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Resolution of 4-(4-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepin-4yl)-1(2H)-phthalazinone enantiomers in plasma with frit-FAB LC–MS using a conalbumin column

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Abstract: A new column-switching method for analysis of drug enantiomers in plasma has been developed with liquid chromatography–frit fast atom bombardment mass spectrometry in combination with a chiral resolution column, which consists of conalbumin (egg-white glycoprotein) immobilized on silica gel and can be used in the reversed-phase separation mode. This method makes it possible to inject a large volume of deproteinized plasma and obtain resolution of drug enantiomers with high sensitivity. The optimum mobile phase, including a non-volatile buffer such as phosphate buffer, for separation of drugs from a large amount of endogenous compounds can be used, because of inclusion of a trapping column with a desalting function. This method is very simple and rapid, and should be very powerful in studies requiring high-sensitivity analysis with chiral separation of drugs from biological samples such as plasma.

Keywords: *Frit-FAB LC–MS; column switching; conalbumin column; direct analysis; drug enantiomers; plasma.*

Introduction

The enantiomers of drugs which have a chiral centre often have different pharmacological activity or toxicity, owing to differences in the behaviour of the enantiomers *in vivo* [1], so the assay of drug enantiomers in biological samples is important. High-performance liquid chromatography (HPLC) is an appropriate tool for separation of drug enantiomers, and many chiral stationary phases have been developed [2–6]. Protein-conjugated columns, such as a bovine serum albumin-conjugated column [7], and α_1-acid glycoprotein-conjugated column [8] and an ovomucoid-conjugated column [9], can separate drug enantiomers with a broad range [10] and can be used in the reversed-phase separation mode [11], which is well suited for the analysis of biological samples [12, 13]. However, these chiral columns cannot necessarily separate the desired compound from its metabolites [14] or endogenous compounds. On the other hand, mass spectrometry (MS) can perform separations based on molecular weight, so the combination of HPLC and MS (LC–MS) is a powerful method for the analysis of drugs and biological substances [15, 16]. But the development of LC–MS has been hindered by the difficulty of connecting an LC system operating at a flow rate of around 1 ml min^{-1} in general with an MS instrument requiring a high vacuum condition. Many of the LC–MS interfaces [17, 18] so far developed do not allow the use of a non-volatile buffer such as phosphate buffer, which is most generally used in conventional LC, because the ionization in MS is subject to interference by non-volatile buffer components. Thus, the optimum mobile phase conditions for separation in LC cannot be used in LC–MS employing these interfaces.

In a recent paper, Asakawa *et al.* [19] described a newly developed LC–MS system based on a column-switching method to overcome the above problem. This LC–MS system in combination with frit-fast atom bombardment MS (frit-FAB LC–MS) uses an analytical column to separate target compounds and an introduction column to pass them into the MS section, and these two columns are connected by sample loops, dilution tubes and a trapping column. In this LC–MS system, the solution

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containing the target compounds that is introduced into the MS section is independent of the mobile phase used on the analytical column. Thus, the optimum mobile phase for separation of target compounds (e.g. a non-volatile buffer such as phosphate buffer) can be selected in order to obtain the best separation on the analytical column, and the optimum mobile phase for high ionization efficiency in MS can also be chosen, without conflict.

We have developed a new column-switching LC–MS method by using a microbore conalbumin column in place of the introduction column in the above frit-FAB LC–MS system. The separatory column in this system is used to separate the desired compounds from endogenous compounds, and the introduction column to separate drug enantiomers and pass them to the MS section. In this study, we used (±)-4-(4-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepin-4-yl)-1(2H)-phthalazinone (AZE) as a model compound (Fig. 1). AZE is an antiallergic drug with a wide spectrum of pharmacological activities. It inhibits the action of many chemical mediators such as leukotriene [21] and histamine [22]. AZE has an asymmetric carbon, and its chiral separation has already been achieved with a conalbumin column in the reversed-phase separation mode using phosphate buffer [20]. In this paper, we will show that this method is useful for resolution of drug enantiomers in plasma with only deproteinization procedure.

Experimental

Materials and reagents

AZE and its enantiomers were prepared in our laboratories. A microbore conalbumin column (150 mm × 1.5 mm i.d.) was prepared as described in a previous paper [20]. Potassium dihydrogenphosphate, phosphoric acid and ammonium acetate of analytical-reagent grade were obtained from Wako Pure Chemical Co. (Osaka, Japan). Other chemicals and solvents were of HPLC grade.

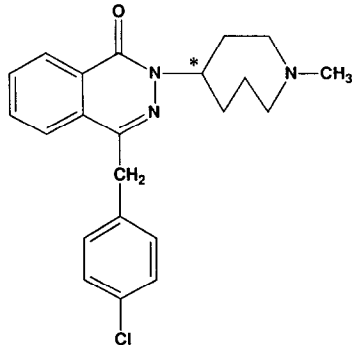

Figure 1
Structure of AZE. The chiral centre is indicated by an asterisk.

Apparatus

A schematic diagram of the column-switching system consisting of three HPLC pumps is shown in Fig. 2.

In LC-1, a pump (P1) (Model LC-9A, Shimadzu, Kyoto, Japan), which was controlled by a gradient controller (Model SCL-6B, Shimadzu), delivered the mobile phase (M1). Samples were injected using a Rheodyne injector (I) (Model 7125, Berkeley, CA, USA) with a 500 μl loop. The first column (C1) was 150 mm × 4.6 mm i.d., Intersil ODS-2 (GL Science, Tokyo, Japan) for separation of the fraction of interest from a plasma sample. The effluent from C1 was monitored with a variable-wavelength UV detector (D1) (Model SPD-6A, Shimadzu).

