High-pressure liquid chromatography/electrochemical detection method for monitoring MDA and MDMA in whole blood and other biological tissues

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The drug Ecstasy (3,4-methylenedioxymethamphetamine (MDMA)) is one of several hallucinogenic amphetamine derivatives reported to be serotonergic neurotoxins. The following is a description of a new high-pressure liquid chromatographic (HPLC) analytical method for the analysis of MDMA, 3,4-methylenedioxyamphetamine (MDA) and *N*-ethyl-3,4-methylenedioxyamphetamine (MDE) from whole blood. Upon separation of MDMA, MDA and MDE by HPLC, quantitation is achieved by use of electrochemical detection. Retention times for MDA, MDMA, and MDE are 6.5, 9.2, and 10.3 min, respectively, allowing for a complete chromatographic run every 15 min. The sensitivity of the method is 1 ng/ml which allows for measurement of MDA, MDMA, or MDE in microsamples of whole blood. The volume of blood required is very small (200 μl); therefore, there is minimal blood loss in repeated blood sampling from small animals. Assay linearity was demonstrated from 1 ng/ml to at least 1 μg/ml. The coefficients of variation for both intra-assay and inter-assay comparisons were less than 9%. Other HPLC methods have been previously described for the analysis of amphetamine derivatives, but this new method offers greater sensitivity, rapid turn-around time and ease of use.

Introduction

The illicit drug Ecstasy (3,4-methylenedioxymethamphetamine (MDMA)) is an hallucinogenic amphetamine derivative which is a serotonergic neurotoxin. The neurotoxicity of MDMA is characterized by: (1) a chronic decrease in brain levels of serotonin and 5-hydroxy indole acetic acid (Schmidt and Taylor, 1986); (2) long-term depression of tryptophan hydroxylase activity in selected brain regions (Schmidt and Taylor, 1987), and (3) decreased density of serotonin uptake sites in serotonergic nerve terminals, which is associated with serotonergic nerve terminal degeneration (Bataglia et al., 1987). Because of the concern for neurotoxicity, MDMA was classified as a schedule I drug by the DEA in 1984. Since then, much research has been conducted to elucidate the mechanisms of neurotoxicity of MDMA and a metabolite of MDMA 3,4-methylenedioxyamphetamine (MDA).

Analyses of MDMA and MDA have been conducted using a variety of analytical methods, in-

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cluding gas chromatography (Lim and Foltz, 1988), high-pressure liquid chromatography (HPLC) (Noggle et al., 1988; Garret et al., 1991), mass spectrometry (Schmidt et al., 1986; Noggle et al., 1988), and immunoassay (Bost, 1988). These methods are useful for the measurement of blood levels, but most of them have limitations when used for quantitation of MDMA or MDA in tissues other than blood. This is due to limited tissue availability as well as low tissue concentrations of drug. In addition, whereas gas chromatography/mass spectrometry can be used to monitor these compounds in low concentrations, a drawback is that expensive equipment is needed and labor intensive procedures are required for the chemistry involved in the analysis (Lim and Foltz, 1988).

With respect to the mechanism of action of MDMA, a relationship has been suggested to involve serotonin and/or dopamine in brain (Schechter, 1989). Thus, a method for measurement of MDMA and MDA is needed to assess tissue levels of these agents in brain tissue where concentrations are low and in a dynamic flux.

The method described in this paper employs HPLC coupled with electrochemical detection (EC) under conditions which allow for measurement of low picogram quantities of drug from whole blood. Because of the sensitivity of the assay and the efficiency of the extraction procedure, the volume of blood samples required for analysis is very small (200 μl). Therefore, repeated blood sampling causes minimal blood loss.

Materials and methods

Chemicals

Racemic 3,4-methylenedioxyamphetamine HCl (MDA), 3,4-methylenedioxymethamphetamine HCl (MDMA), and *N*-ethyl-3,4-methylenedioxyamphetamine HCl (MDE) were generously provided by the National Institute on Drug Abuse. The structures of these compounds are shown in Fig. 1. Analytical grade methanol, ethyl acetate, 1-butanol, *N*-hexane, sodium hydroxide, sodium acetate and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA).

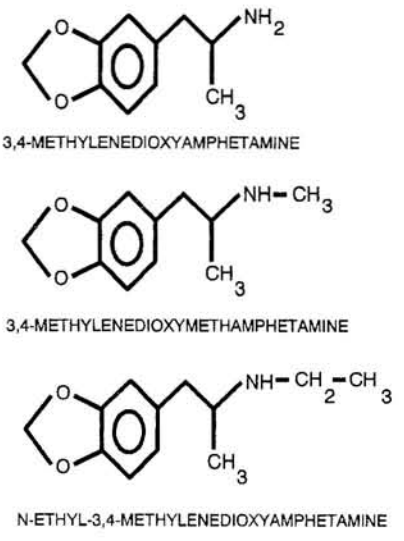

Fig. 1. Structures of MDA, MDMA and MDE (internal standard).

