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The investigation and the use of high flow column-switching LC/MS/MS as a high-throughput approach for direct plasma sample analysis of single and multiple components in pharmacokinetic studies

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Abstract

Recently direct plasma injection LC/MS/MS technique has been increasingly used in pharmaceutical research and development due to the demand for higher throughput of sample analyses. In this work, two on-line extraction methods including high flow LC/MS/MS and high flow column switching LC/MS/MS were investigated. The evaluations were conducted and focused on their performances with respect to peak responses, separation efficiency, and signal to-noise ratio in a multiple-component LC/MS/MS assay. Two HPLC pumps were used-with one for high flow delivery and one for gradient elution. A CTC autosampler was used to inject plasma samples. High flow LC was achieved by the use of 4 ml/min flow rate on a 1×50 mm Waters Oasis column. A 2×100 mm YMC column was coupled via a column-switching valve. The extracted analytes were analyzed in multiple-reaction-monitoring (MRM) mode using a triple quadrupole MS/MS. As a rapid and simple procedure, vortex-mixing plasma and internal standard directly in sample vials completed sample preparation. The high flow column switching method (two-column system) provided sharper peak shape than the conventional high flow method. This effect increased analyte signal-to-noise ratio and sensitivity. Narrower peak width resulted in much better separation efficiency, which was required for multiple compound (*N*-in-1) analysis. A 2 mm I.D. column resulted in better peak shape and resolution than using a smaller I.D. column. The selected method achieved acceptable recoveries for most of the compounds tested, and it was successfully applied to a 10-in-1 pharmacokinetic (PK) study. The results showed that the dynamic range, lower limit of quantitation, assay accuracy and precision were acceptable for all compounds. Rapid sample preparation eliminated labor intensive and time consuming processes and improved productivity. This high throughput on-line extraction high flow column switching method has been proven particularly useful for multiple component analysis in PK studies.

Keywords: Bioanalysis; High flow column switching; Direct plasma injection; Resolution; Extraction efficiency; Pharmacokinetics

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1. Introduction

The increasingly competitive pharmaceutical marketplace has posed challenges to the speed of drug discovery processes, and in turn, to the throughput and productivity of biological sample analysis. The hyphenated technique between high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) has made it possible to not only dramatically shorten method development time but also reduce the chromatographic run time due to its high sensitivity and specificity. However, considering the high cost and limited time of MS/MS detection, it is truly necessary to develop simple, fast and efficient analytical procedures that are suitable for high throughput LC/MS/MS. To meet the great demand on analytical throughput, pharmaceutical research groups have been the pioneers of developing new techniques such as 96 well format [1–4], on-line extraction [5,6], and parallel separation [7–9].

Pharmacokinetic (PK) screening plays an important role in pharmaceutical discovery research and development. Using cassette dosing (N-in-1) of numerous compounds followed by simultaneous multiple component analysis with LC/MS/MS has significantly accelerated the throughput of the PK screening process. Although there are several controversial issues regarding result accuracy of the simultaneous dosing approach in animals, this approach has proven to be one of the most effective ways to rank order a large number of compounds in terms of their PK behaviors [10–14]. For most pharmaceutical research, N-in-1 studies with small pool size have been widely accepted and applied by scientists in drug discovery. Compared to sequential single compound study format, the N-in-1 cassette dosing PK screening has evidently reduced the time on selecting new PK leads by N-fold.

Conventional plasma extraction methods such as solid phase extraction (SPE), liquid–liquid extraction (LLE), and protein precipitation (PP) are time-consuming and labor-intensive. These tedious processes have become bottlenecks and have often limited the throughput of current fast LC/MS/MS analysis. Recently, progress has been made in the development of automated extraction procedures by using different robots and 96-well format to speed up the throughput of the process [15,16]. In the meantime, on-line sample extraction using column switching has become an increasingly appealing approach for high throughput bioanalysis. This technique includes using either a disposable cartridge or a reusable extraction column. The analytes are extracted from a cartridge and subsequently eluted onto an analytical column for separation [17,18]. Some applications simply combined extraction and separation together on a single column and split LC eluent into a micro-flow before MS/MS detection [19,20].

Recently, the use of high flow chromatography (some literature used the term 'turbulent flow') coupled with tandem mass spectrometry (HFLC/MS/MS) has shown great potential for the direct assay of pharmaceutical compounds in plasma and other biological fluids [21–26]. The extraction is performed on a column filled with large packing materials (30–60 μm in diameter). Consequently, large molecules from the plasma sample can readily pass through the column to waste while the analytes of interest can be retained under aqueous conditions. The analytes can then be eluted using a high organic solvent for MS/MS analysis. Although this on-line technique possesses a fast and rugged extraction within a short run time, HFLC/MS/MS often results in peak broadening, poor signal-to-noise (S/N) ratio and low separation efficiency, which are not suitable for analyzing samples in multiple component PK studies due to potential interference. In N-in-1 PK screenings where a large number of compounds ($N \geq 10$) are dosed simultaneously, precursor ions and product ions of similar m/z often interfere with each other. It is critically important to maintain enough resolving power in chromatography in order to eliminate potential interference and signal suppression in MS detection. In addition, the reduced compound dose levels and decreased instrument duty cycle also demand higher analyte sensitivity. The ultimate solution is the com-

bination of HFLC with further analytical separation.

Taking advantage of direct plasma injection and the high throughput afforded HFLC while maintaining separation efficiency, a high flow column switching LC/MS/MS method has been developed and has been widely used for PK and metabolic screening in the authors' laboratories [27]. A similar analytical method was also reported by other groups [28,29], which involved a forward-column-elution instead of a backward-column-elution step on the extraction column. The use of column switching allows performing the entire extraction including load, wash, flush and re-equilibration in a short time period while the separation is in progress. Therefore, by conducting extraction and separation in parallel, many labor intensive and time consuming off-line processes can be eliminated.

In this report, two on-line extraction methods including high flow and high flow column-switching liquid chromatography-tandem mass spectrometry (HFLC/MS/MS and HFCS LC/MS/MS) have been investigated. The evaluations were conducted and focused on their performances with respect to analyte peak responses, separation efficiency, and signal to-noise ratio in a multiple component LC/MS/MS assay format. Several generic drugs with diverse structures were utilized in the evaluation. A few polar compounds were specifically chosen to challenge the efficiency of these on-line extraction processes. Finally, the effectiveness of the HFCS LC/MS/MS method was demonstrated in a 10-in-1 PK study in dog.

