Structural Elucidation of an Uncommon Phenylethylamine Analogue in Urine Responsible for Discordant Amphetamine Immunoassay Results

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Abstract

The present paper describes investigations following the analysis of a urine specimen containing important amounts of an unknown substance detected by gas chromatography–mass spectrometry (GC–MS) analysis. FPIA analysis was positive (cutoff 0.3 mg/L) and Triage™ 8 rapid test was negative (cutoff 1 mg/L) for amphetamines. Considering the GC–MS spectrum, two different molecules, for example, *N*-ethyl-1-(3,4-methylenedioxyphenyl)ethylamine (1) or *N*-ethyl-4-methoxyamphetamine (2), have been suspected. Synthesis of these two compounds was carried out together with spectral (MS, ^{1}H and ^{13}C NMR, IR, UV) and chromatographic (GC) characterization as well as determination of immunological cross reactivities (FPIA and Triage 8). The unknown compound present in the urine specimen has been finally identified as *N*-ethyl-4-methoxyamphetamine (2), an uncommon amphetamine analogue.

Introduction

Amphetamines and the amphetamine-derived designer drugs are considered potent stimulants of the central nervous system or entactogens. Since the beginning of the 1990s they have been increasingly abused, especially among teenagers. Toxicological effects in overdose may include among others hyperpyrexia, seizures, tachycardia, and hallucinations (1). Alertness and a general feeling of well-being are the psychological effects. Death related to the abuse of amphetamines or derivatives is rare, but it has also been reported (2–4).

The rather simple molecular structure of these drugs makes chemical synthesis and purification relatively easy. Many different amphetamines as well as structurally similar compounds have thus been described (5). Immunoassays of urine are generally used as a first presumptive screening. Gas chromatography–mass spectrometry (GC–MS) analysis after extraction and acetylation is one of the most popular confirmation techniques of presumably positive samples. Unambiguous determination of MS data, however, often is a difficult task because of the spectral similarity of many amphetamines, their metabolites, and derivatives. Another problem is the fact that amphetamine or analogues detected in urine may also result from pharmaceuticals that metabolize to amphetamine or methamphetamine (6). The toxicology of amphetamines and amphetaminelike designer drugs has recently been reviewed in two book articles (7,8).

So far, 1-phenylethylamines (1-PEA) and its derivatives have not been reported to be in widespread use, probably because of their low potential for abuse. But, as they are still unscheduled substances, there may be a substantial temptation for the synthesis and marketing of 1-PEAs. Identification and quantitation of 1-phenylethylamine in seized powder samples and in urine specimens have been described (9–11). *N*-Methyl-1-phenylethylamine has been found in kilogram quantities in the United States (12) and in ecstasy pills in Germany (13). It is, however, not known if these 1-PEAs have been synthesized intentionally or if an error in the choice of the starting material for the manufacturing of amphetaminelike designer drugs is responsible for an unintentional synthesis. King et al. (14) described the synthesis and recent seizures of 1-PEA and its derivatives in England, the United States, and in the Netherlands. Pharmacological studies of 1-PEA and its derivatives are rare. In a rather old study (15), (+)-amphetamine has been reported to be 5–6 times more active as a central stimulant than (+)-1-PEA in mice.

The present paper describes our investigations carried out after obtaining an unidentified mass spectrum (Figure 1) from a urine sample by GC–MS analysis. The retention index of the unidentified peak was 1880. Triage 8 gave a negative result, whereas fluorescence polarization immunoassay (FPIA) analysis gave a positive result for amphetamines. These incoherent results made us suspect the presence of an amphetamine analogue.

Experimental

Chemicals

All solvents were obtained from Lab-Scan analytical sciences

(Labscan Ltd., Dublin, Ireland). HCl, NaOH, and pyridine were obtained from UCB (Louvain, Belgium). Ethylamine hydrochloride was obtained from Merck (Merck KGaA, Darmstadt, Germany). Sodium cyanoborohydride and 4-methoxybenzyl methylketone were obtained from Fluka (Fluka Chemie AG, Buchs, Switzerland). 3,4-(Methylenedioxy)acetophenone was obtained from Aldrich (Aldrich Chemie, Bornem, Belgium). Acetic anhydride was purchased from Fluka (Buchs, Switzerland).

