Handbook of ANALYTICAL VALIDATION

Handbook of ANALYTICAL VALIDATION

Michael E. Swartz
Ira S. Krull

CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Version Date: 20120309

International Standard Book Number-13: 978-1-4200-1448-8 (eBook - PDF)

Visit the Taylor & Francis Web site at
http://www.taylorandfrancis.com

and the CRC Press Web site at
http://www.crcpress.com

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Printed in the United States of America on acid-free paper
Version Date: 20120309

International Standard Book Number: 978-0-8247-0689-0 (Hardback)

Visit the Taylor & Francis Web site at
http://www.taylorandfrancis.com

and the CRC Press Web site at
http://www.crcpress.com

Contents

Preface

Back in 1997, in support of some method validation vendor seminars we were doing at the time, Professor Ira Krull and I wrote a 92-page paperback book that was published by Marcel Dekker (now a part of Taylor & Francis). We remain, to this day, surprised at the success of that "little" book and how many people used it and referenced it as their introduction to method validation. We wrote that book to shed light on the subject of method validation from a practical standpoint. It was written in response to requests for clarification and questions we received during our travels teaching and giving seminars on the subject of the guidelines, as many people viewed them as somewhat vague at the time.

In the years since the initial publication, a lot has happened in the world of method validation. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) proposed and approved new guidelines, the US Food and Drug Administration (FDA) published a new draft guideline and implemented the ICH guidelines, and the US Pharmacopeia (USP) has either updated or published new chapters, all related to the topic of method validation. In addition, new journals also appeared on the topic, and many scientific journals now require method validation data in all submissions. Accompanying these developments, a virtual cottage industry of training, consulting, and software development arose to support the newer method validation regulatory emphasis. We have always maintained that validating methods means doing nothing more than what has always been recognized as "good science." But good science can mean different things to different people. So with that thought in mind, and in light of the increased regulatory focus and all of the new developments in the field since we published that short first work, we decided several years ago that an updated and expanded version was called for, culminating in the *Handbook of Analytical Validation*.

The original book included seven chapters that all briefly dealt with method validation and related subjects. The updated handbook includes ten chapters that cover all the original subjects in the first edition in much more detail, and several new chapters on related subjects. It is organized much the same way one might approach method validation in a regulated environment, starting with instrument qualification, followed by method development with validation in mind, and then specific topics pertaining to method validation from a number of perspectives. This second effort also includes many more examples and although many of the examples presented focus on liquid chromatography of one mode or another (it is one of the predominant techniques used in regulated laboratories today), the same principles and techniques apply to all analytical techniques and procedures used in a regulated environment. But please note that the information presented in this text represents the interpretation and opinions of the authors only and not those of any past, present, or future employers.

Just like the first work, we hope that this book provides the reader with help and information that can be used in addition to consulting your companies' SOPs, the many references, and the guidance available to simplify the overall process of method development, optimization, and validation.

Michael Swartz

Ira Krull

1 Introduction to Analytical Method Validation

1.1 INTRODUCTION

The primary focus of this book is analytical method validation (AMV); however, it is important to have a perspective on where AMV fits into the overall process of validation, in addition to how the process is governed and regulated, before going into too much detail. Therefore, this chapter provides a brief overview of the drug development process; the organization and hierarchy of the main regulatory agency, the Food and Drug Administration (FDA); important contributions of the International Conference on Harmonization (ICH); and pertinent AMV guidelines and their purpose. It also discusses the basic concepts of how AMV fits into the overall validation process, how AMV differs depending on the various phases of the drug development process, and addresses the importance of trained personnel in a regulated environment.

1.2 DRUG DEVELOPMENT PROCESS

The stages of drug discovery and development are well defined and can be divided into several distinct phases as outlined in Figure 1.1 [1]. In the drug discovery phase, laboratories are mostly concerned with drug characterization studies, structure determinations, solubility, p*K*a, spectral data, stability, chromatographic content and purity analysis, and related method development. The amount of method validation that is required at this early stage is very limited. As the drug shows more promise for a target in screens, or in cell and tissue assays, additional analytical method development and validation work is pursued and performed. Just as analytical methods must evolve, so too must AMV. The ability to conduct "good science" at the right time with the best use of resources must be balanced against the ability to quickly implement change during drug development. Indeed, much of the work performed this early on in drug development is performed outside of regulatory scrutiny, in a non-Good Manufacturing Practice (GMP) setting or format.

In the preclinical phase, bioanalytical method development and validation from serum, tissue, or other biological matrices often ensues, and Good Laboratory Practice (GLP) regulations apply. The type of method development and validation studies performed at the preclinical stage of drug development is also used in support of pharmacokinetic, toxicokinetic, and drug metabolism studies. Such methods may also be used to support drug formulation and drug delivery (e.g., dissolution studies); and similar to the bioanalytical studies, these studies are performed in a

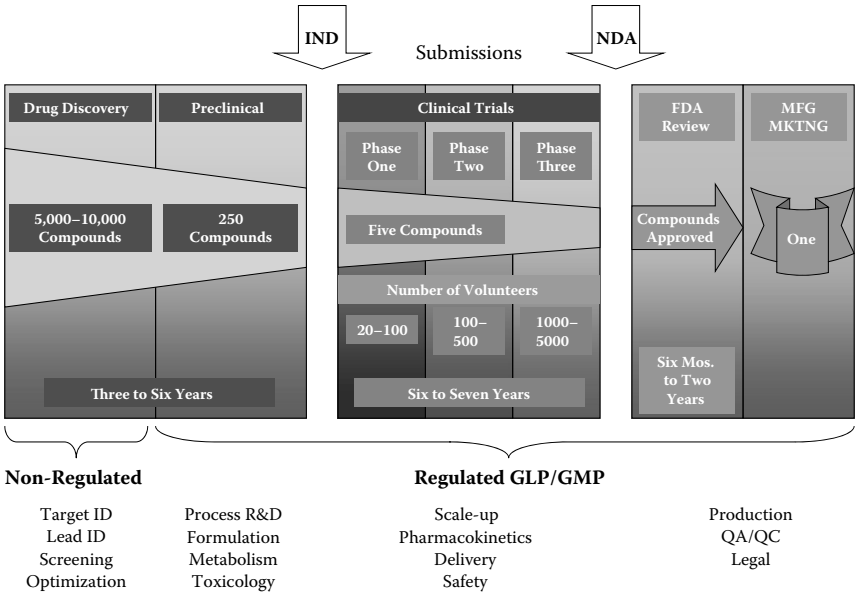


FIGURE 1.1 An overview of the stages in the drug development process.

regulated GMP fashion. It is at this point, following the Preclinical Phase studies, that the Investigational New Drug (IND) application is made to the FDA.

In the clinical phase, containing Phase I–III safety and efficacy studies, there will be human pharmacokinetics studies, which again may need additional method development and validation work to be performed due to the different matrices that might be involved. At this point in time, while the drug moves closer to market, a New Drug Application (NDA) filing is prepared that includes a complete AMV package according to the type of method and its intended use. Complete validation at this point in the process might also include interlaboratory collaborative studies (also known as round-robin studies), involving a number of labs, analysts, instrumentation, and samples to prepare for the transfer of the method, depending on where or how it is implemented.

In the end, the amount or extent of method validation can be correlated with Figure 1.1; that is, the amount of validation increases the further a drug moves along in the development process [2,3]. One of the major goals in method validation is to balance the amount of validation performed to meet United States Pharmacopeia (USP) guidelines and FDA recommendations. As the drug survives the stages indicated in Figure 1.1 and moves toward marketing approval, there is no need to perform a comprehensive or complete validation for a new method on a drug that is early in the discovery or preclinical stages of its life cycle. In early development, only minimal validation work is performed; and if the drug survives these early stages, the amount of validation performed will increase as the drug moves closer to market. Therefore, AMV is an evolving process, largely dependent on where a given drug is in its stages of development.

While the cost of successful development and commercialization of a new drug increases significantly with the amount of time expended in years, there is also a substantial increase in costs from the beginning of the Clinical Phase III trials to the NDA submission, and a phase approach to method development and validation is one way to reduce costs in drug development. Figures have placed the cost of drug development at anywhere from $897 million to upward of $1.7 billion spread out over 8 to 12 years [4,5]. Phased-in or phase-appropriate method development and validation can save a firm time and expense by not performing needless procedures too far in advance. The goal, of course, is to reduce these time requirements as much as possible, and consequently the cumulative costs involved.

In addition to being a GLP/GMP regulatory requirement, a validated method ensures reliable results, reducing the necessity of repeating expensive studies. Once the drug goes to market, there may be reasons to develop simpler, more robust, more reproducible, faster, cheaper, and easier-to-perform methods, sometimes for cost efficiency and effectiveness or in order to take advantage of new technology. In these instances, when the expense is justified, methods may need to be revalidated.

1.3 FDA HIERARCHY AND ORGANIZATION

The pharmaceutical and biotechnology industries are two of the largest and most rapidly growing industries in the world. Some of the most productive and profitable companies in the world are pharmaceutical or biotech based, and their names have become synonymous with big business. These companies are beholden to the FDA from the initial IND application all the way through to the NDA and final marketing approval. The pharmaceutical industry is perhaps the most heavily regulated industry in the world, and researchers from around the world look to the US FDA for guidance on a regular basis.

The FDA's mission statement is: "… protecting the public health by assuring the safety, efficacy, and security of human and veterinary drugs, biological products, medical devices, our nation's food supply, cosmetics, and products that emit radiation. The FDA is also responsible for advancing the public health by helping to speed innovations that make medicines and foods more effective, safer, and more affordable; and helping the public get the accurate, science-based information they need to use medicines and foods to improve their health" [6].

It is required of all pharmaceutical firms, no matter where they are located in the world, to receive FDA approval for initial clinical studies, final clinical studies, and NDAs before marketing their products in the United States. It is the US FDA, in the end, that will decide which products are safe to enter clinical studies, which products are justified in going to market, which products shall remain on the market, and to deal with whatever problems may arise once a product has been on the market for any length of time.

The FDA is an agency within the Department of Health and Human Services and consists of eight centers/offices:

1. Center for Biologics Evaluation and Research (CBER)
2. Center for Devices and Radiological Health (CDRH)

3. Center for Drug Evaluation and Research (CDER)
4. Center for Food Safety and Applied Nutrition (CFSAN)
5. Center for Veterinary Medicine (CVM)
6. National Center for Toxicological Research (NCTR)
7. Office of the Commissioner (OC)
8. Office of Regulatory Affairs (ORA)

The two centers that are most important to any discussion of compliance and validation from a laboratory perspective are the CBER and CDER. The CBER regulates biological products for disease prevention and treatment that are inherently more complex than chemically synthesized pharmaceuticals, including

- Blood and blood products, such as plasma, blood-derived proteins including clotting factors for hemophilia, tests used to screen blood donors, and devices used to make blood products
- Vaccines and allergenic products
- Protein-based drugs, such as monoclonal antibodies and cytokines that stimulate the immune system to fight cancer, and enzyme therapies that stop heart attacks

The CDER promotes and protects health by assuring that all chemically synthesized prescription and over-the-counter drugs are **safe and effective**. The CDER evaluates all new drugs before they are sold, and serves as a consumer watchdog for the more than 10,000 drugs on the market to be sure they continue to meet the **highest standards**.

FDA chemists review proposed analytical procedures submitted as part of NDAs and abbreviated new drug applications (ANDAs). But while the FDA provides guidelines to follow, it is not a research organization designed to improve or troubleshoot a method once submitted. Therefore, proper care and attention must be given so that the validation package submitted is as complete as possible, with no errors or omissions. Having to resubmit a validation package for review is like waving a red flag and is bound to draw the wrong type of attention! Getting it right the first time decreases the time to market and the associated costs.

1.4 INTERNATIONAL CONFERENCE ON HARMONIZATION

For years, many countries around the world have had national regulatory systems to evaluate the quality, safety, and efficacy of pharmaceutical products. While formed on the same basic commitments, detailed technical requirements diverged over time to such an extent that the pharmaceutical industry found it necessary to duplicate many time-consuming and expensive test procedures in order to market new products internationally.

In response to the growing global pharmaceutical market, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) was first conceived in 1990 at a meeting hosted by the European Federation of Pharmaceutical Industries and Associations (EFPIA) in

Brussels. Since this first organizational meeting, a biennial conference has been held, in addition to other periodic conferences and workshops.

Initially, a threefold purpose ("Terms of Reference") was identified:

1. To provide a forum for discussion between regulatory agencies and the pharmaceutical industry on the differences in the technical requirements for product registration in the three member regions
2. To identify areas where changes in technical requirements and agreement on research and development procedures could lead to a more economical use of resources (human, animal, and material) without compromising safety
3. To recommend practical ways to achieve harmonization in the interpretation and application of technical guidelines

These original terms of reference have since been modified, however:

1. To maintain a forum for a constructive dialogue between regulatory authorities and the pharmaceutical industry on the real and perceived differences in the technical requirements for product registration in the EU, the United States, and Japan in order to ensure a more timely introduction of new medicinal products, and their availability to patients
2. To monitor and update harmonized technical requirements leading to a greater mutual acceptance of research and development data
3. To avoid divergent future requirements through harmonization of selected topics needed as a result of therapeutic advances and the development of new technologies for the production of medicinal products
4. To facilitate the adoption of new or improved technical research and development approaches that update or replace current practices, where these permit a more economical use of human, animal, and material resources, without compromising safety
5. To facilitate the dissemination and communication of information on harmonized guidelines and their use such as to encourage the implementation and integration of common standards

The term *ICH*, while originally was meant to denote an international conference on harmonization, has now become more associated with the *process* of harmonization than the actual conferences themselves. Indeed, many of the recommendations or guidelines developed as a result of the ICH processes have been implemented; however, more are forthcoming.

1.4.1 Structure of the ICH

At the inaugural meeting of the ICH, representatives of the regulatory agencies and industry associations of Europe, Japan, and the United States met to establish terms of reference, and created a steering committee that has since met at least twice yearly. These six founding parties (three regulatory and three trade associations) are the direct participants in the ICH process.

1.4.1.1 European Commission of the European Union (EU)

The European Commission represents the fifteen members of the European Union and is currently working, through harmonization, to achieve a single market to allow free movement of products throughout the EU. The European Medicines Agency (EMEA), based in London, was created by the European Commission to provide technical and scientific support for ICH activities.

1.4.1.2 European Federation of Pharmaceutical Industries and Associations (EFPIA)

The EFPIA is based in Brussels and counts member associations in sixteen countries in Western Europe among its members. Members also include all of Europe's major research-based pharmaceutical companies. Much of the federation's work is concerned with the activities of the European Commission and the (EMEA), and is accomplished by a network of experts and country coordinators that ensure that the EFPIA's views within ICH are representative of the European pharmaceutical industry.

1.4.1.3 Ministry of Health and Welfare, Japan (MHW)

In Japan, the MHW is responsible for the improvement and promotion of social welfare, social security, and public health. Within the MHW, the Pharmaceutical Affairs Bureau is responsible for reviewing and licensing all medicinal products and acts as the focal point for ICH activities. Technical advice on ICH matters is obtained through the MHW's expert groups, together with an affiliated organization, the National Institute of Health Sciences.

1.4.1.4 Japan Pharmaceutical Manufacturers Association (JPMA)

The membership of the JPMA represents ninety research-based pharmaceutical manufacturers in Japan. Within the JPMA, ICH activities are coordinated through specialized committees of industry experts who participate in the ICH expert working groups.

1.4.1.5 US Food and Drug Administration (FDA)

The FDA has a wide range of responsibilities for drugs, biologicals, medical devices, cosmetics, and radiological products. The largest of the world's drug regulatory agencies, the FDA is responsible for the approval of all drug products used in the United States, regardless of origin. The FDA consists of administrative, scientific, and regulatory staff organized under the Office of the Commissioner and has several centers with responsibility for various regulated products. Technical advice and experts for ICH activities are drawn from both the CDER and CBER.

1.4.1.6 Pharmaceutical Research and Manufacturers of America (PhRMA)

PhRMA represents the research-based pharmaceutical industry involved in the discovery, development, and manufacture of prescription medicines in the United States. There are also research affiliates, members that conduct biological research related to the development of drugs and vaccines. PhRMA, which was previously known as the US Pharmaceutical Manufacturers Association (PMA), coordinates its technical input to ICH through its Scientific and Regulatory Section. Special committees of experts from PhRMA companies deal specifically with ICH topics.

1.4.1.7 Observers

Since the ICH was initiated, there have been official observers associated with the process to act as a link with non-ICH countries and regions. Each of the observer parties has a seat on the ICH Steering Committee. The observers to ICH include

- The World Health Organization (WHO)
- The European Free Trade Area (EFTA), represented at ICH by Switzerland
- Canada, represented at ICH by the Drugs Directorate, Health Canada

1.4.2 ICH Administration

ICH is administered by the ICH Steering Committee that is supported by the ICH Secretariat. Since ICH was established, each of the six co-sponsors has had two seats on the ICH Steering Committee, which oversees the harmonization activities. The International Federation of Pharmaceutical Manufacturers Association (IFPMA) is a federation of member associations representing the research-based pharmaceutical industry and other manufacturers of prescription medicines in fifty-six countries throughout the world. IFPMA has been closely associated with ICH since its inception to ensure contact with the research-based industry outside the ICH Regions. IFPMA has two seats on the ICH Steering Committee and runs the ICH Secretariat. The Secretariat also participates as a nonvoting member of the Steering Committee. The Secretariat operates from the IFPMA offices, in Geneva, and is primarily concerned with preparations for, and documentation of, meetings of the Steering Committee. The secretariat is also responsible for the coordination of preparations for EWG meetings. The World Health Organization, the Canadian Health Protection Branch, and the European Free Trade Association (EFTA) also nominate participants to attend the ICH Steering Committee meetings. In addition, each of the six co-sponsors has designated an ICH Coordinator to act as the main contact point with the ICH Secretariat. The ICH Coordinator ensures that ICH documents are distributed to the appropriate persons within the area of their responsibility.

1.4.3 ICH Harmonization Process

Topics are selected for harmonization by the ICH Steering Committee on the basis of a concept paper proposed by one of the ICH parties or by the ICH Expert Working Groups. The concept paper identifies the main objective of the proposed harmonization in terms of the perceived problem and desired outcome.

Once initiated, the topic proceeds through a stepwise process, where, if completed, a final draft guideline is created and sent back to the member regulatory agencies for implementation. The stepwise process proceeds as follows.

1.4.3.1 Step 1

In the beginning, a six-party Expert Working Group (EWG) is appointed for the topic, and one of them is chosen as the rapporteur. The EWG holds preliminary discussions on the topic, and the rapporteur prepares a first draft. This draft may be a guideline, policy statement, recommendation, or a "points to consider" document. The draft is

reviewed and revised by the EWG until a consensus is reached on the scientific issues. The draft is then forwarded by the EWG to the steering committee for further action.

1.4.3.2 Step 2

At the next step, the draft is approved by the six ICH parties in the steering committee and is transmitted to the three regional regulatory agencies for formal consultation. This regulatory consultation may include organizations and associations outside the ICH process, as well as the IFPMA, EFPIA, JPMA, and PhRMA, and the observers WHO, EFTA and the Drugs Directorate, Health Canada. This comment period is normally six months, except when there are special circumstances.

1.4.3.3 Step 3

Next, a regulatory rapporteur is designated from the EU, MHW, or FDA to collect comments in the three regions. The rapporteur, in consultation with the other regulatory experts, analyzes the comments and amends the "Step 2" draft if necessary. If, as a result of this process, significant change results and the original consensus is not maintained, one or more regulatory authorities may recirculate the amended parts of the draft for further approval. If amendment is not necessary, the rapporteur prepares a final draft and seeks the approval of the regulatory experts from the other parties. The final draft is "signed off" by experts designated by the regulatory parties before being referred to the ICH Steering Committee for adoption.

1.4.3.4 Step 4

The final draft is discussed within the Steering Committee and "signed off" by the three ICH regulatory parties and recommended for adoption.

1.4.3.5 Step 5

The process is complete when the full recommendations are incorporated into domestic regulations or other appropriate administrative measures, according to national/regional internal procedures. In the United States, during both the comment period (Step 2) and implementation (Step 5), the full text of the guideline is published in the *Federal Register* and eventually will end up in the appropriate compendia, such as the *US Pharmacopeia*. Guidelines are also available via the Internet. Each of the other regulatory parties has its own implementation process, and they make them available via the Internet as well.

1.4.4 ICH Harmonization Initiatives

ICH harmonization topics to date have been divided into several major categories with ICH codes assigned to each topic. The guidelines derived from each topic are commonly referred to by the ICH codes. These categories include Quality topics ("Q"-topics relating to pharmaceutical quality assurance), Safety topics ("S"-topics relating to in vitro and in vivo preclinical studies), Efficacy topics ("E"-topics relating to clinical studies in humans), and Multidisciplinary topics ("M"-topics that defy categorization).

Method validation guidelines fall under the Quality topics, in Section Q2, Validation of Analytical Procedures. The harmonized ICH text of Topic Q2A:

TABLE 1.1
ICH Guidelines Pertaining to AMV

Guideline	Subject	Title
Q2(R1)	Analytical Validation	Validation of Analytical Procedures: Text and Methodology
Q1A(R2)	Stability	Stability Testing of New Drug Substances and Products
Q1B	Stability	Stability Testing: Photostability Testing of New Drug Substances and Products
Q1C	Stability	Stability Testing for New Dosage Forms
Q3A(R2)	Impurities	Impurities in New Drug Substances
Q3B(R2)	Impurities	Impurities in New Drug Products
Q6A	Specifications	Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (including Decision Trees)
Q6B	Specifications	Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

Definitions and Terminology was finalized (Step 4) in October 1994. This guideline identified the validation parameters required for analytical methods. It also discussed the characteristics that must be considered during the validation of analytical procedures that are included as part of the registration process. Q2A: Definitions and Terminology was published in the *Federal Register* in 1995 and is now considered implemented [7]. The harmonized ICH text of Topic Q2B: Methodology was finalized (Step 4) in November 1996. Q2B extended Q2A to include the actual experimental data required, along with statistical interpretation for the validation of analytical procedures. Q2B: Methodology was also published in the *Federal Register* in 1997, and is also considered implemented [8]. In November of 2005, Q2A and Q2B were combined into a single guideline, Q2 (R1), and renamed "Validation of Analytical Procedures: Text and Methodology" [9]. While guideline Q2 (R1) certainly forms the foundation for method or procedure validation, several additional guidelines are also pertinent to any method validation discussion, and these are summarized in Table 1.1. All the ICH guidelines significantly affect people working in the validation area, and should be consulted, as these guidelines have been incorporated into the USP, and federal regulators often reference these documents.

It should be pointed out that the ICH is not a regulatory body, or in the business of generating duplicate guidelines. Rather, the ICH has provided clear (with respect to global compendia) guidance on several topics, with, among others, the FDA as willing participants. This participation has helped to ensure that a single set of current guidelines is adopted and maintained through the normal regulatory process.

1.5 AMV GUIDANCE

Guidelines are documents prepared for both regulatory agency personnel and the public that establish policies intended to achieve consistency in the agency's regulatory

approach, and to establish inspection and enforcement policies and procedures. For example, the FDA guidance provides recommendations to applicants on submitting analytical procedures, validation data, and samples to support the documentation of the identity, strength, quality, purity, and potency of drug substances and drug products, and is intended to assist applicants in assemblingß information, submitting samples, and presenting data to support analytical methodologies [10,11]. The recommendations apply to drug substances and drug products covered in NDAs, ANDAs, biologics license applications (BLAs), product license applications (PLAs), and supplements to these applications. The guidelines are generic; that is, they apply to any analytical procedure, technique, or technology used in a regulated laboratory (e.g., gas chromatography, GC; mass spectrometry, MS; and infrared spectroscopy, IR).

Analytical chemists have practiced method validation for decades without even knowing it. Always striving for repeatability, linearity, accuracy, etc., they called it "good science." In 1987, the FDA issued a guidance document that formally coined the term "method validation" for the first time, and designated the specifications in the current edition of the *United States Pharmacopeia* (USP) as those legally recognized when determining compliance with the Federal Food, Drug, and Cosmetic Act [10–12]. Since the late 1980s, government and other agencies (e.g., FDA, ICH) have issued new and updated guidelines on validating methods. More recently, new information has been published, updating the previous guidelines and providing more detail and harmonization with ICH guidelines. In August of 2000, the FDA updated the original 1987 guidance document in draft form to reflect changes in the agency's approach, the use of new technology, and to be consistent with the ICH guidelines Q2A: Text on Validation of Analytical Procedures and Q2B: Validation of Analytical Procedures: Methodology [11]. USP Chapter <1225> on Validation of Analytical Procedures has also been updated to reflect these changes and the newer Q2 (R1) "combined" guideline" [13]. Table 1.2 is a list of some of the USP and FDA guidance pertaining to AMV. The 2000 FDA draft guidance, in combination with USP Chapter <1225> and ICH Q2 (R1), forms the very foundation of AMV.

The 2000 FDA draft guidance emphasizes the ICH recommendations for noncompendial analytical procedures and elaborates on topics such as types of analytical

TABLE 1.2
General USP/FDA Guidelines Pertaining to AMV

Guideline	Subject/Title (reference)
USP <1225>	Validation of Compendial Procedures
USP <1226>	Verification of Compendial Procedures
USP <621>	Chromatography
USP <1092>	The Dissolution Procedure: Development and Validation
USP <1058>	Analytical Instrument Qualification
USP <1010>	Analytical Data Interpretation and Treatment
FDA 1987	Guideline for Submitting Samples and Analytical Data for Methods Validation [10]
FDA 2000	Draft Guidance for Industry: Analytical Procedures and Methods Validation [11]

procedures, reference standard qualification and characterization, format of analytical procedures submitted in NDAs and ANDAs, validation of noncompendial analytical procedures, compendial analytical procedures, content and processing of validation packages, and revalidation. As of the year 2010, this draft guidance is the most recent guideline available from the FDA, and is briefly outlined here; subsequent chapters of this volume address the guidance in more detail.

1.5.1 Types of Analytical Procedures

The guidelines break methods or procedures into three different types:

1. Regulatory analytical procedures
2. Alternative analytical procedures
3. Stability indicating assays

A regulatory analytical procedure is the analytical procedure used to evaluate a defined characteristic of the drug substance or drug product. Regulatory analytical procedures are found, for example, in the USP.

An alternative analytical procedure is an analytical procedure proposed by the applicant for use instead of the regulatory analytical procedure. A validated alternative analytical procedure should be submitted only if it is shown to perform equal to or better than the regulatory analytical procedure. If an alternative analytical procedure is submitted, the applicant should provide a rationale for its inclusion and identify its use (e.g., release, stability testing), validation data, and comparative data to the regulatory analytical procedure.

A stability indicating assay is a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. It accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities. Additional information on validating stability indicating assays can be found in Chapter 7.

1.5.2 Reference Standards

A reference or primary standard may be obtained from the USP or other source, and when necessary, FDA review staff can be consulted regarding alternative sources for standards. When there is no official source, a reference standard should be of the highest possible purity and be fully characterized. Working, in-house, or secondary standards are also commonly used; however, they should be qualified against the primary reference standard.

1.5.3 Certificate of Analysis

A certificate of analysis (CoA) for reference standards from nonofficial sources should be submitted in the section of the application on analytical procedures and controls. CoAs should include a list of each test performed on the standard, and the methods used to perform the test. The acceptance limits and the results for each test should

also be tabulated. Additional information to look for on a CoA includes information about the lab that did the testing, the expiry date, and the lot number of the standard (or other identifying number). Each CoA should also be uniquely identified.

1.5.4 Characterization of a Reference Standard

Reference standards from the USP and other official sources do not require further characterization. A reference standard that is not obtained from an official source should be of the highest purity that can be obtained by reasonable effort, and it should be thoroughly characterized to ensure its identity, strength, quality, purity, and potency. It is recognized that often the qualitative and quantitative analytical procedures used to characterize a reference standard are different and more extensive than those used to control the drug substance/product itself.

Analytical procedures used to characterize a reference standard should not rely solely on comparison testing to a previously designated reference standard. Generally, characterization information should include

- A brief description of the manufacture of the reference standard, if the manufacturing process differs from that of the drug substance. Any additional purification procedures used in the preparation of the reference standard should be described.
- Legible reproductions of the relevant spectra, chromatograms, thin-layer chromatogram (TLC) photographs or reproductions, and other appropriate instrumental recordings.
- Data that establishes the purity. The data should be obtained by using appropriate tests, for example, TLC, GC, or high-performance liquid chromatography (HPLC).
- Appropriate chemical information, such as the structural and empirical formula and molecular weight, and amount of water and counter-ion salt type and content. Information to substantiate the proof of structure should include appropriate analytical tests, such as elemental analysis, IR, ultraviolet spectroscopy (UV), nuclear magnetic resonance (NMR), and MS, as well as applicable functional group analysis.
- A physical description of the material, including its color and physical form.
- Appropriate physical constants such as melting range, boiling range, refractive index, dissociation constants (pK values), and optical rotation.
- A detailed description of the analytical methods and procedures used to characterize the reference standard.

Although primarily aimed at synthetic drugs, the guidelines also apply to biopharmaceutical product reference standards; however, additional and different tests are important to assess physicochemical characteristics, structural characteristics, biological activity, and immunochemical activity. Physicochemical determinations may include isoform, electrophoretic, and liquid chromatographic patterns, as well as spectroscopic profiles. Structural characterization may include a determination of amino acid sequence, amino acid composition, peptide map, and carbohydrate

structure. Biological and immunochemical activity should be assessed using the same analytical procedures used to determine product potency. These can include animal-based, cell-culture-based, biochemical, or ligand/receptor-binding assays. Additional information on this topic can be found in Chapter 7.

1.5.5 Content and Format for Analytical Methods and Procedures

Any method submitted to the FDA in an NDA, ANDA, BLA, or PLA should be described in sufficient detail to allow a competent analyst to reproduce the necessary conditions and obtain results comparable to the applicant's. As a general rule of thumb, the method should be written so that there is only one possible interpretation for carrying it out. Parts of the method—for example, issues observed during robustness testing—that require special attention should be described, and a cautionary statement included if necessary. A description of analytical procedures from any other published sources should be provided, because the referenced sources may not be readily accessible to the reviewer.

Table 1.3 summarizes the information that would typically be included in an analytical procedure description.

1.5.6 Content and Processing of the Method Validation Package

The method validation package will usually include information copied from pertinent sections of the application. To help the FDA review chemist, the copies should retain the original pagination of the application sections. For ANDA and NDA products, the archival copy and two extra copies (ANDAs) or three extra copies (NDAs) of the method validation package should be submitted with the application. Table 1.4 lists the information that the method validation package should include.

1.5.6.1 Selection and Shipment of Samples

On request from the FDA, an applicant must submit samples of drug product, drug substance, noncompendial reference standards, blanks, internal standards, non-USP reference standards, samples of impurities, degradation products, and unusual reagents so that the suitability of the methods can be evaluated by FDA laboratories. For BLAs and PLAs, representative samples of the product must be submitted, and summaries of the results of tests performed on the lots represented by the submitted sample must be provided. In general, the quantity of samples in each set should be double the amount needed to carry out the testing as performed by the applicant. The submitted drug product samples should be from a batch made with the proposed market formulation. For ANDAs and appropriate supplements, a sample of the finished product from a batch being used to support approval of the submission should be used. For biological products, samples from several consecutively manufactured batches should be submitted.

The drug product should be supplied in its original packaging. Bulk substances (e.g., drug substances, impurities, excipients) should be stored in opaque nonreactive containers. To prevent breakage during shipping, the samples should be adequately packaged in a sturdy container.

TABLE 1.3
Information Typically Included in an Analytical Procedure Description

Section	Comments	
Principle	A statement of the principle or objective of the procedure: for example, isocratic reversed-phase HPLC separation with UV detection	
Sampling	Number of samples, how they are used, number of replicates per sample	
Equipment and Parameters	Listing of all equipment (type, detector, column type/dimensions) and parameters (temperature, flow rate, wavelength) used	
Reagents	List reagents and their grade, directions for preparation, storage conditions, shelf life, directions for safe use,	
System Suitability Testing	Per USP Chapter 621 for chromatography, acceptance criteria are predefined and demonstrate that the system is in working order at the time of the analysis	
Preparation of Standards	Procedures for the preparation of all standards (e.g., stock, working standards, internal standards) should be included	
Preparation of Samples	Sample preparation should be clearly described, including any specific details for specialized sample preparation (e.g., derivatization, solid phase extraction) procedures	
Procedure	A step-by-step description of the procedure should be provided, including injection sequences, start-up parameters, and equilibration times if appropriate.	
Calculations	Representative calculations defining all symbols, constants, etc. Any formulas or transformations should be described in detail.	
Reporting of Results	General:	Procedures for Impurities:
	The format used to report results (e.g., % label claim, v/v, w/w, parts per million (ppm)) should be specified.	Include the name and location identifier (e.g., relative retention time (RRT)), type of impurity, and detection or quantitation limit if appropriate.

Samples shipped from outside the United States should contain the appropriate customs forms to reduce delay in delivery. If special storage precautions (e.g., freezing, use of an inert gas blanket) are required to protect sample integrity, arrangements should be made in advance with the validating laboratory for scheduled direct delivery. If a sample is toxic or potentially hazardous, the container should be prominently labeled with an appropriate warning and precautionary handling instructions.

1.5.6.2 Responsibilities

It is the responsibility of the applicant to provide complete contact information, for FDA contact, sample requests, etc. The FDA review chemist evaluates the application and in coordination with the appropriate FDA laboratory contacts applicants regarding the nowadays unlikely event that a method is actually replicated in the FDA laboratories. The laboratory also communicates any results and comments to the review chemist. Finally, the investigator has the responsibility to inspect the laboratory where the release and stability testing are performed to ensure compliance with GMP.

TABLE 1.4
Information Typically Included in a Method Validation Package

Section	Comments
Tabular list of all samples to be submitted	List should include lot number, identity, package type and size, date of manufacture, and quantity.
Analytical Procedures	A detailed description of each of the analytical procedures listed in the specifications should be submitted.
Validation Data	Appropriate validation data to support the analytical procedures should be submitted, along with individual values and summary tables. Representative instrument output, raw data, and information from stress studies should also be included.
Results	The results obtained for the submitted samples and the dates of the analysis should be provided. Alternatively, a certificate of analysis could also be submitted.
Composition	The components and composition of the drug product should be provided.
Specifications	The specifications for the drug substance and the drug product should be included.
Material Safety Data Sheets (MSDSs)	Material safety data sheets (MSDSs) for all samples, standards, and reagents, as well as any other materials used in the analytical procedures listed in the method validation package, should be included.

1.6 VALIDATION PROCESS

AMV is a critical part of the overall process of validation in any regulated environment. AMV is a part of the validation process that establishes, through laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application and provides an assurance of reliability during normal use, sometimes referred to as "the process of providing documented evidence that the method does what it is intended to do." Regulated laboratories must perform AMV in order to be in compliance with government or other regulators, in addition to being good science. A well-defined and documented validation process can not only provide evidence that the system and method are suitable for the intended use, but also aid in transferring the method and satisfy regulatory compliance requirements.

Validation is also the foundation of quality in the laboratory, and AMV is just one part of a regulatory quality system that incorporates both quality control and quality assurance [12,14]. The terms *quality control* and *quality assurance* often are used interchangeably, but in a properly designed and managed quality system, the two terms have separate and distinct meanings and functions. Quality assurance (QA) can be thought of as related to *process* quality, whereas quality control (QC) is related to the quality of the *product*. In a given organization, it does not matter what the functions are named, but the responsibilities for these two activities should be clearly defined. Both quality assurance and quality control make up the Quality Unit

and are essential to the production of analytical results that are of high quality and are compliant with the appropriate regulations.

QC is the process that determines the acceptability or unacceptability of a product or a product plan, and is determined by the comparison of a product against the original specifications that were created before the product existed. In some organizations, the QC group is responsible for the use of the method to perform analysis of a product. Other tasks related to QC may include documented reviews, calibrations, or additional types of measurable testing (sampling, etc.) and will reoccur more often than activities associated with quality assurance. QC will usually require the involvement of those directly associated with the research, design, or production of a product. For example, in a laboratory-notebook peer-review process, a QC group would check or monitor the quality of the data, look for transcription errors, check calculations, verify notebook sign-offs, etc.

QA is determined by top-level policies, procedures, work instructions, and governmental regulations. At the beginning of the validation process, QA may provide guidance for the development or review of validation protocols and other validation documents. During the analytical stage, QA's job is to ensure that the proper method or procedure is in use and that the quality of the work meets the guidelines and regulations. QA can be thought of as the process that will determine the template and pattern of quality control tasks. As opposed to quality control checks, quality assurance reports are more likely to be performed by managers, by corporate-level administrators, or third-party auditors through the review of the quality system, reports, archiving, training, and qualification of the staff who perform the work.

From a review of the various guidelines, it is evident that AMV is just one part of the overall validation process that encompasses at least four distinct steps as shown in Figure 1.2: (1) software validation, (2) hardware (instrumentation) validation/qualification, (3) analytical method validation, and (4) system suitability. The overall

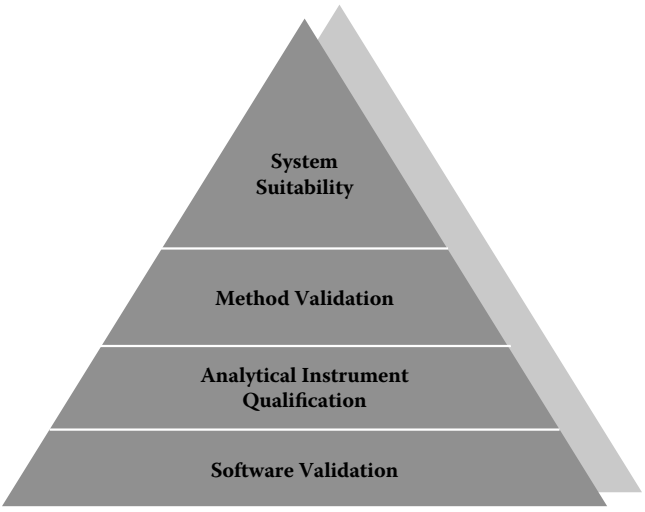


FIGURE 1.2 The basic steps in the validation process.

validation process using trained, qualified personnel begins with validated software and a validated/qualified system; then a method is developed and validated using the qualified system. Finally, the whole process is wrapped together using system suitability. Each step is critical to the overall success of the process.

1.6.1 Software Validation

A comprehensive treatment of software validation is outside the scope of this volume. However, it is an important topic to at least touch upon here as these days every modern laboratory makes use of computerized systems to generate and maintain source data and documentation from a variety of instrumentation. These data must meet the same fundamental elements of data quality (e.g., attributable, legible, contemporaneous, original, and accurate) that are expected of paper records and must comply with all applicable statutory and regulatory requirements. Two FDA guidelines have appeared recently that address the topic of software validation, and should be consulted for more detailed information [15,16]. In addition, in March 1997, the FDA issued 21 CFR Part 11, which provided the original criteria for acceptance of electronic records, electronic signatures, and handwritten signatures executed to electronic records as equivalent to paper records and handwritten signatures executed on paper under certain circumstances [17]. However, after the effective date of 21 CFR Part 11, significant concerns regarding the interpretation and implementation of Part 11 were raised by both FDA and the pharmaceutical industry and, as a result, 21 CFR Part 11 was reexamined [18]. The new Scope and Application Guidance clarified that the FDA intends to interpret the scope of Part 11 narrowly and to exercise enforcement discretion with regard to Part 11 requirements for validation, audit trails, record retention, and record copying. However, most of the other original Part 11 provisions remain in effect.

1.6.2 Analytical Instrument Qualification

Prior to undertaking the task of method validation, it is necessary to invest some time and energy up-front to ensure that the analytical system itself is validated, or *qualified*. Qualification is a subset of the validation process that verifies proper module and system performance prior to the instrument being placed on-line in a regulated environment. In March 2003, the American Association of Pharmaceutical Scientists (AAPS), the International Pharmaceutical Federation (FIP), and the International Society for Pharmaceutical Engineering (ISPE) co-sponsored a workshop entitled "A Scientific Approach to Analytical Instrument Validation" [19]. Among other objectives, the various parties (the event drew a cross-section of attendees; users, quality assurance specialists, regulatory scientists, consultants, and vendors) agreed that processes are "validated" and instruments are "qualified," finally reserving the term *validation* for processes that include analytical methods/procedures and software development.

The proceedings of the AAPS et al. committee have now become the basis for a new general USP chapter, number 1058, on Analytical Instrument Qualification (AIQ) that originally appeared in the USP's Pharmacopeial Forum [20–22]. The chapter details the AIQ process, data quality, roles and responsibilities, software

validation, documentation, and instrument categories. Additional information on AIQ can be found in Chapter 2.

1.6.3 System Suitability

According to the USP, system suitability tests are an integral part of chromatographic methods [23]. These tests are used to verify that the resolution and reproducibility of, for example, a chromatographic system, are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. System suitability is discussed further in Chapter 5.

1.7 TRAINING

In a regulated laboratory, instruments must be qualified and methods must be validated to be suitable for their intended purposes. It is equally important, however, that personnel are properly trained and qualified for the task at hand. The FDA treats the use of untrained or unqualified laboratory personnel in a regulated laboratory the same as an adulteration of the drug substance or drug product. Even so, the FDA still frequently cites firms for a lack of trained personnel; Table 1.5 lists some

TABLE 1.5
Examples of Training Deficiencies from Actual FDA 483 Warning Letters

Failure to assure that the Quality Assurance Unit (QAU) director has adequate education, training, or experience to perform his assigned functions 21 CFR58.29(a)]: "Any individual responsible for the supervision of a non-clinical laboratory study must have education, training, and experience to enable that person to perform his assigned functions. [21CFR 58.29(a)]. You appointed a member of your management team to conduct the responsibilities of the QAU, but your documentation indicates that this person did not have the training and experience to assume these duties.

Procedures for identifying training needs have not been followed [21 CFR 820.25(b)]. Specifically, employee training needs were not addressed and training was not documented.

Persons engaged in manufacturing, processing or packing of drug and device products do not have adequate training to enable those persons to perform the assigned functions. [21 CFR 211.25 (a)] and [21 CFR 820.25 (b)]."

Failure to have adequate laboratory controls. Examples are as follows: Lack of adequate training for laboratory analysts and manufacturing employees.

Failure to establish adequate procedures for identifying training needs and ensuring that all personnel are trained adequately, as required by 21 CFR820.25(b). For example, training procedures did not include: (a) training with regard to defects that might occur from the improper performance of their jobs; (b) training with regard to defects and errors that might be encountered as part of specific job functions; and (c) there was no documentation that QC employees who perform verification and validation activities received training to make them aware of defects and errors that might be encountered within their job functions.

"Our investigator documented deficiencies in your firm's training program, including associated employee training records [21 CFR 606.20(b) and 21 CFR211.25(a)]: You did not retain employee competency test documentation as required by your standard operating procedures."

recent examples of training deficiencies in actual FDA 483 warning letters [24]. Training is an important component of GMP, and to satisfy GMP requirements, training must be focused [12,14]. The two areas that are most relevant from a regulated laboratory job function standpoint are the training requirements themselves and the training that relates directly to the job function. From a GMP requirements standpoint, the training objective should be to enable people to make decisions and interpretations of the guidelines or to ask appropriate questions when there is a lack of clarity regarding any situation within the work environment. For training that relates directly to job function or tasks, the objective is to teach the things personnel need to know to perform their job in an effective manner. But how can these training objectives be accomplished and be meaningful in today's work environment, where everyone feels the pressure to accomplish more, faster? The addition of training as a requirement seems to be an added burden that could further stress an organization or lab. However, when training is done correctly and given serious thought, it can help meet the requirements of GMP as well as benefit the lab by increasing productivity. Therefore, an investment in training is a positive business decision that enables a company to meet the requirements to function in a compliant environment.

Laboratory training for compliance purposes is achieved by ensuring that personnel are trained to understand the regulations to the point that they impact the lab and to accomplish various functions in the lab, such as operating instrumentation.

To help an employee understand the GMP regulations that impact the lab, the training should be specific to an individual's function and focus within the lab. It is of little value to train or educate an employee on all of the regulations if there is no impact on the job that the person fulfills everyday. This thought is obviously mitigated by the idea that there should be a basic level of training that introduces the employee to the company's philosophy and standards for compliance. A new employee might think he is aware of the standards that are acceptable from previous experience. However, this experience might not be relevant in his new environment. It is also true that if only a general focus is given to training, there is more likelihood of causing confusion or clouding of issues. The focus of regulatory training should enable a person to ably and effectively meet the requirements and to understand what those requirements mean from their employer's point of view. The focus should be on their daily needs.

At the same time, there should be someone who has a high-level total-picture view to ensure there is continuity for the overall regulatory or compliance program and that it is aligned with the rest of the organization. This person needs more complete training on the GMP requirements to meet business objectives, and could be a manager within the lab or a quality representative for the company focusing on the lab. This person could also be someone whom the technicians turn to for assistance with questions that are beyond their scope or current training level. The next level of training is related to accomplishing the given functions in the lab, such as operating instrumentation and signing off that analyses were performed as required. It is important to note that it is not enough just to be able to push buttons to make instrumentation function and follow the standard operating procedures. The requirements put pressure on lab management and personnel to understand the background or basics of any analytical technique that is used in the lab. These analytical techniques

are used to assess a product's quality and availability for shipment. The training required for different laboratory functions and levels as well as each individual's training record should be documented and reviewed regularly, and available to audit upon request.

Finally, it is interesting to note that in today's environment, there is pressure to use competency-based testing and training to prove understanding or certification, as opposed to having a record of attendance alone accepted as training. Though this is a step in the right direction, its implications should be considered from the standpoint of cost in both money and time. It should be considered in light of the goals that are to be attained. It also should be understood from the standpoint of any given company and its philosophies that competency-based training should be required at all points in the training process.

1.8 CONCLUSION

In today's global market, the development of a new drug is a long and costly process, involving regulatory, governmental, and sanctioning bodies from around the world. A well-defined and documented validation process is what provides regulatory agencies with evidence that the system (instrument, software, method, and controls) is suitable for its intended use.

REFERENCES

1. http://www.fda.gov/cder/handbook/develop.htm.
2. FDA Guidance for Industry, IND's for Phase 2 and 3 Studies of Drugs, Chemistry, Manufacturing and Controls, Content and Format, Draft Guidance, CBER and CDER, February 1999. See http://www.fda.gov/cder/guidance/guidance.htm.
3. Boudreau, S. P., McElvain, J. S., Martin, L. D., Dowling, T., and Fields, S. M., Method validation by phase of development, an acceptable analytical practice*, Pharm. Technol.*, p. 54, November 2004.
4. DiMasi, J. A., Hansen, R. W., and Grabowski, H. G., The price of innovation: New estimates of drug development costs, *J. Health Economics*, 22, 151, 2003.
5. Tufts Center for the Study of Drug Development, Press release Nov. 9, 2006, and May 13, 2009. See http://csdd.tufts.edu.
6. http://www.fda.gov/opacom/morechoices/mission.html.
7. ICH Q2A: Text on validation of analytical procedures, *International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use,* Geneva, Switzerland, March 1995, *Fed. Reg.*, Vol. 60, March 1, 1995, p. 11260. See also www.ich.org.
8. ICH Q2B: Validation of analytical procedures: Methodology, *International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use,* Geneva, Switzerland, May 1997, *Fed. Reg.*, Vol. 62, No. 96, May 19, 1997, pp. 27463–27467. See also www.ich.org.
9. ICH Q2 (R1): Validation of analytical procedures: Text and methodology, *International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use,* Geneva, Switzerland, November 2005. See also www.ich.org.
10. United States Food and Drug Administration, Guideline for submitting samples and analytical data for methods validation, February 1987. US Government Printing Office: 1990-281-794:20818, or at www.fda.gov/cder/analyticalmeth.htm.

11. Draft Guidance for Industry: Analytical Procedures and Methods Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Division of Research, Rockville, MD, August 2000.
12. Current Good Manufacturing Practice for Finished Pharmaceuticals, 21 CFR Part 211, http://www.fda.gov/cder/dmpq/cgmpregs.htm.
13. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <1225>.
14. Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs, 21 CFR Part 210, http://www.fda.gov/cder/dmpq/cgmpregs.htm.
15. FDA, General Principles of Software Validation; Guidance for Industry and FDA Staff. See http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm085371.pdf.
16. FDA Guidance for Industry, Computerized Systems Used in Clinical Investigations, May 2007. See http://www.fda.gov/cder/guidance/7359fnl.htm.
17. FDA, 21 CFR Part 11, Electronic Records; Electronic Signatures; Final Rule. *Fed. Reg.,* Vol. 62, No. 54, p. 13429, March 20, 1997.
18. FDA, CFR Part 11, Electronic Records; Electronic Signatures—Scope and Application, 2003. See http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072322.pdf.
19. Qualification of analytical instruments for use in the pharmaceutical industry: A scientific approach**,** *AAPS PharmSciTech*, 2004, 5(1) Article 22 (http://www.aapspharmscitech.org).
20. Hertzog, D. L., et al., Developed and validated stability-indicating HPLC method for the simultaneous determination of losartan potassium, hydrochlorothiazide, and their degradation products, *Pharm. Forum*, 31(5), Sept–Oct 2005, pp. 1453–1463.
21. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <1058>.
22. Swartz, M. E., *Analytical Instrument Qualification, Pharmaceutical Regulatory Guidance Book*, Advanstar (publisher), Supplement to LC•6C Magazine Santa Monica CA, pp. 12–16, July 2006.
23. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <621>.
24. www.fda.gov/foi/warning.htm and search for "Training" for more examples.

2 Analytical Instrument Qualification

2.1 INTRODUCTION

In the most general sense, validation refers to a process that consists of at least four distinct components or steps: software, instruments, methods or procedures, and system suitability [1–8]. The system, the software, and the method must all be validated, and system suitability is used to keep the process in check. But while the overall process is called *validation*, some of the steps are also referred to by that same term, as well as others—for example, *qualification* and *verification*. Prior to undertaking the task of method validation, it is necessary to invest some time and energy up-front to ensure that the analytical system itself is validated, or *qualified*. Qualification is a subset of the validation process that verifies proper module and system performance prior to the instrument being placed on-line in a regulated environment.

In March 2003, the American Association of Pharmaceutical Scientists (AAPS), the International Pharmaceutical Federation (FIP), and the International Society for Pharmaceutical Engineering (ISPE) co-sponsored a workshop entitled "A Scientific Approach to Analytical Instrument Validation" [9]. Among other objectives, the various parties (the event drew a cross-section of attendees; users, quality assurance specialists, regulatory scientists, consultants, and vendors) agreed that processes are "validated" and instruments are "qualified," finally reserving the term *validation* for processes that include analytical methods/procedures and software development.

The proceedings of the AAPS et al. committee were originally published as a position paper and also appeared in USP's Pharmacopeial Forum [9]. These proceedings have now become the basis for a new general USP chapter, number 1058, on Analytical Instrument Qualification (AIQ) that originally appeared in the USP's Pharmacopeial Forum [10]. The chapter details the AIQ process, data quality, roles and responsibilities, software validation, documentation, and instrument categories. In addition, because high-performance liquid chromatography (HPLC) is the predominant analytical technique used in the regulated laboratory today, specific examples related to qualifying an HPLC instrument will be discussed.

2.2 COMPONENTS OF DATA QUALITY

The goal of any regulated laboratory is to provide reliable and valid data suitable for its intended purpose. Analysts use validated methods, system suitability tests,

and in-process quality control checks to ensure that the data they acquire is reliable, and there are specific guidance and procedures available to ensure compliance [4–8]. Until recently, however, there was no specific guidance or procedures dictating what constituted AIQ, the process of ensuring that an *instrument* is suitable for its intended application. AIQ is just one component of data quality that also includes software and analytical method validation, system suitability tests, and quality control tests. In general, AIQ and analytical method validation generally ensure the quality of analysis *before* conducting a test; system suitability and quality control checks ensure the quality of analytical results *immediately before* or *during* sample analysis.

While method validation and system suitability are covered in detail in Chapters 4, 6, and 7 of this volume, a brief discussion of system suitability and quality control checks is perhaps necessary here also. System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. According to the USP, system suitability tests are an integral part of chromatographic methods [8]. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. Parameters such as plate count, tailing factors, resolution, and reproducibility are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability "sample" that is typically a mixture of main components and expected by-products.

Quality control check samples are run to make sure the instrument has been properly calibrated or standardized. Instrument calibration ensures that the instrument response correlates with the response of the standard or reference material. Quality control check samples are also often used to provide an in-process assurance of the test's performance during use.

2.3 AIQ PROCESS

Instruments are qualified according to a stepwise process grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ), as outlined in Table 2.1. Another way of looking at the AIQ process is sometimes referred to as a timeline approach; as presented in Figure 2.1, where a true chronological order of events takes place.

2.3.1 Design Qualification

The AIQ process timeline in Figure 2.1 begins with the DQ phase at the vendor's site, where the instrument is developed, designed, and produced in a validated environment according to good laboratory practices (GLP), current good manufacturing practices (cGMP), and ISO 9000 standards. Users should ensure that the instrument is fit for their intended use and that the manufacturer has adopted a quality system for development, manufacturing, and testing, and has adequate support for installation,

TABLE 2.1
Timing, Applicability, and Activities for Each Phase of AIQ

DQ	IQ	OQ	PQ
	Timing and Applicability		
Prior to purchase of a new instrument	At installation of each instrument (new, old, or existing unqualified)	After installation or major repair of each instrument	Periodically at specified intervals for each instrument
	Activities		
Assurance of vendor's DQ	System description**	Fixed parameters**	Preventive maintenance and repairs
Assurance of adequate support from manufacturer	Instrument delivery		SOPs for operation, calibration, maintenance, and change control
Instrument's fitness for use in lab	Utilities/facility/ environment		
	Network data and storage**	Secure data storage, backup, and archive**	
	Assembly and installation		
	Installation verification**	Instrument function tests**	Performance tests**

Note: Activities under each phase are usually performed as given in the table. However, in some cases, it may be more appropriate to perform or combine a given activity with another phase. Activities spanning more than one phase are indicated by the double asterisk.

service, and training. Vendor-supplied documentation and consumer audits of the vendor are usually sufficient to satisfy users' DQ requirements.

2.3.2 Installation Qualification (IQ)

During the IQ phase in Figure 2.1, all the activities associated with properly installing the instrument (new, preowned, or existing) at the users' site are documented. A system description, including manufacturer, model, serial number, etc.; proper site requirements; and the receipt of all the parts, pieces, manuals, etc., necessary to perform the installation are confirmed. Table 2.2 illustrates an example of a form that might be used during IQ to document instrument components. During physical installation, all of the fluidic, electrical, and communication connections are made for components in the system. Documentation describing the instrument and components (e.g., Table 2.2), how the instrument was installed, who performed the installation, and other miscellaneous details should be recorded, signed off, and archived. An installation verification confirming the success of the installation should also be performed before proceeding to the next phase.

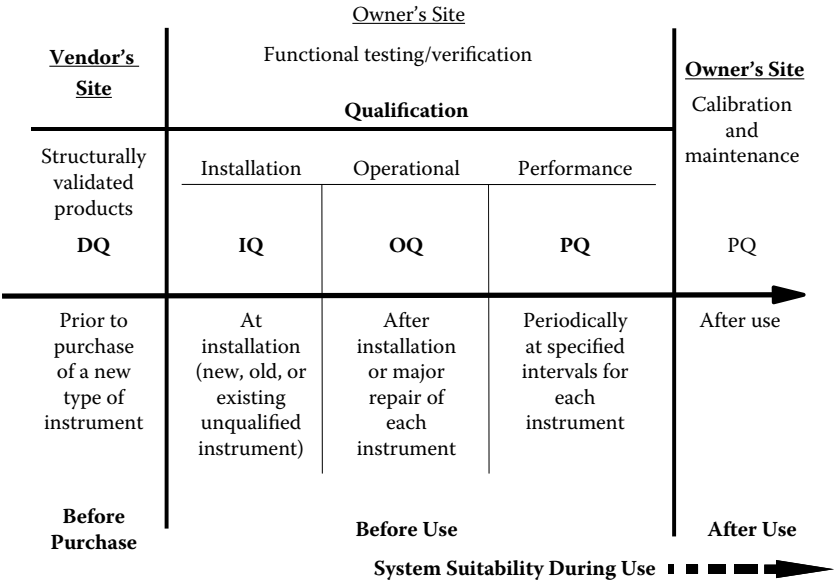


FIGURE 2.1 A timeline approach to AIQ.

