**Article**

# Determination of MDMA and Its Three Metabolites in the Rat Perfused Liver

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**3,4-Methylenedioxymethamphetamine (MDMA) is one of the most commonly abused illicit drugs in the world. We developed a rapid and simple high-performance liquid chromatography with a fluorescence (FL) detector method to determine MDMA and its metabolites, such as 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxyamphetamine (HMA) and its main unstable metabolite 3,4-dihydroxymethamphetamine (HHMA) besides the internal standards, in a perfusion medium. The separation of analytes was performed at 25°C on a Chromolith® C18 (100 × 4.6 mm) column from Merck (Darmstadt, Germany) without any derivatization. The FL detector wavelength was fixed at 285 nm for excitation and at 320 nm for emission. Acetonitrile:phosphate buffer (0.02 M) at pH = 3 (5:95 v/v) was used as the mobile phase. The elution order was HHMA, HMA, MDA and MDMA with a retention time of 1.7, 2.6, 6.1 and 7.4 min, respectively. The method was validated according to the FDA bioanalytical method validation guideline. The limits of quantifications (LOQs) obtained for MDMA, MDA, HMA and HHMA were 1, 1, 1.5 and 5 ng/mL, respectively. The repeatability of relative standard deviation was <11% (except for LOQs). This method was applied successfully to determine MDMA and its metabolites in rat liver perfusion samples. To our knowledge, this is the first method introduced for the determination of HHMA as a free form with an FL detector.**

## Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) was first introduced to physicians as an appetite suppressant in 1940s. However, in 1980s, its usage was forbidden in many countries due to its fatal side effects (1, 2). MDMA is a releaser and presynaptic reuptake inhibitor of serotonin and dopamine. Moreover, it is a potent agonist of trace amine-associated receptor 1 (3). This compound, due to its psychotropic and entactogenic effects such as euphoria, increasing sociability, well-being and stimulation (2), is one of the most commonly abused drugs in the world (4). The use of this compound has increased among young people over the past two decades (1, 5).

*In vitro* and *in vivo* studies revealed that MDMA is metabolized through two main pathways via cytochrome P450 isoenzymes: O-demethylation, which produces 3,4-dihydroxymethamphetamine (HHMA), and N-dealkylation, which produces 3,4-methylenedioxyamphetamine (MDA). CYP2D6 is the main isoenzyme in O-demethylation. CYP1A2 and CYP3A4 play a minor role in this biotransformation. CYP1A2 and CYP2B6 are the most important isoenzymes in the N-dealkylation procedure (2, 6). HHMA is further metabolized via catechol-O-methyltransferase to form 4-hydroxy-3-methoxyamphetamine (HMA). HMA can also be produced from MDA to a small extent (7, 8).

*In vitro* studies showed that HHMA is the main metabolite (up to 70%) of MDMA with a catecholamine structure (9, 10). Studies revealed that MDMA itself is not neurotoxic. It seems that HHMA is responsible for MDMA cell toxicity in abusers. HHMA causes apoptosis and necrosis in rat hippocampal neurons (7, 9). However, data on its role are contradictory. Only few studies have been published about the determination of HHMA in serum, urine and water using high-performance liquid chromatography/electrochemical detection (HPLC/ED) (7), liquid chromatography/mass spectrometry (LC/MS) (6) and capillary electrophoresis methods (11).

MDA, which is also known as "Love Drug," has effects similar to those of MDMA on abusers (12). This compound is the most important and reported metabolite of MDMA in various human fluids, such as blood, serum, urine, vitreous humor (1, 13), oral fluid (5), hair (12) and sweat (8).

