Special Issue: Review

Biomedical Chromatography

Received 25 October 2009, Accepted 26 October 2009 Published online in Wiley InterScience

(www.interscience.wiley.com) DOI 10.1002/bmc.1373

Systematic LC-MS/MS bioanalytical method development that incorporates plasma phospholipids risk avoidance, usage of incurred sample and well thought-out chromatography

Mohammed Jemal*, Zheng Ouyang, Yuan-Qing Xia

ABSTRACT: This treatise summarizes the underlying principle and the road map for systematic LC-MS/MS bioanalytical method development. The three themes that have recently emerged as central to building quality during method development—phospholipids, incurred sample and sound chromatographic considerations—are the main focus of this article. In order to reduce the bioanalytical risk associated with plasma phospholipids, a dual approach involving extraction and chromatography is recommended. The use of incurred sample during method development is essential to avoid interference arising from drug-related components. This is different from the current practice of incurred sample reanalysis, which tests reproducibility during method application. LC column/mobile phase optimization is needed to achieve appropriate peak shape, sensitivity and the separation of the analyte from interfering metabolites and phospholipids. Related to sound chromatographic considerations, we lay out facts and myths related to UPLC, vis-à-vis HPLC. Incorporation of quality during method development avoids the costly experience of finding out by chance about the invalidity of a method after it has been used in support of a number of pivotal clinical and non-clinical studies. To this end, we put forth an outline of a protocol for a systematic LC-MS/MS bioanalytical method development. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: LC-MS/MS bioanalytical method development; incurred sample; metabolite interference; phospholipids matrix effect; phospholipids removal by extraction; chromatographic separation of phospholipids from analyte; LC column and mobile phase optimization; UPLC versus HPLC

Introduction

Since our review article (Jemal, 2000) in this journal a decade ago, there have been notable advances in LC-MS/MS quantitative bioanalysis. Several review articles published since then (Xu *et al*., 2007; Jemal and Xia, 2006; Berna *et al*., 2004; Souverain *et al*., 2004; Naidong, 2003; Tiller *et al*., 2003) have covered the diverse advances achieved in the recent past. In this article, we present, in a systematic manner, themes that have recently emerged as central to building quality during the development of LC-MS/MS bioanalytical methods. Use of quality methods developed in this manner will reduce bioanalytical risk and avoid the costly and nerve-wracking experience of finding out that an invalid method, although 'officially validated', has been used to support pivotal clinical and non-clinical studies. The first theme discussed is the ionization suppression/enhancement (matrix effect) caused by plasma phospholipids. After presenting the chromatographic, mass spectrometric and extraction behaviors of phospholipids, we recommend a dual approach, involving selective sample extraction and chromatographic separation, for incorporation during method development in order to avoid phospholipids-related bioanalytical risk. As a second theme, the importance of using incurred sample (post-dose sample) during method development is discussed. The significance of using incurred sample during method development cannot be overemphasized. This is essential in order to ensure that metabolites and other components present only in the incurred sample, and not in quality control (QC) samples, do not interfere with the accurate quantitation of the drug or any particular metabolite of interest. This is different from the concept of incurred sample reanalysis (ISR), which is currently being widely practiced during the application of a validated method. ISR tests only reproducibility and may not have any relevance to detecting potential metabolite interference. As a third theme, the significance of a systematic selection of LC columns and mobile phases in LC-MS/MS bioanalysis is discussed. In conjunction with sound chromatographic consider-

* Correspondence to: M. Jemal, Bioanalytical and Discovery Analytical Sciences, Research and Development, Bristol-Myers Squibb, Princeton, NJ 08543-4000, USA. E-mail: mohammed.jemal@bms.com

Bioanalytical and Discovery Analytical Sciences, Research and Development, Bristol-Myers Squibb, Princeton, NJ 08543-4000, USA

Abbreviations used: ISR, incurred sample reanalysis; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelins.

ations, we delineate the facts and myths related to the performance of sub-2 µm liquid chromatography, popularly known as UPLC (ultra-performance liquid chromatography), vis-à-vis HPLC (high-performance liquid chromatography). Finally, putting it all together, we put forth an outline of a protocol for a systematic approach to method development in order to reduce bioanalytical risk.

Phospholipids

Background and Structural Summary

Association of plasma phospholipids with ionization suppression or enhancement (matrix effect), which is a major source of bioanalytical risk, has come to the fore in the bioanalytical community only in the last six or seven years thanks to the pioneering work by Bennett, Van Horne, Ahnoff *et al*. (Bennett and Van Horne, 2003; Van Horne and Bennett, 2003; Ahnoff *et al*., 2003). It is thus important to monitor the fate of phospholipids during method development and application. As shown in Fig. 1, plasma phospholipids belong to one of two structural classes of phospholipids: glycerophospholipids and sphingomyelins (Murphy *et al*., 2001; Pulfer and Murphy, 2003; Perterson and Cummings, 2006). Glycerophospholipids are made up of a three-carbon glycerol backbone with different substitutions at the three carbon (C) positions. As shown in Fig. 1, the C-1 position contains a fatty acid ester, an alkyl ether or a vinyl ether, giving rise to phosphatidyl, plasmanyl and plasmenyl (plasmalogen) glycerophospholipids, respectively. The C-2 position may contain a fatty-acid ester group or the C-2 hydroxyl group (OH) may remain free without being esterified, the latter giving rise to the lysophospholipids (lyso) class. The C-3 position OH is esterified with a phosphoric acid, which in turn is esterified to create different polar head groups. Depending on the polar head groups at the C-3 position, glycerophospholipids are classified into different classes: phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS). On the other hand, as shown in Fig. 1, sphingomyelins (SM) contain an alky chain at the C-1 position, with the OH remaining free. While the C-2 position contains a fatty acid amide, the C-3 position contains the same group found in the PC class of glycerophospholipids. The total concentration of all phospholipids in human plasma is about 1.6–3.0 mg/mL (Lehninger, 1982). The PC class of phospholipids accounts for about 60–70% of the total phospholipids found in plasma (Schwarz *et al*., 1977; Pang *et al*., 2008). The other abundant classes are SM, PE, PI, lyso-PC, PS and PG, listed in the order of decreasing concentration (Pang *et al*., 2008).

Mass Spectrometric Monitoring of Phospholipids in Bioanalysis

A timely and well-received technique for monitoring phospholipids during LC-MS/MS bioanalytical method development and application has recently been reported (Little *et al*., 2006). The technique is based on using positive electrospray (ESI) selected reaction monitoring (SRM) of *m/z* 184 → *m/z* 184, with the precursor ion (*m/z* 184) generated in the source due to collision-induced dissociation (CID) of phospholipids. This technique can be used to monitor PC, lyso-PC and SM phospholipids. It cannot be used to monitor the other phospholipids, such as PE, PI, PG, PS and PA, since these phospholipids do not generate the *m/z* 184 ion in the

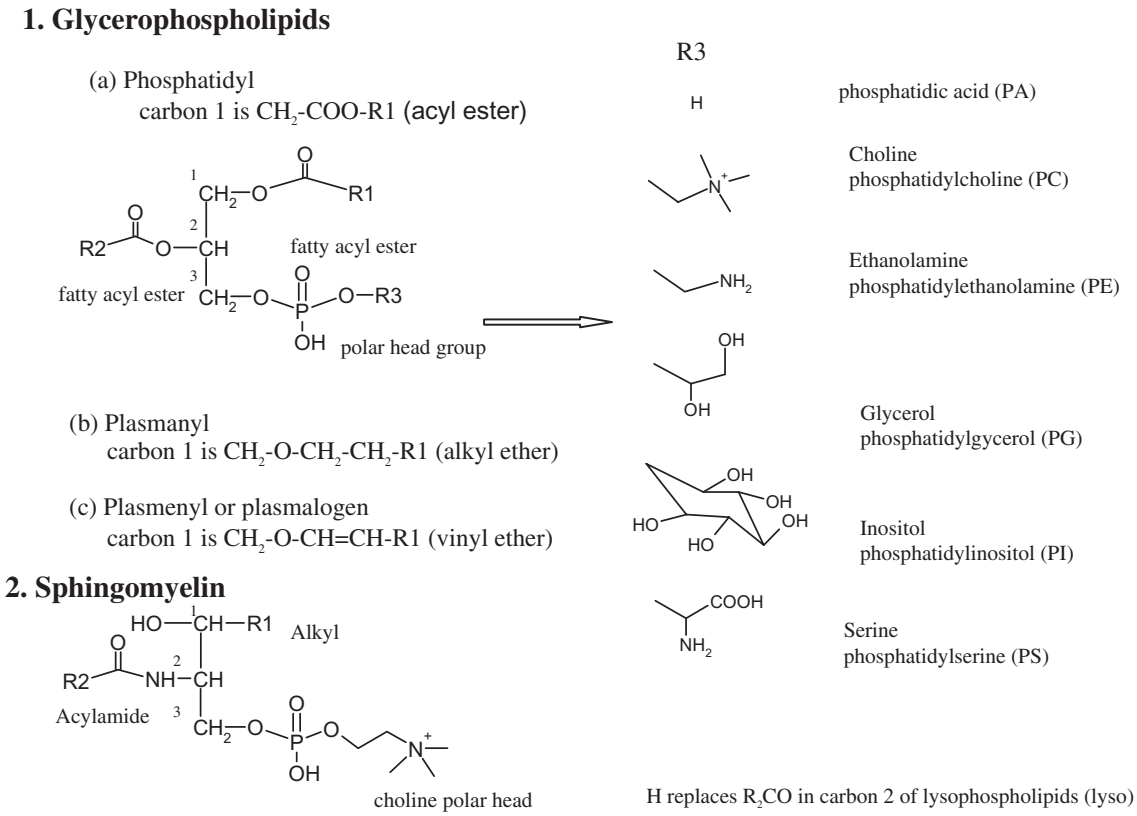

Figure 1. Phospholipids structure. Reproduced from Xia and Jemal (2009b) with permission.

source. We recently evaluated three tandem mass spectrometry (MS/MS) techniques to monitor phospholipids, using positive and negative ESI, and we finally recommended the following all-inclusive technique (Xia and Jemal, 2009b): positive precursor ion scan of *m/z* 184 for the detection of all the PC, lyso-PC and SM phospholipids; positive neutral loss scan of 141 Da for the detection of lysoPE and PE; and negative precursor ion scan of *m/z* 153 for the detection of the remaining phospholipids. As illustrated below, this technique not only can detect all the classes of phospholipids but also possesses other distinct advantages compared with the *m/z* 184 → *m/z* 184 SRM technique.

We demonstrated the application of the all-inclusive technique in the detection of phospholipids in human plasma by injecting the supernatant of acetonitrile-precipitated plasma. As shown in Fig. 2c, using the positive precursor ion scan of *m/z* 184, a number of intensive chromatographic peaks were obtained in the elution window between 2 and 10 min. Post-acquisition interrogation of the data enables the identification of the positive precursor ions corresponding to the chromatographic peaks. The results confirmed the presence of the previously reported (Pang *et al*., 2008; Uran *et al*., 2001; Wang *et al*., 2004; Takatera *et al*., 2006) major lyso-PC phospholipids (*m/z* 496, *m/z* 520, *m/z* 522, *m/z* 524, and *m/z* 544), PC phospholipids (*m/z* 758, *m/z* 760, *m/z* 786) and SM phospholipids (*m/z* 701 and *m/z* 703) in human plasma. The SM phospholipids can be easily distinguished from PC phospholipids as the protonated molecular ion of a SM is expected to be an odd number whereas the protonated molecular ion of a PC is expected to be an even number (nitrogen rule). The lyso-PC phospholipids eluted in the 2–5 min region and the PC and SM phospholipids eluted in the 5–10 min region, with the *m/z* 760 and *m/z* 786 PC phospholipids being among the late eluting components. The phospholipid profile obtained using the SRM of *m/z* 184 → *m/z* 184 (Fig. 2d) is in general similar to that obtained using the positive precursor ion scan of *m/z* 184 (Fig. 2c). A very important advantage of the technique based on the precursor ion scan of *m/z* 184 over the *m/z* 184 → *m/z* 184 technique is that the former not only detects all the phospholipids that have the choline polar head, but it also identifies the precursor ions corresponding to each chromatographic peak, which is not the case with the latter. This feature enables the facile distinction between the PC, lyso-PC and SM phospholipids. This is very important when working under different chromatographic conditions where the elution order of the different phospholipids is not known *a priori*. The results obtained using the positive neutral loss scan of 141 Da (Fig. 2b) confirmed the presence of previously reported (Pang *et al*., 2008; Uran *et al*., 2001; Taguchi *et al*., 2005) PE phospholipids (*m/z* 742, *m/z* 744, *m/z* 768) in human plasma. The results obtained using the negative precursor ion scan of *m/z* 153 are shown in Fig. 2a. The results obtained using the SRM transitions of the individual phospholipids species selected to represent the different classes of phospholipids are shown in Fig. 2e.