In LC-2, a pump (P2) (Model 576, GL Science) delivered the mobile phase (M2) for concentration of the compounds of interest on a trapping column (TC) (10 mm × 4.0 mm i.d., ULTRON ES-OVM, Shinwa Kako, Kyoto).

In LC-3, a pump (P3) (Model LC-9A, Shimadzu) delivered the mobile phase (M3) for resolution of enantiomers. The drug enantiomers were re-chromatographed on the microbore column (C2) (150 mm × 1.5 mm i.d., conalbumin column), monitoring with a variable-wavelength UV detector (D2) (Model 200 Linear, Reno, NV, USA) equipped with a micro-cell (cell volume, 0.6 μl). Switching was done with Rheodyne 7000 (V1, V4) and Rheodyne 7060 (V2, V3, R) switching valves. The introduction into the frit-FAB–MS system was performed by using a pneumatic splitter (S) (Model MS-PNS, JEOL, Tokyo) in order to control the flow rate at 5 μl min^{-1}.

Samples

Sample solutions were prepared by dissolving known amounts of racemic AZE in water or human plasma. A 1 ml plasma sample was deproteinized by adding 100 μl of trichloroacetic acid and was centrifuged at 3000 rpm for 10 min. The supernatant was injected into this system.

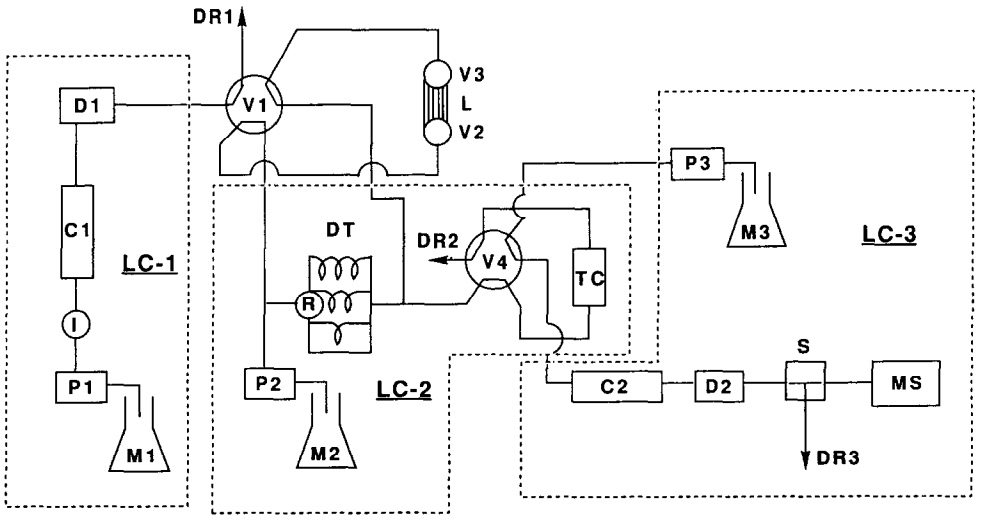

Figure 2
A schematic diagram of the column-switching system for LC–MS. P1 = solvent pump; M1 = mobile phase for separation of drugs from plasma; I = injector; C1 = column for separation of drugs from plasma; D1 = UV detector; P2 = solvent pump for trapping of drugs; M2 = mobile phase for trapping of drugs on the trapping column; TC = trapping column; DT = dilution tubes; L = sampling loops; P3 = solvent pump for chiral separation and introduction into mass spectrometer; M3 = mobile phase for chiral separation; C2 = chiral separation column; D2 = UV detector with micro-cell for monitoring the chiral separation; S = splitter; V1, V4 = six-port switching valves for changing the flow direction; V2, V3 = six-port valves for selection of sampling loops; R = six-port switching valve for changing the dilution ratio; DR1, DR2 and DR3 = drain; MS = mass spectrometer.

HPLC conditions

In LC-1, AZE was monitored by D1 at 254 nm. M1 consisted of methanol–potassium phosphate buffer (pH 3.0; 1 mM) and the gradient was linear for 30 min (10:90–50:50, v/v). M2 was ammonium acetate buffer adjusted to pH 8.0 (5 mM) by adding aqueous ammonia. M3 was methanol–ammonium acetate buffer (pH 5.0; 100 mM) (20:80, v/v) containing 0.5% glycerol. M1 was delivered by P1 at a flow rate of 1.0 ml min^{-1}, M2 by P2 at a flow rate of 2.0 ml min^{-1} and M3 by P3 at a flow rate of 0.1 ml min^{-1}. All operations were carried out at ambient temperature.

Mass spectrometry (MS)

A JEOL JMS-SX102A apparatus with a frit-FAB interface was employed. MS conditions in the FAB mode included a xenon atom beam from a saddle field gun operated at 8 kV and 5.0 mA; the scan range was 25–500 daltons (direct insertion analysis; 0–900) at a scan speed of 2.0 s per decade. The FAB mass spectra were recorded with a normal resolving power of 1000. The source operating pressure was typically 10^{-5} Torr.

Procedure

Samples were injected onto C1, and the eluates were monitored with D1 at 254 nm (LC-1). The fraction containing AZE was switched into the 2 ml sample loop (L) via V1. Then M2 was used to wash the above eluate into TC with dilution (LC-2). Finally, by switching V4, AZE was flushed out by M3 from TC to C2, where enantiomeric separation was performed and detected by MS (LC-3).

