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Development and Validation of HPLC Stability-Indicating Assays

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Part I: Method Development

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1. WHAT IS A STABILITY-INDICATING METHOD?

According to the regulatory definition (1), a stability-indicating method is one of a number of

> Quantitative analytical methods that are based on the characteristic structural, chemical, or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.

Therefore a stability-indicating method is an analytical procedure that is capable of discriminating between the major active (intact) pharmaceutical ingredient (API) from any degradation (decomposition) product(s) formed under defined storage conditions during the stability evaluation period. In addition, it must also be sufficiently sensitive to detect and quantify one or more degradation products. A corollary may be added that the analytical method must be also capable of separating or resolving any other potential interfering peak such as an internal standard. With these criteria, then, the discriminating "nature" of the method indicates the method to be *stability-indicating* as well as *stability-specific*. Later in the discussion we will see that other methods may be stability-specific but not stability-indicating. Stressed testing may be used (1,2) to expedite the decomposition pathway(s) to generate decomposition product(s) for the API. However, stressed testing under forced conditions of oxidation, photolysis, hydrolysis, and varying pH values may form some decomposition products that are unlikely to be formed under accelerated or long-term stability storage conditions. The products generated nonetheless may be useful in developing and validating a suitable stability-indicating analytical method for the analysis of the drug substance and the drug product, expediting the availability of the completed analytical method.

It is paramount that the chosen analytical method used for stability evaluation be validated and discriminating to ensure efficacy of the subsequent stability evaluation. Confidence in the stability data is predicative on time invested up front to ensure a viable procedure as well as to conform to legal and regulatory requirements (2).

2. STRATEGY OF METHOD DEVELOPMENT

Development of a stability-indicating method should be predicated on the method's intended application as well as selecting a suitable technique designed to assess

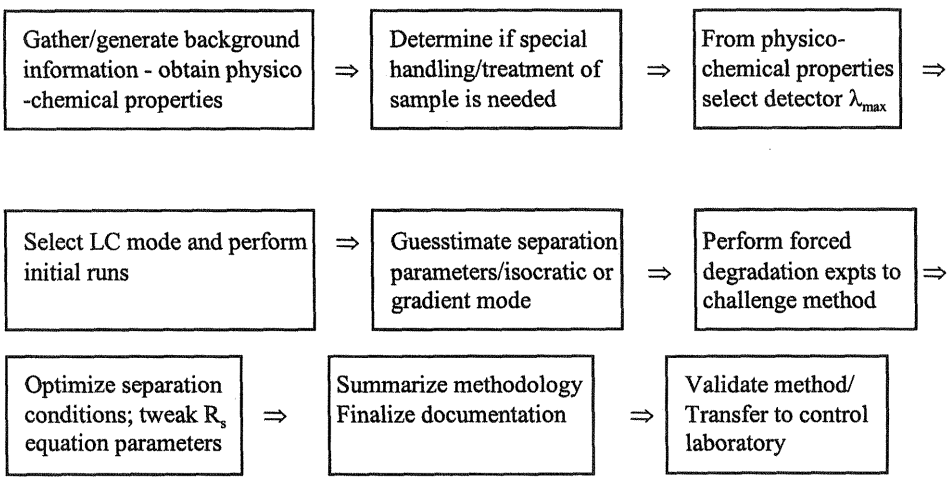
the API's stability requirements. Obviously the intended application of a stability-indicating method is for monitoring the stability of a given drug in a finished product and would require assessment of the method's stability-indicating properties. One specialty application of a stability-indicating method is cleaning validation testing, which would require assessment of its stability-indicating properties, as holding time (of the swaps) would be a critical factor. Other applications such as product release, performance testing (i.e., dissolution testing) and in-process testing do not require this assessment. Some pharmaceutical houses still (but now less commonly due to technological advances and overall industry practice) utilize a non-stability-indicating method such as UV, for product release, and an HPLC method for stability testing. However, whenever there is a hold time issue, common in dissolution or in-process testing, it would be prudent to assess the method for its stability-indicating properties before its intended application.

Other chromatographic separation methods, such as chiral chromatography (CC), thin-layer chromatography (TLC), gas chromatography (GC), and (increasingly) capillary electrophoresis (CE), are stability-indicating and stability-specific methods. Still the most prevalent technique is reversed-phase HPLC alone or coupled with ion-suppression, which accounts for 85% or more of the general pharmaceutical applications.

Nonchromatographic and spectroscopic techniques such as titrimetry, atomic absorption, UV spectrophotometry, and infrared spectroscopy, while precise, are not considered stability-indicating, and as such not suitable for stability assessment applications.

3. OVERVIEW OF THE METHOD DEVELOPMENT PROCESS

Before beginning with actual experimentation it would be advantageous to view method development from a broader perspective. The method development process can be visualized from a high-level process map perspective better to define the general steps encountered to achieving the end product, a stability-indicating method.



4. GETTING STARTED

It is probably best to approach method development with the intention of using the developed method for stability assessment as a final application, after the method has been validated. This approach entails determining the discriminating ability of the selected method up front before investing time and money in evaluating other analytical parameters prior to assessing the stability-indicating element of the method.

Reversed-phase HPLC is the method of choice for stability-indicating and stability-specific methods, although thin-layer chromatography (TLC), gas chromatography (GC), and capillary electrophoresis (CE) are also acceptable choices. Reversed-phase HPLC coupled with ionic suppression account for probably over 85% of stability-indicating methodologies for small molecular weight pharmaceutical entities. This combination is well suited for applications in release testing, in-process as well as stability testing. Additional applications may be in cleaning validation and performance testing. Other techniques such as titration and UV spectroscopy, while commonly used for release testing, are generally considered nonspecific and thus are not considered for stability assessment.

Invariably when one is faced with finding or developing a method, one or two routes may be used depending on the nature of the chemical entity: modification or development. Modification is used when there is information or a method already exists for a similar entity. In this case, the existing method is modified or tweaked to accommodate the new entity. This may or may not be suitable; if not, development (starting from scratch) is the way to go. The goals of the separation should also be considered at this point.

4.1. Background Information

Knowledge of physicochemical properties of the API is invaluable to the method development process. Information on the various properties has been collected, either through a systematic program of generating the appropriate information in support of drug discovery (organic chemistry synthesis) on the one hand, or on the other, from a search of the literature or from company drug profiles, spectral libraries, or reports. Information such as dissociation constants, partition coefficients, fluorescent properties (if any), chromatographic behavior, spectrophotometric properties, oxidation-reduction potentials, formulation stability studies, and solubility studies are all very useful and can expedite the development process.

Dissociation constant and partition coefficients can be used to develop efficient liquid/liquid extraction procedures, and data on fluorescence, spectrophotometric, chromatographic, and oxidation-reduction properties can be used to determine the best means of measuring and quantifying the analyte of interest. Stability studies are performed on the drug substance, in solution and mixed with pharmaceutical excipients as part of compatibility studies. Labile functional groups are identified, and the susceptibility of the drug to hydrolysis, oxidation, thermal degradation, etc. is determined. Compatibility studies are performed to assess the stability of the API when mixed with common excipients and lubricants as well as to determine any interaction between the drug and the (inactive) raw materials. Solubilities should

be determined in a number of solvents covering a range of polarities that are commonly used in method development.

Solubilities should be determined in aqueous and organic solvents, such as

Aqueous	*Organic*
Water	Ethanol/methanol
Buffers	Chloroform
0.1 N HCl	Cyclohexane
0.1 N NaOH	Acetonitrile
	Tetrahydrofuran

Spectral libraries are established, and information gleaned is useful for selection of initial conditions for an HPLC separation. On the other hand, however, sometimes this physicochemical information may not be known or available, so that an initial separation would have to be tried, based on prior experience, in order to determine a course of action for subsequent experimentation.

4.2. What Is Known About the Sample

Ideally, knowledge of the API's nature relative to composition and other properties would be beneficial. For example, information about the compound's synthetic route would shed light on any related product(s) and possible degradation product(s), as well as possible impurities; knowledge of the compound's chemical structure would reveal any possible stereoisomer which in turn would necessitate a different separation strategy, and so forth.

Table 1 shows typical information that would be helpful concerning the nature of the compound. The more information is available, the less empirical the approach to developing a separation method will be.

5. SEPARATION GOALS

To determine the separation goals, which should be clearly defined, a number of questions should be asked to help delineate the end purpose of the separation. Typical questions may include

> What is the overall purpose of the method—quantitative, qualitative, or for isolation/purification of a compound (i.e., content assay, stability, impurities, cleaning assay, or for purification application)?

Table 1 Useful Physicochemical/Related Information Concerning the Compound

Wavelength of absorption (λ_{max})
Identity/number of compounds present (i.e., stereoisomers/chiral centers?)
Chemical structure (functionality); amphoteric
Molecular weight
pK_a values of compounds
Salt form of the drug
Solubility of compound
Purity of compound

What level of accuracy and precision would be needed?
The method is designed for what type of matrix? How many types of sample matrices are encountered?
Is the method developed using certain equipment transferable to the control laboratory, which may not have the same equipment?
Will the method be used for a few samples or many samples?
What chromatographic parameters are needed?
How much resolution is needed?
What is a suitable/acceptable separation time?
What is a suitable column pressure?
How much sensitivity is required?
Is an internal standard needed?
Are there any detection issues? Most analytes absorb in the UV region of the spectrum.
Does integration use peak area or height?
Is the mode isocratic or gradient?

6. SELECTION OF THE CHROMATOGRAPHIC MODE

6.1. The Different Modes of Liquid Chromatographic Methods (HPLC)

While there are a number of HPLC methods available to the development chemist, perhaps the most commonly applied method is reversed-phase. Reversed-phase and reversed-phase coupled with ion-pairing probably account for more than 85% of the applications for a typical pharmaceutical compound. The typical pharmaceutical compound is considered to be an API of less than 1,000 daltons, either soluble in water or in an organic solvent. The water-soluble API is further differentiated as ionic or nonionic which can be separated by reversed-phase. Similarly, the organic soluble API can be classed as polar and nonpolar and equally separated by reversed-phase. In some cases, the non-polar API may have to be separated using adsorption or normal phase HPLC, in which case the mobile phase would be a nonpolar organic solvent. For those "special" compounds that do not fall into this category (API>1000 daltons [biopharmaceuticals], isomers or enantiomers), other chromatographic modes may be necessary for separation. These include ion-exchange and chiral chromatography. In this discussion of developing a stability-indicating HPLC method, only reversed-phase will be discussed.

6.2. Reversed-Phase Chromatography

Thus given the limited number of methods with stability-indicating properties, it is probable that the method selected would be HPLC. Two very advantageous characteristics of HPLC, its discriminating power and its ability to operate at room temperature or at low elevated temperature, would not contribute to the degradation of the analyte. It is further assumed that the API is of low molecular weight (<1000 daltons), organic in nature (versus inorganic), and not a biopharmaceutical. These restrictions apply to a large percentage of the pharmaceuticals and enable them to be readily separated using reversed-phase HPLC, and sometimes with the aid of an ion-suppression agent, in roughly 85%

of the applications. The next question, then, is whether the chromatographic mode would be isocratic or gradient (see Fig. 3, Sec. 15).

6.3. Chiral Chromatography

Within this decade, since 1992, the FDA has published a position paper on the development of new stereoisomeric drugs (3). Prior to this time the majority of chiral synthetic compounds were marketed as racemic mixtures. This is because, until recently, it was not technically possible or economically feasible to separate racemic mixtures into their individual enantiomers. Experience has indicated that the individual enantiomers may exhibit different therapeutic effects. For example, the R-enantiomer of sotalol is antiarrhythmic while the S-enantiomer is a beta-blocker (4); and the dextro isomer *d*-propoxyphene (Darvon®, Lilly) is analgesic while the levo isomer *l*-propoxyphene is antitussive (but never developed into a marketed product) (5). However, with the FDA's position paper and current technological advances such as large-scale chiral separation techniques and asymmetric syntheses, new chemical entities (NCEs) containing a chiral center must be resolved into the different enantiomers and each enatiomer characterized and the drug product be composed of only one enantiomer instead of a racemate.

Thus, as contained in the International Conference on Harmonization (ICH) draft guideline on drug/drug product specifications (6), the tests in the table must be satisfied for new drug substances that are optically active:

Drug substance	*Test/specification requirement*
Impurities	Similar to other impurities
Assay	Enantioselective procedure or achiral method with appropriate means to control enantiomeric impurity
Identity	Test(s) should discriminate the enantiomers
Drug Product	
Degradation products	Control of other enantiomer if that enantiomer is a degradation product
Assay	If enantiomer is not a degradation product, an achiral method is acceptable, but chiral assay is preferred, or alternatively, achiral assay plus means to control the presence of the enantiomer
Identity	Test to verify the presence of the correct enantiomer

As such, in the development of a chiral method, the regulatory requirements must be considered. The reader is referred to decision tree #5 (page 62903) of the same reference for a schematic guide to development strategy and to the Wozniak (7) paper to determine what additional analytical information is needed for the development of chiral drug products.

6.4. Gas Chromatography

Gas chromatography, while *stability-indicating*, is not as versatile as HPLC, as the drug substance may not be volatile. On the other hand, increasing the temperature

to effect volatility may cause degradation as well as effecting racemization. However, there may be a limited number of instances in which this technique would be useful, such as for small nonaromatic compounds that simple are not possible to separate by current HPLC and TLC techniques.

6.5. Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a mature chromatographic technique and is still widely used throughout the pharmaceutical industry in research as well as in the control laboratory. It is used throughout the drug development process for determining the purity of the drug substance, reference standards, and intermediates. It possesses many advantages including simplicity, low cost, and a short run time. It is cost effective. Its main disadvantage is variability. Constanzo (8) has proposed a three-point window approach to optimize resolution, and thus to minimize the variability, by controlling the mobile phase composition.

TLC (limit test) is used to complement a non-stability-indicating procedure as indicated in the FDA Guideline for Submitting Samples and Analytical Data for Methods Validation (2).

6.6. Capillary Electrophoresis and Capillary Electrochromatography

As sciences, both capillary electrophoresis (CE) and capillary electrochromatography (CEC) today are probably where HPLC was 10 years ago. CE is a separations technique based on the mobility of ions through a buffer-filled capillary in an electrically charged environment. This would provide a separation of charged species. When CE is coupled with a stationary phase and high pressure, it is known as CEC, in which the separation is based on electrophoretic migration and chromatographic partitioning enabling the separation of neutral species. Both techniques are more applicable to biological systems, in biopharmaceutical and other R&D applications, than in quality assurance/product specification applications. The techniques are very sensitive and well suited for separations of small amounts of expensive biopharmaceuticals. On the other hand, they have less utility as a product release or stability test methodology, especially in product specification applications of small molecular entities where there is an abundance of samples and where sensitivity is not an issue.

