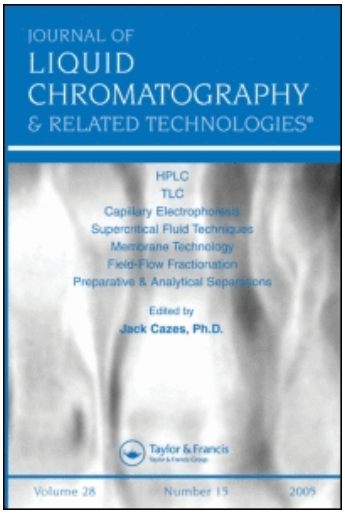

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SEPARATION AND DETERMINATION OF 2,5-DIMETHOXY-4-METHYLAMPHETAMINE ENANTIOMERS IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for quantitation of 2,5-dimethoxy-4-methylamphetamine (DOM) enantiomers in plasma by high-performance liquid chromatography (HPLC) is described. *d*- and *l*-DOM were readily converted to the amides by condensation with a newly developed chiral reagent, succinimidyl ester of *l*-α-methoxy-α-methyl-1-naphthaleneacetic acid. The yielded diastereomers were separated on the μPorasil column with cyclohexane/ethyl acetate (3:1) exhibiting satisfactory k' and R values. The clean-up procedure by use of Sep-pak C_{18} and carboxymethyl Sephadex LH-20 (CM-LH-20) proved to be effective for determination of the drug in biological fluids by HPLC. The plasma levels of *d*- and *l*-DOM after administration of the racemate to the rabbit were determined by the method thus established.

INTRODUCTION

Separation and determination of the enantiomeric drugs in biological fluids is prerequisite for pharmacokinetic studies of the racemate. Application of liquid chromatography to resolution of the enantiomers has been developed in two ways: direct separation of enantiomers on an optically active stationary phase (1) and derivatization with a chiral reagent followed by

chromatography of the diasteromers on a conventional column. The previous papers of this series described synthesis of new chiral derivatization reagents for use in optical resolution of amino acids by HPLC (2,3). Among these, α-methoxy-α-methyl-1-naphthaleneacetic acid appears to be most promising, since optical resolution of the reagent itself is efficiently attained and the resulting diasteromers are readily resolved on a normal phase column. The present paper deals with preparation of an activated chiral reagent and its use for separation and determination of enantiomers of DOM (I), one of hallucinogens in rabbit plasma, by HPLC.

MATERIALS AND METHODS

Reagents

DOM was obtained by the method of Matin *et al.* (4). All the reagents employed were of analytical grade. Solvents were purified by distillation prior to use. Sep-pak C_{18} cartridge supplied by Waters Assoc. (Milford Mass.) was washed thoroughly with ethanol and water before use. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals (Uppsala). CM-LH-20 (1 meq/g) was prepared accoding to the procedure of Setchell *et al.* (5). N-Ethoxycarbonylmethyl-4-nitrophthalimide, an internal standard (IS), was prepared in these laboratories.

Succinimidyl Ester of *l*-α-Methoxy-α-methyl-1-naphthaleneacetic Acid (II)

N-Hydroxysuccinimide (560 mg) in dioxane (16 ml) and dicyclohexylcarbodiimide (1.36 g) were added to a solution of *l*-α-methoxy-α-methyl-1-naphthaleneacetic acid (800 mg) in ethyl acetate (16 ml), and the resulting solution was stirred at room temperature overnight. After removal of the precipitate by filtration, the filtrate was evaporated *in vacuo*. The oily residue was subjected to column chromatography on silica gel (60 g). Elution with cyclohexane/ethyl acetate (3:1) and recrystallization of the eluate

from ether gave II (500 mg) as colorless needles. mp 115-116°. $[\alpha]_D^{15}$+ 85.0° (c=0.1, $CHCl_3$). Anal. Calcd. for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.24; N, 4.28. Found: C, 66.18; H, 5.54; N, 4.28. NMR ($CDCl_3$)δ: 2.22 (3H, s, $-CH_3$), 2.77 (4H, s, $-COCH_2-$), 3.26 (3H, s, $-OCH_3$), 7.36-8.48 (7H, m, Ar-H). The optical purity of this reagent was estimated to be over 99.5% by the method described in the previous paper (2).

