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Direct simultaneous determination of drug discovery compounds in monkey plasma using mixed-function column liquid chromatography/tandem mass spectrometry

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Abstract

A direct injection method based on a single column and high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) was developed for the simultaneous determination of two drug candidates in monkey plasma samples in support of pharmacodynamic studies. Each diluted monkey plasma sample containing internal standard was directly injected into a mixed-function column for sample cleanup, enrichment and chromatographic separation. The proteins and macromolecules first passed through the column while the drug molecules were retained on the bonded hydrophobic phase. The analytes retained on the column with an aqueous liquid mobile phase were then chemically eluted by switching to a strong organic mobile phase at a constant flow rate of 1.0 ml/min. The column effluent was also diverted from waste to mass spectrometer for analyte detection. Samples from two different analyte studies were assayed in one analytical procedure and the calibration curves were prepared using both analytes. The calibration curves were linear over the range of 5–2500 ng/ml for both analytes. The retention times for analytes and the internal standard were found to be consistent and no column deterioration was observed after 200 injections. The apparent on-column recoveries for the test compounds in monkey plasma samples were greater than 90% with 6% CV ($N = 5$). The total analysis time was less than 5 min per sample.

Keywords: Plasma; Chromatography; High performance liquid chromatography

1. Introduction

Today's pharmaceutical industry is undergoing dramatic changes in drug discovery areas as a result of technological advancement of combinatorial chemistry. The large numbers of samples generated from drug discovery experiments have caused increasing demands for rapid sample analysis. The high resolving power of high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) has become the preferred and essential analytical approach for the fast, sensitive, and multi-component determination of real-world samples [1–4]. The LC-MS/MS technique has significantly reduced the need for extensive sample clean-up pro-

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cedures in bioanalytical assays. However, the step to remove macromolecular compounds such as proteins from biological fluids is still essential to prevent instrumental contamination or clogging of the HPLC column from protein precipitation as a result of organic modifier in the mobile phase in reversed-phase chromatography. Sample preparation is often the rate-limiting step in preventing higher throughput LC-MS/MS analysis of drug candidates in biological fluids. Although different techniques including turbulent flow and on-line solid phase extraction for simplified sample preparation procedures have been explored to allow for direct analysis of pharmaceutical compounds in biological samples, they typically include complex analytical configurations [5–10]. Normally, these direct injection methods utilize dual-column LC systems that need one extraction column for on-line purification followed by an analytical column for chromatographic separation.

Previously, we reported a simple, automated direct injection LC-MS/MS method that employed a single column to perform all the functions required for direct analysis of single test compound in guinea pig plasma samples [11]. In this work, we investigated the utility of this direct single column LC-MS/MS system for the determination of multiple drug discovery compounds in monkey plasma samples. In the assay procedure, the calibration curves for two test compounds were generated simultaneously from one set of standard monkey plasma samples and the two sets of study samples were assayed in one analytical procedure. This allows for increased efficiency in using LC-MS/MS system. We also compared results obtained from protein precipitation, off-line solid phase extraction (SPE) and the proposed direct injection method for plasma sample analysis by LC-MS/MS using two test compounds. The monkey plasma samples were obtained from pharmacodynamic experiments that were important studies in selecting biologically active lead compounds for a drug discovery project. The analytical results obtained by the proposed direct injection method were comparable, within 15% difference, with those obtained by protein precipitation method and off-line SPE method. The advantages and performance of the proposed method were evaluated using both study samples and spiked plasma samples.

2. Experimental

2.1. Reagents and chemicals

The compounds **I** and **II** are structural analogs obtained from Schering Plough Research Institute (SPRI). The compound used as an internal standard was also obtained from SPRI as described previously [11]. Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA) and ammonium acetate (99.999%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Deionized water was generated from Milli-Q water purifying system purchased from Millipore Corporation (Bedford, MA, USA) and house high-purity nitrogen (99.999%) was used. Drug-free cynomolgus monkey plasma was purchased from Bioreclamation Inc. (Hicksville, NY, USA). Ammonium acetate solution (0.05 M, pH 6.9 was prepared by dissolving 3.85 g of ammonium acetate in 1.0 l of deionized water. Mobile phase A and B contained 4 mM ammonium acetate in water–acetonitrile (80:20) and 4 mM ammonium acetate in water–acetonitrile (20:80), respectively.

