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Enantioselective high-performance liquid chromatographic assay for determination of the enantiomers of a new anti-ulcer agent, E3810, in Beagle dog plasma and rat plasma

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Abstract

An enantioselective high-performance liquid chromatographic method for the determination of E3810, a new anti-ulcer agent, in Beagle dog plasma and rat plasma has been developed. After extraction from plasma with ethyl acetate, E3810 enantiomers were measured by reversed-phase high-performance liquid chromatography on a Chiralcel OD-R column. The enantiomers were detected by ultraviolet absorbance detection at 290 nm. The recoveries of E3810 enantiomers and internal standard were greater than 91%. The calibration curves were linear from 0.03 to 20 μg/ml for Beagle dog plasma and from 0.1 to 100 μg/ml for rat plasma. The limits of quantification of both enantiomers were 0.03 μg/ml for Beagle dog plasma and 0.1 μg/ml for rat plasma. The intra- and inter-day accuracy and precision data showed good reproducibility of the method. The assay was applied for the analysis of E3810 enantiomers in plasma after intravenous administration of racemic E3810 to Beagle dogs and rats. This method should be very useful for enantioselective pharmacokinetic studies of E3810.

1. Introduction

E3810, (±)-sodium 2-[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]methylsulfinyl]-1H-benzimidazole (Fig. 1), is a new anti-ulcer agent which possesses inhibitory activity against gastric acid secretion as a consequence of inhibition of H^+, K^+-ATPase, and is presently under development [1–4]. E3810 has an asymmetric sulfur atom in the molecule and thus has two enantiomers, *R*(+)-E3810 and *S*(−)-E3810 (see Fig. 1). We have previously reported the pharmacokinetics of racemic E3810 in animals [5,6], while those of the enantiomers have not been investigated.

Recently, it has been reported that some chiral agents show enantioselective pharmacokinetics, and therefore analytical methods to separate the enantiomers are required [7,8]. Furthermore, enantioselectivity of pharmacokinetics sometimes causes each enantiomer to possess different pharmacological or toxic effects, so elucidation

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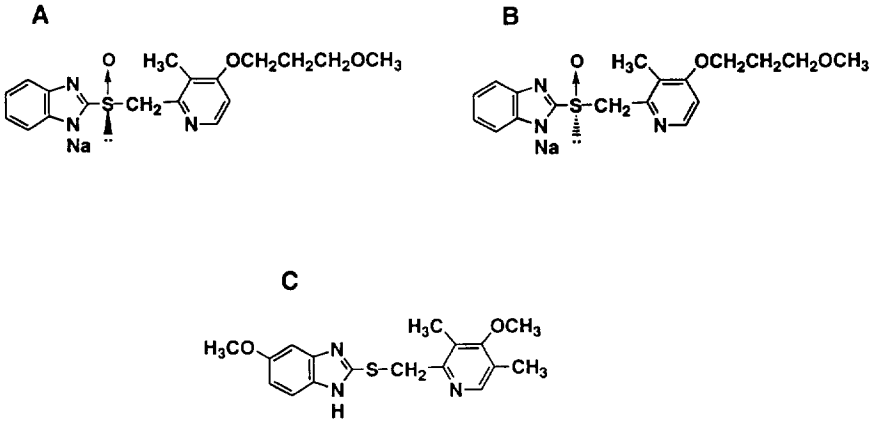

Fig. 1. Chemical structures of *R*(+)-E3810 (A), *S*(−)-E3810 (B) and the internal standard (I.S.) (C).

of enantioselectivity of drugs is important [9–11]. Although we understood that elucidation of the pharmacokinetic profile of individual enantiomers was important concerning E3810, we were unable to study this because of the lack of an analytical method for simultaneous determination of E3810 enantiomers. Other gastric proton pump inhibitors, such as omeprazole and lansoprazole, have a sulfoxide group in the molecule similar to E3810. They are marketed as a racemic mixture, but there is little information on enantioselective assay methods and pharmacokinetic properties of the enantiomers.

Therefore, our purpose was to establish an analytical method to measure the E3810 enantiomers and to elucidate their pharmacokinetics after administration of racemic E3810.

