

Critical Review of Development, Validation, and Transfer for High Throughput Bioanalytical LC-MS/MS Methods

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Abstract: Swift growth in the use of LC-MS/MS for the analysis of drugs in biological matrices has been compelled by the need for timely and high-quality data at many stages in drug discovery and development process: from high throughput screening of drug candidates and rapid data generation for pre-clinical studies to almost 'real-time' analysis of clinical samples. Prompt and rational method development, validation, and transfer play a pivotal role in achieving the goals of "faster, better, and cheaper" for pharmacokinetic studies since this could easily account for more than 50% of the time and labor resources for a moderate-sized project. Strategy for rational method development, validation and transfer has been largely kept as institutional knowledge but rarely appeared in literature. In this review article, strategies for developing and validating robust high throughput LC-MS/MS methods will be critically reviewed and discussed. Automated sample preparation, fast chromatography, minimization of matrix effects, and strategy of narrowing the gap between validation and incurred sample analysis are just a few topics covered in this review. Other interesting approaches for improving method efficiency and ruggedness such as direct injection SPE and liquid/liquid extracts as well as multiplexing of LC columns will also be discussed. Potential pitfalls during method development and validation are pointed out. At the end, the question "how fast is fast enough and how fast is too fast?" will be answered after considering all aspects of the method development and validation.

Keywords: LC-MS/MS, Bioanalytical, Development, Validation, Transfer.

1. INTRODUCTION

The use of LC-MS/MS has grown exponentially in the last decade, due to its un-matched sensitivity, extraordinary selectivity, and rapid rate of analysis. The principle of MS is the production of ions from analyzed compounds that are separated or filtered on the basis of their mass-to charge ratio (m/z). Most of applications for quantitative bioanalysis use tandem mass spectrometers (MS/MS) that employs two mass analyzers – one for the precursor ion in the first quadrupole and the other for the product ion in the third quadrupole after the collision – activated dissociation of the precursor ion in a collision cell. Innovative and successful research efforts in the past decades on the design of an effective interface connection between LC (operated under atmospheric pressure) and MS (operated under a high-vacuum environment) have made LC congenial with MS. Electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI), collectively called atmospheric pressure ionization (API), have matured into reliable interface necessary for quantitative LC-MS/MS bioanalysis. More recently, atmospheric pressure photo-ionization (APPI) also became an interesting alternative ionization source for quantitative LC-MS/MS [1,2]. Yet, analysis of drug candidates in biological matrices using robust LC-MS/MS methods remains a difficult, time-consuming, daunting, and occasionally dismaying challenge. Successful use of LC-

MS/MS requires understanding the mechanism of various sample extraction processes and the underlying principles of both chromatography and MS. Many LC-MS/MS challenges have been reviewed, discussed and published [3-15]. Clinical applications of LC-MS/MS on diagnosis and screening for inherited metabolic diseases were reviewed by Rashed [16]. Our previous publications also addressed the systematic trouble-shooting of LC-MS/MS separations and sample preparations [17,18]. Tiller *et al.* discussed fast LC-MS analysis in the analysis of small molecules from the point of view of chromatographers [19]. A book on high throughput bioanalytical sample preparation was written by Wells [20].

In this review, we will critically discuss rational method development, validation and transfer of robust LC-MS/MS methods with emphasis on important factors impacting the incurred sample analysis. We focused on the mature and established technologies used in most quantitative bioanalytical laboratories. Therefore, emerging new technologies such as chip-based nano electrospray, LC-MALDI, and ultra-performance liquid chromatography (UPLC)- MS/MS are not covered in this review. Though the principles remained unchanged, every 3-5 years, new generations of mass spectrometers with higher sensitivity, better resolution, more ruggedness, and more automation emerged. Review on MS instrument features deserves a separate review and is beyond the scope of the current review. Although discovery studies do not have validation requirement, the practices described in this review is also applicable.

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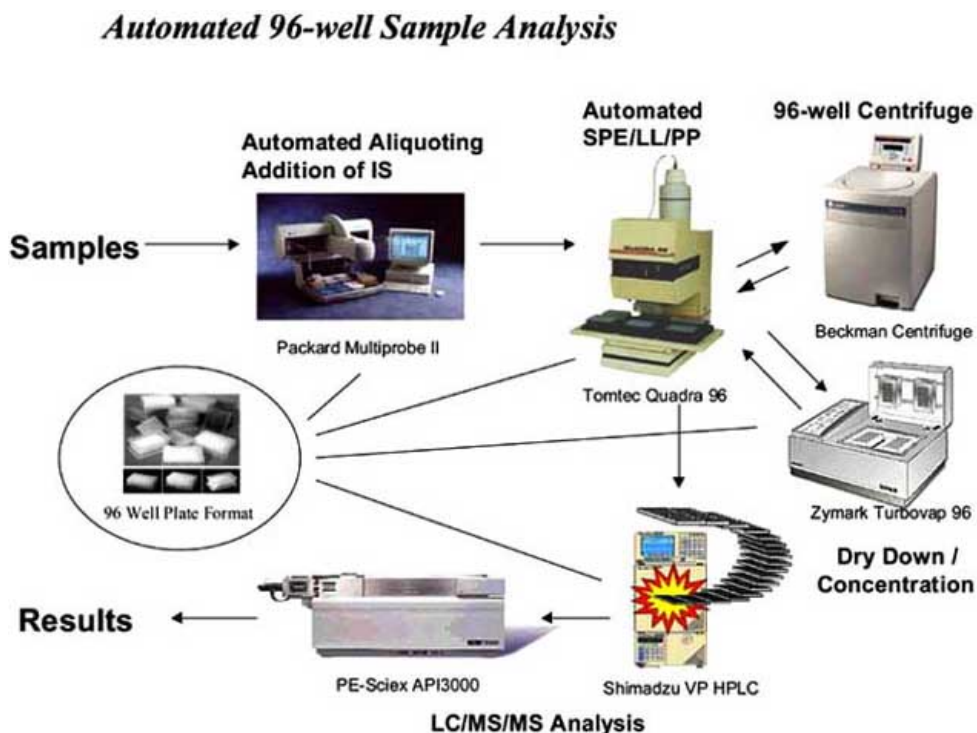


Fig. (1). General Approach for Automated 96-well Sample Analysis.

2. Rational Method Development Strategy

Traditional sequential method development requires sequential optimization of mass spectrometric and chromatographic conditions, sample extraction, recovery of the analyte, and lack of interference and matrix effects for each method. This type of method development is time-consuming, labor and instrument intensive and costly when several different LC-MS/MS methods for various types of analytes need to be developed. This approach has been identified as the major bottleneck for meeting the ever-increasing needs for LC-MS/MS methods. A concept of simultaneous development of multiple bioanalytical LC-MS/MS methods was presented [21]. Optimal conditions of mass spectrometry, chromatography, and extraction were screened and developed for six structurally different analytes. Experimental designs for simultaneously determining and evaluating recovery, matrix effects, and chromatographic interference were proposed. In another presentation, processes were optimized so that a robust LC-MS/MS method was developed in a single working day [22]. Two scientists work simultaneously on the same project in a coordinated way: one focusing on sample preparation and other focusing on instrumentation. Important method parameters such as matrix suppression and recovery were investigated. Janiszewski *et al.* also proposed high-throughput method development approaches by simultaneous testing multiple SPE chemistries using a custom multiple sorbent 96-well plate with optimized extraction conditions for up to five analytes are determined in a single 2.5-h experiment [23]. Sadagopan *et al.* systematically investigated the feasibility of using EDTA anticoagulant in plasma to improve the throughput of LC-MS/MS assays [24]. Use of automated sample transfer

resulted in a three-fold productivity increase over the manual transfer. Avery suggested investigating simultaneously multiple experimental approaches for assessing the matrix effects, thus speeding up method development [25]. Li and Cohen proposed a novel approach by using “surrogate analyte” to analyze endogenous compounds [26]. Method development strategy for sample preparation was also discussed by Wells [20]. Derivatization for enhancing MS sensitivity has been successfully used to develop highly sensitive LC-MS/MS method to measure “hard-to ionize” compounds such as estradiol and ethinyl estradiol [27-29].

3. Sample Preparation

One strategy for high-throughput bioanalytical analysis 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. 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 off-line extraction and sample clean-ups. Automated solid-phase extraction (SPE) [30-37], liquid/liquid extraction (LLE) [38-44], and protein precipitation (PP) [45,46] all can be performed in 96-well format. Both cartridge and disc in 96-well SPE formats have been successfully used. New trends in SPE have been reviewed by Poole [47]. Polson *et al.* discussed optimization of protein precipitation based on effectiveness of protein removal and ionization effect [48]. (Fig. 1) provides a general approach for automated 96-well sample analysis. In comparison to manual operation, automated sample preparation saved at least 50% time. However, the

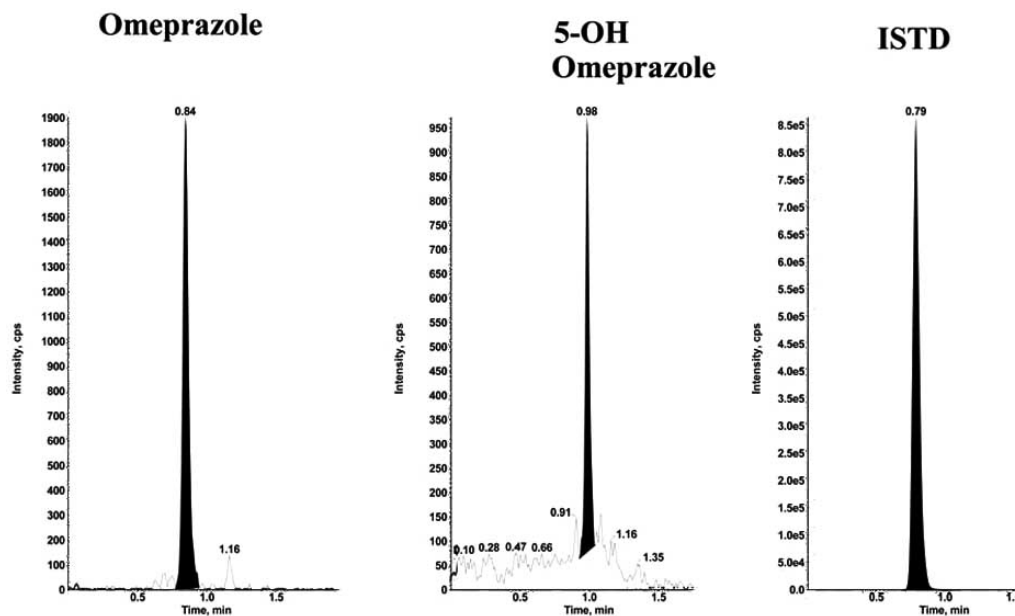


Fig. (2). HILIC-MS/MS of Omeprazole and 5-OH Omeprazole (2.5 ng/ml) in Human Plasma.

