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Simultaneous development of six LC–MS–MS methods for the determination of multiple analytes in human plasma

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Abstract

Traditional sequential single analyte method development is both time-consuming and labor-intensive. In this report, a concept of simultaneously developing multiple liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) methods were proposed. Mass spectrometric and chromatographic conditions as well as sample preparation methods for all analytes were optimized concurrently. Mass spectrometric conditions for six analytes, i.e. clonidine (CLO), albuterol (ALB), fentanyl (FEN), ritonavir (RIT), naltrexone (NAL), and loratadine (LOR), were established simultaneously using the Sciex *Analyst* software. LC–MS–MS sensitivities obtained using gradient elution methods on reversed-phase Inertsil ODS3 and normal phase Betasil silica columns were compared. Sample extraction methods using protein precipitation, liquid/liquid extraction, or solid-phase extraction (SPE) were evaluated. Recovery of analytes was determined. Matrix effects and interference due to endogenous compounds were investigated. Selection of a potential internal standard was discussed.

Keywords: LC–MS–MS; Method development

1. Introduction

Rapid growth of using liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) for the analysis of drugs in biofluids has been driven by the demand for timely, high-quality data at various stages in drug development process: from high throughput screening of drug candidates to rapid data generation for pre-clinical studies to almost 'real-time' analysis of clinical samples [1]. N in one cassette dosing has been used by the pharmaceutical industry. Early pharmacokinetic is facilitated by the administration and analysis of several compounds in the same animal. Desire for fast and more efficient sample preparation has enjoyed special attention for the last several years [2]. Parallel sample preparation methods, such as 96-well solid-phase extraction (SPE), have been adopted by many bioanalytical laboratories [3–6]. Means of increasing the utilization of mass spectrometers such as multiplexing have been introduced [7–9]. Many

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bioanalytical laboratories have adopted a process of multi-shifts (7 days and 24 h operation). While it seems that all the processes and instruments are poised to meet the ever-increasing challenges for faster bioanalytical sample analysis, the speed for LC–MS–MS method development appears to have become the bottleneck, due to both lack of well-trained bioanalytical method developers and lack of sufficient LC–MS–MS instruments. In many organizations, the same instrument must be shared for both routine sample analysis and method development. Traditional sequential single analyte method development requires sequential optimization of mass spectrometric and chromatographic conditions, sample extraction, recovery of the analyte, and lack of interference and matrix effects for each method. This type of method development approach is time-consuming, labor and instrument intensive and costly when several different LC–MS–MS methods for various types of analytes need to be developed.

In this article, we present the concept of simultaneous development of multiple LC–MS–MS methods for assaying analytes of various structures in biological fluids. Clonidine (CLO), albuterol (ALB), fentanyl (FEN), ritonavir (RIT), naltrexone (NAL) and loratadine (LOR) were selected as the test compounds. Chemical structures of these six compounds are shown in Fig. 1. Gradient liquid chromatographic elution on either C18 or silica columns was optimized and their sensitivities were compared. Six sample preparation methods, including protein precipitation, liquid/liquid extraction, and SPE, were evaluated. Blank matrix samples, pre- and post-extraction fortified samples, and neat solution samples were used for the determination of interference, recovery and matrix effects.

2. Experimental

2.1. Chemicals and reagents

CLO hydrochloride (purity 100%) was from USP (Rockville, MD, USA). ALB (purity 99.0%), NAL hydrochloride (purity 100%), FEN citrate (purity 99.0%) and FEN-d_5 citrate (purity 99.0%) were from Sigma (St. Louis, MO, USA). RIT (purity 100%), and LOR (purity 99.9%) were from Custom Synthesis Service (Middleton, WI, USA). Formic acid (FA) and acetic acid were from Aldrich (Milwaukee, WI, USA). Ammonium hydroxide was from Sigma. Water, methanol, aceto-

