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Characterization of a technique for rapid pharmacokinetic studies of multiple co-eluting compounds by LC/MS/MS

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Abstract

A method for rapid pharmacokinetic screening of multiple potential drug candidates has been developed. This technique, based on the ability of liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) to independently monitor multiple components, enables the quantification of substances which may or may not be chromatographically resolved. Our results indicate that the limit of quantitation and accuracy of this multiple-compound LC/MS/MRM quantitation method are comparable to a single-compound LC/MS/MRM quantitation method. No apparent ion suppression due to the existence of extraneous compounds in the analytical solution and biological matrix effect are observed in the range of the calibration curve. The issue of potential residual molecule cross-talk interference existing in the multiple-reaction monitoring mode has been discussed. This multiple-compound LC/MS/MRM quantitation method can be used for high throughput pharmacokinetic screening and to assay mixtures that have co-eluting analytes or similar m/z of precursor/product ion pairs.

Keywords: Pharmacokinetic; Multiple compound dosing; LC/MS/MS; High throughput

1. Introduction

Drug discovery increasingly relies on the ability to rapidly identify molecules at a very early stage that meet a variety of requirements with respect to both activity and pharmacokinetic behavior. Often the speed of determination of pharmacokinetic parameters such as volume of distribution, clearance, oral bioavailability and elimination half-life becomes a limiting factor. The combination of liquid chromatography and tandem mass spectrometry (LC/MS/MS) has been recognized as a specific and sensitive method for the determination of low levels of drugs and their metabolites in biological fluids [1–3]. Recently, LC/MS/MS has been used in simultaneous screening of mixtures of compounds (up to 12 or more), with both high sensitivity and specificity, in biological fluids for increased sample throughput in drug discovery research [4–6]. This strategy has a great impact

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on the screening of large numbers of compounds and identifying potential drug candidates in a high-speed manner. However, there are two major concerns related to this multiple-compounds cassette dosing. First, there is a risk of drug–drug interaction or pharmacokinetic influences when multiple compounds are co-administered to a single subject. The strategy of co-administering compounds from the same therapeutic target class and including a compound with known pharmacikinetic parameters as a 'biological internal standard' in the mixture can help to identify and reduce problems associated with drug–drug interaction including metabolic inhibition or induction. A second concern arises from the possible influences of multiple-compounds study in the LC/MS/MS assay, since structures and the corresponding precursor/product ion pairs from same dosing mixture group are often very close. Complete chromatographic separation of each component is not always straightforward and practically achievable, especially for a very short run times.

Our primary objective was to develop a LC/MS/Multiple Reaction Monitoring (LC/MS/MRM) quantitation method with a LC analysis time less than 5 min and with the flexibility to handle compounds which may co-elute on the chromatographic column and may have similar MRM patterns. Attempts have been made to ensure that the effort required for method development is minimal and the method provided is sensitive and accurate in comparison to a single-compound assay method. The quality of the data generated by this method has been validated. Issues such as possible ion suppression due to the existence of extraneous compounds in the analytical solution, the potential of residual product ion interference problems existing in the mass spectral multiple-reaction monitoring mode and the effect of biological matrix on the sensitivity have been discussed here. It is important to point out that for a high throughput pharmacokinetic screen, the criteria for acceptable data can be less stringent since further evaluation of selected interesting compounds from cassette dosing will follow by dosing as single compounds in the same species.

2. Experimental

2.1. Chemicals

All investigated compounds were synthesized at Merck Research Laboratories, Rahway, NJ. Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ), ammonium acetate from Regis Technologies, Inc. (Morton Grove, IL and pyrogen-free hydroxypropyl-β-cyclodextrin from Cerestar USA, Inc. (Hammond, IN). Oasis™ HLB extraction cartridges (3 ml) were obtained from Waters (Milford, MA). High purity liquid nitrogen (99.999%) was obtained from JWS Technologies (South Plainfield, NJ).

2.2. Standard and sample preparation

Standard stock solutions of either tested compounds or internal standards were prepared as 1 mg ml^{-1} solutions (of the free base) in 50/50 (v/v) acetonitrile/water. A series of standard solutions 4, 20, 40, 80, 200, 400, 800 and 2000 ng ml^{-1} were prepared by the dilution of the stock solution. For the multiple-compounds LC/MS/MRM quantitation method, standard solutions included all components of these related multiple compounds and the concentration of each compound was adjusted to 4, 20, 40, 80, 200, 400, 800 and 2000 ng ml^{-1}. The stock solution of M-6 was prepared to have 1 mg ml^{-1} (free base) of each of the six compounds in 50/50 (v/v) acetonitrile/water. The precursor and product ions of these six compounds are different from compounds A-D. Their retention times in the LC system described below were 0.9, 1.3, 1.4, 1.6 and 1.7, min, respectively.

