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Reliable on-line sample preparation of basic compounds from plasma using a reversed phase restricted access media in column-switching LC

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Abstract

We investigated on-line sample preparation of basic compounds from plasma using a methylcellulose-immobilized reversed-phase restricted-access media in column-switching liquid chromatography (LC). Dilution of the plasma sample with phosphate buffered saline prevented or delayed the formation of fibrin clots at 4 °C and resulted in reproducible on-line sample preparation over a 30-h period. The use of an ion-pair reagent in the extraction LC enhanced recoveries of hydrophilic basic compounds. The ability of the methods to quantify compounds in plasma were validated and the method was successfully applied to the pharmacokinetic study of a hydrophilic basic compound injected into the bloodstream of rats.

Keywords: Plasma; Ion-pair chromatography; Restricted-access media; Basic compound; Fibrin clots

1. Introduction

During the drug discovery process, numerous compounds are evaluated for physico-chemical properties [1]; administration, distribution, metabolism, and excretion (ADME); and toxicity. *In vivo* ADME studies are critical for identifying the most promising potential drug compounds. Furthermore, *in vivo* pharmacokinetic analyses to determine the concentrations of compounds and their metabolites in plasma are needed to define the characteristics of compound and to assess appropriate dosage. In this respect, precise, accurate, sensitive and moderate- or high-throughput analytical procedures are required. Batch sample preparation in the 96-well format with manual or robotic liquid handling has been utilized to increase throughput [2–5]. Direct analysis of compounds, using the column-switching (LC–LC) technique with restricted access media (RAM) [6], large particle supports [7–10] or turbulent flow condition [11–13] have achieved sensitive determination. These strategies of direct analysis offer automated sample preparation with a relatively simple setup. However, sample preparation methodologies, especially robotic liquid handing, remain problematic for plasma analyses due to the frequent formation of plasma clots or aggregates (thrombin or fibrinogen clots). Clots clogging pipet tips during sample preparation or LC tubing during analysis can sabotage the experiment. The effects of storage temperature and anticoagulants on clot formation were tested, but such approaches were unsuccessful in completely eliminating clots in plasma [14]. As an approach to remove clots from plasma samples prior to the sample preparation, a stainless steel wire-mesh screen for 96-well plates was recently described [15].

Insufficient recovery of analytes is another problem encountered in the off- and on-line sample preparation of hydrophilic compounds using reversed-phase material. The use of a cation exchanger would give high recovery of cationic analytes; however, such an approach requires well-designed sample preparation in ionic strength, organic solvent concentration and pH of employed solvents and adaptation with following LC and/or detection principle.

To overcome the problem of fibrin clot formation, we assumed that the formation of fibrin clots in plasma sample could be delayed by lowering fibrinogen concentration and we examined the effect of dilution of the plasma sample with aque-

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ous solution prior to on-line sample preparation on recovery of analytes, its reproducibility and durability. To achieve sufficient recoveries of hydrophilic basic compounds from plasma using reversed-phase material, we investigated the use of an ion-pair reagent during on-line extraction, using a methylcellulose-immobilized-octadecylsilane (MC-ODS) precolumn [16] in an LC–LC system called Shimadzu Co-sense for BA system.

2. Experimental

2.1. Materials

Ammonium acetate and acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). Heptafluorobutyric acid (HFBA) was obtained from TCI (Tokyo, Japan). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). Phosphate buffered saline (PBS) was purchased form Invitrogen Corporation (Auckland, NZ).

All reagents were analytical grade and used without further purification. Water was deionized and purified by a Milli-Q® purification system from Millipore (Bedford, MA, USA). Donepezil HCl (Aricept®), ER-235516, ER-113424, and ER-120867 which is a metabolite of ER-113424 produced *in vivo* were synthesized by Eisai (Tokyo, Japan) and used as model basic compounds. The structures, log $P_{o/w}$, and pK_a values determined by capillary electrophoresis [17] of these compounds are presented in Fig. 1.

