Analysis of 3,4-Methylenedioxymetamphetamine: Whole Blood Versus Dried Blood Spots

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Abstract

Analysis of dried blood spots is an increasingly accepted method in therapeutic drug monitoring, whereas its application by analogy to forensic samples has not been studied in detail. Therefore, we investigated whether determination of 3,4-methylenedioxymethamphetamine (MDMA) and its main metabolite 3,4-methylenedioxyamphetamine (MDA) from dried blood spots (DBS) is as reliable as that from whole blood specimens. Analysis was performed by liquid chromatography–tandem mass spectrometry following liquid–liquid extraction of blood and corresponding DBS samples from 20 volunteers participating in a controlled driving experiment under the influence of MDMA. The assay was checked for carryover, ion suppression/enhancement, linearity of response, lower limits of detection (LLOD) and quantitation, extraction efficiency and the within-run and between-run assay imprecision for both whole blood and DBS. The LLODs were 2.0 and 1.6 ng/mL for MDMA in whole blood and DBS, respectively, using a volume of 100 µL. LLODs of MDA were determined to be 0.25 ng/mL in whole blood specimens and 0.12 ng/mL in DBS. Extraction efficiency and imprecision did not differ significantly between the two methods for both MDMA and MDA. The mean concentration ratio of corresponding whole blood and DBS samples, t-test, and the Bland-Altman difference plot were used to test hypothesis of equality. Statistical analyses revealed that methods did not significantly differ for MDMA or MDA. Thus, DBS analysis has potential as a precise and inexpensive alternative to whole blood analysis of MDMA.

Introduction

Blood is the specimen of choice to detect and quantify a drug concentration if there is suspicion of impairment or intoxication. In cases of driving under the influence of drugs (DUID) it is often necessary to wait for authorized medical personnel to collect an appropriate specimen. Thus, concentrations determined from these samples will not accurately reflect the concentration present in blood at the time of driving.

In 1913, Ivar Bang published a method for the determination of blood glucose in rabbits using dried blood spots (DBS) for analysis (1). DBS have been used for decades in neonatal metabolic screening (2,3). During the last 20 years DBS analysis has established itself as an accepted method in therapeutic drug monitoring (4). Given the advantages, especially with regard to the simple sampling, which can also be performed by non-medical personnel, DBS could be a suitable alternative for the determination of drugs from DUID cases.

United Nations Office on Drugs and Crime (UNODC) data from 2009 show that ecstasy-like drugs, including 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxyethylamphetamine, are used by approximately 12–24 million people worldwide (5). MDMA consumption affects the ability of risk assessment and causes a lack of balance and motor coordination. Because of ecstasy's effect on the cognitive performance related to driving (6,7), detection of the drug from blood specimens is essential in DUID cases (8).

MDMA is metabolized via N-demethylation to MDA, which is pharmacologically active. MDA concentrations found in blood or serum samples are about 5–10% of the corresponding MDMA levels (9).

The aim of the present study was to investigate whether quantitative analysis of MDMA and MDA concentrations in DBS is equivalent and may be an alternative to the respective determination from whole blood specimens.

Materials and Methods

Authentic samples

Blood and corresponding DBS specimens from 20 healthy volunteers in a 4-condition cross-over driving experiment were provided by the University of Groningen, The Netherlands.
Blood spots were prepared by spotting a 100-µL aliquot of blood onto filter paper of a customized collection device manufactured from Whatman 903 specimen collection paper (GE Healthcare, Dassel, Germany), which were subsequently dried at room temperature over night. Then, each sample was packed in a plastic bag together with a desiccant pack and stored at ambient temperature (20–24°C), whereas whole blood samples were kept frozen (–20°C) until analyzed. Analysis was performed by liquid chromatography–tandem mass spectrometry (LC–MS–MS) following liquid–liquid extraction of both media.