The utility of the technique, however, lies in its ability to achieve high sensitivity and resolution through high efficiencies with minimal peak dispersion. Moffatt et al. (9) have reported unusually high efficiencies of up to 2.5 million plates per meter in the capillary electrochromatographic analysis of partially ionized anionic-neutral pyrimidine compounds using a standard C_{18} stationary phase.

The number of manufacturers of CE/CEC equipment are not nearly as many as for HPLC equipment. Major manufacturers include Unimicro Technologies, Thermo Bioanalysis, Beckman Coulter, and Micro-Tech Scientific. The last company's model Ultra-Plus II has an integrated, gradient capillary HPLC/CE/CEC system. This combination of gradient elution and electrophoretic migration provides a rapid analysis with high resolution (10).

7. ROLE OF FORCED DEGRADATION

7.1. Regulatory Basis

The 1987 edition of the FDA stability guidance document (1) stipulates that the API be subjected to a number of forced degradation conditions to include acidic, basic, and oxidative conditions. Workers in the field have also included temperature and light (photostability). The current draft stability guide (11), while not yet official, specifically includes photostability and temperature cycling requirements; no mention of acidic, basic, or oxidative conditions were made, however. The current ICH guidances (Q2A and Q2B) also do not specify how degradation studies are to be conducted; this was left to the discretion of the responsible companies.

7.2. Scientific Basis

Forced degradation should be one of the activities performed early in the development process to ensure that the method is discriminating before a lot of time, effort and money have been expended. The guidance documents do not indicate detailed conditions, so the conditions and interpretations are left up to the development scientist. Suggested forced degradative conditions are summarized in Table 2. Trial and error are needed to find the proper combination of stress agent concentration and time to effect a degradation, preferably in the 20–30% range. Depending on the API, not every stress agent may effect a degradation, but each agent has to be evaluated to determine whether degradation results.

Additional comments are warranted.

Adequate k'. The initially developed method should achieve a suitably retained peak, with a k' of about 4 to 10. This range allows a suitable time space in the chromatogram for degradants to elute before or after the active (major) peak. Since the polarity of the degradants relative to the major peak is not known, the k' of the major peak eluting in the middle of the chromatogram adds some assurance that the degradants would elute on either side of the main peak.

Degradation conditions. Unfortunately this is a trial and error process. Typical degradative conditions involve hydrolysis, photolysis, acid/base reactions, and temperature. The goal is to obtain about 20–30% degradation and not complete degradation of the active compound. Achieving 100% degradation would be too strenuous and could possibly cause secondary degradation, giving degradation products of the degradation product(s), which are not likely to be formed under normal storage conditions. Depending on the API, not all of the degradation conditions effect degradation, and after a reasonable effort (varying concentrations and time) to produce a degradation product with no success, one can move on to the next condition. For example, when chlorhexidine digluconate, an antimicrobial agent in mouthwash, was subjected to each of the above conditions, only degradants were isolated from heat, acid and light (12). While it was impervious to the other conditions, this was not known up front, so each of the conditions had to be tried.

Acid/base. Generally the concentration of the API is doubled to enable the reaction solution to be neutralized before injecting into the HPLC system to prevent damage to the silica-based chromatographic column.

Controls. Refer to Table 2. It is important that corresponding matrices and appropriate controls be treated in a similar fashion to identify possible interferences.

Table 2 Suggested Outline for Performing Forced Degradation Studies

Decide/select matrix for degradation

		Degradation conditions					
Product/matrix	Degradation	Acid	Base	Peroxide	Bisulfite	Photostability	Temperature
Product	Yes	√	√	√	√	√	√
Placebo/vehicle	Yes	√	√	√	√	√	√
API/raw material	Yes	√	√	√	√	√	√
Internal standard	No	—	—	—	—	—	—
Controls							
Product	No	—	—	—	—	—	—
API/Raw material	No	—	—	—	—	—	—
Blank solution	No	—	—	—	—	—	—

Decide/select degradation conditions/agents

Medium	Conditions*
1 N HCl, 10 mL	Reflux 30 minutes, neutralize with base
0.1 N NaOH, 10 mL	Reflux 30 minutes, neutralize with acid
3% Hydrogen peroxide, 10 mL	Reflux 30 minutes
10% Sodium bisulfite, 10 mL	Reflux 30 minutes
Light	Light chamber, 1 lumens (92.9 lux = 1000 ft-candles), 7 days
Temperature (dry heat)	80°C, 7 days

* Strive for 20–30% degradation.

Internal standard. Should the analytical method utilize an internal standard, it is not recommended to degrade the internal standard, but its k' should not interfere (elute) at any of the possible eluting k' peaks.

Evaluation of the degradation mixture is generally performed using a photodiode array detector. Assessing the purity of the major peak is very important and could be difficult in light of possible peak inhomogeneity after the degradation process. One must be assured that there is no degradation peak (hiding) under or unresolved from the major peak of interest. The utility of the diode-array detector is that the analyst can select a whole wavelength range, say from 200 to 350 mm, with a bandwidth of 80 nm. With just one single chromatographic run, all compounds absorbing within this range will be detected. With only one wavelength selected using a conventional UV detector, for instance at 280 nm, any compound not absorbing at this wavelength will not be detected. Figures 1A and 1B depict diode-array chromatograms for assessing peak purity.

Refer to Section 17.4 for further discussion.

8. PEAK PURITY

There is always that nagging question of whether the peak of interest (the major analyte peak) is pure or homogeneous. This is a difficult question, and many investigators have tried to prove the homogeneity of the major peak under stressed conditions during the method development and validation process. Various techniques have been used to characterize peak homogeneity, such as spectral suppression, absorbance ratio, spectral overlay (13), electrospray mass spectrometry (14,15) and dual detection (16).

9. SAMPLE PREPARATION

Sample preparation is a critical step in the overall chromatographic process, and can affect the chromatography if not developed or treated properly. This step encompasses sample filtration, sample extraction as well as sample derivatization, although the latter is not commonly used in the pharmaceutical quality laboratory. The purpose of this step is to prepare the sample so that the drug substance can be readily chromatographed, separated from other materials. Thus, it is a step to remove any interferences, to enhance the detection of the drug substance as well as to protect or enhance the life of the analytical column.

The following considerations are noted:

What is the matrix?
Ensure complete dissolution of the analyte in mobile phase or weaker solvent.
Miscibility and solubility.
Does the analyte precipitate in the buffer?
Some typical treatment modes are
- Direct injection
- Dilution
- Sonication
- Shaking

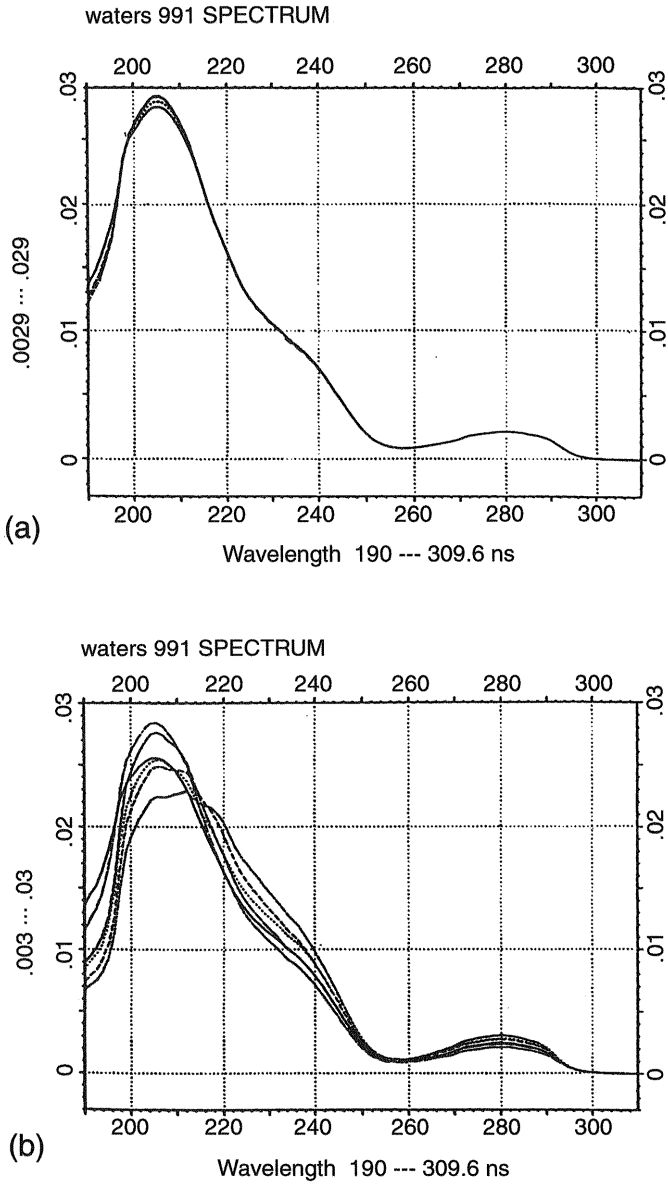


Figure 11.1 (a) No degradation (scans superimposable). (b) Presence of degradation (scans not superimposable).

Filtration/ultrafiltration
Extraction—Liquid or solid phase
Evaporation
Reconstitution
Derivatization
Heating/cooling

On rare occasions, the drug product, in a solution package form, may be injected directly or after an appropriate dilution. Typically, for a solid, such as a tablet or a capsule, the pretreatment would necessitate a comminution step, followed by extraction/sonication, filtration, and dilution. For example, an ointment may necessitate an extraction followed by evaporation, reconstitution, and dilution, or heating to dissolve the analyte/matrix, followed by cooling to precipitate the matrix, and then filtration.

While the typical dosage form—solid (tablet/capsule), semisolid (ointment/cream), or solution (cough syrup/ophthalmic solution)—utilizes a combination of the treatment modes mentioned earlier, solid-phase extraction (SPE) has become a recognized and viable technique for sample preparation methodologies, especially for biosamples and as an alternative to liquid-liquid extractions in many U.S. Environmental Protection Agency (EPA) methods. A recent supplement to *LC/GC* magazine was dedicated to advances in SPE (17).

It is very important that the sample preparation, prior to injection into the liquid chromatograph, be freed of particulate matter, through either filtration or centrifugation, and that the solvent be compatible with the HPLC system. If there is incomplete sample solubility or if the solvent is too polar, band distortions or tailing will result. Ensuring that the sample is completely dissolved in the proper solvent and then diluting the sample in mobile phase will eliminate these problems.

10. DEVELOPING THE SEPARATION—CHOOSING THE EXPERIMENTAL CONDITIONS

From Sec. 5, we assume that separation goals have been determined, such as resolution (at least baseline), reasonable run time (under 10 minutes), and ruggedness. These elements are further discussed below and developed in greater depth in Part II of this chapter under Validation. From Sec. 6.2 above, a case has been made that reversed-phase HPLC is suitable for our API of interest. The next step is to determine whether the API is typical. Referring to Secs. 6.1 and 6.2 above, let us further assume that the API is ionic and acidic. From a listing of generic separation conditions, see Table 3, conditions for an ionic and acidic compound are selected, and an initial exploratory run using gradient elution is made.

At this point, two options may be available to us before performing the exploratory run in the development of the desired stability-indicating procedure. First, there may be a method, either in-house or from the literature, already available for the same API or compound of interest. Useful information may be gleaned from here to modify to suit the specific compound on hand. On the other hand, sometimes established methods may not be optimal, so rather than modifying the method to suit our need, it may be better in the long run to develop a new method that is optimal and rugged.

Exploratory runs can be done manually or with computer software. Both are trial and error methods, but the latter is more systematic, quicker, and requiring fewer injections. When and after an initial exploratory run has been performed, the chromatogram is evaluated before proceeding with the next injection, and subsequent adjustments are made to the mobile phase composition. Each subsequent injection is thus based on the previous conditions, so that after a number of injections

Table 3 General Experimental Conditions for an Initial HPLC Run

	Initial Parameters		
Chromatographic variables	Neutral compounds	Ionic-acidic compounds (carboxylic acids)	Ionic-basic compounds (amines)
Column			
Dimension (length, ID)	25 cm × 0.46 cm	25 cm × 0.46 cm	25 cm × 0.46 cm
Stationary phase	C_{18} or C_8	C_{18} or C_8	C_{18} or C_8
Particle size	10 μm or 5 μm	10 μm or 5 μm	10 μm or 5 μm
Mobile phase			
Solvents A and B	Buffer-acetonitrile	Buffer-acetonitrile	Buffer-acetonitrile
% B (organic) isocratic	50%	50%	50%
% B (organic) gradient	20%–80%	20%–80%	20%–80%
Buffer			
Type	Phosphate	Phosphate	Phosphate
Concentration	50 Mm	50 mM	50 mM
pH	3.0	3.0 & 7.5 (gradient)	3.0 & 7.5 (gradient)
Modifier	10 mM triethylamine and 1% acetic acid, if needed	1% acetic acid	25 mM Triethylamine
Flow rate	1.5–2.0 mL/minute	1.5–2.0 mL/minute	1.5–2.0 mL/minute
Temperature	Ambient to 35°C	Ambient to 35°C	Ambient to 35°C
Sample size			
Volume	10 μL – 25 μL	10 μL – 25 μL	10 μL – 25 μL
Mass	<100 mcg	<100 mcg	<100 mcg

the proper conditions can be found (18). Refer to Sec. 12 below further discussion on software method development.

10.1. Key Variables—Resolution Equation Parameters

In reversed-phase/ion-pair chromatography, there are essentially 8–10 key variables that affect the separation, as depicted in the resolution equation, R:

$$R = \frac{1}{4} \cdot N^{1/2} \cdot (\alpha - 1) \cdot \frac{k'}{1 + k'}$$

where N, α and k' are referred to as the efficiency, selectivity, and retention (capacity) factors, respectively affecting the resolution of the analyte from other components in the separation. The efficiency is affected by the nature of the column, and both selectivity and retention are affected by the solvent. Column variables include length, particle size, and flow. Solvent variables are the nature of the sample, the mobile phase, and the column surface, i.e., bonded-phase (adsorbent type) such as C18, phenyl or cyano, etc.

These key variables include mobile phase strength, solvent type, column type/size, pH, temperature, ion-pair reagent (type and concentration), buffer, and mobile phase flow rate.