Chromatographic Elution Behavior of Phospholipids

It is very important to evaluate the elution behavior of the phospholipids in order to establish a strategy for eliminating bioana-

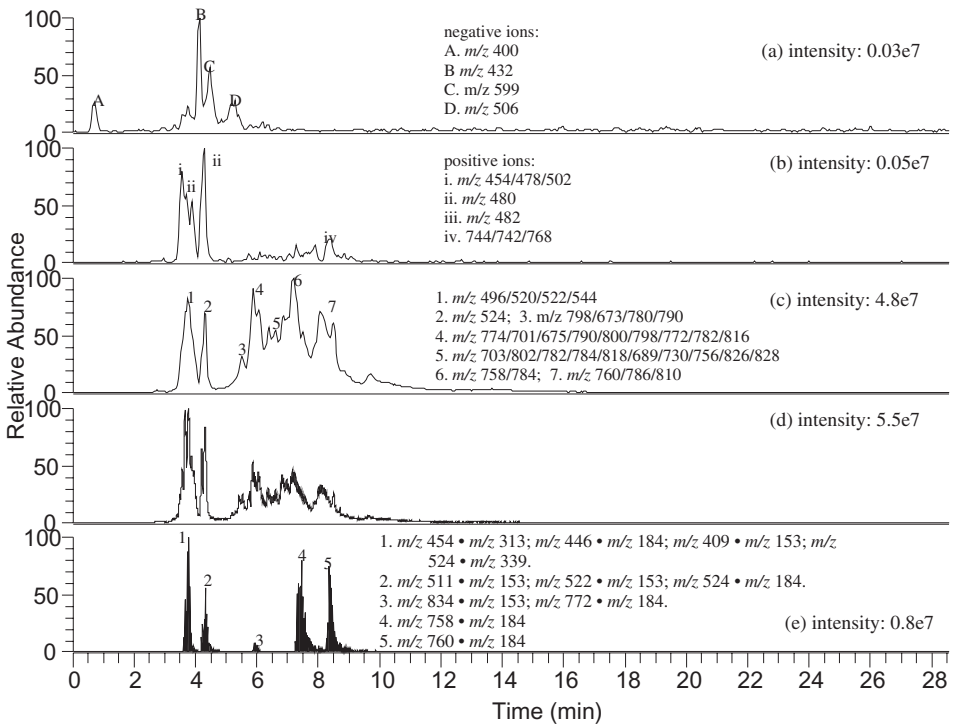

Figure 2. Comparison of three techniques used to monitor human plasma phospholipids from acetonitrile-precipitated human plasma injected into LC-MS/MS system using a gradient elution. Chromatograms obtained using the technique based on the precursor ion/neutral loss scans: (a) negative precursor-ion scan of *m/z* 153; (b) positive neutral loss of 141 Da; (c) positive precursor ion scan of *m/z* 184; (d) chromatogram obtained using the technique based on the in-source-CID *m/z* 184 → *m/z* 184; (e) chromatogram obtained using a technique based on the using SRM transitions of phospholipids species each representing a class of phospholipids. XBridge C_{18} (2.1 × 50 mm, 3.5 µm); flow rate 0.3 mL/min; column temperature 30°C. Gradient elution with eluent A (5 mM ammonium formate and 5 mM formic acid in water, pH 3.2) and eluent B (acetonitrile): start at 30% B and hold for 0.5 min; increase B to 95% in 4.5 min; hold for 23 min. Reproduced from Xia and Jemal (2009b) with permission.

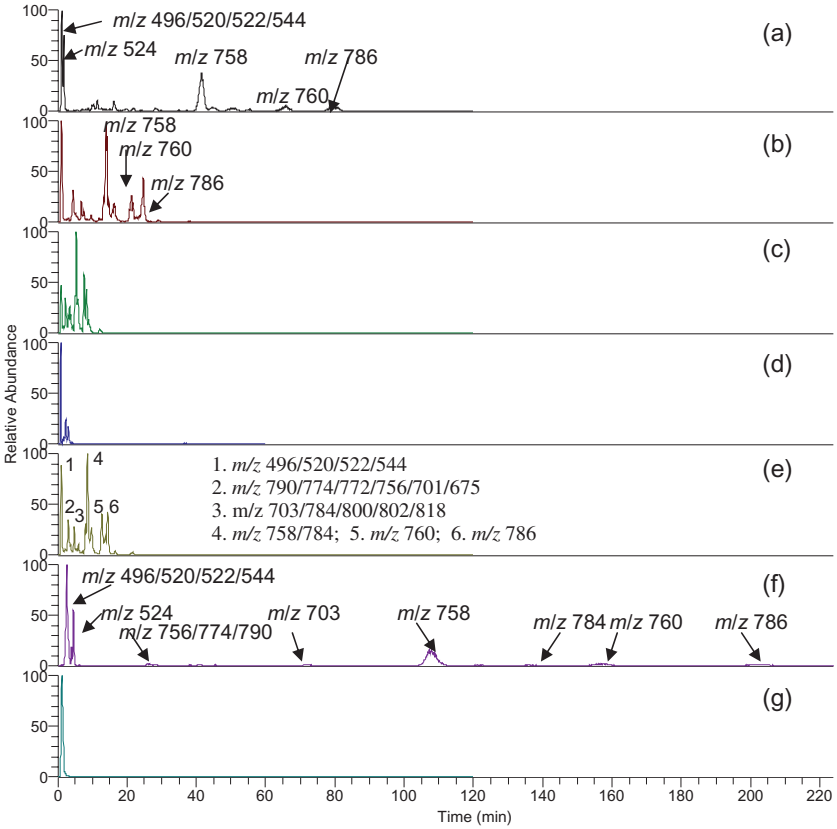

Figure 3. Phospholipids chromatographic elution profiles illustrated with the LC-MS/MS chromatograms obtained from acetonitrile-precipitated human plasma using the positive precursor ion scan of *m/z* 184 for detection of phospholipids, with an isocratic elution using the following mobile phases made up of an aqueous eluent (5 mM ammonium formate–5 mM formic acid in water) and an organic eluent: (a) 65% acetonitrile, (b) 75% acetonitrile, (c) 85% acetonitrile, (d) 95% acetonitrile, (e) 80% acetonitrile, (f) 80% methanol, (g) 80% isopropyl alcohol. Reproduced from Xia and Jemal (2009b) with permission.

lytical risk due to phospholipids that have not eluted completely during the analytical run time. The results obtained in our laboratory (Xia and Jemal, 2009b) using different mobile phases in reversed-phase chromatography, the most commonly used mode of chromatography in LC-MS/MS bioanalysis, are summarized in Fig. 3. Under isocratic conditions, using a mobile phase consisting of 5 mM ammonium formate–5 mM formic acid in water as the aqueous eluent and acetonitrile as the organic eluent in different proportions, phospholipids eluted faster as the acetonitrile percentage was increased (Fig. 3a–d). A total removal of phospholipids required about 80 min for 65% acetonitrile (Fig. 3a), 25 min for 75% acetonitrile (Fig. 3b), 10 min for 85% acetonitrile (Fig. 3c), and 5 min for 95% acetonitrile (Fig. 3d). The results obtained using 20% of the same aqueous eluent (5 mM ammonium formate–5 mM formic acid) and 80% of acetonitrile, methanol or isopropyl alcohol as the organic eluent are shown in Fig. 3e–g. Under these conditions, the total removal of plasma phospholipids required about 20 min for acetonitrile (Fig. 3e), 205 min for methanol (Fig. 3f) and 3 min for isopropyl alcohol (Fig. 3g). Thus, the most effective organic eluent to remove phospholipids from the reversed-phase column used was isopropyl alcohol, followed by acetonitrile and methanol, which is in contrast with a previous report (Little *et al*., 2006), where methanol was indicated to be a stronger eluent than acetonitrile. The difference in the acetonitrile and methanol elution order seen between the two laboratories could be due to the different LC column and gradient elution used in the previous work or due to the two different detection techniques used: the *m/z* 184 → *m/z* 184 SRM vs the precursor ion scan of *m/z* 184. Since the latter technique provides the capability of identifying the precursor ions associated with each chromatographic peak, the bioanalyst can easily ascertain that all the known major lyso PC, PC and SM species have been eluted off the column, even in the absence of *a priori* information on the chromatographic elution order. Our work (Xia and Jemal, 2009b) showed that, using the same organic eluent, different aqueous eluents did not make a significant difference in the elution behavior of phospholipids, which is in contrast with the dramatic effect seen when changing the type and/or percentage of the organic eluent used.

Under the reversed-phase chromatographic conditions used for LC-MS/MS bioanalytical methods, typically 0.3–0.6 mL/min flow rate for a 2 × 50 mm column with a mobile phase consisting of less than 80% methanol or acetonitrile, the lysophospholipids, such as lyso-PC, will elute near the analytes (drugs, metabolites or biomarkers) while the phospholipids, such as PC, will elute much later. This reality puts into question the perceived advantage of column washing via increasing the organic component of the mobile phase to 100% following the elution of the analyte, unless the washing procedure is conducted long enough to remove all the phospholipids. Inadequate column washing may be worse than not washing at all since the washing procedure would cause the otherwise late eluting phospholipids to elute

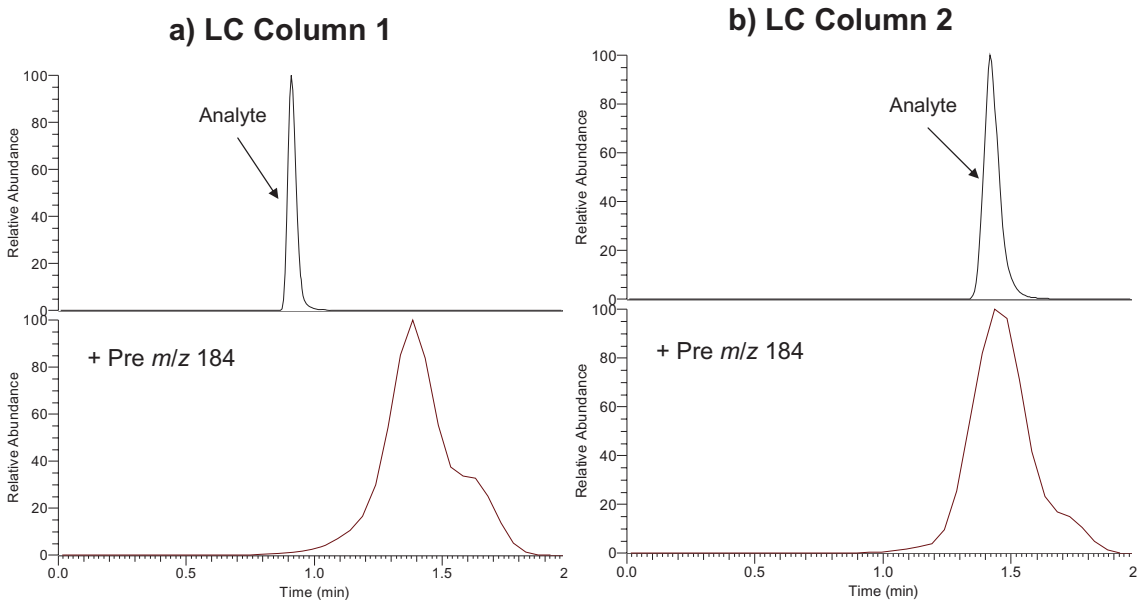

Figure 4. LC column effect on the separation of an analyte from plasma phospholipids with the same mobile phase: (a) column 1; (b) column 2. In each of panel (a) or (b), the top trace depicts the SRM transition of the analyte, and the bottom trace depicts phospholipids monitoring using the positive precursor ion scan of *m/z* 184 (+ Pre *m/z* 184).

sooner as narrower peaks with enhanced probability to cause ionization suppression or enhancement.

Strategy to Avoid Phospholipids Untoward Effect

We recommend a two-part approach to avoid bioanalytical risk related to phospholipids. The first part is to chromatographically separate the analyte from phospholipids, and the second part is to remove the phospholipids during sample extraction.

Chromatographic separation of the analyte from phospholipids. For the past several years, we have adopted the strategy of chromatographically separating the analyte from phospholipids during quantitative LC-MS/MS method development for drugs and their metabolites in plasma. During this period, we dealt with a large number of drugs and metabolites having diverse physicochemical properties. Our experience shows that, using reversed-phase chromatography, appropriate column-mobile phase combinations could be found to achieve the separation of the analytes from all the phospholipids, including the early eluting lyso phospholipids, with the analyte eluting before the lyso phospholipids and the larger phospholipids eluting after the lyso phospholipids. Figure 4 illustrates the effect of the LC column using the same mobile phase in achieving the separation of the analyte from the phospholipids. Under the same chromatographic conditions, the analyte was separated from plasma phospholipids on column 1, while the analyte co-eluted with the phospholipids on column 2.

It is highly desirable that the phospholipids elute after the analyte of interest since this would allow, if so desired, changing the chromatographic conditions to rapidly elute the phospholipids off the column after the analyte elution. When analyzing a large batch of samples, there are different possible approaches to deal with phospholipids eluting slowly after the analyte elution. One approach would be to completely elute all the phospholipids off the column before the next injection of a sequence consisting of a large batch of samples. Following the elution of the analyte, this can be accomplished in a rapid manner by increasing the flow rate and/or raising the organic component of the mobile phase to 100%, or by introducing another organic solvent of greater eluting strength, such as isopropanol. Another approach would be to wait until the completion of the batch analysis and then conduct the column washing. This is illustrated in Fig. 5 for a plasma sample extracted using liquid–liquid extraction (LLE), which removed almost all the phospholipids from the extract. There were no phospholipid peaks detected in the blank plasma LLE extract in the first injection (Fig. 5a) using isocratic elution. On the 201th injection (Fig. 5b), using the same isocratic elution, there were no phospholipid peaks detected from the same LLE extract; however, the overall baseline signal intensity significantly increased, indicating continuous, slow phospholipids bleeding off the column. On the 202nd injection (Fig. 5c), a high organic wash step was applied after the point of the analyte elution. As shown, the wash step achieved the removal of the accumulated phospholipids as indicated by the presence of the *m/z* 758 and *m/z* 786 ions. On the other hand, there was no accumulation of lyso phospholipids.