This study was conducted according to the code of ethics on human experimentation established by the Declaration of Helsinki (latest revision, Seoul 2008). Approval of the protocol was obtained from the Ethics Committee of the University Medical Center of Groningen, The Netherlands, and all participants were screened and provided written informed consent before entering the study.

Samples were taken on 4 different occasions at 1.5-h intervals after oral administration of either 100 mg MDMA or placebo. As 5 participants provided blood on only 3 of the 4 occasions, the final number of samples enrolled in analysis of both blood and respective DBS was 75 specimens.

**Materials**

Deuterated and undeuterated MDMA and MDA were obtained from LGC (Wesel, Germany). High-pressure liquid chromatography (HPLC)-grade acetonitrile, methanol, ethyl acetate (≥ 99.5%), solid NaOH (99%), ammonium acetate (≥ 98%), and acetic acid (100%) were purchased from Roth (Karlsruhe, Germany). HCl (25%) was from Merck (Darmstadt, Germany); and double distilled water was supplied by Braun (Melsungen, Germany).

Drug-free whole blood was obtained from the local blood bank of the University Hospital of Heidelberg (Germany).

**Analysis of MDMA and MDA from DBS and whole blood by LC–MS–MS**

Because of the broad concentration range of MDMA and MDA present in the samples, two different calibration lines were prepared for DBS and blood separately: the lower calibration standards A contained 5, 10, 20, 30, and 40 ng/mL MDMA and 0.25, 0.5, 1, 2, and 3 ng/mL MDA and calibration standards B contained 50, 100, 200, 300, and 400 ng/mL MDMA and 2.5, 5, 10, 20, and 30 ng/mL MDA.

DBS calibrators extracted in each batch were prepared by spotting 100 µL of fortified blood onto filter paper which was dried at room temperature over night. DBS were cut out with a punch (18-mm diameter) and transferred into plastic tubes. Extraction of DBS was performed in the same way as for whole blood specimens and corresponding calibrators.

One milliliter of 0.01 M NaOH and deuterated standards (calibration A: 20 ng/mL MDMA-d₅ and 2 ng/mL MDA-d₅; calibration B: 200 ng/mL MDMA-d₅ and 20 ng/mL MDA-d₅) were added to a 100-µL aliquot of each sample or calibrator. Supplemented samples were extracted with 1.5 mL ethyl acetate and centrifuged (10 min, 4300 × g). The organic layer was transferred to a silanized vial, acidified with 50 µL of methanolic hydrochloric acid (MeOH/HCl, 49:1, v/v), evaporated to dryness under nitrogen at 40°C and reconstituted with 50 µL of the mobile phase (4 mM ammonium acetate buffer pH 3.2/methanol/acetonitrile, 60:8:32, 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). It was interfaced to an HPLC pump equipped with an autosampler (1100 series, Agilent, Waldbronn, Germany). The samples (5-µL aliquots) were eluted from a Zorbax Eclipse XDB-C₈ column (2.1 × 150 mm, 5-µm particle size, Agilent, Waldbronn, Germany) at a flow rate of 220 µL/min. Data were monitored with the following transitions: MDMA m/z 194 → 163* and 194 → 135; MDMA-d₅ m/z 199 → 165; MDA m/z 180 → 163* and 180 → 135; MDA-d₅ m/z 185 → 168. (Transitions marked with an asterisk were used for quantitation.)

Calibration lines were constructed with linear least squares regression using the ratio of the target analyte peak area to the corresponding internal standard peak area. In addition, imprecision, extraction efficiency and bench top stability (24 h) were investigated according to the FDA Guidance for Industry (10). Carryover was checked as described by Bansal and DeStefano (11). Ion suppression or enhancement were determined according to Matuszewski et al. (12). The lower limits of detection (LLOD) and quantitation (LLOQ) were estimated from the calibration curves according to the ICH Guideline for Validation of Analytical Procedures (13).