10.2. Isocratic or Gradient Mode

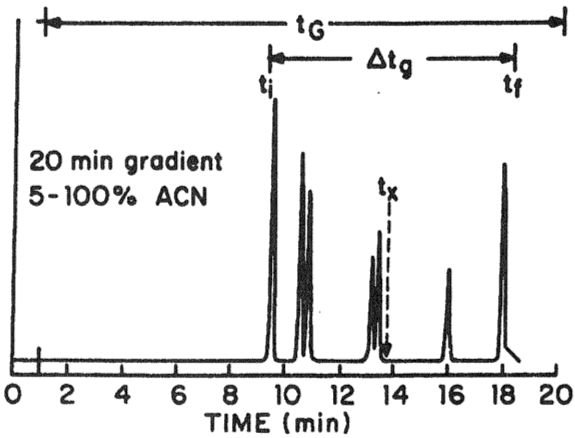
Either isocratic or gradient mode may be used to determine the initial conditions of the separation, following the suggested experimental conditions given in Table 3. Depending on the number of active components to be resolved or separated, the more complex the separation, the more gradient elution would be advantageous over isocratic mode, which is akin to a brute force application when trying to separate a complex mixture. When faced with developing a method to separate a complex mixture, the use of computer software is useful. This is further discussed in Sec. 12.

In deciding whether a gradient would be required or whether isocratic mode would be adequate, an initial gradient run is performed, and the ratio between the total gradient time and the difference in gradient time between the first and last component are calculated. When the calculated ratio is < 0.25, isocratic is adequate; when the ratio is > 0.25, gradient would be beneficial (19) as shown in Figure 2.

For complex mixtures (separations), when there are many degradation products, a long gradient run may be needed. In this case, a compromise may have to be made, using an isocratic method for product release and a gradient method for stability assessment. The isocratic method has generally a shorter run time, say under 15 minutes, and no degradation product would be monitored, assuming that none are formed initially. With time the degradation products are formed and must be monitored, which requires a gradient method to resolve completely the mixture (15 minutes and longer depending on the complexity of the degradation mix). The gradient method, then, would be the stability or regulatory method.

10.3. Role of pH

pH is another factor in the resolution equation that will affect the selectivity of the separation. In reversed-phase HPLC, sample retention increases when the analyte



$$t_x = (t_f + t_i)/2$$

$\Delta t_g/t_G > 0.25 \longrightarrow$ gradient

$\Delta t_g/t_G < 0.25 \longrightarrow$ isocratic

$$\Delta t_g/t_G = \frac{18.0-9.5}{20} = 0.425$$

$\therefore$ gradient required

Figure 11.2 Isocratic or gradient? (From Ref. 19.)

is more hydrophobic. Thus when an acid (HA) or base (B) is ionized (converted from the unionized free acid or base) it becomes more hydrophilic (less hydrophobic, more soluble in the aqueous phase) and less interactive with the column's binding sites.

$$\begin{aligned} HA &\Leftrightarrow H^+ + A^- \\ B + H^+ &\Leftrightarrow BH^+ \\ \text{Hydrophobic} &\quad \text{Hydrophilic} \\ \text{(more retained on column)} &\quad \text{(less retained on column)} \end{aligned}$$

As a result, the ionized analyte is less retained on the column, so that the k' is reduced, sometimes dramatically. When the $pH = pK_a$ for the analyte, it is half ionized, i.e., the concentrations of the ionized and unionized species are equal. As mostly all of the pH-caused changes in retention occur within ±1.5 pH units of the pK_a value, it is best to adjust the mobile phase to pH values at least ±1.5 pH units above or below the pK_a to ensure practically 100% unionization for retention purposes. The pH range most often encountered in reversed-phase HPLC is 1–8, normally considered as low pH (i.e., 1–4) and intermediate pH (i.e., 4–8). Generally, at low pH peak tailing is minimized and method ruggedness is maximized. On the other hand, operating in the intermediate range offers an advantage in increased analyte retention and selectivity. See Sec. 10.7 for further discussion. For a detailed

treatment of retention as a function of pH, the reader is referred to the works of Lewis et al. (20) and Schoenmakers and Tijssen (21).

As stated in Table 1, it is important to know the salt form of the drug substance of interest or whether it is amphoteric. This information is invaluable to the development of the analytical methodology because it will aid in the optimization of the method to effect better separation, resolution, and chromatography. If the drug is amphoteric, the pH can be selected whereby the compound exists as a single species and not a mixture of species. Mixed species will lead to poor separations. On the other hand, if the drug has different salt forms, say the hydrochloride and the napsylate (e.g., *d*-propoxyphene hydrochloride, Darvon®, and *d*-propoxyphene napsylate, Darvon-N®, both drug products marketed by Eli Lilly), the problem is not as critical, for

The salts represent different products and are marketed separately for different pharmacokinetic effects, i.e., different absorption profiles, with the hydrochloride being more soluble, and thus showing a faster absorption and distribution.

In solution, both salts will be dissociated from the organic propoxyphene moiety so that the final analytical methodology is appropriate for the separation and detection (or titration) of the analyte free base. For example, in the USP monographs for the two propoxyphene (hydrochloride and napsylate salts) APIs and their several products (22), the final analytical methods, be they titrimetric or chromatographic, all detect the analyte free base propoxyphene, and the assay percentage is calculated using a molecular weight correction factor.

10.4. Role of Solvent Type

Solvent type (methanol, acetonitrile, and THF) will affect selectivity similarly for ionic and neutral analytes. Hence changing a solvent would be a useful variable in the separation. The choice between methanol and acetonitrile may be dependent on the solubility of the analyte as well as the buffer used. While THF may be the least polar of the three, it has the highest solvent strength. If that property is not essential, its odor and potential peroxide formation may be a deterrent.

10.5. Role of Mobile Phase

The mobile phase composition (percent aqueous to organic) as well as the solvent strength will affect both α (solvent selectivity) and k' (solvent strength). The sample solvent will have a similar effect as well and may lead to peak distortion if the polarity between the mobile phase and the sample solvent is great. Thus, if at all possible, it is best to dissolve the sample in the mobile phase, if not, at least to make the final dilution in the mobile phase.

Chromatographic separations thus vary with solvent properties and are related to sample solubility, polarity, and solvent strength. Solvents that interact strongly with the sample will increase the sample solubility and decrease the chromatographic retention as more sample ions exist in the solvent and are not able to be in equilibrium with the adsorbent surface. Thus changing the organic solvent will change the selectivity. Polarity is the summation of dipole and hydrogen bonding

interactions, and in reversed-phase chromatography, less polar solvents exhibit greater solvent strength than polar solvents. The solvents water (most polar), methanol, acetonitrile, and tetrahydrofuran (THF) are placed in ascending order of polarity but reversed in their order of solvent strength.

These three organic solvents (methanol, acetonitrile, and THF) form the basis of the solvent selectivity triangle and exhibit differences in their relative interactions. They are also miscible with water and possess low viscosity and UV transparency. Collectively these three organic solvents along with water provide a four-solvent mobile phase optimization strategy. Each organic solvent in combination with water or water containing a buffer or additive(s) comprise the mobile phase. Sometimes the mobile phase may contain two organic solvents. The aqueous phase composition is commonly referred to % A and the organic phase as % B.

When the sample is eluted with a mobile phase of 100% B (organic), there is no separation, as the sample is eluted in the void volume. This is because the sample is not retained; but retention is observed when the mobile phase solvent strength is decreased to allow equilibrium competition of the solute molecules between the bonded phase and the mobile phase. When the separation is complex, that is, many components are to be separated, and when the solvent strength is decreased and there is still no resolution between two close peaks, another organic solvent of a different polarity or even a mixture of two organics may need to be tried to effect separation. Additionally, mobile phase optimization can be enhanced in combination with bonded phase optimization (i.e., substituting C18/C8 with cyano or phenyl). A goal for the band spacing of a solute (k') should be in the range of 4 to 9 and a run time of about 15 minutes or 20 minutes at most for most routine product release or stability runs.

10.6. Role of Buffer

When the mobile phase contains only water and organic solvent, it is recommended that the pH of the mobile phase be controlled by using a buffer to provide capacity. Thus when selecting a buffer for a given application, the following considerations are important:

The buffer capacity is dependent on pH, buffer pK_a, and buffer concentration.
UV absorbance—UV transparent to below or at the wavelength of the organic solvent.
Other properties, such as solubility and stability of the buffer and its reactivity to the analyte and hardware components of the chromatographic system.

The buffer concentration, or ionic strength, will affect the selectivity. An increase in the buffer concentration can lead to a decreased retention as the ionic interaction between the analyte and silanols are swapped out by the increased buffer concentration. When selecting a given buffer, additive, or even the solvent, sufficient regard for their compatibility with the analyte or HPLC system must be considered (23).

10.7. Role of the Ion-Pair Reagent

Initially, when deciding whether to select reversed-phase HPLC or reversed-phase HPLC with ion-pairing, a good rule of thumb is to consider the nature of the analyte

of interest. If the sample is neutral, begin with reversed-phase; and if the sample is ionic, use ion-pairing. Thus reversed-phase HPLC and reversed-phase HPLC with ion-pairing are similar except that the latter contains an ion-pair reagent in the mobile phase to improve the selectivity of ionic samples. The use of an ion-pair reagent is suggested only when separation is not adequate with reversed-phase HPLC. This is because using an ion-pair reagent introduces additional experimental parameters that need to be controlled, such as what ion-pair reagent to use and its concentration. Because of this added variable, reversed-phase HPLC should be utilized on any ionic analyte first before trying ion-pair reversed-phase HPLC.

The solubility of the ion-pair reagent may also be affected depending on the organic solvent used in the mobile phase. Methanol is generally preferred over acetonitrile or THF because it provides better solubility for the ion-pair reagent as well as for buffers and salts. In this case a suitable buffer is chosen, and at a concentration of about 25 mM, the pH and ion-pair reagent concentration are varied to provide optimal selectivity to the separation. These variables are not easily altered with commercial ion-pair "kits" as their pH and concentration have been standardized and ready to use.

Table 4 summarizes the types of ion-pair reagents and the conditions for their use.

The mechanism of retention imparted by the ion-pair reagent, such as an alkyl sulfonate, provides a change in the equilibrium between the ionized analyte and the ion-pair reagent that is attached to the silica adsorbent through the hydrophobic alkyl group and the negative charge of the sulfonate ion. The positively charged

Table 4 Listing of Types of Ion-Pair Reagents and Conditions of Use

Ion-pair reagent type and examples	Used for compound class	pH of mobile phase	Concentration of ion-pair reagent
Alkyl sulfonates (sulfonic acid alkyl salts)	Cationic samples (protonated bases)	3.5	0.005 M
Pentane sulfonate Hexane sulfonate Heptane sulfonate Octane sulfonate Examples PIC® B series (Waters) Q-Series (Regis)	Basic compounds		
Alkyl ammonium salts	Anionic samples (ionized acids)	7.5	0.005 M
Tetrabutylammonium phosphate Tetrabutylammonium hydrogen sulfate Examples: PIC® A series (Waters) Q-Series (Regis)	Acidic compounds		

(protonated) analyte ion competes for the negative site of the sulfonate ion. This altered equilibrium in effect imparts a change in the solubility of the analyte sample which in turn alters the retention as the analyte is now "attached" to the adsorbent so that it is eluted at a later time.

The pH of the mobile phase is closely associated with ion-pairing, whether the ion-pair reagent is positively charged (tetrabutylammonium, TBA^+) or negatively charged (C_5- or C_6- sulfonate/C_5- or C_6-SO_3^-), and dependent on whether the analyte is an acid or a base. Cationic samples (protonated base) or bases use the pentane, hexane, or a higher hydrocarbon sulfonate ion-pair reagent. Anionic samples (ionized acids) or acids commonly use tetraethylammonium or tetrabutylammonium hydroxide as the ion-pair reagent. Their optimization is pH dependent. For example, Waters® Chromatography ion-pair reagents operate in the low and intermediate pH ranges: PIC™ A (tetrabutylammonium phosphate) for acids operates at pH 7.5 and PIC™ B5 to B8 (pentane to octane sulfonic acid) for bases operate at pH 3.5.

For selection of the proper ion-pair reagent, alkyl chain lengths must be considered. The length of the alkyl chain enables selective separation of the analyte. The longer the chain, the more hydrophobic the counterion, and therefore, the greater the retention due to equilibrium between the counterion and the column adsorbent. Thus by selecting a reagent with a longer chain, selective solubility is obtained, enhancing the resolution.

10.8. Role of the Column

The HPLC column is the heart of the method, critical in performing the separation. The column must possess the selectivity, efficiency, and reproducibility to provide a good separation. All of these characteristics are dependent on the column manufacturer's production of good quality columns and packing materials. Properties of the silica (backbone) such as metal content and silanol activity produced in the manufacturing and bonding processes determine the properties of the finished bonded phase. A good silica and bonding process will provide the reproducible and symmetrical peaks necessary for accurate quantitation.

Commonly used reversed phases are C18 (octadecylsilane, USP L1), C8 (octylsilane, USP L7), phenyl (USP L11), and cyano (USP L18) (24). They are chemically different bonded phases and demonstrate significant changes in selectivity using the same mobile phase. Their properties vary from manufacturer to manufacturer, but given the state-of-the-art character of the vendor's manufacturing process, they show good quality control and provide batch-to-batch reproducibility. For example, no two L1 columns are the same, they vary from manufacturer to manufacturer relative to their pore volumes, pore sizes, surface areas, particle sizes (average range), carbon loads, whether end-capped or not, and the amount of bonded-phase coverage, as well as varying in their basicity and acidity characteristic. With state-of-the-art developments in column technology, most columns on the market exhibit good quality control and provide excellent column-to-column reproducibility and batch-to-batch reproducibility (25), and in some cases they give the chromatographer the option of using column selectivity as an alternative tool (besides mobile phase selectivity) to optimize the HPLC method development (26).

Column length also plays a role in the separation resolution. As column length changes, the efficiency (N) changes in direct proportion to the ratio of the column length (27). Resolution, as indicated in the resolution equation (vide supra), changes as a function of the square root of the change in N, and an estimate of the change in resolution as a function of column length can be approximated with the equation

$$R_{s2} = R_{s1} \cdot \left(\frac{L_2}{L_1}\right)^{1/2}$$

where R_{s1} is the resolution obtained from column 1 and R_{s2} is the estimated resolution with column 2.

Similarly the run time (RT) and column back pressure (P) will also change in direct proportion to a change in the column length by

$$RT_2 = RT_1 \cdot \frac{L_2}{L_1} \qquad P_2 = P_1 \cdot \frac{L_2}{L_1}$$

where RT_1 is the run time for column 1, RT_2 the run time for column 2, P_1 the pressure for column 1, and P_2 the pressure for column 2.

