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DETERMINATION OF 3,4-METHYLENEDIOXYAMPHETAMINE AND 3,4-METHYLENEDIOXYMETHAMPHETAMINE ENANTIOMERS IN WHOLE BLOOD

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SUMMARY

A method for the determination of the enantiomeric content of 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) in microsamples (200 μl) of whole blood is described. The method involves liquid–liquid extraction of MDA and MDMA from blood and derivatization with the chiral reagent N-trifluoroacetyl-L-prolyl chloride. Separation, identification and quantitation of diastereomeric derivatives is by gas chromatography–mass spectrometry. The analytical range of the assay is from 0.12 ng to 48 ng injected on-column. Details for the synthesis of the enantiomers of MDMA are also provided.

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INTRODUCTION

1-(3,4-Methylenedioxyphenyl)-2-aminopropane (3,4-methylenedioxyamphetamine; MDA) and its N-methyl derivative (3,4-methylenedioxymethamphetamine; MDMA) are ring-substituted amphetamines which have become popular drugs of abuse due to the euphoric effects they produce. Recently, studies have focused on determining the pharmacological and toxicological effects of these drugs. While MDA may be abused alone, it has also been identified as a major metabolite of MDMA in rats and man [1,2]. Schmidt [3] has shown that the enantiomers of MDMA have different toxicities. The *S*(+) isomer causes a long-term serotonin neurotoxicity in rats whereas the *R*(−) isomer of MDMA does not. Others have shown that the enantiomers of MDA and MDMA may have different behavioral effects [4]. For these reasons, we developed a method for separating, identifying and quantitating the enantiomers of MDA and MDMA in microquantities of blood.

The optically active derivatizing reagent N-trifluoroacetyl-L-prolyl chloride (LTPC) forms diastereomers on-column, allowing for separation, identification and quantitation of MDA and MDMA enantiomers. LTPC has been used previously to separate enantiomers of methylphenidate, propranolol, amphetamine and methamphetamine [5–8]. This paper describes the use of LTPC for the quantitative analysis of MDA and MDMA and presents data showing that racemization and kinetic resolution do not occur. Data are also presented showing that an enantiomeric excess, a condition where one optical isomer is present in excess of its enantiomer, does not induce any changes in the amount of derivative formed or in the chromatography of the products.

EXPERIMENTAL

Chemicals

The optical isomers of MDA were prepared as reported by Anderson et al. [9] and were used as starting materials for the synthesis of *S*(+)- and *R*(−)-MDMA.

A solution of *S*(+)-1-(3,4-methylenedioxyphenyl)-2-aminopropane hydrochloride (3.3 g) in water (50 ml) was alkalinized by the addition of solid sodium hydroxide to pH 10. Extraction with diethyl ether (3×50 ml), drying of the combined ether portions with magnesium sulfate and removal of the solvent under reduced pressure afforded 2.5 g of the free base as a pale yellow oil. Ethyl chloroformate (1.7 g, 15 mmol) in dry tetrahydrofuran (20 ml) was added in a dropwise manner to a stirred solution of the free base (2.5 g, 14 mmol) and triethylamine (1.5 g, 15 mmol) in tetrahydrofuran at 0°C. The reaction mixture was allowed to warm to room temperature and stirring was continued for an additional 16 h. The mixture was filtered, the solid material was washed with tetrahydrofuran (3×25 ml) and the combined organic por-

tions were evaporated under vacuum to afford an oil. The oil was dissolved in diethyl ether (100 ml) and the ether solution was washed successively with 10% hydrochloric acid (2×30 ml) and water (2×30 ml). The ether solution was dried with magnesium sulfate and the solvent was evaporated under reduced pressure to give 3.3 g (94% yield) of the carbamate as a pale yellow oil that was homogeneous by thin-layer chromatography. A solution of the carbamate (3.3 g) in anhydrous diethyl ether (100 ml) was added dropwise at 0°C to a stirred suspension of lithium aluminum hydride (1 g, 26 mmol) in anhydrous diethyl ether (30 ml). When the addition was complete, the reaction mixture was heated at reflux for 20 h, cooled to 0°C and excess hydride was decomposed by the careful addition of water. The resulting suspension was filtered and the filtrate was dried with magnesium sulfate and saturated with dry hydrogen chloride gas until precipitate formation ceased. Recrystallization from isopropanol afforded 2.0 g (66% yield) of *S*(+)-MDMA·HCl: m.p. 184–185°C; optical rotation (10% aqueous solution at 24°C): +13.8°.

