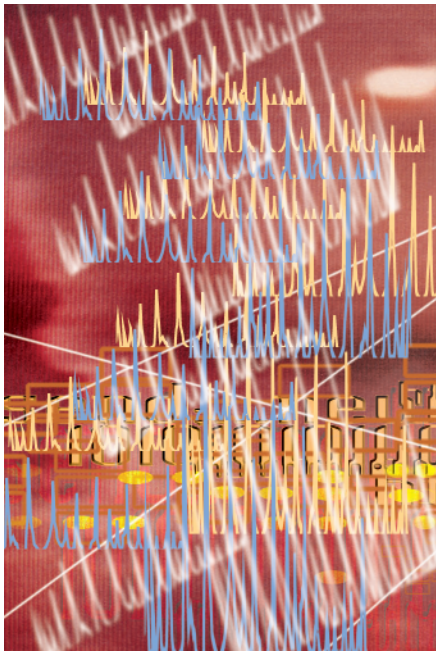

Systematic Troubleshooting for LC/MS/MS

Part 2: Large-Scale LC/MS/MS and Automation

Naidong Weng and Timothy D.J. Halls

Meeting the challenges of large-scale LC/MS/MS can improve the analytical processes that support biopharmaceutical drug development. The conclusion of this article presents troubleshooting techniques for LC/MS/MS and illustrates an automation strategy.

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LC/MS/MS can provide superior sensitivity and selectivity, rapid analysis, maximized development efficiencies, and improved turnaround times — its challenges are in large-scale application. In Part 1 (*BioPharm*, November 2001), we offered troubleshooting techniques for sample preparation and chromatography (1). LC/MS/MS remains one of the most useful tools available for bioanalysis. A rational, strategic approach of developing robust, large-scale, and automated LC/MS/MS methods can reduce slowdowns and bottlenecks in drug development and contribute to synergistic, consistent, long-term performance. Part 2 discusses further troubleshooting techniques and presents an automation strategy that improves method robustness and performance.

Elimination of Carry-Over

Carry-over is probably one of the most commonly encountered problems of LC/MS/MS method development. Sources of problems range from instrument hardware and the selection of appropriate rinse solvents to challenges with chromatography. Frequently, resolving the problem requires a combination of individual experimental solutions and systematic and logical investigation.

Carry-over is a phenomenon that was discovered during method development in LC/MS/MS of ritonavir in human plasma. A cyano column was used with 0.1% acetic acid and an acetonitrile gradient elution. Blanks injected after high-level standards showed increasing carry-over (Table 1). Chromatograms (Figure 1) show both the high-level standard and the blank injected immediately after the high-level standard. Prior injection of the same blank before the high-level standard did not show a ritonavir peak, which confirmed a carry-over effect. The gradient elution was actually determined to be the cause of the carry-over effect.

To address the issue, an isocratic elution with a mobile phase that contained 45% acetonitrile was used to eliminate the carry-over. A new problem surfaced when the original internal standard was not retained on the column in the mobile phase, so a new one had to be used. The new standard had retention properties similar to those of the analyte. Figure 2 depicts the resolution of the carry-over problem. The method was validated and successfully used for sample analysis.

The ritonavir example is one of the more complicated troubleshooting scenarios. Both chromatographic condition and internal standard were changed as a result of the problem and its solution. Occasionally, carry-over problems have simpler solutions. In another example, simply raising the needle height solved the carry-over effect. Carry-over is often avoided by merely minimizing the contact surface between analyte and needle.