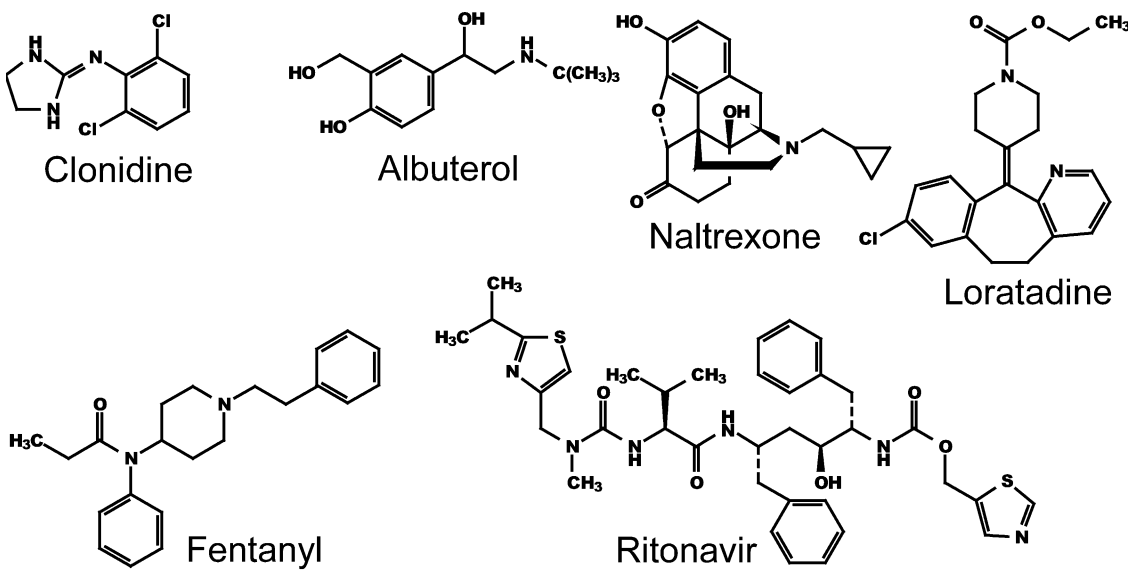

Fig. 1. Chemical structures of CLO, ALB, FEN, RIT, NAL, and LOR.

Table 1
MS parameters

	CLO	ALB	FEN	RIT	NAL	LOR	FEN-d_5
Precursor ion (amu)	230	240	337	721	342	383	342
Product ion (amu)	213	148	188	296	324	337	188
Declustering potential (V)	51	26	46	51	46	56	46
Focusing potential (V)	160	90	140	160	150	160	140
Collision energy (V)	35	27	33	29	31	33	33
Collision cell exit potential (V)	16	10	12	18	20	20	12

Ionization: positive ion electrospray (+ESI); mode: MRM; Turbo Ionspray gas flow rate: 8 l/min; curtain gas setting: 10; Nebulizer gas setting: 10; collision activation gas setting: 4; source temperature: 400 °C; capillary voltage: 5000 V; Q1 resolution: unit; Q3 resolution: unit.

nitrile, hexane, and methyl-tertiary butyl ether (MTBE), all of LC grade, were from Fisher (St. Louis, MO, USA). SPE cartridges used in this study were Bond Elut Certify mixed-mode (1 ml, 25 mg) and C18 (1 ml, 50 mg) from Varian (Harbor City, CA, USA) as well as Oasis HLB (1 ml, 30 mg) from Waters (Milford, MA, USA). Control human plasma using EDTA (K_3) as an anticoagulant was obtained from Biochemed (Winchester, VA, USA).

2.2. LC–MS–MS

The LC–MS–MS system consisted of a Shimadzu series 10AD VP LC system (Kyoto, Japan), and a Perkin–Elmer Sciex API-3000 tandem mass spectrometric detector with electrospray interface (Toronto, Canada). Multiple Reaction Mode (MRM) sensitivities for each analyte were simultaneously optimized by testing on infusion of a methanol–water (1:1, v/v) solution containing 0.1 μg/ml each of the analytes. The important MRM parameters for all analytes are shown in Table 1. To optimize chromatographic conditions, a mixture of 10 ng/ml of each analytes in either 1% FA in water or 1% FA in acetonitrile was injected onto a Inertsil ODS3 or Betasil silica column, respectively, both 50×3 mm^2 I.D., 5 μm, from Keystone Scientific (Bellefonte, PA, USA). The columns were maintained at ambient temperature. The linear gradient elution for the Inertsil ODS3 column was: 0 min, acetonitrile–water–FA (10:90:1, v/v/v); 2.0 min, acetonitrile–water–FA (70:30:1, v/v/v); 2.1 min, acetonitrile–water–FA (10:90:1, v/v/v). The linear gradient elution for the Betasil silica column was: 0 min, acetonitrile–water–FA (90:10:1, v/v/v); 1.0 min, acetonitrile–water–FA (50:50:1, v/v/v); 2.0 min, acetonitrile–water–FA (50:50:1, v/v/v); 2.1 min, acetonitrile–water–FA (90:10:1, v/v/v). The flow rate was 0.5 ml/min and the injection volume was 10 μl.

2.3. Sample extraction

2.3.1. Protein precipitation

To 0.20 ml of a plasma sample, 1.0 ml of acetonitrile was added. After vortex-mix for 1 min, the sample was centrifuged at $1643 \times g$ at ambient temperature for 5 min. The supernatant was transferred to a clean tube and was evaporated to dryness under a stream of nitrogen at 50 °C. The residue was then reconstituted with 0.20 ml of 1% FA in acetonitrile.

2.3.2. Liquid/liquid extraction

To 0.20 ml of a plasma sample, 0.10 ml of 10% ammonium hydroxide solution was added. To the same tube, 2 ml of MTBE or hexane was added. After vortex-mix for 1 min, the sample was centrifuged at $1643 \times g$ at ambient temperature for 5 min. After freezing the aqueous layer, the upper organic layer was decanted into a clean glass tube, evaporated to dryness under a stream of nitrogen, and reconstituted with 0.20 ml of 1% FA in acetonitrile.

