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Direct cocktail analysis of drug discovery compounds in pooled plasma samples using liquid chromatography–tandem mass spectrometry

Yunsheng Hsieh*, Matthew S. Bryant, Jean-Marc Brisson, Kwokei Ng, Walter A. Korfmacher

Department of Drug Metabolism and Pharmacokinetics, Schering Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

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Abstract

Direct plasma injection technology coupled with a LC–MS/MS assay provides fast and straightforward method development and greatly reduces the time for the tedious sample preparation procedures. In this work, a simple and sensitive bioanalytical method based on direct plasma injection using a single column high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) was developed for direct cocktail analysis of double-pooled mouse plasma samples for the quantitative determination of small molecules. The overall goal was to improve the throughput of the rapid pharmacokinetic (PK) screening process for early drug discovery candidates. Each pooled plasma sample was diluted with working solution containing internal standard and then directly injected into a polymer-coated mixed-function column for sample clean-up, enrichment and chromatographic separation. The apparent on-column recovery of six drug candidates in mouse plasma samples was greater than 90%. The single HPLC column was linked to either an atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) source as a part of MS/MS system. The total run cycle time using single column direct injection methods can be achieved within 4 min per sample. The analytical results obtained by the described direct injection methods were comparable with those obtained by semi-automated protein precipitation methods within ±15%. The advantages and challenges of using direct single column LC–MS/MS methods with two ionization sources in combination of sample pooling technique are discussed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Direct cocktail analysis; Drug discovery compounds

1. Introduction

With the development of various higher speed methods of synthesis including both parallel and combinatorial chemistry a variety of potent compounds can be quickly synthesized for drug discovery projects. Oral pharmacokinetic (PK) screening currently plays an important role in the screening paradigm on selecting lead compounds for most drug discovery projects [1]. Rapid and effective ways to provide PK information are desirable to shorten the times required for drug discovery in pharmaceutical industry. Many approaches such as the cassette dosing (*N*-in-one dosing) technique [2–6], parallel

*Corresponding author. Tel.: +1-908-740-5385; fax: +1-908-740-2966.

E-mail address: yunsheng.hsieh@spcorp.com (Y. Hsieh).

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liquid chromatography (LC) [7] and sample pooling [8] have been described previously. While cassette dosing seems to be an efficient way to simultaneously screen multiple new compounds, the potential for drug–drug interactions is a problem even at a low dose [1]. Alternately, sample pooling following one-in-one dosing provides a smaller number of test samples to be assayed while still providing a substantial amount of PK information. However, sample pooling techniques require very sensitive and selective bioanalytical assays for simultaneous determination of drug molecules because of the dilution of the plasma samples and the multiple analytes.

In accelerating the discovery process for pharmaceutical compounds, there is a continuing demand for higher throughput bioanalytical methods. Conventional high-performance liquid chromatography (HPLC) methods for drug assay required tedious sample preparation steps to avoid matrix interference from biological fluids. HPLC combined with atmospheric pressure ionization (API) tandem mass spectrometry (MS/MS) has become an essential tool in today's pharmaceutical industry [9]. The sensitivity of the API sources and the inherent selectivity of tandem mass spectrometry (MS/MS) have significantly reduced the sample preparation steps and the assay development time. However, the step to remove macromolecular compounds such as proteins from biological samples is still unavoidable due to the need to prevent both LC column clogging in reversed-phase chromatography and ion source contamination in mass spectrometry. Sample preparation is often the rate-limiting step in developing higher throughput LC–MS/MS assay for drugs in biological fluids. Although different techniques using turbulent flow and on-line solid-phase extraction for automated sample preparation procedures have been explored to allow direct analysis of pharmaceutical compounds in biological samples, they typically include complex analytical configurations [10–14]. 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. Recently, we successfully demonstrated a simple direct injection method using one mixed-function column to perform both the sample clean-up and chromatographic separation for quantitative analysis of a drug discovery compound in guinea pig plasma samples [15].

