18 Leveraging Advances in HPLC and Sample Preparation to Maximize DMPK Data Quality

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18.1 SUMMARY

The reliable reporting of data from the quantitative analysis of drugs and their metabolites is the backbone of drug discovery and development; therefore, the bioanalytical methods that produce the data should be sufficiently accurate and reproducible. This chapter provides an overview of different technologies that offer opportunities to improve the data quality and efficiency of method development, method validation, and biological sample analysis.

The regulatory requirements of data quality are reviewed. Specifically, regulators require that bioanalytical methods be validated to ensure accurate and precise results. The quality of an analytical method is normally assessed by the performance of standards and quality control samples (QCs). Current regulatory guidance recommends an acceptance criterion of 15% for accuracy and precision of all standards and QCs, with the exception of the lower limit of quantitation (LLOQ), where the acceptance criterion is increased to a 20% deviation.

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Technologies that improve sample cleanup are reviewed because data quality is the sum of effective sample preparation and appropriate chromatographic separation of endogenous matrix components. In other words, poor sample preparation cannot be overcome by high performance liquid chromatography (HPLC). Inadequate sample cleanup and chromatography often leads to matrix effects that are revealed by irreproducible results for some bioanalytical methods. Plasma phospholipids have been identified as playing a major role in causing matrix effects in liquid chromatography-mass spectrometry (LC-MS) bioanalysis. Therefore, in order to minimize the potential for matrix effects, the removal of phospholipids is an important component of any sample cleanup process. Moreover, the process of sample extraction is often a rate-limiting step in the workflow associated with quantitative bioanalysis.

Of the three commonly used extraction techniques for plasma or serum samples, protein precipitation ("dilute and shoot") is the least suited for assays that require low levels of quantitation because of its susceptibility to ion suppression and matrix effects from remaining endogenous components. Liquid–liquid extraction and solid-phase extraction (SPE) are techniques that offer much cleaner sample extracts that in turn serve to make the method more robust and scalable. SPE is particularly powerful in this regard because of its unique ability to utilize a variety of retention mechanisms. The most widely used SPE sorbent phases are those that are based on retention and elution mechanisms that involve polar and/or nonpolar interaction mechanisms. To fulfill the requirements of selectivity and retention, vendors now offer mixed-mode sorbents that afford hydrophobic and hydrophilic interactions in addition to ion exchange. With mixed-mode strong cation- or anion-exchange SPE, significant cleanup is achieved during the 100% organic wash step, where phospholipids bound by reversed-phase interactions are removed along with any other neutral hydrophobic interference.

To reduce time and effort, on-line sample preparation (direct sample injection), using either turbulent flow chromatography (TFC) columns or single-use SPE-packed cartridges, is a viable alternative to off-line sample preparation.

A practical review of chromatographic principles is provided. It is important to maintain chromatographic resolution as flow rates are increased or column particle sizes are decreased; otherwise, no improvement in productivity is realized. The long-term trend toward ever smaller column particle sizes is an effort to achieve the same performance as is possible with larger particles but in a shorter analysis time. The development and application of small particles (sub-2-μm particles) and fused-core (pelicular) high resolution columns is discussed.

Although increasing resolution by using smaller particles is a common approach to enabling faster separations, selectivity has the greatest effect on resolution. Selectivity is governed predominantly by analyte interactions with both the stationary and mobile phases. Of these, the stationary phase is the most important for chromatographic resolution. Selectivity differences may be brought about by changes in hydrogen bonding, dipole–dipole interactions, and/or ionic interactions with the stationary phase. The ionization state of an analyte resulting from the pH of the mobile phase may also alter the degree of interaction with the stationary phase. Column temperature is another easily adjustable parameter that can provide alternative selectivity leading to increased resolution and an overall reduction in analysis times. The conformational changes that occur with long-chain alkyl stationary phases as a function of temperature can produce selectivity differences among analytes.

Parallel or multiplexed LC-MS/MS systems that can analyze the eluent from several LC systems using a single mass spectrometer are a solution to support high throughput production of quality data. Multiplexed systems with ultrahigh performance capable chromatography systems are available. Included in some systems is the ability to alternate between on-line extraction methods and conventional LC methods.

The presence of unidentified metabolites can lead to inaccurate measurement of analyte concentrations because of ionization suppression, interference in peak integration, or, most problematic, in-source regeneration of the parent drug from phase II metabolites, as the metabolites are not present in the calibration standards and QCs. The importance of chromatographic separation in quantitative analysis should never be overlooked.

Minimizing the risk factors that can affect method reproducibility is an important aspect of developing a quality method. Developing a quality method requires that sufficient attention be given to the fundamentals of sample preparation and chromatography in the face of the never-ending drive for higher throughput.

18.2 INTRODUCTION

Analytical methods employed for the determination of drugs and metabolites in biological matrices such as urine, plasma, serum, and even tissue are essential throughout drug discovery and development. Over the last decade, the development of LC-MS technology has revolutionized bioanalysis. The combination of LC and MS/MS offers unparalleled sensitivity and specificity few other techniques can match [1–3]. Indeed, there was a period following the introduction of LC-MS/MS instrumentation that sample cleanup and high resolution chromatography were thought to be superfluous [4,5]. Fortunately, practitioners soon learned that adequate sample cleanup and chromatographic retention were needed to separate matrix interferences and biotransformed products away from the analyte to obtain better specificity, accuracy, and precision for drug determination in biological matrices [6–9]. Two review articles summarize the literature related to the development of fast LC-MS methods [10] and bioanalytical sample preparation [11].

Despite the advantages offered by the LC-MS/MS technology, the development of rugged and reproducible methods for bioanalysis remains a challenging endeavor. Indeed, there are reports that address the systematic troubleshooting of LC-MS/MS methods [12,13]. Therefore, the successful use of LC-MS/MS for bioanalysis requires an understanding of the mechanisms of various sample extraction processes and the underlying principles of both chromatography and mass spectrometry.

The reliable reporting of data from the quantitative analysis of drugs and their metabolites is the backbone of drug discovery and development. As the drug continues through development, the decisions become more critical; therefore, the bioanalytical methods that produce the data should be sufficiently accurate and reproducible. The regulatory agencies of different countries have their respective guidance documents describing the requirements for bioanalytical method validation and the application of these methods to routine drug analysis. For example, the FDA considers the quality of bioanalytical data generated during toxicology studies to be critical in determining the safety of a drug [14] and therefore requires that the bioanalytical methods be validated to ensure accurate and precise results [15–17]. Bioanalytical method validation is the

process that demonstrates that an analytical method used to quantify an analyte in a biological matrix is reliable, reproducible, and suitable for its intended purpose.

18.3 REGULATORY GUIDANCE FOR THE VALIDATION OF QUANTITATIVE CHROMATOGRAPHIC ASSAYS

The pharmaceutical industry is governed by enacted laws and regulations issued under those laws by regulatory agencies of many different countries. These regulatory agencies on occasion offer suggestions of best practices in the form of guidance documents that they will normally consider acceptable, if followed [17–20]. The FDA Guidance on Bioanalytical Methods Validation is one such guidance that describes best practices for the validation and subsequent application of bioanalytical methods [21]. Complementary guidance is available from the European Medicines Agency [22,23]. The fundamental parameters for method validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability [24–27]. The reader is directed to the chapter titled ***Analytical Method Development and Validation in Accordance to the Regulatory Guidelines*** for an in-depth discussion of the regulatory requirements associated with the application of bioanalytical methods.