Using *R*(−)-1-(3,4-methylenedioxyphenyl)-2-aminopropane hydrochloride as starting material, the *R*(−) isomer of MDMA·HCl was prepared in the exact same manner as described above for *S*(+)-MDMA. The reaction yields for the two steps were 96 and 74%, respectively. The crude *R*(−)-MDMA·HCl was recrystallized to constant melting point from isopropanol: m.p. 183–184°C; optical rotation: −13.5°. The melting points of *S*(+)- and *R*(−)-MDMA·HCl are comparable to those reported by Anderson et al. [9]. Racemic MDMA·HCl (m.p. 151–152°C) was prepared in a similar fashion from racemic MDA·HCl and was identical in all respects with an authentic sample obtained from the National Institute on Drug Abuse.

LTPC, 0.1 *M* in chloroform (Lot No. P82-223-3, stated by manufacturer to contain 1.2% of its enantiomer N-trifluoroacetyl-D-prolyl chloride, DTPC) was obtained from Regis (Chicago, IL, U.S.A.). *n*-Hexane, 1-butanol, ethyl acetate and 1% ethanol in chloroform were HPLC grade. (+/−)-Methoxyphenamine·HCl (Lot No. 122F-0815) was purchased from Sigma.

Chromatographic system

Diastereomers were separated on a Hewlett Packard methylsilicone column (12.5 m×0.2 mm I.D., 0.33 μm film thickness). On-column derivatization was accomplished by filling a 10-μl syringe with 3 μl of the extract (chloroform layer), 0.2 μl of air and 1 μl of 0.1 *M* LTPC in chloroform and rapidly injecting into the gas chromatograph. To allow the derivatization reaction to take place the gas chromatograph was equipped with a split liner packed with 5 mm of OV-101 on 80–100 mesh Supelcoport held in place with silanized glass wool. The gas chromatograph was operated in the split mode with a split ratio of 15. Initial oven temperature was 220°C, initial time 2 min, program rate 10°C/min and final temperature 280°C. The injector port temperature was 275°C.

The mass spectra shown in Figs. 2–4 were obtained after autotuning the

mass spectrometer with perfluorotributylamine. For quantitation of MDA and MDMA the mass spectrometer was operated in the selected-ion mode. To increase sensitivity for quantitative analysis, the mass spectrometer was tuned manually optimizing ion abundances of the *m/z* 69 and 219 ions of perfluorotributylamine.

The first peak of the pair of methoxyphenamine-LTPC diastereomers with *m/z* 148 was used as the internal standard for quantitation. MDA-LTPC and MDMA-LTPC diastereomers were quantitated using the *m/z* 162 ion.

Extraction

A 400-ng amount of methoxyphenamine (20 μl of 20 ng/μl in water), the internal standard, was added to 200 μl of whole blood. Samples were made alkaline with 500 μl of 1 *M* sodium hydroxide; 1 ml of extraction solvent (ethyl acetate–*n*-hexane–1-butanol, 10.10:1) was added and the samples were vortexed rapidly for 20 s and centrifuged for 5 min at 1100 *g*. The organic phase was pipetted into microcentrifuge tubes and 250 μl of 0.5 *M* hydrochloric acid were added. Samples were vortexed for 15 s and centrifuged 5 min. The organic layer was pipetted and discarded; 500 μl of 1.0 *M* sodium hydroxide were added to the acid phase and the aqueous phase was extracted with 25 μl of chloroform containing 1% isopropanol. Samples were vortexed for 15 s and centrifuged for 5 min. A 3-μl sample of the chloroform layer was injected as described above.

