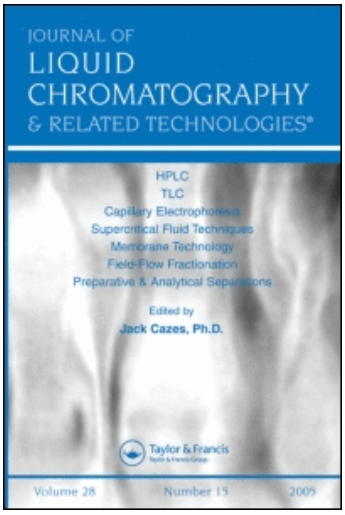

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LIQUID CHROMATOGRAPHIC DETERMINATION OF N-HYDROXY-3,4-METHYLENEDIOXYAMPHETAMINE AND METABOLITE IN PLASMA

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ABSTRACT

The analysis of N-hydroxy-3,4-methylenedioxyamphetamine (NOHMDA) from plasma samples using reversed-phase liquid chromatography is described. The separation of NOHMDA, its metabolite 3,4-methylenedioxyamphetamine (MDA) and the internal standard N-methyl-MDA (MDMA) were well resolved in about twelve minutes using a C_8 stationary phase and an acidic aqueous acetonitrile mobile phase. The isolation procedure involved liquid/liquid extraction of 50 uL of plasma using chloroform followed by HPLC analysis of the resulting residue. The recovery was greater than 90% for NOHMDA and slightly higher for MDA with good reproductibility.

INTRODUCTION

The various N-substituted derivatives of 3, 4-methylenedioxyamphetamine (MDA) have become popular drugs of abuse in recent years (1-3). These drugs are claimed to have a unique ability to facilitate interpersonal communication by reducing the anxiety or fear that normally accompanies the discussion of emotionally painful events (4). The continued designer-drug exploration of the

MDA series has resulted in legislation to upgrade the penalties associated with the clandestine use of these compounds.

MDA was the first of the class of hallucinogenic amphetamine derivatives to show popularity as a recreational drug. Structurally, MDA resembles both amphetamine and mescaline and was categorized as a hallucinogen when it was placed on the DEA Schedule I list. However, it has been reported to act primarily as a central nervous system stimulant that may be hallucinogenic in large doses (5) and its propensity to produce hallucinations has been disputed (6).

Although MDA may lack the sensory disruptions commonly recognized with LSD and mescaline, it has been reported to be much more toxic than mescaline when tested in laboratory animals (7). Of the several phenethylamine derivatives studied by Hardman, (7), MDA was by far the most potent. This additional potency has been attributed to the addition of the 3, 4-methylenedioxy-group coupled with the methyl group substituted on the alpha-carbon of the amine side chain.

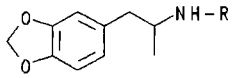
Several of the N-substituted derivatives of MDA have appeared as drugs of abuse and the N-methyl MDA (MDMA), N-ethyl (MDEA) and N-hydroxy (NOHMDA) derivative have been reported to have psychotomimetic activity in humans (8). MDMA is perhaps the most popular of the MDA derivatives and is known by the street names "Ecstasy" or "XTC". This drug has been extensively studied in animals via a variety of techniques including drug discrimination (9) and neurochemical methods (10).

Although clinical studies lend some support to many psychotherapists opinion that MDMA can be safely utilized as an adjunct to psychotherapy, this compound is not without the potential for causing detriment to the casual user. In two separate reports, Hayner and McKinney (11) and Dowling et al. (12), document several case studies involving ingestion of MDMA resulting in both tolerance to the drug and death of the user. Further, doses of MDMA purchased on the street (11) have been found to vary substantially in actual MDMA content (from a low of 16 mg to a high of 150 mg). MDMA has been described (11) as an

unpredictable drug, one that has the potential to kill at previously tolerated doses.

The N-hydroxy derivative of MDA has shown psychotomimetic activity (8) and, as with MDEA, little is known about the chemistry and pharmacology of this compound. However, it has been encountered by forensic scientists in the United States under the street name "Fantasy" (13) and recently has been added to the list of Schedule I drugs. Initial analytical techniques, utilizing gas chromatography, have been less than satisfactory due to the tendency of NOHMDA to undergo thermal degradation (3, 13).

In this work, we report the development of a liquid chromatographic method for the analysis of NOHMDA and MDA from small volume plasma samples. The drugs of interest were isolated from the plasma matrix via solvent extraction with quantitation by an isocratic reversed phase liquid chromatographic procedure.