Removal of phospholipids during sample extraction. It has now been several years since we gave preference to the technique of LLE for the extraction of small-molecule drugs and metabolites from plasma samples over the other techniques of sample extraction, namely, solid-phase extraction (SPE), protein precipitation (PPT) and online extraction. In general, we have found LLE, if properly optimized, to give very clean extracts as gauged by the absence of endogenous peaks interfering with the SRM quantitation of the analyte even at the level of the lower limit of quantitation (LLOQ). The selectivity achieved involving the extraction of a number of drugs and metabolites was, in general, as good as or better than that obtained with SPE. The run-to-run assay reproducibility obtained with LLE was very good, with no untoward effects observed arising from lot-to-lot

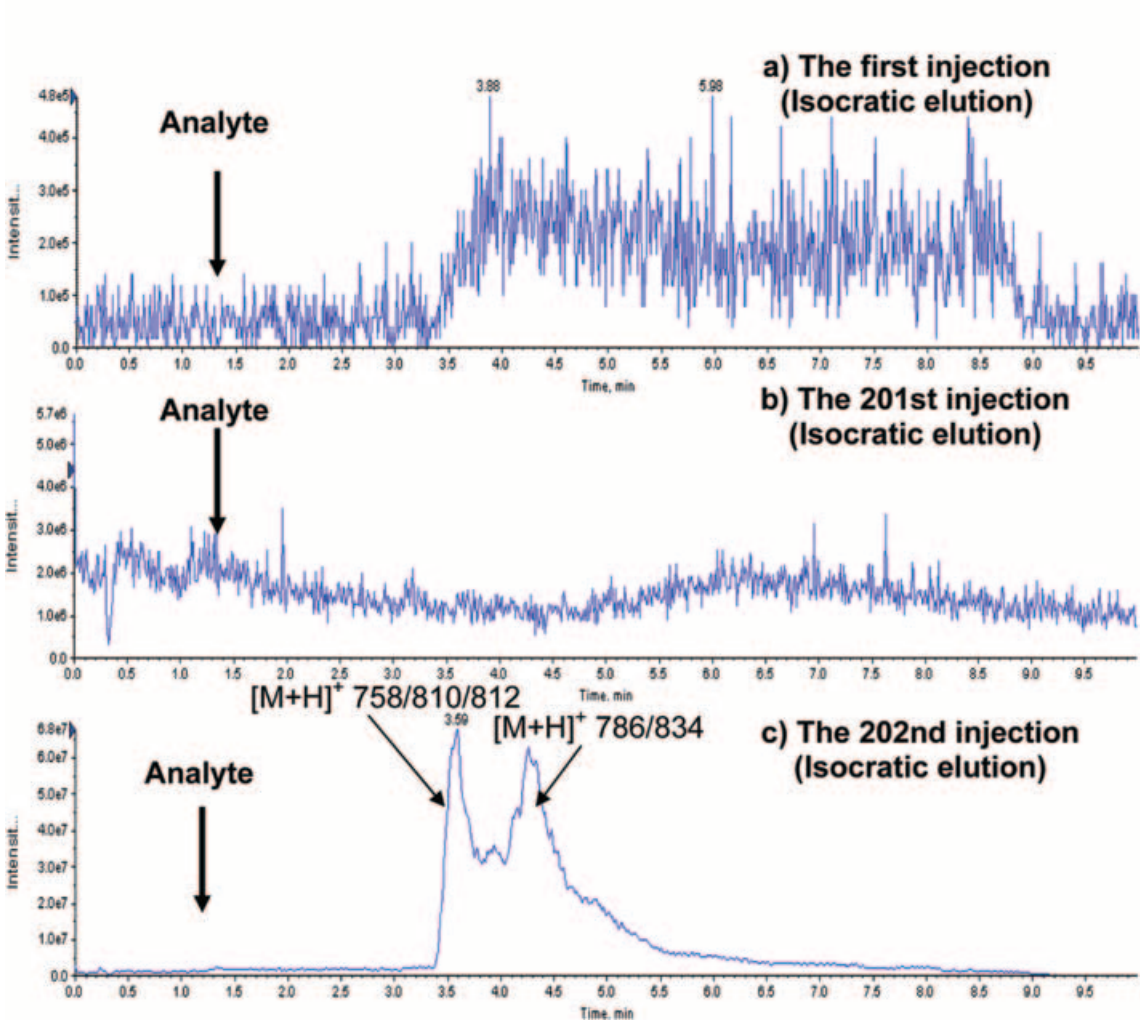

Figure 5. The chromatographic fate of phospholipids, monitored using the precursor ion scan of *m/z* 184, in a blank plasma extract, obtained via liquid–liquid extraction, during 202 runs (injections), all under isocratic conditions except for the final run where isocratic elution was used for the first 3 min and then a steep gradient scheme was applied as a wash step: (a) first injection with isocratic elution; (b) 201th injection with isocratic elution; (c) 202nd injection with isocratic elution for the first 3 min followed by steep gradient elution.

variation that may occasionally be observed with SPE products. LLE-based methods are easy to transfer from one laboratory to another and are of relatively low cost. With the advent of 96-well format devices, automation of LLE has become practical and user-friendly. Traditionally, the extraction of analytes using LLE has been performed by conducting the extraction after adjusting the plasma pH to a value equal to pK_a minus 2 for acidic analytes and to pK_a plus 2 for basic analytes, assuming that the non-ionized analyte species have a better extractability behavior than the ionized species. While this may hold true in general, we have found that, for some analytes, good extraction efficiency could be achieved under conditions where the analyte is apparently largely ionized. A paper published recently (Hendriks *et al*., 2007), which gives an excellent theoretical treatise of extraction efficiency vis-à-vis analyte pK_a, confirms our finding.

Because of the recent enhanced awareness about the bioanalytical risk posed by the plasma phospholipids, we studied the fate of phospholipids during the different LLE procedures normally used for the extraction of drugs and metabolites from plasma. In general, we found that a number of organic solvents commonly used in LLE, such as *n*-butyl chloride (1-chlorobutane) and methyl-*tert*-butyl ether (MTBE), and solvent combinations, such as hexane–ethyl acetate and hexane–2-methyl-1-butanol, are very selective in extracting the analyte and leaving the phospholipids behind (Ouyang *et al*., 2009). In Fig. 6, we show the relative amounts of a lyso phosphatidylcholine (C16:0 lyso-PC) contained in LLE plasma extracts obtained using *n*-butyl chloride, MTBE and ethyl acetate compared with a PPT extract as a benchmark. The extraction of the C16:0 lyso-PC into *n*-butyl chloride, MTBE and ethyl acetate was less than 0.1, 1 and 15%, respectively. While evaluating the extraction of phospholipids into the different organic solvents used in LLE, we conducted limited evaluation of the extraction of the phospholipids using SPE under commonly used conditions and using recently commercialized phospholipids removal plates (Aurand *et al*., 2009 and Yong *et al*., 2009). As shown in Fig. 6, the amount of the C16:0 lyso-PC in the SPE extracts, under the conditions used, was larger than that seen in the *n*-butyl chloride and MTBE LLE extracts. On the other hand, the phospholipids removal plates were as good as *n*-butyl chloride LLE in providing extracts free of the C16:0

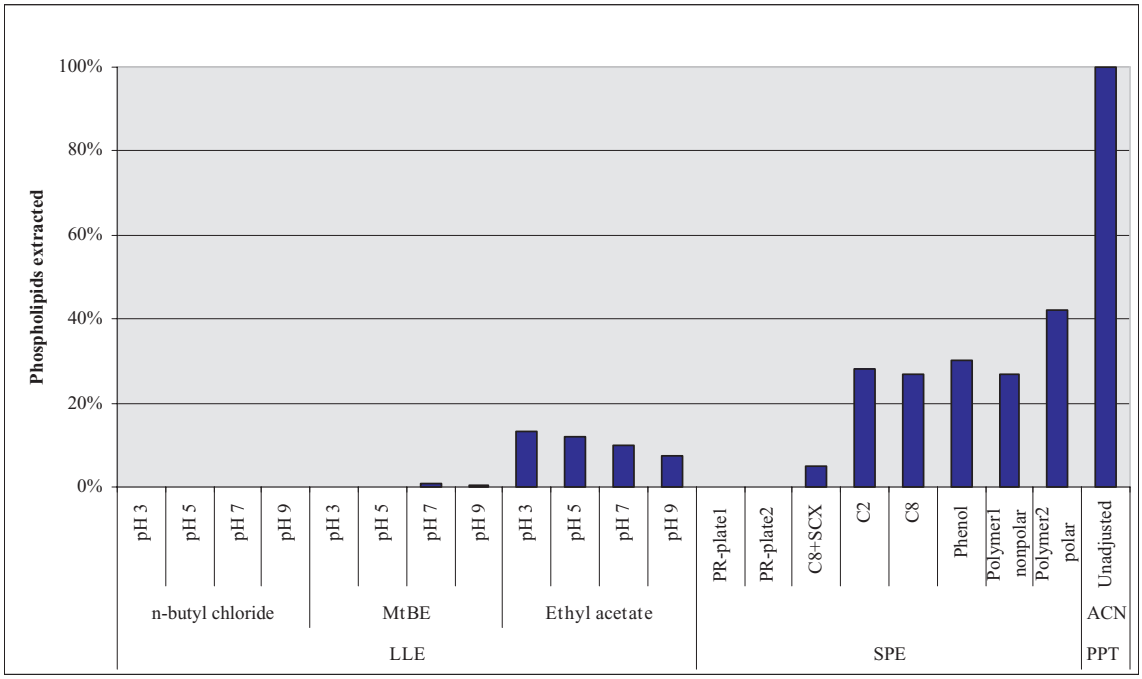

Figure 6. Extraction of C16:0 lyso phosphatidylcholine (C16:0 lyso-PC) from human plasma using liquid–liquid extraction with three different solvents under different pHs, compared with typical solid-phase extraction and two commercialized phospholipids removal sorbents (PR-plate1 and PR-plate2). The lyso-PC was monitored using selected reaction monitoring of *m/z* 496 → *m/z* 184.

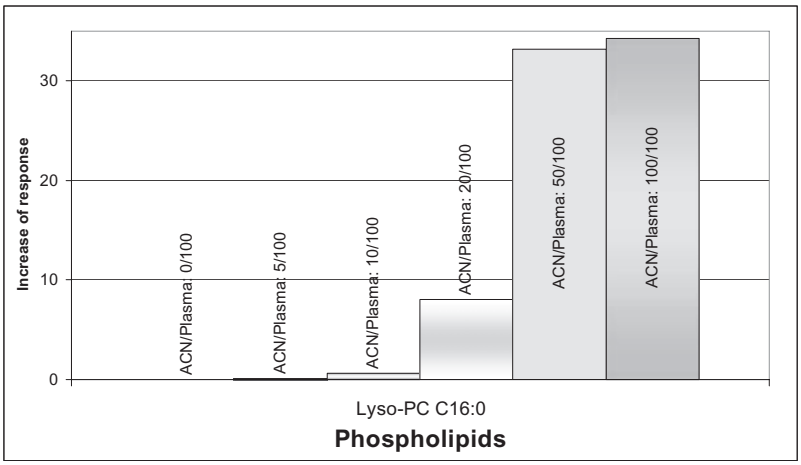

Figure 7. The effect of acetonitrile added to human plasma on the extraction of C16:0 lyso phosphatidylcholine (C16:0 lyso-PC) by liquid–liquid extraction with methyl-*tert*-butyl ether. The acetonitrile:plasma volume ratios are: 0:100, 5:100, 10:100, 20:100, 50:100 and 100:100. The extracted C16:0 lyso-PC increased by as much as 35-fold depending on the volume of the acetonitrile added. The lyso-PC was monitored using selected reaction monitoring of *m/z* 496 → *m/z* 184.

lyso-PC. In LLE, it can be generally concluded that the extraction of the PC and lyso-PC species increases with the increase in the polarity of the organic solvent and the lyso-PC species are extracted to a lesser degree compared with the PC species. It can also be concluded that, considering the minimal extraction of phospholipids, especially the lyso species, and the likelihood of obtaining a decent LLE recovery for the commonly encountered small-molecule drugs and metabolite, *n*-butyl chloride and MTBE are the best single-component solvents for LLE. Although the lyso phospholipids are less abundant in plasma than the phospholipids, the former are more important in LC-MS/MS bioanalysis since they elute earlier and closer to the commonly encountered small-molecule drugs and the associated metabolites under the normally used conditions of reversed-phase chromatography. Hence, it is important that LLE using *n*-butyl chloride and MTBE is especially efficient in removing the lyso phospholipids. It should be noted that other investigators have recently evaluated the extraction behavior of phospholipids during LLE and our findings are in general agreement with those reported by the other investigators (Garofolo *et al*., 2008; Williams *et al*., 2009; Bergeron *et al*., 2009; Liu *et al*., 2009).

Depending on the solvent used for the preparation of an internal standard (IS) working solution used for adding the IS to a plasma sample, a substantial amount of an organic solvent may be introduced to the plasma sample. This could affect the amount of the phospholipids extracted, as illustrated in Fig. 7 for C16:0

lyso-PC using an acetonitrile IS solution and LLE with MTBE. While the effect of adding acetonitrile equal to 5 and 10% of the plasma volume was negligible, the amount of the lyso-PC extracted into MTBE increased nearly 10-fold, compared with the 0% acetonitrile addition, when the added acetonitrile was equal to 20% of the plasma volume. Thus, it is important to be cognizant of the effect of the organic solvent introduced during the IS addition. Consequently, the IS solution used for spiking a plasma sample should contain as little organic solvent as possible, considering the solubility and stability of the compound.

