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Quantitative bioanalysis: an integrated approach for drug discovery and development

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Abstract

An integrated approach to quantitative bioanalysis, incorporating turbulent flow chromatography (TFC) with mass spectrometric detection, was developed to support in-house drug discovery and development efforts. Activities such as metabolic stability screening and pharmacokinetic characterization support are carried out on a single unified platform. Two different TFC column-switching configurations, parallel and serial, are presented. The first, a parallel TFC column configuration, is capable of high-throughput analysis but carryover can reach as high as 0.24%. The characteristics of the instrument operating in the parallel configuration are provided for analysis of samples generated during in vitro metabolic stability assessments, a key screen during the lead optimization phase of drug discovery. Operating in this configuration, the system has the capability of performing on-line solid phase extraction and analysis of approximately 400 samples containing phosphate-buffered saline in approximately 14 h. The second, a serial TFC column configuration, was used to perform direct plasma injection analysis. The advantage of the serial configuration is the relatively low carryover (<0.040%) observed due to increased number of valve washes; however these extra washes lead to increased injection cycle times. A method developed using the serial TFC column configuration for the determination of dihydropyridines in plasma samples is given as an example. Analytical performance criteria examined during method development and validation included linearity, accuracy, precision, and recovery. The robustness of the technique was demonstrated by applying the method in the analysis of over 2500 plasma samples generated during preclinical drug development studies. Further, combined analysis of plasma and brain tissue was performed using acetonitrile precipitation as sample pretreatment for both matrices.

Keywords: Mass spectrometry; Turbulent flow chromatography; Liquid chromatography; Column-switching; Quantitation; Plasma; Brain tissue

1. Introduction

The role of liquid chromatography-mass spectrometry (LC-MS) in drug discovery and development has not changed since Lee and Kerns reviewed the topic approximately 4 years ago [1]. The ubiquitous phrase “doing more with less” features more prominently than ever during lead candidate generation and selection. This situation is further exacerbated in a small pharmaceutical company faced with a limited workforce. Quantitative analytical support ranging from first-stage “pharmaceutical profiling” or “drugability” activities, such as aqueous solubility, in vitro metabolic stability, and permeability screening, to later-stage in vivo pharmacokinetic characterization of promising lead candidates continues to play important roles in guiding lead optimization efforts. Liquid chromatography interfaced with mass spectrometry employing atmospheric pressure ionization has paved the way for the early-stage activities to be performed almost in parallel with the screening of compounds for biological receptor activity. This has prompted one researcher to boldly suggest that these screenings may precede biological receptor activity screening in the future [2].

The use of in vitro drug metabolism approaches for the prediction of various in vivo pharmacokinetic characteristics is widely practiced in the pharmaceutical industry. In particular, in vitro metabolic stability assessment using hepatic subcellular fractions to predict in vivo hepatic clearance is

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employed as part of the initial screening of candidates in a lead optimization program. This is because the liver is the main organ involved in the metabolism of xenobiotics, the process by which most drugs are cleared from the body. The correlation between in vivo hepatic clearance values and the intrinsic clearance values determined from liver microsomal incubation experiments is also well documented [3–6]. The application of this in vitro screening approach consistently generates hundreds of samples for analysis in a single batch, prompting researchers to develop clever analytical techniques involving LC-MS [7–9]. Korfmacher et al. [7] introduced an automated system for quantitative analysis of metabolic stability samples incorporating LC-MS and automated data processing strategies. Samples were analyzed serially using a relatively lengthy HPLC gradient analysis time (10 min). Recently, Xu et al. [8] realized higher throughput by utilizing an eight-channel parallel LC-MS system capable of analyzing eight samples simultaneously. However, careful preselection of analytes must be practiced to ensure mass differentiation within the same set of injections. While these systems can dramatically increase the throughput in quantitative analyses of samples, a dedicated LC-MS platform is required rendering each system less flexible for other bioanalytical support activities.

For in vivo characterization of pharmacokinetics and bioavailability, it is necessary to administer the drug to selected animal species both intravenously and by the intended route of administration (usually oral). Whole blood samples are collected over a predetermined time course after dosing, and the drug is quantified in the harvested plasma by a suitable bioanalytical method. Concurrently, it is also useful to collect plasma/tissue samples from animals tested during in vivo pharmacological models. Based on the concentration/effect (pharmacokinetic/pharmacodynamic) relationship, it may be possible to establish a link between in vitro pharmacologic activity and the behavior of a compound in vivo. Of particular importance for a drug discovery program targeted towards neurodegenerative diseases is the measurement of drug concentration in brain tissue samples to ascertain the extent of brain penetration by the drug candidate. LC-MS plays an unsurpassed role as the enabling technology for high-throughput quantitative bioanalysis in the aforementioned activity. The unmatched selectivity and sensitivity of tandem mass spectrometry (MS/MS) enables sample analysis times of 5 min or less. However, one bottleneck still remains: plasma and tissue samples require the extraction of analyte(s) from endogenous proteins and lipids that would typically obstruct the flow through a liquid chromatography column. Therefore, sample preparation is still a necessary step in the analysis of biological samples and is commonly achieved by protein precipitation, solid phase extraction (SPE), or liquid–liquid extraction. More recently, column-switching extraction approaches in the form of either a reusable extraction column or a disposable cartridge have enjoyed a resurgence [10]. Although the analysis of tissue samples is not performed as routinely as the analysis of plasma samples, drug quantitation in complex tissues have been reported involving the use of LC-MS [11–19], including techniques incorporating on-line SPE with column-switching [14,19]. However, most methods require lengthy optimization and/or sample preparation procedures, which would not be well-suited for the throughput-oriented drug discovery environment.

Turbulent flow chromatography (TFC) with on-line solid phase extraction and column-switching has emerged over the last 6 years as analytical chemists continually strive to reduce time-consuming manual sample preparation. In TFC approaches, separation or extraction of analyte(s) from biological sample matrices is performed in the turbulent flow regime. While the concept of TFC is not new [20,21], Quinn and Takarewski [22] were the first to recognize the viability of this technique to achieve fast separations of small analyte molecules from the larger biomolecules commonly encountered in the biological matrices. The mechanism of separation or extraction, made possible by the large porous particles (e.g., 60 μm) in the extraction column packing material, was discussed extensively in the original patent publication [22]. Briefly, the approach involves using high linear flow rates that are accessible through the use of large particle diameters in a packed column. The higher flow rates causes the typical laminar flow profile to transition into a turbulent flow profile beyond a certain threshold flow rate. Turbulent flow is characterized by a plug profile at the solvent front instead of a parabolic profile for laminar flow. It is widely believed that this turbulent flow profile facilitates higher mass transfer rate through the formation of “eddies” within the flow of solvent. The higher flow rate coupled with increasing analyte diffusion rates within the porous particles combine to give reduced plate heights that are significantly lower than predicted in the Van Deemter equation. While the efficiencies of these separations may not be as high as separation in the laminar flow regime, turbulent flow allows the separation of small analyte molecules from the much larger biomolecules very effectively. This strategy is evident from various research groups that have successfully applied this technique in recent years [23–29].

In our laboratory, an integrated approach employing a single LC-MS platform was used to support quantitative bioanalysis during both metabolic stability screening and pharmacokinetic characterization. Our goal was to minimize sample handling and increase throughput for analysis of complex matrixes, by coupling the capability of on-line turbulent flow technology with the selectivity of mass spectrometric detection. In this paper, the throughput and reproducibility of TFC–LC-MS for analysis of samples generated from the metabolic stability screening protocol is highlighted. We further evaluated overall system performance for direct plasma analysis using a two-analyte method with a structural analog as an internal standard. The direct plasma injection method was evaluated with regards to linearity, accuracy, precision, recovery, and ruggedness. Also presented is a strategy used for combined plasma and tissue sample anal-

ysis, which showcase the inherent flexibility of the system and its ability to function as a single platform for quantitative bioanalysis.