The aim of the present work was to extend our investigation into applying a single column LC–MS/MS system to the direct analysis of pooled plasma samples for the determination of multiple components. We demonstrate a comparison of a protein precipitation method and the direct plasma injection method for analysis of six drug candidates by LC–MS/MS. The advantages and performance of the proposed method were evaluated using both study samples and spiked plasma samples. In addition, while ion suppression due to the matrix effect from plasma samples was initially observed using ESI ionization, it was determined that this problem could be minimized by improving the chromatographic separation and the retention of analytes. The data presented in this paper confirm that the single mixed-function column LC–MS/MS assay is applicable for direct quantitative multi-component analysis of plasma samples with the sample pooling technique.

2. Experimental

2.1. Reagents and chemicals

The compounds I, II, III, IV, V, VI and the internal standard are structural analogs obtained from Schering Plough Research Institute (SPRI). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroethanol, trifluoroacetic acid (TFA) and ammonium acetate (99.999%) were purchase from Aldrich (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 mouse plasma (heparinized) was purchased from Bioreclamation (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 phases A, B and C contained 4 m*M* ammonium acetate in water–acetonitrile (80:20), 4 m*M* ammonium acetate with 0.01% TFA in water–acetonitrile (20:80) and 4 m*M* ammonium acetate in water–acetonitrile (20:80), respectively.

2.2. Equipment

LC–MS/MS analysis was performed using a PE Sciex (Concord, Ontario, Canada) Model API 365 tandem triple quadrupole mass spectrometer equipped with either APCI) or ESI interfaces. The HPLC system consisted of a Leap autosampler with a refrigerated sample tray set to 10 °C from LEAP Technologies (Carrboro, NC), 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 using a 96-well plate. For the traditional LC–MS/MS method, a Symmetry C_{18} column (4.6×50 mm, 5 μm) from Waters Inc (Bedford, MA, USA) was used as the analytical column. For the single column direct injection method, a Capcell MF C_8 column (5 μ, 4.6×50 mm) from Phenomenex Inc (Torrance, CA, USA) was used as pretreatment and analytical column. So far, both columns have shown the same capability in terms of the number of repeatable injections. The backpressure for the Capcell MF C_8 column was around 40 bar. The typical HPLC–MS/MS chromatogram with Symmetry C_{18} column was reported elsewhere [16].

2.3. Sample collection

For each compound dosed, following oral administration at a dose of 25 mg/kg for rapid PK screening 18 study plasma samples from each of three mice/time point were pooled to generate six ''single-pooled'' samples at the six time points (1–6 h post-dose) as described elsewhere [17]. The single-pooled mouse plasma samples were stored in the −20 °C freezer for further analysis. Prior to quantitation, a 10-μl aliquot of each single-pooled plasma study sample from one time point post-dose was transferred and pooled together with other study (additional compounds using one-in-one dosing) samples at the same time points into a 96-well tray; these samples are called ''doubled-pooled'' plasma samples. In the same way, the individual standard plasma samples from the six different test compounds were pooled to make pooled standards. Therefore, a final set of six double-pooled mouse plasma samples was generated from the original 108 individual mouse plasma samples. The sample set was assayed via an abbreviated two-point calibration curve which results in six samples plus eight standards (including two blank plasma samples) and two solvent blanks for all six test compounds.

