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METHOD DEVELOPMENT FOR PRECLINICAL BIOANALYTICAL SUPPORT

MASOOD KHAN[1] AND NAIDONG WENG[2]

[1]*Covance Laboratories Inc., Chantilly, Virginia*
[2]*Johnson & Johnson Pharmaceutical Research & Development, Raritan, New Jersey*

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4.1 PRECLINICAL BIOANALYTICAL SUPPORT USING LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETERS (LC-MS/MS)

4.1.1 Introduction

Preclinical development represents a critical stage in the progression from discovery to marketed pharmaceutical drug candidates that have passed initial discovery screening and are identified to possess some drug-like properties. Serious resources and financial commitments are now being made to vigorously test the drug candidates before they enter clinical trials. From a regulatory point of view, it is also essential to ensure the welfare of the volunteers and patients participating in the clinical trials by vigorously testing the safety using appropriate animal models. A series of questions concerning the toxicity, pharmacokinetic (PK) parameters, safety assessment, formulation optimization, and so on need to be answered.

Bioanalytical support plays a pivotal role in answering these questions. Timely bioanalytical support is essential to improve the success rate of drug candidates moving along the preclinical development pipeline and allows decisions to be made early to modify/improve the drug candidates or terminate the program. By definition, the goal of preclinical development support is attrition of the drug candidates with less favorable drug-like properties. The financial benefit of eliminating candidates from the drug development pipeline has been highlighted by Lee and Kerns [1].

Bioanalytical support at the preclinical development phase presents some unique challenges. The first unique challenge is the *Good Laboratory Practice* (GLP) *requirement*. Unlike discovery support, where bioanalytical support does not typically run under GLP compliance and oftentimes generic fast gradient methods are used to rank the drug candidates, preclinical bioanalytical support is under strict GLP regulations, where the data generated undergo rigorous regulatory scrutiny. The mindset is certainly shifted from the more creative discovery bioanalytical support to more compliance-oriented development bioanalytical support. Cutting-edge technology is only used at preclinical bioanalytical support with caution and only after these technologies meet the challenge of vigorous validations for staying in compliance will they be widely accepted as one of the primary tools for preclinical bioanalytical support.

The second unique challenge is to have good *metabolic selectivity coverage*. Usually at the early stages of preclinical studies, information on metabolites may not be well characterized and a definitive human absorption, distribution, metabolism, and excretion (ADME) study is usually not run until clinical studies. New microdosing paradigms are evolving, which, over time, may garner human metabolism information earlier. Yet, from the bioanalytical point of view, the limited information on metabolites normally available presents a challenge. It is often uncertain

how many metabolites should be included in the assay. Insufficient coverage of metabolite measurement would leave inadequate coverage of the safety margin in clinical trials should a minor metabolite in an animal species become a significant one in humans. On the other hand, methods measuring too many metabolites could result in high failure rates, a highly undesirable feature that is not only costly but also leads to regulatory question regarding the validity of the entire method.

The third unique characteristic of the preclinical bioanalytical support is the very quick turnaround time for *method development, validation, and sample analysis* for multiple animal species (mouse, rat, dog, rabbit, monkey, etc.) and oftentimes for multiple matrices (plasma, urine, tissue, liver, brain, etc.). The requirements for the skill set and infrastructure of a bioanalytical laboratory supporting preclinical work could be different from those supporting clinical bioanalytical works. For clinical bioanalytical support, it is not unusual to have a group of scientists dedicated to method development and validation and then the methods are transferred to other chemists to run large sets of routine sample analysis that could last for several months to several years. For preclinical bioanalytical support, such a transition is also possible but requires a more vigorous method ruggedness test and more frequent communication between the method developer and chemists running the samples. Also, there is a need for a very quick turnaround as well as a heavy load on method development/validation instead of routine sample analysis. It is still desirable to free method developers from routine sample analysis so that they can focus on quality method development. In general, the ratio of method developers to sample analysts should be higher for supporting preclinical LC-MS/MS than for clinical LC-MS/MS.

The fourth unique characteristic is that at this stage one needs to think about a strategy of *streamlining the process at various stages of bioanalytical support*. Table 4.1 summarizes the major characteristics for the discovery, preclinical development, and clinical bioanalytical support. These three stages should not be viewed as three separate and consecutive ones, since each can extend well into the next stage. Streamlining the sample analysis process during the preclinical stage presents unique challenges in comparison with discovery or clinical bioanalytical supports, even though great effort has been made by many scientists in the pharmaceutical industry. In the discovery stage, a few generic methods are applied to many candidates, which make it easier to adopt a universal strategy to streamline and automate the process with the ultimate goal of generating data as quickly as possible to enable eliminating poor candidates. Data review by a quality assurance unit is not needed and reporting is usually kept quite simple. When the projects get transferred to the preclinical stage, an individual unique method needs to be developed and validated for each candidate to ensure the method meets the specific requirements specified in standard operating procedures (SOPs) as well as in regulatory guidance. The method will then be used for analyzing samples. Depending on the nature of the program, the sample numbers can vary quite significantly. The scientist needs to make sure that automation is applicable for real sample analysis [2, 3]. At the preclinical stage, emphasis is usually on method reproducibility and method specificity to ensure the highest quality data. However, one should not overlook the benefit of using automation when appropriate. Automation does reduce human errors and improve method reproducibility. Once the drug candidate moves into the clinical stage, automation and other means of improving sample processing become more important. The cost

TABLE 4.1 Bioanalytical Support for Discovery, Preclinical, and Clinical Studies

	Discovery	Preclinical	Clinical
Regulatory requirement	Non-GLP	GLP	Although GLPs only apply to preclinical studies, clinical falls under the guidance and by practice the same standards as the GLPs
LC-MS/MS method	Generic	Tuned to compound; requires extensive method development	Tuned to compound; extensive method development but could leverage preclinical methods
Validation	Minimal	Extensive validation to multiple matrices in multiple species	Extensive validation but limited to human samples; specificity tests to coadministered compounds
Validation strategy	Abbreviated method validation; minimal stability test	Full validation for one species and partial validation for other species including incurred sample reproducibility; has large curve range that may be problematic due to carry-over or ionization saturation	Full validation including incurred sample analysis; very sensitive method may be needed for high potency candidate
Sample analysis	Small sets of samples per compound but hundreds or thousands of compounds	Moderate numbers of samples for dozens of compounds	Large numbers of samples for very few compounds
Sample analysis strategy	Streamline process from sample collection to data generation; use of generic methods also allows easier set up for automation	Due to relatively small sets of samples and various methods tuned for each compound, automation is feasible but may not be feasible for some methods due to limited sample volumes	Quick turnaround time for sample analysis (automated sample preparation, multiplexing, UPLC) to allow data to be released

of automating clinical bioanalytical methods can easily be justified. The same clinical bioanalytical method can be used by multiple bioanalytical chemists within or even among different organizations to meet the demand of analyzing large sets of samples within a short period of time. The nature of clinical samples also makes automation more amenable for routine sample analysis. Sample volumes are typically several-fold higher than those in preclinical studies and are more suitable for automation. Like discovery bioanalytical support, sample analysis speed is likely to be the rate-limiting step for clinical bioanalytical support. Approaches for expediting sample analysis, such as multiplexing two high-performance liquid chromatography (HPLC) units into one mass spectrometer or ultra-pressure liquid chromatography (UPLC) using sub 2 μm columns, are frequently used.

The literature on general bioanalytical method development, validation, and sample analysis has been extensively reviewed [4–14], but a strategy focused on preclinical bioanalytical support would certainly be useful. In this chapter, we share our practical experience on preclinical bioanalytical support.

4.1.2 Regulatory Requirement

A distinguishing feature of preclinical bioanalytical support is the regulatory compliance. Standard operating procedures (SOPs) are used to address many aspects of the regulatory requirements, ranging from instrument qualification, maintenance, and calibration, to bioanalytical method validation, sample analysis, data management, and sample archiving. The goal of these SOPs is to ensure the highest data integrity that can pass regulatory scrutiny. These SOPs are usually drafted based on specific regulations and guidance from the U.S. Food and Drug Administration (FDA) and international regulatory agencies, based on various federal/state requirements and based on specific policies at each institution. The single most important regulatory guidance is the FDA guidance on bioanalytical method validation [15], which provides a general guideline on establishing important parameters, including method specificity/selectivity, sensitivity, linearity, accuracy, precision, stability, matrix effects, recovery, carry-over, and contamination. The method should also demonstrate reproducibility and accuracy of measurements for incurred samples and be suitable for its intended use. However, it is up to each institution to formulate its own SOPs based on this general guidance. It is well recognized that there is a gap between the spiked quality control (QC) samples and incurred samples [16, 17]. It is generally accepted industry-wide and by the regulatory agencies that the method should meet criteria such as selectivity, sensitivity, linearity, accuracy, precision, and stability using quality control samples that are prepared by spiking known amounts of analytes to the control matrix. However, QC samples may not entirely reflect the nature of the incurred samples and this fact is often neglected by the bioanalytical chemists, resulting in over- or underestimation of analytes due to various factors. Therefore, efforts should be made to address this gap to satisfy the regulatory compliance needs.

4.1.3 Batch Failure Rate

Batch failure rate has been particularly useful to assess the method ruggedness or reliability. High failure rates usually indicate inherent shortcomings of a given

method and could be used by the FDA to reject the submitted data. Excluding execution errors, good bioanalytical methods should achieve at least 80% passing rate. All failed batches should also be documented and reported in both validation and sample analysis reports. Under no circumstances should the failed batches be omitted from the report. If the method has failed to demonstrate the acceptable batch passing rates during sample analysis, analysis should be placed on-hold and an investigation for the root cause should be initiated. Based on the conclusions of the root-cause analysis, the method may need to be modified and revalidated. A decision on analyzing samples in the failed batches should be judicious and the reanalysis should be authorized after an investigation. Testing into compliance by repeatedly reinjecting the failed batch until it passes acceptance criteria is definitely a red flag for regulatory authorities. Excessive manual peak reintegration, especially for standards and quality control samples, is also an indication of inherent problems in method ruggedness or data integrity and this practice should be avoided. One should be aware that, with multiple analytes (drug candidates with multiple metabolites), the overall batch failure rates can be significantly worse even if each single analyte has an acceptable 80% passing rate. Therefore, including too many metabolites in a method could potentially introduce bias to otherwise useful data from the parent compound and its most important metabolites. One approach is to have a separate non-GLP method for those metabolites whose significance to humans in not yet fully understood [18]. For the drug and significant metabolites, bioanalytical methods intended for GLP studies should be vigorously tested and validated to have a thorough assessment of the method reproducibility and ruggedness. It is costly and time consuming to troubleshoot the methods in the middle of sample analysis, especially for preclinical studies where quick turnaround is required.

4.1.4 Narrowing the Gap for Incurred Sample Analysis

Due to potential differences such as protein binding and metabolite conversion, quality control samples, which are prepared by spiking analytes of interest into the control blank matrix, cannot totally mimic incurred samples. This difference may lead to a significant bias for the quantitative bioanalysis and must be carefully evaluated. The importance of assessing method accuracy and reproducibility using incurred samples has been emphasized at the 3rd AAPS/FDA Bioanalytical Method Validation Workshop (commonly referred to as the Crystal City III meeting) [19]. It was suggested that evaluation of assay reproducibility and accuracy for the incurred samples needs to be performed on each species used for GLP toxicology studies. Method reproducibility could be assessed by repeat analysis of individual or pooled incurred samples. Analyzing individual incurred samples will generate a second set of data. A clear written policy should be established for how to handle this second set of data. One approach that avoids generating a second set of data is to use pooled samples. Pooled samples provide the same type of data as individual incurred samples, but they are not reportable as study data. Nevertheless, the argument against this pooling is that bias from individual samples could be averaged out. For example, dosing vehicle that can cause significant ion suppression and therefore biased quantitation is more predominant in the early time points and pooling with later time points may diminish such an effect. One approach to solve this issue is to pool samples from similar time points. Assessment of method accu-

racy for the incurred samples presents a major challenge. It would be extremely difficult to ascertain the method accuracy for the incurred samples since the true value is unknown. The feasible approaches to ascertain some level of method accuracy for incurred samples might be the addition of analytes to the incurred samples (standard addition) or comparison of results from two orthogonal methods. Even with these assessments, situations causing bias for the quantitation of incurred samples could still exist. One such possibility is the instability of the incurred samples during the sample collection procedure and sample storage. This may be even more pronounced for the large molecule therapeutics. Neither the standard addition approach nor the orthogonal method approach would detect such a problem. Therefore, as a bioanalytical scientist, one must be judicious when developing methods and make sure any issues that can cause potential method bias or poor reproducibility are resolved prior to the method validation. One common mistake when developing separate assays for parent compound and metabolite is the failure to investigate selectivity and stability toward the potential influence of the drug and other metabolites on each other. Each individual method may only contain the analytes to be measured in that method which does not typically reflect the entire content of incurred samples. Potential conversion between known analytes or between known and unknown metabolites may occur during the sample collection, storage, preparation, or postextraction, leading to quantitation bias. The extent of the bias depends on the conversion rate and the concentration ratio among these interconverting compounds. Typically, concentrations for the calibration standards and QCs are prepared in such a way that both concentrations increase or decrease proportionally. This approach allows the use of one common stock solution to prepare standards or QCs at lower concentrations. In most cases, this approach would be fine since for most of the drug candidates such a proportioned concentration change is indeed observed (Fig. 4.1a). However, for drug candidates such as prodrugs, the concentration ratio of prodrug versus active drug could be lopsided, as shown in Fig. 4.1b. At early time points, the prodrug concentration can be significantly higher than the drug concentration. Because of the labile nature of the prodrug, the drug concentration may be overestimated because of the conversion of prodrug to drug during storage, extraction, postextraction, and in the LC-MS/MS source. One approach to investigating this problem is to prepare additional quality control samples at nonequivalent concentrations.

4.1.5 Control Animal Samples

One important aspect of bioanalytical preclinical support is to analyze samples from the control animal groups, which are not given a dose of the drug candidate. Any positive finding in these control animal samples indicates potential contamination in the dosing, collection, storage, sample preparation, or analysis process and usually warrants a thorough investigation. In order to minimize potential contamination during the sample preparation and analysis, these control animal samples may be analyzed in a separate batch from the samples from dosed animals. Multiple matrix blank samples are also analyzed along with these control animal samples to assist the investigation into the source of contamination, should it occur. Determining whether the measured concentrations in the control animals follow any pharmacokinetic (PK) profile or the parent/metabolite ratio is consistent with *in vivo* observations

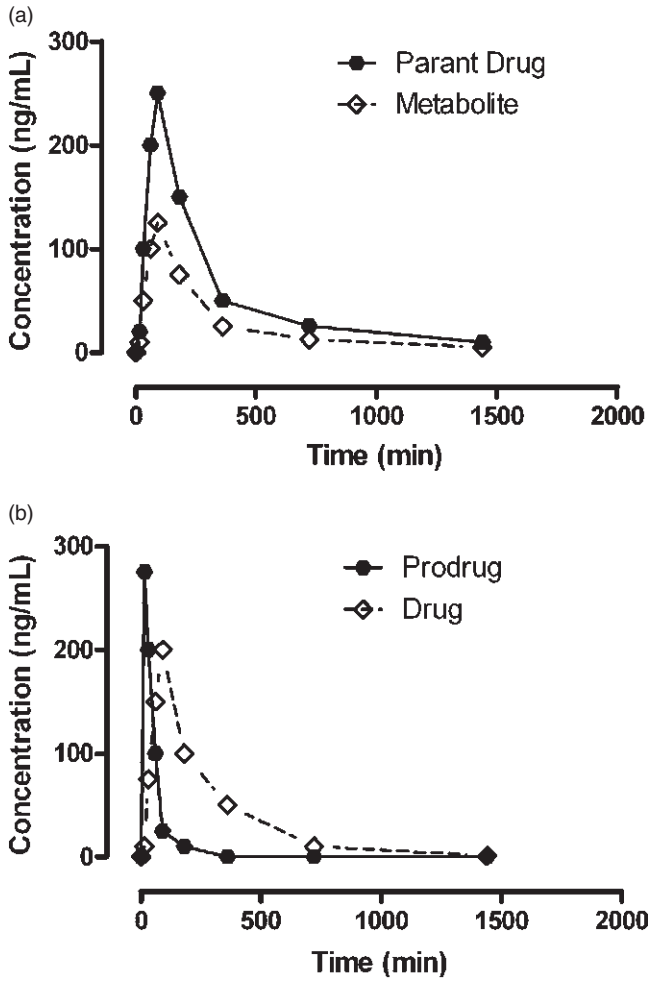

FIGURE 4.1 Typical PK profiles for parent drug/metabolite and prodrug/drug. Note the parallel concentration profile between parent drug and metabolite in (a) and the lopsided concentration profile between prodrug and drug in (b).

also provides good insight into the source of contamination. It might be necessary to have a dedicated laboratory and instruments to analyze the control animal samples if a drug candidate is highly potent and is dosed in very small amount.

4.1.6 Metabolic Selectivity Coverage During Drug Development

An industry report on drug metabolites in safety testing (commonly referred to as MIST) was published in 2002 [20]. After deliberation between industry and the FDA, a draft FDA *Guidance for Industry: Safety Testing of Drug Metabolites* was issued in 2005 [21]. Extensive characterization of the pharmacokinetics of unique and/or major human metabolites would be very useful to correlate metabolites and toxicological observations. At the Crystal City III meeting, it was agreed that a

"tiered" method validation approach could be used for measuring unique and/or major metabolites in early drug development. A tiered validation approach would allow information to be gathered on human and animal metabolites with scientifically valid methods while deferring more labor-intensive GLP validations to later stages in the drug development when significant metabolites are identified. In order to identify which metabolites to measure, metabolites formed in several animal species and human liver microsomes *in vitro* are identified and their concentrations are measured. Yet, there is no guarantee that there is a direct correlation between the metabolites thus observed and the human metabolites observed *in vivo*. If unique human metabolites are found or major metabolites were observed at concentrations without safety margin from toxicology studies, it could present some unique challenges to bioanalytical scientists. It would be beneficial if human metabolites could be identified early during the drug development. With the advancement and availability of the highly sensitive accelerated mass spectrometry (AMS), one can perform exploratory human ADME by using the microdosing scheme suggested in the guidance for exploratory studies or by combining exploratory human ADME with single ascending dosing (SAD) [22].

In order to obtain meaningful results from the study, one also needs to pay attention to the metabolite stability *ex vivo*. Metabolite stability in plasma could be affected by enzymes, pH, anticoagulants, storage conditions, and freeze/thaw cycles. It is well known that acylglucuronides can degrade back to the original drug under neutral or basic pH [23]. Adjusting the pH to 3–5 would stabilize the acylglucuronides. Candidates can also be subject to hydrolysis due to esterases. An enzymatic inhibitor (sometimes more than one) is sometimes needed to stabilize both the parent compound and its metabolite(s). One should be aware that commercially purchased plasma may not have the same enzymatic activity as the plasma harvested from freshly drawn blood. It is advisable to confirm the metabolic stability using fresh matrix.

Another consideration related to metabolic specificity is the in-source fragmentation occurring at the LC-MS/MS interface. Under high temperature (300–600 °C) and high voltage (2–5 kV), phase II glucuronide metabolites may lose the glucuronide to form the original compound, which will show up in the aglycone analyte channel. If there is no chromatographic separation between them, overestimation of the aglycone compound may occur.

4.1.7 Method Development Strategy

Vigorous method development and optimization is essential in order to narrow the gap between spiked QC and incurred samples. The spiked QC samples only contain the added analytes while the incurred samples may contain additional known and unknown metabolites. In the early stages of preclinical bioanalytical support, information related to metabolite characteristics and stability may not be fully developed. As mentioned previously, metabolic specificity of the method needs to be carefully evaluated. Another unique feature of preclinical bioanalytical support is the dosing vehicle effect on the quantitation, especially when limited or minimal sample preparation and fast gradients are used.

With attaining high quality data as the ultimate goal, bioanalytical scientists strive to develop methods with good selectivity, high sensitivity, and faster throughput.

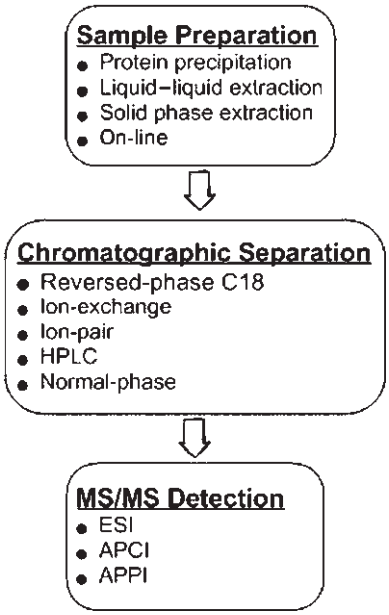

FIGURE 4.2 Three distinguished but interrelated stages (sample preparation, chromatographic separation, and MS/MS detection) of analysis of biological samples using LC-MS/MS.

Bioanalytical methods (chromatographic) typically consist of three important but interrelated parts: sample preparation, chromatography, and MS detection (Fig. 4.2). Many techniques can be employed for each of these parts. When developing a robust method, one needs to consider all three parts as an integrated system. Sometimes trade-offs need to be balanced. For example, if a simple sample preparation procedure, such as protein precipitation, is used, one may need to compensate for the potential ion suppression or in-source conversion of glucuronide metabolites to parent compound by using a more extensive chromatographic separation. Ideally, the mechanism for sample extraction and chromatography should be orthogonal to provide better method selectivity. A combination of reversed-phase solid-phase extraction (SPE) and reversed-phase LC may not provide sufficient separation power of metabolites or interferences. Of course, one will always need to keep in mind the analyte integrity during sample extraction, postextraction, and chromatography. Although liquid chromatography with ultraviolet or fluorescence detection (LC-UV/FLU) or gas chromatography with mass spectrometer detection (GC-MS) methods are still widely used and sometimes offer better methods than LC-MS/MS [24], the use of LC-MS/MS, owing to its intrinsic advantages, has nevertheless grown exponentially in the last decade and is used extensively for the preclinical bioanalytical support for small molecule therapeutics. The principle of MS is the production of ions from analyzed compounds that are separated or filtered based on their mass to charge ratio (m/z). Most applications for quantitative bioanalysis use tandem mass spectrometers (MS/MS) that employ two mass analyzers: one for the precursor ion in the first quadrupole and the other for the product ion in the third quadrupole after the collision–activated dissociation of the precursor ion in a collision cell

(second quadrupole). Between the high pressure LC and the MS operated under a high-vacuum environment, interface connections that operate at atmospheric pressure, such as electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), and atmospheric-pressure photoionization (APPI), have matured into highly reliable systems necessary for quantitative LC-MS/MS bioanalysis. On the chromatographic side, reversed-phase columns are extensively used but separation based on other retention mechanisms can also be used complementarily (e.g., normal-phase chromatography for chiral separations).

Sample Preparation Protein precipitation (PPT), liquid/liquid extraction (LLE), and SPE are frequently used sample preparation techniques in the format of either individual tubes/cartridges or 96-well plates. Analytes of interest are released from protein when a protein precipitation reagent, such as organic solvents (e.g., acetonitrile, methanol), acids, or salt (e.g., ammonium sulfate), is added to the biological samples to denature the protein. These analytes stay in the supernatant after centrifugation and can be analyzed as is or can go through evaporation/reconstitution steps to make the injection compatible with the chromatographic condition. For highly protein-bound compounds, it is frequently necessary to release them from the proteins by adjusting the sample pH; otherwise, the analyte could be trapped in the precipitates. In principle, solid-phase extraction (SPE) is analogous to liquid/liquid extraction (LLE). As a liquid sample passes through the SPE, compounds are adsorbed onto the support or sorbent material. Interferences can then be selectively removed using washing solvents. Finally, the desired analytes may be selectively recovered by an elution solvent, resulting in a purified extract. The eluent can either be injected directly onto LC-MS/MS or go through the evaporation/reconstitution steps for further concentration enhancement and for compatibility with chromatographic conditions. Unlike protein precipitation, where universal extraction and recovery is usually achieved regardless of the chemical structures of the analytes, success using LLE and SPE to extract analytes largely depends on the chemistry of the molecules and the experience of the analyst. Although LLE and SPE usually give cleaner samples than protein precipitation and thus less matrix effects due to ion suppression and enhancement, their selectivity can result in differential recovery among parent compound and metabolites as well as the internal standards. This could place a limit on their use for simultaneous analysis of multiple analytes. Use of stable isotope-labeled (SIL) internal standards could compensate for this deficiency to some extent since the SIL internal standards usually have the same recovery and chromatographic characteristics as the unlabeled analytes.

Chromatography With the initial sample clean-up using SPE, LLE, or PPT, unwanted compounds can still be present in higher concentrations than the analytes of interest. A second stage of cleanup, typically involving LC separation, further separates analytes of interest from the unwanted compounds. Without this further separation, those unwanted and present compounds in the MS/MS are not typically observed but present significant challenges. In the LC-MS interface, these compounds compete with analytes for ionization and cause inconsistent matrix effects that are detrimental to quantitative LC-MS/MS.

Reversed-phase LC has traditionally been used for quantitative LC-MS/MS. There are numerous types of reversed-phase columns, with the C18 column being

the most predominant. With increasing organic solvent concentrations in the mobile phase, the analyte retention decreases. However, one should be aware of the potential bimodal retention on the reversed-phase column due to the residual silanol group [25]. This bimodel retention may cause retention shifts during the run or irreproducibility of the method. Of particular interest is the use of monolithic columns (available in C18 format) operated at a high flow rate. Compared to a particulate column, the monolithic column has a reduced pressure drop but still maintains high separation efficiency at high mobile phase flow rates. This is due to its unique bimodal pore structure, which consists of macropores (2 μm) and mesopores (13 nm) [26]. The mesopores provide the surface area for achieving adequate capacity while the macropores allow high flow rates because of higher porosity, resulting in reduced flow resistance. Monolithic columns have become increasingly popular for use in ultrafast bioanalysis of drug candidates using tandem mass spectrometric detection [27–29]. Another recent development in column technology is the fused-core column, specially designed for hyperfast chromatography under the tradename Halo. Due to the small and uniform 2.7 μm particle size and fused-core technology, the analytes travel in the column with reduced diffusion, resulting in improved column efficiency and reduced column backpressure. Sub 2 μm particle columns used for UPLC has also drawn lots of attention. Special LC units, designed to operate under a backpressure as high as 15,000 psi, allow fast chromatography. Hydrophilic interaction chromatography (HILIC) became a popular choice in the last several years. HILIC is similar to normal-phase LC in that the elution is promoted by the use of polar mobile phase. However, unlike classic normal-phase LC, where the water to the mobile phase has to be kept to minimal but constant levels, water is present in a significant amount (>5%). HILIC also uses water-miscible polar organic solvents such as acetonitrile instead of water-immiscible solvents like hexane and chloroform. LC-MS/MS using HILIC on silica columns has been extensively studied and reviewed [30, 31].

On-Line Extraction and Chromatography On-line extraction is another frequently used technology. With the improvement in instrumentation and column technology [32], on-line sample extraction that is coupled into LC-MS/MS has developed as an important tool in the past few years. In general, this technique employs turbulent flow chromatography, in which extraction columns are packed with retaining particles of large size (e.g., 50 μm) on a commercially available device or on a regular homemade column switch device. Biological samples are injected directly onto the extraction column, usually after dilution with an aqueous buffer. The analytes are then washed either forward or backward onto the analytical column for further chromatographic separation prior to the MS/MS detection. Common on-line extraction systems use typical reversed-phase columns for both the extraction and analysis. In our laboratory, we have successfully employed a weak-cation extraction (WCX) column and HILIC analytical column to analyze an extremely polar quaternary amine compound. This compound has no retention at all on a reversed-phase column but has excellent retention and peak shape on a HILIC column. The analyte in plasma was loaded onto a WCX column with an aqueous mobile phase and the WCX column was then washed with acetonitrile to remove endogenous compounds. The analyte was then eluted off the WCX column,

using acidified acetonitrile, onto the HILIC analytical column for a gradient elution.

