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Mass Selective Detection of Amphetamine, Methamphetamine, and Related Compounds in Urine

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Abstract

A method is presented for the routine analysis of amphetamine, methamphetamine, and related compounds in urine with gas chromatography coupled with mass spectrometry operated in the selective ion monitoring mode. The analytes are isolated by liquid–liquid extraction and are derivatized with trifluoroacetic anhydride. 3,4-Methylenedioxy-methamphetamine-D_5 is employed as the internal standard. Standard solutions are prepared using spiked urine samples, which are subjected to all phases of sample preparation. Disposable deactivated glass containers are employed throughout the process.

Introduction

More than 10,000 urine specimens a year are tested in our laboratory for illicit drugs, with amphetamine and related compounds being found in samples most frequently. Screening for amphetamine and methamphetamine is carried out using polarization fluorescent immonoassay (AxSym, Abbott Diagnostics, Abbott Park, IL), whereas testing for 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxy-amphetamine (MDA), and 3,4-methylenedioxy-*N*-ethyl-amphetamine [MDE(A)] is accomplished by enzyme immunoassay (EIA) performed on a Selectra XL instrument (Vitalab, Vitalscientific NV, Dieren, the Netherlands). Positive screening results are confirmed with gas chromatography (GC)–mass spectrometry (MS) measurements in all cases.

The confirmatory test cut-off value for amphetamine and methamphetamine is 500 ng/mL each, as defined by the United States National Institute on Drug Abuse (NIDA) in the Federal Register (1), with methamphetamine considered positive only if the sample contains at least 200 ng/mL amphetamine at the same time. In our laboratory, however, GC–MS confirmatory test cutoff values for amphetamine and methamphetamine have been defined as low as 200 ng/mL. Yet, samples containing methamphetamine are rendered positive only if they show an amphetamine concentration higher than 200 ng/mL as well.

In the case of MDMA, MDA, or MDE(A), the guidelines of the NIDA recommend that samples be preliminarily considered positive if the screening procedure indicates a higher concentration than 1000 ng/mL for any of these compounds (2). In contrast to this, we have found it useful to perform the confirmatory analysis of samples that contain any of the substances at a higher concentration than 500 ng/mL. As NIDA has not published confirmatory test cut-off values for MDMA, MDA, and MDE(A), these concentrations have been defined as 300 ng/mL for each substance.

Our investigations have proved that a single liquid–liquid extraction allows the detection of amphetamine and related compounds in the concentration range of confirmatory test values. Because amphetamine and related compounds are labile, we prepared their trifluoroacetic (TFA) adducts prior to analysis, as they remain more stable throughout the whole process. Although the electron ionization (EI) spectra of underivatized compounds are identical in the case of a number of analytes, forming TFA adducts allows more selective detection and a verifiable conclusive identification. We also found it important that the column temperature be raised, as this contributes substantially to high resolution and lower interferences in the spectrum. Therefore, we increased the oven temperature at 40°C/min up to 170°C after the initial 2-min isothermal period. We noted that at higher temperatures the majority of the less volatile substances extracted from the urine would either be more rapidly eluted or better equilibrated on the capillary column.

Calibration solutions were prepared by spiking commercially available urine controls with our analytes. Pentadeutero-3,4-methylenedioxy-methamphetamine (MDMA-D_5) was used as the internal standard. The standard solution contained all the analytes, as well as the internal standard, at a concentration of 625 ng/mL in methanol. This allowed us to assess the recovery of analytes from the urine samples. Qualitative and quantitative analysis of ephedrine and norpseudoephedrine could be performed in the same way.

Initially, we attempted to use solid-phase extraction for sample cleanup but found that the eluted extracts contained water in all cases whose elimination proved to cause considerable technical difficulties. This led us to employ liquid–liquid extraction instead.

Experimental

Equipment

An Agilent 6890 GC with an Agilent 5973 MS was used (Agilent Technologies, Wilmington, DE). The analyzer was operated with an installed automatic sampler. The data system was an HP KAYAK XM 600 with an HP MS/MSD ChemStation A3.01 (Hewlett-Packard, Palo Alto, CA) and software called B.E.N. (a program designed to run on Windows Excel determining the analytical parameters of the GC–MS measurement) (3).

Analytical conditions

The GC was operated in the splitless mode with an injector temperature of 250°C and a transfer line temperature of 280°C. A 30-m HP-5MS capillary column (0.25-mm i.d. and 0.25-µm film thickness) was used. The temperature program consisted of an initial isothermal period of 2 min at 60°C, followed by a ramp to 170°C at a rate of 40°C/min, then to 270°C at 8°C/min, held for 1 min, to 300°C at 30°C/min, and held 1 min at this final temperature.