Aliquots of 100 μl normal rat plasma were fortified with 25 μl standard solution and 25 μl internal standard solution for the plasma calibration standards and the plasma QC samples. The calibration curve covered the range of 1–500 ng ml^{-1}. Oasis cartridges were used to isolate analytes of interest from either fortified normal plasma or sample plasma by solid phase extraction (SPE).

The efficiency of the solid phase extraction was measured in the following way. A 100 μl aliquot of normal plasma was spiked with 25 μl analyte solution and 25 μl internal standard solution and then purified by SPE. The same amount of normal plasma spiked only with 25 μl internal standard solution was similarly purified by SPE first, after which 25 μl analyte solution were added. The peak area ratio of the two as determined by LC/MS/MRM was taken as the extraction efficiency.

2.3. Animal dosing

Individual rats (fasted male Sprague Dawley) were simultaneously dosed either intravenously or orally with a four-compound mixture (Compounds A–D) at 2 mg kg^{-1} each compound. The vehicle used for formulation was 25% (w/v) aqueous hydroxypropyl-β-cyclodextrin. Heparinized plasma was prepared from whole blood collected at various time points following the dose over a 24-h period and stored frozen until assayed.

2.4. Instrument

All LC/MS/MS experiments were performed on a PE-Sciex API 300 triple quadruple mass spectrometry (Concord, ON, Canada) which was coupled to a Shimadzu LC-10AD liquid chromatography system. The stationary phase was Spherisorb CN with a 5 micron particle diameter (Keystone Scientific, Inc., Bellefonte, PA). The column size was 2.0 i.d. × 50 mm for turbo ionspray probe and 4.6 i.d. × 50 mm for heated nebulizer probe. The mobile phase flow rate was 0.2 or 1.0 ml min^{-1} for turbo ion spray probe or heated nebulizer probe, respectively. The mobile phase composition consisted of an 80/20 acetonitrile/water mixture containing 10 mM ammonium acetate.

API 300 tuning and calibration were performed at unit mass resolution in the positive-ion mode with polypropylene glycol in 4:1 CH_3CN–NH_4OAc. The mass spectrometer was operated with a dwell time of 200 ms for each transition in the multiple reaction monitoring mode. Either a heated nebulizer probe (400°C) or a turbo ionspray probe (350°C) was used as an interface to a liquid chromatography system.

3. Results and discussion

Because our laboratory is involved in pharmacokinetic screening of compounds in the very early stage of drug discovery, we have screened more than 300 promising compounds covering five different therapeutic target areas by using this multiple-compound LC/MS/MRM quantitation method. The accuracy of the multiple-compound LC/MS/MRM quantitation method is our primary concern. We have evaluated issues such as possible ion suppression, the potential of residual molecule cross-talk problems and the effect of biological matrix on the sensitivity for some representative compounds in each therapeutic areas. Four compounds (A–D) have been chosen as an example to demonstrate the scope and limitation of the co-eluting multiple-compound LC/MS/MRM quantitation method. The structures of these four compounds are very similar (the structures and therapeutic classes of these investigational compounds cannot be disclosed at this time). Each compound was characterized by LC/MS and LC/MS/MS to ascertain its precursor ion and further to select a product ion for use in multiple-reaction monitoring (MRM). The mass spectral information for precursor ions, product ions and retention time of compounds A–D are summarized in Table 1. Compounds A and B have different precursor ions, but co-elute on the LC column and share some of the same product ions. The only intense product ion of Compounds A and B is 259.4 amu which limits the selection of product ion for simultaneous analysis of Compounds A and B by LC/MS/MRM. We have assayed these four compounds both with the traditional single-compound method and our multiple-compound LC/MS/MRM method.

3.1. Cross-talk interference of residual molecules

Cross-talk of residual molecules is common to all mass spectrometers that employ a high pres-

sure collision cell. When successive product ions are monitored in the MRM mode, cross-talk interference can occur if the time used to clear previous ions in the collision cell is not sufficient. For high throughput drug candidate screening with simultaneous dosing of multiple compounds from the same therapeutic class, structures are generally similar and therefore the precursor or product ions of those compounds can be very close or exactly the same. Preventing cross-talk

Table 1
Summary of LC/MS/MS properties of compounds A–D and internal standard[a]

Compound	Precursor ion (amu)	Product ion-1 (amu)	Product ion-2 (amu)	Retention time (amu)
Compound A	519.4	259.4		1.5
Compound B	548.4	259.4		1.5
Compound C	582.4	311.4	272.4	1.5
Compound D	599.4	460.4	443.4	3.4
Internal standard	574.4	434.4		2.7

[a] Product-ion spectra were acquired by LC/MS full scan on PE Sciex API300 heated-nebulizer probe.

