ELSEVIER

Journal of Chromatography B, 719 (1998) 177–189

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic determination of erdosteine and its optical active metabolite utilizing a fluorescent chiral tagging reagent, *R*-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole

Norikazu Muramatsu[a], Toshimasa Toyo'oka[a,*], Kazuo Yamaguchi[b], Satoshi Kobayashi[b]

[a]*Department of Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan*

[b]*Analytical and Pharmacokinetic Department, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co. Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan*

Received 5 June 1998; received in revised form 11 August 1998; accepted 11 August 1998

Abstract

Chiral separation of racemic M1 metabolized from erdosteine was investigated by reversed-phase chromatography. The sensitive determination of M1 and erdosteine with UV detection was difficult because of their low absorptivity in the effective wavelength region. To improve the sensitivity and separatability, one thiol and two carboxyl groups in the M1 structure were labelled with DBD-F and *R*-(−)-DBD-APy, respectively. Non-fluorescent DBD-F quantitatively reacted with thiol in M1 at room temperature for 30 min in borate buffer (pH 9.3) to produce the fluorescent derivative. On the other hand, the labelling of two carboxyls was carried out with a chiral fluorescent reagent, *R*-(−)-DBD-APy, in acetonitrile containing DPPA. The derivatives corresponding to a pair of the enantiomers were completely separated with water–acetonitrile containing 0.1% TFA as the mobile phase by an ODS column. Erdosteine with a carboxyl group was also labelled with *R*-(−)-DBD-APy and separated together with M1 derivatives. The detection limits ($S/N=3$) of erdosteine and M1 were 0.37 and 0.22 pmol, respectively. The proposed derivatization and separation methods were applied to simultaneous determination of racemic M1 and erdosteine in rat plasma after administration of erdosteine. The amounts of both enantiomers of M1 were essentially the same in oral and intravenous administrations. In contrast, total amounts (reduced-form and oxidized-form) of *S*-(−)-M1 in rat plasma were higher than those of *R*(+)-M1 in both administrations.

Keywords: Enantiomer separation; Erdosteine; *R*-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole

1. Introduction

Some biologically active substances play essential roles as single enantiomers in living organisms. Chiral drugs possessing chiral center(s) are also

*Corresponding author. Tel.: +81-54-264-5656; fax: +81-54-264-5593; e-mail: toyooka@ys2.u-shizuoka-ken.ac.jp.

0378-4347/98/$ – see front matter
PII: S0378-4347(98)00393-4

considered to be different compounds in the body. Therefore, the metabolites of their enantiomers administered as a drug often possess diverging activities, toxicities and pharmacokinetic properties, similar to the administered drug, because macromolecular substances such as enzymes have characteristic chiral selectivity in biological systems.

When a drug and its metabolites are determined in biological specimens such as plasma and urine, selective and sensitive determination methods, which are able to resolve a pair of enantiomers in precise, repeatable and reliable, are required.

Many compounds developed as drugs possess various functional groups such as carboxyl (–COOH), amines (–NH, $-NH_2$) and thiol (–SH) in their skeleton. The determination of the compounds strongly absorbed in ultraviolet (UV) at more than 250 nm are relatively easy. When target compounds have no prominent absorption in the effective wavelength region (ca. 250–350 nm), however, trace level detection of them with absorptiometry is generally difficult on account of their wavelength properties. Furthermore, the determination in biological specimens such as blood and urine is impossible with UV detection due to possible interference of intrinsic substances in the samples. Many derivatization reagents which react with various functional groups have been used to improve the properties and detection selectivity. If the target compound is a chiral molecule having an asymmetric carbon, a chiral tagging reagent is adopted for the resolution because the resulting diastereomers are generally separated well by reversed-phase chromatography. Fluorescence generators and fluorescent reagents are more suitable for trace analysis in terms of selectivity and sensitivity [1–5].

Erdosteine, (±)-{[2-oxo-2[(tetrahydro-2-oxo-3-thienyl)amino]ethyl]thio}acetic acid, having both a carboxyl group and a thiolactone ring in the structure is a chiral drug, and mainly metabolized to the ring opening compound, M1, (±)-*N*-(2-carboxymethylthioacetyl)homocysteine, (Fig. 1) [6–10]. The metabolite M1 is endowed with mucolytic, mucomodulator and free radical scavenging properties. Also, it has a therapeutic application in preventing oxidative lung disease caused the elastase inhibitory capacity of α1-anti trypsin induced by cigarette smoke [11,12]. Compared with other known

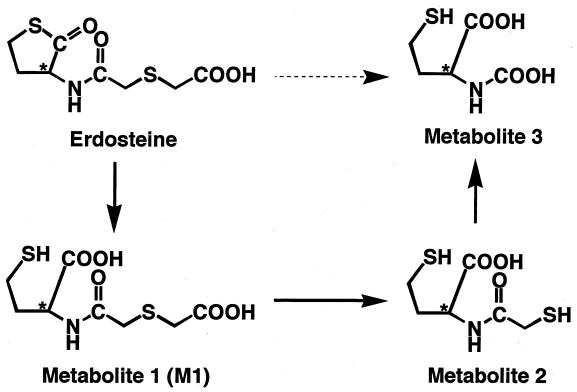

Fig. 1. Metabolic pathway of erdosteine.

anti-oxidant drugs, erdosteine appeared to be the most active [6,7,12,13]. Furthermore, the *S*-(−) enantiomer was more effective than *R*-(+) enantiomer in protecting against lung damage [6].

Erdosteine and its metabolite M1, optically active substances, do not give remarkable absorption more than 230 nm. Therefore, it is difficult to determine the racemic mixture of M1 and erdosteine without any treatment. Thus, chiral separation of M1 metabolized from erdosteine was studied as an example of a chiral molecule for which there is no effective determination method. This paper presents development of the optical resolution method of M1, based upon double-labelling of carboxyl and thiol groups using 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [14] and *R*-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole [*R*-(−)-DBD-APy] [15,16], by high-performance liquid chromatography (HPLC) with fluorescence detection. The proposed procedure is also applied to the simultaneous determination of erdosteine and M1 in rat plasma.

