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A validated gas chromatographic–electron impact ionization mass spectrometric method for methylenedioxymethamphetamine (MDMA), methamphetamine and metabolites in oral fluid

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Abstract

An analytical method to simultaneously quantify amphetamine (AMP), methamphetamine (MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), 3-hydroxy-4-methoxy-methamphetamine (HMMA) and 3-hydroxy-4-methoxy-amphetamine (HMA) in oral fluid is presented. Four hundred microlitres of oral fluid collected via expectoration was extracted by solid phase extraction. GC/MS–EI with selected ion monitoring (SIM) yielded linear curves 5–250 ng/mL for AMP, MAMP, MDMA and MDEA, 5–500 ng/mL for MDA and 25–1000 ng/mL for HMA and HMMA. Recoveries were greater than 85%, accuracy 87–104%, and precision less than 8.3% coefficient of variation. This assay will be used to investigate distribution of sympathomimetic amines into human oral fluid following controlled drug administration.

Keywords: Amphetamine; Methamphetamine; Methylenedioxymethamphetamine; Ecstasy; Oral fluid; Gas chromatography; Mass spectrometry

1. Introduction

Sympathomimetic amines including methamphetamine (MAMP), amphetamine (AMP), methylenedioxymethamphetamine (ecstasy, MDMA), methylenedioxyethylamphetamine (MDEA), and methylenedioxyamphetamine (MDA) are commonly abused. The Drug Abuse Warning Network (DAWN) tracks drug use trends in the United States by surveying drug-related emergency department visits. The most recent DAWN report estimated that during the third and fourth quarters of 2003 there were 42,538 amphetamine and methamphetamine-related visits along with 2,221 MDMA-related visits [1].

Sympathomimetic amines increase alertness, energy and self-confidence, and enhance mood, well-being and euphoria [2]. Effects of the ring-substituted amphetamines (MDMA, MDEA and MDA) include "entactogenic" effects such as extroversion, increased sociability, and perceptual disturbances [2,3]. Dangerous adverse effects of sympathomimetic amines include delirium, seizures, stroke, hyperthermia and heat stroke, which can lead to organ failure [2]. The past decade has seen numerous reports of MAMP-induced neurotoxicty with evidence of damage to dopaminergic and serotonergic pathways in animals and humans [4,5]. There is evidence of MDMA-induced neurotoxicity of serotonin neurons in rats and primates, although in mice toxicity appears specific to dopamine neurons [3].

The primary metabolites of MAMP are amphetamine (AMP) and *p*-hydroxymethamphetamine, which are formed by *N*-dealkylation and aromatic hydroxylation, respectively (Fig. 1, panel A) [6]. MDMA, MDA and MDEA are extensively metabolized by cytochrome P450 2D6 and 3A4 and catechol-*O*-methyltransferase undergoing *O*-demethylation, *N*-dealkylation and *O*-methylation (Fig. 1, panel B) [7–12]. The dihydroxy-species, HHA, HHMA, and 3,4-dihydroxyethylamphetamine can undergo sulfate conjugation, while HMA, HMMA and 4-hydroxy-3-methoxyethylamphetmine can undergo both sulfation and glucuronidation [9]. MDMA metabolites have been implicated in MDMA neurotoxicity due to the fact that peripheral MDMA administration, and not direct injection into rat brain, damaged neurons [8].