Standards

Whole blood was spiked with racemic MDA·HCl and MDMA·HCl to make concentrations ranging from 4000 to 10 ng/ml as the free base. Therefore the range tested for each enantiomer of MDMA and MDA is 2000–5 ng/ml of whole blood. A separate standard curve was generated for each enantiomer analyzed.

Samples used to show that an enantiomeric excess was not inducing a change in the derivatizing reaction, or subsequent chromatography, were made from optically pure standards in methanol. Standards with a ratio of 50:50 were made by adding equal amounts of *S*(+) and *R*(−) enantiomers to obtain a racemic mixture; 75:25 standards were made by adding 75% of one enantiomer to 25% of the other enantiomer. Optically pure standards were made by dissolving optically pure standards in methanol.

RESULTS AND DISCUSSION

Chromatography of methoxyphenamine-LTPC, MDA-LTPC and MDMA-LTPC diastereometers is shown in Fig. 1. Under the conditions employed, the internal standard diastereomers eluted with retention times of 3.34 and 3.45 min. MDA-LTPC diastereomers eluted with retention times of 3.58 and 3.77 min. Resolution of the two MDA peaks was 2.5. MDMA-LTPC eluted with

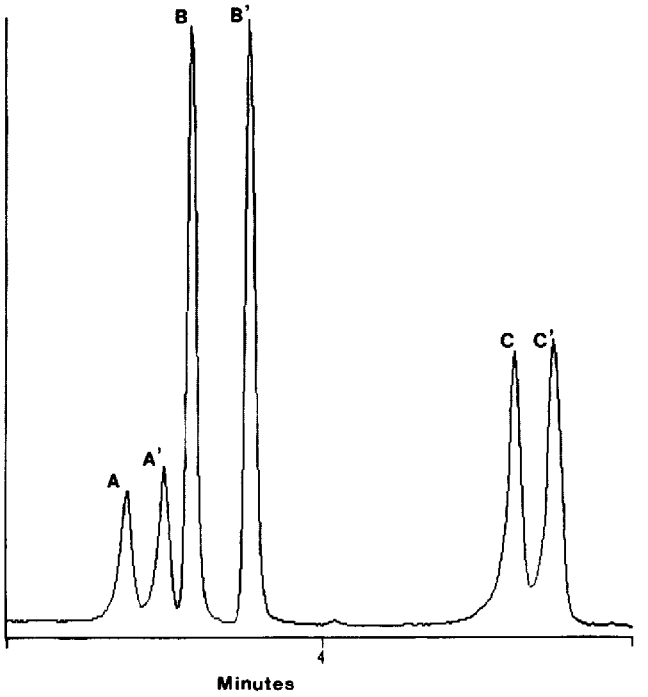


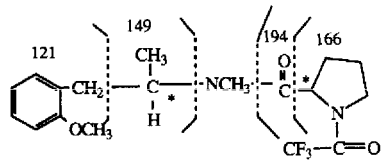
Fig. 1. Chromatogram of the LTPC diastereomers of methoxyphenamine, MDA and MDMA. The methoxyphenamine-LTPC diastereomers (A and A') elute first with retention times of 3.34 and 3.45 min. $R(-)$-MDA-LTPC (B) elutes at 3.58 min and its diastereomer $S(+)$-MDA-LTPC (B') at 3 77 min. $R(-)$-MDMA-LTPC (C) elutes at 4.62 min; $S(+)$-MDMA-LTPC (C') elutes at 4.73 min.

retention times of 4.62 and 4.73 min. Resolution of the two MDMA peaks was 1.0. Resolution was calculated in the standard manner [10]. The $R(-)$ diastereomers of MDA-LTPC and MDMA-LTPC eluted before the $S(+)$ diastereomers.