Instrumentation

The instruments used were Waters Model 6000A solvent delivery system and Model 440 absorbance detector, monitoring at 280 nm, equipped with a Chromatopac-EIA integrator (Shimadzu Co., Kyoto). The test samples were applied to the chromatograph by a Waters Model U6K sample loop injector with an effective volume of 2 ml. The Waters μPorasil column (1 ft. x 1/4 in. I.D.) was used under ambient conditions.

Sample Preparation

A plasma sample (0.5-2 ml) was diluted with 4-fold volume of water, adjusted to pH 4 with 1% HCl, neutralized with 2% Na_2CO_3 and passed through Sep-pak C_{18} cartridge. After successive washing with water (3 ml) and 10% ethanol (2 ml), the drug was eluted with 1% methylamine/80% ethanol (4 ml). Upon evaporation of the solvent, the residue was redissolved in 90% ethanol (0.5 ml) and applied to a column (10 mm x 6 mm I.D.) packed with CM-LH-20 (100 mg). The neutral and acidic materials were removed by elution with 90% ethanol (3 ml), and the desired fraction was collected by elution with 6% methylamine/90% ethanol (3 ml). After evaporation of the solvent with an aid of nitrogen gas stream, the residue was heated with II (200 μg) in pyridine (200 μl) at 60°C for 2 hr. To the reaction mixture was added IS (1 μg) in n-hexane/ethyl acetate (3:1) (1 ml), and the solution was washed with 5% HCl and water. The organic phase was evaporated

under a nitrogen gas stream, and the residue was redissolved in cyclohexane/ethyl acetate (3:1) (150 μl) whose 50-70 μl aliquot was applied to HPLC.

Quantitation

The diastereomeric amides (III) formed from DOM by condensation with II exhibited an absorption maximum at 281 nm with shoulders at 250, 270, and 290 nm in ethyl acetate. Absorbance at 280 nm was used for monitoring. The calibration curve was constructed by plotting the ratio of peak area of *d*- or *l*-DOM to that of IS against the amount of each enantiomer, and good linearity was observed in the range of 25-400 ng/tube, respectively.

Recovery Test for DOM added to Plasma

The test samples were prepared by dissolving 25, 100, or 200 ng each of *d*- and *l*-DOM in human blood plasma (1 ml). The clean-up by use of Sep-pak C_{18} cartridge and CM-LH-20 followed by determination of each enantiomer by HPLC was carried out according to the procedure described above.

Administration of DOM

dl-DOM in saline (0.5 ml) was injected subcutaneously to a male albino rabbit weighing 3.2 kg at a dose of 3 mg/kg. The blood was withdrawn from femoral artery without anesthesia at 10, 20, 30 min, 1, 2, 3, 4, 5 and 6 hr after administration and centrifuged for 10 min at 3,000 rev./min to separate plasma.

RESULTS AND DISCUSSION

Our initial effort was directed to HPLC separation of the diastereomeric amides formed from DOM enantiomers with the chiral reagent. First, suitable conditions for quantitative formation of the covalent bond with the amino group of DOM by use of a derivatization reagent were investigated. There have been several methods

available for the formation of a peptide bond. Among these the acyl imidazolide is most widely used for derivatization of the amine in gas chromatography and HPLC. However, this type of reagent is not so stable and its purification is usually somewhat tedious. The use of the succinimidyl ester, that is, an activated ester, was attempted for converting DOM into the amide (Figure 1). The reaction of l-α-methoxy-α-methyl-1-naphthaleneacetic acid with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide afforded the succinimidyl ester in a fairly good yield. Racemization of the reagent itself did not occur to any extent during the condensation reaction and subsequent processing. When DOM was heated with the activated reagent in pyridine at 60°C, the yielded amount of the amide increased with the prolonged reaction time and arrived at the plateau at 1.5 hr as illustrated in Figure 2.