Recovery and Matrix Effects

LC coupled with MS/MS detection is a specific and sensitive method for drug analysis in biological matrices. Because of the highly sensitive nature of MS/MS detection, extensive chromatographic resolution may not be required, and short run times can be used to obtain very high throughput for sample analysis. Materials in the extracted biological matrix, however, can exist in much higher concentrations than the analyte. Some materials may have the same *m/z* for both father and daughter ions and will be observed on the chromatogram as interference peaks. Though the peaks may be unseen on the LC/MS/MS chromatogram, what happens more often is that material from the extracted biological matrix elutes closely to the analyte. Ionization is affected and results in high imprecision and loss of

sensitivity within a run. For an LC/MS/MS method, it is important to identify whether the loss of sensitivity is due to poor recovery or to matrix suppression, because both causes give the same result. Carefully designed experiments will establish the source of the problem.

Recovery is determined by comparing the peak areas of extracted samples with those of neat solutions spiked (postextraction) into a blank matrix. Because both samples have the matrix ingredients present, the matrix effects can be considered the same for extracted samples and postextraction spiked samples. Any differences in response can now be considered to be due to extraction recovery. The matrix effect is determined by comparing peak areas of neat solutions spiked (postextraction) into blank matrix with those of other neat solutions. Because the analytes are not extracted, the analyte should have the same response in postextraction spiked samples and in neat solutions. The matrix ingredients, therefore, cause whatever differences are noted in the responses.

A useful method to assess matrix suppression is postcolumn infusion of an analyte into the MS detector. The extracted blank matrix is injected by an autosampler onto the analytical column. The setup is shown in Figure 3. The purpose of postcolumn infusion with analyte is to raise the background level so that the suppression matrix will show as negative peaks. This setup has been successfully used to identify and troubleshoot matrix suppression peaks.

During LC/MS/MS method development for analysis of a nucleoside compound and its metabolite, lower and inconsistent signals of the metabolite peak were observed. An aqueous–organic mobile phase and a silica column were used. The extraction method was a simple protein precipitation. The lower signal was due to matrix suppression, which was confirmed by postcolumn infusion portrayed in Figure 4. A broad suppression band was observed around the analyte peak. The problem was overcome by diluting the extracted sample five-fold with the weaker elution solvent, in this case, acetonitrile. The suppression was no longer observed, and the method has since been validated and successfully used for routine sample analysis.

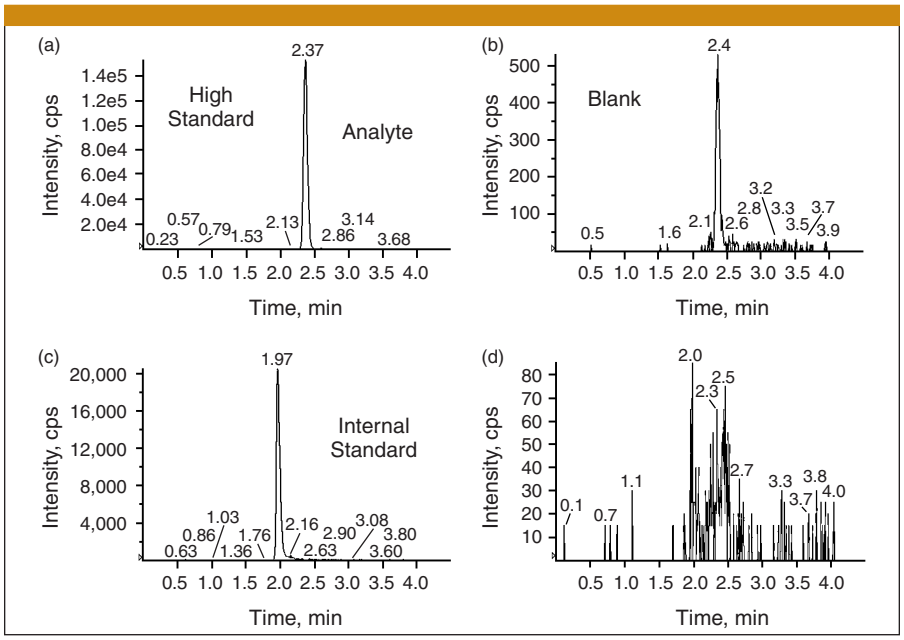

Figure 1. Identification of carry-over problem by injecting a standard at high concentration (left panels) followed by a blank (right panels)