MDA-LTPC and MDMA-LTPC were identified using the 162 and 166 m/z ions. Methoxyphenamine was identified using the 148 and 166 m/z ions. Figs. 2, 3 and 4 show the mass spectra of methoxyphenamine-LTPC, MDA-LTPC and MDMA-LTPC. The fragmentation pattern of MDA-LTPC is consistent with that reported by Lui et al. [11]. Both isomers of MDA, when derivatized with LTPC, had the same major ion fragments. Similarly, both isomers of MDMA, when derivatized with LTPC, had the same major ion fragments.

The extraction efficiency for MDA and MDMA from whole blood is shown in Table I. Extraction efficiency was calculated by comparing peak areas of extracted blood samples relative to unextracted standards in methanol. The extraction efficiency of the internal standard was $52 \pm 11\%$ (mean $\pm$S.D.,

Methoxyphenamine-LTPC



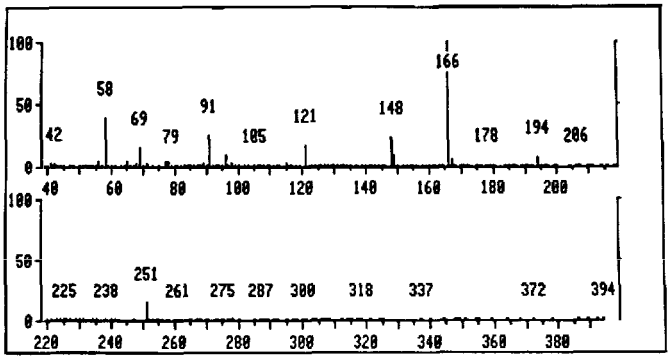
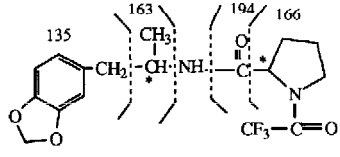


Fig. 2. Mass spectra of methoxyphenamine-LTPC. Dashed lines indicate sites of ion fragmentation. Numbers indicate *m/z* of fragments before rearrangements.

MDA-LTPC



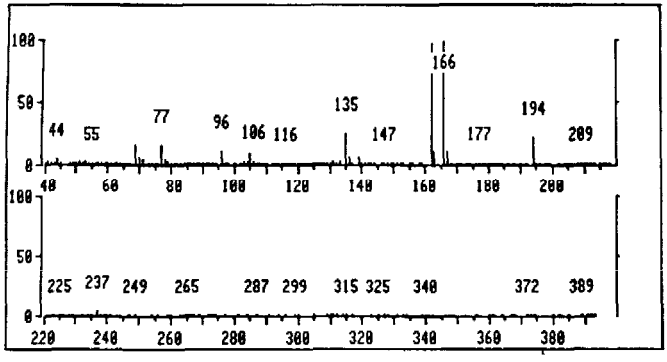
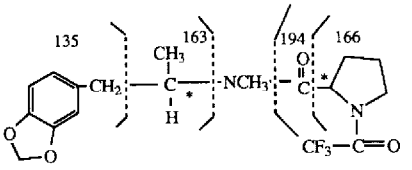


Fig. 3. Mass spectra of MDA-LTPC. Dashed lines indicate sites of ion fragmentation. Numbers indicate *m/z* of fragments before rearrangements.

MDMA-LTPC



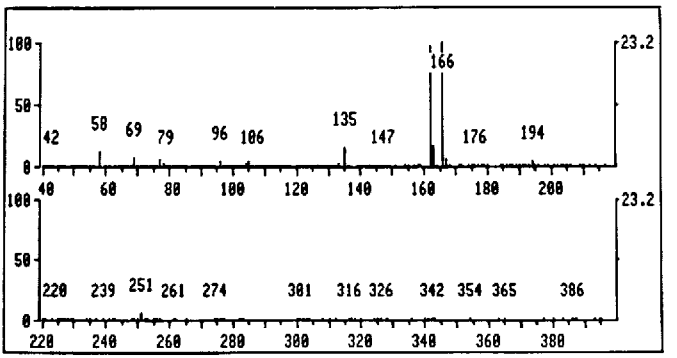


Fig. 4 Mass spectra of MDMA-LTPC. Dashed lines indicate sites of ion fragmentation. Numbers indicate *m/z* of fragments before rearrangements.