In the past 2 years, the HFCS LC/MS/MS method was further developed and refined to be practical for the analysis of different kinds of biological matrices, which included plasma, serum, whole blood, liver microsome, urine, bile, and synovial fluid. In fact, many of the studies were implemented in *N*-in-1 format that contained multiple components in every biological sample. Using plasma analysis as an example, the purpose of this paper was to compare and rationalize different high throughput on-line LC/MS/MS methodologies for direct sample injection.

2. Experimental

2.1. Reagents and chemicals

The test compounds, aminopterin, apomorphine, benzoylcegonine, carbamazepine and temazepam (structures shown in Fig. 1), as well as the mobile phase additive, formic acid (FA), were purchased from Sigma (St. Louis, MO). All HPLC grade solvents were from EM Science (Gibbstown, NJ). Compounds used in the *N*-in-1 PK analysis were synthesized at DuPont Pharmaceuticals Company.

2.2. Equipment

The mass spectrometer used was a Micromass Quattro Ultima triple-quadrupole mass spectrometer (Manchester, UK) equipped with an electrospray ionization (ESI) source (Z-spray™). Two binary high-pressure mixing HPLC pumps were used for the HFCS LC system. A HP1100 pump (Hewlett-Packard, Waldbronn, Germany) was used to deliver a high flow through the extraction column to load and wash the sample and subsequently to flush and equilibrate the extraction column. A Shimadzu LC-10AD-vp pump (Shimadzu, Tokyo, Japan) was used to deliver a gradient flow to elute the analytes from the extraction column and to perform the separation on the analytical column. A CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC) was used to inject plasma samples. The 54-vial sample trays and 96 well plates used can be refrigerated in a sample cooling stack at temperatures of 4–10 °C. This autosampler also allowed for the use of two separate wash solvents for both the syringe and the injector. An aqueous flushing solvent was used before a high organic solvent to avoid possible plugging from PP by high organic solvent. The Waters YMC AQ narrowbore and microbore columns (5 μm, 2×100 and 1×100 mm) were used for separation. A Waters Oasis HLB extraction column (30 μm, 1×50 mm) was used for extraction.

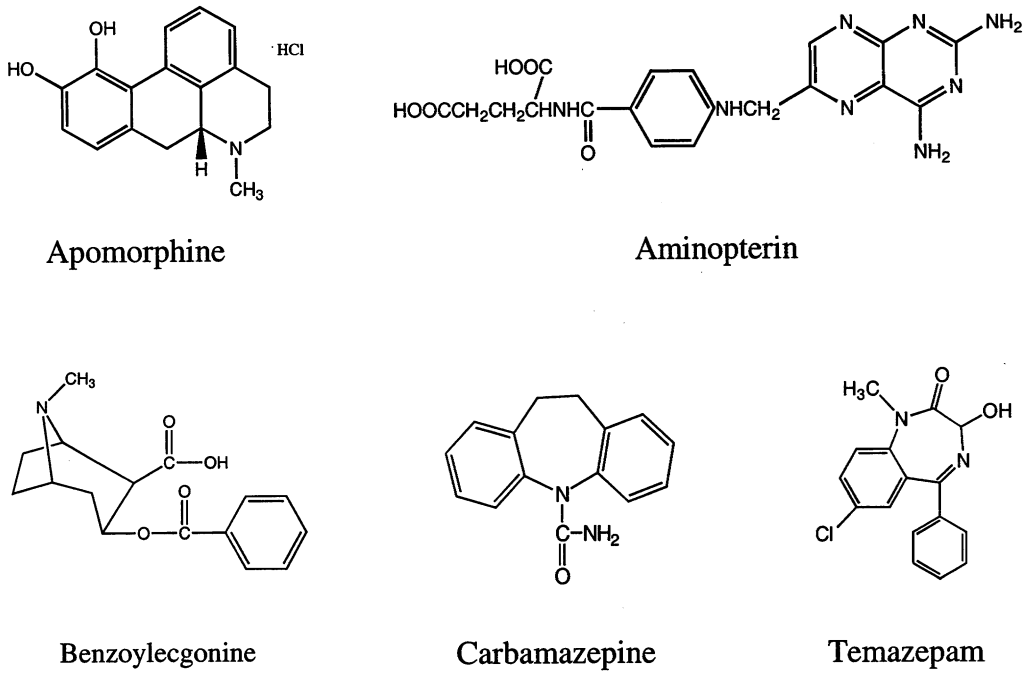

Fig. 1. Chemical structures of the five test compounds used for evaluation of high flow column switching assay.

2.3. Standards and quality control (QC) samples

All primary standard stock solutions of test compounds and cassette dosing compounds (1 mg/ml) were prepared in acetonitrile (ACN). The internal standard (IS) and standard working solutions used in the PK assay were made in acetonitrilel/water (5:95, v:v) solution.

2.4. Plasma sample preparation procedures

The sample preparation procedures involved mixing plasma sample with IS and/or standard working solutions in 12 mm polypropylene plastic HPLC vials for the on-line extraction. The detailed procedures were summarized stepwise in Table 1.

2.5. Chromatographic conditions

The HFCS LC/MS/SM system, as depicted in Fig. 2, was used for the direct injection of plasma sample mixtures. After the plasma sample (60 μl) was injected by the CTC autosampler, the sample was loaded onto the extraction column by solvent A, HPLC grade water, at a fast flow rate gradient (0.5–4 ml/min) that was delivered by the HP1100 pump in the first 1.4 min. While the extraction column was still directed to waste during this time, the plasma sample was extracted as an on-line SPE. The proteins and other hydrophilic

Table 1
Preparation of plasma samples for HFCS on-line extraction

Preparation of Standards and QCs Combined the following in a plastic HPLC vial	Preparation of Plasma Samples Combined the following in a plastic HPLC vial
200 μl blank plasma (or matrix)	200 μl plasma sample (or sample matrix)
20 μl standard working solution	20 μl deionized water
100 μl internal standard solution[a]	100 μl internal standard solution[a]
Sealed with a Teflon-line cap Vortexed	Sealed with a Teflon-line cap Vortexed

[a] The internal standard was only used in quantitation of PK samples.