Column: Betasil Si, 50x3 mm at 40°C

Linear Gradient: water/acetonitrile/formic acid (containing 5 mM ammonium acetate)
(Min 0: 5/95/0.1; Min 2.0: 32/68/0.1)

Flow rate: 1 ml/min

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commonly used solvents for SPE, LLE, and PP have stronger elution strength than the mobile phase of the reversed-phase column. That difference limits the injection volume, and therefore the sensitivity of the assay, since the peak shape depends largely upon the injection solvent [49,50]. In many cases, an evaporation step followed by reconstitution with mobile phase compatible solvent is necessary. Further improvement on throughput for another 50% was made when the organic extracts from SPE [51,52] or LLE [53,54] were injected directly onto the silica column with low aqueous – high organic mobile phases. While the commonly used SPE or LLE extraction solvents are stronger elution solvents (not compatible) than a mobile phase on typical reversed-phase chromatography, they are weaker elution solvents (compatible) on the silica column with low aqueous – high organic mobile phases operated under hydrophilic interaction chromatography (HILIC). (Fig. 2) shows a chromatogram of direct injection of LLE solvent (ethyl acetate) of omeprazole and its metabolite. In this approach, the time-consuming and error-prone solvent evaporation and reconstitution steps are eliminated.

4. Chromatography

With the initial sample clean-up using SPE, LLE or PP, unwanted compounds can be present still in higher concentrations than the analytes of interest. A second stage of clean-up, typically involving LC separation, further separates analytes of interest from the unwanted compounds. Without this further separation, those unwanted and MS/MS unseen compounds present significant challenges. In the LC-MS interface, these compounds compete with analytes for ionization and cause inconsistent matrix effects that are detrimental to quantitative LC-MS/MS performance [55,56].

Reversed-phase LC has been traditionally used for the quantitative LC-MS/MS. With increase of organic solvent concentration in the mobile phase, the analyte retention decreases. However, one should be aware of the potential bi-modal retention on the reversed-phase column due to the residual silanol groups [57]. This bi-model retention may cause retention shift during the run or irreproducibility of the method. Among all the reversed-phase columns, of particular interest is the use of monolithic columns (available in C18 format) operated at a high flow rate. The chemistry and characteristics of the monolithic columns have been studied and reviewed [58,59]. Compared to a particulate column, the monolithic column has a reduced pressure drop but still maintains high separation efficiency. This is due to its unique bimodal pore structure, which consists of macropores (2 μm) and mesopores (13 nm) [60]. The mesopores provide the surface area for achieving adequate capacity while the macropores allow high flow-rates because of higher porosity, resulting in reduced flow resistance. Monolithic columns have become increasingly popular for use in ultrafast bioanalysis of drug candidates using tandem mass spectrometric detection [61-64]. (Fig. 3) shows an ultrafast LC-MS/MS of five analytes within 1.2 min [65].

Polar compounds often have poor retention on a reversed-phase column even with high aqueous mobile phase. Poor analyte on-column retention may result in detrimental matrix effects, which has been identified as one of the major reasons why bioanalytical LC-MS/MS methods fail. High aqueous content mobile phase is also not conducive to achieving the good spray conditions that are critical for sensitivity. Many drugs have basic functional groups, and acidic mobile phases are used and MS in the positive ion mode detects these compounds as the protonated

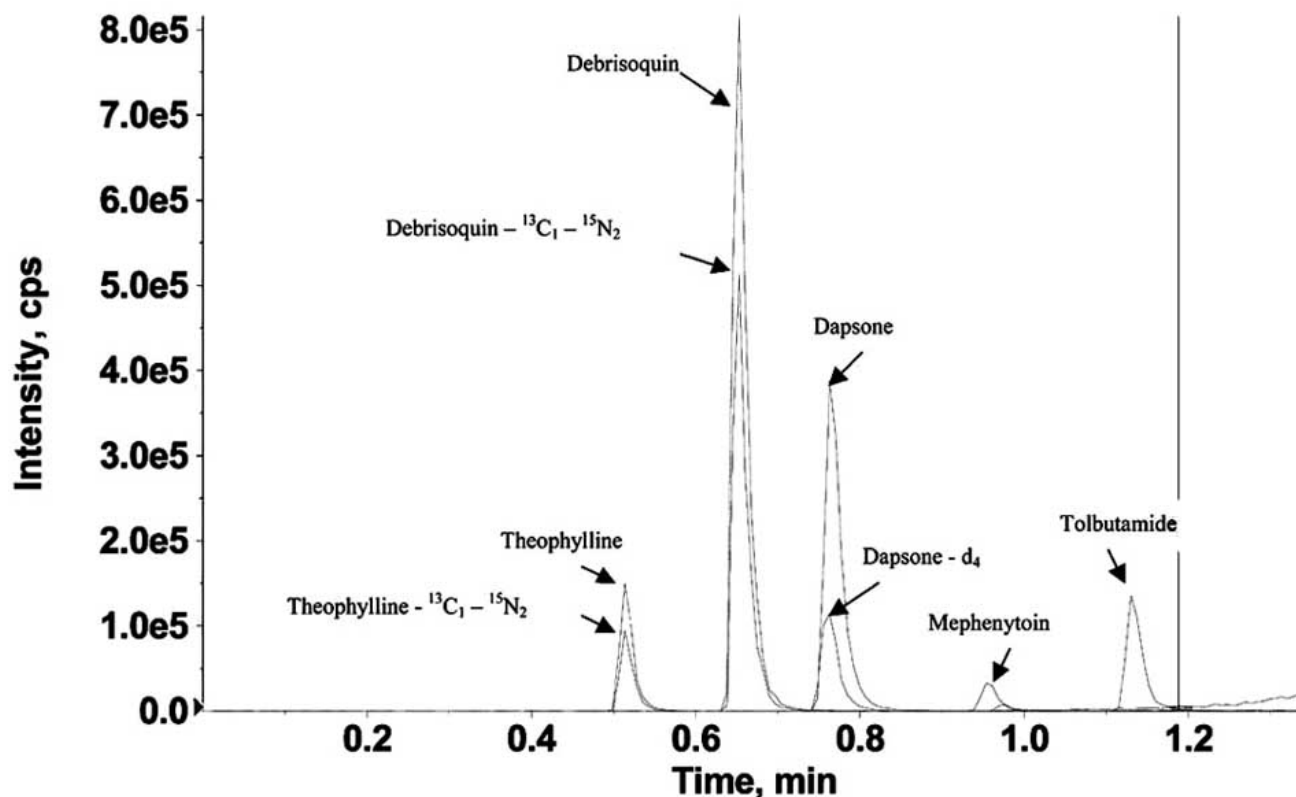


Fig. (3). LC-MS/MS total ion chromatogram of theophylline, tolbutamide, mephentyoin, debrisoquin, and dapsone.

Column: Chromolith SpeedROD RP-18e, 50 x 4.6 mm

Mobile phase: acetonitrile – water – formic acid (linear gradient elution from 10:90:1 at 0 min to 90:10:1 at 1.40 min). The eluent was post-column splitted and approximately 0.5 mL/min was directed to the LC-MS interface.

Flow rate: 2.5 mL/min

Injection volume: 5 μ L of 300 ng/mL of each analyte in a mixture of methanol – water – formic acid (50:50:0.1, v/v/v).

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ions. Ionization of polar compounds further decreases the analyte on-column retention on a reversed-phase column. To overcome this mismatch between reversed-phase LC and MS detection, other chromatographic materials were investigated to achieve better sensitivity and better on-column retention. Zirconia-based column may offer different retention mechanism from silica-based column and has been explored for quantitative bioanalytical LC-MS/MS application [66]. Ion-exchange chromatography and HILIC became popular choices in the last several years. Ion-exchange chromatography is particularly effective for retaining and separating nucleotide compounds [67]. HILIC is similar to normal phase LC in that the elution is promoted by the use of polar mobile phase. However, unlike classic normal phase LC where the water in the mobile phase has to be kept in minimal but constant levels, water is present in a significant amount (>5%). HILIC also uses water-miscible polar organic solvents such as acetonitrile instead of water-immiscible solvents like hexane and chloroform. LC-MS/MS using HILIC on silica column has been extensively studied and reviewed [68-74]. Because of the higher organic content in the mobile phase and the more favorable solution chemistry for ionization (acidic mobile phase for basic compounds and neutral mobile phase for acidic compounds), sensitivity improvement was observed [75]. Besides the feature of

direct injection of SPE and LLE extracts, silica column has very low back pressure under HILIC condition, which facilitates high flow rate chromatography [76]. (Fig. 4) shows a fast gradient elution of nicotinic acid and its six metabolites within one minute [77]. Nicotinic acid and metabolites are extremely polar compounds and have no retention at all on a reversed-phase monolithic column.