TABLE I

EXTRACTION EFFICIENCY OF THE ENANTIOMERS OF MDA AND MDMA FROM WHOLE BLOOD AT DIFFERENT CONCENTRATIONS

The extraction efficiency of the internal standard was 52 ± 11% (mean ± S.D., $n = 15$). Extraction efficiency was determined as described in the text.

Concentration (ng/ml)	Extraction efficiency (mean ± S.D.) (%)			
	R(−)-MDA	*S*(+)-MDA	*R*(−)-MDMA	*S*(+)-MDMA
50	107 ± 10	106 ± 16	93 ± 4	106 ± 13
100	90 ± 13	88 ± 8	95 ± 17	111 ± 13
400	70 ± 9	70 ± 10	63 ± 12	79 ± 14
750	71 ± 16	77 ± 15	63 ± 15	81 ± 16
2000	62 ± 11	63 ± 11	59 ± 12	68 ± 13

$n = 15$). Each entry in Table I reflects an average (± S.D.) of three determinations.

The accuracy of the method is shown in Table II. Accuracy was determined by spiking blood samples with known concentrations of MDA and MDMA, analyzing the samples and calculating the amounts of MDA and MDMA pres-

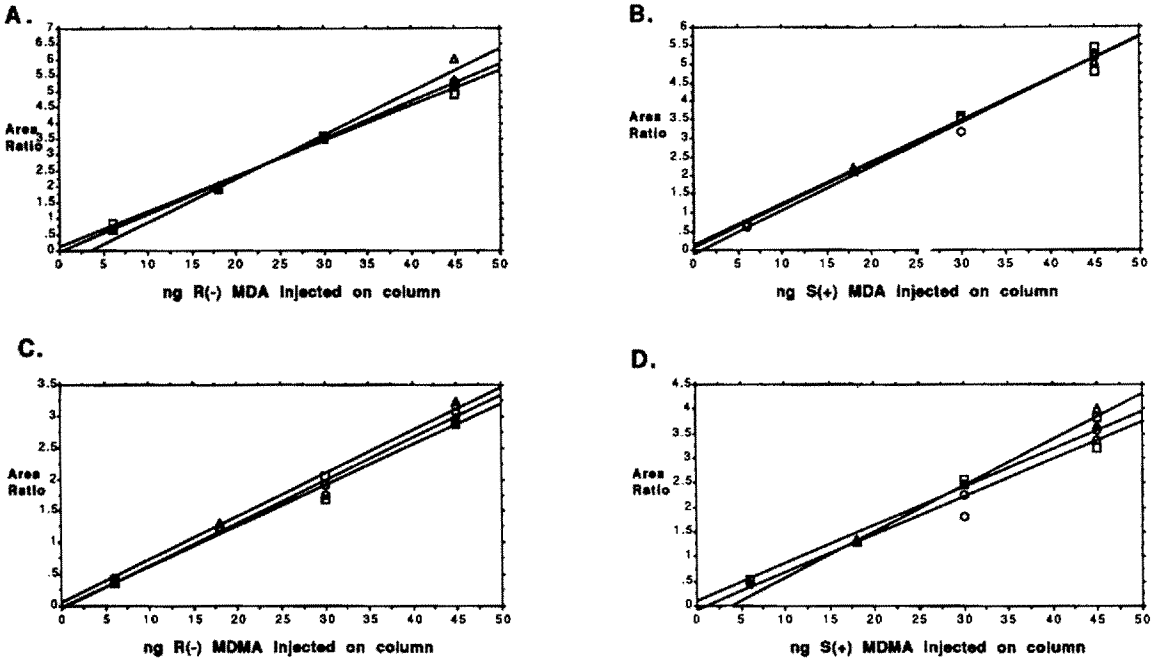


Fig. 5. Graphs showing that enantiomeric excess is not inducing changes in the derivatization reaction or chromatography. Squares represent data from racemic standards. Triangles represent data from optically pure standards. Circles represent data from 75:25 mixtures of enantiomers. (A) $R(-)$-MDA; (B) $S(+)$-MDA; (C) $R(-)$-MDMA; (D) $S(+)$-MDMA. All plots are graphed as area of compounds relative to internal standard area versus amount (ng) of compound injected.

