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Generic approach to high throughput ADME screening for lead candidate optimization

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Abstract

The use of liquid chromatography/mass spectrometry (LC/MS) methodologies has revolutionized the way compounds are analyzed in the pharmaceutical industry due to its high selectivity and high sensitivity. At Cephalon, we have developed a single generic method that we use for our entire high throughput absorption, distribution, metabolism and excretion (ADME) screening. The generic method eliminates the need to develop a new method for each new compound being screened and tremendously reduces sample preparation time by using turbulent flow chromatography for on-line extraction of biological matrices. The combination of several different in vitro and in vivo screens that are all analyzed by a single LC/MS/MS method allows us to generate data for lead candidate selection rapidly with minimum effort.

Keywords: ADME screening; Lead candidate optimization; Generic methods; Turbulent flow chromatography

1. Introduction

There is a growing trend in the pharmaceutical industry toward rapid screening for pharmacological relevant properties early in the drug selection process. The aim is to increase the likelihood of success when compounds move into development by finding ways to improve the drug like character of potential drug candidates. At Cephalon, we run a series of in vitro and in vivo screens to evaluate the chemical and physical properties of new drug candidates. Most of these screens depend on the use of LC/MS due to its high sensitivity and selectivity. Extremely fast run times are achieved by employing multi-reaction monitoring (MRM) because the separation of the various components present in a sample is done by the mass spectrometer. Compounds no longer need to be separated in time chromatographically as they would with conventional HPLC methods [1–4]. The result is 2–3 min run times that are ideally suited to screening large numbers of samples quickly.

The ability to assay samples in a few minutes shifted the bottleneck of high throughput screening away from analysis time to sample preparation. On-line sample cleanup techniques have also come of age in the last ten years. Several types of on-line columns are available that include restricted access media (RAM) [5–8], turbulent flow (TF) [9–18], solid phase extraction (SPE) [19–23], and monolithic [24–30].

A drawback to using on-line cleanup columns alone is the relatively poor chromatography that results from the lower theoretical plates in these types of columns. Large peak tailing is usually observed that results in lower sensitivity and worse reproducibility [31–33]. Dual column methods perform much better, but take more time [10–15]. However, even the dual column methods did not produce good peak shapes when compared to normal HPLC methods. We decided to use isocratic focusing to more efficiently trap compounds eluting from the sample clean-up columns onto the analytical column [16].

The general methodology of isocratic focusing dual column clean-up methods is as follows. The sample is injected onto a clean-up column under turbulent flow conditions (high flow rates) with 100% aqueous mobile phase.

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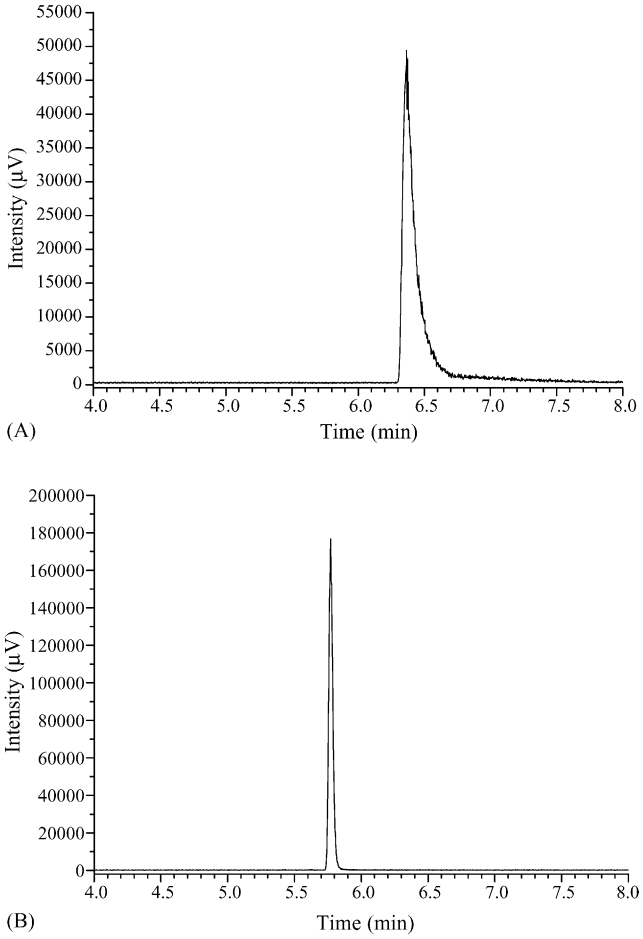


Fig. 1. K252a (100 ng/mL): (A) no isocratic focusing and (B) isocratic focusing.

Small molecules are retained while proteinacious material is washed to waste. Once the compound is extracted from the biological matrix, sample is eluted from the clean-up column with organic mobile phase. The flow from the cleanup column is combined with a 100% aqueous flow from a second HPLC pump prior to reaching an analytical column. The analytical column focuses the compound at the head of the column due to the high aqueous content in the mobile phase after mixing the eluent from the clean-up column with the 100% aqueous flow from the second HPLC pump. Once the compound is transferred to the analytical column, the compound is eluted from the analytical column with a ballistic gradient. A comparison of the dual column methods with and without isocratic focusing is shown in Fig. 1 and Table 1 [11]. Fig. 1 demonstrates a four-fold increase in signal height when using isocratic focusing and Table 1 shows a factor of 2 reduction in %R.S.D. for 10 replicate injections.

