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EMIT-d.a.u. MONOCLONAL AMPHETAMINE/METHAMPHETAMINE ASSAY. II. DETECTION OF METHYLENEDIOXYAMPHETAMINE (MDA) AND METHYLENEDIOXYMETHAMPHETAMINE (MDMA)

ALPHONSE POKLIS[a], ROBERT L. FITZGERALD[b], KAREN V. HALL[a] and JOSEPH J. SAADY[a]

[a]*Department of Pathology and Toxicology Laboratory, Medical College of Virginia Hospitals, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0597 and* [b]*USA Department of Veterans Affairs, Medical Center, San Diego, CA (USA)*

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Summary

The cross-reactivity, stereoselectivity and clinical performance of the EMIT-d.a.u. monoclonal amphetamine/methamphetamine immunoassay (EM) for the detection of methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) in urine was evaluated. The cut-off concentrations of racemic MDA and MDMA were found to be approximately 800 ng/ml and 3000 ng/ml, respectively. The EM assay demonstrated a high selectivity for the S(+) isomer of both MDA and MDMA. Urines collected over a 24-h period from rats administered 20 mg i.v. racemic MDMA were all positive when analyzed by the EM assay. The EM was found vastly superior to the EMIT d.a.u. polyclonal amphetamine/methamphetamine assay for the detection of MDA and MDMA. The EM assay displayed sufficient sensitivity for detection of these drugs following clinical intoxication.

Key words: Immunoassay; Urine; Drug testing; Amphetamine; Methylenedioxyamphetamine; Methylenedioxymethamphetamine

Introduction

3,4-Methylenedioxyamphetamine (MDA) and 3,4 methylenedioxymethamphetamine (MDMA) are psychotropic agents chemically and pharmacologically related to amphetamine and mescaline [1,2]. Clinically both drugs display marked sympathomimetic activity similar to amphetamine demonstrated by peripheral vasoconstrictions, tachycardia, pupillary dilation and effects on other smooth muscles. Central nervous system stimulatory effects also mimic those of amphetamine and in cases of overdose convulsions, hyperthermia and behavior

Correspondence to: Alphonse Poklis, Department of Pathology and Toxicology Laboratory, Medical College of Virginia Hospitals, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0597, USA.

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changes may occur [3,4]. The 3,4-methyleneoxy group on the phenyl portion of the drugs bestows psychopharmacological properties similar to those of mescaline. Both MDA and MDMA produce perceptional distortions and pronounced subject effects including intensification of feelings, a facilitation of self-insight, an overwhelming desire to communicate, profound empathy and euphoria [2,4–6]. At high doses, MDA may cause hallucinations; however, hallucinations are not associated with MDMA intoxication. [7].

Both MDA and MDMA were first synthesized early in this century. MDA was initially tested as an anorexic agent, antitussive or ataractic, although it was never successfully brought to market as such [2]. Both have been used to treat mental disorders such as depression and have been used as an adjunct to psychotherapy [2,4,7]. Both have been associated with illicit drug use in the USA. MDA was a popular 'love drug' in the late 1960s and throughout the 1970s [4,8]. MDMA, as 'Ecstacy', 'XTC' and 'X', has become a widely used drug of abuse beginning in the mid-1980s [9,10]. Presently, both drugs are classified a Schedule 1 compound by the US Federal Drug Enforcement Agency. This present epidemic produces the need for fast, easy-to-use, reliable urine drug screening methods for the detection of MDA and particularly MDMA. Immunoassays designed for the detection of amphetamines such as the polyclonal EMIT-d.a.u. Amphetamine (EP) assay and the TDx fluorescence polarization amphetamine/methamphetamine II assay (TDX) have cross-reactivity for MDA and MDMA [11,12].

Previously, we evaluated the EMIT-d.a.u. amphetamine/methamphetamine monoclonal immunoassay (EM) which is stereoselective in the detection of amphetamine and methamphetamine [13]. The communication presents an evaluation of the degree of cross-reactivity, and stereoselectivity of the EM assay for MDA and MDMA. Additionally, urine specimens collected from rats administered MDMA were also analyzed by the EM and EP assays and confirmed by chiral GC/MS.

Experimental

Immunoassay

All reagents necessary for the Monoclonal EMIT-d.a.u. Amphetamine/Methamphetamine (EM) assay and the polyclonal EMIT-d.a.u. Amphetamine (EP) assay were obtained from Syva Company (Palo Alto, CA 94304): EMIT® antibody/substrate reagent A, enzyme reagent B and EMIT drug assay buffer concentrate. All immunoassay reagents were stored refrigerated at 2°C.