2.3.3. Solid-phase extraction

2.3.3.1. Certify mixed mode SPE. To 0.20 ml of a plasma sample, 0.20 ml of 5% acetic acid in water was added. The sample was then applied to a Bond Elut Certify SPE cartridge which had been pre-conditioned with 1 ml of methanol and 1 ml of water. After the sample being drawn through the SPE bed, the cartridge was washed with 1 ml of 5% acetic acid in water, followed by 1 ml of methanol. The analytes were then eluted with 0.6 ml of 2% ammonium hydroxide in acetonitrile. The eluent was evaporated to dryness under nitrogen, and reconstituted with 0.20 ml of 1% FA in acetonitrile.

2.3.3.2. Bond Elut C18 SPE and Oasis HLB SPE. To 0.20 ml of plasma sample 0.20 ml of water was added. The sample was then applied to a Bond Elut C18 SPE or an Oasis HLB cartridge, both of which had been pre-conditioned with 1 ml of methanol and 1 ml of water. After the sample being drawn through the SPE bed, the cartridge was washed with 1 ml of water, followed by 1 ml of 5% methanol in water. The analytes were then eluted with 0.6 ml of methanol. The eluent was evaporated to dryness under nitrogen, and reconstituted with 0.20 ml of 1% FA in acetonitrile.

2.4. Recovery determination

Recovery was determined by comparing the analyte peak area counts from those samples fortified with analytes at 10 ng/ml prior to extraction, to those samples fortified with analytes at 10 ng/ml post-extraction.

2.5. Matrix effects determination

Matrix effects were determined by comparing the analyte peak area counts from those samples fortified with analytes at 10 ng/ml post-extraction, to those samples from neat solutions at 10 ng/ml. To determine matrix effect profiles, analytes were infused into the mobile phase through a T-connection between the column and the interface while injecting the extracted blank plasma samples.

3. Results and discussion

3.1. Optimization of MS conditions

The auto-tuning functions of Perkin–Elmer Sciex LC–MS–MS systems are powerful, flexible and easy to use. They were incorporated into tuning processes to achieve quick and reproducible optimization of mass spectrometric conditions by simply infusing solutions containing the analytes of interest. The auto-tuning capability offered by the *Analyst* software was used to automatically maximize precursor ions intensity and then identify and maximize their product ions intensity, leading to an immediate generation of optimized instrument parameters for the determination of the analytes. After the optimized instrument parameters had been established, a function called Multiple Ion Optimization was utilized to verify the performance of a system in MRM mode. Six precursor ions were tuned simultaneously and profiles of the product ion were generated (Table 1). The two most intensive product ions of each precursor ion were optimized. The most sensitive product ion was then chosen to construct its MRM channel.

3.2. Optimization of chromatographic conditions

Because these analytes have various polarities, a gradient elution was chosen for the chromatographic separation. Results of gradient elution on the reversed-phase ODS and silica columns were compared. For the ODS column, the gradient elution started with a mobile phase of high percentage of aqueous solution. For the silica column, the elution started with a mobile phase of high percentage of organic solvent. In order to achieve on-column stacking effects, the injection solvents for ODS and silica columns were water with 1% formic acid and acetonitrile with 1% formic acid, respectively [10,11]. The gradient programs were adjusted so that the last eluting analyte was observed within approximately 3 min while the first peak still had sufficient retention away from solvent front. Figs. 2 and 3 show the chromatograms obtained with reversed-phase ODS and silica columns, respectively. Good peak

shapes were obtained on both columns but the sensitivity was better on the silica columns for all analytes. The sensitivity increase by using silica column is 37% for ALB, 69% for CLO, 212% for NAL, 216% for FEN, 282% for LOR, and 673% for RIT. The better sensitivity on the silica column could be attributed to the higher organic content in the mobile phase, and therefore favorable spray condition at the LC–MS–MS interface [12]. The combination of silica columns and aqueous–organic mobile phases has been successfully used by the authors for LC–MS–MS analysis of several compounds in biological fluids [13–17]. The silica column demonstrated excellent stability, reproducibility and compatibility with biological samples. Analytes poorly retained on

