Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 251–261
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneous fast HPLC-MS/MS analysis of drug candidates and hydroxyl metabolites in plasma

Yunsheng Hsieh *, Jean-Marc Brisson, Ganfeng Wang, Kwokei Ng, Walter A. Korfmacher

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

Received 6 May 2002; accepted 24 January 2003

Abstract

A rapid bioanalytical method was evaluated for the simultaneous determination of drug discovery compounds and their potential metabolites in plasma samples within 1 min run time by fast high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS). The fast HPLC-MS/MS system is achieved by using mini-column HPLC coupled to tandem mass spectrometer which is advantageous over regular HPLC-MS/MS systems, such as a shorter chromatographic region of ionization suppression, less solvent consumption and higher throughput. Matrix ionization suppression effect of the test compounds in plasma samples when using fast HPLC-MS/MS method was examined by a post-column infusion technique. In the described example, the proposed approach has been successfully employed to determine the plasma concentration of the test compound and its hydroxyl metabolite (M+16) in monkey in the low ng/ml region. The monkey pharmacokinetic results obtained by the proposed fast HPLC-MS/MS method were in good agreement within 20% error with those obtained by the regular HPLC-MS/MS method based on the same sample preparation procedure.

Keywords: Fast HPLC-MS/MS; Matrix ionization suppression; Ballistic gradient separation; Drug discovery compounds; Hydroxyl metabolites; Pharmacokinetics

1. Introduction

Drug discovery has played a key role in the growth of pharmaceutical companies. Due to the advancement of combinatorial chemistry and parallel synthesis, today's pharmaceutical industry has been able to dramatically increase the rate of production of new chemical entities (NCEs). Consequently, the large numbers of samples derived from in-vitro and in-vivo drug metabolism experiments based on these NCEs have yielded increasing demand for both rapid method development and higher throughput assays. The selection of lead compounds in most drug discovery projects frequently depends on the results of the drug metabolism (DM) and pharmacokinetics

* Corresponding author. Tel.: +1-908-740-5385; fax: +1-908-740-2966.
E-mail address: yunsheng.hsieh@spcorp.com (Y. Hsieh).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00351-0

(PK). Therefore, fast and efficient ways to provide accurate DMPK information on the target compounds and their major metabolites are required for both drug discovery and clinical development efforts [1–3]. The HPLC-MS/MS system has become the instrument of choice for drug assay in biological fluids due to its inherent selectivity and sensitivity resulting in shorter chromatographic run time than the HPLC methods with other detectors [3].

In order to keep pace with the speed of drug discovery, several efforts such as parallel sample introduction [4], sample pooling techniques [5], direct plasma injection [5–7] and fast chromatography [8–13], all using HPLC-MS/MS systems, have been continuously explored to increase sample throughput. In this work, we describe the analytical performance and matrix ionization suppression effects of the fast HPLC-MS/MS conditions currently developed within our laboratory for the simultaneous determination of multi-components in monkey plasma to support in-vivo PK studies. Separation of the analytes was successfully achieved on a reversed phase mini-column using a short gradient time. The column effluent was directly connected either to the atmospheric pressure chemical ionization (APCI) source or the electrospray ionization (ESI) source as part of the tandem mass spectrometer (MS/MS) system. Simultaneous selective reaction monitoring (SRM) of multi-analytes and the internal standard were used for the quantitative determination of the analytes. The matrix effect of ionization suppression in various plasma samples for quantitative fast HPLC-MS/MS analyses was investigated using a post-column infusion technique. Furthermore, the assay accuracy was demonstrated by a direct comparison of the analytical results obtained from regular HPLC-MS/MS methods and the proposed fast HPLC-MS/MS methods for quantitative plasma analysis using a drug candidate and its synthetic hydroxyl metabolite. The plasma concentrations of the dosed compound at individual time-points were also correlated with biological responses. The advantages and performance of the proposed method in terms of chromatographic resolution, matrix ionization suppression and sample throughputs were evaluated using both actual and spiked plasma samples.

2. Experimental

2.1. Reagents and chemicals

The test compound, its synthetic hydroxyl metabolite (M+16) and internal standard (ISTD) are new chemical entities produced from the Schering Plough Research Institute. The chemical structure of the internal standard was published elsewhere [14]. Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA) and ammonium acetate (99.999%) was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Deionized water was generated from Milli-Q water purifying system purchased from Millipore Corp. (Bedford, MA) and house high-purity nitrogen (99.999%) was used. Drug-free rat, dog and monkey plasma samples (with heparin) were purchased from Bioreclamation Inc. (Hicksville, NY). 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 and B were 4 mM ammonium acetate in water–acetonitrile (95:5) and 4 mM ammonium acetate in water–acetonitrile (20:80), respectively.

