



**Naidong Weng and  
Timothy D.J. Halls**

LC/MS/MS provides superior sensitivity and selectivity, rapid analysis, maximized development efficiencies, and improved turnaround times. Although large-scale LC/MS/MS analysis is fraught with challenges, you can learn to overcome the obstacles with careful planning and these troubleshooting techniques. Part 1 presents troubleshooting techniques related to sample preparation and chromatography.

# Systematic Troubleshooting for LC/MS/MS

## Part 1: Sample Preparation and Chromatography

**D**evelopment and validation of bioanalytical methods for pharmaceutical product analysis are common rate-limiting steps in biopharmaceutical product development. A rational, strategic approach to developing robust, automated, validated bioanalytical methods can reduce slowdowns and bottlenecks in drug development and contribute to synergistic, consistent, long-term performance.

Use of liquid chromatography with tandem mass spectrometry detection (LC/MS/MS) for bioanalysis has grown exponentially in the pharmaceutical industry since the 1980s. The popularity of this method is attributed to its superior sensitivity, extraordinary selectivity, and rapid rate of analysis. Adoption of LC/MS/MS has been driven by the need for timely, high-quality data at various stages of the drug development process: from high-throughput screening of drug candidates and prompt data generation for preclinical studies to almost “real-time” analysis of clinical samples (1). Rapid and rational LC/MS/MS methods play an important role in bioanalytical sample analysis and shortening cycle times for biopharmaceutical product development (2).

### LC/MS/MS Rationale

The underlying principle of MS is the production of ions from analyzed compounds that are then separated or filtered based on their mass-to-charge ratio ( $m/z$ ) and detected in a spectrometer. The mass spectrum generated plots the abundance of the produced ions as a function of  $m/z$ . The most dominant applications for quantitative bioanalysis employ tandem mass spectrometers (MS/MS) that use a triple quadrupole instrument. Two mass analyzers are used: one for selection of the precursor (father) ion in the first quadrupole, and the other for

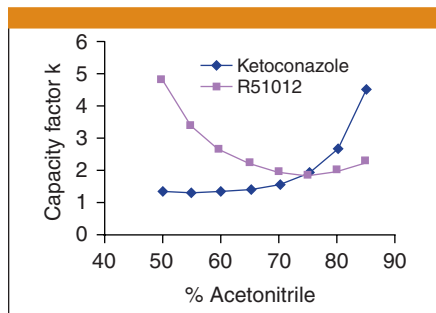
selection of the product (daughter) ion in the third quadrupole after the collision of the father ion in a collision cell (the second quadrupole). That mode of ion selection and detection is called *selected reaction monitoring* (SRM).

**Joining MS and LC.** MS operates in a high-vacuum environment. In contrast, today’s premium separation technology, liquid chromatography (LC), is performed under atmospheric pressure. Historically, its aqueous mobile-phase flow rate was incompatible with MS. However, innovative and successful research efforts on the design of an effective interface connection between LC and MS over the past 25 years have made LC compatible with MS. Electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI), collectively called atmospheric pressure ionization (API), have matured into the reliable interface necessary for quantitative LC/MS/MS bioanalysis.

Biological systems are complicated to analyze, and the development of robust LC/MS/MS methods suitable for routinely analyzing thousands of samples remains a difficult, time-consuming task. The popularity and growth of LC/MS/MS rose so quickly in the early 1990s that some users of the method abandoned sound techniques of sample preparation and clean-up, discarded the basic principles of chromatography, and neglected the fundamentals of chemistry. Such oversights ultimately created problems attributed to the analytical method. Many LC/MS/MS challenges have been reviewed, discussed, and published (3,4).

LC/MS/MS remains one of the most useful tools available for bioanalysis. Successful use requires understanding the extraction process and the underlying principles of both chromatography and MS. A systematic approach for developing robust methods and automation (wherever possible) is necessary to realize long-term benefits and

Corresponding author **Naidong Weng** is associate director of bioanalytical chemistry, and **Timothy D.J. Halls** is vice president of pharmaceutical chemistry at Covance Laboratories Inc., 3301 Kinsman Boulevard, Madison, WI 53704, 608.242.2652, fax 608.242.2735, [naidong.weng@covance.com](mailto:naidong.weng@covance.com), [www.covance.com](http://www.covance.com).