Table 2
Conditions of cross-talk interference in the LC/MS/MRM[a]

Set number	Mass difference (amu)	Compound	Parent (amu)	Product (amu)	Cross-talk (%)	Retention time (min)
1	$\Delta = 1$	1	557.2	145.2	0	2.1
			557.2	385.2	0	
		2	558.2	446.9	0	2.2
			558.2	112.2	0	
2	$\Delta = 2$	3	576.5	374.2	0	1.8
		4	574.3	414.3	0.1	2.4
			574.3	387.2	0	
3	$\Delta = 4$	5	574.3	414.2	0	2.1
			574.3	386.2	0	
		6	578.4	348.2	0	2.0
			578.4	376.2	0	
4	$\Delta = 5$	7	489.2	112.2	0	2.1
		8	494.2	112.2	0.8	2.0
5	$\Delta = 10$	9	495.2	384.2	0	2.2
			495.2	112.2	0	
		10	505.2	394.2	0	2.2
			505.2	112.2	0	
6	$\Delta = 20$	11	475.2	112.2	0.1	2.1
		12	495.2	112.2	0.1	2.0
7	$\Delta = 43$	13	544.2	112.2	0	2.4
			544.2	433.2	0	
		14	501.2	112.2	0	2.5
			501.2	390.2	0	
8	$\Delta = 2$	15	475.2	112.2	50	2.1
			475.2	364.2	50	
		16	477.2	112.2	50	2.0
			477.2	366.2	50	

[a] The cross-talk studies were conducted on PE-Sciex API 300 heated-nebulizer probe.

interference among those multiple compounds is a critical issue for establishing an accurate and reliable LC/MS/MRM quantitation method. We chose a series of compound pairs to address this cross-talk issue. Table 2 is the summary of precursor ions, product ions, retention times and the percentage of cross-talk observed for these pairs of compounds. For each comparison set, equimolar amounts of the test compounds were used. The percentage of cross-talk is defined as the amount of second compound detected in the first compound MRM acquiring conditions. All compound pairs co-elute except Set # 2. These sets can be categorized into three groups and the potential of having cross-talk interference is different for each of these groups. For Sets # 1–3, the mass differences of precursor ions are 1, 2 and 4 amu, respectively, while the mass differences of product ions are relatively large. No significant cross-talk interference was observed in these cases. For Sets # 4–7, the product ions are the same and the precursor ions have 5, 10 and 20 amu differences. No significant cross-talk interference was observed in these cases either. A cross-talk of less than 1% detected in compounds 4, 8, 11 and 12 is probably from impurities. Finally, significant cross-talk interference was observed in the Set # 8 where the differences of both precursor ions and product ions were in the range of 0–2 amu and the retention times of two compounds were very close. Further separation of compound 15 and compound 16 on the column is a way to reduce the risk of cross-talk interference. Other methods for reducing cross-talk interference are the use of a long pause time or an irrelevant ion pair between each MRM transitions of interest to allow the fragments to leave the collision cell before the next MRM transition is monitored. However, a longer pause time or an irrelevant MRM transition can dramatically increase the total scan time when many ions are being monitored. Therefore, the results of Table 2 provide some guideline either on the selection of compounds for co-administration to a single animal, or on the development of a multiple-compound LC/MS/MRM method.

3.2. Ionization efficiency

Peak intensity of an LC/MS/MS signal is related to the ionization efficiency of the individual analyte while passing from the solution phase to the gas phase. For multiple-compound assay, some compounds will co-elute from the column and go through the ionization process simultaneously. Therefore, co-eluting analytes in the solution may compete in the ionization process and change the ionization efficiency of individual analyte [7,8]. The following experiments were designed to study if additional compounds in the chromatographic eluate will affect the peak intensity of the analyte compound. We looked at the possible effects of Compounds B, C, D and a mixture of 6 compounds (M-6) on Compound A. As listed in Table 1 and experimental section, Compounds B and A have the same product ion and LC retention time. For Compounds C, D and M-6, the precursor/product ion pairs are different from Compound A. However, Compound C and some of compounds in M-6 co-elute with Compound A. Compound D has a different retention time compared to Compound A. Fig. 1 (a–d) shows the relative peak intensity of Compound A (peak area ratio of Compound A to internal standard) versus a wide concentration range of additional components, such as Compounds B, C, D and M-6. At both the low concentration (5 ng ml^{-1}) and the high concentration (250 ng ml^{-1}) of Compound A no significant peak intensity suppression was observed when additional components were present in the same solution as the analyte. The results suggest that the ionization efficiency of an analyte in the mixture would not be affected by other co-eluting analytes or close precursor/product ion pairs over the range of linear calibration curve. Therefore, the accuracy of the multiple-compound LC/MS/MRM method can be as good as the single-compound LC/MS/MRM method.