2.2. Animals

Male Sprague-Dawley (SD) rats (7 weeks, 292.1–325.0 g) were purchased from Charles River Laboratories Japan (Yokohama, Japan).

2.3. Sample preparation

Following anti-coagulation with heparin, SD rat plasma was obtained by centrifugation of SD rat blood. The centrifuged plasma was used as the "blank plasma" sample. Primary stock solutions of donepezil and a mixture of ER-113424/ER-120867 were prepared in 30% acetonitrile. A primary stock solution of ER-235516 was prepared in water. The concentration of compounds in these solutions was 100 μg/mL.

Donepezil was added to fresh plasma, freeze-thawed plasma, and fresh plasma diluted with PBS (1:1, v/v) to 10 μg/mL. SD rat plasma containing ER-235516 or a mixture of ER-113424/ER-120867 was prepared by adding the compounds to blank plasma at concentrations of 30, 150, 300, 1500, and 3000 ng/mL. The blank plasma and compound-containing plasma samples were diluted with PBS (1:1, v/v) to 15, 75, 150, 750, and 1500 ng/mL.

The calibration standard solutions were prepared in a mixture of water and acetonitrile (70:30, v/v) using the same compound concentrations as for the spiked plasma samples. No internal standard was used in all experiments.

2.4. Instrumentation and chromatography

For LC–LC–UV, two different LC–LC systems that called "Shimadzu (Kyoto, Japan) Co-sense for BA system" were used. The first was the extraction LC-equipped by-pass line [16,18], which was used for on-line extraction using an extraction pump. The other was the system with dual pump dilution illustrated in Fig. 2. This system consisted of two LC-20AD pumps for extraction LC, an LC-20AB (dual pumps) for analytical LC, an SIL-20AC auto-injector, a DGU-20 degasser, a CTO-20A column oven, an FCV-20AH six-port valve, an SPD-20A UV detector, and a CBM-20A communication bass module. An LC

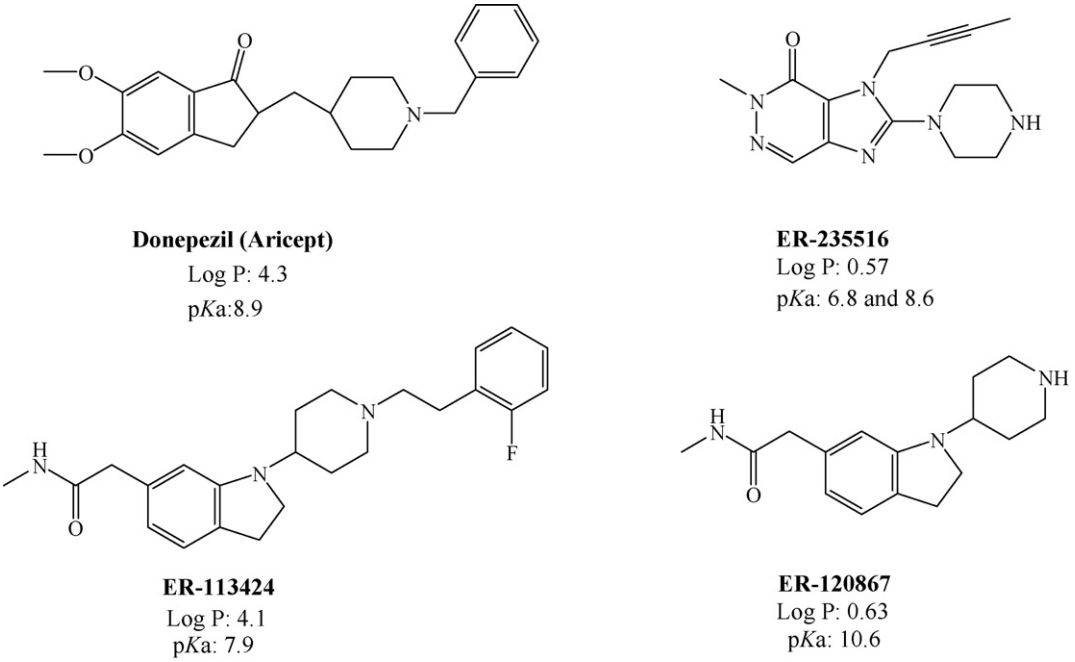

Fig. 1. Structures, partition coefficients, and dissociation constants (pK_a) of proton conjugated acid of donepezil (Aricept), ER-235516, ER-113424, and ER-120867.