**Figure 1.** Unexpected results from reversed-phase chromatography: retention (capacity factor) of ketoconazole and its internal standard R51012 on Hypersil BDS C18 column (50 × 3 μm) using a mobile phase of acetonitrile–water–formic acid (x:(100-x):1, v/v/v) where x is the percentage of acetonitrile in the mobile phase) at a flow rate of 0.5 mL/min

efficiencies. This month we highlight rational method development and validation and includes troubleshooting tips for sound sample preparation, for analyte stability in biological matrices, and for chromatographic conditions and injection solvents. Part 2 will further discuss development strategies for LC/MS/MS analysis of biopharmaceutical products including proteins and peptides. It will present troubleshooting tips for carry-over, recovery and matrix effects, and selectivity; and for automation where possible to improve the overall reliability and efficiency of LC/MS/MS.

**Sound Sample Preparation**

The role of sample clean-up in LC/MS/MS is critical. In addition to assessment of analytes in the usual picogram/mL to nanogram/mL range, biological sample analysis is complicated because the samples contain macromolecules and other compounds such as proteins, endogenous and exogenous compounds, and coadministered pharmaceuticals (collectively referred to as *unwanted* compounds). Those compounds will be at higher concentrations than the analyte.

The first step in sample clean-up is to remove as many of the unwanted compounds as possible without significant loss of the analytes of interest. Solid-phase extraction (SPE), liquid–liquid (LL) extraction, and protein precipitation (PP) are often the techniques of choice. Unwanted compounds can be present still in higher concentrations

**Table 1.** LC/MS/MS methods using silica columns and aqueous–organic mobile phases

Analyte	Extraction	Species	Matrix	LLOQ–ULOQ <sup>a</sup>	Sample Volume <sup>b</sup>
Albuterol	SPE	human	serum	0.05–10	0.4
Clonidine	LL	human	serum	0.01–1	1
Fentanyl	SPE	human	plasma	0.050–5	0.25
Fexofenadine	SPE	human	plasma	5–2,500	0.05
Fluconazole	LL	human	plasma	0.5–100	0.5
Fluoxetine	LL	human	plasma	0.5–250	0.1
Norfluoxetine				0.5–250	
Hydrocodone	SPE	human	plasma	0.1–100	0.3
Hydromorphone				0.1–100	
Ketoconazole	LL	human	plasma	20–10,000	0.25
Loratadine	LL	human	plasma	0.01–1	1
Descarboethoxy-loratadine				0.025–2.5	
Midazolam	LL	human	plasma	0.1–100	0.4
1-OH midazolam				0.1–100	
4-OH midazolam				0.1–100	
Morphine	SPE	human	plasma	0.5–50	
Morphine-3-glucuronide				1–100	
Morphine-6-glucuronide				10–1,000	
Nicotine	LL	human	plasma	1–200	0.25
Cotinine				10–2,000	
Omeprazole	LL	human	plasma	5–2,000	0.2
Oxycodone	SPE	human	plasma	0.1–50	0.4
Oxymorphone				0.1–50	
Noroxycodone				0.1–50	
Paroxetine	LL	human	plasma	0.05–50	0.4
Protease inhibitors	PP	human	plasma		0.1
Amprenavir				10–10,000	
Indinavir				10–10,000	
Nelfinavir				10–10,000	
Ritonavir				10–10,000	
Saquinavir				10–10,000	
Pseudoephedrine	SPE	human	plasma	10–2,500	0.1
		human	urine	10–2,500	0.1
Ribavirin	PP	human	plasma	10–10,000	0.1
Rosiglitazone	LL	human	plasma	1–1,000	0.05
Sertraline	SPE	human	plasma	0.25–100	0.25
Desmethyl-sertraline				0.5–100	
Sildenafil	SPE	human	plasma	1–500	0.35
Desmethyl-sildenafil				1–500	
Triazolam	LL	human	plasma	0.1–50	0.2