The incorporation of on-line sample cleanup shifted the bottleneck in analysis time from sample preparation to methods development. We typically receive many new compounds every week to evaluate in the various screens in place at Cephalon. If new methods were needed for each compound, there would not be enough time left to actually assay the samples. Therefore, to truly take full advantage of high throughput ADME screening, it was necessary to develop a generic method that eliminates the need to develop a new method for each new compound being screened.

Table 1
Comparison of 10 replicate injections over time with and without isocratic focusing

Injection #	Area counts	Standard deviation	%R.S.D.
1–10	8582	157	1.83
100–109	8747	166	1.90
500–509	8259	164	1.98
1000–1009	8955	212	2.37
11–19[a]	7342[a]	310[a]	4.22[a]

[a] No isocratic focusing.

Once we started using isocratic focusing to improve peak shape, it quickly became apparent that isocratic focusing had several other adventitious properties that resulted in our development of a generic method [16]. The isocratic focusing resulted in increasing the column life times of the clean-up columns. The longer column lifetimes are due to the fact that the shape of the peak eluting from the clean-up column becomes irrelevant to what is observed at the detector. There-

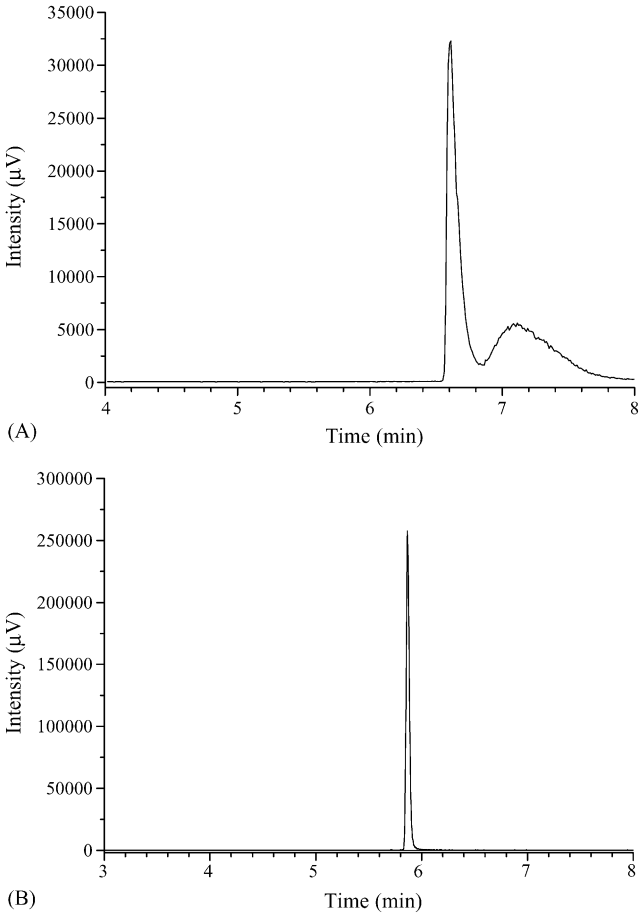


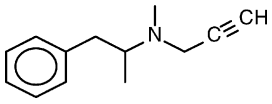
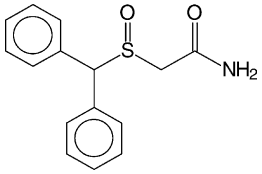
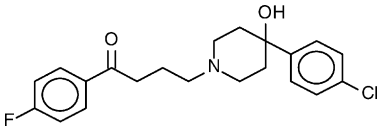
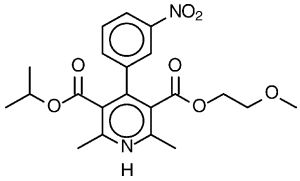
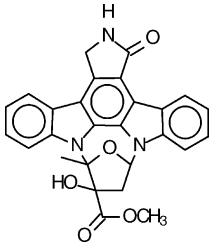
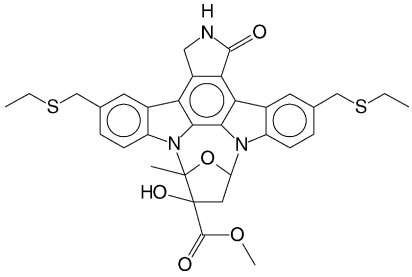
Fig. 2. K252a (100 ng/mL) after 1000 injections of plasma: (A) without isocratic focusing and (B) with isocratic focusing.

fore, once the peak shapes start to deteriorate on the clean-up you can still continue to use them. Fig. 2 illustrates this point. The refocusing of the peak eluting from the clean-up column is responsible for the development of a generic method since the shape of the peak eluting from the clean-up column has no effect on the peak seen by the detector eluting from the analytical column. One only has to guarantee that the entire eluting peak is collected during the transfer from the clean-up column to the analytical column. Therefore, by choosing the appropriate percent organic in the mobile phase for elution from the clean-up column, the hydrophilic compounds will elute quickly while hydrophobic ones will elute as broader peak over time. Both peak shapes will be less then ideal as they elute from the clean-up column, but both types of compounds will produce excellent responses at the detector because of the isocratic focusing done at the head of the analytical column. A wide range of hydrophobicities can be analyzed by the same method as long as the entire peak is collected during the transfer step.