2. Experimental

2.1. Chemicals

Racemic E3810 was synthesized at Eisai (Ibaraki, Japan). *R*(+)-E3810, *S*(−)-E3810 and the internal standard [2-(4-methoxy-3,5-dimethyl-pyridine-2-yl)methylthio-5-methoxybenzimidazole] were synthesized at Tsukuba Research Laboratories of Eisai. The structures of these compounds are shown in Fig. 1. The optical purity of the enantiomers was 98.4% for *R*(+)-E3810 and 98.3% for *S*(−)-E3810. Acetonitrile (HPLC grade), ethyl acetate (HPLC grade), diethylamine (analytical grade) and triethylamine (analytical grade) were obtained from Wako (Osaka, Japan). Ammonium perchlorate was obtained from Kanto Chemical (Tokyo, Japan). Other chemicals were of commercially available, analytical grade. All reagents were used without further purification.

2.2. HPLC apparatus

The LC-10A HPLC system (Shimadzu, Kyoto, Japan), which consisted of a LC-10AD pump, a SPD-10A UV detector, a SLC-10A system controller and a C-R7A (or C-R4A) reporting integrator, was used. Injections were performed using a Waters 717 autosampler (Waters, Milford, MA, USA) at 4°C. A Chiralcel OD-R column (250 mm × 4.6 mm I.D., 5 μm particle size; Daicel Chemical IND, Tokyo, Japan) was used for separation of the enantiomers. The mobile phases were 0.5 *M* ammonium perchlorate (pH 7.8)–acetonitrile (80:20, v/v) (solvent A) and 0.5 *M* ammonium perchlorate (pH 7.8)–acetonitrile (20:80, v/v) (solvent B), and the following linear gradient elution system was utilized: time 0 min: A–B (90:10); 40 min: A–B (10:90); 50 min: A–B (90:10). The chromatographic runs were carried out at room temperature. Detection was performed with an absorbance wavelength of 290 nm.

2.3. Stock solutions

Stock solutions of *R*(+)-E3810 and *S*(−)-E3810 were respectively prepared by dissolving them in methanol containing 0.1% diethylamine. The concentrations of the stock solutions were 200, 100, 30, 10, 3, 1, 0.5, 0.3 and 0.2 μg/ml. I.S. solution was prepared by dissolving I.S. in methanol containing 0.1% diethylamine at a final concentration of 10 μg/ml. These solutions were stored at −20°C.

2.4. Sample preparation

A 50-μl volume of I.S., 100 μl of methanol containing 0.1% diethylamine and 1 ml of Britton–Robinson buffer (pH 10.4) were added to Beagle dog plasma and rat plasma samples. The Britton–Robinson buffer was made by mixing 1/25 *M* acid solution (2.71 ml of 85% phosphoric acid, 2.36 ml of acetic acid, 2.47 g of boric acid/l) and 0.2 *M* NaOH. The samples were extracted twice with 4 ml of ethyl acetate with a mechanical horizontal shaker for 5 min. After centrifugation for 5 min at 2000 *g*, the organic phase was transferred and evaporated to dryness at 40°C under a nitrogen stream. The residue was reconstituted in 100 μl of methanol containing 0.1% diethylamine, and a 30-μl aliquot of Beagle dog plasma sample or a 50-μl aliquot of rat plasma sample was analyzed by HPLC.

2.5. Determination of recovery

The recoveries of extraction efficiency of the Beagle dog plasma and rat plasma extraction procedure for both enantiomers and the I.S. were determined on four samples. The peak heights obtained were compared with those by direct injection of each substance without extraction.

2.6. Calibration curves

To make calibration standards, each 50 μl of stock solution in methanol containing 0.1% diethylamine was added to 0.5 ml of Beagle dog blank plasma or 0.1 ml of rat blank plasma. The spiked concentrations of the calibration standards were 20, 10, 3, 1, 0.3, 0.1, 0.05 and 0.03 μg/ml for Beagle dog plasma and 100, 50, 15, 5, 1.5, 0.5, 0.25, 0.15 and 0.1 μg/ml for rat plasma. The samples were then processed as described in Section 2.4. The calibration curves were constructed by plotting the peak-height ratio of each enantiomer to internal standard versus spiked concentration. The calibration curves were calculated by non-linear least-squares regression analysis using a MULTI program [12]. The used equation in this program was linear: $y = ax + b$, where x is spiked concentration and y is peak height ratio.

2.7. Accuracy and precision of the assay

Standard samples were prepared by spiking plasma with stock solutions of enantiomers to give final concentrations given in Section 2.6. In order to evaluate the intra-day validity, replicate samples were determined for each concentration on the same day. The inter-day validity was evaluated for four or five days. The accuracy was evaluated as percentage error [(found concentration − spiked concentration)/spiked concentration] × 100 (%), and the precision was evaluated by the coefficient of variation (C.V., %).