2. Experimental

2.1. Materials and reagents

Formic acid, methanol (HPLC grade), acetonitrile (HPLC grade), and water (OmniSolv grade) were purchased from EM Science (an affiliate of Merck KgaA, Darmstadt, Germany) through VWR Scientific (West Chester, PA, USA). Potassium phosphate buffer (0.1 M) was obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA). Human liver microsomal preparations were purchased from In Vitro Technologies (Baltimore, MD, USA). The NADPH regenerating system (NRS) containing the appropriate co-factors was obtained from Gentest (Woburn, MA, USA). Cyclone™ HTLC columns were supplied by Cohesive Technologies (Franklin, MA, USA). Phenomenex Luna 3 μm C18(2) analytical cartridge columns (20 mm × 4.0 mm) were purchased from Phenomenex (Torrance, CA, USA). Nimodipine was obtained from Alexis Biochemicals (San Diego, CA, USA). MEM 1003, a Memory Pharmaceuticals development candidate, was supplied by Bayer AG (Wuppertal, Germany). Control (drug-free) animal plasma was purchased from Biochemed Pharmacologicals (Winchester, VA, USA).

2.2. Solutions and standards

All stock solutions for spiking biological samples were prepared in methanol as 1 mg/mL solutions. A separately weighed and prepared stock solution was used for preparation of quality control (QC) samples. These stock solutions were subsequently diluted to give individual intermediate working solutions (typically in 50% methanol:50% water, v/v) for spiking of control plasma. The concentrations of the working solutions were selected such that the volume of methanol introduced would not exceed 3% of the volume of plasma. Calibration standards (numbering between 8 and 10 concentration levels) were prepared in the nominal range from 0.5 to 500 ng/mL. Quality control samples were prepared similarly from separate working solutions to give nominal concentrations in plasma of 0.5, 1.5, 15, and 400 ng/mL. Internal standard stock solution was also prepared at a concentration of 1 mg/mL in methanol and subsequently diluted to produce a working solution at a nominal concentration of 40 ng/mL in water (plasma-only analysis) or acetonitrile (plasma/tissue analysis).

2.3. Sample preparation

2.3.1. In vitro human liver microsomal incubations

An automated in vitro metabolic stability assay using human liver microsomes was carried out on a Tecan Genesis® RSP liquid handling workstation (Research Triangle Park, NC, USA). The workstation was used to combine the microsomes (20 μL of 20 mg/mL preparation) with 0.1 M phosphate buffer (350 μL) in a Costar® 1.2 mL polypropylene 96-cluster-tube plate (Corning, NY, USA) maintained at 37 °C. The test article (5 μL of 50 μM solution in methanol to give a final incubating concentration of 0.5 μM) was then added to the mixture. The reaction was initiated following the addition of NRS (125 μL) to give a final incubating volume of 0.5 mL. The tubes were positioned on an orbital shaker maintained at a temperature of 37 °C and allowed to incubate/shake for up to 60 min. To quench the reaction at preselected intervals, a 100 μL aliquot from the tubes was transferred to a 96-deep-well microtiter plate (kept at 4 °C) containing 100 μL of acetonitrile in each well. Incubation mixtures without NRS or microsomes were used as negative controls. Testosterone was used as a positive control at the same concentration as the test article. Upon completion of the assay, the plates were mixed well and centrifuged at 2000–3000 × *g* for 10 min. The supernatant (10 μL aliquot) was subsequently analyzed.

2.3.2. Plasma-only and plasma/tissue analyses

All samples were prepared in 96-deep-well (1.2 mL) Greiner Masterblock® plates purchased through VWR Scientific. For plasma-only analyses, 50–100 μL of plasma were diluted by the addition of water (1:1 ratio, v/v) containing internal standard. The diluted plasma sample was then mixed well and a 25 μL aliquot was subsequently analyzed.

The tissue sample in the examples given in this paper was brain, which had been previously homogenized. Water was added (3:1 ratio; v/w) during the homogenization process to attain a homogenate consistency that allowed easy sample transfer by means of an air displacement pipette. For combined plasma/tissue analyses, 50–100 μL of plasma or tissue homogenate was diluted by acetonitrile (2:1 ratio, v/v) containing internal standard. The sample was then mixed well and centrifuged at 2000–3000 × *g* for 10 min. The resulting supernatant was isolated and subsequently analyzed (25 μL aliquot).

2.4. Instrumentation

Automated homogenization of brain tissue samples was carried out on a Tomtec Autogizer® (Tomtec Corporation, Hamden, CT, USA). A Tomtec Quadra® 96 Model 320 was used for various liquid transfers involving 96-well microtiter plates.

Samples for analysis (25 μL aliquot) were delivered by a Leap Technologies/CTC HTS PAL autosampler (CTC Analytics, Zingen, Switzerland) equipped with a 100 μL injector syringe, a 50 μL injection loop, and three sample storage drawers, each capable of holding two 96-well microtiter plates. A Cohesive Technologies 2300 HTLC system, which consisted of two separate binary solvent pumps and a valve-switching module comprising two Valco six-port

valves, was used as the sole TFC–LC system. One solvent pump was used for sample loading and extraction in the turbulent flow regime while the other operated as the analyte elution pump. The binary mobile phase of both pumps consisted of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). TFC at a flow rate of 5 mL/min of 100% A was employed during sample loading and extraction, which was facilitated by a Cyclone™ HTLC (50 mm × 1.0 mm) column packed with 60 μm porous particles. Analytes trapped on the HTLC column were reverse-eluted onto a separate analytical cartridge column (Phenomenex Luna C18) using fast gradient liquid chromatography at 1.25 mL/min. A linear gradient of 10–90% B facilitated the elution of most analytes. The 2300 HTLC system was controlled by the 2300 HTLC Version 1.4.1 software from Cohesive Technologies. The column effluent was split in 1:4 ratio, with the smaller fraction directed into the mass spectrometer ion source.

The Cohesive 2300 HTLC was coupled to an Applied Biosystems-Sciex API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA) operating under pneumatically-assisted electrospray (TurboIonSpray®) ionization conditions. Nitrogen delivered from a high-pressure dewar served as the nebulizer, auxiliary, source exhaust, curtain, and collision gas. Calibration of the mass axis was performed using polypropylene glycol. Unit mass resolution (0.7 Da peak width at half-height) was observed for both the first and second mass analyzers. Mass spectrometer data acquisition was controlled via a contact-closure signal received from the Cohesive 2300 HTLC. Peak detection mode was either selected ion monitoring (for metabolic stability) or selected reaction monitoring (for plasma-only or plasma/tissue analyses) with dwell times of 100–150 ms.

Mass spectrometer data acquisition and quantitation were performed using the Analyst software, Version 1.2. For plasma and/or tissue analyses, calibration curves were derived from the peak area ratio of analyte/internal standard, using least squares linear regression of the area ratio versus the nominal concentration of the standards. A weighting of $1/x^2$, with x the concentration of a given standard level, was generally found to give an optimal fit to the concentration/response data. Deviations from the regression line were calculated using the regression equation to back-calculate the expected concentration at each standard level. Quality control (QC) sample concentrations were also calculated from these regression curves, using the observed analyte/internal standard ratio.