2.2. Equipment

HPLC-MS/MS analysis was performed using a PE Sciex (Concord, Ont., Canada) Model API 3000 triple quadrupole mass spectrometer, equipped either with heated nebulizer or electrospray interfaces. The HPLC system consisted of a CTC HTS PAL autosampler with a refrigerated sample compartment (set to 10 °C) from LEAP Technologies (Carrboro, NC), Shimadzu on-line degasser, LC-10AD VP pump and LC-10A VP controller (Columbia, MD). For the fast gradient method, a Synergi C18 column (2.0 × 30 mm, 4 μ) from Phenomenex Inc (Torrance, CA) was used as the analytical column. For the regular method, a Synergi C18 column (4.6 × 50 mm, 4 μ) from Phenomenex Inc. was used as the analytical column. The Quadra 96 (Tomtec, Hamden, CT)

system was used for semi-automated sample preparation with protein precipitation. The experimental mass spectrometric conditions were determined using generic state files for both APCI and ESI interfaces. For the matrix effect studies, the test compound, its hydroxyl metabolite and ISTD were continuously infused into PEEK tubing in between analytical column and mass spectrometer through a tee using a Harvard Apparatus Model 2400 (South Natick, MA) syringe pump. Either a protein precipitation extract of the blank plasma samples or mobile phase B (10 μl) was injected into HPLC column. Effluent from the HPLC columns mixed with the infused compounds and entered either APCI or ESI interfaces.

2.3. Sample collection

The animal dosing experiments were carried out in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act. Study blood samples were collected at specified time-points up to 24-h following oral administration to individual monkeys at 3 mg/kg and chilled on crushed ice. Plasma was then separated by centrifugation and stored frozen (−20 °C) until analysis.

2.4. Standard and sample preparation

Stock solutions of the test compound, its synthetic hydroxyl metabolite and ISTD were prepared as 1 mg/ml solutions in methanol. Analytical standard samples were prepared by spiking known quantities of the standard solutions into blank monkey plasma. The concentration range for both analytes in monkey plasma was 1–2500 ng/ml level.

The monkey plasma samples were prepared using the protein precipitation technique. A 150-μl aliquot of an acetonitrile solution containing 1000 ng/ml of internal standard was added to 50 μl of plasma located in a 96-well plate. After mixing and centrifugation, the supernatant was automatically transferred to a second 96-well plate by the Quadra 96 instrument. For the comparison of assay accuracy, 10-μl aliquots of the extract were injected by the Leap autosampler to four fast HPLC-MS/MS systems for quantitative analysis.

2.5. Chromatographic conditions

Chromatographic separation was achieved using mobile phases A and B. For the fast gradient HPLC method, a ballistic gradient from 10 to 100% mobile phase B was run over 0.3 min, held for 0.6 min and re-equilibrated to 10% B over 0.1 min at a constant flow-rate of 1.0 ml/min. For the regular HPLC method, a linear gradient from 10 to 100% mobile phase B was run over 1.2 min, held for 2.5 min and re-equilibrated to 10% B over 0.3 min at a constant flow-rate of 1.0 ml/min. The effluent from the HPLC systems was connected directly to the mass spectrometer when using the APCI source. The flow between the HPLC columns and mass spectrometer was split at a ratio of 3:1 when using the ESI source. The retention times for the test compound, its hydroxyl metabolite and ISTD with the fast gradient method were 0.88, 0.74 and 0.78 min, respectively and those with the traditional method were 2.82, 2.31 and 2.56 min, respectively.

The chromatographic resolution was calculated as $R_s = 2(T'^{R1} - T'^{R2})/(W_2 + W_1)$, where T'^{R1} and T'^{R2} are the reduced retention times of the analytes 1 and 2, respectively; W_1 and W_2 are the peak widths of analytes 1 and 2, respectively measured by extrapolating the relatively straight sides to the baseline [15].

2.6. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion mode. The heated pneumatic nebulizer probe conditions were as follows: 450 °C temperature setting, 80 psi nebulizing gas pressure, 1.0 l min^{-1} auxiliary gas flow, 0.9 l min^{-1} curtain gas flow-rate. The ESI conditions were as follows: 400 °C temperature setting, 0.8 l min^{-1} auxiliary gas flow, 0.9 l min^{-1} curtain gas flow-rate. The MS/MS reaction selected to monitor the test compound was the transition from *m/z* 406, $[M+H]^+$ ion, to a product ion at *m/z* 360. The hydroxyl metabolite and internal standard were monitored using the transitions from *m/z* 422 →

376 and *m/z* 639→471, respectively. The protonated molecules were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting 5. The collision offset-voltage was set at 35 V. The product ion spectrum of the ISTD was shown elsewhere [7] and the product spectra of both the test compound and its hydroxyl metabolite are given in Fig. 1. For the unknown hydroxyl metabolites, the MS/MS transitions were determined using an intelligent guess based on the fragmentation patterns of the dosed compounds.

3. Results and discussion

The fate of discovery compounds is frequently based on their pharmacokinetic parameters in animal models, which are mainly calculated from the plasma concentrations of the dosed compounds over post-dose time. However, metabolite profiling is also important for interpreting the efficacy outcome, modifying the lead compound and explaining the toxicity of the specific drug in a drug discovery program [1,3]. In this work, our goal was to develop a generic procedure for quantitative screen-type fast gradient HPLC-MS/MS assays for simultaneously monitoring the dosed compounds and their metabolites in plasma samples.