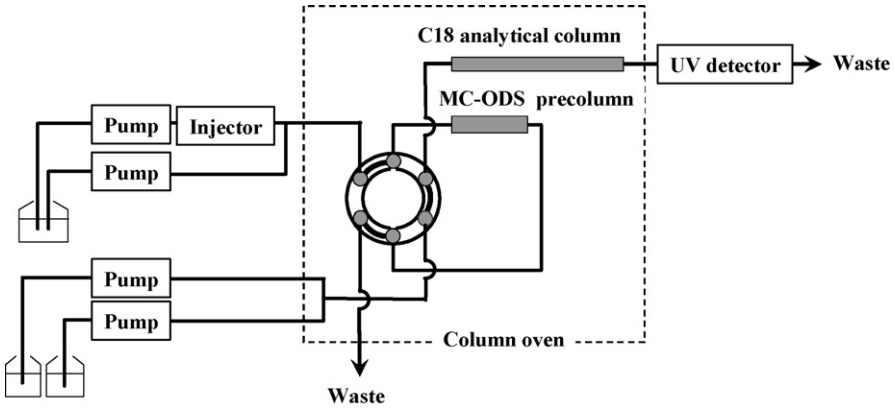

Fig. 2. Schematic diagram of the LC–LC–UV system (Co-sense for BA system).

solution software was used for liquid chromatograph control, data acquisition, and data analysis. The MC-ODS precolumn [16] (Shimadzu Shimpack MAYI-ODS, 10 mm × 4.6 mm i.d., 50 μm particle, 12 nm pore size) was used as an extraction column, and a YMC (Kyoto, Japan) Pro C18 analytical column (75 mm × 4.6 mm i.d., 3 μm particle, 12 nm pore size) was used.

For analysis of donepezil in plasma using the system with dilution by-pass, eight-fold dilutions of samples were prepared to enhance the compound recovery from plasma. The mobile phase consisted of a mixture of water and acetonitrile (950:50, v/v) containing 5 mmol/L of ammonium acetate.

For analysis of ER-235516 and the mixture of ER-113424/ER-120867 using the LC–LC system with dual pump dilution, the injected samples were delivered at 0.4 mL/min from the injector. On-line dilution was conducted with a dilution flow of the same mobile phase at 2.6 mL/min. Solutes were concentrated on the MC-ODS precolumn for 2 min with an extraction mobile phase at 50 °C. Following removal of plasma proteins and other matrix components, the target compounds enriched on the MC-ODS precolumn were transferred to the analytical column by switching a six-port valve under gradient elution mode at a flow rate of 1.0 mL/min at 50 °C. Target compounds were detected by a SPD-10Avp UV detector at a wavelength of 236 nm for ER-235516 and 294 nm for ER-113424 and ER-120867. The mobile phases consisted of (A) a mixture of water and acetonitrile (1000:10, v/v) containing 0.1% TFA and (B) a mixture of water and acetonitrile (100:900, v/v) containing 0.1% TFA.

For analysis of ER-235516, the initial concentration of mobile phase (B) was 0% and rose to 15% over 10 min. For analysis of ER-113424 and ER-120867, the initial concentration of mobile phase (B) was 0% and rose to 30% over 18 min, where it was maintained for 2 min. After each run, both the precolumn and the analytical column were flushed with mobile phase (B).