3.3. Matrix effect on signal intensity

In a manner similar to ionization efficiency, we further evaluated the plasma matrix effect on the intensity of the LC/MS/MS signal. Fig. 2 shows

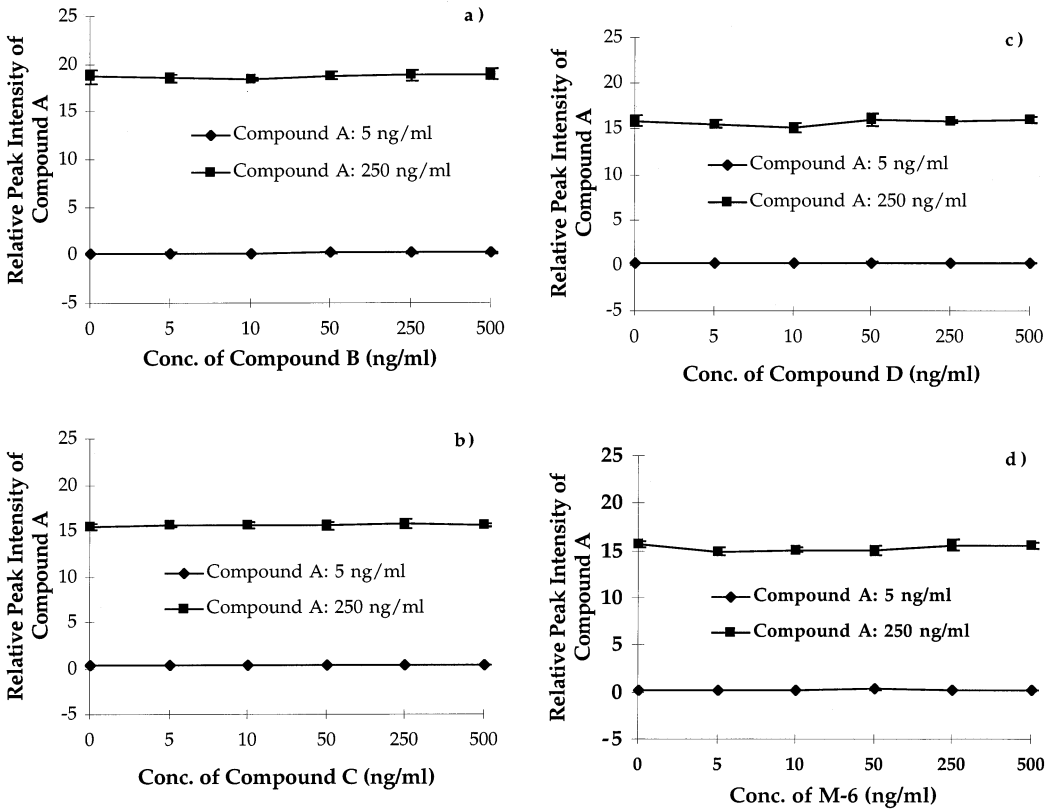

Fig. 1. Effect of single Compound B (a), Compound C (b), Compound D (c) and a Mixture of 6 compounds, M-6 (d) on the peak intensity of Compound A (replicate determinations, $n = 3$).

the comparison between the matrix effect of the single-compound method and the multiple-compound method. Different amounts of solid phase extraction purified normal plasma residue were added back to the aqueous solutions of individual Compounds A, B, C and D and a mixture of Compounds A–D. Fig. 2a and b show the plasma matrix effect on the Compounds A–D when each individual compound was measured by the single-compound method. Fig. 2c and d show the plasma matrix effect on the Compounds A–D measured simultaneously by the multiple-compound method. It appears that matrix effect is concentration (either 10 or 200 ng ml^{-1}) independent. With increasing plasma matrix in the analyte solution, little effect was seen on the peak intensity of the analytes. Overall the degree of the matrix effect on each individual compound can be considered as similar for both the single-compound method and the multiple-compound method. Further experiments will be carried out to elucidate other factors such as the diversity of inter-compound effects.