2.8. Application of the method

Beagle dog

Plasma levels of the E3810 enantiomers in Beagle dogs were determined after intravenous administration of racemic E3810 (3 mg/kg). Six male Beagle dogs (12 months; BMR Research Laboratory, Gifu, Japan) were fasted overnight and used for the study. Taking the stability into consideration, the intravenous solution (6 mg/ml) was prepared by dissolving racemic E3810 in physiological saline immediately before administration. The solution was administered via the cephalic vein. At 5, 15, 30 and 45 min and 1, 1.5, 2, 3, 4 and 6 h after administration, blood (ca. 3 ml) was collected from the cephalic vein. After centrifugation at 2000 *g* for 10 min, a 0.5-ml aliquot of plasma was obtained and immediately added to 100 μl of 1% diethylamine solution in

water. The plasma samples were stored frozen at −20°C until analysis.

Rat

Plasma levels of the E3810 enantiomers in rats were determined after intravenous administration of racemic E3810 (40 mg/kg). Four male Sprague–Dawley rats (8 weeks; Japan SLC, Shizuoka, Japan) were fasted overnight and used for the study. The intravenous solution (40 mg/ml) was prepared by dissolving racemic E3810 in physiological saline–1 *M* NaOH (99:1, v/v) and administered via the femoral vein. At 5, 15, 30 and 45 min and 1, 1.5 and 2 h after administration, blood (ca. 0.3 ml) was collected from the jugular vein. After centrifugation, a 0.1-ml aliquot of plasma was obtained and then treated in the same manner as described for the Beagle dogs

2.9. Calculation of pharmacokinetic parameters

Assayed plasma concentration–time data for each enantiomer were used to determine the pharmacokinetic parameters. The parameters were calculated by the one-compartment model procedure using non-linear least-squares regression analysis (MULTI, Ref. [12]). In this study, $t_{1/2}$ (elimination half-life from plasma), V_d (volume of distribution), AUC (area under the plasma concentration–time curve) and Cl_{tot} (total clearance) were estimated. AUC was calculated by the trapezoidal rule extrapolated to infinity. Cl_{tot} was calculated from the equation: Dose/AUC.

3. Results and discussion

3.1. Chromatograms

Typical chromatograms of the extracted products from the blank samples and the spiked with E3810 enantiomers are shown in Figs. 2A and 2B for Beagle dog plasma and Figs. 3A and 3B for rat plasma. The chromatograms of plasma samples are shown in Fig. 2C for Beagle dog plasma and Fig. 3C for rat plasma following the intravenous administration of racemic E3810. The enantiomers could be separated from each other using a commercially available chiral column (Chiralcel OD-R) with a simple extraction procedure. We have confirmed that chiral conversion of the enantiomers does not occur during the extraction procedure in this assay. The retention times of *R*(+)-E3810, *S*(−)-E3810 and internal standard were 23.7 min, 24.9 min and 35.4 min, respectively, and there was no interference at these retention times. Also, the four major metabolites of E3810 reported (Metabolite I, II, III and IV) [5,6] were found not to interfere with the assay.

3.2. Recovery

The recoveries of extraction efficiency of both enantiomers were performed at two concentrations. The I.S. recovery was determined at the concentration used in the assay procedure. The mean analytical recoveries of the analytes from Beagle dog plasma and rat plasma ranged from 81.4% to 88.8% and from 77.9% to 82.4%, respectively. The I.S. recovery from both plasmas ranged from 97.3% to 97.9% (Table 1).

3.3. Calibration curves

Typical regression lines gave the following equations. Beagle dog plasma: *R*(+)-E3810 and *S*(−)-E3810, $y = 0.761x - 0.00359$ and $y = 0.779x - 0.00179$, respectively, in the range of 0.03–20 μg/ml. Rat plasma: *R*(+)-E3810 and *S*(−)-E3810, $y = 0.144x - 0.00140$ and $y = 0.154x - 0.00064$, respectively, in the range of 0.1–100 μg/ml. Good linearity was observed in every preparation.

