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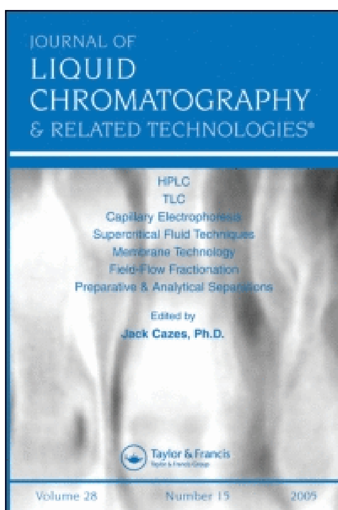

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Separation and Determination of (I)-Ephedrine and (d)-Pseudoephedrine in Plasma by High-Performance Liquid Chromatography with Fluorescence Detection

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SEPARATION AND DETERMINATION OF (l)-EPHEDRINE AND (d)-PSEUDOEPHEDRINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

The separation and determination of (l)-ephedrine (l-Ep) and (d)-pseudoephedrine (d-Ps) in Plasma by high-performance liquid chromatography (HPLC) is described. The newly developed method is based on precolumn derivatization with 5-dimethylaminonaphthalene - 1 - sulfonyl chloride (DNSCl) in 0.03% triethylamine in acetonitrile at 50 C° . The diastereomers formed were separated on a reversed phase column by HPLC with fluorescence detection employing 0.6 % phosphate buffer (pH 6.5)-methanol (3:8,v/v) as mobile phase. Clean-up of the ephedrine stereoisomers in plasma was efficiently attained by the combined use of a Sep-pak C_{18} cartridge and an ion-exchange gel, Carboxymethyl Sephadex LH-20 (CM-LH-20). The detection limit of each ephedrine stereoisomer was 100 pg at a signal-to-noise ratio of 3:1. The plasma-level profile of l-Ep and d-Ps in a guinea pig was investigated by the newly developed method. The determination of l-Ep and d-Ps in a volunteer's plasma after oral administration of Xiao Qing-long Heji [an oriental pharmaceutical preparation clinically provided now in China] was also performed.

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INTRODUCTION

High-performance liquid chromatography (HPLC) is presently being extensively utilized for the separation and determination of isomeric compounds in biological fluids. The application of HPLC for optical resolution of isomers has been developed in two ways: One is the use of a chiral stationary phase or a chiral mobile phase; the other is the use of a chiral reagent for derivatization followed by the separation of the diastereomers using conventional column and mobile phase. Irrespective of the inevitable disadvantages regarding simplicity , the derivatization method is more favorable for the determination of isomeric compounds in biological specimens with respect to both sensitivity and versatility. (l)-Ephedrine and (d)-pseudoephedrine are known to have the same chemical structure but with different pharmacological activities (1, 2). A reliable method for separation and determination of those stereoisomers in biological fluids is therefore required . This paper reported a highly resolving and sensitive methods for separation and determination of those stereoisomers in plasma by a reversed phase HPLC method through a newly developed precolumn dansyl derivatization reaction combined with the use of a Sep-pak C_{18} cartridge and an ion-exchange gel, CM-LH-20, for clean-up of the biological samples.

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MATERIALS

Optically pure (l)-ephedrine hydrochloride , (d)-pseudoephedrine hydrochloride, (l)-norephedrine hydrochloride and (d)-norpseudoephedrine hydrochloride were characterized and supplied by Professor Da-dun Cheng (Nanjing Pharmaceutical Institute ,China). Xiao Qing-long Heji (901101) was provided by Pharmaceutical Institute of Liaozhong (Liaoning , China). Dansyl chloride was purchased from Fluka co.,ltd (Switzerland) . The Sep-pak C_{18} cartridge (Millipore co., USA) was kindly donated by Professor Junichi Goto (Tohoku University, Japan) and washed successively with ethanol , 5% aqueous bovine serum albumin solution and then water prior to use. Carboxymethyl Sephadex LH-20 (CM-LH-20) (0.95 mequiv. /g) was prepared in our laboratory according to the known method. All other chemicals employed were of analytical reagent grade. Solvents were purified by distillation prior to use. All glassware used was silanized with trimethylchlorosilane.