2.3.3 Operational Qualification (OQ)

Once the IQ phase is completed, testing is done to verify that the instrument or instrument modules operate as intended in an OQ phase, as illustrated in Figure 2.1. First, fixed parameters, for example, length, weight, height, voltage inputs, pressures, etc., are either verified or measured against vendor-supplied specifications. Since these parameters do not change over the lifetime of the instrument, they are usually measured just once. Next, secure data handling is verified. Finally, instrument function tests are undertaken to verify that the instrument (or instrument modules) meets vendor and user specifications.

Instrument function tests should measure important instrument parameters according to the instrument's intended use and environment. In HPLC, the following types of tests might be included

- Pump flow rate accuracy
- Gradient accuracy
- Injector accuracy
- Column oven and auto sampler temperature
- Detector wavelength accuracy and linearity
- Detector linearity

The analyst would first verify that all the individual modules in the system performed the start-up diagnostic routines successfully, and then each module is tested individually against predetermined specifications. Relevant OQ tests for each of the

TABLE 2.2
Example IQ Instrument Identification Form

Equipment Name

Manufacturer

Serial Number

Firmware Revision Number

Owner's Equipment Number

Enter N/A in the installed column for any components not applicable to this instrument

Options	**Installed *(Initials)***	**Serial Number**
Sample Heater/Cooler		
Column Heater/Cooler		
Column Selection Valve		
Bar Code Reader		
Other (list)		

Certification

The Performer as signed below attests that the information identifying the system described above is accurate and complete.

Performer	**Signature**	**Date**

The Reviewer as signed below attests that the information identifying the system described above is accurate and complete.

Reviewer	**Signature**	**Date**

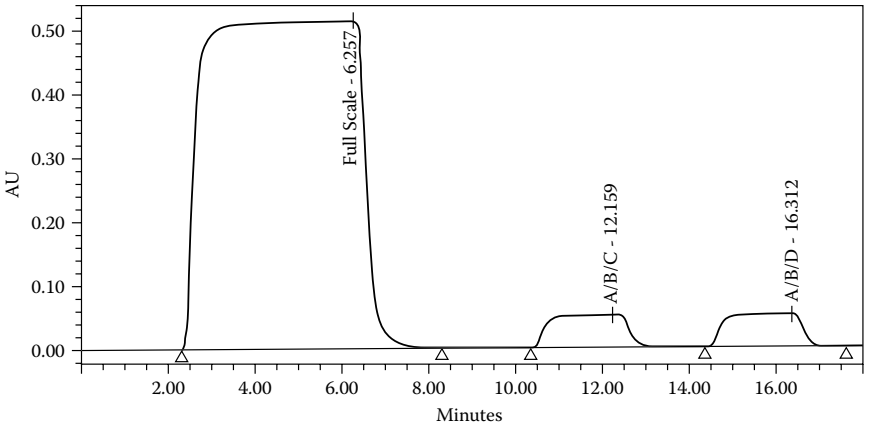
Note: Similar forms may be used for other instruments and components, for example, detectors, fraction collectors, etc.

modules in the system should be repeated whenever the instrument undergoes major repairs or modifications.

2.3.3.1 HPLC Pump Flow Rate Accuracy

Pump flow rate accuracy is typically determined over a range of values, from 0.5 to 5.0 mL/min, for general analytical work, and sometimes lower (down to 0.25 mL/min) for work using mass spectrometry (MS) detection. Methanol is commonly used, and must be thoroughly degassed. A length of tubing or other flow restriction device is attached to the outlet of the pump to provide 500–700 psi of backpressure at the testing flow rate. Once the backpressure has stabilized, a calibrated stopwatch is used to measure the amount of time used to fill a Class A volumetric flask. The flow rate is calculated using the following equation:

$$\text{Calculated flow rate} = \text{Volume of flask}/(\text{Time in seconds}/60)$$



Peak Results

	Name	RT	Height	GPV Percent
1	Full Scale	6.257	517135	100.00
2	A/B/C	12.159	51609	9.98
3	A/B/D	16.312	51216	9.90

FIGURE 2.2 Example of a UV detector response for a gradient accuracy test on a quaternary system. Solvent A is methanol; solvents B, C, and D contain 0.1% concentration of propylparaben in methanol. The first "peak" is from a 50/50 mixture of the A and B solvents; "peaks" two and three are due to adding 10% C solvent and 10% D solvent, respectively.

The values at each flow rate tested are recorded in a form similar to that shown in Table 2.3.

2.3.3.2 Gradient Accuracy

Most HPLC systems in use today are capable of delivering mobile-phase gradients or a change in composition (e.g., % organic) versus time over the course of a chromatographic run. Gradient pumps are commonly binary (capable of delivering two different solvents) or quaternary (capable of delivering up to four different solvents). To test for gradient accuracy, one or more of the solvent lines can be primed with a solvent containing an ultraviolet (UV) absorber (e.g., propylparaben at 254 nm) and a UV detector used to monitor the response. Instrument gradient conditions are used to deliver known proportions of the mobile phase, and the full-scale detector response is measured against predetermined specifications to determine accuracy. Figure 2.2 is an example of a UV detector response for a gradient accuracy test for a system capable of quaternary solvent delivery. The first "peak" is from a 50/50 mixture of the A and B solvents; the subsequent "peaks" two and three are due to adding 10% C solvent, and 10% D solvent, respectively. The results measured as peak heights of 9.98 and 9.90 are well within the stated manufacturer's specifications.

2.3.3.3 Injector Accuracy

Injector accuracy (volume) is typically performed by injecting a set amount of sample, weighing the sample vial before and after. The average volume used per

TABLE 2.3
Example Flow Rate Accuracy Test Result Form

Pump Serial Number

Flow Rate Accuracy Results

Set Flow Rate (mL/min)	Observed System Pressure (psi)	Observed Elapsed Time (s)	Calculated Flow Rate (mL/min)	Flow Rate Specification (mL/min)	Test Results	
					Pass (Initials)	Fail (Initials)
5.000				4.94–5.06		
1.000				0.988–1.012		
0.500				0.489–0.511		
0.250				0.239–0.261		

Comments

Certification

The Performer as signed below attests that the test was performed according to the prescribed procedure and that the results above are accurate and complete.

Performer **Signature** **Date**

The Reviewer as signed below attests that the test was performed according to the prescribed procedure and that the results above are accurate and complete.

Reviewer **Signature** **Date**

Note: Flow rates and specifications are suggestions only, and should be based upon actual use, vendor's specifications, and measurement error.

injection (assuming six injections were performed between weighings) is calculated according to the formula:

$$\text{mg or } \mu\text{L water per injection} = 1{,}000 \times [(W_1 - W_2)/6]$$

Water is often used for this test because its density, 0.99823 g/mL at 20°C and 0.99707 g/mL at 25°C, introduces less than 0.3% error when volume is assumed equal to weight (grams × 1,000 = µL).

2.3.3.4 Column Oven and Auto Sampler Temperature

The temperature of the sample compartment or the column oven is measured using a thermometer probe placed so that it does not come into contact with the surface in either component. A temperature is set that is at least 5°C above or below the ambient temperature, and sufficient time is allowed for the temperature to stabilize before recording the value. The time required to reach and stabilize the set temperature depends on the difference between the set and ambient temperatures.

2.3.3.5 UV Detector Wavelength Accuracy and Linearity

To assess UV detector wavelength accuracy, a National Institute of Standards (NIST) traceable standard of erbium perchlorate is often used. Absorbances at three wavelengths (255, 379, and 522 nm) are measured and compared against vendor specifications, taking into account the additive effects of detector and standard variability (as much as 1.5 nm combined). Some detectors are equipped to handle cuvettes; others can simply be flushed with the required solution using a syringe. A sample detector start-up diagnostics and wavelength accuracy result form is shown in Table 2.4.

Linearity is assessed by either flushing the cell or filling a cuvette with a known concentration of a standard (e.g., propylparaben 5, 10, 20, 25, and 30 mg/mL), and calculating response factors at multiple levels (minimum of five). The response factor %RSD is then compared against a predetermined or vendor-supplied specification. A sample detector linearity result form is shown in Table 2.5.

2.3.4 Performance Qualification (PQ)

Per Figure 2.1, once an IQ and an OQ have been performed, PQ testing is conducted. PQ testing should be performed under the actual running conditions across the anticipated working range. It should be repeated at regular intervals; the frequency

TABLE 2.4
Sample Detector Start-up Diagnostics and Wavelength Accuracy Result Form

Detector Serial Number

Solution ID/Lot Number		**Expiration Date**	
Start-up Diagnostics	Pass (Initials)	Fail (Initials)	
Test Apparatus	Flow Cell (Initials)	Cuvettes (Initials)	
Wavelength Accuracy			
Measured Peak Value (nm)	**Expected Peak Value (nm)**	**Pass (Initials)**	**Fail (Initials)**
	255 (±2.0 nm)		
	255 (±2.0 nm)		
	379 (±2.0 nm)		
	522 (±2.0 nm)		

Comments

Certification

The Performer as signed below attests that the test was performed according to the prescribed procedure and that the results above are accurate and complete.

Performer **Signature** **Date**

The Reviewer as signed below attests that the test was performed according to the prescribed procedure and that the results above are accurate and complete.

Reviewer **Signature** **Date**

TABLE 2.5
Sample Detector Linearity Result Form

Detector Serial Number		
Test Apparatus	Flow Cell (Initials)	Cuvettes (Initials)
Flow Cell Type and Path Length		
Solution ID/Lot Number	**Expiration Date**	
Solution Concentration (mg/mL from CoA)	**Absorbance**	**Sensitivity (Absorbance/ Concentration)**
0		
5		
10		
15		
20		
25		
30		
Sensitivity Mean		
Sensitivity Standard Deviation		
% RSD	**Specification**	<4.5%
Test Result	Pass (Initials)	Fail (Initials)
Comments		

Certification

The Performer as signed below attests that the test was performed according to the prescribed procedure and that the results above are accurate and complete.

Performer	**Signature**	**Date**

The Reviewer as signed below attests that the test was performed according to the prescribed procedure and that the results above are accurate and complete.

Reviewer	**Signature**	**Date**

depends on such things as the ruggedness of the instrument, and the criticality and frequency of use. PQ testing at periodic intervals can also be used to compile an instrument performance history.

In practice, a known method, with known predetermined specifications, is used to verify that all the modules are performing together to achieve their intended purpose. In practice, OQ and PQ frequently blend together in a holistic approach, particularly for injector linearity and precision (repeatability) tests, which can be conducted more easily at the system level. For HPLC, the PQ test should use a method with a well-characterized analyte mixture, column, and mobile phase. Figure 2.3 shows an example of a "vendor" PQ test method HPLC separation that incorporates the

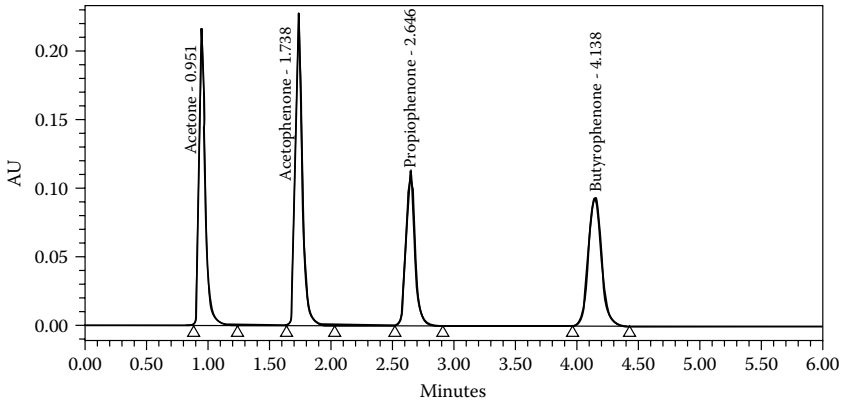


FIGURE 2.3 Example of a vendor PQ test. Separation was performed using a 4.6 by 75 mm 3.5-μ Symmetry C18 column (Waters, Milford, Massachusetts) at 30°C. Mobile phase was 40/60 water/methanol v/v at a flow rate of 1.0 mL/min. A 20-μL injection, and UV detection at 254 nm were used. Peaks are in order: (1) acetone (t_0), (2) acetophenone, (3) propiophenone, and (4) butyrophenone, 0.01 mg/mL each in water.

essence of a holistic OQ and PQ test. Actual user PQ tests should incorporate the essence of the system suitability section of the general chromatography chapter in the USP [8] in order to show suitability under conditions of actual use.

2.3.5 Preventative Maintenance and Repairs

As shown in Figure 2.1, after the instrument is placed on-line in the laboratory, repair (in the case of failure to meet PQ test specifications) or preventative maintenance followed by calibration and standardization may be required. While it is not necessary to requalify an instrument when parts are replaced for minor repairs or routine maintenance or normal repair, some sort of test to verify the repair or replacement should to be performed. However, when major repairs are undertaken, some subset of requalification becomes necessary. Table 2.6 lists typical minor and major repairs.

Major repair items are ones that have the ability to affect the accuracy, linearity, or precision of the system or instrument, for example, a pump CPU board. Because the pump CPU board controls the pump motor drivers that determine proper flow rates, a CPU board's proper functionality is best determined by performing a complete OQ followed by a system PQ. All major repairs should be documented in the maintenance log for the module or other suitable log as appropriate.

Minor repair items are items that are unlikely to affect proper operation of the device, for example, replacing pump seals or detector lamps. Because the problem can be easily diagnosed and readily verified following repair, it is not necessary to perform a complete OQ/PQ; performing system suitability analysis (see Chapter 5) before running any unknowns is usually all that is required. As with any major repair, minor repairs should also be documented in the maintenance log for the module or other suitable log as appropriate, along with any testing performed to verify the repair.

TABLE 2.6
Examples of Major and Minor Repairs

Device	Major Repairs	Minor Repairs
Pumps	CPU/control boards	Front panels
	Driver circuit boards	Power supplies
	Motors	Check valves/cartridges
	Gears	Plungers
	Castings	Seals
	Gradient proportioning valves	Pressure transducers
	Pump control modules	Fans
	Piton drives	Fuses
	Automated gradient controllers	
Injectors	CPU/control boards	Front panels
	Driver circuit boards	Power supplies
	Injector assemblies	Sample positioning drives
	Valve asseblies	Pressure transducers
	Fluid packs	Seal packs and needles
		Valve seals
		Fans
		Fuses
Detectors	Cell assemblies	Front panels
	CPU/control boards	Power supplies
	Analog and preamp control boards	Lamps
	Photodiodes and arays	Cell windows
	Optic benches	Fans
	Gratings	Fuses
	Grating drive devices	Air filters
	Mirrors	
	Beam splitters	
	Photomultiplier tubes	
	Optical slits	
	Filter wheel replacement	
Data Systems	Data acquisition and control boards	Network card
	CPU boards	Interface boards
	Hard drives	Displays
	Reformatting hard drives	Power supplies
	Loading software	Keyboards
	Reloading software	Printers
	Upgrading operating system	Removable media drives
	Installing service packs (operating and data system)	RQAM memory modules
Analog-to-Digital Devices	Analog-to-digital control boards	Fans
		Fuses

Each laboratory should have SOPs in place that define the period of use (usually defined as a reasonable interval during which the instrument operates without any loss in functional performance) and the procedures for placing the instrument online following maintenance (OQ, PQ, or system suitability), as well as for proper maintenance and calibration.

2.4 ROLES AND RESPONSIBILITIES

Although consultants, validation specialists, and quality assurance (QA) personnel will often be involved in the AIQ process, the users are the ones who ultimately "own" the process of AIQ, and are responsible for maintaining the instrument in a qualified state. QA personnel have the responsibility to review the AIQ process to determine that it meets regulatory requirements and to ensure the scientific validity of the process. Manufacturers and developers are responsible for the DQ and for relevant processes used in the manufacturing and assembly of the hardware and software associated with the instrument. Vendors usually make available a summary of these efforts, as well as a test script that can be used to qualify the instrument and software at the user's site. Manufacturers should also notify users about hardware or software defects, offer training, service, and repair.

2.5 SOFTWARE VALIDATION AND CHANGE CONTROL

Just about every piece of hardware used in the modern laboratory today is software driven or controlled. Whether it is firmware (integrated chips), software used for instrument control, data acquisition and processing (e.g., chromatography data systems or CDS), or stand-alone software, such as laboratory information management systems (LIMS), it all must be validated.

Firmware is validated by the manufacturer during DQ; and because it is generally considered part of the instrument itself, when the hardware is qualified, the integrated firmware is also qualified. The same can be said for the CDS; rather than performing a modular validation of the software by itself, the CDS is qualified by the user by qualifying the instrument according to the AIQ process in place.

Change control also follows the DQ/IQ/OQ/PQ process as manufacturers add new features and correct known defects in their instrumentation. The change control process enables the user to determine what (if any) changes should be adopted, and to assess the effects of changes to determine what (if any) requalification is required.

Software reloads or upgrades are considered major repairs, and requalification is necessary. An IQ should be performed whenever loading new software, reloading software (e.g., after a hard disk drive failure), adding service packs, or upgrading to new versions. An OQ should then be performed to verify computational capability.

2.6 AIQ DOCUMENTATION

Two types of documents result from AIQ: static and dynamic. Static documents are generated during the DQ, IQ, and OQ phases and should be kept accessible, either electronically or in a separate qualification binder. Static documents may include

such things as user manuals, site requirement documents, etc. Dynamic documents are generated during the OQ and PQ phases, when actual instrument testing takes place. These documents provide a running record of the instrument's use and maintenance, and should be kept in a system log book with the instrument, available for viewing as necessary by anyone interested (i.e., the FDA). These documents should also be appropriately archived for future reference and protection.

2.7 INSTRUMENT CATEGORIES

Analysts in the regulated laboratory apply a wide range and complexity of instrumentation in their everyday tasks, from balances to mass spectrometers, and the level of complexity dictates the level of qualification. Recognizing the differences, the USP categorized instruments into three groups: A, B, and C. More information on the three categories as they are broken down in the USP, along with some examples and approaches, are outlined in Table 2.7.

Conformance of Group A instruments (the lowest level of qualification) with user requirements is determined by visual observation; no independent qualification process is required. Examples of Group A instruments include spatulas, ovens, magnetic stirrers, microscopes, and vortex mixers.

The conformance of Group B instruments with user requirements is determined according to the instruments' SOP, and their failure is usually readily discernible. Examples of instruments that fall into this category are pH meters, balances, thermometers, refrigerator/freezers, and vacuum ovens.

Group C instruments are defined as highly method-specific, complex instruments with conformance determined by their application. Full qualification is

TABLE 2.7
USP Qualification Criteria and Approaches

Category	USP Classification Criteria	USP Qualification Approach	Examples
A	Standard equipment No measurement capability No calibration requirement	Specifications set at manufacturer Conformance with specifications verified and documented by observation during operation	Centrifuges Sonicators Magnetic stirrers
B	Standard instruments with measurable output or that control physical parameters	User-specified requirements Calibration required Conformance to specifications/ requirements specified in SOPs and IQ/OQ	pH meters Thermometers Pumps Ovens Balances Water baths
C	Complex instruments and computerized systems	Full qualification required specific performance and functions tests	HPLC GC Spectrometers (UV, AA) Dissolution baths

applied to instruments in this group. Installation of instruments in this group can be quite complicated and is often only undertaken by specialists. Examples include HPLC and gas chromatography (GC) instruments, mass spectrometers, and electron microscopes.

One word of caution regarding the instrument groupings: the exact category that an instrument falls into can only be determined by the user and its intended application.

2.8 CONCLUSION

Data quality is built on the foundation of method and software validation, AIQ, and system suitability. Each of these components plays a critical role in the process of validation. In a regulated laboratory, instruments must generate reliable data, and only a proper AIQ process can fulfill this mission. An approach as outlined in the USP and AAPS committee report that focuses on scientific principles rather than simply generating paperwork will increase laboratory efficiency and make the validation process less subjective and easier to defend.

REFERENCES

1. Swartz, M.E. and Krull I.S., *Analytical Method Development and Validation*, Marcel Dekker, New York, 1997.
2. Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs, 21 CFR Part 210, http://www.fda.gov/cder/dmpq/cgmpregs.htm.
3. Current Good Manufacturing Practice for Finished Pharmaceuticals, 21 CFR Part 211, http://www.fda.gov/cder/dmpq/cgmpregs.htm.
4. United States Food and Drug Administration, Guideline for Submitting Samples and Analytical Data for Methods Validation, February 1987. US Government Printing Office: 1990-281-794:20818, or at www.fda.gov/cder/analyticalmeth.htm.
5. Draft Guidance for Industry: Analytical Procedures and Methods Validation. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Division of Research, Rockville, MD, August 2000.
6. ICH Q2 (R1): Validation of analytical procedures: Text and methodology, *International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use,* Geneva, Switzerland, November 2005. See also www.ich.org.
7. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <1225>.
8. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <621>.
9. Hertzog, D.L. et al., Developed and validated stability-indicating HPLC method for the simultaneous determination of losartan potassium, hydrochlorthiazide, and their degradation products, *Pharm. Forum*, 31(5), L10587, Analytics Instrument Qualification Sept–Oct 2005, pp. 1453–1463.
10. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <1058>.
11. Qualification of analytical instruments for use in the pharmaceutical industry: A scientific approach, *AAPS PharmSciTech*, 2004, 5(1) Article 22 (http://www.aapspharmscitech.org).

3 HPLC Method Development and Optimization with Validation in Mind

3.1 INTRODUCTION

Method development and optimization is the foundation of any validated method; a properly developed and optimized method can help to ensure a method's success upon implementation. Though the focus of this chapter is on HPLC methods, by and large, the conceptual steps outlined here for method development and optimization will also be applicable to many analytical procedures performed in a regulated environment, including, but not limited to, gas chromatography (GC), capillary electrophoresis (CE), or mass spectrometry (MS). In this chapter, different approaches to method development and optimization in a regulatory environment are discussed, along with suggested HPLC instrument configurations and software tools.

3.2 HPLC METHOD DEVELOPMENT APPROACHES

An effective analytical method development process involves evaluating and optimizing various method parameters to satisfy the stated goals of the method or procedure. There are many literature reports of the experimental design and approach to method development [1–7], and over the years many different approaches to HPLC method development have evolved. The selection or development of any new or improved method often involves tailoring existing approaches and instrumentation to the current analytes of interest, as well as to the final objectives or requirements of the method. It often also involves robustness (Chapter 5) or "prevalidation" studies, performed to ensure that the resulting method is "validatable." Perhaps the easiest and most straightforward method development approach is to survey the existing literature to see if methods, either exact or related, already exist. In addition to the scientific literature, many instrument and software vendors offer databases of existing applications, some with methods that can be directly downloaded into a chromatography data system (CDS), for example, the D-Library (Dionex Inc., Sunnyvale, California). Another method development approach involves starting with the structures of the analytes and developing the method based on information determined from these structures, either obtained from reference material, or observed or measured. These physical-chemical

TABLE 3.1
Example of Some Commercially Available Method Development Software

SoftwareTitle	Vendor	Comments
AutoChrom	ACD Labs, Toronto, ON, Canada	Several different modes One module uses analyte properties for starting point Uses MS for peak tracking
ChromSword	Agilent, Wilmington, Delaware	Uses analyte structure Has embedded column database Automated or stand-alone
Fusion AE	Waters, Milford, Massachusetts	Quality by design experimental design Automated with the Waters system
DryLab	Molnar Institute for Applied Chromatography, Berlin, Germany	Theory-based

properties of the analytes, for example, solubility, pK_a or pK_b, spectral properties, molecular weight, and polarity, are used to choose rational starting mobile phase and column conditions from which additional fine-tuning or optimization experiments are carried out. Software is available (ACD/ChromGenius or AutoChrom, Advanced Chemistry Development, Toronto, Ontario, Canada) that can utilize structure information to predict retention times and set restrictions on separation conditions. The structure-based physical-chemical property approach is often combined with chemometric software that can also model chromatographic separations. Several software and instrument vendors offer software for chromatographic modeling, including DryLab (Molnar Institute for Applied Chromatography, Berlin, Germany) and ACD/LC and GC Simulator (Advanced Chemistry Development, Toronto, Ontario, Canada).

Software programs, either third party or CDS software itself, can often interact with HPLC instrumentation to automate the entire method development process, some even in a quality-by-design (QbD) framework (Fusion-AE Method Development Software, Waters Corporation, Milford, Massachusetts). Column or method screening approaches take advantage of this interaction to systematically screen different columns and method conditions (mobile phase composition, pH) followed by additional optimization, often in combination with other approaches. Table 3.1 lists some of the common, commercially available software available to assist in method development.

3.3 METHOD GOALS

There are several valid reasons for developing new methods of analysis:

- There may not be a suitable method for a particular analyte in the specific sample matrix.
- Existing methods may be too error-, artifact-, and/or contamination-prone, or they may be unreliable (have poor accuracy or precision).

- Existing methods may be too expensive, time consuming, or energy intensive, or they may not be easily automated.
- Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including improved analyte identification or detection limits, greater accuracy or precision, or better return on investment.
- For legal, compliance, or scientific reasons, there may be a need for an alternative or orthogonal method to confirm the analytical data originally obtained from existing methods.
- Existing method may not be stability indicating.

Before undertaking method development, it is important to have a complete understanding of the goals, objectives, and expectations of the method, and then to translate the goals of the method into a method development design and to define the required analytical performance characteristics for validation.

Goals for a new or improved analytical method might include the following:

- Qualitative identification of the specific analytes of interest providing some structural information to confirm "general behavior" (e.g., retention time, color change, pH)
- Quantitative determination (at trace levels when necessary) that is accurate, precise, and reproducible in any laboratory setting when performed according to established procedures (e.g., SOPs)
- Stability indicating
- Ease of use
- Ability to be automated
- High sample throughput
- Rapid sample turnaround time
- Low cost per analysis
- Sample preparation that minimizes cost, time, effort, materials, and volume of sample consumed
- Direct output of qualitative or quantitative data to laboratory computers in a format usable for evaluation, interpretation, printing, and transmission to other locations via a network/laboratory information system (LIMS)

Reference standards that have been well identified and characterized, and whose purity is already known should be used for initial method development and preliminary evaluation of the goals and requirements of the method, and the initial analytical performance characteristics for validation should be identified, according to the type of method/procedure (Chapter 7).

3.4 HPLC METHOD DEVELOPMENT INSTRUMENTATION

Since the 1970s, the fundamental components of a basic liquid chromatograph have consisted of the same basic parts: a pump, a means of injecting a sample, a column,

a detector, and some type of data recording device. However, during the past decade, and certainly to a greater extent within the past five years or so, the basic components have become much more sophisticated, and specialized systems have emerged for specific applications, including those for method development. There are several key components of any HPLC system, and systems used specifically for method development are really no different. HPLC systems can be modular or integrated, and use either isocratic or gradient solvent delivery. Modular systems consist of separate modules connected in such a way as to function as a single unit, and can provide a degree of flexibility to exchange different components in and out of the system, sometimes necessary for maintenance purposes or experimental requirements. However, in regulated laboratories, this flexibility may not be viewed as an advantage due to compliance issues with analytical instrument qualification.

In integrated systems, the individual components can share electrical, communication, and fluid connections and control, and can operate in ways that provide better solvent and sample management than modular systems. Modern integrated systems are holistically designed to take advantage of managing both the sample and the solvent in ways that can significantly decrease injection cycle time and provide increased precision and accuracy while still providing flexibility in detection choices. HPLC system architecture can be further classified by how the solvents in the mobile phase are blended, as illustrated in Figure 3.1. Traditional high-pressure systems use two or more pumps to blend solvents under high pressures. A separate controller is used to alter the flow rate to blend different proportions of solvent or to generate gradients, using external mixers. While the system has the disadvantage of the cost and maintenance of multiple pumps, high-pressure systems typically have lower system (gradient delay) volumes, important when using smaller diameter, sub-2-μm particle columns and fast LC techniques. High-pressure systems are usually binary systems, although optional solvent-select valves can grant access to more than one solvent at a time per pump. In low-pressure designs, a single pump draws the solvent through a multiport proportioning valve. Software algorithms control and time the opening of the ports with the pump stroke under microprocessor control to blend the solvent or generate a gradient in the pump head under low pressure. Degassing, either by helium sparge or membrane modules, is required to prevent outgassing during solvent blending. The simplicity of a single pump is certainly an advantage, but the pump head and other downstream components contribute to a typically larger system or gradient delay volume than that found in high-pressure systems. However, low-pressure systems offer more flexibility in solvent selection than high-pressure systems, and can be used to generate different mobile phase compositions on-line (as opposed to premixing), adding more flexibility during method development. In addition, in low-pressure systems, any solvent volume change that might occur during mixing is accomplished before the solvent is pressurized; therefore, flow rate changes that might result from this effect in high-pressure systems that do not precompress the solvent are not a problem with low-pressure systems.

In general, gradient systems are preferred over isocratic systems for method development because of their multisolvent capability. Gradient multisolvent systems can be used to prepare mobile phases on-the-fly, often referred to as "dial-a-mix" or "auto blend," providing maximum flexibility for method development (especially

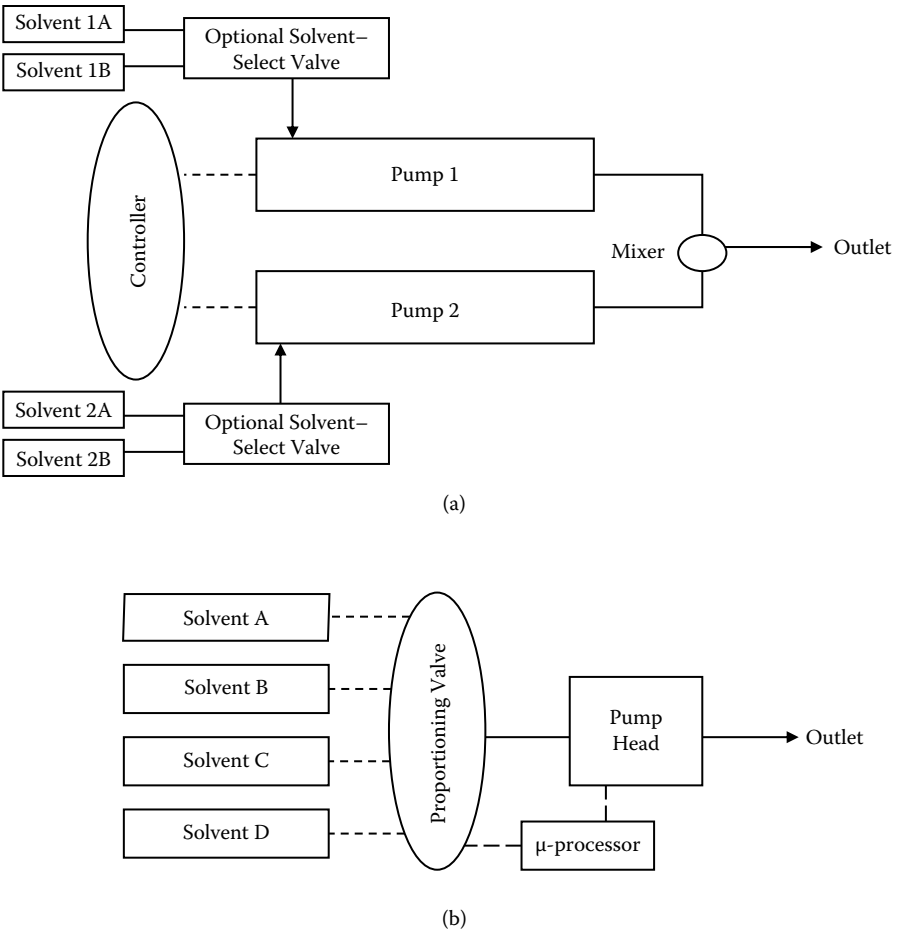


FIGURE 3.1 Diagram illustrating (a) high-pressure and (b) low-pressure mixing system architecture. The high-pressure system uses two separate pumps and a controller; the low-pressure system uses a single pump and a multiport proportioning valve.

for method and column screening approaches), and the mobile phases are often more robust and accurate than premixed mobile phases when methods are in routine use. Figure 3.2 illustrates this capability. Figure 3.2c shows three overlaid chromatographic results from one system, three different chemists, on three different days, using premixed solvents. The chemist-to-chemist reproducibility is seen to be quite variable. In Figure 3.2b, every tenth injection of 100 runs from an experiment using premixed solvents are overlaid. Figure 3.2b illustrates that even on a single system, with a single chemist, premixing solvents can affect repeatability over time. Variability in this instance most likely arises from selective evaporative loss of the organic solvent, as later runs have longer retention times. Finally, in Figure 3.2a, overlaid results are presented for every tenth injection of 100 runs using auto blend or dial-a-mix; that is, using the system to make the mobile phase. As illustrated, the

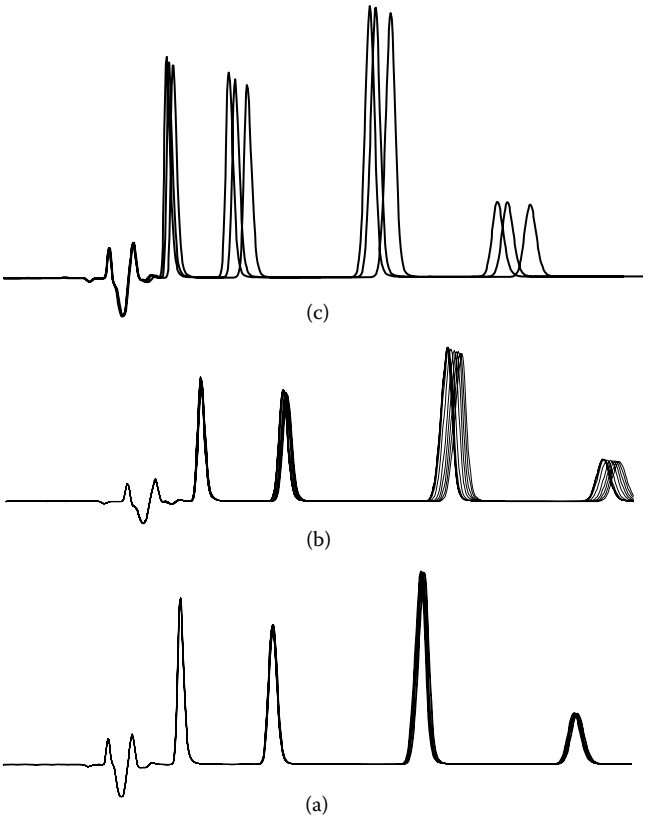


FIGURE 3.2 Comparison of premixing mobile phase solvents to auto blend. Figure 1c: three overlaid chromatographic results from one system, three different chemists, on three different days, using premixed solvents. In Figure 1b, every tenth injection of 100 runs from an experiment using premixed solvents are overlaid. In Figure 1a, overlaid results are presented for every tenth injection of 100 runs using auto blend, or dial-a-mix; that is, using the system to make the mobile phase. (Reprinted from HPLC method development for pharmaceuticals, Volume 8 of *Separation Science and Technology*, S. Ahuja, Editor, Chapter 6, Contemporary liquid chromatographic systems for method development, p. 148, 2007.)

system is far more accurate in preparing the mobile phases than either a single or multiple analysts premixing the mobile phase. Using auto blend, different organic solvent proportions, buffer strength, and pH can be generated using the solvent manager to proportionally mix the appropriate stock solutions to obtain the final mobile phase conditions. Auto blending of this type can be used to the analyst's advantage during method development.

The automated blending of solvents might at first seem a trivial matter. However, automated method development systems depend on precise and reproducible blending in scouting experiments designed to study the effects of different mobile phase conditions on selectivity. In the strictest sense, gradient chromatography is essentially auto blending, albeit over time. The kinds of results obtained in Figure 3.2c

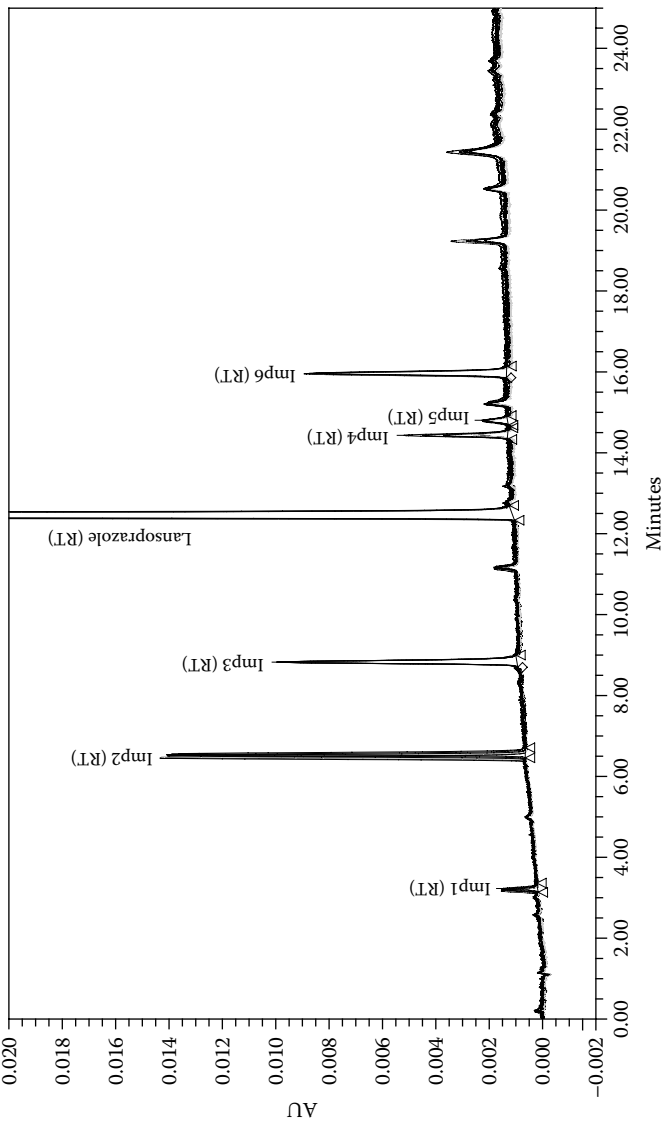


FIGURE 3.3 Six overlaid chromatograms of a method requiring critical resolution of a series of minor components requiring accurate and precise mobile phase delivery. (Reprinted from HPLC method development for pharmaceuticals, Volume 8 of *Separation Science and Technology*, S. Ahuja, Editor, Chapter 6, Contemporary liquid chromatographic systems for method development, p. 150, 2007.)

are critical to the type of separations illustrated in Figure 3.3. Figure 3.3 shows six overlaid chromatograms of a method requiring critical resolution of a series of minor components. Without accurate and precise mobile phase generation and solvent delivery, this critical resolution could not be maintained. Isocratic conditions, if desired for the final method, can be determined from gradient conditions, and of course still be run on the gradient system.

3.4.1 HPLC Systems for Column and Method Scouting

Method and column scouting is a method development approach commonly used to investigate potential starting conditions for further method optimization. A typical HPLC system used to generate the kind of results obtained in Figures 3.2 and 3.3 and run scouting experiments is shown in Figure 3.4. Most major LC manufacturers' systems can be similarly configured into a resulting method development workhorse system to generate the kind of results obtained in Figure 3.3 and run column scouting experiments where the mobile phase can be varied over a range of conditions, including organic content and pH. In addition to the basic solvent and sample manager, systems for method development are often configured with solvent and column switching valves, a column oven, and multiple detector capabilities (Section 3.4.6). For the most part, photodiode array (PDA) and single quadrupole mass spectrometry (MS) are the most useful detectors for method development. Other useful detectors include evaporative light scattering (ELSD) or corona charged aerosol (CAD). Systems configured in this manner are capable of delivering mobile phases consisting of different

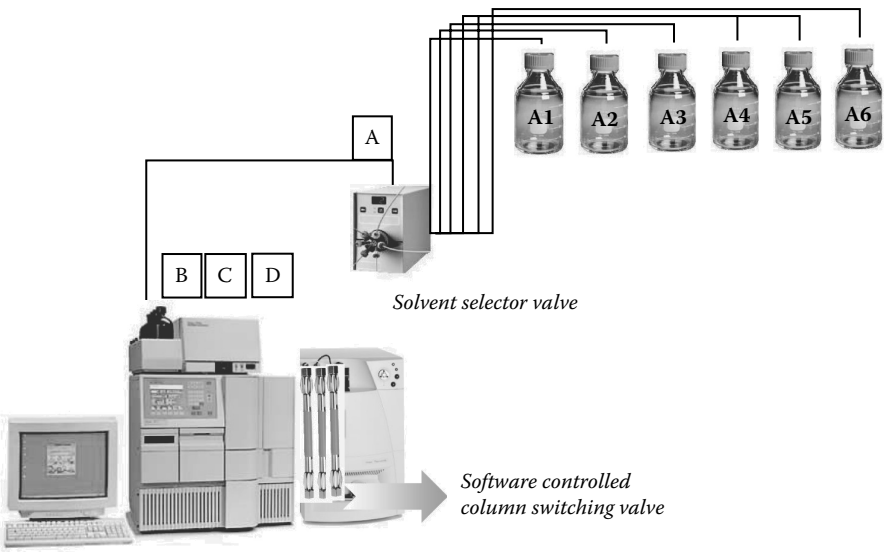


FIGURE 3.4 Example of a typical HPLC system configured for method development. (Reprinted from HPLC method development for pharmaceuticals, Volume 8 of *Separation Science and Technology*, S. Ahuja, Editor, Chapter 6, Contemporary liquid chromatographic systems for method development, p. 151, 2007.)

blends of multiple organic solvents, multiple buffers and pHs, and operating four or more columns at different temperatures. This multiparameter mobile phase and multicolumn capability provides access to many of the potential variables the analyst needs to investigate when manipulating selectivity in method development. In addition, using multiple detectors in combination, there is less dependence on individual analyte properties such as UV chromophores or ionization.

Scouting or screening systems are also often used to identify the most promising conditions (solvent, column pH, etc.) for further optimization (Section 3.5) or development. By considering the physical characteristics and analyte properties, templates (collections of instrument methods) can be written in the chromatography data system (CDS) to generate the various mobile phase conditions, equilibrate or switch columns, perform all the chromatographic runs, and run wash or shutdown procedures for both the columns and the system. These templates are usually written once and then used repeatedly as new methods need to be developed, allowing the analyst to run the system in a semi-automated manner. A typical screening experiment might generate in excess of sixteen chromatograms; for example, using four different columns, high and low pH, and two different solvents (e.g., methanol and acetonitrile) as outlined in Figure 3.5. Of course, multiple additional conditions generate a great deal more chromatographic data, and once generated, all the data must be scrutinized. However, rather than go through each chromatogram individually, the analyst can use the custom reporting features of the CDS relational database to generate summary plots that at a glance reveal which runs gave the greatest number of peaks, most resolution, etc., as illustrated in Figure 3.6.

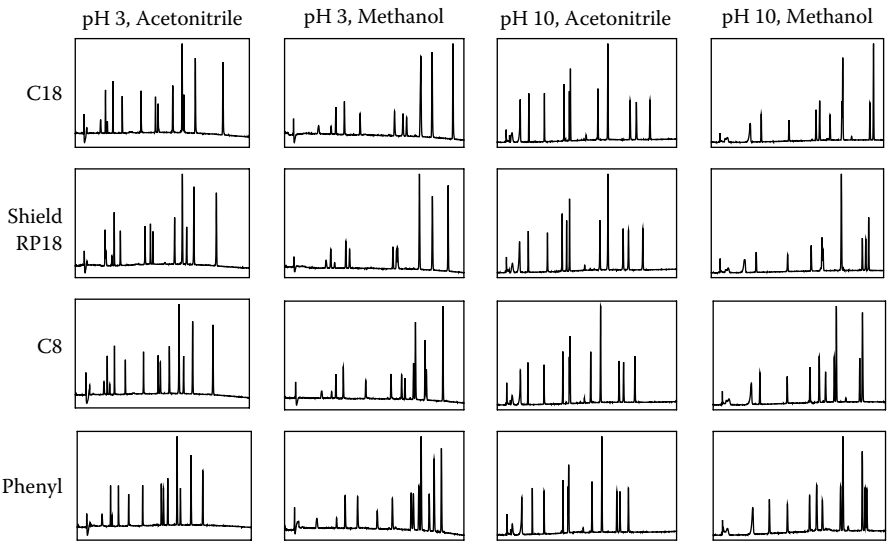


FIGURE 3.5 Example column scouting method development approach. In this example, four different columns, two different buffers (high and low pH), and two different organic solvents (acetonitrile (ACN) and methanol (MeOH)) are methodically scouted to generate a set of conditions that can be chosen for additional optimization if needed.

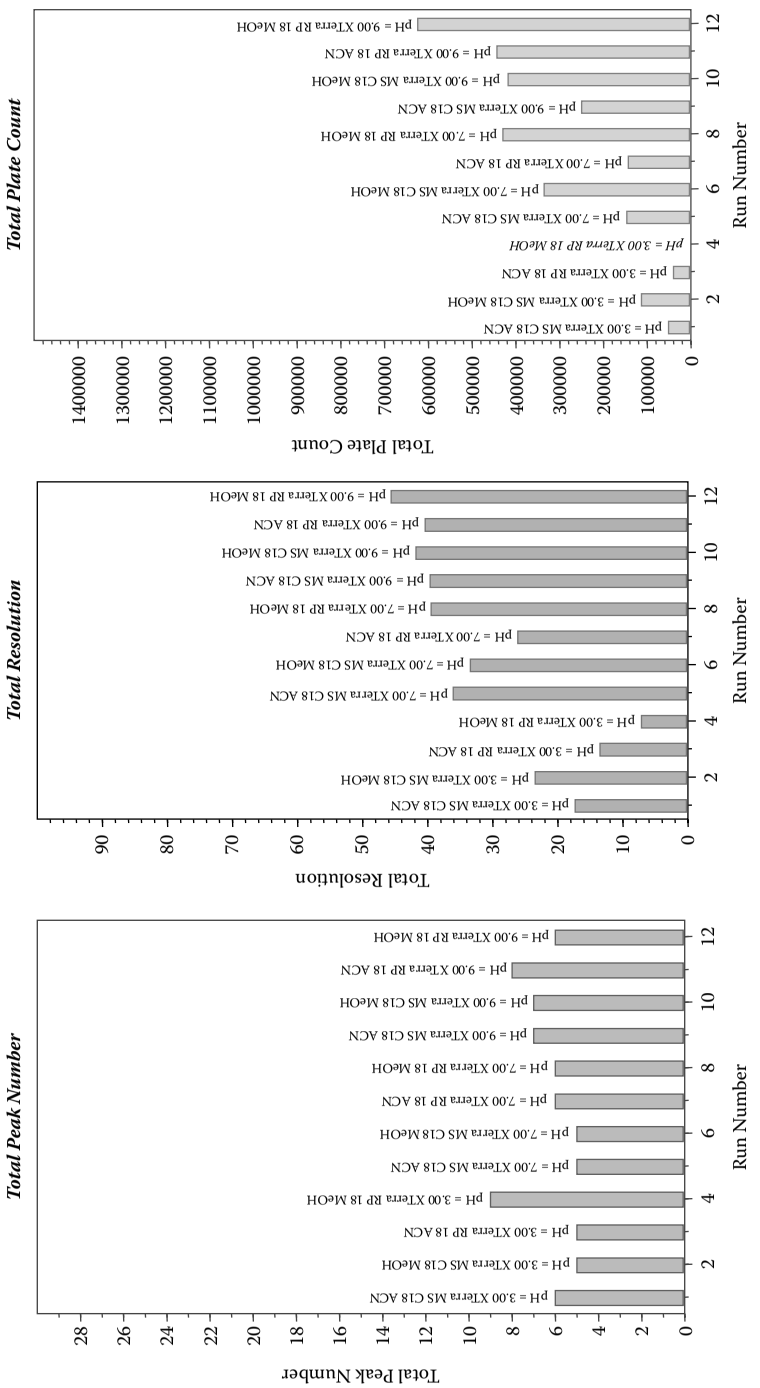


FIGURE 3.6 CDS bar chart data mining. Results from the types of experiments run in Figure 3.2 can be summarized in charts such as these using the CDS, making it much easier to pick out optimum conditions as opposed to reviewing each chromatogram individually.

3.4.2 Automated HPLC Method Development Systems

Automation of the optimization is a natural extension of a methodical, planned method development process. Why automate? The desire to automate method development stems from the simple reason that traditional manual HPLC method development is a labor-intensive, time-consuming, and often-imprecise process, resulting in lost time, money, and productivity because it can take weeks to develop a method manually. Automated method development systems provide an alternative to the traditional slow, manual, and unreliable trial-and-error method development approach and can often reduce method development time to as little as a few hours. In addition, automated systems can often evaluate a larger number of conditions, thus improving the robustness of the method.

Systems have been developed that utilize external modeling software (Table 3.1; e.g., DryLab, Molnar-Institute, Berlin, Germany; or LC Simulator or AutoChrom, Advanced Chemistry Development, Toronto, Ontario, Canada) that either partially or completely automate the HPLC method development process (8–14). These theory-based modeling software programs allow analysts to evaluate a much wider range of experimental conditions than would ever be practical by running experiments in the laboratory, significantly decreasing method development and optimization time. With this type of software, the effects of variables, either alone or in combinations—for example, organic concentration, pH, temperature, gradient slope, and buffer concentration—can be easily observed. In addition, analysts can

- Evaluate method robustness to decrease the cost of revalidating methods
- Transfer gradient methods from one instrument to another, eliminating method redevelopment time
- Model two separation variables simultaneously for faster method development
- Shorten run times to increase sample throughput
- Train new chromatographers and establish laboratory method development SOPs

Figure 3.7 shows a screenshot from DryLab software during the development of a separation of some nitroaromatics. Screens such as this in the software can be used to model separations, including different solvent compositions and column configurations. The underlying software algorithms are based on HPLC theory, and are very accurate in their predictions, as summarized in Table 3.2 for a separation of cocaine, methadone, and related substances.

The critical component in a completely automated system is software that bridges the gap between the modeling software and CDS software that runs the system and generates data. In these systems, the process of method development starts with the help of a Windows-based interface between the modeling software (e.g., AutoChrom, LCD Labs) and the CDS. The software interface asks for specific information about separation needs, and using software protocols, suggests actual starting conditions, including pH, solvent, and column. The software can also facilitate the setup of the method in the CDS and complete the analysis. Systems and software are now available from many vendors that incorporate PDA and MS for peak

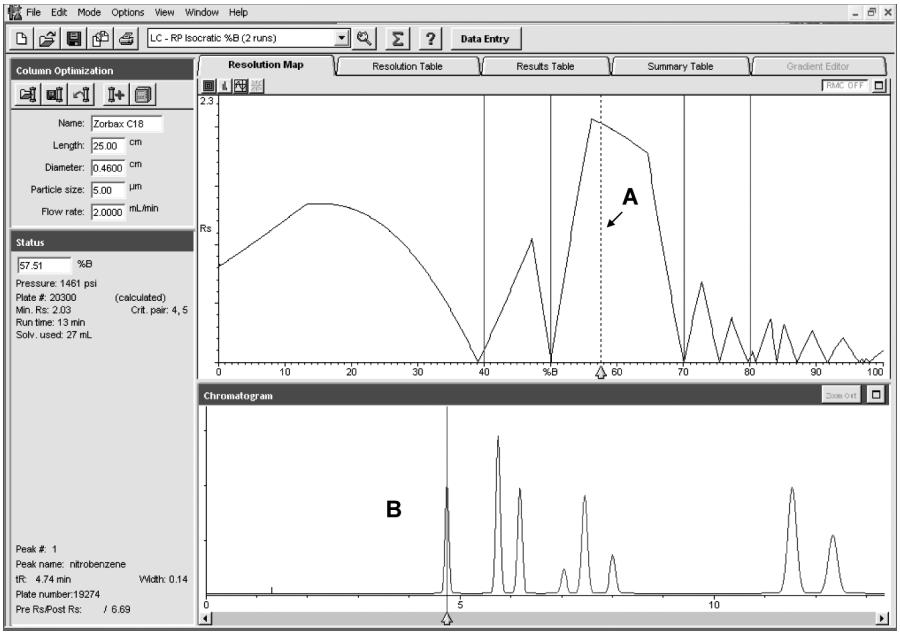


FIGURE 3.7 Example DryLab resolution map. Chromatogram at point B is a prediction of the choice of experiment dictated by the mouse placement in the software at point A. (*Source:* Figure courtesy of Molnar-Institute, Berlin, Germany.)

TABLE 3.2
DryLab Prediction Accuracy

Peak	DryLab Predicted T_r	Experimental T_r	% Error
Cocaine	3.98	3.94	0.70
Benzoylecgonine	4.31	4.23	1.32
Antipyrine	5.08	5.01	0.98
Phenacetin	9.50	9.48	0.17
Dibucaine	9.94	10.05	0.77
Methadone	10.20	10.31	0.73

Source: Data courtesy of Molnar-Institute, Berlin, Germany.

tracking during automation. A PDA or MS spectral-based peak-tracking algorithm allows more accurate identification of sample components during the method development process, identifying peaks as selectivity and therefore elution order changes over the course of a few "chemistry calibration" runs. Once the calibration runs are processed, the chromatography variables are quickly modeled, and an optimized chromatographic method prediction is obtained. Using systems of this type, with intelligent decision-making software, it is not uncommon to optimize a method in as little as four or five chromatographic runs over just a few hours [13,14].

3.4.3 UHPLC in Method Development Systems

One of the primary drivers for the growth and continued use of HPLC has been the evolution of packing materials used to affect the separation. The underlying principles of this evolution are governed by the van Deeter equation:

$$H = A(d_p) + B/u + C(d_p)^2u$$

which is a formula that describes the relationship among H, plate height (HETP or column efficiency); linear velocity, u, (flow rate); and particle size or diameter, d_p.

The "A" term represents eddy diffusion, the "B" term represents longitudinal diffusion, and the "C" term represents resistance to mass transfer in and out of the particle.

According to the van Deeter equation, as the particle size decreases to less than 2 µm, not only is there a significant gain in efficiency, but the efficiency does not diminish at increased flow rates or linear velocities [15]. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits; this has come to be known as Ultra High Pressure LC (UHPLC). UHPLC takes full advantage of chromatographic principles to run separations using columns packed with smaller particles, and/or higher flow rates for increased speed, with superior resolution and sensitivity [16–18].

An example of the use of UHPLC for rapid method development is illustrated in Figure 3.8. The method development process for this rather complex separation was accomplished in twenty-two preliminary runs, including organic composition scouting, and individual injections for peak identification. Due to the short UHPLC run times, the entire method was developed in less than an hour. Attempting to do the same via HPLC could take days to weeks longer, and in the end, HPLC may not be able to accomplish this result due to its inherent lower efficiency and resolving power.

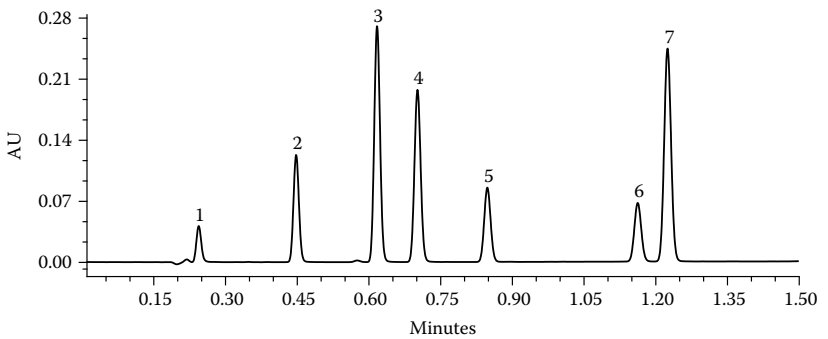


FIGURE 3.8 UHPLC separation of coumarin and related compounds illustrating fast method development. Final conditions included a 2.1 by 50 mm 1.7-µm ACQUITY UPLC BEH C_{18} (Waters Corporation, Milford, Massachusetts) column at 35°C. A 5–80% B linear gradient over 1.0 minute, at a flow rate of 1.0 mL/min was used. Mobile phase A was 0.1% formic acid, and B was acetonitrile. UV detection at 254 nm and 40 pts/s. Peaks are (1) 7-hydroxycoumarin–gluconoride, (2) 7-hydroxycoumarin, (3) 4-hydroxycoumarin, (4) coumarin, (5) 7-methoxycoumarin, (6) 7-ethoxycoumarin, (7) 4-ethoxycoumarin.