It has previously been demonstrated that the diastereomeric amides are efficiently resolved on a normal phase column rather than on a reversed phase one (2,3). Interaction of the amide linkage with the silanol group of silica appears to be effective

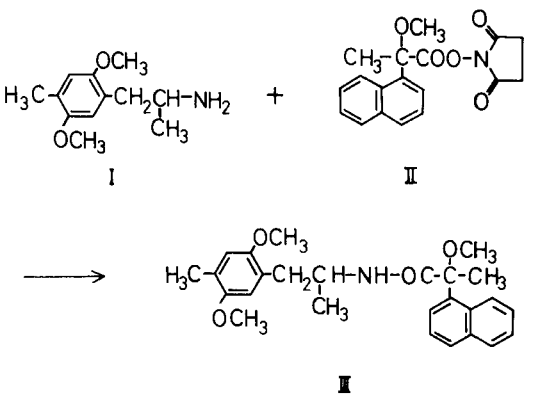

FIGURE 1 Formation of Diastereomeric Amides from DOM with Activated Chiral Reagent

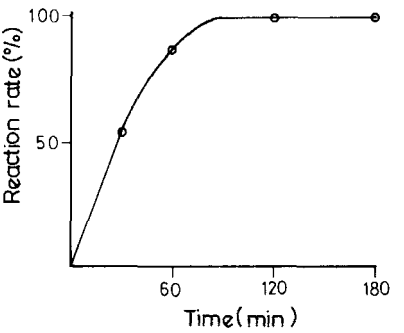

FIGURE 2 Time Course for Derivatization of DOM with Activated Chiral Reagent

for separation of the diastereomers. On the basis of these findings a normal phase column, μPorasil, was chosen in the present study.

The suitable solvent system in respects of capacity ratio (k'), separation factor (α) and resolution factor (R) was investigated by combination of ethyl acetate or tetrahydrofuran with n-hexane or cyclohexane (Figure 3). The eluents other than cyclohexane/tetrahydrofuran showed the appropriate k' value. Satisfactory resolution was obtained with cyclohexane/ethyl acetate and n-hexane/tetrahydrofuran. Of these two, the latter was not suitable for the plasma specimens because the overlapping peaks due to endogenous materials disturbed the chromatogram. These experiments led to the conclusion that, among the four solvent systems, cyclohexane/ethyl acetate (3:1) is most satisfactory to provide appropriate k' values for *d*-DOM (1.86), *l*-DOM (2.51), and IS (3.37) as well as excellent R value for the enantiomers.

Elution of the diastereomeric amides in HPLC was checked by monitoring the absorbance at 280 nm where the detection limit was 5 ng. The calibration curve was constructed by plotting the ratio of peak area of *d*- or *l*-DOM to that of IS against the amount of enantiomeric DOM. The ultraviolet detection showed a