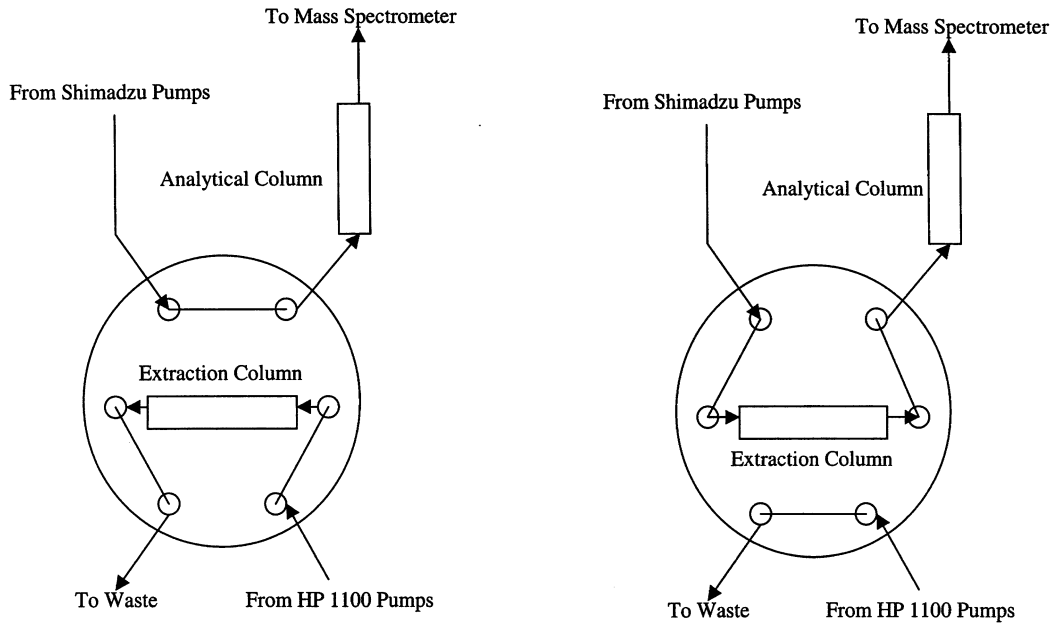

Fig. 2. Schematic diagrams of column switching valve configurations in the high flow column switching LC/MS/MS.

matrix components in the plasma sample were removed while the analytes were retained on the extraction column. This extraction process was somewhat similar to conventional off-line SPE. However, the difference was that the loading and washing steps were continuous and were completed within 1.4 min. At the end of 1.4 min the switching valve was switched to the other position (configuration 0 in Fig. 2). The configuration 0 allowed the extraction column to be connected counter-flow to the analytical column in the flow path of the Shimadzu pump. After 1.4 min the Shimadzu pump started a gradient using solvents C (water with 0.1% formic acid) and D (acetonitrile with 0.1% formic acid) to elute the retained analytes from the extraction column to the analytical column using a flow rate of 0.4 ml/min. The effluent from the analytical column was directed to the mass spectrometer electrospray source without splitting. An elution step of 2 min or more was given before the switching valve was switched back to the original position (configuration 1 in Fig. 2). The HP1100 pump then started to deliver solvent B, methanol with 0.1% formic acid, at a flow rate of 4 ml/min for 2 min to flush the extraction column. During the last 2 min of the run cycle, the HP1100 pump delivered solvent A at 4 ml/min to recondition the Oasis extraction column for the next sample. The run time for the assay of the analyte mixture consisting of five test compounds and the assay of the 10-in-1 PK study were 8 and 12 min, respectively. The goal here was to achieve good separation efficiency and not to push for a short run time. The chromatographic conditions were summarized in Table 2.

2.6. Mass spectrometric conditions

The Micromass Quattro Ultima triple quadrupole mass spectrometer was operated in positive ion electrospray mode for all studies. Unit mass resolution was used for the first (MS1 setting 15) and the second (MS2 setting 15) mass analyzers. The MS detection mode was multiple-reaction-

monitoring (MRM) with a dwell time of 100 ms per ion pair in the *N*-in-1 studies. A dwell time of 200 ms was used for each test compound. The ESI source block temperature and the source disolvation temperature were set to 130 and 370 °C, respectively. Nitrogen was used as both a nebulizing gas and a disolvation gas, and the flow rates were set to 70 and 800 l/h, respectively. The argon collision gas pressure was set to 2.0 mbar. The source cone voltage and collision energy were specifically optimized for each test compound based on its spectral intensity using a microflow infusion. The data were acquired from the mass spectrometer using Micromass Masslynx 3.4 software. The same software was also used for the integration of chromatographic peaks for each channel of ion transition.

For the system synchronization, a pentium 450 MHz Compaq computer with Masslynx 3.4 was used to control the mass spectrometer through a TDAT board and the HP1100 pump via an HPIB board, respectively. The CTC autosampler was controlled by its own keypad. The Shimadzu pump system with a built-in switching valve was controlled only by the Shimadzu controller. The Quattro Ultima MS, HP1100 pump, Shimadzu pump and CTC autosampler were also connected by contact closures to synchronize time events. The CTC autosampler injected the sample and sent a contact closure signal to the HP1100 pump to start its time program. The HP1100 pump then sent out two closure signals to the mass spectrometer and the Shimadzu pump controller to start the data acquisition and the time program on the Shimadzu pump, respectively. Before the end of each run cycle, HP1100 pump sent out another contact closure signal to CTC to inject the next sample.

3. Results and discussion

3.1. Test compounds

To evaluate the analytical performance of HF LC/MS/MS and HFCS LC/MS/MS for direct plasma injection, five commercially available drug compounds were selected. The structures of these compounds are shown in Fig. 1. None of these compounds were structural analogues of each other but they were all considered as relatively polar compounds with some diversity in structure. The objective of this pool selection was to evaluate chromatographic separation efficiency, sensitivity and extraction efficiency of the different methods. In general, polar compounds pose a great challenge to most of the trace level assays in biological fluids. The positive electrospray product ion scan spectra of the protonated molecules $[M + H]^+$ of the five compounds are shown in Fig. 3. The ionization conditions of each molecule were optimized and the following mass

Table 2
A typical gradient programming of an 8 min HFCS LC/MS/MS

HP1100 Pump			Shimadzu Pump		
Time (min)	B% MeOH + 0.1 %FA	Flow Rate (ml/min)	Time (min)	D% ACN + 0.1%FA	Flow Rate (ml/min)
0	0	0.5	0	10	0.4
0.7	0	4	1.4	10	0.4
1.4	0	4	1.4	RV = 0[a]	0.4
1.5	0	0.3	4	RV = 1[a]	0.4
4	0	0.3	4	70	0.4
4.1	100	4	4.5	70	0.4
6	100	4	4.6	10	0.4
6.1	0	4	8	10	0.4
8	0	4			

[a] Six-port switching valve programming.