Results and Discussion

Kajima *et al.* [23] showed that AZE can be separated in the normal-phase mode by using the chiral additive method. However, biological samples cannot be assayed by this method without laborious pretreatments. We have developed a new chiral separation column, a conalbumin-conjugated silica gel column, which can be used in the reversed-phase mode, and we have established the optimum mobile phase condition using a phosphate buffer for separation of AZE enantiomers [20]. However, a non-volatile mobile phase such as phosphate buffer cannot be used directly in LC–MS because the AZE enantiomers are directly introduced into the MS instrument after chiral separation by the conalbumin column. In frit-FAB LC–MS, the flow rate into the MS section must be controlled to

the level of 5 μl min^{-1}, and generally a splitter is placed before the MS to adjust the flow rate. Then, a sample injected into the frit-FAB LC–MS system can be introduced in a larger amount into the MS by decreasing the flow rate in C2. The amount of sample introduced into the MS is 1/20 of the sample injected into this system when the microbore column (150 mm × 1.5 mm i.d.) is used, because the flow rate is only 0.1 ml min^{-1}, so this microbore column can provide 10 times higher sensitivity than a conventional column (150 mm × 4.6 mm i.d.). In addition, diffusion of a sample in the microbore column is the same as or less than that of a conventional column, and consequently higher responses can be obtained in the chromatograms [24]. For these reasons, we used a microbore conalbumin column in this study. First, we examined the mobile phase conditions for the column-switching method.

Studies of HPLC conditions on LC-2 and LC-3

Initially, we examined the effect of pH of M2 (20 mM buffer concentration) for chromatography on C2 by using LC-2 and LC-3 when M3 on C2 was fixed as methanol–acetate buffer (pH 5.0; 20 mM) (20:80, v/v) at a flow rate of 0.1 ml min^{-1} (Fig. 3). The best resolution was obtained at pH 5.0 (Fig. 3(A)). However, in this column-switching system, a large volume of M2 flowed into TC, because M1, which is rich in organic solvent, must be diluted with a large volume of M2 (free from organic solvent) to allow strong adsorption of the target compounds on the front of TC. At pH 5.0, AZE is dissociated, so the adsorption of AZE on TC is very weak and AZE is eluted, because ULTRON ES-OVM used as TC has a reversed-phase property [9]. The hydrophobicity of AZE increases with higher pH, because its pK_a value is about 8.5 [20]. But, as

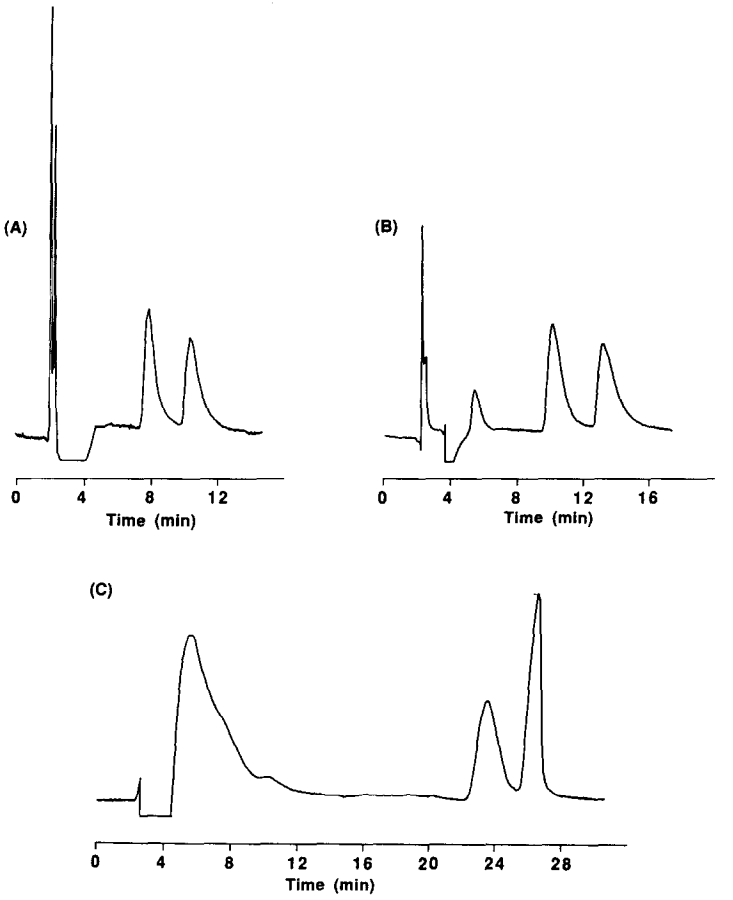

Figure 3
Effect of pH of M2. (A) pH 5.0; (B) pH 5.5; (C) pH 8.0. Chromatographic conditions: TC, ULTRON ES-OVM (1.0 × 4.0 mm i.d.); M2, 20 mM ammonium acetate buffer; flow rate on TC, 2.0 ml min^{-1}; trapping time, 2.0 min; C2, conalbumin column (150 mm × 1.5 mm i.d.); M3, 20 mM ammonium acetate buffer (pH 5.0)–methanol (80:20); flow rate on C2, 0.1 ml min^{-1}; detection, UV 254 nm; injection amount, 50 ng.

shown in Fig. 3, the use of pH 8.0 buffer as M2 resulted in deformation of the peak on C2 (Fig. 3(C)). Thus, the pH of M2 greatly influenced chromatography on C2, because the bed volume of C2 is only a few hundred microlitres, as is that of TC. That is to say, an extreme difference of pH between M2 and M3 produces a phenomenon resembling pH step gradient elution on C2, and the *d*-enantiomer, which is more weakly retained on the conalbumin column, would undergo greater diffusion during pH step gradient elution. We considered that the influence of M2 on the chromatography on C2 could be minimized by the buffer effect of M3 if salt concentration of M2 was kept as low as possible and that of M3 was high. The result obtained by using 5 mM M2 (pH 8.0) and 100 mM M3 (pH 5.0) is shown in Fig. 4. C2 gave a good enantio-separation and the peak shapes did not vary when over 20 ml of M2 was passed onto TC. This result shows that the target compounds can be strongly retained on the front of TC and, therefore, can be passed to C2 without diffusion.