While most analytical columns are standardized to a 4.6 mm id, their lengths vary; they are available in lengths of 5 cm, 15 cm, and 25 cm, whereas the original Waters μBondapak® C18 column measures 30 cm × 3.9 mm id. A good selection of columns illustrating type and sizes can be found in most HPLC vendors' supply catalogs.

Claessens et al. (28) have reported on an extensive study on the effect of buffers on silica-based column stability in reversed-phase HPLC. As the analytical column has a silica-based backbone, it is not stable in alkaline pH. The authors reported that silica-based bonded phase packings variably degrade with buffers as a function of the type of anion, cation, pH, buffer type, and temperature.

10.9. Role of Temperature

While temperature is a variable that can affect selectivity, α, its effect is relatively small. Also, the k' generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. Still, it may have some effect when there is a significant difference in shape and size between samples. Overall, it is better to use solvent strength to control selectivity than to use temperature; its effect is much more dramatic. Snyder et al. (29) reported that an increase of 1°C will decrease the k' by 1 to 2%, and both ionic and neutral samples are reported to show significant changes in α with temperature changes. Because of possible temperature fluctuations during method development and validation, it is recommended that the column be thermostated to control the temperature.

10.10. Role of Flow Rate

Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure. The disadvantage is that when flow rate is decreased, to increase the resolution slightly, there is a corresponding increase in the run time.

11. OPTIMIZATION (OPTIMIZING THE SEPARATION)

Up to this point, efforts to develop a suitable stability-indicating HPLC method have revolved around the resolution equation (see Sec. 10.1). To optimize the method, the chromatographer must tweak the three variables in the equation. The capacity factor, k', can be affected with a change in the solvent. The efficiency factor, N, can be altered with a change in the column dimension, particle size, stationary phase, and flow rate. Lastly, the separation factor, α, can be modified with a change in the solvent, pH, ionic strength of the buffer, stationary phase, mobile phase additives, and temperature. These three factors need to be considered for optimizing the method, conveniently performed utilizing the Plackett–Burman design and computer software. The use of computer software for optimization is becoming more and more common. Refer to Sections 12 and 17.10 for further discussions on these topics.

11.1. Peak Area or Peak Height for Quantitation

The chromatographer can either select peak area or peak height for quantitation assuming that both modes have been properly calibrated and validated. It is suggested, however, that peak area be used for development and peak height for stability for the reasons stated in the table.

Development—Peak Area	*Stability Monitoring—Peak Height*
Suitable for simple, well-resolved mixtures	More accurate/sensitive than peak area
Less frequent standardization required	Requires less resolution of compounds
Generally more precise than peak height	More affected by instrumental variations
Generally best when simple equipment is used	Best suited for complex mixtures
Better suited for nonsymmetrical peaks	Use for trace analysis

11.2. Plackett–Burman Design

Often in method optimization it is necessary to consider various variables, such as environmental and experimental conditions, that affect the ruggedness of a given method. One such experimental design often used in ruggedness testing is the Plackett–Burman design named after the authors that first published their work more than a half a century ago. Refer to Sec. 17.10 in the *Validation* part of this chapter for further discussion on this subject.

12. COMPUTER SOFTWARE FOR METHOD DEVELOPMENT

The discussion in Sec. 10 presumes developing a method by manual trial and error, yet in a systematic manner. That is, the conditions for an initial run are noted, and, based on the outcome of the first run, modifications are made for the second run. Then based on the results of the second run, additional modifications are made for the third run, and so forth until a good separation is obtained. Thus a number

of these trial and error runs may be needed to obtain the desired separation, which may conceivably be time consuming.

In the last fifteen years or so, the use of software for method development in reversed-phased HPLC has increased dramatically, with the intended purpose of separating complex mixtures by shortening the development time and optimizing the resolution based on a limited amount of experimental retention data. A number of these computer systems are commercially available. Many reviews on the subject have been published (30), and many references to using the DryLab^{TM} have been reported (31). DryLab^{TM} is a widely used computer simulation program that after a limited number of actual injections at different conditions can predict an optimal condition or separation at other conditions.

13. OTHER APPLICATIONS

13.1. Analytical Method for Cleaning Assessment

Normally, production equipment is shared to manufacture different pharmaceutical products. Thus cleaning processes following production of pharmaceutical products are critical to prevent cross-contamination. The analytical method used to assess the effectiveness of the cleaning process is usually the same stability-indicating method used for product release and stability monitoring, with some adjustments to increase its sensitivity. How sensitive and specific the method has to be is commonly determined from a joint effort between the pharmaceutical engineer and the analytical chemist to establish the necessary cleaning limit. The method developed must be capable of being validated and rugged enough to meet predetermined specifications consistently. In addition to HPLC, total organic carbon (TOC) analysis has become a widely used method for analyzing cleaning residues, and the Compendia have dedicated General Chapter <643>, Total Organic Carbon, to the subject (32). TOC, however, is not as specific as HPLC. Conductivity has also been used. Generally HPLC is the most accurate, reliable, and specific of all the analytical cleaning methods.

13.2. Physicochemical Characterization Method (Dissolution Method)

A liquid chromatographic method developed for product release or stability monitoring can be adapted for use with a dissolution assay. An HPLC method for dissolution assay testing is optimized for speed and is not intended for determination of degradation products or process impurities. Instead, the real utility of this combination (dissolution with HPLC determination) is that it eliminates interferences from formulation excipients. Assuming that the HPLC method has been developed and validated, the development process is bridged over to developing the dissolution methodology. A preliminary dissolution test is developed very early in the pharmaceutical development process to support formulation development. Primary dissolution parameters for development include selection of the filter, the apparatus type, the rotation speed, and the dissolution medium. Once these parameters have been established, they are to be validated as part of the total validation effort for the HPLC dissolution methodology. The reader is referred to the article by Skoug et al. (33) for an overview of the subject.

13.3. Nonchromatographic Methods

Approaches and guidelines used to develop and validate a chromatographic method can be applied to develop nonchromatographic methods (not stability-indicating) as well. It is equally appropriate to follow the guidelines of USP 23 General Chapter <1225>, Validation of Compendial Method (34), selecting and validating those analytical elements that are needed for a rugged method. These nonchromatographic methods include UV spectrophotometry, atomic absorption, infrared spectroscopy, and titrimetry.

Additional discussions on the validation of various nonchromatographic methods are found in Sec. 19.3.

Part II: Method Validation

14. REGULATORY AND COMPENDIAL BASIS OF METHOD VALIDATION—WHERE TO START

Analytical methods including chromatographic and nonchromatographic techniques are used to generate reliable and accurate data during drug development and post approval of the drug products. The testing, in general, includes the acceptance of raw materials and the release of drug substances and finished products, in process testing, and analysis of stability samples for establishing expiration dating. Therefore test methods that are used to assess the compliance of pharmaceutical products with established acceptance criteria must meet proper cGMP standards of accuracy and reliability as set forth by the regulatory agencies (35).

According to Section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the USP and NF constitute legal standards. Under the Food Drug, and Cosmetic Act, the FDA can enforce the USP/NF standards of strength, quality, purity, packaging, and labeling. Therefore for compliance purposes, every analytical method should be validated according to pharmacopeial standard, because each method could be included in a drug monograph.

Method validation is a regulatory requirement. The Food and Drug Administration and the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use have published a series of guidelines on the validation of analytical procedures (36,37). In USP 23/NF 18, General Chapter <1225> has been allocated for validation of compendial methods (34). This chapter describes in detail as well as in summary how to evaluate particular performance parameters. In general, it is assumed that this chapter is applied to chromatographic methods of analysis, and that for nonchromatographic procedures some alternate guidelines should be used. However, in USP 23/NF 18 no such distinction has been made. Therefore performance parameters given in General Chapter <1225> can be used to evaluate the performance of any analytical method. However, one needs to be careful in selection of performance parameters. Also, methods described in the current USP are not stability-indicating in nature. Therefore for monitoring of stability studies, guidelines given in General Chapter ⟨1225⟩ can be used to validate these methods.

What constitutes validation? The validation of analytical method is the process in determining the suitability of a given methodology by laboratory studies that the method in question can meet the requirements for the method's intended use. Method validation is not simply a measure of procedure; method validation is a measure of performance of the total analytical system. Sections 211.165(c) and 211.194(a)(2) of the cGMP for method validation specify that any method adopted at the product development stage be verified under actual conditions of use, and that subsequent variations on existing methodology are subjected to validation.

General Chapter <1225> states, "Validation is the process of providing documented evidence that the method does what it is intended to do." In other words, the process of method validation ensures that the proposed analytical methodology is accurate, specific, reproducible, and rugged for its intended use.

The articles in the current revision of the Compendia are also recognized to be legal standards when determining compliance with the Federal Food, Drug, and Cosmetic Act. Regulated industries must perform method validation to comply with Compendial or other regulatory requirements, and the data generated becomes a part of the methods validation package submitted to the FDA.

Similarly, the general regulation, which is currently represented in 21 CFR 2.19, states, "Where the method of analysis is not prescribed in a regulation, it is the policy of FDA, in its enforcement programs to utilize the methods of analysis of AOAC as published in the current edition." Further, it is stated in the FDA's current Good Manufacturing Practices for Finished Pharmaceuticals regulations 21 CFR 211.165(e) and 21 CFR 211.194(a)(2) that if a firm is using AOAC-OMA or USP/NF methods of analysis, only minimal additional validation data is required (35).

15. VALIDATION PROTOCOL

While the text of Title 21 CFR Part 211, ICH Guidelines, and General Chapter <1225> all provide terms and definitions, there is no specific discussion of validation protocol and methodology. In ICH Guidelines (Q2B) on Method Validation Methodology, the applicant has been made responsible for the appropriate validation protocol and procedure suitable for their product. Therefore prior to initiating a validation study, a well-planned validation protocol is required. This protocol should consist of experimental design and elements required for validation of the proposed test method that have been reviewed for scientific soundness and completeness by qualified individuals and approved by appropriate company management authority. The validation protocol should include a detailed test procedure, basic experimental design, elements for validation, predefined acceptance criteria, reference of related methods, and management approval.

As mentioned earlier, description of the test method is very significant for successful validation. Therefore a test procedure is a description of the "analytical method" to be used as a guide in validating the method and serves as a basis for the preparation of the validation protocol. It should include

1. A listing of reagents, solvents, and other supplies
2. Instructions for the preparation of standards, samples, and solutions
3. A listing of equipment to be used or equivalents
4. Instrumental parameters and chromatographic conditions

5. System suitability requirements
6. Standard and sample analysis sequence
7. Calculation section to include results formatting

Prior to outlining the experimental design or protocol, however, it is necessary to make some basic assumptions as suggested by Swartz and Krull (38,39). These assumptions are that

Specificity or selectivity for the developed method has been demonstrated (i.e., forced degradation already performed).

The developed method has been optimized to the point where investing time and effort in validation is justified and feasible.

Evaluation of data generated by the developed method is performed by valid statistical approaches to remove some of the subjectivity of method validation.

Keeping in mind these assumptions, the current ICH methodology guidelines, and the requirements for validation depending on the type of analytical method, one can design a stepwise protocol. A typical protocol designed by Swartz and Krull is given in Fig. 3 (38,39).

As shown in Fig. 3, the first parameter to be evaluated is robustness. This parameter is usually evaluated during the method development stage, when the effect

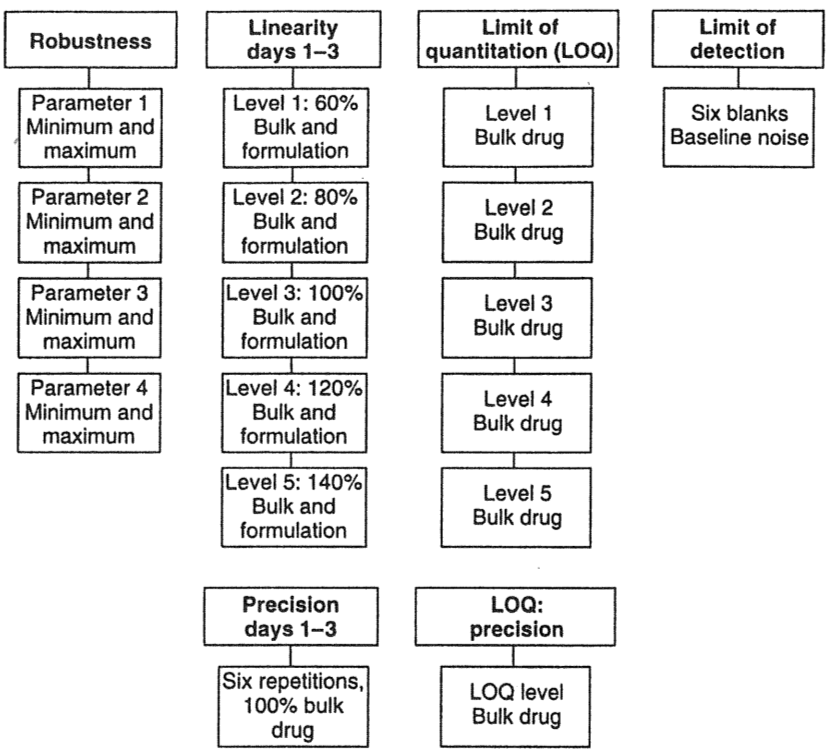


Figure 11.3 Sample method validation protocol. (From Refs. 38, 39.)

of different parameters on selectivity is studied. Method robustness can be evaluated in a stepwise univariate approach or as a part of an experimental design incorporating multivariate parameters.

Next, a linearity test over five levels for both the drug substance and the dosage form is performed. The range is determined according to the test method's intended use (34,36,38,39). Comparison of the results between the drug substance and the dosage form fulfills the accuracy requirements. A minimum of three measurements at each level should be made.

At the end of day 1, a minimum of six repetitions are performed at the 100% level of the drug substance for repeatability.

Steps 1 and 2 are repeated over additional days for intermediate precision. The detection limit (DL) and quantitation limit (QL) can then be determined if required. For calculation of these performance characteristics one can follow criteria given in the USP or ICH guidelines (Q2B). It is stated that this protocol is merely a generic example, and specific protocols or SOPs should be documented and followed for the particular method and its intended use.

16. VALIDATION PARAMETERS

Prior to conducting validation studies it is imperative to decide which parameters are required to be studied. These parameters are termed "analytical performance characteristics" or sometimes "analytical figures of merit." Most of these terms are familiar and are used daily in the laboratory. However, some may mean different things to different laboratory groups. Therefore a complete understanding of the terminology and definitions of these characteristics is important.