The EM assay was calibrated with negative, cut-off and medium calibrators containing 0.0, 1000 and 5000 ng/ml of S(+)-methamphetamine, respectively. The EP assay was calibrated with negative, cut-off and medium calibrators containing 0, 300 and 2000 ng/ml of racemic amphetamine. EM assay urine controls containing 3000 ng/ml and 5000 ng/ml S(+)-methamphetamine were obtained from Quality Assurance Service Corporation (Martinez, GA 60907). Urine controls of 500 ng/ml and 3000 ng/ml of racemic amphetamine for the EP assay were also from Quality Assurance Service Corporation.

Both EM and EP assays were performed on a Syva ETS Analyzer with 3.02 version software [14].

Gas chromatography/mass spectrometry (GC/MS)

The chiral derivatizing reagent *N*-trifluoroacetyl-1-prolyl chloride (LTPC), 0.1 M in chloroform, was purchased from Regis Chemical Co. (Morton Grove, IL 60053). The LTPC was reported to contain 1.2% of its enantiomer, *N*-trifluoroacetyl-*d*-propyl chloride (DTPC). All extraction solvents were HPLC grade. For chiral GC/MS analysis, methoxyphenamine, was purchased from Sigma Chemical Co. (St. Louis, MO 63178). Drug free urine used for the preparation of GC/MS calibrators or 'drug added specimens' in the cross reactivity studies was collected from laboratory personnel and was free of the analytes of interest. Caffeine and nicotine and their metabolites were present in some specimens.

Racemic methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) were purchased from Sigma Chemical Company. Stereoisomers, S(+) and R(−), of MDA and MDMA were synthesized as previously described and supplied by the Department of Medicinal Chemistry, Medical College of Virginia, Richmond, VA 23298 [15]. The optical purity S(+) and R(−)-MDMA and MDA was determined by the chiral GC/MS procedure described below using LTPC as the chiral derivatizing reagent. The peak area of the minor peak due to optical impurities of the derivatizing reagent and the analyte reference material, was compared to the area of the primary isomer peak in order to calculate optical purity. MDA and MDMA stereoisomer reference material was determined to be 99% optically pure.

A stock standard of each racemic mixture or pure isomer was prepared in methanol. This was diluted with methanol to prepare working standards of 1 mg/ml. The working standards were diluted with drug free urine to make GC/MS calibrators of 0.0, 250, 500, 1000, 2000 and 5000 ng/ml for each analyte.

Chiral GC/MS analyses were performed using a Hewlett Packard 5985A GC/MS equipped with a 12-m × 0.2-mm i.d. fused silica capillary column with a 0.22-mm film thickness of cross-linked methyl silicon stationary phase and 7900 and 7920 disc drives with a 21-MXE data system (Avondale, PA 19311). The instrument was operated in the electron ionization (EI) mode, with an ionization potential of 70 eV, at normal autotune® conditions. Scanning was done from *m/z* 50/310. SIM was performed using Answer® software monitoring four ions simultaneously.

Chiral GC/MS analysis of MDA and MDMA was performed as previously described [15]. Briefly, 100 μl of 150 μg/l methoxyphenamine, internal standard, was added to 2.0 ml aliquots of urine which were then made basic with sodium hydroxide and extracted with 100 μl of chloroform. After vortexing and centrifuging, 3 μl of the chloroform were drawn into a 10-μl syringe. Three microliters of LTPC were then drawn into the same syringe and the mixture was injected into the injection port of the gas chromatograph for 'on-column' derivatization. GC conditions were: injection port temperature, 275°C; initial oven temperature, 220°C for 4 min; program rate, 10°C/min to 280°C. Under

the conditions of the assay, the retention times of R(−)-MDA and S(+)-MDA, and S(+)-MDMA and R(−)-MDMA were: 3.58, 3.77, and 4.62, 4.72, respectively. The methoxyphenamine diasteriomers eluted at 3.34 and 3.45 min.

Evaluation protocol

To determine possible methodological bias between the methods, cut-off calibrators and 'low' positive urine controls for the immunoassays were analyzed in duplicate by TFAA achiral GC/MS. Results were ±10% of the labeled values. The cross-reactivity of both EMIT assays was determined by adding racemic MDA and MDMA to drug free urine to make concentrations of 200, 400, 600, 800, 1000, 2000 and 5000 ng/ml.

The stereospecificity of the assays was evaluated by adding specific S(+) and R(−) stereoisomers of MDA and MDMA to drug free urine to make concentrations of 150, 300, 500, 1000, 2000, 5000, 10 000, 25 000 and 50 000 ng/ml. Each set of urines were prepared in duplicate and was simultaneously analyzed by each immunoassay and the appropriate change in absorbance was noted and compared to that of the cut-off calibrator.