For the reasons described earlier, we have recently been using mostly LLE for the LC-MS/MS bioanalytical methods used for drug candidates in late discovery and early development. This has prompted us to investigate means to reduce the amount of phospholipids extracted during plasma sample extraction by LLE using relatively polar solvents, which would be required for extracting relatively polar analytes. The approach being investigated, which is not complete as of this writing, is based on the use of colloidal silica in combination with divalent or trivalent cations, which is a modification of a previously reported approach (Wu *et al*., 2008) that we investigated as part of acetonitrile PPT extraction of plasma samples. A similar approach was recently reported (Schoener and Murakami, 2009). Further investigation in this area is warranted.

Use of Incurred Sample for Method Development

Nowadays, awareness is relatively high among bioanalysts about the risk associated with metabolites contributing to the measured drug concentration in incurred biological samples. In general, there are two types of metabolite interference. The first type is due to mass spectrometric interference of the metabolite with the accurate quantitation of the drug. The second type is due to the generation of the drug from its metabolite that undergoes degradation during the multiple sample handling and clean-up steps involved prior to the LC-MS/MS bioanalysis.

Metabolite Mass Spectrometric Interference in the Absence of Chromatographic Separation

The first type of mass spectrometric interference arises due to in-source conversion of a metabolite to generate the molecular ion of the parent drug, thereby providing the same SRM transition as that used for the drug and thus interfering with the quantitation of the drug, unless there is chromatographic separation between the two. We illustrated this type of interference in our original article on this subject in 1999 and since then there have been a number of publications dealing with the subject matter (Jemal and Xia, 1999; Kapron *et al*., 2005; Jemal, 2005; Vanderhoeven *et al*., 2006; Xue *et al*., 2006, 2008; Schwartz *et al*., 2006). Drugs producing metabolites that can potentially interfere in this manner include those containing lactone, carboxylic acid and sulfhydryl (thiol) functional groups (Jemal and Xia, 1999; Jemal, 2005). The corresponding metabolites, which have hydroxy acid, acylglucuronide and disulfide functional groups, undergo in-source conversion to generate the molecular ion of the corresponding parent drug. Other published examples include analysis of a drug in the presence of its *N*-oxide metabolite (Ayrton *et al*., 1999; Ramanathan *et al*., 2000) and analysis of a primary amine containing drug in the presence of its carbamoyl glucuronide (Liu and Pereira, 2002). A summary of putative metabolites of drugs of different chemical structures and the associated SRM transitions is presented in Table 1 (Jemal *et al*., 2002).

For a hydroxy acid drug that produces a corresponding lactone metabolite, chromatographic separation between the drug and the metabolite may be needed even in the absence of in-source generation of the drug entity (Jemal and Ouyang, 2000). Such a situation arises when both the metabolite and the drug form the $[M + H]^+$ and $[M + NH_4]^+$ ions and the quantitation of the drug is based on its $[M + H]^+$ ion being used as the precursor ion for the SRM transition. The interference arises because of the M + 1 isotopic contribution of the $[M + NH_4]^+$ ion of the lactone metabolite, which is lower than the $[M + H]^+$ ion of the hydroxy acid by only 1 mass-unit. This occurrence is due to the unique feature of a lactone and the corresponding hydroxy acid in that there is 18 mass-unit difference between the two compounds, which is only 1 mass-unit different from the 17 mass-unit difference that exists between the $[M + H]^+$ and $[M + NH_4]^+$ ions of any compound.

Another type of mass spectrometric interference arises from isomeric metabolites such as the *Z*-isomeric metabolite of a drug containing a methyloxime group of *E*-configuration (Jemal, 2005; Xia *et al*., 1999). Epimeric, diastereomeric and enantiomeric metabolites (Testa *et al*., 1993; Xia *et al*., 2006a) also cause the same kind of interference since such metabolites would obviously interfere with the SRM transition used for the quantitation of the drug.

A third type of mass spectrometric interference arises from other metabolites which are isobaric with the drug. A phosphate prodrug, which is used as a drug delivery strategy for enhancing the solubility of drug candidates containing alcohol or phenol groups, hydrolyzes *in vivo* to form the parent drug, which may further undergo conjugation to form a sulfate metabolite (Wu *et al*., 2009). Since the addition of the sulfate or phosphate group increases the mass of the parent drug by the same 80.0 Da, a phosphate prodrug and its sulfate metabolite are isobaric. It should be noted that the sulfate metabolite, if not chromatographically separated, would interfere not only with the isobaric phosphate prodrug but also with the parent drug since the sulfate metabolite would undergo in-source conversion to produce the molecular ion of the parent drug (Wu *et al*., 2009). Recently, when dealing with a drug containing a methyl ether group (RCH_2OCH_3), we encountered a metabolite (RCOOH), which contained the COOH group instead of the CH_2OCH_3 group in the parent drug, with the two groups being isobaric. Chromatographic separation between the drug and the metabolite was required to avoid the interference. In the absence of prior information about this type of metabolite, there is a chance that the metabolite would co-elute with the drug, especially when using an acidic mobile phase, where the metabolite would be retained longer, behaving like the drug, and using rapid chromatography with a low resolving power.

Metabolites with masses which are lower by 1 or 2 Da compared with the corresponding parent drugs will cause interference due to M + 1 or M + 2 isotopic contributions of the metabolites, with the M + 2 contributions enhanced for chlorine or bromine containing drugs. Metabolites lower by 1 Da (compared with the parent drug) could originate via oxidative deamination of the drug ($R_1R_2CHNH_2$) to form a ketone metabolite (R_1R_2CO). Metabolites lower by 2 Da could originate via hydroxylation followed by dehydration ($-H_2$), oxidation of primary alcohol to aldehyde ($-H_2$), and oxidation of secondary alcohol to

Table 1. Putative metabolites of drugs of different chemical structures and the SRM transitions for the metabolites vis-à-vis the SRM transitions of the drug

Drug type	Drug SRM	Metabolite	Metabolite SRM
Carboxylic acid	$[M + H]^+ \rightarrow P^+$	Acylglucuronide	(a) $[M + H + 176]^+ \rightarrow [M + H]^+$ (b) $[M + H + 176]^+ \rightarrow P^+$
γ or δ Hydroxy carboxylic acid	$[M + H]^+ \rightarrow P^+$	Lactone	(a) $[M + H - 18]^+ \rightarrow [M + H]^+$ (b) $[M + H - 18]^+ \rightarrow P^+$
Lactone	$[M + H]^+ \rightarrow P^+$	Hydroxy acid	(a) $[M + H + 18]^+ \rightarrow [M + H]^+$ (b) $[M + H + 18]^+ \rightarrow P^+$
Alcohol or phenol	$[M + H]^+ \rightarrow P^+$	*O*-glucuronide	(a) $[M + H + 176]^+ \rightarrow [M + H]^+$ (b) $[M + H + 176]^+ \rightarrow P^+$
Alcohol or phenol	$[M + H]^+ \rightarrow P^+$	*O*-sulfate	(a) $[M + H + 80]^+ \rightarrow [M + H]^+$ (b) $[M + H + 80]^+ \rightarrow P^+$
Amine	$[M + H]^+ \rightarrow P^+$	*N*-glucuronide	(a) $[M + M + 176]^+ \rightarrow [M + H]^+$ (b) $[M + H + 176]^+ \rightarrow P^+$
Amine	$[M + H]^+ \rightarrow P^+$	*N*-oxide	(a) $[M + H + 16]^+ \rightarrow [M + H]^+$ (b) $[M + H + 16]^+ \rightarrow P^+$
Thiol (sulfhydryl)	$[M + H]^+ \rightarrow P^+$	Disulfide	(a) $[M + M - 1]^+ \rightarrow [M + H]^+$ (b) $[M + M - 1]^+ \rightarrow P^+$
Sulfide	$[M + H]^+ \rightarrow P^+$	*S*-oxide	(a) $[M + H + 16]^+ \rightarrow [M + H]^+$ (b) $[M + H + 16]^+ \rightarrow P^+$

The SRM transitions shown are for electrospray ionization in the positive ion mode. M is the mono-isotopic mass of the drug. P is the product ion in the SRM transition used for quantitation of the drug. For each drug type, the fragmentation exhibited by the metabolite SRM transition designated as (a) can potentially take place within the source of the mass spectrometer as well. If such in-source fragmentation occurs and there is no chromatographic separation between the drug and the metabolite, the concentration of the drug determined by using the $[M + H]^+ \rightarrow P^+$ transition would be inflated. A similar list of SRM transitions can be prepared for electrospray negative ionization, and for atmospheric pressure chemical ionization in the positive or negative ion mode. Reproduced from Jemal *et al*. (2002) with permission.

ketone ($-H_2$). While metabolites higher by 1 or 2 Da would not interfere with the drug quantitation when at least a unit-mass resolution is maintained, it should be noted that the quantitation of such metabolites would be interfered with by the isotopic contribution of the parent drug. Metabolites higher by 1 Da could originate via oxidative deamination of the drug to form an alcohol metabolite, and hydrolysis of a $RCONH_2$ drug to RCOOH metabolite. Metabolites higher by 2 Da could originate via the reduction of ketone or aldehyde to alcohol.

As described above, achieving chromatographic separation between a drug and its metabolite is essential when the metabolite causes mass spectrometric interference with the quantitation of the drug. One additional approach that can be explored to separate the metabolite from the drug is through the use of high-field asymmetric waveform ion mobility spectrometry (FAIMS). A FAIMS system that is physically located between the sprayer and the orifice of a mass spectrometer controls the type of ions entering the orifice of the mass spectrometer, thereby discarding the unwanted ions. The ion selection in FAIMS is achieved by applying a compensation voltage (CV) that is specific to the ion of interest. Ideally, when the selected CV is optimum for the drug, the drug molecular ions pass through the FAIMS system into the orifice of a mass spectrometer, while the metabolite molecular ions are filtered away before reaching the orifice. Consequently, FAIMS provides separation between the drug and the metabolite even in the absence of chromatographic separation. We have demonstrated the separation of an *N*-oxide metabolite (Kapron *et al*., 2005) and an acylglucuronide metabolite (Xia and Jemal, 2009a) from their parent drugs.

Metabolite Interference due to Conversion to the Parent Drug During Biological Sample Collection, Storage, Extraction and Analysis

An unstable metabolite may have untoward effect on the accurate quantitation of the parent drug since it may convert to the drug during the multiple steps of sample collection, handling and preparation that precede the LC-MS/MS analysis. Acylglucuronides are such metabolites and tend to be unstable, especially under alkaline conditions and elevated temperatures, degrading to generate the parent drug (Khan *et al*., 1998; Shipkova *et al*., 2003). Mildly acidic conditions of pH 3–5 tend to be the most desirable pH region for minimizing the hydrolysis of acylglucuronides in biological samples or during the numerous steps involved during sample analysis. Lactone metabolites of hydroxy acid drugs are also prone to degradation to generate the

parent drug. A combination of low temperature and mildly acidic pH of 3–5 have been used to minimize the hydrolysis of the lactone metabolite back to the drug or vice versa (Jemal *et al*., 2000; Zhang *et al*., 2004). Other metabolite functional groups may undergo *E* to *Z* isomerization (or vice versa) due to exposure to light or undesirable pH conditions to generate the parent drug. Such functional groups include *O*-methyloximes (Xia *et al*., 1999) and carbon–carbon double bonds (Wang *et al*., 2003). Metabolites could also undergo epimerization to generate the drug (Testa *et al*., 1993; Won, 1994).

For the quantitation of a drug in the presence of its unstable metabolite which potentially converts to the parent drug, conditions must be optimized to minimize such a conversion. However, even the optimal conditions adopted may not totally prevent conversion of the metabolite to the drug. It is thus essential to design method development appropriately in order to minimize the adverse effect of such a conversion on the accuracy and precision of the method. The significance of proper method design was systematically illustrated using two compounds, pravastatin (a hydroxy acid) and pravastatin lactone, which would undergo interconversion to different degrees depending on the conditions used (Jemal and Xia, 2000). The important attribute of the method design was the use of the appropriate ratios of the concentrations of pravastatin and pravastatin lactone in the quality control (QC) samples. Methods that implemented such an approach of bioanalytical method design include those used for the simultaneous quantitation of simvastatin and simvastatin acid (Jemal *et al*., 2000; Zhang *et al*., 2004).

Methanol is a commonly used organic solvent in different steps of LC-MS/MS bioanalysis, including standard stock preparation, washing steps of SPE, reconstitution following evaporation and chromatography. Metabolites containing ester groups, including acylglucuronide metabolites, may react with methanol to produce the corresponding methyl ester, especially under basic conditions (Khan *et al*., 1998; Ferreirós *et al*., 2007). Thus, the use of methanol should be avoided with a drug containing methyl ester group ($RCOOCH_3$) since such a drug could produce the corresponding carboxylic acid metabolite (RCOOH) and the corresponding acylglucuronide (RCOO–acylglucuronide) metabolite. In the presence of methanol, especially under basic conditions, the acylglucuronide could react with methanol to produce $RCOOCH_3$, which is the drug. Under such conditions, the measured concentration of the drug would be highly inflated if the acylglucuronide concentration was large compared with the drug concentration. On the other hand, it should be noted that for a drug that contains an ester group other than a methyl ester, such as an ethyl ester, a reaction with methanol would cause underestimation of the measured drug concentration since methanol would react with the drug to form the methyl ester analog of the drug (Ferreirós *et al*., 2007).