METHODS

Instruments

The apparatus used for this work was a LC-10AD solvent delivery system (Shimadzu, Kyoto) equipped with a RF-535 fluorescence spectrophotometer (excitation wavelength 316 nm; emission wavelength 486 nm). A Shimpack CLC-ODS column (5 μm, 15 cm x 6.0 mm ID) and a Shimpack G-ODS Guard column (5 μm, 1 cm x 4.0 mm ID) (Shimadzu, Kyoto) were used at ambient temperature.

Procedure for separation and determination of (l)-ephedrine and (d)-pseudoephedrine in plasma

To a plasma specimen (100-500 μl) was added (l)-norephedrine (100 ng) as an internal standard (I.S.). The mixture was diluted with a 0.5 M phosphate buffer solution (pH 7.0, 2 ml) and then passed through a Sep-pak C_{18} cartridge. After successive washing with water (5 ml) and 20% ethanol (3 ml), (l)-ephedrine and (d)-pseudoephedrine were eluted with ethanol (8 ml). The eluate was evaporated down and redissolved in acetonitrile (100μl) and mixed with DNSCl (600 μg) in 0.03% triethylamine in acetonitrile (100 μl). The resulting solution was heated at 50 C° for 20 min and then evaporated down under N_2. The residue obtained was redissolved in 90% ethanol (1 ml) and applied to a column (18 mm x 6 mm ID) of CM-LH-20 (80 mg). Elution was carried out at a flow rate of 0.1 ml/min. After washing with 90% ethanol to remove neutral and acidic compounds, the diastereomers formed were eluted with 0.05 M methylamine in 90% ethanol (6 ml). The dried eluate was redissolved in the mobile phase (50-100 μl) and an aliquot of the solution was injected into the HPLC system.

Administration of (l)-ephedrine and (d)-pseudoephedrine to a guinea pig

(l)-Ephedrine hydrochloride (835 μg/kg) and (d)-pseudoephedrine hydrochloride (855 μg/kg) dissolved in saline was provided i.v. to a male guinea pig weighing 0.92 kg. The blood was withdrawn at 0.5, 1, 2, 4, 6 and 8 hr after injection and centrifuged at 1500 g for 20 min to separate the plasma.

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Administration of Xiao Qing-long Heji to a healthy volunteer

20 ml of Xiao Qing-long Heji (a traditional Chinese pharmaceutical preparation) was orally administered to a healthy male volunteer (0.16 ml/kg). The blood sample was withdrawn at 2 hr after administration and centrifuged at 1500 g for 20 min to separate the plasma.

RESULTS

Determination of (l)-ephedrine and (d)-pseudoephedrine in plasma

Following the established standard procedure, (l)-norephedrine was added to the plasma specimens as I.S. and the ephedrine stereoisomers were extracted with a Sep-pak C_{18} cartridge then treated with DNSCl in the presence of triethylamine in acetonitrile. After purified on a Sephadex LH-20 column, the diastereomers were subjected to HPLC on a Shimpack CLC-ODS column using a mixture of 0.6% phosphate buffer (pH 6.5)-methanol (3:8,v/v) as mobile phase, monitored by fluorescence detection (excitation wavelength 316 nm; emission wavelength 486 nm). A calibration graph was constructed by plotting the ratio of the peak area of (l)-ephedrine and (d)-pseudoephedrine to that of (l)-norephedrine against the amount of those stereoisomers, a linear response to each stereoisomer being 1-800 ng/ml. After providing i.v. (l)-ephedrine and (d)-pseudoephedrine to a guinea pig, the blood specimens were collected at 0.5, 1, 2, 4, 6 and 8 hr, the plasma level of l-Ep and d-Ps was investigated. It is evident from the data on Fig 1 that the pharmacokinetic patterns of (l)-ephedrine and (d)-pseudoephedrine in a guinea pig are closely similar, in spite of the metabolic rate of d-Ps being a little quicker than that of l-Ep. This method was further applied to the determination of (l)-ephedrine and (d)-pseudoephedrine in a volunteer's plasma obtained at 2 hr after oral administration of 20 ml of Xiao Qing-long Heji (a traditional Chinese pharmaceutical preparation). A clear chromatogram was obtained (Fig 2), following the standard procedure. The peaks of (l)-ephedrine and (d)-pseudoephedrine on the chromatogram represent 163 and 73 ng/ml, respectively.

