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Evaluation of online extraction/mass spectrometry for in vivo cassette analysis

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Abstract

An online extraction/mass spectrometry technique was evaluated for direct analysis of plasma samples. A simple user-friendly online extraction system that consists of two pumps, an autosampler, a six-port switching valve and a mass spectrometer is described. The system was controlled by the LC–MS software (Masslynx 3.5, Waters Corporation, Beverly, MA). Various analytical conditions such as extraction column, mobile phases, run time and wash solvent were optimized to establish an analytical method that was simple, easy to set up and generic. Sample preparation effort was minimal, which included dilution of plasma with water and centrifugation conducted in 96-well plate format. The system was used to analyze in vivo plasma samples from rat *n*-in-one cassette dosing studies. Concentration and pharmacokinetic (PK) data obtained from the online extraction method were comparable with data obtained from the protein precipitation extraction method. Overall, the simple, robust online extraction system provides cost savings by minimizing sample preparation and method development time. The system was used to analyze compounds from different structural classes. These studies suggest that calculated lipophilicity of a compound can be used as a tool for pre-selection of extraction column, which would save method development time for early discovery studies.

Keywords: Sample preparation; Bioanalysis; Drug discovery; TFC; Online extraction; Pharmacokinetics

1. Introduction

Bioanalysis of small molecules by liquid chromatography–tandem mass spectrometry (LC–MS/MS) helps effectively meet the needs of fast-paced screening and discovery stages of today's pharmaceutical research [1]. Plasma, bile, urine and tissue are matrices that are often subject to analyses in pharmacokinetic (PK) and drug metabolism studies, of which plasma is the most widely analyzed matrix. Streamlined approaches during quantitation of drugs in biological matrices such as the use of 96-well format for sample collection, use of EDTA as anticoagulant to minimize sample clot formation, semi-automated sample extraction, automated LC–MS/MS method development and automated data processing have shortened bioanalytical turnaround times [2–5]. In addition, strategies such as direct cocktail analysis by sample double pooling have been reported to reduce the overall bioanalysis time [6]. Bioanalytical throughput has also been improved by shortening the LC analysis time with the use of column-switching, fast gradient analysis, high flow rate analysis and staggered parallel LC technology [7–11]. Another approach, online extraction (OLE) has been widely used to reduce the sample preparation time. These techniques have employed polymer-based [12–19] or other restricted access media type columns [20,21] in conjunction with high flow rates/turbulent flow chromatography (TFC) to wash off matrix such as proteins while retaining the analyte on the column and subsequently eluting with a high organic mobile phase onto the MS.

More recently, many researchers have adopted the commercially available instrumentation such as the HTLC 2000 system marketed by Cohesive Technologies to perform turbulent flow extraction of analytes from in vitro or in

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vivo samples [22,23]. In conjunction with the extraction column, an analytical column is also used to obtain better peak shape, improved chromatographic capacity to minimize matrix-related ion suppression and separation of analytes/metabolites [24–26]. The necessity of the use of analytical column is dependent on the problem at hand and the rigor of the analytical method required. The latter is typically determined by the type of study (discovery screening, lead development, candidate development) for which the method will be used. Some experimenters have used offline clean up of samples before using the Cohesive TFC instrumentation, presumably, to enable the analytical method to meet the acceptance criteria [27].

In vivo cassette screening approach is widely used in the pharmacokinetic/drug metabolism groups of the pharmaceutical industry to screen for compounds with good pharmacokinetic properties in early drug discovery [28–31]. Cassette approach, in which one animal is dosed with multiple compounds, increases the throughput of in vivo PK studies. By using cassette dosing, in vivo PK profiles can be evaluated for more compounds in a shorter time period using fewer animals and the study results in fewer samples for analysis. The compounds are then simultaneously quantitated in each sample from a single LC–MS/MS analysis; the inherent specificity, selectivity and sensitivity of the MS allow such analysis. However, this approach has a disadvantage of potential drug–drug interactions that could provide misleading PK results [32,33]. A recent survey has listed the utility of cassette dosing across several pharmaceutical companies [34]. The advantages of cassette dosing from the bioanalytical perspective are maximizing mass spectrometric utilization and screening capacity. Some bioanalytical challenges faced in supporting these studies are the inability to analyze compounds with similar molecular weight or structures due to inter-channel cross talk issues, increased MS and LC method development time and increased data processing and review time. The problem faced with MS cross talk can be overcome by intentionally selecting compounds that are dissimilar in molecular weight or structure whenever possible. The additional time required for method development can be reduced by using automation for profiling of critical MS parameters of compounds and ballistic gradient chromatography [35,36]. Turnaround can also be reduced by reducing the sample preparation time. Our intent was to combine these techniques with a generic OLE system, and thus minimal sample preparation, for analysis of in vivo cassette samples to further reduce our cycle times while maintaining the analytical data quality. In this paper, we describe the evaluation of an OLE–MS technique for direct analysis of plasma samples from in vivo cassette screening studies using a simplified hardware approach.