Analyte Adsorption Issue Loss of analytes during the collection of incurred samples, preparation of quality controls, storage, and analysis due to adsorption to the container should also be considered. Compound adsorption to storage containers often happens in sensitive LC-MS/MS assays, which causes nonlinear response and loss of sensitivity. The use of a zwitterionic detergent such as 3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) as an additive in urine can prevent some analytes from adhering to surfaces during sample collection, storage, and preparation [33]. Protein binding of the drug candidate could also be significantly different for the quality control samples and incurred samples. If the analyte was not released from the protein prior to the extraction, quantitation bias could occur, depending on the extraction method. Xue et al. [34] observed the same issue with a liquid/liquid extraction method. For the plasma extraction method, two incubation steps were required after the addition of 5 mM ammonium acetate and the internal standard (stable isotope labeled) in acetonitrile to release the analyte bound to proteins prior to LLE with toluene.

4.1.8 Method Automation Strategy: From Preclinical to Clinical

As the candidates progress well, method development and validation for supporting human clinical trials will start well before the drugs enter the clinic. A well-established automated process, wherever possible, should be used to replace manual tasks early on in the preclinical stage. Automation results in greater performance consistency over time and in more reliable methods.

Standardization in sample collection can significantly enhance bioanalytical productivity. Preclinical samples could be collected in 96-well format [35] or transferred to 96-well format from a pierceable capped tube fitted with a nonleaking resealable polymer septum [36]. Automated 96-well plate technology for sample preparation is well established and accepted and has been shown to effectively replace manual tasks. The 96-well instruments can execute automated off-line extraction and sample cleanups. Automated SPE [37–40], LLE [41–46], and PPT [47, 48] can be performed in 96-well format. Both packed cartridges and disks in 96-well SPE formats have been successfully used. Further efficiency improvements were made when the organic extracts from SPE [49, 50] or LLE [51, 52] were injected directly onto the silica column with low aqueous–high organic mobile phases. While the commonly used SPE or LLE solvents are stronger elution solvents (not compatible) than a mobile phase on typical reversed-phase chromatography, they are weaker elution solvents (compatible) on the silica column with low aqueous–high organic mobile phases operated under HILIC. In this approach, the time-consuming and error-prone solvent evaporation and reconstitution steps are eliminated.

4.1.9 Matrix Effects and Recovery

The influence of matrix effects on quantitative bioanalytical LC-MS/MS has been well recognized [53, 54]. The U.S. Food and Drug Administration's (FDA) *Guidance*

for Industry on Bioanalytical Method Validation requires the assessment of matrix effects during method validation for quantitative bioanalytical LC-MS/MS methods [55]. In a broad sense, matrix effects represent the impact of the matrix on the analysis of the analytes, including ion suppression/enhancement, differentiated recovery, and altered stability, and is certainly not just limited to quantitative bioanalytical LC-MS/MS. However, it has been generally accepted and proposed in the industry guidance that matrix effects for small molecule bioanalytical analysis is defined as the analyte ionization suppression or enhancement in the presence of matrix components that could originate from endogenous compounds such as phospholipids [56], metabolites, coadministered drugs, internal standards [57], dosing vehicles [58–60], mobile phase additives [61], and plastic tubes [62]. These effects are more pronounced with ESI than with APCI [63, 64]. There are several commonly used ways of measuring matrix effects. One approach is to compare the MS response of the analyte spiked postextraction with that in a neat solution [65–67]. Since there is no extraction involved, any signal loss or enhancement in the postextraction spiked sample will be assumed due to matrix effects. Another useful approach of assessing matrix effect is postcolumn infusion of an analyte into the MS detector. The extracted blank matrix is injected by an autosampler onto the analytical column [68–70]. The purpose of postcolumn infusion with the analyte is to raise the background level so that the matrix-induced suppression will show as negative peaks. An approach for assessing the lack of inconsistent matrix effect among individual samples is to measure the consistency for the results obtained from these types of experiments with samples from individual matrix lots (typically $n > 6$) [71–76].

Constanzer et al. [77] measured both absolute matrix effects by comparing the responses from postextraction spiked samples with those from neat solutions and relative matrix effects by measuring the consistency of the response factors from spiked matrix lots. Results from LC-MS/MS methods were compared to a validated non-MS method, such as LC-UV [78, 79], LC with fluorescence detector [80], enzyme-linked immunosorbent assay (ELISA) [81, 82], and fluorescence polarization assay [83], or to a different separation method such as GC [84]. Comparable results indicate lack of matrix effects or inconsistent matrix effects since this approach does not positively identify the magnitude of the matrix effects. Huang et al. [85] used LC-UV to detect the down-field matrix peaks that caused ion suppression for the analyte of interest and modified the chromatographic condition [85]. Recovery is determined by comparing the MS response of extracted samples with those spiked (postextraction) into a blank matrix. Because both samples have the matrix constituents present, the matrix effects can be considered the same for extracted samples and postextraction spiked samples. Any differences in responses can be considered as caused by extraction recovery.

4.1.10 Effect of Dosing Vehicles

In preclinical studies, dosing vehicles typically are used at high concentrations to dissolve the test compounds. These dosing vehicles, especially polymeric vehicles such as PEG 400 and Tween 80, can cause significant signal suppression for certain analytes when minimal sample cleanup is used. Table 4.2 shows a real-world example of the adverse effects of dosing vehicles. The results obtained using a fast gradient LC-MS/MS method with minimal retention are significantly lower than those

TABLE 4.2 Comparison of Results Obtained from the Same Set of IV Plasma Samples Containing PEG 400 When Two Different Gradient LC-MS/MS Methods Are Used

Time Postdose (h)	Results from the Extended Gradient Method (ng/mL)	Results from the Fast Gradient Method (ng/mL)
0.067	753	261
0.133	712	219
0.2	761	239
0.333	523	176
0.5	394	104
0.75	279	72.0
1	116	28.0
1.5	35.0	7.00
2	6.00	2.00

obtained from an extended gradient LC-MS/MS method. When these two sets of data were fitted into a noncompartment model, values for C_{max}, area under the curve (*AUC*), and clearance (*Cl*) were more than 300% different [59].

Effective means of minimizing this type of effect include better chromatographic separations, better sample cleanup, and alternative ionization methods. An easy way to check dosing vehicle effects is to fortify some quality control samples with the vehicle [86]. Significant differences in the measured values between fortified quality control samples and regular quality control samples indicate potential dosing vehicle effects.

4.1.11 Carry-over and Contamination

Carry-over should be differentiated from contamination. Carry-over is usually caused by liquid handlers for the sample preparation or autosamplers for the injection. Carry-over can be estimated and to some extent managed by prearranging the samples so that the consecutive samples are not impacted by the carry-over [87]. Nevertheless, carry-over should be evaluated during method development and validation and if possible reduced. Contamination could be systematic (such as contamination of a reagent with the analyte) or could be random (such as spillover of extraction solvents of two adjacent wells in a 96-well plate). Contamination is undesirable and is usually unmanageable. Care must be taken to minimize carry-over and contamination so that they will not negatively impact the assay results.

4.1.12 Misconception About Stable Isotope-Labeled Internal Standard

Use of stable isotope-labeled analyte as the internal standard has played a significant role in advancing quantitative bioanalytical LC-MS/MS methodology. Due to the high similarity of the physical and chemical characteristics of labeled and unlabeled analytes, almost identical sample extraction recovery and matrix effects could be expected. Potential loss of analyte during the sample preparation and signal suppression/enhancement due to matrix components can be effectively compensated for. However, the stable isotope-labeled internal standard will not compensate for analyte loss due to instability or adsorption to either container or proteins. Occasionally, stable isotope-labeled internal standards have been chro-

matographically separated from the analyte and have experienced different matrix effects [88].

Yang et al. [89] presented an interesting case study for diagnosis and troubleshooting of problems associated with strong analyte–protein binding. The method was validated using a stable isotope-labeled internal standard with a LLE method using hexane as the extraction solvent. However, upon repeat analysis of the same samples, the concentration values increased fivefold from the original value. The concentration increased with each additional freeze/thaw cycle. It was found that this drug candidate has a strong protein binding and hexane is not sufficient to release the drug candidate from the protein. Freeze/thaw cycles gradually denatured the protein and weakened the binding, resulting in increases in the free and extractable drug candidate concentration. A protein precipitation (PP) method was then used to inhibit the protein binding and to release the analyte. Consistent results were then obtained.

4.1.13 Troubleshooting Strategy

Successful method troubleshooting requires full understanding of all aspects of the bioanalytical method. For example, poor dynamic range can potentially be caused by many factors. A systematic investigation of the root cause would help resolve the issues. Table 4.3 provides a general investigational guide for the troubleshooting of poor linearity. Similar systematic investigations could be made to other important parameters for quantitative bioanalytical methods. One important parameter to

TABLE 4.3 Troubleshooting for Poor Linearity

Observation	Potential Root Cause
Response factors decrease at high end	Poor solubility? Correct reconstitution solvent and volume: analyte soluble in matrix? Saturation due to large curve range—at LC-MS/MS interface? At detector?
Response factors decrease at low end	Adsorption of analytes to container? Adsorption of analytes to 96-well plate? Loss of analytes in evaporation/reconstitution step? Adsorption of analyte to injector/tubing/column/source?
Abnormal high response at low end of the curve	Contamination? Carry-over?
Internal standard (ISTD) response increases with increased analyte concentration	Isotope effects or impurities from analyte? Adsorption of analytes and ISTD to the container?
ISTD response decreases with increased analyte concentration	Suppression from analyte to ISTD?
ISTD not tracking analyte	LC-MS matrix effect? Extraction recovery problem? Instability of analyte or ISTD in extract? Inadequate equilibrium for protein binding of ISTD?

review when performing troubleshooting, especially when troubleshooting a mismatch of calibration standards and quality controls, is the response factor. The response factor is the response ratio of the analyte versus internal standard normalized against the concentrations. Response factors should be consistent throughout the entire calibration range. Table 4.4 shows a troubleshooting example. At first glance, the accuracy results indicate that the two low levels of quality control samples (0.25 ng/mL and 0.75 ng/mL) are biased high based on the calculated accuracy results. However, a further review of the response factors shows that these two

TABLE 4.4 Troubleshooting for Poor Accuracy of Quality Control (QC) Samples[a]

Sample Name	Calculated Concentration (ng/mL)	Response Factor	Accuracy (%)	Sample Name	Calculated Concentration (ng/mL)	Response Factor	Accuracy (%)
CAL-200	205	0.0163	102.6	QC-0.25	0.298	0.01607	119.2
CAL-100	109	0.0173	109.3	QC-0.25	0.298	0.01611	119.4
CAL-50	47.7	0.0151	95.32	QC-0.25	0.296	0.01597	118.5
CAL-20	20	0.0158	100.1	QC-0.25	0.299	0.01617	119.8
CAL-10	10.3	0.0162	102.6	QC-0.25	0.297	0.01601	118.8
CAL-5	4.92	0.0155	98.35	QC-0.25	0.297	0.01601	118.8
CAL-2	2.03	0.0157	101.3	QC-0.75	0.883	0.01774	117.8
CAL-0.5	0.54	0.0157	107.9	QC-0.75	0.902	0.01813	120.3
CAL-0.25	0.241	0.0125	96.49	QC-0.75	0.86	0.01724	114.6
CAL-0.25	0.246	0.0128	98.54	QC-0.75	0.865	0.01734	115.3
CAL-0.5	0.518	0.0150	103.6	QC-0.75	0.878	0.01762	117
CAL-2	1.9	0.0147	95.15	QC-0.75	0.874	0.01754	116.5
CAL-5	4.72	0.0148	94.33	QC-160	166	0.01641	103.5
CAL-10	9.8	0.0155	98.01	QC-160	174	0.01724	108.7
CAL-20	19.8	0.0157	99.13	QC-160	166	0.01650	104
CAL-50	48.8	0.0155	97.64	QC-160	168	0.01666	105.1
CAL-100	97.9	0.0155	97.85	QC-160	167	0.01657	104.5
CAL-200	203	0.0161	101.7	QC-160	170	0.01680	106
Mean		0.01532	100	QC-75	80.6	0.01704	107.5
SD		0.001147	4.13	QC-75	78.7	0.01664	104.9
CV%		7.49	4.13	QC-75	81.1	0.01715	108.2
				QC-75	78.8	0.01665	105
				QC-75	79.2	0.01674	105.6
				QC-8	8.91	0.01757	111.3
				QC-8	8.67	0.01710	108.3
				QC-8	8.7	0.01717	108.8
				QC-8	8.44	0.01665	105.5
				QC-8	8.73	0.01723	109.2
				QC-8	8.8	0.01736	110
				Mean		0.016878	111.3
				SD		0.000576	5.93
				CV%		3.42	5.33

[a]Pay special attention to the lower response factors but good accuracy for CAL-0.25. QC samples have consistent response factors over the entire concentration range but poor accuracy caused by the lower response factor of CAL-0.25. Because this is $1/x^2$ weighted linear regression, the lowest standard (CAL-0.25) has the most significant weighting for the calculation of QC concentrations.

levels of quality control samples have the same response factors as the other levels of quality controls. It also reveals that the response factors dropped for the calibration standards at lower concentrations even though the calculated accuracy for these standards is excellent. Further investigation revealed that the compound in the spiking solution used to prepare calibration standards was impacted by adsorption onto the container. This simple example clearly demonstrates the importance of reviewing the response factors rather than only relying on the calculated accuracy for the troubleshooting.

4.1.14 Conclusion

Bioanalytical support for preclinical studies is complicated. Hence, bioanalytical scientists need to fully understand both the underlying sciences and regulatory requirements. They should always be aware of the potential pitfalls that could occur at any stage of the study. There are a number of parameters requiring evaluation during method development and validation. The method validation strategy should be tailored for the intended use.

4.2 PRECLINICAL BIOANALYTICAL SUPPORT USING LIGAND-BINDING ASSAY

4.2.1 Introduction

Much of the information discussed in Section 4.1 for the bioanalysis of small molecules may also apply to the bioanalysis of large molecule therapeutics as well. However, there are some unique challenges associated with quantification of large molecule therapeutics. These are due mainly to the nature of the molecules and limitations of the technology (e.g., ligand-binding assay (LBA)), used for bioanalysis.

Recombinant proteins and humanized monoclonal antibodies comprise an ever increasing larger proportion of protein therapeutics and, as a result, have posed new challenges and special needs. These molecules may not survive the typical sample handling conditions of the chromatographic assays. Moreover, these drugs are highly potent. Consequently, their blood concentration levels tend to be very low. Hence, the method of quantification for these entities must be very gentle, specific, and highly sensitive. Ligand-binding assays such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are the techniques of choice for quantification of large molecule drugs in biological samples [90, 91].

In addition, due to their high molecular weight, there is always the possibility that large molecule therapeutics may elicit an immune response directed against the drug [92]. A small change in protein structure, even though it may comprise a very small proportion of the total protein, can induce a large immunogenic response. Therefore, regulatory agencies require immunogenicity evaluation for large molecule therapeutics even in the preclinical stage [93]. Consequently, there is an increasing need to develop novel assays that can detect and characterize the anti-drug antibodies (ADA) or immunogenicity of large molecule therapeutics. To accomplish this, the laboratory has to rely on several bioanalytical techniques and technologies to measure these changes including LBA.

Like any other bioanalytical method, the intended use of the LBA helps to determine the course of development and validation of the assay. Ligand-binding assays are used for (1) the quantitation of therapeutic entities to support the toxicokinetic / pharmacokinetic studies [91, 94] and (2) monitoring the level of surrogate biomarkers to support pharmacodynamic evaluation [95]. On the other hand, qualitative or quasiquantitative assays are generally used for the determination of anti-drug antibodies [92, 96]. The LBA developed for the bioanalytical support should not only be robust, reliable, and reproducible but should also be GLP compliant from a regulatory perspective. It can then be used for production, toxicology, and Phase I though IV testing. In view of the small sample volumes available in typical preclinical studies, it is even more compelling to design and develop a robust assay to reduce sample reanalysis.

In this section we provide a brief account of the ligand-binding assay technology, specific challenges associated with the GLP-compliant method development and validation of quantitative assays, and current industry and regulatory perspectives. Method development and validation for detection of anti-drug antibodies [92, 96] or quantification of biomarkers [95] may require slightly different strategies.

4.2.2 Ligand-Binding Assay Technology

A typical ligand-binding assay utilizes an analyte-specific binder (e.g., antibody, binding protein, drug, or receptor) to capture the analyte of interest. The captured analyte is detected by the "detector molecule," which is generally an antibody labeled with a radioisotope (e.g., ^{125}I), an enzyme (e.g., horseradish peroxidase, alkaline phosphatase), or another label (e.g., biotin, avidin). ELISA, the most commonly used ligand-binding assay, generally uses a detector molecule that is labeled with an enzyme. The extent of bound enzyme activity is measured by the changes in color intensity of the substrate solution. The color intensity is directly proportional to the concentration of analyte captured on the microtiter plate (see Fig. 4.3). A detailed description of the ligand-binding assay technology is beyond the scope of this chapter. Several books that provide in-depth information on ligand-binding assays, including ELISA, are included in the references [97, 98].

4.2.3 Using Ligand-Binding Assay in a GLP Environment

To support preclinical studies, an analyte-specific ligand-binding assay must be validated to conform to GLP regulations. In a GLP environment, like any other bioanalytical method, an LBA has a three-phase life cycle: (1) method development, (2) prestudy validation, and (3) in-study validation. Table 4.5 summarizes the assay assessment parameters that are addressed at various stages of the method life cycle. During method development, emphasis is focused on designing a robust and analyte-specific method for the intended use. This requires advanced planning and a careful consideration of (1) assay design and format, (2) critical reagent selection, (3) critical reagent stability evaluation, (4) matrix selection, (5) sample collection conditions, and (6) optimization of assay condition to achieve desired performance characteristics. Once a robust method has been developed, the prestudy validation becomes uncomplicated. The assay characteristics, including precision and accuracy, limits of quantification, and analyte stability, are formally confirmed during the

TABLE 4.5 Summary of Method Validation Assessment Parameters Over the Method Life Cycle

Performance Parameters	Development	Prestudy Validation	*In-Study Validation*
Critical reagents	Identify and procure	Apply	Apply
Assay format / batch size	Establish	Apply	Apply
Matrix of calibrators and controls	Establish	Confirm	Apply
Minimal required dilution	Establish	Confirm	Apply
Analyte stability	Initiate	Establish	Ongoing assessment
Specificity	Establish	Apply	Apply
Selectivity	Evaluate	Confirm	Apply
Calibration curve fitting algorithm	Establish	Confirm	Apply
LLOQ and ULOQ	Evaluate	Establish	Apply
Precision and accuracy	Evaluate (CV and RE)	Establish (CV and RE)	Apply (TE)
Run acceptance QC (low, medium, high)	Evaluate	Establish	Apply
Dilutional linearity	Establish	Confirm	Apply—confirm if extended
Batch size	Evaluate	Establish	Apply—confirm if extended
Robustness/ ruggedness	Evaluate	Establish	Monitor
Parallelism	Evaluate where possible	Evaluate where possible	Establish with incurred samples
Run acceptance criteria	N/A	Runs accepted based on calibration curve acceptance criteria	Runs accepted based on acceptable calibration curve and QC samples following 4-6-X rule

prestudy validation phase. These characteristics are monitored during the in-study validation phase in which study samples are analyzed [91, 94].

4.2.4 Ligand-Binding Assay Specific Challenges

The distinguishing characteristics of ligand-binding assays that separate them from chromatographic (e.g., LC-MS/MS) assays are summarized as follows.

1. The design of a sandwich ligand-binding assay takes advantage of the ability of an antibody to specifically bind to the analyte (drug). The amount of analyte

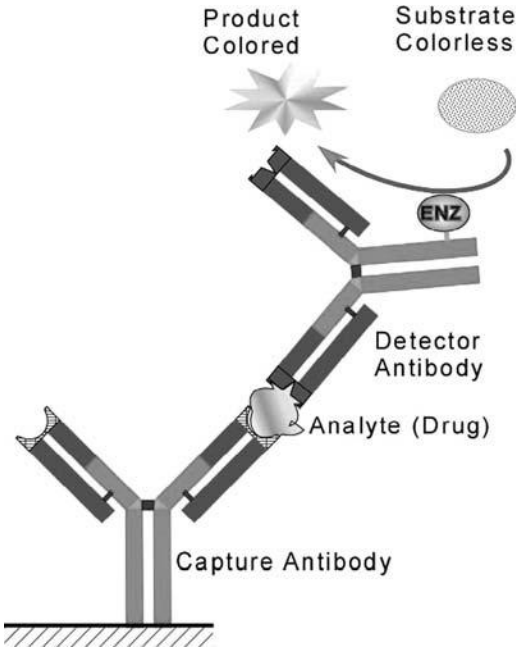

FIGURE 4.3 A sandwich enzyme linked immunosorbent assay (ELISA) with colorimetric endpoint.

is measured indirectly by the binding of a detector antibody conjugated to an enzyme or any other labeling molecule (Fig. 4.3). In a chromatographic assay the analyte is physically separated into distinct peaks. The area under the peak is precisely measured and is directly proportional to the analyte concentration. Due to the indirect measurements in LBA, the results are somewhat less precise, as reflected by coefficient of variation ($CV \leq 20\%$), than in chromatographic assays ($CV \leq 10\%$).

2. The biological samples are generally analyzed without pretreatment in a LBA, as opposed to the process of extracting analyte from samples prior to the chromatographic assay. Consequently, numerous matrix components including binding proteins, drugs, degrading enzymes, heterophilic antibodies, and anti-animal antibodies may have negative or positive interference in the assay performance.
3. Due to the limited analyte-binding capacity of the binder molecule (e.g., capture antibody), the typical calibration curves in these assays are nonlinear, as opposed to the linear curves in chromatographic assays. Consequently, the working range of quantification in an LBA is narrower than in the linear curves of chromatographic assays (Fig. 4.4). In preclinical studies, relatively high doses of drugs are administered. The resulting high concentration of drug in the study samples may require sample dilutions that could be several thousandfold.

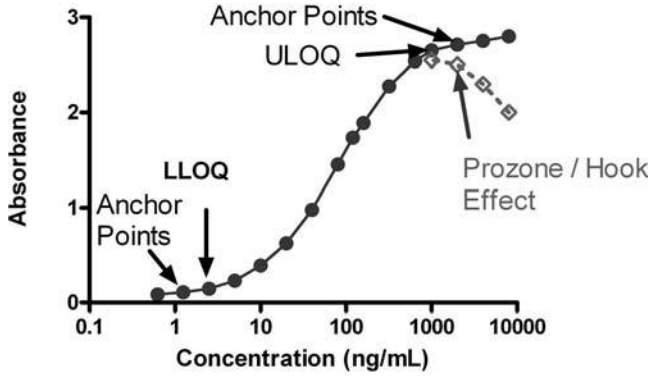

FIGURE 4.4 Prozone or Hook effect Illustration. A typical sigmoid calibration curve for a sandwich ELISA is shown with "anchor points" at both ends of the curve bracketing the calibrators at the LLOQ and ULOQ levels. A high dose Prozone or Hook effect is illustrated by suppression of signal (absorbance) with analyte at concentrations far above the ULOQ level.

4.2.5 Responding to the Challenge of Ligand-Binding Assay

For all assays, the key factor is the accuracy of the reported results [15]. In view of the above characteristics, besides method reproducibility considerations, one must pay special attention to establishing the method **(1)** specificity, **(2)** selectivity, **(3)** minimal required dilution, **(4)** dilutional linearity, and **(5)** analyte (drug) stability in the biological matrix [94]. Evaluation and validation of the method repeatability, precision, and accuracy for the LBA is described in detail elsewhere [91, 94].

Method Specificity Demonstration of method specificity for the LBA could be a challenging task. The LBA is generally developed during the discovery phase when specific antibodies are raised against the drug molecule and are characterized. These antibodies are used to develop the initial ELISA method. Later, the assay is used to support preclinical and clinical studies. The specificity of the method is based mainly on the ability of these antibodies to specifically interact with the drug molecule as opposed to interacting with nondrug (i.e., cross-reactant) molecules.

Antibody specificity is generally evaluated by determining the cross-reactivity of the antibody with nondrug molecules, including coadministered drugs, drug metabolites, or molecules that are structurally related to the drug. To evaluate the antibody specificity, dose–response curves are generated using drugs of interest and potential cross-reactive compounds at significantly higher concentrations than the drug itself [97, 98]. The extent of acceptable cross-reactivity may depend on the relative concentration of the potential cross-reactant present in the study sample. An antibody with low cross-reactivity (e.g., <1%) with a potential cross-reactant may not be suitable if the concentration of the cross-reactant in the sample is at a very high concentration relative to the analyte (e.g., 1000-fold). In such cases, a sample cleanup method may be required to remove the cross-reactants prior to sample analysis. Similarly, an antibody with 20% cross-reactivity with a potential contaminant may be acceptable for use if the analyte itself is expected to be in great excess.

Method Selectivity In a typical LBA, the samples are analyzed without pretreatment. Numerous matrix components that are present in the sample may affect the assay performance. The ability of the method to selectively quantify the analyte of interest in the presence of a variety of matrix components defines the method selectivity [15, 91, 94]. Method selectivity evaluation and validation is one of the most crucial and demanding tasks in establishing the LBA. During method development, at least 10 individual matrix samples from 10 normal animals or subjects (from normal or patient population) are assayed unspiked and spiked with the drug at a concentration near the lower end of the calibration curve. Acceptable recovery of drug (usually within 20% of the nominal concentration) in at least 80% of the samples is considered an indication that the method is selective. At this stage of assay development (where blank matrix is not available), the calibration curve may be prepared in the assay diluent. The results of the initial selectivity screening of matrix samples may be used to identify matrix samples that can be pooled to form a blank matrix pool. This pool can then be used for preparing the calibrators and the validation or QC samples [91, 94]. Method selectivity is confirmed during the prestudy validation, where both calibrators and control samples are spiked in the pooled biological matrix. In these experiments the selectivity is evaluated by assaying at least 10 individual matrix samples unspiked and spiked with the analyte near the lower limit of quantification (LLOQ).

Minimal Required Dilution of Sample In assays where a strong matrix effect is observed, it may be necessary to determine the "minimal required dilution" (MRD) of the sample, that is, the dilution at which matrix interference is minimized to an acceptable level. Since the reportable concentration of analyte is the product of the measured concentration multiplied by the dilution factor, the practical limit of quantification will be greatly affected by the MRD of sample [94]. Hence, the target should be to develop a relatively more sensitive (with lower LLOQ) method where the MRD is higher. A method with smallest MRD that has acceptable interference and sensitivity for the intended use would be ideal.

Dilutional Linearity Due to the narrow range of quantification in ligand-binding assays and the possible high concentration of analyte of interest in the sample (especially in preclinical studies), a number of samples may require dilution prior to analysis. Therefore, to avoid running highly concentrated "dilution controls" with each assay during sample analysis, dilutional linearity of sample at a wide range of dilutions is demonstrated during prestudy method validation. It should be noted that the final content of biological matrix should be identical to that in the calibration curve and/or QC samples. Therefore, dilutional linearity should be established subsequent to "method selectivity" evaluation, MRD establishment, and preparation of a blank matrix pool.