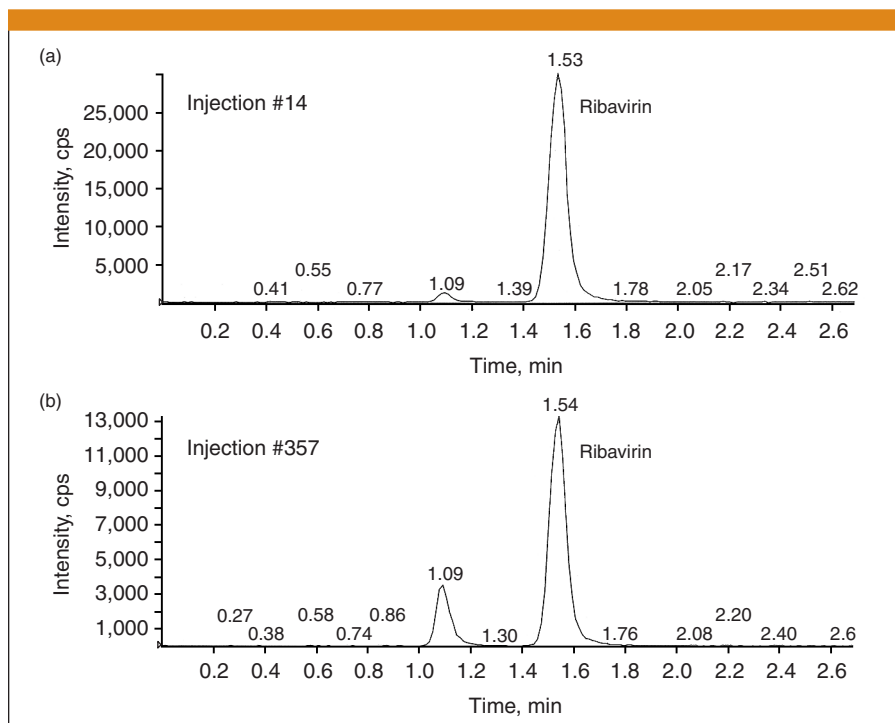
<sup>a</sup>Upper and lower limits of quantitation in ng/mL

<sup>b</sup>in mL

than the analytes of interest after the first clean-up. A second stage of clean-up, typically involving LC separation further separates analytes of interest from the unwanted compounds. MS/MS offers a third stage of separation through selection of appropriate precursor and product ion pairs so that unwanted compounds are not registered (*unseen*) by the detector.

However, those unwanted and MS/MS unseen compounds present significant challenges for LC/MS/MS practitioners. In

the LC/MS interface, unwanted compounds compete with analytes for ionization and can cause inconsistent matrix effects that are detrimental to quantitative LC/MS/MS. If not separated from the analytes, some conjugated metabolites break down in the interface so that analyte concentration is overestimated. LC/MS/MS practitioners must use caution in their analyses and envision that behind every analyte peak, “unseen” MS peaks from contaminants may cause or contribute to potential problems in the assessment.



**Figure 2.** Silica column stability for a LC/MS/MS method of analyzing ribavirin in human serum; column: Betasil silica 50 × 3 μm; mobile phase: acetonitrile–water–trifluoroacetic acid (TFA) (95:5:0.05, v/v/v); sample preparation: protein precipitation

**Analyte Stability**

Certain drug product analytes are subject to degradation. Endogenous enzymes found within biological matrices can accelerate analyte degradation. Information about degraded analytes should be obtained during method development. A general troubleshooting approach to address that problem would be stabilizing analytes by choosing an appropriate anticoagulant, pH, or enzyme inhibitor. If analytes cannot be stabilized, then in situ derivatization of the analytes to more stable forms in biological matrices should be attempted.

Anticoagulants in the plasma can have significant stabilizing effects on the analyte. Some ester-containing analytes are unstable in plasma containing sodium heparin as the anticoagulant, but they may be relatively stable in plasma where sodium fluoride is the anticoagulant (5).