3.4. Method validation

For each compound (A–D), two QC levels were evaluated by both the single-compound (each compound was assayed individually) and the multiple-compound LC/MS/MRM quantitation method. Here, data obtained from the single-compound method is used as a reference for the multiple-compound LC/MS/MRM quantitation method. Table 3 lists the theoretical values and the found values, solid phase extraction sample recovery at two concentration levels and lower level of quantitation (LLQ, S:N = 10) [9] of compounds A–D for both methods. Linear re-

gression with $1/X$ weighting was used to calculate the standard curves. The slope of the calibration curve for each compound determined by both methods was >0.95 and the intercept of y-axis was <0.002 over the concentration range of 1–500 ng ml^{-1} and the correlation coefficients were >0.995. The QC samples at 5 and 100 ng ml^{-1} level were within 75–125% of theoretical values for both methods (see Table 3). Furthermore, as shown in Table 3, the solid phase extraction efficiencies of these four compounds were higher than 90% at two concentration levels for both methods. These data suggest that the accuracy and the solid phase extraction efficiency taken from the multiple-compound method are comparable to the single-compound method.

An LLQ of a particular compound depends on that compound's structure and the instrument used. It will also be affected when more compounds exist in the mixture being assayed. When assaying a mixture of six or more compounds, extensive optimization effort is often necessary to achieve the same LLQ values as those obtained from single-compound method. Fig. 3 (a–d) shows representative chromatograms of LC/MS/MRM for normal plasma and lower level of quantitation (LLQ) of Compounds A–D taken by the multiple-compound method (chromatograms for single-compound LC/MS/MRM are not shown). For Compounds A–D, an LLQ in the range of 0.5–4 ng ml^{-1} was achievable for both the multiple-compound LC/MS/MRM quantitation method and the single-compound LC/MS/MRM quantitation method without extensive optimization effort.

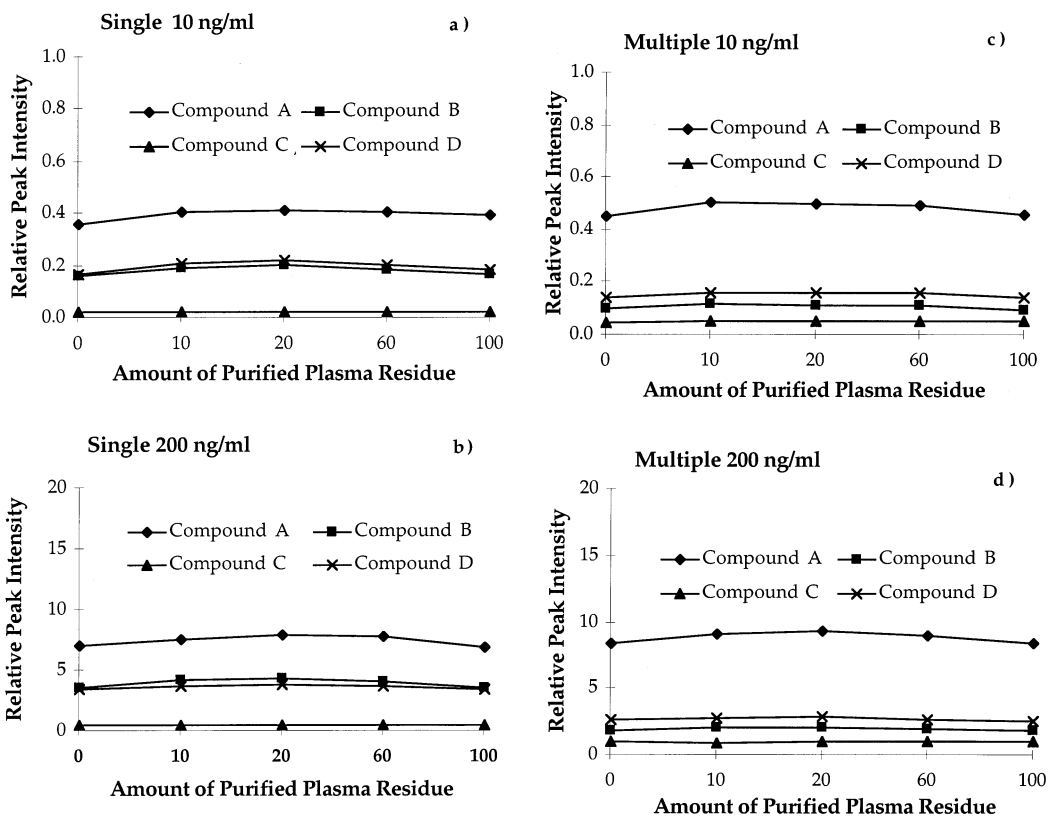

Fig. 2. Comparison of matrix effect on the single-compound and multiple-compound LC/MS/MRM methods: (a–b) were acquired by the single-compound method; and (c–d) were acquired by the multiple-compound method. The concentration level of each compound was 10 ng ml^{-1} for a and c and 200 ng ml^{-1} for b and d.