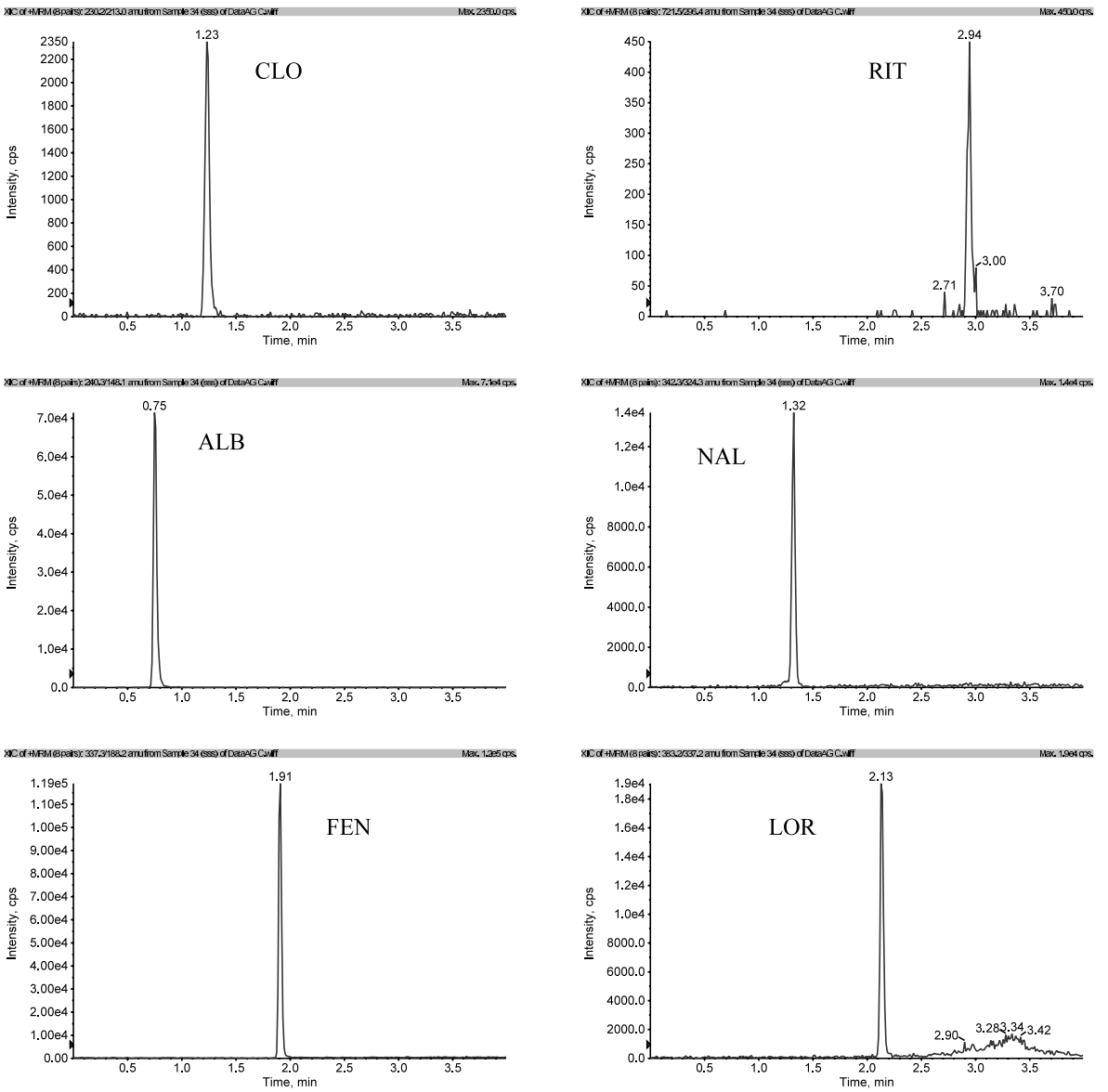

Fig. 2. LC–MS–MS of CLO, ALB, FEN, RIT, NAL, and LOR on an Inertsil ODS-3 column.