The selection of desired performance characteristics would depend on the type of analytical method and its intended use. For example, an assay method designed for finished product release should not be used for the determination of detection or quantitation limits of an active ingredient. However, if the method has been designed to monitor trace quantities of the active ingredient in cleaning validation samples, then knowledge of the detection and quantitation limits are appropriate and necessary.

Therefore, selection of validation parameters for each assay or test method should be made case by case, to ensure that parameters are appropriate for the intended use. This is even more important when validating stability-indicating methods, because such validations are complex, as these involve forced degradation studies, spiking of samples with known degradants and literature searches.

16.1. USP General Chapter <1225>, Validation of Compendial Methods

General Chapter <1225> (34) describes typical analytical performance characteristics, how they are determined, and which subset of data elements is required to demonstrate validity, based on the method's intended use. These performance characteristics can be referred to as the "Eight Steps of Method Validation." These analytical performance characteristics are

Accuracy
Precision
Specificity

Detection limit (DL)
Quantitation limit (QL)
Linearity and range
Ruggedness
Robustness

Compendial test and assay procedures vary significantly in type of analytical method used, and the type of information required for validation of a given analytical method will vary depending on the nature of the method. Consequently in General Chapter ⟨1225⟩ (34), the most common test and assay procedures have been divided into four categories for harmonization with the ICH guidelines:

Category I: Analytical methods for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished dosage forms
Category II: Analytical methods for determination of impurities in bulk drug substances or degradation products in finished dosage forms, including quantitative assays and limit tests
Category III: Analytical methods for determination of performance characteristics (e.g., dissolution, drug release)
Category IV: Identification tests

Analytical variables that are normally required for method validation in each of these categories are listed in Table 5.

An evaluation of performance characteristics shown in Table 5 indicate that for assays in Category I, determination of DL and QL is not required because the major component or active ingredient to be quantitated is present at high levels. All other parameters are evaluated to obtain quantitative information needed. Assays in Category II are further divided into quantitative and limit tests subcategories. If quantitative information is required, measurement for DL is not necessary, but the remaining parameters are evaluated. For limit tests, on the contrary, no quantitation is required. Thus it is sufficient to measure only the DL and demonstrate specificity and ruggedness. The parameters to be determined under Category III are dependent upon the nature of the test. Dissolution testing, for example, falls into this category.

Table 5 USP Data Elements Required for Assay Validation

Analytical performance parameters	Assay Category I	Assay Category II		Assay Category III	Assay Category IV
		Quantitative	Limit tests		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
DL	No	No	Yes	*	No
QL	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

* May be required depending on the nature of the specific test (Ref. 33).

16.1.1. Stability-Indicating Nature of USP Assays

A word of caution. Assays appearing in USP monographs are not always stability-indicating. They may be for the innovator product, as submitted by the innovator company for inclusion as a USP monograph, which then becomes the benchmark. If another company wishes to market the same product, as a generic version, that company must validate the assay according to the validation parameters discussed in USP General Chapter <1225>, because that product is different from the innovator product relative to the source API and formulation.

16.2. ICH Guidelines

ICH Guidelines Q2A (Text on Validation of Analytical Procedures) and Q2B (Validation of Analytical Procedures: Methodology) were developed within the Expert Working Group (Quality) of the Requirements for Registration of Pharmaceuticals for Human Use. These documents present a discussion of the characteristics for consideration during validation of analytical procedures included as part of registration applications submitted within the European Union, Japan, and the United States.

ICH Guidelines Q2A also provides descriptions of typical validation parameters, how these are measured, and which subset of each parameter is suitable for validation of the analytical method, based on its intended use. The discussion of the validation of analytical procedures has been divided into three common categories of analytical procedures:

Identification tests
Quantitative tests for impurity content—Limit tests for the control of impurities
Quantitative tests of the active moiety in bulk drug substance or drug product or other selected component(s) in the drug product

As per ICH Guidelines Q2A, the objective of the analytical procedure needs to be clearly understood since this will govern the validation characteristics that need to be evaluated. Typical validation characteristics, which should be considered, are

Accuracy
Precision
Repeatability
Intermediate precision
Specificity
Detection limit
Quantitation limit
Linearity
Range
Robustness
System suitability

Analytical variables that are normally required for method validation is summarized in Table 6.

Table 6 ICH Validation Characteristics Versus Type of Analytical Procedures

Type of analytical procedure	Identification	Impurity testing		Assay
		Quantitative	Limit tests	
Accuracy	No	Yes	No	Yes
Precision				
Repeatability	No	Yes	No	Yes
Intermediate precision	No	Yes	No	Yes
Specificity	Yes	Yes	Yes	Yes
LOD	No	Yes	Yes	No
LOQ	No	Yes	No	No
Linearity	No	Yes	No	Yes
Range	No	Yes	No	Yes

Source: Refs. 35 and 37.

The difference in the USP and ICH terminology is for the most part one of semantics; however, there is one notable exception. In the ICH Guidelines, system suitability is part of validation, whereas the USP deals with system suitability under chromatography in the USP, called General Chapter <621> Chromatography (24). The FDA is already implementing the ICH Guidelines, and it is anticipated that the ICH definitions and terminology will become a part of the USP chapter on validation. It is probable that USP categories I and II will match the ICH categories of Assay and Impurity testing, respectively. The ICH has not yet chosen to address methods for performance characteristics (USP Category III) but has instead included analytical methods for compound identification. In this ICH category, it is only necessary to show that the method is specific for the compound being identified.

ICH Guidelines Q2B is complementary to ICH Guidance Q2A, which presents a discussion of characteristics that should be considered during the validation of analytical procedures. This guidance gives recommendations on how to consider the various validation characteristics for each analytical procedure. These recommendations will be discussed in detail under definition of validation parameters.

16.3. FDA Reviewer Guidance

The FDA Reviewer Guidance—Validation of Chromatographic Methods provides comprehensive description of typical validation parameters and how these are determined (40). This FDA guidance has similarities to the ICH Guidelines Q2A and Q2B, but has examples in form of tables or figures to demonstrate data representation for validation parameters. The purpose of this guidance is to present the issues to be considered when evaluating chromatographic test methods from a regulatory perspective. Examples of common problems, which can delay the validation process, have been included.

The validation characteristics to be evaluated according to this FDA guidance are

Table 7 Comparison of Analytical Parameters Required for Assay Validation

USP General Chapter <1225>	ICH Q2A Guidelines	FDA Reviewer Guidance
Accuracy	Accuracy	Accuracy
Precision	Precision	Precision
No	Repeatability	Repeatability
		Injection
		Analysis
No	Intermediate precision	Intermediate precision
No	No	Reproducibility
Specificity	Specificity	Specificity/selectivity
Detection limit	Detection limit	Detection limit
Quantitation limit	Quantitation limit	Quantitation limit
Linearity	Linearity	Linearity
Range	Range	Range
Ruggedness	No	No
Robustness	Robustness	Robustness
System suitability[a]	System suitability	System suitability
		Sample solution stability

[a] System suitability discussed separately in USP 23 General Chapter <621>.

Accuracy
Detection and quantitation limits
Linearity
Precision
Repeatability
Injection repeatability
Analysis repeatability
Intermediate precision
Reproducibility
Range
Robustness
Sample solution stability
Specificity/selectivity
System suitability specifications and tests

A comparative discussion of validation parameters given in the FDA and ICH guidelines will be made under Sec. 17, "Definition of Validation Parameters." Analytical parameters needed for method validation as described in the General Chapter <1225>, ICH Guidelines Q2A, and the FDA Reviewer Guidance are summarized in Table 7.

17. DEFINITION OF VALIDATION PARAMETERS

In the literature, there are many articles on definition and interpretation of validation parameters required for assay validation as published by Krull and Swartz (38,39,41,42). Persson et al. (43) have discussed the evaluation of method

validation in an article titled "How good is your method?" In Sec. 17, definition of validation parameters is based on requirements stipulated in the ICH Guidelines Q2A, Q2B, the FDA Reviewer Guidance, and USP General Chapter <1225>.

Though many types of chromatographic techniques are available, the most commonly submitted method in NDAs and ANDAs is reversed-phase HPLC with UV detection. Therefore this method is selected here to illustrate parameters for validation. The criteria for the validation of this technique can be extrapolated to other detection methods and chromatographic techniques. For acceptance, release, or stability testing, accuracy should be optimized, since the need to show deviation from the actual value is of great concern.

17.1. Accuracy

Accuracy is the measure of how close the experimental value is to the true value. It is measured as the percent of analyte recovered by assay or by spiking samples in a blind study. For the drug product, this is performed by analyzing synthetic mixtures (placebos) spiked with known quantities of drug. Accuracy should be established across the specified range (that is, line of working range) of the analytical procedure. For the assay of the drug substance, accuracy measurements are made by comparison of the results with the analysis of a standard reference material or to compare the results obtained from a second well-characterized independent procedure, the accuracy of which is stated and/or defined. For quantitation of the impurity, accuracy is determined by spiking drug substance or drug product with known amounts of available impurities. In case it is impossible to obtain impurities or degradation products, comparison of results to a second well-characterized independent method is acceptable. The response factor of the drug substance can be used. Another approach is to perform specificity studies by forced degradation. This will be discussed under specificity. It should be decided up front how the individual or total impurities are to be reported, e.g., percent weight/weight or area percent, in all cases relative to the major analyte.

The FDA recommends that recovery be performed at the 80, 100, 120% of label claim as stated in the Guideline for Submitting Samples and Analytical Data for Method Validation (2). Recovery data, at least in triplicate at each level (80, 100, and 120% of label claim) is recommended. The data should be calculated as percent label claim, and the mean of the replicates along with % RSD for each level is reported to demonstrate accuracy and sample analysis precision.

ICH Guidelines Q2B recommend assessment of accuracy at three concentration levels covering the specified range (i.e., three concentration levels and three replicates at each level of the total analytical procedure). The data should be reported as the percent recovery of the known amount added or as the difference between the mean and true values with confidence intervals.

17.2. Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. In USP 23/NF 18, General Chapter <1225>, precision is defined as "the degree of agreement among individual test results obtained by repeatedly applying the analytical method to multiple samplings of a homogeneous sample." Thus precision refers to the distribution

of individual test results around their average. Precision is usually expressed as percent relative standard deviation (% RSD) for a statistically significant number of samples. Both the FDA and the ICH recommend that precision be measured at three different levels. No such recommendation is given in the USP.

17.2.1. Repeatability

Repeatability expresses the results of the method operating over a short time interval under the same conditions. Repeatability is also termed intra-assay precision. According to the FDA Reviewer Guidance, repeatability is evaluated for injector performance and analysis of samples. For injector repeatability, there must be a minimum of 10 injections with an RSD of not more than ±1%. Similarly, with the methods for release and stability studies, an RSD of not more than ±1% for at least five injections for the active drug is desirable. For low-level impurities, higher variations in RSD may be acceptable. For analysis repeatability, determinations are made on multiple measurements of a sample by the same analyst under the same analytical conditions. The FDA recommends that the study be combined with accuracy.

The ICH recommends that repeatability should be determined from a minimum of nine determinations covering the specified range for the procedure (e.g., three levels, three replicates each), or from a minimum of six determinations at 100% of the test or target concentration. The target concentration is defined as the concentration of the compound of interest given in the analytical method.

17.2.2. Intermediate Precision

Intermediate precision expresses within-laboratory variations. This was previously evaluated as part of ruggedness. This attribute evaluates the reliability of the method in an environment different from that used during the method development phase. Depending on time and resources, the method can be evaluated on different days, with different analysts and equipment, etc. The FDA recommends performing accuracy on two separate occasions to indicate the intermediate precision of the test method. The ICH recommends using an experimental design (matrix) so that the effects, if any, of the individual variables on the analytical procedure can be monitored.

17.2.3. Reproducibility

Reproducibility is assessed by performing collaborative studies between laboratories. Multiple laboratories are desirable, if possible. According to the FDA Reviewer Guidance, reproducibility is not required if intermediate precision is achieved. The ICH recommends that reproducibility studies be performed for standardization of an analytical procedure, for instance, for inclusion of procedures to pharmacopoeias. The ICH also recommends that documents in support of each type of precision should include the standard deviation (S), the % RSD, the coefficient of variation, and the confidence interval.

17.3. Specificity/Selectivity

The terms specificity and selectivity are often used interchangeably. The term selectivity has been used in General Chapter <1225> of the 1990 edition of the

USP (44), whereas in the 1995 edition the term specificity has replaced selectivity. Specificity is generally used to express a method's response for a single analyte, whereas the term selectivity of a method is a measure of the extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components. However, as both the USP and the ICH currently use the term specificity, it will also be used here to avoid any confusion.

The USP defines specificity as the ability to measure accurately and specifically the analyte of interest in the presence of other components in the sample matrix. These components may include other active ingredients, excipients, impurities, and degradation products. According to the ICH, the validation procedure should be able to demonstrate the ability of the method to assess unequivocally the analyte in the presence of impurities, matrix components, and degradation products. Lack of specificity of an individual procedure may be compensated by other supporting procedure(s) such as TLC.

Specificity has been divided into two separate categories by ICH:

A. IDENTIFICATION. Specificity is demonstrated by the ability to discriminate between compounds of closely related structures, which are likely to be present. The other approach is by comparison of results to a known reference material.

B. ASSAY AND IMPURITY TEST(S). For assay and impurity tests, specificity can be demonstrated by the resolution of the two components which elute closest to each other. Chromatograms obtained should be appropriately labeled to show individual components. For nonspecific assays, overall specificity may be demonstrated by use of other supporting analytical procedures. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used.

The ICH has also addressed issues of specificity for impurities. The approach is similar for both assay and impurities. If impurities are available, then it must be demonstrated that the assay is unaffected by the presence of spiked materials such as impurities and/or excipients. For the impurity test, the discrimination may be shown by spiking drug substance or drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

If the impurities or degradation product standards are not available, then specificity may be demonstrated by comparison of the test results to a second well-characterized pharmacopoeial or independent validated procedure. For the assay, the two results are compared. For the impurity tests, the impurity profiles are compared head to head.