3. Results and discussion

In our laboratory, there were two modes (serial and parallel) of column configuration utilized incorporating TFC. Fig. 1 illustrates the serial TFC–LC-MS–MS configuration, which consists of a single TFC column (e.g., Cyclone™ HTLC) operating at turbulent flow with a single analytical LC column operating at laminar flow coupled to a mass spectrometer. The stepwise methodology that was pre-programmed on the system is given in Table 1. In this serial configuration, diluted plasma is loaded onto the TFC column at 5 mL/min (by loading pump) with mobile phase A (water with 0.1%

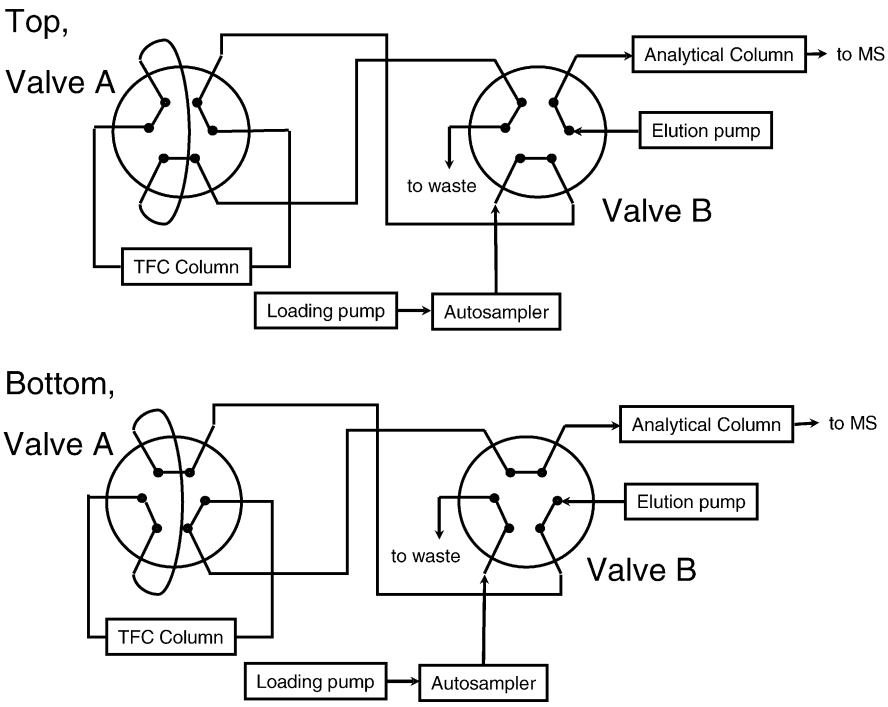

Fig. 1. Schematic of the serial TFC–LC-MS–MS configuration.

Table 1
Serial column TFC–LC-MS–MS methodology (SD = solvent delivery; CD = column flow direction); see corresponding schematic in Fig. 1

Time (min)	Loading pump		Valves module		Eluting pump		Description
	Flow (mL/min)	B (%)	SD	CD	Flow (mL/min)	B (%)	
0	5	–	Load	→	1.25	10	Load sample and discard plasma
0.25	5	–	Load	←	1.25	10	Reverse wash
0.42	5	–	Elute	←	1.25	90	Elute sample to detector
1.42	5	–	Elute	←	1.25	90	Hold
1.92	5	100	Load	←	1.25		Clean column
2.42	5	–	Load	→	1.25	–	Clean column
2.92	5	100	Load	→	1.25	–	Clean column
3.08	5	100	Elute	←	1.25	90	Clean column
3.25	5	100	Load	→	1.25	90	Clean column
3.42	5	100	Elute	←	1.25	90	Clean column
3.58	5	100	Load	→	1.25	90	Clean column
3.75	5	100	Elute	→	1.25	10	Re-equilibration
3.92	5	100	Load	←	1.25	10	Re-equilibration
4.08	5	–	Load	→	1.25	10	Re-equilibration

formic acid) for 15 s (see Fig. 1, top). The TFC column is reverse-washed for 10 s by switching Valve A. The trapped analyte(s) are then reverse-eluted onto the analytical column at 1.25 mL/min using a gradient from 10% to 90% mobile phase B (acetonitrile with 0.1% formic acid) for 1 min and held at 90% mobile phase B for an additional 30 s (see Fig. 1, bottom). Analytes are separated by laminar flow chromatography and introduced into the mass spectrometer. At this time (1.92 min), the loading pump, which had been continuously washing the TFC column with 100% mobile phase A, switches to 100% mobile phase B. The subsequent steps are then used to wash the valves and tubing further. Although the latter steps appear redundant, the combinations of solvent delivery (SD) and valve/column direction (CD) changes were necessary steps to minimize overall system carryover. We systematically investigated the number of steps required and found that a minimum of two load/elute SD cycles was necessary. The resultant overall run time per injection was just over 4 min.

A parallel TFC–LC-MS–MS configuration can also be realized by taking advantage of the unique combination of software that controls the Cohesive 2300 HTLC and the associated valve-switching module. The parallel configuration consists of two TFC columns for on-line extraction at turbulent flow and a single analytical column (Fig. 2). In this configuration, two separate column-switching methods were

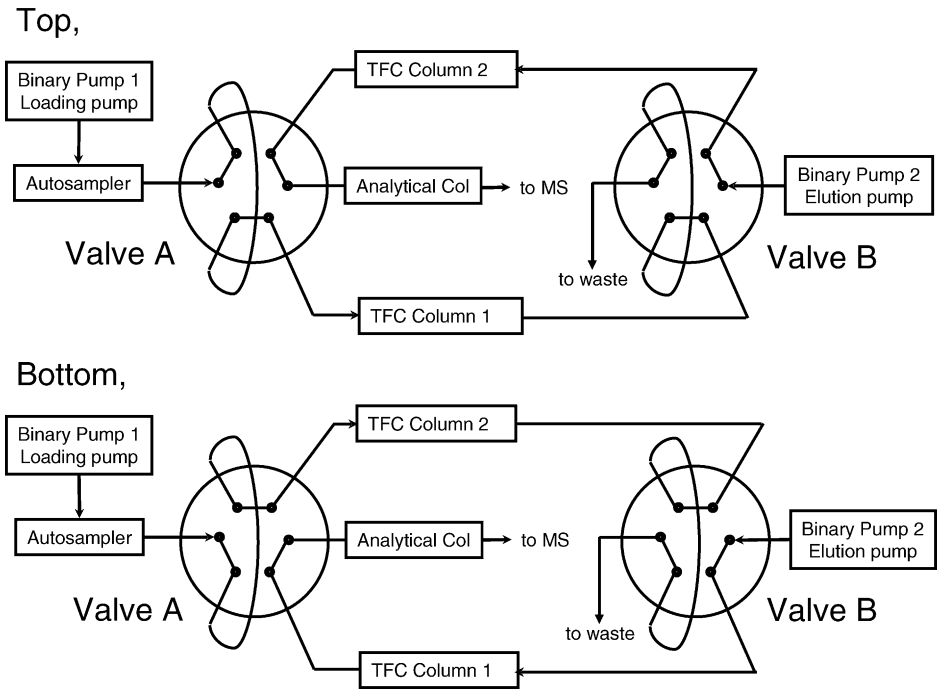

Fig. 2. Schematic of the parallel TFC–LC-MS–MS configuration.