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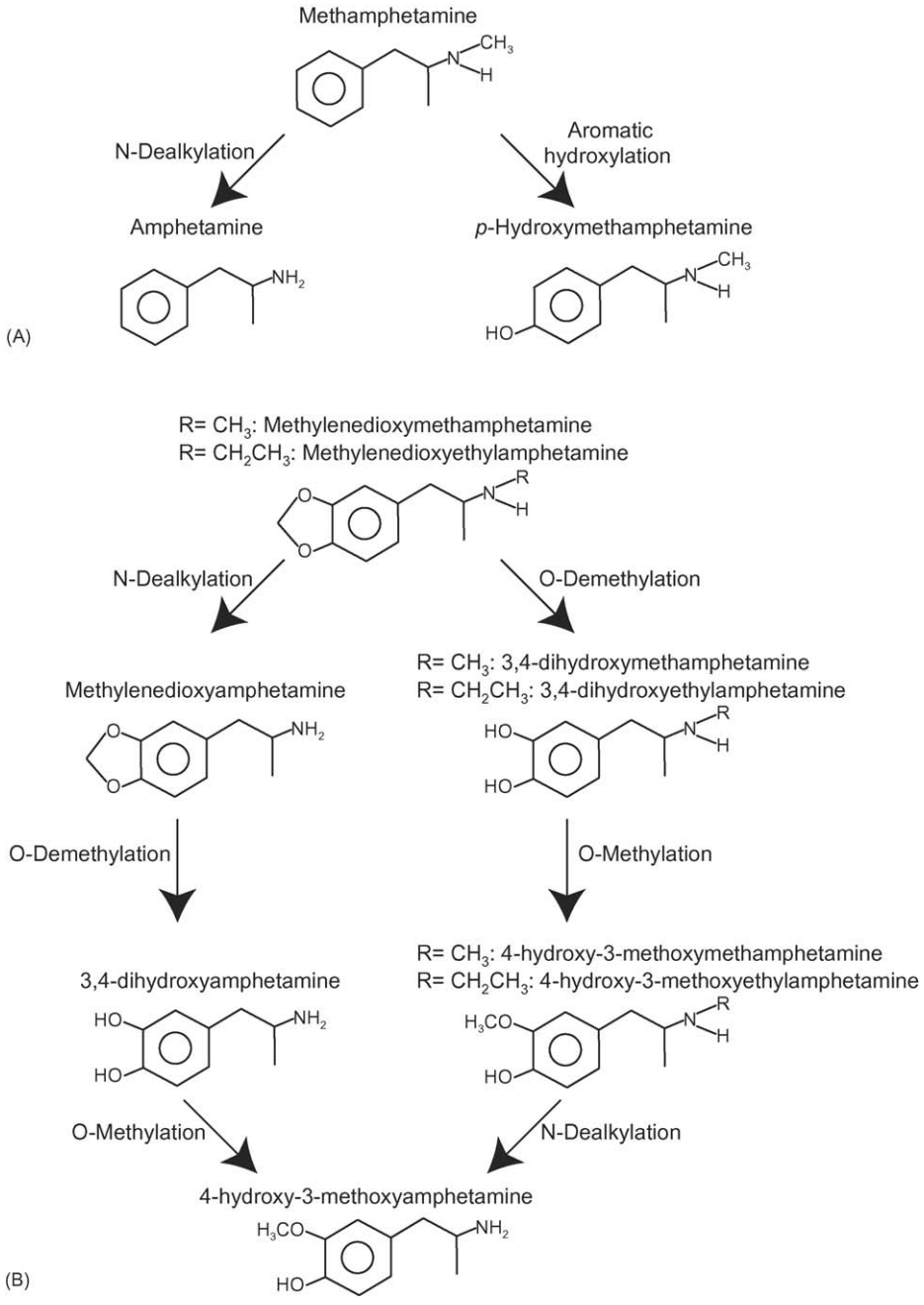

Fig. 1. Metabolic pathways for sympathomimetic amines: (A) metabolism of methamphetamine; and (B) metabolism of ring-substituted amphetamines.

There is interest in testing oral fluid as an alternative to plasma or urine in clinical and forensic settings since oral fluid can be collected non-invasively while under direct observation [13]. In some cases, oral fluid drug concentrations reflect concurrent plasma concentrations [13,14]. Oral fluid is a mixture of oro-naso-pharyngeal secretions and secretions from three major and minor accessory salivary glands. Bacteria, epithelial cells, erythrocytes, leukocytes and food debris also are present; however the major component of oral fluid is water [15,16]. Oral fluid is hypotonic relative to plasma, although ionic concentrations vary considerably between resting and stimulated states [15]. To transfer from blood to oral fluid, drugs must pass through capillary walls, basement membranes and lipophilic membranes of glandular epithelial cells [13,15]. Molecular weight, lipophilicity, degree of ionization and protein binding are important factors influencing drug transfer into oral fluid, with small, lipophilic, non-ionized, unbound drugs being more likely to distribute into oral fluid [13,15].