2.2. Equipment

LC-MS/MS analysis was performed using a PE Sciex (Concord, Ont., Canada) Model API 365 triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface. The HPLC system consisted of a Leap autosampler from LEAP Technologies (Carrboro, NC) which included the sample cooling stack set to 10 °C, Shimadzu on-line degasser, LC-10AD VP pump and LC-10A VP controller (Columbia, MD, USA). The Quadra 96 (Tomtec, Hamden, CT) system was used for semi-automated sample preparation for protein precipitation and solid phase extraction (SPE) methods. An Empore 96-well disk plate with C18-SD sorbent (3M, St. Paul, MN) was used for SPE. A Symmetry C18 column (3.9 × 50 mm, 5 μm) from

Waters Inc (Bedford, MA, USA) was used as the analytical column for both the protein precipitation and SPE methods. For the direct injection method, a single Capcell MF C8 column (4.6 × 50 mm, 80 Å, 5 μm) from Phenomenex Inc (Torrance, CA, USA) was used as both the extraction and analytical column.

2.3. Sample collection

For two pharmacodynamic studies, blood samples were collected into vacutainer tubes at specified time-points up to 24 h following oral administration of the individual test compounds to individual monkeys at a dose of 3 mg/kg. After clotting on ice, plasma was isolated by centrifugation and stored frozen (− 20 °C) until analysis.

2.4. Standard and sample preparation

Stock solutions of test compounds and internal standard were prepared as 1 mg/ml solutions in methanol. The combined analytical standard samples were prepared by spiking known quantities of the two standard solutions into blank plasma. The concentration range for both compounds in plasma was 5–2500 ng/ml.

For the protein precipitation method, 150 μl acetonitrile solution containing 1 ng/μl of internal standard was added to 50 μl of plasma. After mixing and centrifugation the supernatant was transferred to a 96-well plate. Aliquots of 10 μl were injected to LC-MS/MS for bioanalysis. For the semi-automated SPE method, the 96-well extraction disk plate was preconditioned with methanol and water prior to loading 50 μl of plasma. The plate was then washed with 500 μl of water and eluted with 200 μl of methanol directly into a second 96-well plate. Aliquots of 10 μl were injected for LC-MS/MS assay. For the direct injection method, 50 μl of plasma was loaded into a 96-well injection plate and diluted with 50 μl deionized water containing 1 ng/μl internal standard. About 10-μl aliquots of the diluted plasma were then directly injected into the single mixed function column LC-MS/MS system for quantitation.

2.5. Chromatographic conditions

For each assay, the dual-analyte standard curve was assayed in duplicate at the beginning and end of the analytical run. The samples from study 1 (analyte 1) and study 2 (analyte 2) were loaded sequentially between the standard curve samples.

2.5.1. Protein precipitation method and off-line SPE method

Chromatographic separation was achieved using a two-solvent gradient system: A and B as described in detail previously [11]. At a constant flow-rate of 1.0 ml/min a linear gradient from 5 to 95% B was run over 1 min, held for 2 min and re-equilibrated to 5% B over 1.5 min. The effluent from the HPLC system was connected directly to mass spectrometer for detection.

2.5.2. Direct injection method

The instrumental configuration of the proposed single column direct injection method is given in Fig. 1. A 10-μl portion of the diluted plasma sample was transferred and injected by the autosampler into the CAPCELL MF C8 column with a mobile phase A at a consistent flow-rate of 1.0 ml/min. The switching valve was diverted to waste to remove the macromolecules from plasma matrix. After 1.5 min the valve was switched to the mass spectrometer and a linear gradient from

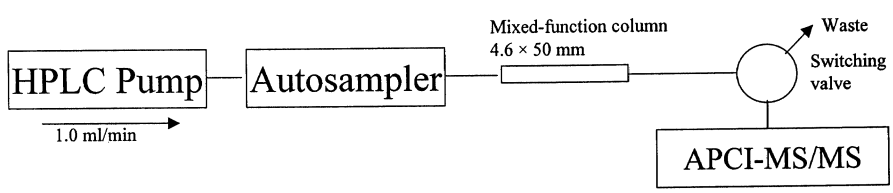

Fig. 1. Schematic diagram of the direct single column LC-MS/MS method.