3.4.4 Solvent Management

LC pumps are sometimes categorized according to the way solvents are blended (Figure 3.1). Low-pressure designs use a single pump to deliver mobile phases generated by an upstream proportioning valve. High-pressure systems use two or more pumps to proportion solvents downstream at high pressure. As illustrated in Figure 3.9, the most significant difference between high-pressure and low-pressure systems is in the system volume. While low-pressure systems usually exhibit less compositional ripple in the chromatographic baseline, high-pressure systems usually have lower volumes. Therefore, if speed or high throughput is desired, high-pressure systems are usually preferred. However, low-pressure systems can usually accommodate a larger number of different mobile phase solvents, and software-configurable solvent-select valves are also frequently used on method development systems to expand capability. Regardless of the type of system used, it is important to remember that a proper determination of the volume is important for any system used in method development. Problems related to method transfer can often be traced to differences in system, dwell, or gradient delay volumes, as no two systems will have exactly the same volume. The volume difference is particularly significant when transferring methods between low-pressure and high-pressure systems. In addition, problems may also result from how the volumes are calculated [19]. Accurate volume determinations for high-pressure systems can be made using a step gradient method because the mobile phase is generated post-pump. For accurate low-pressure system volume determinations, a linear gradient must be used, to take into account the pre-pump volume from the solvent proportioning valve. It is for this reason that many method developers now recommend programming in a short isocratic step at the

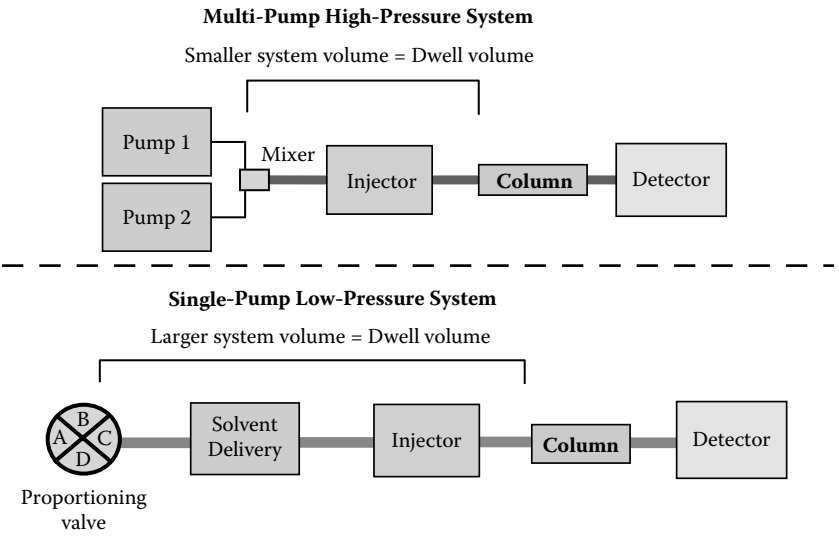


FIGURE 3.9 Schematic diagrams of a low-pressure mixing system using a single pump and a four-position solvent proportioning valve (bottom), and a high-pressure mixing system using multiple pumps (top).

beginning of every gradient to accommodate transfer to systems with differing volumes [20]. When UHPLC systems were first introduced in 2004, only high-pressure mixing systems were available. Recently (2010), low-pressure mixing systems were introduced that combine all the attributes of working with small particles at high pressures with quaternary solvent mixing for method development [21].

3.4.5 Sample Management

Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure; and to protect the column from experiencing extreme pressure fluctuations, the injection process must be relatively pulse-free. The swept volume of the sample manager also must be minimized to reduce potential band spreading. For UHPLC, a fast injection cycle time is needed to fully capitalize on the speed of the analysis, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to realize the increased sensitivity benefits. Temperature control and compatibility with a wide range of sample formats (e.g., vials, microtiter plates) are also desirable features in any sample management device used for method development.

3.4.6 Detection

Detection plays an important role in method development systems, and the most desirable configurations include a variety of complementary detectors to respond to the widest range of analyte attributes. Depending on analyte properties, the most commonly employed detectors in method development systems include UV (PDA), evaporative light scattering (ELSD), corona charged aerosol (CAD), and mass spectrometry (MS-either single or triple quadrupole). Multiple detectors in a system can be configured in series or parallel; often, the choice of which configuration to use depends on whether or not the detector is destructive. Destructive detectors (e.g., ELSD, MS, CAD) must be placed last in the flow path and require splitting of the flow stream.

Photodiode array (PDA) detection is commonly used during method development to determine peak identity and purity/homogeneity. PDAs extend the utility of UV detection by providing spectra of eluting peaks that can be used to aid in peak identification, and to monitor for co-elutions (peak homogeneity or purity), helpful during method development. They can also serve as a multiwavelength UV/VIS detector. The spectra collected at the chromatographic peak apex can be used to create a library that can in turn be used to compare subsequent spectra for identification purposes, and spectra collected across the peak at each data point can be compared to evaluate peak homogeneity or purity. The added spectral resolution of modern PDA detectors, coupled with chromatography data system (CDS) software algorithms, can quickly compare fine differences in the spectra not clearly visible to the eye. Some comparisons are done by a simple direct point-to-point comparison of spectra, while in others, complex vector analysis in multidimensional space is performed to look at spectral fine structure. In order for PDA spectral comparisons to work, the compounds must have some UV absorbance, and there must be some degree of spectral and

chromatographic resolution. Spectra will also be changed if the organic concentration or pH is altered, for example, during method development. The changes in spectra resulting from mobile phase differences often result in a shifting of the spectra, affecting the quality or the "fit" of the match, but not necessarily the information obtained.

Recent improvements in the ability to efficiently nebulize an HPLC column effluent has led to the increased utility and popularity of the evaporative light scattering detector (ELSD). The ELSD works on the principle of evaporation (nebulization) of the mobile phase, followed by measurement of the light scattered by the resulting particles. The column effluent is nebulized in a stream of nitrogen or air carrier gas in a heated drift tube, and any nonvolatile particles are left suspended in the gas stream. Light scattered by the particles is detected by a photocell mounted at an angle to the incident light beam. Carrier gas flow rate and drift tube temperature must be adjusted for whatever mobile phase is used. Detector response is related to the absolute quantity of analyte present; and while decreased sensitivity will be obtained for volatile analytes, unlike the UV detector, no chromophores are required and it has orders of magnitude more response than the refractive index (RI) detector, another common detector in situations where analytes do not have strong chromophores. The ELSD also has the advantage over RI detection in that the response is independent of the solvent, so it can be used with gradients, and is not sensitive to temperature or flow rate fluctuations. Mobile phases, of course, must be volatile, similar to those used for MS detection, as listed in Table 3.3 [6]. Linearity can be limited in some applications, but is certainly quantitative over a wide enough range if properly calibrated. Recent applications of the ELSD have also been extended to UHPLC.

Corona charged aerosol detection (CAD), sometimes referred to as corona discharge detection (CDD), is a unique technology gaining in popularity in which the

TABLE 3.3
Properties of Common Organic Solvents Used in Liquid Chromatography

Solvent	UV Cutoff (nm)[a]	Viscosity (cP)	Boiling Point (°C)
Acetonitrile	190	**0.38**	82
1-Butanol	215	2.98	118
Dimethylformamide	268	0.92	153
Dimethylsulfoxide	268	2.24	189
Heptane	200	0.40	98
Hexane	195	0.31	69
Methanol	205	0.55	65
n-Propanol	210	2.30	97
Tetrahydrofuran	212	0.55	66
Water	190	1.00	100

[a] Wavelength at which solvent absorbs 1.0 AU in a 10-mm cell. (*Source:* Adapted from Snyder, L. R. et al., *Introduction to Modern Liquid Chromatography*, 3rd edition, John Wiley & Sons, Hoboken, NJ, 2010, p. 882.)

HPLC column eluent is first nebulized with a nitrogen (or air) carrier gas to form droplets that are then dried to remove mobile phase, producing analyte particles [22,23]. The primary stream of analyte particles is met by a secondary stream of nitrogen (or air) that is positively charged as a result of having passed a high-voltage platinum corona wire. The charge transfers diffusionally to the opposing stream of analyte particles, and is further transferred to a collector, where it is measured by a highly sensitive electrometer, generating a signal in direct proportion to the quantity of analyte present.

Because the entire process involves particles and direct measurement of charge, CAD is highly sensitive, provides a consistent response, and has a broad dynamic range, which offers advantages when analyzing compounds lacking UV chromophores, as illustrated in Figure 3.10. Often compared to other universal-type HPLC detectors, such as RI and ELSD, CAD has been shown to be much easier to use, and similar to ELSD but unlike RI, can accommodate gradients. In addition, CAD response is not dependent on the chemical characteristics of the compounds of interest, but on the initial mass concentration of analyte in the droplets formed upon nebulization, providing a much more uniform response as opposed to, for example, UV, where responses can vary dramatically according to the wavelength used and the extinction coefficient.

Mass spectrometry is a powerful analytical technique that can be used to confirm, quantify, identify, or characterize compounds of interest. Mass spectrometers measure the mass-to-charge (m/z) ratio of ions in the gas phase, allowing the determination of a compound's molecular weight (to varying degrees of accuracy). By breaking apart molecules into fragments, MS can also be used to analyze smaller portions

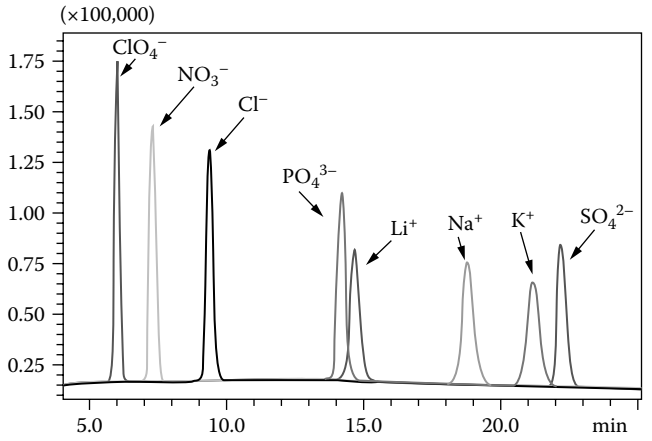


FIGURE 3.10 Simultaneous analysis of anions and cations using HILIC/CAD. Conditions: A Sequant ZIC®-*p*HILIC 5 mm, 4.6 × 150 mm column (The Nest Group, Southborough, Massachusetts) operated at 30°C was used. Gradient conditions: 20 to 70% B over 26 min; mobile phase A: 15% 100 mM ammonium acetate pH 4.68, 5% methanol, 20% IPA, 60% acetonitrile; mobile phase B: 50% 30 mM ammonium acetate pH 4.68, 5% methanol, 20% IPA, 25% acetonitrile, at a flow rate of 0.5 mL/min and a 10-µL injection.

of a molecule. Information from this fragmentation assists in the elucidation of the compound's chemical structure and properties.

Modern mass spectrometers are simple, easy-to-use instruments with a much smaller footprint than their predecessors and can be configured with a chromatographic method development system to provide a wealth of useful information. The basic components of an MS system are shown in Figure 3.11. Because a comprehensive treatment of MS is outside the scope of this chapter, the reader is urged to consult the many excellent detailed reviews of the technology that are available [24,25]. But single quadrupole mass spectrometers are becoming increasingly common in the method development laboratory and is covered here in some detail.

Quadrupole MS uses radio frequency (rf) and direct current (dc) voltages for the separation of ions, and are probably the most widespread mass spectrometers because of their relatively low price and ease of operation. In a quadrupole mass spectrometer, the rf and dc potentials are applied to four rods arranged in a square array, as illustrated in Figure 3.12. Ions are scanned or filtered by varying the DC/RF voltages across the quadrupole rods. Generally speaking, quadrupole analyzers are used to determine the nominal mass of a compound. Nominal mass is often used to confirm the identity of known compounds in method development.

In method development, MS is used in much the same way as the PDA: to identify and track peaks as selectivity changes, and to monitor for co-elution. But unlike PDA, MS provides a positive identity, and can provide deconvoluted total ion chromatograms specific for a molecular weight when co-elution of partial resolution does occur.

Of course, no detector response is universal. MS response is dependent on the ability to ionize a compound, and not all compounds can be ionized under all conditions. In similar respects, not all compounds have UV chromophores, so PDA detection is, of course, limited. However, it is very rare to have both no ionization and the lack of a UV chromophore; therefore, it is increasingly common to use MS and PDA in tandem during method development.

When it comes to MS detection, the low UHPLC system and dwell volume increases peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting), and the added resolution promotes increased source ionization efficiencies, making UHPLC the ideal technology for an MS inlet in a method development system. Higher UHPLC sensitivity also improves the quality of the spectra obtained.

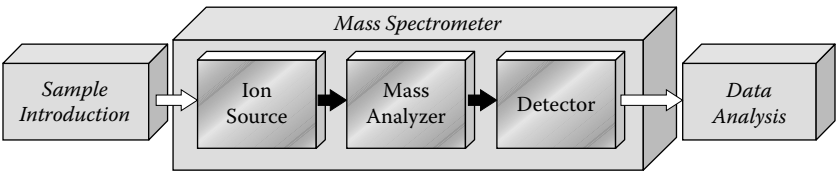


FIGURE 3.11 The basic components of an MS system. (Reprinted from HPLC method development for pharmaceuticals, Volume 8 of *Separation Science and Technology*, S. Ahuja, Editor, Chapter 6, Contemporary liquid chromatographic systems for method development, p. 167, 2007.)

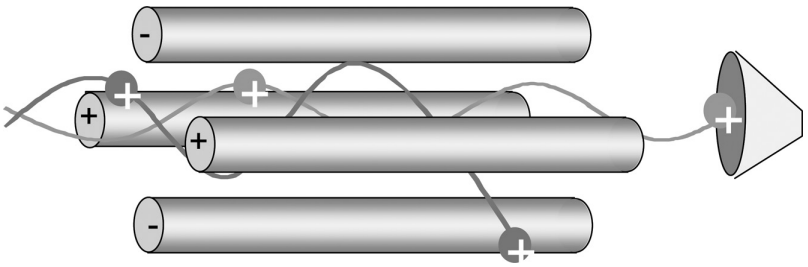


FIGURE 3.12 Quadrupole MS schematic. (Reprinted from HPLC method development for pharmaceuticals, Volume 8 of *Separation Science and Technology*, S. Ahuja, Editor, Chapter 6, Contemporary liquid chromatographic systems for method development, p. 167, 2007.)

However, similar to ELSD and CAD, using a mass detector places constraints on mobile phase selection. Proper selection of the mobile phase and any additives is critical to detection viability (Table 3.3). First and foremost, the mobile phase must be suitable for the ionization, and must be selected depending on the ionization mode (electrospray (ESI) or atmospheric pressure chemical ionization (APCI), positive or negative mode) and the analyte (e.g., p*K*a). The molecular weight of the mobile phase components should also be considered. It is not always possible to analyze compounds whose molecular weight is lower than the one of the mobile phase or any additives. For routine operation, it is easier to use volatile buffers. Acids such as HCl, H_2SO_4, or methane sulfonic acid might damage the instrument and should not be used; volatile organic acids (e.g., TFA, formic, acetic) should be used instead.

Some ions (e.g., Na, NH_4, acetate) from the mobile phase can form adducts. In the case of phosphate, multiple adducts are observed, which can produce complicated mass spectra. The formation of an adduct is usually not a reason for avoiding a mobile phase as adducts can sometimes be used to advantage. Ion pairing reagents can impact the spray formation, the droplet evaporation, and compete in terms of ion formation, and are generally avoided. Buffer concentration is generally kept as low as possible (millimolar range). If the buffer concentration is too high, ion suppression occurs, thus affecting sensitivity.

Common eluents for LC/MS include methanol/water; acetonitrile/water (methanol usually gives a better sensitivity than acetonitrile); pH modifiers (formic, acetic acids, TFA, NH_4, TEA, DEA); and buffers (carbonates, ammonium formate, ammonium acetate, ammonium carbonates, and ammonium phosphate (all nonvolatile)).

The column, while of course providing the separation without using a high concentration of buffers, or ion pairing reagents, must be stable so that the column will not "bleed" or shed interfering compounds. Special low- or no-bleed MS versions of columns are available from most suppliers.

3.4.7 Column Module

Running a column at room temperature, or even "controlled" room temperature, is a thing of the past. Modern requirements for accuracy and precision require that columns be thermostated. Because temperature can also be used as a selectivity tool,

modern method development systems typically require a column heater module. It is also an advantage for the column module to accommodate several columns of various geometries that can be randomly accessed from a software-controlled solvent switching valve. For UHPLC, the column module should also have the capability of adequately preheating the mobile phase without adding too much dispersion to prevent band broadening within the column.

3.4.8 Columns for Method Development

The myriad of column stationary phases available may at first make choosing a column for method development seem like a daunting task. There are, however, a few basic guidelines to keep in mind, and some tools available, which make the task much simpler.

During method development, the primary goal is to manipulate selectivity for the analytes of interest. In order to do that, it is important to choose columns that are orthogonal. For column screening, common columns might include a selection of C_{18}, phenyl, an embedded polar group stationary phase, and perhaps a column selected for highly polar compounds or a C_8. Many column vendors provide column selectivity charts, which can provide valuable information about columns that are similar, or different, in selectivity. The USP also provides information of this type; as shown in Figure 3.13, the USP maintains a searchable database of column evaluations on their website that can also be used to find both equivalent or orthogonal (different) columns (for validation and development, respectively) [26].

PQRI Database

Symmetry C18 (Waters)

You have the option to see the columns that are the most similar to the column of your interest, or the columns that are the most different (for applications in orthogonal methods), by selecting View Different or View Similar.

You are viewing different columns.

View Similar

The database will automatically display the first 10 columns that, theoretically, could be equivalent or very different to/from your column, depending on the option you selected. The column with rank 0 is your column. The smaller the F value more similar are the columns, at least theoretically. The higher the F value more different are the columns.

Rank	F	Column	H	S	A	B	C(2.8)	C(7.0)	Type	USP Designation	Manufacturer
0	0	Symmetry C18	1.052	0.063	0.018	–0.021	–0.302	0.123	B	L1	Waters
1	246.6	EC Nucleosil 100-5 Protect 1	0.544	0.048	–0.411	0.309	–3.213	–0.573	EP		Macherey Nagel
2	230.89	Zorbax Bonus RP	0.654	0.107	–1.046	0.373	–2.971	–1.103	EP		Agilent
3	214.32	Hypersil Prism C18 RP	0.645	0.089	–0.459	0.301	–2.817	–0.716	EP	L1	Thermo Scientific
4	207.39	ZirChrom-PBD	1.284	0.158	–0.384	–0.072	2.188	2.188	Other		ZirChrom
5	200.88	ZirChrom-EZ	1.04	0.117	–0.999	–0.001	2.089	2.089	Other		ZirChrom
6	195.98	Apex II C18 (ODS??)	1.008	–0.074	0.235	0.123	2.039	2.69	A	L1	Grace-Jones
7	193.68	Inertsil CN-3	0.369	0.049	–0.808	0.083	–2.607	–1.297	CN	L10	GL Science
8	185.77	Resolve C18	0.968	–0.127	0.335	–0.046	1.921	2.144	A	L1	Waters
9	173.99	ZirChrom-PS	0.589	–0.232	–0.477	0.062	1.75	1.75	Other		ZirChrom
10	160.06	Supelcosil LC-18	1.018	–0.047	0.181	0.162	1.595	1.752	A	L1	Supelco

FIGURE 3.13 USP Column Equivalency Database. Searchable database of column information that can be used to find orthogonal/different (for method development) or similar (for method validation) columns. (See http://usp.org/USPNF/columnsDB.)

Column lot or batch reproducibility should also be evaluated late in method development or prevalidation; generally, method development is performed on one column lot, and then verified both on another new column from the same lot and a new column from a different lot. These column lot evaluations are often performed either as a part of intermediate precision or robustness (Chapter 4). Column lot reproducibility is less of an issue now compared to earlier, as many column manufacturers now manufacture their columns from scratch instead of buying the base silica, which itself can differ from lot to lot in trace impurities, which can affect chromatography.

One final note on the column front: it is also important that method development be performed using only new HPLC columns as columns can have a memory effect from previous conditions/methods that have been used, resulting in reproducibility issues when the column is eventually replaced.

3.4.9 Mobile Phase Considerations

A rule of thumb for mobile phases used in method validation: the simpler the mobile phase, the more robust it will be. Some of the common organic solvents and their properties used in method development are listed in Table 3.4. Acetonitrile is generally preferred over methanol for method development because it has a lower UV cutoff, resulting in better PDA spectral interpretations. Methanol may be preferred for some MS applications; however, the actual selectivity obtained is more of a driving force for solvent choice. Use of high-temperature (reduced viscosity) and high-pressure technology such as UHPLC (or both) has opened up the range of possible

TABLE 3.4
Properties of Common Mobile Phase Buffers and Additives

Buffer or Additive	pK_a*	Buffer Range	MS Compatibility
Acetic acid (glacial)	4.8		
Ammonium acetate p*K*a 1	4.76	3.8–5.8	Yes
Ammonium acetate p*K*a 2	9.2	8.2–10.2	Yes
Ammonium bicarbonate	9.2, 10.3	8.2–11.3	Yes
Ammonium formate p*K*a 1	9.2	2.8–4.8	Yes
Ammonium formate p*K*a 2	9.2	8.2–10.2	Yes
Ammonium hydroxide	9.2		Yes
Ammonium phosphate, dibasic	7.2, 9.2	6.2–10.2	No
Formic acid	3.8		Yes
Phosphoric acid	2.1		No
Potassium phosphate, monobasic	2.1	1.1–3.1	No
Potassium phosphate, dibasic	7.2	6.2–8.2	No
Potassium phosphate, tribasic	12.7	11.7–13.7	No
Sodium citrate, tribasic	3.1, 4.8, 6.4	2.1–7.4	No
Triethylamine	11.0		Yes
Triethylammonium acetate (TEEA) p*K*a 1	4.76	3.8–5.8	Yes
Triethylammonium acetate (TEEA) p*K*a 1	11.0	10–12	Yes
Trifluoroacetic acid	0.3		Yes

solvents used, to include solvents such as isopropanol that are too viscous to use in conventional LC analyses. Many different buffer types and additives are used in HPLC mobile phases (Table 3.3); however, given the propensity to develop methods compatible with MS, or to use other evaporative-type detectors (ELSD, CAD), volatile components are most often used.

3.5 METHOD OPTIMIZATION

During method optimization, the initial set of conditions that has evolved from the first stages of development can be improved or maximized in terms of selectivity, resolution, peak shape, efficiency, and run or inject to inject cycle time. When optimizing any method, an attempt should be made to provide analytical figures of merit or specifications that are required to meet the assay requirements defined at the initial stages of method development. Results obtained during method development can then be measured against the desired specifications to determine how optimization should proceed. A target must be established; without adequate and definitive requirements or specifications, a method cannot be truly optimized. Evaluating the method against the predetermined specifications at this early stage should reveal the direction additional optimization experiments need to take to meet the method specifications.

If the initial analytical data derived from method development appears promising, it is time to evaluate its performance quantitatively. Initially, most work on method development and optimization is performed with analytical standards. In general, the analytical figures of merit generated to evaluate the method are also derived using standards. The scope of the method evaluation should be broad enough to include generation of information that is immediately usable for confirmation or identification of the analyte in any sample, for example, UV or mass spectra. Method optimization goals include increased sensitivity, peak symmetry and resolution, and a lack of analyte co-elutions.

As with method development, optimization of the method can follow either of two general approaches—manual or computer software driven—and the types of systems and software discussed in Section 3.1 for method development can also be used for method optimization. The manual approach commonly involves varying one experimental variable at a time, while holding all others constant, and recording changes in response. This univariate approach to system optimization is slow, time consuming, potentially expensive, and may miss the effects between variables (e.g., the effects of heat on pH). In the second approach, optimization using computer-driven software, higher efficiency/throughput can be obtained while experimental input is minimized. Automated software approaches can be applied to many applications. In addition, they are capable of significantly reducing the time, energy, and cost of virtually all instrumental method development, and can be useful to verify that the optimized method satisfies the stated goals of the method.

Certain general criteria are often considered a part of a "prevalidation" study:

- Chromatographic resolution is adequate.
- Limits of detection or quantitation that provide an adequate signal-to-noise response.

- Calibration plots are linear over several orders of magnitude, beginning with the quantitation limit.
- Suitable accuracy is obtained (perhaps performed in conjunction with linearity).
- Method- or procedure-appropriate precision is obtained (again, perhaps performed in conjunction with linearity).
- Demonstration of peak homogeneity (e.g., no co-elutions, or a demonstration that the method is stability indicating).

System optimization is one of the most time- and energy-consuming parts of the overall method development procedure. It requires an iterative procedure, constant replication, and the acquisition of a large amount of quantitative data. Too often, optimization results in a method that meets the immediate requirements of the analyst but ignores possible future needs. Ideally, the analyst should optimize each new method to the fullest practical extent in the time available, in order to ensure a broad utility of the method and obviate the repetition of experiments for future method development.

3.6 SUMMARY

Methods can be developed from scratch through scouting approaches, or adapted from existing methods found in the literature or other sources. But one thing is certain: method development is a complex, time-consuming process. Any effort to streamline, automate, and methodically and logically approach the process can pay great dividends in terms of throughput, efficiency, and reducing time to market, as well as producing a method that is easily validated.

REFERENCES

1. *Development and Validation of Analytical Methods*, C. M. Riley and T. W. Rosanske, Editors, Pergamon Press, Elsevier Science, Amsterdam, Holland, 1996.
2. *Pharmaceutical and Biomedical Applications of Liquid Chromatography*, C. M. Riley, W. J. Lough, and I. W. Wainer, Editors, Pergamon Press, Elsevier Science, Amsterdam, Holland, 1994.
3. Snyder, L. R., Glajch, J. L., and Kirkland, J. J., *Practical HPLC Method Development*, John Wiley & Sons, New York, 1988.
4. Snyder, L. R., Glajch, J. L., and Kirkland, J. J., *Practical HPLC Method Development*, 2nd edition, Wiley-Interscience, New York, 1997.
5. Neue, U., *HPLC Columns, Theory, Technology, and Practice*, Wiley-VCH Publishers, New York, 1997.
6. Snyder, L. R., Kirkland, J. J., and Dolan, J. W., *Introduction to Modern Liquid Chromatography*, 3rd edition, John Wiley & Sons, Hoboken, NJ, 2010.
7. Swartz, M. E., Chapter 6: Contemporary liquid chromatographic systems for method development, pp. 145–186, in *HPLC Method Development for Pharmaceuticals*, Ahuja, S., Editor, Elsevier, Burlington, MA, 2007.
8. Dolan, J. W., Snyder, L. R., Djordjevic, N. M., Hill, D. W., Saunders, D. L. Van Heukelemn, L., and Waeghe, T. J. Simultaneous variation of temperature and gradient steepness for reversed-phase high-performance liquid chromatography method development. I. Application to 14 different samples using computer simulation. *J. Chromatogr., A*, 803, 1, 1998.

9. Dolan, J. W., Snyder, L. R., Saunders, D. L., and Van Heukelem, L., Simultaneous variation of temperature and gradient steepness for reversed-phase high-performance liquid chromatography method development. II. The use of further changes in conditions. *J. Chromatogr., A*, 803, 33, 1998.
10. Dolan, J. W., Snyder, L. R., Djordjevic, N. M., Hill, D. W., and Waeghe, T. J., Reversed-phase liquid chromatographic separation of complex samples by optimizing temperature and gradient time. I. Peak capacity limitations. *J. Chromatogr*., 857, 1, 1999.
11. Dolan, J. W., Snyder, L. R., Wolcott, R. G., Haber, P., Baczek, T., Kaliszan, R., and Sander L. C., Reversed-phase liquid chromatographic separation of complex samples by optimizing temperature and gradient time. III. Improving the accuracy of computer simulation. *J. Chromatogr., A*, 857, 41, 1999.
12. Dolan, J. W., Snyder, L. R., Djordjevic, N. M., Hill, D. W., and Waeghe, T. J. Reversed-phase liquid chromatographic separation of complex samples by optimizing temperature and gradient time. II. Two-run assay procedures. *J. Chromatogr., A*, 857, 21, 1999.
13. Molnar. I. Computerized design of separation strategies by reversed-phase liquid chromatography: Development of DryLab software. *J. Chromatogr., A*, 965, 175, 2002.
14. Swartz, M. E., Jones, M., Fowler, P., and Andrews, M., Automated HPLC Method Development and Transfer. *LCGC*, 20(6), 43, June 2002.
15. Jerkovitch, A. D., Mellors, J. S., and Jorgenson, J. W. The Use of Micron-Sized Particles in Ultrahigh-Pressure Liquid Chromatography. *LCGC North America*, 21(7), 2003.
16. The Quest for Ultra Performance in Liquid Chromatography—Origins of UPLC Technology, Waters Corporation. See http://www.waters.com/waters/partDetail.htm?cid=511539&id=38628&ev=10120661&locale=en_US.
17. Beginners Guide to UPLC—Ultra-Performance Liquid Chromatography. Waters Corporation. See http://www.waters.com/waters/partDetail.htm?cid=511539&id=38629&ev=10120684&locale=en_US.
18. Swartz, M.E. and Murphy, B., UPLC: An introduction and review. *J. Liq. Chrom. Related Tech*., 28, 1253, 2005.
19. Swartz, M. E. and Plumb, R., Waters Corporation, Milford, MA, unpublished data.
20. Lukaly, P., Pfizer, Ann Arbor MI, personal communication.
21. Swartz, M. E., HPLC Systems and Components Introduced at Pittcon 2010: A Brief Review. *LCGC*, 28(5), 376, May 2010.
22. Corona Charged Aerosol Detector, brochure 70-6454 Rev A, ESA/Dionex, Chelmsford, MA, 2004.
23. Swartz, M., Emanuele, M., Awad, A., and Hartley, D., Supplement to LC/GC North America, April 2009. See http://chromatographyonline.findanalytichem.com/lcgc/article/articleDetail.jsp?id=592237&sk=&date=&pageID=2.
24. Niessen, W. M. A., *Liquid Chromatography-Mass Spectrometry*, Chromatographic Science Series Vol. 97, 3rd ed., Taylor & Francis, London, 2006.
25. McMaster, M. C., *LC-MS: A Practical Users Guide*, Wiley, Hoboken, NJ, 2005.
26. See http://www.usp.org/USPNF/columnsDB.html.

4 Method Validation Basics

4.1 INTRODUCTION

Method validation is one part of the overall validation process that encompasses at least four distinct steps: (1) software validation, (2) hardware (instrumentation) validation/qualification, (3) method validation, and (4) system suitability, and each step is critical to the overall success of the process. Method validation establishes through laboratory testing that the performance characteristics of the method meet the requirements of the intended analytical application. It provides an assurance of reliability of laboratory studies during normal use, and is sometimes referred to as the process of providing documented evidence that the method does what it is intended to do. In addition to being good science, regulated laboratories must carry out method validation in order to be in compliance with governmental or other regulatory agencies. In addition to providing proof that acceptable scientific practices are used, method validation is therefore a critical part of the overall validation process. A well-defined and documented method validation process not only satisfies regulatory compliance requirements, but also provides evidence that the system and method are suitable for their intended use, and aids in method transfer [1–4].

Many regulated industries have well-defined processes in place for method validation, for example, environmental monitoring laboratories under the oversight of the US Environmental Protection Agency (EPA) [5] and organizations that rely on the International Organization for Standardization (ISO) [6]. This chapter's primary focus is on the validation of analytical procedures employed by the pharmaceutical industry; in addition to the general process of method validation, terms and definitions, protocol, and reporting are discussed. Because HPLC is the predominant technology used in the regulated pharmaceutical laboratory, where possible the examples here will highlight its use, and the information presented here can be applied to other analytical procedures and techniques. Practitioners should also bear in mind, however, that any analytical technique used in a regulated laboratory must be validated for its intended use using the guidelines cited here. In nonregulated industries (e.g., academic laboratories), there is also a need for high-quality methods that provide reliable data. In these instances, method validation is also strongly recommended even when it is not required by regulation, and many peer-reviewed journals these days also require data supporting method validation.

4.2 METHOD VALIDATION GUIDELINES

In the late 1980s, the US Food and Drug Association (FDA) first designated the specifications listed in the current edition of the *United States Pharmacopeia* (USP) as those legally recognized to determine compliance with the Federal Food,

Drug, and Cosmetic Act [1,2], and every USP since has included guidelines on method validation. More recently, new information has been published, updating the previous guidelines and providing more detail and harmonization with International Conference on Harmonization (ICH) guidelines [3,4]. The inclusion and definition of some terms differ between the FDA, USP, and ICH, but as a process, harmonization on a global basis has provided much more detail than was available in the past, and it helps to minimize the differences between global regulatory requirements.

Validation is regulated by the FDA and has roots in manufacturing practice guidelines for the laboratory environment. Two of the most common references to these practices are cGMP [current Good Manufacturing Practice, e.g., (7, 8)] and the International Organization on Standardization (ISO) 9000 Global Management Standards [9] and related ISO documents. The two most important guidelines for any method validation process are USP Chapter 1225: Validation of Compendial Methods [2], and the International Conference on Harmonization (ICH) Guideline: Validation of Analytical Procedures: Text and Methodology Q2 (R1) [6]. Although the main focus of this chapter is on HPLC, both the USP and ICH guidelines are generic; that is, they apply to any analytical procedure, technique, or technology used in a regulated laboratory.

Even though the USP is the sole legal document in the eyes of the FDA, this chapter draws from USP and ICH guidelines for definitions and methodology; and for the most part, the FDA, USP, and ICH guidelines agree. Where the guidelines disagree, it is up to the user to decide upon an appropriate interpretation of the guidelines and a justification. Often, the interpretation and justification are the responsibility of the user's quality assurance unit and may be aided by review of the latest regulatory actions (e.g., FDA-issued Form 483 Inspectional Observations, which are available on the FDA's website). In addition to these guidelines, sometimes a regulatory body publishes other information that can be useful for interpretation of the guidelines. One of these is a "reviewer guidance" [10] published by the FDA. Reviewer guidance is intended to help FDA auditors determine what constitutes a good method, so many users try to adhere to the suggestions in this document to help ensure that their methods will pass regulatory scrutiny.

4.3 TERMS AND DEFINITIONS

Method validation sometimes seems to have a vocabulary of its own, with many terms in common, everyday use in the laboratory, so this chapter begins with a discussion of these terms and definitions. Several parameters, generally referred to as analytical performance characteristics, may be investigated during any method validation protocol. These parameters are listed in Figure 4.1.

Although most of these terms are familiar and are used daily in any regulated HPLC laboratory, they sometimes mean different things to different people. For example, ruggedness, which forms a part of any well-designed precision study, is often confused with robustness, and for this reason the term *ruggedness* is falling out of use. Hopefully, the following definitions taken directly from the guidelines will clear up any confusion.

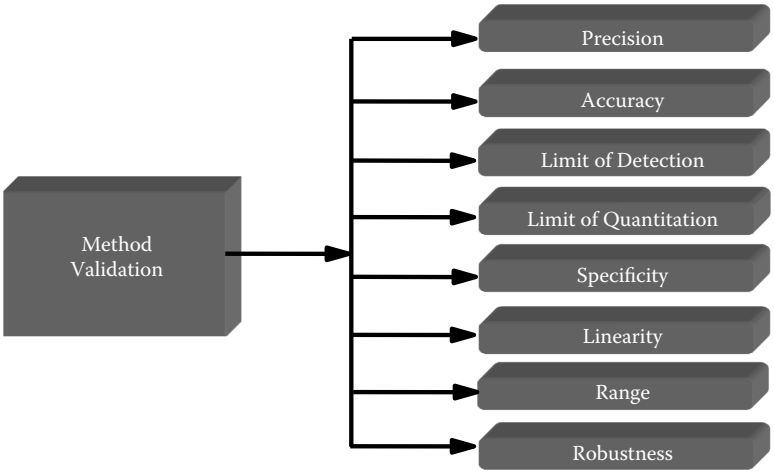


FIGURE 4.1 AMV analytical performance characteristics.

4.3.1 Accuracy

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between an accepted reference value and the value found in a sample. Established across the range of the method, accuracy is measured as the percent of analyte recovered by the assay. For the drug substance, accuracy measurements are obtained by comparison of the results to the analysis of a standard reference material, or by comparison to a second, well-characterized method. For the assay of the drug product, accuracy is evaluated by the analysis of synthetic mixtures spiked with known quantities of components. For the quantification of impurities, accuracy is determined by the analysis of samples (drug substance or drug product) spiked with known amounts of impurities (if impurities are not available, see specificity, Section 4.3.3).

Table 4.1 illustrates the results for a typical accuracy study. To document accuracy, the guidelines recommend that data be collected from a minimum of nine determinations over a minimum of three concentration levels covering the specified range (i.e., three concentrations [e.g., 50%, 100%, and 150% of target value], three replicates each). The data should be reported as the percent recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals (±1 SD). In Table 4.1, data is shown relative to 100%, and the mean recovery for $n = 9$ samples is 98.69% with %RSD = 0.28%. In this example, both the accuracy and precision pass the predefined acceptance criteria of 98% to 102% and ≤2%, respectively.

In most cases, accuracy requires a priori knowledge of the level of analyte present in simulated drug products, or in synthetic mixtures spiked with known amounts or quantities of the components of interest (analytes). When standards are available, it is sufficient to demonstrate that results are unaffected by using spiked samples. However, in situations where the actual level is not known, or if standards of the analyte of interest are not available, then accuracy can only be

TABLE 4.1
Determination of Method Accuracy/Recovery and Precision

	Accuracy/Recovery		
Sample Concentration	**Replicate 1**	**Replicate 2**	**Replicate 3**
1	98.93%	98.89%	98.54%
2	99.05%	98.55%	98.50%
3	98.88%	98.72%	98.14%
Mean	98.69%		
Standard deviation	0.28%		
Relative standard deviation	0.28%		
Acceptance criteria	Accuracy (mean)	Precision (RSD)	
	98%–102%	≤2.0%	
Assessment	Pass	Pass	

determined by comparison to a second validated method, if one exists. In spiking experiments and the preparation of QC samples, reference standards or standard reference materials (SRMs) must be used. A reference standard is a highly purified compound that is well characterized. Because chromatographic methods rely heavily on reference standards to provide accurate data, the quality and purity of the reference standard should be well documented. There are two categories of reference standards: USP reference standards that do not need characterization, and noncompendial standards. Noncompendial standards must be of the highest purity that can be obtained by reasonable effort and should be thoroughly characterized to ensure their identity, strength, quality, and purity. Reference standards are not always available for every analyte of interest. In the absence of other information, it may be necessary to calculate the amount of an impurity based on a comparison of its response to that of the drug substance; the ratio of responses of equal amounts of the impurity and the drug substance (response factor) should be used if known. In other words, it is perfectly acceptable to quantitate an impurity against the drug substance if a suitable impurity reference standard is not available. However, it is highly recommended that authentic standards be either synthesized or isolated for all analytes of interest.

There are, in some instances, SRMs of a specific analyte in a known sample matrix, such as mercury in tuna fish or insulin in a biofluid. These types of SRMs are often commercially available from various sources. Such SRMs come with a Certificate of Analysis (CoA), guaranteeing that the reference material contains a guaranteed amount or level of analyte in a known sample matrix. However, it is not unusual to find that the exact SRMs to meet all expected needs are not always available. Sometimes, SRMs must be prepared in-house or through an outside vendor or contractor, and often require more characterization than the drug substance or product itself. Modern chromatography data systems are capable of documenting, tracking, or trending accuracy using control charts of the type illustrated in Figure 4.2. Control charts of this type can be used to easily observe data that falls out of trend, above or below set predetermined control limits.

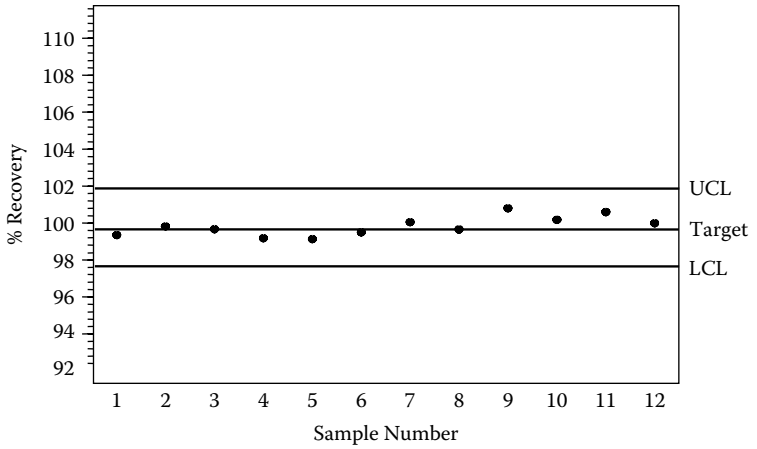


FIGURE 4.2 Example control chart used to track and trend accuracy results. UCL refers to the upper control limit, LCL the lower control limit.

4.3.2 Precision

The precision of an analytical method is defined as the closeness of agreement among individual test results from repeated analyses of a homogeneous sample. Precision is commonly performed on three different levels: repeatability, intermediate precision, and reproducibility.

4.3.2.1 Repeatability

Repeatability refers to the ability of the method to generate the same results over a short time interval under identical conditions (intra-assay precision). It should be determined from a minimum of nine determinations that cover the specified range of the procedure (i.e., three concentrations, three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration. Representative chromatographic repeatability results are summarized in Table 4.2, where results are summarized for six replicate injections of the same sample. The 0.10% RSD easily passes the ≤2% acceptance criterion.

4.3.2.2 Intermediate Precision

Intermediate precision refers to the agreement between the results from within-laboratory variations due to random events that might normally occur during the use of a method, such as different days, analysts, or equipment. Think in terms of parameters that might change, that are normally not written into a method, or are external to the method. To determine intermediate precision, an experimental design should be employed so that the effects (if any) of the individual variables can be monitored. Typical intermediate precision results are shown in Table 4.3. In this study, analysts from two different laboratories prepared and analyzed six sample preparations from one batch of samples and two preparations each from two additional batches (all samples are assumed to be the same concentration); all data from each analyst were pooled for the summary in Table 4.3. Each analyst prepared his or her own standards

TABLE 4.2
Determination of Repeatability by Replicate Injections

Injection	Response
1	626225
2	625890
3	625110
4	625447
5	625666
6	624398
Mean	625456
Standard deviation	642.34
RSD	0.10%
Acceptance criteria (RSD)	≤2%
Assessment	Pass

TABLE 4.3
Measurement of Intermediate Precision

	Amount	
	Analyst One	Analyst Two
Mean	25.9 mg	26.0 mg
Std. Dev.	0.07 mg	0.05 mg
%RSD	0.27	0.19
%Difference (means)	0.39	
Acceptance criteria (RSD)	≤2%	
Assessment	Pass	

and solutions, used a column from a different lot, and used a different HPLC system to evaluate the sample solutions. Each analyst successfully attained the precision requirements of ≤2% RSD, and the %Difference in the mean values between the two analysts was 0.39%, which indicates that there is no difference in the mean values obtained (Student's *t*-test, $P = 0.01$).

4.3.2.3 Reproducibility

Reproducibility refers to the results of collaborative studies among different laboratories. Documentation in support of reproducibility studies should include the standard deviation, relative standard deviation (or coefficient of variation), and the confidence interval. Table 4.4 lists some results typical of a reproducibility study. To generate the data shown here, analysts from two different laboratories (different from the analysts involved in the intermediate precision) prepared and analyzed six sample preparations from one product batch and two preparations each from two additional batches (all samples were assumed to be the same concentration). Each analyst prepared his or her own standards and solutions, used a column from a different lot, and used a

TABLE 4.4
Measurement of Reproducibility

	Amount	
	Lab One	**Lab Two**
Mean	26.0 mg	25.6 mg
Std. Dev.	0.12 mg	0.24 mg
%RSD	0.46	0.94
%Difference (means)	1.53	
Acceptance criteria (RSD)	≤2%	
Assessment	Pass	

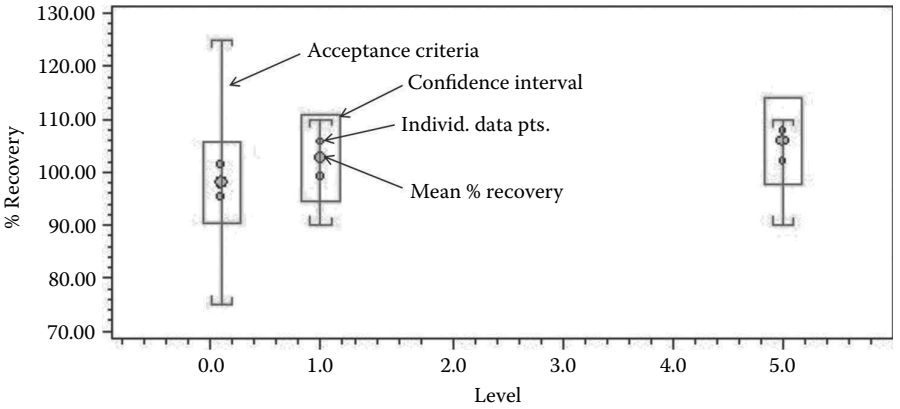


FIGURE 4.3 An example of a chromatography data system Whisker plot, a common way of documenting precision and accuracy. The box represents the upper and lower confidence intervals, the whiskers with up-tics and down-tics represent the user-defined upper and lower acceptance criteria. The small points are the individual data points of percent recovery (at each concentration level); the large points are the mean percent recovery at each concentration level.

different HPLC system to evaluate the sample solutions. Each analyst successfully attained the precision requirements of ≤2% RSD, and the percent difference in the mean values between the two analysts was 1.53%, indicating that there is no difference in the mean values obtained (Student's *t*-test, $P = 0.01$). Figure 4.3 gives additional examples of measuring and documenting precision at various levels.

4.3.2.4 Ruggedness

Ruggedness was defined in past USP guidelines as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analysts, instruments, reagent lots, elapsed assay times, assay temperature, and days. It is a measure of the reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst. The use of the term *ruggedness*, however, is

falling out of favor, and is not used by the ICH, but is instead addressed in guideline Q2 (R1) [4] under the discussion of intermediate precision (Section 4.3.2.2, within-laboratory variations: different days, analysts, equipment, etc.) and reproducibility (Section 4.3.2.3, between-laboratory variations from collaborative studies).

4.3.3 Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample. It takes into account the degree of interference from other active ingredients, excipients, impurities, degradation products, etc. Specificity in a method ensures that a peak's response is due to a single component (no peak overlaps). Specificity for a given analyte is commonly measured and documented by resolution, plate number (efficiency), and tailing factor.

For identification purposes, specificity is demonstrated by either the ability to discriminate between other compounds in the sample or by comparison to known reference materials. For assay and impurity tests, specificity can be shown by the resolution of the two most closely eluted compounds. These compounds usually are the major component or active ingredient and a closely eluted impurity. If impurities *are* available, it must be demonstrated that the assay is unaffected by the presence of spiked materials (impurities or excipients). If the impurities *are not* available, the test results must be compared to a second, well-characterized procedure. For assay, the two results are compared directly; and for impurity tests, the impurity profiles are compared. Comparison of test results will vary with the particular method, but may include visual comparison as well as retention times, peak areas (or heights), peak shape, etc.

Starting with the publication of *USP 24*, and as a direct result of the ICH process, it is now recommended that a peak-purity test based on photodiode array (PDA) detection or mass spectrometry (MS) be used to demonstrate specificity in chromatographic analyses. Modern PDA technology is a powerful tool to evaluate specificity [11]. PDA detectors can collect spectra across a range of wavelengths at each data point collected across a peak, and through software processes, each spectrum can be compared to determine peak purity. Used in this manner, PDA detectors today can distinguish minute spectral and chromatographic differences not readily observed by simple overlay comparisons, even at low levels as shown in Figure 4.4. More information on using PDA detectors to evaluate specificity using spectral contrast techniques can be found in Chapter 7, Section 7.3.1.4.

PDA detectors can be limited in the evaluation of peak purity on occasion by a lack of UV response, as well as by the noise of the system and the relative concentrations of interfering substances. Also, the more similar the spectra are, and the lower the relative absorbances, the more difficult it is to distinguish co-eluted compounds. MS detection overcomes many of these limitations of the PDA, and in many laboratories it has become the detection method of choice for method validation. MS can provide unequivocal peak purity information, exact mass, and structural and quantitative information. The combination of both PDA and MS in a single HPLC instrument can provide valuable orthogonal information to help ensure that interferences are not overlooked during method validation.

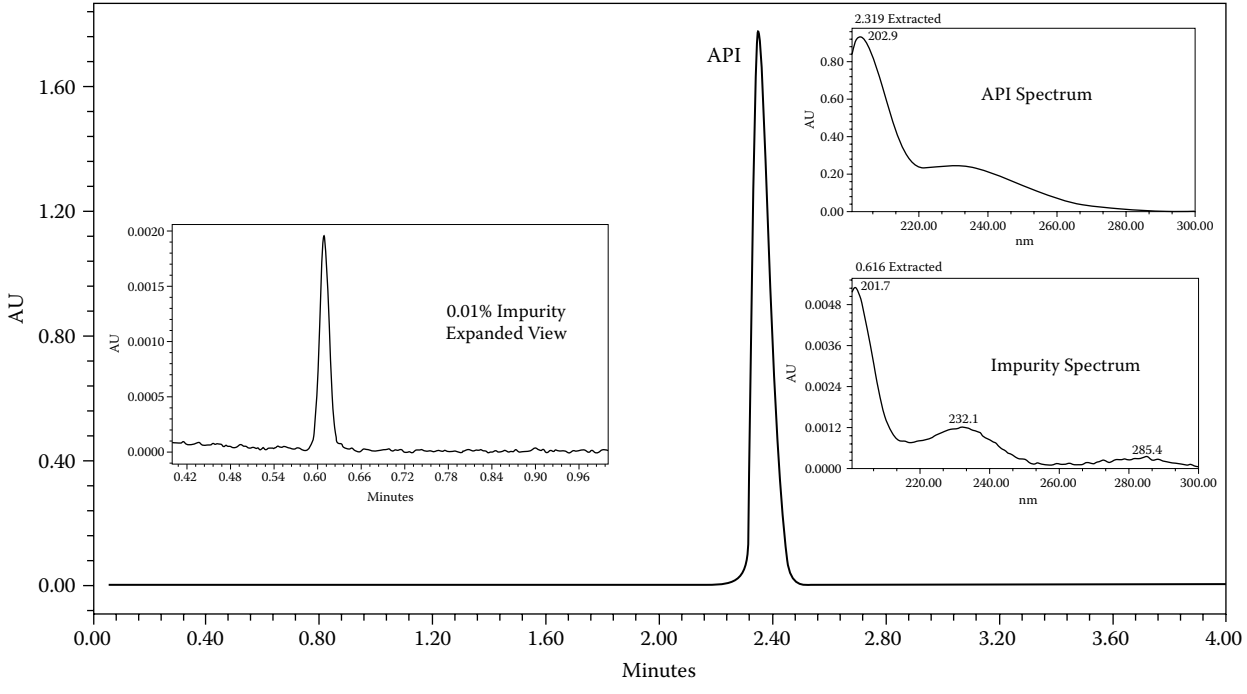


FIGURE 4.4 Chromatography data system residual plot from the data plotted in Figure 4.6. Each residual is an estimate of the error in the data and displays how far the data points fall from the regression line. Each residual is the difference between the observed (or actual) response and the response of the regression line.

4.3.4 Detection Limit

The detection limit (DL—also sometimes referred to as the limit of detection or LOD) is defined as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. Usually expressed as the concentration of the analyte (e.g., percentage, parts per billion) in the sample, the DL can be determined by approaches based on visual examination, signal-to-noise (S/N), or on a calculation based upon the standard deviation of the response and the slope of a calibration curve.

Visual examination can be used in both instrumental and noninstrumental approaches, for example, the presence or absence of a peak in a chromatogram, or a color change in a titration. Visual examinations can be highly subjective, however, and are not in common use.

The S/N approach can be used with analytical procedures that exhibit baseline noise. Determination of the S/N ratio is performed by comparing measured signals from samples of known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. Typically, the signal is measured from baseline to peak apex and divided by the peak-to-peak noise determined from a blank injection. It is important that the noise be measured in the blank chromatogram during the same elution window as the peak of interest. An S/N ratio between 3:1 and 2:1 is generally considered acceptable for estimating the detection limit.

Calculations based on the standard deviation of the response and the slope of a calibration curve is based on the following formula:

$$DL = 3.3*\sigma/S$$

where σ is the standard deviation of the response and S is the slope of the calibration curve. The slope may be estimated from the calibration curve of the analyte, or a separate curve approaching the DL may be prepared. The value of σ may be determined based on the standard deviation of blank injections, the residual standard deviation of response, or the standard deviation of y-intercepts of the regression lines of the calibration curve. Table 4.5 provides a simple example of determining the DL using this formula where the response was determined at five levels (minimum number of levels for linear curve [Section 4.3.6]).

Determination of σ for the standard deviation of blank injections is performed by analyzing an appropriate number of blank samples for the magnitude of analytical background response and calculating the standard deviation of these responses. When using the calibration curve calculation, the standard error of the y-intercept (based on regression analysis with zero not included) is recommended as it is a better indicator of the DL at low concentrations than averages derived at higher concentrations from the residual standard deviation. Although the S/N method is somewhat less subjective than visual determinations, calculations based on a calibration curve are the least subjective and have the least operator bias. Regardless of the method used, multiple samples should be injected at the limit for verification, and the actual method used should be documented.

TABLE 4.5
Calculation of DL and QL

	Conc. (ng/mL)	Response
	1.5	5250
	3	7950
	7.5	16650
	15	31800
	30	58950
R Square	0.9995	
Standard error of intercept	380.71	
Slope	1892.87	

Note: From table values: DL = 3.3(380.7)/1,892.87 = 0.66 ng/mL. *From table values:* QL = 10(380.71)/1,892.87 = 2.01 ng/mL.

4.3.5 Quantitation Limit

The quantitation limit (QL, also sometimes called the limit of quantitation) is defined as the lowest concentration of an analyte in a sample that can be quantitated with acceptable precision and accuracy under the stated operational conditions of the method.

QL can be determined by some of the same procedures used to determine the DL, either by S/N or on a calculation based on the standard deviation of the response and the slope of a calibration curve. For QL, the S/N ratio of 10:1 is used as a rule of thumb because actual QL determinations must take into account the method objectives of accuracy, precision, and the desired quantitative level. Typically, the signal is measured from baseline to peak apex and divided by the peak-to-peak noise determined from a blank injection. It is important that the noise is measured in the blank chromatogram during the same elution window as the peak of interest.

Calculations based on the standard deviation of the response and the slope of the calibration curve are based on the following formula:

$$QL = 10*\sigma/S$$

where σ is the standard deviation of the response and S is the slope of the calibration curve. The slope may be estimated from the calibration curve of the analyte, or a separate curve approaching the QL may be prepared. The value of σ may be determined based on the standard deviation of blank injections, the residual standard deviation of response, or the standard deviation of y-intercepts of the regression lines of the calibration curve. Table 4.5 provides a simple example of determining the QL using this formula where the response was determined at five levels (minimum number of levels for linear curve [Section 4.3.6]).

Determination of σ for the standard deviation of blank injections is performed by analyzing an appropriate number of blank samples for the magnitude of analytical background response and calculating the standard deviation of these responses. As with DL, when using the calibration curve calculation, the standard error of the

y-intercept (based on regression analysis with zero not included) is recommended as it is a better indicator of the QL at low concentrations than averages derived at higher concentrations from the residual standard deviation.

Determining the QL is a two-step process. Regardless of the method used to determine the QL, the limit should be first documented and supported, and then an appropriate number of samples are analyzed at the QL to fully validate the method performance at the QL. That is, a candidate QL is estimated, such as by signal-to-noise ratio or the slope of a calibration curve, and once this value is found, it needs to be confirmed by demonstration of the appropriate response for samples formulated at the LOQ (limit of quantitation).