2.5. Reproducibility of on-line extraction of compounds from plasma

Donepezil was used as a model basic compound. Two hundred μL plasma samples containing 10 μg/mL of donepezil were aliquoted in 200-μL polypropylene sample vials, placed in the autosampler, and stored at 4 °C. These samples were repetitively injected once per vial, and the reproducibility of the peak area observed in analytical LC was evaluated. The plasma samples used for this study were fresh rat plasma, freeze-thawed rat plasma, and fresh rat plasma diluted with PBS (1:1, v/v).

2.6. Effect of an ion-pair reagent in extraction LC on compound recovery

The effect of an ion-pair reagent in the extraction LC mobile phase on the recovery of compounds from the MC-ODS precolumn was evaluated. The mobile phase consisted of a mixture of water and acetonitrile (95:5, v/v) containing 5 mmol/L ammonium acetate, with or without 0.1% HFBA as the ion-pair reagent. Standard solutions (10 μg/mL) of ER-235516 and the mixture of ER-113424/ER-120867 were used for the analysis. Absolute recovery from the MC-ODS precolumn was assessed by comparison of peak areas observed for each compound in LC–UV and LC–LC–UV analyses.

2.7. Effect of extraction temperature on on-line extraction of compounds from plasma

One-hundred μL of blank plasma, standard solution, or plasma sample containing 3000 ng/mL ER-113424/ER-120867 or ER-235516 (stored at 4 °C) were injected into the MC-ODS precolumn. On-line extraction of compounds was conducted at 30, 40, and 50 °C, and the resulted chromatograms were recorded. For this evaluation, the analytical column was maintained at 50 °C using an EYELA (Tokyo, Japan) column heater/cooler.

2.8. Analysis of ER-235516, ER-113424, and ER-120867

Linearities of the standard solutions and plasma samples ($n = 3$) were assessed at plasma concentrations of 30, 150, 300, 1500, and 3000 ng/mL (actual concentrations 15, 75, 150, 750 and 1500 ng/mL). Intra- and inter-day accuracy, precision, and reproducibility were evaluated ($n = 3$ for intra-day, $n = 6$ for inter-day). The accuracy was determined as recovery (%) of

compounds from plasma. The precision was determined as the compound concentration in the plasma sample calculated from the calibration curves of compounds in standard solutions. The reproducibility was determined as the coefficient of variation [CV (%)] of accuracy. ER-113424 and ER-120867 were analyzed simultaneously.

2.9. Pharmacokinetics of ER-235516 in SD rats

ER-235516 (3 mg/kg) in saline solution was injected intravenously into the right jugular veins of two fasting SD rats. Blood samples (about 250 μL) were drawn from the left jugular vein using a heparinized syringe at 0.083, 0.25, 0.5, 1, and 2 h following ER-235516 administration. Blood samples were centrifuged to obtain plasma, diluted with an equal volume of PBS, and analyzed.

3. Results and discussion

3.1. Reproducibility of the on-line extraction of donepezil from plasma

We evaluated the reproducibility of on-line extraction of spiked donepezil in fresh plasma, freeze-thawed plasma, and fresh plasma diluted with PBS (1:1, v/v). Samples were stored at 4 °C in the auto-sampler. For this evaluation, extraction LC with by-pass line [16,18] was used for the on-line dilution of plasma samples due to its sensitivity to plasma clots in the LC tubing between the injector and the six-port switching valve. Fresh plasma and freeze-thawed plasma samples were repetitively injected 25 times over 20 h, and plasma diluted with PBS was repetitively injected 32 times over 30 h. Prior to the evaluation of freeze-thawed plasma, fibrin clots found in the sample were manually eliminated.

The resulting reproducibilities [CV (%)] for donepezil (10 μg/mL) extraction were 42.2% ($n = 25$) for fresh plasma, 2.90% ($n = 25$) for freeze-thawed plasma, and 0.21% ($n = 32$) for fresh plasma diluted with PBS.