Different combinations of MDMA and its metabolites have been determined via various chromatographic methods. The high-tech chromatographic methods such as LC/MS (2, 6, 14) or gas chromatography/mass spectrometry (GC/MS) (8, 15) are sensitive and specific. The applications of these methods have encountered some limitations. The LC/MS is very expensive and every lab cannot afford its expenses. The determination of MDMA and its metabolites via GC/MS needs derivatization prior to analysis, which is time consuming and complicated. The HPLC system equipped with an ultraviolet (UV) detector has low sensitivity. However, HPLC with a fluorescence detector (HPLC/FL) is accessible in almost all labs and has a reasonable sensitivity for the determination of MDMA and its metabolites without derivatization. Buechler *et al.* (10) developed an HPLC/FL method without derivatization in human plasma for the determination of MDA, and Clauwaert *et al.* (13) did it in whole blood, serum, vitreous humor and urine for the determination of MDMA and MDA.

To the best of our knowledge, there is no report of simultaneous determination of HHMA besides MDMA, MDA and HMA as free forms in physiological fluids. However, the determination of the HHMA-free form in various physiological fluids is important to establish the contribution of HHMA to MDMA neurotoxicity in humans. In this study, we developed a specific, sensitive, rapid and simple HPLC/FL method for the simultaneous determination of MDMA, MDA, HMA and HHMA in a perfusion medium. This method was applied to real rat liver perfusion samples after validation.

## Materials and methods

### Chemicals

Acetonitrile and methanol of HPLC grade and all other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from Millipore Direct-Q system. MDMA, MDA, HMA and HHMA, along with four

proposed compounds (1-(3,4-dimethoxyphenyl)-*N*-methylpropan-2-amine (**1a**), 1-(3,4-dimethoxyphenyl)-*N*-methylbutane-2-amine (**2a**), 1-(4-methoxyphenyl)propan-2-amine (**3a**) and 1-(benzo[d][1,3]dioxol-5-yl)-*N*-ethylpropan-2-amine (MDEA, **4a**)) as an internal standard (IS) (Figure 1), were synthesized in the medicinal chemistry department, Faculty of Pharmacy, Tehran University of Medical Sciences, according to a modified, previously reported method that showed the acceptable purity when compared with standard samples purchased from Lipomed Pharmaceutical (Switzerland) (16). The structure of the compounds was confirmed by IR, $^1$H-NMR and $^{13}$C-NMR spectra.

### Apparatuses and chromatographic conditions

The Knauer chromatographic system used consisted of a pump model K-1001, a fluorescence detector model RF-10AXL, a solvent degasser and a 100 μL loop injector. EZChrom Elite software was used for instrument control, data acquisition and analysis. The separation of analytes was performed at ambient temperature (25°C) on the Chromolith® Performance RP-18e (100 × 4.6 mm) column with Chromolith® RP-18e (5 × 4.6 mm) guard from Merck (Darmstadt, Germany). The FL detector wavelength was fixed at 285 nm for excitation and at 320 nm for emission.

### Standard and biological sample preparation

The 1 mg/mL methanolic solutions of MDMA, MDA, HMA, HHMA and ISs were prepared as working standards.

Analytical samples were prepared by dilution of working standards in water and in the perfusion medium. These analytical samples were centrifuged at 14,462 g for 15 min and diluted in the mobile phase where necessary.

The quality control samples were prepared separately from working standards at three different concentrations (limits of quantification (LOQ), 10 and 50 ng/mL) in water and in the perfusion medium for HHMA, MDMA, MDA, HMA and ISs.

Rat liver perfusion samples (500 μL) were centrifuged at 14,462 g for 15 min. The supernatants were then transferred and stored at −20°C. Samples were diluted in the mobile phase where necessary.

### Method development

Method development involved the investigation of flow rates (1–2 mL/min) and mobile phase constitution. As a mobile phase, either acetonitrile or methanol in varying ratios (v/v) was added to potassium dihydrogen phosphate, sodium acetate and ammonium acetate (0.02–0.04 M; pH = 3), separately. Orthophosphoric acid and acetic acid were used for pH adjustment in the case of phosphate buffer and acetate buffer, respectively.