The quality of an analytical method is normally assessed by the performance of standards and QCs. Current guidance recommends an acceptance criterion of 15% for accuracy and precision of all standards and QCs, with the exception of the LLOQ, where the acceptance criterion is increased to a 20% deviation. However, standards and QCs may not adequately reflect the composition of study samples from dosed subjects, that is, incurred samples. The limitation of using spiked control samples to assess "real" samples has been acknowledged for years, and there is general acceptance of the inherent risk of obtaining erroneous results. Confirmatory reanalysis of incurred samples is a recent best practice to determine the reproducibility of a method and ascertain the potential influence of metabolites, protein binding, sample homogeneity, and matrix effects otherwise not determined when using spiked standards and QCs [20,28,29]. Minimizing the risk factors that can affect method reproducibility is an important aspect of developing a quality method and is a primary focus of this chapter. Developing a quality method requires that sufficient attention be given to the fundamentals of sample preparation and chromatography in the face of the never-ending drive for higher throughput.

18.4 SAMPLE PREPARATION CONSIDERATIONS

As LC and MS technologies improve and analysis times reduce, the process of sample extraction becomes a rate-limiting step in the workflow associated with quantitative bioanalysis. For example, reducing overall cycle times is critical for clinical development when multisubject pharmacokinetic (PK) studies can generate a few thousand plasma and urine samples. Moreover, the development of drugs of ever increasing potency will continue to challenge the analytical chemist to lower the levels of quantitation (LLOQ). An LLOQ of 100 pg/mL is a common request for clinical development programs, and when this is coupled with the demand for lower sample volumes, the analytical chemist is well challenged.

Technologies for sample preparation have progressed in lock step with improvements in LC-MS/MS instrumentation. Robotic liquid handling systems with multiple probes for solvent and sample aspirating/dispensing in 96-well plate formats have been developed and commercialized to speed up sample preparation time [30–32]. Parallel processing of samples by protein precipitation or SPE can take anywhere from 10 min to an hour for a single 96-well plate.

The reader is directed to the chapter titled ***Direct Biofluid Analysis Systems*** for a more complete discussion of sample preparation.

18.4.1 Ion Suppression/Enhancement

A very common form of sample extraction for plasma or serum samples is acetonitrile precipitation of proteins followed by chromatographic separation using ballistic gradients on short analytical columns. Cycle times of <2 min are routine. However, it has become apparent that inadequate sample cleanup and chromatography often leads to, what is now termed, *matrix effects* that are revealed by irreproducible results for some bioanalytical methods [5,33]. Matrix effects cause either analyte ionization suppression or enhancement and occur when endogenous components coelute with the analyte [9,34–38]. The more common effect of ion suppression is observed primarily in electrospray ionization (ESI), where analyte ion signal is attenuated by competition from the ionization of bulk ions inside the solution droplets [39].

A number of techniques to visualize ion suppression have been proposed in the literature. The most widely adopted method for the qualitative assessment of matrix effects involves monitoring the MRM (multiple reaction monitoring) transition of a postcolumn-infused analyte while blank matrix is injected [36,40]. Ion suppression is revealed as dips in the constant background ion signal of the analyte. For the quantitative assessment of matrix effect, a different technique is used, wherein the response of the analyte spiked into extracted blank matrix is compared with that of the analyte spiked into matrix-free reconstitution solution [9,35,39,41,42].

Plasma phospholipids have been identified as playing a major role in causing matrix effects in LC-MS bioanalysis [43–45]. The two common structural classes of phospholipids are glycerophospholipids and sphingomyelins [46–48], and their concentrations in human plasma range between 1.6 and 3.0 mg/mL [49]. Although there is significant lipid variation within the population, because of diet and metabolic rate, phosphatidylcholine (PC) is the primary phospholipid circulating in plasma, accounting for about 60–70% of the total phospholipids [50–52]. As a zwitterion, PC can cause ion suppression in both positive and negative ESI modes because of its ability to ionize in both environments. Therefore, in order to minimize the potential for matrix effects, the removal of phospholipids is an important component of any sample cleanup process. The effectiveness of the cleanup can be monitored by using positive ESI selected reaction monitoring of m/z 184 $\rightarrow$ 184, with the precursor ion (m/z 184) generated in the source by collision-induced dissociation (CID) of phospholipids [53]. An alternative approach utilizes either positive ion neutral loss scans of 141 Da or a negative precursor ion scan of m/z 153 [54].

Considerable attention has been given to approaches for the removal of phospholipids during sample extraction. One report evaluated popular and commercially available ion exchange and mixed-mode SPE sorbents for their ability to remove

phospholipids. It was determined that reverse-phase retention mechanisms are detrimental in eliminating ion suppression caused by late-eluting phospholipids. If an analyte and its metabolites can be retained using an ion exchange mechanism alone, mixed-mode extraction phases should be avoided [55]. Moreover, it is recommended that elution of analytes be performed using aprotic solvents such as acetonitrile rather than using protic solvents such as methanol because of the increased solubility of phospholipids in the latter [56].

Sorbents containing lanthanide metal active surface elements were used successfully to remove phospholipids through a two-step cleanup process involving liquid–liquid extraction with methyl *tert*-butyl ether followed by SPE on a proprietary column [57–59]. Similarly, almost 99% of phospholipids were removed from serum by using cerium-modified columns [60]. More recently, a product (Sigma-Aldrich HybridSPE™) became available, which incorporates zirconia-coated particles. The product is available in a 96-well plate format and has demonstrated selective affinity toward phospholipids while remaining nonselective toward a range of basic, neutral, and acidic compounds [61]. More recently, Waters has introduced a sample preparation product called *Ostro*™ with a proprietary packing that selectively removes phospholipids while filtering precipitated proteins after protein precipitation.

It should not be forgotten that formulation excipients such as polyethylene glycol (PEG), sometimes administered in large quantities with the compound, can create ion suppression effects that are not present in control matrices [62–64]. Similarly, significant ion suppression was observed with a formulation containing polysorbate 80 (Tween 80®) [64,65].

Adequate sample cleanup along with sufficient chromatographic separation remain the primary tools to control matrix effects. Even then, it is important to adequately compensate for any alteration of ion signal by using an appropriate internal standard, most often a stable-isotope-labeled (SIL) analog of the drug [66]. An SIL internal standard is chemically identical to the analyte and is therefore considered to be essential in any quantitative assay that uses MS detection to avoid the influence of residual matrix effects on ionization efficiency [42,67]. Nevertheless, the use of an SIL internal standard does not always guarantee correction of matrix suppression [68]. The only way to avoid or minimize matrix effects is to develop a method that uses adequate sample cleanup and chromatographic resolution.

18.4.2 Orthogonal Approaches to Sample Preparation/Chromatography

Of the three commonly used extraction techniques, protein precipitation ("dilute and shoot") is the least suited for routine plasma or serum assays with low LOQs (limits of quantitation) because of its susceptibility to ion suppression and matrix effects. For an alternative methodology, the reader is directed to the chapter titled ***Analytical Method Development and Validation in Accordance to the Regulatory Guidelines*** for a discussion on direct plasma analysis.

Liquid–liquid extraction and SPE are techniques that offer much cleaner sample extracts that in turn serve to make the method more robust and scalable. SPE is particularly powerful in this regard because of its unique ability to utilize a variety of retention mechanisms [69]. Moreover, the availability of SPE products in a 96-well format affords the opportunity for high throughput analysis using automated robotic liquid handlers with minimal operator intervention.

The most widely used SPE sorbent phases are those that are based on retention and elution mechanisms that involve polar and/or nonpolar interaction mechanisms. Specifically, sorbents that incorporate C_{18} ligands are the most popular. However, the combination of polar and nonpolar retention mechanisms is also the least selective because nearly everything is retained from the biological matrices along with the analyte of interest. Although this retentiveness is sometimes an advantage in cases where metabolites and parent drug have very different polarities, in general, methods based on this type of interaction mechanism will then require more refined HPLC conditions to compensate for the lack of sample cleanup selectivity. On the other hand, a sorbent that utilizes an ion exchange interaction can be highly selective for molecules that contain functional groups capable of exhibiting either a positive or a negative charge under appropriate acidic or basic conditions [69]. The drawback with high selectivity is that, on occasion, metabolites with a significantly different pK_a from the parent drug will be difficult to isolate.