2. Experimental

2.1. Materials and reagents

Erdosteine was synthesized by Edmond Pharma. The fluorescent derivatization reagents DBD-F, *R*-(−)-DBD-APy (optical purity 99.8%) [17] and *R*-(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [*R*-(−)-

DBD-PyNCS] were purchased from Tokyo Kasei (Tokyo, Japan). Diphenyl phosphoryl azide (DPPA, Tokyo Kasei), diethyl phosphorocyanidate (DEPC, Wako Pure Chemicals, Osaka, Japan), 2,2-dipyridyl disulfide (DPDS, Tokyo Kasei), triphenylphosphine (TPP, Kanto Chemicals, Tokyo, Japan), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Wako) and pyridine (Wako) were used as received. Chloroform-d [99.8 atom% D contains 1% (v/v) tetramethylsilane (TMS), Aldrich], methanol-d_4 (99.8 atom% D, Merck, Darmstadt, Germany) and dimethylsulfoxide-d_6 (99.8 atom% D, Merck) were used for nuclear magnetic resonance (NMR) measurements. *n*-Capric acid ($C_{10:0}$) and *n*-lauric acid ($C_{12:0}$) were obtained from Tokyo Kasei, and (*S*)-6-methoxy-α-methyl-2-naphthaleneacetic acid [(+)-naproxen] and 1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid (indomethacin) were from Sigma (St. Louis, MO, USA). [2*S*]-1-[3-mercapto-2-methyl-propionyl]-L-proline (captopril) was supplied by Sankyo (Tokyo, Japan). Sodium sulfate (Na_2SO_4) and hydrochloric acid (HCl) were purchased from Wako Pure Chemicals. All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. Apparatus

The HPLC system consisted of two LC-10A pumps and an SCL-10A controller (Shimadzu, Kyoto, Japan). Samples were injected through a SIL-$10A_{XL}$ autoinjector (Shimadzu). The analytical column was a 5 μm Ultron VX-ODS (150×4.6 mm I.D., Shinwa, Kyoto, Japan). The column was maintained at 40°C with a CTO-10A column oven (Shimadzu). A Shimadzu RF-$10A_{XL}$ fluorescence monitor equipped with a 12-μl flow cell was employed for the detection of the eluate from the column. All mobile phases were degassed with an on-line degasser (DGU-12A, Shimadzu). The flow-rate of the eluent was fixed at 1.0 ml/min. The Vstation chromatography software (GL Sciences, Tokyo, Japan) was used for data analysis in HPLC. For solid-phase extraction, a GL-SPE vacuum manifold apparatus (GL Sciences) and Bond Elut Certify II (Varian, Habor City, CA, USA) as extraction column were used. A micro-refrigerated centrifugation system, CST-150MT (Shimadzu) was used for drying. A SPE-200 centrifugal evaporator (Shimadzu) was employed for evaporation of sample solution.

The liquid chromatography–mass spectrometry (LC–MS) system consisted of a Hewlett-Packard HP1100 HPLC system (Yokogawa Analytical Sciences, Tokyo, Japan), constituted from two pumps, a degasser, a column oven, an autosampler and a photodiode array detector, and a Finnigan MAT LCQ MS system equipped with electrospray ionization (ESI) probe. For data analysis in LC–MS, HP ChemStation (Yokogawa Analytical Sciences) and LCQ Rev.1.1 SPI was employed.

Proton NMR (^{1}H-NMR) spectra were recorded on an JNM GSX 500 NMR spectrometer (Jeol, Tokyo, Japan) at 500 MHz using TMS as an internal standard. Melting-points (m.p.s) were measured using a MEL-TEMP micro melting-point apparatus (Cambridge, UK).

2.3. Synthesis of derivative I

Erdosteine (50 mg, 0.20 mmol) was converted into M1 with 50 ml of 0.1 *M* NaOH at 80°C for 30 min. Then, DBD-F (60 mg, 0.25 mmol) dissolved in 25 ml of CH_3CN was added dropwise to a stirred M1 solution. After stirring for 60 min at room temperature (20–30°C) in the dark, the CH_3CN in the reaction mixture was evaporated under reduced pressure. Excess and unreacted DBD-F in the residue was extracted out with 50 ml of ethyl acetate (AcOEt). The same extraction procedure was repeated three times. The aqueous solution containing derivative I was adjusted to pH 1–2 with 36% HCl and then 50 ml of AcOEt was added to the acidic solution to extract derivative I. The same extraction procedure was repeated four times. The combined AcOEt extracts were washed with 20 ml of water, dried over anhydrous sodium sulfate (Na_2SO_4) and evaporated in vacuo.

Derivative I (Fig. 2): pale yellow crystals; m.p. 139–141°C; yield 92%; ^{1}H-NMR (ppm) in DMSO-d_6, 8.51 (1H, d, J=7.5 Hz, 4), 7.93 (1H, d, J=7.0 Hz, b or c), 7.49 (1H, d, J=7.0 Hz, b or c), 4.46 (1H, br, 3), 3.40 (2H, s, 6 or 7), 3.32 (2H, s, 6 or 7), 2.81 (6H, s, g), 2.23 (2H, m, 1), 2.11 (2H, m, 2); ^{13}C-NMR (ppm) DMSO-d_6, 172.6 (8′), 171.1 (8), 169.0 (5), 148.8 (f), 145.3 (e), 135.8 (a), 134.2 (d),

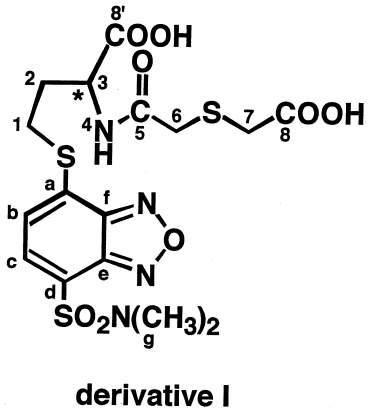

Fig. 2. Chemical structure of derivative I.

123.3 (b), 119.9 (c), 51.1 (3), 37.4 (g), 34.5 (6 or 7), 33.7 (6 or 7), 30.0 (2), 27.2 (1); ESI LC–MS (*m*/*z*), 493 $[M+H]^+$.

2.4. Synthesis of derivative II

R-(−)-DBD-APy (95 mg, 0.30 mmol) dissolved in 25 ml of CH_3CN was added to the stirring solution of derivative I (50 mg, 0.10 mmol) in 25 ml of CH_3CN in the presence of DPPA (500 μl) at room temperature. After stirring at room temperature for 2 h in the dark, CH_3CN in the reaction mixture was evaporated under reduced pressure. To the residue was added 25 ml water and the unreacted *R*-(−)-DBD-APy was extracted out with 20 ml benzene (repeated three times). Then, derivative II in the aqueous layer was extracted with 20 ml chloroform. The same extraction procedure was repeated three times. The chloroform solution containing the derivative was washed with 0.1 *M* NaOH (3×20 ml) and water (3×20 ml). The combined $CHCl_3$ extracts were dried over anhydrous sodium sulfate (Na_2SO_4) and evaporated in vacuo.