3.4. Accuracy and precision

Intra-day and inter-day accuracy and precision of the method were determined at concentrations of 0.03 to 20 μg/ml for Beagle dog plasma (Table 2) and 0.1 to 100 μg/ml for rat plasma (Table 3). Table 2 shows that the C.V. was 0.9–12.5% for *R*(+)-E3810 and 1.6–9.4% for *S*(−)-E3810 at different concentrations in Beagle dog

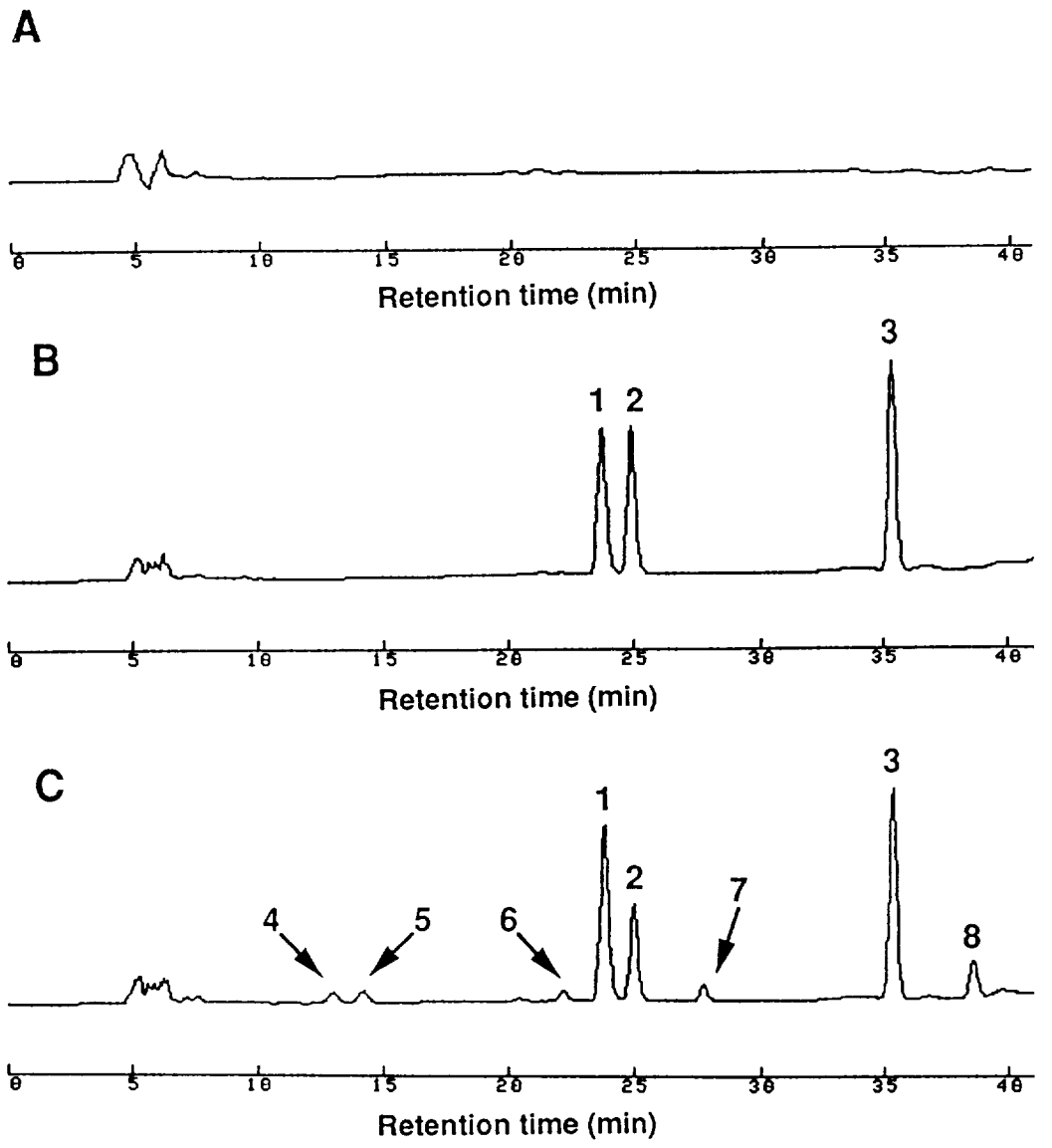

Fig. 2. Typical chromatograms of blank Beagle dog plasma (A), plasma spiked with 1 μg/ml of each enantiomer (B), and plasma from a Beagle dog 30 min after intravenous administration of racemic E3810 (3 mg/kg). Peaks: 1 = *R*(+)-E3810; 2 = *S*(−)-E3810; 3 = internal standard; 4 = metabolite III(*R*); 5 = metabolite III(*S*); 6 = metabolite II; 7 = metabolite IV; 8 = metabolite I.