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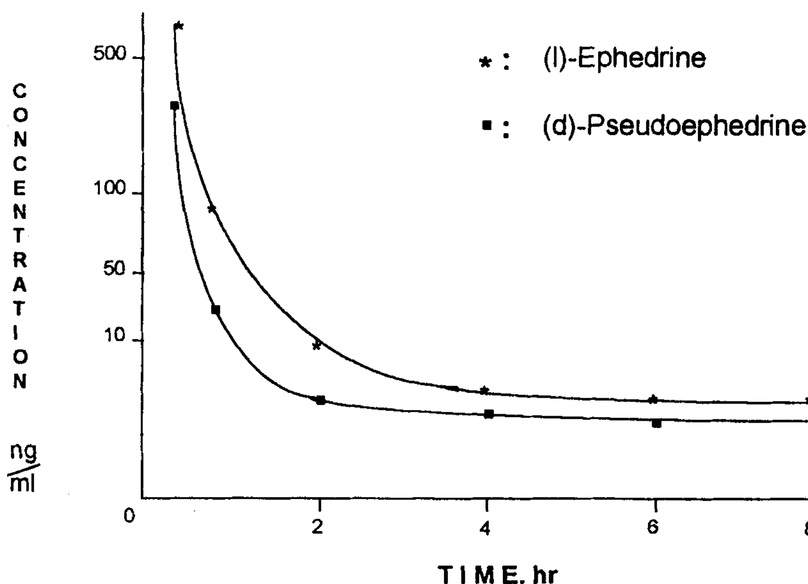

Figure 1. Plasma level of (l)-ephedrine and (d)-pseudoephedrine in a guinea pig after providing i.v. l-Ep and d-Ps at 835μg/kg and 855 μg/kg, individually.

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DISCUSSION

Derivatization of (l)-ephedrine, (d)-pseudoephedrine, (l)-norephedrine and (d)-norpseudoephedrine with DNSCl to form the diastereomers

A number of methods for analysis of ephedrine stereoisomers have been reported in recent years, including nuclear magnetic resonance (NMR)(3), high-performance liquid chromatography (HPLC)(4), enzyme immunoassay (EIA)(5) and other methods. Most of these methods, however, seem to be unsuitable for quantitative determination of ephedrine stereoisomers in plasma, owing to either poor resolution or low sensitivity. To provide a novel diastereomeric methods for separation and determination of ephedrine stereoisomers in plasma with satisfactory resolution and sensitivity was therefore attempted. An easily available reagent 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNSCl) was used as the derivatization