Derivative II (Fig. 3): orange crystals; m.p. 132–135°C; yield 61%; ^{1}H-NMR (ppm) in DMSO-d_6, 8.51 (1H, d, *J*=7.5 Hz, 4), 8.51 (2H, d, d, *J*=6.5 Hz, l′, l″), 7.95 (1H, d, *J*=7.5Hz, b or c), 7.83 (2H, d, d, *J*=8.0 Hz, c′, c″), 7.47 (1H, d, *J*=7.5 Hz, b or c), 6.14 (2H, d, d, *J*=8.0 Hz, b′, b″), 4.52 (1H, br, 3), 3.60–4.30 (2H, br, i′, i″), 3.35 (2H, s, 6 or 7),

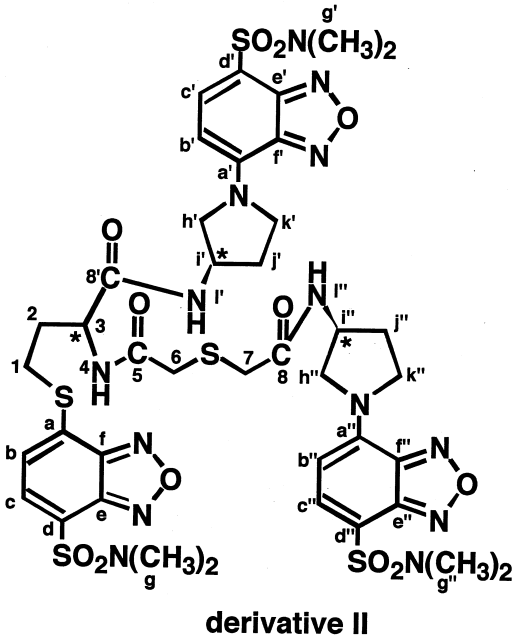

Fig. 3. Chemical structure of derivative II.

3.34 (2H, s, 6 or 7), 3.25 (4H, m, h′, h″), 3.22 (4H, m, k′, k″), 2.84 (6H, s, g), 2.73 (6H, 6H, s, s, g′, g″), 2.30 (2H, m, 1), 2.17 (2H, m, 2), 2.09 (4H, m, j′, j″); ^{13}C-NMR (ppm) in DMSO-d_6, 170.6 (8 or 8′), 168.9 (8 or 8′), 169.0 (5), 148.7 (f), 146.8 (f′, f″), 145.1 (e), 144.8 (e′, e″), 140.6 (a′, a″), 135.7 (a), 139.3 (d′, d″), 134.6 (d), 130.2 (b), 122.9 (b′, b″), 128.8 (c′, c″), 120.0 (c), 55.8 (i′, i″), 51.8 (3), 48.9 (h′, h″), 37.6 (g′, g″), 37.5 (g), 35.1 (6), 35.0 (7), 30.9 (k′, k″), 30.8 (2), 30.4 (j′, j″), 27.2 (1); ESI LC–MS (*m*/*z*), 1079 $[M+H]^+$.

2.5. Spectral measurements of the reagents and their derivatives

One μ*M* of the reagents (DBD-F and DBD-APy) and the synthetic derivatives (derivatives I and II) in CH_3CN were prepared as stock solutions. A 50-μl volume of each stock solution was mixed with 3 ml of water, CH_3CN, water–CH_3CN (500:500) or water–CH_3CN–TFA (500:500:1). The diluted solution was used for the measurement of excitation and

emission spectra with a Hitachi F-3010 spectrofluorophotometer using a 1-cm quartz cell without spectral correction.

2.6. Effect of the reagent concentrations on the derivatization reactions

Fifty-μl volumes of various concentrations of DBD-F in CH_3CN (1, 5, 25, 50, 100, 200 molar equivalents against analyte) and 50 μl of 0.1 m*M* M1 in CH_3CN were reacted in alkaline medium including 50 μl of borate buffer (pH 9.3). The vial was tightly capped and allowed to stand for 30 min at room temperature under protection from light. An aliquot of each reaction solution was injected onto the column and monitored with fluorescence detector at 450 nm (excitation at 390 nm). The yields were calculated based on the fluorescent peak area obtained from synthesized derivative I.

Similarly, 50 μl of *R*-(−)-DBD-APy dissolved in CH_3CN (1, 5, 25, 50, 100, 200, 400 molar equivalents against analyte) was mixed with 50 μl of synthesized derivative I (0.1 m*M* in CH_3CN) and 5 μl DPPA in glass vials. The solutions were filled up to 150 μl with CH_3CN. After derivatization at room temperature for 120 min, a 10-μl volume of the solution was injected onto the HPLC column and fluorometrically detected at 560 nm (excitation at 470 nm). The yields were calculated based on the fluorescent peak area obtained from synthesized derivative II.

2.7. Effect of activation agents on the formation of derivative II

The activation agents [i.e., 5 μl of (A) DPPA or (B) DEPC; (C) 50 μl CH_3CN containing 10 m*M* DPDS and TPP; (D) mixture of 80 μl of 35 m*M* EDC in CH_3CN and 20 μl pyridine] were mixed with 50 μl of 20 m*M* *R*-(−)-DBD-APy in CH_3CN and 50 μl of 50 μ*M* derivative I in glass vials. Then the solution was filled up to 200 μl with CH_3CN. The solution was tightly capped and allowed to stand over 480 min at room temperature or 50°C in the dark. At fixed time intervals, a 10 μl volume of the solution was injected onto the column for HPLC and the fluorescence peak area corresponding to derivative II was detected at 560 nm (excitation at 470 nm).

2.8. Recommended procedure for determination of M1 (SH form) and total M1 (SH and SS forms)

2.8.1. Determination of M1

To 0.1 ml plasma was added 0.5 ml water, 0.56 ml 0.1 *M* borate buffer (pH 9.3), 10 μl of 0.1 m*M* captopril (I.S.) in water, and 0.42 ml DBD-F (0.32 mg) in CH_3CN. After reaction at room temperature for 30 min, CH_3CN in the reaction mixture was evaporated under reduced pressure. Then 2.0 ml of AcOEt was added to the remaining solution, rigorously mixed, and centrifuged at 3000 rpm for 2 min. The AcOEt solution separated was discarded and the same extraction procedure as mentioned above was repeated three times. The aqueous layer containing the DBD-derivative was adjusted to pH 1–2 with 0.1 *M* HCl (ca. 1.5 ml). The derivative in the acidic solution was extracted with AcOEt (three times with 2 ml each). The AcOEt extracts combined was evaporated in vacuo and re-dissolved in 0.1 ml of CH_3CN. To 50 μl of the solution was added 5 μl of DPPA and 45 μl of *R*-(−)-DBD-APy (0.8 mg) in CH_3CN solution. The solution was allowed to stand at room temperature for 2 h. An aliquot of the reaction solution was separated with reversed-phase chromatography and detected fluorometrically at 560 nm (excitation at 470 nm).

2.8.2. Determination of total M1

Oxidized M1 (SS-form) in 0.1 ml plasma was reduced with 20 μl of 10% tri-*n*-butylphosphine (TBP) in dimethylformamide (DMF) at 37°C for 15 min. After reduction, 0.1 ml of water was added to the reaction solution and centrifuged at 10 000 rpm and 4°C for 5 min. The supernatant separated was treated using the procedures described above.