Standards

Stock solutions (1 mg/ml) of MDA, MDMA and MDE were prepared in 0.1 M perchloric acid and frozen until needed. Whole blood working standards were prepared by spiking blood with MDA and MDMA to obtain standard concentrations ranging from 1 ng/ml to 5 μg/ml. The internal standard (MDE) was used at a concentration of 2 μg/ml in 0.1 M perchloric acid.

Extraction

Liquid-liquid extraction was performed on whole blood. To 200 μl of whole blood was added 100 ng of internal standard, MDE (50 μl of 2 μg/ml). The samples were then made alkaline with the addition of 200 μl of 1 M sodium hydroxide, after which 1 ml of extraction solution (ethyl acetate/*N*-hexane/ 1-butanol; 10 : 10 : 1) was added. The samples were vortexed for 60 s and centrifuged for 10 min at $1400 \times g$. The organic phase was separated from the aqueous phase and 50 μl of 0.5 M acetic acid was added to the organic phase. Samples were vortexed for 30 s and centrifuged for 5 min at $1400 \times g$. The organic phase was discarded, and 20 μl of the aqueous phase was injected onto the column for

separation and quantitation. The extraction technique as described above is a modified version of the method used by Fitzgerald (1989).

Separation and quantitation

Separation of MDA, MDMA, and MDE was accomplished with HPLC. The mobile phase consisted of 0.1 M sodium acetate (pH = 4.25)/methanol (10:3). The column used was a Whatman (250 mm × 4.6 mm) 5 μ silica Partisphere column. Mobile phase flow was maintained at 0.8 ml/min with a Perkin-Elmer series 10 HPLC pump. The detector was a Bioanalytical Systems (BAS) electrochemical detector (model LC44). The voltage potential at the working electrode was +1.240 V in reference to an Ag/AgCl electrode. The working electrode used in the assay was a BAS dual glassy carbon electrode. The output signal from the detector was internally filtered at 0.5 Hz to decrease baseline noise, and was connected to a Hewlett Packard integrator (model HP3394). Quantitation of drug levels were calculated by comparing the peak height ratio of drug to internal standard with that from a standard curve consisting of whole-blood standards ranging from 1 ng/ml to 1 μg/ml.

Results

Fig. 2 demonstrates the hydrodynamic voltamogram for MDA, MDMA and MDE. This figure illustrates that the optimal potential for the analysis of these compounds is +1.24 V or greater. However, one must be cautious when working at such a high positive potential when using an aqueous mobile phase because water starts to be oxidized near a potential of +1.20 V. The insert of Fig. 2 demonstrates the relationship of the oxidation of the mobile phase, mainly the oxidation of water, and the potential across the electrochemical cell. One can see that the current passing through the cell rapidly increases at potentials greater than +1.22 V. As the background current increases so does the baseline noise. Therefore as the potential increases the signal-to-noise ratio decreases. The potential setting of +1.24 V yields the greatest signal response, with a background current that produces minimal baseline noise.

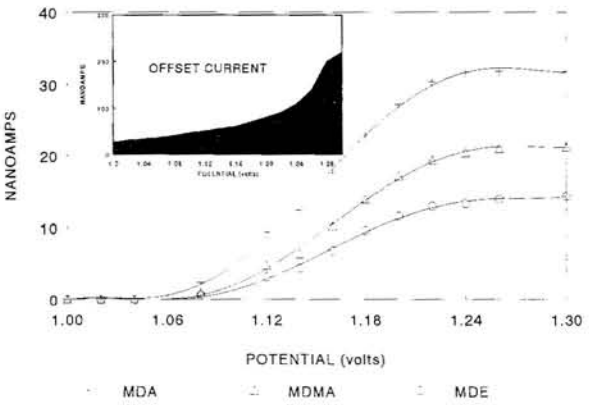

Fig. 2. Hydrodynamic voltamogram for MDA, MDMA and MDE. The insert depicts the relationship of voltage potential across the analytical cell to background current passing through the cell. The concentration of MDA, MDMA, and MDE injected onto the column was 40 ng.

Chromatograms for whole-blood standards spiked with MDA and MDMA are shown in Fig.