Establishing analyte stability in the biological matrix at an early stage of method development is crucial. For example, when a new investigational drug containing ester functional groups was recently tested, using sodium heparin as the anticoagulant, 98% of the analyte was degraded at room temperature. Decreasing the temperature to –20 °C resulted in 25% degradation by

plasma esterase ex vivo in 20 hours. The same analyte showed no degradation at all when the same experiment was conducted using sodium fluoride as the anticoagulant. That information provided critical information for the appropriate design of the clinical study protocols.

Some amine-containing nucleosides are metabolized ex vivo by plasma deaminase. Addition of small amounts of tetrahydrouridine (THU) inhibit deaminase activity and stabilize the analytes (6). Thio- (sulfhydryl-containing) compounds are usually unstable in plasma. In situ derivatization of thio- compounds with *N*-ethylmaleimide (NEM) (7) or with methyl acrylate (MA) (8) lead to stable derivatives, which subsequently can be reliably analyzed.

**Chromatographic Conditions**

Reversed-phase chromatography is universally applied in LC/MS/MS. The advantages of reversed-phase columns with mobile phases of various compositions include excellent stability, column efficiency, and versatility for a wide variety of compounds.

**On-column retention.** Reversed-phase columns do not always behave in a “reversed-phase” way. The characteristics of

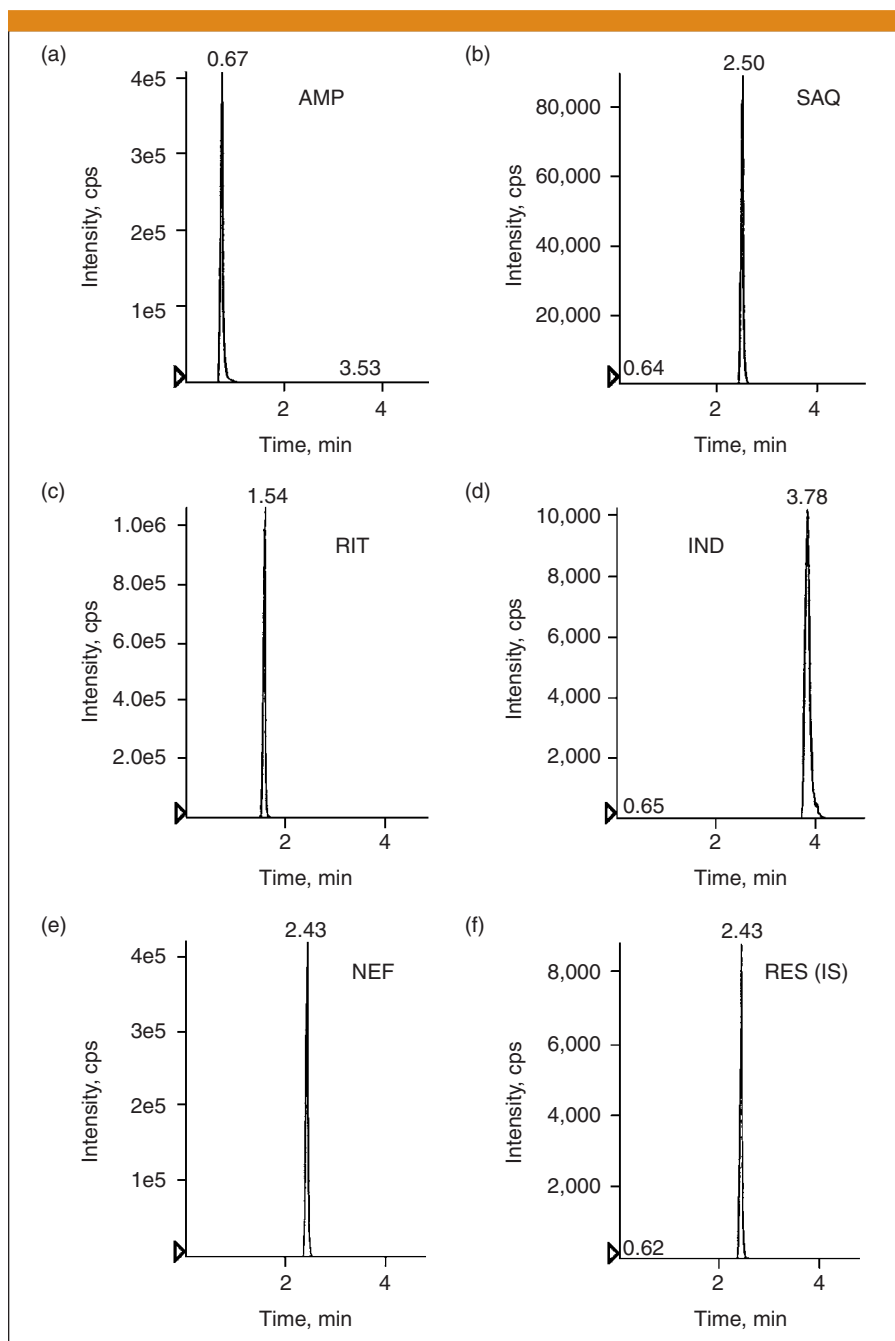
the analyte, the column, and the mobile phase can interact with unexpected results. For example, Figure 1 shows that when organic content of the mobile phase composition was increased (a desirable characteristic for improving sensitivity), the capacity factor (*k'*) decreased initially but increased after the deflection point. That system demonstrated an initial reversed-phase mode with primary retention because of hydrophobic interaction between the analytes and the alkyl chains. It reverted to normal-phase where the primary retention was because of hydrophilic interaction between the analyte and the residual silanol groups. That kind of dual retention behavior was observed for various analytes on different brands of reversed-phase columns. Even the extensively end-capped reversed-phase column had at least 30% nonendcapped residual silanol groups. Analyte on-column retention, therefore, depends on the characteristics and interactions of the analyte, the column, and the mobile phase.