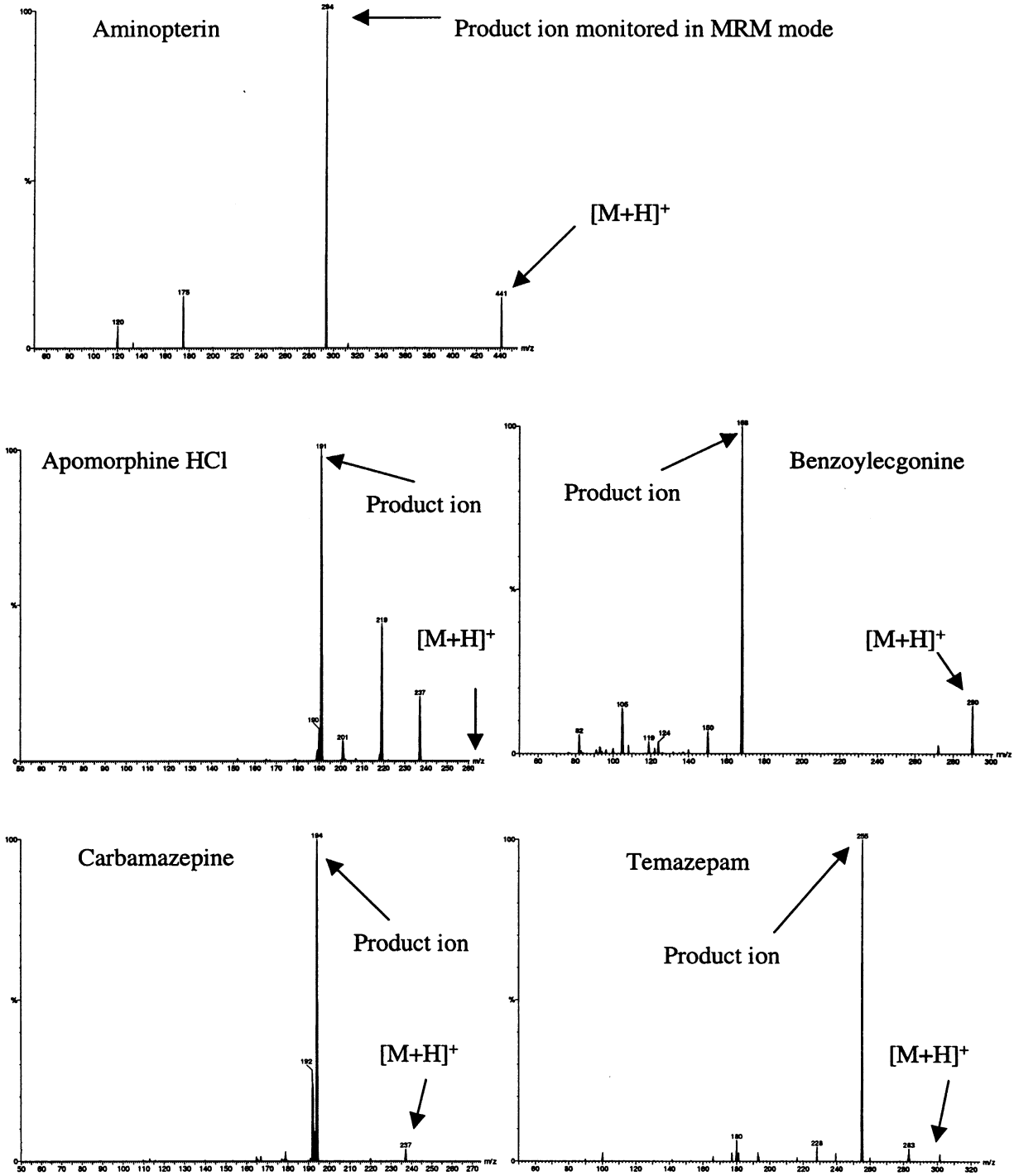

Fig. 3. ESI positive-ion spectra of the five test compounds, MS/MS product-ion spectra.

transitions were chosen for quantitative determination: m/z 441 → 294 for Aminopterin, m/z 268 → 191 for Apomorphine, m/z 290 → 168 for Benzoylcegonine, m/z 237 → 194 for Carbamazepine, and m/z 301 → 255 for Temazepam.

3.2. Comparison of separation efficiency and sensitivity

Two on-line extraction LC/MS/MS methods were evaluated in this experiment. Plasma samples containing four test compounds (1 μg/ml for each) were directly injected onto an extraction column operated under high flow conditions. The first method was HF LC/MS/MS without using an analytical column. The plasma sample was extracted and eluted on the Oasis column (1 × 50 mm) using a solvent gradient at a high flow rate of 4 ml/min, and the eluent was spilt to 0.4 ml/min before it was directed to the MS/MS detector. The second method was HFCS LC/MS/MS which employed both an extraction column and a narrowbore analytical column (YMC AQ 2 × 100 mm) coupled by a column-switching valve. The plasma sample was first extracted on the Oasis column (1 × 50 mm) using a 4 ml/min aqueous solvent, and the extracted analyte bands were back eluted using a solvent gradient at a flow rate of 0.4 ml/min. The plasma samples were prepared identically and they contained four test compounds at a concentration of 1 μg/ml for each test analyte. The injection volume was 60 μl of plasma mixture in both methods. The Ultima MS/MS detection conditions for both methods were identical.

Total ion chromatograms and extracted ion chromatograms for all analytes using HF LC/MS/MS and HFCS LC/MS/MS are shown in Figs. 4 and 5, respectively. The peak to peak signal-to-noise ratios (S/N:PtP) were calculated using Micromass Masslynx software version 3.4. Using the HF LC/MS/MS without an analytical column, the analyte peaks were broad. The calculated resolutions of the adjacent peak pairs were 0, 0.09 and 0.37, respectively. The S/N ratios of Aminopterin, Apomorphine, Benzoylcegonine and Carbamazepine were 360, 987, 1965 and 970, respectively. All four compounds were eluted at the same retention time without any separation. In contrast, using the HFCS LC/MS/MS with an analytical column resolved in much sharper and narrower analyte peaks. The calculated resolutions of the adjacent peak pairs were 1.50, 1.06 and 6.24, respectively. The resulting S/N ratios of Aminopterin, Apomorphine, Benzoylcegonine and Carbamazepine were 5784, 6730, 11 809 and 13 950, respectively. The S/N ratios of these analytes from HFCS LC/MS/MS were significantly enhanced (6–14-fold). Furthermore, every compound was completely separated and baseline resolved. Obviously, the latter method offered a much better sensitivity and separation efficiency.

The sharp peak responses in the HFCS method may be attributed to back elution of a strong gradient solvent from the extraction column into the analytical column. For the elution step, the column diameter changed from 1 to 2 mm while the column pack particle size changed from 30 to 5 μm. Theoretically, the analyte bands were pushed and focused and so were the peak responses during this cross-column separation process. Therefore, using column switching and directing the extracted band to an analytical column significantly improved both the sensitivity and chromatographic separation that are usually not sufficient by using high flow chromatography alone.

