Technical Note

A Comparison Between Experimental and Authentic Blood/Serum Ratios of 3,4-Methylenedioxymethamphetamine and 3,4-Methylenedioxyamphetamine

Regine Garcia Boy[1,*], Joerg Henseler[2], Johannes G. Ramaekers[3], Rainer Mattern[1], and **Gisela Skopp[1]**

[1]Institute of Legal Medicine and Forensic Medicine, University Hospital, Voss-Str. 2, 69115 Heidelberg, Germany; [2]Radboud University, Thomas van Aquinostraat 1, 6525 GD Nijmegen, The Netherlands; and [3]Department of Neuropsychology and Psychopharmacology, Faculty of Psychology and Neuroscience, Maastricht University, Universiteitssingel 40, 6229 ER Maastricht, The Netherlands

Abstract

This paper compares the blood-to-serum distribution (B/S ratio) of 3,4-methylenedioxymethamphetamine (MDMA) and its major metabolite 3,4-methylenedioxyamphetamine (MDA). B/S ratios were determined by liquid chromatography–tandem mass spectrometry analysis following liquid–liquid extraction as a function of the hematocrit value (experimental specimens) and in blood and corresponding serum samples (n = 63) from 16 healthy volunteers participating in a controlled driving experiment (authentic specimens). A regression analysis to calculate the B/S ratio was performed followed by an analysis of covariances (ANCOVA). A linear relationship between the hematocrit value and the B/S ratio of both MDMA and MDA could be established from the experimental data. For MDMA, the regressions provided mean B/S ratios of 1.22 and 1.26 for the experimental setting and the authentic samples, respectively. For MDA, the analysis determined slopes of 1.15 and 1.27 for the experimental setting and field study, respectively. ANCOVA revealed that the method of determination (experimental vs. authentic specimens) did not influence the resulting slopes. A conversion factor of 0.80 may give an adequate estimate to derive the serum concentration for MDMA if only the concentration in whole blood is known, whereas such a definitive factor could not be established for MDA because of its very low levels in authentic samples.

Introduction

The designer drug 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) accounts for about 9 million users of central stimulants world-wide, approximately one-third of them in Europe. This exceeds use levels for cocaine and heroin, combined (1). Among other things, MDMA consumption leads to decreased ability of risk assessment combined with reckless behavior and is thus of interest in driving under the influence (DUI) cases (2). MDMA is metabolized to 3,4-methylenedioxyamphetamine (MDA) via *N*-demethylation, mostly by the cytochrome P450 enzymes CYP2D6 (3) and, to a minor extent, CYP1A2 (4). MDA is still pharmacologically active and the only metabolite found in serum or plasma, with concentrations of typically 5–10% of the corresponding MDMA levels (5).

Many drugs of forensic interest, such as Δ^9-tetrahydrocannabinol (THC) and 11-carboxy-THC (6,7), tricyclic antidepressants (8), benzodiazepines (8–11), and opiates (12–14), show an unequal distribution between blood serum and cellular phase. Ratios may not only vary between different compounds with the same core structure, but also between the parent drug and corresponding metabolites (8) or depend on the hematocrit value (13,15). Except for THC, 11-carboxy-THC (16), ethanol (15), and, more recently, benzodiazepines (11), distribution studies have mostly been performed in vitro on supplemented samples after separation of plasma or serum and red blood cells by either equilibrium dialysis (17) or centrifugation. As plasma or serum samples are seldom available in forensic investigations, MDMA and MDA are generally determined from whole blood samples. Knowing the distribution of MDMA and MDA into the major subcompartments of blood is mandatory in order to reliably compare whole blood to plasma or serum levels that have been derived from controlled pharmacokinetic studies. For MDMA and MDA, as well, it is not known how far off from the true values the in vitro determined ones are likely to be.

This paper compares the blood-to-serum distribution (B/S ratio) of MDMA and MDA in both an experimental setting and using authentic samples from healthy volunteers conducting a DUI experiment under the influence of MDMA.

* Author to whom correspondence should be addressed: Dr. Regine Garcia Boy, Institute of Legal Medicine and Forensic Medicine, Voss-Str. 2, 69115 Heidelberg, Germany. E-mail: regine.garcia@med.uni-heidelberg.de.