Apparatus

Electron impact (EI) mass spectra were recorded with a mass selective detector from Hewlett-Packard (5971A-series II) fitted with a 12-m Ultra-2 capillary column from HP with 0.2-mm internal diameter and 0.33-µm film thickness. Helium was used as the carrier gas at a flow rate of 0.5 mL/min. The ionization voltage was 70 eV, the injector temperature was 260°C, and the detector temperature was 280°C. The initial column temperature was 70°C (2 min); ramp 20°C/min; and final temperature 280°C (11.5 min). The MS spectra were recorded from *m/z* 50 to 650. One microliter of the final solutions were injected into the GC–MS operating in the splitless mode. Infrared (IR) spectra were recorded on a Perkin-Elmer Fourier transform infrared (FT-IR) spectrometer (Paragon 1000 PC) from 600 to 4400 cm^{-1}. UV absorption was measured at pH 3.8 using a Gynkotek UV-vis detector (UVD 340S). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Bruker instrument in $CDCl_3$ or CD_3OD at 500 MHz. Chemical shifts are given in d (expressed in parts per million), coupling constants (J) are given in Hertz (Hz). The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. Immunoassays were performed on an Axsym Fluorescence Polarimeter (Abbott) using the amphetamine/methamphetamine II reagents and with Triage 8 rapid test (Biosite, San Diego, CA) according to the instructions of the manufacturers. For the determination of cross-reactivities, aliquots of a methanolic stock solution were added to negative urine samples to give final concentrations ranging from 0.15 to 8.00 mg/L.

Methods

In a first attempt for the interpretation of the GC–MS spectrum, the presence of the *N*-acetylated derivatives of either *N*-ethyl-1-(3,4-methylenedioxyphenyl)ethylamine (1) or *N*-ethyl-4-methoxyamphetamine (2) (Figure 2) has been suggested. Synthesis and discussion of the MS spectra of underivatized amphetamine 2 were published by Noggle et al. (16). To our knowledge, they have not yet been found in urine from drug abusers. Synthesis of these two compounds was carried out, together with spectral and chromatographic determination and structural elucidation of the unknown compound present in the urine specimen.

N-Ethyl-1-(3,4-methylenedioxyphenyl)ethylamine (1). A mixture of 3.2 g (40 mmol) ethylamine HCl and 1.3 g (8 mmol) 3,4-(methylenedioxy)acetophenone in 30 mL methanol was stirred under reflux for 1 h. After cooling down to room temperature, 0.9 g (15 mmol) sodium cyanoborohydride was added, and stirring was continued for 48 h. The mixture was then poured into 100 mL of H_2O and made acidic by addition of 0.8 mL of 12M HCl (formation of HCN !). The solution was washed with 3 × 20 mL of dichloromethane. The aqueous portion was made alkaline with 1.5 mL of 12M NaOH solution and extracted three times with 30 mL of dichloromethane. The combined organic portions were dried over anhydrous sodium sulphate and concentrated under reduced pressure. Twenty milliliters of an ether/12M HCl (99:1) solution was added to the oily residue (the free base), which precipitated the hydrochloride salt. It decomposes at a temperature exceeding 250°C.

The hydrochloride salt is freely soluble in water and methanol, partially soluble in ethanol and chloroform, and insoluble in diethylether, toluene, ethylacetate, and acetone.

MS (EI) *m/z* (relative intensity): 56 (19), 63 (6), 65 (17), 72 (14), 89 (7), 91 (16), 118 (9), 149 (23), 178 (100), 179 (12), 193 (8). ^{1}H-NMR (500 MHz, CD_3OD): δ (ppm) 1.32 (3H, t, J=7.3); 1.68 (3H, d, J=7.3); 2.86 (1H, m); 3.01 (1H, m); 4.35 (1H, q, J=7.2); 6.04 (2H, s); 6.93 (1H, m); 7.02 (1H, m); 7.07 (1H, m). ^{13}C-NMR (500 MHz, CD_3OD): δ 11.55 (q), 19.70 (q), 41.99 (t), 59.04 (d), 103.02 (t), 108.42 (1C arom.), 109.71 (1C arom.), 122.98 (1C arom.), 131.20 (1C arom.), 149.99 (1C arom.), 150.04 (1C arom.). IR: $1/\lambda$ (cm^{-1}) 2966, 2762, 2483, 1586, 1501, 1470, 1444, 1381, 1248, 1101, 1038, 932, 871, 811. UV: λ_{max} 237, 284 nm.