5. Parallel Analysis

In addition to the high flow rate LC-MS/MS, another approach for achieving high throughput is to employ parallel analysis approach, either using multiple inlets in the mass spectrometer source and multiplexing LC unites into one MS. A four-channel multiplexed electrospray (MUX) ion source (Waters-Micromass, Manchester, UK) allows the non-stop introduction of the eluent from four LC columns into the mass spectrometer. The sampling rotor permits, automatically controlled by the software, only one spray at a time to be admitted to the cone of the mass spectrometer. While the multiple inlets in the mass spectrometer allow four-fold increase in throughput, sensitivity loss and potential cross-inlets contamination are the drawbacks [78]. Multiplexing LC units require a little bit more complicated system set-up but the two shortcomings are overcome. (Fig.

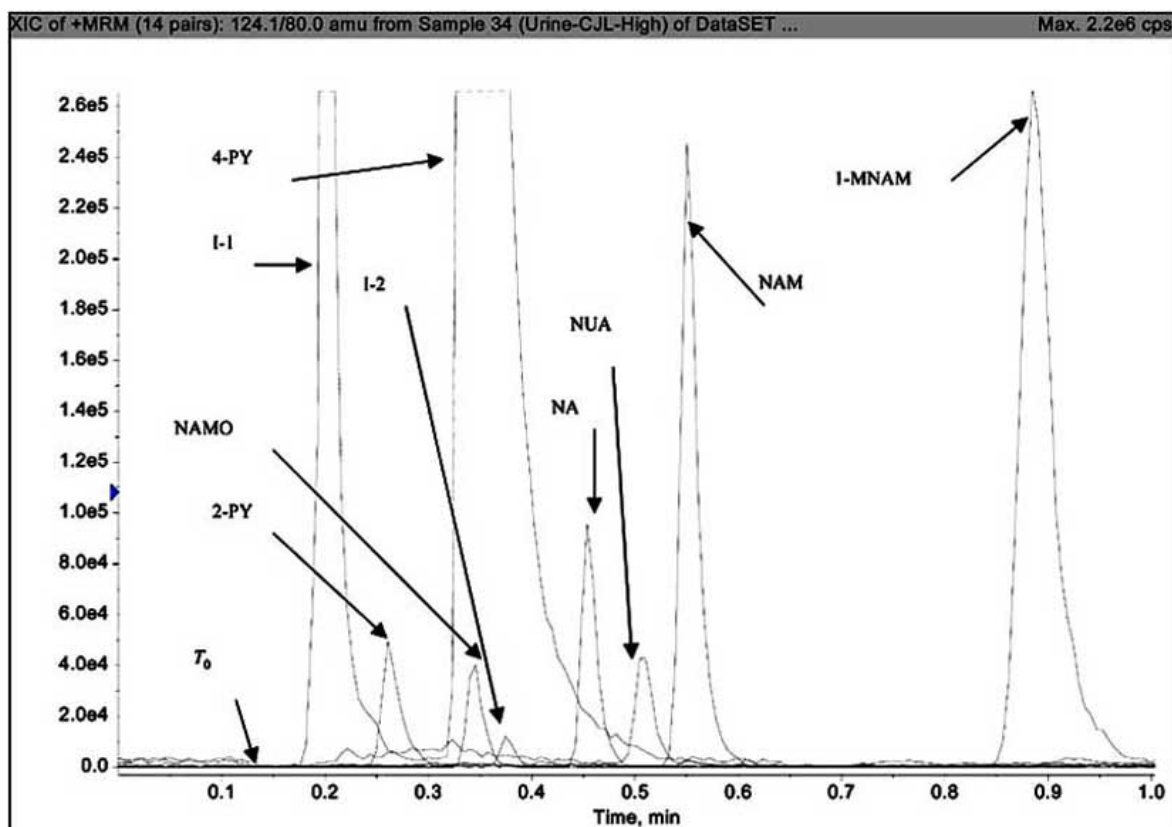


Fig. (4). Reconstructed XIC mass chromatogram from an incurred human urine sample containing nicotinic acid and its metabolites on an Hypersil silica column (50 x 4.6-mm) using a flow-rate of 4 ml/min.

Mobile phase: The mobile phases A and B were water and acetonitrile respectively, both containing 1% formic acid (v/v). Gradient mobile phase: 0.01-0.25 min gradient from 90% B to 65% B; 0.25-0.90 min gradient from 65% B to 50% B; At 0.91 min, back to 90% B.

Peak identification: NA = Nicotinic acid; NAM = Nicotinamide; NUA = Nicotinuric acid; 2-PY = 1-Methyl-2-pyridone-5-carboxamide; I-MNAM = 1-Methyl-nicotinamide; NAMO = Nicotinamide-*N*-oxide; 4-PY = 1-Methyl-4-pyridone-5-carboxamide.

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5) demonstrates that when two LC units feed one MS, the run time is reduced 50%. This approach has been successfully used for analysis of drug candidates in plasma [79-81].

6. On-line Extraction

One of the major bottlenecks in high throughput analysis of biological samples is the sample preparation prior to injection [82]. This step is almost always necessary in order to remove matrix components, such as proteins, salts and other organic compounds, in biological matrices which otherwise may impose interference or ionization suppression to the analyte(s). With the improvement in instrumentation and column technology [83-85], on-line sample extraction that is coupled into LC-MS/MS has developed, in the past few years, as an important tool facilitating direct injection analysis of biological samples, especially for the discovery support. In general, this technique employs turbulent flow chromatography in which extraction columns are packed with retaining particles of large size (e.g., 50 μm), thus providing fast sample extraction under high flow conditions. As compared to the off-line automated solid-phase extraction (SPE) (e.g., with 96-well or 384-well format), on-line

extraction provides more flexibility for troubleshooting and method development. In addition, it also lowers the cost for sample analysis by using extraction columns that can be repeatedly used for hundreds of biological samples.

On-line extraction can be performed with two different configurations on the commercially available CohesiveTM instrument. In single-column extraction, samples are loaded onto extraction column, and extracted directly with the mobile phase of strong solvent (e.g., high organic content for reverse-phase extraction) onto the analytical column. This approach provides a very high sample throughput and low sample carry-over between injections. However, the peak capacity and chromatographic resolution with this method can be low due to the use of the solvents of strong elution strength for both extraction and separation. Single column extraction has thus certain limitations for the applications where drugs, the metabolites and/or endogenous species need to be separated from each other to eliminate interference in mass spectrometric detection. In a dual-column configuration, samples are extracted with the solvent that is previously stored in an extraction loop. The extracts are transferred through a tee to the analytical column into which the analytical pump delivers a weak mobile phase at

Simple Multiplexing

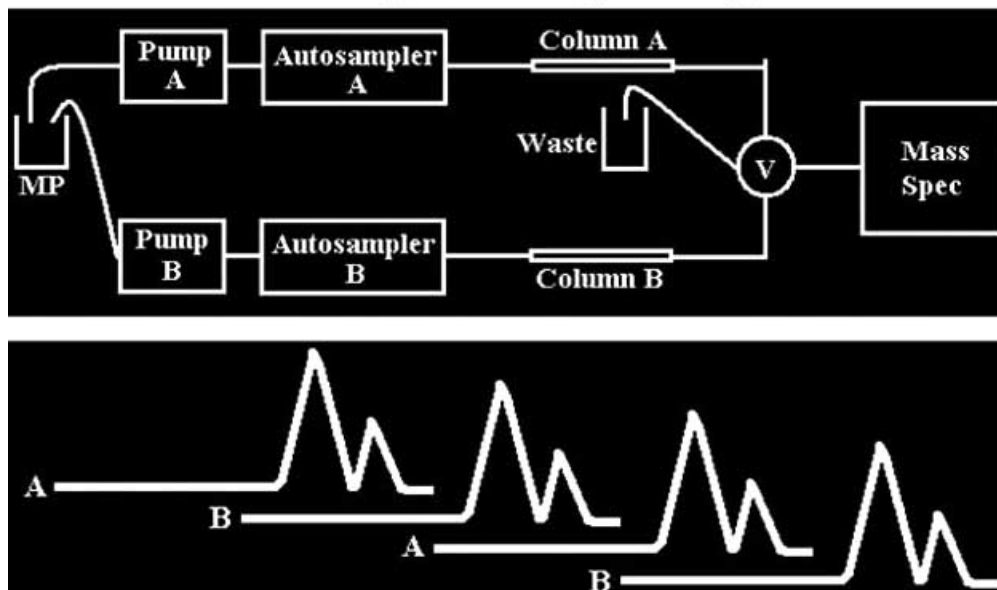


Fig. (5). Simple Multiplexing Double the Throughput.

relatively higher flow rate in order to dilute the extraction solvent and thus focus the extracted compounds on the analytical column. Once the sample is transferred, the compounds are eluted and separated with a gradient on the analytical column (while the extraction loop is being filled with the extraction solvent by the extraction pump for the next extraction). Dual-column extraction LC-MS/MS generally provides better chromatographic separation and good peak shape as compared to the single-column method. In general, the extraction flow rate in the *t*-in step needs to be maintained minimal in order to uphold a desired focusing effect, which along with a gradient elution on the analytical column can certainly reduce the analytical throughput, in comparison with single-column extraction mode. One approach of increasing the throughput is to use the monolithic column as the analytical column, operated under a high flow rate. (Fig. 6) shows the example of dual-column on-line extraction LC-MS/MS of dextrorphan/dextromethorphan in human plasma.