R = H, MDA
R = CH_3, MDMA
R = OH, NOHMDA

EXPERIMENTAL

General: ^{1}NMR Spectra were obtained on a Varian T-60A Spectrometer using DMSO and Tetramethylsilane as solvent and internal standard respectively. All melting points were determined in open glass capillary tubes using a Thomas-Hoover melting point apparatus and are uncorrected. Electron impact mass spectrometer data were obtained on a Finnagan 3300 Mass Spectrometer at 70 ev of ionization energy using a solid probe inlet. Elemental analyses (C, H, N) were performed by Atlantic Microlab Inc., (Atlanta, GA). The liquid chromatographic system consisted of a Waters Associates Model M-45 solvent delivery system, U6K Injector, Model 440 UV dector operated at 280 nm, and a

Houston Instruments' Omniscribe dual pen recorder. Acetonitrile, chloroform, and methanol were HPLC grade and obtained from Fisher Scientific Company (Fairlawn, NJ). All other Chemicals were reagent grade or better and used without further purification. Water used in standard preparation and chromatographic studies was double distilled. MDA, MDMA and NOHMDA were synthesized as previously described (3).

Chromatographic Procedures: Reversed phase separations were accomplished using a Keystone Scientific (Bellefonte, PA) Deltabond 300 Octyl column (15cm X 4.6m mid). Solvent systems utilized for the liquid chromatographic studies consisted of several different combinations of phosphate buffer:acetonitrile solutions. All mobile phases were degassed prior to use by stirring on a magnetic stirrer for one hour. The mobile phase flow rate was 1.0m 4 min. the UV absorbance detector was operated at 0.02 AUFS and all separations were carried out at room temperature.

Extraction Procedure: Fifty microliters of plasma was pipetted into a clean 10.75mm test tube and 500 ng of MDMA was added as an internal standard. Twenty five microliters of dibasic sodium phosphate (0.5M, pH 9.6) was added to aid in the extraction of the amines. 1.0 ml of HPLC grade chloroform was then added and the tube vigorously vortexed for thirty seconds. The sample was then centrifuged at 3500 rpm for three minutes to break any emulsion formed. The bottom organic layer was then transferred to another clean 10.75mm test tube with a pasteur pipet. 1.0 drop of acidified methanol (1.0 drop of conc. HCl/10ml of MeOH) was then added to the extract to prevent any loss of the amines by evaporation under a stream of nitrogen. The samples were then reconstituted with 50 ul of mobile phase and injected onto the chromatographic system that consisted of a pH 4.0 25 mM potassium phosphate buffer:acetonitrile mixture in the ratio of (770:30).

Efficiency of Recovery and Reproductibility: Control plasma was spiked with MDA and N-Hydroxy MDA to achieve concentrations of 1.0, 2.5, 5.0, 10.0, and 20.0 mcg/ml. Identical concentrations were also prepared in methanol using additional methanol to prepare the dilutions and 500 ng of MDMA was added to all tubes as an internal standard. Plasma samples of 50 ng of MDMA was added to all tubes as an internal standard. Plasma samples of 50 ul each were taken through the liquid extraction procedure while 50 ul of each of the methanol standards evaporated to dryness after the addition of 1.0 drop of acidified methanol. Both series of tubes were then reconstituted with 50ul of mobile phase and injected onto the chromatographic system. The mobile phase consisted of a pH 4.0 25 mM phosphate buffer:acetonitrile mixture in the ratio of (770:30). The recovery efficiency was based on the ratio of the response achieved from the plasma specimens versus the response seen with the methanol standards. Reproductibility of the extraction procedure was obtained by spiking plasma with N-hydroxy MDA and MDA to achieve concentrations of 2.0, 6.0, 12.0, and 15.0 mcg/ml. 50 ul samples were aliquoted in sets of three at each concentration. One set was analyzed immediately while the other two sets were frozen at -20°C for subsequent analysis on consecutive days.

RESULTS AND DISCUSSION

The liquid chromatographic separation of MDA, MDMA and NOHMDA is shown in Figure 1. This separation was obtained using a Deltabond C_8 stationary phase and a pH 4.0 phosphate buffer:acetonitrile (770:30) mobile phase. Previous chromatographic studies (14) had shown that buffering the mobile phase pH at 4.0 produced optimal resolution and peak shape for these three compounds. As the pH of the mobile phase was increased to 5.0 and then 6.0, MDA and MDMA retained good peak symmetry with only a slight increase in capacity factors. In contrast, the NOHMDA peak became severely tailed and a dramatic increase in retention was observed. The significant difference in basicity between NOHMDA