To fulfill the requirements of selectivity and retention, vendors now offer mixed-mode sorbents that afford hydrophobic and hydrophilic interactions in addition to ion exchange [55,70]. The advantage of incorporating both of these retention mechanisms is the potential to isolate structurally diverse analytes with adequate selectivity and high recovery [56,71]. Moreover, during mixed-mode strong cation- or anion-exchange SPE, significant cleanup is achieved during the 100% organic wash step, where phospholipids bound by reversed-phase interactions are removed along with any other neutral hydrophobic interference. With a reversed-phase only SPE, the final organic elution step simply releases the hydrophobic interferences along with the analyte of interest into the eluent.

Xue *et al*. [72] developed a simplified protein precipitation and mixed-mode cation-exchange SPE method for the extraction of a basic drug from human plasma. The concept was to directly transfer the protein precipitated supernatant onto an unconditioned Oasis® MCX μElution 96-well extraction plate. The simplified method offered equivalent performance and eliminated the time-consuming conditioning and rinsing steps.

18.4.3 Micro-Solid-Phase Extraction Tips

In order to eliminate the time-consuming steps of excess solvent evaporation followed by sample reconstitution, vendors have developed SPE products with smaller sorbent beds. Examples include the Waters Oasis μElution Plate and Omix® tips from Varian. SPE in pipette tips using monolithic sorbent technology is a particularly interesting development because it offers new automation possibilities not previously available [73,74]. Specifically, the elimination of a vacuum manifold enables true walkaway automation and the micro-SPE tip can perform exhaustive extractions simply by passing the sample back and forth over the sorbent bed. The micro-SPE tips have sufficient capacity to extract up to 100-μL aliquots of plasma, and analytes can be eluted with as little as 60 μL of organic solvent [75,76].

18.4.4 Turbulent Flow Extraction

To reduce time and effort, on-line sample preparation (direct sample injection) based on TFC offers an alternative and TFC on-line with LC-MS/MS has demonstrated its

utility and potential in the bioanalytical support of preclinical [77–80] and clinical studies [81–83].

Under conditions of high linear flow rates in columns packed with large, irregularly shaped particles ranging in size from 35 to 75 μm, the solvent flow profile will change from laminar to turbulent. Under the conditions of turbulent flow, low molecular weight molecules will diffuse faster into particle pores than larger molecular weight molecules. The practical implication is that biological samples can be injected directly onto a column and small molecules are retained at the column head while large macromolecules, such as proteins, and other unretained polar analytes, wash away. This is a highly effective technique to conduct on-line sample extraction [84–86].

Typical conditions for TFC involve flow rates above 4 mL/min with 10 mm i.d. columns and above 1 mL/min for 5 mm i.d. columns. For efficient extraction of analytes, the stationary and mobile phases selected need to provide a retention factor of at least 50 column volumes, preferably 100–200 column volumes [87–90].

Turbulent flow on-line extraction can be performed with two different configurations. For single-column methods [91], samples are loaded onto the column under turbulent flow conditions and then analyte elution occurs with a mobile phase of suitable strength under laminar or turbulent flow conditions. The peak capacity of the chromatographic separation can be low because the same solvent is used for both extraction and separation. Moreover, the high flow rates require splitting of the effluent before introduction into the ion source of a mass spectrometer.

The preferred configuration involves two columns; that is, samples are retained on a turbulent flow column, while proteinaceous material is diverted to waste and then the desired analyte(s) is eluted onto an analytical column (Figs. 18.1 and 18.2) [88]. The eluent is transferred through a tee to the analytical column into which the analytical

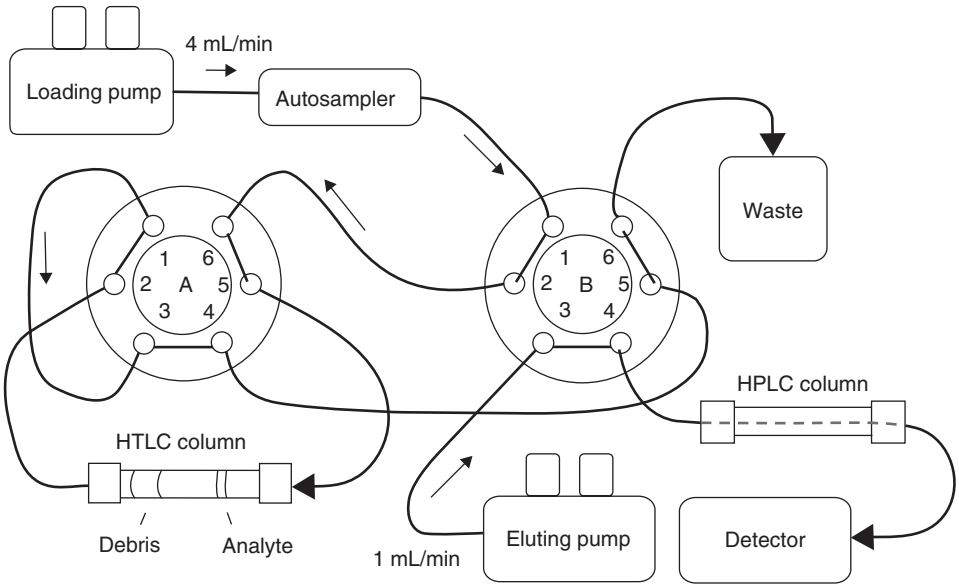

Figure 18.1 Turbulent flow extraction. Cohesive Technologies' "Quick Elute" program showing equilibration and loading steps.

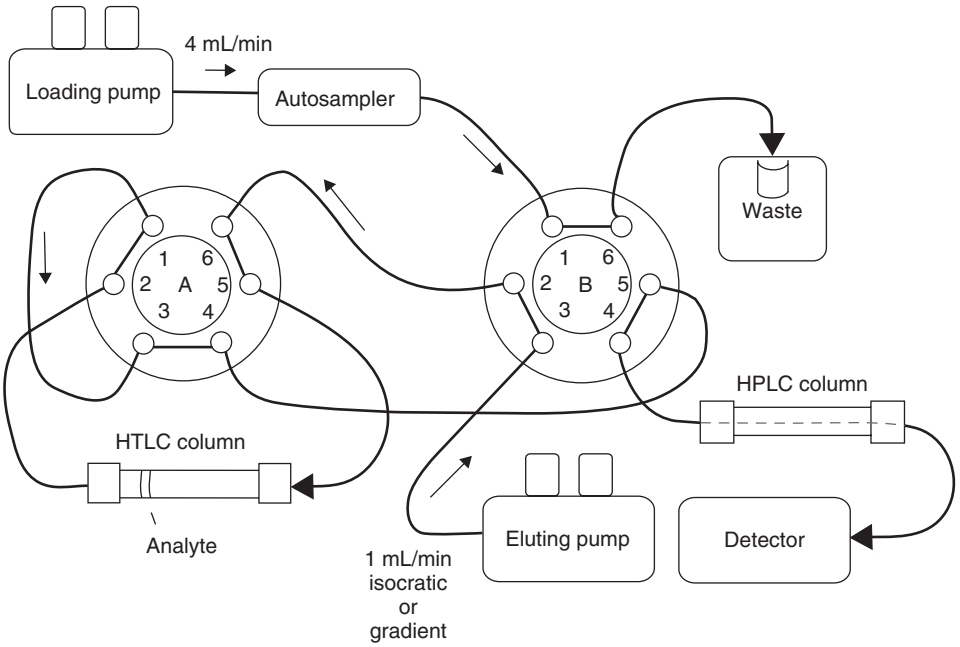

Figure 18.2 Turbulent flow extraction. Cohesive Technologies' "Quick Elute" program showing the elution step.

pump delivers a weak mobile phase at a higher flow rate relative to the extraction flow to focus analytes on the analytical column [92]. In general, the extraction flow rate in the mixing step needs to be minimized in order to maintain the desired focusing effect [93]. Once the sample is transferred, the compounds are eluted and separated with a gradient or step elution on the analytical column. Dual-column extraction normally provides better chromatographic separation and peak shape than single-column methods. It is worth noting that to increase the extraction column lifetime and avoid column and system clogging, samples should be diluted or even extracted before injection.