Other interesting extraction columns often used for on-line extraction are disposable cartridges, restricted access media (RAM), monolithic, and immunoaffinity. Excellent reviews have been written on these extraction columns [86,87].

7. Matrix Effects and Recovery

Although the mechanism and the origin are not fully understood, influence of matrix effects on quantitative bioanalytical LC-MS/MS has been extensively investigated [88-91]. The U.S. Food and Drug Administration's (FDA) Guidance for Industry on Bioanalytical Method Validation requires the assessment of matrix effect during method validation for quantitative bioanalytical LC-MS/MS methods [92]. Our detailed review of all quantitative bioanalytical LC-MS/MS articles published in *Journal of Chromatography - Biomedical Application* from 1/2000 to 8/2004 indicates

<50% of the articles discussed the potential influence of matrix effects on their methods. In 2000, there are only 4 out of total 41 quantitative bioanalytical methods discussed matrix effects to any extent. This ratio has been since gradually increased – 7/47 for 2001, 18/58 for 2002, 37/102 for 2003, and 25/56 for 2004 (up to August). Matrix effects has been defined as the analyte ionization suppression or enhancement at the presence of the matrix components that could originated from the endogenous compounds, metabolites, and co-administered drugs [52]. More recently, matrix effects from internal standards [93], dosing vehicles [94-96], and, mobile phase additives [97], and plastic tubes [98] were also reported. These effects are more pronounced with ESI than with APCI [99] but both ionization modes can experience matrix effects [100]. Although ion suppression due to matrix effects has been most reported in literature, there were a few recent reports discussing ion enhancement due to matrix effects although the mechanism is unclear [101-103]. Several approaches have been used to identify the matrix effects. Results from LC-MS/MS methods were compared to a validated non-MS method such as LC-UV [104-107], LC with fluorescence detector [108], ELISA [109,110], fluorescence polarisation assay [111], or compared to a different separation method such as GC [112]. Comparable results indicate lack of matrix effects or inconsistent matrix effects but this approach does not positively identify the magnitude of the matrix effects. Huang *et al.* used LC-UV to detect the down-field matrix peaks that caused ion suppression for the analyte of interest and modified the chromatographic condition [113]. Another approach for assessing consistent matrix effect among the individual matrix lots is to measure the consistency for the results obtained from sample from individual matrix lot (typically $n > 6$) [114-123]. One could argue that as long as the results (both back-calculated concentration and MS response) are consistent among the tested lots, matrix effects would not likely contribute negatively to the quantitation.

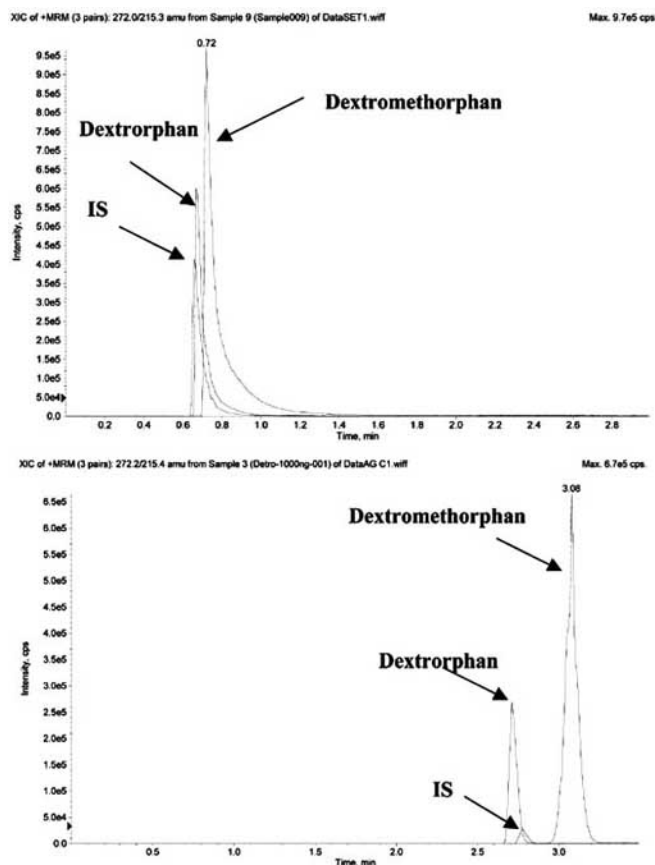


Fig. (6). On-line extraction LC-MS/MS chromatograms of dextroprorphan and dextromethorphan in human plasma samples.

Extraction column: Cohesive Cyclone C-18, 50 x 1.0 mm, 50 μ m;
 Analytical column: Zorbax XDBm C18, 15 x 4.6 mm, 3 μ m.
 Top panel: single-column extraction;
 Bottom panel: dual-column extraction.

This is really what matters for the quantitative analysis. Of course, the magnitude of the suppression or enhancement will still be unknown. There are a couple of commonly used ways of measuring matrix effects. One approach is to compare the MS response of the analyte spiked post-extraction with that in a neat solution [124-129]. Since there is no extraction involved, any signal loss or enhancement in the post-extraction spiked sample will be assumed due to matrix effects. Another useful approach of assessing matrix effect is postcolumn infusion of an analyte into the MS detector. While the analyte is continuously infused through a tee to the mobile phase eluent, the extracted blank matrix is injected by an autosampler onto the analytical column [130-136]. The instrument configuration for post-column infusion experiment is shown in (Fig. 7). The purpose of post-column infusion with the analyte is to raise the background level so that the suppression matrix will show as negative peaks as shown in (Fig. 8). It should be noted that since a gradient elution on a silica column (starting with high organic concentration) was used, the analyte MS response was changed along with the mobile phase change: higher MS response in a mobile phase with high acetonitrile content. Injecting the reconstitution solution generated the "baseline" profile. Any deviation from the "baseline" caused by injecting the extracted matrix blank indicated the existence

of matrix effects. From (Fig. 8), it is obvious that protein precipitation method yielded more matrix suppression than mix-mode SPE. Most of the articles use one of above approaches to investigate matrix effects. Constanzer *et al.* measured both absolute matrix effects by comparing the responses from post-extraction spiked sample with those from neat solutions and the relative matrix effects by measuring the consistency of the response factors from spiked matrix lots [137]. Recovery is determined by comparing the MS response of extracted samples with those spiked (post-extraction) into a blank matrix. Because both samples have the matrix ingredients present, the matrix effects can be considered the same for extracted samples and post-extraction spiked samples. Any differences in responses now can be considered to be caused by extraction recovery.

8. Method Validation and Critical Issues During Sample Analysis

Once a bioanalytical method is developed, various tests (collectively called methods validation) are conducted to prove that the method can be used for its intended application. The FDA guidance defines the relevant bioanalytical terms and acceptance criteria. In addition to the evaluation for precision, accuracy, and stability of the LC-

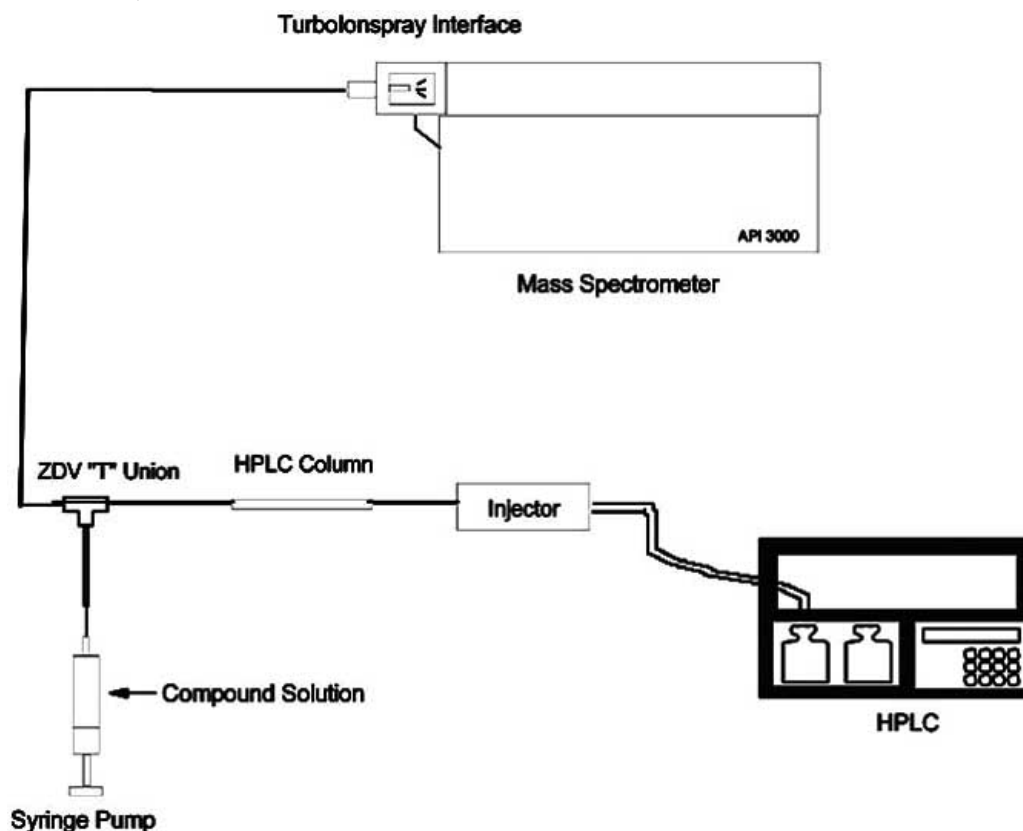


Fig. (7). Instrument Set-up for Using Post-column Infusion to Investigate Matrix Effects.