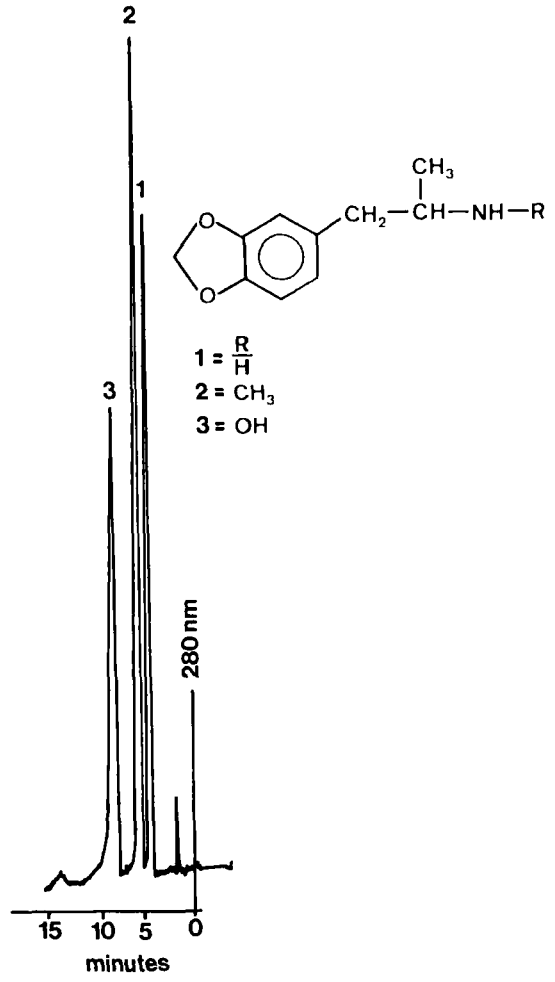


Figure 1. RPLC separation of MDA (1), MDMA (2) and NOHMDA (3) on Deltabond C_8 with pH 4.0 phosphate buffer: acetonitrile (770:30).

(pka=6.2) and MDA (pka=10.04) may account for the reversed phase retention differences between these compounds (14). Variation of the mobile phase pH in the 2.5 to 6.0 range would result in significant change in the degree of ionization of a relatively weak base such as NOHMDA while having little affect on the more basic amines such as MDA and its N-alkyl derivatives. The reversed-phase elution order is a result of the hydrophobicity of the N-substituent and the relative basicity of these three amines. In preliminary studies MDA was detected as a metabolite of NOHMDA, thus any analytical method for biological samples would need to determine both compounds. MDMA was selected as a possible internal standard due to its similar chromatographic and chromophoric properties.

In previous studies NOHMDA has been shown to be unstable at higher temperatures (3) and in solution at pH levels greater than 7.0. Under the high temperatures of gas chromatographic analysis NOHMDA undergoes a disproportional reaction to yield the reduction product MDA and the corresponding oxime oxidation product (3). In aqueous solution NOHMDA is relatively stable at pH 7.0 and below, however its degradation half-life increases with pH to 2.57 ± 0.13 hours at pH 10.0 (14).

The isolation of MDA, MDMA and NOHMDA from serum samples was studied via solvent extraction. The goal of the analytical method was to determine the plasma concentration of MDA and NOHMDA from small volume samples obtained in pharmacokinetic studies in rats. The developed isolation procedure used 50 ul of plasma spiked with internal standard and adjusted to pH 9.6 by the addition of 25 ul of 0.5M phosphate (pH 9.6). The sample was then extracted for 30 seconds with 1.0 ml of chloroform and centrifuged at 3500 rpm for 3 minutes. The organic layer was transferred to a clean tube, mixed with one drop of acidified (HCl) methanol and evaporated to dryness at 37°under a stream of nitrogen. The resulting residue was dissolved in 50 ul of mobile phase for analysis. In all initial studies using blank serum spiked with NOHMDA the HPLC analysis showed no oxime decomposition product in the samples. Thus, the various steps of the assay procedure did not affect the stability of the NOHMDA.