When determining limits chromatographically, the efficiency and lifetime of the column can play a significant role. Figure 4.5 shows two separations performed on two different columns under equivalent conditions. The columns used are both designed for the application; however, one (Figure 4.5a) is more efficient (either from being packed better, or from having fewer secondary interactions leading to peak broadening) than the other. Lower efficiency leads to broader peaks and lowers the signal-to-noise ratio. A loss of efficiency can also occur as a column ages. Depending on the mobile-phase conditions, it is common over time for the base silica to slowly dissolve, or it is possible for some of the surface ligands to be stripped off, either

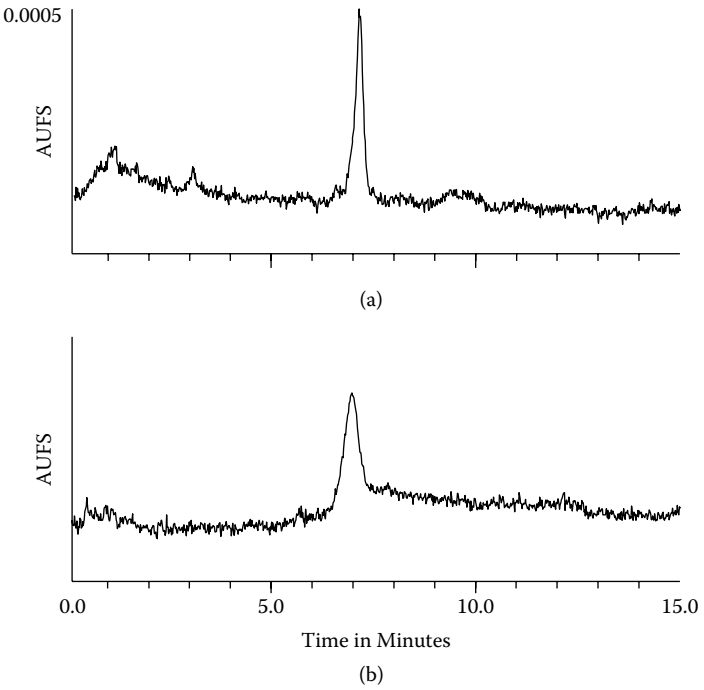


FIGURE 4.5 PDA detector response of the chromatographic analysis of an API and impurity at the 0.01% level. Insets show an expanded view of the chromatogram in the region where the impurity elutes, and the PDA-derived UV spectra for both the API and the impurity.

of which can lead to a decreased number of plates or a loss in efficiency. It is a good idea, prior to setting specifications, or during column use, to be aware of these effects and to take them into account so that chromatographic issues are not misinterpreted as product or sample issues.

4.3.6 Linearity and Range

Linearity is the ability of the method to provide test results that are directly proportional to analyte concentration within a given range. Linearity generally is reported as the variance of the slope of the regression line (e.g., standard error from an Excel regression analysis). Range is the interval between the upper and lower concentrations of analyte (inclusive) that have been demonstrated to be determined with acceptable precision, accuracy, and linearity using the method as written. The range is normally expressed in the same units as the test results obtained by the method (e.g., ng/mL). Guidelines specify that a minimum of five concentration levels be used to determine the range and linearity, along with certain minimum specified ranges depending upon the type of method. Table 4.6 summarizes typical minimum ranges specified by the guidelines. Data to be reported generally includes the equation for the calibration curve line, the coefficient of determination (r^2), and the curve itself, and the residuals as illustrated in Figures 4.6 and 4.7, respectively.

TABLE 4.6
Example Minimum Recommended Ranges

Type of Method	Recommended Minimum Range
Assay	80%–120% of the target concentration
Impurities	From the reporting level of each impurity, to 120% of the specification
Content uniformity	70%–130% of the test or target concentration
Dissolution	±20% over the specified range of the dissolution test

Note: For toxic or more potent impurities, the range should be commensurate with the controlled level.

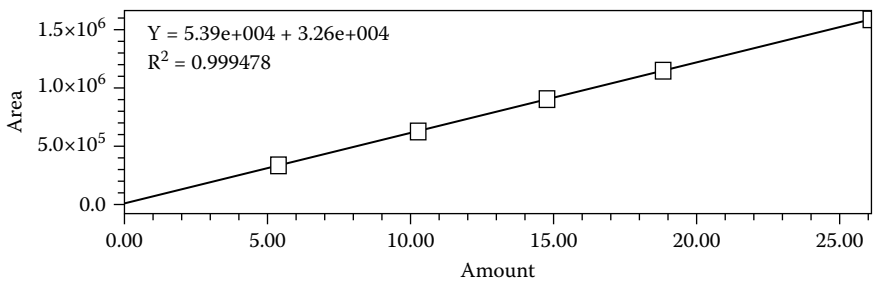


FIGURE 4.6 Effect of column efficiency on DL and QL determinations. The higher-efficiency column used to generate the chromatogram in Figure results in a higher S/N than the lower-efficiency column used to generate the chromatogram in Figure.

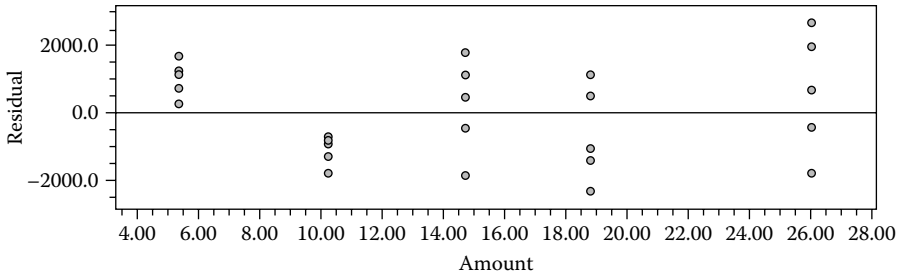


FIGURE 4.7 Chromatography data system linearity plot, showing *y*-intercept, slope, and coefficient of determination.

4.3.7 Robustness

The robustness of an analytical procedure is defined as a measure of its capacity to obtain comparable and acceptable results when perturbed by small but deliberate variations in procedural parameters listed in the documentation. Robustness provides an indication of the method's suitability and reliability during normal use. During a robustness study, method parameters are intentionally varied to see if the method results are affected. The key word in the definition is *deliberate*. Variations should be chosen symmetrically around a nominal value, or about the value specified in the method, to form an interval that slightly exceeds the variations that can be expected when the method is implemented or transferred. For example, if the buffer pH is adjusted by titration and the use of a pH meter, the typical laboratory has an error of ≈±0.1 pH units. To test the robustness of a method to variations in a specified pH 2.5 buffer, additional buffer might be prepared and tested at pH 2.4 and pH 2.6 to ensure that acceptable analytical results are obtained. For instrument settings, manufacturers' specifications can be used to determine variability. The range evaluated during the robustness study should not be selected to be so wide that the robustness test will purposely fail, but rather to represent the type of variability routinely encountered in the laboratory. Challenging the method to the point of failure is not necessary. One practical advantage of robustness tests is that once robustness is demonstrated over a given range of a parameter, the value of that parameter can be adjusted within that range to meet system suitability without a requirement to revalidate the method.

Robustness should be tested late in the development of a method, and if not, is typically one of the first parameters investigated during method validation. However, throughout the method development process, attention should be paid to the identification of which chromatographic parameters are most sensitive to small changes, so that when robustness tests are undertaken, the appropriate variables can be tested. Robustness studies also are used to establish system suitability parameters to make sure the validity of the entire system (including both the instrument and the method) is maintained throughout method implementation and use. In addition, if the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

To measure and document robustness, the following HPLC parameters should be routinely monitored:

- Critical peak pair resolution R_s
- Column plate number N (or peak width in gradient elution)
- Retention time t_R
- Tailing factor TF
- Peak area (and/or height) and concentration

Replicate injections will improve the estimates (e.g., %RSD) of the effect of a parameter change. In many cases, multiple peaks are monitored, particularly when some combination of acidic, neutral, or basic compounds are present in the sample. Often, the results of robustness studies are included in the method development report; such examples, plus corrective instructions, will help to translate robustness studies into practical method implementation. Robustness is discussed in Chapter 5 in greater detail.

4.4 VALIDATION ACCORDING TO METHOD TYPE

Guidelines recognize that it is not always necessary to evaluate every analytical performance parameter for every type of method. The type of method and its intended use dictate which performance characteristics need to be investigated, as summarized in Table 4.7 [2,4]. Both the USP and ICH divide analytical methods into four separate categories that generally apply to drug substances and drug products:

- Category 1: Assays for the quantification of major components or active ingredients
- Category 2: Determination of impurities or degradation products
- Category 3: Determination of performance characteristics
- Category 4: Identification tests

Specific examples of the different types of methods in each category are discussed in more detail in Chapter 7.

4.5 DOCUMENTATION

Validation documentation includes the protocol used to carry out the validation, the analytical test method, and the validation report. These documents should be written as controlled documents as part of a quality system that ensures compliance with appropriate regulations.

4.5.1 Validation Protocol

The validation protocol specifies the requirements (validation procedures and acceptance criteria) to be satisfied. Where possible, the protocol should reference standard operating procedures (SOPs) for specific work instructions and analytical methods.

The protocol must be prepared and approved before the official validation process begins. In addition, the validation protocol typically contains the following:

- Protocol title
- Purpose of the analytical method to be validated
- Description of the test and reference substances
- Summary of the analytical method to be validated, including the equipment, specified range, and description of the test and reference substances (may be referenced or appended to the protocol)
- Validation parameters to be demonstrated
- Establishment and justification of the acceptance criteria for the selected validation parameters
- Dated signature of approval of a designated person and the quality unit

The *protocol title* is a brief description of the work or study to be performed: for example, "Validation of the Test Method for the HPLC Assay of Drug Substance X in Drug Product B." The *purpose* should specify the scope and applicability of the method. The *summary* is the actual written method or procedure, with enough detail to be easily reproduced by a qualified individual. To reduce repetition, the method may be included by reference or as an appendix to the protocol. The specific *validation parameters* to be evaluated are also included in the protocol, because the validation parameters are dependent on the type of analytical method (Section 4.4). *Acceptance criteria* for method validation often are established during the final phase of method development, such as during experiments to show that the method is capable of being validated (sometimes referred to as "prevalidation" experiments). The *designated Quality Unit representative* reviews and approves the protocol to ensure that the proper regulatory regulations will be met and that the proposed work will satisfy its intended purpose.

Experimental work outlined in the validation protocol can be designed such that several appropriate validation parameters are measured simultaneously. For example, linearity uses the accuracy and precision sample preparations and data; LOD and LOQ are determined from the range and linearity data; sample and standard solution stability use the same preparations from accuracy and precision. Executed in this manner, the experimental design makes the most efficient use of time and materials. An example validation protocol can be seen in Appendix A.

4.5.2 Test Method

The test method is the formal document that contains all the necessary detail to implement the analytical procedure on a routine basis. The test method is a controlled document with revision control (the requirement that any document changes are authorized and that all revisions are available for later comparison), approvals at the appropriate levels (including the quality unit), and written with enough detail to warrant only one possible interpretation for any and all instructions. A typical test method will include the following:

- Descriptive method title
- Brief method description or summary

- Description of the applicability and specificity, along with any special precautions (e.g., safety, storage, and handling)
- List of reagents, including source and purity/grade
- Equipment, including the HPLC and any other equipment necessary (balances, centrifuges, pH meters, etc.)
- Detailed instrument operating conditions, including integration parameters
- Detailed description of the preparation of all solutions (mobile phases, diluents), standards, and samples
- System suitability test description and acceptance criteria
- Example chromatograms, spectra, or representative data
- Detailed procedures, including an example sample queue (the order in which standards and samples are run)
- Representative calculations
- Revision history
- Approvals

Once drafted, methods often are subjected to a prevalidation stage, to demonstrate that they are capable of being validated. The prevalidation stage typically consists of an evaluation of linearity and accuracy. Sometimes a test of robustness, if it has not already been evaluated during method development, is then carried out. The validation process usually will proceed more smoothly, and with lower risk of failure, if the ability to pass all the key validation criteria is confirmed during the prevalidation stage. A draft method will become an official test method after a full validation of its intended purpose.

4.5.3 Validation Report

The validation report is a summary of the results obtained when the proposed test method is used to conduct the validation protocol. The report includes representative calculations, chromatograms, calibration curves, and other results obtained from the validation process. Tables of data for each step in the protocol and a pass or fail statement for each of the acceptance criteria are also included. A validation report generally consists of the following sections:

- Cover page with the title, authors, and affiliations.
- Signature page dated and signed by appropriate personnel, which may include the analyst, the group leader, a senior manager, and a quality control and/or a quality assurance representative.
- An itemized list of the validation parameters evaluated, often in the form of a table of contents.
- An introduction or objective.
- Method summary including instrument and solution preparation specifics.
- Validation results in subsections organized by the parameter studied. Each subsection should include a brief summary of the applicable protocol, and the mean, standard deviation, relative standard deviation, acceptance criteria, and assessment (pass or fail).

- Any deviations from the protocol, planned or observed, and the impact (if any) on the validation.
- Any amendments to the protocol, with explanations and approvals.
- Conclusion.

A properly designed validation protocol can serve as a template for the validation report. For example, in the protocol a test can be described and the acceptance criteria listed. For the validation report, this information is supplemented with supporting results, a reference to the location and identity of the raw data, and a pass/fail statement.

4.6 SUMMARY

Method validation constantly evolves and is just one part of the overall regulated-environment process. The validation process starts with instrument qualification (Chapter 2) before an HPLC instrument is placed online, and continues long after method development (Chapter 3), optimization, and transfer (Chapter 9), living on with the method during routine use. A well-defined and documented validation process provides regulatory agencies with evidence that the system and method are both suitable for their intended use. It also ensures that guidelines have been framed to help establish method validation requirements and specifications.

The bottom line is that all parties involved should be confident that an HPLC method will give results that are sufficiently accurate, precise, and reproducible for the analysis task at hand. Formal method validation is just a set of tools to use to accomplish this task. Whether or not a formal validation is required, performance of good, justifiable science as part of an established quality system will help to ensure that the resultant method and the data that it generates will survive the scrutiny of reviewers.

REFERENCES

1. Guideline for Submitting Samples and Analytical Data for Methods Validation, USFDA-CDER (February 1987), http://www.fda.gov/cder/guidance/ameth.htm.
2. Chapter 1225, Validation of Compendial Methods, *United States Pharmacopeia No. 34-NF* 297, (2011).
3. Analytical Procedures and Methods Validation, USFDA-CDER (August 2000), http://www.fda.gov/cder/guidance/2396dft.htm.
4. Harmonized tripartite guideline, validation of analytical procedures, text and methodology, Q2 (R1) *International Conference on Harmonization,* November 2005, http://www.ich.org/LOB/media/MEDIA417.pdf.
5. Guidance for Methods Development and Methods Validation for the Resource Conservation and Recovery Act (RCRA) Program, U.S. EPA (1995), http://www.epa.gov/epawaste/hazard/testmethods/pdfs/methdev.pdf.
6. ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories, 2005, http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=39883.
7. Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs, 21 CFR Part 210, http://www.fda.gov/cder/dmpq/cgmpregs.htm.

8. Current Good Manufacturing Practice for Finished Pharmaceuticals, 21 CFR Part 211, http://www.fda.gov/cder/dmpq/cgmpregs.htm.
9. International Organization for Standardization, http://www.iso.org/iso/home.htm.
10. Reviewer Guidance, Validation of Chromatographic Methods, USFDA (November 1994) http://www.fda.gov/cder/guidance/cmc3.pdf.
11. Snyder, L. R., Kirkland, J. J., and Glajch, J. L. *Practical HPLC Method Development*, 2nd ed., Wiley-Interscience, New York, 1997.

5 Robustness and System Suitability

5.1 INTRODUCTION

The robustness of an analytical procedure is defined as a measure of its capacity to obtain comparable and acceptable results when perturbed by small but deliberate variations in procedural parameters listed in the documentation [1,2]. Robustness provides an indication of the method's suitability and reliability during normal use and is the cornerstone of any good method validation process. One consequence of the evaluation of robustness is the establishment of system suitability parameters to ensure that the validity of the analytical procedure is maintained whenever used. Although not formally a part of method validation according to the USP, system suitability tests are an integral part of chromatographic methods [3]. System suitability tests are used to verify that the resolution and precision of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole, and in this way system suitability can be thought of as the test that pulls together the entire validation process. This chapter discusses both topics from an experimental design and specification-setting standpoint. Both USP Chapter 1225: Validation of Compendial Methods, and the International Conference on Harmonization (ICH) Guideline: Validation of Analytical Procedures: Text and Methodology Q2 (R1), address robustness [1,2]. While the USP is the sole legal document in the eyes of the FDA, this chapter draws from both guidelines as appropriate for definitions and methodology.

5.2 ROBUSTNESS STUDIES FOR METHOD VALIDATION

The ICH and the USP guidelines define the robustness of an analytical procedure as a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the documentation, providing an indication of the method's or procedure's suitability and reliability during normal use. Traditionally, robustness has not been considered a validation parameter in the strictest sense because it is usually investigated during method development, once the method is at least partially optimized. When thought of in this context, evaluation of robustness during development makes sense, as parameters that affect the method can be easily identified when manipulating selectivity for method optimization purposes. Evaluating robustness either prior to or at the beginning of the formal method validation process also fits into the category of "you can pay me now, or you can pay me

later." In other words, investing a little time up-front can save a lot of time, energy, and expense later.

During a robustness study, method parameters are intentionally varied to see if the method results are affected. The key word in the definition is *deliberate*. In liquid chromatography (LC), examples of typical variations include

- Mobile phase composition
  - Number, type, and proportion of organic solvents
  - Buffer composition and concentration
- pH of the mobile phase
- Different column lots
- Temperature
- Flow rate
- Wavelength
- Gradient variations
  - Hold times
  - Slope
  - Length

Robustness studies are also used to establish system suitability parameters to make sure the validity of the entire system (including both the instrument and the method) is maintained throughout implementation and use.

Robustness is often confused with the term *ruggedness*, but the two terms really have different and distinct meanings. Ruggedness was once defined in USP guidelines as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different

- Laboratories
- Analysts
- Instruments
- Reagent lots
- Elapsed assay times
- Assay temperature
- Days

That is, a measure of the reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst. The use of the term *ruggedness*, however, is not used by the ICH, but is addressed in the guideline Q2 (R1) under intermediate precision (within-laboratory variations; different days, analysts, equipment, etc.) and reproducibility (between-laboratory variations from collaborative studies applied to the standardization of the method) [2]. Because of the confusion with robustness, use of the term *ruggedness* (in regard to method validation at least) is falling out of favor. References to ruggedness have been deleted in current USP guidelines to more closely harmonize with ICH, using the term *intermediate precision* instead. Confusion still exists, however, concerning how and when to apply the different term: robustness and intermediate

precision/ruggedness. To clear up any confusion, it may be easier to think of the terms as parameters *external* (ruggedness) or *internal* (robustness) to the method, resulting in an easy rule of thumb: if it is written into the method (e.g., 30°C, 1.0 mL/min, and 254 nm), it is a robustness issue. If it is not specified in the method (e.g., you would never specify: Steve runs the method on Tuesdays on instrument six), it is an intermediate precision/ruggedness issue. In addition, it is a good idea to always evaluate the external intermediate precision ruggedness parameters separate from the internal robustness parameters.

5.3 ROBUSTNESS STUDY EXPERIMENTAL DESIGN

For years, analysts have conducted both optimization and robustness studies according to a univariate approach, changing a single variable or factor at a time. Performing experiments in this manner most likely resulted from being trained as a scientist (one variable at a time!) as opposed to a statistician. This approach, while certainly informative, can be time consuming, and often important interactions between variables, for example pH changes with temperature, may remain undetected. Multivariate approaches allow the effects of multiple variables on a process to be studied simultaneously.

In a multivariate experiment, varying parameters simultaneously rather than one at a time can be more efficient, and allows the effects between parameters to be observed.

There are four common types of multivariate experimental design approaches:

1. *Comparative*, used to choose between different alternatives
2. *Response Surface Modeling*, used to hit a target, minimize or maximize a response
3. *Regression Modeling*, used to quantify dependence of response variables on process inputs
4. *Screening*, used to identify which factors are important or significant

The choice of which design to use depends on the objective and the number of parameters, referred to as factors, that need to be investigated. For chromatographic studies, the two most common designs are Response Surface Modeling and Screening. Response Surface Modeling is commonly used for method development, but the focus of the remainder of this chapter is on screening because it is the most appropriate design for robustness studies. The references include more detail beyond what is presented here, as well as additional information on various experimental designs [4–7].

5.3.1 Screening Designs

Screening designs is an efficient way to identify the critical factors that affect robustness, and are for the larger numbers of factors often encountered in a chromatographic method. There are three common types of screening experimental designs that can be used: full factorial, fractional factorial, and Plackett–Burman designs.

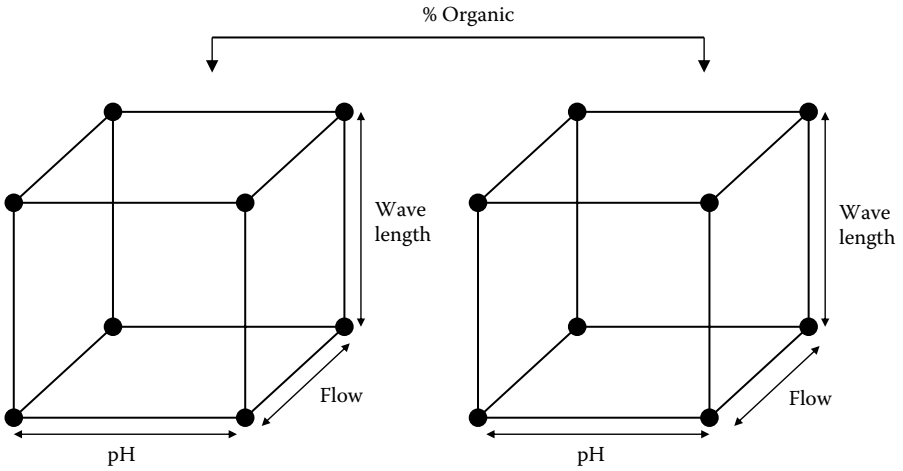


FIGURE 5.1 Full factorial design experiments for four factors: pH, flow rate, wavelength, and percent organic in the mobile phase. Runs are indicated by the dots.

5.3.1.1 Full Factorial Designs

In a full factorial experiment, all possible combinations of factors are measured. Each experimental condition is called a "run," and the results are called "observations." The experimental design consists of the entire set of runs. A common full factorial design is one with all factors set at two levels each, a high and a low value. If there are k factors, each at two levels, a full factorial design then has 2^k runs. In other words, using four factors, there would be 2^4 or 16 design points or runs. To further illustrate the point, Figure 5.1 illustrates a full factorial design robustness study for four factors; pH, flow, wavelength, and percent organic in the mobile phase.

5.3.1.2 Fractional Factorial Deigns

Full factorial design runs can really start to add up when investigating large numbers of factors: for nine factors, 512 runs would be needed! (Without even taking into account replicate injections.) In addition, the design presented in Figure 5.1 assumes linear responses between factors, but in many cases, curvature is possible, necessitating center point runs, further increasing the number of runs. For this reason, full factorial designs are usually not recommended for more than five factors to minimize time and expense.

So, how do analysts investigate more factors, with or without center points? A carefully chosen fraction or subset of the factor combinations may be all that is necessary, which is referred to as fractional factorial designs. In general, a "degree of fractionation (2^{-p})," such as ½, ¼, etc., of the runs called for in the full factorial design are chosen, as shown in Figure 5.2. In the example above with nine factors resulting in 512 runs for a full factorial design, fractional factorial designs can accomplish the same evaluation in as little as 32 runs (using a 1/16 fraction: 512/16, or 2^{k-p}. The latter is arrived at by taking the full factorial $2^k * 2^{-p}$ or 2^{k-p}.)

Fractional factorial design works mostly due to the "scarcity of effects principle" that states that while there may be many factors, few may actually be important,

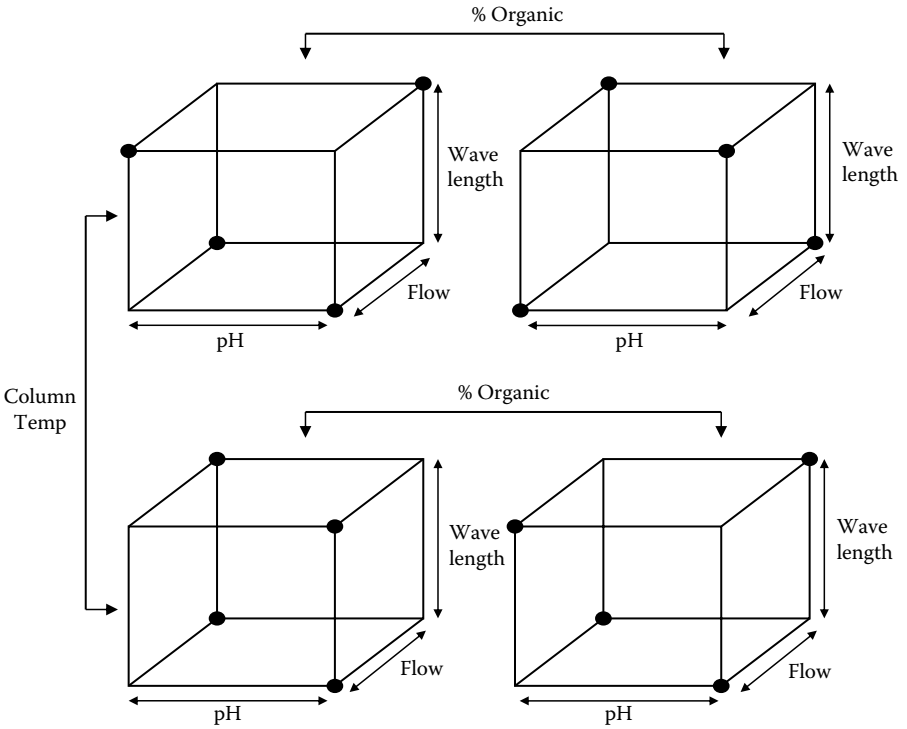


FIGURE 5.2 Fractional factorial design robustness study for a five-factor experiment: pH, flow rate, wavelength, percent organic, and temperature. Runs are indicated by the dots.

and experiments are usually dominated by main effects; not every variable interacts with every other variable. The scarcity of effects principle is further based on the probability that it is rare (low probability) for every factor to be low or high at the same time. Therefore, the most critical issue in fractional factorial design is selection of the proper fraction of the full factorial to study. There is, of course, a price to be paid for reducing the number of runs, and that is that not all factors can be determined "free and clear," but are aliased or confounded with other factors, and the design resolution refers to the degree of confounding. Full factorial designs have no confounding, and have a resolution of infinity. Fractional factorial designs can be resolution 3 (some main effects confounded with some two-level interactions), resolution 4 (some main effects confounded with three-level interactions), and resolution 5 (some main effects are confounded with four-level interactions). In general, the resolution of a design is one more than the smallest order interaction with which a main effect is confounded (aliased). Where possible, important factors should not be aliased with each other. Chromatographic knowledge and the lessons learned during method development (e.g., what affects the separation the most?) are very important for selecting the proper factors and fraction. But do not worry; runs can always be added to fractional factorial studies if ambiguities result.

TABLE 5.1
Plackett–Burman Design in twelve Runs for up to eleven Factors

Run	Pattern	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
1	+++++++++++	1	1	1	1	1	1	1	1	1	1	1
2	−+−+++−−−+−	−1	1	−1	1	1	1	−1	−1	−1	1	−1
3	−−+−+++−−−+	−1	−1	1	−1	1	1	1	−1	−1	−1	1
4	+−−+−+++−−−	1	−1	−1	1	−1	1	1	1	−1	−1	−1
5	−+−−+−+++−−	−1	1	−1	−1	1	−1	1	1	1	−1	−1
6	−−+−−+−+++−	−1	−1	1	−1	−1	1	−1	1	1	1	−1
7	−−−+−−+−+++	−1	−1	−1	1	−1	−1	1	−1	1	1	1
8	++−−−+−−+−+	1	1	−1	−1	−1	1	−1	−1	1	−1	1
9	+++−−−+−−+−	1	1	1	−1	−1	−1	1	−1	−1	1	−1
10	+++−−−+−−+−	1	1	1	−1	−1	−1	1	−1	−1	1	−1
11	−+++−−−+−−+	−1	1	1	1	−1	−1	−1	1	−1	−1	1
12	+−+++−−−+−−	1	−1	1	1	1	−1	−1	−1	1	−1	−1

Note: High (1) and low (−1) values correspond to chromatographic variables or factors.

5.3.1.3 Plackett–Burman Designs

For robustness testing, it usually is sufficient to determine whether a method is robust to many changes, rather than to determine the value of each individual effect, and Plackett–Burman (PB) designs are very efficient screening designs where only main effects are of interest. Plackett and Burman published their now-famous paperback in 1946 describing their economical experimental designs in multiples of four rather than a power of two [8], and PB designs have been frequently reported in the literature for chromatographic robustness studies (see, for example, References 9–12). Table 5.1 shows a generic PB design for the twelve runs needed to evaluate eleven factors, according to the general formula N–1 factors. Figures 5.3a and 5.3b illustrate an example of some actual experimental conditions that might be used for a PB design for an eight-factor HPLC experiment. A PB design is a type of resolution 3 two-level fractional factorial design where main effects are aliased with two-way interactions. PB designs also exist for 20 (19 factors), 24 (23 factors), and 28 (27 factors) run designs, but these are seldom used in chromatography as there is rarely the need to evaluate so many factors. In instances where N–1 factors do not result in a multiple of four, "dummy" factors are used.

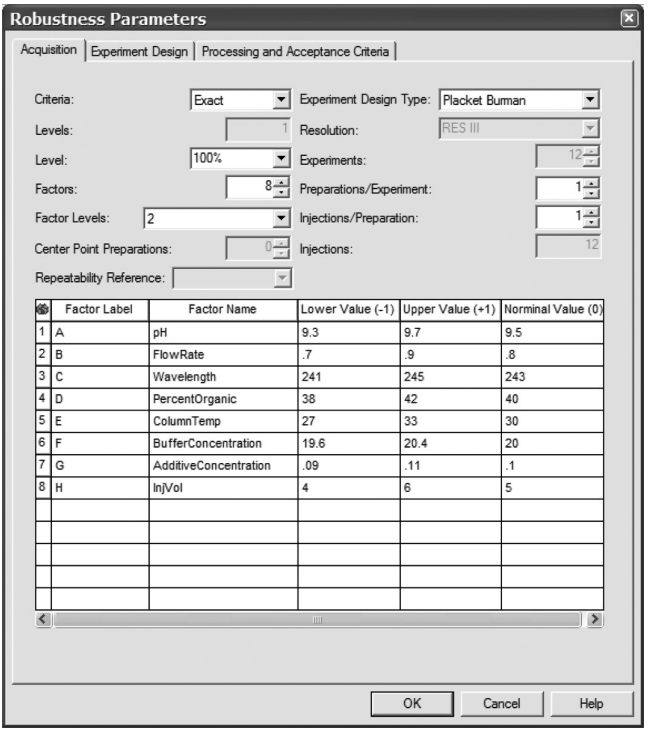
5.3.2 Determining the Factors, Measuring the Results

A typical HPLC method consists of many different parameters that can affect the results. Various instrument, mobile phase, and even sample parameters must be taken into account. Even the type of method (isocratic versus gradient) can dictate the numbers and importance of various factors.

Typically, factors are chosen symmetrically around a nominal value, or the value specified in the method, forming an interval that slightly exceeds the variations

that can be expected when the method is implemented or transferred [13–15]. For example, if the method calls for premixing a mobile phase of 60% methanol, then the high (1) and low (–1) factors might be 58% and 62% methanol, or some similar range expected to bracket the variability a properly trained analyst using proper laboratory apparatus can be expected to measure. In the case of instrument settings, manufacturers' specifications are sometimes used to determine variability. If the instrument is being used to generate the mobile phase, for example, gradients, or set the temperature, then the range should bracket those specifications. Ultimately, the range evaluated should not be selected to be so wide that the robustness test will purposely fail, but rather to represent the type of variability routinely encountered in the laboratory.

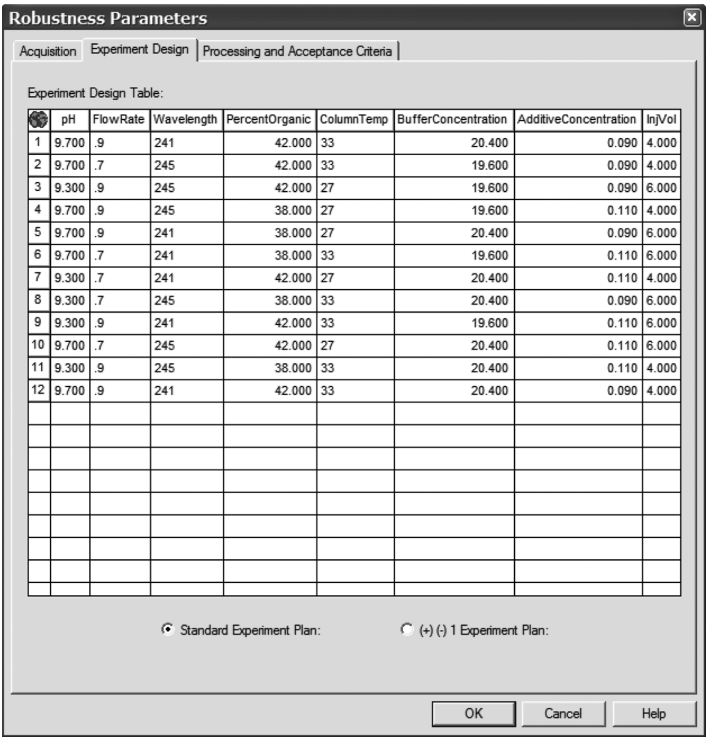
Table 5.2 lists some factor limits for an isocratic method where the mobile phase is premixed. Mobile phase composition, flow rate temperature, and wavelength are all considered. Gradient method factor limits are listed in Table 5.3. Gradient methods represent a slightly different factor selection challenge; in addition to some of the factors that must be considered for an isocratic method, gradient timing should be taken into consideration.



	Factor Label	Factor Name	Lower Value (-1)	Upper Value (+1)	Nominal Value (0)
1	A	pH	9.3	9.7	9.5
2	B	FlowRate	.7	.9	.8
3	C	Wavelength	241	245	243
4	D	PercentOrganic	38	42	40
5	E	ColumnTemp	27	33	30
6	F	BufferConcentration	19.6	20.4	20
7	G	AdditiveConcentration	.09	.11	.1
8	H	InjVol	4	6	5

FIGURE 5.3a Example experimental conditions that might be used for a PB design for an eight-factor HPLC experiment. Figure 5.3a shows the experimental design setup with the factor names, nominal, upper and lower values, and

(*Continued*)



	pH	FlowRate	Wavelength	PercentOrganic	ColumnTemp	BufferConcentration	AdditiveConcentration	InjVol
1	9.700	.9	241	42.000	33	20.400	0.090	4.000
2	9.700	.7	245	42.000	33	19.600	0.090	4.000
3	9.300	.9	245	42.000	27	19.600	0.090	6.000
4	9.700	.9	245	38.000	27	19.600	0.110	4.000
5	9.700	.9	241	38.000	27	20.400	0.090	6.000
6	9.700	.7	241	38.000	33	19.600	0.110	6.000
7	9.300	.7	241	42.000	27	20.400	0.110	4.000
8	9.300	.7	245	38.000	33	20.400	0.090	6.000
9	9.300	.9	241	42.000	33	19.600	0.110	6.000
10	9.700	.7	245	42.000	27	20.400	0.110	6.000
11	9.300	.9	245	38.000	33	20.400	0.110	4.000
12	9.700	.9	241	42.000	33	20.400	0.090	4.000

FIGURE 5.3b (*Continued*) Example experimental conditions that might be used for a PB design for an eight-factor HPLC experiment. Figure 5.3b the suggested experimental design and conditions for the twelve chromatographic runs.

TABLE 5.2
Example Robustness Factor Selection and Limits for an Isocratic Method

Factor	Limit Range (±)
Organic solvent concentration	2%–3%
Buffer concentration	1%–2%
Buffer pH (if adjusted)	0.1–0.2 pH units
Temperature	3°C
Flow rate	0.1–0.2 mL/min
Wavelength	2–3 nm for 5-nm bandwidth
Injection volume	Injection type and size dependent

Note: These limits are examples only and should be chosen according to expected laboratory and instrument variations.

TABLE 5.3
Example Robustness Factor Selection and Limits for a Gradient Method

Factor	Limit Range (±)
Initial hold time[a]	10%–20% of segment time.
Slope and length	The slope is set by the initial %B and the final %B, as well as the gradient length. It is recommended to adjust the lengths by 10%–20% and allow the slope to vary.
Final hold time	Adjusted according to the last eluting compound and varied accordingly.

Note: Factors and limits listed here are in addition to many of the factors considered in an isocratic method.

[a] It is increasingly common for gradient methods to have an initial hold time to accommodate transfer to instruments with different dwell volumes.

For any chromatographic run, there are a myriad of results generated. Typical results investigated for robustness studies include critical pair resolution, efficiency (N), retention time of the main components, tailing factor, area, height, and quantitative results such as amounts. Note that there are different ways of measuring some of these factors; standard operating procedures (SOPs) or other documentation should specify how the results were calculated. If a quantitative result is desired, it is necessary to measure both samples and standards. Replication of the design points can also improve the estimate of the effects. In addition, it is a good idea to measure results for multiple peaks, as compounds will respond differently according to their physicochemical characteristics, for example, ionizable versus neutral compounds that might be present in the same mixture.

5.4 ANALYZING THE RESULTS

Once the design has been chosen, the factors and limits determined, and the chromatographic results generated, the real work starts. All of that data must be analyzed, and at this point, many analytical chemists begin to search out their resident statistician. Ultimately, the limits uncovered by the robustness study, determined in the data treatment or observed in the graphs, are used to set system suitability specifications.

While consulting and collaborating with a good statistician for robustness studies is always a good idea, there are many tools available to assist the analyst in analyzing the results.

Statistical software is available from a variety of sources. There are add-in programs for Excel, and popular commercially available software such as SPSS (Chicago, Illinois or spss.com), JMP (Cary, North Carolina or JMP.com), or Minitab (State College, Pennsylvania, or minitab.com). Third-party software adds to the validation process, as it too must be validated.

Chromatography data systems (CDSs) are also available that perform many of the requisite calculations and reporting for robustness studies [16]. But unlike third-party software, CDSs have the advantage that the data is traceable, validation need only be performed once, and the entire audit trail, relational database, reporting, etc.,

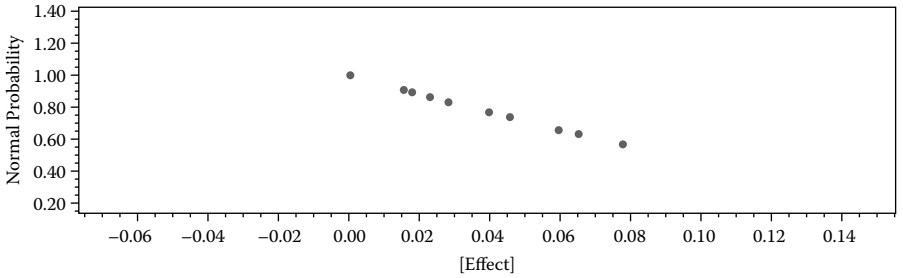


FIGURE 5.4 Example probability plot. In this example, the factor effects are plotted against a linear distribution; departures from the line would indicate robustness issues.

features can be used not just to generate the data, but also to analyze and report the data during method validation.

While a comprehensive statistical discussion is outside the scope of this chapter, several good references are available for more detail [5–7]. Essentially, the analyst must compare the design results, or the results obtained from the experiments run, for example, according to Table 5.1, or Figure 5.3, to the nominal results. Regression analysis and calculation of standard or relative error are common ways to look at the data. Analysis of variance (ANOVA), which is a test that measures the difference between the means of groups of data, is another common way of making the comparison. Sometimes called an F-test, ANOVA is closely related to the t-test. The major difference is that, where the t-test measures the difference between the means of two groups, an ANOVA tests the difference between the means of two or more groups.

The primary goal of any robustness study is to identify the key variables or factors that influence the result or response, and graphs are an easy way to observe the effects at a glance. Effects plots, or probability plots, are two common ways to represent robustness data, and most general-purpose statistical software programs can be used to generate probability plots. As illustrated in Figure 5.4, in a probability plot the data is plotted in such a way that the points should approximately form a straight line. Departures from the straight line indicate deviations in the data that affect robustness. Different types of probability plots, called normal or half-normal probability plots, are used to further qualify the data. Normal probability plots are used to assess whether or not the data set is approximately normally distributed. Half-normal probability plots can identify the important factors and interactions.

An effects plot is another type of graphical representation, as depicted in Figure 5.5. Similar to a bar chart, or histogram, the effects plot can also identify the important factors and interactions.

5.5 DOCUMENTATION AND REPORTING

As the saying goes, if it isn't written down, or documented in a report, it never happened. Proper documentation of the robustness study is, of course, essential to the method validation process. A properly constructed report must include the

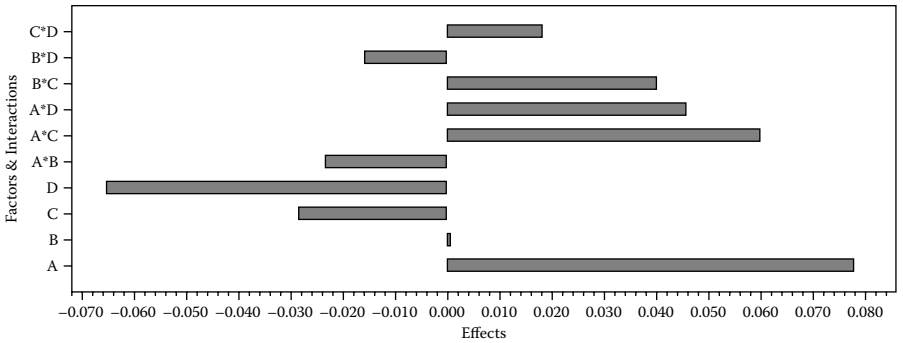


FIGURE 5.5 Example effects plot. Factor effects can be either positive or negative. The magnitude of the bar is an indication of the magnitude of the effect.

experimental design used for the study; all the graphs used to evaluate the data, and tables of information, including the factors evaluated and the levels; and the statistical analysis of the responses. The factor limits, and any system suitability specifications arrived at, should also be tabulated. A precautionary statement should also be included for any analytical conditions that must be suitably controlled for measurements that are susceptible to variations in the procedure.

5.6 SYSTEM SUITABILITY TESTS

System suitability tests are an integral part of chromatographic methods used to verify that the resolution and reproducibility of, for example, a chromatographic system, are adequate for the analysis to be performed [17]. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole.

System suitability is defined as the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factor, resolution, reproducibility (%RSD retention time and area for repetitive injections), and signal-to-noise (S/N) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability "sample," which is a mixture of main components and expected by-products. Specifications for the accuracy of recovery between two standard preparations are also commonly used; the second, separately prepared accuracy standard is often referred to as a "check standard." Table 5.4 lists the terms to be measured and their recommended limits obtained from the analysis of the system suitability standard [18]. In most cases today, chromatography data system (CDS) software can calculate system suitability parameters to provide a review of the separation and to summarize data. Results stored in a relational database can be compared and summarized on a peak-by-peak or system-by-system basis to provide the feedback necessary to determine system performance for troubleshooting. CDS system suitability results can also be used interactively in some systems to prevent analysis of unknown samples following a failed system suitability specification.

TABLE 5.4
System Suitability Parameters and Recommendations

Parameter	Recommendation
Capacity Factor (k′)	The peak should be well resolved from other peaks and the void volume, generally $k' > 2.0$
Repeatability	RSD ≤ 1% for N ≥ 5 is desirable
Relative Retention	Not essential as long as the resolution is stated
Resolution (R_s)	$R_s > 2$ between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing Factor (T)	T ≤ 2
Theoretical Plates (N)	In general, should be > 2000

Source: Center for Drug Evaluation and Research (CDER), Reviewer Guidance: Validation of Chromatographic Methods, US Government Printing Office, 1994, 615-023-1302/02757.

5.7 SYSTEM SUITABILITY STANDARDS

The USP defines a system suitability standard as a sample that consists of a mixture of main components and any additional materials used in the control of the analytical system [17]. The system suitability standard is distinctly different from other samples or standards often used in the method development and validation process, such as quality control samples, or standards and samples used for calibration or quantitation.

At times, the use of the pure analyte of interest standard alone without any sample components present (e.g., blood, formulations, urine, etc.) is justified, for example, during method development and optimization. However, this type of sample should not be considered appropriate for system suitability because it contains but a single component (the standard of the analyte). True system suitability standards are usually a little more complex than analyte quantitation standards because they usually contain several of the expected, major components of the final sample (analyte plus [perhaps]) impurities, metabolites, or degradants).

Quantitation and calibration standards often only contain one major component and as such are not necessarily appropriate for system suitability determinations. System suitability samples must contain at least the major analyte of interest and ideally a closely eluting component or components that might be found in actual unknown samples, at known levels. These other components could be synthetic precursors normally found in the preparation of the analyte itself, thermal or photostability (breakdown) products, metabolites, or impurities. Thus, to prepare a useful system suitability standard, a pure sample of the analyte standard (bulk drug) and at least one of the expected, other major components in the HPLC chromatogram should be used, because it is necessary to have more than one component present in the sample in order to document the parameters called out in the USP [17].

System suitability standards, such as quantitation or calibration standards, are usually prepared from reference standards. Chromatographic methods rely heavily on reference standards to provide accurate data. Therefore, the purity of the reference

standard is very important. The FDA recognizes two categories of reference standards: compendial and noncompendial. Compendial reference standards are obtained from sources such as the USP and do not need further characterization. Noncompendial reference standards are standards of the highest purity that can be obtained by reasonable effort and should be thoroughly characterized to assure its identity, strength, and purity. It is generally recognized that more rigorous tests are often used to define the reference standard than those used to define the drug substance itself. It is also recommended that purity correction factors be included in any method calculations, along with any other correction factors that may be applicable (e.g., moisture).

5.8 SYSTEM SUITABILITY PROTOCOL

System suitability must be performed on a regular basis. However, in spite of this requirement, a review of common method deficiencies on the FDA Internet website indicates that system suitability tests are not always routinely performed. On any given day that unknown samples are to be run, it is customary to perform a system suitability test analyzing unknown samples. Unless otherwise specified in the method or USP monograph, data from five replicate injections are used to calculate the relative standard deviation (RSD) if the requirement is 2.0% or less; data from six replicate injections is used if the RSD requirement is more than 2.0%.

All data should be identical, within experimental error, and if the specifications are not satisfied, then any subsequent quantitative results are suspect. Table 5.5 lists some example system suitability specifications that have been used in the authors' laboratory. Table 5.6 illustrates an example sample queue that utilizes a system suitability test. If the system suitability specifications are met during the execution of the sample queue, the entire sample queue is completed. However, if the specifications are not met, an out-of-specification (OOS) result is generated (Chapter 6), an investigation is launched, and no unknown samples are analyzed until the investigation is complete, the fault resulting in the OOS is corrected, and subsequent system suitability tests are successful.

TABLE 5.5
Example System Suitability Specifications

Parameter	Acceptance Criteria	
System Suitability	Linearity	$r^2 \geq 0.99$
	Resolution between analyte of interest and next peak	≥2.5
	Retention time of analyte	8.0 ± 1 min
	Overall standard precision (5 injections)	Area RSD ≤ 2%
		Retention time RSD ≤ 1%
	Efficiency (plates)	N > 7500
	Tailing factor	$t_f \leq 1.5$
	S/N of low-level analyte peak (e.g., LOQ level)	S/N ≥ 10
	Check std. recovery	98% to 102%

TABLE 5.6
Example Instrument Sample Queue

Sample	# of Injections
Blank (diluent)	At least 1
System suitability	5
Check std.	1
Working std.	1
Blank (diluent)	1
Sample prep 1	1
Sample prep 2	1
Sample prep 3	1
Sample prep 4	1
Sample prep 5	1
Sample prep 6	1
3.0 µg/mL Working std.	1
Blank (diluent)	1

5.9 METHOD ADJUSTMENTS TO MEET SYSTEM SUITABILITY REQUIREMENTS

Adjustments to USP methods have been allowed to satisfy system suitability requirements as often noted in individual monographs. Historically, as long as adjustments to the method are made within the boundaries of any robustness studies performed, no further actions are warranted. Any adjustment outside the bounds of the robustness study constitutes a change to the method, requiring a revalidation. However, many USP methods precede the adoption of robustness studies, or the results were not available.

In 1998, Furman et al. proposed a way to classify allowable adjustments [19]. But it was not until 2005 that guidance appeared on the topic [20–22]. The FDA Office of Regulatory Affairs (ORA) has had guidance in place for a number of years [20], and after some deliberation the USP has also now included guidelines into Chapter 621, Chromatography [17]. Table 5.7 summarizes the maximum adjustments allowed for various LC and GC parameters taken from both the USP and ORA documents. Adjustments outside the ranges listed in Table 5.7 constitute modifications, or changes, which are subject to validation. Chromatographic adjustments in order to comply with system suitability requirements should not be made to compensate for column or system failure, and adjustments to the composition of the mobile phase in gradient methods are not recommended. If adjustments to gradient methods are necessary, only column changes (same packing material) or dwell volume adjustments are recommended. Adjustments should only be made from the existing method as written as a starting point each time the method is run. Multiple adjustments can have a cumulative effect on the performance of the system and should be considered carefully before implementation.

TABLE 5.7
Maximum Specifications for Adjustments to LC Operating Conditions

Parameter	Maximum Specification	Comments
pH	±0.2 units	
Buffer Salt Concentration	±10%	Providing the pH variation is met
Ratio of Components in the Mobile Phase	Components specified at 50% or less: ±30% relative (to the total mobile phase composition) or ±10% absolute	Change in any component cannot exceed ±10% absolute; adjustments can only be made to one component in a ternary mixture[a]
UV Detector Wavelength	No deviations	A validated procedure must be used to verify that the error in the detector wavelength setting is, at most, ±3.0 nm
Column Length	±70%	
Column Inner Diameter LC	±50% (ORA) Adjust as long as linear velocity is kept constant	For USP, see Reference 7
Column Inner Diameter, GC	±50%	
Flow Rate (GC or LC)	±50%	See Section 5.8.2 for formula
Injection Volume	Reduced as far as consistent with accepted precision and detection limits	
Particle Size	Reduced by as much as 50%	
Column Temperature	±10°C	Column thermostating is recommended to improve reproducibility of t_r
GC Capillary Film Thickness	−50% to 100%	
Oven Temperature Program (GC)	±20% (time)	

[a] Examples:

Binary mixtures: For a specified ratio of 50:50; 30% is 15% absolute, exceeding limit of ±10% absolute of any one component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60 to 60:40. For a specified ratio of 2:98, 30% of 2 is 0.6% absolute. Therefore, the maximum allowable adjustment is 1.4:98.6 or 2.6:97.4.

Ternary mixtures: For a specified ratio of 60:35:5, 30% of the second component (35%) is 10.5% absolute, exceeding maximum permitted ±10%; therefore, it may only be adjusted within the range 25% to 45% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient proportion of the first component should be used to give a total of 100%. Therefore, mixture ranges of 50:45:5 to 70:25:5 or 58.5:35:6.5 to 61.5:35:3.5 would meet the requirement.

While the criteria in Table 5.7 seem straightforward, many of them do not completely account for recent advances in LC technology, and additional guidance is forthcoming [23]. Some additional comments are also warranted from an LC method perspective as summarized in the following subsections.

5.9.1 pH Adjustments

As shown in Table 5.7, the pH value of the buffer in the mobile phase can be adjusted to ±0.2 pH units. Adjusting the pH should, however, take into account the p*K* values of the compounds of interest; because near the p*K*, even a 0.1 unit change in the pH can result in significant (>10%) changes in retention time [24]. Studies show that for many compounds only operating at pH extremes (pH > 8 or pH < 4 for basic compounds, pH < 3 or pH > 7 for acidic compounds), generally well away from the compound p*K*, will accommodate the ±0.2 unit allowable change, due to the slope of the pH-versus-retention curve [25]. Any adjustments to the buffer (if used) concentration and temperature should also take into account the effect on pH. In the case of LC column temperature, where a ±10ºC change is allowed, selectivity effects might be encountered.

5.9.2 Column Length, Diameter, and Particle Size Adjustments: Scaling the Separation

Per Table 5.7, column internal diameter can be adjusted provided that a constant linear velocity is maintained; length adjustments up to ±70% are allowed. It is possible to reduce flow rate by up to 50%; however, when column dimensions have been modified, they should be adjusted using the following formula:

$$F_2 = F_1 \times l_2(d_2)^2/l_1(d_1)^2$$

where F_1 is the flow rate indicated in the monograph, in milliliters (mL) per minute; F_2 is the adjusted flow rate, in milliliters per minute; l_1 is the length of the column indicated in the monograph; l_2 is the length of the new column used; d_1 is the column inner diameter indicated in the monograph; and d_2 is the internal diameter of the new column used.

Column length, internal diameter, and particle size adjustments really must be considered together, and when correctly scaled according to well-known theoretical principles, equivalent separations will result. For example, keeping the length-to-particle-size ratio (L/d_p) constant, an identical separation can be obtained for a 5-cm, 1.7-µm column as for a 30-cm, 10-µm column ($L/d_p = 3$ for both) as long as an increase in the flow rate inversely proportional to the particle size is also maintained.

Consider an example of converting or migrating a method from HPLC to newer LC technology that uses sub-2-µm particle size chemistry, termed UHPLC [26–30]. Chemistry (sub-2-µm particles) and instrumentation (systems capable of pressures greater than 6,000 to 19,000 psi) necessary to take advantage of this new

technology are available from a number of commercial vendors. A few easy steps using equations that geometrically scale the original method to the new column packed with sub-2-μm particles using exactly the same mobile phase composition are necessary to achieve equivalent results. These equations take into account the changes in the gradient time (unless using isocratic conditions), flow rate, and injection volume.

The gradient is scaled using

$$L_2/L_1 \times t_{g1} = t_{g2}$$

where $L_1$and L_2 are the lengths of the original and new columns, and t_{g1} and t_{g2} are the times of each gradient step, respectively.

The flow rate is scaled taking into account the difference in the diameter of the two columns:

$$(d_2)^2/(d_1)^2 \times F_1 = F_2$$

where d_2 and d_1 are the column diameters and F_1 and F_2 the flow rates.

To keep the column volumes proportional, the gradient steps should be readjusted for the new flow rate:

$$(F_2 \times t_{g2})/F_3 = t_{g3}$$

where F_2 and t_{g2} are the flow rate and gradient time of the geometrically scaled values and F_3 and t_{g3} are the optimized values. (F_3 is usually increased above that calculated for F_2 [0.5 mL/min in the example below], to better approximate the optimum linear velocity for a sub-2-μm particle.)

The injection volume is scaled taking into account the volumes of the two columns:

$$V_1 \times [(r_2^2 \times L_2)/(r_1^2 \times L_1)] = V_2$$

where r_2^2 and r_1^2 are the radii of the columns, L_1 and L_2 are the lengths of the columns, and V_1 and V_2 are the injection volumes, respectively.

Laboratories might be interested in implementing UHPLC to save time and expense compared to existing standard methods. Figure 5.6a shows an HPLC separation of a series of related caffeic acid derivatives from *Echinacea purpurea*, a natural product. When column reequilibration is taken into account, the run time exceeds 40 min. When properly scaled for injection volume, flow rate, and gradient time, the separation illustrated in Figure 5.6b is obtained. The run time is complete in less than 6 min, including reequilibration, increasing throughput fold approximately 7-, while using about a factor of 10XX less solvent. Proper scaling results in a new separation that is accomplished without changing the look of the original separation; if it was not for the time scale in Figure 5.6, it would be difficult to distinguish between the two separations.