For stability-indicating assays where potency and impurities are determined simultaneously mass balance must be taken into consideration. Any decrease in potency should be explained by mass balance. The following equation can be used to account for any loss of potency:

$$100\% = \text{Drug}\% + \text{Related substances}\% + \text{Water}\% + \text{ROI}\% + \cdots$$

In the FDA Reviewer Guidance, specificity/selectivity is established by showing that the analyte should have no interference from extraneous components and be well resolved from them. A representative chromatogram showing resolution of these

extraneous peaks from the main analyte peak is required for submission. The origins of extraneous peaks in drug substance are process impurities (which include isomeric impurities) from the synthesis process, residual solvents, and other extraneous components from extracts of natural origins. For the drug product, sources of extraneous peaks include any impurities, degradation products, interaction of the active drug with excipients, residual solvents from both the active drug substance and the excipient, and so on.

17.4. Forced Degradation

In previous sections, we have defined specificity as discussed by USP Chapter <1225>, the ICH Guidance, and the FDA Reviewer Guidance. The discussion was limited to specificity studies in the presence or absence of impurities and excipients. A question that arises if nothing (i.e., no extraneous peaks) is observed is, What approach one might use to show the specificity and stability-indicating nature of the proposed method?

Both the FDA and the ICH recommend forced degradation/or stress testing of the drug substance and drug product. For these studies, acid and base hydrolysis, temperature, photolysis, and oxidation are recommended. Neither the ICH nor the FDA guidelines specify how to perform these forced degradation studies. Experimental conditions and the design of these studies have been left to the discretion of pharmaceutical companies. A generic protocol for these studies is shown in Table 2.

To demonstrate that the analyte chromatographic peak obtained after forced degradation or stress studies is a single entity, peak purity tests are recommended by the FDA and the ICH. Photodiode array detection can be used to demonstrate peak purity. The spectra collected across a peak are compared mathematically to establish peak homogeneity.

It is generally recommended that about 20–30% of analyte degradation, at least, in one medium be achieved. For some compounds, severe degradation conditions may be required.

17.5. Detection Limit (DL)

The detection limit (DL) is the lowest concentration of the analyte that can be detected, but not necessarily quantitated, under the stated experimental conditions. It is a parameter of limit test and specifies whether or not an analyte is above or below a certain value. In the current USP General Chapter <1225>, determination of limit of detection is described for instrumental and noninstrumental methods. For instrumental methods, one determines the signal-to-noise ratio by comparing test results from samples with known concentration of analyte with those of blank samples and establishes the lowest concentration at which analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is required. Another approach is to calculate the standard deviation for analysis of a number of blank samples. The standard deviation multiplied by a factor, usually 2 or 3, gives an estimate of limit of detection.

For noninstrumental methods, DL is determined by the analysis of samples with known concentrations of analyte. The minimum concentration at which the analyte can be reliably detected is the limit of detection. The ICH has recognized

the signal-to-noise ratio convention but also lists several other approaches for determining DL, depending on whether the procedure is instrumental or noninstrumental. These approaches are as follows.

A. BASED ON VISUAL EVALUATION. Visual evaluation may be used both for instrumental and noninstrumental methods. It requires analysis of samples with concentrations of analyte and establishing the minimum level at which analyte can be reliably detected. Visual noninstrumental methods can include DL determined by techniques such as TLC or titration.

B. BASED ON THE STANDARD DEVIATION OF THE RESPONSE AND THE SLOPE. The detection limit may be calculated based on the standard deviation (SD) of the response and slope (S) of the calibration curve (a specific curve should be generated by using samples containing analyte in the range of detection limit), according to the formula

$$\text{Detection limit (DL)} = 3.3 \times \text{SD}$$

The SD of the response can be determined from the SD of the blank, the residual SD of the regression line, or the SD of the y-intercept of the regression line. The detection limit and method used to determine the detection limit must be documented and supported, and a suitable number of samples should be analyzed at the limit to validate it. The FDA is of the opinion that expression of the detection limit in terms of a signal-to-noise ratio of 2 or 3 is not very practical. The reason for this is attributed to differences in the noise level on a detector during the method development phase and when samples are analyzed on different detectors. Detector sensitivity can vary with the model number or manufacturer.

17.6. Quantitation Limit (QL)

The quantitation limit is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions of the method. This is a parameter of the quantitative assays for low concentrations of compounds in sample matrices such as impurities in bulk drug substances and degradation products in finished products.

In the current USP General Chapter <1225>, the quantitation limit, QL, which is similar to the detection limit, is expressed as the concentration of analyte in the sample, and precision and accuracy of the measurements are also reported. The QL is dependent on the type of procedure, i.e., instrumental or noninstrumental. For instrumental methods, sometimes a signal-to-noise ratio of 10 : 1 is used to determine the QL. However, it is pointed out that the determination of the QL based on signal-to-noise ratio criteria is a compromise between the concentration and the required accuracy and precision. In other words, as the QL concentration level decreases, the precision increases. For better precision, a higher concentration must be reported for the QL. This compromise is dependent on the analytical method and its intended use.

As with to the limit of detection, the ICH has recognized using a signal-to-noise ratio of 10 : 1 for quantitation. However, this approach can only be applied to analytical procedures that exhibit baseline noise. Again, as with the DL, the ICH lists the same two options that can be used to determine the QL. They are visual evaluation for both noninstrumental and instrumental methods; the latter method can

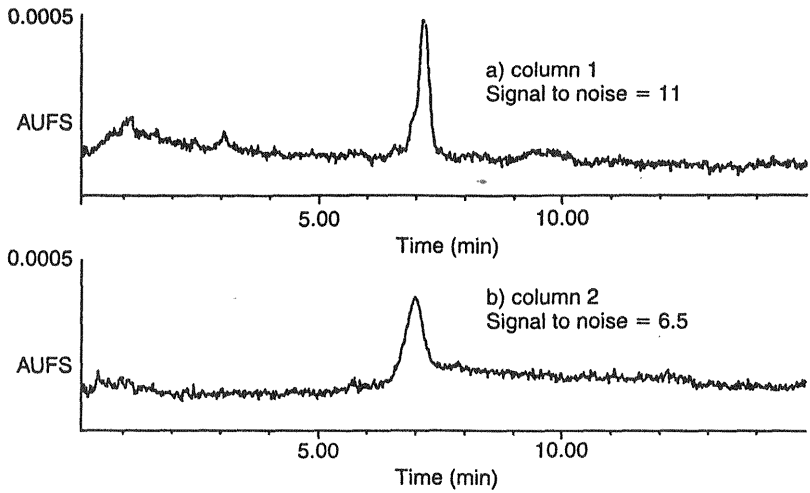


Figure 11.4 Effect of peak shape on LOD and LOQ. (From Ref. 38.)

be based on the standard deviation of the response and the slope. The formula is changed to

$$\text{SD} = 10 \times \frac{\text{SD}}{\text{S}}$$

Determination criteria and requirements for documentation are the same as described under DL in Sec. 17.5, as well as comments by the FDA on the subject. In addition, the FDA Reviewer Guidance recommends that data for analysis repeatability and injection repeatability at the quantitation limit be generated. Further, the Guidance recommends that the use of an additional reference standard at the quantitation limit level be incorporated in the test method.

Additional points regarding the detection and quantitation limits are warranted. These parameters are affected by chromatography. Figure 4 shows the effects of peak shape and efficiency on the signal-to-noise ratio. Sharp peaks will yield a higher signal-to-noise ratio, thus lowering both the DL and the QL. Therefore for the chromatographic determination of these parameters, the age and type of the column and the age of the detector lamp need to be considered. Thus periodic maintenance of the chromatographic detector to maintain optimal results is required.

Finally, the DL and the QL should not be confused with sensitivity. Sensitivity is defined as the slope of the calibration curve, and as such does not usually reference the actual limit of detection or limit of quantitation.

17.7. Linearity

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample within a given range. Linearity is generally reported as the variance of the slope of the regression

line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. The linear range of detection that obeys Beer's law is dependent on the compound analyzed and the type of detector used.

USP General Chapter <1225> gives general directions on the determination of linearity along with handling of the data. However, there are no concentration levels specified to monitor linearity. The ICH also has adopted an approach similar to that of the USP for the determination of linearity and data interpretation. The least squares method is recommended for evaluation of the regression line.

The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be reported. For linearity studies, a minimum of five concentrations is recommended. According to the FDA Reviewer Guidance, the linearity range depends on the intended use of the test method. For content assay, linearity should be performed between 80% and 120% of target concentration. The linearity range for the assay/impurities combination method based on area percent (for impurities) should be greater than 20% of the target concentration down to the limit of quantitation of the drug substance or impurity. A coefficient of correlation (r^2) value, an intercept, and a slope should be reported.

17.8. Range

The range of an analytical method is the interval between the upper and lower concentration levels of analyte (including these concentrations) for which the method as written has been shown to be precise, accurate, and linear. The range is usually expressed in the same units as test results obtained by the analytical method. According to USP General Chapter <1225>, the range of method is validated by verifying that acceptable precision and accuracy is obtained by the analytical method when actual analysis of samples containing analyte is performed throughout the intervals of the range.

The ICH recommends an approach similar to the USP for validation of range. It recommends specific ranges based on the intended use of the method, as follows.

1. For assay of a drug substance or drug product, the minimum specified range is 80% to 120% of the target concentration.
2. For content uniformity testing, the minimum range is 70% to 130%.
3. For the determination of impurity, the minimum range is from the reporting level of an impurity to 120% of the specification.
4. For a combination assay procedure for both active and impurity, where a 100% standard is used, linearity should cover the range from reporting level to 120% of the assay specification.
5. For dissolution testing, the recommended range is ±20% over the specified range of the test. That is, in the case of an extended release product dissolution test with a Q value of 20% after 1 hour, up to 90% in 24 hours, the range for validation will be 0 to 110% of the label claim.
6. For toxic or more potent impurities, the range should be commensurate with the controlled level. FDA recommendations for range are as discussed under the Linearity and Accuracy sections. These ranges can also be applied to other substances such as preservatives.

17.9. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in some parameters and provide an assurance of its reliability during normal usage. The robustness of the method is investigated by varying some or all conditions, e.g., organic composition of the mobile phase, pH, ionic strength, column temperature, age of column, column type. ICH guidelines recommend that robustness studies be performed during the method development stage. Also, if measurements are affected by variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the test method.

Robustness can also be partly assured by good system suitability specification. Therefore, it is important to set tight but realistic system suitability specifications.

17.10. Application of Plackett–Burman Design to Ruggedness Testing

Ruggedness is normally defined as the lack of influence on test results by operational and environmental variables of the analytical method. Ruggedness is a measure of reproducibility of test results under normal operational conditions from laboratory to laboratory and from analyst to analyst. According to ASTM Guidance E 1169-89, "Standard Guide for Conducting Ruggedness Tests" (45), it is necessary to monitor the effects of environmental and experimental factors on the results obtained using the test method to assure method accuracy. Furthermore, the purpose of ruggedness testing is to determine which variables the method is susceptible to and how to control it. Ruggedness testing does not determine the optimum operational conditions for the test method. To determine the ruggedness of the method, the ASTM guidance recommends use of the experimental design as reported by Plackett and Burman. This guidance discusses effects of change on two levels per variable, as this design is easy to use and provide useful information needed for improvement of the test method. An example of ruggedness testing for an HPLC method is given in Tables 8 through 10.

Table 8 shows the various factors and their high and low limits to be considered in ruggedness testing. Table 9 shows the factors and their high and low limits in a +/− format. Lastly, Table 10 summarizes the results obtained when each of the eight combinations (rows across the spreadsheet) are experimentally performed.

Table 8 Ruggedness Testing—Typical HPLC Factors

Factor	Low value	High value
A. pH	3.0	4.0
B. Temperature	35°C	40°C
C. Mobile phase composition	45/55	55/45
D. Buffer concentration	0.05 M	0.1 M
E. Particle size	3 micron	5 micron
F. Column length	3 cm	5 cm
G. Flow rate	1.0 mL/min	1.5 mL/min

Table 9 Ruggedness Testing—Typical HPLC Conditions

Excel	A	B	C	D	E	F	G	H	I
1	Run/factor	A	B	C	D	E	F	G	Result
2	1	−1	+1	+1	+1	−1	−1	+1	99.8%
3	2	+1	−1	+1	+1	+1	−1	−1	101.1
4	3	−1	+1	−1	+1	+1	+1	−1	98.9
5	4	−1	−1	+1	−1	+1	+1	+1	99.5
6	5	+1	−1	−1	+1	−1	+1	+1	99.9
7	6	+1	+1	−1	−1	+1	−1	+1	98.5
8	7	+1	+1	+1	−1	−1	+1	−1	98.0
9	8	−1	−1	−1	−1	−1	−1	−1	97.0
10	Effect	0.575	−0.575	1.025	1.675	0.825	−0.025	0.675	

Table 10 Ruggedness Testing—Typical HPLC Conditions

Excel	A	B	C	D	E	F	G	H	I
1	Run/factor	A	B	C	D	E	F	G	Result
2	1	3	40	55/45	0.1	3	3	1.5	99.8%
3	2	4	35	55/45	0.1	5	3	1.0	101.1
4	3	3	40	45/55	0.1	5	5	1.0	98.9
5	4	3	35	55/45	0.05	5	5	1.5	99.5
6	5	4	35	45/55	0.1	3	5	1.5	99.9
7	6	4	40	45/55	0.05	5	3	1.5	98.5
8	7	4	40	55/45	0.05	3	5	1.0	98.0
9	8	3	35	45/55	0.05	3	3	1.0	97.0
10	Effect	0.575[a]	−0.575	1.025	1.675	0.825	−0.025	0.675	

[a] Content of cell = SUM PRODUCT(B2:B9,$I2:I9)/4. This takes the difference between the average test results for the "+" runs and the average test results for the " − " runs. Conclusion: Eight experiments performed compared to 56 individual experiments. The cell with the "highest" effect value indicates the most variable factor. In this example, it is Factor D, the buffer concentration, followed by Factor C, the mobile phase composition.

Results obtained are placed in a spreadsheet, such as Excel, and the effect calculated. The highest effect (i.e., largest value) in the column listed would indicate that factor to be the most critical, and special attention is needed to control its variability.

For a detailed discussion of Plackett–Burman design experimentation, readers should consult the ASTM guidance (45) and Torbeck (46).

17.11. Stability of Sample and Standard Solutions

The FDA recommends that solution stability of the drug substance (used as sample or in-house standard) or drug product after preparation according to the test method should be evaluated. This is considered critical as most HPLC analyses are automated. For the duration of an analytical run, the standard or sample will stay in solution for hours in the laboratory environment before all the samples are com-

pletely tested. Therefore monitoring of sample or standard stability will ensure that there is no degradation occurring due to hydrolysis, photolysis, or adhesion to glassware over the course of the run period. The FDA recommends that data to support the stability of sample or standard solution under normal laboratory conditions for a minimum period of 24 hours should be generated.