In some cases, it is observed that high concentration of analyte may suppress the signal, resulting in underestimation of analyte [99]. This phenomenon is known as the "Prozone" or "Hook" effect. A typical illustration of the effect is depicted in Fig. 4.4. Dilutional linearity evaluation may help in identifying this effect, especially for assays used to support preclinical studies. If this effect goes undetected, it may result in a gross underestimation of drug concentration in the samples where high concentrations of analyte are expected. A typical example is illustrated in Table 4.6

TABLE 4.6 Demonstration of Hook Effect and Dilutional Linearity[a]

Dilution (Fold)	Measured Concentration (pg/mL)[b]	Computed Concentration (pg/mL)	
Neat	231	231	Hook or Prozone effect
2	365	729	
4	674	2,698	
80	>ULOQ	Not computed	
160	>ULOQ	Not computed	
200	>ULOQ	Not computed	
400	755	302,078	Dilutional linearity[c]
800	369	294,798	
1,600	178	285,226	
3,200	93	297,229	
6,400	50	322,447	
12,800	26	335,578	
25,600	<LLOQ	Not computed	

[a]Data from this table is visually depicted in Fig. 4.5.
[b]Method ULOQ = 25 pg/mL; ULOQ = 1000 pg/mL.
[c]Average concentration (pg/mL) = 306,226 with 6.2% CV.

and Fig. 4.5. A sample from an escalating dose study, where high concentration of drug is expected, may be assayed "neat" (without dilution), giving a low measured concentration. This anomalous observation may call for an investigation. In trouble-shooting experiments, the sample may be re-assayed at a wide range of dilutions. In the illustration, it is observed that with increasing dilutions the measured concentration increased, reaching the ULOQ of the assay. Moreover, subsequent dilutions from 400-fold to 128,000-fold showed dilutional linearity. Computed concentrations, measured in the dilutional linearity range, provided a realistic estimate of the concentration of drug in samples. This scenario clearly illustrates the importance of Prozone or Hook effect evaluation and establishment of the dilutional linearity in an LBA.

Parallelism is another assay parameter that is conceptually similar to the dilutional linearity. It is evaluated when incurred samples become available. Plotting the responses generated by the diluted incurred samples versus the calibration curve gives a visual impression of parallelism. Various approaches to parallelism data have been described in the literature [95, 100]. A test of parallelism demonstrates that the analyte present in the sample is structurally similar to that used for generation of the calibration curve.

Analyte Stability in Biological Matrix Biological matrices may vary from each other with respect to their chemical constituents including proteins and enzymes. Proteolytic enzyme composition may have a distinct effect on the stability of a drug in a biological matrix. Rat or mouse serum or plasma samples should be treated with special care. It has been our experience that an analyte that may be stable in rabbit or monkey serum may very well be highly unstable in rat or mouse serum. Therefore, in such cases, it may be necessary to collect the study samples in the

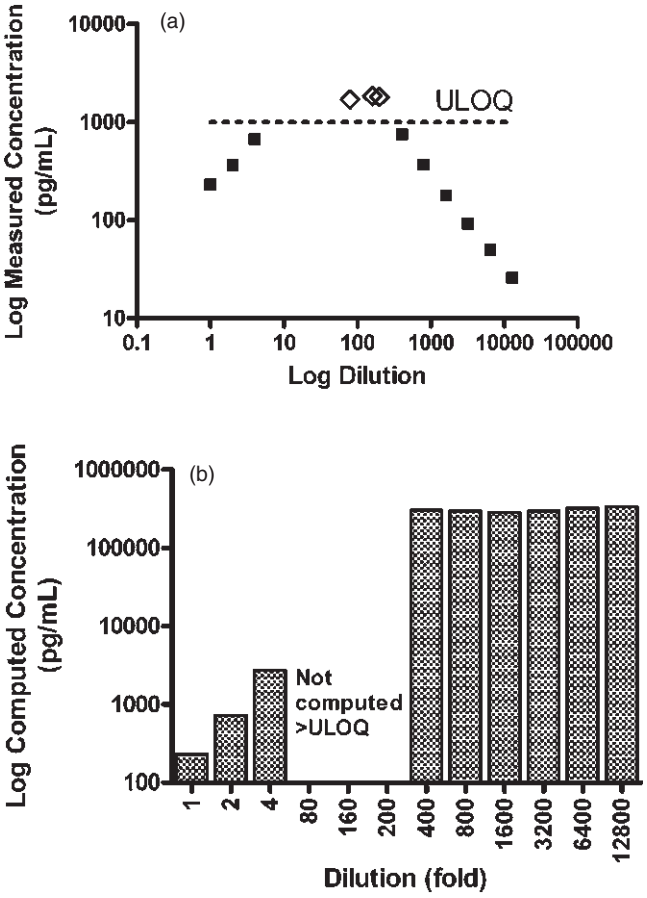

FIGURE 4.5 Illustration of the significance of dilutional linearity evaluation, especially in an assay where Prozone or Hook effect is present. (a) Initially with increasing dilutions the measured concentration in a sample increased, reaching or exceeding the ULOQ of the assay, demonstrating the suppression of response with high concentration of analyte. Subsequently, six dilutions showed a linear decrease in measured concentration with increasing dilutions of the sample. (b) The computed concentrations corrected for the dilution factor from 400-fold to 128,000-fold demonstrated dilutional linearity by showing that computed concentration is independent of the dilution factor in this range.

presence of a cocktail of proteolytic enzyme inhibitors and/or keep the samples on ice during analysis.

4.2.6 Using Commercial Kit Based Assays

Often commercial kits are used for quantification of drugs that are homologs of endogenous compounds or for the measurement of biomarkers in biological samples. Kits are generally designed for use in clinical or research lab environments. In order to adopt commercial kits for GLP-compliant bioanalytical work, one must redesign the composition and format of the control samples that are used to monitor the assay performance and to make assay acceptance decisions. It is of the utmost

importance that a quality control strategy is designed in advance to evaluate and monitor the lot-to-lot variability of kits. Several multiplexed assay kits are also available commercially. Adaptation and validation of these kits for quantification of analytes of interest in biological matrices could be challenging and therefore should be thoroughly explored prior to sample analysis.

4.2.7 Industry and Regulatory Perspective

Historically, the focus of regulatory agencies has been on the chromatographic techniques for the quantification of small molecular weight drug entities that constituted the major proportion of drug development portfolios in the pharmaceutical industry. The first conference on bioanalytical method validation (commonly known as the Crystal City I conference), that was held in Crystal City, Virginia in 1990, addressed in depth the validation of chromatographic assays but paid little attention to nonchromatographic methods including immunoassays [101]. The search for LBA-specific guidance was started soon after the 1990 Crystal City conference. In 1998, one of the authors of this chapter (MK) organized a roundtable at the AAPS annual meeting in San Francisco. This was, perhaps, the first time that LBA-specific challenges were discussed on a public platform. This led to the publication of a white paper giving a comprehensive account of the pharmaceutical industry perspective on the validation of immunoassays for bioanalysis [91]. Soon after this publication in 2000, a historic workshop on bioanalytical method validation for macromolecules was sponsored by the AAPS and the FDA (1–3 March 2000). This workshop, for the first time, addressed the validation of a variety of methodologies for assays of large molecule therapeutics [102]. However, the scope of the workshop was too broad to reach a consensus. In May 2001, the FDA *Guidance for Industry: Bioanalytical Methods Validation* was issued [15]. It was thought that a focused approach was needed to address the special challenges associated with LBAs for large molecule therapeutics. Consequently, a subcommittee of the then newly formed Ligand Binding Assay Bioanalytical Focus Group (LBABFG) at the American Association of Pharmaceutical Scientists (AAPS) was set out to critically review the workshop summary report, evaluate exceptions to criteria in the guidance, and produce a consensus document. The LBABFG white paper was published in 2003 [94]. Around the same time, the AAPS sponsored a workshop (12–13 May 2003) mainly to address the "how to" of the validation of methods used for PK analysis of macromolecules [103]. Over the last four years, LBABFG white paper has provided valuable direction to LBA scientists working in regulatory compliant environments. In May 2006, the 3rd AAPS/FDA Bioanalytical Method Validation Workshop was held. With some changes, this workshop essentially endorsed the LBA method performance acceptance criteria proposed in the LBABFG white paper (Table 4.7). In the workshop, the recommended run acceptance rule of "4-6-30" (i.e., a minimum of 4 out of 6 QC samples are within 30% of their nominal values) has been changed to a "4-6-20" rule as stipulated in the conference report [19].

4.2.8 Conclusions

Ligand-binding assays are the technique of choice for bioanalytical support for preclinical and clinical studies for large molecule therapeutics. In view of the small sample volumes and high drug concentrations that are typically available in preclinical studies, one should design and develop a robust assay where reliable and repro-

TABLE 4.7 Comparison of LBA Specific Acceptance Criteria as Recommended by the LBABFG White Paper and AAPS/FDA 3rd Conference Report

Performance Characteristic	LBABFG White Paper	AAPS/FDA 3rd Conference Report	Recommended Changes in Conference Report
Validation Sample %RE	±20% ±25% at the LLOQ	±20% ±25% at the LLOQ and ULOQ	±25% RE extended to ULOQ level as well
Validation Sample %CV	±20% ±25% at the LLOQ	±20% ±25% at the LLOQ and ULOQ	±25% CV extended to ULOQ level as well
Validation Sample %TE	±30% ±40 at the LLOQ	±30% ±40% at the LLOQ and ULOQ	±40% TE extended to ULOQ level as well
In-study standards (excluding anchor points)	≥75% of standard points are ±20% RE (±25% RE at the LLOQ); at least six valid standard points must remain within limits	≥75% of standard points are ±20% RE (±25% RE at the LLOQ and ULOQ)	±25% RE extended to standard points at ULOQ level as well; number of acceptable points in a standard curve not specified
In-study quality controls	4-6-30[a] rule; at least 50% of QCs are valid at each level	4-6-20[a] rule; at least 50% of QCs are valid at each level; three levels analyzed in duplicate (six results)	Major changes: (1) QC acceptance limit is reduced from 30% RE to 20% RE; (2) three levels of QC samples in duplicate specified

[a]Exceptions allowed with justification.

ducible results are obtained and sample reanalysis is minimized. Moreover, the method specificity, selectivity, dilutional linearity, and reproducibility must be established and validated upfront to avoid potential problems that may be encountered during sample analysis. Method validation performance acceptance criteria for precision of measurements for LBA assays are much wider compared to that of chromatographic assays. Current white papers and workshop reports provide adequate recommendations for development, pre-study and in-study validation, and implementation of these assays to support regulatory compliant studies.

This chapter provides an overview of the current advance and trends in the bioanalytical area to support preclinical studies. It covers both conventional small molecule drugs and macromolecular BIOTEC therapeutic entities.

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5

ANALYTICAL CHEMISTRY METHODS: DEVELOPMENTS AND VALIDATION

IZET M. KAPETANOVIC[1] AND ALEXANDER V. LYUBIMOV[2]

[1]*NIH NCI Division of Cancer Prevention, Chemoprevention Agent Development Research Group, Bethesda, Maryland*

[2]*University of Illinois at Chicago, Chicago, Illinois*

Contents

Preclinical Development Handbook: ADME and Biopharmaceutical Properties, edited by Shayne Cox Gad

5.1 INTRODUCTION

5.1.1 Background

Analytical methodology plays an integral part of drug development from the initial synthesis and manufacture through clinical trials and postmarketing monitoring. In new drug applications (NDAs), abbreviated new drug applications (ANDAs), biologics license applications (BLAs), or product license applications (PLAs), data must be submitted to establish that the analytical procedures used in testing meet proper FDA and ICH guidelines of accuracy and reliability. At the time of submission, the NDA, ANDA, BLA, or PLA should contain method validation information to support the adequacy of the analytical procedures. All analytical methods should be validated (to demonstrate that the analytical procedures are suitable for their intended use) prior to their use. The suitability of a compendial analytical procedure must be verified under the actual condition of use, but full validation is not required. As in all other preclinical pharmacology/toxicology studies, analytical assays should be conducted according to the FDA's Good Laboratory Practices (GLPs) (21 CFR part 58). Selective and sensitive validated analytical methods for the quantitative evaluation of drugs are critical for the successful conduct of preclinical safety and efficacy studies.

5.1.2 Purpose

Analytical methods are required for determination of the following:

- Active pharmaceutical ingredient (API)—manufacturing control, stability testing, shelf-life forecast.
- Synthetic contaminants, degradation products—manufacturing control, stability testing, shelf-life forecast.
- Pharmaceutical excipients (binders, disintegrants, lubricants, antioxidants and preservatives, suspending and dispersing agents, natural or artificial flavorings and colorings, coatings, etc.)—manufacturing control, stability testing. The *United States Pharmacopeia and the National Formulary* (USP-NF) contains information on the identity, strength, quality, and purity for more than 250 excipients including official test methods in analytical testing.
- Drug and metabolites in biological fluids and tissues—bioavailability, pharmacokinetics.

It is important to have validated analytical methods from the start and throughout the drug development process. The generic approach to method development involves three basic steps: sample preparation (clean up), separation, and detection. A schematic flow diagram depicting the most commonly used options is shown in Fig. 5.1. While general approaches to method development are similar, complexity and requirements of time and resources vary. For example, the *United States Pharmacopeia-National Formulary* (USP-NF) provides standard methods for pharmaceutical excipients, obviating a need for analytical method development, and will not be specifically addressed here. On the other hand, analysis in biological fluids and tissues is the most time and labor intensive due to complexity and variability

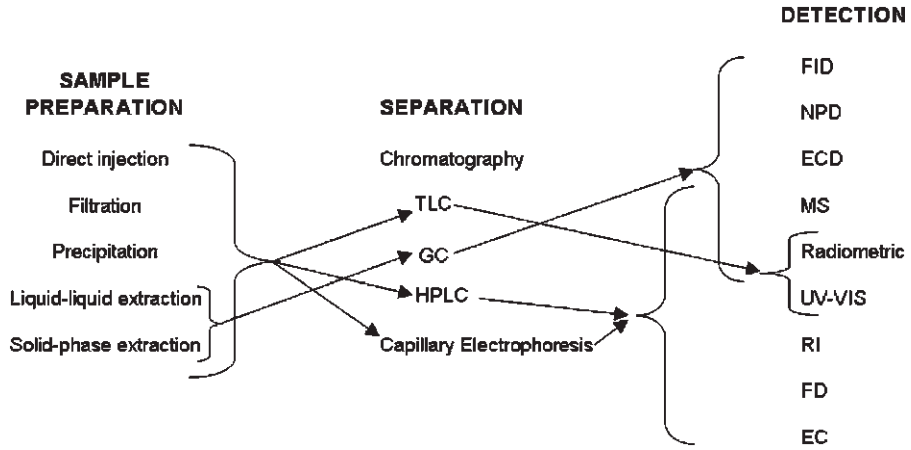

FIGURE 5.1 General scheme for developing an analytical method based on separation techniques. Most commonly used approaches are depicted.

of the biological matrices and the relatively low levels of analytes. Selection of appropriate sample preparation, separation, and detection approaches depends on the nature of the sample, the nature of the sample matrix, the concentration of analyte(s) in the matrix, the laboratory levels of skill and available resources, and so on.

5.2 ANALYTICAL METHOD DEVELOPMENT

5.2.1 Sampling and Handling

The proper sampling and handling of samples is one of the most important factors in a high quality analysis. The reader is referred to an excellent resource, Chapter 7 in *Quality Assurance Principles for Analytical Laboratories* [1], for details of sampling and handling. The main principles are described below.

There are three basic activities involved in solving an analytical problem: (1) collection and handling of the appropriate sample, (2) preparation of sample for analysis, and (3) analysis using an appropriate method.

These activities are independent of each other, but one significantly influences the others. Failure to observe standard procedures for sample collection, handling, and documentation often impairs the intended purpose of the analysis itself. In many cases, the collection of samples falls outside a laboratory's control. However, the laboratory must do all it can to receive appropriate, applicable, and defensible samples.

Key points for sampling are the following:

- The laboratory shall have a sampling plan and procedures for sampling when it carries out sampling of substances, materials, or products for subsequent testing or calibration.

- Sampling plans shall, whenever reasonable, be based on appropriate statistical methods.
- Sampling procedures shall describe the selection, sampling plan, withdrawal, and preparation of a sample or samples from a substance, material, or product to yield the required information.
- The laboratory shall have procedures for recording relevant data and operations relating to sampling.

It is extremely important to perform accurate subsampling (taking the appropriate test portion for analysis) in the laboratory. The number of samples to be analyzed in a given situation is limited by the resources available for the collection of the samples or for their analysis. Analyses of multiple samples always are preferred over single samples since single samples give no information on the homogeneity of the lot that was sampled. In addition, for single samples, the sampling error is also confounded with the analytical error. It is a common practice to collect several samples for test substance and dosage formulation analyses to assess homogeneity. For example, at least three samples from the top, middle, and bottom portions of the dietary admixture or dosage formulation should be collected and usually each portion is analyzed in triplicate to assess homogeneity. However, for a bioanalytical analysis, a single sample is usually analyzed because of limited volume of sample and high cost of each analysis. Each sample should be prepared in a way that it achieves homogeneity and should be handled in a manner that prevents alteration from the original composition. Even seemingly homogeneous materials such as liquids may be subject to sedimentation or stratification.

Shipment or delivery of the sample should be done under appropriate conditions (e.g., temperature) in a proper container package and with a proper label and should be accompanied by meaningful documentation, including Material Safety Data Sheet (MSDS) and instructions for sample storage upon delivery. The documentation consists of a chain-of-custody or similarly named document that accompanies the sample as it moves to and through the laboratory. A dependable record of sample handling is important. The sample shall be accepted by a designated person (a custodian in laboratories of appreciable size) who documents the action by completing a sample accountability record. This document will contain the sample number, the name of the product, and the date received; will indicate who received it; will describe the method of shipment or delivery, the packages received, and their condition; and will provide space for recording various storage locations before and after analysis. Deliveries of the sample, or parts of the sample, to the analyst, and its return, shall also be recorded on this form, as shall the signed statement concerning the final disposition of the reserve sample. A two-part form can be used for this purpose. One copy remains with the custodian and the other moves with the sample through the laboratory and is used by the supervisor for sample management purposes. Some laboratories use a Sample Receiving Log book for sample control. The information entered in the Log book is essentially the same as described for the two-part form.

For proper handling of the test samples and calibration items, the laboratory shall have written procedures for their transportation, receipt, handling, protection, storage, retention, and/or disposal. The laboratory shall have a system for identifying

test and/or calibration items. The laboratory shall also have documented procedures and appropriate facilities to avoid deterioration, damage, loss, or cross-contamination of any test item or sample during storage and handling. All necessary environmental conditions, including special security arrangements for sample integrity as needed for some samples, shall be established, maintained, monitored, and recorded.

5.2.2 Sample Preparation

Sample preparation is a very important first and often critical step in the analytical method, especially when biological samples are involved. Typically, it is the most difficult and time-consuming step. The purpose of sample preparation is to isolate analytes of interest from interfering sample components, concentrate the analytes, and dissolve them in a suitable solvent for subsequent separation and detection. Sample preparation significantly impacts on the recovery, selectivity, sensitivity, reproducibility, and ruggedness of the analytical method. Sample cleanliness or, more often, a lack thereof may have a direct effect (e.g., full or partial chromatographic peak overlap) on detection and quantification of analytes of interest, but also an indirect effect (e.g., contamination of analytical column or mass spectrometry (MS) ion source, ionization suppression in MS). Not only can some of the interferences lead to a loss in sensitivity (e.g., ionization suppression in MS) [2], but this effect may be variable depending on individual samples [3]. However, in some instances, the coextracted materials may be helpful. For example, coextractants can neutralize active sites on glassware, injector, or column and thereby minimize adsorptive effects. Therefore, coextractants may have a positive or negative effect and should be considered during analytical method development. Consequently, it is strongly advisable that all comparisons of extracted biological samples be made to similar extracts of spiked blank biological matrix instead of neat solutions.

Commonly encountered sample problems include (1) lack of compatibility with chromatographic system (e.g., tendency to clog or degrade the analytical column), (2) being too dirty (presence of coextracted materials that interfere with separation or detection of analytes), and (3) being too dilute (sensitivity issue). More common approaches to sample preparation will be discussed later, while the less common ones (turbulent flow chromatography, monolithic chromatography, immunoaffinity, etc.) will not be included here.

Comparison of most commonly employed sample preparation techniques is presented in Table 5.1. In general, simpler, faster methods tend to generate dirtier samples, which tend to limit the number of injections that can be made on a column or into a chromatographic system before chromatographic or system conditions start to deteriorate.

Direct Injection Direct injection approaches have been developed to facilitate and expedite analysis of biological samples and are more suitable for liquid as opposed to gas phase separation methods. They include direct injection with or without prior dilution, column switching, and restricted access medium (RAM) or internal surface reversed-phase (ISRP) column methods.

A simple dilution and injection approach is not commonly used because it requires high analyte concentration and/or high detector response to an analyte.

TABLE 5.1 Relative Comparison of Commonly Used Sample Preparation Methods

Factor	Direct Injection	Filtration	Precipitation	LLE	SPE
Simplicity	++++	+++++	+++++	+++	+++
Speed	+++	++++	++++	+	+++
Resultant sample cleanliness	++	+	+	+++++	+++++
Resultant analyte concentration	+	+	+	+	+++
Selectivity	+	+	+	+++	++++
Solvent consumption	++	+	+++	+++++	++
Possible injections per column	++	++	++	+++++	+++++

This straightforward approach may be suitable for analysis of simple matrices (e.g., API or formulated pharmaceutical product) but generally is not useful for the analysis of biological samples due to low analyte concentration and numerous complex interferences.

Column switching for separation and enrichment of analytes of interest has been in use for a long time [4]. Hagestam and Pinkerton [5] introduced an online cleaning method for HPLC analysis with a RAM/ISRP column [6–8]. RAM columns operate via both size exclusion and phase partitioning modes. Prefiltered samples are injected onto a RAM column, which retains small molecules in the inner hydrophobic pores while larger ones go to waste unretained. Subsequently, small molecules are eluted from the RAM column onto an analytical reversed-phase column, where they are concentrated prior to elution with an appropriate strength mobile phase. A number of manufacturers offer RAM columns with similar basic principles but some variations on the theme (e.g., http://www.registech.com/ram/#SPS). The advantages of this method include speed, simplicity, automation, potential increased safety for analysts due to lesser exposure, and applicability to varied biological, botanical, and environmental matrices. However, the online sample cleaning methods are, in general, subject to carry-over and injector clogging problems. Another drawback of the RAM method is a relatively low sensitivity. However, recent improvements in HPLC column technology and evolution of LC-MS and LC-MS/MS instrumentation have provided additional selectivity and sensitivity [8–10]. Hogendoorn et al. [11] demonstrated suitability of RAM columns for LC-MS/MS analysis of analytes with a wide range of polarities in human serum. Other similar techniques include size exclusion chromatography and turbulent flow chromatography (narrow bore columns packed with large particles) but will not be further discussed here.

The column switching approach utilizes switching valves to redirect the flow of a mobile phase between two columns—a cleanup column and an analytical separation column. Limitations include a requirement of mobile phase compatibility between the two columns. An example of column switching includes online solid phase extraction (SPE, to be discussed later). Good sensitivity and precision were reported in a comparison of online and offline SPE methods for measurement of six isoflavones and lignans in urine [12]. However, the online method was found to be more sensitive, precise, reproducible, and cost effective. Another variation involves a ternary-column system [13], which utilizes dual extraction columns in a

parallel configuration. This allows use of one column for extraction while the other is being equilibrated and thereby decreasing the injection cycle time and increasing the sample throughput.

Filtration Various filtration methods serve similar purposes and have similar advantages and disadvantages to those of protein precipitation (see next section). There are basically two types of filters: depth and screen. Depth filters are randomly oriented fibers that retain particles throughout the matrix rather than just on the surface. They have a higher load capacity than screen filters [14]. Screen filters are polymeric membranes that have a uniform distribution of pore sizes. They are relatively thin so that there is a minimal amount of liquid retention. Screen filters clog more rapidly than depth filters.

In developing a method that requires filtration, adsorption of the analyte onto the filter must be taken into account. For dilute solutions of adriamycin, more than 95% is adsorbed to cellulose ester membranes and about 40% to polytetrafluoroethylene membranes [15]. For more concentrated solutions, as would be encountered in bulk formulation testing, filter adsorption is not as important a concern. Nevertheless, the common practice is to discard the first several milliliters of the filtrate. For protein-based products, there is significant nonspecific binding to nylon-based microporous membranes and minimal binding to hydrophilic polyvinylidene fluoride membranes [16]. Ultrafiltration removes protein and protein-bound drug, which makes it suitable for plasma-free drug concentration measurements. Dialysis, on the other hand, is primarily useful for protein binding studies.

Precipitation Protein precipitation or deproteinization (commonly acetonitrile, perchloric acid, or methanol) is also a relatively simple method of sample preparation and minimally eliminates interferences from large molecules. However, interferences from small molecules remain and this approach is more suited for analytical methods that offer additional selectivity such as LC-MS and LC-MS/MS. Precipitation with acetonitrile or methanol may be combined with subsequent extraction and may be used for HPLC or LC-MS/MS analytical methods, which employ these solvents as mobile phases. In addition, matrix-based interferences can create indirect problems in MS detection due to interference with the ionization process. Furthermore, sensitivity may also be an issue as this method does not include a preconcentration step. Matrix interferences place additional strain on the analytical system. Occasionally, sample dilution prior to precipitation may be needed to avoid trapping analytes within precipitated matrix. One significant advantage of protein precipitation is that it extracts all analytes regardless of their polarity. This overcomes limitations of other extraction methods (e.g., LLE, SPE), which may not effectively extract analytes with a wide range of polarities [17]. The precipitation approach is amenable to automation.

Liquid–Liquid Extraction (LLE) LLE is one of the oldest and widely used methods for sample preparation. It is based on analyte (solute) partitioning between immiscible solvents according to relative analytes' solubilities in the solvents. In order to optimize separation in terms of recovery, selectivity, and sample cleanliness, several extractions with the same or different solvent combinations may be necessary. For example, acidic analytes can be extracted from acidified aqueous solution

into organic solvent (e.g., methyl-*t*-butyl ether), followed by their extraction from methyl-*t*-butyl ether into a basic aqueous solution (e.g., ammonium hydroxide), which is neutralized, and analytes are back-extracted into methyl-*t*-butyl ether. Commonly used extraction solvents include hexane, methyl-*t*-butyl ether, ethyl acetate, chloroform, methylene chloride, and dilute acid or base. Modifiers such as a small percentage of isopropanol can also be added. Fine tuning in LLE can be carried out by judicious selection of solvents, solvent modifiers, solvent volumes, pH, and so on. Not all extractions need to involve aqueous and organic phases. There are some organic solvent combinations that are immiscible and can be used in LLE. For example, the acetonitrile : hexane combination is immiscible and can be used to clean somewhat polar analytes from lipophilic components in a biological matrix. LLE is labor intensive, slow, and not easily amenable to automation. Liquid handling stations are making automation more feasible. LLE generally requires large solvent consumption and solvent evaporation, which have environmental, safety, and health concerns. Finding a suitable solvent system and conditions may be difficult for simultaneous extraction of analytes with different polarities. There is also a tendency for emulsion formation, which introduces further complications. Adsorptive losses may result during evaporation and it may be necessary to pacify the active surface by sylation. Some solvents have a tendency for peroxide formation (e.g., diethyl ether, ethyl acetate) that can lead to analyte stability problems. Methyl-*t*-butyl ether has roughly similar polarity but a lesser propensity for peroxide formation and can often be substituted for those solvents.