Table 2
Parallel column TFC–LC-MS–MS methodology (SD = solvent delivery; CD = column flow direction); see corresponding schematic in Fig. 2

Time (min)	Loading pump		Valves module		Eluting pump		Description
	Flow (mL/min)	B (%)	SD	CD	Flow (mL/min)	B (%)	
0	5	–	Elute	←	1.25	10	HTLC column 1 load
0.50	5	100	Load	→	1.25	90	Elute sample to detector
1.25	5	–	Load	→	1.25	90	Hold
1.75	5	100	Elute	←	1.25	90	Hold
1.92	5	100	Load	→	1.25	–	Clean column
2.08	5	–	Load	→	1.25	10	Re-equilibration
0	5	–	Elute	→	1.25	10	HTLC column 2 load
0.50	5	100	Load	←	1.25	90	Elute sample to detector
1.25	5	–	Load	←	1.25	90	Hold
1.75	5	100	Elute	→	1.25	90	Hold
1.92	5	100	Load	←	1.25	–	Clean column
2.08	5	–	Load	←	1.25	10	Re-equilibration

actually used in an alternating sequence. Table 2 outlines the two sequential methods. Diluted plasma is loaded onto TFC column 1 for 30 s at 5 mL/min while analytes elute from TFC column 2 to the analytical column (see Fig. 2, top). At the next step, the valve is switched and the trapped analytes are reverse-eluted from TFC column 1 onto the analytical column using a gradient from 10% to 90% mobile phase B for 45 s and held at 90% mobile phase B for an additional 30 s (see Fig. 2, bottom). The subsequent steps are used to wash and re-equilibrate both TFC columns to prepare for the next sample. The cycle then repeats itself using the same method although the column directions are reversed relative to the preceding injection. The cycle time per injection is essentially halved as one TFC column is washed while the other is in-line with the analytical column and mass spectrometer. It is worthwhile to note that although the parallel configuration allows higher throughput, the individual valves cannot be isolated for additional cleaning unlike the serial configuration.

The absence or presence of memory effects or carryover is perhaps the single most important parameter to consider when evaluating a technique employing direct plasma analysis and column-switching. Whereas the mass spectrometer is considered to be relatively free of carryover effects, the same cannot be expected for the Cohesive 2300 HTLC or the autosampler. Consequently, the two configurations of the Cohesive 2300 HTLC system described above were subjected to a carryover evaluation. For this test, water-diluted plasma (1:1 ratio, v/v) was used as the sample matrix although we have subsequently observed no difference in the degree of carryover between water-diluted plasma and plasma-free organic solutions. The carryover effect of injecting a high concentration (1 μg/mL) followed by a blank sample prepared in the same matrix was tested. Because we have observed that carryover was compound-dependent, we used the "stickiest" compound, nimodipine, that we had encountered. Results obtained from the serial configuration showed a lower carryover (0.045%, based on peak area of carryover peak to the peak area of high concentration) relative to the parallel configuration (0.24%). We attributed this to the extra washes that were possible in the serial configuration.

3.1. Metabolic stability screening (parallel TFC–LC-MS)

Samples from in vitro experiments such as metabolic stability assessments were analyzed using the parallel configuration since starting concentrations were known a priori and carryover effects of less than 0.50% were not considered significant. Although it may not be necessary to carry out additional sample pretreatment beyond quenching with acetonitrile or an acidic solution, we have found that it is often a better practice to introduce samples that are relatively free of non-volatile buffer components into the mass spectrometer in order to maintain continuous mass spectrometer operation. This practice has the added benefit that subsequent metabolite profiling can be carried out using the same system and sample for candidates that were shown to be less metabolically stable.

As part of the validation of the overall metabolic stability protocol, testosterone was used as a positive reference control at two starting incubation concentrations, 0.5 and 5 μM. The procedure involved quadruplicate sampling at 0, 15, 30, and 60 min post-incubation. The resulting acetonitrile-quenched samples were analyzed, and the percent of testosterone remaining (normalized against the 0 min concentration) was monitored as a function of time. Because the procedure involved automated liquid transfers and incubation, the precision of the results obtained reflects not only the analytical merits, but also the reproducibility of the liquid handling workstation used for this purpose. Further, as part of on-going quality control, the percent of testosterone remaining at 60 min was tabulated for each batch of incubation. From the precision results as measured by the relative standard deviation (R.S.D.) obtained for two incubation concentrations at the selected sampling times and across multiple analytical batches/days, two observations could be made (Table 3). First, excellent precision (R.S.D. < 9%) was obtained by simply using relative peak area comparisons, obviating the need for internal standard addition (Table 3A). Second, the interbatch testosterone incubation data (Table 3B) showed that after 24 (5 μM) and

Table 3
Percent remaining of testosterone (0.5 and 5 μM) showing (A) intrabatch precision as measured by relative standard deviation after 0, 15, 30, and 60 min, and (B) interbatch precision tabulated for the 60 min value

Time (min)	Mean peak area	Remaining (%)	R.S.D.
(A)			
Incubation concentration (5 μM)			
0	2.02E + 07	100	2.2
15	1.77E + 07	88	2.4
30	1.52E + 07	75	3.4
60	1.35E + 07	67	8.2
Incubation concentration (0.5 μM)			
0	2.41E + 06	100	3.2
15	2.14E + 06	89	1.0
30	1.92E + 06	80	5.3
60	1.74E + 06	72	6.2

Interbatch statistics (60 min value)	Testosterone incubation concentration	
	5 μM	0.5 μM
(B)		
Number of batches	24	41
Mean % remain (60 min)	67	69
S.D.	8.5	8.8
R.S.D.	13	13

41 (0.5 μM) consecutive batches of incubations, the mean percent remaining at 60 min was 67% (R.S.D. = 13%) and 69% (R.S.D. = 13%), respectively. The results suggest that overall reproducibility in the metabolic stability protocol was acceptable for batch-to-batch comparison of compound stability. If further refinement in reproducibility were needed, then one could envision using percent remaining values normalized to testosterone values for cross-comparison.

The parallel TFC–LC-MS–MS configuration operating under a simple gradient provided the throughput necessary for rapid metabolic stability screening by facilitating the analysis of almost 400 samples (4 × 96-well microtiter plates) in a single, overnight batch. In addition, initial metabolite profiling of relatively unstable compounds could be undertaken the following day. Identification of metabolically labile sites on these compounds is an aid to the medicinal chemist in further structural optimization. This approach is similar to one recently introduced by Lim et al. [25] in which possible metabolites are profiled by data-dependent full-scan product ion experiments in conjunction with simultaneous monitoring of parent drug metabolic stability using an ion-trap mass spectrometer. The authors showed that direct analysis by TFC coupled with fast-gradient laminar-flow chromatography was sufficient for the resolution of a drug and its regioisomeric metabolites.

3.2. Plasma-only analysis (serial TFC–LC-MS–MS)

It is often believed that a technique for quantitative analysis of plasma samples is not considered fully mature unless it has been validated according to the guidelines set forth in the bioanalytical method validation guidance for industry [30] and applied extensively for analysis of study samples. Hence, an example is provided in which the various aspects of selectivity, linearity, accuracy, precision, and recovery are evaluated for a method involving the measurement of two dihydropyridines (nimodipine and MEM 1003, a Memory Pharmaceuticals development candidate) in mouse plasma. A method based on the serial TFC–LC-MS–MS configuration was utilized in the analysis of plasma samples because this configuration minimized carryover effects. Although similar methods for rat and dog plasma were also developed, mouse plasma analysis highlights an advantage of using on-line HTLC extraction. The mouse yields the least total volume of plasma per unit time among the three animal species; therefore, a limited sample volume is available for analysis. Only 50 μL of mouse plasma was needed in this analysis because pre-concentration was carried out on-line, and no reconstitution in a separate volume of solvent was necessary. Although the stability of both nimodipine and MEM 1003 was evaluated in different plasma matrices and under varying conditions, for brevity, it will not be reported here.

3.2.1. Selectivity

Analytes and internal standard (nitrendipine, a structural analog) were detected by tandem mass spectrometry using selected reaction monitoring under negative ionization conditions. The full-scan negative ion mass spectrum and product ion (of the $[M-H]^-$ ion) mass spectrum of nimodipine are shown in Fig. 3. Similar fragmentations were observed for MEM 1003 and nitrendipine. The selected transitions for nimodipine, MEM 1003, and nitrendipine were m/z 417 → 122, m/z 431 → 136, and m/z 359 → 122, respectively. To evaluate the selectivity of the overall method, six different sources of control or blank plasma samples were evaluated for interference at the retention times of interest. The chromatogram of each blank plasma sample was scrutinized for potential peaks that could interfere with quantitation, but none were found for any of the compounds. In addition, the risk of "crosstalk" or the ability of one SRM channel causing a false positive peak in another was examined by injecting a 100 ng/mL solution of each compound separately. The absence of crosstalk was verified by the absence of a quantifiable chromatographic peak in the SRM channels other than the compound that was injected.