Oral fluid drug concentrations are influenced by variations in salivary flow rate and pH [13,14]. Salivary secretion is controlled via both sympathetic and parasympathetic pathways. Salivary flow varies according to the time of day, i.e. circadian rhythm, and by other stimuli including eating, chewing and administration of sympathetic and parasympathetic drugs [15]. The complex control of salivary secretion can produce variations in oral fluid concentrations within and between subjects,

making interpretation of oral fluid testing results challenging [14].

Interpretation of oral fluid testing is also complicated by the method of oral fluid collection. Oral fluid can be collected by simple expectoration or using a marketed oral fluid collection device. An important factor that can complicate interpretation of oral fluid testing is whether oral fluid secretion is stimulated during collection [13]. Oral fluid secretion can be mechanically stimulated (chewing on parafilm, Teflon rubber bands or chewing gum) or by acidic stimulation produced by acidic candy. Stimulation can reduce oral fluid drug concentrations due to dilution produced by increased oral fluid production. Oral fluid drug concentrations could also be effected by oral fluid pH fluctuations resulting from stimulation [13,16]. Oral fluid secretion is not substantially stimulated by collection devices unless acidic components are incorporated in the device [13,16]. However, presence of the device in the mouth alone may slightly stimulate oral fluid secretion [13]. Additionally, some drugs adsorb to the collection devices [17,18].

Analytical sensitivity is a significant concern when conducting oral fluid drug testing; decreased salivary secretion is a side effect of many drugs including sympathomimetic amines yielding small volumes of oral fluid available for analysis [19]. Metabolite concentrations are often much lower than parent drug concentrations, further elevating sensitivity concerns [20]. Navarro et al. reported mean maximum oral fluid concentrations for the MDMA metabolite MDA of 45 ng/mL, 4–5% of peak MDMA concentrations in eight participants after a 100 mg oral MDMA dose [21]. Similarly, mean maximum AMP oral fluid concentrations 2–12 h after oral administration of 20 mg MAMP to five participants were 14.2 ng/mL, approximately 7% of peak MAMP concentrations [22]. We are interested in measuring metabolites of MAMP and MDMA in oral fluid to evaluate pharmacokinetic variations, to test whether metabolites can be useful for establishing time of last drug use, and to determine the contribution of active metabolites to pharmacodynamic effects.

Analysis of oral fluid for sympathomimetic amines has been detailed in several publications, employing electron impact ionization gas chromatography-mass spectrometry (GC/MS–EI) [23,24] or liquid chromatography tandem mass spectrometry (LC–MS/MS) [25–27]. Two GC/MS–EI methods for MAMP, MDMA and metabolites employed heptafluorobutyric acid anhydride derivatization after either liquid-liquid extraction or solid phase extraction (SPE). Although these methods required small oral fluid sample volumes of 100 and 250 μL, the limits of quantification (LOQs) for MAMP, AMP, MDMA, MDEA and MDA were 20 and 25 ng/mL, respectively [23,24]. A report by Navarro et al. describes a GC/MS method requiring 1 mL of oral fluid that achieved LOQs of 5.7, 1.0 and 2.9 ng/mL for MDMA, MDA and HMMA, respectively, but did not include other sympathomimetic amines [21]. LC–MS/MS methods using 50–200 μL of oral fluid achieved LOQs of 0.5–2.0 ng/mL for MAMP, AMP, MDMA, MDEA and MDA [25,26]. None of the existing GC/MS or LC–MS/MS methods for sympathomimetic amines included the MDMA metabolite HMA.

Cristoni et al. detailed development of an LC–MS/MS assay for sympathomimetic amines in urine employing ion trap tandem mass spectrometry and reported considerable difficulties obtaining efficient ionization of MDA using electrospray ionization or atmospheric pressure chemical ionization [28]. Preliminary LC–MS/MS investigations by our group encountered similar ionization efficiency difficulties for MDA and HMA, which limited assay sensitivity for these analytes. The sympathomimetic amine target analytes in this assay are readily derivatized, making them amenable to GC/MS analysis. Therefore, we elected to pursue development of a GC/MS assay for sympathomimetic amines in oral fluid.