Rationale and Strategy for the use of Incurred Sample for Method Development

The accepted practice of developing and validating LC-MS/MS bioanalytical methods is based on using calibration standards and QC samples prepared by spiking the drug into a blank biological matrix, as described in the 2001 FDA guidance for bioanalytical method validation (Food and Drug Administration, 2001) and the conference report of the third AAPS/FDA bioanalytical workshop held in 2006 (Viswanathan *et al*., 2007). However, as described above, metabolites found in incurred samples could affect the accuracy and precision of the drug measurement. Ideally, the QC samples used for method development and validation should contain the metabolites in addition to the drug. However, it is not practically possible to obtain the reference standards of all metabolites at the time of method development and validation, especially for drugs in early development. In 2002, a strategy was put forth (Jemal *et al*., 2002) based on using incurred sample, known to contain the drug metabolites, to challenge a method previously validated in the traditional manner using spiked QC samples. It was proposed that the strategy be applied to methods used for the analysis of samples from first-in-human (FIH) studies. As soon as the FIH samples from high dose panels become available, a pooled incurred sample would be prepared to test the method for metabolite interference. If the method were found not to be valid due to metabolite interference, the method was to be re-optimized and revalidated. Consequently, all the samples previously analyzed by the invalid method were to be reanalyzed using the newly validated method shown to be free of metabolite interference. It should be noted that the reanalysis may not be feasible if the presence of an unstable metabolite would have required stabilization of the sample at collection.

Our current strategy is based on using a pooled incurred sample during method development, not based on using a pooled incurred sample to challenge an already validated method as proposed in 2002 (Jemal *et al*., 2002). The new strategy is to be used in late drug discovery or early drug development. For the development of methods intended to support animal studies, a pooled incurred sample is obtained from earlier animal discovery studies conducted for pharmacokinetics and other discovery studies. For methods intended to support FIH studies, incurred sample is obtained from selected pre-IND (Investigative New Drug) animal studies. Later, when appropriate human incurred samples are available, the FIH method is to be retested using a human pooled incurred sample. The proposed use of a pooled incurred sample for method development is completely different from the ISR currently implemented in bioanalytical laboratories providing data for submission to regulatory agencies. The concept of ISR was adopted in the third AAPS/FDA bioanalytical workshop held in 2006, as described in the conference report (Viswanathan *et al*., 2007), and the details of how to conduct ISR were discussed in a workshop dedicated to ISR in 2008, as described in the conference report (Fast *et al*., 2009). The stated purpose of ISR is to demonstrate assay reproducibility by analyzing a set of incurred samples on two different occasions using the same validated method in exactly the same manner. Unfortunately, an invalid method, namely, a method that does not accurately measure the drug analyte due to the metabolite interference described above, may exhibit excellent ISR reproducibility. A clear case in point is an incurred sample containing a stable metabolite which causes mass spectrometric interference and co-elutes with the drug. Thus, it is essential to build quality during method development before the initiation of method validation. One important way of incorporating quality is through the use of a pooled incurred sample during method development. There are two categories in the strategy of using a pooled incurred sample for method development. For both categories, a pooled incurred sample is prepared by taking aliquots from high-dose-panel samples, including different animals or human subjects and different post-dosing time points, in order to obtain an adequate volume of the pooled sample.

In category 1, the objective is to ensure the chromatographic separation of a drug from its metabolites that show response in the SRM channel of the drug. First, a portion of the pooled incurred sample is extracted using acetonitrile precipitation. The extract is then analyzed using the chromatographic conditions of the method under development using the SRM transitions of known and potential metabolites, in addition to that of the drug. This procedure is then repeated using modified chromatographic conditions that give a larger retention factor (*k*), using a weaker mobile phase by lowering the organic amount, or using a shallower gradient. Third, the procedure is repeated using a different column/mobile phase combination to achieve orthogonal chromatography. The purpose of using the modified chromatographic conditions is to test if an additional peak would appear in the drug SRM channel, which would indicate the presence of a metabolite which is not separated from the drug under the initially used chromatographic conditions. With isomeric metabolites such as diastereomers, epimers and *E*/*Z* isomers, unlike metabolites that undergo in-source conversion to produce the drug molecular ion, there are no additional SRM channels that can distinguish the metabolites from the drug and hence chromatographic separation is essential (Xia *et al*., 1999, 2006a; Testa *et al*., 1993). It is impossible to know the chromatographic conditions or chromatographic run times required to achieve the chromatographic separation of the drug from such metabolites in the absence of authentic reference standards. In general, it is recommended that a retention factor of at least 5 be achieved for the drug. It should be mentioned that this does not address the problem caused by the presence of an enantiomeric metabolite. It should also be noted that an incurred sample may contain a metabolite that has the same SRM transition as the internal standard (IS), especially when a structural analog of the drug is used as the IS (Matuszewski *et al*., 1998). Therefore, it is prudent to analyze a pooled incurred sample without the IS and show that the IS SRM channel is clean at the retention time of the IS.

In category 2, the objective is to ensure that the drug is not generated from its metabolites during sample handling, storage, preparation and extraction. First, a 24-hour room temperature stability evaluation of the incurred sample is conducted vis-à-vis a QC sample treated in the same manner. The incurred and QC samples are then extracted identically and analyzed by the LC-MS/MS method under development. Second, the effect of the conditions used for extraction on the incurred sample is evaluated. For an LLE extraction procedure, this would involve adding the requisite buffer and keeping the sample at room temperature for at least 1 h before conducting the LLE extraction. Third, the stability of the extract, obtained from the incurred sample, is tested for at least 24 h.

Chromatography

One of the most important recent advances in HPLC is in the area of enhancing speed and efficiency as measured by plate count (*N*). To this end, various techniques have been introduced, including the use of sub-2 µm particles, high temperature, monolithic columns and superficially porous stationary phases (Carr *et al*., 2009; Zhang *et al*., 2009; Poppe, 1997; Plumb *et al*., 2007; McNeff *et al*., 2007; Fekete *et al*., 2010; Guillarme *et al*., 2009). The term UHPLC, which stands for ultra high-performance liquid chromatography, may be used to indicate any one of these techniques. The technique based on using sub-2 µm particles, popularly known as UPLC to indicate ultra performance liquid chromatography, has recently been widely used in different areas, including LC-MS/MS bioanalysis. The introduction of the concept of the 'Poppe plot' (Poppe, 1997), where the plate time (t_0/N) is plotted against *N* (with t_0 designating the column dead time) to display the compromise between efficiency and speed, has been an invaluable contribution to chromatography. This concept supplements the kinetic characterization of columns in terms of their van Deemter flow curves. The main advantage of the 'Poppe plot' approach is to enable the chromatographer to find the optimal conditions, such as optimal column length and flow rate, under given separation conditions, such as fixed analysis time and maximum pressure.

UPLC as Practiced in LC-MS/MS Bioanalysis

In LC-MS/MS bioanalysis using UPLC, sub-2 µm particle size columns, typically 2 × 50 mm, 1.7–1.9 µm, are used instead of the 2 × 50 mm, 3–5 µm columns and the flow rate is normally 0.6–1.0 mL/min. A plot of plate *N* as a function of flow rate (a form of van Deemter curve) is ideally expected to show that *N* increases with flow rate, reaches a maximum, which is considered to be the optimum flow rate, and then gradually decreases. A UPLC column is ideally expected to give a higher optimum flow rate and a flatter curve beyond the optimum flow rate. Since *N* is inversely proportional to the particle size, UPLC columns are expected to give higher *N* at the optimum flow rate. Thus, better resolution and narrower peaks are expected, which should translate to better sensitivity.

We were interested in determining how well the popularly claimed advantages of UPLC—enhanced N, sensitivity, resolution and speed—are realized under the conditions normally used for LC-MS/MS bioanalysis, namely a short run time (1–3 min), low retention factor (*k*) and relatively high flow rate. Thus, we evaluated *N* as a function of flow rate with UPLC columns using different analytes (Xia and Jemal, 2008). Representative plots of *N* against flow rate are shown in Fig. 8. We found that the expected ideal relation between *N* and flow rate, as exemplified by the plot shown for toluene at *k* of 21 in Fig. 8, was obtained only for certain analytes that eluted at a relatively high *k*. Otherwise, the plots obtained were significantly different from the expected ideal plots, depending on the analyte and the *k* used, as exemplified in Fig. 8 by the pravastatin plot at *k* of 2. Under non-ideal analyte/*k* combinations, the *N* and optimum flow rates obtained were significantly lower than the theoretically expected values. Our observations obtained for the different analytes are in line with a recent publication which reported that kinetic performance of a chromatographic system is analyte dependent (de Villiers *et al*., 2009). Thus, the *N* values and the relation between *N* and flow rate obtained for new columns using test compounds and reported by column manufacturers may not reflect the performance achievable with pharmaceutical compounds.

We also evaluated *N* as a function of *k* for a UPLC column at a fixed flow rate of 0.6 mL/min and the data obtained for eight analytes are shown in Fig. 9. The results show that *N* increases with *k*, with *N* reaching a plateau at around *k* = 10 for some analytes, but not for others. This observed phenomenon, which is in general agreement with a previous report (Neue, 1997), is important since LC-MS/MS bioanalysis is normally conducted in the low *k* region. Another observation is that the maximum *N*, obtained at a higher *k*, is analyte-dependent, as can be seen by comparing the maximum *N* obtained for lidocaine and reserpine (Fig. 9). A

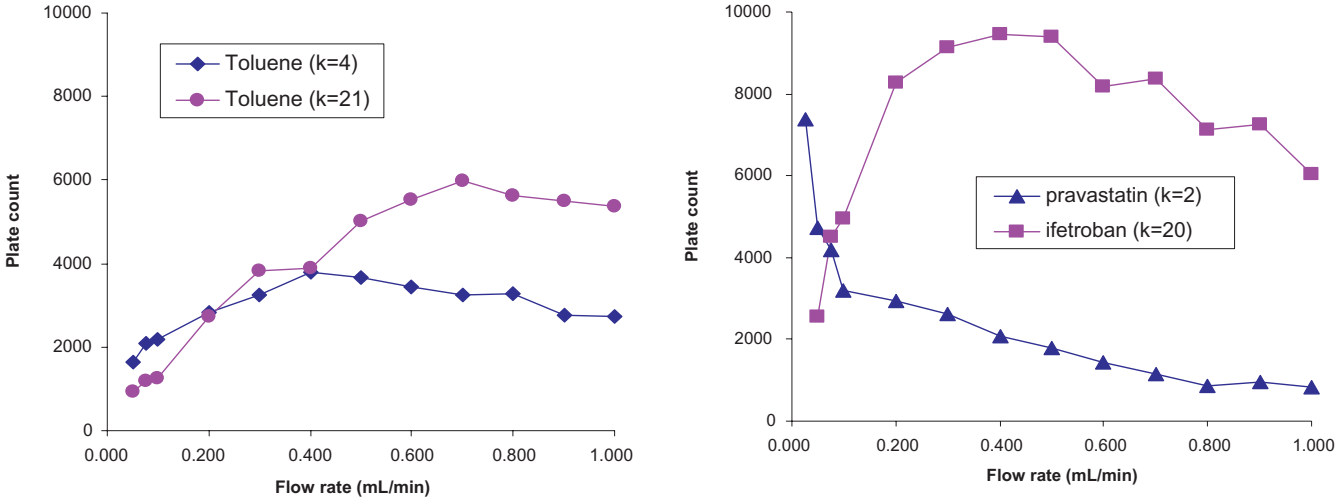

Figure 8. *N* as a function of flow rate obtained with a UPLC column using different analytes demonstrated with representative plots for three analytes: left panel for toluene at retention factor (*k*) of 4 and 21; right panel for pravastatin at $k = 2$ and ifetroban at $k = 20$. Column: 1.9 µm, 2 × 50 mm. Isocratic elution; mobile phase:water/acetonitrile with 0.1% formic acid. Detection: UV at 254 nm for toluene and positive ESI selected reaction monitoring.

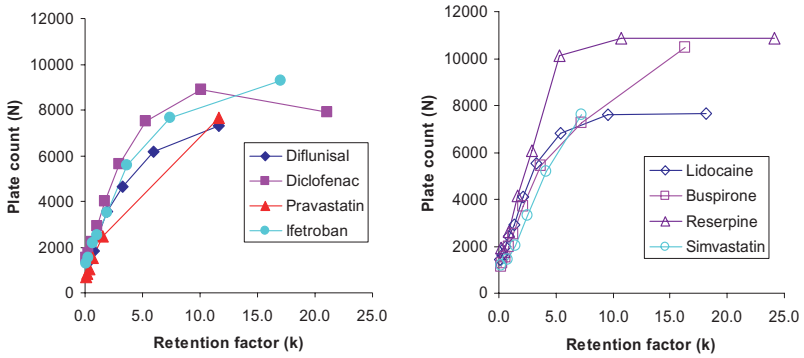

Figure 9. *N* as a function of retention factor (*k*) obtained with a UPLC column at a fixed flow rate of 0.6 mL/min using eight analytes: left panel for four analytes; right panel for additional four analytes. Column: 1.9 µm, 2 × 50 mm. Isocratic elution; mobile phase: water/acetonitrile with 0.1% formic acid or ammonium bicarbonate/hydroxide. Detection: positive ESI selected reaction monitoring.

third important observation is that the *N* obtained at $k = 1.0$ could be as low as one-quarter of the *N* obtained at a larger *k*. Thus, small-*k* chromatography—not uncommon in LC-MS/MS bioanalysis—has the disadvantage of providing a lower *N* and a lower optimum flow rate. Since resolution is proportional to $(k/k + 1)$ and $N^{1/2}$, using a lower *k* decreases the resolution directly and, additionally, via the lowering of *N*.