Solid-Phase Extraction (SPE) SPE is analogous to offline gradient liquid chromatography and has overtaken LLE in popularity as it lacks a number of the drawbacks associated with LLE. It is relatively easy to automate, requires small solvent volumes, can concentrate samples without evaporation, and yields cleaner extracts and higher recoveries for polar analytes. However, as mentioned earlier, there are cases where SPE may not be suitable for analytes with a broad range of polarities [17]. Earlier manufacturing problems in batch-to-batch reproducibility of the column materials have mostly been resolved. Cartridge columns with a wide variety of bonded phase packing materials (phases or sorbents) are commercially available (or custom prepared, if desired). Cartridge columns are obtainable in different sizes (e.g., 1 mL, 3 mL, 6 mL, 12 mL) with different amounts of packing material (e.g., 30 mg, 50 mg, 60 mg, 100 mg, 150 mg, 200 mg, 500 mg) to accommodate different sample sizes and not to mass or volume overload SPE columns. As a rough guide, nonionic silica-based sorbents can retain a mass of solute approximately equivalent to 1–5% of sorbent mass or 1–5 mg of solute per 100 mg of sorbent. Polymeric sorbents have a larger surface area and can accommodate solute up to 15% of sorbent mass. However, keep in mind that solute represents analytes plus coextracted material. As a rule of thumb, solvent volumes between 4 and 8 times the bed volume are needed to ensure appropriate conditioning, washing, and elution. Silica-based SPE columns have approximately 150 μL bed volume per 100 mg of sorbent, and polymeric sorbents about 250 μL per 100 mg of sorbent. Most commonly used sorbents comprise siloxane-bonded materials with different functional groups to provide normal-phase, reversed-phase, ion exchange, or mixed-mode chromatographic separation. General characteristics of different phases are summarized in Table 5.2. Some of the phases can operate in different modes. For

TABLE 5.2 Characteristics of Common Silica-Based SPE Phases

	Normal	Reversed	Ion Exchange
Sorbent functional group	Silica Cyano Diol Amino	Octadecyl (C18) Octyl (C8) Methyl (C1) Ethyl (C2) Phenyl	Strong cation exchange (SCX; pK_a 2.0–3.0): benzene sulfonylpropyl Strong anion exchange (SAX; pK_a 11.0–12.0): quarternary amine Amine (pK_a 9.5–10.5)
Analytes	Polar	Nonpolar	Ionized
Elution factor(s)	Solvent strength	Solvent strength	Ionic interaction (pH, ionic strength, counterion strength)
Increasing elution strength	Hexane Toluene Chloroform Methylene chloride Tetrahydrofuran Ethyl acetate Acetone Acetonitrile Isopropanol Methanol Water	Water Methanol Isopropanol Acetonitrile Acetone Ethyl acetate Tetrahydrofuran Methylene chloride Chloroform Toluene Hexane	Cations: Li^+ Na^+ NH^+ K^+, Mg^{2+} Ca^{2+} Anions: Hydroxide, fluoride, propionate Acetate, formate Phosphate, carbonate Sulfate Nitrate Citrate

example, cyano phase can be used either in normal-phase or reversed-phase mode and amino phase can be used in normal phase or anion exchange phase. Undesirable secondary interactions can occur with free silanol moieties and can be minimized by end-capping.

SPE cartridges require prior conditioning to remove any impurities from them and make the sorbent compatible with the sample solvent and allow sample retention. Two conditioning washes are commonly used (each 1–2 mL per 100 mg of sorbent): the first to clean the column and the second (similar to or weaker than the sample solvent) to make the column conducive to sample retention. Sample application and elution should not be performed too rapidly as sufficient residence time must be allowed for effective analyte–sorbent interaction. In order to avoid channeling, it is also important not to allow sorbent to dry between conditionings and prior to sample application. After cartridge conditioning, liquid sample (in the weakest strength solvent possible) is applied and allowed to pass through the

column via gravity, pressure, or vacuum. Sample retained on the column should be washed with a stronger solvent than the one used in sample loading but weaker than solvent that would cause its elution. Elution of analytes should be performed with solvent strong enough to allow elution in a relatively small volume. This may avoid or minimize a need for a concentration step (e.g., evaporation) prior to sample analysis. Solvent combination may sometimes be necessary for elution (e.g., methanol : water) in order to minimize the elution volume and/or coelution of undesirable substances. Ion exchange phases require additional considerations. For effective analyte retention, sample pH should be at least 2 pH units lower or 2 pH units greater than the pK_a of the corresponding cation or anion so approximately 99% of analyte remains ionized. In addition, low ionic and high solvent strength promote retention and elution, respectively. The SPE phase and the solvent system can be selected depending on specific goals: analyte elution (interference retention), interference elution (analyte retention with subsequent elution; most common), or analyte concentration. While evaporation may not be necessary when using small elution volumes, it may be advantageous in order to further increase assay sensitivity and improve chromatographic behavior (e.g., peak shapes) by dissolving the extracted sample in the mobile phase or a weaker solvent. Chromatographic artifacts can be encountered when injecting samples, especially larger sample volumes, in a solvent significantly different from the mobile phase.

More recently, miniaturized SPE formats (SPE disks and pipette tips) have been developed. These allow use of smaller sample and elution volumes, provide large flow area, and avoid channeling. SPE disks can easily be adapted to an online 96-well approach.

Comparison of plasma sample purification by LLE, automated LLE (96-well collection plate with robotic liquid handling system), and automated SPE (96-well collection plate with robotic liquid handling system) [18] found total time for automated SPE to be slightly less than that for automated LLE, and both were three times faster than manual LLE. Total time for the automated SPE could be reduced further by a factor of 2 if the evaporation step was omitted. Analyst time was lower by a factor of 25 for the two automated methods as compared to the manual LLE. Overall results in terms of accuracy and precision were comparable for the three methods.

Advantage of SPE over simpler methods like precipitation is shown in Fig. 5.2. More effective cleanup yielded a cleaner chromatogram, decreased ionization suppression by coeluting matrix components, and thereby improved signal to noise by more than tenfold.

Solid-phase microextraction (SPME) represents another recent variation on the SPE method [19]. This method of preparation does not use solvent and the separation takes place on a fused-silica fiber, which is coated with a suitable stationary phase. The fiber is inserted into the sample and allowed to extract analytes via absorption and/or adsorption. Analytes are then eluted either thermally or via mobile phase. This technique can be used in conjunction with GC, CE, or HPLC and is nicely suited for MS.

Derivatization Derivatization may sometimes be required in order to improve volatility, stability, chromatographic behavior, sensitivity, and selectivity. Ideally, derivatization reactions should be rapid, simple, and mild and should yield a single

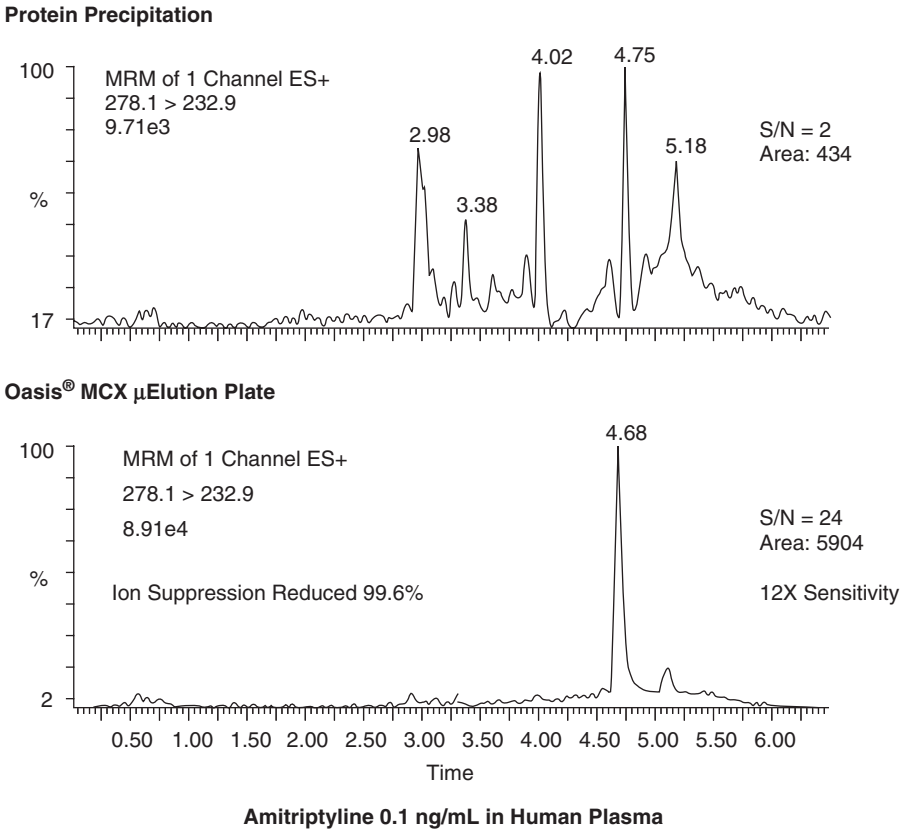

FIGURE 5.2 Effect of sample cleanup on sensitivity in LC-MS. Upper chromatogram depicts sample after precipitation cleanup, while the lower tracing is for analogous sample following a SPE cleanup. (Used with permission from Waters Corp.)

product. Choice of derivatizing moiety depends on the need (e.g., stability, sensitivity, chirality), available derivatizable groups on the analyte (e.g., carboxyl, amine, hydroxyl, thiol), separation method (e.g., GC, HPLC), and detection method (e.g., electrochemical, ultraviolet, fluorescent). Tables 5.3 and 5.4 contain examples of commonly used derivatizing agents for GC and HPLC, respectively. In addition, derivatization can also improve ionization efficiency in atmospheric pressure ionization mass spectrometry (API-MS) and thereby improve sensitivity, which may be compromised by coextracted substances [20]. Derivatization can also help identify and characterize metabolites. For example, *N*-methyl-*N*-(*tert*-butyldimethylsilyl) derivatives tend to yield stable molecular (or quasimolecular) ions in mass spectrometry and thereby allow determination of molecular weight. Ions and fragments can be identified easier by using a mixture of deuterated and nondeuterated (or other stable labeled derivatizing agent) agents. A number of reviews have been published on various derivatizing techniques for the most common separation techniques including GC [21–24], HPLC [25–32], and capillary electrophoresis (CE) [26–28, 33, 34]. Other derivatization approaches have also been reported (e.g., combining sample preparation and derivatization) [35]. While derivatization offers pos-

TABLE 5.3 Examples of Commonly Used GC Derivatizing Agents

Functional Moiety	Derivatizing Agent	Enhanced Detector Response
Alcohols	Heptafluorobutyric anhydride	ECD
	Pentafluorobenzyl chloride	ECD
	Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	
	N-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)	
Amines (primary)	Chloroacetic anhydride	ECD
	Pentafluorobenzoyl chloride	ECD
	Pentafluorobenzaldehyde	ECD
	Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	
	N-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)	
Amines	Heptafluorobutyric anhydride	ECD
	Pentafluorobenzyl chloride	ECD
	Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	
	N-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)	
Amino acids	Acetic acid	
	Heptafluorobutyric anhydride	ECD
	Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	
	N-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)	
Carboxylic acids	Pentafluorobenzyl chloride	ECD
	Pentafluorobenzyl alcohol	ECD
	Diazomethane	
	Hydrochloric acid + methanol	
	Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	
	N-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)	
Phenols	Heptafluorobutylimidazole	ECD
	Pentafluorobenzyl chloride	ECD
	Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	
	N-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)	

sibilities for significant analytical improvements, appropriate caution needs to be exercised in order to avoid potential pitfalls, such as various artifacts with trimethylsilylation [36].

5.2.3 Analytical Techniques for Method Development

Nonseparation Methods

Absorption and Emission Spectroscopy

ULTRAVIOLET (UV)–VISIBLE (VIS) Spectrophotometry is a technique of detecting and measuring absorption of electromagnetic radiation by molecules with π or unshared electrons. The UV-VIS spectrum is divided into 200–380 nm (UV) and 380–800 nm (VIS) regions. The Beer–Lambert law is used for measurement of ana-

TABLE 5.4 Examples of Commonly Used HPLC Derivatizing Agents

Functional Moiety	Derivatizing Agent	Enhanced Detector Response
Alcohols	Benzoyl chloride	
	4-Dimethylamino-1-naphthoyl nitrile	FD
	3,5-Dinitrobenzyl chloride	
	p-Nitrobenzoyl chloride (4-NBCl)	
	p-Nitrophenyl chloroformate	
	p-Iodobenzensulfonyl chloride	
Amines (primary)	Benzoyl chloride	
	4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)	FD
	Dansyl chloride (DnS-Cl)	FD
	Flourescamine	FD
Amines	Benzoyl chloride	
	4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)	FD
	Dansyl chloride (DnS-Cl)	
	3,5-Dinitrobenzyl chloride	
	p-Nitrobenzoyl chloride (4-NBCl)	FD
	o-Phthaldialdehyde (OPT)	FD
Amino acids	Dansyl chloride (DnS-Cl)	FD
	Flourescamine	FD
	Pyridoxal	FD
	o-Phthaldialdehyde (OPT)	FD
Carboxylic acids	Benzyl bromide	
	p-Bromophenacyl bromide	
	4-bromomethyl-7-methoxycoumarin (Br-Mmc)	FD
	9,10-Diaminophenanthrene	FD
	Phenacyl bromide	
Ketones/aldehydes	*p*-Nitrobenzylhydroxylamine hydrochloride	
	2-Diphenylacetyl-1,3-indandione	FD
	2,4-Dinitrophenyl hydrazine	
Phenols	4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)	FD
	Dansyl chloride (DnS-Cl)	FD
	3,5-Dinitrobenzyl chloride	
Thiols	4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)	FD
	N-(9-Acridinyl)maleimide	FD

lytes. While UV-VIS detectors are commonly used for HPLC analysis, UV-VIS spectrophotometry without prior chromatographic separation is not well suited for complex biological samples. It is frequently used for analysis of API or other substances in the manufacturing process and in USP methods [37]. Further improvements in UV-VIS spectrophotometry are being made, for example, UV derivative spectrophotometry [38].

INFRARED (IR) IR is a technique of detecting absorption of energy resulting from transitions between vibrational and rotational energy levels of molecules. It is in the

0.5–200 μm (20,000–50 cm^{-1}) region of the electromagnetic spectrum. IR is commonly used for characterization of API and in the manufacturing process [39, 40].

ATOMIC ABSORPTION SPECTROMETRY (AAS) Primary applications of AAS in the pharmaceutical area involve quantification of metal ions, metal-containing organic compounds, and inorganic molecules (e.g., arsenic trioxide, different platinum analogues, silver, mercury, copper, palladium) [41–48] in drug formulations, tissues, and biological liquids. It is also used for trace element control in natural plant products and in synthesized drug substances [49–52]. AAS is also used for indirect measurements of organic substances based on formation of ion-paired complexes with the drugs [53–56].

AAS detects absorption or emission of light in thermally vaporized and extensively atomized molecules. Atomization is achieved via either flame aspiration or electrothermal atomization (graphite furnace). The former also allows emission analysis while the latter ashes components and diminishes matrix effects. Graphite-furnace atomic absorption spectrometry (GF AAS) is well suited for analysis of biological samples as it only requires very small sample size (20 μL) and provides increased sensitivity due to a longer residence time. More details on HGAAS (hydride generation atomic absorption spectrometry) can be found in Dědina and Tsalev [57]. All aspects of the theory, instrumentation, and practical usefulness of electrothermal atomization for analytical atomic spectrometry can be found in Jackson [58]. There are also some general guides on the theory and practice of AAS [59–62]. Recently, a fully validated GF AAS method has been described for the cancer chemotherapeutic oxaliplatin [48].

Ligand-Binding Assays A ligand-binding assay (LBA) is a technique that uses specific antigen or antibody, capable of binding to the analyte, to identify and quantify substances. The antibody can be linked to a radioisotope (radioimmunoassay, RIA), to an enzyme that catalyzes an easily monitored reaction (enzyme-linked immunosorbent assay, ELISA), or to a highly fluorescent compound by which the location of an antigen can be visualized (immunofluorescence).

Because LBAs are indirect assays dependent on binding interactions, factors such as lipemic and hemolyzed samples, binding proteins, and anticoagulants that interfere with this process will destabilize the assay.

ELISA is a useful and powerful method in estimating ng/mL to pg/mL of test articles and metabolites in the solution, such as serum, urine, and culture supernatant. It is a relatively easy task to develop ELISA if "suitable" antibodies against materials of interest such as proteins, peptides, and drugs are readily available. ELISAs combine the specificity of antibodies with the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. There are two main variations on this method: ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen. An ELISA is a five-step procedure: (1) coat the microtiter plate wells with antigen; (2) block all unbound sites to prevent false-positive results; (3) add antibody to the wells; (4) add IgG conjugated to an enzyme; and (5) react the substrate with the enzyme to produce a measurable colored product, thus indicating a positive reaction.

One of the most common types of ELISA is the "sandwich ELISA." The sandwich ELISA measures the amount of antigen between two layers of antibodies. The antigens to be measured must contain at least two antigenic sites, capable of binding to antibody, since at least two antibodies act in the sandwich. So sandwich assays are restricted to the quantitation of multivalent antigens such as proteins or polysaccharides. Sandwich ELISAs for quantitation of antigens are especially valuable when the concentration of antigens is low and/or they are contained in high concentrations of contaminating protein. When two "matched pair" antibodies are not available for the target, another option is the competitive ELISA. An advantage to the competitive ELISA is that nonpurified primary antibodies may be used. In order to utilize a competitive ELISA, one reagent must be conjugated to a detection enzyme, such as horseradish peroxidase. The enzyme may be linked to either the immunogen or the primary antibody. There are several different configurations for competitive ELISAs. The example below uses a labeled immunogen as the competitor. Briefly, an unlabeled purified primary antibody is coated onto the wells of a 96-well microtiter plate. This primary antibody is then incubated with unlabeled standards and unknowns. After this reaction is allowed to go to equilibrium, conjugated immunogen is added. This conjugate will bind to the primary antibody wherever its binding sites are not already occupied by unlabeled immunogen. Thus, the more immunogen in the sample or standard, the lower the amount of conjugated immunogen bound. The plate is then developed with substrate and color change is measured.

Ligand-binding assays, including immunoassays/antibody assays, are required to meet the established requirements for chemical assays. However, due to the nature of the ligand-binding assays, certain additional elements such as specificity must be more explicitly established and other elements such as accuracy and precision may meet a lesser standard, or be evaluated on a sample-to-sample basis. The principal elements of a method validation are discussed in ICH Q2A and Q2B and in the U.S. FDA guidance on bioanalytical method validation. The exceptions or additional elements as they apply to validation of ligand-binding assay are described [63]. The criteria for acceptance of method validation, prestudy validation, and in-study validation are described in great detail in this document. In contrast to other analytical methods, LBA are inherently nonlinear. Because four- and five-parameter logistic curves are used to create calibration curves, a large number of calibrators should be used to most accurately describe the curve. The LBA should additionally be validated for cross-reactivity to metabolites and comedications. This evaluation should be performed at high levels and in combination to thoroughly demonstrate selectivity or to determine specific cross-reactivity for each competing analyte.

Greater latitude in precision is allowed for these types of assays. The precision and accuracy should be evaluated during the method validation by analyzing 4 sets of QC samples at LLOQ, low, medium, and high levels in duplicate in 6 different batches. For assays not capable of meeting the nominal acceptance criteria, greater criteria can be set but the precision should be evaluated for each sample analyzed by preparing and analyzing multiple aliquots of each sample. For accuracy in each batch, 4 out of 6 QC samples must be within ±15% of nominal concentration but the two failed QC samples may not be at the same level (4-6-15 rule). When separation (or cleanup) is used for samples but not for calibrators, the recovery of this separation or cleanup step must be determined and used to correct reported sample

concentrations. Possible approaches to assess recovery are the use of a radiolabeled tracer analyte or an internal standard not recognized by the antibody and measured using another technique. Assessment of analyte stability should be performed in whole matrix, not treated, stripped, or prepared matrix. Matrix effects are particularly troublesome in immunoassay methods. The effect of matrix and nonspecific binding must be evaluated and documented in a number of different ways during the method validation: (1) serial dilution of reference analyte with matrix and then the evaluation of response to known concentration; (2) the calibration curve in matrix versus buffer; (3) parallelism between diluted samples and reference analyte; and (4) non specific binding. For the in-study validation, acceptance criteria should follow the 4-6-30 rule (4 out of 6 samples should be within ±30% of nominal concentration).

More recently, recommendations for the development and implementation of bioanalytical method validation for macromolecules in support of pharmacokinetic and toxicokinetic assessments were published in detail [64]. Briefly, values of ±20% (25% at the lower limit of quantification (LLOQ)) are recommended as default acceptance criteria for accuracy (% relative error (RE), mean bias) and interbatch precision (% coefficient of variation (CV)). In addition, as the secondary criterion for method acceptance, it was proposed that the sum of the interbatch precision (%CV) and the absolute value of the mean bias (%RE) be less than or equal to 30%. This added criterion is recommended to help ensure that in-study runs of test samples will meet the proposed run acceptance criteria of 4-6-30. Exceptions to the proposed process and acceptance criteria are appropriate when accompanied by a sound scientific rationale.

Usually, if possible, the LBA should be cross-validated with a chemical method such as LC/MS [65–67]. LBAs are widely used for macromolecular measurements in blood, urine, and other biological liquids [68–72], monitoring of drug–protein interactions [73], and small molecular measurements in different biological fluids [65, 74–76].

Isotope Assays

RADIOISOTOPE ANALYSIS Isotopes of elements contain nuclei with the same number of protons, but different numbers of neutrons. In general, atoms where the number of protons does not equal the number of neutrons tend to be unstable. Some isotopes are stable and some are not. Both stable and unstable isotopes are used in analytical methods. An unstable isotope has an excess of neutrons in its nucleus. To eliminate the surplus, the nucleus decays to a different nucleus by emitting particles and energy. Radioactive nuclides are also known as radioisotopes.

Radioisotopes have proved to be an indispensable tool in biomedical research and have played a pivotal role in the investigation of absorption, distribution, metabolism, and excretion (ADME) properties of new chemical entities over the past several decades [77–79]. The main advantage of using radioisotopes in studying the disposition of new drug candidates is the ease of detection and the achievement of high sensitivity, especially when compounds with high specific activity are used. There are two main types of radiochemical analysis: (1) isotope dilution assay and (2) neutron activation analysis.

Isotope Dilution Assay (IDA) The IDA is a method of quantitative analysis based on the measurement of the isotopic abundance of a nuclide after dilution by mixing with one or more of its isotopes [80]. The activity ratio or isotopic ratio of the mixture defines the concentration of the analyte, which is a tremendous advantage for measurement since quantitative separation of the analyte from nonradioactive components is not required.

Reverse dilution analysis involves addition of nonradioactive (cold) drug or metabolite to a sample containing low levels (per weight basis) of radiolabeled drug in order to isolate a radioactive substance. Subsequent purification (usually via crystallization and recrystallizations) to constant specific activity demonstrates the original presence of the compound added.

The principal limitation to IDA is the availability of a suitable spike substance or tracer [81]. The half-life and type of radiation emitted by a radiotracer are very important, as is the purity. The half-life must be long enough so that sufficient activity is available during the analysis for good counting statistics. However, too long half-lives can be a problem because of low specific activities and storage and disposal problems. The IDA can be used as the analytical technique to quantify the absolute abundances of the isotopes excreted in the experiment.

An injection of a radioisotopically labeled drug can be used to determine blood, plasma, or urine levels and their temporal profile by liquid scintillation or gamma counting and measurement of activities in serially derived samples. Liquid scintillation counters can be used to measure total sample radioactivity. In addition, radioactivity of individual components can be determined after chromatographic separation by using an online radiometric chromatographic detector or by collecting chromatographic fractions and using a liquid scintillation detector.

More recently, accelerator mass spectrometry (AMS) has also been introduced into the field of pharmacology and toxicology [82–84]. The sensitivity is due to the fact that this technique measures radioactive atoms directly instead of merely their decay, and thus AMS is approximately one million times more sensitive than liquid scintillation. The main advantage of this technique is its exquisite sensitivity in the attamole range. High sensitivity allows for microdosing studies and human Phase 0 studies.

Positron Isotopes Among beta-emitters, at least 95% of all samples used in metabolism and toxicology studies are labeled with either tritium (^{3}H) or carbon-14 (^{14}C). Perhaps 1–3% of samples contain phosphorus-32 (^{32}P) and less than 1% include all other beta-emitters combined. Of these samples, probably 99% contain one isotope by itself with 1% or less being a mixture of two isotopes, almost always $^{3}H + {}^{14}C$. Other commonly used positron isotopes are ^{11}C, ^{13}N, ^{15}O, and ^{18}F.

When beta-emitters such as ^{3}H and ^{14}C decay, they release into their surroundings an electron (beta particle), which may have different kinetic energy.

Carbon-14 is a weak β-ray emitter (155 keV). A maximum specific activity of 62.4 mCi per milliatom of carbon or 4460 mCi/g is more than sufficient for most PK studies, where typical doses of carbon-14-labeled drugs are approximately 10–20 μCi. Long physical half-life (5.7×10^{3} yr) is an advantage that allows calculating specific activity without having to consider losses due to spontaneous decay. Biological half-life for a bound compound is 12 days and 40 days for an unbound isotope. Many carbon-14-labeled drugs can be prepared in the analyst's laboratory.

Tritium (^{3}H) is a weak β-ray emitter (i.e., maximum = 18.6 keV) with significantly lower energy than ^{14}C. Tritium-labeled drugs of higher specific activity than those labeled with carbon-14 must be used to achieve equivalent sensitivities in detection. Tritium (^{3}H) has a long physical half-life of 12.3 yr and usually a biological half-life of 10–12 days, which depends on the nature of the labeled molecule.

Sulfur-35 is an artificially produced β-emitting isotope. The β particles' energy of ^{35}S exceeds that of ^{14}C. This has prompted the use of ^{35}S-labeled drugs in pharmaceutical analysis. The short physical half-life (87.9 days) and low number of drugs containing sulfur limits the overall utility of ^{35}S in the analysis of drugs and metabolites in biologic fluids.

Phosphorus-32 is another artificially produced β-emitting isotope. The β particles' energy of ^{32}P exceeds that of ^{14}C. Because of a short physical half-life (14.3 days), it requires calculation of loss of radioactivity due to spontaneous decay. Phosphorus occurs in an extremely small number of drugs and therefore is of low importance in biopharmaceutical analysis.

Gamma Isotopes The most commonly used gamma isotopes are ^{125}I, and ^{99m}Tc. Other commonly used gamma isotopes are ^{111}In,^{123}I, and ^{153}Sm.

Gamma-emitters, upon their decay, give off discrete packets of energy (gamma rays) in the form of photons rather than particles. Gamma emission is monoenergetic as contrasted to the broad energy distribution of beta-emitters. Thus, for a radioisotope such as iodine-125 (^{125}I), all of the gamma emissions have the identical energy of 35 keV. Iodine-125 has been most useful in the development of radiolabeled antibodies for certain radioimmunoassays. It is also used in labeling proteins and hormones. Since a small number of therapeutic agents have iodine atoms as a part of their molecular structure, ^{125}I labeling is of little direct benefit in preparing radiolabeled small molecule drugs.

Technetium-99m is used primarily in imaging analysis. A short biological half-life (6 hours) and sufficient physical half-life (1 day) make it ideal for gamma imaging resolution.

Positron emitters are more appropriate for the direct labeling of drug molecules since the gamma-emitting radionuclides are rarely an integral part of the drug molecule structure. The positron (β-emitting) radionuclides are used for *in vitro* and *in vivo* experiments. The gamma-emitting radionuclides are useful for *in vivo* imaging.

The following are the most frequently used radiolabeling techniques: isotope exchange reactions, introduction of a foreign label, labeling with bifunctional chelating agents, biosynthesis, recoil labeling, and excitation labeling. Details and recommendations for radiolabeling can be found in Saha [85].

High chemical and radioactive purity of the labeled drugs is very important for successful ADME and PK studies. The loss of efficacy of a labeled compound over a period of time may result from radiolysis and depends on the physical half-life of the radionuclide, the solvent, any additive, the labeled molecule, the nature of emitted radiations, and the nature of the chemical bond between the radionuclide and the molecule. Radiolysis is decomposition by radiation of labeled compounds emitted by the radionuclides present in them. Radiolysis introduces a number of radiochemical impurities in the sample of labeled material and one should be cautious about these unwanted products. Autoradiolysis occurs when the chemical bond

breaks down by radiation from its own molecule. Indirect radiolysis is caused by the decomposition of the solvent by the radiation, producing free radicals that can break down the chemical bond of the labeled compounds. To help prevent indirect radiolysis, the pH of the solvent should be neutral because more reactions of this nature can occur at alkaline or acidic pH. Higher specific activity results in greater effect of radiolysis. The longer the half-life of the radionuclide, the more extensive the radiolysis will be. Usually a period of three physical half-lives or a maximum of 6 months is suggested for shelf life.

Detection Different detection means are employed for beta and gamma measurement but after the initial detection both employ essentially the same electronics to count the number of events detected as well as the same software to process the recorded results.

For beta detection, because the beta particles that are most often used have such low penetrating power, in the process known as "liquid scintillation counting," a discrete sample or a column eluate is usually mixed with special energy transfer solvents and chemicals ("scintillators"), which produce light when a radioactive decay takes place. Alternatively, a column eluate, but never a discrete sample, is allowed to flow over particulate solid ("solid scintillator") matter, which emits light when a radioactive decay occurs on the surface or in close proximity to it.