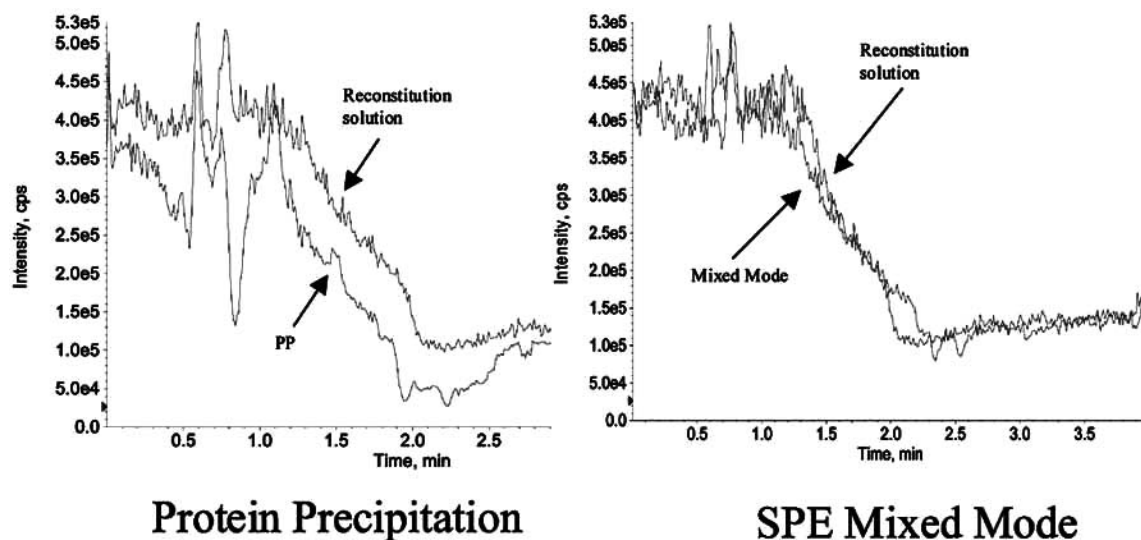


Fig. (8). Post-column Infusion Chromatograms for Protein Precipitation and Mixed-mode SPE. Reprint from [21], with permission from Elsevier.

MS/MS methods, an investigation of matrix effects should be performed. Analyte extraction recovery and potential carry-over of the extraction apparatus or autosampler should also be carefully examined. The pharmaceutical industry and regulatory agencies generally recognize and accept that the validation of a bioanalytical method is performed using standards and quality control samples in which the analytes have been fortified with a blank biological matrix. However, the incurred samples may or may not be the same as the

fortified quality control samples. This difference may lead to a significant bias for the quantitative bioanalysis and must be carefully evaluated [138,139].

In the discovery and preclinical studies, dosing vehicles typically are used at high concentrations to dissolve the test compounds. These dosing vehicles, especially polymeric vehicles such as PEG 400 and Tween 80, can cause significant signal suppression for the IV samples from small rodents. Fig. 9 shows a typical response of the internal

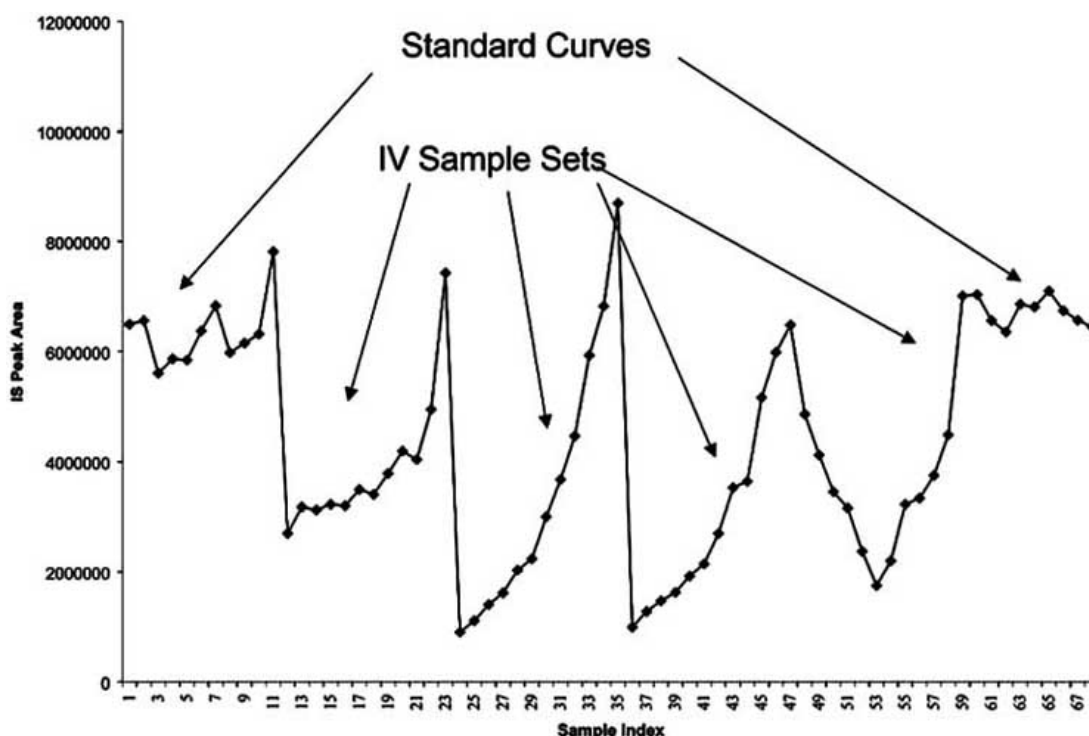


Fig. (9). Suppression of IS by PEG 400 in Study Samples.

standards obtained from quality control samples and incurred samples. The internal standard responses in the incurred samples, especially the early time points, are significantly suppressed. Effective means of minimizing this type of effect includes better chromatographic separations, better sample cleanup, and alternative ionization methods [95]. An easy way to check dosing vehicle effects is to fortify some quality control samples with the vehicle [140]. Significant differences of the measured values between fortified quality control samples and regular quality control samples indicate potential dosing vehicle effects.

The drug and metabolite(s) are assumed to follow the same pharmacokinetic profile and therefore have roughly the same proportion for their concentration profiles. Quality control samples are therefore prepared in such a way that the concentration ratio for drug and metabolite is constant over the entire curve range. In many cases, however, this is not true and lopsided concentration profiles can be observed, especially for prodrugs that typically have much shorter half-life than the active drug. This phenomenon has been observed by Lee *et al.* [141].

Protein-binding of the drug candidate and protein could be significantly different for the quality control samples and incurred samples. This can also cause quantitation bias, depending upon the extraction method. Ke *et al.* used a chelating agent (disodium EDTA) to release protein-bound analytes during 96-well liquid-liquid extraction [142]. Yang *et al.* presented an interesting case study for diagnosis and trouble-shooting of this type of problem [143]. The method was validated using stable-labeled internal standard with a LLE method using hexane as the extraction solvent. However, upon repeat analysis of the same samples, the

concentration values increased to five folds of the original value. The concentration increased with each additional freeze/thaw cycle. It was found that this drug candidate has a strong protein-binding and hexane is not sufficient to release the drug candidate from the protein. Freeze/thaw cycles gradually denatured protein and weakened the binding, resulting in increase the free and extractable drug candidate concentration. PP method (Protein-binding was disturbed) was then used and consistent result was obtained. Two lessons learned here – A more selective extraction method (here LLE versus PP) may not be the one most suitable for the study; Stable-labeled internal standard is not an automatic guarantee for a method suitable for routine sample analysis. The later point of view was also expressed by Jemal *et al.* [91]. Recently, Fura *et al.* also discussed the shift in pH of biological fluids during storage and processing as well as its impact on bioanalysis [144]. Compound adsorption to storage container often happens in sensitive LC-MS/MS assays for urine analysis, which causes non-linear response and loss of sensitivity. The use of a zwitterionic detergent 3-[3-Cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS) as an additive in urine can prevent analyte from adhering to surfaces during sample collection, storage, and preparation [145].