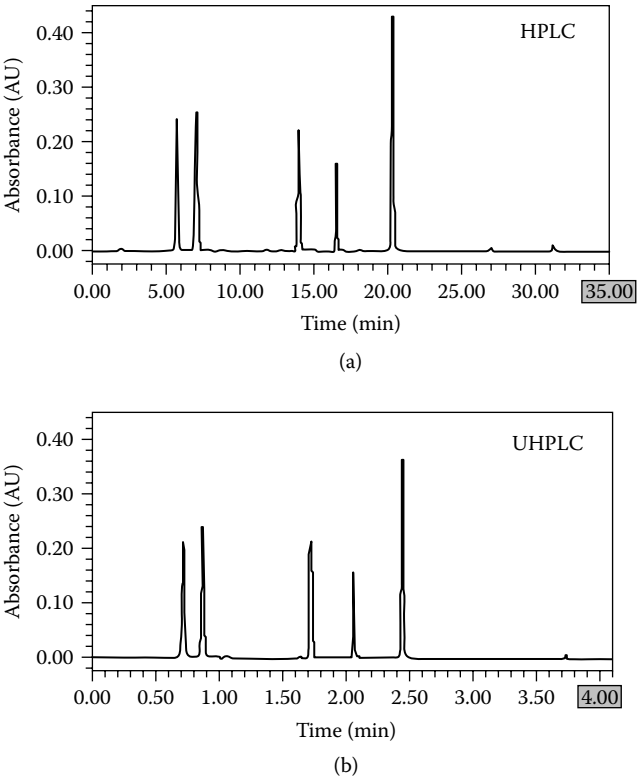


FIGURE 5.6 Separation properly scaled from a (a) 5.0-µm to a (b) 1.7-µm column. (a) Original HPLC separation of caffeic acid derivatives from *Echinacea purpurea*, a natural product. Column: 4.6 by 150 mm, 5.0-µm XTERRA® MS C_{18} Column (Waters Corporation) at 40°C. A 8-50%B linear gradient over 32 min, followed by a 3-min step to 90%B, and a 6 min reequilibration to starting conditions, at a flow rate of 1.0 mL/min, was used. Mobile phase A was 0.1% CF_3COOH in H_2O, Mobile Phase B: 0.08% CF_3COOH in acetonitrile, UV detection at 330 nm. Peaks are in order: Caftaric acid, chlorogenic acid, cynarin, echinacoside, cichoric acid, 0.1 mg/mL each in 50:50 H_2O: MeOH with 0.05% CF_3COOH, 10 µL injection. (b) Resulting 1.7-µm particle separation of caffeic acid derivatives from *Echinacea purpurea*, a natural product, after scaling the HPLC separation. Column: 2.1 by 50 mm 1.7-µm ACQUITY™ BEH C_{18} Column at 40°C. A 8-50%B linear gradient over 4.45 min, followed by a 0.41 min step to 90%B, and a 1.14-min reequilibration to starting conditions, at a flow rate of 0.5 mL/min, was used. Sample and mobile phase conditions were identical to Figure 5.1a. A 1.0-µL injection was used.

5.10 CONCLUSION

A properly designed, executed, and evaluated robustness study is a critical component of any method validation process. In a development laboratory, a robustness study can provide valuable information about the quality and reliability of the method, and is an indication of how good a job was done in developing and

optimizing the method, indicating whether or not further development or optimization is necessary.

When performed early in the validation process, a robustness study can provide feedback on what parameters can affect the method if not properly controlled, and help in setting system suitability parameters for when the method is implemented.

REFERENCES

1. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <1225>.
2. International Conference on Harmonization, Harmonized tripartite guideline, validation of analytical procedures, text and methodology, Q2 (R1), November 2005. See www.ICH.org.
3. ISO/IEC 17025, General Requirements for the Competence of Testing and Calibration Laboratories, (2005), http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=39883.
4. Box, G. E. P., Hunter, W. G., and Hunter, J. S., *Statistics for Experimenters*, John Wiley & Sons, New York, 1978.
5. Miller, J. C. and Miller, J. N., *Statistics for Analytical Chemistry*, Ellis Horwood Publishers, Chichester, UK, 1986.
6. *NIST/SEMATECH e-Handbook of Statistical Methods*, http://www.itl.nist.gov/div898/handbook.
7. Meier, P. C. and Zund, R. E., *Statistical Methods in Analytical Chemistry*, John Wiley & Sons, New York, 1993.
8. Plackett, R. L. and Burman, J. P., The Design of Optimum Multifactorial Experiments. *Biometrika*, 33, 305, 1946.
9. Ragonese, R., Mulholland, M., and Kalman, J., *J. Chromatogr. A*, 870, 45, 2000.
10. Nguyet, A. N. M., Tallieu, L., Plaizier-Vercammen, J., Massart, D. L., and Heyden, Y. V., Validation of an HPLC method on short columns to assay ketoconazole and formaldehyde in shampoo. Correct reference to read volume 32. *J. Pharm. Biomed. Analysis*, 21, 1–19, 2003.
11. Sun, S-W. and Su, H-T., Validated HPLC method for determination of sennosides A and B in senna tablets. *J. Pharm. Biomed. Analysis*, 29, 881–894, 2002.
12. Heyden, Y. V., Nijhuis, A., Smeyers-Verbek, B. G. M., and Massart, D. L., Guidance for robustness/ruggedness tests in method validation. *J. Pharm. Biomed. Analysis*, 24, 723–753, 2001.
13. Heyden, Y. V., Questier, F., and Massart, D. L., Ruggedness testing of chromatographic methods: selection of factors and levels. *J. Pharm. Biomed. Analysis*, 18, 43–56, 1998.
14. Questier, F., Heyden, Y. V., and Massart, D. L., RTS, a computer program for the experimental set-up and interpretation of ruggedness tests. *J. Pharm. Biomed. Analysis*, 18, 287–303, 1998.
15. Lukalay, P. and Morgado, J., Automation of the chromatographic analytical method validation process. *LCGC*, 24(2), 150–156, February 2006.
16. Empower 2 Software: Method Validation Manager, April 2007, 720001488EN LB-PC, Waters Corporation, Milford, MA.
17. *The United States Pharmacopeia 34/ National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <621>.
18. Center for Drug Evaluation and Research (CDER), Reviewer Guidance: Validation of Chromatographic Methods, US Government Printing Office, 1994, 615-023-1302/02757.
19. Furman, W. B., Dorsey, J. G., and Snyder, L. R., System Suitability Tests in Regulatory Liquid and Gas Chromatographic Methods: Adjustments Versus Modifications. *Pharm. Technol*. 22(6), 58–64, 1998.

20. FDA ORA Laboratory Procedure, #ORA-LAB.5.4.5, Revised 09/09/2005. See also http://www.fda.gov/ora/science_ref/lm/vol2/section/5_04_05.pdf.
21. *Pharm. Forum*, 31(3), <621> Chromatography 825, May–June 2005.
22. *Pharm. Forum*, 31(6), <621> Chromatography 1681, Nov.–Dec. 2005.
23. Neue, N. D., McCabe, D., Ramesh, V., Pappa, H., and DeMuth, J., Stimuli to the revision process: Transfer of HPLC procedures to suitable columns of reduced dimensions and particle sizes. *Pharm. Forum* 35(6). April 2010.
24. Swartz, M. E., Unpublished data on the analysis of tricyclic amines at pH 7.2.
25. Swartz, M. E. and Krull, I. S., Developing and Validating Stability Indicating Methods. *LCGC* 23(6), 46, June 2005.
26. Swartz, M. E., UPLC: An introduction and review. *J. Liq. Chromatogr. Rel. Technol.*, 28(7–8), 1253–1263, 2005.
27. Separation Science Re-Defined, Supplement to LCGC, May 2005.
28. Mazzeo, J., Neue, U., Kele, M., and Plumb, R., Advancing LC Performance with Smaller Particles and Higher Pressure. *Anal. Chem.*, 77(23), 460A–467A, 2005.
29. The Quest for Ultra Performance in Liquid Chromatography—Origins of UPLC Technology, Waters Corporation. See http://www.waters.com/waters/partDetail.htm?cid=511539&id=38628&ev=10120661&locale=en_US.
30. Beginners Guide to UPLC—Ultra-Performance Liquid Chromatography. Waters Corporation. See http://www.waters.com/waters/partDetail.htm?cid=511539&id=38629&ev=10120684&locale=en_US.

6 Setting Specifications and Investigating Out-of-Specification Results

6.1 INTRODUCTION

Specifications establish the criteria to which a drug substance or a drug product should conform to be considered acceptable for its intended use. Setting specifications requires justifications, and the application of different types of tests under different circumstances, each with their own acceptance criteria.

The most common source of questions on the subject of method validation concerns setting specifications. Specifications that establish tests, procedures, and acceptance criteria play a major role in assuring the quality of new drug substances and products at release and during shelf life. But who determines specifications? How does one find or establish acceptance criteria? In a regulated laboratory, setting specifications and acceptance criteria is generally left up to the originator. That is, only the originator (applicant or manufacturer) can determine and justify what is appropriate for a particular product, test, or procedure for eventual approval (hopefully!) by a regulatory agency.

While specifications define a pharmaceutical product's essential characteristics and establish the criteria for releasing it for further use, during routine use of a validated analytical method, results will occasionally fall outside the defined specifications. When results do not meet specifications, an investigation should be triggered so that corrective action can be taken; and as with many processes in the regulated laboratory, there is a recommended way to carry out an investigation. However, guidance is available. This chapter discusses the general concepts highlights some specific guidelines and testing used in the process of setting specifications, and addresses potential sources and ways to avoid and investigate out-of-specification (OOS) results.

6.2 GUIDANCE FOR SETTING SPECIFICATIONS

In 2000, the FDA adopted an International Conference on Harmonization (ICH) guideline on specifications for new drug substances and products [1,2]. This guideline addresses the process of selecting tests and methods, setting specifications for the testing of drug substances and dosage forms, and includes several

flowchart decision trees for different types of tests. The guideline was written to establish global specifications for marketing approval of new drug substances and products of synthetic chemical origin, and new drug products produced from them, that have not been previously registered in the United States, European Union, or Japan.

So, just what exactly is a specification? Quite simply, a specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria for the test described, for example, numerical limits, ranges, or other criteria to which a drug substance or product should conform to be considered acceptable for its intended use. Specifications ensure product quality and consistency, and are just one part of a total control strategy in addition to thorough product characterization during development, and adherence to good manufacturing practices (e.g., suitable facilities, validated manufacturing processes and test procedures, raw material testing, stability testing, etc.). When proposing specifications, justification is needed for each procedure and acceptance criterion. Justification should include related data from development, pharmacopeial standards, test data from toxicological and clinical studies, and results from accelerated and long-term stability studies. When justifying a specification, normal or acceptable analytical or manufacturing variability should be taken into consideration. The final goal is "conformance to specifications," which means that the drug substance or drug product, when tested according to the documented analytical procedure, satisfies the listed acceptance criteria.

6.3 GENERAL CONCEPTS FOR DEVELOPING AND SETTING SPECIFICATIONS

An understanding of several different types of testing concepts is necessary in order to develop and set specifications. These concepts include the following: Limited Data Available at Filing, Periodic or Skip Testing, Release versus Shelf-life Acceptance Criteria, In-process Tests, Parametric Release, and Pharmacopeial Tests. Not all of these tests are universally applicable, but each must be considered as circumstances warrant. Test design and development considerations should also take into account data and experience acquired during the development of a new drug substance or product. Sometimes this experience can lead to justifying exclusion or replacement of specific tests.

6.3.1 Limited Data Available at Filing

Often, only a limited amount of data is available when an application is filed, and the basis for setting acceptance criteria focuses on safety and efficacy. Until additional data and experience manufacturing a drug substance or product are obtained, it may be necessary to propose revised acceptance criteria. This situation necessitates reviewing initial approved tests and acceptance criteria as more data is collected. After review, modifications involve loosening and tightening the acceptance criteria as appropriate.

6.3.2 Periodic or Skip Testing

Periodic or Skip Testing is the performance of specific tests on preselected batches at predetermined intervals as opposed to testing every batch. Of course, it is understood that all the untested batches must still conform to the acceptance criteria for that product. Batch selection and intervals must be justified and approved by the regulatory authorities prior to test implementation. Because often only a limited amount of data is available when an application is filed, this concept is generally implemented post-approval.

6.3.3 Release versus Shelf-Life Acceptance Criteria

Sometimes, for drug products, more restrictive acceptance criteria are set for release of the drug product than are applied to the shelf-life. This concept is sometimes applied to assay and impurity (degradation product) testing levels. Sometimes an applicant may choose to have tighter in-house limits at the time of product release to provide additional assurance that the product will remain within the regulatory acceptance criteria throughout its shelf-life.

6.3.4 In-Process Tests

In-process tests are performed during the manufacture of the drug substance or product, as opposed to the traditional prerelease testing. When the acceptance criteria are identical to or tighter than the release specification, the in-process test can be included in the release specification. However, this approach must be validated to show that the characteristics of the product do not change from the in-process stage to final release. In-process tests that are used only to adjust process parameters within an operating range are not normally included in the specifications.

6.3.5 Parametric Release

Parametric release testing involves monitoring of specific batch parameters (e.g., temperature, pressure, time) as an alternative to routine release testing. Appropriate physical or chemical laboratory tests may also be included in parametric release testing. Sometimes, these parameters can be more easily controlled and measured than, for example, sterility. The parametric release process should be maintained in a validated state, as demonstrated by revalidation at established intervals, and the attribute that is indirectly controlled together with the associated parametric test procedures should be included in the specifications.

6.3.6 Pharmacopeial Tests

Wherever they are appropriate, pharmacopeial procedures should be followed. One of the main goals of the ICH process is harmonization of procedures on a global basis, and the United States, Japan, and European pharmacopeias have all expressed a commitment. Eventually, all three pharmacopeias will be considered equivalent and interchangeable.

6.4 UNIVERSAL TESTS/CRITERIA

There are some tests that are considered universal for setting specifications for new drug substances and products. These universal tests include Description, Identification, Assay, and Impurities. Implementation of tests in this category should also take into account general method validation guidelines found in other USP and ICH documents [3,4]. A *Description* constitutes a qualitative statement about the state and color of the new drug substance. *Identification testing* should be able to discriminate between compounds of closely related structure that might be present, and should be specific for the new drug substance. Chromatographic retention time, for example, is not specific; however, the addition of an advanced detection technique such as photodiode array (PDA) or mass spectrometry (MS) is generally acceptable. An *Assay* to determine the new drug substance content should be specific, and stability indicating. Impurities (organic and inorganic impurities and residual solvents) are governed by additional ICH guidelines; organic impurities that are degradants of the new drug substance, and process-related impurities from the new drug product should be monitored, with acceptance limits (5–10).

For many of these tests, reference standards are used that in most cases are more stringently characterized than the substance being regulated. Reference standards should be accompanied by a certificate of analysis from a reputable source and have a quality appropriate for their intended use, including control of impurities, often by procedures not routinely applied in routine testing.

6.5 SPECIFIC TESTS/CRITERIA: NEW DRUG SUBSTANCES

In addition to the foregoing general tests, the following specific tests may be considered for new drug substances.

6.5.1 Physicochemical Properties

Used to measure properties such as pH, melting point/range, and refractive index, these tests are determined by the physical nature of the drug substance and its intended use.

6.5.2 Particle Size

For many formulations, particle size can have a significant effect on dissolution rates, bioavailability, and stability. Testing should be carried out using an appropriate procedure, and acceptance criteria should be provided.

6.5.3 Polymorphic Forms

Differences in polymorphic forms can, in some cases, affect quality or performance of the product, as different crystalline forms can alter physical properties. In the cases where differences are known to exist, the appropriate solid state should be specified. Physicochemical techniques such as melting point (including hot-stage microscopy), solid-state IR, x-ray powder diffraction, thermal analysis (procedures

such as differential scanning calorimetry and thermogravimetric analysis), Raman spectroscopy, and solid-state NMR are often used to determine if multiple forms exist.

6.5.4 Chiral-Drug Substance

When a new drug substance is developed as one enantiomer, the other enantiomer is generally treated in the same way as other impurities. However, sometimes technical limitations prevent the same limits of quantitation from being applied. Nevertheless, an assay for the enantioselective determination of the drug substance should be a part of the specification [11]. The determination can be achieved either by a chiral assay procedure or by the combination of an achiral assay together with an appropriate method to control the enantiomeric purity. For a drug substance developed as a single enantiomer, criteria should be included in the specification when the specific test has an impact on quality for batch control. Identity tests should be capable of distinguishing both enantiomers and the racemic mixture. In general, there are two cases where a stereospecific test is appropriate for release/acceptance testing: where there is a significant possibility that the enantiomer might be substituted for the racemate; and where there is evidence crystallization might lead to unintentional production of a nonracemic mixture.

6.5.5 Chiral-Drug Product

Unless racemization has been shown to be insignificant during manufacture of the dosage form and on storage, stereospecific control for the analysis of degradation products is necessary. On assay, where there is no racemization, an achiral assay may be sufficient. Otherwise a chiral assay should be used, or alternatively the combination of an achiral assay plus a validated procedure to control stereospecificity. For identification, a stereospecific test is not generally employed unless racemization is a concern. Then it is more appropriately covered at the drug substance stage.

6.5.6 Water Content

When the new drug substance is known to be hydroscopic, a test for water content is important. Justification of the specification should include data on the effects of hydration and moisture absorption. A detection procedure specific for water (e.g., Karl Fischer titration) is preferred, but in some cases a loss on drying procedure may be sufficient.

6.5.7 Inorganic Impurities

Inorganic impurities commonly arise from catalysts used in the manufacturing process. The need for tests and acceptance criteria is usually determined during development based on knowledge of the process. Pharmacopeial procedures and acceptance criteria exist for sulfated ash/residue on ignition; other appropriate techniques, such

as atomic absorption spectroscopy, are also commonly used for other inorganic impurities.

6.5.8 Microbial Limits

Where needed, pharmacopeial procedures are used to specify parameters such as the total aerobic microorganism count, the total count of yeast and molds, and the absence of specific objectionable bacteria. The choice of the type of microbial tests and acceptance criteria is based on the nature of the drug substance and method of manufacture.

6.6 SPECIFIC TESTS/CRITERIA: NEW SOLID ORAL DRUG PRODUCTS

For some new drug products, additional testing may be needed, depending on the dosage form. The specific dosage forms highlighted in the guidance include solid and liquid oral drug products and parenterals. For solid oral drug products, specific additional tests include dissolution, disintegration, hardness/friability, and uniformity of dosage units.

6.6.1 Dissolution

Specifications for solid oral dosage forms usually include a test to measure release of the drug substance from the drug product by dissolution. For immediate release formulations, single point determinations are commonly used. For modified release formulations, appropriate test conditions and sampling procedures must be established. In general, multiple time-point rate release curves are called for when testing extended or delayed release formulations. In instances where the rate of release can be demonstrated to significantly affect bioavailability, batch tests that can discriminate between acceptable and unacceptable bioavailability are needed. In this instance, in vitro/in vivo correlation may be used to establish acceptance criteria. In practice, the variability in mean release rate at any given time point should not exceed a total difference of ±10% of the labeled content of the drug substance (i.e., a total variability of 20%; a requirement of 50% means a range from 40% to 60%).

6.6.2 Disintegration

Disintegration may be substituted for dissolution for rapidly dissolving (dissolution >80% in 15 min at pH 1.2, 4.0, and 6.8), highly soluble (dose/solubility volume <250 mL from pH 1.2 to 6.8) new drug products. Disintegration is also appropriate where a relationship to dissolution has been documented.

6.6.3 Hardness/Friability

Hardness/friability is normally performed as an in-process control (addressed previously). It is usually only necessary to include these attributes in the specification if

the characteristics of hardness/friability have a critical impact on product quality (e.g., chewable tablets).

6.6.4 Uniformity of Dosage Units

Uniformity of dosage units in this context refers to both the mass of the dosage form and the content of the active ingredient in the formulation. In general, pharmacopeial methods should be used [12].

6.7 SPECIFIC TESTS/CRITERIA: NEW ORAL LIQUID DRUG PRODUCTS

For oral liquid drug products (and powders intended to be reconstituted as oral liquids), many of the same tests as for solid dosage forms are still appropriate (e.g., uniformity, dissolution, water content), but additional specific additional tests include pH (acceptance criteria and proposed range justified), antimicrobial and antioxidant preservative contents, extractables, alcohol content, particle size distribution, redispersability, rheological properties, and reconstitution time. More details concerning each of these tests can also be obtained directly from the guidelines as space allows only a brief summary here.

For formulations using an antimicrobial or antioxidant, criteria for preservative content should be established. The establishment of criteria for preservative content is usually established by shelf-life stability testing according to established guidelines [13].

Extractables are normally evaluated during development and stability; and after levels are shown to be consistently below acceptable and safe values, elimination of the test is acceptable.

For products that contain alcohol as declared on the label, the content should be specified and quantitative results obtained by assay or calculation.

Some liquid dosage forms can settle on storage, necessitating specifications for redispersability, requiring either mechanical or manual shaking for a predetermined length of time.

For viscous solutions or suspensions, specifications governing rheological properties, such as viscosity, may be appropriate. Both the test and the acceptance criteria should be stated.

A reconstitution specification is appropriate for powder products that require reconstitution. The choice of diluents should be justified.

6.8 SPECIFIC TESTS/CRITERIA: PARENTERAL DRUG PRODUCTS

In addition to some of the foregoing tests, several tests specific to parenteral products must be considered, including a test for endotoxins and pyrogens (typically a limulus amoebocyte lysate test), particulate matter (an acceptance criteria for visible particulates and solution clarity), functionality testing of delivery systems (test procedures and acceptance criteria for the functionality of prefilled syringes or cartridges), and osmolarity.

6.9 DECISION TREES

In an attachment to the specifications guideline, several decision trees are included to help determine appropriate courses of action to establish acceptance criteria. These decision trees are excellent sources of protocol. Table 6.1 summarizes the eight decision trees included in the guidance. Figures 6.1 and 6.2 show examples of two of the decision trees: Figure 6.1 for establishing acceptance criteria for a specified impurity in a new drug substance, and Figure 6.2 for establishing identity, assay, and enantiomeric impurity procedures for chiral new drug substances and products containing chiral drug substances.

6.10 OOS BACKGROUND

It seems to happen in every laboratory, sooner or later, no matter how good a job is done validating the method, no matter how robust; inevitably a result is obtained

TABLE 6.1
Summary of Decision Tree Attachment to the Guideline for Setting Specifications and Acceptance Criteria

Decision Tree	Title	Comments
1	Establishing Acceptance Criterion for a Specified Impurity in a New Drug Substance	Relevant batches are those from development, pilot, and scale-up studies.
2	Establishing Acceptance Criterion for a Degradation Product in a New Drug Product	Refers back to decision tree #1.
3	Establishing Acceptance Criterion for Drug Substance Particle Size Distribution	Helps to determine if acceptance criterion for particle size is required.
4	Investigating the need to set Acceptance Criteria for Polymorphism in Drug Substances and Drug Products	Establishes criteria or justification for exclusion of test.
5	Establishing Identity, Assay, and Enantiomeric Impurity Procedures for Chiral New Drug Substances and New Drug Products Containing Chiral Drug Substances	Numerous footnotes that should be consulted for more detail.
6	Microbiological Quality Attributes of Drug Substance and Excipients	Establishes criteria or justification for exclusion of test.
7	Setting Acceptance Criteria for Drug Product Dissolution	Justification for dissolution versus disintegration; acceptance criteria for single versus multiple-point rate release profiles.
8	Microbiological Attributes of Nonsterile Drug Products	Establishes criteria or justification for skip-lot testing or exclusion of test.

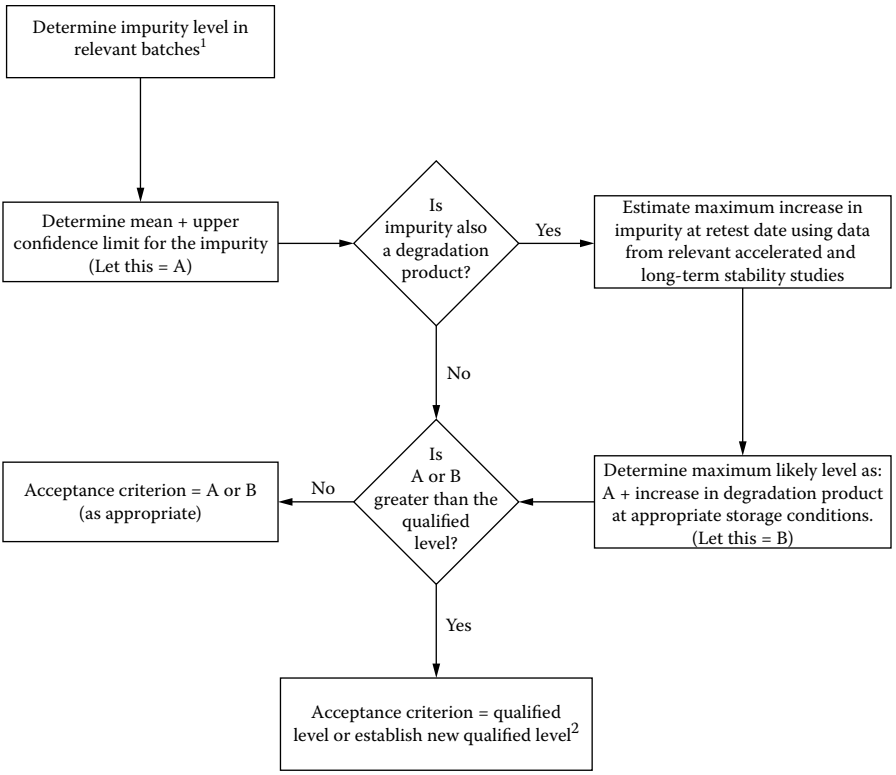


FIGURE 6.1 Establishing acceptance criterion for a specified impurity in a new drug substance. [1]Relevant batches are those from development, pilot, and scale-up studies. [2]Refer to ICH guidelines on Impurities in New Drug Substances [6].

that falls outside the specification or acceptance criteria. While steps can be taken to decrease the frequency of OOS (out of specification) results, it is rare that they can be completely prevented. FDA regulations require that an investigation be conducted whenever an OOS test result is obtained. Therefore, it is essential in a regulated laboratory to have a standard operating procedure (SOP) in place that describes the actions to take to determine the cause of the OOS result, and the corrective action that must be undertaken. A thorough SOP will ensure that correct decisions are made regarding the acceptance or rejection of a batch. And batch rejection does not negate the need to perform an investigation. Thorough and systematic investigation of an OOS result not only leads to scientifically sound decisions, but also is mandated by law in the Code of Federal Regulations (CFR) and by the court's decision in the now-infamous 1993 case of U.S. FDA versus Barr Labs. Indeed, FDA guidance is available on the topic of OOS investigations; and while this chapter will discuss the FDA guidance in some detail, the reader is encouraged to consult the references for additional details [14,15]. FDA guidance documents are always a good source of information because they are prepared for review staff and establish policies intended to achieve consistency in the FDA's policy and regulatory approach, and

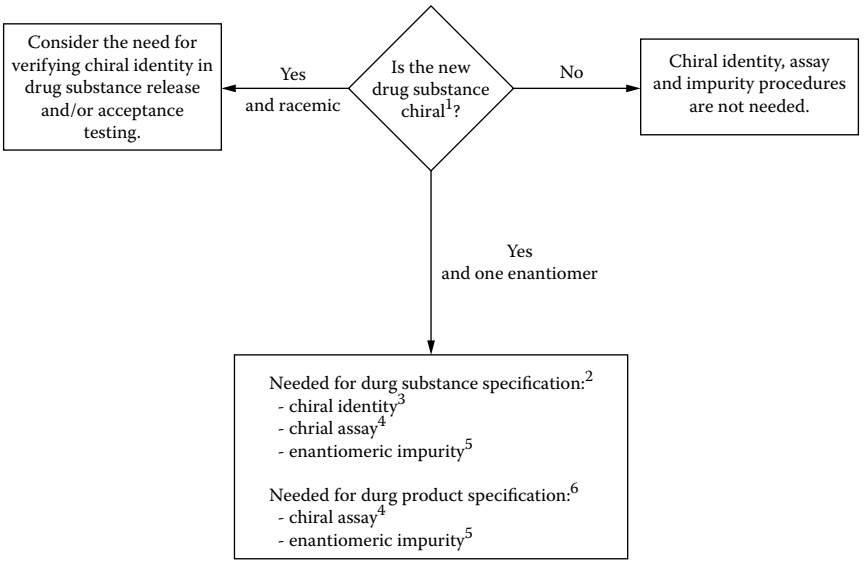


FIGURE 6.2 Establishing identity, assay, and enantiomeric impurity procedures for chiral new drug substances and new drug products containing chiral drug substances. [1]Chiral substances of natural origin are not addressed in this guideline. [2]As with other impurities arising in and from raw materials used in drug substance synthesis, control of chiral quality could be established alternatively by applying limits to appropriate starting materials or intermediates when justified from developmental studies. This essentially will be the case when there are multiple chiral centers (e.g., three or more), or when control at a step prior to production of the final drug substance is desirable. [3]A chiral assay or an enantiomeric impurity procedure may be acceptable in lieu of a chiral identity procedure. [4]An achiral assay combined with a method for controlling the opposite enantiomer is acceptable in lieu of a chiral assay. [5]The level of the opposite enantiomer of the drug substance may be derived from chiral assay data or from a separate procedure. [6]Stereospecific testing of drug product may not be necessary if racemization has been demonstrated to be insignificant during drug product manufacture and during storage of the finished dosage form.

establish inspection and enforcement policies and procedures. FDA good guidance practices state that official procedures should be followed when communicating new or different regulatory expectations that are not readily apparent from current regulations to a broad public audience [16]. However, similar to the recently published FDA guidance on method validation [17,18], guidelines on OOS investigations first appeared in draft, not final form [14]. Draft guidance represents the FDA's current thinking on a particular topic and opens it up for public comment [19]. By issuing draft guidance, the FDA can update guidelines based on advances in technology and knowledge, changes in regulatory requirements, and policy mandates.

The FDA's OOS guidance applies to active pharmaceutical ingredients, excipients, and other components and the testing of finished products to the extent that current good manufacturing practices apply. It discusses how to investigate suspect, or OOS results, including responsibilities, the laboratory phase of the investigation,

additional testing that might be necessary, when to go beyond laboratory investigations, and the final evaluation of test results.

6.11 PREVENTING OOS RESULTS

OOS results can come from laboratory, operator, or process/manufacturing errors. But the best way to minimize the occurrence of OOS results is to prevent them from happening in the first place, and the best way to do that is to have proper laboratory controls in place. The integrity of laboratory testing and record keeping is of fundamental importance to the FDA in pharmaceutical production and control. Proper laboratory controls must include

- Standard operating procedures (SOPs)
- Validated analytical methods
- Properly trained and supervised personnel
- Properly qualified and calibrated instrumentation

SOPs are written for many laboratory activities, including sampling methods, sample handling, test methods, and calibration and maintenance of instrumentation. They are written to ensure uniformity, and are necessary for assuring compliance with regulatory requirements. The SOP covering OOS results must define the responsibility for the investigation and provide clear direction to laboratory personnel.

By validating an analytical method, documented evidence is obtained that the method accomplishes or is suitable for its intended purpose. Compendial USP methods need not be validated, but can simply be verified for suitability under actual conditions of use. Noncompendial methods must be validated with respect to several parameters, including accuracy, precision, linearity, limit of quantitation or detection, robustness, specificity, and range. Both the USP and ICH provide guidelines for validating noncompendial methods [3,4].

Properly trained and supervised laboratory personnel are needed to effectively carry out laboratory operations according to established procedures. Having an adequately trained laboratory staff can cut down on the frequency of retests, investigations, and staff turnover, which are red flags for the FDA.

Similar to a method, an instrument must be suitable for its intended use. Instrument validation, referred to as qualification, is accomplished by performing installation, operational, and performance qualifications, along with documented routine calibrations. By documenting qualification, calibration, and maintenance procedures, demonstrating that the instrument can meet a set of predetermined specifications, one variable (the instrument) can be ruled out in any subsequent investigation. In addition, all analytical methods have system suitability requirements, and systems not meeting these requirements should not be used.

6.12 IDENTIFYING AND ASSESSING OOS TEST RESULTS

So, what constitutes an OOS result? For the purposes of this discussion, we can use the FDA guidance definition: "OOS results include all suspect results that fall

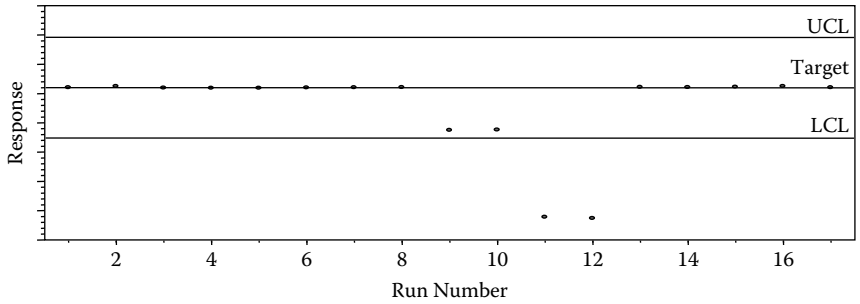


FIGURE 6.3 Typical control chart illustrating OOS results. Charts such as this can be used to monitor for OOS results. By setting an upper control limit (UCL) and a lower control limit (LCL) around a target or average value according to specifications, OOS samples, injections, batches, etc., can be easily observed.

outside the specification or acceptance criteria established in new drug applications (NDAs), official compendia, or by the manufacturer" [15]. Control charts, such as the example shown in Figure 6.3, can be used to easily inspect for OOS results. OOS results can also observed, for example, in chromatography, from system suitability results. Once an OOS result is obtained, an investigation must be launched to determine the cause, and each step of the investigation must be documented. The first phase of the investigation should include an initial assessment of the accuracy of the data, before test solutions are discarded. It is the responsibility of the analyst to review the data for compliance with specifications, and in cases where unexpected results are obtained and no obvious explanation exists, retain test solutions, and inform the supervisor. The supervisor's assessment should be objective and timely, and include the following steps:

1. Discussing the test method with the analyst to confirm that the proper procedure was performed.
2. Examine the raw data, to identify potentially anomalous or suspect information.
3. Confirmation of instrument performance by reviewing qualification and system suitability data.
4. Verify that proper reference standards, solvents, reagents, and other solutions were used, and that they meet quality control specifications.
5. Compare the test method performance to ensure that it is performing to the standard expected based on method validation data.
6. Documented evidence of the assessment.

Examining retained samples promptly is important to facilitate assigning a cause to OOS results. For example, reinjection where a transient instrument malfunction is suspected can provide strong evidence to rule out sample or sample preparation anomalies. However, laboratory error should be relatively rare. Frequent errors suggests inadequate training, poorly maintained or calibrated instruments, or careless work. Whenever laboratory error is identified, corrective action should be taken to prevent the problem from reoccurring.

6.13 INVESTIGATING OOS TEST RESULTS

It should not be assumed that failing test results are attributable to laboratory error without performing and documenting an investigation. If the OOS result cannot be completely attributed to laboratory error, then a full-scale failure investigation must be initiated, with the objective of identifying the source. Varying test results can indicate problems in the manufacturing process, or result from sampling problems, and therefore should be given the highest priority.

6.13.1 General Investigative Principles

The failure investigation should be conducted by the quality control unit involving all other departments that could be implicated. It should consist of a timely, thorough, and well-documented review, and follow these general steps:

1. Clearly identify the reason for the investigation.
2. Summarize the manufacturing process sequences that may have caused the problem.
3. Provide the results of the documentation review with the assignment of actual or probable cause.
4. Determine if the problem has occurred previously.
5. Describe any corrective actions taken.
6. Include a list of other batches and products possibly affected and any required corrective actions, and comments and signatures of appropriate personnel.

6.13.2 Laboratory Phase of an Investigation

During an OOS laboratory investigation, there are three possible outcomes:

- The suspect result is determined to in fact be accurate (or correct) and is no longer suspect.
- The suspect result is determined to be inaccurate (or incorrect) due to an assignable cause.
- The suspect results are determined to be inaccurate (or incorrect) and no assignable cause can be established.

In the latter case, a retest may provide sufficient justification to invalidate or to confirm the OOS. During the laboratory investigation, these outcomes can give rise to three possible scenarios: there is an assignable cause, a retest invalidates the result, or no assignable cause is found and the result is confirmed. Figure 6.4 illustrates an example result investigation summary that might be used during the laboratory investigation. A written outcome, along with any documentation generated during the course of the investigation (e.g., chromatograms, spectra, calculations, observations), should be included with the summary form.

Title, Study or Project # _____

Name of Study Director _____

Sample ID _____

Preinvestigation Phase

☐ Yes or ☐ No—Was the error readily apparent to the analyst or supervisor, for example, was the error a result of transcription, miscalculation, incorrect or incomplete transfer of solutions, incorrect dilution, incorrect setting of instrument parameters, etc.? Where "yes" is indicated, list the cause on the raw data records, analyst and supervisor initial and date, and continue with the analysis without further consideration of the invalid results.

Investigation Phase

☐ Yes or ☐ No—The analyst is knowledgeable in the performance of the procedure, skilled in the tasks required to complete the procedure, and properly executed the correct procedure.

☐ Yes or ☐ No—The raw data (including chromatograms, spectra, etc.), transcriptions, and calculations were reviewed and found to be free of errors or aberrant information.

☐ Yes or ☐ No—The reagents, reference standards, test substances, and the solutions prepared from the same were found to be appropriate.

☐ Yes or ☐ No—The performance of the instrument was found to be adequate (system suitability, check standards, bracketed standards, etc.) and free of sporadic failure.

☐ Yes or ☐ No—The analytical method used to perform the analysis was reviewed and found to be adequate for the intended purpose.

☐ Yes or ☐ No—An assignable cause was identified, and the OOS result is invalidated based on laboratory error.

_____ ____

Area Supervisor/Functional Area Manager or Study Director **Date**

FIGURE 6.4 Example result investigation summary.

6.13.2.1 Assignable Cause

A laboratory investigation may uncover the fact that an error was made or that there was equipment malfunction, and in this case the test result may be invalidated due to an assignable cause. In this case, the analysis is rerun according to the approved procedure, and the new result is reported. In this instance, the laboratory does not have a current good manufacturing practice (cGMP) obligation to report the initial

invalidated result. However, appropriate corrective action should be determined and implemented to prevent any future occurrences.

6.13.2.2 No Assignable Cause/Retest

A retest can provide justification that the suspect result should be invalidated; the investigation may fail to yield an assignable cause, but the result is still considered to be an aberrant or suspect result. In this case, when the analysis is rerun according to an approved written retest plan and is successful (result meets the preestablished acceptance criteria identified in the retest plan), the original results are invalidated pending acceptable review of the retest plan justification.

6.13.2.3 No Assignable Cause/Result Is Confirmed

Even if the investigation fails to yield an assignable cause, the result may still be aberrant or suspect. In this instance the analysis is rerun according to the written retest plan, and the result from reanalysis confirms the original result and must be considered accurate.

During the laboratory phase of an investigation, a number of practices are used. These include retesting a portion of the original sample, testing a new specimen from the collection of a new sample from the batch (resampling), and using outlier testing.

6.13.3 Retesting

Sometimes the investigation may involve retesting a portion of the original sample. Retesting is often indicated when investigating instrument or sample handling problems, for example, a suspected dilution error. The retest sample should be taken from the same homogeneous material that originally produced the OOS result. Decisions to retest should be based on testing objectives and sound scientific judgment, and should always be performed by a second analyst (i.e., not the one who originally obtained the OOS result!). The number of retests should be specified in the SOP, to avoid "testing into compliance," or repeated retesting until a passing result is obtained. If the OOS result is found to be a laboratory error, the retest results are substituted for the original results. The original results must be archived, however, and all explanations documented with the proper sign-offs of all involved. Software that provides for electronic signature sign-off and audit trails helps to maintain regulatory compliance in this regard. If no laboratory or statistical (mathematical) errors can be identified, the original OOS results cannot be invalidated, and must be reported along with the retest results.

6.13.4 Resampling

Resampling is different from retesting because it involves analyzing a new specimen from the collection of a new sample from the batch, as opposed to the analysis of the original sample. Resampling is used when it is suspected that the original sample was not prepared properly, or not representative of the batch. Resampling should be performed by the same qualified, validated methodology used for the original sample.

6.13.5 Averaging (Resampling Testing Data)

Averaging of test data can be a valid approach, depending on the sample and its purpose [20]. In some analytical techniques, several discrete measurements are often averaged to report a test result. For example, an HPLC result may be determined by averaging the peak response from replicate injections of the same sample preparation. In this instance, the average result is considered one test and one result. Reliance on averages has the disadvantage of masking variability among individual test results, however. For this reason, unless averaging is specified by the SOP, all individual test results should be reported, along with a statistical treatment of the variability. This is common, again, in content uniformity assays, where the standard deviation is also reported.

6.13.6 Outlier Tests

cGMP regulations require that statistically sound control criteria include acceptance or rejection levels [21,22]. A result may qualify as a statistical outlier if it is markedly different from the others in a series of results obtained by a validated method. The use of an outlier test should be determined in advance, and again, documented in the SOP, and it should specify the minimum number of results required to obtain a statistically significant assessment. Because an outlier test is only a statistical analysis, it cannot be used to invalidate the data, but is useful for the evaluation of the significance of the result for batch evaluations. One note of caution: in cases where the variability of the product is what is being measured (i.e., content uniformity), an outlier test should not be applied, because a measurement thought to be an outlier may in fact be an accurate result! Table 6.2 provides an example of outlier testing.

6.14 CONCLUDING THE OOS INVESTIGATION

Now it is decision time. To conclude the investigation, following the SOP, the results should be evaluated, the batch quality determined, and a release decision should be made. The goal of the investigation is to arrive at one of two conclusions: either the batch fails and should be rejected (i.e., the OOS result is confirmed), or the OOS result is invalidated and an assignable cause is revealed. The OOS result can only be invalidated upon the observation and documentation of a test result that can reasonably be determined to have caused the OOS result. If the OOS result is confirmed, the batch is rejected.

Of course, there is one other possible outcome. Despite all the controls in place, assessing, identifying, and investigating results may still be inconclusive. In cases where an investigation does not reveal a cause or confirm the OOS result, the OOS result should be retained in the record and taken into account in the batch or lot disposition decision.

Finally, for those products that are the subject of applications, regulations require submitting a field alert report (within three working days) concerning any failure of a batch to meet any of the specifications established in an application. As the saying goes, no job is finished until the paperwork is done!

TABLE 6.2
Summary of Statistics for Testing of Outliers

Outlier: A value in a set of observations that is so different from the rest that it is considered a member of another set or population.

The most common method for testing of outliers is probably Dixon's Q test[a], which reads as follows:

$$Q = [\text{suspect value} - \text{nearest value}]/(\text{largest value} - \text{smallest value})$$

where [suspect value – nearest value] represents an absolute difference, no sign is intended (±). The suspect value is always that number which is farthest from the mean or average value of the set.

Critical values for Q for any probability value ($P = 0.05$ and $P = 0.01$) are found in various tables.[a]

For example: Th.e following values were obtained for the nitrite concentration (mg/L) in a sample of river water: 0.403, 0.410, 0.401, 0.380

The last measurement is suspect; should it be entirely rejected?

We have

$$Q = [0.380 - 0.401]/(0.410 - 0.380) = 0.021/0.030 = 0.7$$

From any table of Q values, the critical value for Q is 0.831 ($P = 0.05$). Because the calculated value of Q does not exceed this, the suspect measurement should be retained.

[a] J.C. Miller and J.N. Miller, *Statistics for Analytical Chemistry*, Halsted Press, Division of John Wiley & Sons, New York, 1986.

6.15 CONCLUSION

Many of the concepts and tests in this chapter are important in the development of harmonized specifications. They are not universally applicable, or necessarily all-encompassing. Tests other than those listed here and in the guidelines may be needed in particular situations or as new information becomes available. New analytical technologies are constantly being developed, and their use is always encouraged where justified. In general, proposals to implement the concepts outlined here and in more detail in the guideline should be justified by the applicant and approved by the regulatory agency prior to implementation.

Whether the topic is setting acceptance criteria, out-of-specification results, system qualification, or method validation, the discussion eventually turns to suitability and acceptability for intended use. And as with all validation topics, the common denominator is doing good science.

REFERENCES

1. ICH Q6A: Specifications for new drug substances and products: Chemical substances. *International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use*, Geneva, Switzerland, March 1995. See also www.ICH.org under topic of Quality guidelines.

2. ICH Q6A: Specifications: Test procedures and acceptance criteria for new drug substances and new drug products: Chemical substances, *Fed. Reg.*, 65(251), 83041–83063, December 29, 2000, Notices. See also www.fda.gov/cder/guidance/index.htm under the topic of ICH Quality Guidelines.
3. Harmonized tripartite guideline, validation of analytical procedures, text and methodology, Q2 (R1). *International Conference on Harmonization*, November 2005, http://www.ich.org/LOB/media/MEDIA417.pdf.
4. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeia Convention, Inc., Rockville, MD, 2011, Chapter <1225>.
5. ICH Q2A(R): Impurities in new drug substances, *Fed. Reg.*, 68(68), 6924–6925, Tuesday, February 11, 2003.
6. ICH Q3B(R): Impurities in new drug products**,** *Fed. Reg.*, 68(220), 64628–64629, Friday, November 14, 2003.
7. ICH Q3C**:** Impurities: Residual solvents, *Fed. Reg.*, 68(219), 64352–64353, Thursday, November 13, 2003.
8. *The United States Pharmacopeia 34/National Formulary 29,* The United States Pharmacopeia Convention, Inc., Rockville, MD, 2011, Chapter <467>.
9. Orr, J. D., Krull, I. S., and Swartz, M. E., Validation of impurity methods. Part 1, *LC/GC Mag.*, 21(7), 626, 2003.
10. Orr, J. D., Krull, I. S., and Swartz, M. E., Validation of impurity methods. Part 2, *LC/GC Mag*., 21(12), 1146, 2003.
11. FDA's policy statement for the development of new stereoisomeric drugs. *Chirality*, 4, 338–340, 1992.
12. *The United States Pharmacopeia 34/National Formulary 29,* The United States Pharmacopeia Convention, Inc., Rockville, MD, 2011, Chapter <905>.
13. ICH Q1A (R2), Stability testing of new drugs and products**,** *Fed. Reg.*, 68(225), Friday, November 21, 2003, pp. 65717–65718.
14. Guidance for Industry, Investigating Out of Specification (OOS) Test Results for Pharmaceutical Production, U.S. Department of Health and Human Services FDA, September 1998.
15. Guidance for Industry, Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production, U.S. Department of Health and Human Services FDA Center for Drug Evaluation and Research (CDER), October 2006. See also http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070287.pdf.
16. 21 CFR 10.115, *Fed. Reg.*, 65(182), 56,468–56,480, September 19, 2000.
17. Analytical procedures and methods validation, *Fed. Reg.* 65(169), 52,776–52,777, August 30, 2000.
18. Brown R., Caphart, M., Faustino, P., Frankewich, R., Gibbs, J., Leutzinger, E., Lunn, G., Ng, L., Rajagopalan, R., Chui, Y., and Sheinin, E., *LCGC*, 19(1), 74–79, January 2001.
19. See http://srpub.phrma.org/letters/12.14.98.oos.commentary.html.
20. Kuwahara, S. S., Validation of impurity methods. Part1, *LC/GC Mag*., *Biopharm Int.*, 11, 31–37, 2007.
21. Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or Holding of Drugs, 21 CFR Part 210, http://www.fda.gov/cder/dmpq/cgmpregs.htm.
22. Current Good Manufacturing Practice for Finished Pharmaceuticals, 21 CFR Part 211, http://www.fda.gov/cder/dmpq/cgmpregs.htm.

7 Validation by Type of Method

7.1 INTRODUCTION

Regulatory guidelines recognize that is it not always necessary to evaluate every analytical performance parameter for every type of method or analytical procedure, or for both drug substances (active pharmaceutical ingredient [API]) and drug products, as discussed in Chapter 4 [1,2]. Several different types of methods are used to measure the API or impurities, degradants, excipients, and additional related substances either in the raw material or finished product during various stages of drug development. The methods discussed in this chapter are impurity (related substances) or stability indicating methods (SIMs), dissolution, bioanalytical methods, peptide mapping, and methods used in support of cleaning validation. The type of method and its intended use, as well as the phase of development, dictate which performance characteristics should be investigated, as summarized in Table 7.1. Both the USP and ICH divide analytical methods into four separate categories:

- Category I: Assays for the quantitation of major components or active ingredients
- Category II: Determination of impurities or degradation products
- Category III: Determination of performance characteristics
- Category IV: Identification tests

These methods and categories generally apply to drug substances and drug products, as opposed to bioanalytical samples, covered in Section 7.5.

In this chapter, the level of validation required corresponding to the type of method and the category, along with some method-type and phase-specific validation criteria are discussed. Some specific methods are addressed, including impurity (related substances) or stability indicating methods (SIMs), dissolution, bioanalytical methods, peptide mapping, and methods used in support of cleaning validation.

7.1.1 Category I Methods

Category I tests target the analysis of major components, and include methods such as content-uniformity and potency-assay analyses. A method used for *assay* is one that measures the active ingredient concentration in a drug product or substance. A *content uniformity* method is similar to an assay method, but specifically targets the measurement of the variability in drug concentration within a batch of

samples. The latter methods, while quantitative, are not usually concerned with low concentrations of analyte, but only with the amount of the API in the drug product. Because of the simplicity of the separation (the API must be resolved from all interferences, but any other peaks in the chromatogram need not be resolved from each other), emphasis is on speed over resolution. For assays in Category I, limit of detection (LOD) and limit of quantitation (LOQ) evaluations are usually not necessary because the major component or active ingredient to be measured is normally present at high concentrations. However, because quantitative information is desired, all the remaining analytical performance parameters are pertinent.

7.1.2 Category II Methods

Category II tests target the analysis of impurities or degradation products (among other applications). An *impurity* test measures the minor components generally unintentionally in the substance or product, originating from the raw material manufacturing, product manufacturing, or degradation during storage or processing. These assays usually look at much lower analyte concentrations than Category I methods, and as seen in Table 7.1 are divided into two subcategories: Quantitative and Limit Tests. If quantitative information is desired, a determination of LOD is not necessary, but the remaining parameters are required. The situation reverses itself for a Limit Test.

TABLE 7.1
Data Elements Required for General Procedure Validation (from USP Chapter 1225)

Analytical Performance Parameter	Category I: Assays	Category II: Impurities Limit Tests		Category III: Specific Tests	Category IV: I.D.
		Quant. Tests	Limit Tests		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
LOD	No	No	Yes	*	No
LOQ	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	No	*	No
Robustness	Yes	Yes	No	Yes	No

Note: Category I: Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products. Category II: Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests. Category III: Analytical procedures for determination of performance characteristics. Category IV: Identification tests. An asterisk indicates the parameter may be required, depending on the nature of the test. For additional details, see Reference 5.

Because quantitation is not required, it is sufficient to measure the LOD and demonstrate specificity and robustness. For a Category II limit test, it is only necessary to show that a compound of interest is either present or not; that is, above or below a certain concentration. Methods used in support of stability studies (referred to as SIMs; Section 7.3) are an example of a quantitative Category II test. A SIM is used to quantify the presence of impurities generated through a forced degradation of the API; it is assumed that this test will enable measurement of any impurities generated during normal or accelerated shelf-life testing of a drug substance or product. Methods in support of cleaning validation and environmental EPA methods often fit into this category, as well as residual solvent testing [3]. Although, as seen in Table 7.1, it is never necessary to measure both LOD and LOQ for any given Category II method, it is common during validation to evaluate both characteristics (more out of tradition than necessity) because often methods are used to fulfill requirements of more than one category.

7.1.3 Category III Methods

The parameters that must be documented for methods in USP-assay Category III (specific tests or methods for performance characteristics) are dependent on the nature of the test. Dissolution testing (Section 7.4) is an example of a Category III method. A *dissolution* assay measures the concentration of API in a solution designed to simulate release of the drug from a formulation under the conditions of administration of the drug (e.g., in simulated stomach fluids). Because it is a quantitative test optimized for the determination of the API in a drug product, the validation parameters evaluated are similar to a Category I test for a formulation designed for immediate release. However, for an extended-release formulation, where it might be necessary to confirm that none of the active ingredient has been released from the formulation until after a certain time point, the parameters to be investigated would be more like a quantitative Category II test that includes LOQ. Because the analytical goals may differ, the Category III evaluation parameters are very dependent on the actual method, as indicated in Table 7.1.

7.1.4 Category IV Methods

Category IV identification tests are qualitative in nature, so only specificity is required. Identification can be performed, for example, by comparing the retention time or a spectrum to that of a known reference standard. Freedom from interferences is all that is necessary in terms of chromatographic separation.

7.2 VALIDATION OF IMPURITY METHODS

To ensure that the data from impurity methods are reliable (precise and accurate), regulated laboratories are expected to validate impurity methods for the API and latter stage key synthetic intermediates. As outlined in Table 7.1, accuracy, precision, linearity/range, specificity, and robustness should all be considered. In addition to these parameters, it is also recommended that sample solution stability should be examined and an appropriate system suitability test established to verify the

proper functioning of the system used for the analyses (2, 4, 5). For example, the stability of a stock reference standard solution is typically evaluated (duplicate injections) at time 0, 3, and 7 days following storage at both room temperature and refrigeration by preparing a fresh dilution at the target concentration level from the stored stock standard and assaying it against freshly prepared system suitability standards prepared from a freshly prepared stock solution. Sample solution stability is evaluated in the same way (duplicate injections) at time 0, 3, and 7 days following storage at both room temperature and refrigerated conditions by assaying a stored sample against freshly prepared system suitability standards.

7.2.1 Classification of Impurities

Active pharmaceutical ingredient (API) batches comprise a number of various substances. For chemically manufactured APIs, the major component is the API itself; however, if the API is a salt, the counterion will also comprise a significant portion of the batch. Other substances (impurities) from various sources are also commonly present at different levels as listed in Table 7.2. The ICH guidance on Impurities in Drugs Substances was accepted by the FDA in 2000 and provides a comprehensive view of what sorts of impurities to expect, how to test for them (in general), how to list them in specifications, and how to qualify their biological safety [6]. These impurities can be organic, inorganic, or solvent related. The nature of the API and the impurities present influence the choice of analytical procedures used in the quantification of impurity levels.

TABLE 7.2
Classification of Impurities

Impurity Type	Examples	Typical Origin
• Organic	• Starting materials • By-products • Intermediates • Degradants • Reagents • Ligands	• Chemical process • Degradants may come from API
• Inorganic	• Reagents • Ligands • Catalysts • Residual metals • Inorganic salts • Filter aids	• Chemical process • Processing equipment • Processing aids (i.e., filter aids)
• Solvents	• Reaction solvents • API isolation solvents • Chromatographic solvents	• Chemical reaction • Crystallization • Precipitation • Extraction or partition • Chromatographic purification

Organic impurities can come from the chemical process or can arise during storage [7]. These impurities may include starting materials, by-products, intermediates, degradation products, reagents, ligands, and catalysts. These may or may not be identified, may or may not be volatile, and may or may not have UV absorption properties similar to the API. Because many organic impurities found in APIs are amenable to HPLC analysis, many impurity methods utilize this technique coupled with UV detection. Because impurities and APIs do not all absorb UV light equally, selection of detection wavelength is important, and an understanding of the UV light absorptive properties of the organic impurities and the API is very helpful. Some organic impurities or APIs, however, do not appreciably absorb UV light. In such cases, HPLC coupled with alternate methods of detection should be employed. Techniques are available such as evaporative light scattering, refractive index, mass spectrometric, and fluorescence detection, and various other element-specific detectors. Each detection technique has its own advantages and limitations. Knowledge of the nature of the API and its impurities is very helpful when selecting the appropriate impurity analytical technique. Application of this sort of knowledge will better ensure the development of precise and accurate impurity methods.

When the API is produced as a salt and the counterion is inorganic, the major inorganic component of the batch is the counterion; however, minor inorganic impurities are typically present in APIs and must also be controlled. Inorganic impurities that can result from the manufacturing process are typically known and identified. They include reagents, ligands, catalysts, heavy or other residual metals, inorganic salts, and other materials such as filter aids. Inorganic impurities are normally detected using procedures found in pharmacopeia or other standard references [6]. Alternative procedures used for the detection of inorganic impurities not listed in the foregoing general literature should always be validated. Based on knowledge of the manufacturing process, one can determine which inorganic impurities may be present in the API. Known metals, used as catalysts, for example, should be controlled during the manufacturing process, if possible. If the desired degree of removal is not achieved prior to API isolation, then metal levels in the API must be determined. Typical techniques for this include atomic absorption spectroscopy and inductively coupled plasma emission spectroscopy. To quantify levels of other inorganic impurities in the API of unknown nature, typically a residue on ignition technique is utilized [8].

Finally, API batches are typically harvested or isolated from a solvent or a mixture of solvents. Solvents used in the API synthesis are generally of known toxicity, and capillary gas chromatography is typically used to quantify levels of residual solvents in APIs [3]. Residual solvents are considered impurities and are listed in three classifications: 1, 2, and 3. Class 1 solvents should be avoided. They are known (or strongly suspected) human carcinogens and environmental hazards such as carbon tetrachloride and benzene. Class 2 solvents should be limited; they are not genotoxic carcinogens but possibly cause irreversible toxicities such as neurotoxicity and teratogenicity. For example, acetonitrile and methylene chloride are class 2 solvents. Class 3 solvents have low toxic potential and include substances such as ethanol, whose permissible daily limit (PDL) allows for APIs containing 0.5% ethanol.