2.9. Drug administration study

Erdosteine (268.32 mg, 1.08 mmol) was dissolved in 10.73 ml of water containing equivalent molar amount of $NaHCO_3$ (stock solution A, concentration, 25 mg/ml). Stock solution B (concentration, 20 mg/ml) was prepared with addition of 2 ml water

containing an equivalent molar amount of $NaHCO_3$ to 8 ml of stock solution A.

Rats were anesthetized with diethyl ether and inserted cannula to femoral vein before administration. After rats awakened from anesthesia, an erdosteine solution was administrated orally at a dose of 100 mg/kg (5 ml/kg of stock solution B) and intravenously at a dose of 25 mg/kg (1 ml/kg of stock solution A), respectively. After administration at 15, 30, 60, 90, 120, 150, 180, 210 min (orally), or 5, 10, 15, 30, 60, 120, 180 min (intravenously), 0.6–0.7 ml blood was collected by a cannula and placed in microtubes with 5 μl of heparin and centrifuged immediately at 15 000 rpm and 4°C for 10 min. Plasma separated was stored at −80°C just prior to use.

3. Results and discussion

3.1. Strategy for determination of M1 enantiomers

Erdosteine is rapidly metabolized to racemic M1 in body (Fig. 1). Since M1 enantiomers have one thiol (–SH) and two carboxyl (–COOH) groups in their structure, chiral separation seems to be possible with chromatographic determination of diastereomers produced from chiral derivatizing agent(s) toward these functional groups. Initially, the tagging of the thiol group with *R*-(−)-DBD-PyNCS [3] was studied for the separation of M1 (Fig. 4). However, the separation was unsuccessful owing to long distance between the two chiral centers and free rotation of the labelled fluorophore.

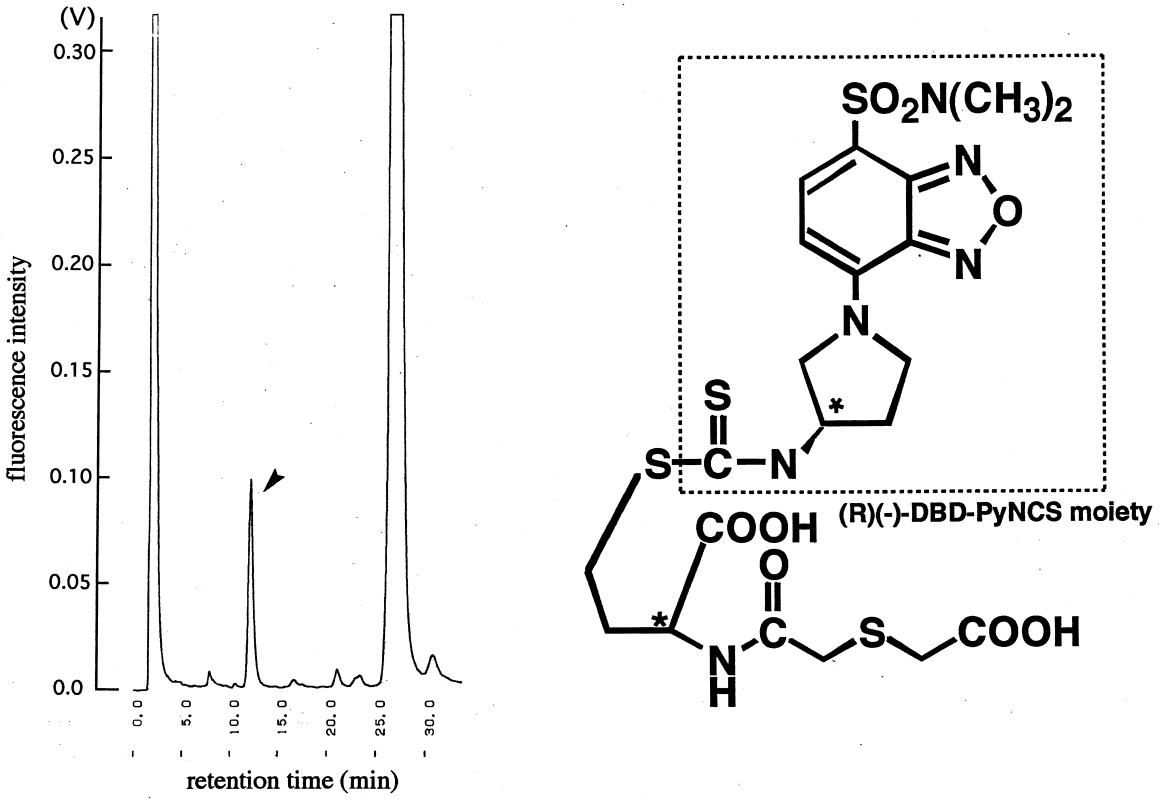

Fig. 4. Chromatogram of M1 labelled with *R*-(−)-DBD-PyNCS. HPLC conditions: column, Ultron VX-ODS (150 mm×4.6 mm I.D., 5 μm); mobile phase, water–CH_3CN–TFA (70:30:0.1); column temperature, 40°C; FL detection, 568 nm (exitation at 455 nm); flow-rate, 1.0 ml/min.

The labelling of the carboxyl group at α-position of an asymmetric carbon may be most effective for the separation of the resulting diastereomers because of the short distance between both chiral centers of the resulting diastereomers. Therefore, a fluorescent chiral reagent, *R*-(−)-DBD-APy, was selected for the labelling of two carboxyl groups; one is the α-position of chiral center and the other is the ω-position. Furthermore, erdosteine seems to be fluorometrically determined with the labelling of the reagent, as is M1. Since the thiol group is generally unstable in liquid, it is recommended to protect the thiol group with a selective tagging reagent, DBD-F, before labelling of carboxyl groups. Thus, the determination procedure of erdosteine and the racemic metabolite M1 was established. The strategy of the determination of M1 enantiomers is shown in Fig. 5.

3.2. Synthesis of derivatives I and II

To determine fluorescence properties and reaction yields, two derivatives of M1 were synthesized utilizing two fluorescence tagging reagents, DBD-F and *R*-(−)-DBD-APy. The fluorophore of M1 labelled with DBD-F is represented as derivative I (labelled compound of a thiol group), whereas derivative II (labelled compound of one thiol and two carboxyl groups) is the fluorophore labelled with DBD-F and *R*-(−)-DBD-APy (Fig. 5).

Erdosteine was quantitatively converted to M1 (more than 95%) at 80°C for 1 h in 0.1 *M* NaOH. Since it was difficult to obtain M1 as crystals because M1 was hygroscopic and easily decomposed in solid form, the synthesis of M1 derivative was carried out without isolation of M1 eliminated from erdosteine in strong alkaline medium. The structures of derivatives I and II were identified with instrumental data such as NMR and MS spectra as described in Section 2.