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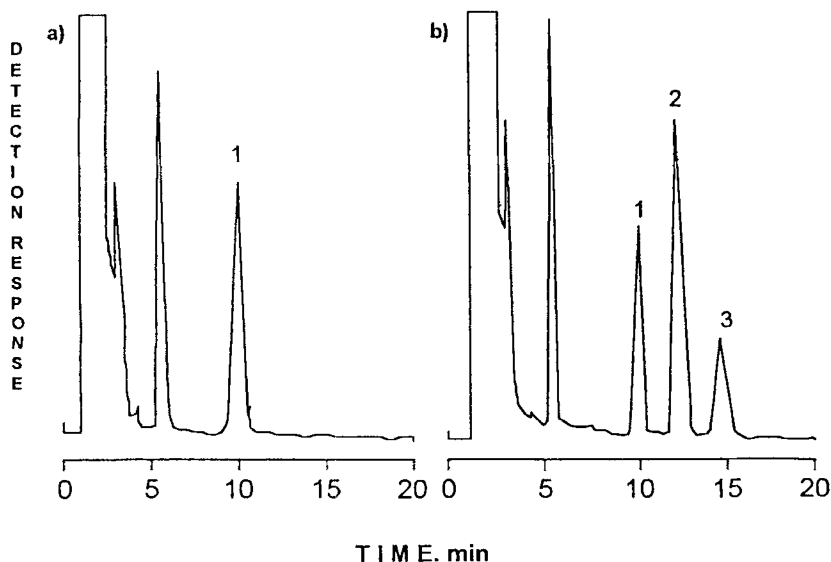
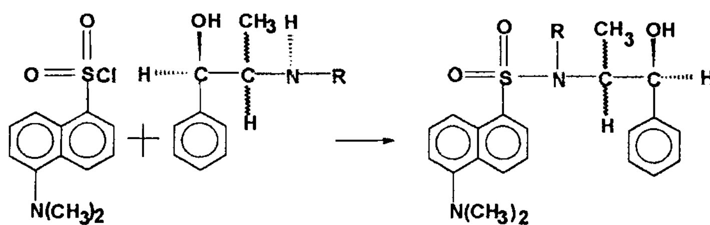


Figure 2. Chromatograms of (l)-ephedrine and (d)-pseudoephedrine in plasma Conditions: column, Shimpack CLC-OD column (5 μm, 15 cm x 6.0 mm i.d.) guarded with a Shimpack G-ODS column (5μm, 1cm x4.0 mm i.d.) mobile phase, 0.6% phosphate buffer (pH 6.5)-methanol / 3:8; flow rate,1.3ml/min; a): blank sample spiked with l-NE as I.S. b): plasma sample obtained from a volunteer 2 hr after oral administration of Xiao Qing-long Heji (0.16 ml/kg); peaks: 1=l-NE (I.S.), 2=l-Ep, 3=d-Ps .



R = CH_3 : (l) - Ephedrine (l - Ep) ; (d) - Pseudoephedrine (d - Ps)

= H : (l) - Norephedrine (l - NE); (d)-Norpseudoephedrine(d - NP)

Figure 3. Derivatization of ephedrine stereoisomers with 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNSCl).

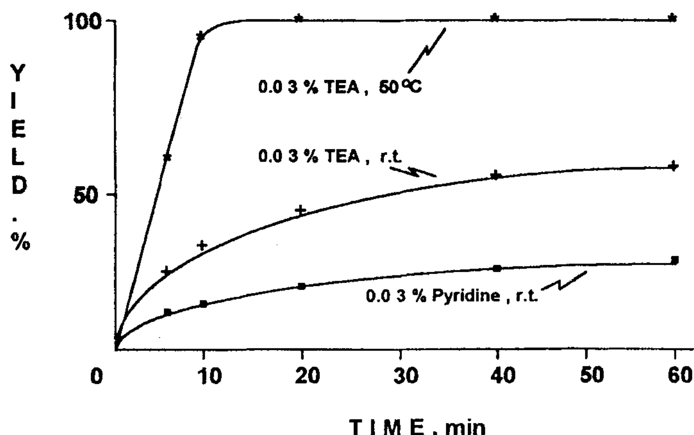


Figure 4. Time course for the derivatization of ephedrine stereoisomers with 5-dimethylaminonaphthalene-1-sulfonyl chloride(DNSCl)