Additionally, twelve urine specimens collected over 24 h from four male rats following i.v. injection of 20 mg/kg MDMA were simultaneously analyzed by each immunoassay and chiral GC/MS.

Results and Discussion

The cross-reactivity of the EM assay with MDA and MDMA was found to be approximately 800 ng/ml and 3000 ng/ml, respectively, (Fig. 1). The greater sensitivity of the assay for MDA compared to MDMA is consistant with our previously reported enhanced sensitivity of the EM assay for amphetamine com-

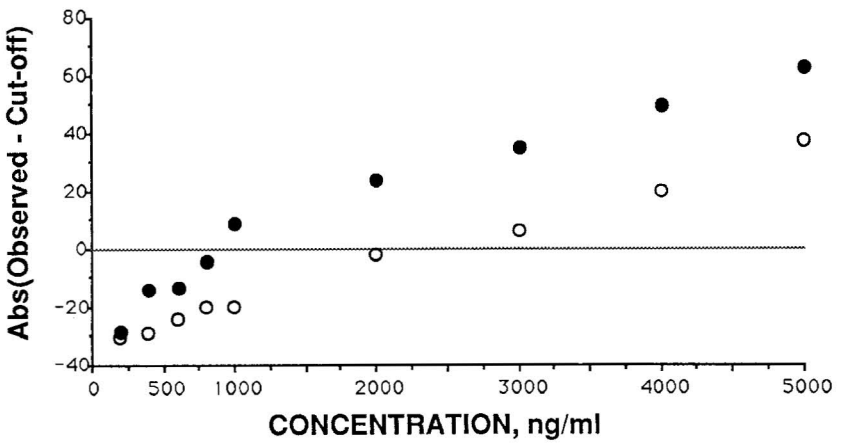

Fig. 1. Cross reactivity of the EMIT-d.a.u. monoclonal amphetamine/methamphetamine assay to methylenedioxyamphetamine (●) and methylenedioxymethamphetamine (○). Any specimen giving a change in absorbance greater than zero is positive.

pared to methamphetamine [13]. Both MDA and amphetamine have a phenylisopropylamine group. The antibodies present in the assay are less sensitive to the *N*-methyl substituted MDMA and methamphetamine. The PE assay gave negative results for both MDA and MDMA at concentrations of 5000 ng/ml. Ruangyuttikan and Moody have previously reported the low cross-reactivity of EP with MDA and MDMA [11]. They site the concentrations necessary to produce positive PE results are 10 000 – 13 000 ng/ml of MDA and 5000 – 8000 ng/ml for MDMA.

As methamphetamine is biotransformed to amphetamine, similarly MDMA is *N*-demethylated to MDA [16,17]. Therefore, ingestion of MDMA will result in urinary excretion of both MDMA and MDA. Previously, we reported that presence of both methamphetamine and amphetamine in urine at concentrations less than the cut-off value of either drug can yield positive EM results [13]. To investigate the effect on MDMA and MDA mixtures on the EM assay, a series of urines containing varying concentrations of the drugs was prepared and analyzed. Urines containing 200 ng/ml MDA and 2000 ng/ml MDMA yielded negative results. However, urines containing 400 ng/ml MDA and 2000 ng/ml of MDMA were positive. Pharamacologically effective doses of MDMA are typically 50 – 125 mg, that is at least ten times the effective dose for amphetamine or methamphetamine [2,4,6]. Verebey, et al. have reported that in man, 65% of MDMA is excreted as parent drug and 7% as MDA within 3 days of ingestion [18]. Therefore, the sensitivity of the EM described above should be sufficient to detect the drug following clinically significant ingestions.

The EM assay was stereoselective for MDA and MDMA isomers, however, the EP assay was found not to be stereoselective for isomers of either drug. The selectivity of the ME assay for S(+) MDA was two to three times greater than for the R(–) isomer, Fig. 2. The EM demonstrated a high degree of selectivity for

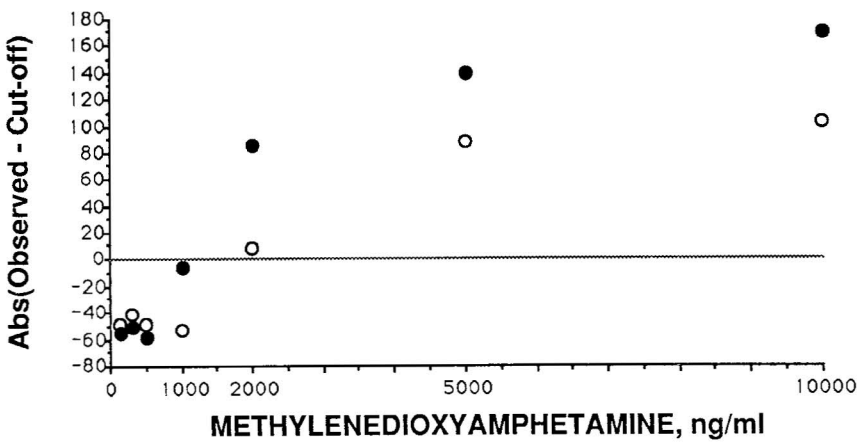

Fig. 2. Stereoselective response of the EMIT-d.a.u. monoclonal amphetamine/methamphetamine assay to S(+)-methylenedioxyamphetamine (●) and R(–)-methylenedioxyamphetamine (○). Any specimen giving a change in absorbance greater than zero is positive.