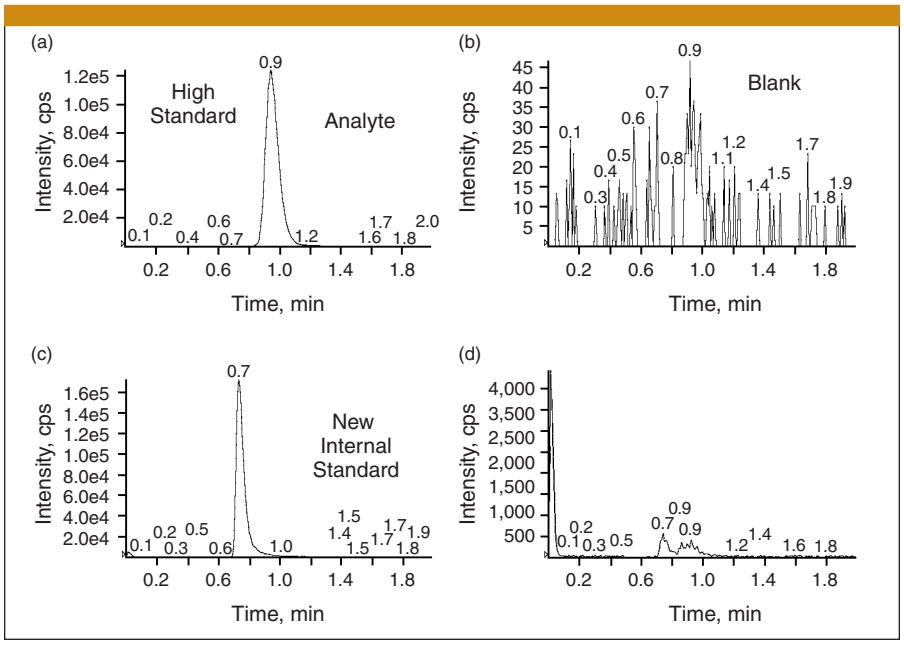

Figure 2. Carry-over problem was resolved as evidenced by no analyte peak in the blank (right panels) injected immediately after the standard at high concentration (left panels)

Selectivity

Although MS/MS is highly selective for discriminating analytes from interference peaks, the blank screen test routinely performed for HPLC-UV is inadequate for LC/MS/MS methods. Because of simplified extraction procedures (a benefit of the high-selectivity of MS/MS detection), many endogenous compounds, metabolites, or coadministered medicines can coelute with the analytes of interest and not show up as

Table 1. Carry-over test of the analyte by injecting standards at high concentration and at blanks

Test	Analyte Area
Standard 500	606,022
Blank	954
Standard 500	595,334
Blank	1,486
Standard 500	608,066
Blank	1,935

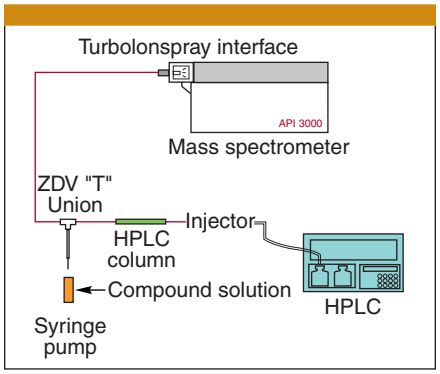

Figure 3. General set-up for identifying matrix effect: postcolumn infusion of compound while injecting extracted blank