2.4. Standard and sample preparation

Stock solutions of six test compounds and internal standard were prepared as 1 mg/ml solutions in methanol. Analytical standard samples were prepared by spiking known quantities of the standard solutions to blank plasma at an abbreviated three-point calibration curve, 50, 500 and 5000 ng/ml levels. Data are reported for samples within the analytical range established by two appropriate standards (depending upon their estimated plasma concentrations, e.g. 50–500 ng/ml or 500–5000 ng/ml) and (2×) above and (0.4×) below this range (e.g. for 50 and 500 ng/ml, up to 1000 ng/ml and down to 20 ng/ml); samples with values below 20 ng/ml were reported as zero. For the protein precipitation method, 150 μl acetonitrile solution containing 1 ng/μl of internal standard was added to 50 μl of the double-pooled study plasma samples and standard plasma samples in a 96-well plate. After vortexing and centrifugation the supernatant was transferred to another 96-well plate using the Tomtec Quadra 96 system. Aliquots of 10 μl were injected for LC–MS/MS analysis. For the direct injection method, 60 μl of double-pooled plasma and standard plasma samples were loaded into a 96-well plate and diluted with 60 μl deionized water containing 1 ng/μl internal standard. Then, 10-μl aliquots of the diluted plasma were directly injected onto a single column coupled to MS/MS system for analysis.

2.5. Chromatographic conditions

For the protein precipitation method, chromatographic separation was achieved using a two-solvent gradient system: mobile phases A and C. At a constant flow-rate of 1.0 ml/min at a linear gradient from 5 to 95% C was run over 1 min, held for 2 min and re-equilibrated to 5% A over 1.5 min. The effluent from the HPLC system was connected directly to the mass spectrometer for detection. The instrumental configurations for the direct plasma

injection method with APCI and ESI interfaces are reported elsewhere [18]. The LC flow-rate was constant at 1 ml/min for each system. A 10-μl portion of the pooled plasma sample was injected by the autosampler directly onto the CAPCELL MF C8 column with a mobile phase A and the divert valve (post-column) switched to waste to remove the macromolecules from the plasma matrix. Macromolecules such as protein passed quickly through the column due to the restricted access to the surface of the packing while the column retained the drug molecules on the bonded hydrophobic phase. With the APCI interface system, after 1.5 min the column effluent was diverted from waste to the mass spectrometer for analyte detection and the LC pump delivered mobile phase B over 2.5 min to elute and separate all test compounds and the internal standard. The separation stages were followed by switching the HPLC pump from mobile phase B to A. The retention times for analytes and internal standard were all less than 4 min. With the ESI interface system, after 2 min the divert valve was switched to the mass spectrometer and a linear gradient from 0 to 100% B was run over 3.5 min, held for 1 min to elute and separate all test compounds and internal standard. The flow between the divert valve and mass spectrometer was split at a ratio of 3:1 to provide a stable ion current. The separation stages were followed by the equilibration stage with the valve switched back to waste and mobile phase changed from B to A. The retention times for analytes and internal standard were all less than 6 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 p.s.i. nebulizing gas pressure, 1.0 l min^{-1} auxiliary gas flow, 0.9 l min^{-1} curtain gas flow-rate. The ESI conditions are as follows: 420 °C temperature setting, 80 p.s.i. nebulizing gas pressure, 0.7 l min^{-1} auxiliary gas flow, 1.0 l min^{-1} curtain gas flow-rate. The MS/MS reaction selected to monitor compound I, was the transition from m/z 550, $[M+H]^+$ ion, to a product ion at m/z 320 with collision energy of 35 eV. The compounds II, III, IV, V, VI and internal standard were monitored using the transitions from m/z 510→m/z 208, m/z 516→m/z 286, m/z 530→m/z 300, m/z 630→m/z 400, m/z 568→m/z 320 and m/z 639 to m/z 471, respectively. The experimental mass spectrometric conditions were determined using a generic state file obtained by infusion of a test compound to mass spectrometer.

3. Results and discussion

The protein precipitation method 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 [19]. The enhanced throughput by sample pooling technique is directly proportional to the number of animals and test compounds being pooled. The single polymer-coated mixed-function (PCMF) column (CAPCELL column) LC–MS/MS system developed in our laboratory [15] has been shown to be a powerful analytical tool for direct determination of plasma samples in support of pharmacokinetic (PK) studies. The roles of PCMF column are to remove macromolecules such as protein and to maintain small drug molecules longer by interacting with the small bonded hydrophobic groups. 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 rapid discovery PK analysis. The single PMCF column LC–MS/MS system had demonstrated many advantages over dual-column LC–MS/MS systems in terms of simplicity and reliability [15].