The run time was 22 min. Helium was used as carrier gas at a 1-mL/min flow rate. The MS was operated in the selective ion monitoring (SIM) mode at 70 eV of electron ionization energy, with an ion source temperature of 200°C and a guard temperature of 150°C. The instrument was autotuned daily using perfluorotributylamine. Information on the analyzed compounds is provided in Table I.

Materials

Amphetamine (100 µg/mL), methamphetamine (100 µg/mL), MDA (100 µg/mL), MDE(A) (100 µg/mL), MDMA (100 µg/mL), and MDMA-D_5 (100 µg/mL) were obtained from Cerilliant Company (Radian International, Austin, TX). TFAA was purchased from Sigma-Aldrich Hungary Kft. (Budapest, Hungary).

Blank human urine was obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals and solvents were acquired from Merck Kft (Budapest, Hungary).

Working solutions

Two hundred-microliter aliqouts of blank human urine were spiked with 4.0, 7.5, 12.5, and 30.0 µL of 10-µg/mL solutions of amphetamine, methamphetamine, MDA, MDE(A), and MDMA in order to obtain working solutions containing the agents at 200, 375, 625, and 1500 ng/mL concentrations. Twelve and a half microliters of a 10-µg/mL MDMA-D_5 solution was added to each working solution as the internal standard. A quality control sample was prepared by spiking 200 µL blank human urine with 12.5 µL of MDMA and MDMA-D_5 solutions (10 µg/mL). Drug-free control urine was analyzed as negative control in each assay. The working solutions were prepared daily and stored in 2-mL deactivated vials.

To investigate recovery, a methanol solution containing 625 ng/mL amphetamine, methamphetamine, MDA, MDE(A), MDMA, and the internal standard MDMA-D_5 was used.

Extraction procedure

Two hundred microliters of urine was placed in a 2-mL deactivated vial and spiked with 12.5 µL of the internal standard MDMA-D_5 solution (10 µg/mL). To this, 50 µL of 0.1 mol/L NaOH and 1 mL of ethyl acetate were added. The vial was sealed airtight, and the mixture was vortexed for 60 s with a Super-Mixer (LAB-LINE Instruments, Melrose Park, IL). The sample was then centrifuged at 5000 rpm using an Eppendorf 5804 centrifuge (Eppendorf GmbH, Hamburg, Germany). The upper layer was transferred to another vial, 100 µL methanol containing 1% hydrochloric acid was added to it, and the solvent was evaporated using a Reacti-Vap Evaporator (Pierce Co., Rockford, IL) at 30°C under a stream of nitrogen gas.

Sample preparation

Following evaporation, 100 µL TFAA was added to the residue. The vial was sealed airtight and held at 60°C for 30 min. Finally, the sample was cooled to room temperature and transferred to a 100-µL microvolume glass tube placed into a 12- × 32-mm, 2-mL autosampler vial, which was sealed airtight. Samples were analyzed within 24 h or held at a constant –5°C until measurement.

Results and Discussion

The method was validated in a two-step process. First, the analysis of each sample series was repeated seven times. We then tested the solutions once a day over a span of seven working days.

The results of our tests were judged according to the German Industrial Standards (DIN) 32645 rules (4). The average processed results are presented in Tables II–VI. The recovery of the compounds ranged 62–68%, but we found that the repetition of the extraction of the samples increased the recovery beyond 80%. However, this also raised the levels of sample matrix compounds in the spectra, thus deterring the selectivity of the method.

The calibration curve was obtained by plotting the quotient of the abundance of the analyte's target ion and that of the internal standard's target ion against the nominal concentration of the analyte in the working solution (5). In our tests, the calibration curve correlation coefficient (r^2) was larger than 0.999, except in the case of MDA, indicating a close relationship between signal intensity and concentration. Statistical tests were performed with a confidence level of 95%. Table II lists the

Table I. Compound Information for Database

Compound	Retention time (min)	Target ion	Qualifier ion 1 (%response)	Qualifier ion 2 (%response)
Amphetamine–TFA	5.93	140	118 [46]	91 [23]
Metamphetamine–TFA	6.59	154	110 [40]	118 [50]
MDMA–TFA	9.21	154	135 [90]	162 [40]
MDA–TFA	8.14	135	162 [36]	275 [3]
MDE(A)–TFA	9.64	168	162 [110]	140 [90]
MDMA-D_5–TFA	9.18	158	136 [120]	164 [15]

recovery and r^2 values.