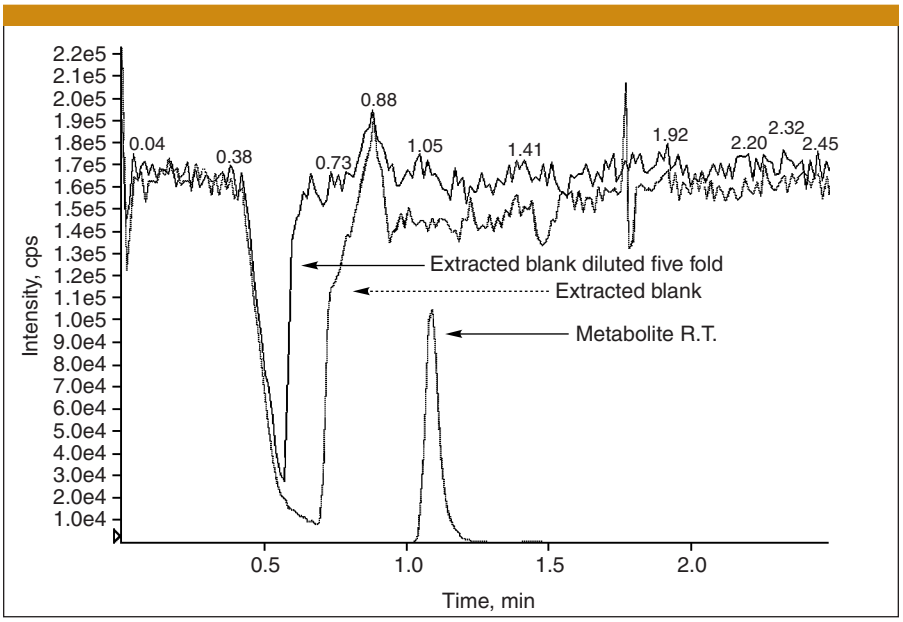

Figure 4. Postcolumn infusion to determine the matrix suppression profiles of original extraction and modified extraction: matrix suppression was eliminated by diluting the extracted samples five fold

interference peaks at the *m/z* channel of the analytes. Those unseen peaks cause matrix suppression and inconsistent signals. The selectivity of LC/MS/MS can be compromised even more by a breakdown of metabolites (especially conjugated metabolites) at the LC/MS interface. Those breakdown products are usually the analytes of interest. Without chromatographic retention and resolution, the conjugated metabolite, and therefore the in-interface breakdown products, can elute with the same retention as the analyte. Those breakdown products have the same precursor–product *m/z* as the analyte and can be falsely quantified as analytes of interest,

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which can result in severe overestimation of the analyte (2).

Long retention times do not necessarily translate into large capacity factors (k′), which are the true measurement of on-column retention. A long narrow-bore column at a low flow rate will have longer retention times even though the analytes may actually elute at void volume. Approximately the same k′ values (k′=1) can be obtained for an analyte with one-minute retention on a 5 cm × 3 mm column at a flow rate of 0.5 mL/min, as for a five-minute retention on a 25 cm × 4.6 mm column at a flow rate of 1.0 mL/min for the same analyte.

One approach to selectivity is to ensure that blanks are truly blank and also to ensure that good accuracy is obtained for different lots of matrix spiked with analytes of interest. Coadministered medicines and known metabolites should be spiked to QC samples to demonstrate that their presence does not cause quantitation bias from either interference or matrix suppression.

Automation

Efficiency and speed bottlenecks exist in every organization, whether a commercial enterprise or an academic laboratory. Laboratory layouts and design issues contribute to many of the inefficiencies because most labs have not been purposefully built for efficient work flow.

One strategic approach to overcoming bottlenecks for high-throughput bioanalysis is to use well-established instrumentation; rigorous, standardized techniques; and automation, wherever possible, to replace manual tasks. Automation results in greater performance consistency over time and in more reliable methods transfer from site to site. Constant assessments of processes, technologies, and procedures are required for continued and incremental process improvements.

For example, automated 96-well plate technology is well-established and accepted and has been shown to effectively replace manual tasks. The 96-well instruments can execute automated offline extractions and sample cleanups. They take advantage of parallel processes, replace manual techniques, and offer consistent, standardized methods.