### Method validation

The method was validated for intra- and inter-day precision, accuracy, limits of detection (LOD), LOQ, linearity, recovery and stability according to the FDA bioanalytical method validation guideline (17).
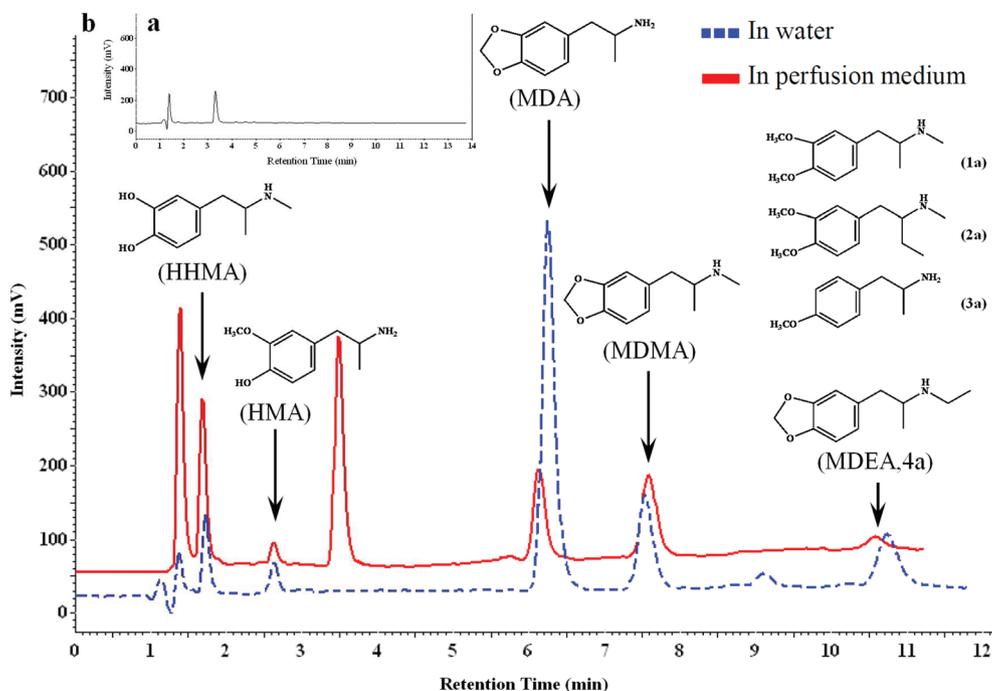


**Figure 1.** (a) The HPLC/FL chromatogram of the blank perfusion medium which was passed through the isolated liver for 30 min. (b) The HPLC/FL chromatogram of MDMA, MDA, HMA, HHMA and MDEA (as IS) in water and perfusion medium along with their structures. The concentrations in water for MDMA, MDA, HMA, HHMA and MDEA are ~18, 50, 10, 25 and 20 ng/mL, respectively. The concentrations in perfusion medium for MDMA, MDA, HMA, HHMA and MDEA are ~15, 15, 6, 34 and 5 ng/mL, respectively. The structures of proposed compounds as an IS are also demonstrated: (1-(3,4-dimethoxyphenyl)-*N*-methylpropan-2-amine (**1a**), 1-(3,4-dimethoxyphenyl)-*N*-methylbutan-2-amine (**2a**), 1-(4-methoxyphenyl)propan-2-amine (**3a**) and 1-(benzo[d][1,3]dioxol-6-yl)-*N*-ethylpropan-2-amine (**4a**, MDEA)).

For intra-day precision, the relative standard deviation (RSD) of five replicates at LOQ, 10 and 50 ng/mL for MDMA, MDA and HMA in the perfusion medium and for HHMA in water was calculated. The RSD of these concentrations was calculated for five replicates over 3 days to establish inter-day precision.