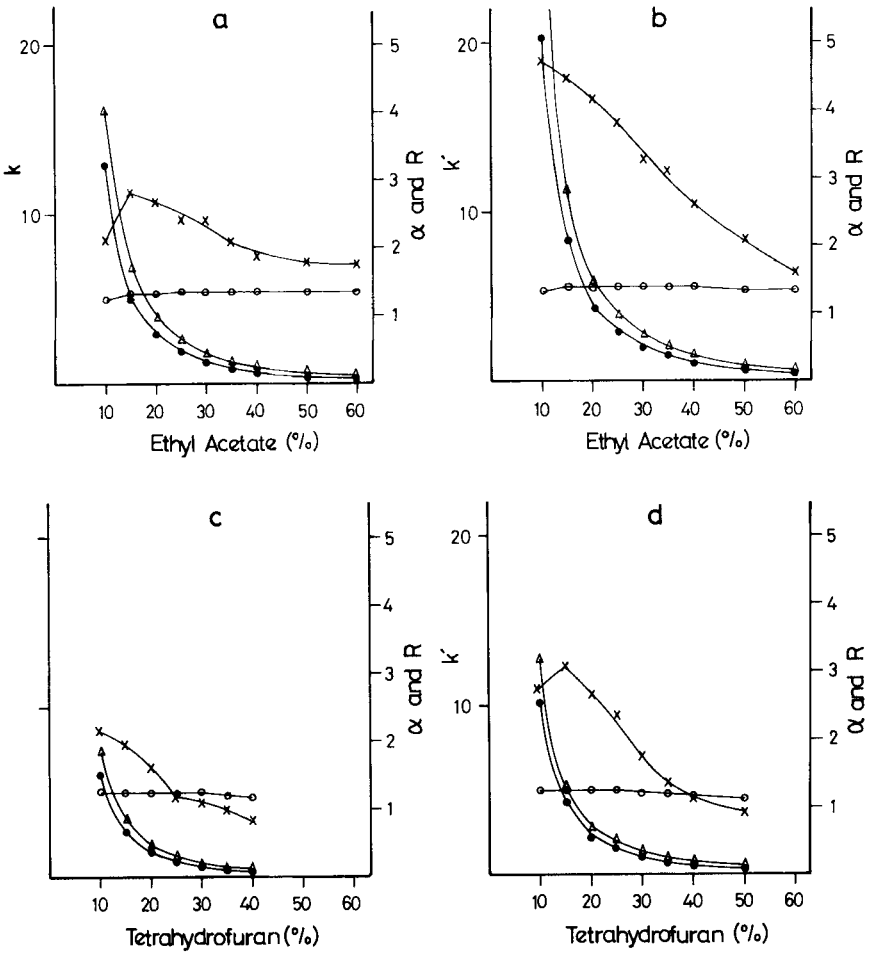

FIGURE 3 Effects of Solvent Systems on k'(*d*:-●-●-, *l*:-Δ-Δ-), α (-o-o-) and R (-x-x-) Values of Diastereomeric Amides formed from DOM Enantiomers
a) cyclohexane/ethyl acetate; b) n-hexane/ethyl acetate; c) cyclohexane/tetrahydrofuran; d) n-hexane/tetrahydrofuran

linear response to each enantiomer in the range of 25-400 ng as shown in Figure 4.

The recovery of a drug in biological fluids is significantly influenced by the clean-up method employed. Treatment of blood

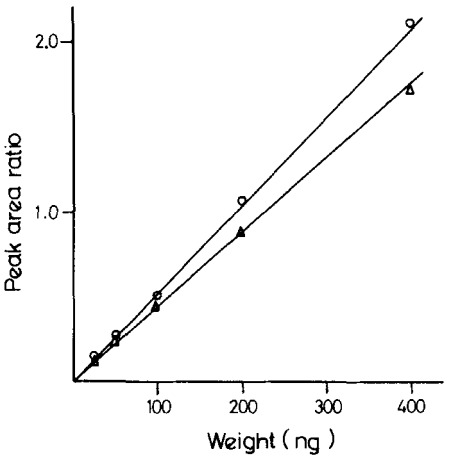

FIGURE 4 Calibration Curves for *d*-DOM (-o-o-) and *l*-DOM (-Δ-Δ-)

plasma with alcohol or acid is commonly used for deprotenization prior to HPLC. Extraction with the organic solvent as well as chromatography on Amberlite XAD-2 resin are also effective for this purpose. These procedures, however, were not applicable owing to the insufficient recovery rate and occurrence of the interfering peaks on the chromatogram. Therefore, the use of Sep-pak C_{18} cartridge was attempted for purification of DOM in plasma. The drug was readily adsorbed on Sep-pak C_{18} and eluted almost quantitatively with 1% methylamine/80% ethanol. Preceding washing with 10% ethanol was effective for removal of the polar substances in plasma. Elimination of the neutral and acidic materials in biological fluids was then undertaken employing lipophilic cation-exchanger, CM-LH-20. The interfering substances were almost entirely removed by eluting with 90% ethanol and the desired DOM was quantitatively recovered with 6% methylamine/90% ethanol. The efficient clean-up procedure for HPLC of DOM in plasma was thus established and an excellent chromatogram of the enantiomers without any interfering peak was obtained.