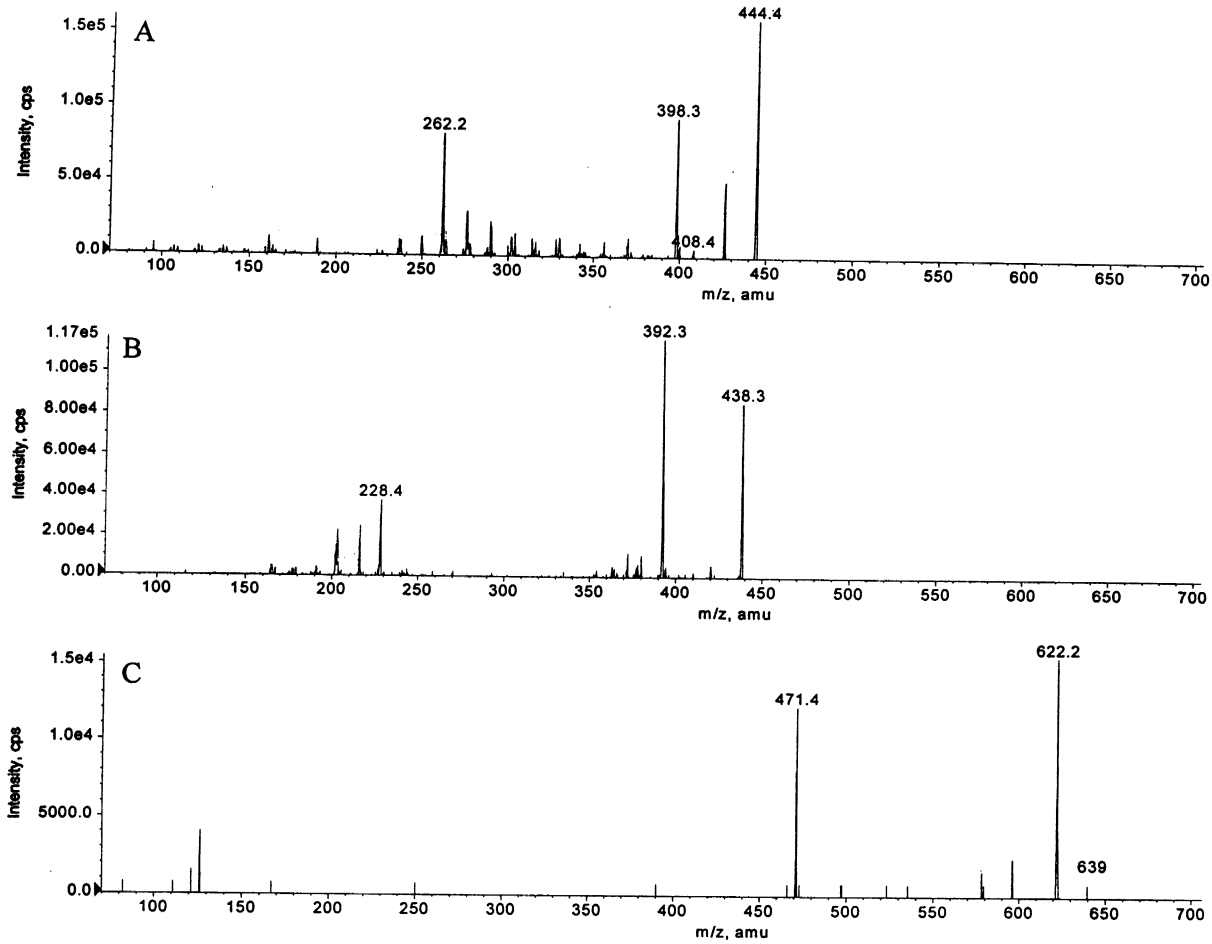

Fig. 2. Product ion spectra of (A) compound **I**; (B) compound **II**; and (C) internal standard.

0 to 100% B was run over 1 min, held for 1.5 min to elute and separate test compounds and internal standard. The separation stages were followed by the equilibration stage with the divert valve switched back to waste and the mobile phase changed from B to A. The retention times for both compounds and the internal standard were 2.88 and 2.81 min, respectively. The total run cycle time was about 4.5 min.