This manuscript details the development and validation of the first GC/MS–EI assay for simultaneous analysis of the seven analytes, AMP, MAMP, MDMA, MDEA, MDA, HMMA, and HMA in oral fluid. Application of this method to specimens collected during controlled MDMA or MAMP administration studies should prove useful for examining drug and metabolite distribution into oral fluid, which will assist interpretation of driving under the influence of drugs and other toxicological investigations.

2. Experimental

2.1. Reagents

AMP-d_0, MAMP-d_0, MDMA-d_0, MDA-d_0, MDEA-d_0, AMP-d_{11}, MAMP-d_{14}, MDMA-d_5, MDA-d_5, and MDEA-d_6 were purchased as racemic mixtures from Cerilliant Corporation (Round Rock TX, USA). Racemic HMMA-d_0 and HMA-d_0 were purchased from Lipomed Inc. (Cambridge, MA, USA). ACS reagent grade Tris [hydroxymethyl] aminomethane base, Tris [hydroxymethyl] aminomethane hydrochloride, triethylamine (99.5% purity), and GC grade *n*-heptane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Concentrated hydrochloric acid, acetic acid, ammonium hydroxide, ACS reagent grade potassium phosphate monobasic and potassium phosphate dibasic, and HPLC grade solvents were obtained from JT Baker (Phillipsburg, NJ, USA). Heptafluorobutyric acid anhydride (HFAA) was from Pierce Chemical Co. (Rockford, IL, USA). Fritted filters (10 μm pore, 4 mL reservoir volume) used in preparing oral fluid samples for solid phase extraction were purchased from United Chemical Technologies (Bristol, PA, USA). SPEC C18AR/MP1, 3 mL reservoir/30 mg bed mass, mixed mode monolithic silica disc solid phase extraction columns were purchased from Varian Inc. (Lake Forest, CA, USA).

p-Methoxymethamphetamine, *p*-methoxyamphetamine, ephedrine, pseudoephedrine, norpseudoephedrine, phenylpropanolamine (norephedrine), dextromethorphan, diphenhydramine, nicotine, 4-hydroxybutyric acid sodium salt, cocaine, benzoylecgonine, ecgonine, methyl ester, 6-acetylmorphine, morphine, codeine, methadone, oxycodone, 11-hydroxy-Δ9-tetrahydrocannabinol, phencyclidine and fenfluramine used for evaluation of potential interferences were purchased from Cerilliant Corporation. Acetylsalicylic acid, 4-acetamidophenol, brompheniramine, caffeine, chlorpheni-

ramine and ibuprofen standards used for interference studies were obtained from Sigma-Aldrich. Δ9-Tetrahydrocannabinol, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, cannabinol and cannabidiol interference standards were purchased from Alltech Associates, Inc. (Deerfield, IL). *p*-Hydroxyamphetamine and *p*-hydroxymethamphetamine interference standards were from the National Institute on Drug Abuse's drug inventory maintained by Research Triangle Institute, Inc. (Research Triangle Park, NC).

2.2. Calibrator and quality control solutions

One milligram per millilitre solutions of AMP, MAMP, MDMA, MDA, MDEA, HMMA and HMA were diluted using methanol to prepare calibration solutions. A stock solution containing all seven analytes at 10 μg/mL was prepared in methanol and stored at −20 °C. Serial dilutions of the calibration stock solution, using methanol, created calibrators of 5–1000 ng/mL when 40 μL of each calibration solution was added to 400 μL blank oral fluid.

Quality control (QC) solutions were prepared in methanol using different ampules of reference standards than were used for preparing calibration standards. Serial dilutions produced low, medium and high quality control solutions spread across the linear dynamic range of each analyte. Fortified oral fluid samples for quality control were prepared by addition of 40 μL low, medium or high QC solution to 400 μL of blank oral fluid. Low, medium and high QC oral fluid concentrations were 15, 100 and 200 ng/mL for all analytes, respectively, except for HMMA and HMA that were 60, 400 and 800 ng/mL, respectively. Methanolic low, medium and high QC solutions were stored at −20 °C.

One hundred microgram per millilitre solutions of deuterated AMP, MAMP, MDMA, MDA and MDEA were diluted in methanol to prepare internal standard solution containing all five deuterated analogs at 1000 ng/mL in methanol. 1000 ng/mL internal standard solution was stored at −20 °C. Ten microlitres of 1000 ng/mL internal standard solution was added to 400 μL oral fluid to yield an internal standard concentration of 25 ng/mL.