Six compounds with a large range of hydrophobicites were chosen to illustrate the utility of a generic method [16]. Their structures are shown in Table 2. The MRM chromatogram for each compound at 5 ng/mL in rat plasma is shown in Fig. 3. All the compounds have good sensitivities, high recoveries (>90%) and excellent peak shapes. The lack matrix effects are demonstrated in Fig. 4 [16], as no differences are observed in the chromatography from the biological matrix.

The method has been used successfully on over 4000 compounds with better then 97% applicability (no changes). Drug substances in the presence of plasma, urine, brain ho-

Table 2
Molecular structures and mass spectral parameters [16]

Compound	MW	Structure	Transition	Cone voltage	Collision energy (eV)
Deprenyl	187		188 → 91	30	20
Modafinil	273		274 → 167	20	20
Haloperidol	375		376 → 165	40	20
Nimodipine	418		419 → 343	30	10
K252a	467		468 → 336	60	40
CEP-1347	615		616 → 554	20	15

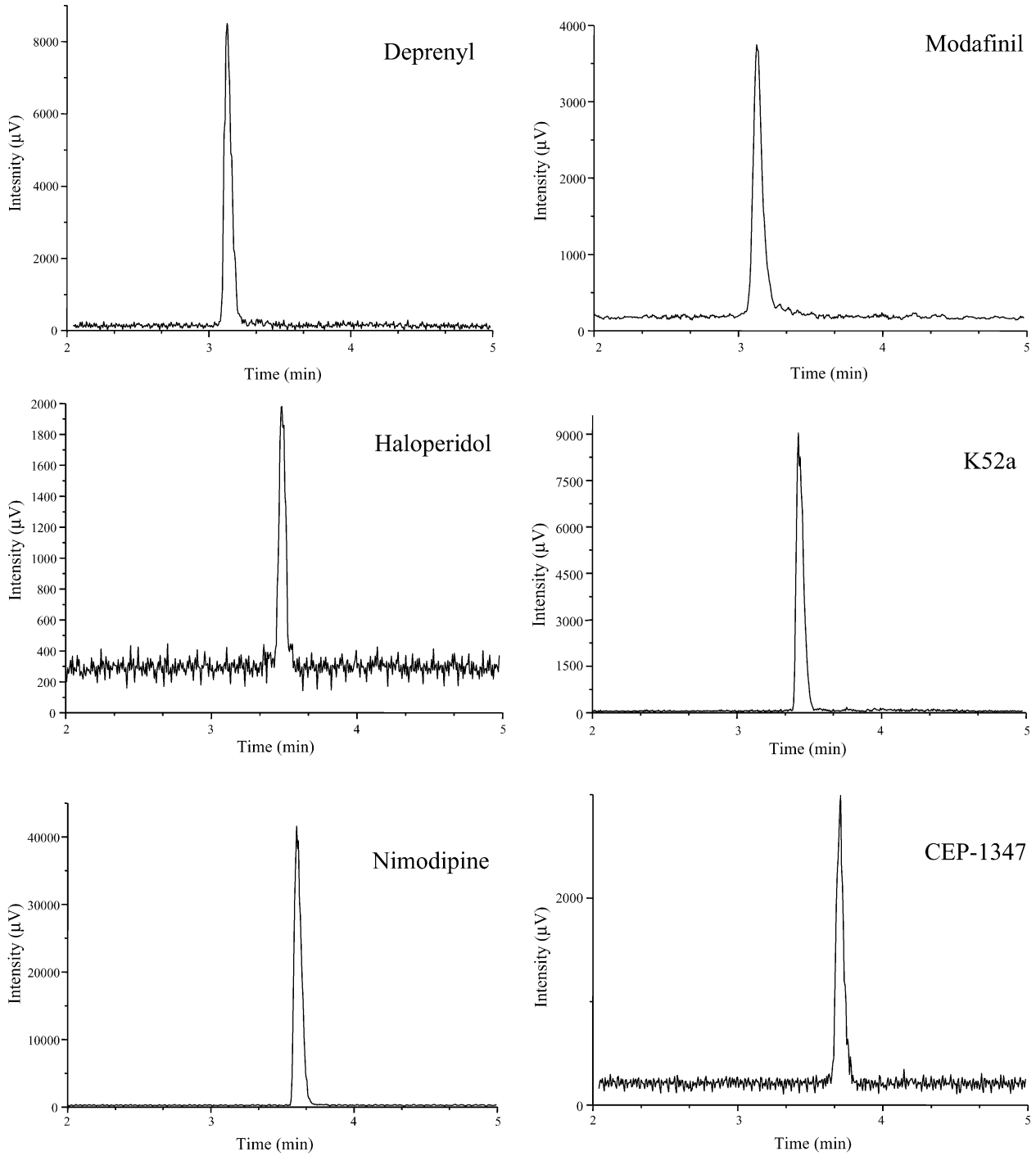


Fig. 3. MRM chromatograms from Deprenyl, Modafinil, Haloperidol, Nimodipine, K252a, and CEP-1347 at 5 ng/mL in rat plasma [16].

mogenate, liver homogenate, intestinal perfusates and cerebrospinal fluid have all been analyzed. Column lifetimes are 1000–1500 injections.