Incorporating a disciplined information technology (IT) strategy into the laboratory is essential for productivity improvements and consistent performance. The IT strategy should be integrated into automation processes whenever and wherever possible. State-of-the-art IT systems (such as customized Laboratory Information Management Systems or LIMS) allow analytical labs to maximize efficiencies and improve the turnaround time of quality-control data for clients. LIMS-type systems track samples and provide validated data streams, reducing turnaround times and the substantial time once allotted to quality control and data checking.

Figure 5 provides a general approach for automated 96-well sample analysis and its benefits. Automated solid-phase extraction (SPE) and liquid–liquid (LL) and protein precipitation (PP) extraction can all be performed in the 96-well format. The chromatograms of extracted low limit of quantitation (LLOQ) and blank plasma or serum samples of three examples are shown in Figure 6. In that example, the Multiprobe liquid handling station (Packard, www.packardbioscience.com) was used to aliquot samples and add internal standards. The Quadra 96-320, a 96-well workstation from Tomtec (www. tomtec.com) was used for SPE (fentanyl), LL (fluconazole), or PP (ribavirin) extractions. Aqueous–organic mobile phases on silica columns were used to achieve the excellent peak shapes and sensitivity.

Table 2 summarizes the blank screen and matrix effect tests. No interference peaks were observed in any of the lots tested. No matrix

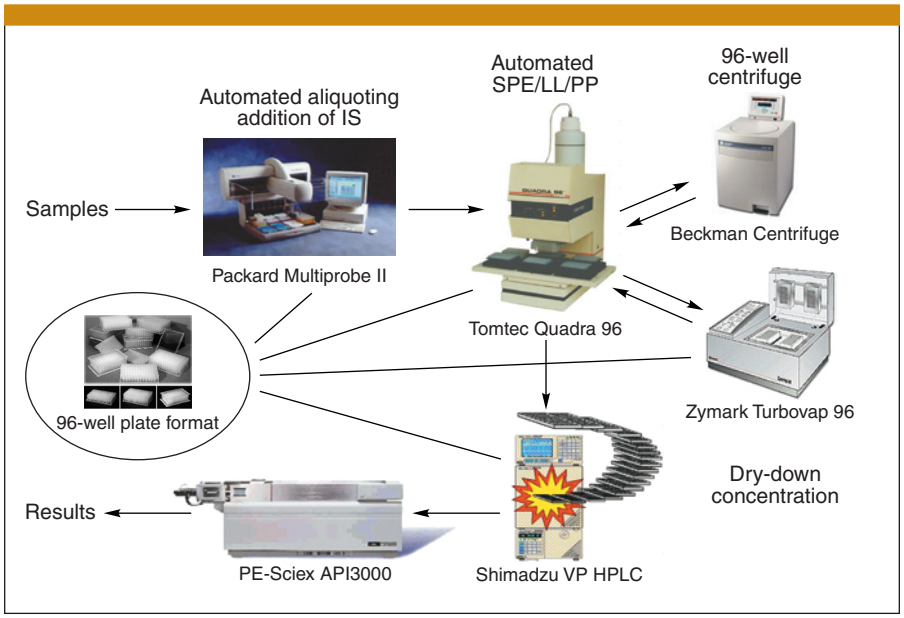

Figure 5. Automated 96-well sample analysis

Table 2. Blank screen and matrix effects test for fluconazole (liquid–liquid extraction) in human plasma; fentanyl (SPE) in human plasma; and ribavirin (protein precipitation extraction) in human serum