7.2.2 Impurity Method Validation Documentation

Impurity method validation begins with the preparation of a validation protocol that should be reviewed and approved by the appropriate departments (such as Analytical Chemistry, Quality Control, and Quality Assurance) [(9); for an example of a generic method validation protocol, see Appendix A]. The validation protocol should describe the test method, performance parameters to be validated, exactly how the performance parameters will be validated (i.e., descriptions of how test samples will be prepared and analyzed), and validation results acceptance criteria. The validation report should cross-reference the validation protocol, should describe the results obtained, and the conclusions made (including the passing or failing of predetermined acceptance criteria). Furthermore, deviations from the validation protocol should be documented and justified as well. The HPLC (or other instrument technique, e.g., GC, etc.) impurity method report (also referred to as the impurity method SOP) should be attached to the validation protocol. It should describe exactly how to execute the test method. It should include a list of instrumentation and related supplies (i.e., column) to use (including acceptable instrument manufacturers and models), a list of the reagents and solvents for use (including the grade and manufacturer), the exact sample preparation instructions (including blank, reference standard, system suitability, and test samples), a description of the instrument operating conditions (sample injection volume, flow rate, gradient parameters and column re-equilibration time [if applicable], detection wavelength, run time, etc.), injection sequence, instructions for calculating system suitability and test sample analysis results (with example calculations), system suitability acceptance criteria, and figures of sample and blank chromatograms clearly indicating how to integrate each impurity peak.

Although not an ICH or FDA requirement, experience has shown that it is also of value to have HPLC impurity method reports reviewed and approved by an analyst and supervisor in the department to which the method will be transferred. The value of this review is that the method recipients have an early opportunity to review and provide constructive feedback about the method well before it is ever transferred to their department. This review has a significant and positive impact on method transfer (Chapter 8).

7.2.2.1 Reporting Impurity Content of API Batches

Once an impurity method has been successfully validated and the validation report has been written and approved, the method is suitable for use in the analysis of clinical API batches. The ICH guidelines address reporting of impurity content in API batches [6]. Organic impurity levels are typically determined by an HPLC impurity assay. Patients must be protected from exposure to significant levels of impurities whose toxicities have not been qualified (through biological testing). If the toxicology lot is manufactured separately (as is often the case) from any of the clinical lots (Phase I, II, or III), then the clinical lot impurity profile must be compared to that of the toxicology lot. This is needed to ensure that patients are not exposed to unacceptable levels of unqualified impurities.

It is therefore very important to understand acceptable means of reporting API batch impurity levels, because this information is used to determine, in many

TABLE 7.3
Impurity Reporting Thresholds

Maximum Daily Dose	Reporting Threshold	Identification Threshold	Qualification Threshold[a]
≤2 g/day	0.05%	0.10% or 1.0 mg per day (whichever is lower)	0.15% or 1.0 mg per day (whichever is lower)
>2 g/day	0.03%	0.05%	0.05%

[a] Qualification in this context refers to safety/toxicological testing.

cases, if an API batch intended for clinical use is acceptable. Impurities are classified in an HPLC impurity profile by retention time (or retention time relative to the API) and structure (if known). Quantitative results should be presented numerically. Individual impurities and total impurity levels greater than 1% should be reported to one decimal place (for example, 1.4%). On the other hand, impurities present at levels less than 1% should be reported to two decimal places (for example, 0.23% and 0.07%). Results should be rounded using conventional rules as described in the ICH and USP guidelines [6,10]. The ICH guidelines state that all impurities at a level greater than the reporting threshold should be summed and reported as total impurities [6]. Information on impurity-reporting thresholds is shown in Table 7.3.

The specifications for APIs at the New Drug Application (NDA) stage should include a list of impurities to be controlled, based on those observed in API batches manufactured with the proposed commercial process. The structures of these impurities may be known or unknown. By NDA filing, a rationale for impurity limits based on appropriate safety (toxicology) or human clinical studies should also be proposed.

7.2.3 Specificity in Impurity Methods

Specificity testing is probably the most complex but also the most interesting part of impurity method validation. The goal is to design an analytical method that separates all impurities from each other and the API peak. Impurity methods must be specific to ensure that levels of all impurities in the API are accurately measured. For an impurity method to be acceptable, impurity and API peaks should be well resolved from each other. Because method specificity must be satisfied by the conditions chosen in the separation, it makes the most sense to examine method specificity first, before moving on to other validation criteria. If the current chosen chromatographic conditions do not satisfy method specificity requirements, then the analytical method will require further optimization. Only once the method has been optimized to satisfy method specificity, can the remaining validation characteristics be addressed with confidence.

During method development and prior to the Investigational New Drug Application (IND) filing, the analytical researcher must first identify API samples that contain impurities that are expected to be in the toxicology and clinical API

batches. The impurity challenge depends, in part, upon the chemical process utilized to synthesize the API. By working closely with colleagues in the process chemistry department, representative samples containing impurities to be expected in the API can usually be obtained. Degradants can be obtained from forced degradation studies or from actual stability samples [11,12].

Two main challenges exist at this first stage: resolving impurities from each other and resolving impurities from the API. Peak resolution is verified by inspecting the chromatograms. Resolution from the API is verified by inspecting the chromatograms (to ensure the peaks are Gaussian), analyzing peaks for homogeneity by photodiode array (PDA) detection, liquid chromatography/mass spectrometry (LC/MS), and by chromatographic methods of alternate selectivity. For additional information on the use of PDA and MS detection in impurity analysis, see Section 7.3.1.4.

7.2.4 Accuracy in Impurity Methods

Guidelines recommend that accuracy be assessed on samples spiked with known amounts of impurities [1,2,6]. In ideal cases, increasing amounts of known impurities are simply spiked into the API test sample, and the closeness of the obtained result to that of the known amount of each impurity in the sample plus the added amount are measured. This determination takes place individually for each impurity.

However, early in the drug development process, impurity and degradant standards are not always available. In such cases, it is acceptable to compare impurity values from the procedure under validation to an alternative impurity assay, such as an alternative impurity assay designed during the method specificity testing. In this instance, the impurity content value for each impurity from one procedure is compared to those obtained from a second, well-characterized procedure.

When authentic impurity standards are not available, it is acceptable to use the API response factor when measuring impurity levels. In these cases, the accuracy of the impurity measurement relies on the closeness of the impurity response factor to that of the API. Finally, the method should specify how the individual or total impurities should be determined, for example, by wt./wt. assay (versus external, authentic impurity standards, or the API diluted to a concentration close to that of the expected individual impurity levels), or by area-%. In all cases, impurity levels should be expressed with respect to the major analyte.

7.3 STABILITY INDICATING METHOD

Stability testing is performed during drug development to provide evidence of how the quality of a drug substance or drug product changes over time in response to a variety of environmental factors. Factors such as temperature, humidity, and light are studied to establish shelf life for the drug product and recommended storage conditions or packaging [11,12]. But before stability studies can be initiated, a SIM must be developed and validated to quantitatively measure potency and impurity levels, to provide the type of information ultimately used for the validation of impurity/degradant methods (Section 7.2).

7.3.1 Developing and Validating SIMS

A SIM is a quantitative analytical procedure used to detect a decrease in the amount of the active pharmaceutical ingredient (API) present due to degradation. According to FDA guidelines, a SIM is defined as a validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences such as degradation products, process impurities, excipients, or other potential impurities, and the FDA recommends that all assay procedures for stability studies be stability indicating [4]. During stability studies, HPLC is routinely used to separate and quantitate the analytes of interest. There are three components necessary for implementing a SIM: sample generation, method development, and method validation.

7.3.1.1 Generating the Sample

SIMs are routinely developed by stressing the API under conditions exceeding those normally used for accelerated stability testing. In addition to demonstrating specificity in SIMs, chemical stress testing, also referred to as forced degradation, can also be used to provide information about degradation pathways and products that could form during storage, and help facilitate formulation development, manufacturing, and packaging. Stressing the API in both solutions and in solid-state form generates the sample that contains the products most likely to form under most realistic storage conditions, which is in turn used to develop the SIM. In simplest terms, the goal of the SIM is to baseline resolve all the resulting products (the API and all the degradation products) each from the other (i.e., no co-elutions). Table 7.4 lists some common conditions used in conducting forced degradation studies for drug substances [13].

Samples should be stored in appropriate vessels that allow sampling at timed intervals (if desired), and that protect and preserve the integrity of the sample. Thermostated and humidity-controlled ovens should also be employed. Generally, the goal of these studies is to degrade the API from 5% to 10%; any more, and relevant compounds can be destroyed, or irrelevant degradation products produced (e.g., degradation products of the degradation products!); any less, and important

TABLE 7.4
Common Conditions Used in Forced Degradation Studies

Study	Conditions
Acidic pH	0.1 N HCl
Neutral pH	pH 7.0 phosphate buffer
Basic pH	0.1 N NaOH
Oxidation	O_2 Atmosphere, or H_2O_2
Photolysis (UV)	1000 watt-h/m^2 (ICH-Ref. 2)
Photolysis (Fluorescence)	6×10^6 lux h (ICH-Ref. 2)

Note: Acid and base solutions should be neutralized before analysis. Initial sample concentrations in the range of 1–10 mg/mL are normally used.

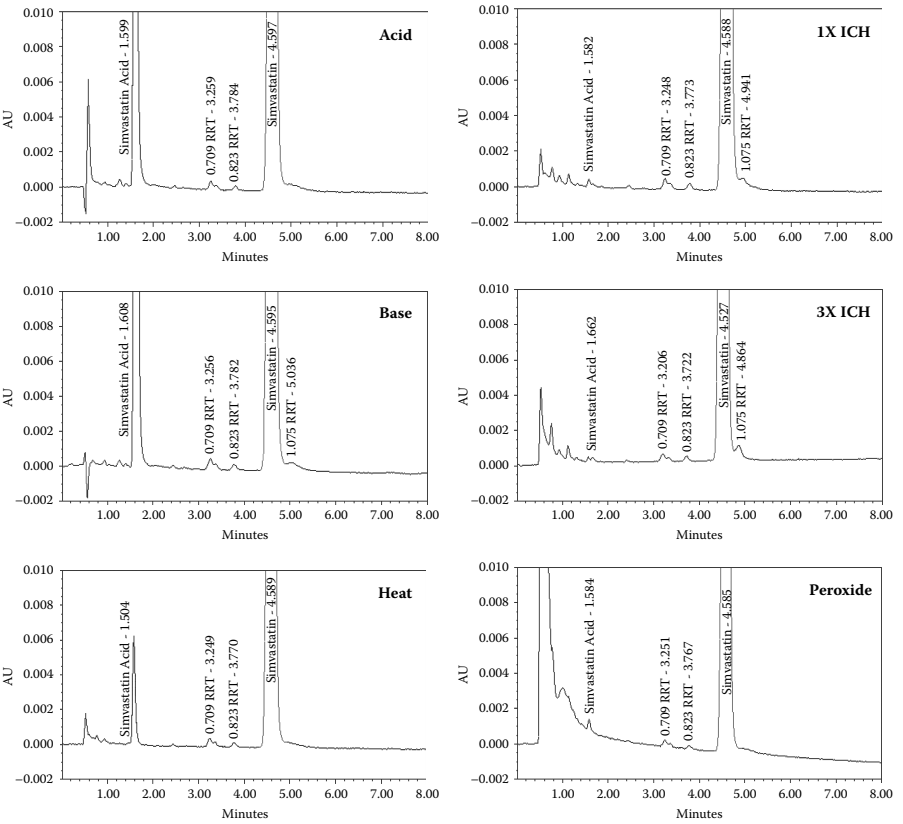


FIGURE 7.1 Example chromatographic comparison of forced degradation experiments. Chromatographic conditions used an isocratic mobile phase of 45/55 ammonium acetate pH 4.5/acetonitrile at 0.6 mL/minute. Column was a 3.0 by 75 mm 2.7 µm C_{18} Halo (MAC-MOD Analytic, Chadds Ford, Pennsylvania). A 5-µL injection and UV detection at 238 nm were used. Test compound is simvastatin, subjected to the kind of forced degradation conditions outlined in Table 7.4.

products might be missed. Experience and data obtained from studies performed previously on related compounds should also be used when developing new protocols. Figure 7.1 illustrates some comparison chromatograms of a forced degradation study using conditions similar to those outlined in Table 7.4.

7.3.1.2 Developing the LC Method

Once the sample is generated through the use of a properly designed and executed forced degradation, it can be used to develop the HPLC method. Nowadays, HPLC method development is often performed on gradient systems capable of automated column and solvent switching, and temperature control. Systems and software that automate the process, some with decision making built in, have also been reported [14,15]. Scouting experiments are often run, and then conditions chosen for further

optimization. Resolving power, specificity, and speed are key chromatographic method attributes to keep in mind during method development. More details on HPLC method development process can be found in Chapter 3, and excellent resources are available to anyone not already schooled in the art [15]. The following sections, however, focus on a few highlights and new developments specific to developing SIMs.

7.3.1.3 Manipulating Chromatographic Selectivity during Method Development

Selectivity can be manipulated by any one or a combination of different factors that include solvent composition, type of column stationary phase, and mobile phase buffers and pH. Chromatographers for the most part are comfortable changing solvents and column stationary phases to generate a separation. However, advances in HPLC column technology have recently made possible the use of pH as a true selectivity tool for the separation of ionizable compounds [16,17]. These hybrid chemistry columns take advantage of the best of both the silica and polymeric column worlds. They are manufactured using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups, resulting in columns that are mechanically strong, with high efficiency, and operate over an extended pH range.

The graphics in Figure 7.2 illustrate why pH can be such a useful tool. As seen in Figure 7.2, acidic compounds are more retained at low pH, while basic compounds are more retained at higher pH (neutral compounds are, of course, unaffected). At pH values utilized traditionally (pH 4–8), a slight change in pH would result in a dramatic shift in retention (upslope or downslope of curve). However, by operating at pH extremes, not only is there 10- to 30-fold difference in retention that can be exploited in method development, but the method can be made more robust as well, a desirable outcome with validation in mind. Indeed, the selectivity differences afforded by a

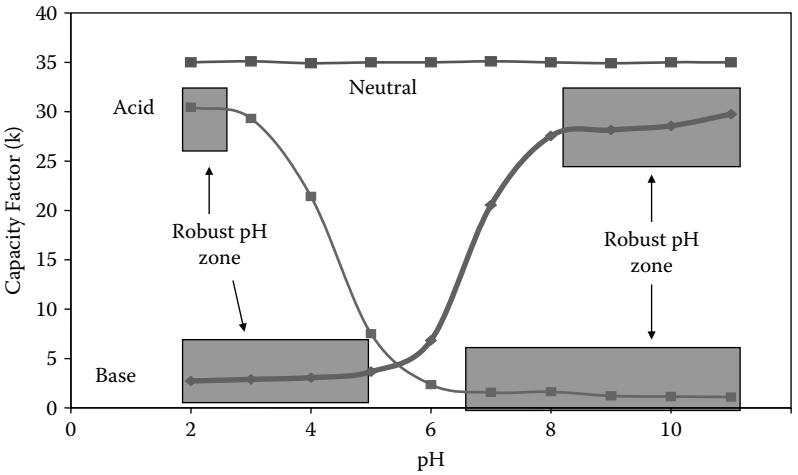


FIGURE 7.2 Reversed-phase retention behavior as pH is varied.

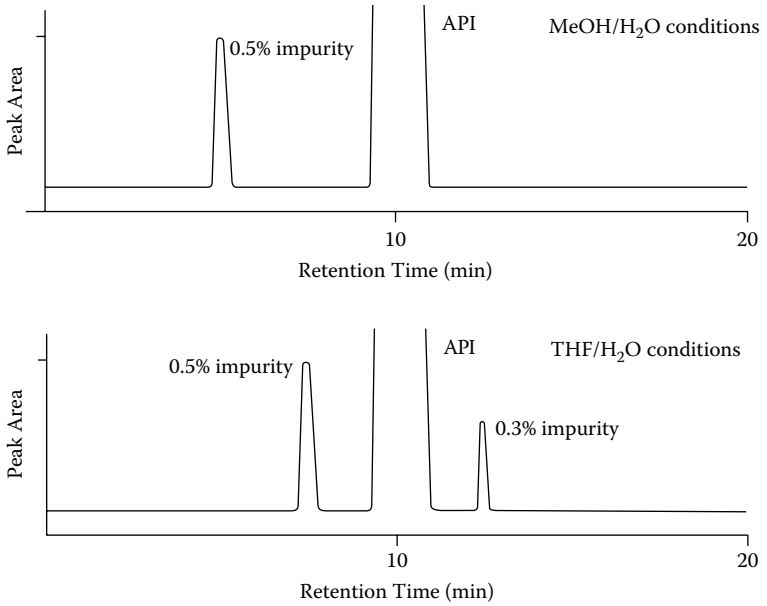


FIGURE 7.3 Hypothetical example: Improved resolution achieved by changing mobile phase selectivity.

change in pH are the equivalent of a 20% change in the organic solvent composition, and are often underutilized.

Another valuable, yet traditional approach to manipulating method specificity utilizes methods with alternate or orthogonal selectivity, as illustrated in Figure 7.3. In this example, the preferred analytical method is 20 min long, and utilizes an isocratic elution of a C_{18} HPLC column with 50/50 MeOH/H_2O. However, only one impurity (0.5%) is observed by this method, and it elutes at a retention time of 5 min, while the API elutes at a retention time of 10 min, and accounts for 99.5% of the integrated chromatogram peak area; no other impurities are observed, and all impurities elute within the run time. To determine if any impurities are co-eluting with the API, the method selectivity was then changed. Changing the elution conditions from 50/50 MeOH/H_2O to 30/70 THF/H_2O, the API peak still elutes at 10 min retention time, but the 0.5% impurity now shifts to 7.5 min, and a new impurity, present at a level of 0.3%, is observed at 12.5 min. Furthermore, the API peak now only represents 99.2% of the integrated chromatogram peak area. This strongly suggests that the 0.3% impurity co-eluted with the API peak when using the H_2O/MeOH conditions. If the 0.3% impurity was an API stereoisomer with the same mass spectrometric fragmentation pattern, then this approach, while traditional, would have been the best one to use to solve this problem.

Other approaches to changing selectivity include changing columns, using different modes of HPLC (e.g., hydrophilic interaction chromatography, or HILIC), or employing entirely different techniques orthogonal to HPLC, such as capillary electrophoretic techniques, gas chromatography, and thin layer chromatography.

7.3.1.4 Evaluating Specificity during SIM Development

Another key parameter to evaluate during SIM development is specificity. The USP and various ICH guidelines define specificity as the ability of a method to unequivocally assess the analyte of interest in the presence of potential interferences (1, 2). In the past, it was acceptable to evaluate resolution, peak shape, and tailing factors to measure and document specificity. However, starting with USP 25, and as a direct result of the ICH process, it was recommended that a peak purity test based on photodiode array (PDA) detection or mass spectrometry (MS) be used to demonstrate that a given peak was pure—that nothing co-elutes.

Modern PDA technology is a powerful tool for evaluating specificity. PDA detectors can collect spectra across a range of wavelengths at each data point collected across a peak, and through software manipulations involving multidimensional vector algebra, compare each of the spectra to determine peak purity. In this manner, PDA detectors today can distinguish minute spectral and chromatographic differences not readily observed by simple overlay comparisons [18–20]. To be successful, three components are required:

1. A UV chromophore, or some absorbance in the wavelength range selected
2. Some degree of chromatographic resolution
3. Some degree of spectral difference

Figure 7.4 shows an example of a partial reversed-phase LC separation, where, by all appearances, the peaks are certainly well resolved, sharp, and symmetrical.

An examination of peak two indicated the peak was pure. However, a close examination of the spectral information related to peak one reveals a different situation.

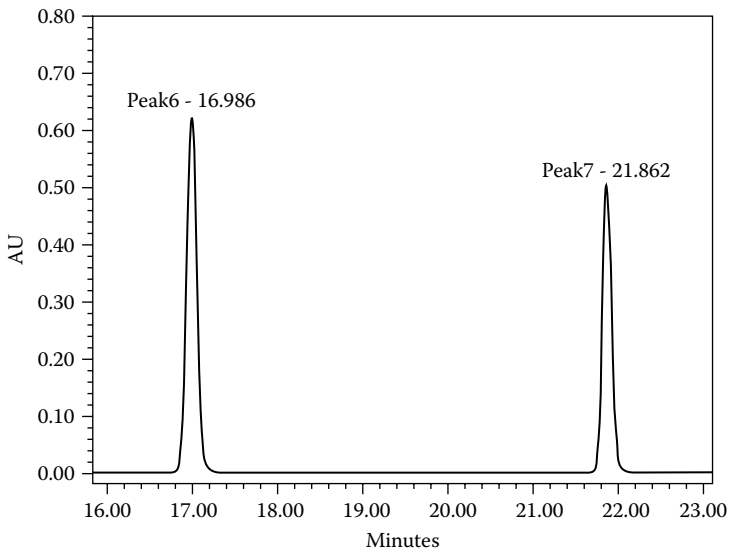


FIGURE 7.4 Example PDA chromatogram used to evaluate specificity/peak purity.

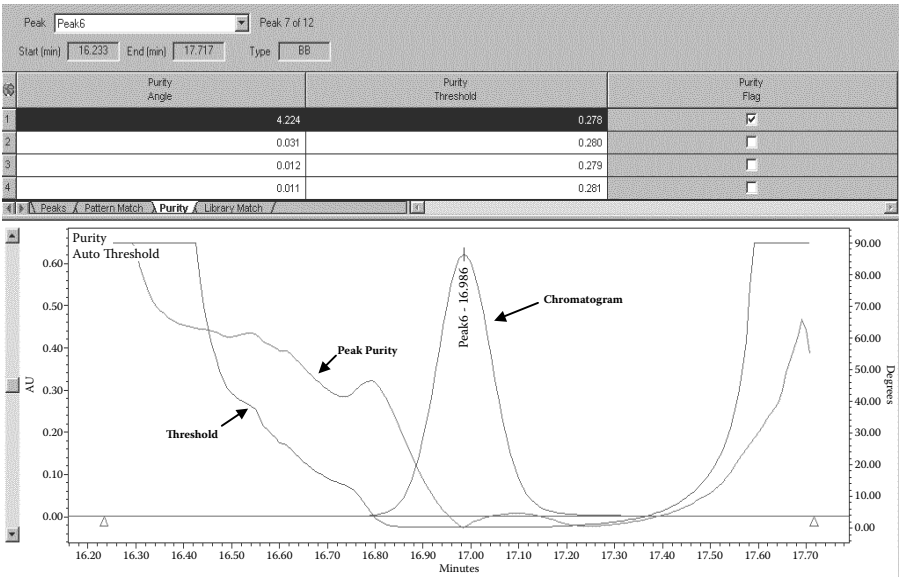


FIGURE 7.5 Example PDA peak purity plot for peak 6 in Figure 7.4.

In Figure 7.5, the calculated peak purity is plotted against the noise threshold, both superimposed on the chromatographic trace. The purity plot clearly indicates a co-elution in the front of the peak as the purity plot exceeds the threshold, and more method development work is necessary.

PDA detectors can be limited on occasion in evaluating peak purity, governed by the three foregoing required components, as well as the noise of the system, and disparate levels of absorbance responses. If impurities co-eluting with the API are present at low levels (0.1%–0.5%), it will be difficult for the peak purity software to detect the very subtle API peak spectra changes even if the impurity chromophores differ from the APIs. This limitation is a significant concern because impurities greater than or equal to 0.10% not qualified for safety by toxicology testing (i.e., not present in the toxicology batch) are typically not permissible in clinical API batches unless their structures and toxicities are known. The more similar the spectra and the lower the relative absorbances, the more difficult it may be to distinguish co-eluting compounds.

Liquid chromatography/mass spectrometry (LC/MS) is a very powerful tool to use for peak purity analysis, and mass spectroscopy (MS) detection has become the detector of choice for even routine method development in many laboratories today. MS has come a long way from the days in which many companies had a dedicated central MS lab and staff. Modern mass spectrometers are smaller, simpler, and operate from the same software used to operate the chromatographic system or other detectors commonly utilized, thus decreasing the learning curve. MS can provide unequivocal peak purity, exact mass, structural, and quantitative information depending on the type of instrument used. MS is also a very useful tool to track peaks as they move around in response to selectivity manipulations in method development. Figure 7.6 is an example of how mass spectra can be extracted from a

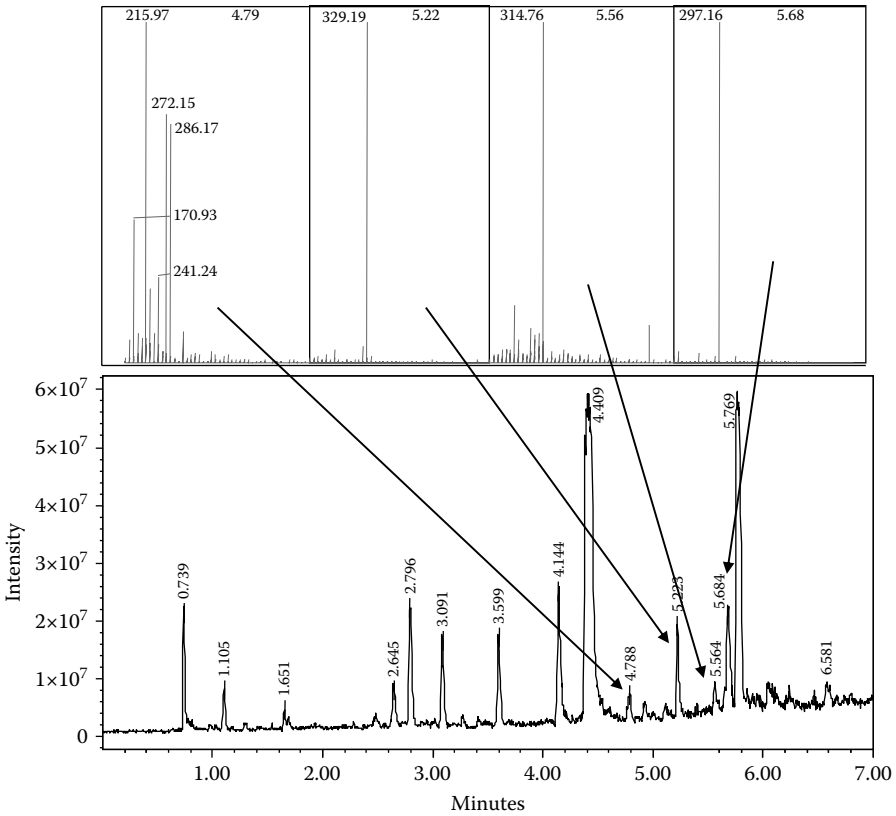


FIGURE 7.6 Extracted single quadrupole mass spectra from impurity peaks in a UHPLC separation of ranitidine and related substances. Conditions: 2.1 × 100 mm, 1.7-µm C18 column, 50°C, 0.45 mL/min. UV detection at 230 nm, 1.0 µL injection. Mobile Phase A: 20 mM ammonium bicarbonate pH 9.0, Mobile Phase B: Methanol. Gradient from 4% to 90% B in 7 min. (*Source:* Figure courtesy of Waters Corporation.)

SIM result and used to identify some of the degradation products in an API, even at trace levels.

Selective ion monitoring by LC/MS allows for the detection of single substances as they elute, based on their mass-to-charge ratio and fragmentation pattern. This tool gives the researcher a greater ability to detect peak inhomogeneity. For example, very low-level impurities co-eluting with the API peak (with different masses and/or fragmentation patterns) may be detected.

Once method specificity for the API and impurities has been established, method specificity must be examined to ensure degradants are also well resolved. Success with this part of the validation is needed to ensure the method will be stability indicating. Typically, during stability testing, the API will partially degrade under the more stressful (forced degradation) conditions (Section 7.3.1.1). The interesting part of this research is designing conditions that will only partially degrade the API. This is where the analytical researcher has the opportunity to employ chemical

knowledge as it relates to the API structure, in order to achieve these degradations. After API partial degradation, the samples are quenched, if necessary, to stop further reaction, and are analyzed to ensure that all impurity and degradants peaks are resolved and all degradants are resolved from each other and the API (utilizing peak purity analyses approaches, as described earlier).

Only the combination of both PDA and MS on a single instrument and software platform provides the type of valuable orthogonal information required when evaluating specificity and developing SIMs.

7.3.1.5 New Technology for SIM Development

Resolving power, specificity, and speed are key chromatographic method attributes to keep in mind during SIM development. Recently, new chromatographic technology has been introduced that capitalizes on small, 1.7-µm particle column packings that can dramatically impact the analysis (method development and validation) of degradation products by providing much-improved resolution and sensitivity [21–24]. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time in the gradient mode) can be extended to new limits, termed Ultra High Performance Liquid Chromatography, or UHPLC. Using UHPLC, it is possible to take full advantage of chromatographic principles to run separations using shorter columns or higher flow rates for increased speed with superior resolution and sensitivity, important attributes for SIMs. Figure 7.7 is an example of a SIM developed for the analysis of a drug-product-related degradation study.

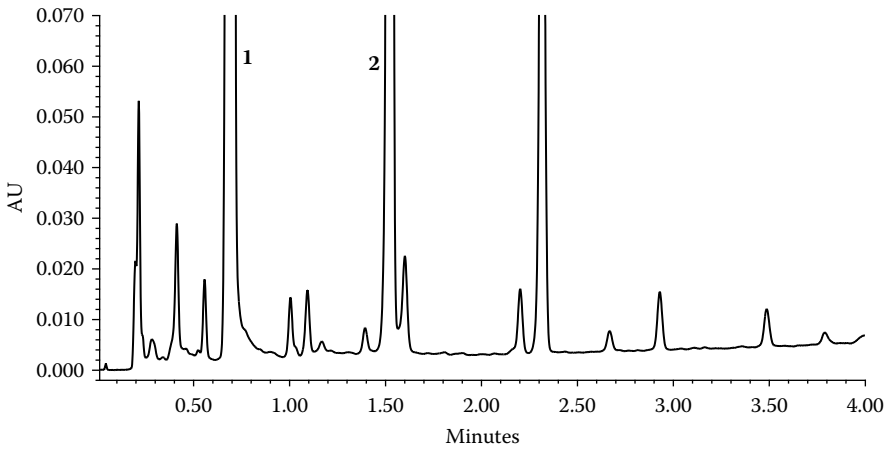


FIGURE 7.7 UHPLC separation of a hydrocodone/acetaminophen formulation subjected to forced degradation at accelerated temperature. A 1.7-µm 2.1 by 50 mm ACQUITY UPLC BEH C_{18} Column (Waters, Milford, Massachusetts) at 30°C was used, and a 3%–20%B linear gradient over 0.8 min, at a flow rate of 0.8 mL/min. Mobile phase A was pH 2 sodium phosphate; B was acetonitrile. UV detection at 233 nm. Injection volume 5.0 µL. Peak 1 is hydrocodone; peak 2 is acetaminophen. The remaining peaks are unidentified degradation products. (Courtesy of Waters Corporation.)

A comparable HPLC separation would take 60 min to perform, not including column reequilibration. UHPLC accomplishes the same separation in much less time (4 min) and with better resolution and sensitivity. And because UHPLC uses the same theories and principles as HPLC, due to the high speed of the UHPLC analyses, methods can be developed much faster. For more details on UHPLC, see Section 3.3.3.

7.3.2 SIM Validation

SIMs fall into the quantitative division of Category II (Section 7.1.2); and as such, all analytical performance parameters must be determined except for the limit of detection (limit of quantitation would apply instead because SIMs need to be quantitative; however, it is commonplace to determine both, as reporting and quantitation levels of impurities can differ). Details of each of the remaining analytical performance parameters (definitions, measurement, and documentation) have been summarized previously (see Chapter 4), and it is really not necessary to repeat them here. However, it should be noted that the hyphenated techniques (e.g., LC/PDA, LC/MS, and LC/MS/MS) and new technology (e.g., UHPLC) outlined previously for method development (Chapter 3) can also be used to great advantage in method validation. For example, specificity (selectivity), while certainly a goal in method development, must also be demonstrated for proper validation. Therefore, all of the foregoing discussion relating to specificity (selectivity) in method development would also apply to validating the method.

7.4 DEVELOPING AND VALIDATING DISSOLUTION PROCEDURES

In vitro dissolution performance tests for solid oral dosage forms, such as tablets and capsules, are used to assess the lot-to-lot quality of a drug product, guide development of new formulations, and ensure the product quality and performance after changes in the manufacturing process, for example, moving to a different site or scale-up. As with any performance test performed in a regulated environment, the dissolution procedure must be properly developed and validated. Dissolution testing is an example of a Category III test (Table 7.1, Section 7.1.3).

The dissolution performance test is a required test for all solid oral dosage forms for product release testing. It is also commonly used as a predictor of a drug product's in vivo performance. To help satisfy dissolution requirements, the USP provides information by way of a general chapter on dissolution, as well as related chapters on disintegration and drug release [25–28]. These USP chapters also provide guidelines on development and validation of dissolution procedures, and should be consulted for additional details.

In vitro dissolution data, together with bioavailability, and chemistry, manufacturing and control (CMC) data, is a critical component of any new drug application submitted to the FDA. A dissolution test is really a simple concept; a tablet or capsule is placed in a known volume of media, and as it dissolves the resulting solution is sampled over time, and assayed (often by HPLC, but also by spectrophotometry) for the level of API present. However, the design, development, and the validation of the procedure can be quite involved, especially when one considers that not only

must the dissolution procedure be developed and validated, but also any analytical technique used for the assay.

7.4.1 Qualification and Calibration

Prior to undertaking the task of dissolution procedure development and validation, it is necessary to invest some time and energy up-front to ensure that the dissolution system itself is validated, or *qualified*. Qualification is a subset of the overall validation process that verifies proper module and system performance prior to the instrument being placed on-line in a regulated environment, and additional information about Analytical Instrument Qualification (AIQ) can be found in Chapter 2 of this volume. Analysts for years have used prednisone and salicylic acid tablets to qualify and "chemically" calibrate dissolution instruments. Figure 7.8 illustrates example HPLC methods commonly

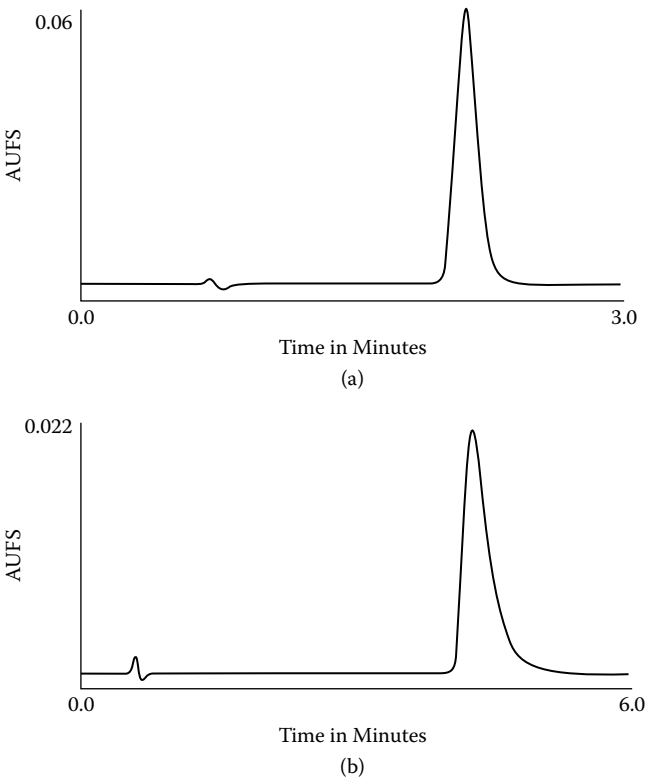


FIGURE 7.8 Example chromatograms from methods used for the chemical calibration or qualification of a dissolution system by HPLC. (a) HPLC separation of a 20-µL injection of a 0.01 mg/mL (in water) prednisone USP standard. Column: 3.9 by 50 mm C_{18}. A mobile phase of water/methanol 50/50, at a flow rate of 1.0 mL/min was used. Detection was by UV at 254 nm. (b) HPLC separation of a 20-µL injection of a 0.1 mg/mL (in water) salicylic acid USP standard. Column: 3.9 by 50 mm C_{18}. A mobile phase of 1.6% acetic acid/methanol 85/15, at a flow rate of 1.0 mL/min was used. Detection was by UV at 270 nm.

used for this purpose. Calibrator tablets and reference standards are available from the USP with lot-specific specifications to aid in qualifying dissolution baths and associated equipment. Recent FDA guidelines suggest that alternative mechanical calibrations can also be used, and when properly executed, satisfy cGMP requirements [29,30].

7.4.2 Dissolution Procedure Development

The dissolution procedure has several distinct components. These components include a dissolution medium, an apparatus, the study design (including acceptance criteria), and the mode of assay. All these components must be properly chosen and developed to provide a method that is reproducible for within-laboratory day-to-day operation, and robust enough to enable transfer to another laboratory.

7.4.2.1 Dissolution Medium

Selection of the most appropriate media conditions is based on discriminatory capability, robustness, stability of the analyte in the test medium, and relevance to in vivo performance, where possible. When selecting the dissolution medium, physical and chemical data for the drug substance and drug product must be considered, for example, the solubility and solution state stability of the drug as a function of the pH value. Other critical drug product properties include the release mechanism (immediate, delayed, or modified) and disintegration rate as affected by formulation hardness, friability, presence of solubility enhancers, and presence of other excipients. When selecting the composition of the medium, the influence of buffers, molarity, pH, and surfactants on the solubility and stability of the drug need also must be evaluated.

The most common dissolution medium is dilute hydrochloric acid; however, other media used includes buffers in the physiologic pH range of 1.2 to 7.5, simulated gastric or intestinal fluid (with or without enzymes), water, and surfactants (with or without acids or buffers) such as polysorbate 80, sodium lauryl sulfate, and bile salts. The use of aqueous–organic solvent mixtures, while generally discouraged, can also be used if justified. Enzymes are also sometimes used in the media when testing gelatin capsule products.

Media volumes are typically in the range of 500 to 1000 mL, with 900 mL being the most common volume. Volumes as high as 2 to 4 L have been used, and as low as 100 mL for high-potency (low dosage strength) drug formulations. Media deaeration is usually required, and can be accomplished by heating the medium or (more commonly) filtering the medium or placing it under vacuum for a short period of time. USP Chapter <711> contains additional information on deaeration [26]. During method development, results from dissolution samples run in a nondeaerated medium versus a deaerated medium should be compared to determine whether deaeration is necessary.

When developing a dissolution procedure, one general goal is to have "sink" conditions. Sink conditions are defined as the volume of medium that is at least three times that required in order to form a saturated solution of drug substance. Dissolution results will more accurately reflect the properties of the dosage form when sink conditions are present.

7.4.2.2 Dissolution Apparatus

USP Chapter <711> lists seven different types of dissolution apparatus [26]. The choice of apparatus is based on the dosage form performance in the in vitro test system. For solid oral dosage forms, the most frequently used apparatus are Apparatus 1 (basket) and Apparatus 2 (paddle). Additional apparatus used include Apparatus 3 (reciprocating cylinder), which is especially useful for bead-type modified-release dosage forms; Apparatus 4 (flow-through cell), which has advantages for modified-release dosage forms that contain active ingredients with limited solubility (both Apparatus 3 and Apparatus 4 may also have utility for soft gelatin capsules, bead products, suppositories, or poorly soluble drugs); Apparatus 5 (paddle over disk) and Apparatus 6 (rotating cylinder) have been shown to be useful for evaluating and testing transdermal dosage forms; and Apparatus 7 (reciprocating holder), which has been shown to have application to nondisintegrating oral modified-release dosage forms, as well as to transdermal dosage forms. Recommendations for the type of apparatus recommended for novel or special dosage forms are available [31]. These recommendations are summarized in Table 7.5. While changes to the approved apparatuses are allowed, justification must be provided.

For some dosage forms, particularly capsules that might float on the media surface, "sinkers" may be required. While sinkers are mentioned in USP Chapter <711> [26], USP Chapter <1092> provides additional detail for constructing and using them [28]. If sinkers are required, steps must be taken in method development to evaluate different types and construction, as sinkers can significantly affect dissolution.

Agitation is also an important part of the dissolution procedure. Apparatus 1 (baskets) at 100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are most commonly used. Other agitation speeds and apparatus are acceptable with appropriate justification obtained during method development. Higher or lower rates are usually inappropriate because of the inconsistency of hydrodynamics below 25 rpm and increased turbulence above 150 rpm. Coning or mounding problems can be solved by increasing the paddle speed or using peaked vessels. If justified, 100 rpm may be used, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may also be justified if the dissolution profiles better reflect in vivo performance, or

TABLE 7.5
Apparatus Recommendations for Novel or Special Dosage Forms

Type of Dosage Form	Release Method Apparatus
Conventional solid oral dosage form	Basket, paddle, reciprocating cylinder of flow-through cell
Oral suspensions	Paddle
Orally disintegrating tablets	Paddle
Chewable tablets	Basket, paddle, or reciprocating cylinder with glass beads
Transdermal patches	Paddle over disk
Semisolid topical preparations	Franz cell diffusion system
Suppositories	Paddle, modified basket, or dual-chamber flow-through cell

Note: For additional details, see Reference 16.

if the method results in better discrimination between bulk batch properties without adversely affecting method reproducibility.

Apparatus site selection is also important; vibrations from doors closing or pumps (e.g., mass spectrometry instrument vacuum pumps) can cause significant variability.

7.4.2.3 Dissolution Study Design

Dissolution is evaluated by measuring rate release profiles, or the amount dissolved over time. Single or multiple points in time can be measured, depending on the dosage type or data desired. For immediate-release dosage forms, the procedure duration is usually 30 to 60 min; and in most cases, a single time-point specification is adequate. However, for formulation development comparison purposes, profile comparisons are required, and it is common to collect data from numerous time points, for example, every 2 min or less over the course of the test. For profile comparisons, a sufficient number of time points should be selected to adequately characterize the dissolution curve's rise and plateau.

For an extended-release dosage form, at least three test time points are typically chosen to characterize the in vitro drug release profile. An early time point, usually 1 to 2 h, is chosen to show that there is little probability of dose dumping (too much drug product dissolving too soon). An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show the essentially complete release of the drug. Test times and specifications are usually established on the basis of an evaluation of drug release profile data. For products containing more than a single active ingredient, drug release is to be determined for each active ingredient.

Sampling is another important experimental design consideration. For many tests, particularly immediate-release formulation tests using one time point over a short (less than 1 h) period, sampling can be done manually. For extended tests, tests with multiple sampling times, or to increase throughput, automated sampling is a useful alternative. When automated sampling is employed, it is important to determine that no bias versus the manual method has been introduced. Regardless of the method of sampling, the sampling site must conform to specifications in the USP [26]. Any hydrodynamic disturbance of the vessels by the sampling probes should also be considered, and adequate validation performed to ensure that the probes are not introducing a significant change in the dissolution rate.

Filtration should also be considered during the method development or experimental design. Dissolution sample filtration is usually necessary to prevent undissolved drug particles from entering the analytical sample and further dissolving, skewing the test results. Also, filtration removes insoluble excipients that may otherwise cause high background or turbidity in the assay technique.

Acceptance criteria must also be considered during test development. The acceptance criteria should be representative of multiple batches from the same nominal composition and manufacturing process, include key batches used in pivotal studies, and batches that are representative of the drug product performance in stability studies. Acceptance criteria in the form of "Q-factors," or the percentage of the labeled content, are derived, that specify a certain amount dissolved at a given time. Dissolution tests can have a single Q-factor or may have multiple Q-factors in, for

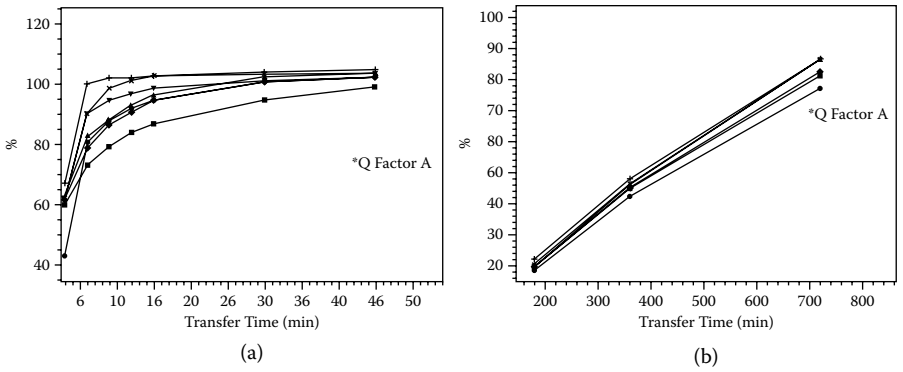


FIGURE 7.9 Example dissolution rate release curves. (a) Phenylpropanolamine HCl immediate-release tablets. Six vessel dissolution test conditions: Apparatus 2 (paddle) at 50 rpm, Q factor >75% released at 45 min. Assay was performed by reversed-phase HPLC. (b) Phenylpropanolamine HCl extended-release tablets. Six vessel dissolution test conditions: Apparatus 1 (basket) at 100 rpm, Q factor >70% released at 720 min. Assay was performed by reversed-phase HPLC.

example, an extended-release formulation, and are typically in the range of 75% to 80% dissolved. A Q value in excess of 80% is not generally used, because allowance must be made for assay and content uniformity ranges. Figure 7.9 illustrates some example rate release dissolution profiles for both an immediate-release and an extended-release formulation of the same drug substance as determined by HPLC analyses.

Finally, the dissolution test procedure should be discriminating enough to be capable of distinguishing significant changes in a composition or manufacturing process that might be expected to affect in vivo performance. In general, a properly designed dissolution test should result in reproducible data. Too much result variability can make it difficult to identify trends, true batch differences, or effects of formulation changes. If too much variability is observed, the usual remedies include changing the apparatus type, speed of agitation, or deaeration; consideration and examination of sinker type; and changing the composition of the medium. During routine testing of the product, variability outside the expected range should be investigated from analytical, formulation, and processing perspectives.

7.4.2.4 Assaying the Results

There are two common ways of analyzing dissolution test samples: spectrophotometric (UV) determinations and HPLC. UV determinations are the most common method of analysis because they are faster, simpler, and require less solvent than HPLC. Typically, the drug substance UV spectrum is observed to choose the optimum wavelength for analysis. Cells with path lengths ranging from 0.02 to 1 cm are typically used; the smaller path length cells are used to avoid diluting the sample once acceptable linearity and standard error are demonstrated.

HPLC methods, however, have distinct advantages, particularly when there is significant interference from excipients or between multiple active ingredients in the

formulation, when increased sensitivity is required, or when there is a desire to automate the dissolution test procedure. HPLC instruments can be used in a flow injection mode when separations are not necessary, and HPLC also has the advantage of different modes of detection (conductivity, fluorescence, and MS, for example) for both sensitivity (molecules lacking chromophores) and selectivity purposes. When developing a dissolution procedure that includes an HPLC assay, the compatibility of the dissolution media with the mobile phase must be considered, especially if large injector volumes (over 100 μL) are needed. Single injections of each vessel time point with standards throughout the run constitute a typical run design. Regardless of the mode of assay utilized, however, the procedure must be validated.

7.4.3 Dissolution Procedure Validation

Dissolution testing is an example of a USP Category III test (Section 7.1.3 and Table 7.1). Because dissolution is a quantitative test, all the analytical performance characteristics apply, with the exception of the limit of detection. In addition, for HPLC-based assays, system suitability is always required [(32); and Chapter 5]. However, in a dissolution test, in addition to the procedure used to perform and assay the test results, some individual "subprocedures" (e.g., filtration, solution stability) must also be validated. And while the various validation performance characteristics listed in the USP are well defined in a general sense, the specifics of how the analytical performance characteristics apply to dissolution testing deserve a little more focus.

7.4.3.1 Specificity/Placebo Interference

To evaluate specificity in dissolution procedures, it is necessary to demonstrate that the results are not affected by placebo constituents, other active drugs, or degradants in the drug product. A proper placebo should consist of everything in the formulation except the active ingredient; all the excipients and coatings (inks, sinker, and capsule shell are also included when appropriate), other actives, etc. In some instances, placebo interference may be evaluated by weighing samples of a placebo blend and dissolving or dispersing it into the dissolution medium at concentrations that would normally be encountered during testing. The interference generally should not exceed 2%.

For extended-release products, a placebo version of the actual drug product may be more appropriate to use than blends, because this placebo formulation will release the various excipients over time in a manner more closely reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate potential interference at multiple sampling points in the release profile.

If the placebo interference exceeds 2%, then method modification, such as (1) choosing another wavelength, (2) baseline subtraction using a longer wavelength, or (3) using HPLC, may be necessary in order to avoid the interference.

Absence of interfering peaks in the placebo chromatogram or lack of absorbance by the placebo at the analytical wavelength demonstrates specificity. Additional general information on determining specificity can be found in Chapter 4, Section 4.3.3.

7.4.3.2 Linearity and Range

Linearity and range are established by preparing solutions of the drug, ranging in concentration from below the lowest expected concentration to above the highest concentration during release. Typically, solutions are made from a common stock using serial dilutions. A range should be chosen (through appropriate dilutions as necessary) so as not to exceed the linearity limits of the instrument.

Sometimes, organic solvents are necessary in the preparation of standards; however, no more than 5% (v/v) of organic solvent in the final solution should be used.

Linearity is typically calculated and reported by least-squares linear regression analysis of the curve generated from a minimum of five points. Typically, a square of the correlation coefficient ($r^2 \geq 0.98$) demonstrates linearity. In addition, the y-intercept must not be significantly different from zero. The ICH recommends that for dissolution testing, linearity should be demonstrated ±20% over the range of the dissolution test. For example, for a controlled-release drug product with a multiple Q-factor of 20% after 1 h, and 80% after 24 h, the validated range should be from 0% to 100% of label claim [6]. Additional general information on determining linearity and range can be found in Chapter 4, Section 4.3.6.

7.4.3.3 Accuracy and Recovery

Accuracy and recovery can be established by preparing samples containing the drug and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from below the lowest expected concentration to above the highest concentration during release. The ICH recommends a minimum of nine determinations over a minimum of three concentrations, for example, three concentrations, three replicates each. An amount of stock solution equivalent to the targeted label claim may be added to the vessel instead of the drug substance, particularly for very low strengths, as it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts. The measured recovery is typically 95% to 105% of the amount added. Often, accuracy and recovery experiments are carried out at the same time as linearity, using data from the same samples. Additional general information on determining accuracy can be found in Chapter 4, Section 4.3.1.

7.4.3.4 Precision

For dissolution method validation purposes, precision is measured over two levels: repeatability and intermediate precision. Repeatability refers to the application of the procedure within one laboratory over a short period of time by one analyst using one instrument. Repeatability is determined by replicate measurements of standard or sample solutions. It can be measured by calculating the RSD of the multiple HPLC injections (peak area and retention time) or spectrophotometric readings for each standard solution. Repeatability can also be measured from the same samples used in the accuracy, recovery, and linearity experiments.

Intermediate precision is evaluated to determine the effects of random events on the precision of the analytical procedure. This evaluation is typically done later in the development of the drug product. The use of an experimental matrix design

is encouraged to study the effects of different days, analysts, and equipment on precision.

The dissolution profiles on the same sample may be run by at least two different analysts, each analyst preparing the standard solutions and the medium. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers; and they perform the test on different days.

Acceptance criteria are often calculated from the difference in the mean value between the dissolution results at any two conditions, and specified to not exceed an absolute 10% at time points with less than 85% dissolved and not to exceed 5% for time points above 85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used. Additional general information on determining precision can be found in Chapter 4, Section 4.3.2.

7.4.3.5 Robustness

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small but deliberate variations in parameters internal to the procedure (Chapter 4, Section 4.3.7; and Chapter 5). For dissolution testing, parameters to be varied include medium composition (e.g., buffer or surfactant concentration), pH, volume, agitation rate, and temperature. These parameters would be investigated in addition to those typically evaluated during validation of the assay method, either spectrophotometrically or by HPLC.

7.4.3.6 Remaining Validation Tests

In addition to the common analytical performance characteristics normally evaluated for procedure validation, standard and sample solution stability and filter validation must also be evaluated.

Solution stability is important given the conditions and length of time of some dissolution tests. The standard and sample solution should be stored under conditions that ensure stability. Solution stability is analyzed over a specified period of time, using freshly prepared solutions at each time interval for comparison. The acceptable range for solution stability is typically between 98% and 102%.

If the solution is not stable, refrigeration may be needed prior to sample analysis, or protection against photodegradation. A time period for analysis should also be specified.

Filter validation is accomplished by preparing a suitable standard solution or a completely dissolved sample solution at the appropriate concentrations. For standard and sample solutions, the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions can be compared.

7.5 BIOANALYTICAL METHODS

Bioanalytical methods are methods that are used for the analysis of drugs and metabolites in biological samples, most commonly plasma, urine, or tissues. They are used in clinical pharmacology, bioavailability, toxicology, bioequivalence, and other studies that require pharmacokinetic evaluation in support of various drug applications.

Bioanalytical methods must be validated to demonstrate that they are reliable and reproducible for their intended use (as for any other analytical method).

Analytical methods for finished product, raw materials, or active pharmaceutical ingredients (APIs) each have their own development and validation challenges. Bioanalytical methods are further complicated by the nature of the sample matrices, the trace concentrations of drug and metabolites encountered, and (potentially) the complexity of the required instrumentation.

Preclinical and clinical pharmacology studies rely on the sensitivity and selectivity of bioanalytical methods. Industry and regulatory conferences have been held over the past several years to discuss bioanalytical method validation [33–35], and after two early conferences, in May of 2001 the FDA issued a guidance document for validating bioanalytical methods [36]. Bioanalytical method regulations are listed as "guideline", the general interpretation of these guideline documents is that if methods are developed that adhere to their recommendations, there will be less likelihood of a negative regulatory action. In other words, if the recommendations of the guidelines are *not* followed, you should be sure to develop a logical and scientifically supported statement to show that alternative performance criteria are justified.

Regulated bioanalysis usually involves an HPLC system coupled to a triple-quadrupole mass spectrometer (LC-MS/MS). The sensitivity and selectivity of the LC-MS/MS allows for the quantitation of analytes with acceptable precision and accuracy at concentrations lower than most other HPLC detectors. Short, small-particle columns (e.g., 30–50 × 2.1 mm i.d. packed with ≤3-µm particles) are typically used for the fast separations needed for the large number of samples generated by clinical studies. An example is presented in Figure 7.10. Isocratic or gradient techniques may be used with run times commonly less than 5 min. Sample preparation

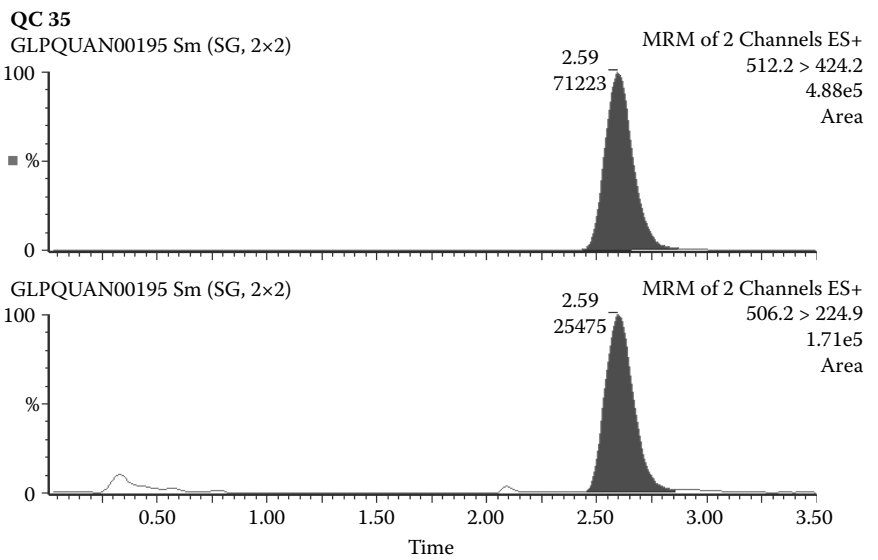


FIGURE 7.10 Example LC-MS/MS chromatogram used to generate the plot in Figure 7.11.

to remove excess protein and other potential interferences can be as much work to develop as the chromatographic method. Automation of both sample preparation and analysis is common.