**Polar ionic retention.** For analysis of polar ionic compounds, reversed-phase LC/MS/MS can be problematic. Anion formation occurs when the pH is higher than the *pK<sub>a</sub>* in acidic compounds, and cation formation occurs when the pH is lower than the *pK<sub>a</sub>* of basic compounds. Column retention decreases matrix effects. Ionization of polar analytes decreases column-retention, and promotes matrix effects that can impact analysis bioanalysis. To retain polar ionic compounds, highly aqueous mobile phases or ion-pair chromatography is required. However, neither of those methods is conducive to the spray conditions required

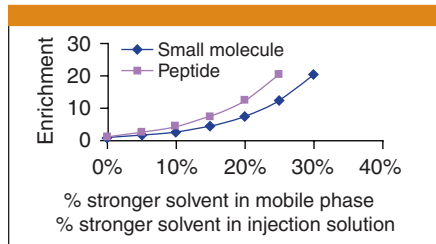
**Table 2.** Reproducibility of gradient elution on silica column for four injections

Retention Time (minutes)					
AMP	RIT	NEL	IS	SAQ	IND
0.67	1.54	2.43	2.43	2.50	3.78
0.69	1.54	2.42	2.44	2.50	3.80
0.69	1.54	2.42	2.42	2.50	3.78
0.69	1.52	2.42	2.44	2.50	3.78

<sup>a</sup> column: Betasil silica 50 × 3 μm; mobile phase: linear gradient elution of acetonitrile–water–formic acid from 95:5:0.2 to 30:70:0.2 in two minutes; flow rate: 0.5 mL/min; sample preparation: protein precipitation  
<sup>b</sup> AMP: amprenavir; RIT: ritonavir; NEL: nelfinavir; IS: internal standard; SAQ: saquinavir; IND: indinavir



**Figure 3.** Chromatograms of gradient elution of five protease inhibitors on silica column with aqueous–organic mobile phase; column: Betasil silica 50 × 3 μm; mobile phase: linear gradient elution of acetonitrile–water–formic acid from 95:5:0.2 to 30:70:0.2 in two minutes; flow rate: 0.5 mL/min; sample preparation: protein precipitation; AMP: amprenavir; RIT: ritonavir; NEL: nelfinavir; IS: internal standard; SAQ: saquinavir; IND: indinavir



**Figure 4.** Theory of analyte enrichment on analytical column

to achieve good LC/MS/MS sensitivity. Good spray conditions are necessary for strong, stable analyte signals.