Materials and Methods

Experimental (in vitro) setting

Fresh blood was drawn by venous puncture from a healthy volunteer (S-Monovette, Sarstedt, Nümbrecht, Germany). After determination of the hematocrit value by centrifugation, the sample was split. A smaller aliquot was put aside while the remaining sample was centrifuged for 10 min at 3000 × *g* (Megafuge 1.0 R, Heraeus Sepatech, Osterode, Germany). The supernatant and packed erythrocytes were separated; liquid and cellular phases were combined to yield different hematocrit values (n = 3) largely covering the physiological range. The original and the combined samples were spiked with MDMA and MDA [200 (one sample) or 250 ng/mL]. A preliminary study revealed that the B/S ratio was not dependent on the concentration (50–500 ng/mL; data not shown). Following equilibration (30 min), about one-third of the sample was then directly extracted; the remaining liquid was again centrifuged (10 min, 3000 × *g*). Five aliquots of whole blood and recovered serum were extracted independently after addition of internal standards (MDMA-d_5 and MDA-d_5). Mean values and standard deviations were recorded.

Authentic samples (field study)

Blood and corresponding serum samples from 16 healthy volunteers of a driving experiment were provided from the Experimental Psychopharmacology Unit, Maastricht University. This study was conducted according to the code of ethics on human experimentation established by the declaration of Helsinki (1964) and amended in Edinburgh (2000). All subjects were experienced MDMA users and gave their informed consent to participate in the study in writing. Approval for the study was obtained from the Medical Ethics committee of the Academic Hospital of Maastricht and the University of Maastricht. A permit for purchase, storage, and administration of MDMA was obtained from the Dutch Drug Enforcement Administration.

Samples were taken on four different occasions, 2 h after oral administration of either MDMA (25, 50, or 100 mg) or placebo. One participant provided blood and serum samples on only 3 of the 4 occasions, and 63 samples in total were obtained for blood and serum. Samples were stored frozen at –20°C for approximately two weeks until analysis. Hemolyzed blood and corresponding serum were extracted twice on two different occasions following the protocol described.

Materials

Deuterated (100 µg/mL in methanol) and undeuterated (1 mg/mL in methanol) MDMA and MDA were obtained from Promochem (Wesel, Germany). High-pressure liquid chromatography (HPLC)-grade acetonitrile, methanol, and ethyl acetate (≥ 99.8%) were purchased from Roth (Karlsruhe, Germany); acetic acid (100%), ammonium acetate (≥ 98%), solid NaOH, and concentrated HCl were from Merck (Darmstadt, Germany); and double-distilled water was supplied by Braun (Melsungen, Germany).

Extraction procedures, instrumentation, and tandem mass spectrometry (MS) conditions

NaOH (0.1 M) and deuterated standards (10 ng) were added to a 100-µL aliquot of samples or calibration standards. Supplemented samples were extracted with ethyl acetate and centrifuged. The organic layer was transferred to a silanized vial, acidified with 50 µL of methanolic hydrochloric acid (MeOH/HCl, 49:1, v/v), and evaporated. The residue was reconstituted with mobile phase [50 µL of 4 mM ammonium acetate buffer (pH 3.2)/methanol/acetonitrile (65:7:28, v/v/v)].

Analysis was performed on an API 4000 tandem MS with a TurboIon ionization source operated in the positive-ion mode (Applied Biosystems, Darmstadt, Germany) interfaced to an HPLC pump equipped with an autosampler (1100 series, Agilent, Waldbronn, Germany). The samples (10-µL aliquots) were eluted from a Zorbax Eclipse XDB-C8 (2.1 × 150 mm, 5-µm particle size, Agilent, Waldbronn, Germany) at a flow rate of 220 µL/min. Data were acquired in multiple reaction monitoring mode (MDMA, m/z 194 → 163; MDA, m/z 180 → 163; MDMA-d_5, m/z 199 → 165; and MDA-d_5, m/z 185 → 168). The transitions were also used for quantification (linear curve fit). Calibration lines (5–1000 ng/mL for each analyte) were assessed for linearity of response using regression analysis. In addition, ion suppression or enhancement, carryover, and benchtop stability (24 h) were checked. The lower limits of detection (LLOD) and quantitation (LLOQ) were estimated from the calibration curves according to DIN 32465 at a probability of 95% (17).

Statistical analysis

A regression analysis to calculate the B/S ratio was performed followed by an analysis of covariances (ANCOVA) to determine whether the two pools of B/S ratios (experimental and authentic) were significantly different from each other. The analyses were performed independently for each of the two analytes using SPSS Version 16.0.1 (SPSS, Chicago, IL).