Because gamma radiation is pure energy and has no mass, it is much more penetrating than beta-particle emission of the same energy. Therefore, the preferred detector for gamma-rays is a cylindrical block of specially activated sodium iodide, one face of which is optically coupled to a photomultiplier with the sides and other face, and the photomultiplier as well, enclosed in a thin aluminum shell. Gamma radiation penetrates the shell, is stopped by the very dense sodium iodide producing light, which is then measured by the photomultiplier. Light production by this means is approximately five times that of a comparable energy beta-particle with liquid scintillator.

Whole-body autoradiography (WBA) is a routine and traditional method of detection on X-ray film. It is successfully used for tissue distribution studies [86, 87]. Whole-body radioluminography is a new method of radiation detection based on a phosphorus imaging technique, offering particular benefits of being much more sensitive and having much wider linear measurement range than traditional X-ray film techniques. This method is successfully and extensively used for tissue distribution studies [78, 88] and is especially attractive in combination with different chromatographic methods on extracts of tissues for studying the metabolic fate of the radiolabel [86].

Neutron Activation Analysis (NAA) NAA is one of the most sensitive techniques used for qualitative and quantitative elemental analysis of multiple major, minor, and trace elements in various samples [89, 90]. For many elements, NAA offers sensitivities that are superior to those possible by any other technique. Moreover, the accuracy and precision of the technique are such that NAA is still one of the primary analytical methods used by the National Institute of Standards and Technology (NIST) to certify the concentration of elements in Standard Reference Materials (SRMs) [91]. In typical NAA, stable nuclides (^{A}Z, the target nucleus) in the sample undergo neutron capture reactions in a flux of (incident) neutrons. The

radioactive nuclides (^{A+1}Z, the compound nucleus) produced in this activation process will, in most cases, decay through the emission of a beta particle (β–) and gamma-ray(s) with a unique half-life. A high-resolution gamma-ray spectrometer is used to detect these "delayed" gamma-rays from the artificially induced radioactivity in the sample for both qualitative and quantitative analysis. One of the principal advantages of NAA is that biological samples in the liquid or solid state can be irradiated and little or no cleanup is necessary. Samples are typically collected, irradiated, and analyzed directly in plastic holders. Care must be taken to avoid contact with metal equipment or other contaminants containing trace levels of metals. NAA is nearly free of any matrix interference effects as the vast majority of samples are completely transparent to both the probe (the neutron) and the analytical signal (the gamma-ray). Moreover, because NAA can most often be applied instrumentally (no need for sample digestion or dissolution), there is little if any opportunity for reagent or laboratory contamination. However, interferences can occur when different elements in the sample produce gamma-rays of nearly the same energy. Usually this problem can be circumvented by choosing alternate gamma-rays for these elements or by waiting for the shorter-lived nuclide to decay prior to counting. Other interferences can occur if another type of nuclear reaction concurrently produces the radionuclide of interest. The accuracy of NAA is generally high, although precision values in the range of ±5–10% (RSDs) have been quoted for this technique.

The instrumentation used to measure gamma-rays from radioactive samples generally consists of a semiconductor detector, associated electronics, and a computer-based, multichannel analyzer (MCA/computer). Most NAA labs operate one or more hyperpure or intrinsic germanium (HPGe) detectors.

Over the last several years there has been a growing interest in the use of *in situ* radiotracers to test new pharmaceuticals and dosage forms being developed for commercial distribution [92–94]. These methodologies offer significant advantages in the evaluation of encapsulations, time release, clearance, and the distribution of the pharmaceutical in animal and human models [95]. Recent work [96] evaluated the sensitivity, accuracy, and precision of NAA to measure serum iohexol (contrast agent) at concentrations necessary for estimating GFR. NAA is also used for elemental analysis of some herbal plants [97].

Readers are referred to *Fundamentals of Nuclear Pharmacy* by G. B. Saha [85] for details on theory and practice of radioisotope methods. This is a comprehensive book describing radiopharmaceuticals currently in use, covering radioactivity, radiation detectors, production of radionuclides, generators, radiopharmaceuticals, and practical recommendations on radiolabeling.

Properties of some radioactive isotopes useful in biology are presented in Table 5.5.

Safety Radioactivity users must follow the institutional Radiation Safety Office instructions, complete special training, and work in a licensed laboratory. The Radiation Safety Office conducts periodical inspections in the user's lab. As with all laboratories, no eating or drinking is allowed in labs used for radioactive work.

When radiation passes through matter, it forms ions by knocking electrons loose from their atoms. This separation of electrons from their atoms is called ionization. Ionization makes X-rays and gamma-rays different from other electromagnetic

TABLE 5.5 Properties of Some Radioactive Isotopes Useful in Biology

Element	Half-life	Beta Energy[a]	Gamma Energy[a]
Barium (^{137}Ba)	2.6 min	None	0.662
Calcium (^{45}Ca)	165 days	0.258	0.0125
Carbon (^{14}C)	5730 years	0.156	None
Cesium (^{137}Cs)	30 years	0.511, 1.176	0.661
Chlorine (^{36}C1)	3.1×10^5 years	0.714	None
Cobalt (^{60}Co)	5.26 years	0.315, 0.663, 1.49	1.17, 1.33, 2.16
Hydrogen (^{3}H)	12.26 years	0.01861	None
Iodine (^{131}I)	8.07 days	Several (600 keV)	Several (364 keV)
Iodine (^{125}I)	60 days	None	35 keV
Iron (^{59}Fe)	45 days	1.573, 0.475, 0.273	Several (1292 keV)
Lead (^{210}Pb)	21 years	0.015	0.0465
Phosphorus (^{32}P)	14.3 days	1.710	None
Phosphorus (^{33}P)	25 days	0.249	None
Potassium (^{42}K)	12.4 hours	3.52, 1.97	Several
Sulfur (^{35}S)	88 days	0.167 (similar to ^{14}C)	None
Zinc (^{65}Zn)	243.6 days	Positron = 0.325	0.34, 0.77, 1.12

[a]Maximum energies in million (mega) electron volts. Where the term "several" is listed, individual values can be obtained from the *Handbook of Chemistry and Physics* [188]. Specific activity is the rate of disintegration per unit quantity of the isotope and is frequently expressed as Ci/mole, mCi/mmole, or dpm/mole

radiation such as light rays or radio waves. Ionization changes the nature of biological molecules. As the amount of energy released (as the radioisotope naturally decays) increases, ionization effects increase, as well as the amount of required protection. For instance, ^{3}H is a very low-energy radioisotope (18 keV) and paper can be used as a shield. ^{32}P is a relatively low-energy radioisotope (1.71 MeV) and requires Plexiglas shields. ^{125}I also is a low-energy isotope (35 keV), but because it also emits gamma-rays, lead shields are required for protection.

Radiation can cause somatic and genetic effects in biological organisms. All radioisotopes are hazardous when they get inside the body. Gamma- or high-energy beta-emitters are external hazards.

STABLE ISOTOPE ANALYSIS Stable isotopes are nonradioactive chemical tracers. Stable isotope ratios are measured by mass spectrometric analyses of either bulk phases or specific compounds. Jasper [98, 99] discussed ten major points regarding the properties and uses of stable isotopes in pharmaceuticals, including the suggestion of "isotope product authenticity" of APIs and drug products.

Stable isotopes are also very useful in pharmacokinetic studies. Stable labeled and nonlabeled drug can be simultaneously administered by different routes in order to determine absolute bioavailability. Stable labeled drug can also be administered while the subject is at steady state to determine the steady-state pharmacokinetic profile without requiring drug withdrawal [100]. Stable isotope tagging methods are also being used in proteomic research [101].

The isotope dilution assay using a stable isotope is suitable for pharmacokinetic and pharmacodynamic studies of proteins and polypeptides. This method involves the use of target protein analogues labeled with stable isotopes (such as deuterium

or ^{15}N). These nonradioactive analogues have different molecular masses and act as internal standards. In a typical experiment, a known amount of the analogue is added to the biological sample to be analyzed prior to any sample handling. After appropriate extraction and purification processes, the final mass spectrometric analysis allows the direct identification of the target compound and its analogue by virtue of their respective molecular masses. In addition, it enables a precise quantification on the basis of the relative intensities of the observed signals (principle of isotope dilution).

The IDA completely avoids the use of radioactive material and is not susceptible to errors arising from immunological cross-reactivity with closely related compounds (e.g., precursors, analogues, maturation or degradation fragments). The measured molecular masses further allow the unambiguous identification of the target compound at a sub-pM level with a clear distinction between its endogenous and injected forms, thus allowing for simultaneous *in vivo* quantitative investigations of both native endogenous and injected compounds [102, 103].

Separation Methods Relative comparison of different aspects of most commonly employed separation techniques is presented in Table 5.6 and characteristics of each will be discussed next.

Chromatography Chromatography is an analytical technique used to separate components of a mixture based on their partitioning between a flowing mobile (gaseous or liquid) phase and a stationary phase. *LC/GC* journal (http://www.lcgcmag.com/lcgc/) is a good practical source of articles about chromatographic issues and applications and periodically distributes a very helpful wallchart guide to LC (or GC) troubleshooting.

Quantification of chromatographically separated peaks is accomplished by measurements of peak areas or peak heights. For well behaved peaks, areas tend to be more precise than heights, but heights tend to be less dependent on flow rate than areas. Band broadening by neighboring peaks and noisy baselines may also affect accuracy of area measurements. Column degradation and method of integration (peak deconvolution algorithm used to identify start and stop integration markers and connection between them) may also affect accuracy.

Internal standard or external standard methods can be used for calibration and quantification. In the internal standard method, a known amount of one or more compounds with similar physicochemical properties to the analyte(s) is added

TABLE 5.6 Relative Comparison of Most Commonly Used Separation Methods

Factor	TLC	GC	HPLC	CE
Resolution	+	++++	+++	++++
Clean sample requirement	+	++++	++	++
Potential sensitivity	+	++++	++++	+++
Need for derivatization	+	++++	+	+
Different separation mode options	+	+++	++++	++++
Available sensitive/selective detectors	+	++++	++++	++++
Automation	+	++++	++++	+++
System cost	+	++++	++++	+++

(spiked) along with known amounts of analyte(s) to calibration samples prior to sample processing. Calibration is based on peak height or area response ratio of analyte to the internal standard. When using mass spectrometric detection, stable labeled (nonradioactive) compounds make ideal standards due to their physicochemical similarity to the analytes. External standard calibration is based on samples only spiked with known amounts of analytes and therefore this approach requires reproducible recovery, dilutions, injections, and constant instrument behavior.

Carry-over is not an uncommon problem in automated chromatographic methods [104]. It can have a significant negative impact on all aspects of method development and validation. Causes for carry-over vary, for example, hardware problems (presence of dead volume in chromatographic plumbing), the nature of the solvent, or analyte solubility. The latter can often be eliminated by appropriate modification of the solvent, its pH, the ionic strength, or the addition of an organic modifier. In some cases, a wash step may be required to minimize any carry-over. While neither ICH nor FDA guidelines specifically address carry-over, chromatographers consider less than 20% of the lowest level of quantitation (LLOQ) as the general acceptance criterion.

Another potential problem during analytical procedures concerns analyte stability, including analyte interconversion [105, 106]. For example, retinoids may undergo stereoisomerization and some statins may undergo interconversion between lactone and open hydroxy carboxylic acid forms. In order to avoid analytical artifacts, appropriate analytical measures and QC samples need to be employed [105, 106].

THIN LAYER CHROMATOGRAPHY (TLC) TLC is a simple, quick, and inexpensive procedure that gives a rapid answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f (R_f = distance of migration of the sample zone/distance of the mobile phase front) of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate). Pharmaceutical applications of TLC include analysis of starting raw materials, intermediates, pharmaceutical raw materials, formulated products, and drugs and their metabolites in biological media [107, 108].

In conventional TLC, the stationary phase is a powdered adsorbent that is fixed to an aluminum, glass, or plastic plate. The mixture to be analyzed is loaded near the bottom of the plate. The plate is placed in a reservoir of solvent so that only the bottom of the plate is submerged. This solvent is the mobile phase; it moves up the plate by capillary action, causing the components of the mixture to distribute between the adsorbent on the plate and the moving solvent, thus separating the components of the mixture so that the components are separated into separate "spots" appearing from the bottom to the top of the plate. The stationary phase (the adsorbent: silica gel or alumina) is polar, and the polarities of both the component of the mixture and the solvent used as the mobile phase are the determining factors in how fast the compound travels.

When the solvent has reached the top of the plate, the plate is removed from the developing chamber and dried, and the separated components of the mixture are visualized. Automation of locating spots is possible with commercially available spotters and plate readers.

Zone identification is confirmed by offline or online coupling of TLC with visible/ultraviolet (UV), Fourier transform infrared (FTIR), Raman, and mass spectrome-

try (MS). The most common method for detection is the use of a densitometer, which measures diffusely reflected light from the sample spot in the UV-VIS range. Video-densitometric quantitation is also used [109]. Instrumental development methods, such as overpressure layer chromatography (OPLC) or automated multiple development (AMD), can provide separations with increased resolution.

Additional information about the sample is gained by using two-dimensional TLC with two different mobile phases. After the first separation and evaporation of the mobile phase, the plate is turned by 90 degrees and a second TLC run is performed using a different type of mobile phase. A further development is the use of high performance thin layer chromatography (HPTLC). Reduction of layer thickness (down to 100 μm) and particle size (down to 5 μm) of the stationary phase leads to an improved separation within a shorter time. An HPTLC procedure takes approximately 10 min (conventional TLC up to 15–20 min). A major disadvantage is the smaller sample capacity. The very small layer thickness of approximately 10 μm and the absence of any kind of binder in combination with the framework of this stationary phase lead to new and improved properties of this ultrathin layer chromatographic (UTLC) silica-gel plate [110].

TLC elution patterns usually extrapolate to column chromatography elution patterns. Since TLC is a much faster procedure than column chromatography, TLC is used to determine the best solvent system for column chromatography. As you increase the polarity of the solvent system, all mixture components move faster (and vice versa with lowering the polarity). The ideal solvent system is simply the system that separates the components. For instance, in determining the solvent system for a flash chromatography procedure, the ideal system is the one that moves the desired component of the mixture to a TLC R_f of 0.25–0.35 and that will separate this component from its nearest neighbor by the difference in TLC R_f values of at least 0.20. Therefore, a mixture is analyzed by TLC to determine the ideal solvent(s) for a flash chromatography procedure.

Comparative studies have often found that HPTLC is comparative in accuracy and precision but superior to HPLC in terms of total cost and time required for pharmaceutical analyses [107, 108]. Recently, several HPTLC methods were developed and completely validated for different classes of pharmaceuticals in herbal extracts [108, 111, 112], bulk drugs and dosage forms [113–117], and plasma [118, 119].

Some advantages of the offline arrangement (HPTLC) as compared to an online process, such as column high performance liquid chromatography (HPLC), have been outlined [107, 117] and include the following:

1. There is the availability of a great range of stationary phases with unique selectivities for mixture components.
2. Ability to choose solvents for the mobile phase is not restricted by low UV transparency or the need for ultrahigh purity.
3. Repetition of densitometric evaluation can be achieved under different conditions without repeating the chromatography in order to optimize quantification since all sample fractions are stored on the plate.
4. High sample throughput can be achieved since many samples can be chromatographed simultaneously.

5. The cost of solvent purchase and disposal is minimal since the required amount of mobile phase per sample is small.
6. Accuracy and precision of quantification is high because samples and standards are chromatographed and measured under the same conditions on a single TLC plate.
7. Sensitivity limits of analysis are typically at nanogram (ng) to picogram (pg) levels.

Despite these advantages, HPLC is the preferred method of the majority of analytical laboratories for routine drug measurements because of its higher resolution, better automation, and superior quantitative detection. More detailed information on the principles, theory, practice, instrumentation, and applications of TLC and HPTLC can be found in the literature [107, 120]. A lot of practical tips are described by Elke Hahn-Deinstrop [121].

GAS CHROMATOGRAPHY (GC) Gas chromatography separates volatile mixture components by partitioning between the inert gas mobile phase and the liquid stationary phase on a solid support. Older glass or steel packed columns (2–6 m × 2–4 mm i.d.) have for the most part been superseded by thin fused-silica capillary columns (10–30 m × 0.05–0.53 mm i.d.). Capillary columns provide much greater separation efficiency (resolution) but are susceptible to sample overloading (as evidenced by long tailing peaks). Wide-bore capillary columns (0.53 mm i.d.) can be used to minimize overloading problems. Suitable choice of capillary injector (split, splitless, on-column) depends on the nature of the mixture to be analyzed (sample cleanliness, analyte concentration, analyte stability, etc.). Split injection is the most common. Only about 1% or less of the sample goes on the column. The splitless injection technique allows application of the entire sample onto a column and is useful for relatively clean samples containing very small amounts of analytes. On-column injection is made directly onto the column at a low temperature and also allows the sample to condense in a narrow band. It is useful for thermally labile analytes that may decompose in the injection port.

Resolution and run times can be optimized by appropriate column temperature programming. Some commonly observed GC problems include tailing, fronting and ghost peaks, and column bleed. Tailing peaks are usually indicative of undesirable secondary interaction of analyte(s) with the system, for example, active or contaminated surfaces (injector inlet liner, septum, transfer lines), poor column cut, or cold spots. Active or contaminated surfaces need to be pacified and/or cleaned. Column overload usually causes fronting peaks (gradual rise and abrupt peak fall). It can be avoided by decreasing the analyte amount and/or increasing the thickness of the stationary phase, internal diameter of the column, or the column temperature. Ghost peaks commonly arise from a contaminated system, most commonly the injector, and may be resolved by appropriate cleaning. Column bleed is always present to some degree but is increased by excessive column temperature, system (mainly injector and column) contamination, or system leaks allowing column exposure to oxygen. Column conditioning or correction of those problems may be needed.

TABLE 5.7 Relative Comparison of Most Commonly Used GC Detectors

Factor	FID	NPD	ECD	MS
Sensitivity	100 pg	10 pg	100 fg	10 pg
Selectivity	+	++	+++	++++
Dynamic range	++++	++	+	+++
Compound sensitivity	General	Nitrogen, phosphorus	Halogens, nitrates	General
Structural information	+	+	+	++++

GC techniques generally offer better resolution and sensitivity than other chromatographic techniques, including HPLC. However, requirements for volatile and thermally stable analytes introduce a limitation for GC analysis of some compounds. This drawback can be overcome with prior derivatization to impart volatility and/or stability.

Detection The most common detection methods are flame ionization, nitrogen phosphorus, electron capture, and mass spectrometry. Comparison of commonly used GC detectors is presented in Table 5.7.

1. *Flame Ionization Detector (FID).* FID detects most organic compounds by ionizing the column eluate in a hydrogen-air flame. It is the most commonly used GC detector and exhibits good sensitivity and wide dynamic range.
2. *Nitrogen Phosphorus Detector (NPD).* NPD, also known as a thermionic detector, responds almost exclusively and in a very sensitive manner to compounds containing nitrogen or phosphorus. It differs from FID by having a rubidium or cesium silicate bead, which when heated emits electrons. Nitrogen or phosphorus compounds affect the resultant thermionic emission, generating a signal. The bead is consumable and requires periodic replacement.
3. *Electron Capture Detector (ECD).* ECD utilizes a nickel-63 beta (electron) emitting source, which ionizes a carrier gas. Electronegative compounds (e.g., halogens, nitrates) interact with the generated ions, leading to detection of these compounds. ECD is extremely sensitive to electronegative compounds but has a somewhat limited dynamic range. It requires a general radioactive license and periodic wipe testing.
4. *Mass Spectrometer (MS).* MS will be discussed in the HPLC section below.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) HPLC separates the dissolved components of a mixture by their equilibration between the liquid mobile phase and a stationary phase. Separation may occur via adsorption, partitioning, ion exchange, or size exclusion. The principles described earlier for SPE sorbent phases are generally applicable to HPLC column packings. Table 5.8 describes the physical properties of commonly employed HPLC solvents. Commonly used mobile phases are described in Table 5.9.

Reversed-phase (partition) and normal-phase (adsorption) separations are most commonly employed in HPLC analyses. Silica gel and alumina adsorption chromatography has been mostly superseded by the emergence of bonded (derivatized) silicas. Bonded phases contain a constant amount of liquid phase coated on an inert solid support. This technological improvement was instrumental in resolving prior reproducibility problems. Present normal-phase technology utilizes cyano, amine, or diol moieties bonded to the stationary phase via siloxane bridges. Normal-phase

TABLE 5.8 Physical Properties of Solvents Commonly Used in HPLC

Solvent	BP (°C)	Viscosity (mPa·s) (20 °C)	Polarity Index (P')	Density (g/mL)	UV Cutoff (nm)
Acetic acid	118	1.31	6.2	1.049	255
Acetonitrile	82	0.34	5.8	0.78	190
Chloroform	61	0.57	4.1	1.49	245
Ethyl acetate	77	0.46	4.3	0.90	255
n-Hexane	69	0.31	0.06	0.66	192
Methanol	65	0.55	5.1	0.79	206
Methyl-*t*-butyl ether	55	0.28	2.5	0.76	210
Methylene chloride	40	0.41	3.4	1.33	233
Potassium phosphate					215
1-Propanol	97	2.26	4.0	0.80	210
2-Propanol	82	2.86	4.3	0.79	205
Tetrahydrofuran	66	0.55	4.2	0.88	212
Triethylamine		0.38			
Trifluoroacetic acid	72			1.54	210
Water	100	1.00	10.2	1.00	180

TABLE 5.9 Commonly Used HPLC Mobile Phases

Compound	Reversed Phase	Normal Phase	Ion Exchange	Size Exclusion
Primary solvents (adjust mobile phase strength)	Methanol Acetonitrile Tetrahydrofuran (Propanol)	Methylene chloride Acetonitrile Methanol Methyl-*t*-butyl ether Ethyl acetate	Water	Water
Secondary solvents (diluents)	Water	Hexane	Buffers: Phosphates Acetate Citrate Trifluoroacetate	Buffer
Modifiers	Buffers: Phosphate Acetate Triethylamine EDTA (Ion pairing: Octyl sulfonate, Tertiary butyl ammonium) TFA			

HPLC employs a nonpolar mobile phase and a polar stationary phase. The mobile phase consists of organic solvents and more lipophilic compounds elute first. Some of the drawbacks of normal-phase HPLC include irreversible adsorption, poor reproducibility (sensitive to water content; deactivation), slow equilibration, and peak tailing.

Reversed-phase column packings employ different length alkyl chains (e.g., C_1, C_2, C_4, C_8, C_{18}, C_{30}), phenyl, or cyano moieties bonded to the stationary phase via siloxane bridges. Reversed-phase HPLC employs a polar mobile phase and a nonpolar stationary phase. Elution order, at least in a first approximation, follows hydrophobicity, where polar compounds elute first. The main mode of separation is via partitioning, but there is also the possibility of secondary interactions due to residual silanol groups. These weakly acidic residual silanols can interact with analytes via ion-exchange, hydrogen bonding, or dipole–dipole mechanisms and can cause peak tailing and long retention times. Using end-capped columns (smaller groups bonded to residual silanols) or a basic modifier like triethylamine can minimize unwanted secondary interactions. Modifiers can also be used to minimize undesirable interactions with stationary phase or control analyte ionization. Ion pairing can be used to neutralize ionic or partially ionized species. Alkyl (methane, heptane) sulfonic acids or tetra alkyl (methyl, butyl) ammonium salts are commonly used as ion-pairing reagents (5–10 mM) for bases and acids, respectively. Tetra alkyl ammonium salts can also be used to block residual silanols. Addition of EDTA to the mobile phase can eliminate problems due to minor metal cation impurities.

Advantages of reversed-phase over normal-phase HPLC include the option to use optically transparent solvents (e.g., methanol, acetonitrile), greater compatibility with biological samples and electrochemical detection, relatively rapid equilibration, possibility of gradient elution, and control of secondary equilibria (equilibrium of solute in the mobile phase or stationary phase, i.e., ionization, ion pairing). The most commonly used solvents in reversed-phase HPLC are methanol and acetonitrile. Acetonitrile offers certain advantages over methanol, for example, lower UV cutoff, lower viscosity and thus lower backpressures, lesser tendency for out-gassing (bubble formation), and higher elution strength. On the other hand, buffer salts and ion-pairing agents are more soluble in methanol. The most commonly used buffers are phosphate and acetate. Phosphate buffers have a larger pH buffering range and greater transparency in UV, but lower solubility in organic solvents and lesser volatility. Gradient elution (stepwise or continuous) in reversed-phase HPLC offers a possibility of improved resolution and sensitivity, reduced run time, and better peak shapes. However, gradient chromatography can lead to baseline drifts. Reversed-phase HPLC is a workhorse for biomedical applications.

Ion-exchange chromatography is based on a reversible exchange of ions between stationary and mobile phases. Column packing uses polystyrene resins cross-linked with divinylbenzene, sulfonated to form strong cation exchanger (SCX), or quarternized amine to form strong anion exchanger (SAX). Similar principles apply to ion-exchange HPLC as already discussed for the SPE sorbent phases and will not be further discussed here.

Size exclusion (gel filtration) chromatography uses an aqueous mobile phase for separation of large organic molecules (proteins, polyptides) based on analyte molecular weight. Retention is mostly determined by size of the pores in the stationary phase. Larger molecules elute first because they tend to be excluded from the pores. Columns are selected such that pore size is consistent with the molecular weight composition of the sample.

Some of the more common HPLC problems include high, low, or cycling pressure; leaks; retention time shifting; and peak shape problems. High pressure is commonly

caused by blockage in the system (e.g., inline filter or a frit on the guard column or analytical column). This may be due to coextracted material or buffer salt precipitation. Cleaning or replacement of a filter or frits may resolve these problems. Low pressure may be caused by bubbles in the pump or a leak. Bubbles may be due to improperly degassed mobile phases or leaky check valves, which may need to be cleaned or replaced. Cycling pressure is usually caused by bubbles in a pump head or dirty check valves and may be resolved by degassing the mobile phase and purging the pump or cleaning or replacing a check valve. Retention problems may also be due to leaks, bubbles, and check valve problems. Changes in chemistry (e.g., mobile phase composition or flow) would also cause similar symptoms. Unidirectional, time-related changes in retention times are most likely due to column aging and may require column cleaning with stronger solvents or its replacement. Column aging may also cause peak shape problems and may also require column cleaning or replacement. Peak shape problems may also be due to the mobile phase (e.g., improperly prepared), lack of modifier to minimize undesirable secondary column interactions, or use of a strong injection solvent. Leaks may be due to improperly cut or tightened fittings, excessive system pressure, or worn pump or injector rotor seals.

There are several considerations to be made in selecting a suitable HPLC system, including solubility of analytes, nature of the sample matrix, nature of the functional groups on the analyte(s), required sensitivity and selectivity, and nature of the inherent detectability or ability to impart selective detectability. A good reference book on HPLC method development is by Snyder et al. [122].

Detection The most common HPLC detection methods are ultraviolet/visible, fluorescent, mass spectrometric, and infrared. Comparison of the most commonly used HPLC detectors is presented in Table 5.10. Desirable detector characteristics include high sensitivity, low drift, low noise, low dead volume, fast response, wide dynamic range, reliability, simplicity, and relative insensitivity to operational conditions (temperature, mobile phase composition, flow rate, etc.).

Sensitivity and specificity may be enhanced by appropriate derivatization. Post column derivatization allows separation to be independent of the derivatizing

TABLE 5.10 Relative Comparison of Most Commonly Used HPLC Detectors

Factor	UV/VIS	FD	EC	RID	MS
Sensitivity	1 ng	1 pg	5 pg	100 ng	5 pg
Selectivity	++	+++	+++	+	++++
Dynamic range	+++	++	+	+	++++
Compound sensitivity	General	Aromatics with conjugated pi electrons	Phenols, catechols, nitrosamines	General	General
Structural information	+++	++	++	+	++++
Environmental sensitivity	+	+++	++++	++++	++

moiety, which could minimize separation and also avoid on-column stability issues (e.g., *o*-phthalaldehyde).