The limits of detection, identification, and quantitation are presented in Table III. The limit of identification is the concentration of the analyte where the unequivocal qualitative identification of a compound is possible with the applied analytical method, but quantitative evaluation is not (6). Using our method, MDA can be identified quantitatively at a 95% confidence level above a concentration of 130.45 ng/mL. Table IV shows the results obtained in the intraseries analysis (7 samples were processed on the same day), while those acquired from the interseries analysis (one series of samples processed on seven consecutive working days) are displayed in Table V. The average concentration of the working solution was 200 ng/mL (7). Relative standard deviations (RSDs) and relative errors (%) are included in the tables. Relative error was the highest for amphetamine and the lowest for methamphetamine. For these two compounds, the statistical parameters obtained from interseries assessments were better than those obtained from the intraseries assessment results. The tables also indicate that the MDMA, MDE (A), and MDA tests provided worse results than amphetamine and methamphetamine, but were still found to be acceptable (8).

We paid special attention to selectivity and the potential interference from other compounds during the validation of the method. Purity tests of solvents, reagents, and samples without matrices were performed by taking them through the entire analytical procedure (9). Reagents were also tested for potential interference and proved to have no peaks at the retention times of the analytes. We repeatedly took blank urine through the testing procedure, as well, and found no interferences. Following validation, we continued to check the selectivity of the method, along with possible interferences on a regular basis by assessing samples found to be negative by using polarization fluorescent immunoassay. We also examined if other sympathomimetic amines (ephedrine, phenylpropanolamine, and pseudoephedrine) would interfere with methamphetamine peaks, as mentioned in some articles (10). We found no interference from these compounds either. In order to further reduce the chance of misidentification caused by interference occurring at the retention time of the analytes, narrow time reference peak windows (± 0.5 %) were set (11).

Conclusion

Most of the laboratories performing the analyses of illicit drugs in urine use complex and time-consuming sample preparation techniques such as base–acid–base liquid–liquid back extraction combined with the freezing of water. In the presented method, single liquid–liquid extraction is performed for the extraction of the drugs of abuse from urine. The matrix load is as low as 200 µL urine for each 100 µL of analytical sample, in contrast to the majority of methods used in other laboratories, which require 1–2 mL urine to be extracted in order to obtain 100 µL analytical sample (12).

Because the analytical sample includes matrix compounds at a very low concentration, no further cleanup is required fol-

Table II. Recovery and Correlation Coefficients

Compound	Correlation coefficient (r^2)	%Recovery
Amphetamine	0.9996	68
Metamphetamine	0.9999	64
MDMA	0.9997	65
MDA	0.9980	65
MDE(A)	0.9992	62

Table III. Limits of Detection, Identification, and Quantitation

Compound	Limit of detection (ng/mL)	Limit of identification (ng/mL)	Limit of quantitation (ng/mL)
Amphetamine	21.07	42.15	90.01
Metamphetamine	22.49	44.99	95.88
MDMA	25.51	51.00	108.23
MDA	30.99	61.98	130.45
MDE(A)	24.84	49.69	105.52

Table IV. Accuracy Measurement and Intraseries (n = 7)*

Compound	C_1† (ng/mL)	C_M‡ (ng/mL)	RSD	Relative error (%)
Amphetamine	200	202.87	3.9	1.44
Metamphetamine	200	206.34	3.5	3.17
MDMA	200	200.80	3.7	0.40
MDA	200	205.00	0.4	2.50
MDE(A)	200	207.00	0.7	3.5

* n = 7 is the number of repeated measurement.
† C_1 = first working solution concentration, nominal value.
‡ C_M = average of measurement values.

Table V. Accuracy Measurement and Interseries (n = 7)

Compound	C_1 (ng/mL)	C_M (ng/mL)	RSD	Relative error (%)
Amphetamine	200	199.80	2.7	0.10
Metamphetamine	200	198.90	4.0	0.05
MDMA	200	206.43	1.5	3.22
MDA	200	204.30	1.4	2.15
MDE(A)	200	206.50	1.6	3.25

lowing extraction. We found that centrifugation adequately separates the organic phase from the rest of the sample without including water (13).

Our method is simple and easy to learn and can be applied for the routine analysis of human urine samples. The analytical parameters (detection limit, accuracy, and reproducibility) show that the confirmatory analysis of amphetamine, methamphetamine, and related compounds is feasible using the described procedure (14).

Liquid–liquid extraction using ethyl acetate has the following benefits: (*a*) it is simple and easy to perform and can be fitted into analytical procedures related to drugs of abuse; (*b*) time and labor force required for sample preparation is minimal; (*c*) it is economical regarding the use of chemicals and equipment; (*d*) it allows sequential analysis with great capacity, limited only by the performance of the analytical instrument (GC–MS); (*e*) it can be validated within an analyte concentration range of 200–1500 ng/mL with a correlation coefficient higher than 0.99; (*f*) short and long-term RSD is lower than 5%; (*g*) analyte recovery is approximately 60–70%; (*h*) the difference between the measured and real value is smaller than 5%; and (*i*) all employed solvents are environmentally friendly.

Based on these benefits, we endorse this sample preparation method for the detection of amphetamine and related compounds in biological samples.

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