N-Ethyl-4-methoxyamphetamine (2). The synthesis of compound 2 was carried out by modification of the procedure given by Shulgin and Shulgin (17). Ethylamine HCl (6.1 g, 75 mmol) and 4-methoxybenzyl methylketone (2.5 g, 15 mmol) were stirred under reflux in 50 mL of methanol for 1 h. After cooling down to room temperature, 1.3 g (20 mmol) of sodium cyanoborohydride was slowly added and the pH of the solution was adjusted to 6 by adding 12M HCl. The mixture was stirred for two days, then poured into 200 mL of water and acidified by addition of 0.5 mL 12M HCl (formation of HCN !). The solution was washed three times with 20 mL of dichloromethane. The aqueous layer was alkalinized with 5 mL of 12M NaOH and extracted three times with 30 mL of dichloromethane. The organic portions were pooled, dried over anhydrous sodium sulphate and concentrated under reduced pressure. The hydrochloride was precipitated by adding 50 mL of a solution ether/12M HCl (99:1). The crude product was filtrated and recrystallized in 30–40 mL of an ether/ethanol (3:1, v/v)

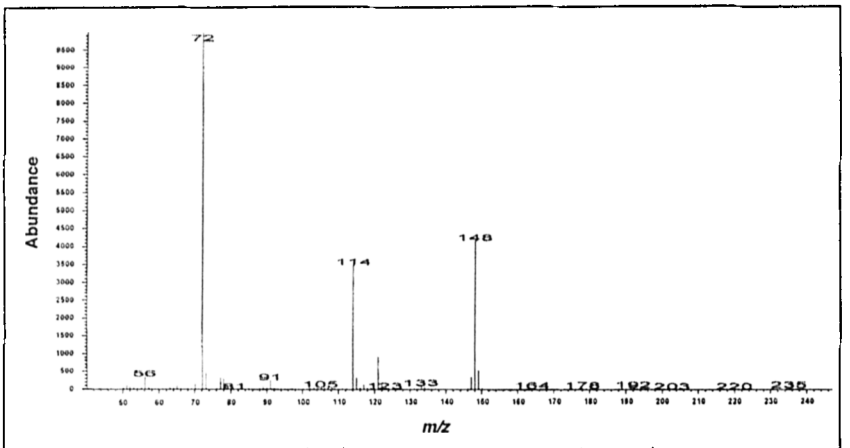

Figure 1. MS spectrum of compound 2 identified in urine specimens after acetylation.

solution. The hydrochloride salt has a melting point of 160–161°C, is freely soluble in water, methanol, ethanol, and chloroform, and is insoluble in diethylether, toluene, ethylacetate, and acetone.

MS (EI) *m/z* (relative intensity): 72 (100), 121 (11). ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 1.34 (3H, d, J=6.5); 1.53 (3H, t, J=7.3); 2.81 (1H, m); 3.07 (1H, m); 3.14 (1H, m); 3.33 (1H, m); 3.49 (1H, m); 3.78 (3H, s); 6.83 (2H, m); 7.13 (2H, m); 9.58 (2H, m). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 11.54, 15.55, 38.57, 40.09, 55.25, 55.64, 114.16, 128.41 (2C arom.), 130.31 (2C arom.), 158.63. IR: 1/λ (cm^{-1}) 2970, 2802, 2480, 1612, 1516, 1300, 1250, 1032, 815. UV: λ_{max} 224, 274 nm.

Extraction of urine specimen

Extraction with 2 mL of dichloromethane/isopropanol (85:15, v/v) of 2 mL of the urine specimen was carried out at pH 5.5 using acetate buffer and at pH 9.5 after alkalinization with ammonium buffer. Two milliliters of the urine was also hydrolyzed at 90°C for 15 min with concentrated HCl, then alkalized to pH 9.5 and extracted with dichloromethane/isopropanol (85:15, v/v). The three extracts were pooled after filtering over anhydrous sodium sulfate. Five microliters of a methanol/HCl solution (99:1, v/v) were added in order to avoid losses of volatile substances by forming the hydrochloride salts. After evaporation to dryness under nitrogen, the residue was acetylated at 90°C for 25 min using a mixture of pyridine and acetic anhydride. After evaporation, the final residue was dissolved in 100 µL of ethylacetate/methanol (90:10, v/v).