Recently, we have made some improvements to previously reported methods to address some of the problems we encountered. This article will address the improvements in analysis time, mobile phase consumption and carry over, as well as the types of in vitro and in vivo assays we use for lead candidate.

2. Experimental

2.1. Sample preparation

Samples are prepared by adding 200 μL of acetonitrile containing an internal standard to 100 μL of plasma. The sample is centrifuged for 10 min at 10,000 rpm and the supernatant is placed into an autosampler vial.

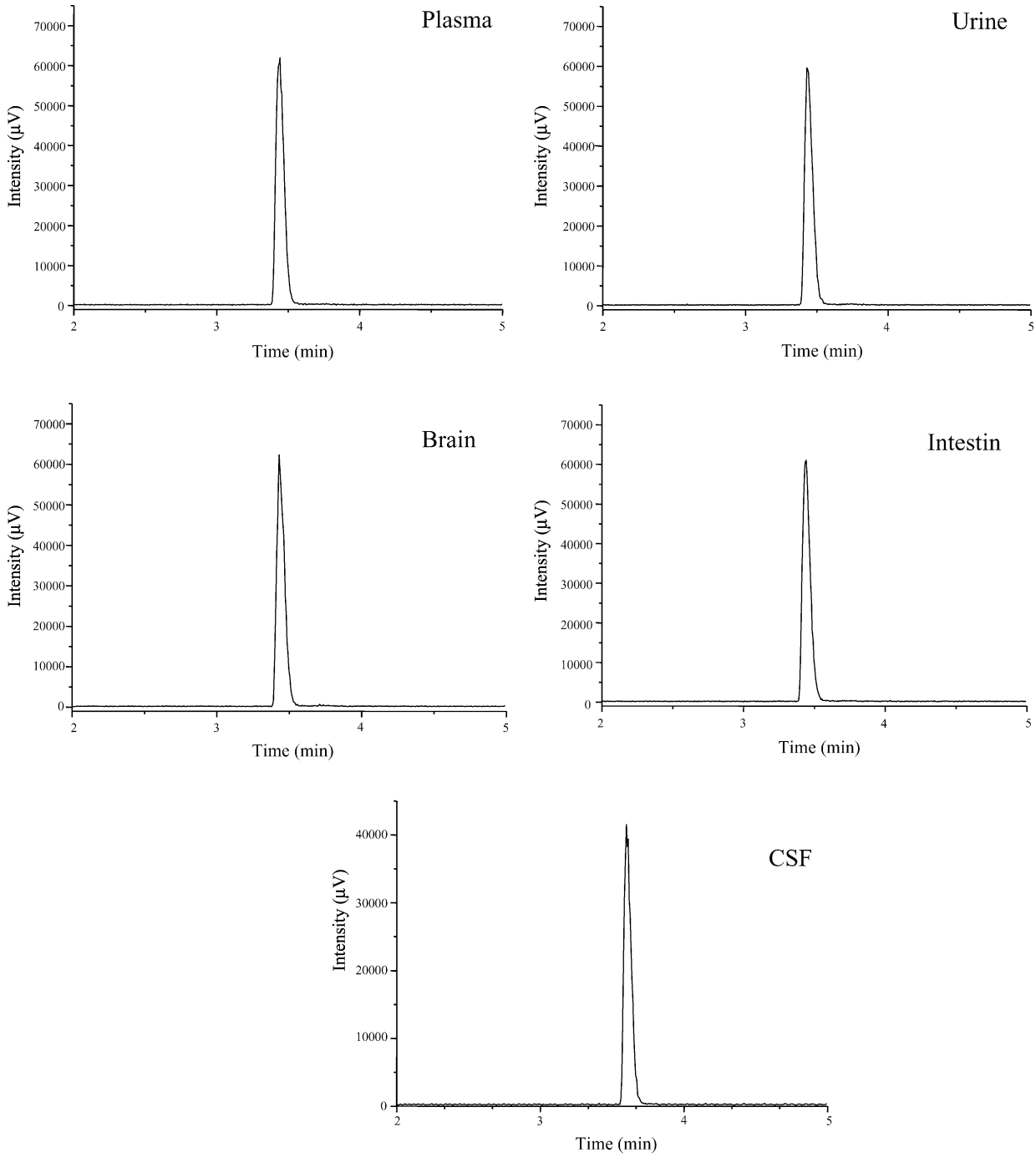


Fig. 4. K252a (50 ng/mL) in rat plasma, urine, brain homogenate, intestinal perfusates, and cerebral spinal fluid [16].