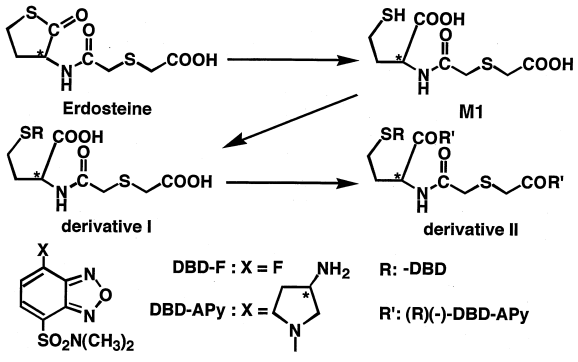

Fig. 5. Strategy for determination of M1 enantiomers.

3.3. Fluorescence characteristics of the derivatives

In order to detect the resulting derivatives effectively, fluorescence properties (maximal wavelengths of excitation and emission, and intensity) in some solvents were compared with those of the reagents used. As shown in Table 1, both intensities and maximal wavelengths of derivative II were almost same as those of *R*-(−)-DBD-APy, in spite of the insertion of three fluorophores. Such a phenomenon

Table 1
Fluorescence properties of the tagging reagents and their derivatives

Compound	Solvent	Maximal wavelength (nm)		RFI
		Ex.	Em.	
DBD-F	A	392	454	0.61
	B	392	446	0.91
	C	388	445	0.76
	D	386	446	0.83
R-(−)-DBD-APy	A	450	575	15.5
	B	458	551	872
	C	450	561	102
	D	451	559	111
Derivative I	A	399	523	22.7
	B	397	494	41.0
	C	397	512	32.4
	D	397	509	37.3
Derivative II	A	468	564	32.9
	B	467	548	429
	C	468	563	96.1
	D	468	563	100[a]

[a] The fluorescence intensity of derivative II in solvent D was tentatively taken as 100.
Solvents: A=water; B=acetonitrile; C=water–acetonitrile (500:500); D=water–acetonitrile–TFA (500:500:1).

is observed in various samples such as DNS derivative. Since DBD-F itself is a non-fluorescent compound, the slight fluorescence observed seems to be due to trace hydrolysate (DBD-OH) of the reagent. Although derivative I labelled with DBD-F fluoresced at around 500 nm (excitation at around 400 nm), the intensities in the solvents tested were lower than those of derivative II. It might be due to the difference of the fluorescence quantum yields (ϕ). The fluorescent properties of the derivatives (I and II) were essentially the same in the presence and the absence of TFA. From the results, it is possible to use the separation of the derivatives with acidic solvent as the mobile phase for chromatography.

The labelling of the thiol group is recommended not only for protection of the SH group, but also for identification of the existence of free SH. Furthermore, the fluorescence at long wavelengths is another advantage for the determination of M1 enantiomers with biological samples, because the likelihood of interference by intrinsic substances might be less.

3.4. Derivatization reaction of M1

The labelling of thiol compounds with DBD-F efficiently proceeds under the conditions at room temperature for 30 min in borate buffer (pH 9.3) containing CH_3CN [14]. In the present study, the conditions were also adopted for the derivatization of SH groups in M1. The derivative corresponding to derivative I quantitatively produced with more than 100 molar excess DBD-F (Fig. 6). On the other hand, derivative II was obtained from the reaction of DBD-labelled M1 (derivative I) with *R*-(−)-DBD-APy under non-aqueous medium in the presence of activation agent such as DPPA and DEPC [18,19]. Fig. 7 shows the time course of the formation of derivative II in the range of 10–480 molar equivalent of *R*-(−)-DBD-APy. Judging from the results in Fig. 7, it is obvious that 400 molar excess of the reagent is necessary for quantitative label of two carboxyls.

Since the labelling of carboxyls essentially required an activation catalyst, the effect of the species on the reaction was examined next. Among the activation reagents tested, DPPA gave quantitative yield after a 2-h reaction at room temperature (Fig. 8). The time course suggested that the derivative is

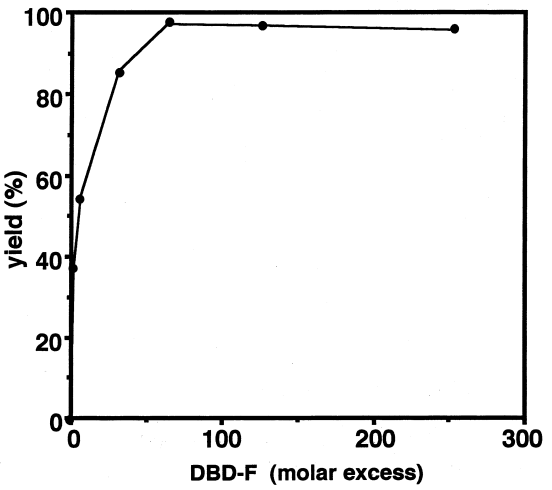

Fig. 6. Effect of DBD-F concentration on labelling of thiol group.

stable for at least 4 h. Although initial reaction rate with DEPC was fastest, the final yield at room temperature was approximate 80%. To increase the yield, the reaction was tested under heating at 50°C. However, the yield was decreased against our expectation. It might be due to the decomposition of the catalyst at elevated temperature. Similar phenomena were also obtained from the reaction in the presence of EDC/pyridine and DPDS/TPP. Consequently, the reaction time of 2 h at room temperature in the presence of DPPA (5 μl) was selected for the

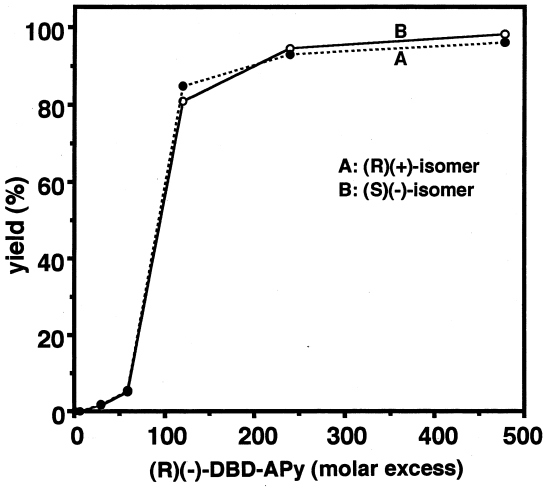

Fig. 7. Effect of *R*-(−)-DBD-APy concentration on labelling of two carboxyl groups in the presence of DPPA.

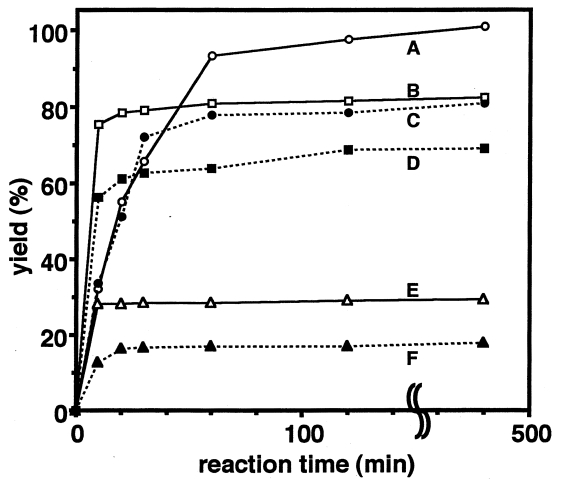

Fig. 8. Effect of catalyst on labelling of two carboxyl groups. Conditions for derivatization: (A) with DPPA at room temperature; (B) with DEPC at room temperature; (C) with DPPA at 50°C; (D) with DEPC at 50°C; (E) with EDC/pyridine at room temperature; (F) with DPDS/TPP at room temperature.

labelling of carboxyl groups in M1 with *R*-(−)-DBD-APy.