A number of drugs have basic functional groups, and MS in the positive ion mode often detects such components as protonated ions. Protonation is the most important means of ionization in positive-ion electrospray mass spectrometry. Acidic mobile phases

### Injection Solution Selection Criteria

These criteria should be considered when selecting an injection solution:

- analyte adsorption, enrichment, retention, solubility, and stability
- retention mechanism(s)
- solvent evaporation

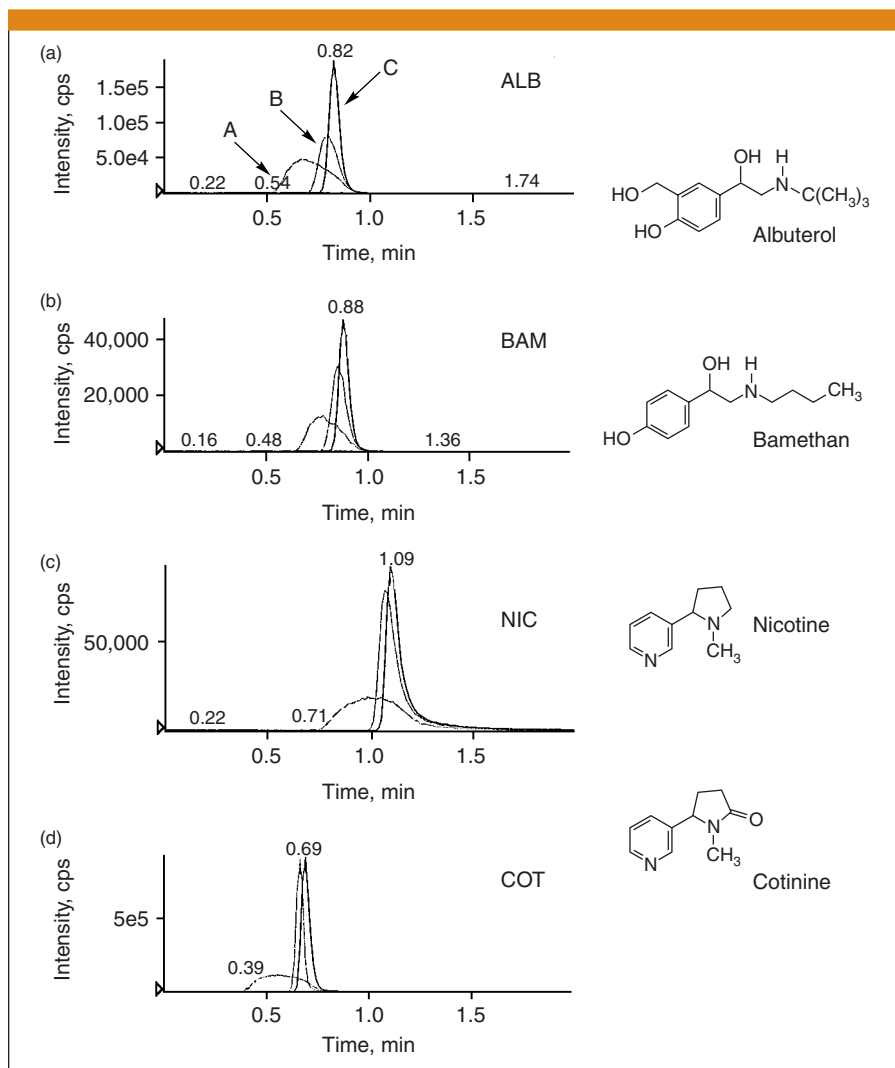
often are used to ensure that analytes are in their protonated forms. However, the charged analytes will have poorer retention on reversed-phase columns. That is undesirable because detrimental matrix effects can obscure the analysis.

That problem can be overcome by capturing polar ionic compounds on a silica column (9). Depending on the conditions, sensitivity on silica columns can increase five- to eightfold. Figure 1 illustrates dual retention on a reversed-phase column through the use of silanol to retain polar analytes. Various compound analytes (Table 1) have been successfully eluted using a silica column (9–20). Basic compounds are eluted with an acidic mobile phase to create cations for electrospray and ion detection.