Continuing with our evaluation of UPLC under conditions normally used for LC-MS/MS bioanalysis, we compared 1.8 and 5.0 µm columns under identical conditions. Figure 10 shows the comparison of the chromatograms obtained using the two columns under a flow rate of 1.0 mL/min and a gradient elution. The important points from this and another run conducted under a flow rate of 0.3 mL/min (chromatograms not shown) are summarized in Table 2. The results show that, both for the 0.3 and 1.0 mL/min flow rate, the column particle size made only a modest difference in the peak height, peak width or resolution obtained, the difference for each parameter being less than a factor of 2. On the other hand, the flow rate caused a more pronounced difference in the performance of the two columns. The peak height increased by 6–7-fold and the peak width decreased by about 3-fold when using the faster flow rate. Thus, the significant increase in sensitivity popularly claimed for UPLC is largely due to the high flow rate used and not the UPLC column per se. The performance comparison of different particle sizes (5.0, 3.5 and 1.8 µm) was also evaluated under isocratic elution. As shown in Fig. 11 and Table 3, the peak height, peak width and resolution results, obtained under a flow rate of 0.6 mL/min, are in agreement with those obtained under the gradient elution. Thus, the difference in the three parameters between the 1.8 and 5.0 µm columns is less than 2-fold. The difference in *N* between the two columns was 2.4, which is approximately equal to the ratio of the particle sizes ($5.0/1.8 = 2.8$), as theoretically expected. In general, it should be noted that the *N* obtained with sub-2 µm particles is not as high as theoretically expected and that the difference between the theoretical and experimental *N* increases as the particle size decreases (Fekete *et al.*, 2010).

In reality, the not-so-significant improvement in the resolution we observed with the 1.8 µm column compared with the 5.0 µm column is not surprising. It should be recalled that resolution is a function of $N^{1/2}$, $(k/k + 1)$ and $(\alpha - 1/\alpha)$, where α denotes selectivity. Therefore, the 2.4-fold difference in *N* observed between the two columns would translate to only a 1.6-fold difference in resolution ($2.4^{1/2} = 1.6$). Such a modest level of improvement can

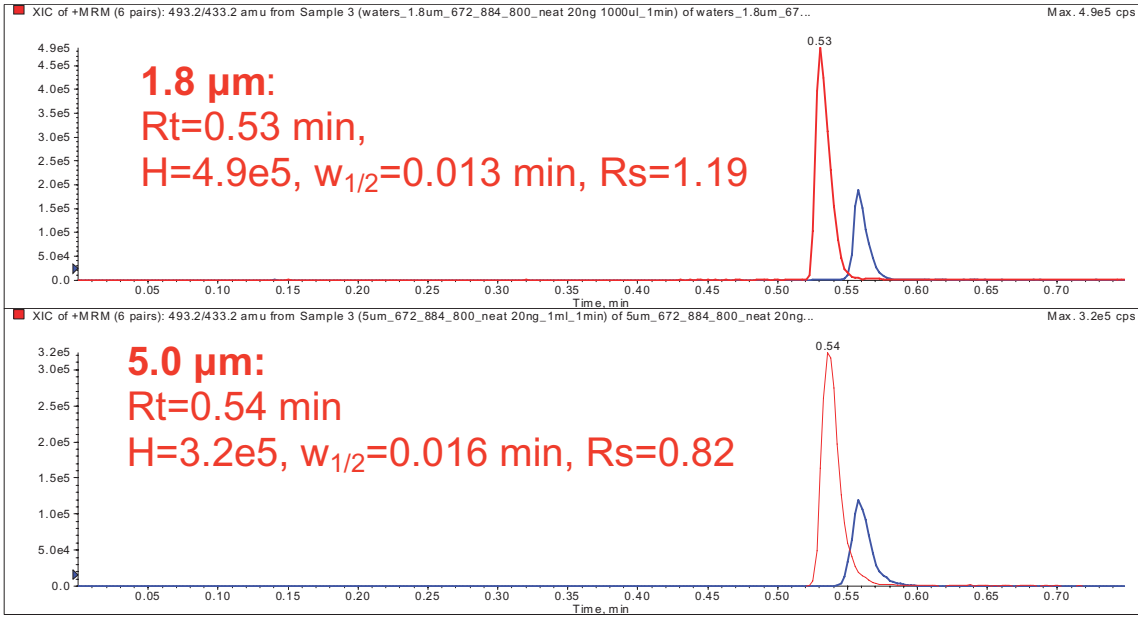

Figure 10. Chromatograms obtained with 1.8 and 5.0 µm columns under identical flow rate of 1.0 mL/min and gradient elution. The retention time (*Rt*), peak height (*H*) and peak width ($w_{1/2}$) values shown are for the earlier eluting peak (0.53 or 0.54 min). Gradient: 0–0.1 min 5% B; 0.1–0.5 min 5–95% B; 0.5–0.75 min 95% B; 0.75–0.78 min 95–5% B; 0.78–1.0 min 5% B, using 10 mM ammonium acetate in water (pH 6.5) as eluent A and 10 mM ammonium acetate in 10% water–90% acetonitrile as eluent B. The results obtained from the chromatograms depicted here and those obtained at a flow rate of 0.3 mL are summarized in Table 2.

Table 2. Results obtained with 1.8 and 5.0 µm columns under two flow rates (0.3 and 1.0 mL/min) and gradient elution[a]

	Retention time, min	Peak height	Peak width at half height	Resolution
1.8 µm, 0.3 mL/min	2.06	7.7×10^4	0.039	1.26
5.0 µm, 0.3 mL/min	2.20	4.5×10^4	0.050	0.85
1.8 µm, 1.0 mL/min	0.53	4.9×10^5	0.013	1.19
5.0 µm, 1.0 mL/min	0.54	3.2×10^5	0.016	0.82

[a] For the 1.0 mL/min flow rate, the chromatograms are shown in Fig. 10.

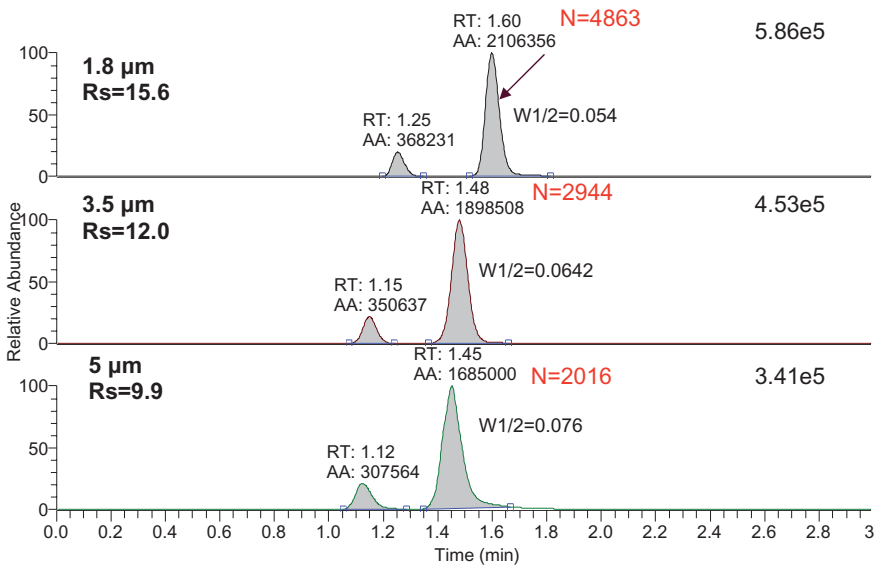

Figure 11. Chromatograms obtained with 1.8, 3.5 and 5.0 µm columns under identical flow rate of 0.6 mL/min and isocratic elution. The retention time, peak height and peak width ($w_{1/2}$) values considered are for the major peak at 1.60, 1.48 or 1.45 min. Mobile phase consisted of 1:1 water and acetonitrile with 0.1% formic acid. The results obtained from the chromatograms depicted here are summarized in Table 3.

Table 3. Results obtained with 1.8 and 5.0 μm columns under identical flow rate of 0.6 mL/min and isocratic elution[a]

	Retention time, min	N	$N^{1/2}$	Peak height	Peak width at half height	Resolution
1.8 μm	1.60	4863	69.7	5.9×10^5	0.054	15.6
5.0 μm	1.48	2944	54.3	4.5×10^5	0.064	12.0
5.0 μm	1.45	2016	44.9	3.4×10^5	0.076	9.93

[a] The chromatograms are shown in Fig. 11.

be achieved by affecting α through the use of a different mobile phase and/or column chemistry/brand. It should be noted that achievement of rapid analysis time is solely a function of the flow rate used, independent of the particle size. On the other hand, bioanalytical chemists have greatly benefited from the introduction of the UPLC technology. The hardware, including the high pressure pump, is a significant addition that allows the use of high-flow-rate chromatography even with the regular columns to realize UPLC-like results.

Column Stationary Phase and Mobile Phase Selection

Reversed-phase (RP) chromatography remains the technique of choice for the analysis of pharmaceutical compounds. Manufacturers are continuously introducing new RP stationary phases to meet the demand for columns that give retention selectivity and symmetrical, sharp peaks (Euerby and Petersson, 2003; Van Gyseghem *et al*., 2006; Stella *et al*., 2007; Marín and Barbas, 2006). Differences in the performance of seemingly similar commercial columns may lie in both the nature of the silica support and the technique used to produce the bonded phase. Factors that can influence retention selectivity and peak shape include surface area, pore size, trace metal activity, bonded phase surface activity, bonding chemistry and silica deactivation process. Extensive efforts have been expended characterizing silica-based RP columns in terms of their surface coverage, hydrophobic selectivity, shape selectivity, hydrogen bonding capacity and ion-exchange capacity at different pH values and with different mobile phases. The objective of such characterizations, using appropriate acidic, basic and neutral probe analytes of different polarities, is to aid in selecting the appropriate column for a given application. Despite such tests and the claims made by the column manufacturers, it is still difficult to predict *a-priori* the right column without some level of experimentation. Commercially available RP column types include those packed with alkyl, cyano, phenyl, perfluorinated, polar embedded, polar/hydrophilic endcapped and a variety of novel phases. The different modifications of the RP phases not only provide different retention behaviors but also extend the usable pH range and allow the use of highly aqueous mobile phases. For certain analytes, perfluorinated phases have been shown to exhibit not only RP characteristics but also normal-phase-like characteristics, especially in a mobile phase of high organic percentage.

In LC-MS/MS bioanalysis, the selection of columns and mobile phases is further complicated since a mobile phase could significantly affect the nature and intensity of the analyte mass spectrometric response obtained using the same column (Jemal, 2000; Jemal and Xia, 2006; Delatour and Leclercq, 2005; Xia *et al*., 2006b; Patring and Jastrebova, 2007; Grujic *et al*., 2008; Peng and Farkas, 2008). Although not as widely reported, there have also been reports of observations that the type/brand of the column used could affect the analyte response using the same mobile phase (Xia *et al*., 2006b; Piovan *et al*., 2004; Ouyang *et al*., 2005). Thus, during method development, it is prudent to incorporate some level of column and mobile phase screening in order to obtain appropriate mass spectrometric response, in addition to achieving the requisite chromatographic separation with symmetrical and efficient peaks. Such screening and optimization could be achieved efficiently using automated systems (Xia *et al*., 2006b; Krisko *et al*., 2006; Biswas *et al*., 2009).

A Protocol for Systematic Method Development

Heretofore, we discussed the underlying principle on selected themes, thereby presenting the rationale for the centrality of these themes to quantitative LC-MS/MS bioanalysis. Herein, putting it all together, we give an outline of a protocol for the systematic development of an LC-MS/MS bioanalytical method in plasma in order to reduce bioanalytical risk. This protocol would ideally be applicable in late drug discovery or early drug development, where substantial information on the physicochemical properties and the metabolism of the drug candidate is already available.

Before initiating method development, a variety of information is assembled from appropriate groups working on the drug candidate of interest. Thus, information related to different physicochemical properties are obtained, such as stability in aqueous solutions at different pHs, photostability, aqueous solubility at different pHs, solubility in selected water-miscible and water-immiscible solvents, pK_a, log$D$ and log$P$. Information is also obtained on *in-vitro* and *in-vivo* metabolism, serum protein binding and partitioning between plasma and red blood cells. It is also essential to have detailed information related to any bioanalytical methods previously used in support of any earlier discovery studies so that any lessons learned can be incorporated into the new method under development. It is also important to obtain the realistic lower limit of quantitation (LLOQ) required for the planned non-clinical and clinical studies.

Method development starts with the optimization of the acquisition of the positive and negative ESI full-scan spectra of the drug candidate and any metabolites for which reference standards are available. These experiments are conducted by infusing each analyte prepared in water–acetonitrile (50:50, v/v) into a mobile phase flowing into the mass spectrometer. Typically, a mobile phase consisting of 40% water (with 0.1% formic acid) and 60% acetonitrile is used for positive ESI, and a mobile phase consisting of 40% water (with 0.005% formic acid) and 60% acetonitrile is used for negative ESI. Using the protonated or deprotonated molecular ion as the precursor ion, product ion spectra are obtained and then SRM transitions are selected. At this stage

of method development, it is essential to utilize at least two SRM transitions since a co-eluting metabolite or an endogenous component may interfere with one or more of the selected SRM transitions. The MS parameters, such as sprayer voltage, capillary temperature, tube lens potential, skimmer voltage, collision energy, sheath gas and auxiliary gas pressure are optimized using the selected SRM transitions.