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reagent to react with two pairs of ephedrine stereoisomers (Fig 3) and the suitable derivatization conditions were investigated. DNSCl reagent (600 μg) was added to a solution of ephedrine stereoisomers (each 300 ng) in various concentrations of triethylamine or pyridine, in acetonitrile (100 μl) at different temperatures. An aliquot of the solution was applied to HPLC and the yields of fluorescent products were estimated by comparing the peak areas with those of synthetic standard samples. As shown in Fig 4, 0.03% triethylamine in acetonitrile at 50 C° is the most desirable pattern, since the reaction rate increased along with the incubation time up to 15 min, resulting in a quantitative formation of diastereomeric compounds. Based on these results, two pairs of ephedrine stereoisomers (l-Ep/d-Ps and l-NE/d-NP) were treated with 5-dimethylaminonaphthalene-1-sulfonyl chloride in 0.03% triethylamine in acetonitrile at 50 °C for 20 min.

Separation of diastereomeric ephedrines

Various combinations of organic solvents were examined for a suitable mobile phase on a Shimpack CLC-ODS column. The 0.6 % phosphate buffer and

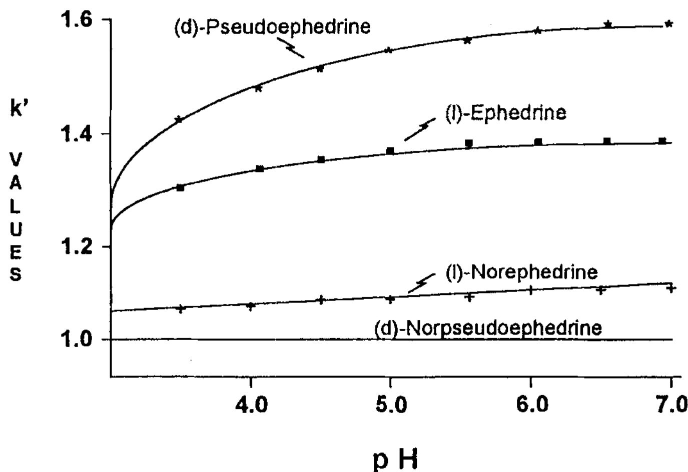

Figure 5. Effects of pH of mobile phase on k'values of (l)-ephedrine, (d)-pseudoephedrine and (l)-norephedrine relative to (d)-norpseudoephedrine.

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methanol system appeared to be promising , since no significant leading and / or tailing peak was observed on chromatograms. Accordingly, the effect of pH of the mobile phase on the capacity ratio (k') was investigated with the 0.6% phosphate buffer-methanol (3:8,v/v) system. As shown in Fig 5, the k' values were influenced by pH of the mobile phase. The k' values of 1-Ep, d-Ps and 1-NE relative to d-NP increased along with the increasing of pH up to 7.0, beyond which the k' values decreased. On the base of above results, pH 6.5 was chosen as a preferred condition for the HPLC. Chromatographic separation of diastereomeric ephedrines was monitored by fluorescence detection (excitation wavelength 316 nm; emission wavelength 486 nm) with satisfactory resolution (Tab 1), the limit of detection being 100 pg (at signal-to-noise ratio = 3). The interesting finding is that among the two pairs of stereoisomers, (1)-ephedrine was eluted earlier than (d)-pseudoephedrine , in contrast, (1)-norephedrine was eluted later than (d)-norpseudoephedrine (Fig 6), which is in good accordance with the elution order of the underivatized forms under the same chromatographic system. This suggests that there may exist some identical characteristics on configuration both for the earlier eluted

Tab.1 HPLC Separation of Diastereomers Derived from Ephedrine Stereoisomers with Dansyl Chloride

	d-NP		*l-NE*		*d-Ps*		*l-Ep*
K'	7.6		9.2		13.1		16.7
α		1.18				1.24	
R		*2.13*				*2.27*	

Conditions : column, a Shimpack CLS-ODS column and a Shimpack G-ODS Guard column ; mobile phase ,0.6% phosphate buffer (pH 6.5)-methanol (3:8); flow rate, 1.3 ml/ min