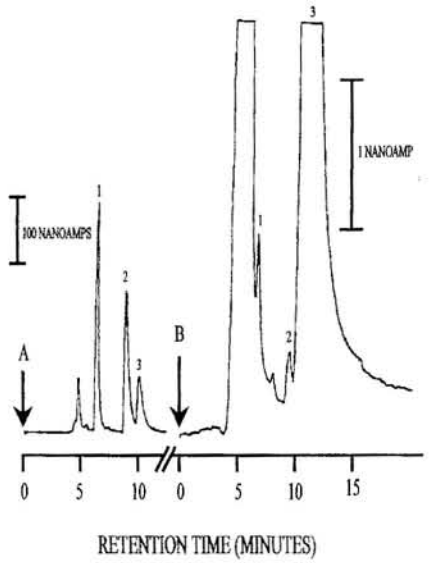

Fig. 3. Chromatograms from whole blood spiked with 500 ng/ml MDA and MDMA with the detector sensitivity at 1 μA/V (A); whole blood spiked with 1 ng/ml MDA and MDMA with the detector sensitivity at 5 nA/V (B). The retention times for MDA (1), MDMA (2) and MDE (3) are 6.5, 9.2 and 10.3 min, respectively.

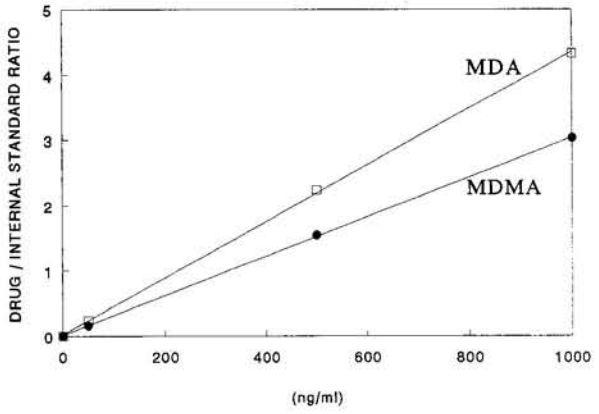

Fig. 4. Calibration curves for MDA and MDMA. Curves were constructed using four whole-blood standards ranging from 1 ng/ml to 1 μg/ml. The Y axis represents the ratio of peak heights of drug over internal standard. The correlation coefficient for the MDA curve is 0.9998 with a Y intercept of 0.00433, and the correlation coefficient for the MDMA curve is 0.9999 with a Y intercept of 0.00303.

3. The peaks for MDA, MDMA and MDE are sharp, symmetrical, and well resolved with baseline separation. Under the conditions employed, the retention times for MDA, MDMA and MDE were 6.5, 9.2 and 10.3 min, respectively. Fig. 4 demonstrates the typical calibration curves for MDA and MDMA. The curves are linear from 1 ng/ml to at least 1 μg/ml. The correlation coefficients of these standard curves are 0.9998 and 0.9999 for MDA and MDMA, respectively.

Extraction efficiencies for MDA and MDMA are demonstrated in Table I. The extraction efficiency was calculated by comparing the amperometric response of equal concentrations of drug extracted to unextracted aqueous standards.

TABLE I

EXTRACTION EFFICIENCY OF MDA AND MDMA FROM WHOLE BLOOD

Results are listed as means ± SD. Extraction efficiency for the internal standard (MDE) is 66% ±6 ($n = 22$)

Concentration	MDA (mean ± SD)	MDMA (mean ± SD)
500 ng/ml	88% ± 6	100% ± 7
10 ng/ml	82% ± 13	91% ± 17

TABLE II

INTRA-ASSAY VARIATIONS FOR MDA (A) AND FOR MDMA (B)

Values are means ± SD. Total number of samples for each target concentration is 8

Target concentration (ng/ml)	Experimentally determined concentration (mean ± SD)	n	Coefficient of variation (%)	Average SE (%)
A: MDA				
2000	2018 ±75	10	3.73	2.7
500	499.8 ± 9.41	10	1.89	1.2
10	9.92 ± 0.57	10	5.74	4.64
B: MDMB				
2000	2031 ±68	10	3.34	3.2
500	500.4 ± 3.85	10	0.77	0.61
10	10.11 ± 0.57	10	5.64	4.29

The precision and accuracy characteristics for the assay are summarized in Tables II and III for intra-assay and inter-assay, respectively. For inter-assay determinations, the time between successive assays was 1 day. The coefficients of variation (standard deviation expressed as a percentage of the mean) for both inter-assay and intra-assay variations were less than 9%.