Step 2 of method development involves the screening of LC mobile phases and stationary phases using an automated system consisting of multiple mobile phases and columns packed with different stationary phases. Typical aqueous eluents used include 0.1% formic acid (pH 2.7), 10 mM ammonium formate with 0.1% formic acid (pH 3.2), 10 mM ammonium acetate with 0.05% acetic acid (pH 4.2), and 10 mM ammonium bicarbonate with 0.1% ammonium hydroxide (pH 9.5). The organic eluents are acetonitrile and methanol, each used in conjunction with the aqueous eluents. A variety of RP columns from different manufacturers are used, including those packed with C_8, C_{18}, phenyl, perfluorinated, polar endcapped and polar embedded stationary phases. Typically, 2 × 50 mm columns are used and the particle sizes range from sub-2 µm (1.7–1.9 µm) to 3.5 µm, depending on the brand of the column used. The analyte solution, prepared in water–acetonitrile or water–methanol (70:30, v/v), is injected under isocratic or gradient conditions into the screening system for detection by the SRM transitions selected above. Based on the analyte peak shape (symmetry), efficiency (*N*), response (peak height), retention factor and resolution between critical pairs (if known at this stage), two or three column/mobile phase combinations are selected.

In step 3, the selected column/mobile phase combinations are tested for the separation of the analytes from plasma phospholipids under the isocratic or gradient conditions used above. This is achieved by injecting the supernatant of acetonitrile-precipitated plasma and monitoring phospholipids by the all-inclusive technique described earlier, which involves the positive precursor ion scan of *m/z* 184, positive neutral loss scan of 141 Da and negative precursor ion scan of *m/z* 153. With the creation of a database for the elution of the phospholipids under different isocratic and gradient elution conditions using different column/mobile phase combinations, this step may be skipped relying on the information obtained from the database.

Step 4 involves the use of pooled incurred sample to test the specificity of the method in the presence of drug-related metabolites, endogenous plasma components and possibly formulation agents. For this purpose, portions of plasma samples from high-dose panels of one or more *in-vivo* studies are combined in order to obtain an adequate volume of a pooled incurred sample. The pooled sample should include aliquots from at least three regions of the pharmacokinetic profile, including the early and late time points and around the maximum concentration region. Ideally, the pooled sample should be from a species known to contain a high concentration of potentially problematic metabolites. If high-concentration plasma samples are not available, urine, bile and *in vitro* samples, such as liver microsomes and hepatocytes, may be used for spiking plasma to generate a metabolite-rich plasma sample. An aliquot of the pooled incurred plasma sample is then extracted using acetonitrile precipitation and the supernatant is used for the analysis. A large number of SRM transitions, as many as the mass spectrometric system allows, are created to cover all the potential metabolites of the drug candidate. The SRM transitions of all known and expected metabolites are included. This can be easily accomplished if the mass spectrometric system is equipped with a metabolite SRM table builder; otherwise, the table has to be created manually. It is very important to include the SRM transitions of all known and potential phase I and II metabolites that may undergo in-source CID and hence give response in the SRM channels used for the drug. It is also important to include metabolites with masses lower than that of the parent drug by 1 or 2 Da. The incurred sample extract is analyzed using the created metabolite SRM table along with the drug SRM transitions using the isocratic or gradient elution conditions of the method. The chromatograms corresponding to the SRM transitions used for the drug are then carefully examined for the presence/absence of chromatographic peaks at different retention times. If these chromatograms show any peaks eluting before or after the drug peak, a determination should be made whether or not these peaks originate from the metabolites by examining the chromatograms corresponding to the metabolite SRM channels. Obviously, it is essential to make a determination of whether or not a metabolite SRM chromatogram shows a peak at the retention time of the drug. The analysis of the incurred sample extract is repeated using the same column/mobile phase combination with a shallower gradient scheme (for a gradient method) or with a lower organic percentage mobile phase (for an isocratic method). The purpose of this analysis is to demonstrate that the single peak attributed to the drug under the faster elution conditions still remains as a single peak, confirming the absence of another component co-eluting with the drug. For the same purpose, the analysis is repeated again using a column/mobile phase combination orthogonal to the first column/mobile phase combination.

After the completion of steps 1–4, the column/mobile phase combination and the gradient or isocratic conditions are fixed for the method. In step 5, different selective plasma sample extraction procedures are evaluated in the presence of an internal standard (IS), preferably a stable isotope-labeled analog of the analyte. The analyte extraction recoveries of the different extraction procedures are then compared. An extraction procedure that gives adequate analyte recovery, depending on the sensitivity required, and at the same time achieves efficient removal of the plasma phospholipids is selected. The use of LLE makes step 5 relatively easy since the phospholipid removal information of the different organic solvents used in LLE can be easily obtained and documented under the relatively limited number of the buffer/pH combinations normally used. Thus, if adequate analyte recovery, say 40%, is obtained with *n*-butyl chloride extraction at pH 9.0, this extraction procedure would be selected, since it has been documented that *n*-butyl chloride extracts are nearly 100% free of phospholipids

In the final step, a few more experiments are conducted before the method is considered suitable for a formal validation. Under the chromatographic conditions selected and using the selected extraction procedure, post-column infusion of the analyte is conducted with the injection of a blank plasma extract to demonstrate the absence of ion suppression/ionization at the retention time of the analyte. In addition, the effect of different reconstitution solvents (injection solvents) on peak shape and peak response is evaluated. A 24 h bench-top room-temperature stability of the incurred sample is also conducted and compared with a zero-time incurred sample. As a control, a QC sample is also subjected to the same stability testing regimen. If the analyte response in the incurred sample shows a significant increase with time, the presence of a conjugate metabolite that is degrading to generate the parent drug is indicated. A similar test is

conducted to test the stability of the incurred sample in the aqueous buffer used for buffering the plasma sample during extraction. The stability of the extract obtained from the incurred sample should also be evaluated. Another quick test conducted is the evaluation of the effect of the internal standard equilibration time in plasma. Finally, method qualification is conducted to gauge the accuracy, precision and ruggedness of the method. For accuracy and precision determination, a set of calibration standards and QC samples are analyzed. Ruggedness is determined by performing at least 200 injections, consecutively, from a single pooled QC sample extract, at a concentration within the first quartile of the standard curve range, portioned into several wells of a 96-well plate. Ruggedness is gaged by the relative standard deviation (RSD) of the peak area ratios (analyte/IS) across the entire ruggedness run, as well as the RSDs of the analyte and IS peak areas. If the assay qualification shows the method will meet the required assay performance criteria for sensitivity, accuracy, precision, ruggedness and linearity, the method is deemed ready for a formal validation.

Conclusion

In order to avoid the costly and nerve-wracking experience of finding out by chance that an LC-MS/MS bioanalytical method, validated as per the currently accepted industry practice, is invalid after it has been used to support a number of pivotal clinical and non-clinical studies, it is important to incorporate quality during method development. The protocol for systematic method development recommended herein, which is based on the use of incurred sample, column/mobile phase optimization and phospholipids avoidance, goes a long way in enhancing the quality of the developed method.

Acknowledgements

We acknowledge and are grateful for the contribution of our colleague, Dr Jian Wang, to the chromatography section and his enthusiastic participation in the discussion of the other topics.

References

Ahnoff M, Wurzer A, Lindmark B and Jussila R. Characterisation of serum albumin and lysoPCs as major contributors to plasma sample matrix effects on electrospray ionisation efficiency. *51st ASMS Conference on Mass Spectrometry*, Montreal, Canada, 2003.

Aurand C, Trinh A, Brandes HK, Bell DS and Ye M. Increased bioanalytical throughput using selective phospholipid depletion. *57th ASMS Conference on Mass Spectrometry*, Philadelphia, PA, 2009.

Ayrton J, Clare, RA, Dear, GJ, Mallet, DN and Plumb R.S. Ultra-high flow rate capillary liquid chromatography with mass spectrometric detection for the direct analysis of pharmaceuticals in plasma at sub-nanogram per milliliter concentrations. *Rapid Communications in Mass Spectrometry* 1999; **13**: 1657–1662.

Bennett PK and Van Horne KC. Identification of the major endogenous and persistent compounds in plasma, serum and tissue that cause matrix effects with electrospray LC/MS techniques. *American Association of Pharmaceutical Scientists Annual Meeting and Exposition*, Salt Lake City, UT, 2003.

Bergeron M, Lahaie M, Furtado M and Garofolo F. Selection of buffer additives for liquid–liquid extraction (LLE) procedure to remove matrix effect due to phospholipids in LC-MS/MS. *57th ASMS Conference on Mass Spectrometry*, Philadelphia, PA, 2009.

Berna MJ, Ackermann BL and Murphy AT. High-throughput chromatographic approaches to liquid chromatographic/tandem mass spectrometric bioanalysis to support drug discovery and development. *Analytica Chimica Acta* 2004; **509**: 1–9.

Biswas KM, Castle BC, Olsen BA, Risley DS, Skibic MJ and Wright PB. A simple and efficient approach to reversed-phase HPLC method screening. *Journal of Pharmaceutical and Biomedical Analysis* 2009; **49**: 692–701.

Carr PW. Wang X and.Stoll DR. Effect of pressure, particle size, and time on optimizing performance in liquid chromatography. *Analytical Chemistry* 2009; **81**: 5342–5353.

Delatour C and Leclercq L. Positive electrospray liquid chromatography/mass spectrometry using high-pH gradients: a way to combine selectivity and sensitivity for a large variety of drugs. *Rapid Communications in Mass Spectrometry* 2005; **19**: 1359–1362.

De Villiers A, Lynen F and Sandra P. Effect of analyte properties on the kinetic performance of liquid chromatographic separations. *Journal of Chromatography A* 2009; **1216**: 3431–3442.

Euerby MR and Petersson P. Chromatographic classification and comparison of commercially available reversed-phase liquid chromatographic columns using principal component analysis. *Journal of Chromatography A* 2003; **994**: 13–36.

Fast DM, Kelley M, Viswanathan CT, O'Shaughnessy J, King SP, Chaudhary A, Weiner R, DeStefano AJ and Tang D. Workshop report and follow-up—AAPS workshop on current topics in GLP bioanalysis: assay reproducibility for incurred samples—implications of Crystal City recommendations. *AAPS Journal* 2009; **11**: 238–241.

Fekete S, Ganzler K and Fekete J. Facts and myths about columns packed with sub-3 μm and sub-2 μm particles. *Journal of Pharmaceutical and Biomedical Analysis* 2010; **51**: 56–64.

Ferreirós N, Dresen S, Alonso RM and Weinmann W. Hydrolysis and transesterification reactions of candesartan cilexetil observed during the solid phase extraction procedure. *Journal of Chromatography B* 2007; **855**: 134–138.

Food and Drug Administration. *Guidance for Industry: Bioanalytical Method Validation* 2001; 1–25. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research: Rockville, MD.

Garofolo F, Mess J-C and Cote CS. Guidelines for method development of HILIC-LC-MS/MS: choice of columns and chromatographic conditions to handle matrix effect due tphopholipids. *American Association of Pharmaceutical Scientists Annual Meeting and Exposition*, Atlanta, GA, 2008.

Grujic S, Vasiljevic T, Lausevic M and Ast T. Study on the formation of an amoxicillin adduct with methanol using electrospray ion trap tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2008; **22**: 67–74.

Guillarme D, Grata E, Glauser G, Wolfender JL, Jean-Luc Veuthey JL and Rudaz S. Some solutions to obtain very efficient separations in isocratic and gradient modes using small particles size and ultra-high pressure. *Journal of Chromatography A* 2009; **1216**: 3232–3243.

Hendriks G, Uges DRA and Franke JP. Reconsideration of sample pH adjustment in bioanalytical liquid–liquid extraction of ionisable compounds. *Journal of Chromatography B* 2007; **853**: 234–241.

Jemal M. High-throughput quantitative bioanalysis by LC/MS/MS. *Biomedical Chromatography* 2000; **14**: 422–429.

Jemal M. Pitfalls in quantitative LC-MS/MS: metabolites contributing to measured drug concentrations. In *Identification and Quantification of Drugs, Metabolites and Metabolizing Enzymes by LC-MS*, Chowdhury SK (ed.). Elsevier: Oxford, 2005.

Jemal M and Ouyang Z. The need for chromatographic and mass Resolution in liquid chromatography–tandem mass spectrometric methods used for quantitation of lactones and corresponding hydroxy acids in biological samples. *Rapid Communications in Mass Spectrometry* 2000; **14**: 1757–1765.

Jemal M and Xia Y-Q. The need for adequate chromatographic separation in the quantitative determination of drugs in biological samples by high-performance liquid chromatography with tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 1999; **13**: 97–106.

Jemal M and Xia Y-Q. Bioanalytical method validation design for the simultaneous quantitation of analytes that may undergo interconversion during analysis. *Journal of Pharmaceutical and Biomedical Analysis* 2000; **22**: 813–827.

Jemal M and Xia Y-Q. LC-MS development strategies for quantitative bioanalysis. *Current Drug Metabolism* 2006; **7**: 491–502.

Jemal M, Ouyang Z and Powell M. Direct-injection LC-MS-MS method for high-throughput simultaneous quantitation of simvastatin and simvastatin acid in human plasma. *Journal of Pharmaceutical and Biomedical Analysis* 2000; **23**: 323–340.

Jemal M, Ouyang Z and Powell M. A strategy for a post-method-validation use of incurred biological samples for establishing the acceptability of a liquid chromatography/tandem mass-spectrometric method for quantitation of drugs in biological samples. *Rapid Communications in Mass Spectrometry* 2002; **16**: 1538–1547.