plasma. In addition, the accuracy was estimated to be within 6.7% for *R*(+)-E3810 and 6.7% for *S*(−)-E3810. Similar results were obtained for rat plasma, as shown in Table 3. The C.V. was 1.3–9.4% for *R*(+)-E3810 and 0.6–7.8% for *S*(−)-E3810; the accuracy was within 8.0% for *R*(+)-E3810 and 8.0% for *S*(−)-E3810. These data suggest that the method is accurate and reproducible for the enantioselective assay of plasma samples. The limits of quantitation of both enantiomers were 0.03 μg/ml for Beagle dog plasma and 0.1 μg/ml for rat plasma; these limits are sufficient for pharmacokinetic studies.

Intra-day ($n = 4$) accuracy and precision of the method when different concentrations of enantiomers [*R*(+)–*S*(−) 3:1 or 1:3] were spiked in

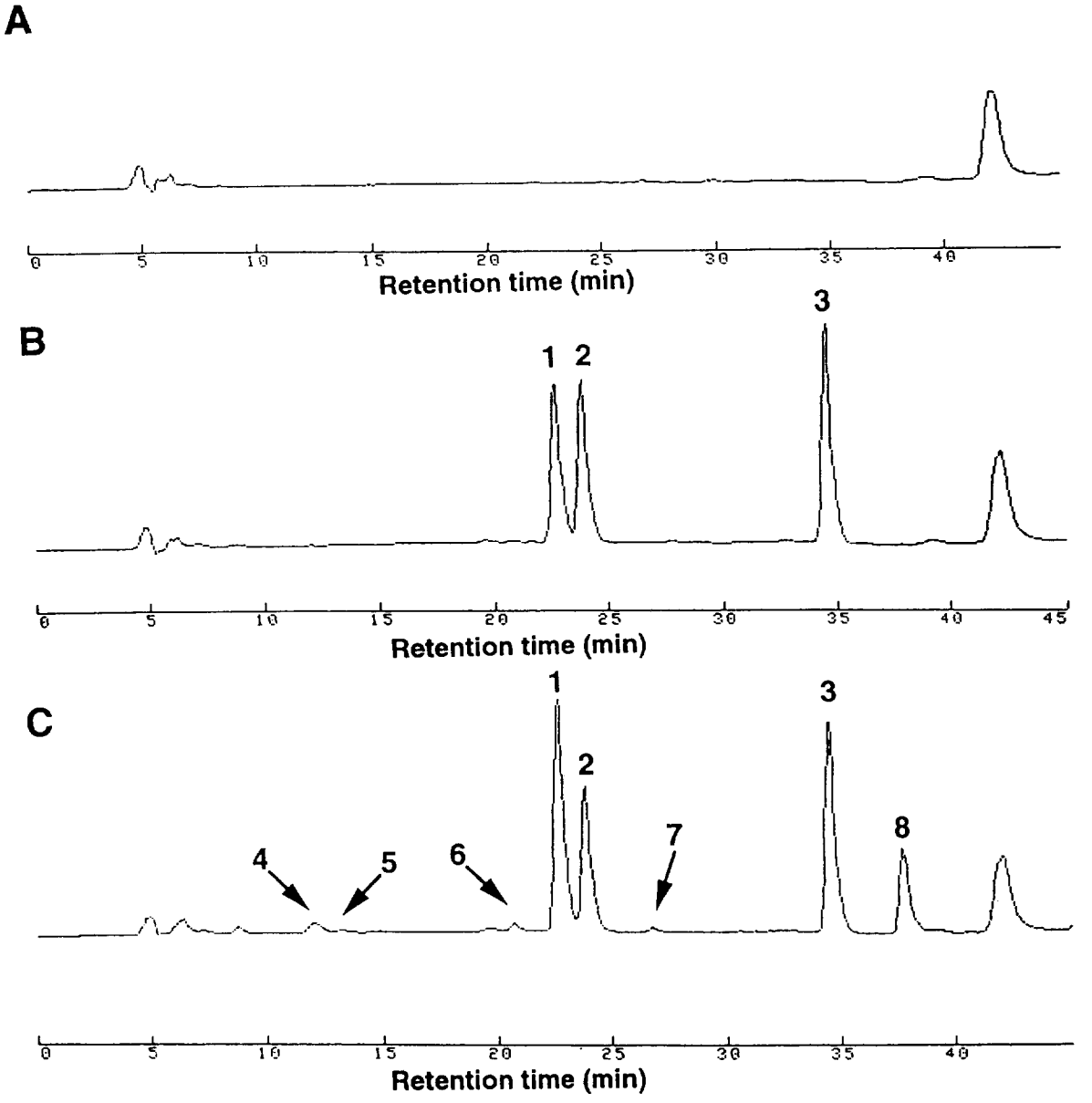

Fig. 3. Typical chromatograms of blank rat plasma (A), plasma spiked with 5 μg/ml of each enantiomer (B), and plasma from a rat 15 min after intravenous administration of racemic E3810 (40 mg/kg). Peaks: 1 = *R*(+)-E3810; 2 = *S*(−)-E3810; 3 = internal standard; 4 = metabolite III(*R*); 5 = metabolite III(*S*); 6 = metabolite II; 7 = metabolite IV; 8 = metabolite I.

the Beagle dog plasma and rat plasma are summarized in Table 4. These data suggest that the method is accurate and precise when different ratios of enantiomers are encountered in the experimental samples.

3.5. Stability

The standard solutions of E3810 enantiomers were stable for more than 4 months if stored at −20°C. No inversion of the enantiomers

Table 1
Analytical recoveries of E3810 enantiomers and I.S. from Beagle dog plasma and rat plasma

Compound	Beagle dog plasma ($n = 4$)		Rat plasma ($n = 4$)	
	Added (μg/ml)	Recovery (%) (mean ± S.E.M.)	Added (μg/ml)	Recovery (%) (mean ± S.E.M.)
R(+)-E3810	0.3	88.8 ± 3.2	0.3	77.9 ± 1.6
	3	81.4 ± 0.6	3	79.9 ± 0.6
S(−)-E3810	1.5	84.2 ± 2.9	1.5	80.1 ± 1.4
	15	84.0 ± 1.5	15	82.4 ± 1.1
I.S.	1.05	97.9 ± 3.1	5.25	97.3 ± 2.0