Due to the superior selectivity offered by the tandem mass spectrometric detection, fast LC-MS/MS has been frequently used to speed up the sample analysis. This can be achieved either by increasing the flow rate or by increasing the elution strength of the mobile phase. Increase of flow rate may slightly decrease the column efficiency but to a large extend the capacity factor is not sacrificed. Of course, one has to find suitable column amenable to the high flow rate,

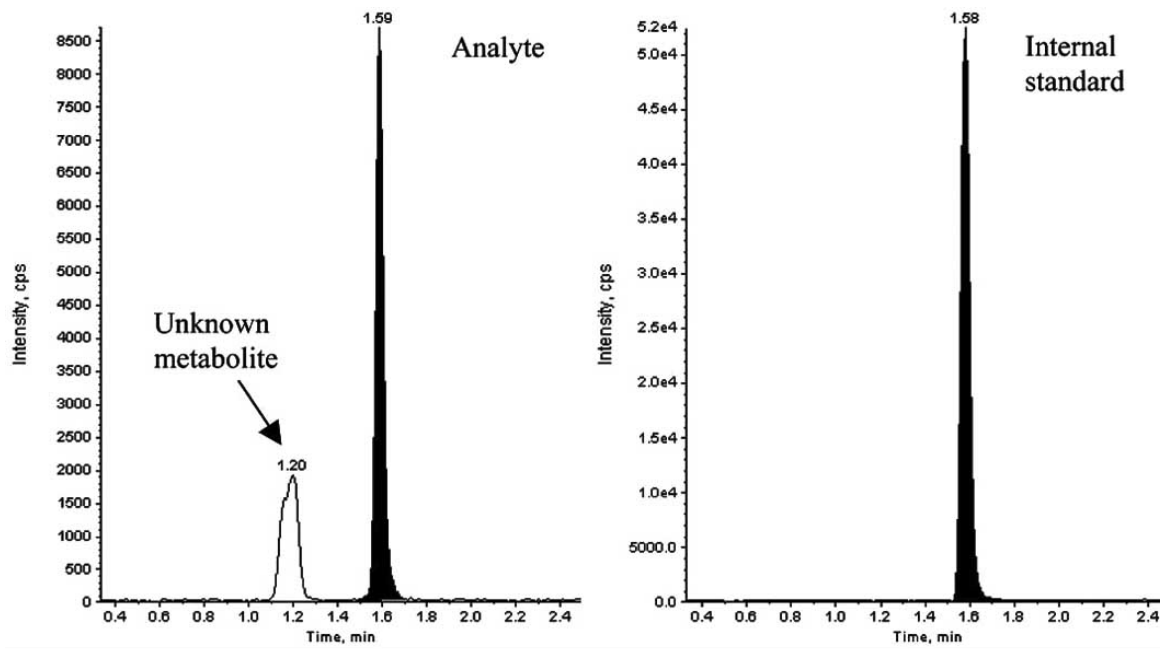


Fig. (10). Chromatogram of an Incurred Sample with a Unknown Metabolite.

not always an easy task. Increasing elution strength will certainly decrease capacity factor and the analyte of interest may fall into the suppression or interference zones. How fast is too fast? Phase II metabolites (glucuronide, and sulfate- and glutathione-conjugation products) and pro-drugs are notoriously unstable in the LC-MS interface and are easily fragmented back to the drugs [146-148]. Without adequate chromatographic retention and separation between the drug and its Phase II metabolites or pro-drugs, the drug concentration can be easily over-estimated for the incurred samples. This bias will not be easily detectable since neither the calibration standards nor the quality control samples may contain these Phase II metabolites. Fig. 10 shows the chromatogram of an incurred sample for which an unknown metabolite was observed in the same channel as the analyte. A very fast LC using stronger elution strength would mask the interference peak with the analyte, causing an over-estimation of the analyte's concentration. Generally in our organization, a fast and adequate quantitative LC-MS/MS analysis should have the retention time $\geq 2 t_0$, at least 10 acquisition points per peak, and good separations from possible interferences or matrix effect. Also, the analysis cycle is limited by the auto-sampler speed as well as the adequate injector wash time in order to reduce carry-over.

9. Method Transfer

Timely method transfer plays an important role in expediting drug candidates through development stages. Method transfer is not a trivial task and requires careful planning and constant communication between the laboratory personnel involved in the transfer. Method transfer could occur within the same organization or between pharmaceutical companies and analytical service providers. To have a successful transfer, the bioanalytical method itself must be robust and the equipment differences between the

delivering and receiving parties should be carefully evaluated. Use of standardized automation equipment has shown to be advantageous during the method transfer. Unfortunately very limited information on method transfer can be found in the literatures [35,149]. Typically in our organization, before the method transfer, scientists from both sites go through the method details very carefully. We found that even some apparently trivial differences such as the hood air flow speed and brands of vortex machines can sometime cause method performance difference. Every method generated in our laboratory contains a very detailed discussion about the method robustness and any potential factors that can affect the method performance.

CONCLUSION

"Fast LC-MS/MS" for the quantitative bioanalysis can only be achieved after considering all aspects of the analysis cycle. LC-MS/MS practitioners should never take any doctrine for granted and should always ask the questions "How fast is too fast?" and "Can this validated method be used successfully for sample analysis". Only with these questions in mind, can we as bioanalytical practitioners always be aware of the potential pitfalls and culprits that could occur in any stage of the study. Otherwise, a seemingly perfect method can generate wasted data, and even worse, poor medicines that could impair public health. Hopefully, this critical review summarizes the advances in this field and points out the current critical view of quantitative bioanalytical LC-MS/MS.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Wilson Shou of Covance Drug Metabolism Department for providing Figure 9.