3.5. HPLC separation of the derivatives of erdosteine and M1

The separation of the derivatives of erdosteine and M1 was performed with reversed-phase chromatography. Since the addition of an internal standard seems to be essential in real sample analysis, a few compounds were tried in terms of versatility, separatability and sensitivity. The derivatives of two fatty acids and indomethacin were eluted later than those of M1 enantiomers, while naproxen derivative was overlapped with the peak caused from DPPA. Among the candidate compounds, i.e., *n*-capric acid ($C_{10:0}$), *n*-lauric acid ($C_{12:0}$), indomethacin, naproxen and captopril, captopril, similar in structure to M1, was selected as the internal standard considering the reactivity with two tagging reagents [DBD-F and *R*-(−)-DBD-APy], the detectability and the separatability of the derivative with the other peaks. Fig. 9 shows the chromatogram obtained from the reaction mixture of erdosteine, M1 and the internal standard (I.S.). As shown in the chromatogram, complete separation of their adducts and reagents was achieved under the proposed chromatographic conditions. Of course, a couple of peaks derived from M1 enantiomers were completely separated using a conventional ODS column with water–acetonitrile containing 0.1% TFA as the mobile phase (R_s=2.43). The peaks corresponding to erdosteine, I.S. and M1 were identified from the on-line HPLC–ESI-MS. The positive ions (m/z) of 543, 736 and 1079 were based on $[M+H]^+$ of erdosteine, captopril (I.S.) and M1, respectively. Both diastereomers derived from M1 enantiomers showed the same MS pattern as each other. The results are strong evidence that the peaks of B and C are derived from M1 enantiomers. With respect to the elution order of the derivatives, *R*-(+)-M1 derivative is faster than that of *S*-(−)-M1. No racemization of the M1 derivatives was observed under the proposed conditions, because the peak corresponding to opposite enantiomer after derivatization of each M1 enantiomer did not appear on the chromatograms.

Erdosteine is a chiral molecule possessing an asymmetric carbon in the structure. The racemic separation is theoretically possible because erdosteine also produces a pair of diastereomers after tagging with *R*-(−)-DBD-APy. However, the resulting derivatives appeared as a single peak on the chromatogram. This is due to the long distance between the two chiral centers, because it is well known that the distance between chiral centers is important for diastereomer separation.

3.6. Simultaneous determination of erdosteine and M1 in rat plasma

For precise and reliable determination of erdosteine and M1 in rat plasma, it is essential to remove intrinsic impurities. Two extraction methods were examined; one is solvent extraction and the other is solid-phase extraction (SPE). Table 2 shows extraction efficiency of AcOEt which is the common solvent used for solvent extraction. High recovery over 95% was obtained from the extraction of 6 ml AcOEt without plasma, while the mean recovery of the derivative added to plasma was ca. 80%. The extraction method using AcOEt may be excellent for the determination in real samples because of its good repeatability (relative standard deviation, R.S.D., less than 2%).

SPE using Bond Elut Certify II was also tried to

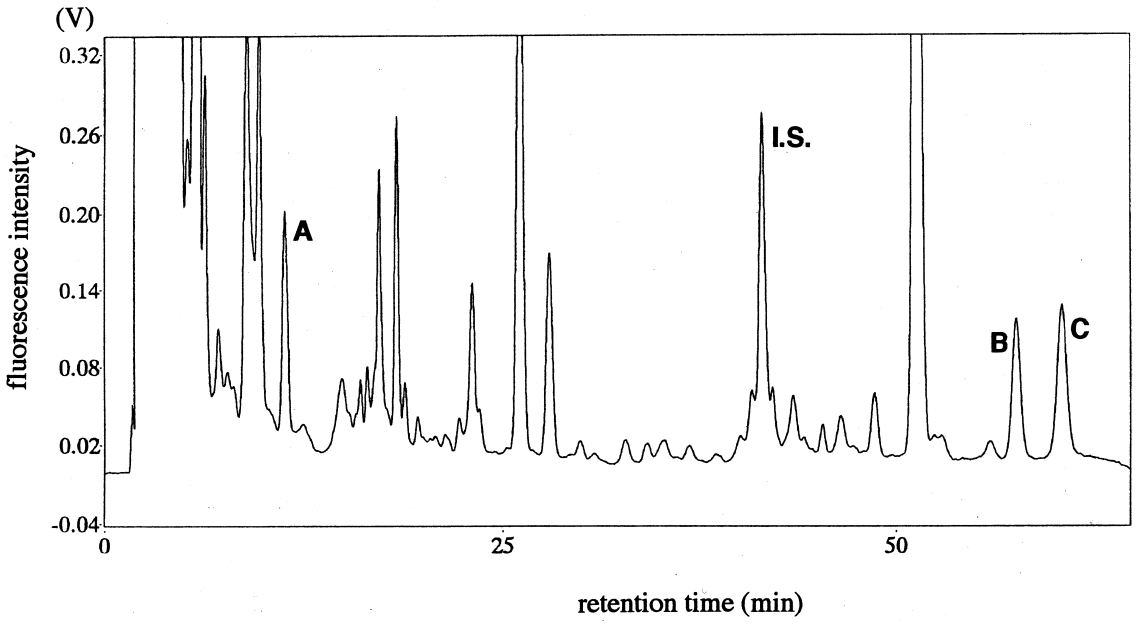

Fig. 9. Chromatogram obtained from plasma spiked with mixture of M1, captopril (I.S.) and erdosteine. Each peak corresponds to 30.7 pmol of M1, 11.3 pmol of captopril and 22.3 pmol of erdosteine, respectively. (A) Erdosteine; (B) *R*-(+)-M1; (C) *S*-(−)-M1; I.S.=captopril. HPLC conditions: column, Ultron VX-ODS (150 mm×4.6 mm I.D., 5 μm); mobile phase, water–CH_3CN–TFA; gradient elution: (30:70:0.1) 0–10 min, (37:63:0.1) 10–35 min, (43:57:0.1) 35–65 min; column temperature, 40°C; FL detection, 563 nm (excitation at 468 nm); flow-rate, 1.0 ml/min.

remove impurities containing in the sample. The recoveries with 3 ml of water–acetonitrile containing 0.1% TFA as the elution solvent are listed in Table 3. As shown in Table 3, 60% acetonitrile in water containing 0.1% TFA gave the most effective extraction (ca. 97%). The extraction ratio was decreased with lower and higher concentrations of acetonitrile (<20% and >80%). Although the SPE method effectively removes impurities in samples, as compared with solvent extraction, complicated handling procedures are required to obtain good results. Thus, solvent extraction using AcOEt was adopted for the following determination of erdosteine and M1 in real sample analysis.