The carryover observed for nimodipine in the serial TFC–LC-MS–MS configuration was 0.045%, as measured by injection of a blank sample after the highest standard. Less carryover (0.025%) was observed for MEM 1003. These values correspond to approximately 45% and 25% of the response of the lowest standard or lower limit of quantitation (LLOQ) for nimodipine and MEM 1003, respectively. A common practice for bioanalytical laboratories is to have a pre-defined limit for carryover as a percentage (i.e., 20–30%) of the response of the LLOQ from which to designate a method as carryover-free. However, declaration of analysis which meets the criterion above as carryover-free would be risky even if freedom from carryover were defined as 20% the response of the LLOQ since the concentrations of toxicolog-

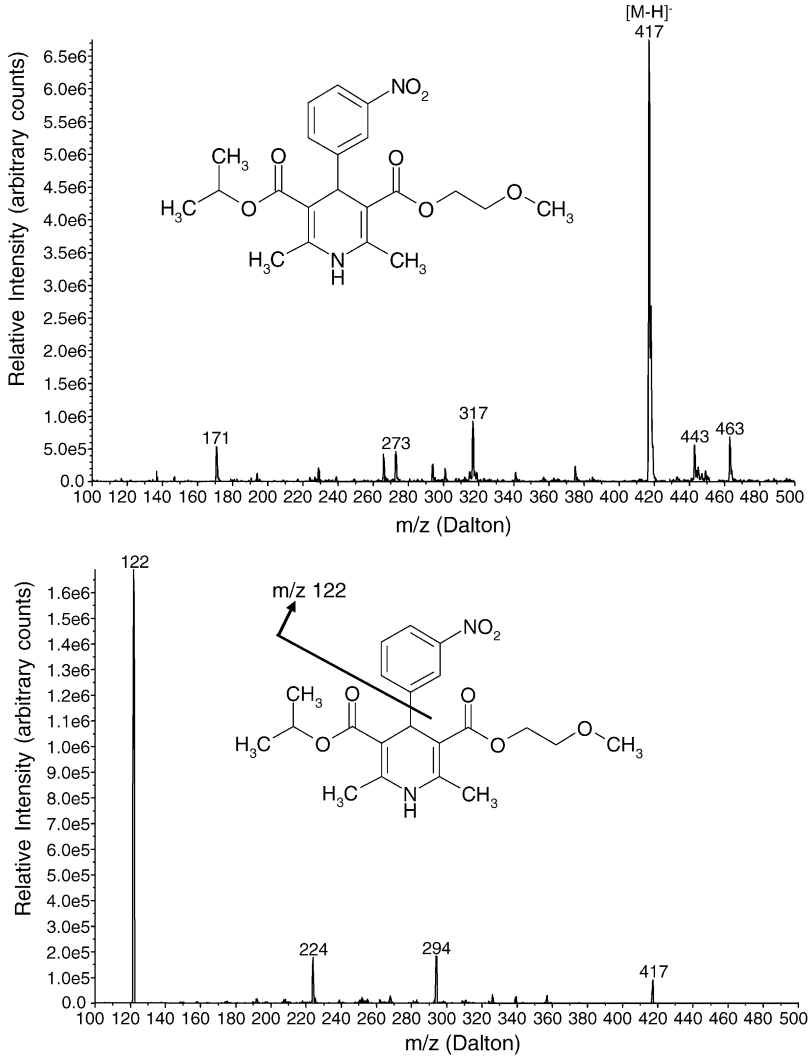

Fig. 3. Mass spectrum of nimodipine (top) and product ion mass spectrum of corresponding $[M-H]^-$ (*m/z* 417 Da) ion.

ical study samples may span a large range and a sample with more than twice the concentration of the highest standard would contribute significantly to the next sample at or close to the LLOQ. Therefore, we believed it was more prudent to quantify the percentage of carryover, and use this value to determine if a high concentration (i.e., above the highest calibration standard) determined for one sample will affect quantitation of the subsequent sample.

3.2.2. Linearity

Control (blank) plasma was spiked with aqueous solutions of analytes to produce calibration standards with known concentrations of analytes in the nominal range of 0.5–500 ng/mL. The SRM chromatograms of a blank, the lowest (0.48 ng/mL or 6 pg on-column) and highest standard (480 ng/mL or 6 ng on-column) for nimodipine and MEM 1003 are given in Figs. 4 and 5, respectively. It is important to note that although the signal-to-noise ratio at the LLOQ suggested that a lower LLOQ would be attainable, we chose a nominal range of 0.5–500 ng/mL to allow sufficient dynamic range for determination of the high concentrations anticipated from dose-range finding toxicological studies. Calibration curves were calculated from the peak area ratio of analyte/internal standard, using least squares linear regression of the area ratio versus the theoretical concentration of the standards and were found to be linear with correlation coefficient values better than 0.997.

3.2.3. Accuracy and precision

Intrabatch (also known as within batch) and interbatch accuracy and precision were assessed by analyzing six replicates of each level of quality control (QC) samples in each of three separate batches. The QC levels are at the lower limit of quantitation (LLOQ QC, 0.48 ng/mL), low (QCL, 1.4 ng/mL), middle (QCM, 14 ng/mL), and high (QCH, 398 ng/mL) concentrations of the corresponding calibration curve. The overall accuracy and precision of the QC samples were evaluated for the different batches and the results