REFERENCES

- [1] Hsieh, Y.; Merkle, K.; Wang, G.; Brisson, J.-M.; Korfmacher, W.A. *Anal. Chem.*, **2003**, *75*, 3122.
- [2] Yang, C.; Henion, J. *J. Chromatogr. A*, **2002**, *970*, 155.
- [3] Brewer, E.; Henion, J. *J. Pharm. Sci.*, **1998**, *87*, 395.
- [4] Lee, M.S.; Kerns, E.D. *Mass Spectrom. Rev.*, **1999**, *18*, 187.
- [5] Jemal, M. *Biomed. Chromatogr.*, **2000**, *14*, 422.
- [6] Brockman, A.H.; Hiller, D.L.; Cole, R.O. *Curr. Opin. Drug Disc. Dev.*, **2000**, *3*, 432.
- [7] Oliveira, E.J.; Watson, D.G. *Biomed. Chromatogr.*, **2000**, *14*, 351.
- [8] Law, B.; Temesi, D. *J. Chromatogr. B*, **2000**, *748*, 21.
- [9] Plumb R.; Dear, G.J.; Mallett, D.N.; Highton, D.M.; Pleasance, S.; Biddlecombe, R.A. *Xenobiotica*, **2001**, *31*, 599.
- [10] Kyranos, J.N.; Cai, H.; Wei, D.; Goetzinger, W.K. *Curr. Opin. Biotechnol.*, **2001**, *12*, 105.
- [11] Triolo, A.; Altamura, M.; Cardinali, F.; Sisto, A.; Maggi, C.A. *J. Mass Spectrom.*, **2001**, *36*, 1249.
- [12] O'Connor, D. *Curr. Opin. Drug Disc. Dev.*, **2002**, *5*, 52.
- [13] Ackermann, B. L.; Berna, M.J.; Murphy, A.T. *Current Topics in Medicinal Chemistry*, **2002**, *2*, 53.
- [14] Bakhtiar, R.; Ramos, L.; Tse, F.L.S. *J. Liq. Chromatogr. Related Technol.*, **2002**, *25*, 507.
- [15] Hopfgartner, G.; Bourgoigne, E. *Mass Spectrom. Rev.*, **2003**, *22*, 195.
- [16] Rashed, M.S. *J. Chromatogr. B*, **2001**, *758*, 27.
- [17] Weng, N.; Halls, T.D.J. *Biopharm*, **2001**, 28.
- [18] Weng, N.; Halls, T.D.J. *Biopharm*, **2002**, 22.
- [19] Tiller, P.R.; Romanyshyn, L.A.; Neue, U.D. *Anal. Bioanal. Chem.*, **2003**, *377*, 788.
- [20] Wells, D.A. High Throughput Bioanalytical Sample Preparation – Methods and Automation Strategies. Progress in Pharmaceutical and Biomedical Analysis Volume 5, **2003**, Elsevier, Amsterdam, The Netherlands.
- [21] Naidong, W.; Bu, H.; Chen, Y.-L.; Shou, W.Z.; Jiang, X.; Halls, T.D.J. *J. Pharm. Biomed. Anal.*, **2002**, *28*, 1115.
- [22] Pan, J.; Junga, H.; Sun, H.; Jiang, X.; Weng, N. *51th Annual ASMS Conference*, Montreal, Quebec, Canada, June 8-12, **2003**.
- [23] Janiszewski, J. S.; Swyden, M.C.; Fouda, F. G. *J. Chromatogr. Sci.*, **2000**, *38*, 255.
- [24] Sadagopan, N.P.; Li, W.; Cook, J.A.; Galvan, B.; Weller, D.L.; Fountain, S.T.; Cohen, L.H. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 1065.
- [25] Avery, M.J. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 197.
- [26] Li, W.; Cohen, L.H. *Anal. Chem.*, **2003**, *75*, 5854.
- [27] Nelson, R.E.; Grebe, S.K.; O'Kane, D.J.; Singh, R.J. *Clin. Chem.*, **2004**, *50*, 373.
- [28] Anari, M.R.; Bakhtiar, R.; Zhu, B.; Huskey, S.; Franklin, R.B.; Evans, D.C. *Anal. Chem.*, **2002**, *74*, 4136.
- [29] Shou, W.S.; Jiang, X.Y.; Naidong, W. *Biomed. Chromatogr.*, **2004**, *18*, 414.
- [30] Allanson, J.P.; Biddlecombe, R.A.; Jones, A.E.; Pleasance, S. *Rapid Commun. Mass Spectrom.*, **1996**, *10*, 811.
- [31] Janiszewski, J.; Schneider, R.; Hoffmaster, K.; Swyden, M.; Wells, D.; Fouda, H. *Rapid Commun. Mass Spectrom.*, **1997**, *11*, 1033.
- [32] Simpson, H.; Berthemy, A.; Burhrman, D.; Burton, R.; Newton, J.; Kealy, M.; Wells, D.; Wu, D. *Rapid Commun. Mass Spectrom.*, **1998**, *12*, 75.
- [33] Zweigenbaum, J.; Heinig, K.; Steinborner, S.; Wachs, T.; Henion, J. *Anal. Chem.*, **1999**, *71*, 2294.
- [34] Rose, M.J.; Merschman, S.A.; Eisenhandler, R.; Woolf, E.J.; Yeh, K.C.; Lin, L.; Fang, W.; Hsieh, J.; Braun, M.P.; Gatto, G.J.; Matuszewski, B.K. *J. Pharm. Biomed. Anal.*, **2000**, *24*, 291.
- [35] Shou, W.Z.; Jiang, X.; Beato, B.D.; Naidong, W. *Rapid Commun. Mass Spectrom.*, **2001**, *15*, 466.
- [36] Shou, W.Z.; Pelzer, M.; Addison, T.; Jiang, X.; Naidong, W. *J. Pharm. Biomed. Anal.*, **2002**, *27*, 143.
- [37] Eerkes, A.; Addison, T.; Naidong, W. *J. Chromatogr. B*, **2002**, *768*, 277.
- [38] Steinborner S.; Henion, J. *Anal. Chem.*, **1999**, *71*, 2340.
- [39] Jemal, M.; Teitz, D.; Ouyang, Z.; Khan, S. *J. Chromatogr. B*, **1999**, *732*, 501.
- [40] Bolden, R.D.; Hoke II, S.H.; Eichold, T.H.; McCauley-Myers, D.L.; Wehmeyer, K.R. *J. Chromatogr. A*, **2002**, *772*, 1.
- [41] Ramos, L.; Bakhtiar, R.; Tse, F.L.S. *Rapid Commun. Mass Spectrom.*, **2000**, *14*, 740.
- [42] Shen, Z.; Wang, S.; Bakhtiar, R. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 332.
- [43] Eerkes, A.; Shou, W.Z.; Naidong, W. *J. Pharm. Biomed. Anal.*, **2003**, *31*, 917.
- [44] Ji, Q.C.; Reimer, M.T.; El-Shourbagy, T.A. *J. Chromatogr. B*, **2004**, *805*, 67.
- [45] Watt, A.P.; Morrison, D.; Locker, K.L.; Evans, D.G. *Anal. Chem.*, **2000**, *72*, 979.
- [46] Shou, W.Z.; Bu, H.-Z.; Addison, T.; Jiang, X.; Naidong, W. *J. Pharm. Biomed. Anal.*, **2002**, *29*, 83.
- [47] Poole, C.F. *Trends in Analytical Chemistry*, **2003**, *22*, 362.
- [48] Polson, C.; Sarkar, P.; Inledon, B.; Raguvaran, V.; Grant, R. *J. Chromatogr. B*, **2003**, *785*, 263.
- [49] Cheng, Y.-F.; Neue, U.D.; Woods, L.L. *J. Chromatogr. B*, **1999**, *729*, 19.
- [50] Naidong, W.; Chen, Y.-L.; Shou, W.; Jiang, X. *J. Pharm. Biomed. Anal.*, **2001**, *26*, 753.
- [51] Naidong, W.; Shou, W.Z.; Addison, T.; Maleki, S.; Jiang, X. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 1965.
- [52] Li, A.C.; Junga, H.; Shou, W.Z.; Bryant, M.S.; Jiang, X.; Naidong, W. *Rapid Commun. Mass Spectrom.*, **2004**, *18*, 2343.
- [53] Zhou, W.; Zhou, S.; Weng, N. 52nd ASMS Conference on Mass Spectrometry and Allied Topics, Nashville, Tennessee, 23-27 May **2004**.
- [54] Naidong, W.; Zhou, W.; Song, Q.; Zhou, S. *Rapid Commun. Mass Spectrom.*, accepted.
- [55] Matuszewski, B.K.; Constanzer, M.L.; Chavez-Eng, C.M. *Anal. Chem.*, **1998**, *70*, 882.
- [56] Weng, N.; Halls, T.D.J. *Pharmaceutical Technology*, **2002**, *26*, 102.
- [57] Chen, Y.-L.; Felder, L.; Jiang, X.; Naidong, W. *J. Chromatogr. B*, **2002**, *774*, 67.
- [58] Griitti, F.; Piatkowski, W.; Guiochon, G. *J. Chromatogr. A*, **1999**, *832*, 51.
- [59] Bidlingmaier, B.; Unger, K.K.; von Doehren, N. *J. Chromatogr. A*, **1999**, *832*, 11.
- [60] Lubda, D.; Cabrera, K.; Kraas, W.; Schaefer, C.; Cunningham, D. *LCCG*, **2001**, *19*, 1186.
- [61] Wu, J.T.; Zeng, H.; Deng, Y.; Unger, S.E. *Rapid Commun. Mass Spectrom.*, **2001**, *15*, 1113.
- [62] Hsieh, Y.; Wang, G.; Wang, Y.; Chackalamannil, S.; Brisson, J.-M.; Ng, K.; Korfmacher, W.A. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 944.
- [63] Deng, Y.; Wu, J.T.; Lloyd, T.L.; Chi, C.L.; Olah, T.V.; Unger, S.E. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 1116.
- [64] Dear, G.; Mallett, D.N.; Highton, D.M.; Roberts, A.D.; Bird, S.A.; Young, H.; Plumb, R.S.; Ismail, I.M. *Chromatographia*, **2002**, *55*, 177.
- [65] Chen, Y.-L.; Junga, H.; Jiang, X.; Naidong, W. *J. Sep. Sci.*, **2003**, *26*, 1509.
- [66] Hsieh, Y.; Merkle, K.; Wang, G. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 1775.
- [67] Mao, D.; Zhou, S.; Weng, N.; Jiang, X. Proceedings of the 52nd ASMS Conference on Mass Spectrometry and allied Topics; Nashville, Tennessee, 23-27 May **2004**.
- [68] Naidong, W.; Shou, W.Z.; Chen, Y.-L.; Jiang, X. *J. Chromatogr. B*, **2001**, *754*, 387.
- [69] Brown, S.D.; White, C.A.; Bartlett, M.G. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 1871.
- [70] McKeown, A.P.; Euerby, M.R.; Lomax, H.; Johnson, C.M.; Ritchie, H.J.; Woodruff, M. *J. Sep. Sci.*, **2001**, *24*, 835.
- [71] Koc, H.; Mar, M.-H.; Ranasinghe, A.; Swenberg, J.A.; Zeisel, S.H. *Anal. Chem.*, **2002**, *74*, 4734.
- [72] Conte Jr., J.E.; Lin, E.; Zhao, Y.; Zurlinden, E. *J. Chromatogr. Sci.*, **2002**, *40*, 113.
- [73] Paek, I.B.; Moon, Y.; Ji, H.Y.; Kim, H.-H.; Lee, H.W.; Lee, Y.-B.; Lee, H.S. *J. Chromatogr. B*, **2004**, *809*, 345.
- [74] Naidong, W. *J. Chromatogr. B*, **2003**, *796*, 209.
- [75] Naidong, W.; Eerkes, A. *Biomed. Chromatogr.*, **2004**, *18*, 28.
- [76] Shou, W.Z.; Chen, Y.-L.; Eerkes, A.; Tang, Y.Q.; Magis, L.; Jiang, X.; Naidong, W. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 1613.
- [77] Li, A.C.; Chen, Y.-L.; Junga, H.; Shou, W.Z.; Jiang, X.; Naidong, W. *Chromatographia*, **2003**, *58*, 723.
- [78] Yang, L.; Wu, N.; Rudewicz, P.J. *J. Chromatogr. A*, **2001**, *926*, 43.