Results

Method validation

The extraction efficiency for MDMA and MDA was always > 95% of the spiked concentration, for blood and serum. Linearity was given within the calibration range, with correlation coefficients r > 0.995 for both MDMA and MDA in blood and serum. All calibrators were within a 10% range of the target concentration; standard deviations were below 7%. The LLODs were 5.5 and 5.6 ng/mL for MDMA and 1.9 and 2.9 ng/mL for MDA in blood and serum, respectively. Findings where the concentrations or either blood or serum or both were below the LLOD were not considered for calculations; those with concentrations between the LLOD and the LLOQ were labeled "positive" but not considered for calculations either. Ion suppression/enhancement and carryover could not be observed, and analytes' concentration did not decrease within 24 h.

Correlation between hematocrit and B/S ratio (supplemented samples)

B/S ratios for MDMA and MDA increased with increasing hematocrit. For MDMA, the ratios ranged from 1.00 to 1.42

(mean: 1.26, SD: 0.09), for MDA from 0.98 to 1.41 (mean: 1.15, SD: 0.08). Linear regression analyses through the origin predicting the whole blood value for a given value of serum revealed regression coefficients of 1.22 (SD: 0.03, R^2 = 0.992) for MDMA and 1.15 (SD: 0.02, R^2 = 0.993) for MDA. Adding the constraint that a value of zero in serum must coincide with a value of zero in whole blood leads to more clearly robust estimates of B/S ratios.

Authentic samples

MDMA concentrations in the 63 authentic samples ranged from not detectable to 310 or 236 ng/mL in blood or in serum, respectively. For all samples where MDMA could be determined in the serum, it was also present in the blood, and vice versa. Two samples showed concentrations between the LLOD and the LLOQ in both serum and blood. The B/S ratio for MDMA ranged from 0.84 to 1.35, the average being 1.16 ± 0.13. Of the total 42 samples in which MDMA could be determined and quantified, 36 (86%) showed a B/S ratio > 1.0, and for 6 samples (14%), the ratio was < 1.0. A simple linear regression through the origin predicting the whole blood value for a given value of serum revealed a regression coefficient of 1.26 (SD: 0.02, R^2 = 0.981) for MDMA. Figure 1 shows a scatter plot with blood and corresponding serum concentrations in supplemented and authentic samples.

The MDA concentration was between the LLOD and the LLOQ in 27 serum samples, whereas for the blood samples, a positive finding could be obtained in 15 cases. MDA concentrations were up to 10.4 ng/mL in blood and 16.3 ng/mL in serum. All 10 samples from which MDA could be determined and quantified had a B/S ratio > 1.0. The average B/S ratio was 1.27± 0.20 (range: 1.01–1.77). A simple linear regression through the origin predicting the whole blood value for a given value of serum revealed a regression coefficient of 1.27 (SD: 0.06, R^2 = 0.953) for MDA.

Comparison of supplemented and authentic samples

The B/S ratios were calculated by means of regression for both studies (supplemented and authentic samples). For MDMA, the regressions provided slopes (B/S ratios) of 1.22 (confidence interval 1.16; 1.28) and 1.26 (confidence interval 1.22; 1.30) for the supplemented and the authentic samples, respectively. For MDA, the analysis determined slopes of 1.15 (confidence interval 1.11; 1.19) and 1.27 (confidence interval 1.15; 1.39) for the supplemented and authentic samples, respectively.

An ANCOVA revealed that the method of determination (experimental vs. authentic) did not influence the resulting slopes. The differences in slope were 0.03 for MDMA and 0.12 for MDA; neither of them turned out to be significant (MDMA: p = 0.358, not significant; MDA: p = 0.925, not significant).

Discussion

The B/S ratio for MDMA and MDA using an experimental setting or authentic human samples has not yet been determined, to the authors' knowledge. De Letter et al. (18) reported MDMA blood to plasma (B/P) ratios in rabbits to range, depending on the time of sampling after intravenous application, between 1.2 and 1.3, which comes very close to the values obtained in the present study. Nonetheless, it is disputable whether B/P ratios in rabbits after i.v. application can be compared to those in humans after administration of MDMA by the oral route.