TABLE III

INTER-ASSAY VARIATIONS FOR MDA (A) AND MDMA (B)

Time between successive analysis is 1 day. Values are listed as means ± SD. Total number of samples for each target concentration is 10

Target concentration (ng/ml)	Experimentally determined concentration (mean ± SD)	n	Coefficient of variation (%)	Average SE (%)
A: MDMA				
2000	1987 ±47	8	2.35	1.26
500	497.19 ± 3.89	8	0.78	0.64
10	9.83 ± 0.87	8	8.85	6.29
B: MDA				
2000	1988 ±43	8	2.18	1.2
500	499.1 ± 3.71	8	0.74	0.62
10	9.81 ± 0.70	8	7.14	5.95

Discussion

HPLC coupled with amperometric detection is a very powerful tool for the quantitation of low levels of drugs. One prerequisite for the use of amperometric detectors in HPLC is that the drugs of interest must be able to be oxidized or reduced. Amperometric detectors operate by setting a fixed potential across 2 electrodes in a thin-layer electrochemical cell and measuring the electric current passing through the cell. As compounds pass through the electrochemical cell they may be oxidized or reduced, depending on the fixed potential across the cell. For the method described in this paper, the potential was +1.24 V. At this high positive potential MDA, MDMA, and MDE are oxidized. Electrons that are liberated from the molecules being oxidized are transferred to the working electrode of the electrochemical cell. This, in turn, sets up a current passing through the cell. The current passing through the cell is linearly converted to a voltage output by the detector which is connected to an integrator where the chromatographic runs are resolved.

A direct relationship exists between the background noise level and the potential across the analytical cell. As mentioned earlier the high positive potential setting used in this method causes a high background current (approximately 120 nA). However this does not present a problem in that the BAS EC detector (model LC44) possess superior filtering capacity. Consequently, under conditions of the assay the background noise was very small resulting in a stable, quite baseline.

A relationship also exists between the high positive potential and equilibration time. The setting of +1.24 V requires at least 6 h for equilibration. Consequentially, the system may be operated continuously to avoid delays for equilibration of the detector. In this study, the system was operated 24 h/day to avoid lengthy start-up time. During periods when analysis was not being conducted the mobile phase was recirculated at a low flow rate (0.5 ml/min).

The column used in the assay was a Whatman silica column. In comparing different sources of silica columns, the Whatman Partisphere 5 μm silica column provided the best separation. Great care was taken when using the column with an aqueous mobile phase because silica is slightly soluble in water. To avoid column damage, the pH of the mobile phase was made acidic, decreasing the solubility of silica in water, and a Solvocon silica saturator column (Whatman) was installed between the pump and the injector.

Concentrations of standard as low as 1 ng/ml were employed in the analysis which is considered to be the sensitivity of the assay employing a signal-to-noise ratio of 6:1. The signal-to-noise ratio was calculated by comparing the peak height of the signal of interest to the height of baseline noise. Baseline noise was defined as the peak-to-peak disturbance in the response of the detector on the recorder strip chart when only mobile phase is passing through the electrochemical cell (Duda, 1993). The assay was linear at all concentrations ranging from 1 ng/ml to at least 1 μg/ml. Because of the linearity, this assay will be useful in monitoring levels of MDA and MDMA in specific tissue compartments as well as blood. Since brain levels are reported to be in the low nanogram per milligram of tissue range, and since the current method allows for the quantitation of 1 ng/ml, monitoring of MDA and MDMA in target tissues such as brain is possible. Preliminary analysis of MDA and MDMA in tissue extracts were well within the linear range of this assay.

In the studies dealing with the extraction of MDA and MDMA from blood it was found that extraction efficiencies for all compounds were very reproducible from assay to assay. Therefore, as expected, the accuracy and precision for the assay was high. The highest coefficient of variation for the extraction of MDA and MDMA from blood standards was 8.85%. Thus, the assay was both highly accurate and precise.

The method as described appears to be superior to previously described HPLC techniques for the analysis of MDA and MDMA because of the low sensitivity (1 ng/ml) and the ability to quantitate drug levels from microsamples of tissue. Garret et al. (1991) published a highly sensitive HPLC method for the analysis of MDA and MDMA in

plasma. This method is sensitive to levels of 2.7 ng/ml for MDA and 1.6 ng/ml for MDMA. The drawback of the method described by Garrett et al. is that it requires 1 ml of plasma for extraction. The method described in this paper can quantitate comparable drug levels from as little as 200 μl of whole blood.

Consequently, this new HPLC/EC procedure should allow for measurement of MDA and MDMA in biological tissues other than blood where tissue samples are small and drug concentrations are low.

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