occurred. In Beagle dog plasma and rat plasma, both enantiomers were stable for more than 6 months if stored at −20°C. Additionally, extracts of Beagle dog plasma and rat plasma reconstituted in methanol containing 0.1% diethylamine were stable at 4°C for more than 7 days in the autosampler.

3.6. Application

This enantioselective assay method was applied to pharmacokinetic studies. Fig. 4 shows the plasma concentration–time profile of *R*(+)-E3810 and *S*(−)-E3810 after intravenous administration of racemic E3810 to male Beagle dogs and male rats. In Beagle dogs, after intravenous administration of racemic E3810 (3 mg/kg), elimination of *S*(−)-E3810 from plasma was faster than that of *R*(+)-E3810. The half-time ($t_{1/2}$) for *R*(+)-E3810 and *S*(−)-E3810 was 24.0 ± 0.7 and 13.4 ± 0.3 min, and AUC was 131 ± 11 and 69 ± 7.2 μg min ml^{-1}, respectively.

Table 2
Intra-day and inter-day validation for E3810 enantiomers in Beagle dog plasma

Compound	Concentration in spiked sample (μg/ml)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
		Concentration found (mean ± S.D.)	C.V. (%)	Accuracy (%)	Concentration found (mean ± S.D.)	C.V. (%)	Accuracy (%)
R(+)-E3810	20	19.876 ± 0.733	3.7	−0.6	20.429 ± 0.192	0.9	2.1
	10	9.775 ± 0.634	6.5	−2.3	10.158 ± 0.318	3.1	1.6
	3	3.048 ± 0.117	3.8	1.6	2.984 ± 0.113	3.8	−0.5
	1	0.995 ± 0.038	3.8	−0.5	0.993 ± 0.019	1.9	−0.7
	0.3	0.294 ± 0.022	7.5	−2.0	0.291 ± 0.003	1.0	−3.0
	0.1	0.097 ± 0.008	8.2	−3.0	0.100 ± 0.002	2.0	0.0
	0.05	0.052 ± 0.005	9.6	4.0	0.052 ± 0.002	3.8	4.0
	0.03	0.032 ± 0.004	12.5	6.7	0.030 ± 0.001	3.3	0.0
S(−)-E3810	20	19.970 ± 0.625	3.1	−0.2	20.658 ± 0.833	4.0	3.3
	10	9.838 ± 0.510	5.2	−1.6	9.964 ± 0.298	3.0	−0.4
	3	3.066 ± 0.126	4.1	2.2	3.007 ± 0.056	1.9	0.2
	1	1.006 ± 0.039	3.9	0.6	0.992 ± 0.016	1.6	−0.8
	0.3	0.302 ± 0.023	7.6	0.7	0.297 ± 0.005	1.7	−1.0
	0.1	0.100 ± 0.008	8.0	0.0	0.100 ± 0.002	2.0	0.0
	0.05	0.051 ± 0.003	5.9	2.0	0.049 ± 0.002	4.1	−2.0
	0.03	0.032 ± 0.003	9.4	6.7	0.030 ± 0.001	3.3	0.0