2. Experimental

The analytical instrumentation consisted of two Shimadzu LV-10ADVP (Kyoto, Japan) pumps A and B, a Shimadzu SIL-HTC autosampler (Kyoto, Japan) equipped with a chilled tray compartment (operated at 10 °C) or a CTC-PAL autosampler (Leap Technologies, Carboro, NC), and a Micromass Quattro Micro or a Quattro Ultima (Waters Corporation, Beverly, MA) mass spectrometer. The aqueous phase 1% formic acid at a flow rate of 2.0 mL/min was delivered by pump A, while the organic phase 100% acetonitrile at a flow rate of 0.8 mL/min was delivered by pump B. Other aqueous phase compositions such as 0.1% formic acid and 0.1% acetic acid were evaluated. Similarly, organic phases containing acetonitrile with up to 5–20% of 0.1% formic acid were tested. During the optimization of the experimental conditions, aqueous flow rates of 0.5, 1, 2, 3 and 4 mL/min and organic flow rates of 0.3–1 mL/min in 0.1 unit increments were tested. Online extraction phase (loading and washing with the aqueous phase) was evaluated at both 0.5 and 1 min. The wash solvent consisted of 50:50 acetonitrile/0.1% formic acid. Other needle wash solutions such as 50:50 methanol/0.1% formic acid, methanol/0.1% acetic acid were evaluated. Cyclone HTLC, Cyclone P and Polar Plus (60 μm median particle size, 1 mm × 50 mm dimension, Cohesive Technologies, Franklin, MA) were used as the online extraction columns. A six-port switching valve (VICI, Houston, TX) was used to divert the flows to either extraction column or waste. A diagram depicting the flow path of the OLE–MS system using the switching valve set-up is shown in Fig. 1. The mass spectrometer was operated in electrospray positive or electrospray negative ion modes. Nitrogen was used as the cone and drying gas and argon was used as the collision gas. The capillary voltage was set at 3.5 kV, while cone voltage and collision energy settings were compound-dependent. The instrument was operated under unit resolution. Masslynx 3.5 (Waters Corporation, Beverly, MA) was used as the system control and quantitation software. The optimum conditions for the generic OLE–MS method were: 10 μL injection volume, 1% formic acid at a flow rate of 2.0 mL/min as the aqueous/loading mobile phase, a 0.5 min washing period, 100% acetonitrile at a flow rate of 0.8 mL/min as the organic/elution mobile phase and 50:50 acetonitrile/1% formic acid in water mixture as the needle wash.

A typical in vivo cassette study consisted of dosing cannulated male Sprague–Dawley rats ($n = 3$ per route of administration) intravenously (IV) and/or orally (PO) with a mixture of compounds in appropriate formulation at 1 and 5 mg/kg, respectively. Plasma samples were collected using EDTA as the anticoagulant at 0, 0.25, 0.5, 1, 2, 4, 6 and 8 h post-dose following a single PO administration; and at 0, 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 6 and 8 h post-dose following a single IV administration. Blank Sprague–Dawley rat plasma was obtained from Bioreclamation (East Meadow, NY). The standard curve was prepared by serial dilution in rat plasma (EDTA anticoagulant) from a 100 μg/mL stock in acetonitrile while maintaining >95% plasma matrix in a 96-well plate. Control matrix blank and matrix blanks without IS were analyzed in each run. Fifty microliters of standard or sample was diluted with 100 μL of water containing in-