Effect of dilution ratio

In LC-1, AZE was separated from endogenous compounds in plasma by use of a gradient method with an ODS column. Hence, the fraction including AZE contained organic solvent (methanol) at a high concentration. Thus, AZE passed through TC when the M2 condition was 5 mM ammonium acetate buffer (pH 8.0) and the M3 condition was methanol–100 mM ammonium acetate buffer (pH 5.0) (20:80). This occurred because TC, ULTRON ES-OVM, has a reversed-phase property, so that drugs are eluted more quickly with increase of organic solvent content in the mobile phase [9]. In this column-switching system, the dilution ratio can be freely altered by changing the length of the resistance tube [19]. We therefore examined the effect of dilution ratio on the trapping and resolution of AZE enantiomers. The chromatograms obtained in this experiment are shown in Fig. 5, and peak area ratios of enantiomers are given in Table 1. When the dilution ratios (M1:M2) were less than 1:7, peak shapes were broad as shown in Fig. 5 and the retention time became long, though the peak area ratios (*d*-AZE:*l*-AZE) were not much changed when the dilution ratio was more than 1:4. This fact was due to diffusion of AZE enantiomers on TC, so we concluded that the dilution ratio should be at least 1:10.

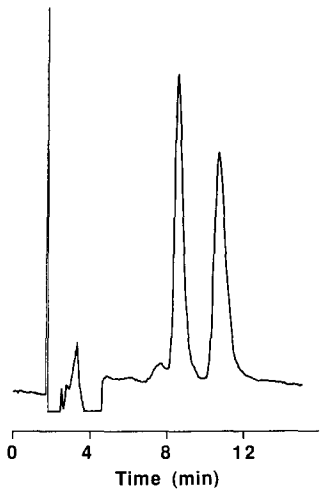

Figure 4
Chromatogram of AZE enantiomers on C2. Chromatographic conditions: M2, 5 mM ammonium acetate buffer (pH 8.0); M3, 100 mM ammonium acetate buffer (pH 5.0)–methanol (80:20); other conditions were the same as given in the legend to Fig. 3.

Figure 6 shows chromatograms obtained with C1 and the enantiomeric resolution with C2, where M2 consisted of 5 mM ammonium acetate buffer adjusted to pH 8.0 and the fraction containing AZE was diluted 1:10 with M2. It is clear that this system can analyse AZE enantiomers.

FAB-MS of d-*AZE from a glycerol matrix*

First, *d*-AZE was analysed by FAB-MS using a direct insertion probe with a glycerol matrix. The spectrum of *d*-AZE is shown in Fig. 7(A). The FAB-MS of *d*-AZE gave the molecular ion (MH^+) at *m/z* 382 as a base peak.

Mass chromatogram and FAB-MS spectrum of AZE enantiomers in the frit-FAB LC–MS system

In LC-1, 100 ng of AZE enantiomers was injected into C1, and the column-switching procedure was carried out under the conditions described in the Experimental section. The mass chromatogram is shown in Fig. 8 and the mass spectrum of *d*-AZE is shown in Fig. 7(B). This spectrum is almost the same as that obtained by direct analysis (Fig. 7(A)) and the molecular ion at *m/z* 382 was clearly observed. In this analysis, a phosphate buffer (non-volatile buffer) was used as M1, but this non-volatile buffer was completely removed with TC, and so did not interfere with the ionization