Table 3
Comparison of the precision, LLQ and the plasma sample recovery of single-compound LC/MS/MRM and multiple-compounds LC/MS/MRM methods[a]

Compound	QC	Single-compound method			Multiple-compounds method		
	(Theoretical value) (ng ml^{-1})	QC (Found value) (ng ml^{-1})	SPE[b] Sample recovery (%) ($n=3$)	LLQ[b] (ng ml^{-1})	QC (Found value) (ng ml^{-1})	SPE[c] Sample recovery (%) ($n=3$)	LLQ[c] (ng ml^{-1})
Compound A	5.0	3.8	95.0 ± 1.0	0.5	4.8	93.9 ± 5.0	0.5
	100.0	95.5	93.2 ± 5.8		106.4	94.0 ± 0.9	
Compound B	5.0	3.9	88.8 ± 2.0	1	5.2	95.0 ± 3.8	1
	100.0	108.8	97.4 ± 1.5		102.5	92.7 ± 0.6	
Compound C	5.0	4.7	90.7 ± 0.9	1	5.0	95.6 ± 3.4	1
	100.0	97.3	97.8 ± 1.2		103.9	92.2 ± 0.7	
Compound D	5.0	5.1	107.8 ± 2.7	4	4.5	97.6 ± 3.0	4
	100.0	112.9	94.6 ± 1.3		108.0	92.7 ± 2.0	

[a] All experiments were acquired on PE-Sciex API 300 turbo ionspray probe.
[b] Each QC sample only included one single compound.
[c] The QC samples included a mixture of Compound A–D with equal amount at 5.0 and 100.0 ng ml^{-1}.