Data analysis was done using Microsoft Excel. A t-test was performed to test whether the analyte concentrations in whole blood and corresponding DBS are significantly different or not; the threshold chosen for statistical significance was 0.05. Agreement of the two methods was further assessed by a Bland-Altman plot (14).

**Results**

**Evaluation of the analytical assay**

No ion suppression/enhancement or carryover was observed. MDMA and MDA concentrations of extracted samples did not decrease within 24 h at ambient temperature. Extraction efficiencies of MDMA and MDA were always higher than 85% and 95%, respectively, of the spiked concentration for both media. Linearity was assessed for the high and low calibration curves of whole blood and DBS, respectively, with coefficients of determination > 0.995. All calibrators were within a 10% range of the target concentration; standard deviations were below 7%. LLODs were determined to be 2.0 and 1.6 ng/mL for MDMA and 0.25 and 0.12 ng/mL for MDA in blood and DBS. LLOQs were 7.0 and 5.7 ng/mL for MDMA and 0.93 and 0.40 ng/mL for MDA in blood and DBS, respectively. Findings where the concentrations in blood or DBS or both were below the LLOQ were not considered for calculations; those with concentrations between the LLOD and the LLOQ were labelled “positive” but not considered for calculations either.

Within-run imprecision of MDMA analysis ranged from 2.46 to 2.79% in whole blood samples and from 2.83 to 3.21% in DBS specimens, respectively. Between-run imprecision was
determined to be from 2.82 to 3.32% in whole blood and 3.88 to 4.99% in DBS. For MDA, within-run imprecision was estimated to range from 2.23 to 2.89% in whole blood and from 2.29 to 3.91% in DBS. Between-run imprecision was 2.88–3.60% and 2.29–4.93% in whole blood and DBS, respectively.

A comparison of the results determined from either whole blood or DBS
Quantitative results of MDMA were obtained from 35 whole blood samples as well as from 35 DBS specimens. Concentrations ranged from 11.46 to 444.25 ng/mL (mean 182.89 ng/mL, median 174.75 ng/mL) in whole blood and from 11.07 to 447.50 ng/mL (mean 186.44 ng/mL, median 173.65 ng/mL) in DBS, respectively. MDA was quantifiable in 30 whole blood specimens and 34 DBS samples with concentrations ranging from 1.12 to 24.44 ng/mL (mean 9.39 ng/mL, median 9.64 ng/mL) in whole blood and from 0.54 to 24.10 ng/mL (mean 8.36 ng/mL, median 7.92 ng/mL) in DBS.

The mean ratio of MDMA concentrations determined from whole blood samples to that determined from corresponding DBS specimens was 0.99 (relative standard deviation 2.41%) and ranged from 0.92 to 1.03. For MDA, the mean ratio was 0.99 (relative standard deviation 5.52%) with a range of 0.92 to 1.18.

A paired t-test was used to determine whether a difference between mean values determined from blood or DBS exists or not. The absolute test value of MDMA was 0.91, compared with the critical value of 2.00. An absolute test value of 0.53 could be calculated for MDA, which is far below the critical value of 2.00.

The differences between MDMA and MDA values from DBS and whole blood were further analyzed using the Bland-Altman difference plot (Figure 1) (14). For MDMA, the mean difference between the methods calculated from 35 paired whole blood and DBS specimens was –3.55 ng/mL (SD 5.15 ng/mL); and, provided that the differences are normally distributed, 95% of all differences lie between the limits of agreement (mean ± 1.96 SD, –14.34 and 7.25 ng/mL). The mean difference of MDA between the methods calculated from 30 paired whole blood and DBS specimens was 0.02 ng/mL (SD 0.70 ng/mL), and the limits of agreement were –1.36 and 1.40 ng/mL.