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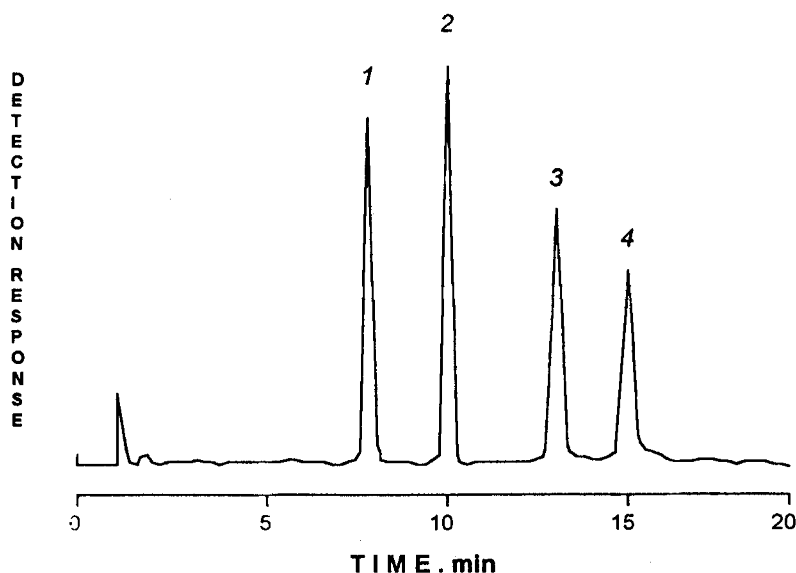

Figure 6. Chromatogram of diastereomeric ephedrines derived from ephedrine stereoisomers with DNSCl Conditions:column,Shimpack CLC-ODS column (5μm,15 cm x 6.0 mm i.d.)guarded with a Shimpack G-ODS column(5μm,1cm x 4.0mm i.d.);mobile phase,0.6% phosphate buffer (pH 6.5)-methanol/3:8; flow rate,1.3 ml/min peaks:1=d-NP, 2=l-NE, 3=l-Ep, 4=d-Ps.

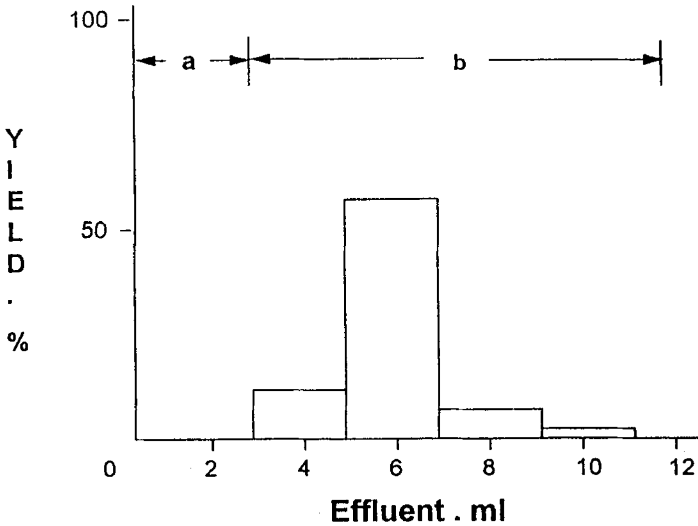

Figure 7. Elution pattern of ephedrine stereoisomers on a Sep-pak C_{18} cartridge Eluent: a) 20% ethanol, b) ethanol.