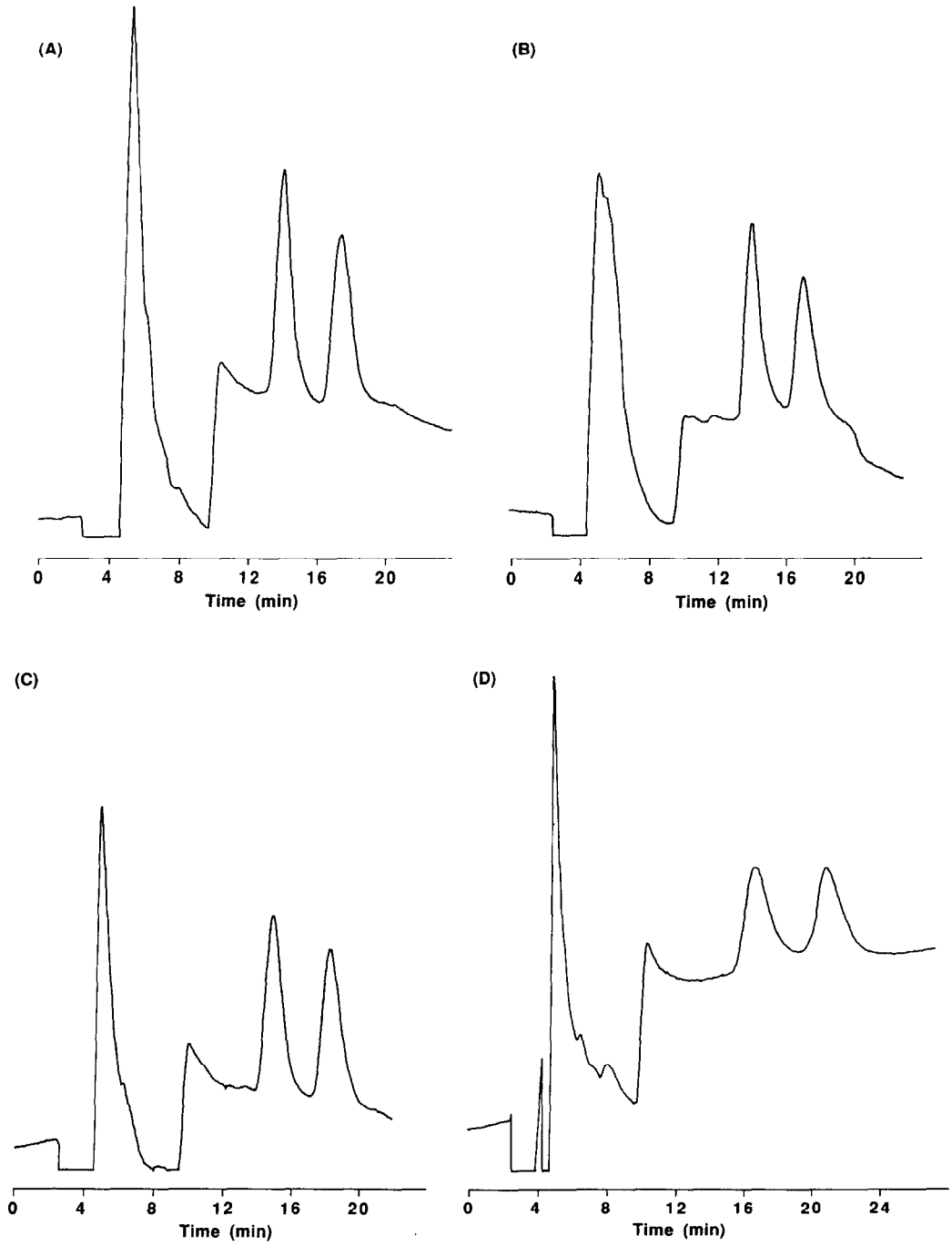

Figure 5
Effect of dilution ratio. The eluate containing AZE was diluted 1:x with M2. (A) x = 16 (B) x = 10 (C) x = 7 (D) x = 4 (E) x = 0. Chromatographic conditions: C1, Inertsil ODS-2 (150 mm × 4.6 mm i.d.); M1, 1 mM potassium phosphate buffer (pH 3.0)–methanol (90:10–50:50/30 min), other conditions are the same as given in the legend to Fig. 4.

of AZE in MS. These results confirm the effectiveness of chiral separation with this LC–MS system.

Detection limit

Figure 9 shows the selected-ion recording when 100 ng of AZE enantiomers was injected into this LC–MS system. The limit of detection for AZE in the selected-ion monitoring (SIM) mode was 500 pg at a signal-to-noise ratio of 15. However, when a large volume of sample such as 500 μl was injected into LC-1, the peak

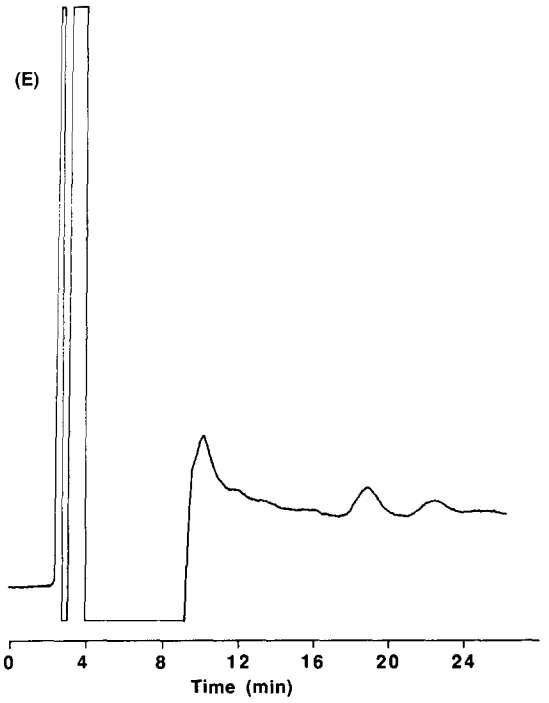

Figure 5
Continued.

Table 1
Effect of dilution ratio on peak area ratios of AZE enantiomers resolved on the microbore conalbumin column

M1:M2	*d*-AZE:*l*-AZE
1:16	51.0:49.0
1:10	51.9:48.1
1:7	50.9:49.1
1:4	51.1:48.9
1:0	70.8:29.2

All conditions are the same as given in the legend to Fig. 5.

shape of each enantiomer on the microbore conalbumin column (C2) was almost the same as that in the case of 10 μl injection (data not shown) because of the use of the gradient method in LC-1 and the concentration of AZE enantiomers on TC. So the detection limit with this system is better than 1 ng ml^{-1}. These results show that this method offers high sensitivity.