The difference between the true value and the measured concentration of five replicates at LOQ, 10 and 50 ng/mL for MDMA, MDA and HMA in perfusion medium and for HHMA in water was used for the determination of accuracy.

For linearity study, the calibration curves were constructed for MDMA, MDA and HMA in a perfusion medium and for HHMA in water at concentrations between LOQ and 50 ng/mL, by linear regression, without weighting. Peak areas were used to construct the calibration curves.

The LOD was defined as the lowest concentration of analytes that produced a response three times higher than the background noise. The LOQ was also defined as the lowest concentration of analytes that produced a response 10 times higher than the background noise.

The stability of the samples (LOQ, 10, 50 and 100 ng/mL) of MDMA, MDA, HMA, HHMA and ISs in water or perfusion medium were studied at $-20°C$ for 3 months, at $4°C$ for 3 days and at ambient temperature $(25°C)$ for 24 h. The obtained results were compared with those obtained from the freshly prepared samples.

### Method application

This method was applied for the determination of MDMA and its three metabolites in rat liver perfusion samples.

The Krebs buffer (6.903 g/L NaCl, 0.335 g/L KCl, 0.162 g/L $KH_2PO_4$, 0.163 g/L $MgSO_4$, 0.305 g/L $CaCl_2$, 2.1 g/L $NaHCO_3$ and 1 g/L glucose, the pH was adjusted to 7.2–7.4) was used as a perfusion medium. The JMS perfusion pump model OT-711 was used for the circulation of the buffer through the isolated rat liver with the flow of 10 mL/min. During the perfusion period, the buffer was oxygenated by 95% $O_2$ + 5% $CO_2$, and the buffer temperature was kept at $37 \pm 1°C$.

A male Sprague–Dawley rat with a weight of 328 g was used. It was housed in an animal care room of the Faculty of Pharmacy, Tehran University of Medical Sciences. It was kept at $25 \pm 1°C$ temperature with 12 h light/dark cycle and had free access to food and water. The rat was anesthetized by IP injection of a ketamine/xylazine mixture (ketamine 100 mg/kg and xylazine 10 mg/kg). An IV cannula was introduced into the portal vein and inferior vena cava. The liver was washed with a perfusion medium for 10 min. After the washing period, 200 ng/mL solution of MDMA in a perfusion medium was circulated in the isolated rat liver for 180 min. The perfusion was done in a recycle mode, and samples were taken at the appropriate time (0.1, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150 and 180 min) from the inferior vena cava and analyzed. The study protocol was performed according to the animal ethical codes of the Faculty of Pharmacy, Tehran University of Medical Sciences.

## Results and discussion

### Method development

The retention time and area under the curve (AUC) of analytes peaks were affected by the flow rate changes. In flow rates >1.5 mL/min, the HHMA and HMA were not separated reasonably from interfering peaks. When the flow rate increased, the AUC of analytes peaks decreased. This effect was in accordance with the Chen *et al.* report (18). Substituting potassium dihydrogen phosphate with sodium or ammonium acetate slightly changed the retention time of analytes. Methanol had a similar effect with acetonitrile on the separation of analytes, in higher ratios (about two times of acetonitrile ratio). When the ratio of acetonitrile in mobile phase increased, from 5 to 10% (v/v), the time of analysis decreased from 7.4 to 6.2 min. However, the HHMA peak was not separated properly from the solvent peak. If the acetonitrile component was removed from the mobile phase, the HHMA peak separated from the solvent peak reasonably, but the run time exceeded 20 min. The results showed that the mixture of potassium dihydrogen phosphate (0.02 M) at pH = 3 and acetonitrile (95:5 (v/v)), with the flow rate of 1.5 mL/min in isocratic mode, can separate MDMA and its metabolites properly. Changing the ratio of acetonitrile to 6 or 4% (v/v) did not affect the separation of analytes significantly. In the selected chromatographic condition, the elution order of analytes was HHMA, HMA, MDA and MDMA. The separation of MDMA and its metabolites was performed in <8 min, which was shorter than the previously reported HPLC/FL methods (10, 13). Among the four compounds proposed as the IS, only compounds **1a** and **4a** had a suitable retention time of 12.7 min and 10.8 min, respectively (Figure 1). The selected ISs had the most structural similarity to the analytes; structural similarity is one of the most important criteria in IS selection.