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Tab.2 Recovery of (l)-Ephedrine and (d)-Norpseudoephedrine Added to Human Plasma

Isomer	Amount (ng/ml) Added	Found	Recovery ± S.D.(%)*
(l)-Ephedrine	30.1	26.0	86.3 ± 9.1
	298.3	270.6	90.7 ± 6.2
(d)-Norpseudo-ephedrine	28.7	25.6	89.2 ± 8.4
	290.6	271.4	93.4 ± 5.9

* n=8

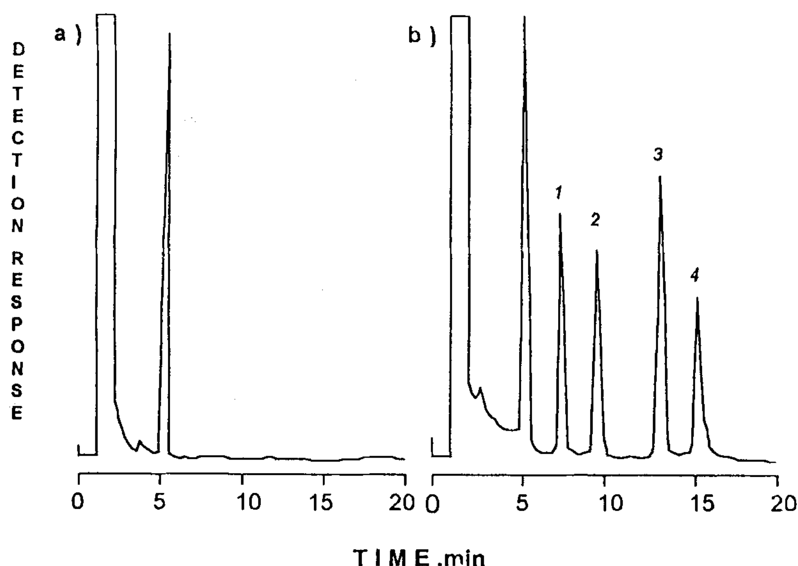

Figure 8. Separation of (l)-ephedrine/(d)-pseudoephedrine and (l)-norephedrne/(d)-norpseudoephedrine in plasma Conditions:column,Shimpack CLC-ODS column (5 μm, 15 cm x 6.0 mm i.d.) guarded with a Shimpack G-ODS column(5 μm,1cm x 4.0mm i.d.);mobile phase,0.6% phosphate buffer (pH 6.5)-methanol/3:8; flow rate,1.3 ml/min;a):blank b):plasma sample; peaks: 1=d-NP, 2=l-NE, 3=l-Ep, 4=d-Ps.

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out parts l-Ep and d-NP and/or for the later eluted out parts l-NE and d-Ps, which is hoped to be useful for further study on those stereoisomers.

Clean-up of (l)-ephedrine/(d)-pseudoephedrine and (l)-norephedrine/(d)-norpseudoephedrine in plasma

In previous study, a Sep-pak C_{18} cartridge was reported for extraction of enantiomeric propranolols, having the similar functional groups as ephedrine stereoisomers, in plasma successfully (6). In this paper, we tried to use this cartridge combined with the use of an ion-exchange gel, Carboxymethyl Sephadex LH-20 (CM-LH-20), for efficient clean-up of the ephedrine stereoisomers in plasma. Ephedrine stereoisomers

in phosphate buffer (0.5 M, pH 7.0) was applied to the cartridge. After washing with water and 20 % ethanol to remove co-existing inorganic salts and other polar substances, the elute obtained with ethanol was separated and analyzed by HPLC. As illustrated in Fig 7, the ephedrine stereoisomers were recovered at a rate of more than 90 % in an initial 8 ml of the effluent. After derivatization with DNSCl, the reaction products were dissolved in 90 % ethanol and applied to a column of CM-LH-20. The neutral and acidic interfering compounds were almost entirely removed by eluting with 90 % ethanol and the desired basic diastereomers were quantitatively recovered with 0.05 M methylamine in 90% ethanol (Tab 2). An excellent chromatogram for these stereoisomers was thus obtained following this procedure (Fig 8).

The newly developed diastereomeric HPLC method by using an easily available derivatization reagent DNSCl together with a novel clean-up procedure proved to be effective for the separation and determination of secondary, or primary, amide type of ephedrine stereoisomers in plasma. The separation and determination of ternary amide type of ephedrine stereoisomers like methylephedrine in plasma were undergoing in this laboratory and the results will be published elsewhere.

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