2.3. Blank oral fluid

Blank oral fluid used for preparation of calibration and QC samples was collected from six non-amphetamines-using volunteers by expectoration into a polypropylene tube. Oral fluid was centrifuged at 2000 × *g* at room temperature for 6 min. A 400 μL aliquot was fortified with internal standard, extracted by SPE and analyzed by GC/MS–EI according to the described method. None of the volunteers' oral fluid contained peaks that interfered with analytes of interest. Six volunteers' oral fluid was pooled, stored at −20 °C, and used in the preparation of calibrators and QC samples. Later, oral fluid specimens from three additional non-amphetamines-using volunteers were evaluated, yielding a total of nine unique oral fluid specimens tested for endogenous interferences.

2.4. Extraction and derivatization procedures

Four hundred microlitres of oral fluid was transferred to a 4 mL, 10 μm fritted filter reservoir placed inside a 13 mm × 100 mm glass test tube. Forty microlitres of calibration or QC solution was added followed by 10 μL of 1000 ng/mL internal standard solution. Samples were vortexed briefly, diluted with 2 mL of 0.1 M potassium phosphate buffer, pH 6.0, and centrifuged through the filters at 1000 × *g* for 5 min at room temperature. Sample filtrates were applied to SPEC C18AR/MP1 columns preconditioned with 1 mL methanol followed by 1 mL distilled water. Columns were washed with 1 mL 0.1 M acetic acid followed by 2 mL methanol. Columns were dried under vacuum for 1 min after acetic acid and methanol washes. Analytes were eluted from the columns into clean 5 mL glass centrifuge tubes with two 750 μL aliquots of freshly prepared elution solvent; ethyl acetate:methanol:ammonium hydroxide (78:20:2, v/v/v). Fifteen microlitres of 120 mM hydrochloric acid dissolved in methanol was added to each sample eluate prior to drying under nitrogen at 35 °C in a Zymark TurboVap.

Residues were reconstituted using 100 μL of 0.05M triethylamine in heptane, followed by addition of 10 μL of HFAA. Tubes were capped, vortexed briefly and incubated at 60 °C for 20 min. After samples cooled to room temperature, 200 μL of 0.05 M Tris buffer, pH 7.4, was added and samples mixed for 2 min using a multi-tube vortex apparatus. Samples were centrifuged at 1855 × *g* for 5 min at room temperature, organic (upper) layers were transferred to auto-sampler vials containing 0.1 mL inserts and vials were crimp sealed.

2.5. Chromatographic instrumentation and analytical conditions

An Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) with mass selective detector (Agilent 5973) operated in electron impact mode was used for sample analysis. The injection port temperature was 250 °C. Injection into the GC–MS system was performed in the pulsed splitless mode with a 3 μL injection volume, pulse pressure of 34.5 kPa (5 psi) and a pulse time of 0.5 min. The GC capillary column was an Agilent HP-5 ms (30 m length × 0.32 mm inner diameter, 0.25 μm film thickness). The GC oven temperature program had an initial temperature of 70 °C, was increased at 25 °C/min to 170 °C and held for 0.2 min, increased at 5 °C/min to 195 °C, and held for 0.5 min, and finally increased at 30 °C/min to 300 °C and held for 1 min. High-purity helium (99.999%) was used as the carrier gas at a flow rate of 1.5 mL/min. The temperatures of the quadrupole, ion source and mass-selective detector interface were 150, 230 and 280 °C, respectively. The MS system was operated in selected-ion monitoring mode with the electron multiplier set to 200 V relative to the daily tune value. The following ions were monitored (with quantitative ions in parentheses): AMP-d_0 91, 118, (240); AMP-d_{11} 98, (244); MAMP-d_0 118, 210, (254); MAMP-d_{14} 213, (261); MDA-d_0 135, (162), 375; MDA-d_5 (167), 380; HMA-d_0 163, (240), 360; MDMA-d_0 162, 210, (254); MDMA-d_5 213, (258); HMMA-

d_0 210, (254), 360; MDEA-d_0 162, 240, (268) and MDEA-d_6 244, (274).