Kapron J, Jemal M, Duncan G, Kolakowski B and Purves R. Removal of metabolite interference during liquid chromatography/tandem mass spectrometry using high-field asymmetric waveform ion mobility spectrometry. *Rapid Communications in Mass Spectrometry* 2005; **19**: 1979–1983.

Khan S, Teitz DS and Jemal M. Kinetic analysis by HPLC–electrospray mass spectrometry of the pH-dependent acyl migration and solvolysis as the decomposition pathways of ifetroban 1-*O*-acyl glucuronide. *Analytical Chemistry* 1998; **70**: 1622–1628.

Krisko RM, McLaughlin K, Koenigbauer MJ and Lunte CE. Application of a column selection system and DryLab software for high-performance liquid chromatography method development. *Journal of Chromatography A* 2006; **1122**: 186–193.

Lehninger AL. *Principles of Biochemistry*. Worth Publishers, New York, 1982; 707.

Little JL, Wempe MF and Buchanan CM. Liquid chromatography–mass spectrometry/mass spectrometry method development for drug metabolism studies: examining lipid matrix ionization effects in plasma. *Journal of Chromatography B* 2006; **833**: 219–230.

Liu DQ and Pereira T. Interference of a carbamoyl glucuronide metabolite in quantitative liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 2002; **16**: 142–146.

Liu G, Snapp HM, Ji Q and Arnold ME. Method Development of a UPLC Assay for the Bioanalysis of Ketoconazole in Human Plasma. *57th ASMS Conference on Mass Spectrometry*, Philadelphia, PA, 2009.

Marín A and Barbas C. Systematic comparison of different functionality columns for a classical pharmaceutical problem. *Journal of Pharmaceutical and Biomedical Analysis* 2006, **40**: 262–270.

Matuszewski BK, Chavez-Eng CM. and Constanzer ML. Development of high-performance liquid chromatography–tandem mass spectrometric methods for the determination of a new oxytocin receptor antagonist (L-368,899) extracted from human plasma and urine: a case of lack of specificity due to the presence of metabolites. *Journal of Chromatography B* 1998; **716**: 195–208.

McNeff CV, Yan B, Stoll DR and Henry RA. Practice and theory of high temperature liquid chromatography. Journal *of Separation Science* 2007; **30**: 1672–1685

Murphy RC, Fiedler J and Hevko J. Analysis of nonvolatile lipids by mass spectrometry. *Chemical Reviews* 2001; **101**: 479–526.

Naidong W. Bioanalytical liquid chromatography tandem mass spectrometry methods on underivatized silica columns with aqueous/organic mobile phases. *Journal of Chromatography B* 2003; **796**:209–224.

Neue UD. *HPLC columns: Theory, Technology, and Practice*. Wiley-VCH: New York, 1997; 58.

Ouyang Z, Jemal M and Shen X. Does LC column type/brand affect LC/MS response? *53th ASMS Conference on Mass Spectrometry*, San Antonio, TX, 2005.

Ouyang Z, Wu S and Jemal M. Biological sample phospholipids clean up: A comparison of sample preparation techniques. *57th ASMS Conference on Mass Spectrometry*, Philadelphia, PA, 2009.

Pang LQ, Liang QL, Wang YM, Ping L and Luo GA. Simultaneous determination and quantification of seven major phospholipid classes in human blood using normal-phase liquid chromatography coupled with electrospray mass spectrometry and the application in diabetes nephropathy. *Journal of Chromatography B* 2008; **869**: 118–125.

Patring JDM and Jastrebova JA. Application of liquid chromatography–electrospray ionisation mass spectrometry for determination of dietary folates: effects of buffer nature and mobile phase composition on sensitivity and selectivity. *Journal of Chromatography A* 2007; **1143**: 72–82.

Peng L and Farkas T. Analysis of basic compounds by reversed-phase liquid chromatography–electrospray mass spectrometry in high-pH mobile phases. *Journal of Chromatography A* 2008; **1179**: 131–144.

Perterson BL and Cummings BS. A review of chromatographic methods for the assessment of phospholipids in biological samples. *Biomedical Chromatography* 2006; **20**: 227–243.

Piovan A, Filippini R, Borsarini A and Caniato R. Flow injection analysis mass spectrometry, a tool to investigate the problems in the quantitative analysis of hypericin using reversed-phase high-performance liquid chromatography. *Rapid Communications in Mass Spectrometry* 2004; **18**: 131–132.

Plumb R, Mazzeo JR, Grumbach ES, Rainville P, Jones M; Wheat T, Neue UD, Smith B and Johnson KA. The application of small porous particles, high temperatures, and high pressures to generate very high resolution LC and LC/MS separations. *Journal of Separation Science* 2007; **30**: 1158–1166.

Poppe H. Some reflections on speed and efficiency of modern chromatographic methods. *Journal of Chromatography A* 1997; **778**: 3–21.

Pulfer M and Murphy RC. Electrospray mass spectrometry of phospholipids. *Mass Spectrometry Reviews*, 2003; **22**: 332–364.

Ramanathan R., Su AD, Alvarez N, Blumenkranz N., Chowdhury SK, Alton, K and Patrick J. Liquid chromatography/mass spectrometry methods for distinguishing *N*-oxides from hydroxylated compounds. *Analytical Chemistry* 2000; **72**: 1352–1359.

Schoener D and Murakami S. The use of liquid–liquid extraction along with colloidal silica and polyvalent cations to remove phospholipids that produce ESI matrix effects. *57th ASMS Conference on Mass Spectrometry*, Philadelphia, PA, 2009.

Schwartz MS, Desai RB, Bi S, Miller AR and Matuszewski BK. Determination of a prostaglandin D_2 antagonist and its acyl glucuronide metabolite in human plasma by high performance liquid chromatography with tandem mass spectrometric detection—a lack of MS/MS selectivity between a glucuronide conjugate and a phase I metabolite. *Journal of Chromatography B* 2006; **837**: 116–124.

Schwarz HP, Dahlke MB and Drelsbach L. Phospholipid composition of blood plasma, erythrocytes, and 'ghosts' in sickle cell disease. *Clinical Chemistry* 1977; **23**: 1548–1550.

Shipkova M, Armstrong VW, Oellerich M and Wieland E. Acyl glucuronide drug metabolites: toxicological and analytical implications. *Therapeutic Drug Monitoring* 2003; **25**: 1–16.

Souverain S, Rudaz S and Veuthey J-L. Restricted access materials and large particle supports for on-line sample preparation: an attractive approach for biological fluids analysis. *Journal of Chromatography B* 2004; **801**: 141–156.

Stella C, Rudaz S, Gauvrit JY, Lantéri P, Huteau A, Tchapla A and Veuthey JL. Characterization and comparison of the chromatographic performance of different types of reversed-phase stationary phases. *Journal of Pharmaceutical and Biomedical Analysis* 2007; **43**: 89–98.

Taguchi R, Houjou T, Nakanishi H, Yamazaki T, Ishida M, Imagawa M and Shimizu T. Focused lipidomics by tandem mass spectrometry. *Journal of Chromatography B* 2005; **823**: 26–36.

Takatera A, Takeuchi A, Saiki K, Morisawa T, Yokoyama *N* and Matsuo M. Quantification of lysophosphatidylcholines and phosphatidylcholines using liquid chromatography–tandem mass spectrometry in neonatal serum. *Journal of Chromatography B* 2006; **838**: 31–36.

Testa B, Carrupt PA and Gal J. The so-called 'interconversion' of stereoisomeric drugs: an attempt at clarification. *Chirality* 1993; **5**: 105–111

Tiller PR, Romanyshyn LA and Neue UD. Fast LC/MS in the analysis of small molecules. *Analytical and Bioanalytical Chemistry* 2003; **377**:788–802.

Uran S, Larsen A, Jacobsen PB and Skotland T. Analysis of phospholipid species in human blood using normal-phase liquid chromatography coupled with electrospray ionization ion-trap tandem mass spectrometry. *Journal of Chromatogrraphy B* 2001; **758**: 265–275.

Vanderhoeven SJ, Lindon JC, Troke J, Nicholson JK and Wilson ID. NMR spectroscopic studies of the transacylation reactivity of ibuprofen 1-*β*-*O*-acyl glucuronide. *Journal of Pharmaceutical and Biomedical Analysis* 2006; **41**: 1002–1006.

Van Gyseghem E, Jimidar M, Sneyers R, De Smet M, Verhoeven E and Vander Heyden Y. Stationary phases in the screening of drug/impurity profiles and in their separation method development: Identification of columns with different and similar selectivities. *Journal of Pharmaceutical and Biomedical Analysis* 2006; **41**: 751–760.

Van Horne KC and Bennett PK. Preventing matrix effects by using new sorbents to remove phospholipids from biological samples. *American Association of Pharmaceutical Scientists Annual Meeting and Exposition*, Salt Lake City, Utah, 2003.

Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG, Weiner R. Workshop/conference report—quantitative bioanalytical methods validation and implementation:

best practices for chromatographic and ligand binding assays. *AAPS Journal* 2007; **9**(1): E30–42.
Wang C, Xie S, Yang J, Yang Q and Xu G. Structural identification of human blood phospholipids using liquid chromatography/quadrupole–linear ion trap mass spectrometry. *Analytica Chimica Acta* 2004; **525**: 1–10.
Wang CJ, Pao LH, Hsiong CH, Wu CY, Whang-Peng JJK and Hu OYP. Novel inhibition of *cis/trans* retinoic acid interconversion in biological fluids—an accurate method for determination of *trans* and 13-*cis* retinoic acid in biological fluids. *Journal of Chromatography B* 2003; **796**: 283–291.
Williams L, Lindenmuth M, Jones R, Desbrow C, Caulfield J, Dowthwaite G, Calverley R, Jordan S and Lodder H. Phospholipid removal: a comparison between traditional liquid–liquid extraction (LLE) and supported liquid extraction (SLE) using LC-MS/MS analysis. *57th ASMS Conference on Mass Spectrometry*, Philadelphia, PA, 2009.
Won CM. Epimerization and hydrolysis of dalvastatin, a new hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor. *Pharmaceutical Research* 1994; **11**: 165–170.
Wu ST, Schoener D and Jemal M. Plasma phospholipids implicated in the matrix effect observed in liquid chromatography/tandem mass spectrometry bioanalysis: evaluation of the use of colloidal silica in combination with divalent or trivalent cations for the selective removal of phospholipids from plasma. *Rapid Communications in Mass Spectrometry* 2008; **22**: 2873–2881.
Wu ST, Cao K, Bonacorsi Jr SJ, Zhang H and Jemal M. Distinguishing a phosphate ester prodrug from its isobaric sulfate metabolite by mass spectrometry without the metabolite standard. *Rapid Communications in Mass Spectrometry* 2009; **23**: 3107–3113.
Xia Y-Q and Jemal M. Evaluation of UHPLC column efficiency at different flow rates and retention factors: implications of the not-so-expected results**.** *56th ASMS Conference on Mass Spectrometry*, Denver, CO, 2008.
Xia Y-Q and Jemal M. High-field asymmetric waveform ion mobility spectrometry for determining the location of in-source collision-induced dissociation in electrospray ionization mass spectrometry. *Analytical Chemistry* 2009a; **81**: 7839–7843
Xia Y-Q and Jemal M. Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and the association of phospholipids with matrix effects. *Rapid Communications in Mass Spectrometry* 2009b; **23**: 2125–2138.
Xia Y-Q, Whigan DB. and Jemal M. A simple liquid–liquid extraction with hexane for low-picogram determination of drugs and their metabolites in plasma by high-performance liquid chromatography with positive ion electrospray tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 1999; **13**: 1611–1621.
Xia Y-Q, Jemal M, Zheng N, Shen X. Utility of porous graphitic carbon stationary phase in quantitative LC-MS/MS bioanalysis: quantitation of diastereomers in plasma. *Rapid Communications in Mass Spectrometry* 2006a; **20**: 1831–1837.
Xia Y-Q, Ouyang Z and Jemal M. Automated column/mobile phase screening system to achieve optimum chromatographic separation and sensitivity during LC-MS/MS bioanalytical method development. *54th ASMS Conference on Mass Spectrometry*, Seattle, WA, 2006b.
Xu RN, Fan L, Rieser MJ and El-Shourbagy TA. Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. *Journal of Pharmaceutical and Biomedical Analysis* 2007; **44**: 342–355.
Xue YJ, Simmons NJ, Liu J, Unger SE, Anderson DF and Jenkins RG. Separation of a BMS drug candidate and acyl glucuronide from seven glucuronide positional isomers in rat plasma via high-performance liquid chromatography with tandem mass spectrometric detection. *Rapid Communications in Mass Spectrometry* 2006; **20:** 1776–1786.
Xue YJ. Akinsanya B, Raghavan *N* and Zhang D. Optimization to eliminate the interference of migration isomers for measuring 1-*O*-*β*-acyl glucuronide without extensive chromatographic separation. *Rapid Communications in Mass Spectrometry* 2008; **22**: 109–120.
Yong B, Hudson W, Jones D and Chen Y-L. A simple way to remove phospholipids from bioanalytical samples. *57th ASMS Conference on Mass Spectrometry*, Philadelphia, PA, 2009.
Zhang N, Yang A, Rogers JD and. Zhao JJ. Quantitative analysis of simvastatin and its *β*-hydroxy acid in human plasma using automated liquid–liquid extraction based on 96-well plate format and liquid chromatography–tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 2004; **34**: 175–187.
Zhang Y, Wang YX, Mukherjee P and Petersson P. Critical comparison of performances of superficially porous particles and sub-2 μm particles under optimized ultra-high pressure conditions. *Journal of Chromatography A* 2009; **1216**: 4597–4605.