2.2. HPLC

A Cohesive Technologies 2300 HTLC system in the two-column configuration is used. The HPLC parameters are listed in Table 3. Figs. 5–8 illustrate the system configuration during sample cleanup, sample transfer, sample elution, and column equilibration, respectively.

Samples are cleaned for 30 s at 1 mL/min with 100% aqueous mobile phase. The flow is then slowed to 0.3 mL/min for 6 s prior to transferring the sample to the analytical column. Samples are then back-flushed off the clean-up column onto the analytical column at 0.3 mL/min with the 40% organic mobile phase stored in the loop of valve 1. The 0.3 mL/min flow is connected to a tee inside valve 2 to a 1.2 mL/min 100% aqueous mobile phase and concentrated on the analytical column for 60 s. The mobile phase in the analytical column is 8% organic during the transfer step, which is sufficiently weak enough for most compounds to be retained. Once the sample is transferred to the analytical column, the samples are eluted with a ballistic gradient from 100 to 0% aqueous in

Table 3
HPLC experimental conditions

Time (min)	A (%)	B (%)	Valve position	Flow (mL/min)
Pump 1				
0.00	100	0	Load	1.0
0.50	100	0		0.3
0.58	60	40	Inject	0.3
1.57	60	40		0.3
1.58	60	40	Load	1.5
2.08	0	100		1.5
3.08	100	0		1.5
4.83	100	0		1.5
Time (min)	A (%)	B (%)	Tee position	Flow (mL/min)
Pump 2				
0.00	100	0	Out	1.2
0.58	100	0	In	1.2
1.58	100	0	Out	1.0
3.07	5	95		1.0
3.57	5	95		1.0
3.58	100	0		1.2
4.83	100	0		1.2

Pump 1: clean-up column, Cyclone P HTLC, 0.5 mm × 50 mm; injection volume, 25 μL; solvent A, 0.05% formic acid in H_2O; solvent B, 0.05% formic acid in ACN. Pump 2: analytical column, Eclipse XDB C18, 4.6 mm × 15 mm, 3 μm, 120 A; solvent A, 0.05% formic acid in H_2O; solvent B, 0.05% formic acid in ACN.

90 s at 1 mL/min. During the elution step the loop on valve 1 is refilled with 40% organic mobile phase and the turbulent flow column is washed. The system is then re-equilibrated for 75 s.

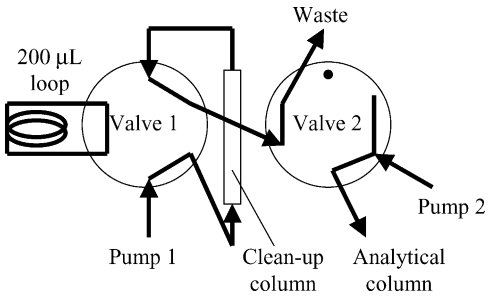


Fig. 5. Valve configuration during sample loading and clean-up (0 min): valve 1, load; valve 2, out [16].

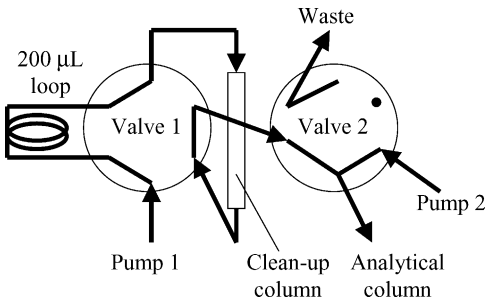


Fig. 6. Valve configuration during sample transfer (0.58 min): valve 1, inject; valve 2, in [16].

2.3. Mass spectrometry

A Micromass Quattro II with a Z-spray source is used. Standards of each compound are tuned on using Multi-Reaction Monitoring (MRM) with Argon as the collision gas in order to optimize the MS conditions.

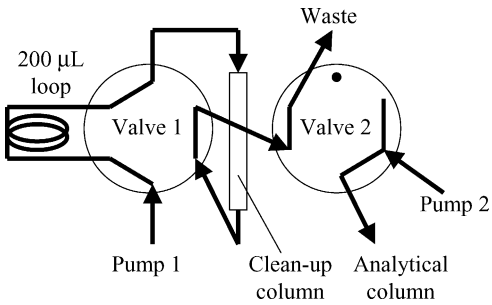


Fig. 7. Valve configuration during sample elution and loop fill (1.58 min): valve 1, inject; valve 2, out [16].

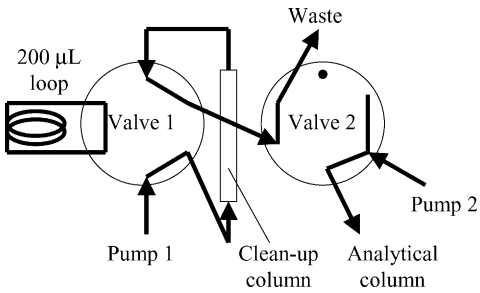


Fig. 8. Valve configuration during column equilibration (3.07 min): valve 1, load; valve 2, out [16].