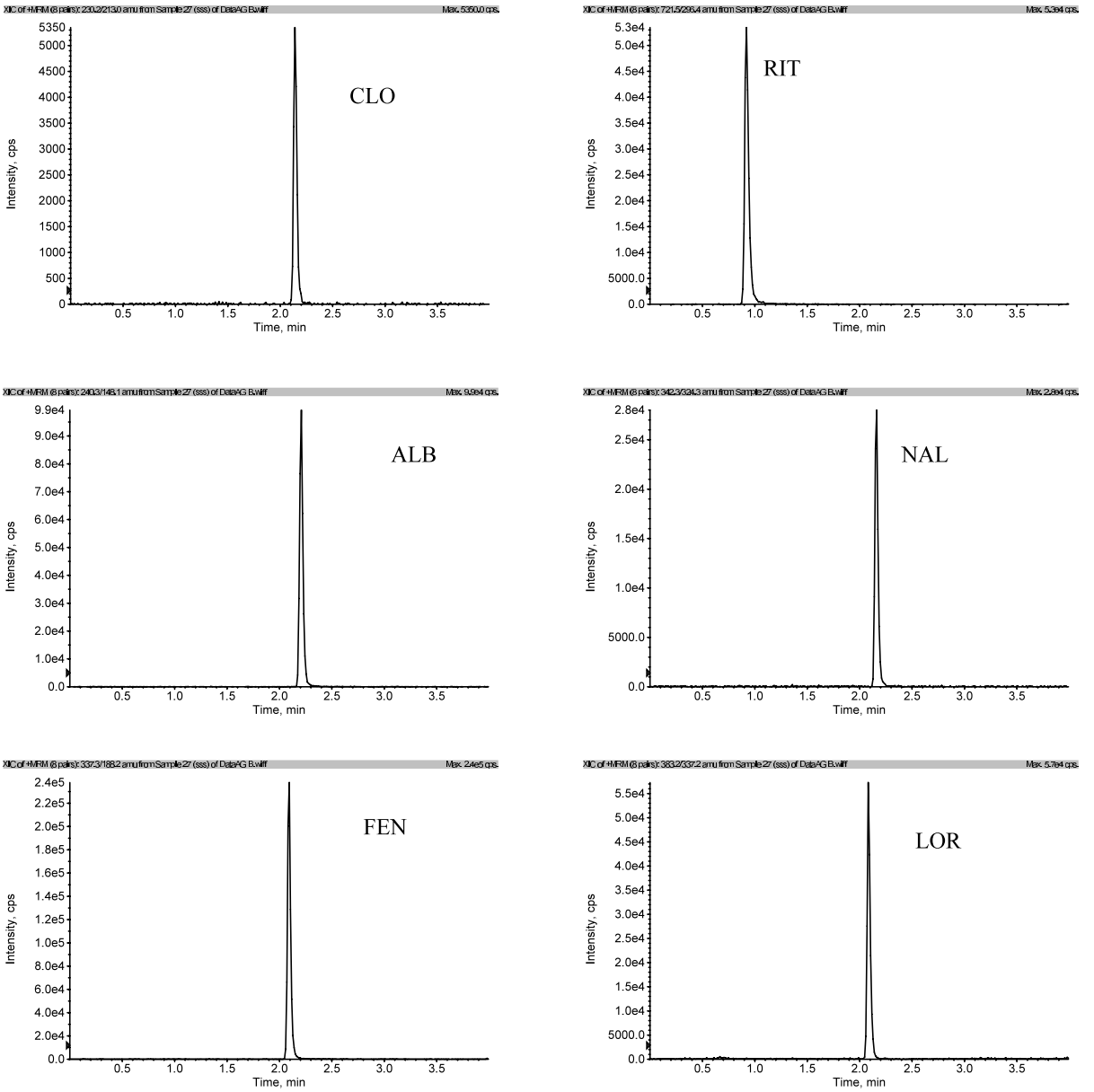

Fig. 3. LC–MS–MS of CLO, ALB, FEN, RIT, NAL, and LOR on a Betasil silica column.

reversed-phase column can be well retained on the silica column using mobile phase rich of organic solvent. Since better sensitivity for all analytes were obtained on the silica column, all further experiments were performed using the silica column.

3.3. Optimization of extraction methods

After optimization of MS and chromatographic conditions, several generic extraction methods, including protein precipitation, liquid/liquid extraction, and SPE were screened. Selection of these

extraction conditions was based on our experiences dealing with other compounds. A good extraction method would have acceptable recovery as well as minimal matrix effects. The detrimental results of matrix effects have been well described in the literature [18–20].

Using the procedures described in Sections 2.4 and 2.5, the recovery and matrix effects were then determined. The results are summarized in Table 2. Protein precipitation method in general gave acceptable recovery but the matrix effects were severe. The greater matrix effects observed for protein precipitation was not a surprise since this method can be considered more generic and analytes as well as many endogenous compounds were non-selectively extracted. On the other end, liquid/liquid extraction using hexane and SPE using C18 were more selective and resulted in little matrix effect. Analytes were more selectively extracted by using these methods. Liquid/liquid extraction using MTBE and SPE using Certify mixed mode SPE were a good compromise between recovery and matrix effects. Both polar and non-polar analytes can be extracted by MTBE as long as they are in the unionized form. The mixed-mode SPE has a different mechanism from either Oasis HLB or C18 SPE methods. The analytes are initially retained on the cartridges by the hydrophobic retention, which is the same as C18 and Oasis HLB. However, after washing the cartridges with an acid, the positive charged analytes interact with the sorbent through ion–ion interaction and the cartridges can then be washed with a organic solvent to remove any compounds retained on sorbent only through hydrophobic retention. Therefore, mixed-mode SPE can be used to selectively extract analytes with basic function groups. In our laboratory, all six extraction methods described here have been used successfully for analyzing various analytes in biological fluids. No single extraction method should be considered superior to others. As long as the recovery and the matrix effects are consistent among various lots of tested biological matrices and the sensitivity is adequate for the particular application, any of the extraction methods can be used successfully. However, a method with better selectivity, higher recovery and lower matrix effects is always more desirable. Liquid/liquid extraction with hexane and SPE with C18 are more selective and had little matrix effects but recoveries were lower for the most of analytes versus the extraction methods. In other words, to achieve optimal recovery for hexane or C18 SPE extraction methods, one needs to spend more time fine tuning the method. Often times, in order to