18.4.5 On-Line SPE

Another alternative to be considered for automated on-line SPE is the Symbiosis™ system from Spark Holland. The Symbiosis system evolved from earlier Prospekt™ systems and consists of a Reliance™ autosampler, a dual high pressure dispenser (HPD) solvent delivery system with six-port valves, a gradient pump set, and dual automated SPE cartridge exchange (ACE) system. Significantly, the system uses single-use (i.e., disposable) SPE cartridges, thereby reducing the potential for column and/or system clogging. Samples do not normally require sample dilution before injection.

The automation features offered by the Symbiosis system have allowed for automated and unattended method development that has proven very useful for rapid method development of robust assays [94]. The authors were able to complete method development within 4 h in a fully automated mode.

The Symbiosis system has also been used to provide a fully integrated approach to bioanalysis [95–97]. The Vial to File™ process involves placing study samples into

an autosampler and then all subsequent operations related to data generation occurs in a hands-free, 21CFR11 compliant environment. Custom-designed sample vials from Sarstedt that incorporate septum piercable caps were used to provide cryogenic storage of unknowns and compatibility with the Symbiosis autosampler. The extraction protocol featured microliter pickup of internal standard concurrent with the sample aliquot.

A recent application of the Symbiosis system is on-line extraction of dried blood samples using sorbent sampling cartridges [98,99]. The technique requires the application of 5–10 μL of blood onto a fibrous material packed into a Symbiosis compatible cartridge. Blood samples absorbed on these cartridges are subsequently eluted on-line using the Symbiosis system for SPE and LC-MS/MS analysis. The continued development of this strategy could result in a significant decrease in the use of animals in drug development studies; specifically, with low blood volume requirements, the characterization of a drug candidate's PK could be performed using a single animal rather than with the current practice of composite PK from multiple animals. Indeed, determining PK from a single animal would most likely provide a better representation of the actual kinetics.

18.4.6 Supported Liquid Extraction

An efficient alternative to traditional liquid–liquid extraction for sample preparation is supported liquid extraction (SLE) offering aqueous sample volumes of up to 400 μL [100,101]. SLE is very efficient, often delivering higher analyte recoveries and cleaner extracts than an equivalent liquid liquid extraction (LLE) method. Moreover, SLE in a 96-well plate format is easily automated with no manual steps such as capping, mixing, centrifugation, and decapping required. SLE plates are most commonly packed with an optimized grade of diatomaceous earth to provide reproducible flow characteristics from well to well.

18.5 A PRACTICAL REVIEW OF THE PRINCIPLES OF CHROMATOGRAPHY

18.5.1 Resolution

The performance of a column can be measured in terms of the height equivalent to a theoretical plate (H), which can be calculated from the column length L and the column efficiency, or theoretical plate number, N:

$$H = \frac{L}{N}$$

The number of theoretical plates is calculated from the equation

$$N = 16\left(\frac{t_R}{W}\right)^2$$

or

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

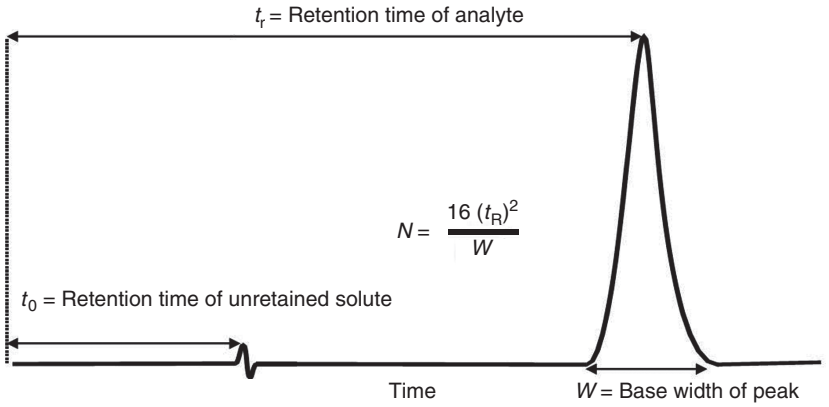

Figure 18.3 Column efficiency expressed as the number of theoretical plates (N).

where t_R is the analyte's retention time, W is the peak width, and $W_{1/2}$ is the peak width at half height (Fig. 18.3). A lower plate height indicates a more efficient column, and a minimum plate height of about twice the particle diameter is generally expected for an efficient column. The van Deemter equation describes H in terms of its dependence on the linear flow velocity (μ):

$$H = A + \frac{B}{\mu} + C\mu$$

where A, B, and C are the coefficients of eddy diffusion, longitudinal diffusion, and axial diffusion (resistance to mass transfer through and between mobile and stationary phases), respectively (Fig. 18.4). Peak (band) broadening results from an analyte diffusing through a column by following random paths with different lengths. The A term can be related to particle size, how well the column is packed, and the internal diameter of the column. An analyte diffuses from the center of the column out to the

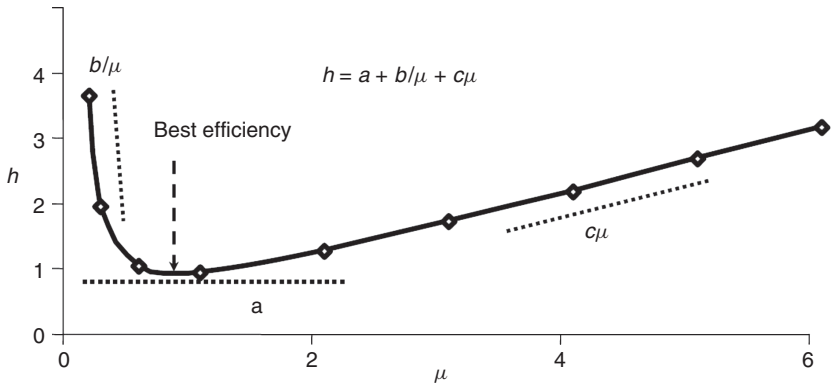

Figure 18.4 van Demeter plot for laminar flow chromatography. The van Deemter curve is a composite of three curves—a (eddy diffusion), b/μ (longitudinal diffusion), and $c\mu$ (axial diffusion, resistance to mass transfer through and between mobile and stationary phases).

edges, and as the velocity of the mobile phase increases, the analyte spends less time on the column, thereby decreasing the effects of longitudinal diffusion. Resistance to mass transfer (axial diffusion) is the partitioning that takes place between the solvent, column packing, and the analyte. If the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase, and the analyte band becomes broadened. In this case, the higher the velocity of the mobile phase, the broader the band.

Chromatographic separation of two analytes, also called *resolution* (R_S), is related to the number of plates in the column, selectivity of the chromatographic system, and the retention of each analyte:

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{k' + 1}\right) \tag{18.1}$$

where N is peak efficiency, α is the selectivity, and k' is the retention (capacity) factor. To obtain high resolution, the three terms must be maximized. For example, increasing N by lengthening the column leads to an increase in retention time and increased band broadening, which is not desirable for high throughput bioanalysis. The capacity factor might be optimized by changing the column temperature (Section 18.7). Column selectivity is normally optimized by changing the mobile phase composition, column temperature, or stationary phase.

Resolution between two peaks in a chromatogram can be determined by

$$R_S = 2\left[\frac{(t_R)_A - (t_R)_B}{(W_A - W_B)}\right] \tag{18.2}$$

where, t_R is the retention time of an analyte and W is the Gaussian curve width of the analyte. When resolution is unity, acceptable resolution is achieved, whereas baseline resolution between two peaks requires $R_S \geq 1.5$.