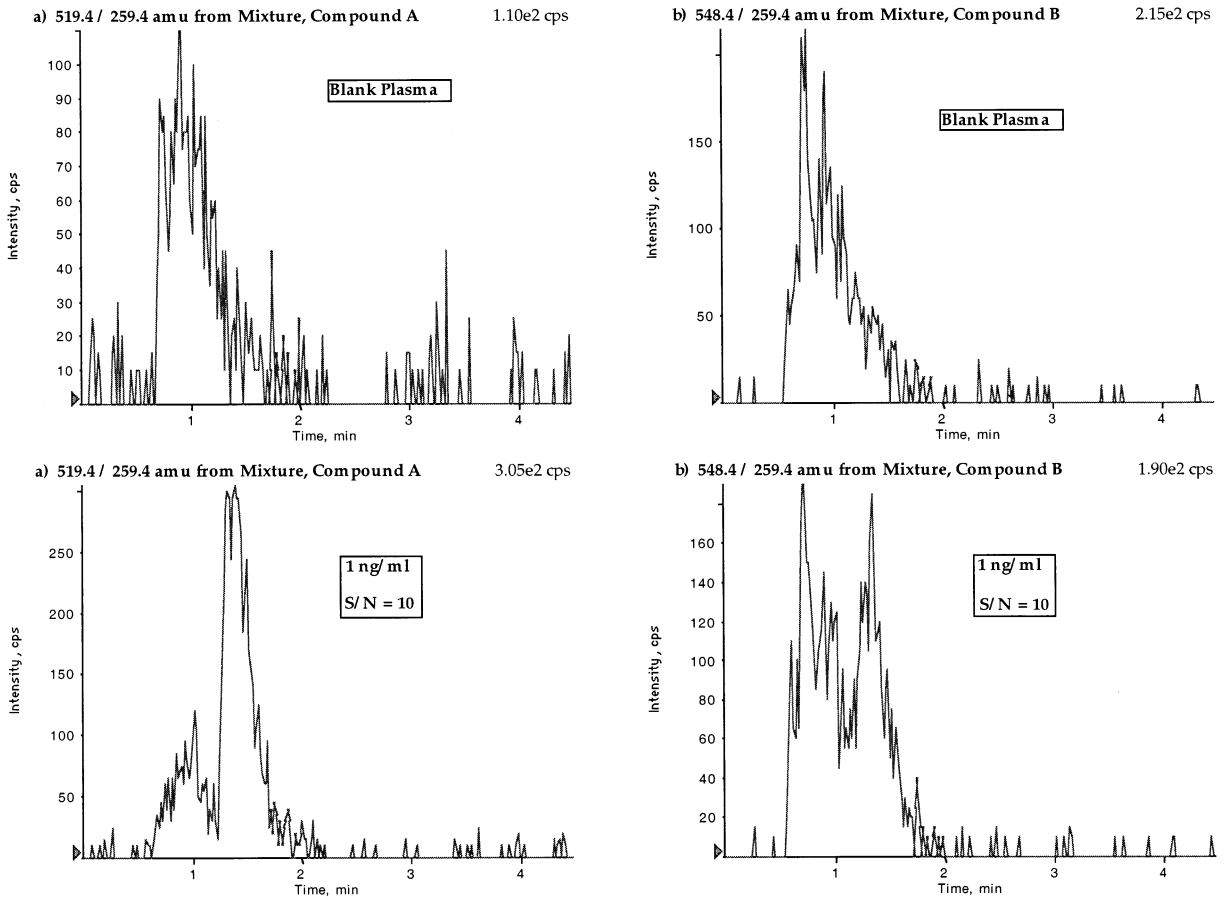

Fig. 3. Representative LC/MS/MRM chromatograms for the mixture of Compounds A–D from the multiple-compounds method: blank plasma and in plasma fortified at 1 ng ml^{-1} (LLQ): (a) Compound A; (b) Compound B; (c) Compound C; and (d) Compound D.

3.5. Pharmacokinetic analysis

To illustrate the practical applicability of this multiple-compound method, Fig. 4 (a–d) shows the plasma pharmacokinetic profiles of Compounds (A–D) co-administered to rats with the resulting plasma samples assayed by the multiple-compound LC/MS/MRM method. Table 4 summarizes the pharmacokinetic parameters for both i.v. and oral dosing. Rapid determination of these parameters is extremely useful to medicinal chemists engaged in the drug discovery process. In the example shown here high plasma clearance is a serious problem. Among the four, Compound D appears to be the least problematic. On the other hand, from a bioavailability consideration Compound A appears to be only one with potential as an orally dosed medication.

4. Conclusion

In general, in a pharmacokinetic evaluation of a series of compounds, multiple-compound mixture study can significantly reduce the sample preparation time, LC/MS instrument time, the number of animals used for dosing and animal handling time. Our results, chosen from many experiments, represent a general trend of possible LC/MS/MRM assay influences of one component on oth-

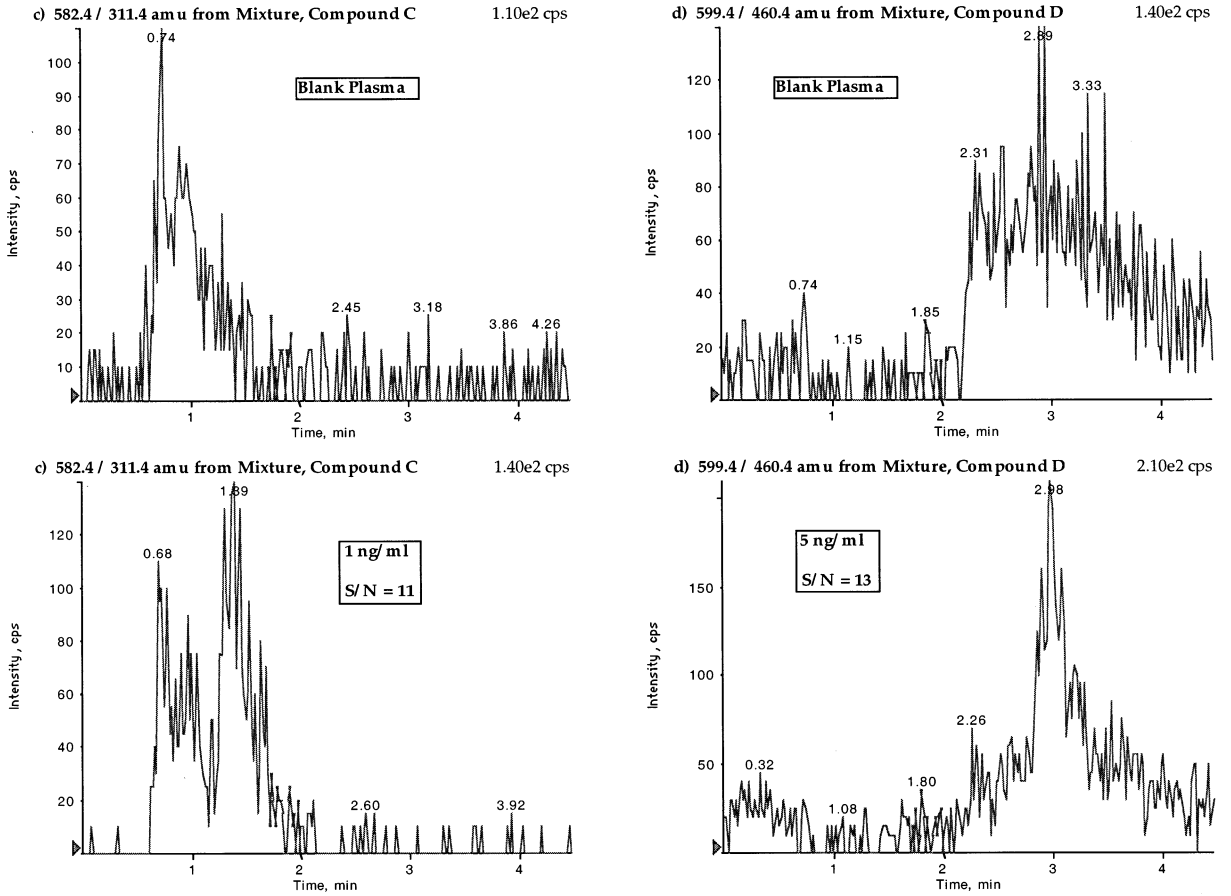

Fig. 3. (*Continued*)