The reproducibility of on-line extraction of donepezil from fresh plasma was unacceptable. For this sample, the donepezil peak was absent two times, and a low recovery (≤50%) was observed three times during the experiment. These phenomena might be caused by clogging of the LC line between the injector and the six-port switching valve with a plasma clot. This assumption is based on the observations that no significant pressure buildup of the system was detected, and the system worked for 20 h without sabotage. Acceptable reproducibility was observed for freeze-thawed plasma, and a drastic improvement in reproducibility was observed for fresh plasma diluted with PBS and the recoveries of analytes were almost full in each examination. In addition, significant pressure buildup was not observed in the LC–LC system. These results indicated that dilution of the plasma sample prevented or delayed the formation of fibrin clots. Dilution of freeze-thawed plasma after eliminating plasma clots was also effective, and 10 mmol/L ammonium acetate could be applied to the dilution solvent (data not shown).

Based on these results, for further investigations we made 1:1 dilutions of plasma samples with PBS prior to analysis.

3.2. Effect of an ion-pair reagent in extraction LC on compound recovery

In this study, we employed three model compounds: ER-235516, with a piperazine ring in its structure; and ER-113424 and ER-120867, with piperidine rings in their structures. ER-235516 and ER-120867 exist in ionic forms at acidic or neutral pH. On-line sample preparation with reversed phase material at below neutral pH resulted in insufficient recoveries for these compounds. The absolute recovery of ER-113424 from the extraction LC with a mixture of water and acetonitile (950: 50, v/v) containing 5 mmol/L of ammonium acetate (pH 7) was almost complete, as determined by comparison with LC–UV analysis of the same sample. Recovery was not affected by the presence of an ion-pair reagent (HFBA) in the extraction mobile phase. On the other hand, the absolute recoveries of ER-235516 and ER-120867 without ion pair reagent were 10% and 25%, respectively, compared to the LC–UV analysis. When the mobile phase consisted of a mixture of water and acetonitrile (950: 50, v/v) containing 5 mmol/L of ammonium acetate and 0.1% of HFBA (pH 2.6), both compounds were fully recovered from extraction LC. These results indicate that the use of HFBA as an ion-pair reagent is effective to enhance the recoveries of hydrophilic basic compounds from extraction LC using reversed-phase material.

Similar attempt using 0.1% of TFA was not successful incompletely for ER-235516, however the use of other ion-pair reagents with longer carbon chain would gave satisfactorily results.

While the RAM function of the MC-ODS was effective even with HFBA in the extraction mobile phase, the background peak originating in plasma matrices components slightly differed from that in the chromatogram in the case of without HFBA in the extraction mobile phase. This phenomenon was probably due to changes in retention of plasma components resulting from the altered pH of the extraction mobile phase and the effect of HFBA.

Thus, for further investigations we employed a mobile phase consisting of water and acetonitrile (95:5, v/v), 5 mmol/L ammonium acetate, and 0.1% HFBA (pH 2.6).

3.3. Effect of temperature on extraction LC

The effect of the extraction temperature (30, 40, or 50 °C) was investigated using the MC-ODS precolumn and a mobile phase mentioned above, the recoveries of compounds from extraction column were not affected by temperature (30, 40, or 50 °C) and the column temperature of 50 °C necessarily shortened the retention times of compounds in analytical LC without loss of separation efficiency. Hence, we employed an on-line purification temperature of 50 °C for further analyses. In addition the column temperature of analytical column was also controlled at 50 °C in a column oven.