1. *Ultraviolet/Visible (UV/VIS) and Photodiode-Array (PDA) Detector.* UV/VIS detectors are analyte property (as opposed to bulk property) detectors. They can be based on single (filter), variable (monochromator), or multiple wavelengths (multiple diodes). A fixed wavelength detector (discrete source, mostly 254 nm) is generally more sensitive than a variable one.

A variable wavelength detector (continuum source with monochromator), on the other hand, allows selection of a wavelength of maximum sensitivity and/or specificity.

A PDA (rapid scanning) provides the most information as it allows quantification at wavelength(s) of maximum sensitivity and/or selectivity, spectral resolution of chromatographically overlapping components, and collection of absorption spectra for peak identification. It collects the entire UV spectrum (all wavelengths simultaneously) several times during the elution of a chromatographic peak and provides three-dimensional data information: time, wavelength, and absorbance unit response. The PDA is commonly used for peak purity determination (Fig. 5.3).

2. *Fluorescence Detector (FD).* Fluorescent molecules absorb light energy and emit it at a lower frequency as electrons return to the ground state from the excited singlet state. An FD is several orders of magnitude more sensitive for compounds with appropriate functional groups (e.g., rigid aromatic compounds with conjugated pi electrons) than the UV detector. It also provides enhanced selectivity, as less than 15% of organic compounds fluoresce. Additional selectivity can be achieved with variable excitation and emission (two monochromators). Further fine-tuning can be accomplished by judicious selection of excitation and emission wavelengths. While an FD offers greater selectivity and sensitivity for fluorescent compounds, it is also more sensitive to environmental factors (e.g., temperature, pressure).

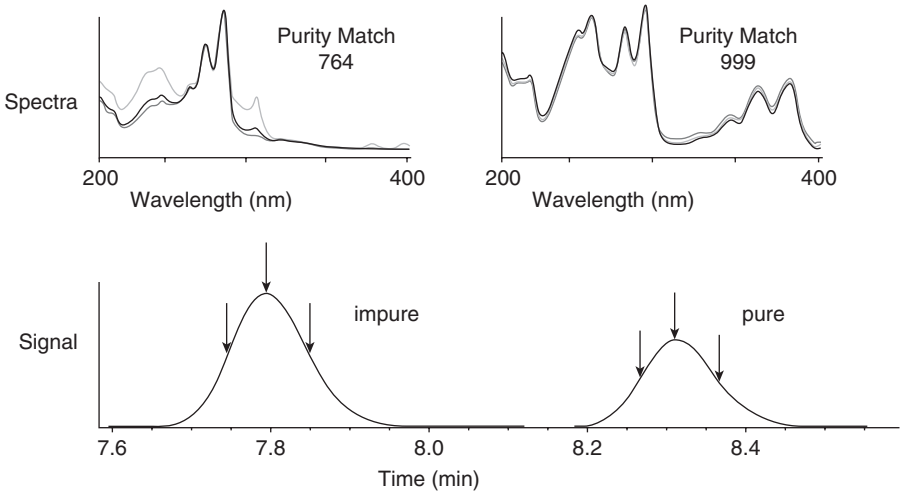

FIGURE 5.3 Use of a PDA for peak purity determination. A PDA (top traces) can detect impurities even when they are not evident from the UV chromatographic signal (bottom traces). (Used with permission from C. Huber, LabCompliance.)

3. *Electrochemical (EC) Detector.* Detection with EC is based on oxidation/reduction reactions at a suitable detector. EC provides enhanced sensitivity and selectivity for analytes with appropriate functional groups (e.g., phenols, catecholamines, nitrosamines). For example, EC detection can be employed in *in vitro* drug metabolism studies where a small amount of phenolic metabolite is being formed in the presence of huge excess of substrate—the latter being transparent in this detector [123]. An EC detector can be operated in either the oxidative or reductive mode, but the former is more commonly employed and is not subject to interferences by oxygen. Electrodes require frequent cleaning, depending on the nature of the samples and mobile phase. In the most prevalent mode, amperometric, a fixed potential applied to the electrode (most commonly glassy carbon) oxidizes (or reduces) the analyte with only a small fraction of analyte (~10%) being involved in electron transfer. In coulometric mode, 100% of the analyte is involved in electron transfer and thus is independent of mobile phase flow. While it offers absolute quantitation by Faraday's law, the coulometric mode requires a large-area electrode, which is more susceptible to contamination. Resultant noise offsets 100% analyte conversion without improvement in signal-to-noise (S/N) ratio.

4. *Refractive Index Detector (RID).* While being a universal detector, this bulk property detector has several inherent limitations: it is very temperature sensitive, it requires the same mobile phase composition throughout the run, the baseline is often difficult to stabilize, and it has relatively poor sensitivity.

5. *Radiometric Detector.* A radiometric detector is primarily used for detection of beta-emitters (^{3}H, ^{14}C, ^{32}P), soft gamma-emitters, or positron-emitters. Radioactive label imparts specificity and allows detection and quantification in biological matrices without concern about nonradioactive interferences. It is especially useful for metabolite detection and pharmacokinetic studies.

6. *Mass Spectrometer Detector.* Mass spectrometric detection adds another dimension to selectivity based on mass/charge (m/z) ratio. In addition to quantification, it also allows identification and characterization of analytes and metabolites [124]. Increased sensitivity and selectivity for quantification can be obtained with selected ion monitoring (SIM) in which only a few selected characteristic ions are monitored. Several ionization modes are possible and are selected to best fit the analyte and sample. Electron impact (EI) is the oldest ionization mode and provides extensive fragmentation and yields important and reproducible structural information. Positive chemical ionization (PCI) provides soft ionization (little excess energy in the molecule), causing lesser fragmentation than EI, and yields information on protonated molecular ions or adducts and molecular weight. It can provide additional sensitivity and specificity over EI. Negative chemical ionization (NCI)—electron capture—provides extreme sensitivity for suitable molecules (containing halogens or nitro groups) or molecules that can be appropriately derivatized (e.g., pentafluorobenzyl) to make them amenable to NCI. Atmospheric pressure ionization (API-MS) techniques, electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) are popular because they combine solvent elimination and ionization in a single step, but some compounds do not ionize using this approach. ESI is a softer ionization than APCI and can be useful for ionization of polar and less volatile components. APCI, on the other hand, is more robust and less susceptible to ion suppression by coeluting substances. Both

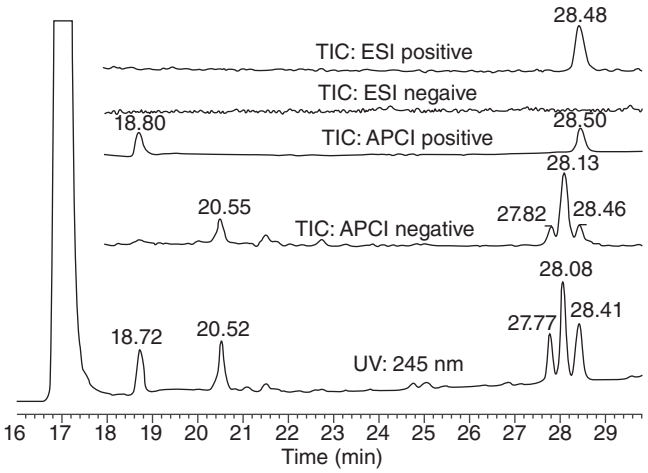

FIGURE 5.4 Effect of different modes of API-MS on detection. The bottom UV trace (245 nm) indicates presence of three impurity components eluting between 27 and 29 min. The top four traces represent corresponding total ion current (TIC) response in different modes of API-MS. (Used with permission from the publisher [190].)

ESI and APCI can operate in either positive or negative mode. Sometimes significant improvements in the sensitivity of API-MS methods can be achieved by derivatization (introduction of permanently charged or readily ionized moieties in ESI or high proton or electron affinities in APCI) or mobile phase modification to facilitate adduct formation [20]. It is also important to keep in mind that response in any ionization mode is compound specific as illustrated in Fig. 5.4. As shown for three unknown compounds eluting between 27 and 29 minutes, response varied markedly using different modes in API-MS. Additional structural information or sensitivity/selectivity is achievable using tandem mass spectrometry or MS/MS techniques (see later discussion).

Several mass spectrometric analyzers are used for drug analyses. The most prevalent is a quadrupole. It has a mass range up to 4000 amu (atomic mass units) and represents a reasonable mixture of mass resolution, linear range, ease of use, and cost. It is also available in a triple quad configuration, TQMS (tandem mass spectrometry), where the middle quadrupole serves as a collision cell to allow for collision-induced dissociation (CID) and operation in MS/MS mode. Parent ions from the first quadrupole undergo further fragmentation in the collision cell to yield daughter ions detectable in the third quadrupole. The process is also known as selected reaction monitoring (SRM) and provides increased selectivity and sensitivity and helps overcome background interferences. MS/MS mode is also useful in peak purity assessment, as shown in Fig. 5.5. HPLC UV and MS monitoring showed a single chromatographic peak. Two largely abundant ions were coeluting in the MS tracings. Further fragmentation in the MS/MS mode of the larger mass ion did not generate the other coeluting smaller mass ion, suggesting that the latter was not spontaneously generated from the former and that it belongs to another species (i.e., the chromatographic peak was impure).

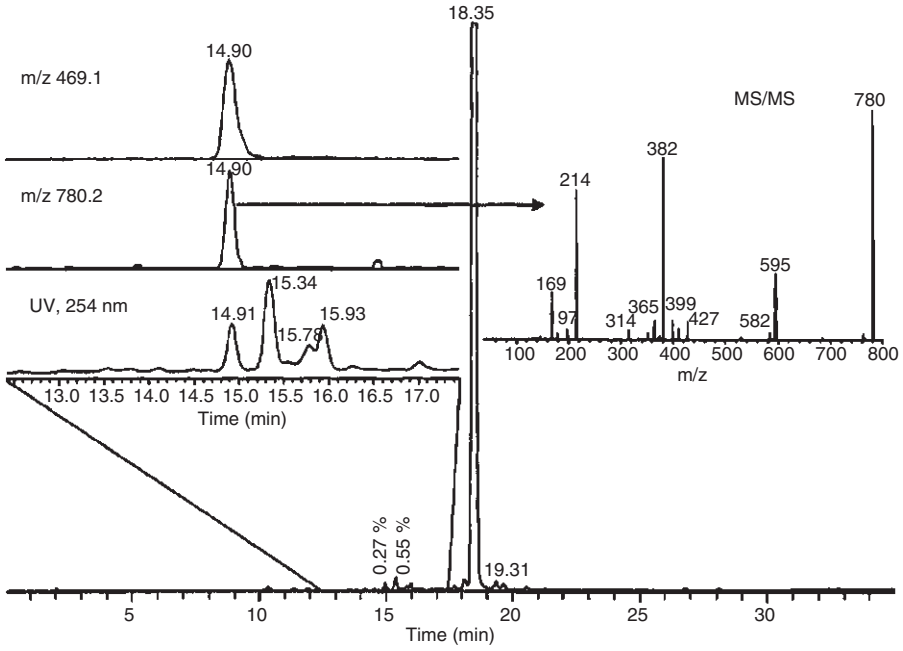

FIGURE 5.5 Detection of coeluting impurity using MS/MS. Left panel shows a UV trace and expanded/enlarged UV and MS (two coeluting ions) traces. Right panel depicts MS/MS spectrum of the larger mass ion that lacks the lower mass ion. (Used with permission from the publisher [190].)

An ion trap represents another quadrupole configuration. Advantages of the ion trap include compact size and ability to trap and accumulate ions and thereby increase signal-to-noise ratio. Trapped ions can be further fragmented and ion trap instruments provide an economical alternative to tandem mass spectrometers. Serial trapping and fragmentation theoretically allow MS^n. Time-of-flight (TOF) analyzers offer several advantages, including high mass resolution, very high mass range, and high mass accuracy. Enhanced mass resolution with TOF instruments provides improved selectivity. A TOF analyzer [124] can also be used in MS/MS mode by combining it with another TOF, a quadrupole, or an ion trap analyzer. Use of LC/MS in the pharmaceutical arena has been reviewed in the literature [8, 125].

As already discussed, matrix effects (presence of coeluting substances) leading to ionization suppression can cause serious problems in mass spectrometric analyses, especially the EIS [2, 3].

While LC-MS offers sensitivity and selectivity and is presently a very popular technique, it is not suitable for all analytical applications [126] and is not a substitute for expertise in chemistry and chromatography.

Capillary Electrophoresis (CE) CE comprises a family of techniques capable of separating a variety of compounds. A recent review [127] describes this separation technique and includes several application examples. The unifying principle in CE is electrical field-driven separation, with migration rate based on analyte size and

charge in the presence of an applied voltage. An electric field is applied across a fused silica capillary with the front end of the capillary immersed in the sample. There are a number of different modes of performing CE separation, including capillary zone electrophoresis (CZE), micellar kinetic electrochromatography (MKEC), and capillary electrochromatography (CEC). CZE is most widely used due to its simplicity and versatility and is applicable to separation of any charged species, including positively and negatively charged species in the same run, but not neutral species. MKEC incorporates pseudopartitioning and thereby also allows separation of neutral species. CEC is a hybrid of CZE and HPLC. CE detectors include UV, fluorescence, electrochemical, and mass spectrometers. In general, some advantages of CE include high resolution, speed, high throughput, small sample size requirement, small reagent consumption, ability to separate most molecules (small and large, even whole organisms), feasible miniaturization, and chiral analysis. It is applicable to both small [128] and large molecules [129]. The major deficiency of CE relative to HPLC has been the higher limit of quantification resulting from small sample volumes and short path length. However, sensitivity improvements (online derivatization, preconcentration, electrokinetic injection, etc.) have been and are being made [128].

Development of various multichannel microfluid devices is under way in order to increase speed and throughput and reduce sample size requirements [130–132] in CE. These include multiplexed CE [131] and microchip electrophoresis [130].

Chiral Methods The common occurrence of chirality (e.g., sugars, amino acids, enzymes) in nature has been known for several centuries, and more recently the stereoselective nature (three-dimensional drug–receptor interaction) of drugs has also become more appreciated and addressed. Recent advances in synthetic [133] and analytical methods (see later discussion) are making development of enantiomeric drugs more technically feasible. The FDA (http://www.fda.gov/cder/guidance/stereo.htm) and other regulatory agencies worldwide are recommending that enantiomers with the best drug characteristics be developed instead of racemates. Enantiomers have identical physical and chemical properties but may behave very differently in an asymmetric milieu. Stereoisomers are frequently distinguished by biological systems and may have different qualitative and quantitative pharmacokinetic and pharmacodynamic (pharmacological and/or toxicological) properties [134]. Therefore, efficacy and safety issues may exist with enantiomers [135]. For example, only the *d*-enantiomer of methylphenidate is active for treatment of the attention deficit hyperactivity disorder and is also significantly more bioavailable. Racemic terodiline has two enantiomers with similar activity for treatment of urinary incontinence. However, cardiotoxicity due to its *R*-isomer led to its withdrawal from the market. In addition, enantiomers may also have a very different metabolic profile and may affect each other's metabolism [136]. There is a growing consensus that new chiral drugs should be developed as single enantiomers (eutomers) and older racemic drugs should be reevaluated and used as pure active enantiomers if supported by clinical data [137]. During drug development, analysis of the formulated racemate should include stereochemical composition. Phase 1 or 2 pharmacokinetic data should address potential interconversion between enantio-

mers. Conventional analytical methods cannot separate enantiomers without introduction of an asymmetric environment. Direct (chiral chromatography or addition of chiral modifier to the mobile phase) and indirect (chromatography following chiral derivatization) analytical approaches can be employed for analyses of chiral compounds and have recently been reviewed [138–141]. Different separation methods (including TLC, GC, CE, and HPLC) have been employed for chiral analysis [141]. Currently, EC and HPLC are most commonly employed for chiral analysis and their application has been the subject of a number of recent reviews [140, 142–149]. Different chiral derivatization approaches have also been used [22, 24, 29, 31]. Miniaturization efforts are under way to improve throughput and speed and reduce sample volume size and include microchip EC [130] and nanotechnology [150].

5.3 ANALYTICAL METHOD VALIDATION

5.3.1 Installation, Operation, Performance Qualification, and Maintenance of Instrumentation

Current regulations do not provide clear and authoritative guidance for validation/qualification of analytical instruments. Therefore, the American Association of Pharmaceutical Scientists, the International Pharmaceutical Federation (FIP), and the International Society for Pharmaceutical Engineering (ISPE) cosponsored a workshop entitled "A Scientific Approach to Analytical Instrument Validation," held in Arlington, Virginia, on 3–5 March 2003. The report describing conclusions of this workshop is published in [151].

The various parties at this workshop agreed that processes are "validated" and instruments are "qualified." Therefore, the phrase "analytical instrument qualification (AIQ)" will be used in lieu of "analytical instrument validation" [151]. Analytical instrument qualification (AIQ) is documented evidence that an instrument performs suitably for its intended purpose and that it is properly maintained and calibrated. AIQ is often broken down into four main phases (Fig. 5.6):

- Design qualification (DQ) for setting functional and performance specifications (operational specifications).
- Installation qualification (IQ) for performing and documenting the installation in the selected user environment.
- Operational qualification (OQ) for testing the equipment in the selected user environment to ensure that it meets the previously defined functional and performance specifications.
- Performance qualification (PQ) for testing that the system consistently performs as intended for the selected application.

The *design qualification* **(DQ)** activity is most suitably performed by the instrument developer/manufacturer. Since the instrument design is already in place for commercial (off-the-shelf) systems, the user does not need to repeat all aspects of

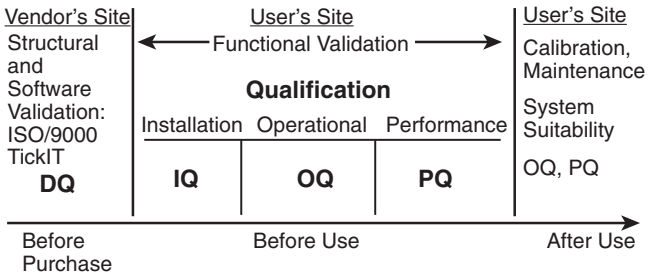

FIGURE 5.6 The validation timeline. Activities under each phase are usually performed in each phase indicated in the figure. In some cases, it may be more appropriate to combine a given activity or perform it with another phase. If performed under the other phase, it is not necessary to repeat the activity under the phase where the activity is listed.

DQ. However, users should ensure that instruments have all the necessary functions and performance criteria that will enable them to be successfully implemented for the intended application and that the manufacturer has adopted a quality system for developing, manufacturing, and testing. Users should also establish that manufacturers and vendors adequately support installation, service, and training. As part of the design qualification process, the vendor should be qualified. This may be done by review of established and documented quality systems (e.g., ISO 9001) at the supplier or by a direct audit.

Installation qualification **(IQ)** is a documented collection of activities needed to install an instrument in the user's environment. The IQ process can be divided into two steps—*preinstallation* and *physical installation*. During preinstallation, all the information pertinent to the proper installation, operation, and maintenance of the instrument is reviewed. Site requirements and the receipt of all of the parts, pieces, and manuals necessary to perform the installation are confirmed. During physical installation, serial numbers are recorded, and all of the fluidic, electrical, and communication connections are made for components in the system. Documentation describing how the instrument was installed, who performed the installation, and other miscellaneous details should be archived. Only after a successful IQ is the instrument ready for OQ testing.

Operational qualification **(OQ)** is the process of demonstrating that an instrument will function according to its operational specification in the user's environment. OQ ensures that the specific modules of the system (or entire system) are operating according to the defined specifications for accuracy, linearity, and precision. This process may be as simple as verifying the module self-diagnostic routines, or may be performed in more depth by running specific tests to verify, for example, detector wavelength accuracy, flow rate, or injector precision. OQ tests can be modular or holistic. Modular testing of individual components of a system may facilitate interchange of such components without requalification and should be done whenever possible. Holistic tests, which involve the entire system, are acceptable in lieu of modular testing [152]. Having successfully completed OQ testing, the instrument is qualified for use in regulated samples testing.

The sample type of tests possible during OQ, which apply to a high performance liquid chromatography (HPLC) unit, can be applied: pump flow rate, gradient linearity, detector wavelength accuracy, detector linearity, column oven temperature, peak area precision, and peak retention time precision. A more intensive list of tests for HPLC is proposed by Grizanti and Zachowski [153]. Bansal et al. [151] consider that OQ tests may not be required to be repeated at a regular interval. Rather, when the instrument undergoes major repairs or modifications, relevant OQ tests should be repeated to verify whether the instrument continues to operate satisfactorily.

Performance qualification **(PQ)** is the process of demonstrating that an instrument consistently performs according to a specification appropriate for its routine use. PQ testing is conducted under actual running conditions across the anticipated working range. In practice, however, OQ and PQ frequently blend together, particularly for linearity and precision (repeatability) tests, which can be conducted more easily at the system level [153]. For HPLC, the PQ test should use a method with a well-characterized analyte mixture, column, and mobile phase. It should incorporate the essence of the System Suitability section of the general chromatography chapter ⟨621⟩ in the USP. Again, proper documentation should be archived to support the PQ process. PQ tests are performed routinely, for example, each time the instrument is used. PQ tests should be performed independent of the routine analytical testing performed on the instrument [151]. In practice, PQ can mean system suitability testing, where critical key system performance characteristics are measured and compared with documented, preset limits. For example, a well characterized standard can be injected five or six times and the standard deviation of amounts are then compared with a predefined value. The analysis of quality control (QC) samples with construction of quality control charts has been suggested as another way of performing PQ. Control samples with known amounts are interspersed among actual samples at intervals determined by the total number of samples, the stability of the system, and the specified precision. The advantage of this procedure is that the system performance is measured more or less continuously under conditions that are very close to the actual application [154].

Documented procedures should exist that instruct the operators on what to do if the system does not meet the criteria. When PQ test(s) fail to meet specifications, the instrument requires maintenance or repair. For many instruments a periodic preventive maintenance (PM) may also be recommended. Relevant PQ test(s) should be repeated after the needed maintenance or repair to ensure that the instrument remains qualified.

Suggested PM and PQ procedures and their recommended frequency for several analytical instruments are contained in Appendix A of *Quality Assurance Principles for Analytical Laboratories* [1] and in *Analytical Method Validation and Instrument Performance Verification* [155].

On completion of equipment qualification, documentation should be available that consists of:

- Design qualification document
- Vendor qualification checklist

- Installation qualification document (includes description of hardware and software)
- Procedures for testing
- Qualification test reports with signatures and dates
- Entries on instrument ID in the laboratory's instrument database
- PQ test procedures and representative results

Software used for analytical work can be classified into the following categories:

- Firmware
- Instrument control, data acquisition, and processing software
- Stand-alone software

1. *Firmware.* The computerized analytical instruments contain integrated chips with low-level software (firmware). Such instruments will not function without properly operating firmware, and users usually cannot alter the firmware's design or function. Firmware is thus considered a component of the instrument itself. Indeed, qualification of the hardware is not possible without operating it via its firmware. So when the hardware (i.e., analytical instrument) is qualified at the user's site, it essentially qualifies the integrated firmware. No separate on-site qualification of the firmware is needed. Any changes made to firmware versions should be tracked through change control of the instrument.

2. *Instrument Control, Data Acquisition, and Processing Software.* Software for instrument control, data acquisition, and processing for many of today's computerized instruments is loaded on a computer connected to the instrument. Operation of the instrument is then controlled via the software, leaving fewer operating controls on the instrument. Also, the software is needed for data acquisition and post-acquisition calculations. Thus, both hardware and software are critical to providing analytical results.

The manufacturer should perform the DQ, validate this software, and provide users with a summary of validation. At the user's site, holistic qualification, which involves the entire instrument and software system, is more efficient than modular validation of the software alone. Thus, the user qualifies the instrument control, data acquisition, and processing software by qualifying the instrument according to the AIQ process defined earlier.

3. *Stand-Alone Software.* An authoritative guide for validating stand-alone software, such as Laboratory Information Management System (LIMS), is available [156]. The validation process is administered by the software developer, who also specifies the development model appropriate for the software. It takes place in a series of activities planned and executed through various stages of the development cycle. The user-site testing is an essential part of the software development cycle. However, the user-site testing, though essential, is only part of the validation process for stand-alone software and does not constitute complete validation. Refer to this guide for activities needed to be performed at the user's site for testing stand-alone software used in analytical work.

Instrument Categories Based on the level of qualification needed, it is convenient to categorize instruments into three groups—A, B, and C, as defined below [151]. Each group is illustrated by some example instruments. The list of instruments provided as illustration is not meant to be exhaustive, nor can it provide the exact category for an instrument at a user's site. The exact category of an instrument should be determined by the user for the specific instrument or application.

1. *Group A Instruments.* Conformance of Group A instruments to user requirements is determined by visual observation. No independent qualification process is required. Example instruments in this group include light microscopes, magnetic stirrers, mortars and pestles, nitrogen evaporators, ovens, spatulas, and vortex mixers.

2. *Group B Instruments.* Conformance of Group B instruments to user requirements is performed according to the instruments' standard operating procedures. Their conformity assessments are generally unambiguous. Installation of Group B instruments is relatively simple and causes of their failure are readily discernible by simple observations. Example instruments in this group include balances, incubators, infrared spectrometers, melting point apparatus, muffle furnaces, pH meters, pipettes, refractometers, refrigerator-freezers, thermocouples, thermometers, titrators, vacuum ovens, and viscometers.

3. *Group C Instruments.* Conformance of Group C instruments to user requirements is highly method specific, and the conformity bounds are determined by their application. Installing these instruments can be a complicated undertaking and may require the assistance of specialists. A full-qualification process, as outlined in this document, should apply to these instruments. Example instruments in this group include atomic absorption spectrometers, differential scanning calorimeters, densitometers, diode-array detectors, electron microscopes, elemental analyzers, flame absorption spectrometers, gas chromatographs, high-pressure liquid chromatographs, inductively coupled argon plasma emission spectrometers, mass spectrometers, microplate readers, near-infrared spectrometers, Raman spectrometers, thermal gravimetric analyzers, UV/VIS spectrometers, and X-ray fluorescence spectrometers.

5.3.2 Validation Parameters and Definition

Validation is a constant, evolving process starting before an instrument is placed online, and continuing long after method development and transfer. A well defined and documented validation process provides regulatory agencies with evidence that the system and method are suitable for their intended use.

The purpose of method validation [157] is to demonstrate that an analytical method is acceptable for its intended use in terms of the criteria below. Assay qualification differs from assay validation in that its intent is to demonstrate that an accepted method (e.g., compendial method from the USP) is suitable for the intended analysis under actual conditions of use. USP provides regulatory guidance for compendial method validation (USP 25-NF 20). When using USP or NF methods, it is not required to demonstrate their accuracy and reliability. Criteria to be used in

TABLE 5.11 ICH Validation Characteristics

Procedure (ICH Q2A) Product Specification	ID Test Present/ Absent	Quantitative Limit Test ≤20%	Qualitative Limit Test ≤5%	Content Purity ≥80%	Content Range 40–60%
Accuracy	No	Yes	No	Yes	Yes
Repeatability	No	Yes	No	Yes	Yes
Specificity	Yes	Yes	Yes	Yes	Yes
Linearity	No	Yes	No	Yes	Yes
Range	No	Yes	No	Yes	Yes
LOD	No	No	Yes	No	No
LOQ	No	Yes	No	No	No

method validation are described in ICH (International Conference on Harmonisation) guidelines Q2A (http://www.fda.gov/cder/guidance/ichq2a.pdf) [158] and Q2B (http://www.fda.gov/cder/guidance/1320fnl.pdf) [159]. Their brief descriptions are included below and details, including tolerances and acceptance criteria, can be found in those guidelines. In addition, FDA Draft Guidance 2396 (http://www.fda.gov/cder/guidance/2396dft.pdf) [160] provides additional information on analytical procedures and methods validation in CMC (chemistry, manufacturing, and controls) documentation and FDA Guidance 4252 provides information on bioanalytical method validation (http://www.fda.gov/cder/guidance/4252fnl.pdf) [161]. Systematic approaches to the acceptance/rejection decision for chromatographic methods and hypothesis testing have been reviewed [162]. ICH validation characteristics for different tests are summarized in Table 5.11.