Table 3
Intra-day and inter-day validation for E3810 enantiomers in rat plasma

Compound	Concentration in spiked sample (μg/ml)	Intra-day ($n = 4$)			Inter-day ($n = 4$)		
		Concentration found (mean ± S.D.)	C.V. (%)	Accuracy (%)	Concentration found (mean ± S.D.)	C.V. (%)	Accuracy (%)
R(+)-E3810	100	107.613 ± 10.097	9.4	7.6	104.115 ± 3.461	3.3	4.1
	50	51.517 ± 2.090	4.1	3.0	50.391 ± 0.940	1.9	0.8
	15	14.653 ± 1.035	7.1	−2.3	15.438 ± 0.360	2.3	2.9
	5	5.270 ± 0.284	5.4	5.4	5.086 ± 0.172	3.4	1.7
	1.5	1.571 ± 0.043	2.7	4.7	1.494 ± 0.019	1.3	−0.4
	0.5	0.510 ± 0.029	5.7	2.0	0.468 ± 0.026	5.6	−6.4
	0.25	0.263 ± 0.016	6.1	5.2	0.238 ± 0.014	5.9	−4.8
	0.15	0.152 ± 0.010	6.6	1.3	0.151 ± 0.012	7.9	0.7
	0.1	0.105 ± 0.007	6.7	5.0	0.108 ± 0.010	9.3	8.0
S(−)-E3810	100	106.573 ± 8.262	7.8	6.6	102.474 ± 3.741	3.7	2.5
	50	48.422 ± 2.283	4.7	−3.2	51.368 ± 1.260	2.5	2.7
	15	14.833 ± 0.650	4.4	−1.1	15.415 ± 0.388	2.5	2.8
	5	5.035 ± 0.147	2.9	0.7	5.075 ± 0.211	4.2	1.5
	1.5	1.478 ± 0.064	4.3	−1.5	1.504 ± 0.009	0.6	0.3
	0.5	0.511 ± 0.013	2.5	2.2	0.460 ± 0.017	3.7	−8.0
	0.25	0.259 ± 0.008	3.1	3.6	0.246 ± 0.012	4.9	−1.6
	0.15	0.148 ± 0.009	6.1	−1.3	0.152 ± 0.009	5.9	1.3
	0.1	0.097 ± 0.007	7.2	−3.0	0.103 ± 0.008	7.8	3.0

There was a ca. 2-fold difference between the Cl_{tot} values of the enantiomers (Table 5). In rats, after intravenous administration of racemic E3810 (40 mg/kg), elimination of each enantiomer from plasma was similar, with $t_{1/2}$ for *R*(+)-E3810 and *S*(−)-E3810 being 9.5 ± 0.5 and 10.4 ±

Table 4
Intra-day validation for E3810 enantiomers in Beagle dog plasma and rat plasma when different concentration of enantiomers were spiked in the plasma samples

Matrix	*R*(+)-E3810 ($n = 4$)				*S*(−)-E3810 ($n = 4$)			
	Spiked (μg/ml)	Found (μg/ml) (mean ± S.D.)	C.V. (%)	Accuracy (%)	Spiked (μg/ml)	Found (μg/ml) (mean ± S.D.)	C.V. (%)	Accuracy (%)
Beagle dog plasma	1	0.982 ± 0.031	3.2	−1.8	0.3	0.300 ± 0.007	2.3	0.0
	0.3	0.306 ± 0.003	1.0	2.0	1	0.971 ± 0.015	1.5	−2.9
	10	10.103 ± 0.147	1.5	1.0	3	3.171 ± 0.051	1.6	5.7
	3	3.092 ± 0.081	2.6	3.1	10	9.978 ± 0.350	3.5	−0.2
Rat plasma	5	5.074 ± 0.570	11.2	1.5	1.5	1.556 ± 0.195	12.5	3.7
	1.5	1.710 ± 0.022	1.3	14.0	5	5.364 ± 0.099	1.8	7.3
	50	57.500 ± 1.211	2.1	15.0	15	18.248 ± 0.280	1.5	21.7
	15	18.072 ± 0.653	3.6	20.5	50	56.865 ± 1.281	2.3	13.7