The described double-pooled approach demands a more sensitivity assay than is required for the single-pooled method because of the 6-fold dilution of plasma samples. The large loading capacity of plasma samples by the mixed function columns supports sensitive bioanalytical assays by accepting a relatively large volume of biological fluids. The maximum sample loading capacity for the PCMF column was not investigated for this effort because 10 μl of the untreated plasma sample was enough to

meet the quantitation limit requirement of 20 ng/ml for this ''rapid mouse'' PK screening program. However, we had previously reported that this column could accept plasma volumes up to 80 μl at a time [15]. The stability of small drug molecules in plasma samples is always the concern on utilizing any direct plasma injection method. However, this concern can be reduced using the described sample pooling approach requiring much shorter overall assay times. The double-pooled plasma samples were arranged in between two sets of standard curve plasma samples. We observed no changes in peak responses of all test compounds in the first set of standard samples and the second set of the standard samples within a total 1.5 h assay time.

The main objective of the study was to investigate the utility of a single column direct injection system for multiple component determination in the double-pooled plasma samples. The performance of the PCMF column was examined by using six drug compounds at early discovery stage throughout the experiments. Both APCI and ESI ionization sources for the tandem mass spectrometer are commonly used for quantitative determination of small molecules in biological fluids. The mass chromatograms with APCI interface of six compounds from pooled standard plasma samples at 500 ng/ml are given in Fig. 1. The compounds sharing the same LC retention times can be easily distinguished by MS/MS detection. The ESI interface provides soft ionization and is more vulnerable to ion suppression due to matrix effects [20]. We initially applied the same LC conditions used for the APCI source to the ESI source and observed ion suppression with ESI source presumably due to the plasma matrix caused by unknown coeluting components. This matrix ionization suppression effect was indicated by inconsistent responses (integrated peak areas) of the internal standard from both spiked standard and study plasma samples (data not shown). To overcome this phenomenon, we improved the chromatographic separation and prolonged the retention of analytes by using a longer gradient separation to help eliminate the ion suppression. After this change, higher and consistent responses of the internal standard for all analytical runs were obtained. The matrix effect with the protein precipitation–ESI method was not investigated in this work. The mass chromatograms of six test compounds and internal standard using the direct-ESI method are shown in Fig. 2.

Comparison of the peak area responses of the test compounds in the spiked plasma standards using direct injection method (through CAPCELL column) with those from the spiked supernatant solution obtained from the protein precipitation technique (through CAPCELL column) provided an indication of the apparent on-column recovery for the on-line column extraction procedure. The apparent on-column recoveries (using APCI) of compounds I through VI were studied with mouse plasma samples spiked at the 500 ng/ml concentration level. The recovery values for compounds I through VI (five sample injections) using the direct injection method with the APCI source were determined to be 91% (%C.V.=3.4), 98% (%C.V.=6.4), 94% (%C.V.=2.7), 100% (%C.V.=2.2), 90% (%C.V.=1.5) and 97% (%C.V.=1.5) in mouse plasma, respectively. These values are acceptable for a bioanalytical method in drug analysis and superior to typical SPE or liquid–liquid extraction recoveries [21,22].