3. Results and discussion

3.1. Mobile phase consumption

One of the criticisms of TF, SPE, and monolithic columns is the large amount of mobile phase used when flowing at 4 or 5 mL/min. In addition, to elute directly into a mass spectrometer at these high flow rates, a split flow is required. To achieve turbulent flow conditions at lower flow rates, a narrower bore TF column is now being used. The reduction to a 0.5 mm column i.d. allows turbulent flow conditions to be reached a 1 mL/min. The smaller bore column also reduces the column volume such that the transfer time from the TF column to the analytical column can be reduced (using the same flow rate during the transfer step) and the equilibration times are shorter. The result is a shorter run time (<5 min, injection to injection) and lower mobile phase consumption.

3.2. Carryover

In early drug screening, carryover is not a major concern. Typical carryover with our generic method is 0.1–0.2%. However, about 10–12% of the compounds, we analyze have larger carryover that can be 1–2%, which will not significantly impact rank ordering in early lead candidate optimization studies. For example, if the oral bioavailability of a compound was measured at 20%, then it is not critical whether it is actually 19 or 21%. The fact that the bioavailability is not 2 or 80% is what is needed to decide how compounds compare to others. Furthermore, the compounds in a disease area usually are fairly similar and behave equivalently such that carryover effects within a class are all about the same. However, if one wants to move this type of assay into the good laboratory practices (GLP) environment, carryover issues must be addressed.

There are basically two ways in which carryover can be reduced or eliminated; however, both require some methods development specific to the problematic compound. In a GLP environment, method validation is needed so spending a little time on the method does not really slow you down. For early screening purposes, the methods need to be generic with minimal time spent on carryover issues unless carryover becomes greater then 2%, which we have not observed.

One major source of carryover, excluding the obvious issues such as worn rotor seals, is the wash solvents used to clean the injector. This situation is more problematic to those who use straight plasma since protein precipitation is possible. We perform a crude protein precipitation during sample preparation (see Section 3.3). Methanol is used as wash solvent one and acetonitrile is used as wash solvent two. We do not encounter any protein precipitation because of our sample preparation. However, if unacceptable carryover is encountered, then the appropriate wash solvents for the compound will be required. Other solvents being used include 50/50 isopropanol/acetonitrile or 10% DMSO in methanol. The appropriate pH will help for acidic or basic compounds.

The second major source of carryover is from the turbulent flow column itself. Thorough cleaning of the column is necessary, since the mobile phase may not be sufficient to clean the column between runs. Use of a quaternary pump instead of a binary pump will allow the use of other mobile phases to wash the turbulent flow column between samples. An example of this approach been published by Grant et al. [34].

3.3. Sample preparation

While neat biological fluids can be analyzed directly with turbulent flow columns, we have found that some sample preparation is necessary to be truly generic in the approach. There are several criteria that need to be met to inject neat biological fluids in a high throughput screening mode.

First, the compound of interest must be stable in the biological matrix being used. Since there is no time to perform stability studies on every compound in all matrices, the safer approach is to destroy the enzymes that may be present by adding some organic. It is not necessary to achieve a thorough protein precipitation since the sample will be cleaned by turbulent flow column; however, the enzymatic activity should be destroyed. When validated GLP studies are needed, there will be time to investigate the stability of compounds in the biological matrices, and therefore, this step may not be needed. We chose to err on the side of caution for compounds where the stability is not known, and thus, spike in some organic.

Second, many of our compounds have extremely low water solubility (<100 ng/mL). We cannot make standards and QC samples by spiking directly into the biological matrix without using some organic.

Third, most of our samples are frozen. When the samples are thawed, there is some solid proteinaous material present. Centrifugation to remove the supernatant is needed; therefore, adding organic, which will precipitate some proteins, does not add any additional steps to the sample preparation. Therefore, our sample preparation consists of adding internal standard in acetonitrile 2:1, centrifuging and removing the supernatant. This approach is a limited protein precipitation that does not perform a thorough cleanup of the sample. Fig. 9 shows an example of samples prepared with and without the use of the turbulent flow column. It is quite apparent how much cleaner the turbulent flow method is relative to injecting the same sample preparation directly onto the analytical column.

If the compound of interest is stable in the biological matrix and standards can be spiked directly into the matrix, then neat biological fluids can be used. The column lifetimes are shortened by factor of 2 (500–600 injections) when using neat biological fluids.