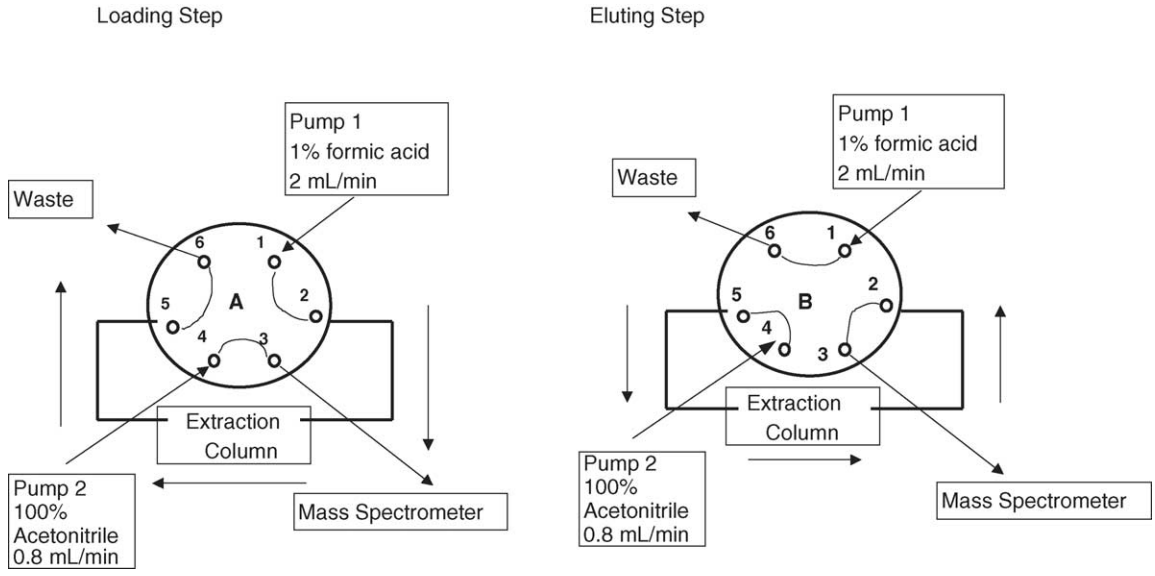


Fig. 1. Schematic of the online extraction system.

ternal standard (a structurally similar compound typically at 100 ng/mL concentration). Various sample/IS dilution ratios such as 1:1, 1:2 and 1:3 were evaluated. The plate was then vortexed for 1 min, and centrifuged at 4000 rpm for 15 min. The plasma samples were injected on to the OLE–MS system. Injection volumes evaluated ranged from 5 to 50 μL. Total recovery of the OLE–MS was evaluated using 10 test compounds. The test compounds were analyzed at concentrations of 5000, 1250 and 156 ng/mL from both neat samples and spiked plasma samples. The ratio of the peak areas of the compound in plasma versus neat was calculated to obtain total recovery of the OLE–MS technique. For protein precipitation, a 50 μL aliquot of the plasma sample was precipitated with 150 μL of acetonitrile containing the internal standard (a structural analog typically at 100 ng/mL). The samples were then centrifuged at 3500 rpm for 15 min and the supernatant was transferred to a 96-well plate for injection on the LC–MS system. The protein-precipitated samples were analyzed on HPLC columns of the dimension 2.0 mm × 50 mm. The stationary and mobile phases (typically a combination of acetonitrile, methanol and water with modifiers such as formic acid, and/or ammonium formate) varied based on the compounds that were analyzed. However, the analytical conditions were maintained to obtain a capacity factor of ≥2. Proprietary compounds analyzed were synthesized in-house (Pfizer Global R&D, Ann Arbor, MI). Ten representative compounds that would be dosed as two different cassettes (i.e. each cassette consisting of five compounds) were chosen to assess the precision and accuracy of the OLE–MS method. A 10-point standard curve was analyzed eight times for each of the cassette using the generic conditions to assess accuracy and precision of the analysis as well as the robustness of the system. Acceptance criteria included a correlation coefficient ≥0.99 for the calibration curve with at least six points included in the curve and a relative error of ≤30% for standards. The molecular weights of the compounds used in the different studies ranged from 150 to 600 Da. *c* log *P*, a measure of lipophilicity, of the compounds was calculated using Daylight Chemical Information Systems software version 4.72 (Mission Viejo, CA).

3. Results and discussion

A number of parameters, that provided the highest sensitivity, lowest carryover, least peak tailing were optimized to establish a generic extraction method. Fig. 2A shows the chromatogram of a test compound after optimization and with an analytical column. Our initial attempts of using an analytical column between the extraction column and the mass spectrometer resulted in excessive peak tailing. By removing the analytical column from the flow path and back flushing the analyte directly on the mass spectrometer, no peak tailing was observed as shown in Fig. 2B. Eliminating the use of analytical column minimizes the number of variables to be optimized and hence the time required for method development. However, one should be cautioned that the lack of an analytical column could result in co-elution of metabolites along with the analyte. This could potentially result in erroneous quantitation of the analyte if the selected reaction monitoring (SRM) transitions of the analyte and metabolite are similar (e.g. acylglucoronide metabolites that undergo in-source fragmentation) [37].