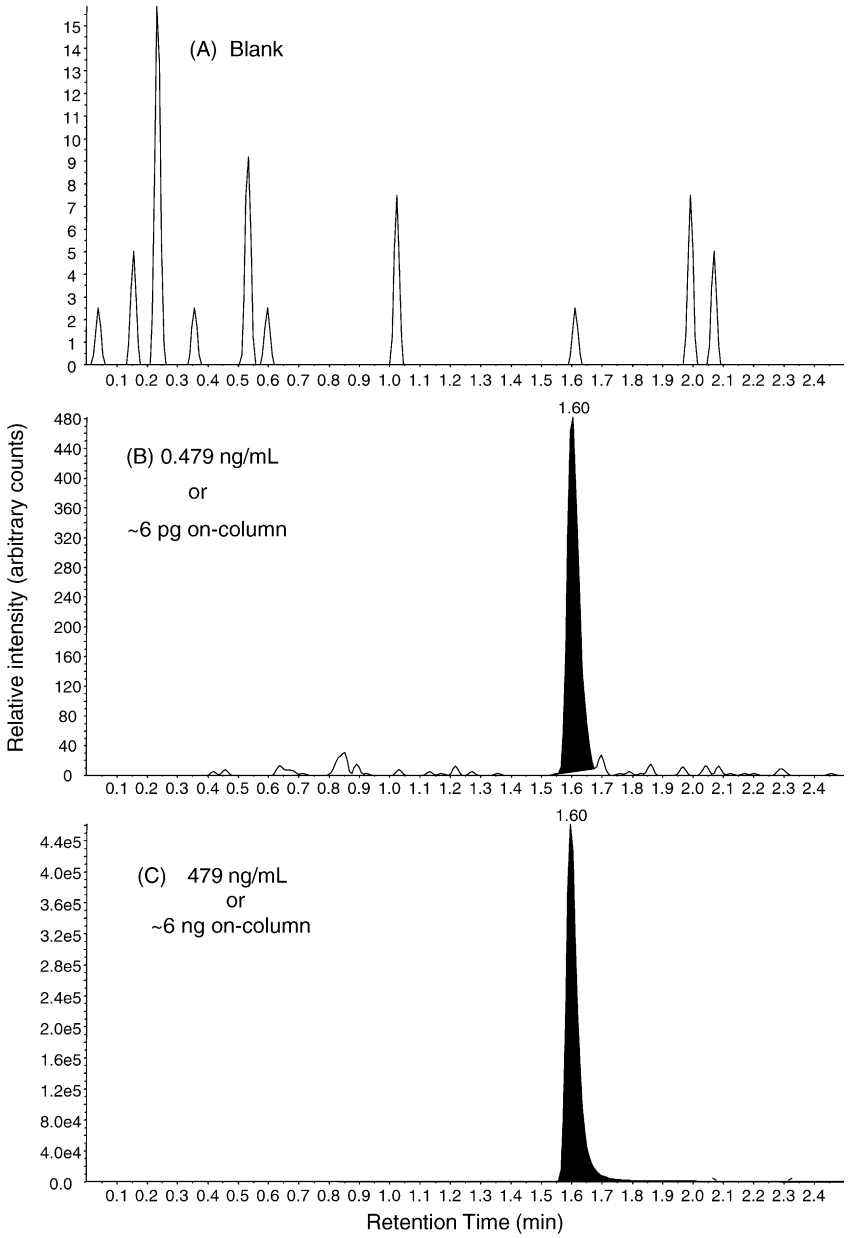

Fig. 4. Selected reaction chromatogram (417/122) of (A) blank, (B) lowest, and (C) highest calibration standards of nimodipine in water-diluted plasma.

are tabulated in Tables 4 and 5 for nimodipine and MEM 1003, respectively. For nimodipine, the intrabatch accuracy and precision values calculated on each of the three separate batch runs for QC samples easily met the validation acceptance criteria of $100 \pm 15\%$ (and $\pm 20\%$ for the LLOQ QC) for accuracy and <15% (and <20% for the LLOQ QC) R.S.D. Mean interbatch (from three separate batches) accuracy values ranged from 95.2% to 102%, while the R.S.D. values ranged from 3.55% to 9.37%. For MEM 1003, similar results were observed for intrabatch accuracy and precision values. Mean interbatch accuracy values ranged from 97.6% to 106%, while the R.S.D. values ranged from 3.81% to 9.44%. The interbatch statistics were well within acceptable limits of accuracy and precision. These results suggest that the method is reliable for the measurement of nimodipine and MEM 1003 in mouse plasma. Further evidence of method robustness is presented in study sample analysis.

3.2.4. Recovery

To determine the extraction efficiencies or recoveries for the analytes and internal standard, the peak area ratios measured for plasma QC samples using TFC extraction were compared to the peak area ratios measured by direct injection (bypassing TFC extraction) of aqueous QC samples. For the direct injection experiment, no valve-switching was needed. The elution pump was used to apply the gradient with a minor hold time adjustment added at the beginning

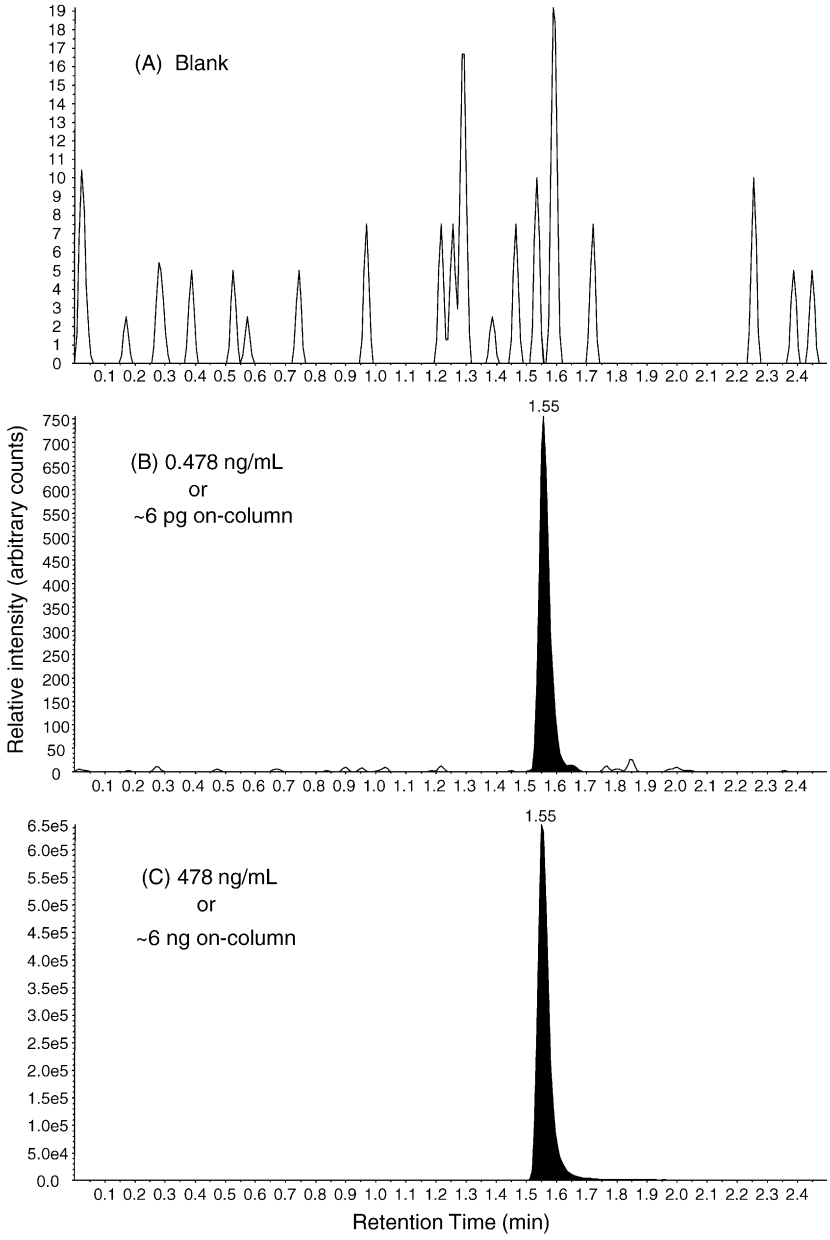

Fig. 5. Selected reaction chromatogram (431/136) of (A) blank, (B) lowest, and (C) highest calibration standards of MEM 1003 in water-diluted plasma.

to account for the absence of extraction. Peak area ratios of analyte/internal standard were used in the recovery calculations so that the recovery of the internal standard could be evaluated concurrently as this method quantifies the analytes using nitrendipine as the internal standard. For nimodipine, the overall mean recovery calculated from the recoveries of QCL, QCM, and QCH samples was 84.4% with an R.S.D. of 8.19% about the mean across the three QC concentration levels. For MEM 1003, the overall mean recovery was 89.5% with a R.S.D. of 6.14% about the mean across the three QC concentration levels. In general, the results suggested that consistent recoveries were obtained across the different concentrations.