3.4. Exceptions

Compounds that do not lend themselves to this method fall into two categories. First, very hydrophilic compounds

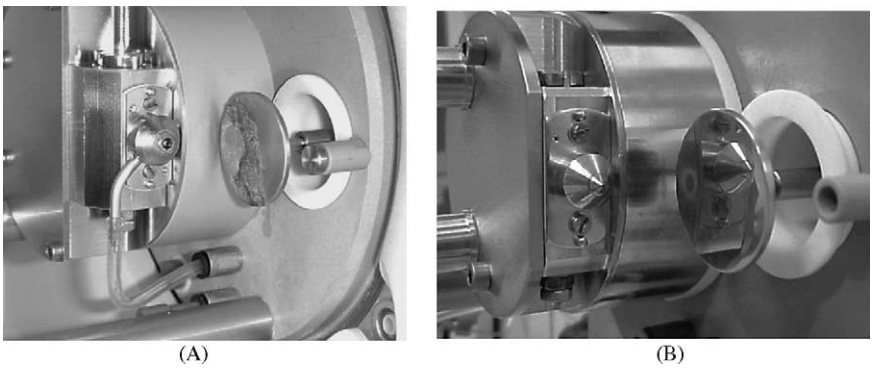


Fig. 9. Comparison of protein precipitated plasma: (A) 200 injections without turbulent flow cleanup and (B) 800 injections with turbulent flow cleanup.

may not be retained by either the Cyclone P HTLC column or on the analytical column at 8% organic. We have not screened a compound to date that was not retained during the clean-up step on the Cyclone P HTLC column. However, 2–3% of the compounds we have tested are not completely retained on the analytical column during the transfer step. The result is tailing on the front end of the peak due to partial migration of the compound through the column isocratically at 8% organic during the transfer step. An example is shown in Fig. 10 for caffeine. The peak tailing introduces more error (less reproducibility) and lowers sensitivity due to peak broadening. However, if sensitivity and reproducibility requirements (LOQ of 1 ng/mL and all standards and QC's within 20% of expected) are met, then the method is used with no revisions. The peak tailing can be eliminated by transferring the compound from the clean-up column to the analytical column with less organic in the mobile phase or by selecting an analytical column that will retain hydrophilic compounds more strongly.

Second, extremely hydrophobic compounds may not be completely transferred to the analytical column at 40% organic, thereby reducing recovery, and thus, sensitivity. Only 0.3% of the compounds we have tested did not meet our sensitivity requirement due to this problem. Increasing the percent organic in the loop used to transfer the sample onto the analytical column to 60% allowed these compounds to be transferred efficiently.

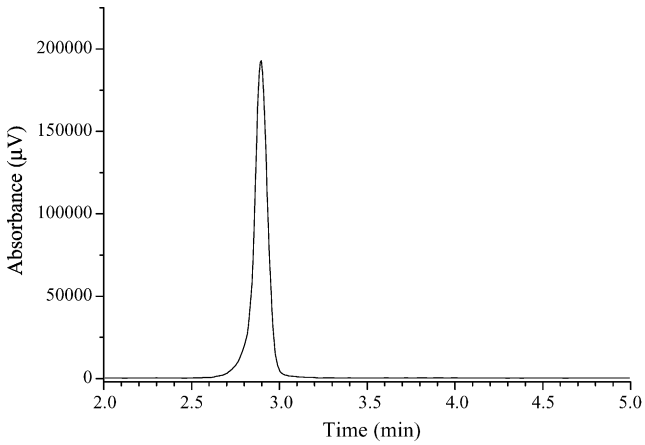


Fig. 10. MRM chromatogram of caffine at 5 ng/mL in rat plasma.

3.5. *In vivo methods*

An excellent review of the uses of LC/MS in drug development can be found in a book written by Lee in 2002 [35]. The details of the methods we employ for characterizing new drug entities are discussed in this book and it is recommended reading for those who want to get a more in depth description of these methods and approaches. Our contribution to these assays is that we use the same generic method for quantifying drug substance regardless of how the samples were generated. The types of assays performed at Cephalon using our generic method will be outlined briefly below.

The advantages of using our method on biological samples are obvious. The on-line sample cleanup removes matrix interference and drastically reduces sample preparation time. The nature of the biological matrix does not affect the utility of the method [16].

In vivo screens performed at Cephalon that use this generic method include the following: oral bioavailability is measured by comparing plasma levels from IV and oral dosing. These studies are initially done in rats but mice, dogs, and monkeys are used as compounds successfully move further along in the screening process. Dose escalation studies are then done to determine if the plasma levels are proportional to the dose. This becomes critical for later toxicology studies. Target tissue organ levels are also measured. The blood brain barrier is the predominate type of target tissue study done at Cephalon but any tissue can be targeted depending on disease being investigated. Compounds that show promise in the previous screens go through formulation comparison and route of administration screening assays. Once the route is chosen, efficacy studies are preformed to determine the projected dose. Finally, 10-day tolerability studies are preformed to evaluate toxicity. All of these screens are done using the same generic method. Compounds that have survived past the

10-day tolerability studies are now candidates for elevation into drug development at which point GLP methods will be needed.

3.6. In vitro methods

In addition to the in vivo methods, several in vitro tests are preformed using the same generic method. While the need to clean samples does not have the same urgency as it does with biological samples, there are still benefits to sample cleanup for in vitro methods and the ability to run all samples with the same analytical method is priceless. The in vitro screens are designed to reduce the amount of compounds that require animal studies by predicting possible ADME problems. Most of these studies do not require LC/MS analysis, but there are two types of studies where we use the same generic method that we use in the in vivo studies. These studies are parallel artificial membrane permeability assays (PAMPA) and metabolism assays.