3.1. Development of fast gradient LC-MS method

One of the common goals in the pharmacokinetic area is to perform shorter separation times for a single component determination in biological samples. Typical strategies for achieving rapid chromatography include: the utilization of shorter columns with small particles and inner diameter, using mobile phase solutions with reduced viscos-

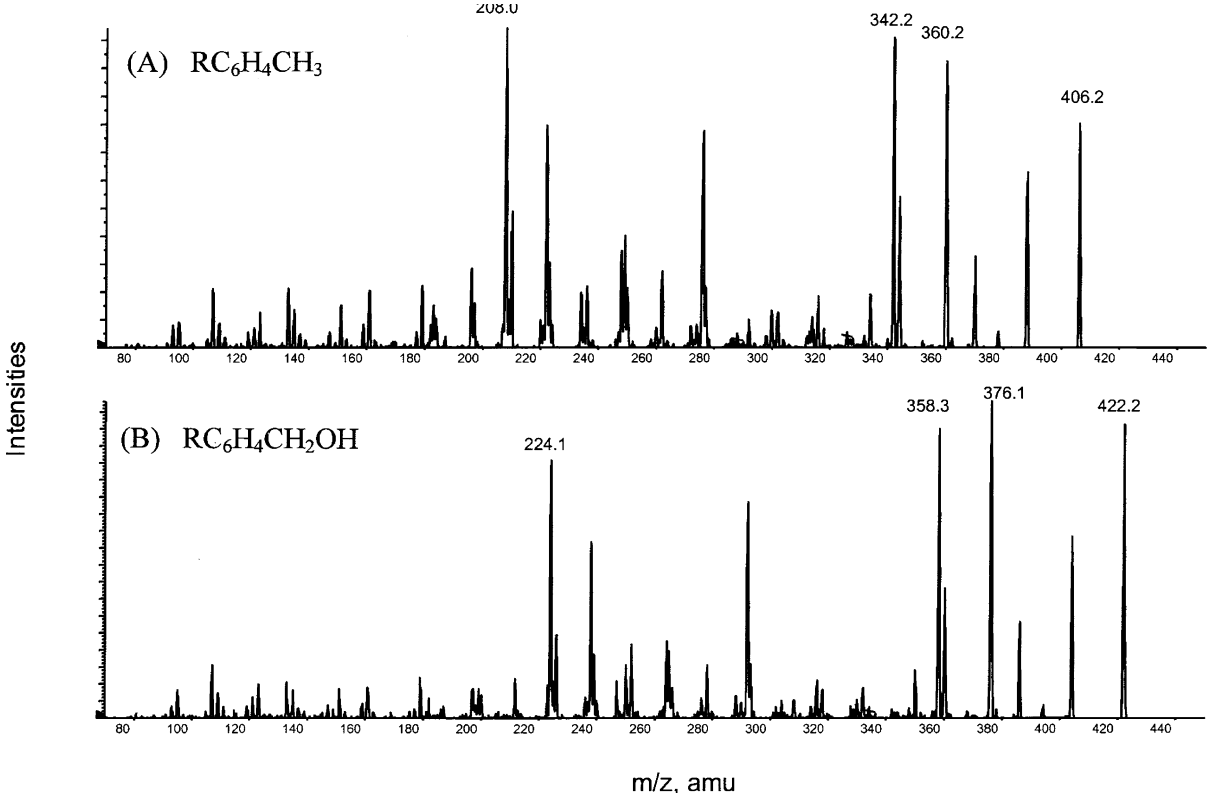

Fig. 1. The product ion spectra of (A) the test compound and (B) its hydroxyl metabolite.

ity and elution strength and increasing flow rate with isocratic separation [10–12]. In this work, we had first developed fast HPLC-MS/MS methods for simultaneous quantitation of drug components and their active metabolites in monkey plasma samples using a ballistic gradient, a short and narrow HPLC column (2 × 30 mm). The performance of the fast chromatographic methods versus the traditional method with a regular 4.6 mm I.D. column was then compared using the test compound and its hydroxyl metabolite.

Fig. 2(A, B) show the HPLC-APCI/MS/MS chromatograms of the test compound and its M+16 metabolite using the regular column and the mini-column methods, respectively. The regular HPLC method demonstrated longer retention times for both the test compound and its M+16 metabolite and provided more chromatographic separation resolution ($R_s = 64$) than the proposed fast HPLC method ($R_s = 30$) for the test compound and its M+16 metabolite. Both analytes were well-resolved on each method. Sufficient chromatographic resolution in the quantitative determination of the dosed compound and its metabolites in biological samples using HPLC-MS/MS is recommended to avoid possible interferences from drug-related biotransformation products [16]. The increase in peak capacity (smaller peak width) compensated for the shorter retention time with the proposed fast HPLC method. Good selectivity of the fast HPLC method was indicated by the appearance of other minor mono-oxygenated metabolites in Fig. 3, which shared the same mass transition range as the authentic hydroxyl metabolite and was chromatographically separated after injection of monkey plasma extract. The other predictable metabolites in the monkey plasma using an intelligent guess of multiple reaction monitoring (MRM) were simultaneously monitored and not observed. The conjugates of