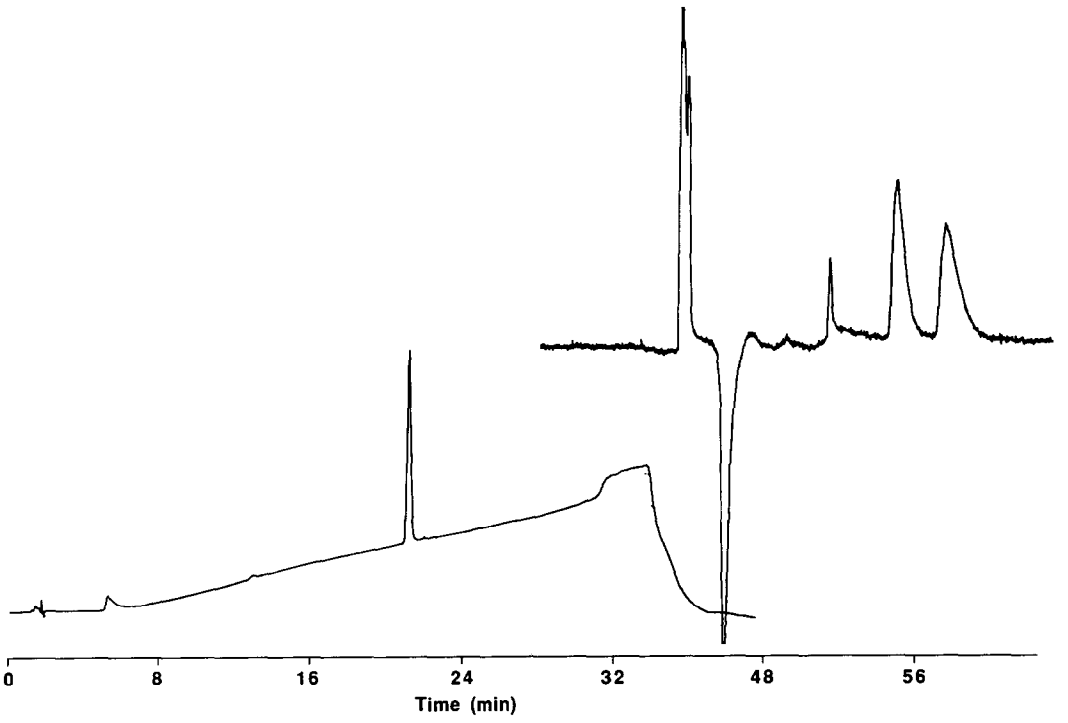

Figure 6
Representative chromatograms obtained with the achiral reversed-phase column (lower chromatogram) and the conalbumin column (upper chromatogram). Chromatographic conditions: dilution ratio, x = 10; other conditions were the same as given in the legend to Fig. 5.

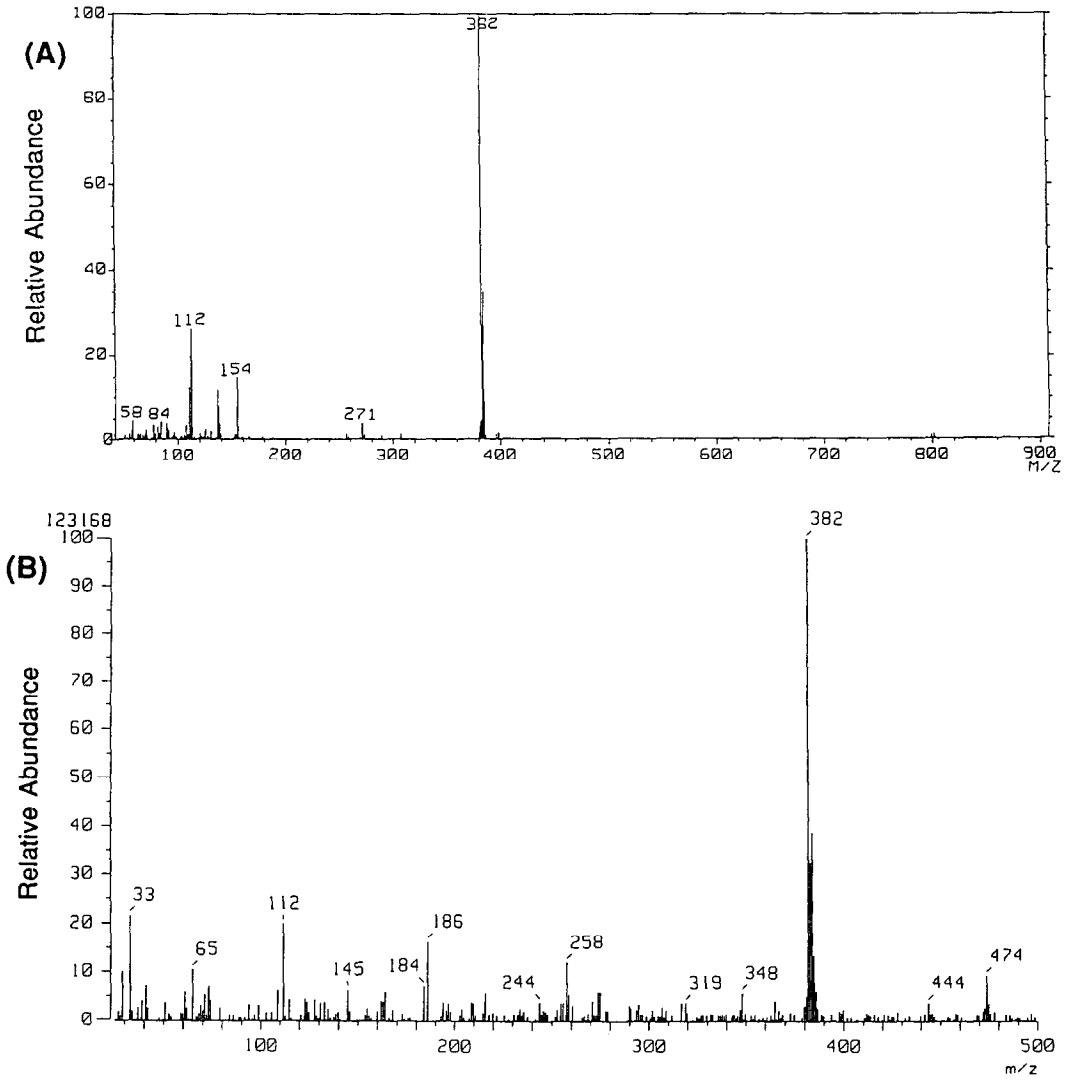

Figure 7
FAB mass spectra of AZE. (A) FAB glycerol mass spectrum of about 1 μg of *d*-AZE using the direct insertion probe. (B) FAB mass spectrum of 100 ng of *d*-AZE obtained using frit-FAB LC–MS.

Plasma sample

This column-switching has the great advantage that laborious pretreatments are not required, because the target compounds (including metabolites) can be separated from endogenous compounds in plasma samples on C1 [25]. This method can analyse drug enantiomers by UV detection without MS in the case of a small volume of sample. However, when a large amount of volume is injected into LC-1 for highly sensitive detection, relatively large amounts of endogenous compounds may influence the background in the chromatography on C2. However, MS is a powerful detector, and a large amount of endogenous compounds would have little effect on detection with SIM. Figure 10(A) shows the chromatogram obtained by injection of 200 μl of a plasma sample containing 50 ng of racemic AZE on LC-1 with a UV detector, and Fig. 10(B) shows the selected-ion recording of the same sample. A good chromatogram with chiral separation was obtained without any influence of endogenous compounds. Thus, our method using a microbore conalbumin column allows the analysis of AZE enantiomers in the presence of a large number of endogenous compounds.