Table 2
Results of recovery and matrix effects for different extraction methods

		PP (%)	LL		SPE		
			MTBE (%)	Hexane (%)	Mixed-mode (%)	Oasis HLB (%)	C18 (%)
CLO	Recovery	60	91	14	84	86	42
	Matrix suppression	60	<5	<5	8	19	<5
ALB	Recovery	65	28	<5	75	25	43
	Matrix suppression	50	<5	<5	<5	<5	<5
FEN	Recovery	62	86	67	83	71	32
	Matrix suppression	67	<5	<5	<5	19	<5
RIT	Recovery	76	98	12	71	85	29
	Matrix suppression	22	<5	<5	<5	25	<5
NAL	Recovery	92	80	<5	71	84	42
	Matrix suppression	66	<5	<5	<5	7	<5
LOR	Recovery	69	87	80	88	84	44
	Matrix suppression	64	5	<5	<5	15	<5

PP, protein precipitation; LL, liquid/liquid extraction; SPE, solid-phase extraction.

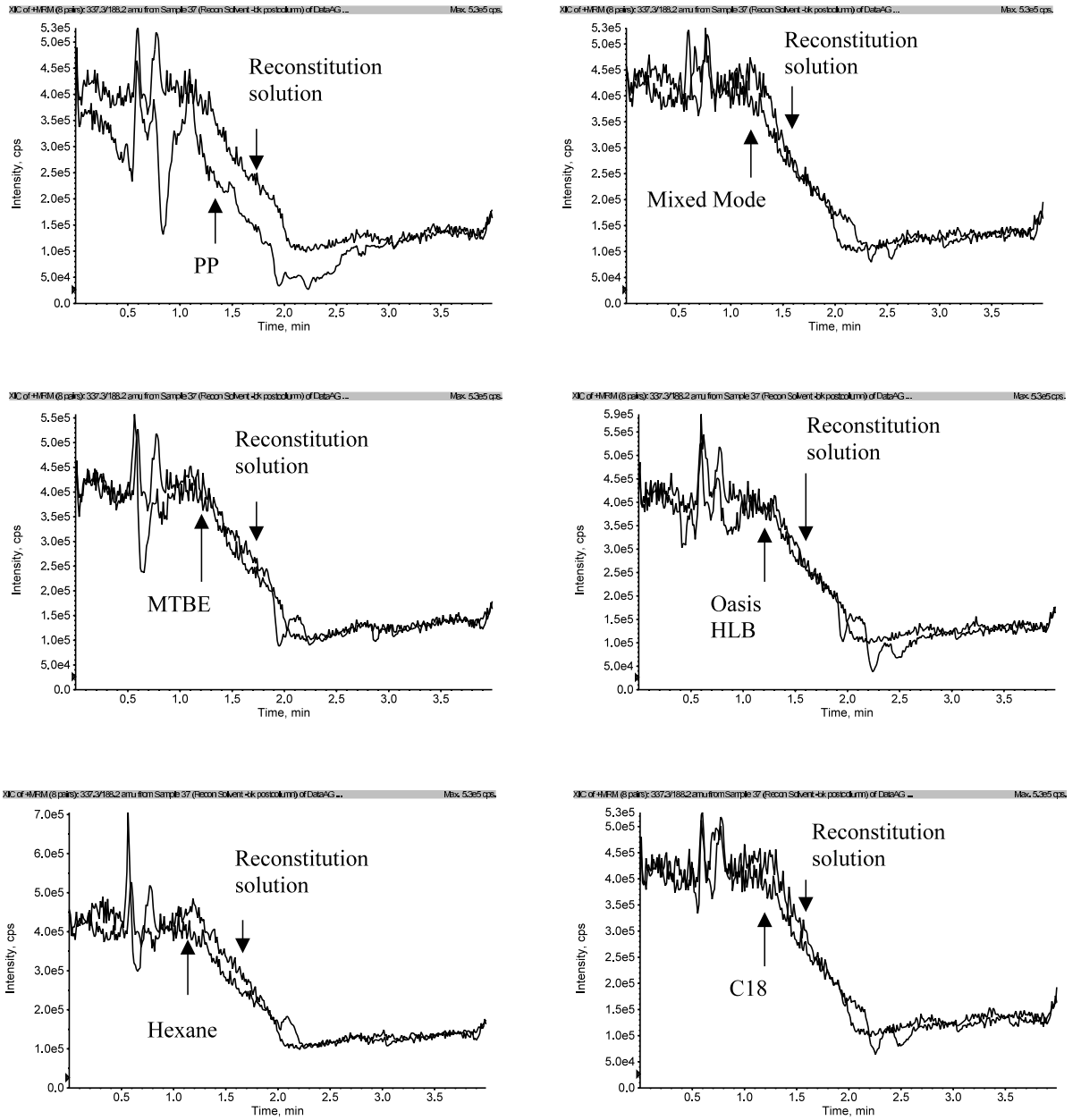

Fig. 4. Comparison of matrix effects profiles by post-column infusion of FEN while injecting extracted matrix blanks.

develop methods quickly enough to support the ever-increasing demand, one has to balance recovery and matrix effects, without compromising the quality of the method.