### Method validation

The validation parameters related to inter- and intra-day precision, recovery, linearity, accuracy, LOD and LOQ for MDMA, MDA and HMA in the perfusion medium and for HHMA in water are presented in Table I. The repeatability RSDs for these analytes were <11% (except at the LOQ level).

**Table I**

Validation Parameters for MDMA, MDA and HMA in Perfusion Medium and for HHMA in Water

|  | HHMA | HMA | MDA | MDMA |
|---|---|---|---|---|
| Calibration range (ng/mL) | 5–50 | 1.5–50 | 1–50 | 1–50 |
| Calibration points | 7 | 7 | 7 | 7 |
| Correlation coefficient ($r$) | 0.994 | 0.996 | 0.996 | 0.991 |
| Slope | 19982.5 | 56512.5 | 196119.6 | 181211.2 |
| Intercept | 4493.1 | −14981.6 | −49749.2 | 258234.6 |
| Limit of quantification (LOQ) (ng/mL) | 5 | 1.5 | 1 | 1 |
| Limit of detection (LOD) (ng/mL) | 1.5 | 0.5 | 0.3 | 0.3 |
| Recovery (%) | 96 | 99 | 97 | 99 |
| Precision (RSD) |  |  |  |  |
| Intra ($n = 5$) |  |  |  |  |
| 50 (ng/mL) | 7.9 | 5.0 | 2.5 | 10.2 |
| 10 (ng/mL) | 8.0 | 8.5 | 8.7 | 9.7 |
| LOQ | 11.7 | 13.2 | 16.3 | 19.6 |
| Inter ($n = 15$) |  |  |  |  |
| 50 (ng/mL) | 6.3 | 5.1 | 3.2 | 5.9 |
| 10 (ng/mL) | 9.4 | 5.9 | 5.3 | 13.6 |
| LOQ | 9.5 | 15.4 | 18.9 | 19.8 |
| Accuracy (%) |  |  |  |  |
| Intra ($n = 5$) |  |  |  |  |
| 50 (ng/mL) | 2.1 | 0.6 | 1.1 | 0.8 |
| 10 (ng/mL) | 2.4 | 4.2 | 8.3 | −0.4 |
| LOQ | 1.0 | 9.6 | 6.9 | 2.1 |
| Inter ($n = 15$) |  |  |  |  |
| 50 (ng/mL) | −2.4 | 0.1 | −0.1 | −7.5 |
| 10 (ng/mL) | 0.6 | 5.4 | 0.5 | −3.0 |
| LOQ | 4.6 | 0.7 | 14.9 | 3.7 |
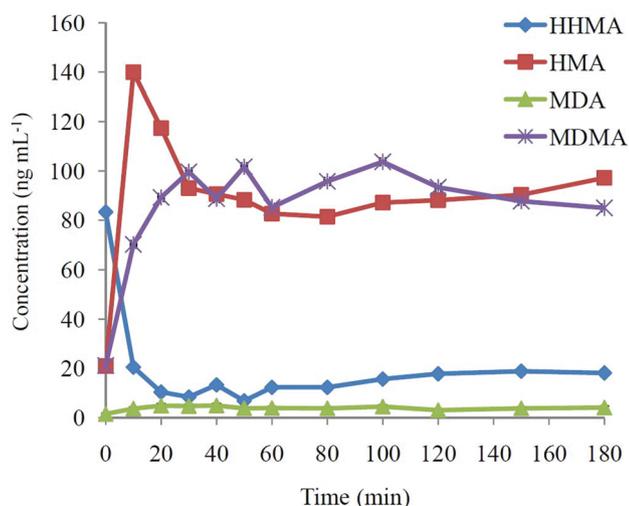
**Figure 2.** The concentration changes of MDMA and its metabolites when 200 ng/mL solution of MDMA in perfusion medium was recycled in the isolated rat liver for 180 min. Samples were taken at 0.1, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150 and 180 min from output cannula. The data of one rat was demonstrated as a sample.