Productivity can be defined as being proportional to resolution per unit time. In other words, it is important to maintain resolution as flow rates are increased; otherwise, no improvement in productivity is realized. The long-term trend toward ever smaller column particle sizes is an effort to achieve the same performance as is possible with larger particles but in a shorter analysis time [102–105].

18.5.2 Optimizing Chromatographic Performance

18.5.2.1 Selectivity. Although increasing resolution by using smaller particles (i.e., increasing N) is a common approach to enabling faster separations, the selectivity term, α, has the greatest effect on resolution. Selectivity is governed predominantly by analyte interactions with both the stationary and mobile phases. Of these, the stationary phase is still the most important consideration for chromatographic resolution. Selectivity differences may be brought about by changes in hydrogen bonding, dipole–dipole interactions, and/or ionic interactions with the stationary phase. Nevertheless, hydrophobic (reverse phase) separations on C_{18} stationary phases remain the most popular choice in most part because of familiarity and availability.

Fortunately, column vendors continue to introduce unique stationary phases that offer the potential for dramatic improvements in selectivity. Examples include biphenyl

stationary phases that show excellent selectivity for aromatic or fused-ring compounds or pentafluorphenyl propyl stationary phases that are very selective and retentive for organohalogens or other compounds containing basic or electronegative functionalities.

18.5.2.2 pH. In reversed-phase chromatography, hydrophobic interactions are the primary mechanism of selectivity between the stationary phase and the analytes of interest. The ionization state of an analyte may alter the degree of interaction with the stationary phase. When a molecule is ionized, it is more likely to participate in hydrogen bonding. In a reversed-phase, aqueous-rich mobile phase, the solute will spend less time participating in hydrophobic interactions with the stationary phase and more time hydrogen bonding in the aqueous portion relative to the neutral species. The result is less retention. When there are significant concentrations of both ionized and neutral forms, the ionized form will have less hydrophobic interaction with the stationary phase while the neutral form will have more hydrophobic interaction. The result is broad or asymmetrical peaks. Moreover, an analyte in its ionized state may participate in unwanted ionic interactions with exposed silanol groups or metal contaminants present in the silica. These ionic interactions may lead to poor peak shape or irreproducibility.

It is possible to predict the ionization state of an analyte relative to the mobile phase pH from the analyte pK_a. The ionization state can then be controlled by simply altering the mobile phase pH. It should be noted that for nonionizable compounds, the retention characteristics remain relatively unchanged throughout the pH range, although the use of different buffers may influence their peak shape.

Partial dissociation of weakly acidic or basic analytes occurs when the pH range of the mobile phase is within two units (above or below) the pK_a of the molecule. Therefore, to avoid unpredictable secondary interactions, the mobile phase pH should be maintained at least two units above (bases) or below (acids) the pK_a of the molecule.

A general observation is that an acidic functionality will tend to have greater retention and efficiency at a pH below its pK_a value and less retention at a pH above its pK_a value. Basic compounds follow the opposite trend; that is, basic functionalities are often less retained at a pH below their pK_a values and demonstrate greater retention and better peak shape at a pH above their pK_a values.

18.5.2.3 Buffer Selection. Next to organic solvent, buffer selection is the most important variable in the development of HPLC method [106]. For example, when separating mixtures containing acids and/or bases by reversed-phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer to achieve reproducible results. Optimum buffering capacity occurs at a pH equal to the pK_a of the buffer. In general, adequate buffering capacity occurs only within ± 1 unit of their buffer's pK_a. Beyond that, buffering capacity will be inadequate. Table 18.1 lists some commonly used buffers for reversed-phase HPLC. Other criteria such as ionic strength, ion-pairing properties, and mass spectrometer compatibility should be considered before selecting any mobile phase modifier. The concentration of the mobile phase buffer usually has little effect on retention in reversed-phase HPLC, provided the buffer concentration is sufficient to control pH; buffer concentrations above 10 mM are usually effective. It should be noted that in situations where ion exchange interactions are occurring between basic analytes and acidic silanols on the surface of silica stationary phases, analyte retention can be affected by buffer concentration. Increasing buffer concentrations may then be used to suppress unwanted ion exchange interactions.

TABLE 18.1 Commonly Used Buffers for Reversed-Phase HPLC

Buffer	pK_a	Buffer Range
Phosphate	2.1	1.1–3.1
	7.2	6.2–8.2
	12.3	11.3–13.3
Formic acid[a]	3.8	2.8–4.8
Acetic acid[a]	4.8	3.8–5.8
Citrate	3.1	2.1–4.1
	4.7	3.7–5.7
	5.4	4.4–6.4
Tris	8.3	7.3–9.3
Triethylamine[a]	11.0	10.0–12.0
Pyrrolidine	11.3	10.3–12.3

[a] Volatile buffers suitable for LC-MS/MS.

18.6 EMERGING TECHNOLOGIES

18.6.1 Hybrid Silica Particles

Silica particles possess excellent mechanical strength and efficiencies, but they have a limited range of pH stability. When mobile phases are used with pH < 2, the bonded phase is susceptible to hydrolysis [107], and at pH > 8, particle erosion can occur because of the dissolution of the base silica particle, which can result in a loss of column efficiency, an increase in column back pressure, and bed collapse of the silica packing material [108]. Chromatographic columns based on organic polymers, graphitic carbon, alumina, titania, and zirconia have been demonstrated to have little or no performance degradation when employed with high pH mobile phases (pH 8–14) at elevated temperatures (60–120°C) [109–111]. Unfortunately, the use of these nonsiliceous materials presents their own limitations. For example, organic polymers are generally less mechanically strong and exhibit significantly reduced efficiencies. Graphitic carbon and metal oxides show strong adsorption for many polar compounds resulting in poor peak shapes.

In an effort to maintain the predictable chromatographic performance afforded by silica-based materials, column vendors have turned their attention to the development of hybrid particles that offer high efficiencies and mechanical strength with extended pH stability.

In 1999, Waters launched the XTerra® family of HPLC columns featuring a first generation of hybrid particle technology. The hybrid particle was synthesized by replacing one out of every three silanols with a methyl group. The hydrophobicity introduced by the methyl group was distributed through the entire structure of the particle backbone, resulting in a rugged hybrid (inorganic/organic) particle. The presence of 33% fewer residual silanols (after endcapping and bonding) provided higher efficiency chromatography for basic compounds. In 2005, Waters introduced a second-generation hybrid material that utilized an ethylene bridged hybrid (BEH) structure [112]. Compared to the first-generation methyl hybrid particle of XTerra columns, the BEH particle offered vastly improved efficiency, mechanical strength,

and pH range. The BEH hybrid particle is one of the key enablers behind Waters Ultra Performance LC (UPLC™) technology.

Introduced by Phenomenex in 2004, the Gemini product line was the first media to use Two-In-One (TWIN™) technology. Phenomenex used an organosilica grafting process to incorporate highly stabilizing ethane cross-linking. The organic groups are evenly distributed into the grafted layers on the silica surface of a pure silica core. This provides extended pH stability, while maintaining the high efficiency and mechanical strength of a silica particle.

18.6.2 Chiral Columns

Substances that differ only in the spatial arrangement of their atoms are called *stereoisomers*. Certain stereoisomers that differ only in their capacity for rotating the plane of polarized light are termed *optically active* or *chiral*. Optically active isomers are called *enantiomers*. Enantiomers can differ in their potency and selectivity and may exhibit different PK. Indeed, toxicity can be linked to one of the enantiomers [113,114]. Studies of the differences in the pharmacological activities of stereoisomers of a chiral drug are necessary in the drug development process and also required by the regulatory bodies [115].