The development and use of a bioanalytical method can be divided into three parts:

1. Reference standard preparation
2. Method development and validation
3. Application of the validated method to routine drug analyses

7.5.1 Reference Standard Preparation

Reference standards are necessary for quantitation of the analyte in a biological matrix. They are used to generate standard curves, to check method performance, quality control, QC, or samples. Reference standards can be one of three types: (1) standards whose purity is certified by a recognized organization (e.g., USP compendial standards), (2) reference standards obtained from another commercial source (e.g., a company in the business of the sales of general or specialty chemicals), and (3) custom-synthesized standards. Whenever possible, the reference standard should be identical to the analyte, or at least an established chemical form (e.g., free acid or base, or salt). In each case, the purity of the standards must be demonstrated through appropriate documentation, usually in the form of a certificate of analysis (CoA). Supporting documentation such as the lot number, expiration date, and evidence of identity and purity should be kept with other method data for regulatory inspection. Compounds used for internal standards (often, isotopically labeled drug) must have similar data to support purity.

7.5.2 Bioanalytical Method Development and Validation

The key bioanalytical performance parameters that must be validated for each analyte of interest in the matrix include accuracy, precision, selectivity, range, reproducibility, and stability. In practice, to develop the method and validate the method, four areas are investigated:

1. Selectivity
2. Accuracy, precision, and recovery
3. Calibration/standard curve
4. Bioanalytical sample stability

From each of these investigations, data is gathered to support the remaining parameters.

7.5.2.1 Selectivity

The selectivity of a bioanalytical method shows that the analyte can be accurately measured in the presence of potential interferences from other components in the sample (including the sample matrix). Interferences can take the form of endogenous matrix components (proteins, lipids, etc.), metabolites, degradation products, concomitant

medication, or other analytes of interest. The FDA guidelines recommend the analysis of blank samples of the appropriate biological matrix from at least six different sources. For example, six different sources of plasma should be spiked with known concentrations of analyte at the lower limit of quantification (LLOQ) to show that accurate results can be obtained. Similarly, a blank extract of each matrix should be analyzed to show the absence of interferences. In cases of a rare or difficult-to-obtain matrix (e.g., plasma from an exotic species or human tissue), the six-matrix requirement is relaxed.

7.5.2.2 Accuracy, Precision, and Recovery

The *accuracy* of a bioanalytical method is defined as the closeness of test results to the true value as determined by replicate analyses of samples containing known amounts of the analyte of interest; results are reported as deviations of the mean from the true value. The FDA guidelines recommend the use of a minimum of five determinations per concentration, and a minimum of three concentrations over the expected range (a minimum of fifteen separately prepared samples). The guidelines further recommend that the mean value be within ±15% of the actual value except at the LLOQ, where ±20% is acceptable.

The *precision* of a bioanalytical method measures agreement among test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. As in recent ICH guidelines, precision can be further divided into repeatability (within-run or intrabatch) determinations, and intermediate (between-run or interbatch) precision [2]. The FDA guidelines recommend the use of a minimum of five determinations per concentration, and a minimum of three concentrations over the expected range. The imprecision measured at each concentration level should not exceed 15% RSD, except for the LLOQ, which should not exceed 20% RSD. Usually, the same data is used to determine both precision and accuracy.

The assay recovery relates to the extraction efficiency and is determined by a comparison of the response from a sample extracted from the matrix to the reference standard (with appropriate adjustments for dilution, etc.). The recovery of the analyte does not necessarily need to be 100%, but it must be quantitative. That is, it should be precise and reproducible. Recovery experiments should be carried out at three concentrations (low, medium, and high), with a comparison of the results for extracted samples versus unextracted samples (adjusted for dilution). Sometimes, it is impractical to analyze unextracted samples (e.g., injection of unextracted plasma will ruin most HPLC columns), so creative ways to show recovery may need to be devised. For example, a liquid–liquid extraction of spiked matrix might be compared to extraction of a matrix-free aqueous solution; or recovery from a solid-phase extraction might be determined by calculation of volumetric recovery and comparison of the response from an extracted sample to a known concentration of reference standard.

7.5.2.3 Calibration/Standard Curve

A calibration curve or standard curve illustrates the relationship between the instrument response and the known concentration of the analyte, within a given range based on expected values. The simplest model that describes the proportionality should be used (e.g., a linear fit is preferred over quadratic curve-fitting function).

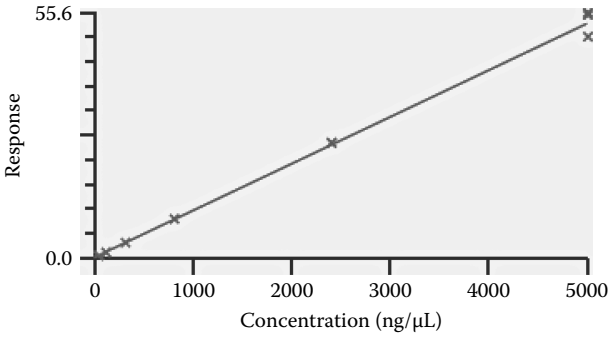


FIGURE 7.11 Example calibration plot obtained for the LC–MS/MS analysis of standard at 10, 30, 100, 300, 800, 2400, and 5000 ng/L. Column: 100 mm 2-mm C18; mobile phase: 55% acetonitrile, 45% water, 0.1% formic acid; flow rate: 0.3 mL/min; injection volume: 20 μL. MS detection: Quatro Ultima (Waters Corp.) MS/MS with a positive-ion electrospray Z source; cone voltage: 100 V; collision energy: 18 eV; collision gas pressure: 2.5 mbar argon.

Because a significant amount of sample manipulation takes place in the typical sample preparation procedure, internal standards are preferred for most bioanalytical methods. At least four out of six nonzero standards (67%) should fall within ±15% of the nominal concentration (±20% at the LLOQ). The calibration curve should be generated for every analyte in the sample, and prepared in the same matrix as the samples by addition of known concentrations of the analyte to blank matrix. The FDA guidelines suggest that a calibration curve should be constructed from six to eight nonzero samples that cover the expected range, including the LLOQ. In addition, noninterference is shown by the analysis of a blank sample (nonspiked matrix sample processed without internal standard) and a zero sample (nonspiked matrix processed with internal standard). Two conditions must be met to determine the LLOQ: (1) analyte response at the LLOQ should be >5-times the blank response, and (2) the analyte peak should be identifiable, discreet, and reproducible with an imprecision of ≤20% and an accuracy of at least 80%–120%. Figure 7.11 shows an example calibration curve for an LC–MS/MS experiment.

7.5.2.4 Bioanalytical Sample Stability

Stability tests determine that the analyte (and internal standard) does not break down under typical laboratory conditions, or if degradation occurs, it is known and can be avoided by appropriate sample handling. Many different factors can affect bioanalytical sample stability; these include the chemical properties of the drug, the storage conditions, and the matrix. Studies must be designed to evaluate the stability of the analyte during sample collection and handling, under long-term (at the intended storage temperature) and short-term (benchtop, controlled room temperature) storage conditions, and through any freeze–thaw cycles. The conditions used for any sample-stability studies should reflect the actual conditions the sample may experience during collection, storage, and routine analysis, including working and stock

solutions. Stock solutions should be prepared in an appropriate solvent at known concentrations. The stability of stock solutions should also be ascertained at room temperature over at least 6 hours, and storage-condition stability (e.g., in a refrigerator) should be evaluated as well. In addition, because samples commonly will be left on a bench-top or in an autosampler for some period of time, it is also important to establish the stability of processed samples (e.g., drug and internal standard extracted from sample matrix) over the anticipated run time for the batch of samples to be processed. Working standards should be prepared from freshly made stock solutions of the analyte in the sample matrix. Appropriate standard operating procedures (SOPs) should be followed for the experimental studies as well as the poststudy statistical treatment of the data.

The FDA guidelines recommend a minimum protocol that includes freeze and thaw stability plus short- and long-term temperature stability. For freeze–thaw stability, three spiked-matrix sample aliquots at each of the low and high concentrations should be exposed to three freeze–thaw cycles. The samples should be kept at the storage temperature for 24 h and then thawed at room temperature (without heating). When completely thawed, the samples should be refrozen for 12 to 24 h, then thawed again; this procedure is repeated a third time. Analysis of the sample then proceeds after completion of the third freeze–thaw cycle.

For short-term temperature stability, three aliquots (at each of the low and high concentrations) are thawed and kept at room temperature for a period of time that is equal to the maximum time (e.g., 4–6 h) the samples will be maintained at room temperature prior to their analysis.

The storage time for a long-term stability evaluation should bracket the time between the first sample collection and the analysis of the last sample (often 12 months or more); the sample volume reserved should be sufficient for at least three separate time points. At each time point, at least three aliquots (at each of the low and high concentrations) stored under the same conditions as the study samples (e.g., –20°C or –70°C) should be tested. In a long-term stability study, the concentration of the stability samples should be determined using freshly made standards. The mean of resulting concentrations should be reported relative to the mean of the results from the first day of the study.

7.5.3 Routine Application of the Bioanalytical Method

Once the bioanalytical method has been validated for routine use, system suitability and QC samples are used to monitor accuracy and precision, and to determine whether to accept or reject sample batches. QC samples are prepared separately and analyzed with unknowns at intervals according to the number of unknown samples for a sample batch. Duplicate QC samples (prepared from the matrix spiked with the analyte) at three concentrations (low, near the LLOQ, midrange, and high) are normally used. The minimum number of QC samples (in multiples of three—low, midrange, and high concentration) is recommended to be at least 5% of the number of unknown samples, or six, whichever is greater. For example, if 40 unknowns are to be analyzed, 40 × 5% = 2, so 6 QCs are run (2 low, 2 midrange, 2 high); or for 200 samples, 200 × 5% = 10, so 12 QCs are run (4 each, low, midrange, and high). At

TABLE 7.6
Example Bioanalytical LC-MS/MS QC Results

	Measured Concentration (pg/mL)				
	QC Sample 1 10 ng/μL	**QC Sample 2 35 ng/μL**	**QC Sample 3 1000 ng/μL**	**QC Sample 4 4400 ng/μL**	**QC Sample 5 5000 ng/μL**
Run 1	11.8	35.7	1009.8	4670.3	5425.0
Run 2	11.1	37.1	1036.0	4796.4	5334.5
Run 3	11.4	35.4	1047.2	4684.9	5180.9
Run 4	10.4	36.0	975.8	4964.3	5241.6
Run 5	10.8	34.6	1047.8	4628.6	5285.6
Run 6	10.9	34.9	986.5	4564.3	5049.0
Run 7	10.9	33.6	971.8	4491.9	5009.2
Run 8	10.8	32.6	960.4	4404.1	4883.7
Run 9	11.3	33.2	956.7	4539.5	5170.8
Run 10	11.4	34.4	977.8	4558.6	4802.7
n	10	10	10	10	10
Target	10.0	35.0	1000.0	4400.0	5000.0
Mean	11.1	34.8	997.0	4630.3	5138.3
Std. Dev.	0.402	1.37	35.45	160.5	199.4
% RSD	3.6	3.9	3.6	3.5	3.9
% Bias	+11.0	-0.6	-0.3	+5.2	+2.8

[a] Data representative of typical results obtained for the analysis of quality control samples at 10, 35, 1000, 4400, and 5000 ng/μL. For experimental conditions, see Figure 7.11. The coefficient of variations (≤4.1%) and biases (≤10.8%) at all concentration levels were within the validation guidelines.

least four out of every six QC sample results should be within ±15% of their respective nominal value. Data representative of typical results obtained by LC-MS/MS for the analysis of QC samples (at concentrations of 10, 35, 1000, 4400, and 5000 pg/mL of plasma) is listed in Table 7.6. As mentioned previously, for acceptable method validation, both the imprecision at each concentration level (%RSD), and the accuracy (%Bias) must be ≤15% (≤20% at the LLOQ). In Table 7.6, the %RSD (≤3.9%) and %Bias (≤11.0%) values at all concentration levels were well within the validation guidelines.

System suitability, sample analysis, acceptance criteria, and guidelines for repeat analysis or data reintegration should all be performed according to an established SOP. The rationale for repeat analyses, data reintegration, and the reporting of results should be clearly documented. Problems from inconsistent replicate analysis, sample processing errors, equipment failure, or poor chromatography are some of the issues that can lead to a need to reanalyze samples. In addition, recent interpretations of bioanalytical guidelines indicate that a certain number of samples be reanalyzed on a routine basis to ensure method performance (sometimes referred to as "incurred sample reproducibility") [36].

7.5.4 Bioanalytical Method Documentation

Good record keeping and documented SOPs are an essential part of any validated method. Once the validity of a bioanalytical method is established and verified by laboratory studies, pertinent information is provided in an assay validation report. Data generated during method development and QC should be available for audit and inspection. Documentation for submission to the FDA should include (1) summary information, (2) method development and validation reports, (3) reports of the application of the method to routine sample analysis, and (4) other miscellaneous information (e.g., SOPs, abbreviations, and references).

The *summary information* should include a tabular listing of all reports, protocols, and codes. The documentation for *method development and validation* should include a detailed operational description of the experimental procedures and studies, purity and identity evidence, method validation specifics (results of studies to determine accuracy, precision, recovery, etc.), and any protocol deviations with justifications. Documentation of the *application of the method to routine sample analysis* is usually quite extensive. It should include

- Summary tables describing sample processing and storage
- Detailed summary tables of analytical runs of preclinical or clinical samples
- Calibration curve data
- QC sample summary data including raw data, trend analysis, and summary statistics
- Example chromatograms (unknowns, standards, QC samples) for up to 20% of the subjects
- Reasons and justification for any missing samples or any deviations from written protocols or SOPs
- Documentation for any repeat analyses, or reintegrated data

7.6 VALIDATING PEPTIDE MAPPING METHODS

Peptide mapping is one of the preferred techniques for the comprehensive characterization of biopharmaceutical products and is often the analytical method of choice for studying a protein's primary structure. It is a very common application in the biopharmaceutical laboratory and has become a true workhorse technique [37]. Similar to any other analytical technique used in a regulated environment, methods for peptide analysis must also be validated. Although most of the underlying principles still apply, the validation of a peptide map includes some additional considerations when it comes to LOD, robustness, and precision, and depends on the stage of the regulatory process. While mostly involving comparative testing, when properly validated, a peptide map can be used to accomplish its intended purposes: to confirm the primary structure of a protein, to detect whether or not alterations have occurred, and to demonstrate process consistency. Typically used as an identity test for proteins, especially those obtained by r-DNA technology, a peptide map is generated by a chemical or enzymatic treatment of a protein followed by a reproducible high-resolution

chromatographic separation and is capable of identifying single amino acid differences. This "fingerprint," sensitive to even the smallest change in a protein's structure, makes it an extremely valuable tool for identity testing and process monitoring. Because of this sensitivity, a peptide map can be used not just for the identification of proteins based on the elution pattern of the peptide fragments in the separation, but also for the determination of posttranslational modifications, the confirmation of genetic stability, and the analysis of protein sequence when interfaced to a mass spectrometer.

While it is necessary to resolve each peptide fragment into a single peak, peptide mapping also represents a significant chromatographic challenge due to the large number of peptides that are generated from the enzymatic digest of a protein, and the significant number of alternative peptide structures (posttranslational modifications from proteolysis, phosphorylation, *N*-terminal acetylation and glycosylation, oxidations, etc.) that can also be obtained.

Similar to any other analytical procedure, when used in a biopharmaceutical laboratory, the method used to generate or evaluate the peptide map must be validated.

Applicable guidance is available from both the United States Pharmacopeia (USP) and the International Conference on Harmonization (ICH) on method validation in general [1,2]. However, more specific USP guidance for peptide mapping validation is also available, and should certainly be examined for more detailed information [38]. Section 7.6 highlights the USP guidance; however, for more information and background, the reader is encouraged to review the appropriate USP chapters.

7.6.1 Biocharacterization of Peptides

Peptide mapping involves comparative testing of specific maps for each unique protein (the test sample) against a reference standard or reference material treated in an identical fashion. It is the end product of one of several potential chemical processes that ultimately provide information about the protein under study. The process of generating a peptide map consists of four steps: isolation and purification of the protein, selective cleavage into the resulting peptides, the chromatographic separation, and the final analysis and identification of the peptides.

Isolation and purification are necessary for dosage forms or bulk drugs that may have excipients or additional active ingredients that may interfere with the protein of interest. When an isolation or purification step is employed, quantitative recovery should be validated against a reference standard.

7.6.1.1 Selective Cleavage of the Protein Peptide Bonds

The cleavage approach used is very dependent on the protein test sample. Cleavage can be either enzymatic or chemical, and each type has multiple cleavage agents, as summarized in Table 7.7. Complete cleavage is more likely to occur with enzymes compared to chemical agents. However, the overall goal is simply to have enough peptide fragments to be meaningful. If there are too many fragments, the map will

TABLE 7.7
Cleaving Agent Examples (Not an Exhaustive List)

Type	Agent	Specificity
Enzymatic	Trypsin	*C*-terminal side of Arg and Lys
	Chymotrypsin	*C*-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin	Nonspecific
	Lysyl endopeptidase	*C*-terminal side of Lys
	Glutamyl endopeptidase	*C*-terminal side of Glu and Asp
	Peptidyl-Asp metallo-endopeptidase	*N*-terminal side of Asp
	Endoproteinase Asp-N	*N*-terminal side of Asp
	Clostripain	*C*-terminal side of Arg
Chemical	Cyanogen bromide	*C*-terminal side of Met
	2-Nitro-5-thio-cyano-benzoic acid	*N*-terminal side of Cys
	o-Iodosobenzoic acid	*C*-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

lose its specificity, and it could become more difficult to discern differences. The purity and activity of the cleavage agent should also be examined.

Sometimes pretreatment of either the sample or the cleavage agent is necessary. It may also be necessary to selectively protect the sample protein to guard against generating too many peptides, or it may be necessary to concentrate the protein or to separate it from added substances or stabilizers that may be used in some drug formulations. Sometimes, chaotropic agents (e.g., urea, guanidine, or surfactants) may be added to unfold the protein to allow full access to cleavage sites.

The cleavage process also must be optimized, and the factors that affect the completeness and effectiveness of protein digestion are the same as those that would affect any chemical or enzymatic reaction. Temperature, pH, time, and the amount (ratio of enzyme/protein) of cleavage reagent used are all important factors. Generally, a temperature between 25°C and 37°C is adequate for most digestions, and a pH is chosen appropriate for the cleavage agent, enzyme (a pH will not denature the enzyme before it can react with the protein), and preservation of the integrity of the sample protein. Time can vary considerably, and if there is enough sample available, it is advisable to perform a time course study. The amount of cleavage agent used should be enough to accomplish a reasonably fast digestion time, but not so much that it interferes with the chromatographic map pattern. There is a compromise between using too much or too little of the cleavage agent, in order to avoid autodigesting the enzyme while generating an adequate map, and overdigesting and losing the "true" map and its information content.

Typically, a protein-to-cleavage reagent ratio between 20:1 and 200:1 is used, and blank determinations are also made using a digestion control with all reagents included except for the protein of interest. Immobilized enzyme reagents have also

been used to digest proteins for peptide mapping, as a way to avoid autodigestion of the enzymes [39].

7.6.1.2 Chromatographic Separation

Many different techniques, as well as different modes of chromatography, are used to separate peptides for mapping. These include various forms of polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE), and reverse phase high performance liquid chromatography (RP-LC), ion exchange (IEC), and hydrophobic interaction chromatography (HIC).

RP-HPLC is arguably the most common technique employed, and the column, mobile phase, and gradient or (rarely) isocratic conditions used can be critical to the success of the separation.

Columns used for peptide maps are generally porous silica, 1.7 to 5.0 µm in size, pore sizes ranging from 100Å to 300Å, with ligands of C_{18} (USP column characterization L1) or C_8 (USP column characterization L7). Temperature control of the column is important for good repeatability. The most common mobile phases used consist of water and acetonitrile, with various additives, for example, trifluoroacetic acid (TFA) or formic acid. If a buffer must be used, phosphate buffers provide the most flexibility for the selection of pH, although some thought must be given to alternative buffers if MS detection is used. Due to the complexity of the resulting sample, shallow gradient separations are generally recommended, with segments sometimes optimized using step functions or different slopes to give better resolution of important regions. Detection at low UV wavelengths, for example, 200 to 230 nm, is typically due to limited chromophores. An example chromatogram of a peptide map is illustrated in Figure 7.12.

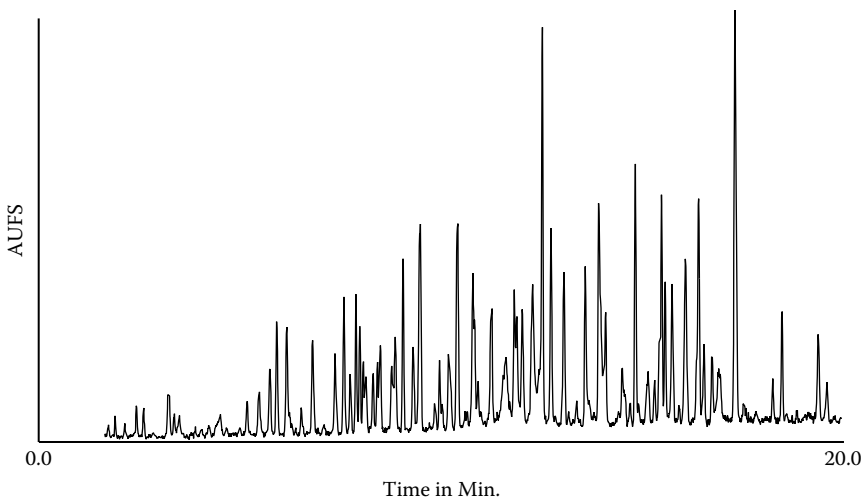


FIGURE 7.12 Example of a peptide map separation of a phosphorylase digest. A 2.1 by 100 mm 1.7-µm C18 column and a linear gradient of acetonitrile were used, with TFA as a modifier. Detection was UV at 214 nm.

7.6.2 Validation of Peptide Mapping Methods

There are several critical factors that must be considered to validate a method used for peptide mapping, and each of the factors, along with the acceptance criteria, should be designed into a protocol or SOP.

The critical factors include robustness, the limit of detection, specificity, linearity, range, accuracy, and precision. Recovery and reagent stability are also important to consider during method validation. Recovery can be addressed by performing either quantitative amino acid analysis, spiking studies, or radiolabeling. Many of the validation parameters must not only address the separation, but also the fragmentation or digestion, particularly when considering robustness studies. The protocol should also include written test procedures that give a detailed description of the analytical method. Because there is a wealth of general validation guidance available, discussion will be restricted here to areas where peptide mapping validation might differ from other types of methods (e.g., methods for synthetic drugs).

7.6.2.1 Robustness

In order to evaluate a peptide map against a standard, the chromatographic separation must be robust. While general chromatographic robustness is covered in Chapter 5 of this volume and should be consulted for more details, the significance and role of pH and mobile phase composition in peptide map robustness should not be overlooked. However, there are additional issues to consider in a peptide map method, and these include (enzyme) reagent quality or purity, and digest stability.

When determining the robustness of the reagents used for digestion, it is common to evaluate a protein reference standard of known composition with cleavage agents from different lots. The number of peaks obtained, their shape, and the peak areas are all compared in the resulting chromatograms. Because in some cases, chromatographic run times can by quite long, the length of time and the conditions under which a digest can be stored before being analyzed must also be evaluated as part of a robustness study. Digest stability is usually evaluated by looking for significant differences in the map resulting from the analysis of several aliquots of a single digest stored at different conditions. It may also be desirable to investigate stability through to several freeze–thaw cycles.

Column considerations must also be made during any proper robustness study, because it is a well-known fact that no two chromatographic columns are created equal. Although manufacturers today have much better control of their processes than in the past, minor column differences can have a significant effect on the separation of these complex samples. It is a good idea to evaluate the reference standard on several different column lots, and to evaluate column lifetime, because as a column ages, the separation can be affected. It is also a common precaution to examine alternative columns and to make slight modifications to the gradient profile where necessary to achieve equivalency.

7.6.2.2 Limit of Detection (LOD)

The LOD in a peptide map is determined by the ability of the method to distinguish changes in the map, for example, the presence or absence of a peak. Experiments

can be carried out to intentionally modify the target protein, and then a digest of the modified protein is mixed with a control digest or standard reference material in varying proportions. Ideally, a decrease in peak response for the unmodified peptide and a corresponding increase for the modified peptide is observed. Peptides modified by oxidation, deamidation, or other mutations usually have reported LODs in the range of 2 to 15 mole percent [40]. The particular chemical modifications chosen to evaluate LOD should take into account both the protein and the expression host.

7.6.2.3 Precision

Precision in peptide mapping is measured on two different levels: repeatability, and reproducibility intratest and intertest reproducibility experiments. Repeatability is measured by running six replicate injections of a single pooled digest of the reference standard. When repeatability is performed in this manner, all variability from the sample and reagents are eliminated, and the true instrument or system component of precision can be measured and used to help set system suitability criteria.

Intra- and intertest measurements are the more important parameters to be evaluated during validation, however. Intratest precision is the reproducibility of the fragmentation (digestion) and the chromatographic separation. Acceptable precision is obtained when the peak retention times and areas are constant from chromatograms obtained from consecutive tests of a series of separately prepared digests of the test protein. The average standard deviation of the retention times and areas should not exceed a predetermined specified acceptance criterion.

Intertest precision is what has been traditionally referred to as intermediate precision or true reproducibility. It is a measure of the reproducibility of the peptide map when the analysis is run according to an experimental design made to measure the effects of the test run on different days, by different analysts, in different laboratories on different systems, and different column lots. For intertest precision, the experimental design should include comparisons using peak retention times and areas relative to an internal standard peak within the same chromatogram. By using relative values, the need to make adjustments for things like injection volume differences, column volumes, and instrument gradient delay volumes is eliminated.

In general, it can be expected that %RSD for peak retention times and areas will be greater for the intertest compared to the intratest precision, which in turn will be greater than the repeatability results. Additional general information on determining precision can be found in Chapter 4, Section 4.3.2.

7.6.2.4 System Suitability

System suitability guidance can be found in the USP chapter on chromatography [(32); and Chapter 5]. Similar to any other method, the acceptance criteria for system suitability of a peptide map depend on the identification of the critical test parameters that affect data interpretation and acceptance. System suitability limits (for both recovery and chromatography) are determined by running a reference standard in parallel with the test protein and looking for indicators that monitor, for example, that the desired endpoint was reached in the digestion, normally selectivity and precision. However, the consistency of the pattern obtained is best defined

by peak-to-peak resolution. Additional chromatographic parameters such as peak width, tailing factors, and column efficiency may also be used.

The parallel study (reference standard and test protein) is also used to visually compare each peak's relative retention time, response (retention time and area), the number of peaks, and the overall elution pattern. This comparison is often complemented by mixing the two samples (1:1, v/v) and evaluating the peak response ratios and elution pattern. If all peaks in this mixed sample have the same relative retention times and peak response ratios, then the identity of the protein test sample can be confirmed. Significantly different retention times are also an indication of system variability, while the appearance of new or broader peaks indicates nonequivalence.

Computer-aided pattern recognition software and other automated approaches have been used on occasion to examine the degree of difference or similarity when comparing two different peptide maps, but have not gained routine acceptance.

7.6.3 Mass Spectrometry in Peptide Mapping

At the Investigational New Drug (IND) phase, limited validation is necessary—typically, only an approved test procedure that includes system suitability as a test control. Sometimes termed *qualification*, complete characterization of the individual peaks is not needed. As the regulatory process proceeds, a partial validation may be needed to give assurance that the method performs as intended in the development of a map for the test protein. However, validation of peptide mapping in support of further regulatory submissions requires a rigorous characterization of each of the individual peaks in the map. Methods that are used to characterize the peaks in a map commonly use mass spectrometry (MS).

In Figure 7.13, the LC/MS separation of a tryptic digest of alpha-1 acid glycoprotein is an example of how MS can be used to characterize a peptide map. The MS detection was performed with a quadrupole time-of-flight (Q-TOF) mass

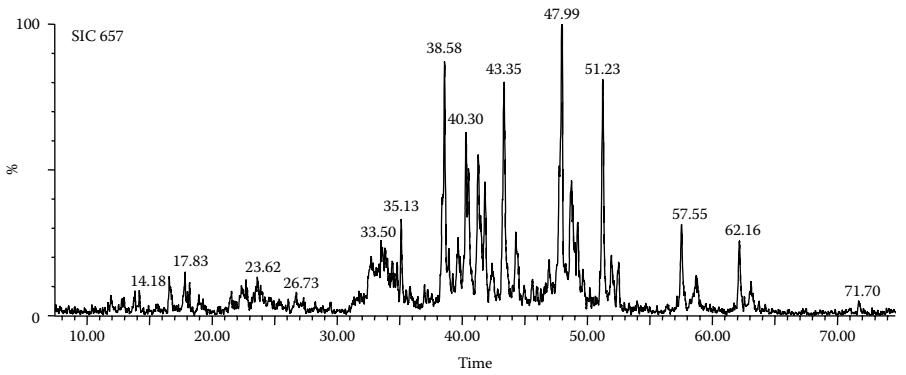


FIGURE 7.13 LC/MS separation of a tryptic digest of alpha-1 acid glycoprotein. Conditions are similar to Figure 7.11, except using a longer gradient and formic acid as a modifier. Data is plotted as a selected ion chromatogram for m/z 657, a signature ion for glycopeptides resulting from carbohydrate fragments.

spectrometer, which is well suited for glycopeptides due to its extended mass range. Glycosylation is a posttranslational modification that plays a critical role in determining the efficacy and safety of a therapeutic protein. Glycosylation can be analyzed on the intact protein by mass spectrometry, as released glycans by a variety of techniques, or as glycopeptides in LC/MS peptide maps. When glycosylation can be characterized with LC/MS of the glycopeptides, the site of attachment can be directly determined and structural information can be obtained through MS/MS experiments. The example in Figure 7.13 shows that LC combined with ESI/TOF (electrospray ionization/time-of-flight) mass spectrometry is a powerful tool for the rigorous characterization of peaks in a peptide map.

7.7 CLEANING METHOD VALIDATION

Cleaning validation is the process of assuring that effective procedures are used to remove residue from pharmaceutical manufacturing equipment. The main rationale for requiring clean equipment is to prevent contamination or adulteration of drug products. A proper cleaning validation program provides documented proof that one can consistently and effectively clean a system or piece of equipment.

The required use of clean equipment is not new. Good Manufacturing Practice (GMP) regulations in place as early as 1963 stated that "equipment shall be maintained in a clean and orderly manner" [41]. In 1978, updated GMP regulations included a similar section on equipment cleaning, which states that "equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination that would alter the safety, identity, strength, quality, or purity of the drug product" [42]. More recently, the development of highly active drug substances, commonly referred to as "potent compounds," has placed more emphasis on cleaning validation to address issues of containment, cleaning, and decontamination [43]. This section briefly discusses cleaning validation and relates it to various method validation approaches. Several references are available on this subject and should be consulted for additional detail [45–49].

7.7.1 General Requirements

In two recent publications, the FDA has outlined certain general requirements for compliance with cleaning validation regulations [50,51]. Written general procedures on how cleaning processes will be validated must be prepared. Sometimes referred to as a cleaning validation master plan, this document should address responsibilities, facilities, cleaning strategies, analytical strategies, residue limit justifications, and change control procedures [52]. The cleaning validation protocol should describe the equipment to be cleaned, methods, materials, and extent of cleaning, parameters to be monitored and controlled, and analytical methods. The protocol should also indicate the collection and type of samples (boil-outs, rinses, or swabs), and how they are labeled, stored, and transported to the laboratory for analysis.

During the cleaning validation process, various cleaning agents and the means of measuring residues, and the means for determining drug-product-related residues

TABLE 7.8
Potential Sources of Contamination Targeted During Cleaning Validation

- Active ingredients
- Synthesis precursors/starting materials
- Reaction by-products
- Degradation products
- Excipients
- Organic solvents
- Microbes (*E. coli*)
- Endotoxins
- Cleaning agents (solvents, surfactants)
- Buffers
- Media
- Lubricants
- Particulates
- Filtering agents
- Residual rinse water
- Environmental dust
- Wear products from equipment

are evaluated. A list of potential contaminants, etc., is provided in Table 7.8. To monitor drug product residues, companies generally test for the active ingredient. Endotoxin testing may also be performed. Measuring the residue of cleaning solutions can be problematic because they can contain more than one active component. One can either develop a test for the most abundant component or use a whole-product approach. In the whole-product approach, a determination is made as to whether any residue is present, without regard for its origin. A standard whole-product approach assay is commonly performed by measuring Total Organic Carbon (TOC), which can detect virtually every product and cleaning agent residue. HPLC is another commonly used technique.

7.7.2 Equipment Cleaning

Another important consideration is evaluating how cleaning is best performed. Equipment can be cleaned by various procedures: Clean in Place (CIP), Clean out of Place (COP), and manual cleaning can all be utilized. CIP is performed by circulating cleaning solutions through the equipment. Cleaning solutions can be recirculated or immediately drained. When practical, or necessary, CIP systems should be disassembled during cleaning validation to facilitate inspection and sampling of inner surfaces for residues or contamination. For COP, whether manual or automated, equipment is removed and cleaned remotely. Although pharmaceutical companies generally prefer automated approaches, human intervention for manual cleaning is still necessary in some instances. Obviously, any time manual cleaning is implemented, personal hygiene must be taken into account.

7.7.2.1 Sampling Methods

Sampling methods can include swabbing, rinsing, boil-outs, and direct extractions, as appropriate, to detect both soluble and insoluble residues. The sampling method used should be capable of quantitatively measuring levels of residues remaining on the equipment surfaces after cleaning. From the FDA perspective, any direct method of sampling the surface of the equipment is the most desirable. The most common direct-sampling method is swabbing. A swab sample is obtained by wiping a surface with solvent-moistened cotton gauze. Using swabs, equipment areas that are hardest to clean but that are reasonably accessible can be evaluated, and a level of residue or contamination per surface area can be determined. Also, dried-out or insoluble residues can be sampled by physical removal. It is important, however, to determine that the sampling medium and the solvent used for extraction from the medium do not interfere with sample analyses. For example, the adhesive used in some swabs has been found to interfere with sample analysis [51].

In some cases, rinsing may be preferred to swabbing. Rinsing can sample a larger surface area and inaccessible equipment areas. However, if the residue or contaminant is not soluble in the rinse solution, ineffective cleaning will result. During validation, therefore, one must still examine the equipment (the proverbial squeaky-clean test!), not just the rinse solution used for cleaning. Boil-outs are a variation on rinsing that involve the refluxing of a solvent to clean internal surfaces. However, boil-outs are not always feasible for piping or portable equipment. CIP sprayers or misting nozzles can facilitate the cleaning of hard-to-reach areas.

7.7.3 Validating Cleaning Methods

Validated analytical methods must be used to detect residues and contaminants. Validation of analytical methods used to assay residues and contaminants employs the same principles as those used for the finished product. In general, equipment-cleaning methods should provide consistent and reproducible results. The quantitation limit for each analytical method should be sufficiently low enough to detect the established level of the residue or contaminant. If residue or contaminant levels are not detected, it does not mean that they are not present in the sample. The analytical method should be challenged in combination with the sampling methods to show the level at which the compounds of interest can be determined. A negative test may just be the result of a poor sampling technique. The recovery and specificity of the method should also be established. Appropriate documentation should be maintained to prove that critical parameters, such as time, temperature, turbulence, cleaning agent concentration, number of rinse cycles, etc., are achieved with each cleaning cycle.

Several different types of analytical methods are used to evaluate equipment cleanliness. In general, methods are classified as either residue nonspecific or residue specific. Residue nonspecific methods include TOC, colorimetric assays (for protein residuals), UV/VIS absorbance, and conductivity testing. As mentioned previously, TOC analyses are commonly used because most contaminants contain organic carbon. TOC is relatively easy to perform and is compatible with most sampling techniques; however, it is not very specific. One potential drawback to TOC testing is that

individual contaminants cannot be identified or quantitated. In addition, residues must be at least somewhat water soluble to be detected. Colorimetric assays, while still classified as residue nonspecific, are more specific than TOC assays. Assays such as the Biuret, Bradford, and Lowery tests can quantify proteins in the presence of other organic residues. UV/VIS absorbance testing is usually used to detect compounds in final rinse water samples. Conductivity testing, sometimes performed on-line, is useful for measuring inorganic or ionic residues. Many buffer and media components and cleaning agents can be easily detected by this technique.

Residue-specific methods include USP purified water assays, endotoxin testing, bioassays, and TLC or HPLC. USP purified water assays include testing for chloride, sulfates, ammonia, carbon dioxide, calcium, heavy metals, oxidizable substances, total solids, pH, conductivity, and TOC. Water assays are commonly performed on rinse samples and are quite useful in assessing the efficacy of a cleaning regimen. The main disadvantage of USP purified water testing is that it only detects a limited number of inorganic compounds that must be water soluble. Also, USP purified water testing is usually a limit test and therefore does not provide quantitative data. Endotoxin testing, also performed on rinse water samples, is used to detect and quantify bacterial cell wall fragments. It can be quite accurate and precise to low levels, but is susceptible to interferences. Bioassays are used to assess the specific biological activity of a given molecule. This is the least commonly used method to assess equipment cleanliness due to time, expense, and a high experimental error. Also, it is unlikely that many biologically active compounds remain so even after a cursory cleaning. On the other hand, HPLC is one of the more commonly used residue specific assays. HPLC can detect and quantitate a broad range of compounds, from proteins and peptides to smaller molecules and detergents. It provides the required specificity, accuracy, and repeatability required for cleaning validation testing.

7.7.4 Setting Residue Limits

The calculation of cleanliness for a piece of equipment is based on the assumption that all surfaces are uniformly contaminated. However, in reality, this is not always the case. Residue limits should be practical, achievable, verifiable, and based on the most deleterious component [52]. Limits may be established based on the minimum known pharmacological or physiological activity of the active ingredient and must be scientifically justifiable.

There are several methods (formulas) used to calculate cleaning effectiveness. These formulas include the 10 PPM formula, the Acceptable Daily Intake formula, and the 1/1000 of Therapeutic Level formula [44,51]. The 10 PPM formula allows for no more than 10 PPM of cross-contamination between successive products. The Acceptable Daily Intake formula allows no more cross-contamination than what the acceptable daily intake indicates. The 1/1000 of Therapeutic Level formula allows no more than 1/1000 of product A's minimum daily therapeutic dose into product B's maximum daily dose. Cleaning criteria are usually based on the formula that results in the lowest number. Obviously, the more potent a given compound, the lower the cleaning criteria. The low levels that often must be measured during cleaning

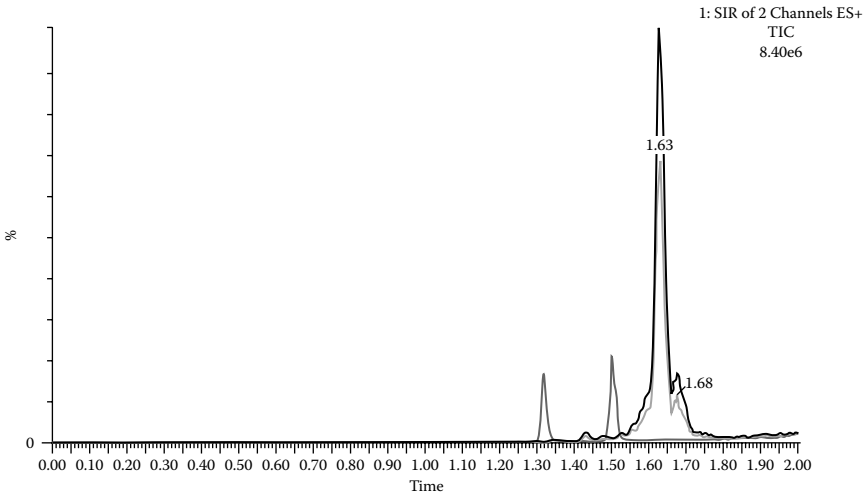


FIGURE 7.14 Example UHPLC/MS analysis of an extracted cleaning swab samples. Figure shows an overlay of a separation of two APIs, and two swab extracts using different extraction solvents. A reversed-phase separation with a 2.1 by 50-mm C18 column and a formic acid/acetonitrile gradient was used, with single quadrupole MS detection in single ion recording mode (two channels). The peaks at 1.3 and 1.5 min are from the APIs of interest; the large peaks at 1.6 min are from the swab.

validation place great demands on the sensitivity of the analytical technique used. For this reason, increasingly more sensitive detection techniques are required, and Figure 7.14 shows an approach using single quadrupole UHPLC/MS detection necessary to provide the requisite sensitivity.

Many companies are experimenting with new technology and methods, especially given today's more potent and complex biotechnology-derived therapeutics. Emerging trends include more specific assays, compound-specific (instead of whole-product) cleaning protocols, and increased automation. In addition, many companies have modified or are moving away from the use of cleaning agents. Aqueous-based cleaning agents free of organic solvents (or even hot water alone) have become more prevalent. Pressurized rinsing, visualization with fiber optics and video cameras, and more specific and sensitive detectors for residue assay methods are also being explored.

By knowing the product, the process, and the ingredients used and their properties, a greater probability of success is all but guaranteed.

7.8 CONCLUSION

The degree, amount, or level of method validation depends on the type of method, its intended use, and the phase of development. The type of method or procedure and its intended use dictate which performance characteristics must be evaluated; the phase of development dictates the amount, or in some cases the level of scrutiny

placed on specifications used to evaluate the methods suitability for use. In today's global market, validation can be a long and costly process, involving regulatory, governmental, and sanctioning bodies from around the world. A well-defined and documented validation process provides regulatory agencies with evidence that the system (instrument, software, method, and controls) is suitable for its intended use. All parties involved should be confident that a method will give results that are sufficiently accurate, precise, and reproducible for the analysis task at hand, and method validation is just one of tools to use to accomplish this task.

REFERENCES

1. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <1225> Validation of compendial procedures.
2. Harmonized tripartite guideline, validation of analytical procedures, text and methodology, Q2 (R1), *International Conference on Harmonization*, November 2005, http://www.ich.org/LOB/media/MEDIA417.pdf.
3. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <467> Organic volatile impurities.
4. Draft Guidance for Industry: Analytical Procedures and Methods Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Division of Research, Rockville, MD, August 2000.
5. Reviewer Guidance: Validation of Chromatographic Methods, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, MD, November 1994.
6. ICH Q3A(R): Impurities in new drug substances, *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, February 2002.
7. Repič, E., in *Principles of Process Research and Development in the Pharmaceutical Industry*, John Wiley & Sons, New York, 1998, pp. 1–54.
8. *The United States Pharmacopeia 24/National Formulary 19*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 1999, Physical Tests, <281> Residue on ignition, p. 1862.
9. ICH Q7A: Good manufacturing practice guide for active pharmaceutical ingredients, *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, November 2000.
10. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharacopeial Convention, Inc., Rockville, MD, 2011, General notices, significant figures and tolerances.
11. ICH Q1A(R2): Stability testing of new drug substances and products, *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, February 2003.
12. ICH Q1B: Stability testing: Photostability testing of new drug substances and products, *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, November 1996.
13. Reynolds, D. W., Forced degradation of Pharmaceuticals, *Am. Pharm. Review*, May/June, 56–61, 2004.
14. Swartz, M. E., Jones, M. D., and Andrews, M. A., Automated HPLC method development and transfer. *LCGC Application Notebook*, 66–67, June 2002.

15. Swartz, M. E., Chapter 6: Contemporary liquid chromatographic systems for method development, in *HPLC Method Development for Pharmaceuticals*, Volume 8, Ahuja, S., Editor, Elsevier, Boston, MA 2007.
16. Neue, U. D., Walter, T. H., Alden, B. A., Jiang, Z., Fisk, R. P., Cook, J. T., Glose, K. H., Carmody, J. L., Grassi, J. M., Cheng, Y.-F., Lu, Z., and Crowley, R. J., Ethylene bridged hybrids and their use in liquid chromatography, Part 1. *Am. Lab*., 31, 36, 1999.
17. Cheng, Y.-F., Walter, T. H., Lu, Z., Iraneta, P., Gendreau, C., Neue, U. D., Grassi, J. M., Carmody, J. L., O'Gara J. E., and Fisk, R. P., Ethylene bridged hybrids and their use in liquid chromatography, Part 2. 18, 1162, 2000.
18. Gorenstein, M. V., Li, J. B., Van Antwerp, J., and Chapman D., Detecting coeluted impurities by spectral comparison. *LCGC* 12(10), 768–772, 1994.
19. Young, P. M. and Gorenstein, M. V., Tryptic mapping by reversed-phase HPLC with photodiode-array detection incorporating the spectral-contrast technique. Elsevier, NY, 12(11), 832–838, 1994.
20. Warren, Wm. J., Stanick, W. A., Gorenstein, M. V., and Young, P. M., HPLC analysis of synthetic oligonucleotides using spectral contrast techniques. *BioTechniques*, Informa, London, 18(2), 282–297, 1995.
21. Swartz, M. E., UPLC: An introduction and review. *J. Liq. Chrom*., 28(7&8), 1253–1263, 2005.
22. Swartz, M. E. and Murphy, B., New frontiers in chromatography; Introducing UPLC. *Am. Lab*. 37(3), 22–27, 2005.
23. The Quest for Ultra Performance in Liquid Chromatography—Origins of UPLC Technology, Waters Corporation. See http:// www.waters.com/ waters/ partDetail. htm? cid=511539&id=38628 &ev= 10120661&locale=en_US. Last accessed February 2012.
24. Beginners Guide to UPLC—Ultra-Performance Liquid Chromatography. Waters Corporation. See http://www.waters.com/ waters/partDetail.htm? cid=511539&id= 38629&ev=10120684&locale=en_US.
25. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <701>, Disintegration.
26. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <711>, Dissolution.
27. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <724>, Drug Release.
28. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <1092>.
29. Guidance for Industry: The Use of Mechanical Calibration of Dissolution Apparatus 1 and 2—Current Good Manufacturing Practice (CGMP), FDA Draft Guidance, October 2007. See www.fda.gov/cder/guidance/7232dft.htm.
30. FDA Division of Pharmaceutical Analysis, Mechanical Qualification of Dissolution apparatus 1 and 2, Document # DPA-LOP.002, Version 2.0, June 2006. See http://www.fda.gov/cder/offices/otr/dissolution.pdf.
31. *AAPS PharmSciTech* 2003; 4(1) article 7 (see www.pharmscitech.org).
32. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011, Chapter <621>, Chromatography.
33. Shah, V. P., Midha, K. K., Dighe, S., McGilveray, I. J., Skelly, J. P., and Yacobi, A., Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference Report. *Pharm. Res.,* 9, 588, 1992.
34. Shah, V. P., Midha, K. K., Findlay, J. W. A., Hill, H. M., Hulse, J. D., McGilveray, I. J., McKay, G., Miller, K. J., Patnaik, R. N., Powell, M. L., Tionelli, A., Viswanathan, C. T., and Yacobi, A., Bioanalytical method validation—a revisit with a decade of progress. *Pharm. Res.*, 17, 1551, 2000.

35. Viswanathan, C. T., Bansal, S., Booth, B., DeStafano, A. J., Rose, M. J., Sailstad, J., Shah, V. P., Skelly, J. P., Swann, P. G., and Weiner, R., Workshop/Conference Report—Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays. *AAPS J.*, 9(1), 2007, E30. See also www.aapsj.org.
36. Guidance for Industry, Bioanalytical Method Validation, USFDA-CDER (May 2001), http://www.fda.gov/cder/guidance/4252fnl.pdf.
37. Viswanathan, C. T., Bansal, S., Booth, B., DeStafano, A. J., Rose, M. J., Sailstad, J., Shah, V. P., Skelly, J. P., Swann, P. G., and Weiner, R., *AAPS J.*, 9(1), 2007, E30. See also www.aapsj.org.
38. Hancock B, Editor. *New Methods in Peptide Mapping for the Characterization of Proteins*. CRC Press, Boca Raton, FL, 1996.
39. *The United States Pharmacopeia 34/National Formulary 29* (The United States Pharmacopeial Convention, Inc., Rockville, MD, 2011), Chapter <1047>.
40. Mark-Varga, G. and Oroszlan, P., Editors, in *Emerging Technologies in Protein and Genomic Material Analysis*, *Journal of Chromatography Library*, 68, 188, 2003, Elsevier, Amsterdam.
41. Bongers, J., Cummings, J. J., Ebert, M. B., Federici, M. M., Gledhill, L., Gulati, D., Hilliard, G. M., Jones, B. H., Lee, K. R., Mozdzanowski, J., Naimoli, M., and Burman, S., Validation of a peptide mapping method for a therapeutic monoclonal antibody: what could we possibly learn about a method we have run 100 times? *J. Pharm. Biomed. Anal*., 21, 1099, 2000.
42. Code of Federal Regulations (CFR) 133.4, 1963.
43. Code of Federal Regulations (CFR) 211.67, 1978.
44. Valvis, I. I. and Champion, W. L. Jr., Cleaning and decontamination of potent compounds in the pharmaceutical Industry. *Org. Process Res. Dev.*, 3, 44–52, 1999.
45. Harder, S. W., The validation of cleaning procedures. *Pharm. Technol*. 8(5), 29–34, 1987.
46. Smith, J. A., A modified swabbing technique for validation of detergent residues in clean-in-place systems. *Pharm. Technol*. 16(1), 60–66, 1992.
47. Fourman, G. L. and Mullen, M. V., Determining cleaning validation acceptance limits for pharmaceutical manufacturing. *Pharm. Technol*. 17(4), 54–60, 1993.
48. Zeller, A. O., Cleaning validation and residue limits: A contribution to current discussions. *Pharm. Technol*. 17(10), 70–80, 1993.
49. I. Losoxantrone., Shea J. A., Shamrock, W. F., Abboud C. A., Woodeshick R. W., Nguyen L. Q., Rubino J. T., and Segretario, J., Validation of cleaning procedures for highly potent drugs. *Pharm. Dev. Technol*. 1(1), 69–75, 1996.
50. Cleaning Validation Reprints from the *Journal of Validation Technology*, published by the Institute of Validation Technology, Royal Palm Beach, FL, 1998.
51. Guide to Inspections; Validation of Cleaning Processes, at www.fda.gov/ICECI/inspections/inspectionguides/ucm074922.htm.
52. Guidance for Industry; Manufacturing, Processing, or Holding Active Pharmaceutical Ingredients, p. 9–10, at www.fda.gov/downloads/drugs/guidancecomplianceregulatory information/guidances/ucm070289.pdf.

8 Analytical Method Transfer

8.1 INTRODUCTION

The objective of a formal method transfer is to ensure that the receiving laboratory is well trained, qualified to run the method in question, and gets the same results—within experimental error—as the initiating laboratory. The development and validation of robust methods and strict adherence to well-documented standard operating procedures (SOPs) is the best way to ensure the ultimate success of the method. This chapter examines the analytical method transfer process, including protocol, documentation, and some possible chromatographic pitfalls to avoid.

8.2 TERMS, DEFINITIONS, AND RESPONSIBILITIES

In a regulated environment, it is rare for the laboratory that develops and validates a method to perform routine sample testing. Instead, once developed and validated (in the originator, or "sending" laboratory), methods are commonly transferred to another laboratory (the "receiving" laboratory) for implementation. However, the receiving laboratory must still be able to get the same results, within experimental error, as the originating laboratory. The process that establishes documented evidence that the analytical method works as well in the receiving laboratory as in the originator's lab, or the transferring laboratory, is called *analytical method transfer* (AMT). The topic of AMT has been addressed by both the American Association of Pharmaceutical Scientists (AAPS: in collaboration with the FDA, EU regulatory authorities) and the Pharmaceutical Research and Manufacturers of America (PhRMA) [1–3]. The PhRMA activities resulted in what is referred to as an Acceptable Analytical Practice (AAP) document that serves as a suitable first-step guidance document for AMT [3]. In their various publications, both the AAPS and PhRMA have described the AMT process in some detail, and this chapter discusses these publications, some of the available guidance, and introduces a few additional thoughts concerning potential chromatographic pitfalls. However, as always, the reader is encouraged to consult these and other references for more information [4–8]. In essence, the AMT process is what qualifies a laboratory to use an analytical testing procedure, and regulators want documented proof that this process was completed successfully; only then can the receiving laboratory obtain GMP "reportable data" from their laboratory results. A typical AMT would take place between a research group that developed the method and a quality control group responsible for releasing finished product, although any time information moves from one group

TABLE 8.1
Sending Laboratory Responsibilities and Receiving Laboratory Provisions

Originating/Transferring Laboratory Responsibilities	Receiving Laboratory Provides
Create the transfer protocol	Qualified instrumentation
Execute training	Personnel
Assist in analysis	Systems
Acceptance criteria	Protocol execution

Note: Both laboratories are responsible for issuing the final report.

to another (e.g., to a contract laboratory), proper AMT should be observed. Both the originator and the receiving laboratories have certain responsibilities and must make certain provisions in the AMT process; these are listed in Table 8.1.

Before initiating AMT, there are a few pretransfer activities that must take place. If not previously involved with the method, the receiving laboratory should have an opportunity to review the method prior to the transfer, and to actually run the method to identify any potential issues that may need to be resolved prior to finalizing the transfer protocol. The originator should provide the receiving laboratory with all the validation results, including robustness study results, as well as documented training.

8.3 ANALYTICAL METHOD TRANSFER OPTIONS

Similar to many things in life, a little up-front investment prevents a multitude of problems later on. The foundation of a successful AMT is a qualified instrument (Chapter 2), properly developed and validated method or procedure (Chapters 3 and 4), and a good robustness study (Chapter 5) is certainly a development and validation cornerstone [9–12]. The AMT process needs to involve more than the receiving laboratory obtaining expected results from a representative sample, because a single test is no indication of how a method will perform over time. A single test also does not generate the data necessary to perform proper statistical evaluations, and compensating errors could also be masked. A formal AMT is not always necessary, however. Compendia methods (e.g., Karl Fisher, residue on ignition), in-process tests, or research methods do not require a formal transfer. In the latter two cases, a system suitability test is employed as the basis for the transfer.

There are several different AMT options. These include comparative testing, complete or partial method validation or revalidation, co-validation between the two laboratories, and the omission of a formal transfer, sometimes called a *transfer waiver.* The choice of which option to use depends on the stage of development the method is being used in (early or late stage), the type of method (i.e., compendia versus noncompendia, simple or complex), and the experience and capabilities of the laboratory personnel.

8.3.1 Comparative Testing

Comparative testing is the most common AMT option used. It is accomplished when two or more laboratories perform a preapproved protocol that details the criteria used to determine whether or not the receiving laboratory is qualified to use the method being transferred. The data resulting from the joint exercise is compared against a set of predetermined acceptance criteria. Comparative testing can also be used in other postapproval situations involving additional manufacturing sites or contract laboratories. In general, comparative testing is most often used for late-stage methods and the transfer of more complex methods.

8.3.2 Co-Validation between Laboratories

Traditionally, a validated method is a prerequisite to AMT. However, another option for AMT is to involve the receiving laboratory from the beginning in the actual validation of the method to be transferred. By completing a co-validation study, the receiving laboratory is considered qualified to perform the method for release testing. To perform this transfer option, the receiving laboratory must be involved in identifying the intermediate precision validation parameters to be evaluated and the experimental design [11]. By including data from all laboratories involved in the study, it is possible to have the validation report stand as proof of AMT.

8.3.3 Method Validation and Revalidation

A third option for AMT is method validation and revalidation. This option involves the receiving laboratory repeating some or all of the originating laboratory's validation experiments. As discussed previously, by completing any type of validation study, the receiving laboratory is considered qualified to perform routine release testing.

8.3.4 Transfer Waiver

In addition to the times when a formal AMT is not needed (e.g., compendia methods), certain other situations might also warrant omitting a formal AMT. They include

- The receiving laboratory is already testing the product and is thoroughly familiar with the procedures.
- A method or procedure exists for a comparable dosage form relative to the existing product.
- The analytical method (or one very similar) is already in use.
- A new method that involves changes that do not significantly alter the use of the method.
- The personnel accompany the transfer of the method from one laboratory to another.

When a transfer waiver is indicated, the receiving laboratory can use the method without generating any comparative data. However, the reasons for the waiver must be documented.

8.4 ELEMENTS OF AMT

Many interrelated components are necessary to achieve a successful AMT. As in any validation process, documentation is pervasive; both for the process and the results. Starting with a protocol and ending with a transfer report, everything must be documented for compliance purposes.