The proposed direct injection method was applied to the quantitation of six sets of mouse plasma samples from rapid mouse oral PK experiments. The mass chromatograms of blank plasma sample showed no interference peaks for all test compounds (data not shown). Validation of bioanalytical methods for quantitation of drug candidates at drug discovery stage is not required [18]. The plasma concentrations of the test compounds and the calculated area under the curve (AUC) for each compound are estimated for screening purpose. Good drug candidates need to demonstrate acceptable oral mouse PK in this project for further efficacy study. We examined the accuracy of the proposed direct injection methods by comparing the analytical results with protein precipitation method. Fig. 3 shows the rapid mouse PK profiles of compounds I through VI using direct LC–ESI–MS/MS method. The $AUC_{(0\rightarrow 6\ h)}$ for compounds I through VI obtained by direct LC–APCI–MS/MS method with double-pooled sample, direct LC–ESI–MS/MS method with double-pooled sample, the protein precipitation method with double-pooled sample and the protein precipitation method with single-pooled sample were summarized in Table 1. Table 1 shows that for all six samples, there was good agreement on the AUC values regardless of the

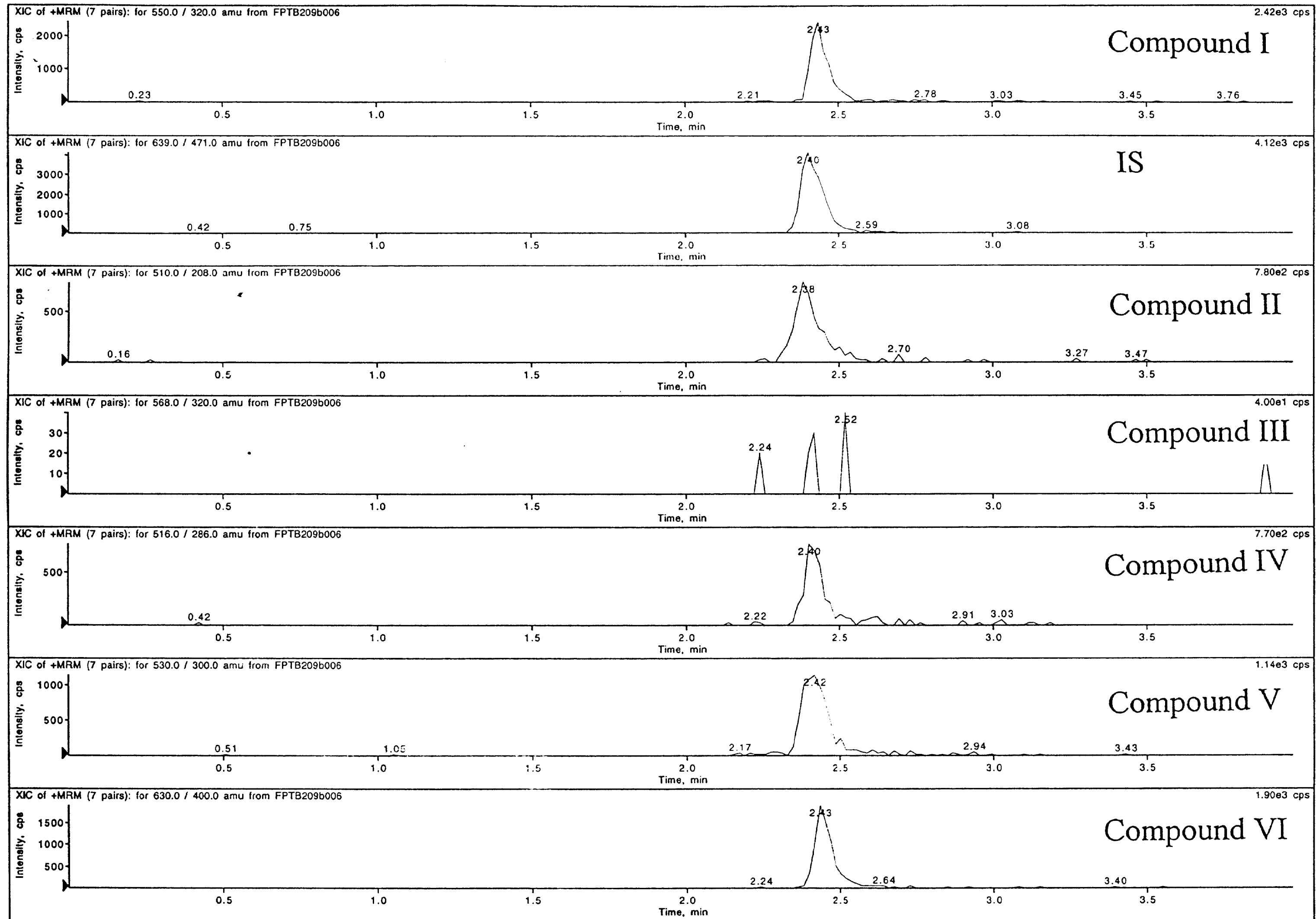

Fig. 1. Mass chromatograms from the double-pooled standard plasma containing six test compounds and the internal standard using direct LC–APCI–MS/MS system.