Results and Discussion

The unidentified MS spectra presented a base peak at *m/z* 72, and a molecular peak was observed at *m/z* 235 with an intensity of only 1%. Other prominent peaks were at *m/z* 114 and 148. Based on an analogy with the fragmentation pattern of acetylated MDMA (18), we tentatively attributed the spectrum to the acetylated derivative of *N*-ethyl-1-(3,4-methylenedioxyphenyl)ethylamine (1), a compound not previously described as drug of abuse. In order to confirm our hypothesis, compound 1 has been synthesized. IR, ^{1}H-NMR, and ^{13}C-NMR spectra of 1 are described in the experimental section. The GC–MS spectrum of acetylated 1, however, has a RI of 1900 (as compared to 1880 for the unidentified peak), and its mass spectrum differed significantly from the unknown compound (Figure 3). The base peak was at *m/z* 164, the molecular peak has an intensity of 38% and other important fragments were found at *m/z* 91 (35%), 149 (52%), and 206 (23%).

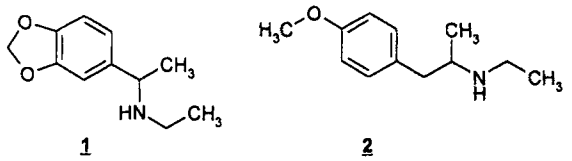

Figure 2. Hypothetic structures for the interpretation of the unidentified MS spectrum.

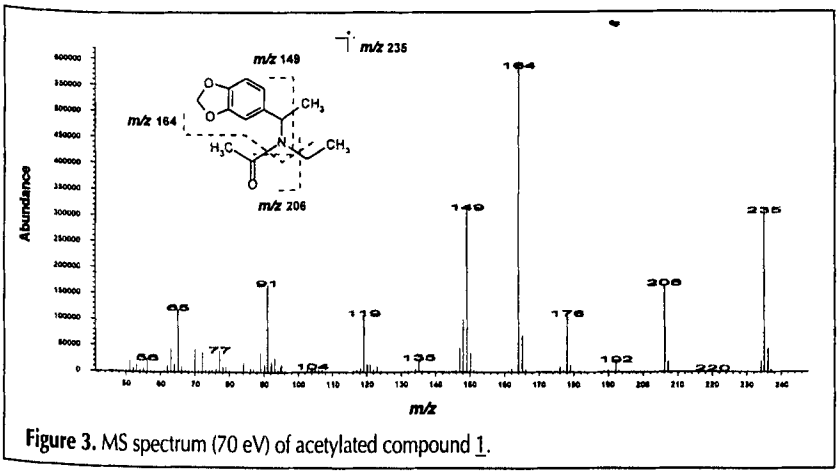

Figure 3. MS spectrum (70 eV) of acetylated compound 1.

The well-documented fragmentation pathway of amphetamines and analogues (19) was not observed with compound 1. This may be due to the higher dissociation energy of the alkyl-phenyl (sp^3-sp^2) bond in 1-PEAs as compared to the alkyl-alkyl (sp^3-sp^3) bond in amphetamines. As a result, an important molecular ion peak was observed for *N*-ethyl-1-(3,4-methylenedioxyphenyl)-ethylamine. The base peak at *m/z* 164 was assigned to a loss of the ethyl group (*m/z* 206) followed by deacetylation.

Considering the weak molecular ion peak (*m/z* 235) and the overall similarity with the amphetamine fragmentation pattern, the amphetamine analogue 2 with identical molecular weight was proposed as another possible candidate. GC–MS analysis of a spiked urine specimen confirmed the identical nature (retention index and mass spectrum) of 2 and the unknown compound (see Figures 1 and 4 for fragmentation).

Immunoassay results are summarized in Table I. The urine specimen containing the unknown substance gave a positive result using FPIA (considering our cutoff of 0.3 mg/L) and a negative result when using Triage 8 (cutoff 1 mg/L). Five blank urine specimens were spiked with 1 or 2 at concentrations ranging from 0.15 to 8.00 mg/L, and cross reactivities have been measured for the amphetamine group. FPIA and Triage 8 rapid test were both negative for *N*-ethyl-1-(3,4-methylenedioxyphenyl)ethylamine at all concentrations. This is probably due to the absence of the 2-phenylethylamine structure, necessary to produce antigen-antibody reaction. The compound 2, however, shows important cross reactivities in FPIA at low concentration (almost 300% at 0.15 mg/L) which then exponentially decrease with increasing concentration (about 40% at 8 mg/L).

Because of the very small amounts of available patient urine, it unfortunately was not possible to carry out quantitative GC–MS or high-performance liquid chromatography measurements with this specimen. Extrapolation of the FPIA results, however, indicates a concentration of approximately 3.0 mg/L for the urine sample. As the cross reactivities of metabolites are not known, this result remains a crude estimation.