The separation of chiral drug candidates generally relies on the formation of transient diastereomeric complexes with the stationary phase, resulting in differing chromatographic properties of the enantiomers. Complexes can be formed by hydrogen bonding, ion pairing, metal-ion-to-ligand attraction, π-acid/π-base interactions, or van der Waals attractions. Resolving enantiomers by forming inclusion complexes with cyclodextrin-based stationary phases is perhaps the most widespread approach in the field [116–118]. Of these, triacetate, tribenzoate, and triphenylcarbamate derivatives of cellulose and amylose derivatives are most common. Several reports, however, have demonstrated the utility of β-cyclodextrin as a chiral mobile phase additive for the separation of enantiomers using reversed-phase HPLC [119–122]. The use of chiral mobile phase additives (CMPAs) provides the flexibility to choose from a variety of achiral stationary phases including sub-2-μm particles. Indeed, by combining the CMPAs with fused-core particles and ultrahigh performance liquid chromatography (UHPLC) instrumentation, fast/ultrafast enantiomeric separations are possible [123].

Efforts to increase the efficiency of chiral separations have led investigators to utilize supercritical fluid chromatography (SFC) because of the low viscosity, high diffusivity, and low surface tension of the eluent [124–127]. Super- and subcritical carbon dioxide modified with methanol or other solvents easily outperforms normal-phase HPLC because flow rates are typically five times faster with no loss of resolution. Moreover, SFC offers an environmentally friendly alternative to the conventional solvents used in HPLC. It should be noted that some basic compounds and, in particular, amines do not resolve well by using SFC [128]. The effect of the modifier on a chiral separation varies greatly with analyte, but it has been reported that 2-propanol, rather than the more common modified methanol, often leads to better selectivity [129].

Protein-based chiral columns offer an end user the ability to perform chiral separations in a reversed-phase environment where there is applicability to an extremely broad range of chiral compounds, including amines, acids, esters, sulfoxides, amides, and alcohols. Examples of proteins that have been immobilized onto 5-μm spherical silica particles for chiral chromatography include human serum albumin, α1-acid

glycoprotein, and the stable enzyme, cellobiohydrolase. The retention and enantioselectivity of these phases can be regulated by changes in pH, buffer concentration, and the nature and concentration of organic modifier.

18.6.3 Monolithic Columns

Modern monoliths emerged between the late 1980s and the mid-1990s to be used as columns for HPLC [130–132]. A monolith is a polymerized porous support structure that fills entirely the column volume without any interparticular voids typical of packed columns [133–135]. Moreover, a monolith is not packed into a column because they are normally prepared *in situ* by polymerization. Monolithic columns have proven to be a very good alternative to particle-packed columns for high efficiency separations in HPLC [136–139]. Because of their small-sized skeletons and wide through pores, these columns provide lower flow resistances and can be operated at high flow rates. Increases in efficiency, however, are moderate because there is greater contribution of a mobile-phase mass-transfer term to band broadening [140]. Also, solvent consumption is considerably higher than that in conventional packed columns and might be an issue for cost-conscious laboratories.

To reduce sample preparation and analysis time, the direct injection of biological samples or diluted supernatant resulting from protein precipitation onto an HPLC column is highly desirable [141,142]. However, the analysis of neat or diluted matrix samples is normally impractical because of the severe buildup of matrix components on HPLC columns made of particulate sorbents. Monolithic columns have proven to be more resilient to this type of approach because of their higher permeability along with the absence of traditional column frits. These differences allow biological matrix components such as proteins to easily pass through the column not affecting column lifetime. Nevertheless, it is always advisable to utilize a higher ratio of dilution in a "dilute and shoot" paradigm to ensure robust LC-MS methods with reduced potential for ionization matrix effects.

18.6.4 Hydrophilic Interaction Chromatography

Reverse-phase LC remains the most widely used technique in HPLC. Molecules are separated based on their hydrophobic interactions between the nonpolar stationary phase and the organic functionalities of typical analytes. However, the retention of polar analytes often requires a highly aqueous mobile phase to achieve retention. Highly aqueous systems sometimes lead to problems such as phase collapse (dewetting) [143,144], decreased MS ionization efficiency because of poor mobile phase desolvation, and yet may still not allow retention of very polar analytes. Some specialized phases were developed to allow the use of highly aqueous systems such as polar embedded phases, hydrophilically endcapped reversed-phase bonded silica, wide-pore low density bonded silica, short-chain phases, and other special designs [143].

Hydrophilic interaction chromatography (HILIC) is a mode of chromatography that can address these issues. HILIC requires mobile phases that are highly volatile (80% organic), are ideal for compound ionization by ESI-MS, and can retain highly polar analytes. The term *hydrophilic interaction chromatography* was first coined by Andrew Alpert to distinguish this technique from normal-phase chromatography [145]. HILIC is run on polar stationary phases such as silica [146–148], amino [149–151], diol

[152], polyhydroxyethylaspartamide, and cyclodextrin-based packing [145,153]. A high organic, low aqueous mobile phase is used to retain analytes with increasing orders of hydrophilicity. According to Alpert, retention is proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase [145]. On silica columns, the mechanism of HILIC involves various combinations of hydrophilic interaction, ion exchange, and reversed-phase retention by the siloxane on the silica surface [154]. This combination of retention mechanisms results in a unique selectivity that allows for polar retention [154–157].

Conventional SPE methods often contain an elution step that uses a high concentration of an organic solvent. This SPE eluent is not always compatible with reversed-phase analysis. A typical sequence would be to evaporate and then reconstitute into a solvent that more closely matches the initial mobile phase conditions [158]. In HILIC, the high organic eluent is ideal for direct injection, thus eliminating the need for lengthy evaporation and reconstitution steps and increasing throughput [146,154].

18.6.5 Sub-2-μm Chromatography

18.6.5.1 The Promise of van Deemter. As early as the 1950s, van Deemter realized that peak efficiency could be improved by reducing particle size [159]. Since then, particle technology has continued to advance, whereby column vendors are now able to offer a variety of stationary phases with sub-2 μm diameters and with the requisite mechanical strength [160–162]. The development of hybrid silica particles was a clear enabler for this technology.

From the resolution equation it can be seen that resolution is proportional to the square root of N. Because N is inversely proportional to particle size, as the column particle size is reduced, resolution increases by the square root of the difference. Similarly, with N being inversely proportional to the square of the peak width, as particle sizes decrease, peak widths become narrower; that is, there is an increase in concentration [163–165]. These improvements, though, come at a cost. Because the pressure required to pump mobile phase through a column is inversely proportional to the square of the particle diameter, the backpressures required for the use of these small-particle columns become quite high [165,166]. Indeed, pressures of up to 4000 bar were used with small-diameter columns packed with sub-2-μm particles to achieve extraordinary separations [163].

In 2004, Waters Corporation introduced the ACQUITY UPLC that became the first commercially available system capable of pumping mobile phase at pressures up to 15,000 psi. Subsequent introductions of higher pressure capable systems came from Agilent Technologies (1200 RRLC series), Thermo Scientific (Accela 1250), Flux Instruments (Rheos Allegro), PerkinElmer (Flexar™ FX-15), and Dionex (UltiMate™ 3000 RSLC).

An important consideration that limits the use of even smaller particles and higher operating pressures is the occurrence of viscous heating. Viscous heating occurs from the dissipation of the frictional forces when liquid passes through the column bed. The generation of heat results in an increase in mobile phase temperature along the column axis [167,168]. This physical phenomenon produces band spreading, increasing peak width, and produces fronting and tailing. Indeed, the frictional heating of the

mobile phase is thought to be the primary reason for the underperformance of sub-2-μm columns when compared to 3.5- and 5-μm columns [169–172]. Therefore, the use of narrower bore columns with sub-2-μm particles is an important consideration to reduce the impact of radial temperature gradients and maximize chromatographic efficiency.