Specificity Specificity (some guidances use the term *selectivity*) is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present (e.g., impurities, degradants, matrix).

Accuracy and Precision The accuracy represents an agreement between measured and theoretical values. Precision refers to variability in the data from replicate determinations. Precision and accuracy are depicted schematically in Fig. 5.7. Five samples per concentration should be used to validate a bioanalytical method in terms of accuracy and precision. The mean value should be within 15% of the theoretical value with a coefficient of variation (CV) of 15%, except for LLOQ, where these should be within 20%. QC samples should represent low, middle, and high concentrations of experimental values.

Limit of Detection (LOD) and Limit of Quantification (LOQ) LOD refers to the lowest amount of analyte that can be detected (distinguishable from background) but not adequately accurate or precise to be quantified. It is commonly based on 3:1 signal-to-noise ratio (SNR) or as 3 times the standard deviation of the response divided by the slope of the calibration curve.

LOQ refers to the lowest and the highest concentrations that can be quantified with adequate accuracy and precision. It is commonly based on 10:1 SNR or as 10 times the standard deviation of the response divided by the slope of the calibration curve.

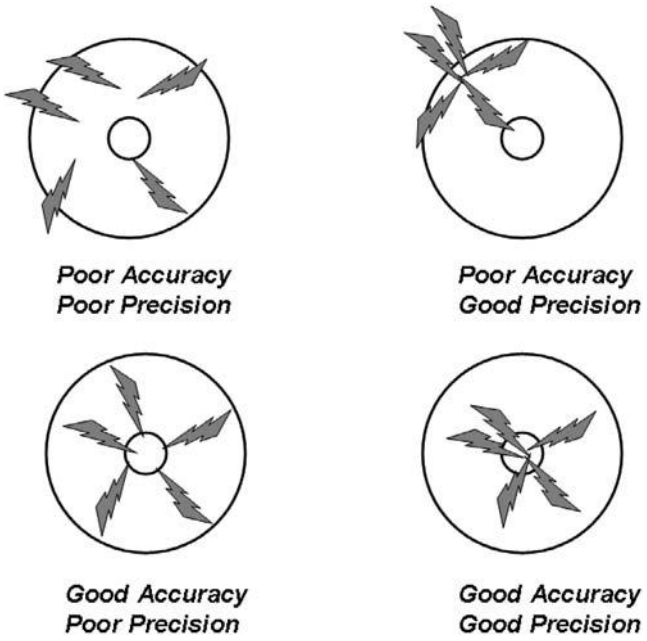

FIGURE 5.7 Schematic depiction of accuracy and precision.

Linearity and Range Linearity refers to direct proportionality between measurement values (response) and analyte concentrations. Commonly 5 to 8 concentrations are used to generate the standard curve. Mathematical transformations to allow linearity may be applied if shown scientifically appropriate.

Range refers to concentrations between the low and high limit of quantification and should bracket the concentration in experimental samples.

Stability Potential degradation of samples, standards, controls, and critical reagents during storage (including freeze–thaw cycles when relevant) should be evaluated and attention paid to expiration dates. Stress studies can be performed by exposing analytes to acid, base, heat, UV light, oxidizers, and so on in order to assess their stability.

Ruggedness Ruggedness refers to reproducibility under normal but variable conditions (e.g., different instruments, operators, laboratories, reagent lots, analytical column lots).

Robustness Robustness refers to the analytical method's ability to remain unaffected by small changes in operational parameters (e.g., temperature, mobile phase composition or flow rate, injection volume) and is used to define acceptable tolerances.

Transferability and Revalidation After a method has been validated, it is ready to be transferred to other laboratories that will be using the method. Documentation of the method should include a detailed written procedure, a method validation

TABLE 5.12 System Suitability Parameters and Recommendations[a]

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

[a]Details of determination of these parameters are presented in Ref. 189.

report, system suitability criteria, and a plan for the method's implementation. General acceptance criteria for the method's intended purpose should also be included. The new user should plan on spending time verifying method performance prior to the method being implemented.

At some time during the lifetime of a method, for one reason or another, it may become necessary to revalidate the method. Revalidation can be carried out in a reactive or proactive manner. Reactive revalidation may be performed in response to changes in incoming raw material, manufacturing batch changes, formulation changes, or any other changes (dilutions, sample preparation) in the method. A total revalidation from scratch is usually not necessary in these instances, but enough revalidation should be performed to address the issues at hand as dictated by the needs and use of the method. Proactive revalidation may be undertaken to take advantage of new technology, or perhaps to automate a previously complex, labor-intensive, and/or time-consuming manual procedure. In such cases, revalidation may be more comprehensive, depending on the undertaking. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole.

System Suitability System suitability is determined by checking a system to ensure system performance before or during the analysis of unknowns. For example, for HPLC, parameters such as plate count, tailing factors, resolution, and reproducibility (%RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability "sample" that is a mixture of main components and expected by-products. Table 5.12 lists the terms to be measured and their recommended limits obtained from the analysis of the system suitability sample for the HPLC analytical method.

5.3.3 Guidelines: ICH, FDA, AOAC, USP, ISO 9000, and ISO 17025

When the ICH was first established, one of the objectives was to organize an International Conference on Harmonization, hence the name given to the initiative. The name of ICH has now, perhaps, become more associated with the process of harmonization, than the actual conferences, although these have been extremely important

for ensuring that the process of harmonization was carried out in a transparent manner and that there was an open forum in which to present and discuss ICH recommendations. ICH is a joint initiative involving both regulators and industry as equal partners in the scientific and technical discussions of the testing procedures that are required to ensure and assess the safety, quality, and efficacy of medicines.

The focus of the ICH has been on the technical requirements for medicinal products containing new drugs. The vast majority of those new drugs and medicines are developed in Western Europe, Japan, and the United States of America and therefore, when the ICH was established, it was agreed that its scope would be confined to registration in those three regions.

The ICH Topics are divided into four major categories and ICH Topic Codes are assigned according to these categories: *Quality Topics*, that is, those relating to chemical and pharmaceutical Quality Assurance, which are relevant to this chapter, are divided into the following subcategories: Q1 Stability Testing, Q2 Validation, Q3 Impurity Testing, Q4 Pharmacopoeias (not implemented yet), Q5 Quality of Biotechnological Products, and Q9 Risk Management.

Q1A (R2): Stability Testing of New Drug Substances and Products (Second Revision) This guideline [163] has been revised a second time in order to accommodate for the consequences of Q1F and reached Step 4 of the ICH process on 6 February 2003. This guideline provides recommendations on stability testing protocols including temperature, humidity, and trial duration. Furthermore, the revised document takes into account the requirements for stability testing in Climatic Zones III and IV in order to minimize the different storage conditions for submission of a global dossier.

Q1B: Photostability Testing of New Drug Substances and Products The tripartite harmonized ICH guideline [164] was finalized (Step 4) in November 1996. This forms an annex to the main stability guideline and gives guidance on the basic testing protocol required to evaluate the light sensitivity and stability of new drugs and products.

Q1C: Stability Testing for New Dosage Forms The tripartite harmonized ICH guideline [165] was finalized (Step 4) in November 1996. It extends the main stability guideline for new formulations of already approved medicines and defines the circumstances under which reduced stability data can be accepted.

Q1D: Bracketing and Matrixing Designs for Stability Testing of Drug Substances and Drug Products The tripartite harmonized ICH guideline [166] was finalized (Step 4) in February 2002. This document describes general principles for reduced stability testing and provides examples of bracketing and matrixing designs.

Q1E: Evaluation of Stability Data The tripartite harmonized ICH guideline [167] was finalized (Step 4) in February 2003. This document extends the main guideline by explaining possible situations where extrapolation of retest periods/shelf-lives beyond the real-time data may be appropriate. Furthermore, it provides examples of statistical approaches to stability data analysis.

Q1F: Stability Data Package for Registration Applications in Climatic Zones III and IV The tripartite harmonized ICH guideline [168] was finalized (Step 4) in February 2003. This document provides guidance on specific stability testing requirements for Climatic Zones III and IV. Besides proposing acceptable storage conditions for long-term and accelerated studies, it gives guidance on data to cover situations of elevated temperature and/or extremes of humidity. The referenced literature provides information on the classification of countries according to climatic zones.

The most important for this chapter are Q2A and Q2B ICH guidelines already mentioned.

Q2A: Text on Validation of Analytical Procedures The tripartite harmonized ICH text [158] was finalized (Step 4) in October 1994. This identifies the validation parameters needed for a variety of analytical methods. It also discusses the characteristics that must be considered during the validation of the analytical procedures, which are included as part of registration applications.

Q2B: Validation of Analytical Procedures—Methodology The tripartite harmonized ICH text [159] was finalized (Step 4) in November 1996. It extends the guideline Q2A to include the actual experimental data required, along with the statistical interpretation, for the validation of analytical procedures.

Q3A(R): Impurities in New Drug Substances (Revised Guideline) First recommended for adoption at Step 4 of the ICH process on 30 March 1995, the guideline [169] was revised under Step 2 of the ICH process on 7 October 1999 and recommended for adoption under Step 4 on 7 February 2002 by the ICH Steering Committee.

The guideline addresses the chemistry and safety aspects of impurities, including the listing of impurities in specifications, and defines the thresholds for reporting, identification, and qualification. Revision of the guideline has allowed clarification of some inconsistencies, revision of the decision tree, harmonizing with Q3B, and addressing some editorial issues.

Q3B(R): Impurities in New Drug Products (Revised Guideline) This guideline [170] was revised and finalized under Step 4 in February 2003. It complements the guideline on impurities in new drug substances and provides advice in regard to impurities in products containing new, chemically synthesized drug substances. The guideline specifically deals with those impurities that might arise as degradation products of the drug substance or from interactions between drug substance and excipients or components of primary packaging materials. The guideline sets out a rationale for the reporting, identification, and qualification of such impurities based on a scientific appraisal of likely and actual impurities observed, and of the safety implications, following the principles elaborated in the parent guideline. Threshold values for reporting and control of impurities are proposed, based on the maximum daily dose of the drug substance administered in the product.

Q3C: Impurities—Guideline for Residual Solvents The tripartite harmonized ICH guideline [171] was finalized (Step 4) in July 1997. This recommends the use

of less toxic solvents in the manufacture of drug substances and dosage forms, and sets pharmaceutical limits for residual solvents (organic volatile impurities) in drug products.

Q5C: Quality of Biotechnological Products—Stability Testing of Biotechnological/Biological Products The tripartite harmonized ICH guideline [172] was finalized (Step 4) in November 1995. This document augments the stability guideline (Q1A above) and deals with the particular aspects of stability test procedures needed to take account of the special characteristics of products in which the active components are typically proteins and/or polypeptides.

Q9: Quality Risk Management The tripartite harmonized ICH guideline [173] was finalized (Step 4) in November 2005. This guideline provides principles and examples of tools of quality risk management that can be applied to all aspects of pharmaceutical quality including development, manufacturing, distribution, and the inspection and submission/review processes throughout the lifecycle of drug substances and drug (medicinal) products, biological and biotechnological products, including the use of raw materials, solvents, excipients, packaging and labeling materials.

Two FDA guidelines—FDA Draft Guidance 2396, *Analytical Procedures and Methods Validation, Chemistry, Manufacturing and Controls Documentation* [160], and FDA Guidance 4252, *Bioanalytical Method Validation* [161]—are important documents regulating analytical methods validation in addition to ICH guidelines.

United States Pharmacopoeia 26, *National Formulary 21*, *Chapter 1225* describes validation of compendial methods [174]. In 2005 the USP proposed revisions to the method validation guidelines published in Chapter 1225 [175]. For the most part, the revisions were made to continue to harmonize with ICH terminology, for example, using the word "procedures" instead of "methods" [176]. The term "pharmaceutical products" is replaced by the term "pharmaceutical articles" to indicate that the guidelines apply to both drug substances and drug products. Another major change is the use of the term "intermediate precision" and the deletion of the section and use of the term "ruggedness." Use of the term ruggedness has been falling out of favor ever since implementation of the original ICH guideline on terminology [158].

Also in 2005, the USP published a proposed new chapter, Chapter 1226, entitled "Verification of Compendial Procedures" [177]. The purpose of this new general information chapter is to provide guidelines for verifying the suitability of a compendial procedure under conditions of actual use. This new chapter summarizes what is necessary to confirm that the compendial procedure works for a particular drug substance, excipients, or dosage form by *verifying* a subset of validation characteristics rather than completing a full validation. The intent is to provide guidance on how to verify that a compendial procedure that is being used for the first time will yield acceptable results utilizing the laboratory's personnel, equipment, and reagents. Verification consists of assessing selected "analytical performance characteristics," described in Chapter 1225, to generate appropriate relevant data as opposed to repeating the entire validation process.

The ISO 9000 family [178–180] was published on 15 December 2000 by the International Organization for Standardization (ISO). These revised standards are

identified by the "2000" in their designation. ISO 9001:2000 [179] specifies requirements for a quality management system for any organization that needs to demonstrate its ability to consistently provide product that meets customer and applicable regulatory requirements and aims to enhance customer satisfaction. ISO 9001:2000 has been organized in a user-friendly format with terms that are easily recognized by all business sectors. The standard is used for certification/registration and contractual purposes by organizations seeking recognition of their quality management system. ISO 9001:2000 specifies requirements for a quality management system where an organization (1) needs to demonstrate its ability to consistently provide product that meets customer and applicable regulatory requirements, and (2) aims to enhance customer satisfaction through the effective application of the system, including processes for continual improvement of the system and the assurance of conformity to customer and applicable regulatory requirements.

All requirements of this international standard are generic and are intended to be applicable to all organizations, regardless of type, size, and product provided. Recently, the ISO has published a new edition of the standard in the ISO 9000 family that defines the vocabulary and describes the fundamentals of quality management systems. ISO 9000:2005, *Quality Management Systems—Fundamentals and Vocabulary* [181], introduces no changes to the descriptions of the fundamentals of quality management systems (QMSS), as presented in the previous edition published in 2000. However, some definitions have been added and explanatory notes expanded or added.

ISO/IEC 90003:2004 [182] provides guidance for organizations in the application of ISO 9001:2000 to the acquisition, supply, development, operation, and maintenance of computer software and related support services. It replaces the old ISO 9000-3 1997 software standard. The American Society for Quality (ASQ) published ANSI/ISO/ASQ Q9000-2000 Series—*Quality Management Standards* [183], which are American National Standards on quality management and quality assurance that are internationally recognized as being identical to the ISO 9000:2000 quality standards.

The ISO/IEC Guide 25, *General Requirements for the Competence of Calibration and Testing Laboratories* [184], has been the internationally recognized basic document for accreditation of laboratories including chemical analysis. The attainment of accreditation is mandatory for some regulatory work areas and frequently is the basis of contracts for analytical work. In 2000 the draft was replaced by ISO/IEC DIS 17025, *General Requirements for the Competence of Testing and Calibration Laboratories* [185]. A new version of ISO/IEC 17025 was issued on 15 May 2005 (ISO/IEC 17025:2005) [186]. The American Association for Laboratory Accreditation (A2LA) provides accreditation and certification in accordance with ISO/IEC 17025:2005.

The ISO 17025 standard states that if testing and calibration laboratories comply with ISO 17025, they also operate in accordance with ISO 9001 or ISO 9002. However, calibration against ISO 9001 and 9002 does not itself demonstrate the competence of the laboratory to produce technically valid data and results. While ISO/IEC Guide 25 was mainly focused on technical controls, the new guide includes more management and administrative controls from the ISO 9001 and ISO 9002 quality standards. ISO/IEC 17025 is the most specific document for quality systems for laboratories. The U.S. FDA is implementing it in their labs and all regulated

laboratories may look at ISO/IEC 17025 as a basis for their operation as many elements will also be subject to FDA audits.

The impact of ISO/IEC 17025 on analytical instrumentation and test methods is similar to the impact GLP and GMP regulations have. Instrument hardware should be calibrated, tested, and verified for performance, and it should be maintained to assure a continuous performance. The performance of hardware should be periodically reverified according to a well documented schedule. Software should be validated by the user or vendor firm and verified by the end-user for proper performance. Analytical methods must be validated if nonstandard methods are used.

Specific to some extent to ISO 9000 and ISO/IEC 17025 is the requirement to trace calibration devices such as reference standards or reference test devices back to national or international standards. All tools used for calibration and performance verification of instruments should be well maintained and calibrated. Training records should be kept for all individuals who perform the calibration and verification.

5.3.4 Good Laboratory Practice (GLP)

Analytical laboratories should follow GLPs for all preclinical drug development studies.

The basic GLP requirements for analytical laboratories include the following:

- Established, controlled, *standard operating procedures* (SOPs) for sample handling, instrument operation, test methods, data handling, and analyst training.
- Use of *labeled, traceable reagents* properly stored and used within expiration dates.
- Use of *traceable reference standards* as testing controls.
- Maintenance of *controlled, complete laboratory notebooks*, including raw data archiving and retrieval systems.
- Having *calibrated, regularly maintained equipment* for every step in sample storage, handling, and testing.
- *Documentation* of all activities performed in operations related to GLP samples, including personnel qualifications and training records.

Attention to five steps of data acquisition for product analysis can help to focus on the goals of compliance activities: analyses should be planned, performed, monitored, recorded, and reported. Viewing these as distinct activities can greatly enhance a facility's ability to follow GLPs.

1. *Planning.* Designate a study protocol and a study director. The protocol should include descriptions of the nature and purpose of the study, sample information, dates, test methods used, justifications, and references. Any modifications to the protocol must be signed and dated. The study director is the party responsible for adhering to compliance activities. Contract laboratories should designate a study director to coordinate efforts with the client's in-house preclinical study director.

2. *Performing.* Facilities where the analytical work is done should be of suitable size, design, and construction for analyses. SOPs should describe all aspects of sample handling (such as receipt, identification, labeling, and storage) and instrument use (such as maintenance, calibration, and monitoring—including refrigerators, freezers, water baths, incubators, etc.). Equipment should be of adequate capacity and suitably located. Personnel should be experienced in the methods they perform and have documented training.

3. *Monitoring.* Analysis systems need to be examined by a quality assurance unit, who will review database information, data analysis, and the final report. A statement should be attached to the final report documenting review by this unit.

4. *Recording.* This includes documentation, data generation and analysis, and data storage and retrieval. It includes everything from the study protocol, procedures, final reports, and audits, to personnel qualifications, equipment records, and sample records.

5. *Reporting.* A final study report should be delivered to the client, describing the nature and purpose of the study, the protocols used, the samples tested, the results, references to raw data (including computer files), and any applicable literature references. All data should be signed and dated, with corrections in the protocol or report included as amendments.

5.3.5 Method Validation Protocol

A validation plan is a written plan stating how validation will be conducted, including test parameters, product characteristics, production equipment, and decision points on what constitutes acceptable results. USP Chapter 1225 [174] on validation of analytical methods specifically addresses terms and definitions, but leaves protocol and methodology open for interpretation. The ICH guidelines on method validation methodology, ICH Q2A and ICH Q2B [158, 159], and the U.S. FDA *Guidance for Industry: Bioanalytical Method Validation* [161] fill this gap. Full or partial validation may be required. Full validation (usually on three different days) is performed when developing and implementing a method for the first time, when a new drug entity is used, and when metabolites are added to an existing assay for quantification. Partial validation (may be performed in 1–2 days) is used for modification of already validated methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to nearly full validation. Before outlining an experimental design or protocol, however, it is necessary to make some basic assumptions. These assumptions include:

1. Selectivity has been previously demonstrated or is measured and documented during the course of the validation protocol.
2. The method has been developed and optimized to the point where it makes sense investing time and effort in validating the method. Indeed, robustness should be the first parameter investigated.
3. Once data are generated, statistically valid approaches are used to evaluate it and make decisions, thus removing some of the subjectivity of method validation.

Given that the above three steps are addressed in one way or another, the following stepwise protocol can be proposed for method validation (each specific protocol depends on the nature of the method).

Step 1. On day 1, a linearity test (except for LBAs) over five levels for both the drug substance and dosage form is performed. Comparison of the results between the drug substance and dosage form fulfills the accuracy requirement.

Step 2. At the end of day 1, six repetitions are performed at 100% of the drug substance for repeatability.

Step 3. Steps 1 and 2 are repeated over two additional days for intermediate precision.

Step 4. LLOQ is evaluated (as needed) by analyzing the drug substance over five levels, plus six repetitions for precision.

Step 5. Baseline noise is evaluated over six repetitions of blank injections for the determination of LLOD (if required).

In this manner, a logical stepwise approach to method validation using ICH methodology can be performed. As stated previously, the use of the appropriate statistical tests (Students-*t*, Cochran, Dixon, and Fisher tests) allows for less subjective decisions to be made regarding the data, reducing method validation to a much more objective undertaking. For example, the Grubbs Procedure described in Ref. [187] may be used to exclude outliers. A rigorous use of statistics may in turn lend itself to some degree of automation in the future.

5.4 CONCLUSION

Analytical technologies continue to evolve, leading to further improvements in sensitivity and selectivity. There are many analytical tools (instrumentations, techniques, computations) presently available to analysts. Selection of tools to be used should be based on the nature of the analyte(s), analytical goals (sensitivity, selectivity, etc.), sample matrix, and sample stability. Tools should not be used blindly but with an analyst's understanding of theory, techniques, instrumentation, and their inherent limitations. While high throughput is desirable, analyses should not become only a matter of inputting samples into analytical machines and merely accepting numerical machine output. Analytical methods need to be subject to appropriate method validation (based on requirements of the most recent guidelines), including periodic instrument testing and calibration, and should incorporate appropriate quality control measures. In addition, stability of analyte(s) needs to be considered from the time of sample collection through final analytical measurement.

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6

CHEMICAL AND PHYSICAL CHARACTERIZATIONS OF POTENTIAL NEW CHEMICAL ENTITY

ADEGOKE ADENIJI AND ADEBOYE ADEJARE*
Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, Philadelphia, Pennsylvania

Contents

6.1 INTRODUCTION

A new chemical entity (NCE) is a compound that has not been approved as a pharmaceutical agent for a particular indication. In the United States, the approving body is the Food and Drug Administration (FDA) and most countries have a similar agency [1]. The compound may be an already approved drug being evaluated for a

**Corresponding author*.

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new indication or a novel compound. New regulations, the ever increasing demand for drugs worldwide, and an unprecedented need to drive drug prices lower challenge the pharmaceutical companies and point to the need to put more lead compounds into the development pipeline [2]. While these chemical entities are constantly being synthesized or isolated from natural sources in various academic and industrial laboratories throughout the world, very few compounds make it to the market as drugs. It is estimated that only one out of every 5000 synthesized compounds ends up on the market as a therapeutically useful drug [3].

Elucidation of the chemical and physical properties of a potential NCE is critical to drug discovery efforts as part of "pharmaceutical profiling." Knowledge of these properties could give insight into how the compound acts or binds to the target receptor to elicit desired or undesired effects and also help with formulation studies [4, 5]. Based on these properties, some compounds could be dismissed as possible drug candidates. For instance, a compound being proposed for intravenous administration that has very poor aqueous solubility might not be favorably considered because of envisaged problems with formulation and bioavailability. Indeed, the fate of many NCEs are decided immediately after the chemical and physical properties of the compounds are determined. These properties often serve as indices by which a compound will be judged for its "drugability" and likelihood of eliciting the desired response. Thus, the journey to the market for a compound that becomes a drug begins with determination of its basic chemical and physical properties such as the mass, structure, solubility, and stability. Further physicochemical characterizations, for example, if the compound has different polymorphs, may follow should the compound exhibit a desired biological profile. However, those may not be warranted if a "cut" decision is made at an earlier stage. A stage-appropriate characterization is therefore critical for every potential NCE for maximization of resources. The most common chemical and physical characterization methods for a novel potential NCE and two examples will be described. If the potential NCE is already approved for an indication, then many pertinent chemical and physical properties would have been determined and are likely to be in literature. This brief chapter focuses on small molecules although many of the methods described have been adapted to characterize therapeutic proteins and other macromolecules.

6.2 DETERMINATION OF MASS

Most drugs are small molecules with mass in the range 100–1000 amu. There are, however, notable exceptions such as lithium salts at the lower end, while peptides, proteins, and antibodies being used in therapy could have masses much greater than 1000 amu. The mass of a compound is the sum of the atomic masses of its constituent elements. It is generally expected that a non-ionic compound with low molecular mass will be absorbed more readily than one with high mass under similar conditions, such as being in the same chemical class and dosage form. This is particularly true for orally administered drugs. Lipinski's rule of five [6, 7] indicates that a molecular mass less than or equal to 500 is essential for passive absorption of an orally administered drug. There are several methods used to determine the molecular mass of a compound. These methods include vapor density, vapor pressure osmometry, cryoscopy, elemental analysis, and undoubtedly the most popular and

important, mass spectrometry [8–11]. Mass spectrometry (MS) and elemental analysis are the two major techniques used in mass determination. MS is a very versatile tool that is applicable in several fields of science and new applications are being found. It involves a high energy bombardment of the molecule of interest to generate charged fragments, which are detected based on the mass/charge ratio. The mass of the compound is established by the mass of the molecular ion or those of its fragments. The molecular formula and the structure of the compound can also be obtained by studying the fragmentation pattern of the compound [9, 10]. MS has also been widely used in determining the structures of proteins and other macromolecules. Quite often, the molecular masses of such peptides and proteins can be inferred from fragment ions.

Elemental analysis, on the other hand, determines the composition of a sample by giving the amount (usually percentage weight) of each element that is present in the sample. It is often based on oxidation of an organic compound at elevated temperatures to produce gaseous molecules of the elements of interest, which are then separated and analyzed [11]. It is very useful as it gives values from which the empirical formula of the compound can be calculated. The molecular formula can then be determined from the empirical formula and molecular weight. By determining the proportion of the constituent elements of a compound, the purity of the compound can also be established.

6.3 STRUCTURE ELUCIDATION

Most drugs are weakly acidic or basic organic compounds [12]. The determination of structure helps to identify the functional groups on the compound, validate the synthetic or isolation pathway, and initiate structure–activity relationship studies that are very important in drug discovery [13]. It also helps to determine physicochemical parameters. The process of structure elucidation involves determining the elements present and their bonding arrangements in the compound and establishes the identity of the chemical entity. Several methods are used for structure elucidation, most of which are spectroscopic. Of these, nuclear magnetic resonance (NMR) is the most versatile and is thus a key tool in determining structures of molecules. NMR exploits the ability of certain nuclei to align themselves either with or against the field when placed in a magnetic field. Since the effective magnetic field experienced by these nuclei is influenced by the electronic environment in their proximity (e.g., the presence of a neighboring strongly electronegative atom), information about atom connectivity can be deduced from spectra analysis [14]. Both ^{1}H- and ^{13}C-NMR are widely used but several other nuclei such as ^{19}F and ^{31}P are also utilized [14–16].

Ultraviolet/Visible (UV/Vis) spectroscopy and particularly infrared (IR) spectroscopy are also widely used. UV/Vis spectroscopy gives information about the presence of chromophores (i.e., conjugated double bonds), which are responsible for selective absorption of radiation in a given wavelength range [14]. IR spectroscopy gives information about functional groups in the molecule. Both methods are based on the ability of compounds to absorb a fraction of incident radiation. In IR spectroscopy, the absorption frequency confirms the presence of particular functional groups (e.g., carbonyl, hydroxyl, and aryl) since these values are consistent

and only slightly influenced by adjacent groups in the molecule. There are charts and tables in the literature that list the absorption frequencies of common organic functional groups and they can be used as reference. The electronic collection of spectra and the availability of spectra simulating software have increased the usefulness of these and other spectroscopic techniques.

Polarimetry measures the ability of a sample to rotate plane polarized light. It is critical for the characterization of optically active or chiral compounds. The specific rotation of a chemical compound. $[\alpha]_D^T$ is defined as the observed angle of rotation α, when light of 589 nm wavelength (the sodium D line) is passed through a sample with a pathlength of 0.1 m and a sample concentration of 1 g/ml at temperature T. It is usually measured at 20 °C. It is applicable for both qualitative and quantitative purposes since the extent of light rotation is dependent on the molecular structure and the concentration of the sample. Thus, it can be used to determine enantiomeric purity of a sample.