3.3. Effects of analytical column diameter on HFCS LC/MS/MS chromatography

Five test analytes were used in this assay. Two Waters YMC ODS-AQ columns, a 1 × 50 mm microbore column and a 2 × 50 mm narrowbore column, were selected as analytical columns to evaluate the impact of their diameters on liquid chromatography. The injection volume of the plasma sample was 60 μl for both analytical columns. The Ultima MS/MS detection conditions for both methods were identical.

Total ion chromatograms and extracted ion chromatograms for all five analytes using different analytical columns with HFCS LC/MS/MS are shown in Fig. 6. Using the 1 mm microbore

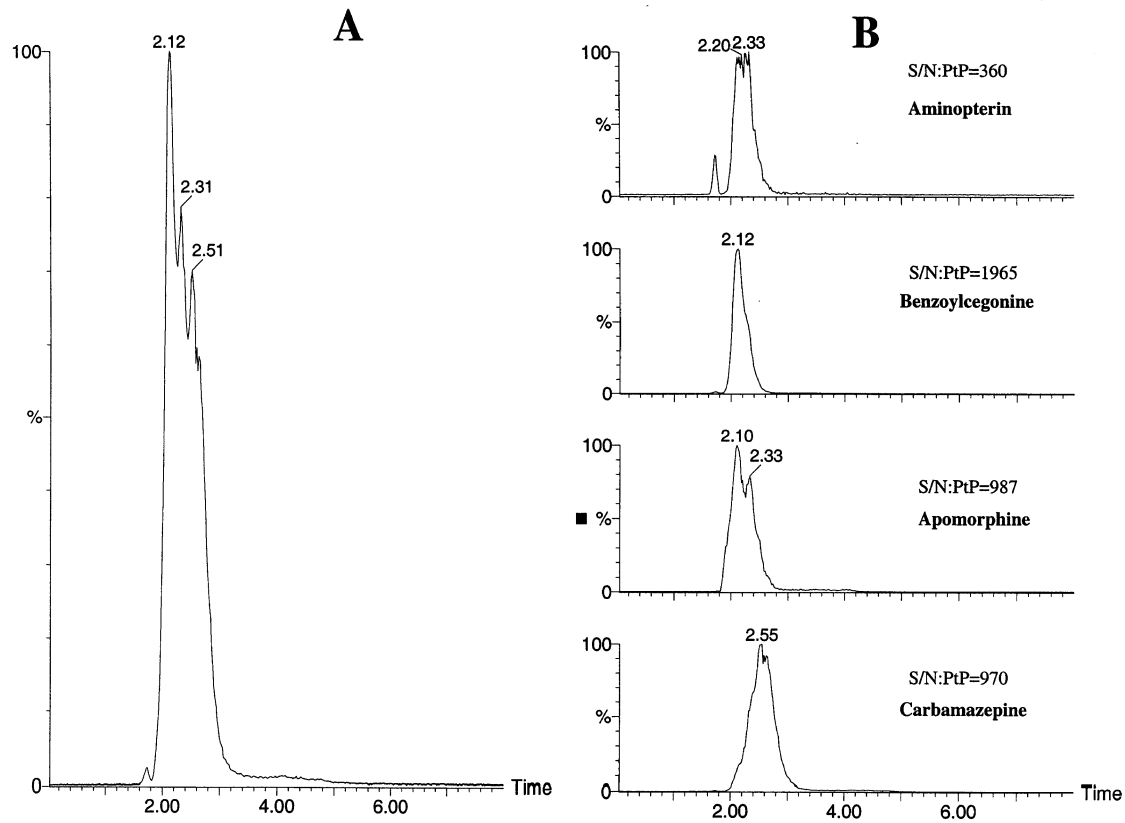

Fig. 4. Total ion chromatogram (A) and extracted ion chromatograms (B) of HFCS LC/MS/MS assays for a 60 μl direct injection of 1 μl test compounds in dog plasma without an analytical column. A 1 × 50 mm Waters Oasis cartridge was used as the HF extraction column. The S/N ratios of all compound responses were shown.

column, the analyte responses in peak area were higher; however, the peak shapes were broader, resulting in lower separation efficiencies. The calculated resolutions of the adjacent peak pairs were 0.32, 0.26, 0.76 and 0.38, respectively. In contrast, using a larger diameter analytical column (2-mm narrowbore) resulted in narrower analyte peaks and consequently, enhanced separation efficiencies. The calculated resolutions of the adjacent peak pairs were 1.00, 1.14, 4.90 and 1.65, respectively. All five compound peaks were baseline resolved. Although the peak areas of the analytes were decreased about 3 fold in average, the S/N ratios of each analyte did not drop by using the 2 mm analytical column.

3.4. Estimation of extraction efficiency of HFCS LC/MS/MS

It is difficult to measure absolute extraction recoveries for plasma samples using the HFCS LC/MS/MS method. Therefore, the relative extraction efficiency was estimated by comparing with the same standard samples analyzed by conventional gradient LC/MS/MS method. The reference standards, were prepared by using post-extraction (extraction performed on Oasis SPE cartridges, 30 mg) spiked with analyte standards at the same concentrations as those contained in the plasma sample for direct injection in HFCS. The injection volumes in these tests were

also kept the same (60 μl). Results of on-line extraction efficiency estimations are shown in Table 3.

The compounds with relatively low polarity exhibited higher efficiencies than the polar compounds using the on-line extraction HFCS LC/MS/MS method for direct plasma sample injection. For example, two relatively less polar compounds in the group, carbamazepine and temazepam, have excellent extraction efficiencies over the more polar compounds. On the other hand, two relatively polar compounds, aminopterin and benzoylecgonine, both possessed a carboxylic group, showed low recoveries at the same conditions. Therefore, change of chemistry in the extraction cartridge could be necessary for analyzing highly polar molecules that are difficult to be retained on the extraction column.

3.5. Application of HFCS LC/MS/MS method for the analysis of 10-in-1 PK samples

Since the HFCS LC/MS/MS method has been evaluated as a good choice for high throughput bioanalysis with direct plasma sample injection, it has been applied to many *N*-in-1 PK studies in the authors' laboratories since early 1999. Usually the number of compounds, *N*, can vary from 5 to 20 in different PK studies in animals. The major goal of this 10-in-1 study was to screen and identify new leads possessing better PK profiles in a group of structural analogues. The least information drawn from this kind of study should rank a clear order based on PK parameters and keep the confirmed winners for comparison in the future.