It is not known whether the *N*-ethyl-4-methoxyamphetamine itself is a fragment or a metabolite resulting from a still bigger parent compound. In fact, GC–tandem MS (Finnigan TSQ 700) operating in the positive chemical ionization mode

indicates the presence of a molecule presenting the 4-methoxyamphetamine structure with a molecular weight of *m/z* 279 (results not shown) (20). The exact nature of this compound, however, remains undetermined.

Conclusions

Determination of molecular structures on the basis of GC–MS data alone often remains uncertain when no reference substances are available. In the special case of amphetamines or analogues (i.e., similar structures), together with unspecific fragmentation pattern (dominant peak at *m/z* 72) and the possibility of pharmaceutically derived amphetamine or methamphetamine moieties, unambiguous determination is often time-consuming and work-intensive. In this work, the presence of the *N*-ethyl-4-methoxyamphetamine (2) was confirmed on the basis of MS spectra, GC retention index, and cross-reactivities in immunoassays. Finally, IR and NMR spectra complete the characterization of this compound. To the best of our knowledge it is the first time this substance (2), possibly a new designer drug, has been found in urine. It had, however, been described previously as a potential drug of abuse by Noggle et al. (15). The interference of degradation products from pharmaceuticals must always be considered and can therefore not be excluded in the present case.

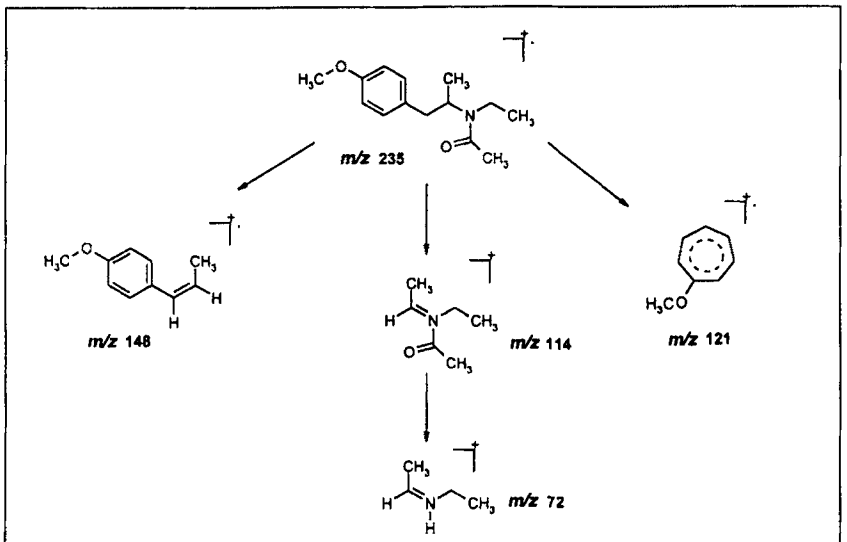

Figure 4. Fragmentation pathway of acetylated compound 2 (19).

Table I. FPIA and Triage 8 Results for Compounds 1 and 2 at Different Concentrations in Spiked Urine and Patient Urine Specimens

	Amount added (mg/L)	FPIA result for amphetamine (mg/L)	Triage result for amphetamine (cutoff 1 mg/L)
Urine sample	–	2.16	negative
N-Ethyl-1-(3,4-methylene dioxyphenyl)ethylamine (1)	0.00	0.01	negative
	0.15	0.03	negative
	0.30	0.02	negative
	1.00	0.02	negative
	3.00	0.04	negative
	8.00	0.05	negative
N-Ethyl-4-methoxy amphetamine (2)	0.00	0.03 ± 0.03	negative
	0.15	0.44 ± 0.03	negative
	0.30	0.63 ± 0.14	negative
	1.00	1.10 ± 0.11	negative
	3.00	2.14 ± 0.38	positive
	8.00	3.27 ± 0.39	positive

Acknowledgments

The authors would like to thank Dr. P. Roesner (LKA Schleswig-Holstein, Kiel, Germany) for GC–tandem MS measurements and NMR analysis (Institute for Organic Chemistry, University of Kiel) and for helpful discussion. We also thank Kristjana Helgadottir from Icelandic Pharmaceuticals Ltd. and Dr. Weiss from Merck KGaA (Darmstadt, Germany) for providing the urine specimen, which was obtained from a suspected drug user, containing the unknown substance.

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Manuscript received December 7, 1998;
revision received April 15, 1999.