3.4. Post-column infusion of analytes to determine matrix effects

Post-column infusion of analytes into the mo-

bile phase while injecting extracted blank matrix is a very useful tool to pinpoint the location of the interference peaks that cause matrix effects [21]. Fig. 4 shows the comparison of the matrix effects profiles for FEN obtained by the six extraction methods described here. The gradient elution on a silica column with an aqueous–organic mobile phase as described in Section 2.2 was used. It should be pointed out that since a gradient elution was employed, the analyte MS response was changed along with the mobile phase change: higher MS response in a mobile phase with higher acetonitrile content. With a mobile phase containing 50% acetonitrile, the analyte MS response was about 25% of that obtained with a mobile phase containing 90% acetonitrile. Injecting the reconstitution solution, in this case 1% FA in acetonitrile generated the 'baseline' profile. Any deviation from the 'baseline' caused by injecting the extracted matrix blank indicated the existence of matrix effects. Evidently, compared to the profile obtained by injecting 1% FA in acetonitrile, protein precipitation extraction yielded the most matrix suppression. This is in agreement with the results shown in Table 2. Similar matrix effects profiles were also observed for the other five compounds.

3.5. Selection of internal standard

A good internal standard should mimic the analyte in the entire sample extraction, chromatographic elution, and mass spectrometric detection. It should track the analyte during the extraction and compensate for any potential recovery inconsistency. It will elute together with the analyte on the column and compensate for any potential inconsistent response due to matrix effects. It will not cause interference to the analyte and vice versa. Stable isotope internal standards (deuterated and C_{13} labeled analyte) are ideal candidates for meeting the above criteria. However, stable isotopes are not always easily accessible due to the prohibitive high cost or due to the technical difficulty in synthesizing them. A non-stable isotope internal standard is still needed. The method development strategy used here can also be used to find a suitable internal standard. From Table 2, acceptable recovery and minimal matrix effects were obtained for FEN using liquid/liquid extraction (MTBE and hexane) and SPE (Certify mixed mode and Oasis HLB) methods. FEN also eluted close to other analytes, as shown in Fig. 5. The deuterated stable isotope FEN-d_5 was then chosen as the internal standard for further studies.

3.6. Selectivity, limit of quantitation, linearity and accuracy

Duplicate sets of blank plasma, and blank plasma fortified with 50, 100, 200, 500, 1000, 2000, 5000, 10 000, 20 000, 50 000 and 100 000 pg/ml of each analyte were extracted using liquid/liquid extraction with MTBE or hexane, and mixed mode SPE with Certify. Table 3 summarizes the results for the limit of quantitation, linearity and accuracy. It should be pointed out that several methods could give almost identical results for the same compound. For example, for FEN, the same LLOQ, and very comparable linearity and accuracy were achieved by using liquid/liquid extraction with either MTBE or hexane, or mixed-mode SPE. Results from only one extraction method were reported, in an arbitrary order of SPE, liquid/liquid extraction with hexane, and liquid/liquid extraction with MTBE. Acceptable results were obtained for all analytes. Chromatograms of LLOQ and blank plasma for each analyte using the extraction method corresponding to Table 3 are shown in Fig. 5. No significant interference was observed in the blank plasma samples. The signal to noise ratio obtained at the LLOQ is at least 10:1.

4. Conclusion

The traditional approach of sequential single analyte method development is both time-consuming and labor-intensive. This approach has been identified as the major bottleneck for meeting the ever-increasing needs for LC–MS–MS methods. The concept of simultaneous development of multiple bioanalytical LC–MS–MS methods was presented and discussed. Optimal conditions of mass spectrometry, chromatography, and extraction were screened and developed for six structurally different analytes, i.e. CLO,