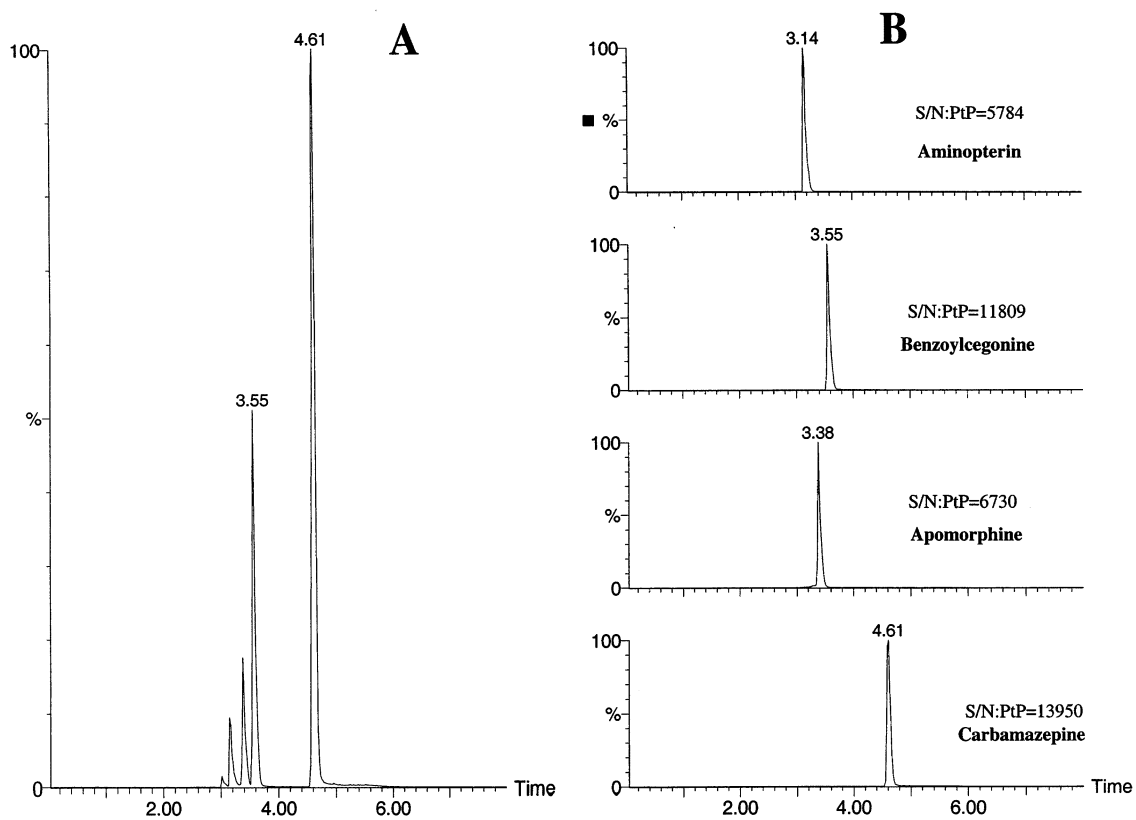

Fig. 5. Total ion chromatogram (A) and extracted ion chromatograms (B) of HFCS LC/MS/MS assays for a 60 μl direct injection of 1 μg/ml test compounds in dog plasma with an analytical column, YMC ODS-AQ 2 × 100 mm (5 μm). A 1 × 50 mm Waters Oasis cartridge was used as the HF extraction column. The S/N ratios of all compound responses were shown.