Injection volume is a critical parameter that affects the peak shape. Overloading the column with a large excess of analyte produces peaks that are broad and also results in significant carryover. Although injection volumes of up to 200 μL have been reported [38], in our case an injection volume of 10 μL provided the best peak shape with adequate sensitivity and less carryover.

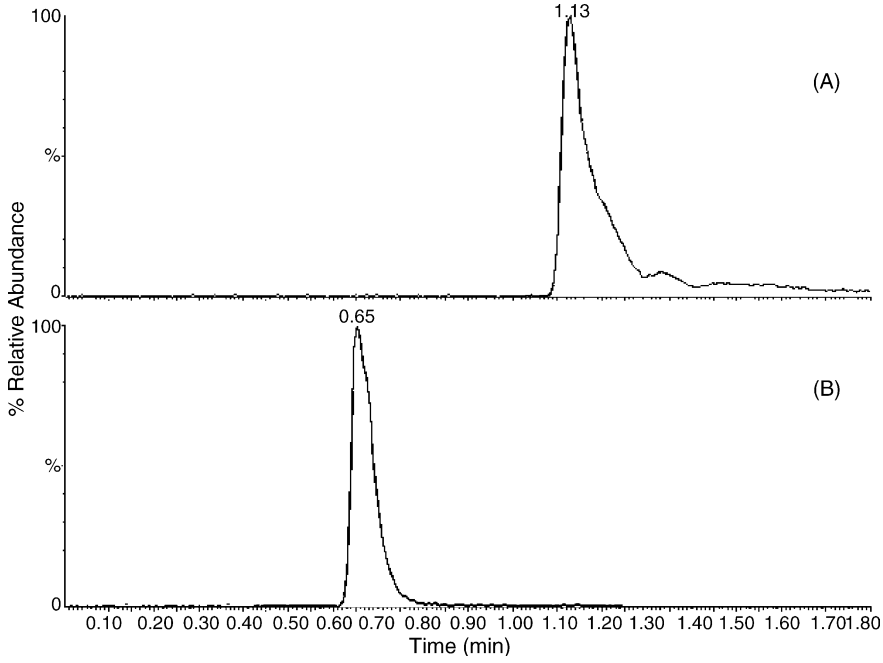


Fig. 2. Chromatogram of a test compound after optimization of the online extraction condition: (A) with an analytical column; (B) without an analytical column.

The matrix injected in OLE is diluted plasma which contains lipids and proteins; due to this the analyte sticks to the tubing and valve material to a greater extent than the offline LC–MS analysis where the analyte is extracted into an organic medium and lipids/proteins are largely removed. Hence, carryover is an inherent problem in OLE techniques. The carryover material, i.e. the analyte that is retained in the common fluid path after an injection, can be carefully washed off by using more than one wash solvent, adjusting the wash solvent composition, and number of washes. The observed carryover of the system resulting from the Shimadzu SIL-HTC autosampler and the rest of the fluid path was less than 0.01%. This was determined by comparing the peak area of a high standard analyzed using an extraction column with the peak area from subsequent analysis of a blank on a new/unused extraction column. However, the column memory effect was compound-dependent. Column memory effect is contributed by the analyte that remains on the extraction/separation column after analysis of a single sample. This was determined by comparing the peak area of the analyte in the blank analyzed after the analysis of the highest standard (5000 ng/mL). The range was between 0.13% and 0.65% depending on the structural class of the compound and the extraction column (Cyclone HTLC or Polar Plus) used. The wash solvent of 50:50 acetonitrile/0.1% formic acid was sufficient to obtain low carryover (<0.1%). However, the combination of carryover and column memory effect could not be controlled to <20% of the limit of quantitation (LOQ) in some cases. The carryover/column memory effect issues may be worked around by placing blanks after the highest standard and introducing blanks in between the samples (at least those such as the early time points from IV dosing) that would have high analyte concentration.