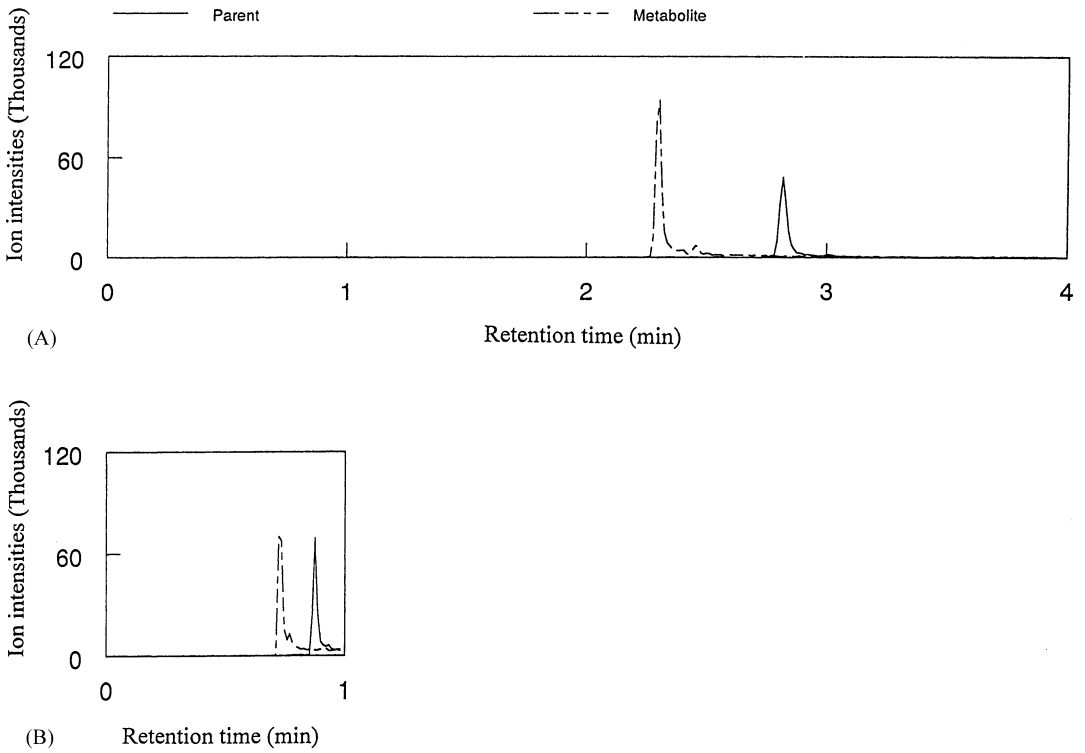

Fig. 2. APCI/MS/MS chromatograms of the test compound and its hydroxyl metabolite with (A) the regular HPLC method and (B) the fast HPLC method.

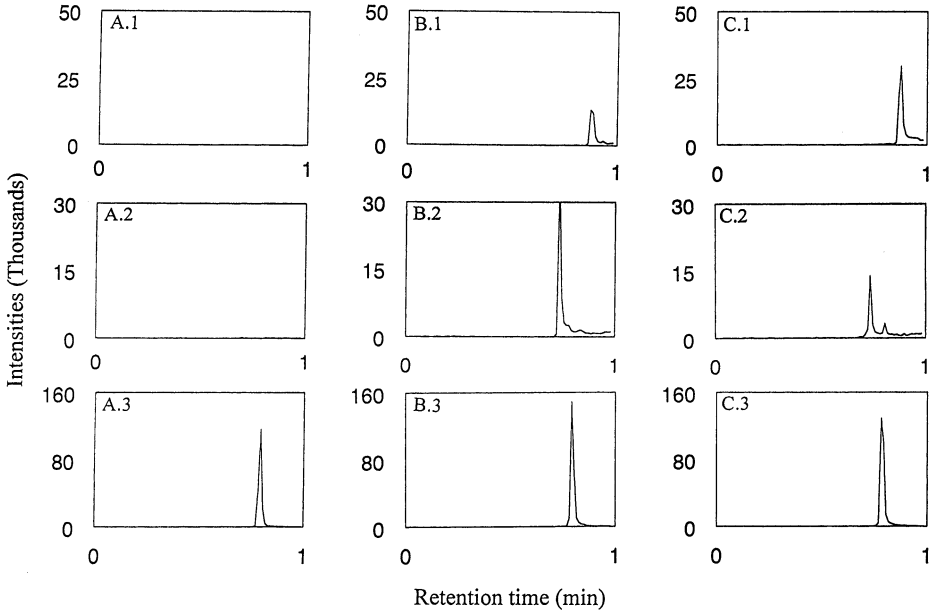

Fig. 3. HPLC-APCI/MS/MS chromatograms of (top row) the test compound, (middle row) metabolite and (bottom row) internal standard in (left column) blank monkey plasma, (center column) spiked standard monkey plasma and (right column) study monkey plasma.

hydroxyl metabolites in plasma extract might cause mass spectrometric interference based on their product ion mass spectra. Normally these conjugated metabolites demonstrate stronger polarity and shorter retention time than their hydroxyl metabolites in a reversed phase chromatography that were not observed in the study monkey plasma samples.