2.6. Mass spectrometric conditions

The mass spectrometer was operated in positive ion mode. The heated pneumatic nebulizer probe conditions were as follows: 470 °C temperature setting, 80 psi nebulizing gas pressure, 1.0 l/min auxiliary gas flow, 0.9 l/min curtain gas flow-rate. The MS/MS reaction selected to monitor compound **I** was the transition from m/z 444, the $[M+H]^+$ ion, to the most abundant product ion at m/z 398. Compound **II** and the internal standard (IS) were monitored using the transitions from m/z 438 $\rightarrow$ m/z 392 and m/z 639 $\rightarrow$ m/z 471, respectively. The molecular ions were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting 4. The collision-offset voltage was 35 V. The product ion spectra of compounds **I**, **II** and IS were given in Fig. 2.

3. Results and discussion

The protein precipitation technique has been chosen as routine sample preparation procedure for LC-MS/MS analysis in our laboratory because of its simplicity and effectiveness in protein removal [4]. Solid phase extraction (SPE) is one of the most common and effective sample preparation techniques for extracting analytes and for removing macromolecules from complex biological sample. However, off-line SPE often involves tedious steps like eluate collection, evaporation, and reconstitution [12]. In addition, the optimization of off-line SPE is also time-consuming. Although the use of extraction column for on-line sample cleanup has become popular and been successfully linked to LC-MS/MS analysis, it provides unsatisfactory chromatographic separation and typically requires a high flow-rate (4 ml/min).

The principal objective of this study was to expand the use of a polymer-coated mixed-function (PCMF) column for direct simultaneous LC-MS/MS analysis of two related components in monkey plasma samples. The roles of PCMF column are to exclude macromolecules such as protein and to retain small drug molecules longer by interaction of the small bonded hydrophobic group [11]. The surface structure of PCMF phase allows large molecules to pass through the column quickly due to the restricted access to the surface by large bonded hydrophilic groups. The PCMF column was not expected to offer large plate numbers for analyte separation. However, it provides sufficient chromatographic efficiency for high speed LC-MS/MS analysis.

The performance of the PCMF column for direct simultaneous determination was examined using compounds **I** and **II** throughout the experiments. Fig. 3A–C show the mass chromatograms for compounds **I**, **II** and the internal standard, respectively. In Fig. 3, the left column represents the signals from the blank monkey plasma indicating no interference peaks for compounds **I** and **II**. The retention time for the internal standard observed in blank plasma (left column), standard plasma (center column), and study plasma (right column) was found to be consistent. Compounds **I** and **II** had the same retention time but can be easily distinguished by MS/MS at different mass transition settings for quantitative determination. The retention times and peak shape, as shown in Fig. 3 for dual-analyte and internal standard were found to be reproducible over 500 injections. The loading capacity of the PCMF column for the untreated plasma sample was indicated by the proportional relation of both compounds based on peak areas from 10 to 80 μl injection volumes (data not shown). We also observed that maintenance of the PMCF column in good condition could be enhanced by washing the column with trifluoroethanol when the column pressure increased as suggested elsewhere [13].

Comparison of the peak area responses of the test compounds in spiked plasma with those from the spiked supernatant solution with protein precipitation technique provided an indication of recovery for each drug candidate for this on-line column extraction procedure, which can be referred to as 'apparent on-column recovery'. Comparison of the peak responses from the spiked plasma with those obtained with the analytes prepared directly in methanol solution (analytical standard) showed a reduction on the integrated peak responses (less than 10%). The loss of signal was due to the sum of losses from the extraction efficiency of the analytes through the PMCF column and the other negative effects such as matrix ion suppression. The apparent on-column recoveries of compounds **I** and **II** were studied with monkey plasma samples spiked at the 250 ng/ml concentration level. The apparent on-column recovery values for compounds **I** and **II** (5 sample injections) using the direct injection method were determined to be 99.8% (%CV = 5.7) and 90.3% (%CV = 5.4) in monkey plasma, respectively, although 99% of protein binding in monkey plasma for both compounds was observed. These recovery values are acceptable for a bioanalytical method in drug analysis and superior to typical SPE or liquid–liquid extraction recoveries [14,15].