Fluconazole (ng/mL)			Fentanyl (ng/mL)			Ribavirin (ng/mL)		
Plasma Lot #	Std 0.000	Std 0.500	Plasma Lot #	Std 0.0000	Std 0.0500	Plasma Lot #	Std 0.0	Std 30.0
7496	0.000	0.505	6307	0.0000	0.0503	6065	0.0	31.8
7497	0.000	0.506	6308	0.0000	0.0479	6066	0.0	30.3
7498	0.000	0.518	6309	0.0000	0.0535	5978	0.0	29.1
7499	0.000	0.487	6310	0.0000	0.0486	5979	0.0	31.0
7500	0.000	0.459	6311	0.0000	0.0514	5980	0.0	31.5
7501	0.000	0.522	6313	0.0000	0.0505	5981	0.0	25.9
Mean	0.000	0.499	Mean	0.0000	0.0504	Mean	0.0	29.9
RSD%	NA	4.7	RSD%	NA	4.0	RSD%	NA	7.3
RE%	NA	−0.1	RE%	NA	+0.7	RE%	NA	−0.2

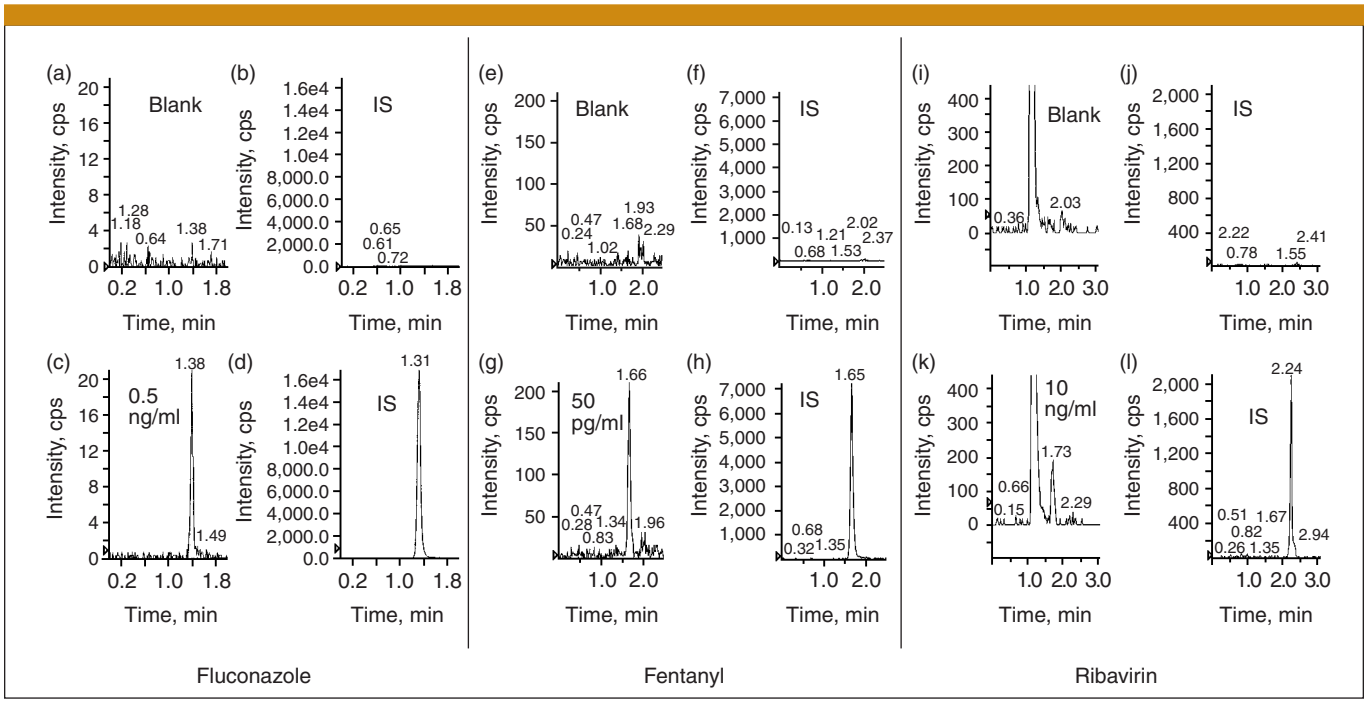

Figure 6. Chromatograms of blank (upper panels) and low limit of quantitation (lower panels) for fluconazole (96-well liquid–liquid extraction), fentanyl (96-well SPE extraction), and ribavirin (96-well protein precipitation extraction)