Discussion

DBS analysis represents a simple and low-cost method for the determination of different drugs in blood samples. It enables blood sampling from subjects with limited venous access or by non-medical personnel, which significantly reduces the time between suspicion and blood sampling resulting in a sample that will more closely reflect actual impairment. It is quite possible to use capillary instead of venous blood (15). If so, the magnitude of variation may be increased by sampling capillary instead of cubital-vein blood; a phenomenon that has been studied in detail for ethanol (16). In contrast to whole blood specimens, DBS can be sent by regular mail because their shipment is possible in sealed envelopes with desiccant packs, but without requiring leak-proof containers or additional cooling. Additionally, storage of DBS is very space saving in comparison with whole blood samples.

In forensic case work, whole blood is still the most often analyzed matrix despite of the risk of infections with HIV or other blood-borne viruses. A great benefit of DBS compared to a whole blood specimen is the decreased risk of infections. For example, HI-viruses and the hepatitis C virus lose their infectivity in DBS, because drying leads to a disruption of their envelope (17). Analytes in DBS are also less prone to degradation by hydrolysis because of the absence of water molecules (18,19). Hence, DBS can be stored frozen or at ambient temperature but protected from light and humidity for up to several years without the extensive degradation that can occur in whole blood specimens (20).

At present, DBS analyses have been published for a very few substances of forensic interest. Elian (21) published a method for the detection of flunitrazepam and its metabolites in DBS using 1-mL DBS samples. Henderson et al. (22) detected benzoylecgonine in residual DBS samples from newborn screenings via radioimmunoassay. In 2001, benzoylecgonine, ephedrine methyl ester, and cocaine were quantified in DBS samples using GC–MS (18). Schuetz et al. (23) used bloodstains found at the scene for detection of drugs in criminal cases and thus
could give crucial information on the chronological order of the events. Determination of morphine and 6-acetylmorphine from DBS has already been proven to be congruent to the measurement in corresponding whole blood samples (24).

The present investigation points once more to the potential of DBS analysis of forensic samples. MDMA and MDA could be extracted, identified, and quantified from DBS as rapidly and precisely as from whole blood samples. The small sample volume used for DBS analysis may give rise to higher LLOQs and LLODs than in whole blood analysis, which uses sample volumes of about 500 µL (25). This is not a disadvantage in DUID case analysis, for impaired drivers show MDMA blood concentrations of 50–580 ng/mL (8), which are significantly higher than the LLOQ determined for the present method. Also, LC–MS–MS allows sensitive measurement, leading to lower LLODs (26). Within-run and between-run imprecision did not differ significantly for either MDMA or MDA analysis. There is evidence from the means of the two methods that determination of MDMA and MDA in DBS closely fits that from whole blood specimens.

The blood/DBS ratio should ideally be 1.0 if the two methods are expected to agree exactly. The present study revealed blood/DBS ratios of 0.99 for both MDMA and MDA, which shows that the two methods give comparable results. In addition, the low relative standard deviations (SD) show the small differences in pairs.

A trend of the differences over the whole concentration range could not be observed for either MDMA or MDA. The mean difference (bias) of MDMA concentrations estimated from analyses of whole blood and corresponding DBS samples was –3.55 ng/mL or 1.94% of the mean value determined from whole blood, which means that determination of MDMA from DBS gives a slightly higher reading compared to that from whole blood. This small difference, however, may not cause problems in forensic interpretation. The calculated SD of the mean difference as a degree of the random error is also likely to be satisfactory.

The bias of the MDA results determined from the two different media was so small, 0.02 ng/mL or 0.17% of the mean value determined in whole blood specimens, that it will not affect interpretation of the analytical results. Again, an SD of the mean difference of 0.70 ng/mL or 7.50% of the mean value of all whole blood specimens indicates that individual measurements of DBS and respective whole blood samples are not far apart.

Conclusions

Estimation of blood/DBS ratios of MDMA and MDA as well as the Bland-Altman plot show that the methods are not significantly different from each other. The differences were small enough to be confident of DBS analysis as a precise and inexpensive option for the determination of MDMA and MDA from a small sample volume. With respect to the mentioned advantages of DBS analysis, determination of further analytes of forensic interest will be established.

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References


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