Samples were loaded on the extraction column using 1% formic acid in water as the mobile phase. The concentration of formic acid was varied from 0.1% to 2%; 1% provided the best sensitivity and peak shape. Based on the sensitivity differences, the presence of acid ($\geq$1%) in the mobile phase enabled better analyte extraction, compared to little or no acid in the mobile phase. Online clean up time, the time for which the aqueous phase is pumped through the column was optimized to be 0.5 min. This was experimentally optimized to obtain best sensitivity and peak shape as well. Longer washing time such as 2 min resulted in peak broadening and low sensitivity. Similarly the aqueous flow rate was optimized to be 2 mL/min. The experimental results showed that 2.0 mL/min provided the highest sensitivity and best peak shape. At 1 mL/min, even though the peak shape was acceptable, the sensitivity was low, while at flow rates of 3 and 4 mL/min peak tailing was an issue. Peak tailing and broadening increased with increase in flow rate. Although turbulent flow phenomenon is expected to occur only at 4 mL/min for a column with 60 μm median particle size and 50 mm × 1 mm dimension [22], 4 mL/min was not the best flow rate to provide us adequate sensitivity and peak shape. The elution mobile phase was 100% acetonitrile. Acetonitrile, at a flow rate of 0.8 mL/min, was used to back flush the analyte plug from the head of the extraction column on to the mass spectrometer. The mass spectrometer was able to handle the 0.8 mL/min flow rate without any visible change in the electrospray plume and without a significant drop in sensitivity. Increased desolvation gas flow rate

and desolvation temperature (≥300 °C) than those used for typical flow rates such as 0.25 mL/min provided sensitivity to at least 10 ng/mL. When 100% methanol was used as the elution solvent, the peak shape was broader, possibly due to the lower elution strength of methanol.

Sample preparation involved addition of 100 μL of water containing the internal standard to 50 μL of sample or standard. The internal standard chosen was a structural analog from the chemical template. Addition of the internal standard in the aqueous phase also served as the dilution step for the samples. Of the different ratios of sample/IS evaluated, a dilution ratio of 1:2 provided the best and adequate sensitivity. Centrifugation of the plasma samples helped prevent clogging of the needle, since much of the plasma protein was palletized resulting in a robust analytical run. Use of the chilled autosampler tray in the Shimadzu autosampler was helpful in minimizing clot formation and analytical run failures due to clogged needle. In the case where chilling was not available (Leap autosampler), clogging of needle was more frequent. This was overcome by adding additional syringe washes.

Accuracy and precision studies using 10 representative compounds were conducted. The mean concentration data at each level, relative standard deviation (%RSD) and relative error (%RE) for one representative compound, as shown in Table 1, were <6% and within 5%, respectively. These results were representative of the other compounds (%RSD and %RE were ≤20%) analyzed as well. The limit of quantitation for the various compounds was 5 ng/mL and the linear dynamic range was 5–5000 ng/mL with correlation coefficients of 0.99 for the calibration curves. Assay selectivity for the analyte and IS was demonstrated with control matrix blanks analyzed. No matrix interference was observed for the different analytes or the internal standards in the blanks. Total recovery of the OLE–MS technique ranged from 30% to 75% and was compound-dependent. Total recovery of the technique is a cumulative quantitation of both the extraction efficiency and matrix effect [39]. Since both parameters, especially matrix effect, are compound-dependent, this result is acceptable.

The generic OLE–MS method was used to quantitate compounds from plasma samples obtained from in vivo cassette dosing studies. Compounds from several different structural templates (structures not shown due to confidentiality) were analyzed. Sample OLE–MS chromatograms from one of the cassettes (0.5 h IV time point) are shown in Fig. 3. Note that the total cycle time for analysis is 1.2 min, which includes a 0.5 min of loading/washing, 0.5 min of elution and 0.2 min equilibration period. The symmetrical peak shape with at least 20 points across the peak was achieved without optimizing the analytical conditions further. This was observed in most cases when the analyte's lipophilicity was compatible with the extraction column chemistry.

Table 1
Accuracy and precision of the online extraction/mass spectrometry technique

Standard concentration (ng/mL)	Mean	SD	%RSD	%RE
9.76	10.0	0.20	2.00	2.46
19.5	19.0	0.77	4.04	−2.48
39.1	39.1	1.58	4.05	0.00
78.1	74.4	3.25	4.38	−4.80
156	150	9.34	6.21	−3.57
313	327	12.3	3.75	4.60
625	637	17.3	2.72	1.87
1250	1276	15.1	1.19	2.06
2500	2537	95.6	3.77	1.46
5000	4930	253.6	5.15	−1.41

The concentration data obtained, for several compounds that were dosed as intravenous (IV) and/or oral (PO) cassettes, using the OLE–MS technique were compared with the concentration data obtained from the offline protein precipitation (PP) LC–MS technique. Fig. 4 shows overlay of mean concentration data ($n = 3$) at different time points using either technique (PP and OLE) for two representative compounds that were part of an IV cassette containing four compounds and belonged to the structural class A. Relevant PK parameters derived from the plasma concentrations for these two compounds are shown in Fig. 4 as well. Fig. 5 shows overlay of mean concentration data ($n = 3$) at different time points using either technique (PP and OLE) for two example compounds that were part of a PO cassette containing four compounds and belonged to the structural class B. Relevant PK parameters derived from the plasma concentrations for these two compounds are shown in Fig. 5 as well. The graphs show that the IV and PO profile for the compounds using concentration obtained by either method are very similar. The intra-technique difference of ≤25% in the calculated PK parameters shows that there is good agreement between the two techniques. A similar comparison was observed for other compounds tested as part of these cassettes.