In addition to the determination of absolute recovery, the effect of the plasma matrix on the response for each analyte was evaluated for suppression or enhancement effects. Matrix effects were examined by comparing peak area ratios for plasma versus aqueous QC samples using TFC extraction. Any significant deviation from 100% recovery would suggest that the plasma matrix was contributing to the suppression (<100%) or enhancement (>100%) of analyte response. For nimodipine, the overall mean recovery calculated was 79.9% with an R.S.D. of 2.35% about the mean across the three QC concentration levels suggesting that the lower overall recovery obtained for the compound was most likely due to suppression effects from the matrix. For MEM 1003, the overall

Table 4
Intrabatch and interbatch statistics from three separate batches of the measurement of nimodipine in water-diluted mouse plasma

	Calculated nimodipine concentration (ng/mL)			
	LLOQ QC 0.479	QCL 1.44	QCM 14.4	QCH 399
Batch 1				
Replicate 1	0.496	1.45	13.7	370
Replicate 2	0.465	1.60	14.0	404
Replicate 3	0.474	1.28	13.7	403
Replicate 4	0.459	1.46	14.2	368
Replicate 5	0.546	1.46	13.2	406
Replicate 6	0.503	1.47	14.2	396
Mean	0.491	1.45	13.8	391
S.D.	0.0322	0.102	0.383	17.5
R.S.D. (%)	6.56	7.01	2.77	4.48
Mean accuracy	102	101	96.1	98.0
Batch 2				
Replicate 1	0.506	1.52	13.4	362
Replicate 2	0.519	1.27	13.3	381
Replicate 3	0.533	1.33	13.5	374
Replicate 4	0.514	1.39	13.3	378
Replicate 5	0.471	1.40	13.6	375
Replicate 6	0.556	1.55	14.0	379
Mean	0.517	1.41	13.5	375
S.D.	0.0284	0.108	0.264	6.79
R.S.D. (%)	5.49	7.65	1.95	1.81
Mean accuracy	108	97.9	93.9	93.9
Batch 3				
Replicate 1	0.457	1.40	13.4	390
Replicate 2	0.579	1.31	13.2	353
Replicate 3	0.439	1.76	13.7	354
Replicate 4	0.448	1.49	13.1	419
Replicate 5	0.442	1.61	14.4	418
Replicate 6	0.408	1.48	14.9	401
Mean	0.462	1.51	13.8	389
S.D.	0.0596	0.159	0.719	29.7
RSD (%)	12.9	10.5	5.22	7.63
Mean accuracy	96.5	105	95.7	97.5
Interbatch statistics				
Number of values	18	18	18	18
Mean	0.490	1.46	13.7	385
S.D.	0.0459	0.125	0.486	20.5
R.S.D. (%)	9.37	8.57	3.55	5.32
Mean accuracy	102	101	95.2	96.5

Table 5
Intrabatch and interbatch statistics from three separate batches of the measurement of MEM 1003 in water-diluted mouse plasma

	Calculated MS11003 concentration (ng/mL)			
	LLOQQC 0.478	QCL 1.43	QCM 14.3	QCH 398
Batch 1				
Replicate 1	0.522	1.42	14.4	388
Replicate 2	0.485	1.64	14.3	433
Replicate 3	0.444	1.51	13.8	427
Replicate 4	0.501	1.50	15.0	400
Replicate 5	0.515	1.53	13.9	424
Replicate 6	0.487	1.42	14.4	422
Mean	0.492	1.50	14.3	416
S.D.	0.0279	0.0816	0.429	17.6
R.S.D. (%)	5.66	5.43	3.00	4.23
Mean accuracy	103	105	100	104
Batch 2				
Replicate 1	0.443	1.52	13.4	383
Replicate 2	0.453	1.36	13.8	392
Replicate 3	0.377	1.47	13.4	391
Replicate 4	0.490	1.39	13.8	378
Replicate 5	0.463	1.51	13.8	386
Replicate 6	0.550	1.42	14.3	396
Mean	0.463	1.45	13.8	388
S.D.	0.0569	0.0653	0.333	6.59
R.S.D. (%)	12.3	4.52	2.42	1.70
Mean accuracy	96.8	101	96.2	97.4
Batch 3				
Replicate 1	0.590	1.56	13.5	408
Replicate 2	0.524	1.38	14.2	379
Replicate 3	0.489	1.83	13.9	335
Replicate 4	0.517	1.74	12.6	411
Replicate 5	0.494	1.50	14.2	394
Replicate 6	0.503	1.55	14.4	414
Mean	0.520	1.59	13.8	390
S.D.	0.0370	0.164	0.666	30.0
R.S.D. (%)	7.12	10.3	4.83	7.69
Mean accuracy	109	111	96.5	98.0
Interbatch statistics				
Number of values	18	18	18	18
Mean	0.492	1.51	14.0	398
S.D.	0.0464	0.123	0.532	23.2
R.S.D. (%)	9.44	8.11	3.81	5.83
Mean accuracy	103	106	97.6	100

mean recovery was 94.9% with an R.S.D. of 5.46% about the mean across the three QC concentration levels indicating that there was negligible matrix effect on the quantitation of the compound.

3.2.5. Study sample analysis

Similar methods were developed for the determination of nimodipine and MEM 1003 in rat and dog plasma. These methods were successfully applied in the analysis of over 2500 samples from separate mouse, rat, and dog preclinical studies. Overall, five TFC columns were used during the course of sample analysis, which translated to approximately 500 water-diluted plasma study samples (excluding calibration standards and QC samples) analyzed on a per TFC column basis. The percent accuracy of 242 QC sets or 726 individual QC samples accumulated from the different studies supported were tabulated and graphically represented in Fig. 6, in which a QC set represents three QC concentration levels (low, middle, and high concentrations). The results indicated that the method based on TFC was sufficiently robust to support the quantitative analysis of diluted plasma samples.

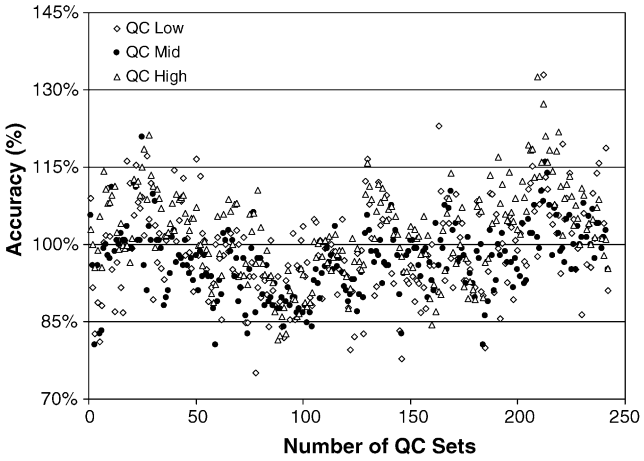

Fig. 6. Percent accuracy of QC samples tabulated from the execution of methods developed for the measurement of nimodipine and MEM 1003 in rat, dog, and mouse plasma (in support of various preclinical studies).

3.2.6. Comparison with protein-precipitated plasma concentrations

It has been suggested that plasma concentrations determined using TFC represents free drug (not bound to plasma proteins) concentrations [10]. This situation may hold true if physiological conditions, such as pH and ionic strength, were maintained throughout the course of analysis. However, the conditions employed during sample loading include exposing samples to 0.1% formic acid at a flow rate of 5 mL/min for 30 s, which we believe would disrupt most non-covalent protein–drug interactions. Nevertheless, an experiment was conducted to determine if there was any difference in concentrations measured by injection of water-diluted plasma (no precipitation) relative to concentrations measured after acetonitrile precipitation of plasma proteins. Fig. 7 shows a plot of MEM 1003 concentrations for study samples determined using non-precipitated plasma versus precipitated plasma subjected to the same analytical procedure following precipitation. A $y = mx$ line, where the slope, m, is equal to 1, is also plotted on the same graph as a visual aid. There was essentially no difference between values measured with and without acetonitrile protein precipitation. This result suggested that loading with water containing 0.1% formic acid at 5 mL/min was sufficient to disrupt non-covalent interactions between the MEM 1003 and plasma proteins.