Conclusions

A new measurement system for enantio-

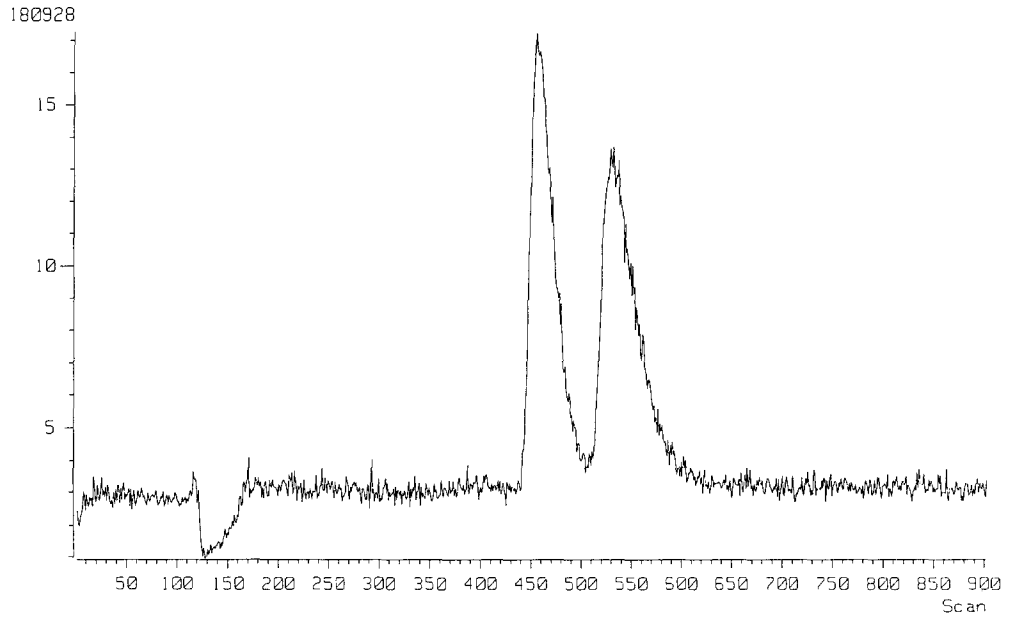

Figure 8
Mass chromatograms at *m/z* 382 of AZE obtained using frit-FAB LC–MS. Conditions: M3, 100 mM ammonium acetate buffer (pH 5.0)–methanol (80:20) containing 0.5% glycerol; other conditions were the same as given in the legend to Fig. 6.

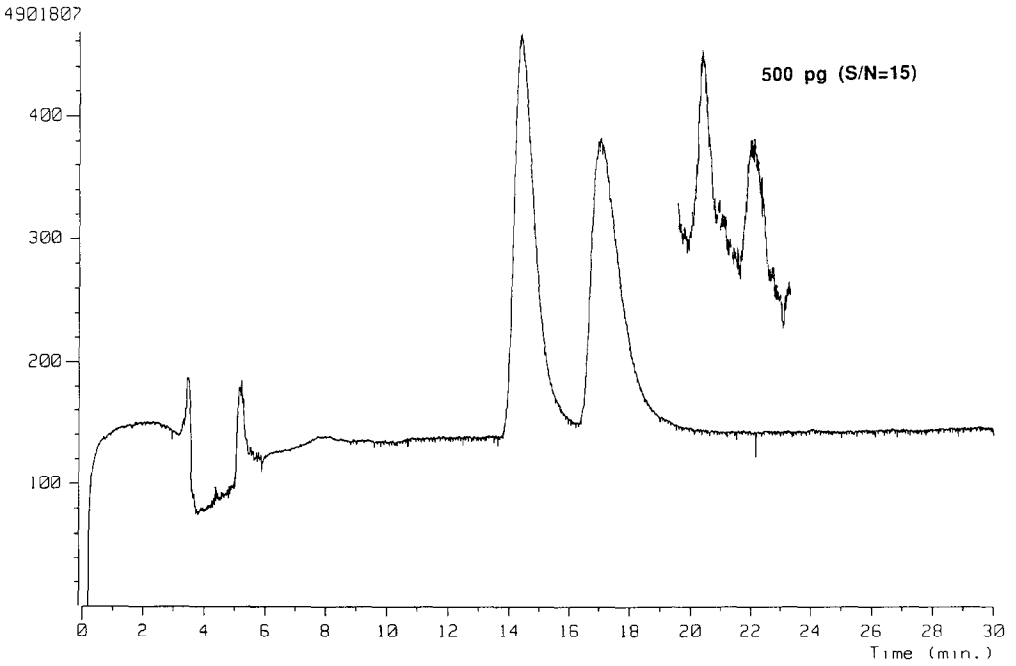

Figure 9
Selected-ion recording obtained with the microbore conalbumin column (C2) for 100 ng of AZE enantiomers. Conditions: C1, Intersil ODS-2 (150 mm × 4.6 mm i.d.); M1, 1 mM potassium phosphate buffer (pH 3.0)–methanol (90:10–50:50/30 min); flow rate on C1, 1.0 ml min^{-1}; TC, ULTRON ES-OVM (1.0 mm × 4.0 mm i.d.); M2, 5 mM ammonium acetate buffer (pH 8.0); flow rate on TC, 2.0 ml min^{-1}; C2, conalbumin column (150 mm × 4.6 mm i.d.); M3, 100 mM ammonium acetate buffer (pH 5.0)–methanol (80:20) containing 0.5% glycerol; flow rate on C2, 0.1 ml min^{-1}; dilution ratio, $x = 10$; Recording *m/z* (SIM mode), 382.