18.6.5.2 Optimizing Sub-2-μm Chromatography. The goal of any LC analysis is to optimize the resolution between critical pairs of analytes in the shortest time. Unit resolution may be acceptable for many applications, whereas increasing the resolution to 1.5 is considered appropriate for nearly all quantitative analyses. Optimizing selectivity by proper choice of stationary phase and chromatography conditions (e.g., organic modifier, pH, gradient) is far more powerful at improving resolution than increasing separation efficiency with smaller particles. Systematic evaluation of chromatographic conditions can be facilitated by using an automated LC method screening tool.

The transfer of an HPLC assay to UPLC is reasonably straightforward with the following recommendations [173,174]:

- By taking advantage of the high resolution potential of sub-2-μm columns, mobile phase solvent strength can be increased, thereby reducing run times.
- By maintaining optimum mobile phase linear velocities to ensure adequate peak resolution while operating at no more than 80% of the maximum rated pressure of the system. Flow rates are simply scaled in proportion to the difference in column dimensions in order to maintain the same linear velocity.
- Column re-equilibration times can be reduced as a result of low system dwell volumes.
- Injection volume should be appropriately adjusted for the column diameter to achieve good peak shapes. The smaller injection volumes are more than compensated by the increased peak height resulting from the use of sub-2-μm particles.
- By limiting sample volume injections to ~50% of the total loop volume. For systems that utilize air-gap sandwiching of the sample, reducing sample volume allows better utilization of the sample loop and higher injection precision.

18.6.5.3 Linear Velocity. Optimum linear velocity is inversely proportional to particle size, whereas the pressure required to operate at the optimum velocity is inversely proportional to the *cube* of the particle diameter. The pressure limits of the LC pump will therefore restrict the length of column if packed with sub-2-μm particles and operated at optimal flow velocity (~0.5 cm/s). Nevertheless, a significant advantage of using sub-2-μm particle columns is the ability to use higher linear velocities without detrimental effects on separation quality. It should be noted that maintaining a separation under gradient conditions when the mobile phase linear velocity is increased requires that the gradient times (t_G) also be adjusted accordingly:

$$t_{G_2} = t_{G_1} \times \frac{\nu_2}{\nu_1} \times \frac{F_1}{F_2} \tag{18.3}$$

where t_G is the gradient time, ν is column volume, and F is flow rate.

18.6.6 Pelicular (Fused-Core) Silica

The recent introduction of fused-core silica particle technology has unlocked opportunities for developing higher throughput assays [175–177]. Examples include MAC-MOD Analytical Halo® and Phenomenex® Kinetex columns. Using sol–gel processing techniques that incorporate nanostructuring technology, a durable, homogeneous porous shell is grown onto a solid silica core. The solid silica cores range in diameters from 1.25 to 1.9 μm and are surrounded by 0.23–0.5 μm superficially porous shells for total particle sizes between 1.7 and 2.7 μm. The highly optimized growth process produces an extremely narrow particle size distribution and dramatically reduces two major sources of peak dispersion, namely, eddy diffusion and resistance to mass transfer. In practice, these fused-core silica particles have shown substantial improvement in chromatographic peak efficiencies over fully porous particles [178–180]. Specifically, the reduction in axial diffusion allows the use of higher flow rates without sacrificing chromatographic performance [181,182]. Compared to sub-2-μm particles, the fused-core particles offer similar efficiency and/or peak capacity at a fraction of the backpressure [183,184]. The adoption of fused-core silica particle technology has been rapid in the pharmaceutical industry for both early discovery [185,186] and late development programs [187].

18.7 ELEVATED COLUMN TEMPERATURES

18.7.1 Theory

Method developers often seek compromise rather than optimization. An often overlooked parameter is temperature that is an easily adjustable parameter and can provide alternative selectivity leading to increased resolution of critical isomer pairs along with an overall reduction in analysis times.

The benefits of using high temperature liquid chromatography (HTLC) were recognized in the 1960s when pioneers such as Giddings and Snyder demonstrated that increasing column temperature could lead to decreased separation times and altered selectivity [188,189]. Recent developments in technology and a better understanding on how temperature can enhance selectivity have created renewed interests in this area [190–192]. As early as 1988, Antia and Horvath [192] proposed in a theoretical study that up to a 100-fold increase in separation efficiency for HPLC can be achieved at significantly elevated column temperatures. A recent publication from Plumb *et al*. described an application utilizing a sub-2-μm HPLC column at elevated temperature to identify metabolites in urine samples. The authors reported a peak capacity of ~700 for a 10-min analysis and greater than ~1000 in 1 h in gradient elution mode [193]. A number of articles have reviewed the recent advances and applications of HTLC [194–197].

18.7.1.1 Van't Hoff Equation. Increasing temperature alters analyte retention by changing the free energy (enthalpy and entropy) between the analyte and the stationary phase [198,199]. The temperature-dependent free energy changes can be represented using a Van't Hoff plot (i.e., ln retention factor vs 1/temperature). Van't Hoff plots are often linear, and the slope of the line can be used to estimate enthalpy, assuming the analyte retention is governed by only one retention mechanism. Several studies, however, have suggested that changes in temperature affect multiple retention mechanisms,

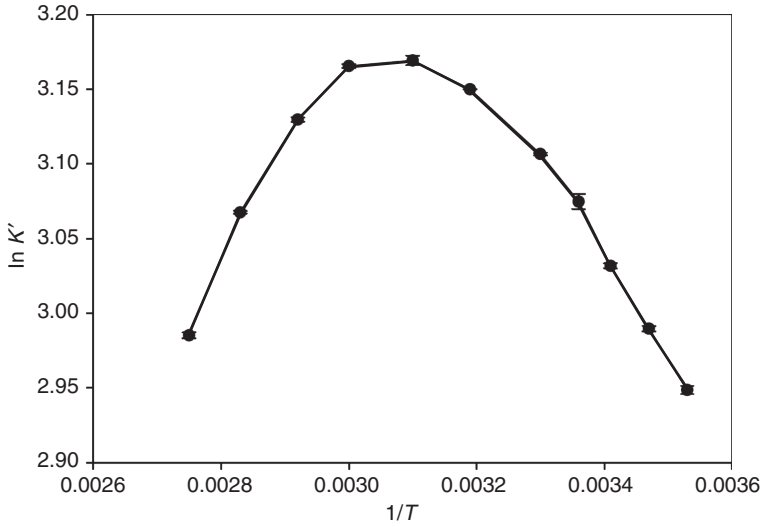

Figure 18.5 The effect of temperature on resolution. Retention factor of an analyte measured as a function of temperature and represented in a Van't Hoff plot.

including acid–base dissociation rate variations and varying adsorption/desorption kinetics in the stationary phase. The end result is nonlinear Van't Hoff plots, and the phenomenon is particularly evident for ionizable analytes (Fig. 18.5) [200–202].

In general, chromatographic peak efficiency increases as temperature increases, especially at higher flow rates [190]. On the other hand, selectivity can either increase or decrease depending on temperature, and with several other parameters also affecting selectivity (pH, mobile phase, stationary phase, etc.), it is very difficult if not impossible to predict how selectivity will change with temperature. The decrease in linear velocity and increase in the diffusion coefficient with temperature usually results in a decrease in k' (although not always). The decrease in k' would suggest a decrease in resolution with increasing temperature. With the effects of efficiency, selectivity, and retention factor combined, the end result is that resolution generally decreases with increasing temperature [191].

18.7.2 Reality

The conformational changes that occur with long-chain alkyl stationary phases as a function of temperature can produce selectivity differences among analytes. The promise of modulating selectivity by adjusting column temperature is certainly an appealing notion. Shen *et al*. [191] demonstrated enhanced resolution of diastereomers using elevated column temperatures of 100°C and an ion-pairing reagent. As a result of the increased column temperature, the authors achieved a twofold reduction in cycle time and a fivefold improvement in sensitivity.