Table 2
Recovery by solvent extraction using ethyl acetate

Sample	Recovery (mean, %)	R.S.D. (%)
Water	95.2	0.32
Plasma	79.6	1.18

Mean values were obtained from five attempts.

Each enantiomer of M1 and erdosteine in plasma was quantified from the calibration curves. The calibration curves were obtained by plotting the ratio of peak areas of the I.S. against amounts of erdosteine or M1. As shown in Fig. 10, good linearity passing through the origin was obtained from all calibration curves. The slight difference of the slopes in the calibration curve Fig. 10A seems to be due to the differences of the fluorescence quantum yields of the diastereomers. However, it is not obvious that the

Table 3
Recovery obtained from plasma by SPE

Acetonitrile (%)	Recovery (mean, %)	S.D. (%)
0	0.51	0.04
20	0.58	0.08
40	63.8	3.1
60	97.2	1.1
80	15.4	1.3
100	12.5	0.56

Mean values were obtained from five attempts. Eluent was water–acetonitrile containing 0.1% TFA and the elution volume was 3 ml.

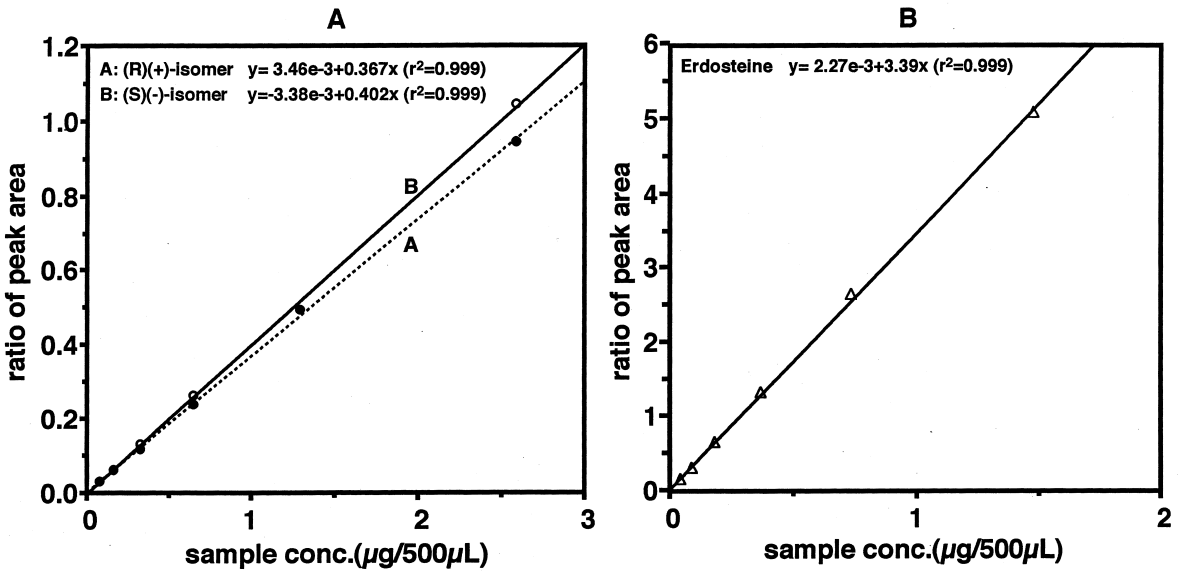

Fig. 10. Calibration curve for M1 enantiomers and erdosteine. (A) M1; (B) erdosteine.

difference is stemming either from the derivatization yields, fluorescence quantum yields and/or elution times of the diastereomers. Since the difference is slight and the linear relations are obtained from the curves, however, it may be no problem to determine M1 enantiomers. The detection limits (signal-to-noise ratio of 3) for M1 and erdosteine were 0.37 and 0.22 pmol on the chromatogram, respectively. On the other hand, the limits of determination were about 10-times higher than those of the detection limits.

As one application of the proposed method, the determination of erdosteine and M1 in rat plasma was performed after administration of erdosteine. The doses of oral and intravenous administration are 100 and 25 mg/kg, respectively. Fig. 11A and B show the plasma levels of erdosteine and a pair of M1 (reduced-forms) at each sampling time. Maximum concentrations of erdosteine and M1 were attained at 30 min after oral administration, and then gradually decreased with progress time (Fig. 11A). The amounts of M1 enantiomers were about one-tenth of those of erdosteine, and were almost comparable at each sampling time. In the case of intravenous administration, the concentrations of erdosteine and M1 were highest just after injection (Fig. 11B). However, both the concentrations and the ratios of erdosteine and M1 showed no significant difference in oral and intravenous administrations (Fig. 11A and B).

Total concentrations of M1 enantiomers, that is, calculated as amounts of M1 obtained after reduction of oxidized-M1 (M1-S-S-M1) with TBP, were also determined at each time after administration (Fig. 12). The results of erdosteine obtained from oral and intravenous administration were similar to those without reduction treatment. In contrast, the concentrations of *S*-(−)-M1 were higher than those of *R*-(+)-M1 in both oral and intravenous administrations (Fig. 12). The results might indicate that large amounts of M1 exist as disulfide with 2 molar M1 and/or covalently bound with plasma proteins. These results suggest that the ratio of free SH form in total concentration of M1 is easily analyzed with the present method. The method involving derivatization, separation and detection is applicable to the determination of racemic M1 in human plasma because the standard level of M1 after dosing of erdosteine tablet is approximately 0.1–0.2 μg/ml.

In conclusion, double labeling technique of different functional groups using two species of reagents is useful for trace analysis of M1. The mild conditions of the method are essential for the resolution of M1 enantiomers because of negligible racemization.