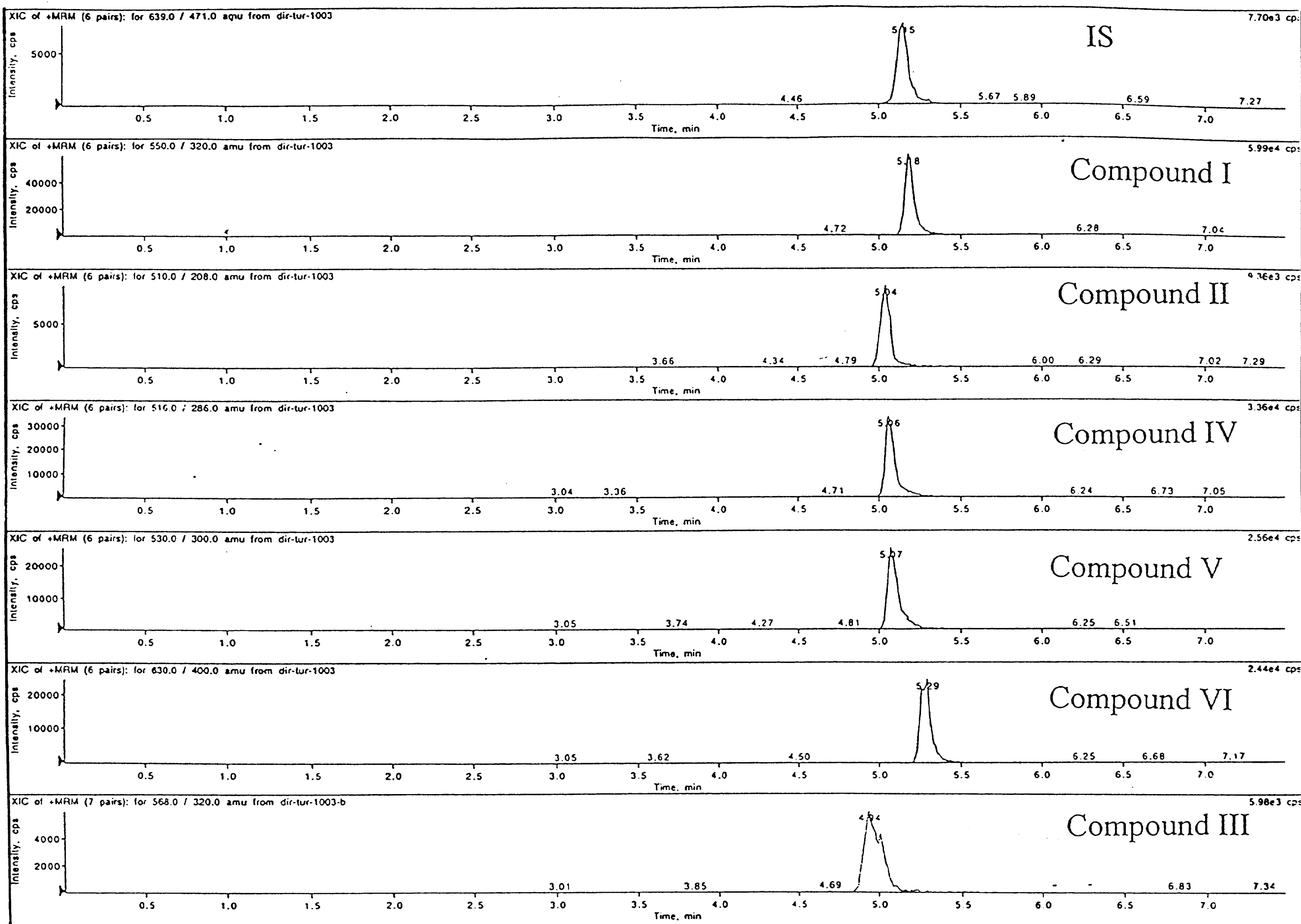

Fig. 2. Mass chromatograms from the double-pooled standard plasma containing six test compounds and the internal standard using direct LC–ESI–MS/MS system.

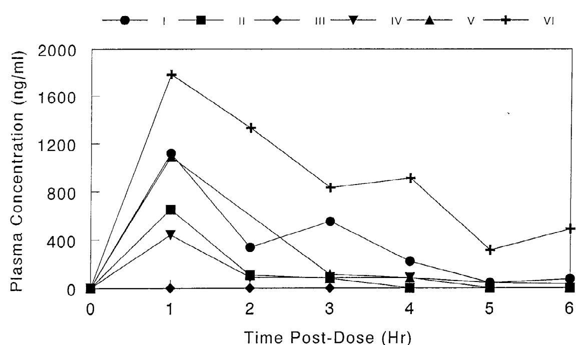

Fig. 3. Rapid mouse PK profiles of compound I through VI using direct LC–ESI–MS/MS method.

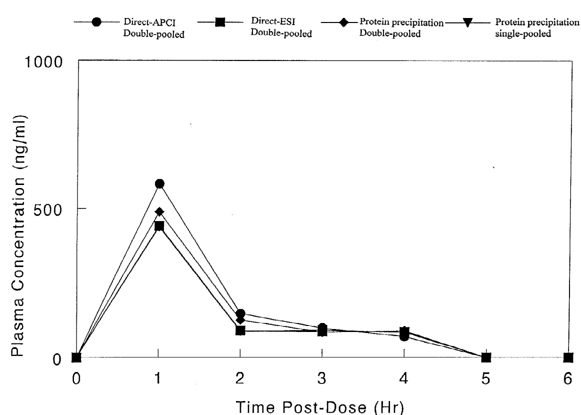

Fig. 4. Plasma concentration vs. time profiles of compound IV in the nude mouse using direct LC–APCI–MS/MS, direct LC–ESI–MS/MS, protein precipitation methods with double-pooled plasma samples and protein precipitation method with single pooled plasma samples.

analytical methods used. Fig. 4 shows the individual plasma concentrations of compound IV as a function of post-dosing time obtained by four aforementioned bioanalytical methods; this figure shows that similar data were obtained regardless of the analytical approaches employed. Furthermore, the correlation of analytical results obtained by direct LC–APCI–MS/MS method against those obtained by direct LC–ESI–MS/MS method, the protein precipitation method with double-pooled samples and the protein precipitation method with single-pooled samples is demonstrated in Fig. 5A, B and C, respectively. The Student's *t*-test ($\alpha<0.05$) indicated that the analytical results in between these methods had no significant difference. The results demonstrate that the proposed direct analysis method provided data equivalent to conventional LC–MS/MS method. In addition, the proposed direct multi-component analysis method combined with double-pooled samples provides a simple analytical method which significantly reduces the off-line sample preparation time thereby enabling higher throughput biological sample determination capability.