ers in multiple-compound mixture dosing. The main features of this multiple-compound LC/MS/MRM quantitation method are simplicity, speed, accuracy, sensitivity and minimal effort required for the method development process. Mixtures of equal amounts of four to six compounds are routinely co-administered to rats and the compounds in the resulting plasma samples are analyzed simultaneously using this method. Analysis of a mixture including more than six compounds is feasible in terms of analytical technology. However, because of the increased risk of drug–drug interaction, dosing more than six compounds simultaneously to a single animal is not often applied. The possible pharmacokinetic influences of one compound on another in a mixture will be discussed in a future paper in which pharmacokinetic data of the same compounds dosed both individually or in a mixture will be compared. Overall, multiple-compound LC/MS/MRM quantitation method significantly increases compound throughput and decreases the turn around time for medicinal chemists to access important in vivo pharmacokinetic information.

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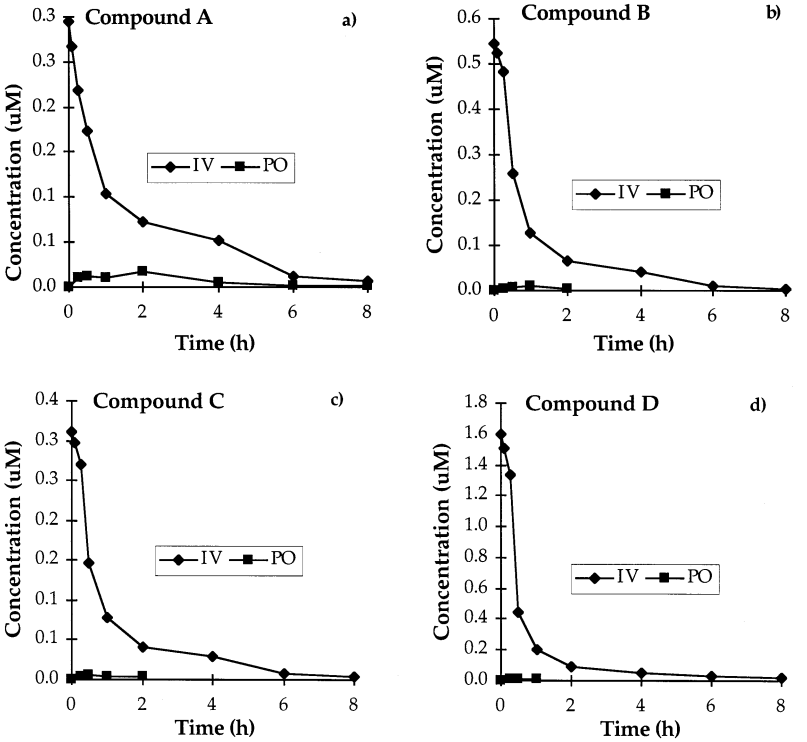

Fig. 4. The plasma pharmacokinetic profiles of co-administered Compounds A–D in rat as determined by the multiple-compound LC/MS/MRM quantitation method.

Table 4
Summary of pharmacokinetic parameters determined by multiple-compounds LC/MS/MRM method

Compound	M.W.	I.V. dose (mg kg^{-1})	AUC 0–∞ (mg min ml^{-1})	C_{lp} (ml min kg^{-1})	$T_{1/2}$ (h)	V_{dss} (L kg^{-1})
IV dose						
A	555.166	2.25	16.5	136.6	2.5	21.2
B	661.703	2.11	23.0	91.9	1.5	9.3
C	663.778	2.09	14.5	144.2	1.9	17.3
D	635.256	2.21	44.4	49.7	2.7	1.9
Compound	M.W.	P.O. dose (mg kg^{-1})	AUC 0–∞ (mg min ml^{-1})	C_{max} (ng ml^{-1})	T_{max} (h)	F (%)
Oral dose						
A	555.166	2.25	2.2	9.3	2.0	13.5
B	661.703	2.11	0.4	5.8	1.0	1.7
C	663.778	2.09	0.9	3.5	0.5	6.5
D	635.256	2.21	n/a	n/a	n/a	0

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