For the quantitative determination, study samples from two different studies (two individually dosed compounds) were assayed in one analytical procedure, thereby providing an improved assay efficiency. Only one standard curve containing

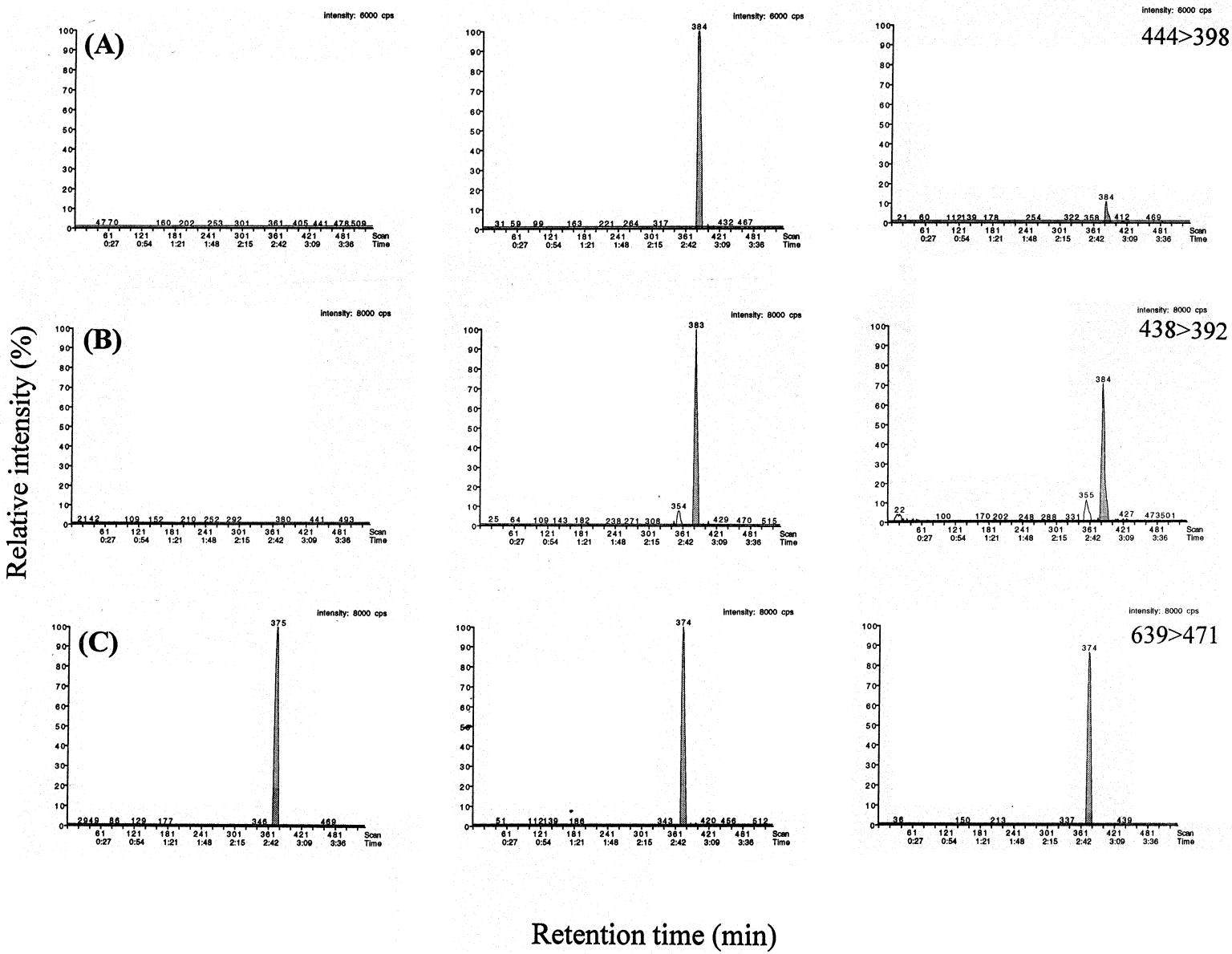

Fig. 3. Direct single column LC-APCI-MS/MS chromatograms (relative intensity verse retention time) of (A) compound **I**; and (B) compound **II**; and (C) internal standard from (left) blank monkey plasma, (center) standard plasma samples at 250 ng/ml, and (right) two study plasma samples.