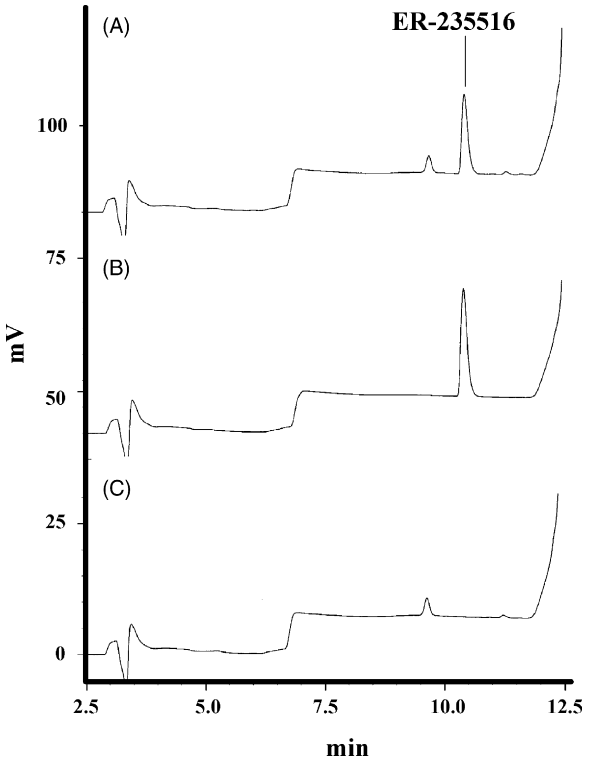

Fig. 3. Typical HPLC–UV chromatograms for ER-235516 obtained using the LC–LC–UV system. (A) Plasma sample 5 min after 3 mg/kg intravenous administration of ER-235516 in rats. (B) ER-235516 standard (1500 ng/mL). (C) Blank plasma (pre-administration).

3.4. HPLC analyses of ER-235516, ER-113424, and ER-120867

Analyses of ER-235516 and the mixture of ER-113424/ER-120867 plasma samples were performed using the MC-ODS precolumn in the LC–LC–UV system and compared with the standards and the blank plasma. Typical HPLC chromatograms for the blank plasma, the standard, and the pharmacokinetic sample of ER-235516 are shown in Fig. 3A–C. ER-235516 was clearly separated from the background interference matrices peaks, indicating sufficient specificity of the ER-235516 peak. In the chromatogram for the mixture of ER-113424/ER-120867 in plasma, compound peaks were likewise separated from the background peak.

The calibration curves ($n = 3$) obtained by plotting peak area versus compound concentration were linear over plasma concentrations of 30–3000 ng/mL, with correlation coefficients (r) above 0.999 for every compound. The equations for the calibration plots were $y = 262.0228x - 1138.32$ for the ER-23556 standard, $y = 262.1115x - 1463.18$ for ER-23556 in plasma, $y = 144.774x + 210.0664$ for the ER-120867 standard, $y = 142.2467x - 832.959$ for ER-120867 in plasma, $y = 74.88504x - 393.8$ for the ER-113424 standard, and $y = 76.56786x - 393.8$ for ER-113424 in plasma. The slopes of the calibration curves for compounds in plasma were quite comparable to that of standards that indicated the effective on-line purification of compounds and sufficient quantification ability of the methods.

The intra- and inter-day precision, accuracy, and reproducibility of detection of the three compounds in plasma were acceptable (Tables 1 and 2). These results demonstrate that on-line sample preparation with an ion-pair reagent in extraction LC using the MC-ODS precolumn provides a reproducible assay for these basic compounds in plasma.

3.5. Pharmacokinetics of ER-235516 in rats

To confirm the applicability of these procedures developed above, a method for ER-235516 was applied to the pharmacokinetic study in rats as an example. Plasma samples obtained from a single intravenous injection of ER-235516 in male SD rats ($n = 2$) were analyzed. Typical HPLC chromatograms of plasma samples prior to and at 5 min after 3 mg/kg intravenous administration of ER-235516 are shown in Fig. 3A and C.

No interference peaks for ER-235516 were observed in the chromatograms, indicating specificity of the ER-235516 peak.