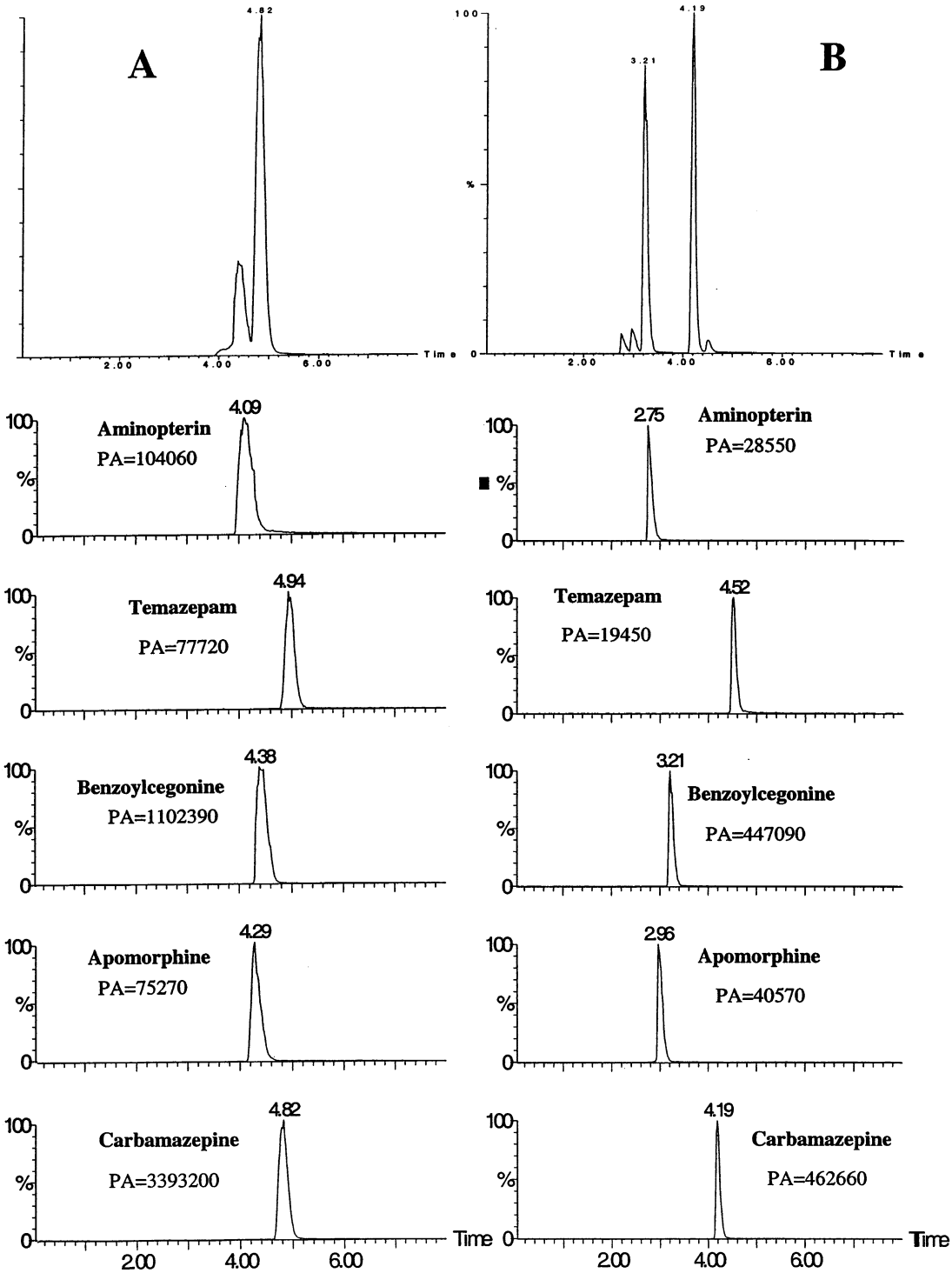

Fig. 6. Effects of analytical column diameter on HFCS LC/MS/MS assay. (A) five test compounds were analyzed using a 1 × 50 mm YMC AQ column at a flow rate of 0.1 ml/min. (B) five test compounds were assayed using a 2 × 50 mm YMC AQ column at a flow rate of 0.2 ml/min. The total ion chromatogram of each, their extracted ion chromatograms and peak areas (PA) were shown above.

Table 3
Recoveries ($N = 3$) of the test compounds using HFCS LC/MS/MS

Compound	1000 ng/ml	500 ng/ml	100 ng/ml	Average Rec.
Aminopterin	26%	25%	22%	24%
Apomorphin HCl	24%	20%	19%	21%
Benzoylecgonine	55%	47%	43%	48%
Carbamazepine	104%	85%	103%	97%
Temazepam	104%	96%	107%	102%

Fig. 7 illustrates the total ion chromatogram and extracted ion chromatograms of a directly injected plasma sample (100 μl) containing eleven compounds including one internal standard. The blood sample was collected 1 h after IV administration of 10 compounds simultaneously into a dog. In general, detection interference happens when analytes share an identical product ion and the molecular weight of their precursor ions are similar. This is often the case for a pool of structural analogs. In this 10-in-1 case, compounds **C2**, **C3** and **C6** showed a major product ion of m/z 501 or 502. Particularly the parent ions of compounds **C2** and **C3** differ by only 1 Da. Interference could be serious if they were not chromatographically separated. However, the separation efficiency provided from HFCS LC/MS/MS successfully resolved both analytes by 0.8 min (HPW 0.2 min) in retention time to ensure they were free of interference.

Table 4 shows the intraday validation of ten compounds in dog plasma using HFCS LC/MS/MS. Most of the validated plasma concentration ranges were from 1 to 2500 nM, with one compound dynamic range from 1 to 500 nM. The intraday accuracy ($\leq 15\%$) and precision ($\leq 15\%$) were acceptable for every compound. In addition, the lower limit of quantitation (ILOQ) of 1 nM (≈ 0.5 ng/ml) was successfully achieved for all ten compounds.

The results of plasma concentration versus time profiles of the ten compounds assayed by HFCS LC/MS/MS method are shown in Fig. 8. The compounds were administered to a male beagle dog at a dose of 0.5 (mg/kg)/compound with intravenous infusion. With the great sensitivity provided by the HFCS LC/MS/MS method, low concentrations at 24 h were all accurately measured. As illustrated by the concentration versus time profiles, the screening and ranking can effectively differentiate compounds with different PK profiles. Compounds **4** and **2** were identified as leads due to their low systemic clearance and volume of distribution. They also showed relatively longer half-lives and higher oral bioavailability.

Overall, as an on-line analytical technique that eliminates labor and time involved in tedious sample extraction, HFCS LC/MS/MS assay successfully combines the speed and ruggedness of HF extraction with the sensitivity and separation efficiency of HPLC method. Based on our observation, the use of this on-line switching approach resulted in less cumulated source contamination than HF LC/MS/MS or PP LC/MS/MS methods. The ESI sampling cone was cleaned once a week after about 1000 injections In addition, the cost for HFCS extraction has the advantage over 96-well plate extraction since most of the Oasis column can be repeatedly used for about 800 injections. Therefore, it is about one-fourth of the cost of 96-well SPE method. Furthermore, the flexibility and simplicity of HFCS on-line extraction makes it more appealing than other methods. For example, if any sample is above the quantification level (AQL) of the validated range, it can be diluted with plasma containing IS (the same ratio as in the original sample preparation) and re-injected with standards and QCs immediately. No second extraction process is needed. It is estimated that the preparation time for 100 plasma samples is less than 1 h while other methods take over 2 h. With the increasing use of robotic liquid handlers, the preparation of sam-

Table 4
Intraday validation accuracy (%CV) and precision (%Diff.) of 10-in-1 assay with HFCS LC/MS/MS

N-in-1 Compound	Dynamic range (nM)	QC1		QC2.5		QC10		QC500		QC1000		QC2500	
		Cv[a] (%)	Diff[b] (%)	CV (%)	Diff (%)	CV (%)	Diff (%)	CV (%)	Diff (%)	CV (%)	Diff (%)	CV (%)	Diff (%)
Compound 1	1–500	1.7	2.0	3.2	7.6	1.9	1.9	15.6	−14.7				
Compound 2	1–2500	5.2	14.0	2.8	−5.6	2.4	−7.8	2.6	3.5	6.7	7.5	4.1	0.9
Compound 3	1–1000	5.6	8.0	5.1	−13.6	1.2	11.7	8.4	−8.8	1.2	−9.8		
Compound 4	1–2500	6.9	−3.0	9.8	13.2	4.6	12.8	4.3	7.8	7.3	3.2	7.0	−5.2
Compound 5	1–2500	3.2	2.0	3.8	1.2	3.5	−3.5	11.1	−15.5	5.5	−15.9	3.6	−12.1
Compound 6	1–1000	7.6	15.0	7.8	8.4	3	14.5	4.6	−1.1	2.5	−7.8		
Compound 7	1–2500	7.2	13.0	8.7	14.4	1.7	12.8	2.1	4.4	4.2	1.0	8.2	−3.1
Compound 8	1–2500	9.3	3.1	5.1	11.6	2.2	7.7	7.2	−3.9	3.2	−3.8	0.7	−11.2
Compound 9	1–1000	4.4	1.0	9.4	11.6	1.6	11.1	2.2	−9.1	2.3	15.8		
Compound 10	1–2500	10.1	−14.0	4.1	1.2	4.0	5.2	10.3	−11.5	6.2	−10.4	1.8	−12.3

[a] CV, coefficient of variation in percentage.
[b] Diff., percent difference.

ples for HFCS LC/MS/MS analysis can be further improved by using robot for a faster throughput in the future.