both compounds **I** and **II** were made. The LC-MS/MS system was set to assay both analytes in one method. In one analytical procedure, both sets of study samples were analyzed. One of the concerns on adapting the direct injection method was the stability of the drug molecules in biological fluids. Therefore, we arranged the two study plasma samples in between two sets of the standard plasma samples to test for possible stability problems. The peak responses from first set of the standard samples and those from the second set of the standard samples were found to match for both compounds within 15% error. This indicated that the compounds were stable in monkey plasma at 10 °C over 12 h assay time. Validation of bioanalytical methods for the determination of drug candidates in the drug discovery stage is not required [4,16]. However, we examined the accuracy of the proposed direct injection method by comparing the analytical results with a conventional LC-MS/MS method using either the protein precipitation technique or the off-line SPE technique for sample preparation. Each of the three methods was applied for the simultaneous quantitation of compounds **I** and **II** in monkey plasma samples from two pharmacodynamic experiments. With the direct single column LC-MS/MS method, as illustrated in Fig. 4A and B, the calibration curves obtained from duplicate standard samples at each concentration level was linear with a correlation coefficient $r^2 = 0.993$ and 0.992 for compounds **I** and **II**, respectively. The accuracy (% bias from back calculation) was good with error less than 15% at all concentrations, 5–2500 ng/ml for both analytes spiked into monkey plasma samples.

Fig. 5A and B show the plasma concentration versus time profiles following oral administration at 10 mg/kg dosing for compounds **I** and **II**, respectively. The plasma levels for both compounds were determined by the protein precipitation, the off-line SPE, and the proposed direct injection method. It was clear that compound **I** had lower plasma levels than compound **II**. The Student's t-test ($\alpha = 0.05$) indicated that statistically there were no significant differences in the results obtained from the aforementioned analytical methods for compound **I**. The area under the curve, $AUC_{(0\rightarrow 24\ h)}$ of compound **I** from the direct injection method, the protein precipitation method, and the SPE method was 667 (h × ng/ml) (3.9% difference from the protein precipitation method), 694 (h × ng/ml), and 569 (h × ng/ml) (18% difference from protein precipitation), respectively. For compound **I**, the above results showed that the proposed direct analysis method was equivalent to the conventional LC-MS/MS method using two sample preparation techniques in terms of accuracy. In addition, the direct injection method significantly reduced the off-line sample preparation time to provide higher throughput for biological sample analysis. For compound **II**, we observed that the plasma concentrations obtained by the SPE assay were consistently lower than the other two assays explored in this work as demonstrated in Fig. 5B. The Student's t-test ($\alpha = 0.05$) suggested that the analytical results for compound **II** obtained by the SPE assay differed significantly from those obtained by the other two methods. The $AUC_{(0\rightarrow 24\ h)}$ of compound **II** from the direct injection method, the protein precipitation method, and the SPE method was 9530 (h × ng/ml) (8.9% difference from the protein precipitation method), 10 500 (h × ng/ml), and 4410 (h × ng/ml) (58% difference from the protein precipitation method), respectively. The reasons for this difference are not clear, but one possibility could be due to inconsistent recovery from study and standard plasma samples with off-line SPE method. In contrast to the SPE method, by using the PCMF column connected directly to the mass spectrometer, we were able to automatically optimize elution conditions that provided consistent recovery and appropriate separation resulting in the best ionization efficiency for analyte measurement. These data demonstrated that the direct plasma injection method provided rapid and more reliable analytical results than the off-line SPE method, which were similar to those obtained via a traditional LC-MS/MS method based on protein precipitation for sample preparation.

4. Conclusion

An efficient bioanalytical method based on mixed-function column HPLC-MS/MS for on-

line purification and separation has been demonstrated for the direct simultaneous determination of two drug candidates using the 'double assay' procedure for the analysis of two monkey studies. The direct injection method provides minimized sample preparation resulting in higher sample throughput. The direct injection LC-MS/MS method was shown to provide accurate results when compared with those obtained using a conventional LC-MS/MS method based on protein precipitation procedure.