The generic OLE method using the Cyclone HTLC extraction column was not universally applicable to compounds of all classes. Compounds that were extremely polar ($c \log P < 0$) were not retained on the Cyclone HTLC column and were flushed from the column during the loading/washing phase. They required the use of an extraction column, Polar Plus that contained a different stationary phase. Although the cycle time and most other conditions were the same as those used for a Cyclone HTLC column, the mobile phase pH was adjusted with ammonium acetate in order to obtain good sensitivity while using the Polar Plus column. This indicates that the Polar Plus column is suitable for extremely polar compounds while Cyclone HTLC is suitable for compounds that are moderately polar and those that are moderately non-polar. The Cyclone HTLC column contains 60 μm polymer particles while the Polar Plus contains coated 50 μm silica particles [40]. Although we initially evaluated the Oasis HLB extraction cartridge from Waters Corporation, in an effort to evaluate columns from different manufacturers, these were not available during the later stages of our evaluation.

Of the various physicochemical properties of the compounds (pK_a, $c \log P$, MW, hydrogen bonding characteristics), a correlation between the $c \log P$ of the compound to

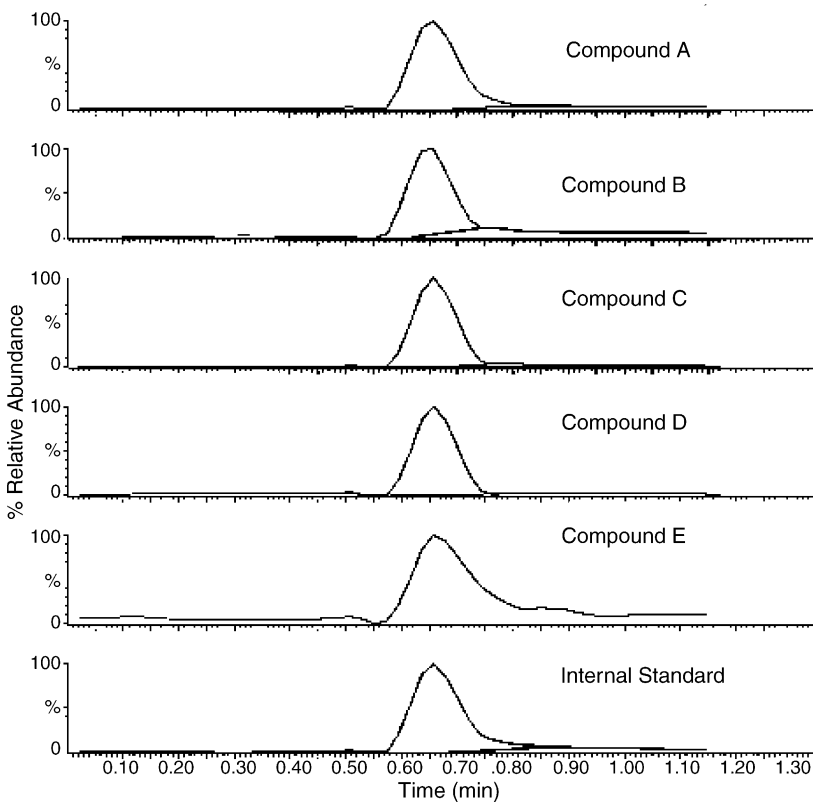


Fig. 3. Online extraction/mass spectrometry chromatograms of five compounds from an intravenously cassette dosed sample at 0.5 h time point.