Table 3
Linearity and accuracy of the calibration standards

Theoretical value (pg/ml)	CLO (mixed-mode SPE)		ALB (mixed-mode SPE)		FEN (mixed-mode SPE)		RIT (liquid/liquid MTBE)		NAL (liquid/liquid MTBE)		LOR (liquid/liquid hexane)	
	Measurement 1	Measurement 2	Measurement 1	Measurement 2	Measurement 1	Measurement 2	Measurement 1	Measurement 2	Measurement 1	Measurement 2	Measurement 1	Measurement 2
50	BLOQ	BLOQ	BLOQ	BLOQ	40	59	51	49	BLOQ	BLOQ	BLOQ	BLOQ
100	BLOQ	BLOQ	BLOQ	BLOQ	102	103	104	91	BLOQ	BLOQ	BLOQ	BLOQ
200	BLOQ	BLOQ	BLOQ	BLOQ	194	185	223	190	203	211	199	181
500	464	527	BLOQ	BLOQ	482	529	528	546	471	473	492	510
1000	1134	956	1007	1130	982	1002	983	888	922	880	1027	975
2000	1948	1903	2542	1860	1860	1875	1850	2082	1640	2266	1996	1901
5000	4816	5065	4760	5619	4962	5045	5199	5098	4764	5063	5270	5005
10 000	9372	9596	8740	8938	9977	9758	10 014	10 128	9743	10 251	10 327	9658
20 000	21 078	18 073	23 563	19 356	20 697	21 065	18 495	18 104	19 119	19 568	19 947	20 847
50 000	57 316	49 210	54 867	52 671	52 711	48 965	50 037	52 680	55 122	51 424	49 835	52 043
100 000	102 969	101 336	101 291	89 602	101 896	106 296	93 563	110 113	98 017	109 297	101 759	103 347
r	0.9965		0.9945		0.9967		0.9972		0.9969		0.9983	

BLOQ: below limit of quantitation (signal/noise ratio <10 or measured concentration not within 80–120% of nominal values). A weighted [$(1/x^2)$ where x is the concentration of the analyte] linear regression was used.

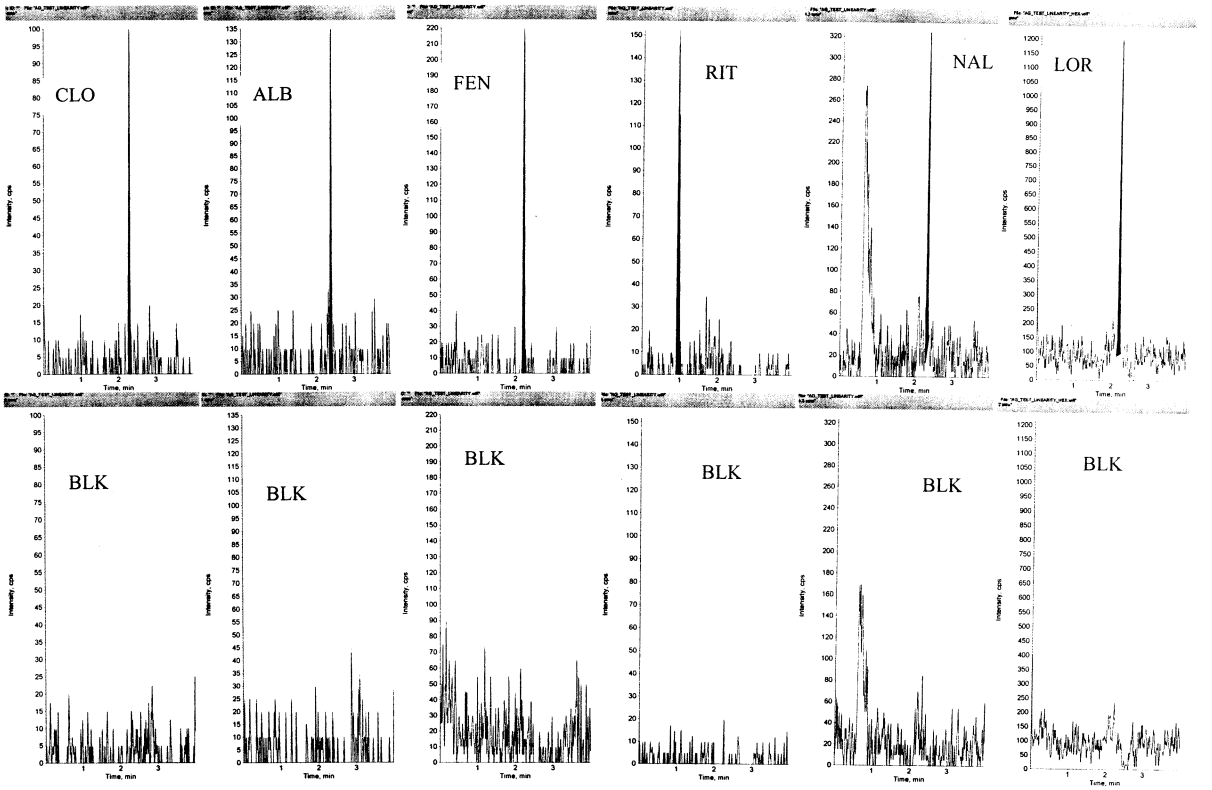

Fig. 5. LC–MS–MS of CLO (500 pg/ml), ALB (1000 pg/ml), FEN (50 pg/ml), RIT (50 pg/ml), NAL (200 pg/ml), and LOR (200 pg/ml) in human plasma (upper panels) and blank human plasma (bottom panels) using gradient elution on Betasil silica column and extraction methods used in Table 3.

ALB, FEN, RIT, NAL, and LOR. Experimental designs for simultaneously determining and evaluating recovery, matrix effects, and chromatographic interference were proposed. Selection of a non-stable isotope internal standard was discussed.

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