8.4.1 Preapproved Test Plan Protocol

Before implementing an AMT, an approved document must be in place that describes both the general transfer process, as well as the acceptance criteria. This document usually takes the form of a standard operating procedure (SOP) that describes the details of the AMT protocol or test plan specific to the product and method. This document should clearly define the scope and objective of the AMT, all of the respective laboratories responsibilities, list all the methods that will be transferred, and provide a rationale for any methods not included (i.e., the transfer waiver). It should also include the selection of materials and samples to be used in the AMT. Representative, homogeneous samples should be used that are identical for both laboratories. Selection of proper materials or samples is very important; usually pre-GMP materials or a "control lot" is chosen so that an out-of-specification (OOS) investigation is not triggered. Remember that the purpose of the method transfer is to assess method performance, not changes in samples or matrix. The protocol should also include certificates of analysis (CoA) for any samples and reference materials used.

Instrumentation and associated parameters should also be described. A best-case scenario would have each laboratory using common instrumentation; if this is not the case, and it rarely is, then the originator should consider running the method on instrumentation common to the receiving laboratory to identify any potential issues prior to a formal AMT. Intermediate precision validation studies also commonly take instrument differences into account. The protocol should also include a description of procedures, requirements, and their rationale, as well as acceptance criteria as outlined later.

8.4.2 Description of Method/Test Procedures

The method description should include not just the mechanics of performing the method, but also validation data and any idiosyncrasies in the method. Any precautions that must be taken to ensure successful results should also be included in the method description. The method should be written in a way as to ensure only one possible interpretation, for example, volume-to-volume (v/v) if volume measurements are made, etc. Clear equations and calculations, if appropriate, should be specified.

8.4.3 Description and Rationale of Test Requirements

Specific information on the number of lots, replicates, and injections, among other parameters, should also be included in the method description, as well as the rationale

for how each parameter was chosen. This section should also describe any system suitability parameters established for the method (Chapter 5).

8.4.4 Acceptance Criteria

The acceptance criteria stipulate how the results will be evaluated. Because statistical evaluations are usually employed, clear instructions on the number of batches, replicates, etc., are needed. It is common for simple statistics such as the mean and standard deviation from repeated use of the method in the originating/transferring laboratory to be used for acceptance criteria. More sophisticated statistics, such as the F-test or t-test, are also commonly applied. The proper use of statistics can provide an unbiased objective view of the comparison results of the transfer, and any approach used should be a part of the overall protocol documentation. While a comprehensive statistical discussion is outside the scope of this chapter, several good references are available for more detail [13–15]. Because specifications are completely method, instrument, sample, etc., dependent, hard and fast specifications are not listed in the PhRMA guidance. However, a partial summary of the AAPS publications list of recommended experimental design and acceptance criteria is presented in Table 8.2.

8.5 DOCUMENTATION OF RESULTS: AMT REPORT

Once completed, the results are summarized in an AMT Report. The report certifies that the acceptance criteria were met, and that the receiving laboratories are fully trained and qualified to run the method. In addition to summarizing all of the experiments performed and the results obtained, it should also list all of the instrumentation used in the transfer. Similar to any laboratory exercise, an important aspect of the AMT report is observations made while performing the method and should also be included. Observations in the form of feedback can be used to further optimize a method or to address special concerns that might not have been anticipated by the originating laboratory.

Of course, sometimes the receiving laboratory may not meet the acceptance criteria in the AMT protocol. When this situation arises, they should be addressed by a policy that dictates how the situation should be handled. An investigation should be initiated and documented in the summary report, and any corrective action taken justified.

8.6 POTENTIAL AMT PITFALLS

Many of the common pitfalls encountered during AMT can be prevented with a little up-front work. It cannot be stressed enough that the robustness studies performed during late method development or early method validation play a critical role in the success of AMT. During robustness studies, the critical method parameters have been identified and noted as a precautionary statement in the method. Intermediate precision validation studies also can serve to identify potential AMT issues. By anticipating that, for example, instruments, experience and training, and procedure interpretations can all differ from laboratory to laboratory, many of the common pitfalls can be avoided.

TABLE 8.2
Experimental Design and Acceptance Criteria for AMT

Type of Method	# Analysts	# Lots or Units	Acceptance Criteria	Notes
Assay	2	3 lots in triplicate	A two one-sided t-test with intersite differences of ≤2% at 95% CI	Each analyst should use different instrumentation and columns, if available, and independently prepare all solutions. All applicable system suitability criteria must be met.
Content uniformity	2	1	Include a direct comparison of the mean, ±3% and variability of the results, (%RSD), that is, a two one-sided t-test with intersite differences of ≤3% at 95% CI.	If the method for content uniformity is equivalent (e.g., same standard and sample concentrations, LC conditions, and system suitability criteria) to the assay method, then a separate AMT is not required.
Impurities, degradation products	2	3 Lots in duplicate (triplicate if done together with the assay)	For high levels, a two one-sided t-test with intersite differences of ≤10% at 95% CI. For low levels, criteria are based on the absolute difference of the means, ±25%.	All applicable system suitability criteria should be met. The LOQ should be confirmed in the receiving laboratory, and chromatograms should be compared for the impurity profile. All samples should be similar with respect to age, homogeneity, packaging, and storage. If samples do not contain impurities above the reporting limit, then spiked samples are recommended.
Dissolution	NA	6 units for immediate release, 12 units for extended	Meet dissolution specifications in both laboratories, and the two profiles should be comparable, or based on the absolute difference of the means, ±5%.	A statistical comparison of the profiles (e.g., F2) or the data at the Q time points similar to that performed for the assay may be performed.
ID		1 unit	Chromatography: confirm retention time. Spectral identification and chemical testing can also be used, assuming operators are sufficiently trained and the instrumentation can provide equivalent results.	
Cleaning validation		2 spiked samples, one above, one below spec.	Spiked levels should not deviate from the spec by an amount 3X the validated standard deviation of the method, or 10% of the spec, whichever is greater.	Essentially a limit test. Low and high samples to confirm both positive and negative outcomes are required.

8.6.1 Instrument Considerations

Many adverse effects encountered during AMT can be traced to the instrument. Injectors differ in design and cycle time, detectors can have different filters, time constants, wavelength accuracy, detector cell volumes, and parameters such as resolution and wavelength range (if appropriate), and HPLC pumps deliver solvents in different ways (e.g., low- versus high-pressure mixing systems). One of the more significant issues involves gradient separations in much more common use today than in times past. When transferring gradient methods between different systems, gradient delay volume must be taken into account, otherwise the situation illustrated in Figure 8.1 can cause significant problems. The top chromatogram in Figure 8.1 is a representation of the original method. Note that the fourth peak is eluted at the start of the gradient, the others in the isocratic segment. The bottom-left chromatogram could result upon transferring the method to a system with a smaller gradient delay volume. Due to the smaller volume, the gradient reaches the column before the isocratic elution of the third peak, compressing it into the fourth peak. In contrast, the bottom-right chromatogram could result upon transferring the method to a system with a larger gradient delay volume, effectively resulting in a longer isocratic hold time. The first three peaks still are eluted as in the original system; the fourth peak, however, is now eluted later. Because LC systems on the market today can differ by as much as 100 μL to over 1 mL in system volume, it is often useful to build in an isocratic hold at the beginning of the gradient for delay volume compensation. If the target system has a smaller system volume, an additional isocratic hold is added at the beginning of the gradient. If the target system has a larger system volume, no exact compensation is possible, nor is it necessarily needed. One word of caution: there are many ways to measure system gradient delay volume. Methods involving linear gradients are preferred because step gradients will not take into account proportioning valve volume in low-pressure systems or actual chromatographic

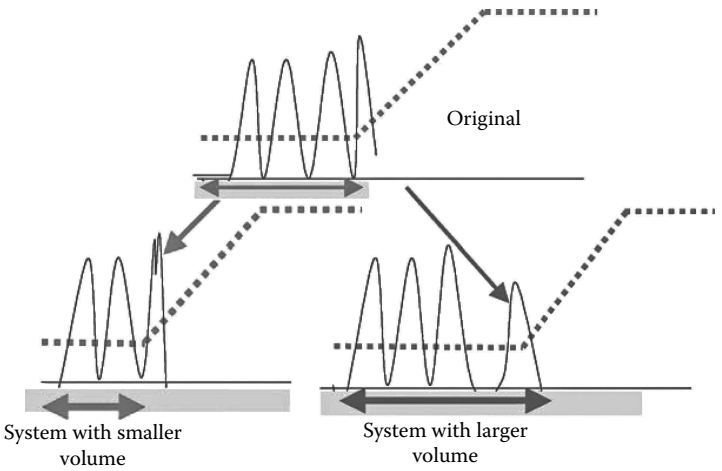


FIGURE 8.1 The effect of different gradient delay volumes on chromatography.

conditions [16,17]. Extra column band broadening from improperly made fittings or longer tubing lengths also can contribute variability.

8.6.2 Column Considerations

Historically, LC columns have been a significant source of variability. However, recently, manufacturers have vastly improved the reproducibility on both a column-to-column and a batch-to-batch basis by having complete control over the manufacturing process and raw materials. It is now a recommended practice to specify the brand and other details of the column used. If additional columns have been tested and approved for the method, they also should be specified by name. Using the blanket statement "or equivalent" should be avoided, given the variability of columns between manufacturers, even if they are of the same type (e.g., C_{18}). The United States Pharmacopeia (USP) has also addressed this issue by creating a database that uses chromatographic tests to classify columns according to various parameters [18]. When finalized, users will be able to cross-reference columns that can be equivalent to the one currently in use.

Column temperature is another source of variability. The days of room temperature or "controlled" room temperature separations are long past; to achieve the kind of reproducibility demanded by today's separation columns and in some cases, mobile phase temperature control is critical. Nowadays, columns are thermostated at least a few degrees above the highest room temperature to compensate for both intra- and interlab temperature variability, either with (preferred) or without solvent preheating, and some column control modules are also capable of cooling to temperatures below room temperature. The accuracy and precision of the set temperature between different column heaters (particularly between different manufacturers) also can vary and should also be considered.

Proper column equilibration also should be observed, either before being used for the first time (isocratic mode) or between runs (gradient mode). A common rule of thumb is to equilibrate a column with ten column volumes of mobile phase, or a total volume equivalent to five times the column volume and three times the system volume.

8.6.3 General Considerations

There are other sources of variability that can lead to pitfalls. Training can be addressed at any time, but it is not uncommon to train new users of the method before formal AMT. Sometimes despite all the upfront work, errors are still made; either honest mistakes or errors in procedure that result from method ambiguities. Procedures should be written so that there is only one possible interpretation of how to perform the method, with enough detail so that nothing is left to chance.

8.7 CONCLUSION

The development and validation of robust methods and strict adherence to well-documented SOPs is the best way to ensure the ultimate success of the AMT. A

proper statistical evaluation of the results of the AMT measured against predetermined acceptance criteria as outlined in the AMT protocol is critical to the process to ensure method success upon implementation in the receiving laboratory.

REFERENCES

1. *ISPE Good Practice Guide: Technology Transfer*, March 2003. www.ispe.org/ispe-good-practice-guides/technology-transfer.
2. *PhRMA Analytical Research and Development Workshop*, Wilmington, DC, September 20, 2000.
3. Scypinski, S., Roberts, D., Oates, M., and Etse, J., *Pharm. Technol.*, March 2002, p. 84.
4. Mcgonigle, E., Assay validation and interlaboratory transfer in *Development and Validation of Analytical Methods*, Riley, C. M. and Rosanske, T. W., Editors, Elsevier, New York, 1996, p. 3.
5. Scypinski, S., Analytical method transfer, in *Handbook of Modern Pharmaceutical Analysis*, Ahuja, S. and Scypinski, S., Editors, Academic Press, San Diego, CA, 2001, p. 485.
6. Lister, A.S., in *Handbook of Pharmaceutical Analysis by HPLC*, Ahuja, S. and Dong, M., Editors, Elsevier, New York, 2005, p. 212.
7. Snyder, L. R., Kirkland, J. J., and Glajch, J. L., Chapter 15: Completing the method: Validation and transfer, in *Practical HPLC Method Development*, John Wiley & Sons, New York, 1997, p. 685.
8. Etse, J., *Am. Pharm. Rev.*, January/February, 8(1), 74, 2005.
9. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeia Convention, Inc., Rockville, MD, 2011, Chapter <1225>.
10. *International Conference on Harmonization*. Harmonized tripartite guideline, validation of analytical procedures, text and methodology, Q2(R1), November 2005. See www.ICH.org.
11. Swartz, M. and Krull, I., Method Validation and Robustness. *LCGC* 24(5), 480, May 2006.
12. Swartz, M., Analytical Instrument Qualification. *Pharmaceutical Technology Special Supplement*, July 2006, p. 12.
13. Miller, J. C. and Miller, J. N., *Statistics for Analytical Chemistry*, Ellis Horwood Publishers, Chichester, UK, 1986.
14. *NIST/SEMATECH e-Handbook of Statistical Methods*, http://www.itl.nist.gov/div898/handbook.
15. Meier, P. C. and Zund, R. E., *Statistical Methods in Analytical Chemistry*, John Wiley & Sons, New York, 1993.
16. Snyder, L. R., Kirkland, J. J., and Glajch, J. L., *Practical HPLC Method Development*, John Wiley & Sons, New York, 1997.
17. M. Swartz, unpublished data.
18. Pappa, H. and Marques, M., Presentation at *USP Annual Scientific Meeting*, Denver, September 28, 2006. See also http://www.usp.org/USPNF/columns.html.

9 Implementing New Technology in a Regulated Environment

9.1 INTRODUCTION

Over the course of a drug product's lifetime (as many as 15 to 20 years or more), many significant advances in technology will occur. Implementing new analytical techniques (e.g., capillary electrophoresis) or advances in existing technology (e.g., sub-2-mm particle columns for HPLC) can significantly impact business practices (return on investment) and product quality. However, in a regulated laboratory, implementing new technology can present many challenges. This chapter discusses some industry-accepted practices for implementing new technology, FDA guidance available on the subject, and the process to consider when adopting new technology for use in approved, standard, or validated analytical methods.

9.2 CHANGES TO AN APPROVED METHOD

First, a disclaimer: This chapter is a summary of accepted practice and interpretation of regulatory guidelines from the authors' experience, as well as the authors' opinions derived from an informal poll of industry and regulatory resources; regulating change is something of a "gray" area, but there is guidance available. Please consult with the proper authorities before implementing any plan based on this chapter. The bottom line, as always, is that good, justifiable science is always the desired solution, and it is a good idea to have standard operating procedures (SOPs) in place to follow.

Standard or validated methods can be found in a number of places, for example, the United States Pharmacopeia (USP), or in the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC). Methods in both new (NDA) and abbreviated (ANDA) drug applications are also considered standard validated methods.

To use a standard method "as is" for the first time, a laboratory must perform a verification to demonstrate that both instrument and method performance criteria are met [1–3]. However, to implement new technology, an adjustment, or a modification or change, to a standard method might be needed.

In April of 2004, the FDA published a guidance that makes recommendations to holders of both NDAs and ANDAs that desire to make postapproval changes [4]. It is important that analysts refer to this guidance to determine what type of changes-being-effected

supplement is recommended. In the guidance, the FDA provided reporting categories depending on the type of change, or the potential to have an adverse effect on the identity, strength, quality, purity, or potency of a drug product.

A *major change* is a change that has a substantial potential to have an adverse effect. In the case of a major change, a *Prior Approval Supplement* (PAS) is required. As the name implies, FDA approval is necessary prior to distribution of the drug product made using the change. A *moderate change* has a moderate potential for an adverse effect, and requires the submission of a supplement called a *Supplement-changes being effected in 30 days*, often referred to as a CBE-30. For changes in this category, the drug product cannot be distributed if the FDA informs the applicant within 30 days that a PAS is required. CBE supplements are also used without the 30-day window for some moderate changes that do not relax acceptance criteria or for those that provide the same or increased assurance of identity, strength, quality, purity, or potency of a drug product. There have been cases where the FDA has not been able to complete a CBE-30 review within 30 days. If a firm were to implement the procedure after 30 days without FDA review, they are within the regulation, but at risk. There have also been cases where the FDA has notified firms that a review will not be completed within 30 days, and has asked the firm not to implement the method. Each of these cases is an example of where it is important to have a good dialog with the FDA.

A *minor change* is a change that has minimal potential for an adverse effect, and these changes are described in the applicant's next *annual report*.

A supplemental or Annual Report must include a list of the detailed description for all the changes; for supplements, the information must be summarized in the cover letter; in annual reports, included in the summary section. Applicants are also encouraged to submit a comparability protocol that describes test, studies, and acceptance criteria used to demonstrate the absence of any adverse effects, and guidance on this topic has appeared in draft form [5]. However, if a comparability protocol was not included in the original application, then it must be submitted as a PAS. The type of supplement is also dictated somewhat by the types of sample, for example, drug product versus intermediate. Verification (see Section 9.4.1) data and any statistical evaluation of equivalence should be included in the annual report.

9.3 WHAT CONSTITUTES A CHANGE TO A METHOD?

Adjustments to USP methods have always been allowed to satisfy system suitability requirements and are often noted in individual monographs. However, at what point does an adjustment become a change? After all, a change in the method triggers a revalidation, and at least some level of reporting as outlined previously (and more on this later!). Historically, as long as adjustments to the method are made within the boundaries of any robustness studies performed, no further actions are warranted. However, any adjustment outside the bounds of the robustness study constitutes a change to the method, thus requiring a revalidation.

In 1998, Furman et al. proposed a way to classify allowable adjustments [6]. But it was not until 2005 that guidance appeared on the topic [3, 7, 8]. The FDA Office of Regulatory Affairs (ORA) has had guidance in place for a number of years [3], and

Chapter 5 discusses this subject in more detail. But once a change has been made to a method, an analyst can be faced with a number of situations:

1. Implement it as an existing standard method (USP, AOAC, or method in an approved NDA or ANDA).
2. Implement it as an existing standard method with adjustments.
3. Implement it as an existing standard method with modifications or changes.

9.4 IMPLEMENT AN EXISTING STANDARD METHOD

To implement an existing standard method, or to determine the suitability of the method under actual conditions of use, verification is necessary to confirm that the method works for a particular drug substance, excipients, or dosage form by *verifying* a subset of validation characteristics per USP Chapter 1226, Verification of Compendial Procedures, rather than completing a full validation [9]. The USP says the purpose of this new general information chapter is to provide guidelines for verifying the suitability of a compendial procedure under conditions of actual use. USP Chapter 1226 summarizes what is necessary to confirm that the compendial procedure works for a particular drug substance, excipients, or dosage form by verifying a subset of validation characteristics, rather than completing a full validation. It is considered an extension of Chapter 1225, and both chapters use similar terminology. The intent of Chapter 1226 is to provide guidance on how to verify that a compendial procedure that is being used for the first time will yield acceptable results utilizing the laboratories' personnel, equipment, and reagents. Verification consists of assessing selected analytical performance characteristics described in Chapter 1225 to generate appropriate relevant data, as opposed to repeating the entire validation process. In draft versions, tables were included in Chapter 1226 outlining specific recommended validation performance characteristics to verify a method for both drug products and drug substances. However, the current version of Chapter 1226 provides only general recommendations.

9.4.1 Verification

Verification is a recent term that refers to the suitability of a compendial procedure under actual conditions of use. The International Organization for Standardization (ISO) puts it this way: validation is confirmation, through the provision of objective evidence that the requirements for *a specific intended use or application* have been fulfilled [10]. Verification is confirmation, through the provision of objective evidence that *specified* requirements have been fulfilled [11]. Put simply, compendial methods are verified, and noncompendial or alternative analytical procedures are validated.

USP methods have always been assumed to be validated, but not knowing what may have passed for validation when the method was submitted often leads analysts down a path of partial- or re-validation, and Chapter 1225 does not provide any guidance on how to verify procedures in the absence of a full validation protocol. Verification is not required for basic compendial test procedures unless there is an indication that the compendial procedure is not appropriate for the sample or matrix

tested. Examples include loss on drying, residue on ignition, various wet chemical procedures such as acid value, and simple instrumental methods such as pH measurements. New or different sample-handling or solution-preparation requirements should also be taken into consideration if used, and may require verification.

The verification process is made up of several individual components: laboratory personnel, an approved procedure or protocol, data comparison, acceptance criteria evaluation, the final summary documentation, and corrective action, if necessary.

9.4.1.1 Laboratory Personnel

Laboratory personnel need to have the appropriate experience, knowledge, and training to be able to carry out the procedure [12]. They must be able to accomplish the given functions in the lab, such as operating instrumentation and signing off that analyses were performed as required. It is important to note that it is not enough just to be able to push buttons to make instrumentation function and follow the SOPs (standard operating procedures). GMP requirements put pressure on lab management and personnel to understand the background or basics of any analytical technique that is used in the lab [13,14]. But in spite of these requirements, the FDA still frequently cites firms for a lack of trained personnel.

9.4.1.2 Approved Procedure

An approved procedure in the form of a verification document or SOP is needed that describes the procedure to be verified, establishes the number and identity of lots or batches of articles that will be used in the verification, details the analytical performance characteristics to be evaluated, and specifies the range of acceptable results. The document should also establish the acceptance criteria that will be used to determine that the compendial procedure performs suitably.

9.4.1.3 Verification Documentation

Once samples are analyzed, the data must be scrutinized and compared to the predetermined acceptance criteria in the approved verification document. The final summary documentation should include a summary of the data, the assessment of the results compared to the acceptance criteria, and a decision of whether or not the data is acceptable, which is a final indication that the laboratory personnel are capable of successfully performing the compendial procedure in the particular laboratory. Acceptable results are final proof that the USP procedure will perform as intended.

If the acceptance criteria are not satisfied, it may be necessary to identify the source of the problem, take corrective action, amend the verification document if necessary, and repeat the analysis. The initial unacceptable results, the probable cause, and any corrective actions implemented should also be described in the final summary document.

Of course, there is another possible outcome where, after several attempts, the verification of the compendial procedure cannot be made. If the source of the problem cannot be identified and rectified, then it may be concluded that the procedure may not be suitable for use with the article being tested. It may then be necessary to revise the current procedure, or redevelop and validate an alternative procedure. In any case, the final verification document should summarize the inability to verify the compendial procedure and describe the action taken.

9.4.1.4 Verification Process Examples

Assessing specificity is often critical to verifying that a compendial procedure is suitable for use in assaying drug substances and drug products. Specificity for a chromatographic method may be verified by conformance with system suitability resolution requirements if they are specified in the method. However, drug substances from different suppliers may have different impurity profiles that are not addressed by the compendial test procedure. Similarly, the excipients in a drug product can vary widely among manufacturers and may have the potential to directly interfere with the procedure or cause the formation of impurities that are not addressed by the compendial procedure. In addition, drug products containing different excipients, antioxidants, buffers, or container extractives may potentially interfere with the compendial procedure. In these cases, a more thorough assessment of specificity may be required to demonstrate suitability of the method for the particular drug substance or product, for example, to include photodiode array and mass spectral analysis.

Figure 9.1 shows a separation used to verify a stability indicating compendial procedure for the analysis of a drug product and its major degradants. In addition to specificity, precision and the quantitation limit were also evaluated. Specificity was evaluated using photodiode array peak purity; Table 9.1 summarizes the precision results, and Table 9.2 the results from the determination quantitation limit. Figure 9.1 illustrates the actual separation at the quantitation limit used to verify the calculated quantitation limit.

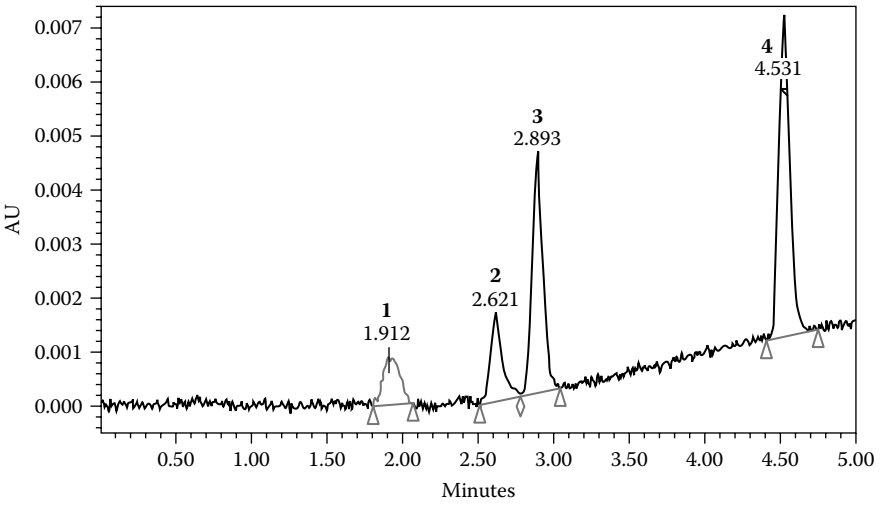


FIGURE 9.1 Verification of compendial procedure quantitation limits. Separation was performed on an Alliance 2695 Separations Module (Waters, Milford, Massachusetts) using a 4.6 by 100 mm 3.5-μ Xterra RPC18 column at 34°C. Mobile phase A was 10 mm pH 9.0 ammonium carbonate, B was methanol run at a 15%–90% B linear gradient over 5 min at 1.0 mL/min. A 20-μL injection and UV detection at 280 nm were also used. Peaks are in order: (1) NTAP (highlighted in red), (2) ACBS, (3) HCT, and (4) TMT.

TABLE 9.1
Summary of Precision and System Suitability Results for an HPLC Stability Indicating Assay with Two Active Ingredients and Two Degradation Products

Component	Avg. Area	%RSD Area	Avg. Rt.	%RSD Rt.	Avg. Rs.	%RSD Rs.	Avg. N	%RSD N
NTAP	298826	0.5	1.926	0.2	—	—	1291	0.9
ACBS	239073	0.5	2.622	0.1	3.92	0.286	5748	1.2
HCT	642003	1.0	2.889	0.1	1.97	0.416	7149	1.2
TMT	1018352	0.8	4.535	0.1	11.77	0.499	17997	1.4

Note: Triamterene (TMT) and hydrochorothiazide (HCT) are the active ingredients, while 5-nitroso-2,4,6-triaminopyrimidine (NTAP) and 4-amino-6-chloro-1,3-benzenesulfanamide (ACBS) are the related substances called out in the compendial procedure. Average of six replicates at 0.63 mg/mL each. Rt. is retention time, Rs. is resolution, and N is efficiency or plates.

TABLE 9.2
Verification of the Quantitation Limit

	Level 1 Response	Level 2 Response	Level 3 Response	Level 4 Response	Level 5 Response
1	8164	13585	21296	42395	61740
2	8130	13785	20936	42467	63528
3	8734	13195	21220	41976	62152
Mean	8343	13522	21151	42279	62473
STDev.	339.3	300.0	189.8	265.1	936.3

Average standard deviation: 406.1.
Slope of calibration curve: 4760000.
Calculated quantitation limit: 0.00085 mg/mL.
The quantitation limit was calculated according to established USP and ICH guidelines by the formula: QL = 10STD/S, where STD is the average standard deviation of the response and S is the slope of the calibration curve [14].

9.5 IMPLEMENT AN EXISTING STANDARD METHOD WITH ADJUSTMENTS

When implementing an existing standard method with adjustments, the main thing to keep in mind is that as long as the adjustments are within system suitability guidelines (Chapter 5) or within the bounds of a robustness study (Chapter 5), it is not necessary to perform a revalidation. According to the ORA guideline, the modified procedure should not adversely affect the precision and accuracy of the data generated as measured against the performance specifications of the method [3].

Changes in column length, diameter, and particle size fit into this category, and additional USP guideline revisions are under way to accommodate the implementation

of this new column technology [15]. Adjustments in this category are usually accommodated in an Annual Report, but some companies, acting conservatively, may use a CBE or CBE-30 document. Actual determination of which document to file may be part of a risk assessment, or an existing SOP. And again, as mentioned previously, the type of supplement is also dictated somewhat by the types of sample, for example, drug product versus drug substance.

9.6 IMPLEMENT AN EXISTING STANDARD METHOD WITH CHANGES

When a new method is implemented in a regulated laboratory, it must be revalidated. Validation is also required when the existing standard method is modified enough to change it, and also a good idea when the existing method is applied to a sample matrix significantly different from that for which the original method was intended. There are many reasons to change a method, and changes to a method can be either reactive or proactive. Reactive situations might exist if there were significant changes to incoming raw material or the manufactured batch, or formulation changes. If it becomes necessary to modify a method to satisfy system suitability requirements so much that it becomes a change, it may also be necessary to perform an out-of-specification (OOS) investigation [16,17].

Many laboratories are proactive with method changes, and implementing new technology fits into this category. New columns, column chemistry, and other method improvements occur frequently, and business case studies are undertaken to determine what changes might be made as cost-cutting or time-saving options. With new technologies resulting in analysis times as short as a minute, with improved sensitivity and no loss in resolution, and software tools available to analyze the data more quickly, revalidation time can be significantly reduced. Some will take the course of action "if it ain't broke, don't fix it," but many companies take advantage of new technology because it makes sense from a business standpoint, even if it means changing the method, investing additional time and resources, and doing a little extra paperwork.

Changes themselves can be of different magnitudes and result in different approaches for implementation. If the changes are so drastic that the applicant is essentially establishing a new or alternative analytical procedure, a PAS is required as this situation falls into the major change category. The same is true in the instance where a change is being made to relax specifications.

However, when adopting, for example, new HPLC technology, analysts generally operate under the assumption that a change is always being made for the better; that is, not relaxing specifications but providing an *increased* assurance of identity, strength, purity, or potency of the material being tested. It can also be further argued that adopting new LC technology is not equivalent to adopting an alternative procedure. A change in column chemistry or scale should not be rated the equivalent of changing from an HPLC method to a titration method, or to NMR or IR, or vice versa. So a change in this category might also be satisfied by either type of CBE document. Of course, the standard method is the legal control procedure. Any change to the procedure would require a CBE-30 submission.

One final recommendation: when implementing a change, equivalency studies with the old method should always be undertaken to identify potential bias. Method equivalency is particularly important if the method is changed in between points in a long-term stability study. The applicant should provide information to explain why the new method is preferred to the original, including supporting data.

9.7 CONCLUSION

While it is certainly easier to adopt new technology with new methods rather than to revalidate current standard methods, implementing new technology can have a significant return on investment that can make a method modification a very worthwhile pursuit. By consulting the guidelines and adhering to a few basic principles, change can be implemented in a painless exercise that can result in faster, more sensitive, and more robust information that may even reveal a wealth of new useful information.

REFERENCES

1. Swartz, M. E. and Krull, I. S., *LCGC* 23(10), 1100–1109, October 2005.
2. In-process revision, *Pharm. Forum*, 31(2), 555, Mar.–Apr. 2005.
3. FDA ORA Laboratory Procedure, #ORA-LAB.5.4.5, Revised 09/09/2005. See also http://www.fda.gov/ora/science_ref/lm/vol2/section/5_04_05.pdf.
4. Guidance for Industry, Changes to an Approved NDA or ANDA, FDA Center for Drug Evaluation and Research, Rockville MD, April 2004. See also http://www.fda.gov/cder/guidance/index.htm.
5. Guidance for Industry, Comparability Protocols-Chemistry, Manufacturing, and Controls Information, FDA Center for Drug Evaluation and Research, Rockville MD, February 2003. See also http://www.fda.gov/cder/guidance/index.htm.
6. Furman, W. B., Dorsey, J. G., and Snyder, L. R., *Pharm. Technol*. 22(6), 58–64, 1998.
7. *Pharm. Forum*, 31(3), 825, May–June 2005.
8. *Pharm. Forum,* 31(6), 1681, Nov.–Dec. 2005.
9. *The United States Pharmacopeia 34/National Formulary 29*, The United States Pharmacopeia Convention, Inc., Rockville, MD, 2011, Chapter <1226>.
10. ISO 9000:2000 clause 3.8.5.
11. ISO 9000:2000 clause 3.8.4.
12. Swartz, M. E., Krull, I. S., and McCabe, J., *LCGC*, 22(9), 906, 2004.
13. Current Good Manufacturing Practice for the Manufacture, Processing, Packing, or Holding of a Drug Product, 21 Code of Federal Register (CFR) Part 211; Subpart A: General Provisions 211.1-Scope.
14. Current Good Manufacturing Practice for the Manufacture, Processing, Packing, or Holding of a drug Product, 21 Code of Federal Register (CFR) Part 211; Subpart B: Organization and Personnel 211.25-Personnel Qualifications.
15. Neue, N. D., McCabe, D., Ramesh, V, Pappa, H., and DeMuth, J., *Pharm. Forum*, in press, April 2010.
16. Swartz, M. E. and Krull, I. S., *LCGC*, 23(6), 26, June 2004.
17. Guidance for Industry, Investigating Out of Specification (OOS) Test Results for Pharmaceutical Production. U.S. Food and Drug Administration, Department of Health and Human Services, Rockville, MD, September 1998.

Glossary of Terms Related to Chromatographic Method Validation

Acceptance Criteria: Numerical limits, ranges, or other criteria to which test results for a drug substance, product, or other sample should conform to be considered acceptable for its intended use.

Accuracy: The closeness of test results to the true value.

Active Pharmaceutical Ingredient (API or Drug Substance): Any substance or mixture of substances intended to be used in the manufacture of a drug (medicinal) product that, when used in the production of a drug, becomes an active ingredient of the drug product.

Analytical Instrument Qualification (AIQ): The process of ensuring that an *instrument* is suitable for its intended application. AIQ is just one component of data quality, which also includes software and analytical method validation, system suitability tests, and quality control tests.

Analytical Performance Characteristics: Parameters assessed during method validation, including accuracy, precision, linearity (range), limit of quantitation or detection, and robustness. Actual parameters assessed depend on the type of method and its intended use.

Analysis of Variance (ANOVA): A statistical test that measures the difference between the means of groups of data. Sometimes called an F-test, ANOVA is closely related to the t-test. The major difference is that where the t-test measures the difference between the means of two groups, an ANOVA tests the difference between the means of two or more groups.

Assay (Content or Potency): An exact result that allows an accurate assessment of the content or potency of the analyte in a formulation.

Assignable Cause: The laboratory investigation following an out-of-specification (OOS) result that determines the suspect result is due to a known cause such as operator or instrument error.

Asymmetry: Factor that describes the shape of a chromatographic peak. Theory describes Gaussian symmetrical peak shapes. Peak asymmetry is measured as the ratio of the distance between the peak apex and the back side of the peak to the distance between the front side of the peak. A value >1 is a tailing peak, while a value <1 is a fronting peak.

Audit: A documented independent review performed on a periodic basis to verify compliance with a quality system.

Batch: A quantity of material produced during one manufacturing cycle using the same specification.

Biologic: A virus, therapeutic serum, toxin, antitoxin, or related substance used for human treatment or disease prevention.

Biological Matrix: Samples of biological origin, typically blood, serum, plasma, urine, feces, saliva, sputum, and tissues.

Biotechnology: The use of living organisms or other biological systems in the manufacture of drugs or other products or for environmental management, for example, waste recycling.

Blank: A sample that does not include the analytes of interest, used to assess the specificity of a method. Examples include mobile phase, diluent, and procedural blanks.

Calibration: Ensures that the instrument response correlates with the response of the standard or reference material. Calibration should be carried out by documented written and approved procedures, using traceable certified standards.

Capacity Factor: A chromatographic parameter (k′) that measures the degree of retention. Calculated from the equation: $k' = (t_R - t_0)/t_0$, where t_R is the retention time and t_0 is the retention time of an unretained peak.

Certification: A documented statement or written guarantee by qualified individuals that an instrument, computer, test, or system complies with specified requirements.

cGMP: Current Good Manufacturing Practice. See Title 21, Code of Federal Regulations, Section 210, 211, and 212.

Change Control: A monitoring system of managing and implementing changes that may affect the status of a validated process. Change control is a way to determine the need for corrective actions that might be necessary to correct or redesign systems or, for example, upgrading software while maintaining a validated state.

Coefficient of Determination: The square of the correlation coefficient.

Coefficient of Variation (CV): The sample standard deviation divided by the sample average, multiplied by 100. Sometimes called *relative precision* or *relative standard deviation*.

Confidence Interval/Limits: Usually expressed as a percentage (e.g., 95%) referring to the range of values around an observed value that will include the expected value.

Confidence Level: Usually expressed as a percentage (e.g., 95%) referring to the probability of precision measurements. A 95% confidence level means a 95-in-100 chance of being correct or a 5-in-100 chance of being wrong in predicting that the precision falls into a specified range.

Conformance to Specifications: The sample, when tested according to a documented analytical procedure, satisfies the listed acceptance criteria.

Corrective Action: Action to eliminate the cause of a detected nonconformity to prevent recurrence.

Correlation Coefficient, r: Degree of correlation between two variables, ranges from −1 to +1. A +1 value indicates a perfect correlation with both values increasing; a value of −1 also indicates a perfect correlation, with one value increasing while the other is decreasing. A zero r value indicates no correlation.

Dead Volume: The volume (V_d) of the chromatographic system not including the column packing. Includes the column interstitial volume and extra column volume contributed by the injector, detector, tubing, and connections. Determined by injecting an inert compound (e.g., acetone).

Design Qualification (DQ): DQ ensures that an instrument is designed and produced in a validated environment according to good laboratory practices (GLPs), current good manufacturing practices (cGMPs), and/or ISO 9000 standards.

Detection Limit (DL or LOD): Characteristic of limit tests, the DL is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value.

Documentation: The organized collection of written or electronic records that describes the structure, purpose, operation, maintenance, and data requirements for systems, instruments, or tests. Includes manuals, procedures, specifications, operating records, final reports, data, etc.

Efficiency: The number of theoretical plates (N) in a chromatographic separation. There are several ways to calculate N; one common way is $N = 16(t_R - t_0)^2$, where t_R is the retention time and t_0 is the retention time of an unretained peak. *See also* HETP.

Error: Any deviation of the observed value from the true value.

External Standard: The analyte itself, in high chemical purity, which is not added to the actual samples, but is used to generate an external standard calibration plot then used to quantitate the analyte in a sample matrix. In order to successfully utilize the external standard method, it is demonstrated, in a reproducible manner, the recovery efficiency of the analyte in the actual sample matrix. Recovery is evaluated by spiking a placebo of the sample matrix with the external standard and showing how much has been recovered. It can then be assumed that the recovery of the same analyte in an actual sample will have the same recovery efficiency.

F Test: A variance ratio test that describes if two independent estimates of variance can reasonably be accepted as being two estimates of the variance of a single, normally distributed sample.

Good Laboratory Practices (GLP): The organizational process and the conditions under which laboratory studies are planned, performed, monitored, recorded, and reported. See, for example, 21 Code of Federal Regulations (CFR) part 58.

Gradient Elution: Increasing mobile-phase strength versus time in a chromatographic run. Gradients can be continuous or stepwise.

HETP: Height equivalent to a theoretical plate, a measure of a chromatographic column's efficiency (N). HETP = L/N, where L is the column length and N is the number of theoretical plates.

Identification: Ensuring the identity of an analyte.

Impurity: Any component present in the intermediate or API that is not the desired entity.

Impurity Profile: A description of the identified and unidentified impurities present in an API.

Installation Qualification (IQ): IQ ensures that all the activities associated with properly installing the instrument (new, preowned, or existing) at the users' site are documented.

Intermediate: A material produced during steps of the processing of an API that undergoes further molecular change or purification before it becomes an API.

Intermediate Precision (formerly referred to as ruggedness): Precision results from within-laboratory variations due to random events such as different days, analysts, equipment, etc. Experimental design should be employed so that the effects (if any) of the individual variables can be monitored.

Internal Standard: There are any number of ways to perform absolute quantitation in demonstrating accuracy and precision in the validation steps. One is to use an internal standard, which is placed into the sample before any extraction or isolation steps are performed. This standard, in known chemical purity, mimics the analyte of interest in efficiency of extraction from the sample matrix, chromatographic/UV/MS performance properties, and it elutes close to the analyte of interest but distinct from it in UV/MS profiles. A useful internal standard will have about the same recovery efficiency as the analyte itself from the sample matrix. The absolute, ideal internal standard would be an isotopically (nonradioactive) labeled analyte, in known chemical and isotopic purity, commercially available at reasonable cost, which will then have all the same chromatographic/UV/MS properties as the analyte itself. But, because it is resolvable in the MS from the analyte itself, it can serve as the perfect internal standard to quantitate recovery and levels of the analyte itself.

Isocratic: Use of a constant-composition mobile phase in chromatography.

Linear Regression: A method that determines the best-fit line through a collection of data points that represent the paired values of both an independent and dependent variable.

Linear Velocity (μ): The velocity of the mobile phase moving through the column, in cm/s. Related to flow rate by the cross-sectional area of the column.

Linearity: The ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range.

Lot: A collection of units of a single type or composition manufactured under identical conditions that are expected to have the same quality and uniformity within specifications.

Mean: The average of a series of measurements.

Mean or Standard Distribution: The average of the deviations of individual measurements from the average of the group.

Median: For observations arranged in order of magnitude, the median is the value for which an equal number of observations are above it and below.

Method Transfer: Comparison of key validation parameters between two testing sites.

Method Validation: The process by which it is established, through laboratory studies, that the performance characteristics of the method meet the requirements for its intended purpose.

Multiple Regression Analysis: A type of linear regression in which two or more multivariate independent variables are fit to a linear model of one dependent variable.

Nominal Concentration: Theoretical or expected concentration.

Normal or Gaussian Distribution: A sampling of data defined by a mean and a standard deviation that exhibits the frequency of a bell-shaped or Gaussian curve.

Observation: Experimentally derived data.

Operational Qualification (OQ): OQ testing is done to verify that the instrument and/or instrument modules operate as intended.

Out-of-Specification (OOS) Results: All suspect results that fall outside specifications or established acceptance criteria.

Outlier: Data that fails to meet a statistical test for acceptance.

Partial Validation: Validation of the affected performance characteristics of a method or procedure resulting from changes to the method or test substances.

Percent Recovery: The observed or assay value divided by the true or theoretical value multiplied by 100.

Performance Qualification (PQ): PQ testing is performed under the actual running conditions across the anticipated working range. In practice, a known method, with known, predetermined specifications is used to verify that all the modules are performing together to achieve their intended purpose. In practice, OQ and PQ frequently blend together in a holistic approach. For HPLC, the PQ test should use a method with a well-characterized analyte mixture, column, and mobile phase. Actual user PQ tests should incorporate the essence of the system suitability section of the General Chromatography Chapter 621 in the USP in order to show suitability under conditions of actual use.

Precision: The degree of agreement among individual test results when an analytical method is used repeatedly to multiple samplings of a homogeneous sample. *See* Repeatability, Intermediate Precision, and Reproducibility.

Procedural Blank: A sample of known composition (e.g., placebo) that does not contain the analyte of interest that is processed, prepared, or handled in the same way (procedure) as an unknown sample or standard.

Procedure: A specified way to perform an activity by execution of an approved document intended to produce a result defined by a specification.

Purity Tests: Analytical procedure that accurately assesses the impurity content of an analyte, for example, related substances test, heavy metals, and residual solvent tests.

Qualification: A subset of the validation process that verifies proper module and system performance prior to the instrument being placed on-line in a regulated environment.

Quality Assurance (QA): The sum total of the organized arrangements made with the object of ensuring that all APIs are of the quality required for their intended use and that quality systems are maintained.

Quality Control (QC): Checking or testing that specifications are met.

Quality Control Samples: Samples run to make sure the instrument has been properly calibrated or standardized. Quality control samples are also often used to provide an in-process assurance of the test's performance during use.

Quantitation Limit (QL or LOQ): The lowest concentration of an analyte in a sample that can be determined (quantitated) with acceptable precision and accuracy under the stated operational conditions of the method.

Range: The interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the method as written.

Reanalyze: Repeating the analysis or performing different analyses on the original sample preparation, reference standards, or reagents.

Recovery: Extraction efficiency generally reported as a percentage of the known amount of an analyte.

Repeatability: Precision results of the method operating over a short time interval under the same conditions (inter-assay precision). Generally, the criteria of concern in USP procedures.

Reproducibility: Precision results of collaborative studies between laboratories.

Resample: To obtain a new sample aliquot from the original test substance source. The sample is used for retesting and should be taken from the same homogeneous material that yielded the out-of-specification (OOS) result.

Resolution (R_S): The separation of two chromatographic peaks that takes into account the retention times and peak widths. Calculated by the equation: $r_S = 2(tr_2 - tr_1)/(w_{b1} + w_{b2})$, or by

$$Rs = \frac{\sqrt{N}}{4}\,\frac{\alpha - 1}{\alpha}\,\frac{k}{k+1}$$

A value of 1.0 is considered to be the minimum for a separation to occur, values of 1.5 or better for good quantitation, and values of 2.0 or higher for robustness or disparate levels such as those found in impurity profiles.

Retest: Repeating the analytical procedure on a resampled aliquot. *See* Resample.

Robustness: The capacity of a method to remain unaffected by small, *deliberate* variations in method parameters; a measure of the reliability of a method. Often used to set the system suitability parameters of a method.

Signature (signed): The record of the individual who performed a particular action or review. This record can be initials, full handwritten signature, personal seal, or authenticated and secure electronic signature.

Specification: A list of tests, references to analytical procedures, and appropriate acceptance criteria for the test described.

Specificity: Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix.

Stability: Degree or rate of degradation of an analyte in a given matrix under specific conditions over a given time interval.

Stability Indicating Method (SIM): A validated, quantitative analytical procedure used to accurately, precisely, and selectively detect a decrease in the amount

of the active pharmaceutical ingredient (API) from potential interferences such as degradation products, process impurities, excipients, or other potential impurities.

Standard Additions: First used in trace metals analysis in atomic absorption or emission spectroscopy and for organic analytes; a method of quantitating an analyte in complex sample matrices at low levels. The method relies on first dividing the sample solution into approximate equal fractions or volumes, analyzing the first sample unspiked, and then spiking each additional fraction with known amounts of the authentic analyte at varying levels (1/2x, x, 2x, 3x, etc.).

Standard Curve: Relationship between the analytical concentration and the experimental response value (also referred to as a *calibration curve*).

Stock Solutions: Solutions prepared from reference material used for preparation of working solutions (also referred to as *primary stock solutions*).

System Suitability: System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. System suitability tests are an integral part of chromatographic methods, and are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. System suitability parameters are established as a direct result of robustness studies.

Tailing: Situation where a normally Gaussian peak has an asymmetry >1.

Validation: A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result that accomplishes its intended purpose, meeting predetermined acceptance criteria.

Validation Protocol: A written plan stating how validation will be conducted and defining acceptance criteria.

Verification: An assessment of selected *Analytical Performance Characteristics* of method validation to generate appropriate relevant data as opposed to repeating the entire validation process. Verification demonstrates that acceptable results utilizing the laboratories' personnel, equipment, and reagents can be obtained.

Working Solution: Stock solution dilution used for the preparation of calibration standards and quality control samples (also referred to as *secondary stock solutions*).

Appendix: Example Method Validation Protocol

The following is an example method validation protocol template that might be used to validate a method internally, or in concert with an outsourcing laboratory. A document of this type would typically include a table of contents; a detailed listing of all the testing to be performed; the actual methods used (either as a part of the protocol or as an attachment); specific instructions on sample and standard preparations; concentrations, numbers of samples, numbers of injections and the sequence; how to treat the data; etc. The document should also include preestablished specifications. Formats can vary: sometimes acceptance criteria are listed along with the method attributes, or separately as presented here. Regardless of the format, the protocol should be a numbered and controlled document.

Title:	Validation of Test Method No. XXXX (Method Title)
Study Sponsor:	Company Name and Address
Study Monitor:	Name, Title, and Contact Information
Test Facility:	Company Name and Address
Study Director:	Name, Title, and Contact Information
Study Number:	
Proposed Experimental Date:	Initiation and Termination Dates

Authors/Approvals, etc. Signatures:

Study Monitor
Name, Title, Affiliation

Study Director
Name, Title, Affiliation

Quality Control/Assurance
Name, Title, Affiliation

A.1 INTRODUCTION

A.1.1 Purpose

This section should describe the purpose of the study; for example, type of method and its intended use, phase of development, required level of regulatory control. It should also include the number and description of the test method, the analytical performance parameters that will be investigated, and any ancillary tests (e.g., solution stability, filter studies).

A.2 TEST AND REFERENCE SUBSTANCES

Each test and reference substance used in the validation should be described in this section, along with chain of custody information. Items such as the source, lot numbers, identification numbers, storage conditions and location, expiry dates, etc., should also be included.

A.3 JUSTIFICATION OF THE TEST SYSTEM

This section describes the justification for the test procedure or system (e.g., HPLC, GC) explaining its use and applicability. Any out-of-the-ordinary methodology or techniques (e.g., chromatographic modes or detection) should also be described.

A.4 MATERIALS AND METHODS

A.4.1 Analysis

In this section, list the test method that will be used for sample analysis, and provide the draft method as an attachment. State how standard and sample preparation will be carried out (if according to the method, just specify as such). If a draft method document is not available, all method details should be described in this section. Typical injection sequences should also be described. For example,

Solution Preparation	Number of Injections
Diluent Blank	2 or more
Sensitivity Standard 1	1
Sensitivity Standard 2	1
Working Standard	5
Check Standard	1
Working Standard	1
Six Samples	1×6
Working Standard	1
Six Samples	1×6
Working Standard	1

A.4.2 System Suitability

Describe how system suitability will be evaluated. For example, system suitability parameters will be evaluated per the test method (Attachment) prior to performing each of the discrete components of the validation described in the following sections. When multiple validation components are analyzed in the same sample sequence, a single system suitability evaluation may be performed, and for robustness studies system suitability can be performed at each condition. System suitability criteria should also be listed in this section, as well as in the draft method.

A.4.3 Specificity

Describe in this section how specificity will be evaluated. For example, specificity will be evaluated by comparing the chromatographic results of individual solutions (at concentrations described) of all available standards and related substances (listed in the table) and a diluent blank. If different from above, sample and standard solution preparation should be described. If utilized, mass spectrometry and photodiode array methods used to evaluate peak purity (lack of coelutions) should be described, particularly if validating a stability indicating method. Any forced degradation/chemical stress studies performed within the scope of the study should also be described in this section, along with specific conditions and sample treatments.

A.4.4 Linearity and Range

This section should describe the concentrations and range (e.g., 80% to 120% of nominal concentration) of the samples to be evaluated for linearity, and how they should be prepared (e.g., according to the test method). The number of sample preparations (minimum of five levels), injections, and data treatment (averaged?) should be specified.

A.4.5 Accuracy

This section should describe specific conditions used (numbers of samples, levels/concentrations) to determine the accuracy of the procedure. Accuracy is typically evaluated by preparing three sets of samples at three levels (e.g., 80%, 100%, and 120% of nominal), and is often combined with linearity determinations. Accuracy is routinely performed at both the assay (100% of nominal) and at the impurity level (0.1% of nominal), depending on the method's intended use.

A.4.6 Precision (Repeatability and Intermediate)

Repeatability is performed by one analyst preparing and analyzing six separate sample solutions according to the method. Intermediate precision is demonstrated by a second analyst preparing six separate additional sample solutions according to the method using the same lot of standard as the first analyst and analyzing the samples on a second column lot and a second instrument on a second day (relative to the first analyst).

TABLE A.1
Example Robustness Parameters and Conditions

Parameter	Nominal Value	Condition 1	Condition 2
Column temperature	30°C	27°C	33°C
Concentration acetonitrile in MP	40%	38%	42%
Mobile phase pH	3.8	3.6	4.0
Mobile phase flow rate	1.0 mL/min	0.90 mL/min	1.10 mL/min

A.4.7 Method Robustness

Method robustness is evaluated by measuring method performance toward typical or normal variations in the method and detector operating parameters, for example, column temperature, mobile phase flow rate, and mobile phase composition, pH, buffer concentration, etc. The effect (if any) of these variations can be determined by evaluating the system suitability criteria. Example parameters and conditions that might be tested are listed in the Table A.1. If any varied condition results in a failure to meet requirements, it must be documented in the method and suitable controls put in place.

Any experimental design used to evaluate the different parameters should be described.

A.4.8 Degradant LOD

The LOD will often be established at 0.05% of the nominal concentration and evaluated with at least one sample prepared at that concentration.

A.4.9 Degradant LOQ

The LOQ will often be established at 0.1% of the nominal concentration, and evaluated by measuring precision and accuracy of six separate samples prepared at the target concentration.

A.4.10 Solution Stability

The stability of the stock reference standard and sample solutions is evaluated (duplicate injections) at established intervals following storage at both room temperature and refrigeration by assaying against freshly prepared standards prepared from a freshly prepared stock solution.

A.5 DATA EVALUATION AND REPORTING

A.5.1 System Suitability

This section should include any system suitability criteria to be measured, for example, peak retention time and area (%RSD), resolution (peaks specified), the check standard recovery, tailing, etc.

A.5.2 Specificity

Specificity is demonstrated by the separation of the peak of interest and any related substances from each other and the absence of detectable peaks in the diluent blank that would co-elute with any of the peaks of interest. Example chromatograms should be provided.

A.5.3 Linearity and Range

The linearity is typically determined by regression analysis of the five concentrations using the method of least squares. The correlation coefficient, coefficient of determination, *y*-intercept, slope of the regression, and the residual sum of squares are typically reported. The range of the method will be obtained from the linearity analysis.

A.5.4 Accuracy

Results will be calculated for each sample (accuracy/recovery). The percent recovery in each sample will be determined based on the actual sample concentrations. The mean, standard deviation, and %RSD are calculated and reported for each concentration level.

A.5.5 Precision (Repeatability and Intermediate)

Repeatability results are calculated from the six samples prepared by the first analyst. The mean, standard deviation, and %RSD will be calculated and reported.

For intermediate precision, the mean, standard deviation, and %RSD will be calculated for each set of experiments (analyst A/column A/system A versus analyst B/column B/system B).

A.5.6 Robustness

The effect (if any) of the variables tested will be determined by the system suitability criteria.

A.5.7 Degradant LOD

Visual observance of a chromatographic peak establishes the LOD. Example chromatograms should be provided.

A.5.8 Degradant LOQ

The mean, standard deviation, and %RSD will be calculated from the analysis of the six samples prepared at the 0.1% level. Example chromatograms should be provided.

A.5.9 Solution Stability

Stability of the stock standard solution will be evaluated by comparing the average assay value (duplicate injections) of a peak in a freshly prepared dilution of the stored stock standard solution (at both refrigerated and room temperature conditions) versus the freshly prepared working standard solutions at each time interval.

The sample stability will be evaluated by calculating the percent assay at each time interval and comparing the results against the assay value at time 0, and must not differ by more than 30%.

A.6 EXAMPLE ACCEPTANCE CRITERIA

Parameter	Acceptance Criteria	
System Suitability	Linearity	$r^2 \geq 0.995$
	Resolution	≥2.5
	Retention Time	6.5 ± 1 min
	Overall Standard Precision (6 injections)	Area RSD ≤ 10%
	Check Std. Recovery	90% to 110%
Specificity	All peaks of interest are resolved from one another.	
	Lack of detectable peaks co-eluting with the peaks of interest in the chromatograms of a diluent blank	
Accuracy	Assay level	97% to 103% NMT 2% RSD
	Impurity level	75% to 125% NMT 25% RSD
Precision (repeatability)	RSD ≤ 2% for both retention time and area	
Precision (intermediate)	Assessed for equivalence using the Student's t-test for establishing the equivalence of means. The test will be applied at the 95% confidence level.	
LOD	Detectable peak	
LOQ	Recovery 75% to 125% with NMT 25% RSD for the analysis of six samples	
Robustness	Meet system suitability criteria for all experiments and samples ≤30% difference from original method conditions	
Solution Stability (Stock standard and samples)	The Reference Standard and Sample Preparations stored at both ambient and 2°C–8°C should remain within 100% ± 2% of the initial assay value at each storage time interval	

A.7 STATEMENT OF EXPECTED RESULTS

It is expected that the results obtained during this validation will demonstrate the suitability of the test method to accomplish its intended purpose.

A.8 DATA RECORDING

All sample data will be recorded in accordance with standard operating procedures and quality systems. It is sometimes customary to refer to notebooks, data forms, or other recording means (e.g., electronic laboratory notebooks). If data forms are used, examples should be attached to the protocol as an appendix, or included as part of the method.

A.9 PROTOCOL AMENDMENTS

This section should describe how amendments and deviations to the protocol should be handled, how approved, etc.

A.10 REGULATORY COMPLIANCE

This section should describe what level of regulatory compliance is applicable (cGMP, cGLP, research only).

A.11 REFERENCES

Include all regulatory, SOP, literature, and internal references as appropriate.

A.12 APPENDICES

This section should include the draft method if available (and not detailed in the body of the protocol), data forms used to record data, analyst notes, and any other pertinent information necessary to carry out the work instructions in the protocol.

Index

P

Q

R

V

W