- [79] Korfmacher, W.A.; Veals, J.; Dunn-Meynell, K.; Zhang, X.P.; Tucker, G.; Cox, K.A.; Lin, C.C. *Rapid Commun. Mass Spectrom.*, **1999**, *13*, 1991.
- [80] van Pelt, C.K.; Corso, T.N.; Schultz, G.A.; Lowes, S.; Henion, J. *Anal. Chem.*, **2001**, *73*, 582.
- [81] King, R.C.; Miller-Stein, C.; Magiera, D.J.; Brann, J. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 43.
- [82] Rule, G. and Henion, J. *Am. Soc. Mass Spectrom.*, **1999**, *10*, 1322.
- [83] Jemal, M.; Xia, Y.; and Whigan, D. B. *Rapid Commun. Mass Spectrom.*, **1998**, *12*, 1389.
- [84] Zhou, S.; Larson, M.; Weng, N.; and Jiang, X. Proceedings of the 50th ASMS Conference on Mass Spectrometry and allied Topics; Orlando, **2002**.
- [85] Herman, J.L.; *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 421.
- [86] Wu, J-T.; Wells, D.A. in *High Throughput Bioanalytical Sample Preparation – Methods and Automation Strategies*, Wells, D.A., Ed.; Elsevier, Amsterdam, **2003**; pp. 505-573.
- [87] Veuthey, J-L.; Souverain, S.; Rudaz, S. *Ther. Drug Monit.*, **2004**, *26*, 161.
- [88] Buhrman, D.L.; Price, P.I.; Rudewicz, P.J. *J. Am. Soc. Mass Spectrom.*, **1996**, *7*, 1099.
- [89] Fu, I.; Woolf, E.J.; Matuszewski, B.K. *J. Pharm. Biomed. Anal.*, **1998**, *18*, 347.
- [90] Matuszewski, B.K.; Constanzer, M.L.; Chavez-Eng, C.M. *Anal. Chem.*, **2003**, *75*, 3019.
- [91] Jemal, M.; Schuster, A.; Whigan, D.B. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 1723.
- [92] FDA, Guidance for Industry: Bioanalytical Methods Validation (US Department of Health and Human Services, Center for Drug Evaluation and Research, and Center for veterinary Medicine, May 2001), available at <http://www.fda/cder/guidance/index.htm>.
- [93] Sojo, L.E.; Lum, G.; Chee, P. *Analyst*, **2003**, *128*, 51.
- [94] Tong, X.S.; Wang, J.; Zheng, S.; Pivnichny, J.V.; Griffin, P.R.; Shen, X.; Donnelly, M.; Vakerich, K.; Nunes, C.; Fenyk-Melody, J. *Anal. Chem.*, **2002**, *74*, 6305.
- [95] Shou, W.Z.; Naidong, W. *Rapid Commun. Mass Spectrom.*, **2003**, *16*, 1538.
- [96] Schuhmacher, J.; Zimmer, D.; Tesche, F.; Pickard, V. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 1950.
- [97] Mallet, C.R.; Lu, Z.; Mazzeo, J.R. *Rapid Commun. Mass Spectrom.*, **2004**, *18*, 49.
- [98] Mei, H.; Hsieh, Y.; Nardo, C.; Xu, X.; Wang, S.; Ng, K.; Korfmacher, W.A. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 97.
- [99] Pommier, F.; Frigola, R. *J. Chromatogr. B*, **2003**, *784*, 301
- [100] Dams, R.; Huestis, M.A.; Lambert, W.E.; Murphy, C.M. *J. Am. Soc. Mass Spectrom.*, **2003**, *14*, 1290.
- [101] Schneider, M.J.; Donoghue, D.J. *J. Chromatogr. B*, **2002**, *779*, 83.
- [102] Wiesner, J.L.; Sutherland, F.C.W.; van Essen, G.H.; Hundt, H.K.L.; Swart, K.J.; Hundt, A.F. *J. Chromatogr. B*, **2003**, *788*, 361.
- [103] Liang, H.R.; Foltz, R.L.; Meng, M.; Bennett, P. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 2815.
- [104] Zhou, L.; Glickman, R.D.; Chen, N.; Sponsel, W.E.; Graybill, J.R.; Lam, K-W. *J. Chromatogr. B*, **2002**, *776*, 213.
- [105] Parise, R.A.; Ramanathan, R.K.; Zamboni, W.C.; Egorin, M.J. *J. Chromatogr. B*, **2003**, *783*, 231.
- [106] Croubels, S.; de Baere, S.; de Backer, P. *J. Chromatogr. B*, **2003**, *788*, 167.
- [107] Lakso, H.; Norström, A. *J. Chromatogr. B*, **2003**, *794*, 57.
- [108] Yang, J.Z.; Bastian, K.C.; Moore, R.D.; Stobaugh, J.F.; Borchardt, R.T. *J. Chromatogr. B*, **2002**, *779*, 269.
- [109] Inoue, K.; Wada, M.; Higuchi, T.; Oshio, S.; Umeda, T.; Yoshimura, Y.; Nakazawa, H. *J. Chromatogr. B*, **2002**, *773*, 97.
- [110] Eriksson, K.; Östin, A.; Levin, J-O. *J. Chromatogr. B*, **2003**, *794*, 115.
- [111] Keevil, B.G.; Lockhart, S.J.; Cooper, D.P. *J. Chromatogr. B*, **2003**, *794*, 329.
- [112] Stokes, P.; O'Connor, G. *J. Chromatogr. B*, **2003**, *794*, 125.
- [113] Huang, Y.; Zurlinden, E.; Lin, E.; Li, X.; Tokumoto, J.; Golden, J.; Murr, A.; Engstrom, J. Conte Jr., J. *J. Chromatogr. B*, **2004**, *799*, 51.
- [114] Jemal, M.; Huang, M.; Mao, Y.; Whigan, D.; Schuster, A. *Rapid Commun. Mass Spectrom.*, **2001**, *15*, 1023.
- [115] Zeng, W.; Wang, A.Q.; Fisher, A.L.; Musson, D.G. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 2475.
- [116] Chavez-Eng, C.M.; Constanzer, M.L.; Matuszewski, B.K. *J. Chromatogr. B*, **2000**, *748*, 31.
- [117] Constanzer, M.L.; Chavez-Eng, C.M.; Matuszewski, B.K. *J. Chromatogr. B*, **2001**, *760*, 45.
- [118] Carnerup, M.A.; Akesson, B.; Jonsson, B.A.G. *J. Chromatogr. B*, **2001**, *761*, 107.
- [119] Chen, Y-L.; Hanson, G.D.; Weng, N.; Powala, C.; Zerler, B. *J. Chromatogr. B*, **2003**, *794*, 77.
- [120] Needham, S.R.; Ye, B.; Smith, R.; Korte, W.D. *J. Chromatogr. B*, **2003**, *796*, 347.
- [121] Jemal, M.; Rao, S.; Gatz, M.; Whigan, D. *J. Chromatogr. B*, **2003**, *795*, 273.
- [122] Verhaeghe, T.; Diels, L.; de Vries, R.; de Meulder, M.; de Jong, J. *J. Chromatogr. B*, **2003**, *789*, 337.
- [123] Frerichs, V.A.; Tornatore, K.M. *J. Chromatogr. B*, **2004**, *802*, 329.
- [124] Choi, B.K.; Hercules, D.M.; Gusev, A.I. *J. Chromatogr. A*, **2001**, *907*, 337.
- [125] Lin, Z.J.; Desai-Krieger, D.; Shum, L. *J. Chromatogr. B*, **2004**, *801*, 265.
- [126] He, X.; Batchelor, T.T.; Grossman, S.; Supko, J.G. *J. Chromatogr. B*, **2004**, *801*, 281.
- [127] Zhou, Z.; Lin, X.; Li, K.; Xie, Z.; Cheng, Z.; Peng, W.; Wang, F.; Zhu, R.; Li, H. *J. Chromatogr. B*, **2004**, *802*, 257.
- [128] Salem, I.I.; Idrees, J.; Al Tamimi, J.I.; Farina, P. *J. Chromatogr. B*, **2004**, *803*, 201.
- [129] Arnold, R.D.; Slack, J.E.; Straubinger, R.M. *J. Chromatogr. B*, **2004**, *808*, 141.
- [130] Bonfiglio, R.; King, R.C.; Olah, T.V.; Merkle, K. *J. Chromatogr. B*, **1999**, *13*, 1175.
- [131] Borges, V.; Yang, E.; Dunn, J.; Henion, J. *J. Chromatogr. B*, **2004**, *804*, 277.
- [132] Wang, Y.; Hochhaus, G. *J. Chromatogr. B*, **2004**, *805*, 203.
- [133] Liang, H.R.; Foltz, R.L.; Meng, M.; Bennett, P. *J. Chromatogr. B*, **2004**, *806*, 191.
- [134] Barbarin, N.; Mawhinney, D.B.; Black, R.; Henion, J. *J. Chromatogr. B*, **2003**, *783*, 73.
- [135] Hsieh, Y.; Merkle, K.; Wang, G. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 1775.
- [136] Nelson, M.D.; Dolan, J.W. *LCGC*, **2002**, *20*, 24.
- [137] Constanzer, M.L.; Chavez-Eng, C.M.; Dru, J.; Kline, K.F.; Matuszewski, B.K. *J. Chromatogr. B*, **2004**, *806*, 243.
- [138] Jemal, M.; Ouyang, Z.; Powell, M.L. *Rapid Commun. Mass Spectrom.*, **2002**, *16*, 1538.
- [139] Hua, S.; Weng, N. *Pharmaceutical Technology*, **2003**, *27*, 74.
- [140] Leahy, M.; Felder, L.; Goytowski, K.; Shou, W.; Weng, N. 52nd ASMS Conference on Mass Spectrometry and Allied Topics, Nashville, Tennessee, 23-27 May, **2004**.
- [141] Lee, J.; Son, J.; Lee, M.; Lee, K.T.; Kim, D-H. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 1157.
- [142] Ke, J.; Yancey, M.; Zhang, S.; Lowes, S.; Henion, J. *J. Chromatogr. B*, **2000**, *742*, 369.
- [143] Yang, L.; Wu, N.; Clement, R.P.; Rudewicz, P.J. *J. Chromatogr. B*, **2004**, *799*, 271.
- [144] Fura, A.; Harper, T.W.; Zhang, H.; Fung, L.; Shyu, W.C. *J. Pharm. Biomed. Anal.*, **2003**, *32*, 513.
- [145] Tang, Y.Q.; Tollefson, J.A.; Beato, B.D.; Weng, N. 2003AAPS Annual Meeting, Salt Lake City, Utah, Oct. 26-30, **2003**.
- [146] Jemal, M.; Xia, Y.Q. *Rapid Commun. Mass Spectrom.*, **1999**, *14*, 422.
- [147] Naidong, W.; Lee, J.W.; Jiang, X.; Wehling, M.; Hulse, J.D.; Lin, P.P. *J. Chromatogr. B*, **1999**, *735*, 255.
- [148] Naidong, W.; Jiang, X.; Newland, K.; Coe, R.; Lin, P.P.; Lee, J.W. *J. Pharm. Biomed. Anal.*, **2000**, *23*, 697.
- [149] Pan, J.; Song, Q.; Shi, H.; King, M.; Junga, H.; Zhou, S.; Naidong, W. *Rapid Commun. Mass Spectrom.*, **2004**, *18*, 2549.