Silica columns demonstrate excellent stability. Typically, one column can be used for at least 500 injections of extracted samples. Figure 2 shows column stability of the LC/MS/MS analysis of ribavirin in human plasma after 350 injections. Even with the protein precipitation extraction method, no retention-time changes were observed. The combination of an aqueous–organic mobile phase and a silica column also demonstrated an excellent, reproducible gradient elution. Reequilibration time was comparable with that of the reversed-phase column. Table 2 lists retention times for four consecutive injections of five different protease inhibitors. The resulting chromatograms demonstrate excellent peak shape and resolution, shown in Figure 3.

### Injection Solvents

A trend toward faster, more efficient LC/MS/MS has prompted the use of columns with smaller dimensions (50 μm × 2 μm). In contrast to conventional columns (250 μm × 4.6 μm), a one column offers advantages that include faster analysis time, better concentration sensitivity, and lower solvent consumption.



**Figure 5.** A common mistake: assuming that  $C_{18}$  columns are always reversed-phase; column: Hypersil BDS  $C_{18}$   $50 \times 2 \mu\text{m}$ , mobile phase: water–acetonitrile–formic acid (20:80:0.2, v/v/v); injection solution: A, water; B, mobile phase; C, acetonitrile

However, when smaller quantities of packing material are used, the chromatography can be more easily disturbed. Mismatches between the injection solution and the mobile phase are a common problem in compounds that demonstrate early elution peaks (21).

Choosing a reconstitution solvent compatible with the mobile phase can increase sensitivity. When the injection solution has a weaker eluting strength than the mobile phase, the effects of the injection solution on peak shape and chromatography efficiency can be enriched. Figure 4 shows a typical enrichment profile. Maximum chromatography efficiency can be achieved using injection solution at the weakest elution strength. From a practical point of view, better chromatography efficiency can

be obtained by diluting the sample with the weaker component in the mobile phase and increasing the injection volume.

**$C_{18}$  columns.** Before choosing a diluting solvent, you should determine the retention mechanism. Retention mechanisms dictate whether water or organic solvent will be stronger for elution. For reversed-phase LC, water is weaker than organic solvents, such as acetonitrile, are stronger. For normal-phase LC, the solvent strengths are reversed: Water is the stronger elution solvent, and organics the weaker. Even the name “reversed-phase  $C_{18}$  column” can be misleading. The retention mechanism on a  $C_{18}$  column can be complicated by the dual-retention mechanism, as shown in Figure 1. The actual retention mechanism depends on the analyte itself, the mobile phase

composition, and the stationary phase. If retention on the  $C_{18}$  column were blindly assumed to be reversed-phase, water would be used as the weaker elution solvent and, therefore, the injection solution. Very poor peak shapes would result. The influence of injection solvents on chromatography is demonstrated in Figure 5.

If an analyte is unstable in the mobile phase but stable in a weaker component of it, the mobile phase can still be used, as long as the weaker solvent is used for reconstitution. For example, omeprazole is unstable in acidic solution. To obtain good peak shape and sensitivity, an acidic mobile phase can be used. After extraction, omeprazole can be reconstituted in acetonitrile and injected onto a silica column with an acidic aqueous–organic mobile phase (13). Because of the short run time (three minutes), on-column degradation of omeprazole is not observed. In that situation, both analyte stability and on-column stacking are achieved because acetonitrile is a weaker solvent for the silica column (see the “Injection Solution Selection Criteria” box).

### Looking Ahead

LC/MS/MS provides superior sensitivity, selectivity, and rapid analysis. Method development for large-scale LC/MS/MS analysis is fraught with challenges, however. Obstacles can be prevented through careful planning and the application of logical problem-solving techniques. Automation and integration of information systems into bioanalytical laboratory processes and platforms have been shown to provide synergistic improvements in consistency, performance, and error reduction. Part 2 will present troubleshooting tips for carry-over, recovery and matrix effects, and selectivity; and for automation where possible to improve the overall reliability and efficiency of LC/MS/MS.

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