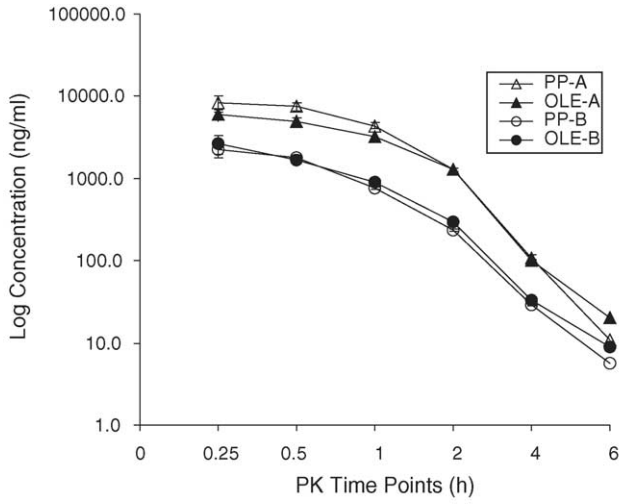
the type of column was apparent. Based on the data from this training set (compounds of different structures; proprietary information), the *c* log *P* of the compounds can be used as a guide when choosing the extraction column. Table 2 shows the *c* log *P* of compounds that we have tested and the corresponding extraction column that was suitable. There was not an observable trend between the acidity/basicity the compounds to the type of suitable extraction column. Cyclone HTLC can be used as the column of choice when the *c* log *P* of the compound is >0, while Polar Plus can be used as the column for compounds with *c* log *P* < 0. Columns such as Cyclone P are also available from Cohesive Technologies for compounds with moderate polarity, however, Cyclone HTLC was able to provide the same extraction for these compounds. These results show that, the difference in the stationary phase between the Cyclone HTLC (polymer-based particle) versus the Polar Plus (silica based particles) renders the differences in their interaction with compounds that have *c* log *P* > 0 (moderately hydrophilic to hydrophobic) and *c* log *P* < 0 (extremely hydrophilic). The *c* log *P* criteria, a calculated property and is readily available in many cases, can thus be used as a column pre-selection guide in order to save method development time in a fast-paced discovery setting.

Typically, an extraction column had a lifetime of 1000 injections. Poor chromatographic peak shape, loss of sensitivity and high system backpressure were some of

Table 2
List of *c* log *P* of compounds and the corresponding extraction column

Compound	Rule of 5[a]	MW	*c* log *P*	Extraction column	Structural type
A	Ok	191.25	−1.2	Polar Plus	Neutral
B	Ok	169.25	−1.0	Polar Plus	Basic
C	Ok	159.26	−0.9	Polar Plus	Basic
D	Ok	171.27	−0.7	Polar Plus	Acidic
E	Ok	244.34	−0.3	Polar Plus	Acidic
F	Ok	211.3	0.8	Cyclone	Acidic
G	Ok	479.55	1.0	Cyclone	Basic
H	Ok	402.45	1.2	Cyclone	Basic
I	Ok	467.53	2.9	Cyclone	Basic
J	Ok	435.9	3.4	Cyclone	Acidic
K	Ok	479.64	4.0	Cyclone	Neutral
L	Ok	422.53	4.4	Cyclone	Basic
M	Ok	406.53	4.4	Cyclone	Acidic
N	Ok	463.64	4.8	Cyclone	Basic
O	Ok	436.56	4.9	Cyclone	Acidic
P	Ok	420.56	4.9	Cyclone	Basic
Q	Ok	431.59	5.1	Cyclone	Basic
R	Ok	477.67	5.3	Cyclone	Acidic
S	Ok	491.7	5.7	Cyclone	Acidic

[a] The Rule of 5 is an approximate measure of whether the solubility and permeability of the compound exceeds levels for a "typical" drug molecule [41]. A "typical" drug molecule consists of molecular weight <500.0, *c* log *P* < 5.0, hydrogen bond acceptors <10, hydrogen bond donor <5.

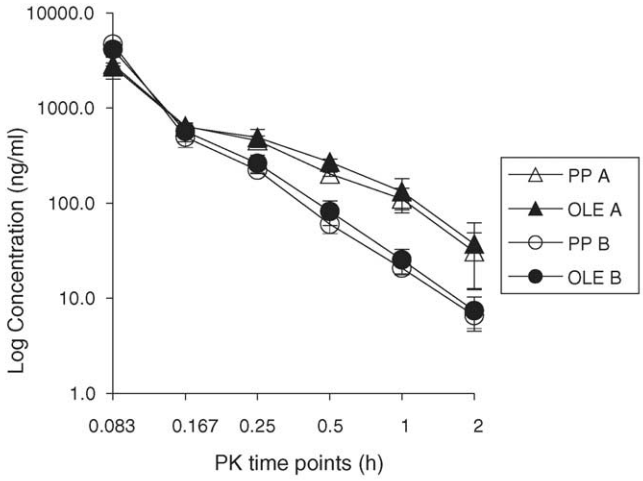


Dose 5 mg/kg PO	Compound A			Compound B		
PK parameter	Protein Precipitation	Online Extraction	%Difference	Protein Precipitation	Online Extraction	%Difference
C_{max} (ng/mL)	5480	3940	28.1	2270	2530	-11.5
AUC $_{(0\text{-}8)}$ (ng*h/mL)	6870	5310	22.7	2110	2320	-10.0
Mean Residence Time (h)	0.975	1.09	-12.2	0.89	0.93	-4.5