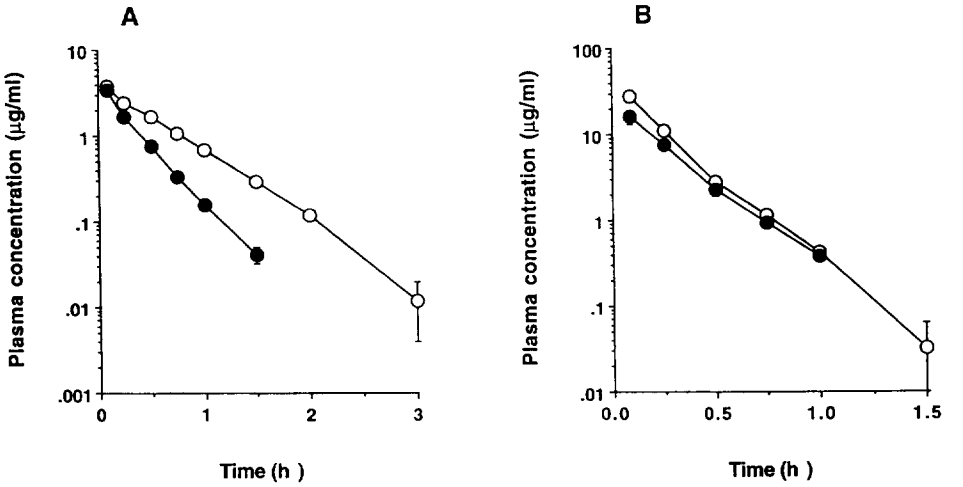

Fig. 4. Plasma concentration–time curves for $R(+)$-E3810 (○) and $S(-)$-E3810 (●) after intravenous administration of racemic E3810 to male Beagle dogs (3 mg/kg) (A) and male rats (40 mg/kg) (B). Each point represents mean ± S.E.M. (A) $n = 6$, (B) $n = 4$

0.5 min, respectively. The V_d values for $R(+)$-E3810 and $S(-)$-E3810 were 692 ± 147 and 1142 ± 316 ml/min, respectively. There was a ca. 1.5-fold difference between the Cl_{tot} values of the enantiomers (Table 5). These results suggest enantioselective pharmacokinetics of E3810. A different enantioselective pharmacokinetic profile was observed between Beagle dog and rat. These findings were acquired using administration of racemic E3810, and there is no other information about the pharmacokinetics of each enantiomer at present. Additional pharmacokinetic studies after administration of each respective enantiomer are needed to clarify the mechanism of the enantioselectivity.

4. Conclusion

An assay method for the simultaneous HPLC determination of E3810 enantiomers in Beagle dog and rat plasma has been developed. This method was used to investigate the enantioselec-

Table 5
Pharmacokinetic parameters after intravenous administration of racemic E3810 to male Beagle dogs (3 mg/kg) and male rats (40 mg/kg)

Species	Enantiomer	$t_{1/2}$ (min)	V_d (ml/kg)	Cl_{tot} (ml min^{-1} kg^{-1})	$AUC_{(0-\infty)}$ (μg min ml^{-1})
Beagle dog	$R(+)$-E3810	24.0 ± 0.7	392 ± 30	11.8 ± 0.9	131 ± 11
	$S(-)$-E3810	13.4 ± 0.3	419 ± 33	23.0 ± 2.1	69 ± 7.2
	R/S ratio	1.79	0.94	0.51	1.90
Rat	$R(+)$-E3810	9.5 ± 0.5	692 ± 147	51.4 ± 9.2	420 ± 58
	$S(-)$-E3810	10.4 ± 0.5	1142 ± 316	79.7 ± 15.9	276 ± 41
	R/S ratio	0.91	0.61	0.64	1.52

Beagle dog: each value represents mean ± S.E.M. ($n = 6$). Rat: each value represents mean ± S.E.M. ($n = 4$)

tive pharmacokinetics in plasma after intravenous administration of racemic E3810 to Beagle dogs and rats.

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