TABLE II

ABSOLUTE ERROR IN QUANTITATING THE ENANTIOMERS OF MDA AND MDMA IN WHOLE BLOOD

Concentrtion (ng/ml)	Amount injected on-column (ng)	Absolute error (mean ±S.D., $n=3$) (%)			
		$R(-)$-MDA	$S(+)$-MDA	$R(-)$-MDMA	$R(+)$-MDMA
5	0 12	9±7	4±1	2±12	1±9
10	0.24	5±3	1±5	2± 3	3±8
50	1.2	11±2	9±4	15± 6	11±3
100	2.4	5±1	6±3	6± 5	3±3
400	9.6	9±4	8±5	8± 3	5±2
750	18	5±3	4±3	7± 5	6±4
2000	48	8±3	7±3	1± 2	6±3

ent. Accuracy was defined as (true value−experimental value)/true value×100%. Table II reflects the average (±S.D.) of the absolute value of accuracy for three determinations.

Optical purity of the derivatizing reagent was checked by chromatographing optically pure standards of $S(+)$-MDA and calculating the area of the minor peak, due to $S(+)$-MDA-DTPC, relative to the major peak, $S(+)$-MDA-LTPC. The optical purity of LTPC was found to be 96.1%. For samples that do not contain a 50:50 ratio of enantiomers it is necessary to correct for the DTPC according to the following equations [5]:

$$X = X_{\text{chrom}} + AX - AY$$

$$Y = Y_{\text{chrom}} + AY - AX$$

where X and Y represent concentrations of $S(+)$ and $R(-)$ enantiomers, respectively, that would be obtained if no DTPC were present in the LTPC. X_{chrom} and Y_{chrom} are the concentrations calculated from peak areas in the chromatograms of the $S(+)$ and $R(-)$ enantiomers, respectively, when working with LTPC contaminated with DTPC. A is the percentage of DTPC in the reagent.

In order to demonstrate that kinetic resolution was not occurring during the flash derivatization, racemic MDA and MDMA were derivatized and chromatographed, and the resultant areas (combination of m/z 142, 162 and 166 ions) of peaks from racemic standards were compared. The area of the $R(-)$ and $S(+)$ peaks were not statistically different using a paired Student's t-test with a p value of 0.05 ($n=6$).

It has been reported that an enantiomeric excess can induce separation of enantiomers in achiral chromatographic systems [12–15]. To show that enantiomeric excess-induced changes were not occurring during the derivatizing

reaction or chromatography, three groups of standards (optically pure, racemic and a 75:25 ratio of enantiomers) were derivatized and chromatographed. Fig. 5 presents the results of linear regression analysis for each of the enantiomeric standards. Analysis of covariance was used to check the assumption of homogeneity of slopes. After showing that the lines generated from optically pure, racemic and 75:25 standards had the same slope, the three different groups were compared using adjusted sums of squares. An F test showed that there was no significant difference between the standard curves generated from the three different groups [16]. Analysis of covariance demonstrated that in the range tested, 6–45 ng injected on-column, derivatization and chromatography were not significantly different regardless of enantiomeric composition.

It has been reported that LTPC from commercial sources racemizes during the derivatization process and that LTPC commercially prepared contains 8–15% DTPC [17]. The extent of racemization depends on the conditions used for the reaction. Use of triethylamine in the derivatization reaction causes racemization of LTPC [18,19] and by avoiding the use of this reagent in this procedure no racemization is observed. The company that manufactures LTPC states that milder conditions are being employed in LTPC synthesis yielding a derivatizing reagent with optical purity of 97–99% [20].

In conclusion, an on-column derivatization procedure was developed that allows for rapid quantitative analysis of the enantiomers of both MDA and MDMA in small samples of whole blood.

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