the isomers of MDMA as the selectivity for the S(+) isomer was 15–20 times greater than the R(–) isomer, Fig. 3. These results are consistant with those previously reported for the EM assay concerning selectivity of amphetamine and methamphetamine stereoisomers [13]. In each instance the ME assay favors the (S)+ isomer.

At present, chiral analysis is not routinely performed on confiscated illicit MDA or MDMA, as the drugs are assumed to be sold as racemic mixtures. There are significant differences in pharmacokinetics, biotransformation and pharmacology/toxicology between the stereoisomers of the drugs [7,16,17,19,20]. S(+) MDA is thought to be amphetamine-like in activity, while the R(–)isomer is hallucinogen-like [19]. S(+)-MDMA is associated with long-term neurotoxicity, while such effects are not evident with R(–) isomer [7,20]. While stereoselectivity of ME assay is not presently important in clinical or forensic urine screening for MDA or MDMA, it may prove relevant in the future.

The superior sensitivity and stereoselectivity of the EM assay for MDA and MDMA as compared to the EP assay, produced strikingly different results when urine specimens from experimental animals administered MDMA were analyzed. Urine samples from 12 rats were collected following a 20-mg/kg i.v. injection of racemic MDMA. Administration of racemic MDMA results in urinary excretion of S(+) and R(–)-isomers of both MDMA and its major metabolite MDA [16–18]. Therefore, physiological specimens contain varying amounts of both MDMA and MDA isomers. Chiral GC/MS analysis yielded the following range of isomer concentrations; R(–) MDA, 16–61 ng/ml; S(+)-MDA, 100–140 ng/ml; R(–) MDMA, 780–15 000 ng/ml and S(+)-MDMA, 600–10 000 ng/ml. The EM assay was positive for all twelve specimens, while the EP assay yielded positive results for only 4/12. The EP assay was negative for specimens containing a total of up to 11 600 ng/ml MDMA and MDA. EP positive results were only obtained with specimens containing greater than 17 000 ng/ml total MDMA and MDA.

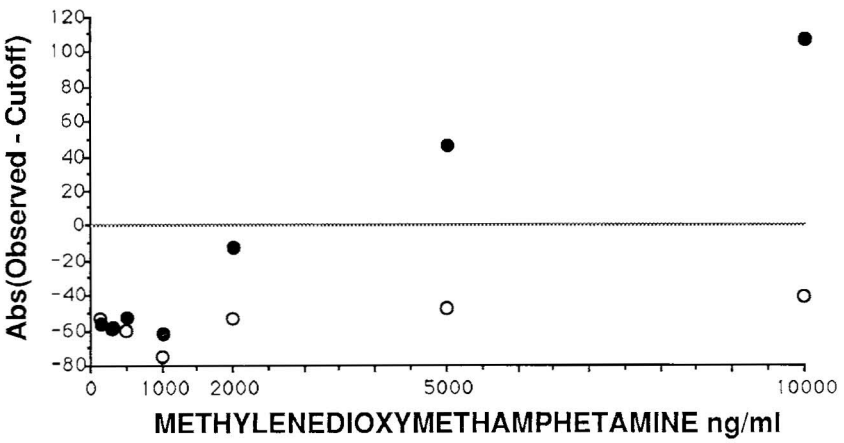

Fig. 3. Stereospecific response of the EMIT-d.a.u. monoclonal amphetamine/methamphetamine assay to S(+)-methylenedioxymethamphetamine (●) and R(–)-methylenedioxymethamphetamine (○). Any specimen giving a change in absorbance greater than zero is positive.

These results are in agreement with the studies of drug added urine samples presented above.

In conclusion, if the amphetamine derivatives, MDA or MDMA, are the drugs primarily sought by urine testing, the monoclonal EMIT-d.a.u. amphetamine and methamphetamine assay provides sufficient cross-reactivity to readily detect these drugs. MDA and MDMA maybe readily distinguished from amphetamine and methamphetamine during confirmational testing by numerous analytical methodologies including spectrophotometry, chromatography and mass spectometery.

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