Applicability of the present method for separatory determination of *d*- and *l*-DOM in plasma was then examined. A known amount of the racemate was added to plasma and the recovery rates were determined. As listed in Table 1, *d*- and *l*-DOM were recovered at a rate of 93-100% with a standard deviation of 2.1-5.7%. It is evident from these data that the proposed method is satisfactory in accuracy and precision.

Simultaneous determination of plasma levels of the enantiomers was carried out according to the procedure thus established. Blood specimen was collected at 10, 20, 30 min, 1, 2, 3, 4, 5 and 6 hr after administration of *dl*-DOM to the rabbit. As illustrated in Figure 5 there was seen a little difference in the plasma level between *d*- and *l*-DOM. A typical chromatogram of the enantiomers in plasma obtained at 30 min is shown in Figure 6.

McGraw *et al.*(6) demonstrated, in the incubation study with rabbit liver microsomes, that the half-life of *l*-DOM was somewhat longer than that of the *d*-enantiomer. In addition, Matin *et al.* (4) reported that the excreted amount of *l*-DOM in urine was much

TABLE 1

Recovery Test for DOM added to Plasma

	Added (ng/ml)	Found (ng/ml)	Recovery ± S.D.* (%)
d-DOM	25.0	23.5	93.8 ± 3.5
	100.0	100.4	100.4 ± 3.7
	200.0	198.0	99.0 ± 2.1
l-DOM	25.0	23.4	93.6 ± 3.5
	100.0	100.5	100.5 ± 5.7
	200.0	191.0	95.3 ± 3.5

* n=6

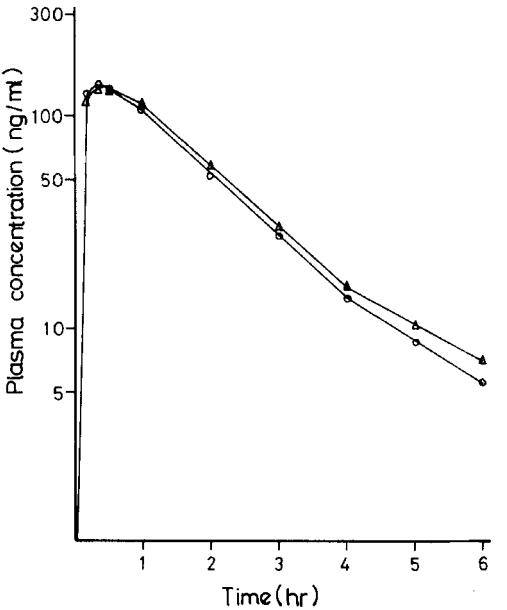

FIGURE 5 Plasma Levels of *d*-DOM (-o-o-) and *l*-DOM (-Δ-Δ-) after Administration of the Racemate to the Rabbit

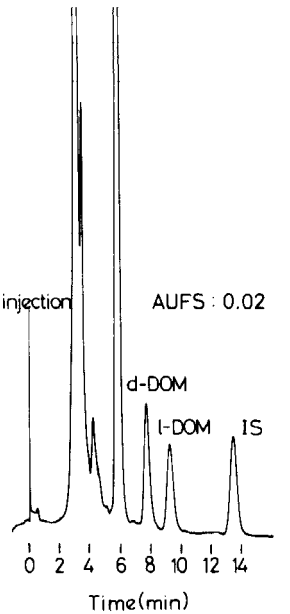

FIGURE 6 High-Performance Liquid Chromatogram of DOM Enantiomers in Rabbit Plasma

larger than that of the *d*-enantiomer when the racemate was administered to the rabbit. In both experiments, quantitation of the enantiomers was carried out by means of gas chromatography and the plasma level of the drug was not determined. The present study revealed that the half-life of *l*-DOM is somewhat longer than that of the *d*-enantiomer in the rabbit. It is to be noted that disappearance of the biologically more active *l*-enantiomer in blood is delayed after the *d*-enantiomer.

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