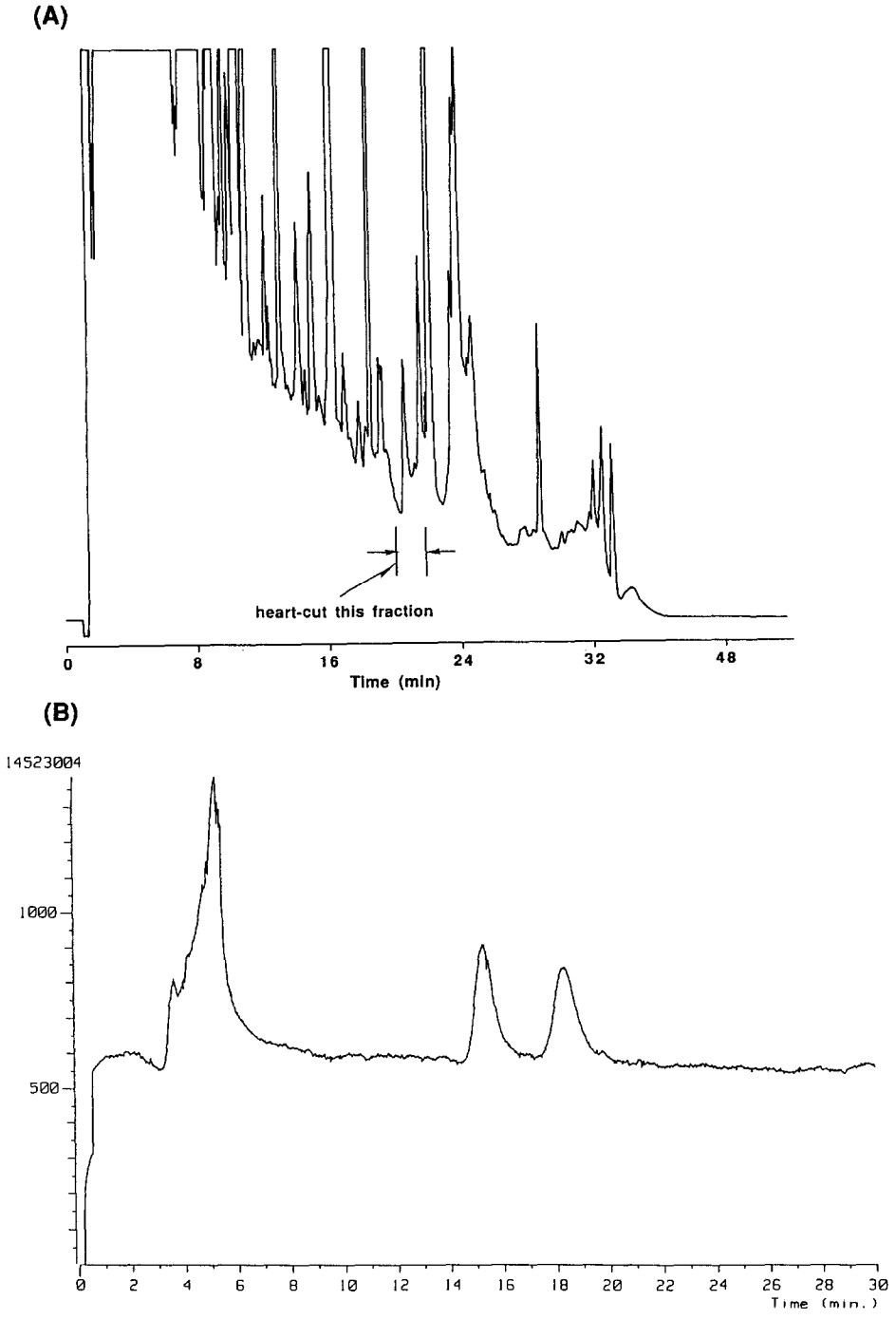

Figure 10
Chromatogram obtained with 200 μl of a plasma sample spiked with 50 ng of AZE enantiomers. (A) Chromatogram obtained with the achiral reversed-phase column (C1) with UV detection (254 nm). (B) Selected-ion recording obtained with the microbore conalbumin column (C2). Conditions: C1, Inertsil ODS-2 (150 mm × 4.6 mm i.d.); M1, 1 mM potassium phosphate buffer (pH 3.0)–methanol (90:10–50:50/30 min); flow rate on C1, 1.0 ml min^{-1}; TC, ULTRON ES-OVM (1.0 mm × 4.0 mm i.d.); M2, 5 mM ammonium acetate buffer (pH 8.0); flow rate on TC, 2.0 ml min^{-1}; C2, conalbumin column (150 mm × 4.6 mm i.d.); M3, 100 mM ammonium acetate buffer (pH 5.0)–methanol (80:20) containing 0.5% glycerol; flow rate on C2, 0.1 ml min^{-1}; dilution ratio, x = 10; Recording m/z (SIM mode), 382.

meric drugs in plasma samples has been developed by using a combination of a new column-switching system including a chiral separation column and an LC–MS system. This system could perform analysis of drug enantiomers in deproteinized plasma, and allows the use of a non-volatile buffer such as a phosphate buffer for separation of drugs from endogenous compounds, because TC, located between C1 and C2, removes the non-volatile buffer completely. Therefore the optimum mobile phase conditions for separation of drugs from endogenous compounds can be chosen without restriction from the viewpoint of inhibition of ionization in MS. This system should be a very powerful tool for direct analysis of drug enantiomers in biological samples such as plasma, urine and bile.

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