The present study revealed B/S ratios > 1 for both MDMA and MDA and slightly lower distribution ratios for MDMA than for MDA, no matter whether results were derived from supplemented or authentic samples. A B/S ratio > 1 demonstrates an additional binding to, or greater solubility in, the red blood cell than can be accounted for simple distribution in the subcompartments of blood. The plasma protein binding of MDMA and MDA is in a low-to-medium range of 34–40% (19) and thus favors distribution into erythrocytes. Statistical analysis showed, that MDMA and MDA partitioning under in vitro and in vivo conditions gave equivalent results that are in accordance with passive diffusion of the analytes as the underlying process.

A linear relationship between the hematocrit value and the B/S ratio of both MDMA [$y = 0.0097x + 0.8661$, x: hematocrit value (%), y: B/S ratio] and MDA ($y = 0.0083x + 0.8393$) could be established from the experimental data. The hematocrit values in blood drawn from healthy adults ranges from 35 to 54% depending on sex (20). Based on the previously mentioned relationship, B/S ratios are expected to range from 1.21 to 1.39 for MDMA and from 1.13 to 1.29 for MDA within the normal range. These estimates are quite in line with the range of B/S ratios covered by the authentic samples.

The concentration range found in the authentic samples (0–310 ng/mL; mean: 78.4 ng/mL; median: 45.6 ng/mL) turns out to be lower than that found in the blood of DUID suspects in a study by Jones et al. (21) (mean: 230 ng/mL; median: 100 ng/mL). This can be explained by the lower-range dosage chosen to minimize potential side effects even though the study participants had previously used MDMA.

In forensic work, serum or plasma is less often analyzed, and determination of the hematocrit value will generally not be performed. Instead, samples of whole blood are submitted for analysis, and these are often hemolyzed and sometimes contain clots. In our case, the hematocrit determination was not possible in the authentic samples because of beginning hemolysis, despite the fact that routine phlebotomy protocols were followed

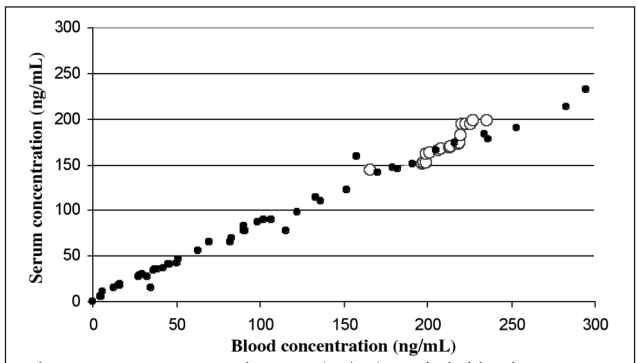

Figure 1. Concentration of MDMA (ng/mL) in whole blood specimens (hematocrit values between 26 and 45%) supplemented with 250 ng MDMA/mL and corresponding serum (open circles, n = 20) and authentic samples from a drug and driving study (black circles, n = 63).

during sample collection. Thus, the whole blood samples from the field study largely addressed the conditions of forensic specimens. ANCOVA statistical analysis confirmed the equivalency of the two methods; the MDMA serum concentration could thus be estimated from whole blood samples with adequate accuracy using a factor of 0.80. If it is advisable to consider inherent biological variations in the B/S relationship, for law enforcement purposes, for example, the SD or confidence intervals as indicated in the results section should be considered.

For MDA, this process bears certain crucial limitations. The concentrations determined from field study specimens were very low, within the first order of magnitude above the limit of detection. The concentration used in the in vitro setting was much higher in order to get reliable and quantifiable results. Although the experimental setting would suggest a factor of about 0.80 to calculate the serum concentration on the basis of a whole blood sample, the distribution of B/S ratios determined from the authentic samples was scattered over a rather large range of values (0.55–0.99). Nevertheless, reports of MDA concentration being 5–10% of the corresponding MDMA blood or serum levels could be confirmed, and statistical analysis using regression analysis followed by ANCOVA revealed that the two methods are, in fact, not significantly different from each other.

Conclusions

The B/S ratios of MDMA obtained in an experimental setting are not significantly different from those of healthy volunteers obtained during a controlled drug and driving study following administration of MDMA by the oral route. A high correlation between the hematocrit value and the B/S ratio of both MDMA and MDA could be established in the experimental setting. Dividing the concentration of MDMA in blood by 1.26 gives a reasonably good estimate of the coexisting concentration in serum. For MDA, higher blood levels would be needed to make reliable predictions.

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