Table 1
Intra-day precision, accuracy, and CV (%) of analyte detection in plasma

Analyte	Concentration (ng/mL)	Precision (ng/mL)	Accuracy (%)	CV (%)
ER-235516	30	32.7 ± 0.3	100 ± 1.2	1.17
	150	151.2 ± 0.4	96.9 ± 0.3	0.30
	300	303.6 ± 1.1	100.5 ± 0.4	0.36
	1500	1478.3 ± 2.0	99.9 ± 0.1	0.13
	3000	3009.6 ± 3.2	100.0 ± 0.1	0.11
ER-113424	30	29.2 ± 0.2	82.4 ± 0.9	1.03
	150	148.8 ± 0.8	91.9 ± 0.5	0.58
	300	275.7 ± 0.5	93.9 ± 0.2	0.18
	1500	1499.9 ± 127.8	101.2 ± 8.7	8.55
	3000	3062.5 ± 117.4	101.8 ± 3.9	3.84
ER-120867	30	25.2 ± 0.1	93.1 ± 0.4	0.39
	150	150.7 ± 0.4	96.1 ± 0.2	0.23
	300	281.5 ± 0.4	96.1 ± 0.1	0.13
	1500	1454.7 ± 99.3	96.4 ± 6.6	6.82
	3000	2947.2 ± 58.7	98.3 ± 2.0	1.99

Table 2
Inter-day precision, accuracy, and CV (%) of analyte detection in plasma

Analyte	Concentration (ng/mL)	Precision (ng/mL)	Accuracy (%)	CV (%)
ER-235516	30	31.9 ± 0.9	100.9 ± 1.2	1.22
	300	300.7 ± 3.4	100.0 ± 0.6	0.61
	3000	3011.8 ± 5.2	100.2 ± 0.3	0.30
ER-113424	30	28.4 ± 0.9	87.6 ± 5.8	6.59
	300	290.0 ± 16.1	97.6 ± 4.2	4.33
	3000	3026.4 ± 85.2	100.7 ± 2.8	2.74
ER-120867	30	28.2 ± 3.3	93.1 ± 0.4	0.48
	300	289.6 ± 8.9	100.4 ± 6.3	6.30
	3000	2980.6 ± 52.3	100.5 ± 2.6	2.62

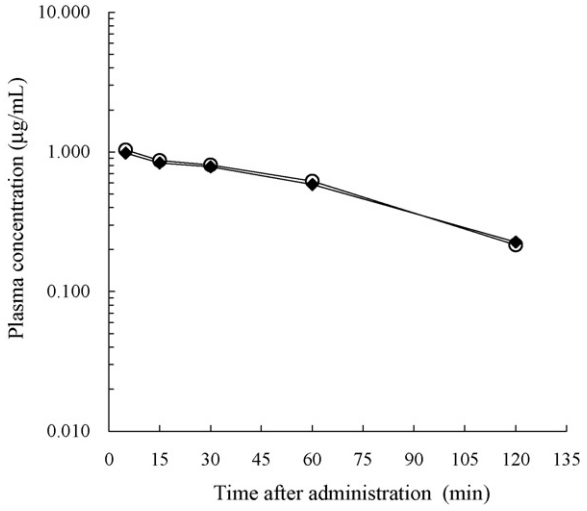

Fig. 4. Plasma concentration vs. time profile for ER-235516 in male SD rats. (◯) Subject 1, (♦) Subject 2.

The resulting plasma concentration versus time profile for ER-235516 in rats is shown in Fig. 4. These results indicate that the on-line sample preparation ability of the MC-ODS precolumn with HFBA as an ion-pair reagent in a column-switching LC system has been successfully applied to pharmacokinetic studies of ER-235516.

4. Conclusion

A rational and effective ion-pair liquid chromatographic sample extraction procedure for basic compounds has been developed using the MC-ODS precolumn in a column-switching LC system. Sufficient recoveries of basic compounds were accomplished, and the method was validated for specificity, precision, accuracy, and reproducibility. Furthermore, the method was successfully applied to the pharmacokinetic analysis of ER-235516 in rats. The methodology presented in this paper will contribute to effective sample preparation for broad basic compounds in biological sample analysis.

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