PAMPA has only been commercially available for the last couple of years but has become very effective in predicting permeability in the GI tract [36–41]. The measured flux is related only to the passive diffusion through a membrane. Active transport and Eflux limited events will not be predicted. Cephalon uses the PAMPA system sold by pIon Inc. (Woburn, MA) with a few changes from the commercially available package. First, we use a soy lethin extract for the phospholipid membrane rather then the blackbody one originally supplied by the vendor. It has been shown that the soy lecithin membrane is a better predictor of permeability in the GI tract [42,43]. Even pIon, Inc. has suggested this change and supplies the soy lecithin as well. Second, and perhaps more importantly, we have found the UV plate reader normally used to quantify the samples in the PAMPA assay to be completely inadequate for many compounds.

There are two types of compounds that result in sensitivity problems for the UV plate reader; compounds that have weak chromophores and compounds with low solubility in water. Sensitivity is not an issue for these compounds using LC/MS methods.

Employing MRM methods also eliminates purity problems since only the compound of interest is detected. The UV plate reader does not distinguish between impurities and the compound of interest and, since many of the initial screens are done on compounds that are only 90% pure, the presence of impurities could change what is measured in the PAMPA assay. Furthermore, stability problems are also eliminated as the degradation products will not be detected in the MRM experiment but can interfere with the UV response.

Permeability through any cell membrane can be investigated by choosing the appropriate phospholipids to mimic the barrier of interest. There are many articles on mimicking the GI tract and commercially available instruments for high throughput screening are available [36–43]. Blood brain barrier (BBB) penetration is extremely important at Cephalon

Table 4
Comparison of measured brain to plasma ratios with the permeability predicted by PAMPA

Compound	B/P (%)	PAMPA
Poor permeability		
1	1	0.01
2	2	0.47
3	2	0.52
4	3	0.01
5	3	2.64
6	5	2.14
7	6	0.60
8	8	11.32
9	9	0.38
10	10	0.44
Acceptable permeability		
11	11	1.20
12	12	1.92
13	12	2.70
14	13	3.65
15	14	1.32
16	14	15.99
17	15	0.85
18	17	1.07
19	22	1.62
20	24	6.59
21	24	1.70
22	28	8.88
23	35	1.70
24	36	6.59
25	37	9.97
26	40	4.66
27	45	4.96
28	48	1.19
Good permeability		
29	50	7.75
30	51	15.08
31	53	1.59
32	55	5.21
33	74	0.34
34	75	0.7
35	76	10.25
36	87	14.73
37	102	2.65
38	112	16.67
39	112	20.8
40	124	17.46
41	125	10.17
42	172	22.8
43	187	22.8
44	219	8.06
45	229	eq (high)
46	231	3.96
47	252	16.65
48	271	14.32
49	271	5.97
50	294	25.22
51	496	14.21
52	841	eq (high)

eq, equilibrated; B/P, measured brain/plasma ratio; PAMPA, permeability in 10^{-6} cm/s.

Table 5
Correlation of permeability predicted by PAMPA to actual measured blood brain barrier measurements

Actual	Predicted by PAMPA		
	Poor	Acceptable	Good
Poor	7	2	1
Acceptable	1	12	5
Good	2	3	19

<1, poor; 1–5, acceptable; >5, good.

since one of the major focuses is on central nervous system (CNS) therapeutics. Di et al. [44] have developed an excellent method for assaying permeability through the BBB. Cephalon has developed a similar BBB PAMPA method. A whole brain extract is used at Cephalon rather then the polar extract Dr. Di uses. A comparison of measured BBB adsorption (brain to plasma ratio) to the predicted PAMPA assay is shown in Tables 4 and 5. The assay is 90% correct at the high and low ends. Compounds predicted with medium permeability are less accurate (75%). All of the false negatives were structurally similar and believed to be actively transported.

Metabolism screens are done with S9 liver preparations (whole liver enzymes) or with selected liver microsomes. Both assays are preformed in a 96 well plate format and analyzed with the generic LC/MS method. The S9 screens are done on all compounds to look for gross metabolism liabilities. Only the concentration of parent drug is measured at this stage. Identification of the metabolites formed will not be preformed until the compound is elevated to development status. Later in the screening process, when half a dozen lead compounds are being evaluated for final selection, individual microsomes are investigated for possible metabolic routes and potential drug/drug interactions. Once again, only the concentration of the parent drug is measured in order to identify possible metabolic liabilities.

4. Conclusion

A generic method was developed at Cephalon that takes advantage of turbulent flow chromatography in conjunction with LC/MS/MS techniques for high throughput screening of drug candidates in the presence of biological matrices. The method has been used to screen over 4000 compounds thus far and has met our sensitivity and reproducibility criteria on over 97% of the compounds tested. The advantage of this method is that development time is no longer needed to investigate new drug candidates, and little, if any, sample preparation time is required. The result is increased productivity through higher sample throughput.

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