There were no interfering peaks in the chromatogram of blank perfusion medium, blank perfusion medium which was passed through the isolated rat liver and spiked media with analytes (Figure 1).

The LOQs obtained for MDMA, MDA and HMA in this method were two times lower than the reported values in the GC/MS study by Scheidweiler and Huestis (15), whereas these values were comparable with those reported by LC/MS/MS (14, 19). This is the first time that HHMA has been determined via HPLC/FL. The previously reported LOQs for HHMA were 9 and 32 ng/mL for plasma and urine, respectively, which had been obtained by HPLC/ED (7). By this method, the LOQ obtained for HHMA was 5 ng/mL for the perfusion medium.

All calibration curves showed an acceptable linearity in the selected ranges. The differences between the slopes of calibration curves and zero were significant ($P < 0.05$) in all cases. Moreover, the intercepts of calibration curves showed no significant differences with zero ($P > 0.05$).

The samples (LOQ, 10, 50 and 100 ng/mL) of MDMA, MDA and HMA in water and in the perfusion medium were stable under all investigated conditions. No significant differences between the AUCs of freshly prepared and stored samples were observed ($P > 0.05$). The stability results for aqueous solutions of MDMA and MDA were in accordance with the results of Clauwaert *et al.* (1) and in contrast to the results published by Concheiro *et al.* (5), which reported MDMA and MDA degraded in water, and this degradation was not temperature dependent. The aqueous solutions of HHMA were also stable in all investigated conditions, while HHMA solutions in the perfusion medium were unstable. Our results indicated that, after 2 h, almost 100% of the HHMA was degraded at ambient temperature ($K_{degradation} = 0.037$ min$^{-1}$ $(0.030-0.047)$ at 25°C) and a new small peak appeared in the chromatogram at a retention time of ~3 min. This new peak separated reasonably from the peak of the HMA and, due to the very small area, did not interfere with the HMA. The reduction in the temperature to 0°C lowered the rate of degradation, and after 2 h, ~20% of the HHMA degraded

($K_{degradation} = 0.002$ min$^{-1}$ $(0.0006-0.003)$ at 0°C). For further investigation of this effect, we developed a separate well-designed study to clear the instability of HHMA in perfusion medium. The results of that new study (20) demonstrated that calcium cation and alkaline pH were the main causes of HHMA instability in perfusion medium.

### *Method application*

The perfusion was started by a 200 ng/mL concentration of MDMA, a concentration close to the MDMA maximum plasma concentration in humans who abused ecstasy pills (21). The real rat liver perfusion samples were analyzed and the data are shown in Figure 2. The MDMA concentration increased over the first 30 min and did not change until the end of the liver perfusion. The data indicated that 50% of MDMA changed to metabolites. The concentration of MDA, which is a minor metabolite, was ~4% of MDMA at the plateau phase. The maximum concentration of HMA was 140 ng/mL in the first 30 min of liver perfusion. However, its production decreased after this period. The reduction in the HHMA concentration may be due to its spontaneous degradation and/or to the reduction in the production of this metabolite. These data specified that after 30 min, the metabolism of MDMA (200 ng/mL) was reduced in isolated rat liver. Since liver failure is the most-addressed cause of MDMA fatality, this effect may be due to liver toxicity (22). On the other hand, this reduction in MDMA metabolism may be due to the reported auto-inhibition produced by this compound (23).