Fig. 4. Overlay of mean concentration–time profile and comparison of pharmacokinetic parameters of two compounds (structural class A) from an intravenous cassette dosing; concentrations ($n = 3$ per time point) were determined by online extraction/mass spectrometry technique and protein precipitation LC–MS technique.

the indicators of a degrading column. Lot to lot variability between extraction columns was high. In some instances, even with a new column, the backpressure of the system was high and a different column had to be used. A typical cassette PK study resulted in ∼80 samples for analysis. Since the flow rate (2 mL/min) was lower than a typical turbulent flow analysis (4 mL/min), the solvent waste output was manageable. Smaller dimension columns (0.5 mm) with the same stationary phases have subsequently become available from Cohesive Technologies, which are designed to reduce the solvent consumption. The run time was 1.2 min, which was less than a typical isocratic analysis run time of 2.5 min on a 2.1 mm × 50 mm analytical column. The cost associated with an extraction column is reasonable, however, the cost associated with the analytical column was eliminated in this method.

There is a timesaving both during sample preparation and sample analysis when using online extraction technique. During sample preparation, the typical supernatant transfer step is eliminated and the run time for sample analysis is reduced by half. The significant timesaving is apparent in the method development front since the $c \log P$ values can be used as a guide for column selection and the mobile phases can be used generically for most compounds. As with any analytical tool, the method described is not a one size fits all, but was developed to be as universal as possible. The limitations such as lack of HPLC separation and carryover/column memory effect should be considered and the method should be used in context with the problem at hand. Since a continuous feedback loop approach to building drug-like properties into new chemical entities simultaneously with improving the pharmacological potency, has replaced the traditional linear process, the relevance and capacity of in vivo PK studies has developed as a key issue in early stage of drug discovery. Such early discovery projects were throughput is critical can benefit from using approaches such as cassette dosing in conjunction with online extraction techniques as described in this paper, where typically compounds are binned or rank ordered with respect to a particular ADME property. In most cases, the promising candidates will be further studied for the determination of their complete PK profile using more definitive methods. Hence, the risk versus benefit



Dose 5 mg/kg IV	Compound A			Compound B		
PK parameter	Protein Precipitation	Online Extraction	%Difference	Protein Precipitation	Online Extraction	%Difference
C_{max} (ng/mL)	2870	2710	5.6	4880	4140	11.5
AUC $_{(0-8)}$ (ng*h/mL)	573	617	-7.7	512	493	3.7
Clearance (mL/min/kg)	29	27	6.9	33	34	-3.0
Volume of Distribution (mL/kg)	721	744	-3.2	248	302	-21.8
Half-life (h)	0.39	0.41	-5.1	0.23	0.24	-4.3

Fig. 5. Overlay of mean concentration–time profile and comparison of pharmacokinetic parameters of two compounds (structural class B) from an oral cassette dosing; concentrations ($n = 3$ per time point) were determined by online extraction/mass spectrometry technique and protein precipitation LC–MS technique.

should be considered prior to use of such high throughput techniques.

4. Conclusion

A simple user-friendly online extraction system has been described, that can be set up with two pumps, an autosampler, a six-port switching valve and a mass spectrometer. The system can be run with no additional commercial software besides the LC–MS system software. Various analytical conditions such as extraction column, mobile phase, run time, wash solvent were optimized to establish a analytical method that was simple, easy to set up and generic. No additional sample preparation effort is needed besides dilution of the plasma sample with water and centrifugation in 96-well plate format. The system was used to analyze in vivo cassette samples for various structural templates. Concentration and PK data obtained from the online extraction method were compared with the protein precipitation method. The difference in the calculated PK parameters between the two methods was within 30%, which is considered acceptable for early discovery projects. The system was suitable for analysis of compounds with $c \log P > 0$ when using the Cyclone HTLC extraction column. However, the extraction column and mobile phase conditions were modified for analysis of compounds with $c \log P < 0$. Compound lipophilicity can be used as a tool for column pre-selection, which saves method development time for early discovery studies. Overall, this simple, robust online extraction system provides cost savings by minimizing the sample preparation and method development time.

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