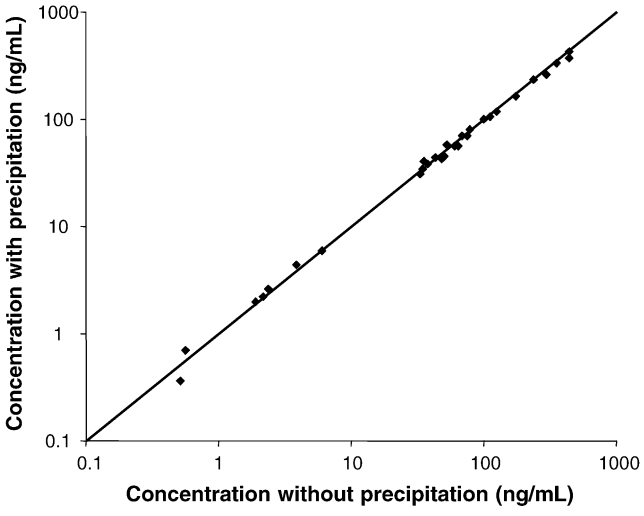

Fig. 7. Plot of sample concentrations measured by acetonitrile-precipitated plasma versus water-diluted plasma (line is shown for $y = mx$, where the slope, m, is equal to 1 is plotted for visual aid).

3.3. Plasma/tissue analysis in drug discovery (serial or parallel TFC–LC-MS–MS)

In general, during the lead optimization stage of drug discovery, a bioanalytical laboratory is challenged with the measurement of a myriad of compounds ranging from low to high lipophilicities in complex biological matrices. Therefore, judicious choice has to be made regarding the extent of method development and optimization for a given compound. A generic liquid chromatrographic gradient method that encompasses most or all analytes is preferred and tandem mass spectrometric detection in the SRM mode is relied upon for selectivity.

In our laboratory, measurement of analyte concentrations in plasma and brain were of paramount importance to support in vivo pharmacology experiments. While it is possible to optimize a method for each analyte to be measured in plasma and/or brain homogenate samples using calibration standards prepared in the corresponding matrix, it is often not pragmatic to do so. One reason is because the number of samples does not justify extensive optimization. Instead, it is essential to have a reliable method in terms of accuracy and precision in the analysis. In addition, although it is prudent to utilize the same matrix for preparation of calibration standards, our goal was to minimize the number of animals that needed to be sacrificed to obtain sufficient blank brain tissue. Therefore, we wanted to apply the same sample preparation and analysis procedure for both plasma and brain homogenate to allow for quantitation of samples from both matrices in a single analytical batch.

Heinig and Bucheli [19] recently reported that by careful method optimization and judicious selection of internal standard, human plasma can be used as calibration standards for quantitation of several drugs in rat plasma and various rat tissue samples. In our laboratory, since most of the initial pharmacological studies were tested in rodent models, rat plasma was readily available and was used to prepare calibration standards for quantitation of plasma and brain samples. For analyses involving plasma/brain samples, a single unified methodology was applied; that is, plasma and brain tissue homogenates were precipitated with acetonitrile as sample pretreatment prior to analysis. As described in the experimental section, brain tissue had to be homogenized by the addition of three-fold amount of water to achieve a consistency comparable to plasma and to provide a suitable suspension of homogenized tissue for subsequent solubiliza-

tion with acetonitrile. Although a two-fold amount of water would be preferable in order not to dilute the original sample concentration in the crude brain tissue, it has been our experience that the viscosity of the resulting homogenate made it difficult to aliquot.

Samples were analyzed by serial or parallel TFC–LC-MS–MS depending on the extent of carryover, which was compound-dependent. To assess the accuracy and precision of each analysis, quality control samples prepared both in plasma and brain homogenate were included in each analytical batch containing plasma calibration standards. Basically, this allowed direct comparison of plasma and brain homogenate QC samples calibrated against plasma standards. Each batch typically comprised low (approximately three to five times the lowest standard) and high (approximately 75–90% of the highest standard) QC samples prepared in brain homogenate, whereas an additional middle QC level (approximately midway in the calibration range) was included for QC samples prepared in plasma. The reason for this difference was primarily due to the availability of plasma relative to brain tissue.

Data was tabulated for 60 compounds from batches in which both plasma and brain samples were analyzed. The results are presented in graphical format in Fig. 8, which plots the number of compounds analyzed and the corresponding mean accuracy (in percentage relative to theoretical concentration) of brain QC samples. The mean accuracy reported in Fig. 8 represents the average of all QC samples and was normalized to the mean percent accuracy from the plasma QC samples of the same batch. The graph showed that approximately one-third of the compounds did not fall within an arbitrarily set acceptance criterion of 75–125% mean accuracy (for discovery support). For these compounds, the brain samples were re-analyzed using calibration standards prepared in brain homogenate. In general, data obtained from the original analysis (using plasma standards) compared favorably with data from the re-analysis (using brain standards) after adjusting for the difference using the percent accuracy normalized to plasma QCs. While not every compound was amenable to this combined approach for plasma/tissue analysis, the overall data suggested that TFC–LC-MS–MS was a flexible analytical platform capable of supporting quantitative bioanalysis of biological matrices.

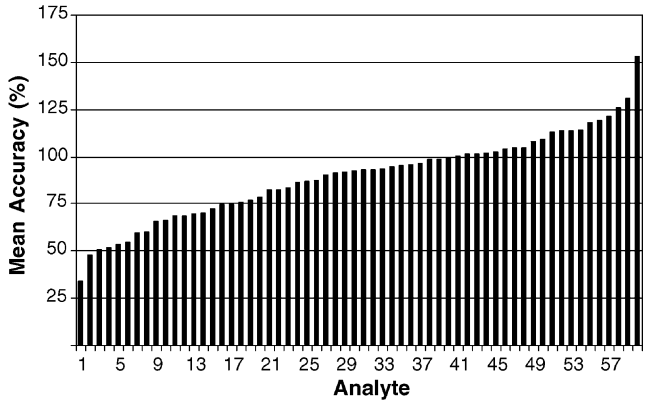

Fig. 8. Mean percent accuracy of brain QC samples measured against plasma calibration standards for 60 different compounds.

4. Conclusion

Our goal was to develop an integrated approach to quantitative bioanalytical support with minimal sample preparation prior to analysis. Through the use of automated liquid handling workstations, most tedious sample transfer steps can be minimized. The Tecan Genesis robotic workstation is capable of generating four 96-well microtiter plates in 2 h from in vitro liver microsomal incubation assays and alleviating most repetitive manual sample preparation steps. For analysis, TFC using the Cohesive 2300 HTLC coupled to the AB-Sciex API 3000 triple quadrupole mass spectrometer was the enabling technology in achieving this goal. In the parallel HTLC column mode, the system was capable of analyzing the almost 400 samples in a single overnight batch or approximately 14 h with minimal ion source contamination.

Beginning with a generic template for plasma and/or tissue analysis, simplified plasma and brain tissue analytical methods (as separate or combined methods) were developed for over 99% of the compounds that we have encountered during lead optimization in drug discovery in the past 3 years. By providing continued bioanalytical support through drug development, knowledge on the analytical nuances that tend to differ from compound-to-compound was preserved. This strategy, in turn, facilitated more rapid method development and validation as a compound progressed into becoming a viable lead candidate for preclinical safety studies.

In addition to the activities discussed above, the system has also been used to support analysis of samples generated from protein binding experiments [31], permeability (Caco-2) screening, and aqueous solubility measurements. In the latter assay, the TFC–LC-MS–MS system, also equipped with a parallel UV/vis diode-array detector, is operated in a third configuration. The configuration was a straightforward (no turbulent flow) liquid chromatography with ultraviolet and mass spectrometric detection system requiring only a single binary pump for generating a gradient for separation. Switching between the different configurations to accommodate the varying functions require only minutes with the high flow rates facilitating rapid re-equilibration. These functions serve to further support the truly multi-tasking options accessible with this single instrumental platform.

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