4. Conclusions

There are many advantages such as improved throughput, sensitivity in combination of direct injection method with sample pooling approach. An efficient bioanalytical method based on mixed-function column HPLC–MS/MS for on-line purification and separation has been demonstrated for the simultaneous determination of early drug discovery

Table 1
Comparison of $AUC_{(0\rightarrow6\ h)}$ of compounds I through VI obtained by direct LC–APCI–MS/MS method with double-pooled sample, direct LC–ESI–MS/MS method with double-pooled sample, protein precipitation method with double-pooled sample and protein precipitation method with single-pooled sample

Compound	Dynamic range (ng/ml)	$AUC_{(0\rightarrow6\ h)}$ ($h\times ng/ml$),			
		Direct-APCI Double-pooled	Direct-ESI Double-pooled	Non-direct-APCI Double-pooled	Non-direct-APCI Single-pooled
I	50–5000	2920	2410	2540	2760
II	50–500	1090	840	1140	1080
III	50–500	0	0	0	0
IV	50–500	729	951	807	740
V	50–5000	1710	1860	1590	1360
VI	500–5000	4810	4860	5140	3950

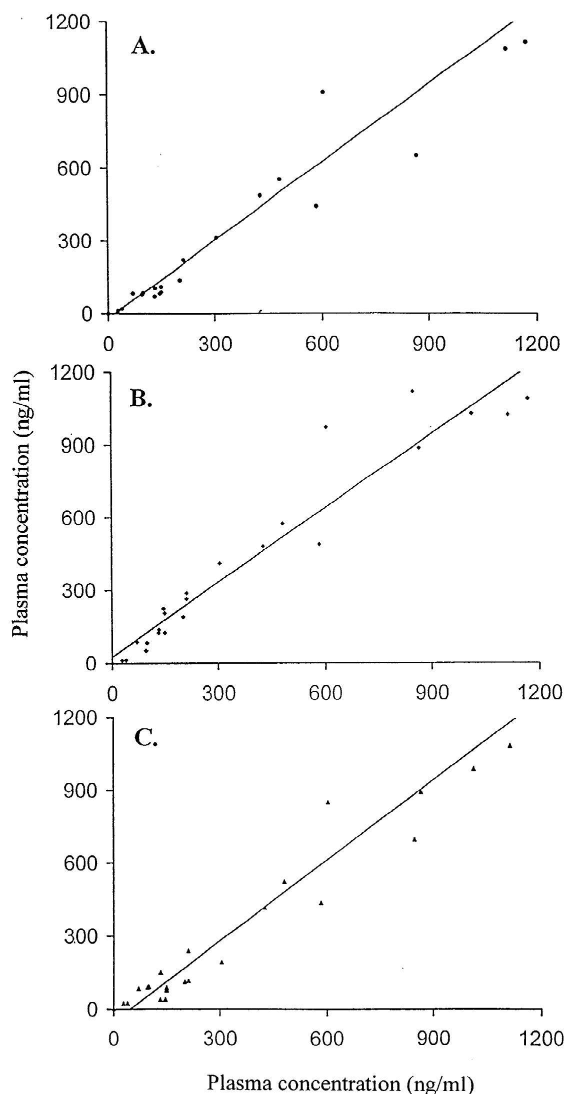

Fig. 5. Correlation of analytical results obtained by: (A) direct LC–APCI–MS/MS method with double-pooled samples vs. protein precipitation methods with double-pooled plasma samples; (B) direct LC–APCI–MS/MS method with double-pooled samples vs. direct LC–ESI–MS/MS method with double-pooled plasma samples; and (C) direct LC–APCI–MS/MS method with double-pooled samples vs. protein precipitation methods with single-pooled plasma samples.

compounds in the double-pooled mouse plasma. The proposed method has been proven to be simple, sensitive, reproducible and reliable for rapid PK screening. The on-line purification column also served as separation column. The proposed technique has shown the potential for direct multi-drug analysis in plasma samples.

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