X-ray crystallography gives vital information about the crystal structure of a compound using diffraction techniques. It allows for measurement of atomic connectivity, bond lengths, bond angles, and torsion angles, thereby giving very detailed information about the structure of a compound [14]. The compounds, however, need to be crystallizable, which is sometimes very difficult, especially for large macromolecules such as protein and DNA complexes. For small molecules, it can be used to determine absolute configurations at chiral centers. This can become very important in discovery efforts as it can help explain compound reactivity and binding to receptors (proteins), which contain many chiral centers.

6.4 PURITY

The presence of impurities in an NCE can not only affect the physicochemical properties but could also preclude correct assessment of the bioactivity and toxicity profiles of the target compound. It is generally desirable that the compound be available in a very pure form before incorporation into any dosage form for *in vitro* or *in vivo* studies. Several methods including some that have been discussed earlier can be used to assess the purity of a compound. These methods include NMR, mass spectrometry, chromatography, UV spectroscopy, IR spectroscopy, and polarimetry. However, in many cases, the very first test to determine the purity is melting point (mp) for solids and boiling point (bp) for liquids. A broad range of values for either parameter is usually indicative of impurity [17]. The mp and bp as well as the refractive index and density are defining characteristics of a compound. Refractive index measures the ratio of the velocity of light in air to that in the sample at a particular wavelength, temperature, and pressure.

Chromatography separates mixtures based on the different rates of movement of the components along an adsorbent medium. Ever since its invention by the Russian botanist Mikhail Tswett in the early twentieth century [18], its application has increased tremendously to earn it a place of pride in analytical science. From the relatively simple thin layer chromatography (TLC) to the more technical gas chromatography (GC) and high performance liquid chromatography (HPLC), chromatographic techniques allow for separation of the components of a sample based on the ease of mobility through a stationary phase. The presence of a single band

in TLC or a single peak in HPLC or GC suggests high purity of the new compound. Various detectors can be used, from UV to fluorescence to MS. Chromatographic techniques are used not only to determine purity but also in purification [19]. The high sensitivity shown by the low limit of detection of these techniques has been employed in quantitative compound determination in plasma and other matrices, where they have been used to detect nanogram/milliliter concentrations of drugs [20, 21]. A major advantage of chromatographic techniques is that they can be adapted for quantitative determinations and/or joined with other techniques to give hyphenated methods (e.g., LC-MS). HPLC has also been used in determining purity of enantiomers [22, 23]. In such cases, a chiral column or derivatization technique to give diastereomers is employed.

6.5 SOLUBILITY AND LIPOPHILICITY

Solubility of a sample in a solvent refers to the amount of the sample that will dissolve in the given solvent at a particular temperature. It is generally desirable for a drug molecule to have some level of water solubility. Poor water solubility can present many problems from reliability and reproducibility of several *in vitro* tests to significant formulation issues [7, 24]. Solubility is typically measured using the shake flask method. The shake flask method involves adding an excess amount of a solid sample to a flask containing a specified amount of buffer; the saturated solution is then agitated until it attains equilibrium, after which the undissolved sample is separated from the solution and the concentration of the sample is determined usually by UV spectroscopy or another applicable technique [25].

Since most drugs are weak acids or bases [11], the pK_a is an important parameter used to study and predict the behavior of an NCE in a physiological environment. The pK_a or *dissociation constant* is a measure of the acid strength of a compound. It allows for determination of the predominant state of an ionizable compound at any given pH and therefore affects solubility of the compound. In general, aqueous solubility increases with ionization. The pK_a can be determined using several methods such as capillary electrophoresis, liquid chromatography, and potentiometry.

The relevant equations are

$$\mathrm{p}K_a = -\log K_a \tag{6.1}$$

Acids: $HA \rightleftharpoons H^+ + A^-$

$$K_a = [H^+][A^-]/[HA], \quad \text{where } [\,] = \text{molar concentration} \tag{6.2}$$

Bases: $HB^+ \rightleftharpoons H^+ + B$

$$K_a = [H^+][B]/[HB^+] \tag{6.3}$$

Lipophilicity refers to the tendency of a compound to partition into nonpolar (organic) versus aqueous environments [26]. Log *P* refers to the logarithm of the partition coefficient and it is the most common measure of lipophilicity of a compound. The partition coefficient is a constant that defines the ratio of a compound in aqueous medium to the compound in an immiscible solvent at equilibrium. The

most commonly used organic solvent for biological purposes is 2-octanol. The partition coefficient is probably one of the most important physicochemical properties that infer the crossing of a drug across biological membranes, binding to proteins, and, consequently, the activity and the ease of excretion from the body. The partition coefficient has been shown to correlate with the activity of many classes of drugs [27, 28]. A compound with log $P > 5$ is usually not considered a good candidate in drug development [6, 7] because it is considered to be too lipophilic.

$$\log P = \log_{10}(\text{Partition coefficient}) \tag{6.4}$$

where Partition coefficient, P = [Organic]/[Aqueous].

It is generally desirable for an NCE to possess enough water solubility so that it can dissolve in body fluids while also possessing sufficient lipophilicity to cross biological membranes. Log P describes the partitioning for a neutral and un-ionizable molecule, which is often not the case. For ionizable compounds, log P is expected to change with pH. The relationship between log P and pH is described by the log distribution coefficient (log D), also called the apparent partition coefficient. Log D, unlike log P, changes with pH as it depends on the acidic or basic nature of the compound. The log D at pH 7.4 is often used as an index of the behavior of a sample in plasma.

$$\text{Distribution coefficient, } D = [\text{Un-ionized}]_{(o)}/[\text{Un-ionized}]_{(aq)} + [\text{Ionized}]_{(aq)} \tag{6.5}$$

$$\log D = \log_{10}(\text{Distribution coefficient}) \tag{6.6}$$

$$\log D_{(\text{pH})} = \log P - \log(1 + 10^{(\text{pH} - \text{p}K_a)}) \quad \text{for acids} \tag{6.7}$$

$$\log D_{(\text{pH})} = \log P - \log(1 + 10^{(\text{p}K_a - \text{pH})}) \quad \text{for bases} \tag{6.8}$$

6.6 STABILITY

Physical and chemical stability considerations become essential to estimate how long the NCE can be stored without significant decomposition. Stability is usually determined by monitoring particular properties of the compound over a period of time, usually days or weeks and sometimes months, under specified storage conditions, which are often designed to be more unfavorable (stress testing) than the envisioned storage conditions [29, 30]. The stability of the compound is assessed in the presence of elevated temperature, light, high relative humidity (75% or higher), varying pH range, and an oxidative environment.

6.7 PROTEINS AND PEPTIDES

Peptides usually exhibit poor *in vivo* stability, pharmacokinetics, and bioavailability. These often pose a major challenge to drug discovery and development efforts. Therefore, most drugs are small molecules or peptidomimetics [31]. With advances in drug delivery and more sophisticated equipment, an increasing number of peptides and proteins are being used in therapy [32, 33].

6.8 MISCELLANEOUS TECHNIQUES

There are many other techniques used to decipher other chemical and/or physical properties of an NCE. Examples include circular dichroism (CD), Raman spectroscopy, and differential scanning calorimetry (DSC). CD is a type of absorption spectroscopy often used to determine optical properties and secondary structures of molecules. While it can be used for any optically active molecule, it is more critical in cases of biomolecules such as sugars, amino acids, peptides, and proteins when embarking on structural determination. It measures the difference in the absorption of right- and left-circularly polarized light as a function of wavelength. It is used extensively to study secondary protein structures: how the secondary structure of a molecule changes as a function of temperature or the concentration of denaturing agents, thus making it a vital tool in assessing the physical and chemical stability of peptides and proteins. Raman spectroscopy is a technique that is used to study the vibrational, rotational, and low frequency modes in a system. Its use derives from the Raman effect, which is the inelastic scatter of photons following light incidence. Its principal use is to identify a compound based on a fingerprint that is unique to the compound, since vibrational information is very specific for chemical bonds in molecules. The fingerprint region of organic molecules is in the range of 500–2000 cm^{-1}. More uses are being discovered for MS. High resolution MS is being accepted as a substitute for elemental analysis by several journals. Purity of amines is sometimes difficult to determine using chromatographic techniques. An MS-based technique that can help identify amine impurities using hydrogen/deuterium exchange has been developed [34].

DSC is a thermoanalytical method that measures the difference in heat absorbed by a chemical compound and a reference as a function of temperature when the two are subjected to the same regulated temperature conditions. It measures the changes in heat flow as the sample and reference undergo any physical transformation, such as phase transition. It is related to several parameters of a compound and is thus used mainly to establish identity and purity of chemical compounds among other uses such as polymorph characterization [35, 36].

6.9 CASE STUDIES

For illustration, we now examine the chemical and physical characterizations of two new potential NCEs.

Example A. 4-Fluoro-*N*-(adamantan-2-yl)-benzenesulfonamide (Fig. 6.1) is a novel compound that was designed and synthesized as a gamma secretase inhibitor [37, 38]. Gamma secretase is an enzyme involved in amyloid precursor protein processing to give amyloid peptides, which are the building blocks of the plaques found in the brains of patients with Alzheimer's disease (AD). It has been hypothesized that gamma secretase inhibitors can halt the onset and/or progression of AD. After synthesis, the melting point for the target compound was determined to be 150–152 °C. The narrow range suggested good purity for the sample. Elucidation of the structure was carried out using IR spectroscopy and NMR, the results of which

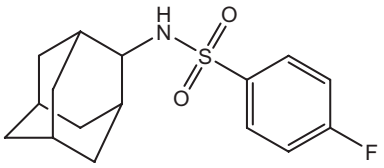


FIGURE 6.1 Structure of 4-fluoro-*N*-(adamantan-2-yl)-benzenesulfonamide.

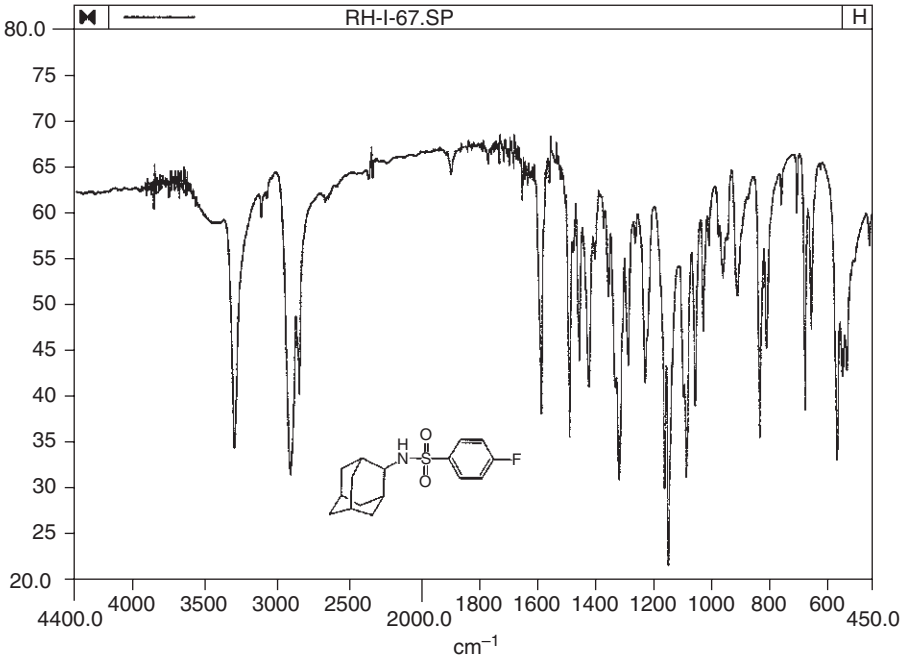


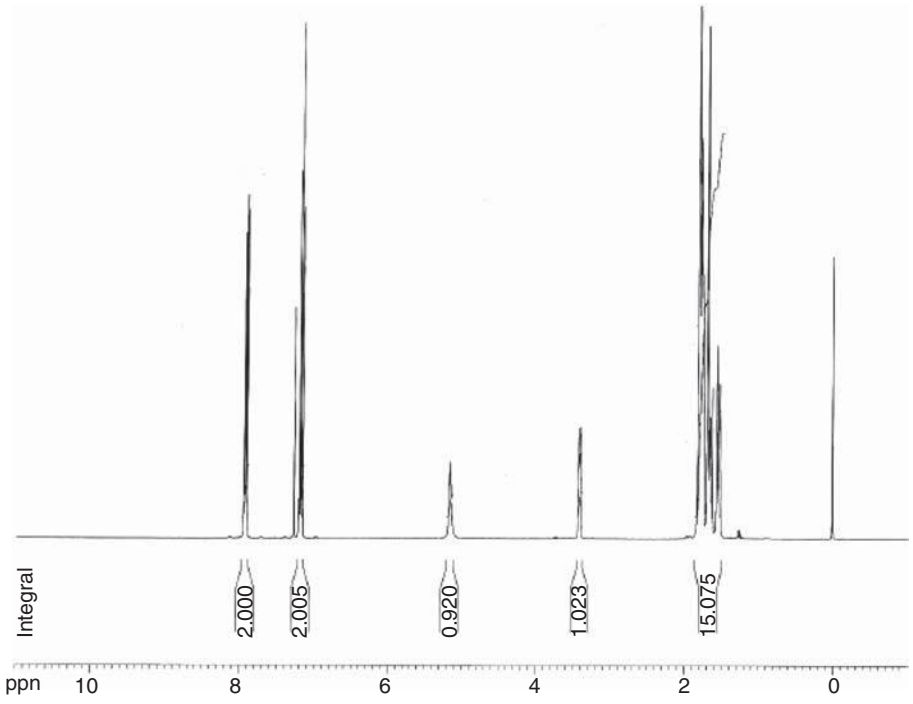
FIGURE 6.2 FT-IR spectrum of 4-fluoro-*N*-(adamantan-2-yl)-benzenesulfonamide in KBr.

confirmed the presence of the expected functional groups. IR (KBr) cm^{-1}: 3300 (NH); 1322, 1166 (O=S=O); 1234 (Ar—F) (Fig. 6.2). The ^{1}H-, ^{13}C-, and ^{19}F-NMR results further confirmed the presence of the expected functional groups; the results are shown in Fig. 6.3. The molecular mass determination using mass spectrometry and elemental analysis, the results of which are shown below, also support the proposed structure of the compound.

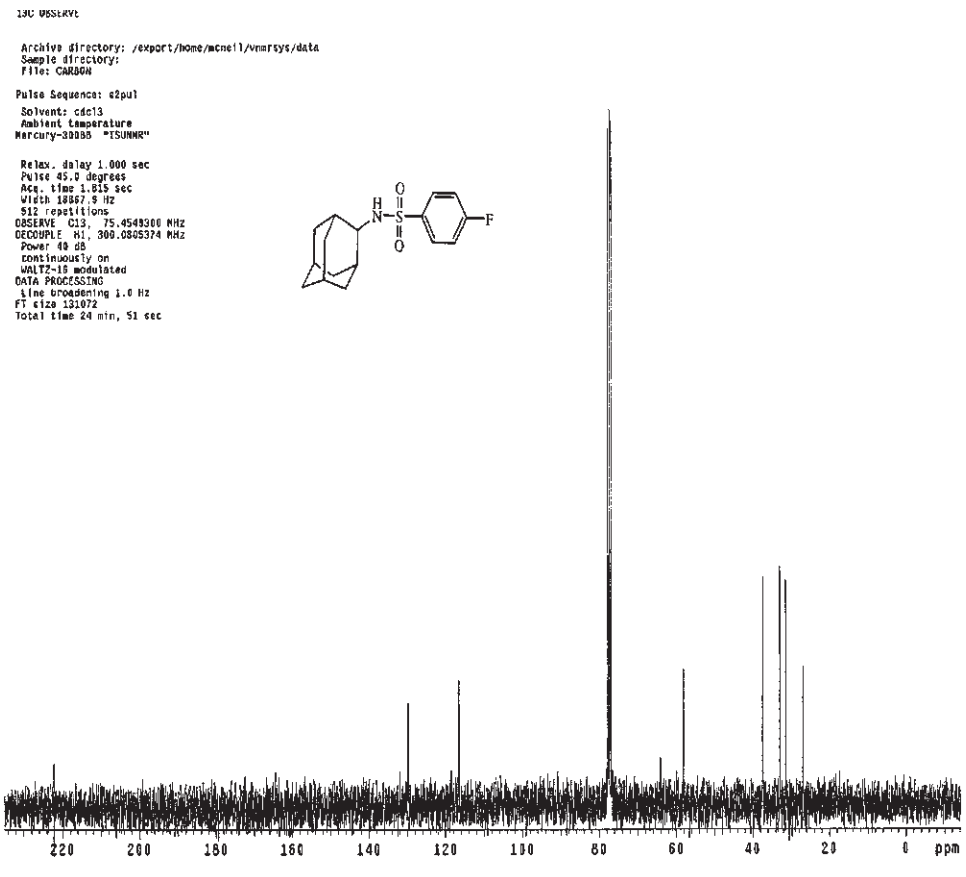
MS ESI (*m/z*, species, %): 308.3, $[M—H]^-$, 100% as shown in Fig. 6.4.

The elemental analysis of the compound showed the proportion of the constituent elements to be the following. Calculation for $C_{16}H_{20}FNO_2S$: C, 62.11; H, 6.52; F, 6.14; N, 4.53. Found: C, 62.06; H, 6.49; F, 6.43; N, 4.37. The "found" is within ±0.4 of the calculated values and thus consistent with the proposed structure.

^{1}H-NMR ($CDCl_3$) δ: 7.9 (s, 2H, 2 × Ar—CH), 7.4–7.1 (d, 2H, 2 × Ar—H), 4.9 (s, 1H, 1 × NH), 3.4 (s, 1H, 1 × CH), 1.8–1.5 (m, 15H, 5 × CH_2, 5 × CH).

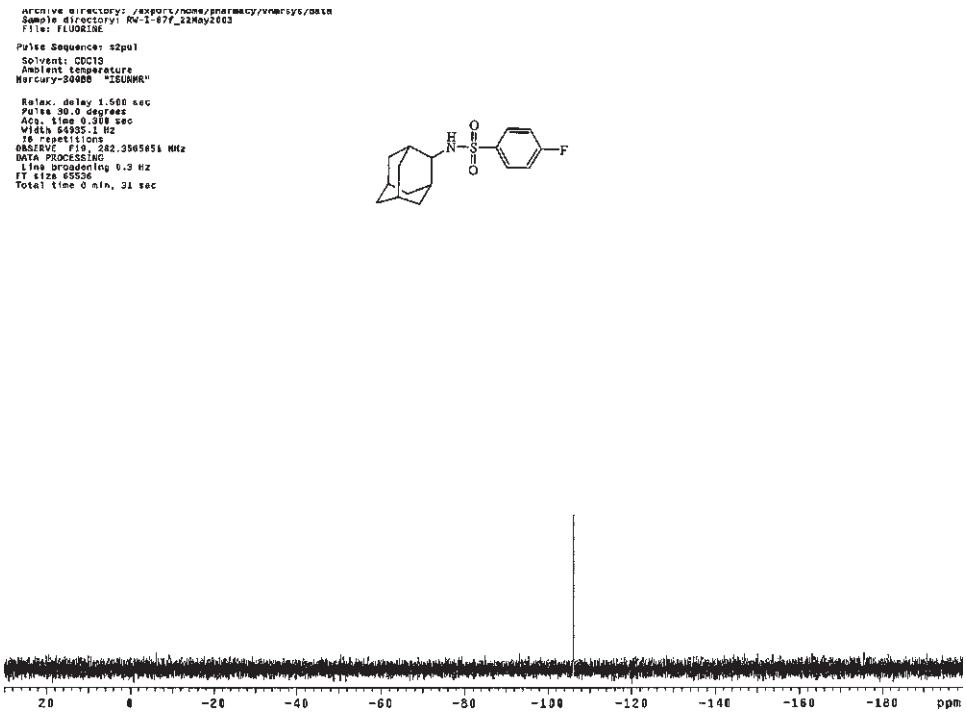


(a)



(b)

FIGURE 6.3 (a) H-NMR, (b) ^{13}C-NMR, and (c) ^{19}F-NMR of 4-fluoro-*N*-(adamantan-2-yl)-benzenesulfonamide in $CDCl_3$.



(c)

FIGURE 6.3 *Continued*

^{13}C-NMR ($CDCl_3$) δ: 130 (s, 2C, 1 × Ar—CF, 1 × Ar—CSO_2), 116 (s, 4C, 4 × Ar—CH), 58 (s, 1C, 1 × CHN), 37 (s, 2C, 2 × CH), 33 (s, 2C, 2 × CH), 31 (s, 4C, 4 × CH_2), 27 (s, 1C, 1 × CH_2).

The equilibrium solubility of this compound in water and phosphate buffers (pH 7.4 and 11.50) were determined at 23.0 ± 1.0 °C by dissolving the compound in 2 mL of the appropriate solvent, shaking, and then centrifuging at 220 rpm for 48 h. The concentration of the drug was measured by UV spectrophotometry.

Determination of the pK_a was conducted using the shake flask method, while the log *P*(octanol/water) was determined using both the shake flask method and HPLC with UV detection. It was also calculated using commercially available software, ChemDraw Ultra®. The agreement of the values gives some confidence in terms of getting a good handle on the lipophilicity of the compound. The results of the solubility, pK_a, and log *P* determination are given in Table 6.1.

In summary, the compound possesses adequate water solubility to dissolve in intestinal fluid and the partition coefficient of 3.4 suggests that the compound possesses acceptable lipophilicity. The compound also has molecular weight of 309.4. It has only one hydrogen bond (HB) donor group (less than the maximum of 5) and less than 10 hydrogen bond acceptor groups, with a generous count reflecting 4. Based on Lipinski's rule of five (Fig. 6.5), one could conclude that this compound

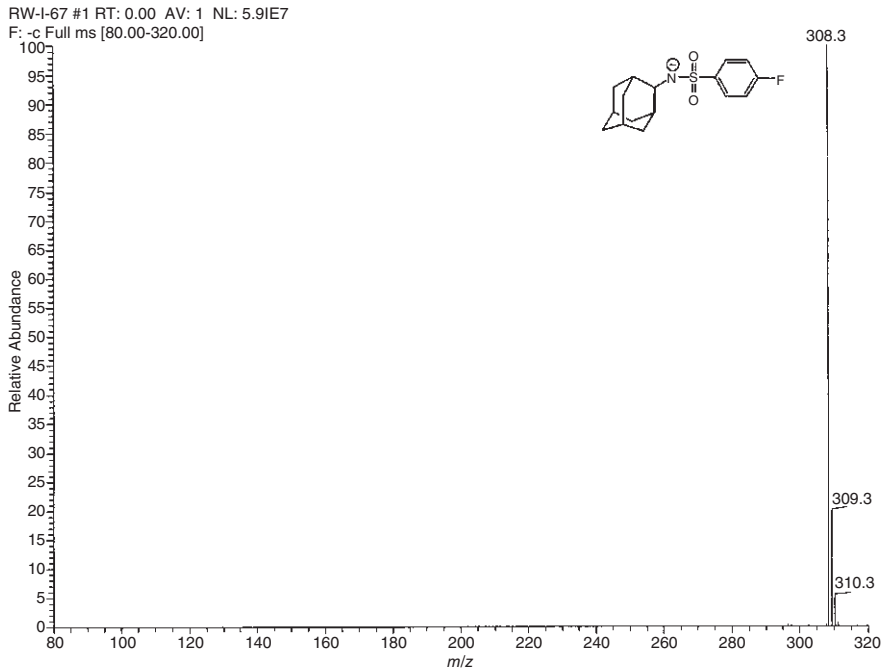


FIGURE 6.4 Electrospray mass spectrum of 4-fluoro-*N*-(adamantan-2-yl)-benzenesulfonamide *m*/*z* 308.3, in MeOH.

TABLE 6.1 Solubility, log *P*, and pK_a of 4-Fluoro-*N*-(adamantan-2-yl)-benzenesulfonamide

Solubility (23.0 ± 1.0 °C) (μg/mL)	
Water	120 ± 50
Phosphate buffer (pH 7.4)	200 ± 30
Phosphate buffer (pH 11.50)	270 ± 60
Log partition coefficients	
Calculated	3.44
Shake flask method	3.36 ± 0.16
HPLC	3.31 ± 0.01
pK_a	10.36 ± 0.11

falls "inside the box" and thus has the appropriate physicochemical properties necessary for absorption in an oral formulation.

Example B (2*R*)-3-(4-fluoro-1-naphthyloxy)-1-(*tert*-butyl-amino) 2-propanol (Fig. 6.6) was synthesized to determine the effect of fluorine substitution on the aromatic ring on adrenergic receptor activities [23, 39]. Similar compounds are potent beta-adrenergic receptor antagonists and are used in the control of blood pressure. Upon completion of synthesis, the melting point of the free base was found to be 56–57 °C, while that of the hydrochloride salt was 165–166 °C. The sharp melting points were indicative of good purity. The structure of the compound was determined using data

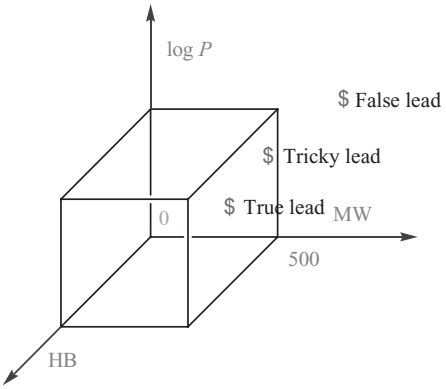


FIGURE 6.5 Optimal properties for orally active compounds are found within the "log *P*-HB-MW" box.

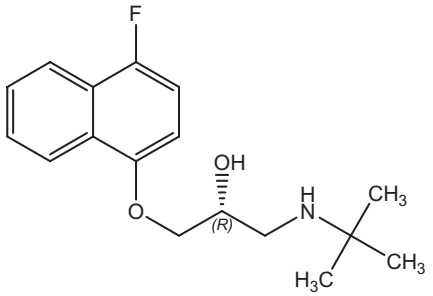


FIGURE 6.6 Structure of (2*R*)-3-(4-fluoro-1-naphthyloxy)-1-(*tert*-butyl-amino) 2-propanol.

from the proton NMR analysis of the sample: ^{1}H-NMR ($CDCl_3$) δ: 8.27–7.40 (m, 4H, aryl H), 7.0–7.10 (t, 1H aryl H), 6.75–6.65 (dd, 1H aryl H), 4.10 (s 2H, C**H**$_2$CHOH), 2.95–2.40 (m, 3H, C**H**OHC**H**$_2$N), 1.11 (s, 9H, C(C**H**$_3$)$_3$). This was supported by the molecular mass determination using mass spectrometry. The base peak was at 292 (M + 1) with the next major peak being 236 (M + 1 – 56), which is due to the cleavage of the *t*-butyl group. The elemental analysis of the hydrochloride salt gave $C_{17}H_{23}NFO_2Cl$, which further supported the structure proposed. The optical rotation values of the free base and hydrochloride salt were determined to be $[\alpha]_D^{25} = +3.71°$ (c = 1.96, EtOH) and $[\alpha]_D^{25} = +13.2°$ (c = 0.81, EtOH), respectively. Enantiomeric purity of the compound was evaluated by HPLC with UV detection. There was a major peak at 3.7 minutes for the *R*-enantiomer (98%) and a very minor peak at 3.15 minutes for the *S*-enantiomer.

6.10 CONCLUSION

As the cost of drug discovery and development continues to rise, the need to make important "cut" decisions as early in the process as possible is more pertinent now than ever. Along with potency and efficacy of the compound to elicit the desired

biological effects, the importance of determining appropriate chemical and physical properties cannot be overemphasized, as they could play a key role in the "cut" decisions. Advances in technology have produced several methods, many of which have been highlighted in this chapter and are at the disposal of the medicinal chemist to characterize a potential new chemical entity.

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