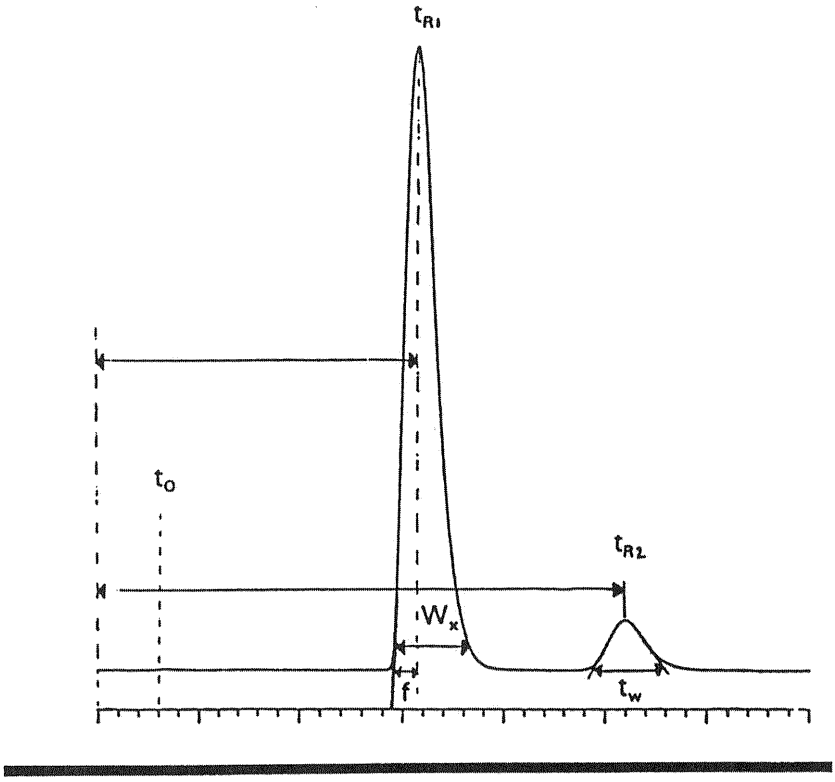
17.12. System Suitability Specifications and Tests

The accuracy and precision of HPLC data collected begin with a well-behaved chromatographic system. The system suitability specifications and tests are parameters that provide assistance in achieving this purpose. According to the ICH and the USP, system suitability testing is an integral part of chromatographic procedures. These tests are used to determine that the resolution and reproducibility of the system are adequate for the analysis to the performed. The basis for these tests is that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as a whole. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. In USP 23 General Chapter <621>, Chromatography, a section has been devoted to system suitability requirements. It is important to know what are regulatory requirements for system suitability tests and specifications for method validation. As stated earlier, system suitability involves checking a system to ensure it is performing adequately before or during the analysis of unknowns. To establish these required parameters [i.e., plate count, tailing factor, resolution (if by-products or impurity standards are available; otherwise a chromatogram from forced degradation studies may be used)], the reproducibility (% RSD) of five or six replicates is calculated and compared to predetermined specification limits. System suitability tests are performed prior to analysis of actual samples. These parameters are studied by analysis of a system suitability sample that is a mixture of main active drug and expected by-product or a known impurity. Table 11 summarizes the parameters to be measured and their recommended regulatory limits for the system suitability tests and specifications (38,40). Definition of terms for system suitability parameters is shown in Figure 5.

Table 11 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 $\leq 1\%$ for $N \geq 5$ is desirable.
Relative retention	Not essential so long as the resolution is stated.
Resolution (R_s)	R_s of >2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.).
Tailing factor (T)	T of ≤ 2.
Theoretical plates (N)	In general should be >2000.

Source: Ref. 37 and 39.



Where
W_x = width of the peak determined at either 5% (0.05) or 10% (0.10) from the baseline of the peak height
f = distance between peak maximum and peak front at W_x
t_o = elution time of the void volume or non-retained components
t_R = retention time of the analyte
t_w = peak width measured at baseline of the extrapolated straight sides to baseline

Figure 11.5 Definition of terms for system suitability parameters. (From Ref. 40.)

For accuracy and precision of analysis, all system suitability parameters play a significant role. Therefore, a critical evaluation of these parameters and their effect on a chromatographic separation are required. As an example, the effects of peak tailing and different resolution values on quantitation are depicted in Figs. 6, 7, and 8.

Resolution is a measure of how well peaks are separated from each other. For reliable quantitation, well-resolved peaks are essential. This parameter is very useful in determining if peaks can interfere in individual quantitation. As shown in Fig. 6, with a small resolution, accuracy of analysis will decrease.

Tailing peaks affect quantitation. With an increase in peak tailing the accuracy of quantitation decreases due to improper peak integration (the area under the peak will not be accurate). The effect of peak tailing is shown in Figs. 7 and 8.

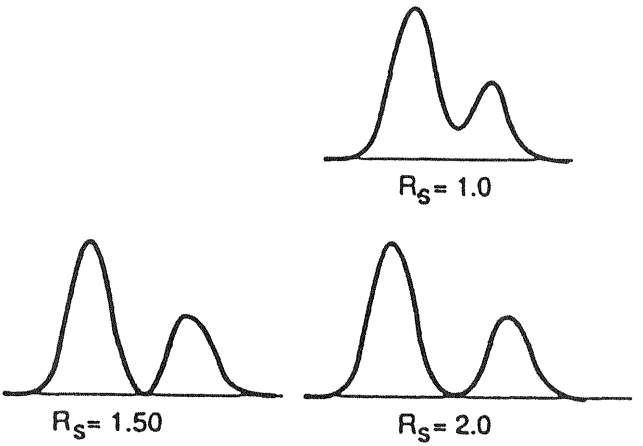


Figure 11.6 Separation of peaks as indicated by R. (From Ref. 40.)

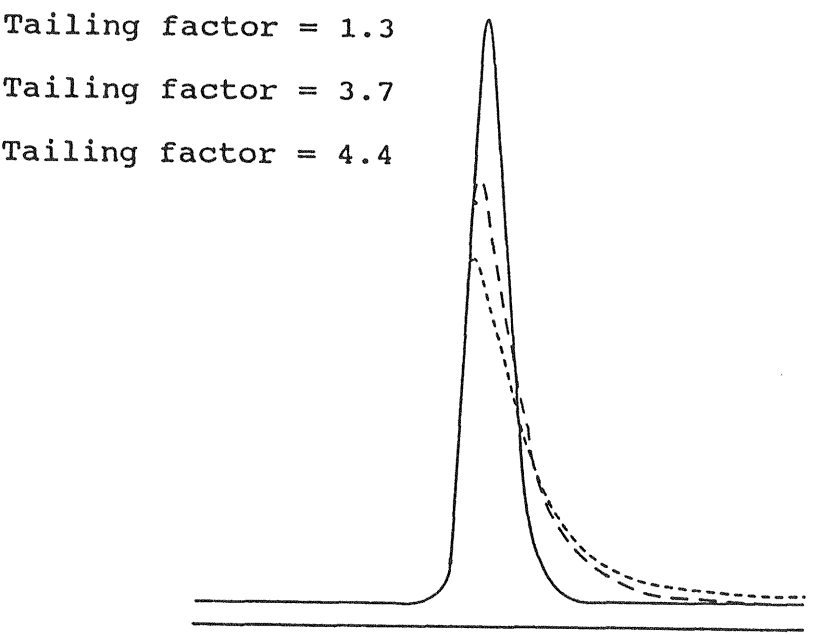


Figure 11.7 HPLC peak with various tailing factors. (From Ref. 40.)

18. POST VALIDATION ISSUES

The validation process does not end after experimental evaluation of the analytical parameters. Data must be evaluated to determine whether validation was successful or not. Does all the data generated meet the specified requirements or not? In the following subsections, steps required to finalize the validation process will be discussed.

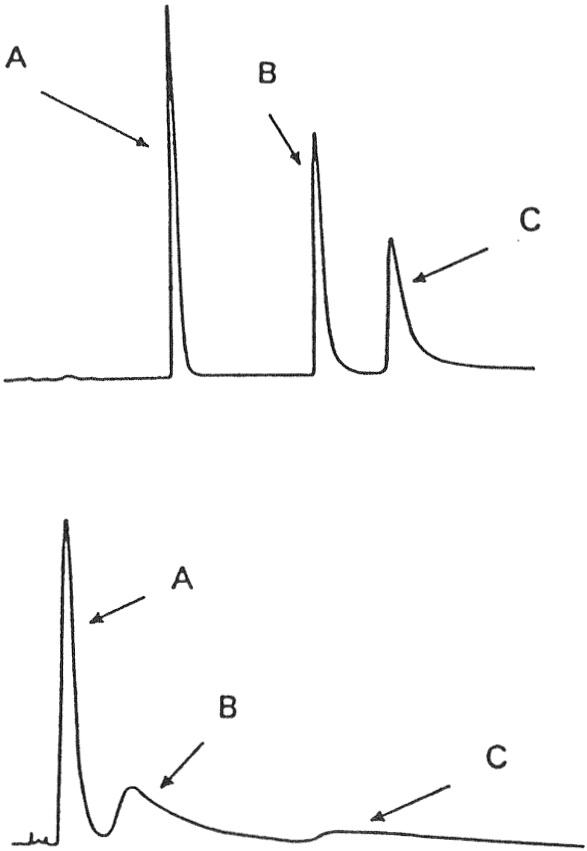


Figure 11.8 Effect of peak tailing on quantitation. (From Ref. 40.)

18.1. After the Laboratory Work

After completion of the laboratory work and documentation of the data in the analyst's notebook, it is very important that all data be carefully reviewed and audited by a qualified person. This process will ensure that data generated through the validation process is correct and meets all the requirements. Only after this step is completed can the next phase of the validation process be implemented.

18.1.1. Validation Report

The method validation report is a regulatory requirement and needs to be submitted to the FDA. The method validation report should be written by the method development group. The format for the report should be agreed upon at the onset of the validation process. This report must describe all the experimental procedures including equipment used, detector type, columns, information on reference standard, chemicals and composition of placebo for accuracy studies. All chromatograms and figures should be labeled properly. For forced degradation studies, conditions used and how this was performed must be explained.

18.1.2. Acceptance Criteria

For each step of the validation analytical parameters, acceptance criteria to determine success or failure of validation, are required. These acceptance criteria should be based on the intended use of the method. Also, regulatory implications should be taken into consideration. For acceptance criteria, it is imperative that responsible personnel with backgrounds in method development be involved. All validation steps should be evaluated against these acceptance criteria. Similarly, system suitability parameters should also accompany acceptance criteria. General acceptance criteria for each validation parameter have been discussed in Sec. 17.

18.1.3. Generation of the Test Method

Generation of the test method is the responsibility of the method's development group. The test method must include a detailed written procedure. For example, for a chromatographic procedure, preparation of mobile phase, column type, detector type, wave length, injection volume, flow rate, reference standard (USP/in-house), preparation of standard and sample solutions, reagent grade, and filters used for standard and sample solutions should be documented. If the method is designed to quantitate the main analyte and impurities simultaneously, then relative retention times for impurities should be given. If the main bulk active is light sensitive, then a precautionary note is required in the test method. Similarly, it should be reflected in the test method whether ambient or elevated temperature is required.

18.2. Revalidation

At some time during the lifetime of the method, for one reason or another, revalidation of the method may become necessary. For revalidation, reactive or proactive approaches may be used. Reactive validation will be required for changes in incoming bulk drug active, manufacturing batch changes, formulation changes, or other changes such as dilutions or sample preparation in the method. Recently, method change versus an adjustment has been the subject of discussion between regulatory agencies and industry (36,38,47).

This distinction is critical, as a process change requires method revalidation, whereas an adjustment does not. As a result of these discussions, limit changes for chromatographic changes do not require revalidation. Changes have been proposed under the following categories:

Aqueous buffer pH

1. Analytes without ionizable groups: ±1 unit
2. Analytes with basic or acidic groups and the buffer pH = pKa±2 units: ±0.2 unit
3. Analytes with basic or acidic groups and the buffer pH ⟨or⟩ pKa±2 units: ±1 unit

In each case a reference standard must be used to demonstrate that there is improvement in chromatography due to pH adjustment. No pH adjustment is allowed if standards are not available for all analytes of interest

Column dimensions

1. Length: ±70%
2. Inner diameter: ±25%, provided a constant linear flow velocity is maintained.

In addition, flow rate changes up to ±50% have been proposed. Work on limits for changes for mobile phase solvent ratios is underway. It is proposed that any method adjustment within these limits will not require additional validation. Proactive revalidation takes into consideration the availability of new technology or perhaps the automation of previously complex or time-consuming manual procedures. In such cases, revalidation may be more comprehensive, depending on the scope of the project.

18.3. Method Transfer

Method transfer is dependent on the intended use of a validated method. If other laboratories such as quality control or stability group are going to use this validated method, then a proper method transfer will be required. Under ideal conditions all laboratories involved should use an interactive approach to achieve method development, optimization, and validation goals in an efficient manner. If the end user has been involved in the development and validation process from the onset of this process as a participant or an observer, then it is convenient to place this method on line in a timely manner. Otherwise, reasonable time and effort will be required for the transfer process to be completed in a timely manner. Validation of a method demonstrates suitability of the method, whereas the method's evaluation and validity is approved by the end user.

The first step in a method transfer is to design a protocol, which is a document consisting of elements as outlined in the validation protocol, and other additional elements such as acceptance criteria, report format, and approval signatures of both the originating and the receiving laboratories. In addition, a detailed test procedure, design of experiments, sampling plan, analyst and equipment, interday and intraday ruggedness, and method transfer report form should also be included for method transfer studies.

Studies required for method transfer include system suitability, linearity, precision (day-to-day, within-day, analyst-to-analyst, analysis of multiple lots), collaboration of laboratories, developer user agreement on split sample results, and use of appropriate statistical standards, e.g., F-Test and t-Test, for evaluation of the method transfer process. The receiving laboratory should allocate enough time for the transfer, participate in interlaboratory studies, anticipate problems, and have a checklist ready of questions for the originating laboratory. For a successful method transfer, it is important to compare equipment or instrumentation in both laboratories. For example, for a chromatographic method, the age of the detector, the column, and the internal diameter of connecting tubing will play significant roles in the generation of comparable chromatograms.

Finally, where is method transfer required? In general, method transfer will be required for a new laboratory, a new method, new personnel, significant changes in a method, from company to a contract laboratory and from research and development group to quality control laboratory and stability group.