### Conclusion

To the best of our knowledge, the present method is the first that can determine HHMA as a free form, together with HMA, MDA and MDMA using HPLC/FL, without prior derivatization and extraction in physiological fluids. The shorter run time that was achieved by this method, compared with previously reported FL detection methods, can reduce the analysis cost. The reasonably selected ISs, suitable sensitivity and simplicity of this method enable researchers to use it for pharmacokinetic studies in isolated animal organs to reveal the unknown kinetics aspect of MDMA and its metabolites. The results of the perfusion study presented in this paper should be confirmed by another study with a higher rat population. This method can be used for the determination of these compounds in other media after some modifications.

### Acknowledgments

### References

1. Clauwaert, K.M., Van Bocxlaer, J.F., De Leenheer, A.P. (2001) Stability study of the designer drugs "MDA, MDMA and MDEA" in water, serum, whole blood, and urine under various storage temperatures. *Forensic Science International*, **124**, 36–42.
2. Shima, N., Kamata, H., Katagi, M., Tsuchihashi, H., Sakuma, T., Nemoto, N. (2007) Direct determination of glucuronide and sulfate

of 4-hydroxy-3-methoxymethamphetamine, the main metabolite of MDMA, in human urine. *Journal of Chromatography B*, **857**, 123–129.

3. Sotnikova, T.D., Caron, M.G., Gainetdinov, R.R. (2009) Trace amine-associated receptors as emerging therapeutic targets. *Molecular Pharmacology*, **76**, 229–235.

4. Mc Fadden, K., Gillespie, J., Carney, B., O'Driscoll, D. (2006) Development and application of a high-performance liquid chromatography method using monolithic columns for the analysis of ecstasy tablets. *Journal of Chromatography A*, **1120**, 54–60.

5. Concheiro, M., de Castro, A., Quintela, O., Lopez-Rivadulla, M., Cruz, A. (2005) Determination of MDMA, MDA, MDEA and MBDB in oral fluid using high performance liquid chromatography with native fluorescence detection. *Forensic Science International*, **150**, 221–226.

6. Meyer, A., Mayerhofer, A., Kovar, K., Schmidt, W.J. (2002) Enantioselective metabolism of the designer drugs 3,4-methylenedioxymethamphetamine ('ecstasy') and 3,4-methylenedioxyethylamphetamine ('eve') isomers in rat brain and blood. *Neuroscience Letters*, **330**, 193–197.

7. Segura, M., Ortuno, J., Farre, M., McLure, J.A., Pujadas, M., Pizarro, N. *et al.* (2001) 3,4-Dihydroxymethamphetamine (HHMA). A major in vivo 3,4-methylenedioxymethamphetamine (MDMA) metabolite in humans. *Chemical Research in Toxicology*, **14**, 1203–1208.

8. De Martinis, B.S., Barnes, A.J., Scheidweiler, K.B., Huestis, M.A. (2007) Development and validation of a disk solid phase extraction and gas chromatography–mass spectrometry method for MDMA, MDA, HMMA, HMA, MDEA, methamphetamine and amphetamine in sweat. *Journal of Chromatography B*, **852**, 450–458.

9. Felim, A., Neudorffer, A., Monnet, F.P., Largeron, M. (2008) Environmentally friendly expeditious one-pot electrochemical synthesis of bis-catechol-thioether metabolites of ecstasy: in vitro neurotoxic effects in the rat hippocampus. *International Journal of Electrochemical Science*, **3**, 266–281.

10. Buechler, J., Schwab, M., Mikus, G., Fischer, B., Hermle, L., Marx, C. *et al.* (2003) Enantioselective quantitation of the ecstasy compound (R)- and (S)-N-ethyl-3,4-methylenedioxyamphetamine and its major metabolites in human plasma and urine. *Journal of Chromatography B*, **793**, 207–222.