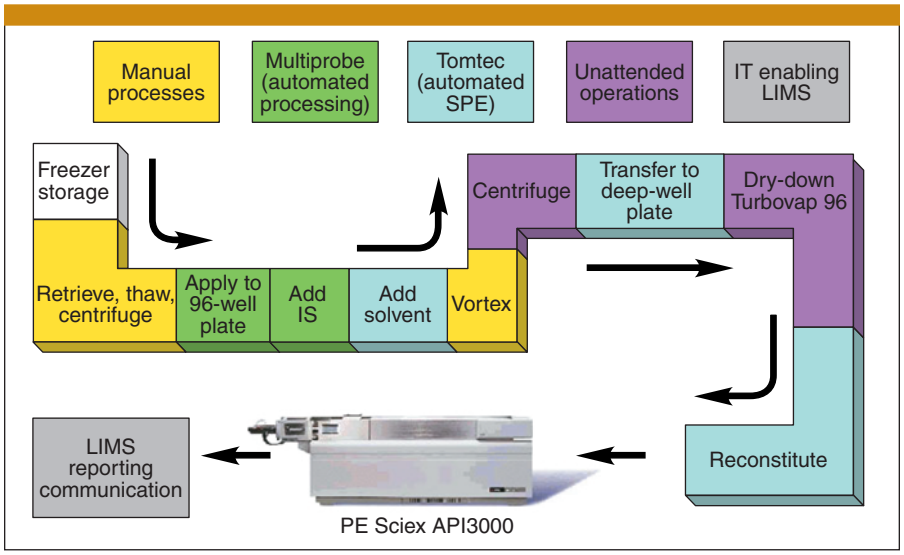

Figure 7. Benefits of automation (arrows indicate where LIMS is applied)

lot-to-lot differences were observed because all the spiked samples were back-calculated within 15% of the theoretical values.

Automation benefits are shown in Figure 7. The arrows in the Figure indicate the stages where the samples are tracked by the LIMS. Manual involvement is kept to a minimum.

Proteins and Peptides

Method development strategies are well suited for LC/MS/MS analysis of proteins and peptides. Reversed-phase LC/MS/MS of proteins and peptides have been extensively used in biotechnology analytical laboratories (3). Typically, a gradient elution on a reversed-phase column is used with an organic solvent that ranges in concentration from 0% to 40%–60%. Small amounts of trifluoroacetic acid (TFA), and to a lesser extent formic acid, are included in the mobile phase to improve the peak shape. Because of the gradient elution and characteristics of proteins and peptides, the carry-over problem can be significant and should be carefully controlled. In some cases, it may even be necessary to inject a blank after each sample to circumvent the problem.

Proteins and peptides also present additional challenges for LC/MS/MS practitioners. Proteins and peptides easily form adducts with various types of salts. The adducts formed can significantly change the charge of the molecules, which may result in poor quantitation reproducibility because of the shifted *m/z* values. Salts can also suppress the LC/MS/MS signals of analytes (4). Therefore, before LC/MS/MS, a desalting process is important using either offline dialysis or online desalting procedures. In the online desalting technique, the analytical column is typically washed by the aqueous mobile phase for several minutes before gradient elution.

Meeting the Challenges

LC/MS/MS provides superior sensitivity, selectivity, and rapid analysis. Automated 96-well technology has significantly improved turnaround times and has opened up new opportunities to maximize efficiencies and to enhance drug development.

Method development for large-scale LC/MS/MS analysis is fraught with challenges, however. Obstacles can be

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overcome through careful planning and through the application of logical problem-solving techniques. Automation and integration of information systems into bioanalytical lab processes and platforms have been shown to provide synergistic improvements in consistency, performance, and error reduction.

Acknowledgments

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