Table 1

Recovery of MDA and N-Hydroxy MDA from Plasma Specimens
Utilizing Liquid/Liquid Extraction

Compound	Amount Added (ug/mL)	Amount Recovered (ug/ml) Run 1	2	3	mean +/- SD	%Rec
NOHMDA	1.00	0.89	0.91	0.91	0.90 +/- 0.01	90.0
	2.50	2.21	2.36	2.33	2.30 +/- 0.08	92.0
	5.00	4.61	4.58	4.73	4.64 +/- 0.08	92.8
	10.00	9.31	9.63	9.54	9.49 +/- 1.17	94.9
	20.00	18.57	18.89	19.06	18.84 +/- 0.25	94.2
MDA	1.00	0.95	0.96	0.98	0.96 +/- 0.02	96.0
	2.50	2.43	2.45	2.50	2.46 +/- 0.04	98.4
	5.00	5.10	4.80	4.85	4.92 +/- 0.16	98.4
	10.00	10.00	10.03	9.70	9.91 +/- 0.18	99.1
	20.00	18.99	19.03	19.59	19.20 +/- 0.34	96.0

Table 1 shows the results of a series of recovery studies for NOHMDA and MDA from blank plasma spiked at levels of 1.0, 2.5, 5.0, 10.0 and 20.0 ug/ml. The results were consistently above 90% for both compounds with the recovery of MDA slightly greater than that of NOHMDA. The reproductibility studies in Table 2 were conducted by spiking relatively large plasma volumes at various concentrations from 2 to 15 ug/ml and analyzing individual 50 ul portions at various times. The samples were stored at -20°C and analyzed on three consecutive days. The individual results and the mean and standard deviation of all runs are shown in Table 2.

The chromatograms in Figure 2 illustrate the results obtained in the analysis of plasma samples. Figure 2a is a blank plasma extract spiked with

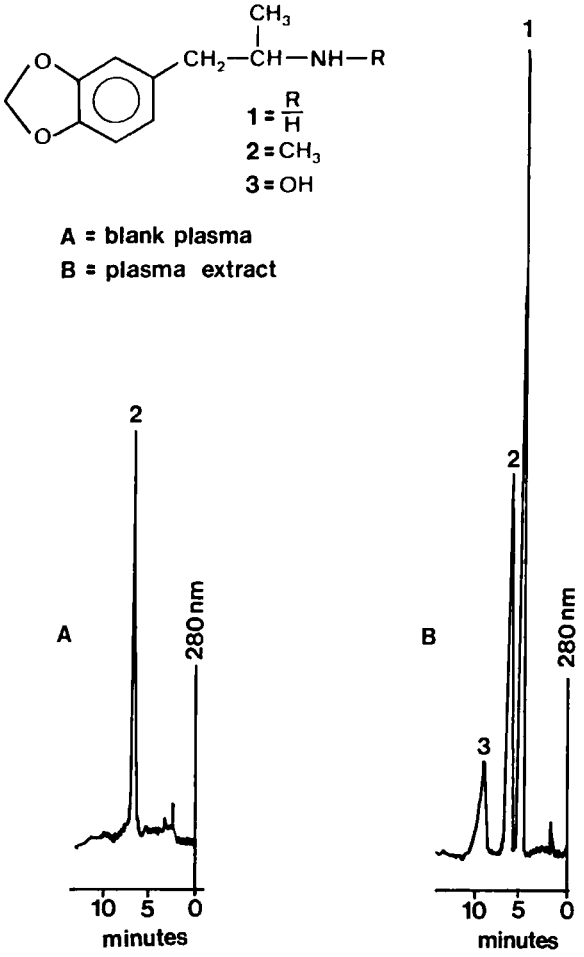


Figure 2. RPLC analysis of plasma containing MDA (1), MDMA (2) and NOHMDA (3). A=blank; B=sample.

Table 2

Reproducibility of Liquid/Liquid Extraction Procedure

Compound	Amount Added (ug/ml)	Amount Recovered (ug/ml) Run 1	2	3	mean +/- SD
N-Hydroxy MDA	2.00	1.79	1.81	1.95	1.85 +/- 0.09
	6.00	5.38	5.41	5.36	5.38 +/- 0.03
	12.00	10.80	11.21	10.96	10.99 +/- 0.21
	15.00	14.30	13.89	14.00	14.06 +/- 0.21
MDA	2.00	1.96	1.94	2.05	1.98 +/- 0.06
	6.00	6.00	5.58	5.89	5.82 +/- 0.22
	12.00	11.50	11.89	11.68	11.69 +/- 0.20
	15.00	14.96	14.89	14.50	14.78 +/- 0.25

internal standard (MDMA) and 2b is an actual plasma sample containing both NOHMDA and its metabolite MDA. The chromatogram in Figure 2b was obtained from a plasma sample carried through the extraction procedure and analyzed by isocratic HPLC analysis using the C_8 Deltabond column and the pH4 buffer - acetonitrile mobile phase. The entire procedure is rapid and very convenient for the analysis of a large number of samples involved in pharmacokinetic and metabolic studies.

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