So far, more than 500 different analytes and their various metabolites in more than 10,000 plasma samples have been quantitatively determined in this laboratory showing reproducible peak areas and the retention times with the proposed fast HPLC-MS/MS methods. The mobile phase consumption per sample with the proposed approach was about four times less than the conventional approach used in our laboratory.

3.2. Matrix ionization suppression studies

A well-accepted concern about assay reliability when developing HPLC-MS/MS methods is the increased likelihood of encountering matrix ionization suppression [17]. Furthermore, matrix ionization suppression is considered more likely a problem when using the protein precipitation method for sample preparation compared to the liquid–liquid and the solid phase extraction methods [18]. In spite of this concern, the protein precipitation method has been chosen as the major sample preparation procedure for LC-MS/MS assays in our laboratory due to its simplicity [3,5]. In order to observe the matrix ionization suppression effects using fast HPLC-MS/MS techniques on plasma protein precipitation extracts from several plasma samples, we monitored the variability of the APCI and ESI responses for the test compound, its M+16 metabolite and ISTD using the post-column infusion scheme. Any changes in the APCI or ESI responses of the infused compounds were assumed to be due to matrix ionization suppression caused by the sample-related materials eluting from the HPLC columns.

We focused on exploring the impact of matrix ionization suppression effects on various plasma samples using the fast HPLC-MS/MS method.

The infusion HPLC-ESI/MS/MS chromatograms of the test compound after a 10-μl injection of mobile phase or rat, dog and monkey plasma extracts using fast gradient HPLC method are shown in Fig. 4(A–C, respectively). The differences in the infusion chromatograms between the mobile phase injection and the plasma extract injection were considered to be caused by ionization suppression due to plasma sample extract constituents eluting from the column. Fig. 4 demonstrate that the degree of loss of ESI response and the length of time required for the ESI response to return to its pre-sample injection signal were repeatable for three different plasma samples in this study. For accurate quantitative determination, it is strongly recommended that the retention times of all analytes should be in the region of little or no matrix ion suppression. The objectives of the post-column infusion experiments were to measure the extent of ionization suppression and to define the 'safer' portion of the chromatographic assay time window yielding little or no ionization suppression when using the proposed fast HPLC-MS/MS system.

As shown in Fig. 4, the proposed fast gradient HPLC method appeared to have short and identical time window of the matrix ion suppression region (12 s) from three kinds of plasma samples. Figs. 5 and 6 show the infusion HPLC-ESI/MS/MS chromatograms of the metabolite compound and ISTD, respectively, after injection of mobile phase, rat, dog and monkey plasma extracts using fast gradient HPLC method. The same time frame of observing matrix ionization suppression for both analytes and the ISTD suggests that all three compounds are affected by matrix ionization suppression to a similar degree. Gradient elution is our first choice for HPLC conditions because it provides better chromatographic resolution of mixtures and loading capacity than isocratic elution, although it requires longer analysis time for column re-equilibration. While isocratic conditions offer faster analyses, it is more difficult to

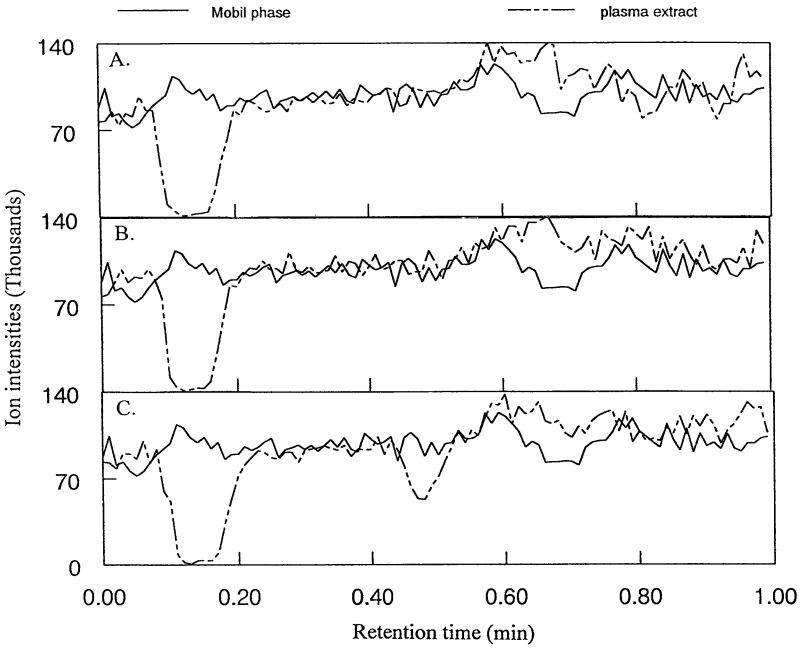

Fig. 4. The infusion HPLC-ESI/MS/MS chromatograms of the test compound in (A) rat, (B) dog and (C) monkey plasma, respectively, obtained by the fast gradient approach, following (solid line) mobile phase and (dot line) blank plasma precipitation extract injections.