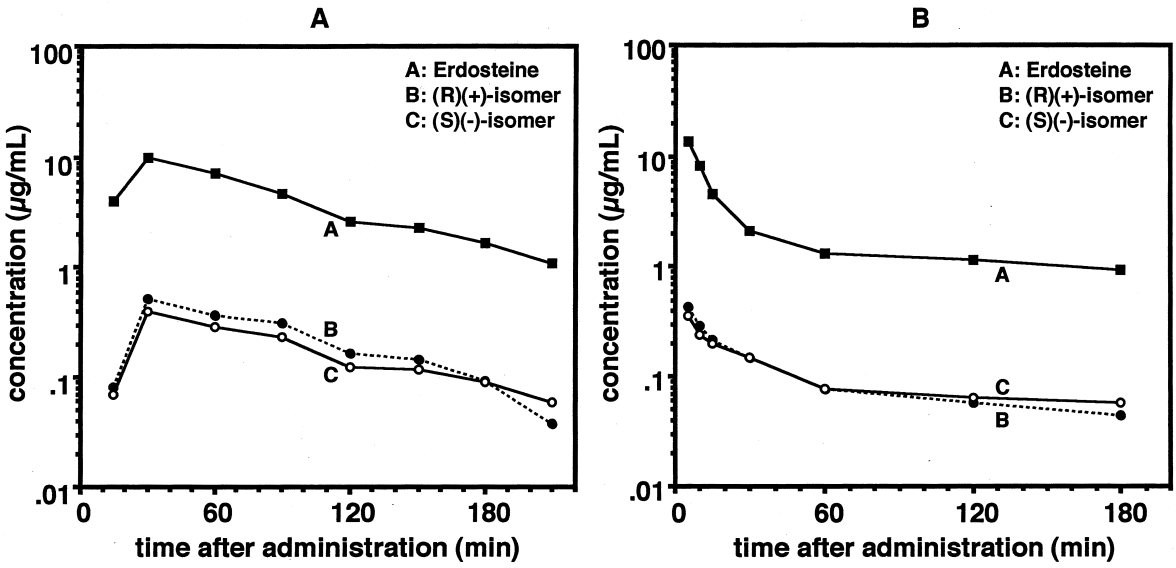

Fig. 11. Concentration of erdoteine and M1 in rat plasma after administration of (A) 100 mg/kg p.o.; (B) 25 mg/kg i.v.

Furthermore, the long maximal wavelengths of excitation and emission are another advantage in real sample analysis, because interference of intrinsic substances in samples is avoided with detection at long wavelengths. The application to monitoring of erdosteine and the metabolite M1 in human samples such as plasma and urine are planed in our laboratory together with method validation. According to our preliminary experiments, it was possible to separate the erdosteine enantiomers with a chiral stationary phase (CSP) column. However, the sensitivity was not sufficient for real samples due to the insensitivity

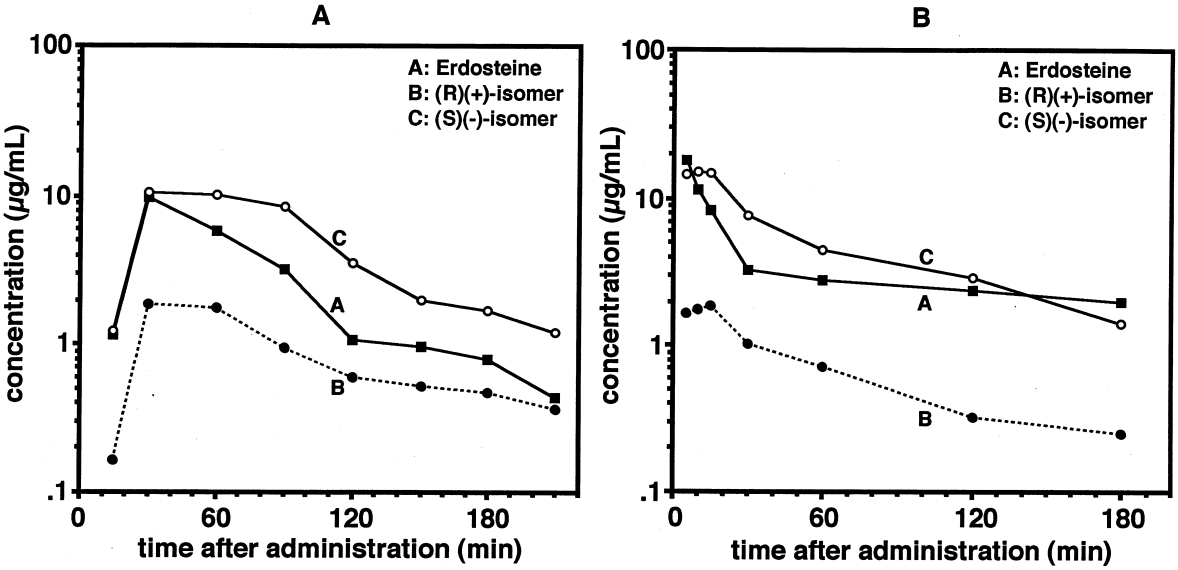

Fig. 12. Concentration of erdoteine and total M1 in rat plasma after administration of (A) 100 mg/kg p.o.; (B) 25 mg/kg i.v.

of UV detection. The sensitive chiral separation of erdosteine has not been achieved with the proposed method. That is the next subject in our project.

References

[1] Y. Yasaka, Y. Ono, M. Tanaka, J. Chromatogr. A 810 (1998) 221.
[2] O.P. Kleidernigg, W. Lindner, J. Chromatogr. A 795 (1998) 251.
[3] D. Jin, T. Toyo'oka, Analyst (London) 123 (1998) 1271.
[4] Y.-M. Liu, M. Schneider, C.M. Sticha, T. Toyo'oka, J.V. Sweedler, J. Chromatogr. A 800 (1998) 345.
[5] A. Karcher, Z. EI Rassi, Electrophoresis (Weinheim) 18 (1997) 1173.
[6] M. Ingresi, M. Nicola, G.B. Fregnan, S. Bradamante, G. Pagani, Farmaco 49 (1994) 703.
[7] G. Ricevuti, A. Mazzone, E. Uccelli, G. Gazzani, G.B. Fregnan, Thorax 43 (1989) 585.
[8] R. Scuri, P. Giannetti, A. Paesano, Drugs Exp. Clin. Res. 14 (1988) 693.
[9] L.D. Giovanni, G.B. Fregnan, C. Rabitti, G. Murari, A. Amato, A. Sovera, I.M. Civello, Int. J. Clin. Pharmacol. Ther. Toxicol. 29 (1991) 269.
[10] D. Olivieri, M.D. Donno, A. Casalini, R. D'Ippolito, G.B. Fregnan, Respiration 58 (1991) 91.
[11] G. Gazzani, G.B. Fregnan, G. Vandoni, Respiration 55 (1989) 113.
[12] G.L. Biagi, G.B. Fregnan, G. Gazzani, G. Vandoni, Int. J. Clin. Pharmacol. Ther. Toxicol. 27 (1989) 235.
[13] M. Vagliasindi, G.B. Fregnan, Int. J. Clin. Pharmacol. Ther. Toxicol. 27 (1989) 238.
[14] T. Toyo'oka, T. Suzuki, Y. Saito, S. Uzu, K. Imai, Analyst 114 (1989) 413.
[15] T. Toyo'oka, M. Ishibashi, T. Terao, Analyst 117 (1992) 727.
[16] T. Toyo'oka, M. Ishibashi, T. Terao, J. Chromatogr. 625 (1992) 357.
[17] T. Toyo'oka, M. Ishibashi, T. Terao, Anal. Chim. Acta 278 (1993) 71.
[18] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu, K. Imai, J. Chromatogr. 588 (1991) 61.
[19] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Imai, Analyst 116 (1991) 609.