11. Wada, M., Nakamura, S., Tomita, M., Nakashima, M.N., Nakashima, K. (2005) Determination of MDMA and MDA in rat urine by semi-micro column HPLC-fluorescence detection with DBD-F and their monitoring after MDMA administration to rat. *Luminescence*, **20**, 210–215.

12. Martins, L.F., Yegles, M., Samyn, N., Ramaekers, J.G., Wennig, R. (2007) Time-resolved hair analysis of MDMA enantiomers by GC/MS-NCI. *Forensic Science International*, **172**, 150–155.

13. Clauwaert, K.M., Van Bocxlaer, J.F., De Letter, E.A., Van Calenbergh, S., Lambert, W.E., De Leenheer, A.P. (2000) Determination of the designer drugs 3, 4-methylenedioxymethamphetamine, 3,4-methylenedioxyethylamphetamine, and 3,4-methylenedioxyamphetamine with HPLC and fluorescence detection in whole blood, serum, vitreous humor, and urine. *Clinical Chemistry*, **46**, 968–1977.

14. Cheze, M., Deveaux, M., Martin, C., Lhermitte, M., Pepin, G. (2007) Simultaneous analysis of six amphetamines and analogues in hair, blood and urine by LC-ESI-MS/MS: application to the determination of MDMA after low Ecstasy. *Forensic Science International*, **170**, 100–104.

15. Scheidweiler, K.B., Huestis, M.A. (2006) A validated gas chromatographic–electron impact ionization mass spectrometric method for methylenedioxymethamphetamine (MDMA), methamphetamine and metabolites in oral fluid. *Journal of Chromatography B*, **835**, 90–99.

16. Pizarro, N., de la Torre, R., Farre, M., Segura, J., Llebaria, A., Joglar, J. (2002) Synthesis and capillary electrophoretic analysis of enantiomerically enriched reference standards of MDMA and its main metabolites. *Bioorganic and Medicinal Chemistry*, **10**, 1085–1092.

17. (2001) *FDA Guidance for Industry, Bioanalytical Method Validation*. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatory Information/Guidances/ucm070107.pdf. (19 September 2011, date last accessed).

18. Chen, C., Uckun, F.M. (2000) Highly sensitive liquid chromatography–electrospray mass spectrometry (LC–MS) method for the determination of etoposide levels in human serum and plasma. *Journal of Chromatography B*, **744**, 91–98.

19. Concheiro, M., Simoes, S.M., Quintela, O., de Castro, A., Dias, M.J., Cruz, A. *et al.* (2007) Fast LC–MS/MS method for the determination of amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB and PMA in urine. *Forensic Science International*, **171**, 44–51.

20. Jamali, B., Ardakani, Y.H., Rouini, M.R., Foroumadi, A., Amidi, S., Aghdamd, V.H. *et al.* (2013) Determination of the role of calcium on instability of neurotoxic metabolite of ecstasy by HPTLC-mass. *DARU Journal of Pharmaceutical Sciences*, **21**, 9.

21. Baumann, M.H., Zolkowska, D., Kim, I., Scheidweiler, K.B., Rothman, R.B., Huestis, M.A. (2009) Effects of dose and route of administration on pharmacokinetics of ( ± )-3,4-methylenedioxymethamphetamine in the rat. *Drug Metabolism and Disposition*, **37**, 2163–2170.

22. Kłys, M., Rojek, S., Woźniak, K., Rzepecka-Woźniak, E. (2007) Fatality due to the use of a designer drug MDMA (Ecstasy). *Legal Medicine*, **9**, 185–191.

23. Van, L.M., Swales, J., Hammond, C., Wilson, C., Hargreaves, J.A., Rostami-Hodjegan, A. (2007) Kinetics of the time-dependent inactivation of CYP2D6 in cryopreserved human hepatocytes by methylenedioxymethamphetamine (MDMA). *European Journal of Pharmaceutical Sciences*, **31**, 53–61.