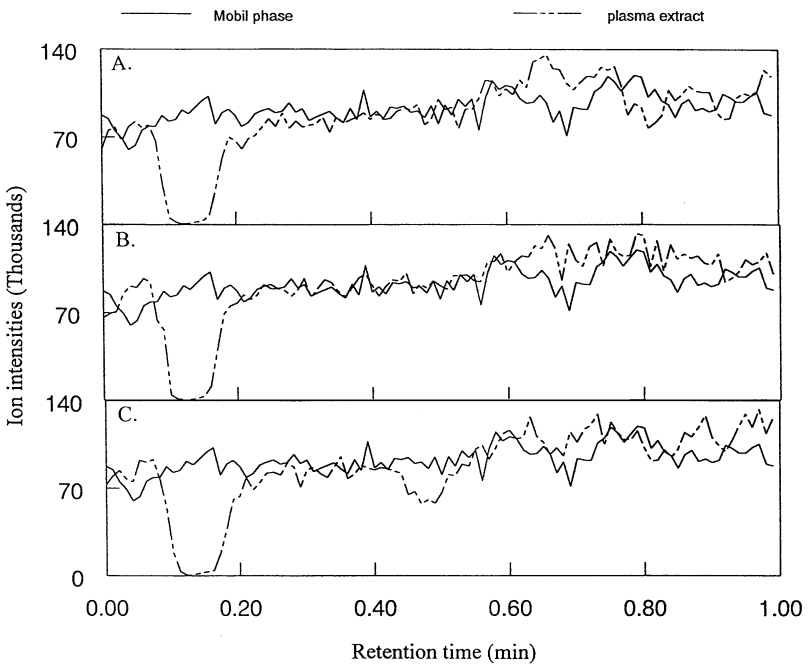

Fig. 5. The infusion HPLC-ESI/MS/MS chromatograms of the metabolite in (A) rat, (B) dog and (C) monkey plasma, respectively, obtained by the fast gradient approach, following (solid line) mobile phase and (dot line) blank plasma precipitation extract injections.

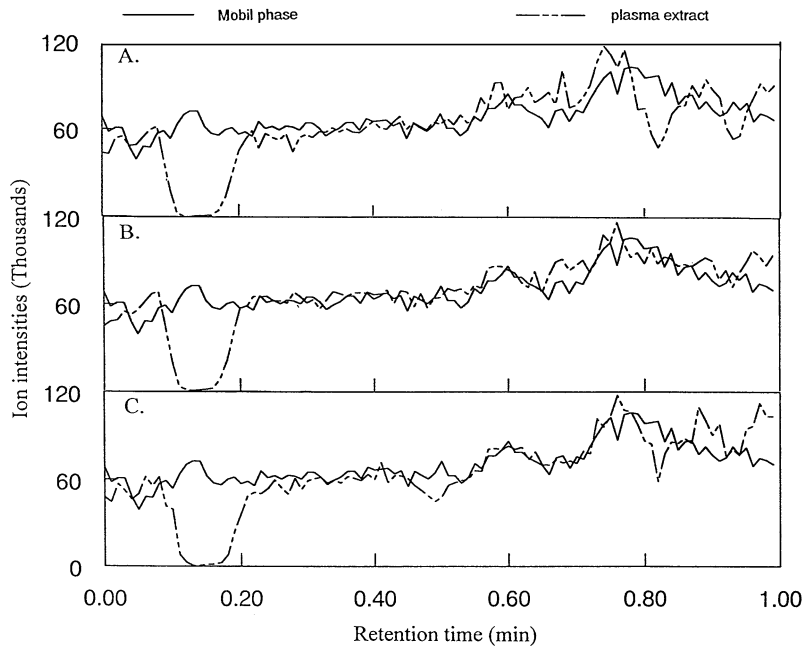

Fig. 6. The infusion HPLC-ESI/MS/MS chromatograms of the internal standard in (A) rat, (B) dog and (C) monkey plasma, respectively, obtained by the fast gradient approach, following (solid line) mobile phase and (dot line) blank plasma precipitation extract injections.

resolve analytes from salts or endogenous nonvolatile sample materials, which may cause ionization suppression. In this work, we employed a ballistic gradient approach to eliminate interferences from the salts such as sulfates and phosphates and polar nonvolatile compounds in plasma extracts, which are likely to be contributors to ionization suppression [19].

The infusion LC-APCI/MS/MS chromatograms of the test compound are given in Fig. 7. Fig. 7 shows that the APCI response of the test compound with three kinds of plasma protein precipitation extracts was less sensitive to the matrix ionization suppression effect compared to ESI interface (10 vs. 100% suppression). This phenomenon was also applied to the metabolite and ISTD (data not shown). These findings correlate with other researchers' report indicating that typically, the APCI source is less vulnerable than ESI source in terms of matrix ionization suppression effects [18]. The recovery time for the APCI response to return to its pre-sample injection level was about half that with the ESI interface.