4. Conclusions

Two high throughput on-line LC/MS/MS bioanalytical methods were investigated for the direct determination of pharmaceutical compounds in plasma. The use of the HFCS LC/MS/MS with an additional analytical column was shown to have greatly improved sensitivity and separation efficiency compared with HF LC/MS/MS. With these advantages, the method is able to provide high quality results while maintaining a high sample throughput in multiple component assays. In addition, analyte interference and suppression effects in LC/MS/MS were minimized, which provided better conditions to achieve the required resolutions for *N*-in-1 PK analyses. By performing on-line extraction and separation in parallel, both labor and time spent on manual sample extraction processes can be minimized.

The on-line extraction HFCS LC/MS/MS method has been successfully used not only in the plasma assays of *N*-in-1 PK studies but also in urine and synovial fluid assays where the biological fluids contain dirty matrices. In fact, good sensitivity and separation efficiency were achieved from all biological fluids including directly injected plasma, serum, whole blood, urine, and bile samples. The resulting dynamic range, lower limit of quantification, QC accuracy and precision were well within the acceptable range for discovery research and development. As a result, this tech-

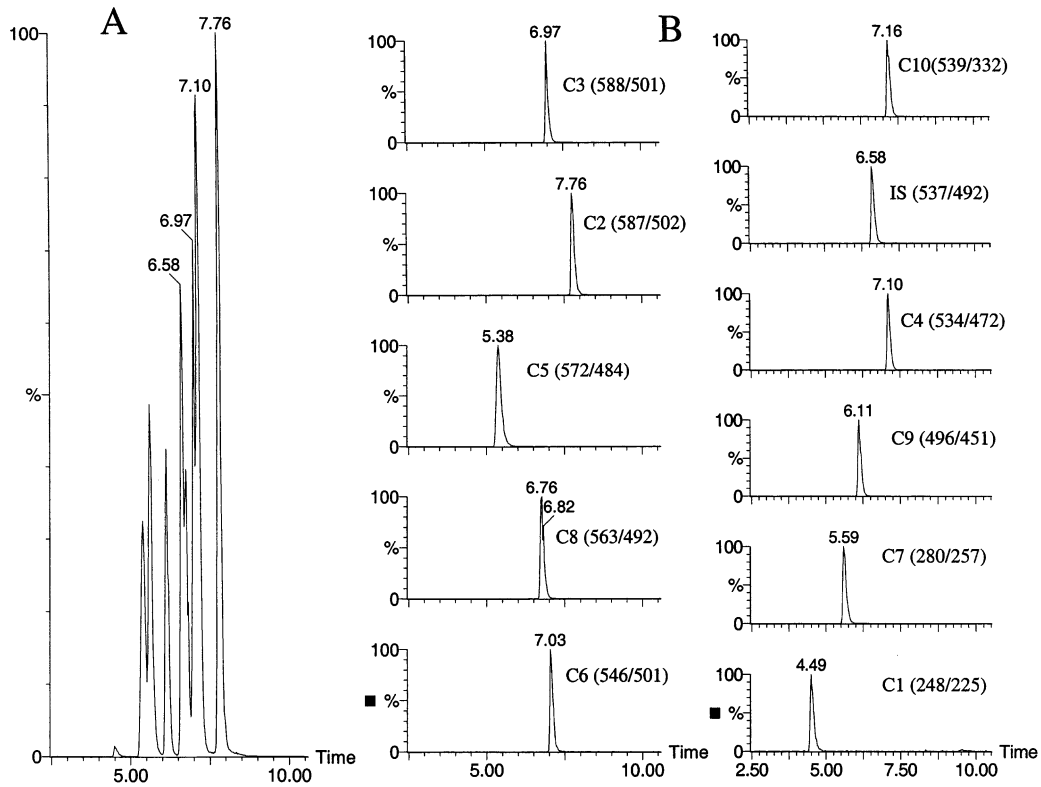

Fig. 7. Total ion chromatogram (A) and extracted ion chromatograms (B) of HFCS LC/MS/MS assays via direct injection of 100 μl of dog plasma sample collected 1 h after IV administration of 10 compounds to a dog. A Waters Oasis 1 × 50 mm, 30 μm cartridge and a YMC basic 2 × 100 mm, 5 μm column were used as the extraction column and the analytical column, respectively.

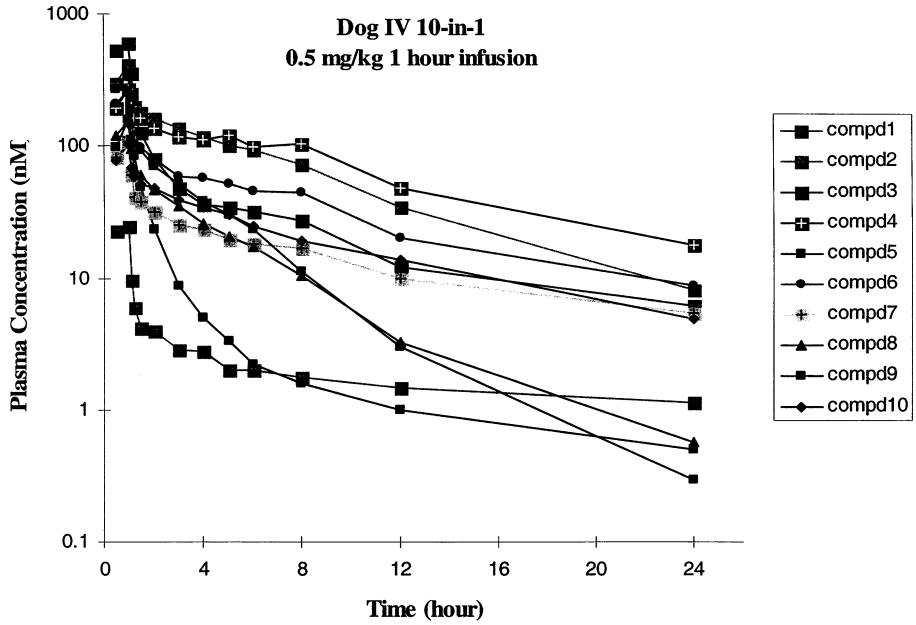

Fig. 8. Plasma concentration versus time profile for 10 compounds in a 10-in-1 PK study after a 1 h intravenous infusion at 0.5 mg/kg to a beagle dog.

nique has been routinely used for both multiple and discrete component analyses for PK studies in the authors' laboratories.

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