Still others have employed temperature programming (thermal gradients) as an alternative to solvent composition gradients for the analysis of pharmaceutical compounds [203–206].

Given the difficulty in predicting the effect of temperature on selectivity, it is recommended that chromatography be evaluated over a range of temperatures. Automation

of the process by using chromatographic methods that slowly increment temperature overnight can reduce the overall development time. Concerns regarding analyte thermal stability and irreproducible chromatography because of column thermal instability remain, and they can be alleviated only by controlled investigations.

18.8 ION-PAIR REAGENTS

Volatile ion-pairing reagents such as perfluorinated carboxylic acids are useful because of their compatibility with both LC and MS [207–209]. Perfluorinated carboxylic acids such as trifluoroacetic acid (TFA), heptafluorobutanoic acid, and nonafluoroheptanoic acid have been used with LC-MS and were found to provide similar ion-pair behaviors when compared to the more conventional sodium heptane sulfonate additive. The use of perfluorinated carboxylic acids as ion-pairing reagents to extend the retention time of very polar analytes in reverse-phase chromatography has been reported. In one recent report, Gao *et al*. [209] used perfluorinated carboxylic acids as additives in the reconstitution solution of bioanalytical samples and discovered that the retention times of polar molecules, such as methadone, were significantly extended. The use of perfluorinated carboxylic acids to enhance diastereomer separations has also been reported [191].

Unfortunately, the use of TFA is known to cause ion suppression and must be dealt with if this acid is used in the mobile phase [210]. The use of surface-tension-lowering modifiers to modify the mobile phase and mitigate ion suppression is a common solution [211]. Postcolumn addition of acids and solvent carriers to aid ionization is known as the *TFA Fix* [212].

18.9 MULTIPLEXING LC-MS/MS

Automation of the pipetting and incubation steps of *in vitro* assays makes it possible for a single analyst to generate many more samples in an 8-h period than can be analyzed by current LC-MS/MS systems. Similar improvements in automation of sample extraction have given the analyst the ability to prepare large batches of *in vivo* samples for LC-MS/MS analysis. Once again, the LC-MS/MS interface is the bottleneck and other options are required. It is essential that whatever processes are established that maximize throughput, they must do so without compromising quality.

Parallel or multiplexed LC-MS/MS systems that can analyze the eluent from several LC systems using a single mass spectrometer are one solution to support the high throughput production of quality data.

Waters MUX technology uses indexed LC–MS interfaces from multiple LC systems running in parallel with MS sampling time split between the LC systems [213,214]. The MUX–electrospray interface incorporates either four or eight nebulization-assisted electrospray probe tips arranged radially about the sampling cone. The spray from each tip is successively admitted to the sample cone via a hole in a sampling rotor driven by a programmable stepper motor. The sampling rotor has a supply of heated dry nitrogen to facilitate desolvation of the analyte from the spray selected. An optical encoder on the shaft of the rotating assembly is used to ensure that each mass spectrum is correctly

assigned to the corresponding liquid stream. The inherent duty cycle of this approach, however, limits the MUX system for fast chromatography with multiple components.

The Cohesive Technologies multiplexing system consists of four fully independent HPLC systems fed by two injection syringes on a twin-arm HTS PAL autosampler. Samples are introduced into the atmospheric pressure ionization MS interface via a selection valve. Timing and triggering for injection, gradient start, and data collection are all synchronized by the Aria software. Each system operates independently, permitting multiple methods to run simultaneously, with the MS remaining idle for <4% of the time. With each system operating independently, it is possible to analyze standards, QCs, and study samples on the same hardware. The Aria system uses a modified Twin PAL autosampler from CTC. The two arms and four injectors allow the system to keep up with the required injection speed while maintaining thorough wash cycles for both the syringes and the injectors.

The latest iteration of the system combines the power of on-line extraction with the speed of UHPLC. The Transcend™ system uses Cohesive Technologies' patented TurboFlow® technology with ultrahigh performance capable chromatography systems. The system can alternate between on-line extraction methods and conventional LC methods in a multiplexed manner.

18.10 METABOLITE INTERFERENCES IN QUANTITATIVE ASSAYS

Drugs are xenobiotics to living organisms, which therefore biotransform them to less toxic, less active, and more hydrophilic forms to enhance their excretion. Metabolic pathways are categorized as either phase I or phase II reactions, and both classes of reaction may occur. In phase I reactions, enzymes (e.g., cytochrome P450) modify the parent compound by hydrolysis, oxidation, and/or reduction [215–217]. Phase II reactions conjugate polar groups to the parent or phase I modified compound. Glucuronidation is the main phase II reaction in mammals [218], with other pathways including sulfation, methylation, acetylation, and conjugation with amino acids or glutathione [219,220].

Metabolite identification is central to many of the activities in the discovery and development of drugs. However, quantitative analysis of parent drugs during the early stages of the pipeline often occurs without prior knowledge of the major metabolites of the target analyte. With a requirement for rapid, high throughput bioanalysis, it should not be surprising that a number of methods are developed, wherein the target analyte not only coelutes with endogenous components of biological fluids but may also coelute with biotransformed products of the drug. The presence of unidentified metabolites can lead to inaccurate measurement of the analyte because of ionization suppression, interference in peak integration, or, most problematic, in-source regeneration of the parent drug from phase II metabolites, as the metabolites are not present in the calibration standards and QCs [221]. Common examples of metabolites that undergo in-source regeneration are *N*-oxides [222,223], and both *O*- and *N*-glucuronides [224–227].

The importance of chromatographic separation in quantitative analysis using LC-MS/MS should never be overlooked. Without prior knowledge of the metabolic pathway of a target analyte, it would be best practice to screen a few unknown samples, if available, for metabolites before method validation. The goal of the screening would be to ensure that multiple analytes with similar structures and masses are free of cross

talk [228]. Endogenous isobaric interference from the sample matrix should also be considered [229].

Jemal *et al*. recommended a set of procedures using incurred samples to establish the validity of an LC-MS/MS method for analyzing samples collected from dosed subjects [230]. Specifically, the stability of incurred samples should be investigated before and after sample preparation and SRM (single reaction monitoring) transitions attributable to putative metabolites be scanned using both the same and chromatographically modified LC-MS/MS methods.

18.11 CONCLUSIONS

High throughput bioanalytical methods are essential to support the rapid discovery and development of drugs in the pharmaceutical industry. Nevertheless, the data being produced by these methods needs to be reliable in order to support decision-making processes, that is, the data should be sufficiently accurate and reproducible. Because LC-MS/MS offers high sensitivity and selectivity, rigorous chromatographic resolution and/or tedious sample extraction protocols may not be required; however, appropriate attention still needs to be paid to the quality of the methods to avoid issues with routine sample analysis. A number of sample preparation methodologies have now matured and automation is commonplace. Nevertheless, with chromatographic analysis, times of frequently <3 min, the bottleneck in sample analysis remains with sample preparation.

The importance of sample preparation stems from three major concerns—removing interferences from the biological sample matrices, concentrating analyte(s) of interest, and improving analytical system performance. The choice of sample preparation method should be dependent on the quality of the data required. It makes little sense to invest a month of development time on a method that affords pg/mL sensitivity with accuracy and precision of $<15\%$ each for a drug discovery screen that simply requires data that indicates one candidate has better bioavailability than 10 other candidates in a cassette-dosed animal model. On the other hand, it may be important to invest such time to develop and validate a method for a lead drug candidate undergoing human safety assessments when such data is subject to regulatory scrutiny.

The final method should be orthogonal to maximize selectivity and reduce ion suppression. If sample extraction utilized C_{18} SPE, then the analyst should consider something other than C_{18} chromatography, for example, a phenyl stationary phase. Alternatively, if strong cation-exchange SPE was selected for sample extraction, any reverse-phase LC method could be utilized. The method should be assessed for recovery, selectivity, precision, accuracy, and ruggedness. Formal validation may yet be required.

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