3.3. Analysis of monkey plasma samples

As an example, the proposed HPLC-MS/MS method with the 1 min sample analysis time was applied for simultaneous determination of the dosed compound and its M+16 metabolite in monkey plasma to demonstrate the realistic suitability of fast analyses. Fig. 3(A–C) show the mass chromatograms for the dosed compound, its M+16 metabolite and the internal standard, respectively. In Fig. 3, the left column represents the signals from the blank monkey plasma indicating no endogenous or interference peaks for each analyte in monkey plasma samples. The retention times and peak areas for the internal standard observed in blank plasma (left column), spiked standard plasma (center column) and study plasma (right column) was found to be consistent through the experiment ($\approx$300 injections). The retention times, as shown in Fig. 3 for both analytes and internal standard, in standard plasma and study plasma samples were also found to be reproducible ($<$5% CV).

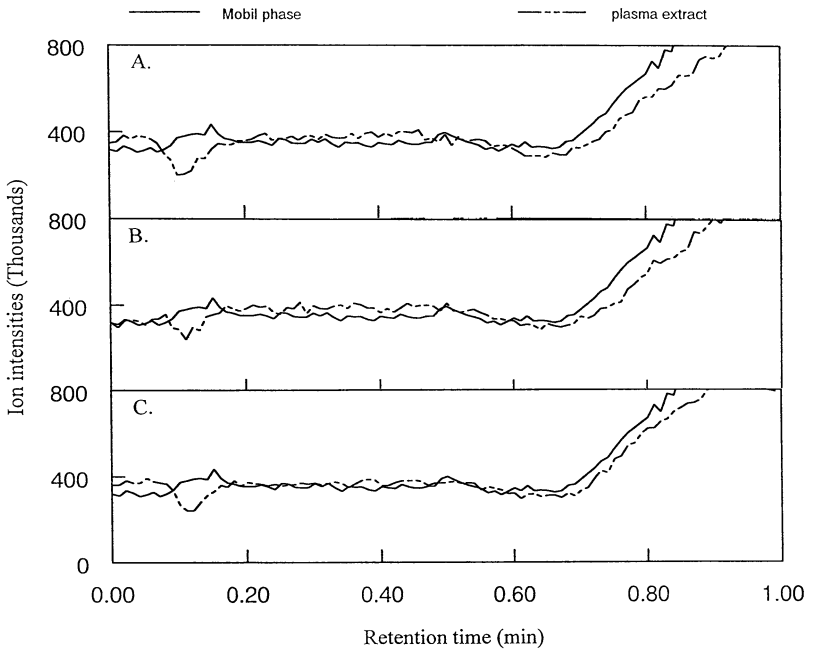

Fig. 7. The infusion HPLC-APCI/MS/MS chromatograms of the test compound in (A) rat, (B) dog and (C) monkey plasma, respectively, obtained by the fast gradient approach, following (solid line) mobile phase and (dot line) blank plasma precipitation extract injections.

The calibration curves for both the dosed compound and its hydroxyl metabolite obtained from duplicate standard monkey plasma samples at each concentration level were linear with a correlation coefficient, $r^2 > 0.99$ (graph not shown). Accuracy (% bias) was $< 15\%$ at all concentrations, 1–2500 ng/ml. The plasma concentration-time profiles of both analytes monitored by fast HPLC-MS/MS and regular HPLC-MS/MS methods using either APCI or ESI interfaces following an oral administration at 3 mg/kg delivered with 20% HPBCD are shown in Fig. 8. The area under the curve, $AUC_{(0\rightarrow 24)}$,values of both analytes obtained by the fast HPLC and regular HPLC-MS/MS methods were in good agreement ($< 15\%$ difference). The Student's t-test results indicated no significant difference of plasma concentrations at each time point of both the dosed compound and its hydroxyl metabolite determined by the aforementioned HPLC-MS/MS methods with 95% confidence ($\alpha = 0.5$). The above results concluded that the proposed fast HPLC-MS/MS methods were equivalent with the regular HPLC-MS/MS methods in terms of accuracy but about four times faster in terms of assay time. In clinical or development support, the recommended compound from drug discovery is normally investigated extensively for a long period of time based on a well-developed assay for quantitative measurements. In discovery stage, most bioactive compounds are tested only once or twice in different species. In addition, to the best of our knowledge, the animal PK data of the recommended compounds obtained using the described fast HPLC-MS/MS method are reproducible compared to those obtained using the other validated HPLC-MS/MS method from our development efforts.