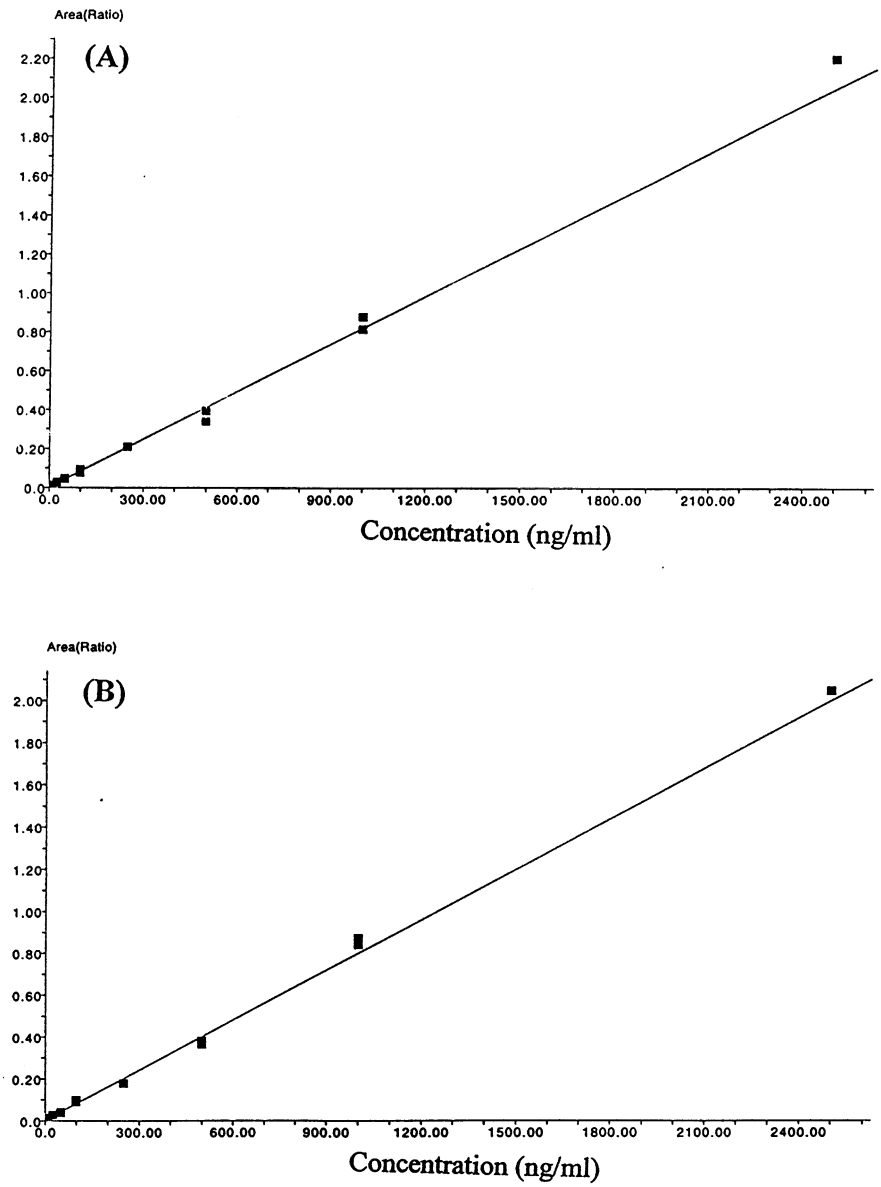

Fig. 4. Calibration curves of (A) compound **I**; and (B) compound **II** in monkey plasma obtained by direct LC-APCI-MS/MS method

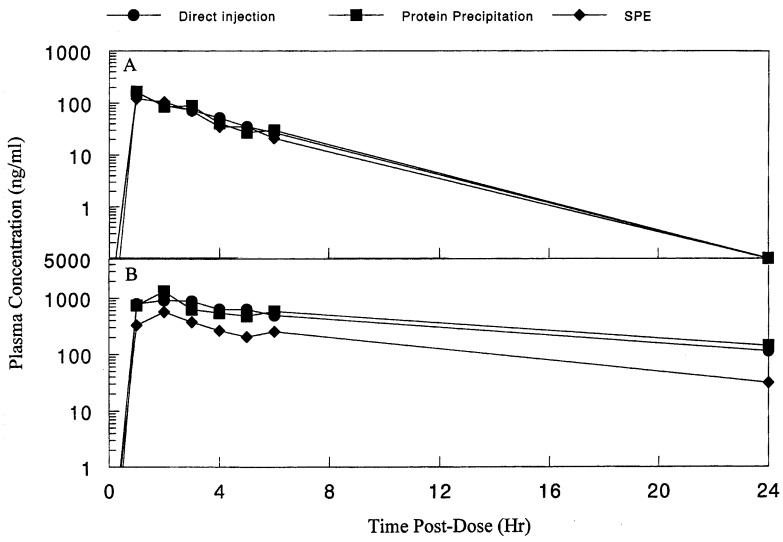

Fig. 5. Plasma concentration versus time profiles of (A) compound **I**; and (B) compound **II** obtained by the protein precipitation, SPE, and the direct single column LC-MS/MS methods.

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