19. APPLICATION OF VALIDATION PRINCIPLES TO OTHER ANALYTICAL TECHNIQUES

19.1. Cleaning Method

To discuss cleaning validation in detail is beyond the scope of this work, as this has become an independent field. However, important steps required for cleaning validation will be briefly described. Readers are encouraged to research relevant publications on this subject. Recently, Kirsch has published an excellent review article on this subject (48). Cleaning validation is a regulatory requirement. The FDA has published a document titled "Guide to Inspections, Validation of Cleaning Processes" on this subject for field application. It is common industry practice to use the same equipment for production of a variety of products. The FDA has placed an increased emphasis on the cleanliness of the equipment to eliminate or minimize the risk of cross-contamination and adulteration of drug products.

Several analytical methods have been used by the pharmaceutical industry to demonstrate the cleanliness of process equipment surfaces. For low-level residues in rinse samples, the electronic conductivity technique are used. This technique is applicable to samples such as detergents and cleaning agents, which contain one or more ionic species. However, this technique is nonspecific and cannot be applied to neutral or highly polar compounds. Also, the FDA has specified a requirement that a correlation must be established between measurable conductance and concentration around the cleaning limit, which is a time-consuming process and not always possible for all analytes in a given formulation.

UV-visible spectrophotometry is another approach used for the detection of residue in rinse samples. This technique is sensitive but is dependent on the presence of a strong chromophore in the analyte for trace level determination. This is also a nonselective technique and not discriminating if more than one UV-active analytes are present in same sample.

Total organic carbon (TOC) has gained wide acceptance for cleaning applications (33,48). This technique is highly sensitive and specific for organic carbon-bearing analytes. TOC may be used in tandem along with conductivity, pH, and perhaps titrimetry to demonstrate the absence of both acid and alkaline detergents used for cleaning. TOC is only applicable to aqueous samples, and extra caution is required during sample acquisition and preparation to avoid bias in results due to carbon contamination.

High performance liquid chromatography (HPLC) has successfully been applied for cleaning residue samples. HPLC is sensitive for many pharmaceutical actives, and the necessary specificity can be obtained by this technique. For this technique, there are a variety of detection modes, such as spectrophotometric, electrochemical, fluorescence, and refractive index, to handle the diversity of pharmaceutical compounds.

Before the validation process begins, the appropriate predetermined level of cleanliness, i.e., at or below the limit at which equipment is considered clean, the final solvent used for the cleaning of equipment, and the type of swabs to be used should be chosen in consultation with the manufacturing group. It is critical that the limit agreed upon is practical and routinely achievable when an appropriate cleaning assay method is followed. Additionally, an acceptance limit that assures

that the next product manufactured on the same piece of equipment is not adulterated or contaminated to the extent that its fitness for use is compromised, must be established. For determination of cleaning residues by HPLC, an appropriate laboratory procedure must be developed using available methodology and validated to meet certain acceptance criteria. For validation, a written protocol will be required.

The method should be sensitive enough to detect the analyte of interest at levels below or above the acceptance limit. The following studies are suggested for validation of the cleaning assay method:

1. Linearity of response for a wide range depending upon cleaning limit. Generally 50% of the cleaning limit to 10 times this concentration.
2. Specificity or selectivity to prevent false passing or failing results.
3. Precision and accuracy to assure correct results.
4. Limits of detection and quantitation.
5. Analyte stability before and after extraction from swab or in rinse samples.
6. The solvent used in the final rinse should be compatible with the assay mobile phase.

Since cleaning validation is considered a limit test, validation requirements may be less rigorous than an HPLC method used for bulk drug active, finished product, and stability samples.

For accuracy, swab and surface recovery approaches should be utilized and evaluated. In swab recovery, an appropriate number of swabs (minimum three) are spiked with amounts of analyte in rinse solution equivalent to the amount of analyte that should theoretically be removed from a known surface area. The spiked analyte is allowed to dried on the swab followed by extraction in a known volume of extracting solvent. Recovery studies should be performed at least for three levels, i.e., limit level, 50% of limit level, and 100% of limit level. The percentage recovery is calculated by using an external standard prepared at limit level concentration. The percent recovery obtained should not be less than 80%.

In surface recovery, 316 stainless steel or inert glass plates are used. A known surface area is spiked with a known amount of analyte by uniformly spreading analyte in rinse solution over the known surface area. The plate is dried under ambient conditions. The drying time will depend on the solvent used. However, overnight drying is preferable. The dried analyte residue is then swabbed using a premoistened swab across the spiked area. Swabbing can be done by horizontal, vertical, and zigzag motion of the swab. For better recoveries more than one swab may be required for removing analyte from the plate surface. The swabs are then placed in a 50 mL tube and extracted with the extracting solvent specified in the method. Recovery calculations are done against an external standard prepared at limit level. Recoveries can be performed at limit level, 50% of limit level, and 100% of limit level. It is pointed out that in the surface recovery approach, recovery values obtained are usually about 70 to 80%. Loss in recovery value is attributed to analyte solubility, analyte–metal binding strength, reactivity of surface with analyte, and swabbing technique. Also, streaking effects encountered in swabbing surfaces are detrimental and result in loss of the active.

19.2. Physicochemical Characterization Method (Dissolution)

USP 23 General Chapter <1225> designates dissolution testing under Category III. The validation parameters recommended in the Compendia are precision and ruggedness studies. Other studies are left to the discretion of the end user. It is common industry practice to verify a USP dissolution method by performing studies such as linearity of standard solutions, placebo interference, capsule shell interference (if a capsule) and reproducibility of response at specified times(s) for release. However, for an in-house developed dissolution method, proper validation studies are required. These studies would include specificity (interference from placebo), precision, linearity, system suitability, filter adsorption, and sample and standard stability. For automated dissolution systems, in addition to filter adsorption, there should be evidence of nonadsorption to active tubing used for delivery throughout the system and carryover effects.

It is difficult to perform recovery studies for dissolution, as spiking of placebo in vessels is not practical. Placebo excipients have a tendency to float on top of the dissolution medium. In addition, it is difficult to make single tablets unless a hand-held press is used. Hand filled capsules lack uniformity, and the procedure is tedious. In another approach, placebo along with label claim amount of active are placed in a 900 mL volumetric flask. The flask is filled to volume with dissolution medium and a magnetic stir bar is used to stir this mixture on a magnetic plate for the specified time period. Calculations of recovery are done against an external standard prepared in the dissolution medium. Acceptance criteria for precision, specificity, system suitability, and linearity are similar to assay validation.

19.3. Nonchromatographic Methods

There are a variety of guidelines available for the validation of the chromatographic procedures, but comparatively little information is available on validation of nonchromatographic methods. In general, it is assumed that USP General Chapter <1225> on analytical validation is only applicable to chromatographic methods. This assumption is incorrect, as USP General Chapter <1225> does not state that the validation parameters given in this chapter cannot be used for nonchromatographic techniques. By careful selection of parameters, a validation protocol can be designed for validation of nonchromatographic methods. Brittain has discussed validation issues and data elements required for validation of nonchromatographic methods (49).

19.3.1. UV Spectrophotometry

For UV spectrophotometric methods for assay, one needs to study parameters such as precision, accuracy, specificity, and linearity (49). For precision, a sufficient number of individual sample preparations should be assayed to permit the calculation of a statistically valid relative standard deviation. Accuracy can be determined by spiking a mixture of excipients (placebo) with known amounts of drug active at different concentration levels. Spike levels are, in general, similar to the linearity range. Spiked samples are prepared by following the "sample preparation" procedure and assayed against an external standard at the target level concentration. The accuracy is calculated from the test results as the percentage

of analyte recovery by the assay. For specificity studies, intrinsic differences in chemical or physical properties are used to ensure accurate determination of analyte even in complex sample mixtures.

The purpose of a specificity study is to demonstrate that the method will yield reliable results even in the presence of interfering species. One approach to determine any possible bias in an assay is by comparison of results of assay value obtained in the presence of placebo excipients to assay value without placebo excipients. Assay bias is evaluated by calculating the percentage agreement between these two results by the formula (49)

$$\text{Percent agreement} = \frac{\text{TP}}{\text{TA}} \times 100$$

where TP = test results in the presence of placebo and TA = test results in the absence of placebo. A 100% agreement with show the absence of bias due to placebo or the potential interfering species. Agreement values >100% indicate positive bias, while agreement values <100% indicate negative bias in the assay procedure.

If standards for impurities or degradation products are not available, then the specificity can be determined by analyzing the samples containing the impurities or degradation products (from bulk drug) and comparing the results with those obtained by another independent and validated assay procedure. The independent assay is considered as the reference assay, and the degree of agreement between these two test results will dictate the specificity for the intended method. Calculations are similar to that described above. A percent agreement of 100% will be required for the absence of any bias in the intended method.

Linearity should be performed at least for five levels, including target level as 100%. Other concentrations should be 50%, 75%, 125%, and 150%. It is important that linearity responses obey Beer's law. Statistical evaluation of linearity is similar to that (as explained under linearity studies) for the chromatographic method validation.

19.3.2. Atomic Absorption Spectroscopy

Atomic absorption is used to determine heavy metals present in the drug substance. Heavy metals fall into the category of a limit test. Therefore rigorous validation may not be required. However, as these metals are present at trace levels, determination of limits for detection and quantitation is of significant importance for the validation of atomic absorption methods. Other validation parameters such as linearity, precision, specificity, and accuracy may be performed as described under Sec. 19.3.1.

The limits of detection and quantitation are determined by analyzing a number of samples prepared at low levels such as 2 ppm, 5 ppm, and 10 ppm. For each concentration level, multiple analysis is performed and standard deviation (SD) is calculated. All standard deviations are then averaged to calculate the mean standard deviation (MSD). To obtain an estimate of the noise level, the MSD is then divided by the slope of the calibration curve. For the detection limit the noise level obtained is multiplied by a factor of 3, whereas for the quantitation limit, a factor of 10 is used.

19.3.3. Infrared Spectroscopy (IR)

Infrared is used for identification of compounds. Currently in USP 23 there is a scarcity of monographs describing the use of IR for quantitation of analytes. For example, IR quantitation is used for the analysis of simethicon bulk drug active, tablets, oral suspensions, and capsules. As such, validation parameters required may be limited to interference studies. This interference may be due to the compound itself. For example, in an infrared spectrophotometric identification test, polymorphism may produce interference. Therefore, for compounds that exhibit polymorphism, it is critical that test samples and the reference standard have similar crystalline form. It then becomes obvious that for the infrared identification test, one should demonstrate that the method is insensitive to any polymorphic form of the material, or that the polymorphic effects have been taken into account. It is pointed out that unlike the chromatographic procedure, there are no official guidelines available on the validation of an infrared technique at present. An article by Ciurczak, "Validation of Spectroscopic Methods in Pharmaceutical Analyses," gives an overview of this subject (50).

19.3.4. Titration

USP 23 has several monographs that stipulate using titrimetry for release of bulk actives. These procedures are nonspecific and may not give accurate results in the presence of reactive impurities or degradation products. Therefore, for validation of these procedures an innovative approach will be required. The parameters to validate a titrimetric method include linearity, accuracy, blank determination, and insensitivity of the method to the amount of indicator used.

For linearity studies, different weights of the compound should be titrated, and the actual and theoretical results should agree. Alternatively, the titration could be done using a narrow range of compound weight, and then it should be stated in the method that the weight of the sample must be within this range. The accuracy should be studied by showing that the volumes of titrant for replicate titrations are very close to each other. In other words, small differences in volume of titrant required to reach an end or equivalence point does not introduce any significant error into the results.

As stated earlier, titrimetric procedures are nonspecific and cannot be used for simultaneous assay of active and impurities. In this case, impurities should be monitored by another independent validated procedure. For bulk active assay, comparison of results obtained by an alternate validated method and those obtained by the titrimetric procedure will demonstrate the validity of the titrimetric method.

19.4. General Considerations

The accuracies of chromatographic methods rely heavily on the purity of reference standards. Therefore a well characterized and highly pure standard is important. The FDA recognizes two categories of reference standards, i.e., compendial and noncompendial. The USP is the source of compendial standards. As these standards are well characterized, no further characterization is required. Noncompendial standards are also of high purity and can be obtained by reasonable effort and should be

thoroughly characterized to ascertain their identity, strength, quality, and purity. Testing requirements for the reference standards are more rigorous as compared to bulk drug substance. The purity correction factors for non-USP standards should be included in any method calculations.

Quantitation of actives in chromatographic methods is based on either external or internal standards. An external standard method is used when the standard is analyzed on a separate chromatogram from the sample. Quantitation in this case will be by comparison of the sample and reference standard responses, i.e., peak area or peak heights for HPLC and GC or spot intensity in TLC for a given analyte of interest. External standard methods are generally used for samples with a single target concentration and narrow concentration ranges (acceptance and release tests). Simple sample preparation procedures or longer run times for detection of extraneous peaks, e.g., impurity test, HPLC methods for stability, and TLC methods also use external standards. For internal standard methods, a compound of known purity is added directly to the sample. However, it must be ensured that the compound being used as an internal standard does not interfere with any analyte of interest or degradation products in the sample. The response ratio between internal standard and analyte of interest in the sample is compared to the ratio of the internal standard and the analyte in the standard that is used for quantitation purposes. Internal standard methods are widely used for quantitation in biological samples and for low and wide sample concentration ranges, e.g., in pharmacokinetics studies.

There are some basic points that should be addressed in the test method.

1. The sample and the standard should be prepared in the mobile phase. If this is not possible, then the level of organic solvent used in the preparation of the sample and the standard must be lower than that present in the mobile phase.
2. The sample and standard concentrations should be close to each other.
3. Sample preparations often require filtration prior to injection onto the system. Filtration removes particulate matter that may clog the column. However, analyte adsorption on the filter can take place. This adsorption effect is important for low-level impurities. Therefore, data to validate this aspect will be required.

In conclusion, method validation is a dynamic process and should not be considered a one-time situation. The design and validation of the method should be such that they ensure its ruggedness or robustness throughout the life of the method. The accuracy of the data is affected by variations in the manufacturing process, the preparation of samples in the laboratory, and the instrument performance. With a well-designed validation and tight chromatographic system suitability criteria, the reliability of the data can be significantly improved. Variations, except from the drug product manufacturing process, can and should be minimized. Good, reliable validated methods will generate data that is trustworthy.

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APPENDICES

1. List of Guidance Documents, CDER. http://www.fda.gov/cder/guidance/index.htm
2. Useful websites:
   www.pharmweb.net
   www.waters.com
   www.usp.org
   www.ich.org
   www.aoac.org