4. Conclusion

An efficient bioanalytical method based on fast gradient HPLC-MS/MS method has been demonstrated for the simultaneous determination of a drug candidate and its phase I metabolite in monkey plasma. The proposed method has been proven to be sensitive, cost-effective and reliable for high throughput PK screening and showed equivalent accuracy to the analytical results ob-

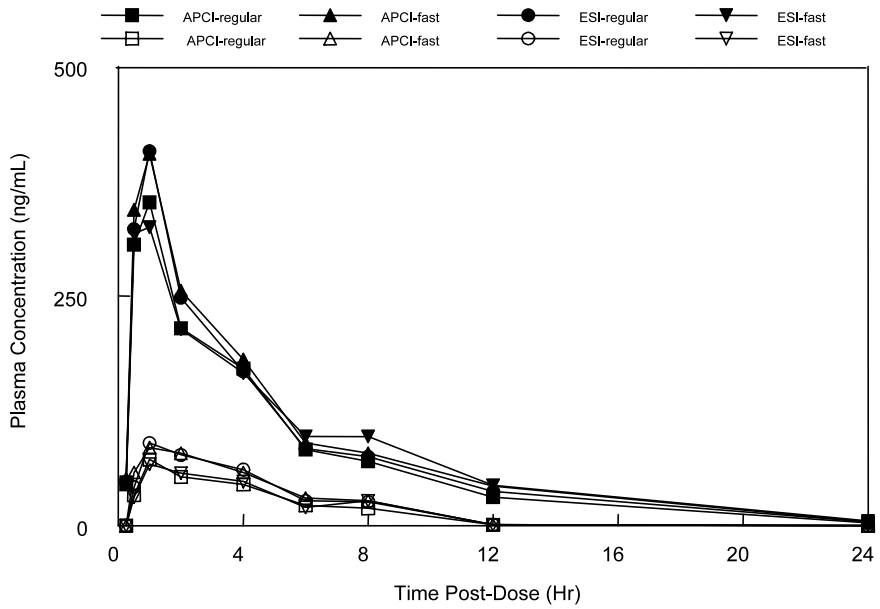

Fig. 8. Pharmacokinetic profiles of the test compound and its hydroxyl metabolite from an individual dosed monkey after oral administration obtained using four HPLC-MS/MS methods with the same plasma extracts.

tained using the regular HPLC-MS/MS method. It was also demonstrated that under the proposed fast gradient HPLC conditions, we are able to achieve short chromatographic run time while maintaining appropriate separation power and avoiding matrix ion suppression problem.

Acknowledgements

The authors thank the Medicinal Chemistry group for synthesizing the test compounds and the Department of Pharmacology for planing the animal studies presented in this work.

References

[1] K.A. Cox, R.E. White, W.A. Korfmacher, Comb. Chem. High Through. Screen. 5 (2002) 29–37.
[2] J.H. Lin, A.Y. Lu, Pharmacol. Rev. 49 (1997) 403–449.
[3] W.A. Korfmacher, K.A. Cox, M.S. Bryant, J. Veals, K. Ng, R. Watkins, C. Lin, Drug Discov. Today 2 (1997) 532–537.
[4] W.A. Korfmacher, J. Veals, K. Dunn-Meynell, X. Zhang, G. Tucker, K.A. Cox, C. Lin, Rapid Commun. Mass Spectrom. 13 (1999) 1991–1998.
[5] Y. Hsieh, J. Brisson, K. Ng, W.A. Korfmacher, J. Chromatogr. B 767 (2001) 353–362.
[6] Y. Hsieh, M.S. Bryant, G. Gruela, J. Brisson, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 14 (2000) 1384–1390.
[7] Y. Hsieh, J. Brisson, K. Ng, W.A. Korfmacher, J. Pharmaceut. Biomed. Anal. 27 (2002) 285–293.
[8] L.A. Romanyshyn, P.R. Tiller, J. Chromatogr. A 928 (2001) 41–51.
[9] J. Ayrton, R. Plumb, W.J. Leavens, D. Mallett, M. Dickins, G.J. Dear, Rapid Commun. Mass Spectrom. 12 (1998) 217–224.
[10] Y. Cheng, Z. Lu, U. Neue, Rapid Commun. Mass Spectrom. 15 (2001) 141–151.
[11] L.A. Romanyshyn, P.R. Tiller, R. Alvaro, A. Pereira, C.E.C.A. Hop, Rapid Commun. Mass Spectrom. 15 (2001) 313–319.
[12] K. Heinig, J. Henion, J. Chromatogr. B 732 (1999) 445–458.
[13] Y. Hsieh, M. Chintala, H. Mei, J. Agans, J. Brisson, K. Ng, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 15 (2001) 2481–2487.
[14] M. Liu, M.S. Bryant, J. Chen, S. Lee, B. Yaremko, P. Lipari, M. Malkowski, E. Ferrari, L. Nielsen, N. Prioli, J. Dell, D. Sinha, J. Syed, W. Korfmacher, A. Nomeir, C. Lin, L. Wang, A. Taveras, R. Doll, G. Njoroge, A. Mallams, S. Remiszewski, J. Catino, V. Girijavallabhan, P. Kirschmeier, R. Bishop, Cancer Res. 58 (1998) 4947–4956.
[15] J.J. Kirkland (Ed.), Modern Practice of Liquid Chromatography, Wiley, New York, 1971.
[16] M. Jemal, Y. Xia, Rapid Commun. Mass Spectrom. 19 (2000) 1757–1765.
[17] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.
[18] C. Miller-Stein, R. Bonfiglio, T. Olah, R. King, Am. Pharmaceut. Rev. 3 (2000) 54–62.
[19] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2001) 942–950.