2.6. Validation experiments and acceptance criteria

Calibration by internal standardization with deuterated analogs was performed. Calibration curves were prepared by adding AMP-d_0, MAMP-d_0, MDMA-d_0, MDA-d_0, MDEA-d_0, HMMA-d_0, HMA-d_0 (fortified oral fluid concentrations of 5–1000 ng/mL) and AMP-d_{11}, MAMP-d_{14}, MDMA-d_5, MDA-d_5, and MDEA-d_6 (fortified oral fluid concentrations of 25 ng/mL) to 400 μL pooled blank oral fluid. A calibration curve was constructed using peak area ratios of standard to internal standard versus known amounts of non-deuterated analytes. Curves were fit to calibration points using linear least squares regression with a $1/x$ weighting factor applied to compensate for heteroscedasticity. In the absence of commercially available stable isotopes for HMA and HMMA, MDA-d_5 and MDMA-d_5 were used as internal standards for HMA and HMMA, respectively.

Limit of detection (LOD) was evaluated in triplicate and defined as the concentration producing a peak eluting within ±0.02 min of the analyte's retention time for the lowest calibration standard, a signal-to-noise ratio of at least 3:1, Gaussian peak shape, and qualifier ion ratios within ±20% of the mean qualifier ion ratios of the calibrators. Limit of quantification (LOQ) also was evaluated in triplicate and defined as the concentration that met LOD criteria, signal-to-noise was at least 10:1 and measured concentration was within 20% of target in three replicates.

Recovery from fortified oral fluid was evaluated by analyzing two sets of QC samples, each consisting of six replicates at low and high QC concentrations. Set A consisted of pooled blank oral fluid fortified with non-deuterated QC solution prior to filtration (fortified at low and high QC concentrations) and 25 ng/mL deuterated internal standards. Set B consisted of blank oral fluid with deuterated internal standard added to blank oral fluid; low and high non-deuterated QC solutions were added to the eluate after SPE. Comparison of mean non-deuterated analyte peak areas, (set A/set B) × 100, gave the overall analyte recoveries expressed as a percentage.

Precision and accuracy were evaluated over the linear dynamic range of the method at low, medium and high QC concentrations. Precision was expressed as percent coefficient of variation (% CV). Intra-assay precision was evaluated by six determinations per concentration in one batch, inter-assay precision by six replicates in four batches ($n_{total} = 24$). Accuracy was determined comparing mean measured concentrations of six analyses to target, and expressed as percent of target concentration ($n_{total} = 24$).

Dilution integrity was investigated by diluting fortified high QC oral fluid samples with 0.1 M phosphate buffer, pH 6.0. 90 and 50% (v/v) dilutions were achieved by diluting a 40 or 200 μL aliquot of high QC oral fluid with 2.36 or 2.20 mL of 0.1 M potassium phosphate buffer, pH 6.0 prior to SPE ($n = 4$ for each dilution). Ten microlitres of 1000 ng/mL internal standard solution was added prior to SPE. Measured concentrations of diluted samples were corrected by dilution factor (×2 or ×10) and compared to expected undiluted high QC concentrations to evaluate dilution integrity.

We evaluated potential endogenous interfering peaks in oral fluid specimens from nine non-amphetamines using volunteers. We also examined potential drug interferences by adding compounds, structurally similar to the analytes of interest or commonly present in forensic or clinical specimens, to low concentration QC samples, 15 ng/mL for all analytes of interest except HMA and HMMA (60 ng/mL). We tested 1000 ng/mL of *p*-hydroxymethamphetamine, *p*-hydroxyamphetamine, *p*-methoxymethamphetamine, *p*-methoxyamphetamine, ephedrine, pseudoephedrine, norpseudoephedrine, phenylpropanolamine (norephedrine), acetylsalicylic acid, 4-acetamidophenol, ibuprofen, dextromethorphan, diphenhydramine, chlorpheniramine, brompheniramine, caffeine, nicotine, 4-hydroxybutyric acid, cocaine, benzoylecgonine, ecgonine methyl ester, 6-acetylmorphine, morphine, codeine, methadone, oxycodone, Δ9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, 11-hydroxy-Δ9-tetrahydrocannabinol, cannabinol, cannabidiol, phencyclidine, phentermine and fenfluramine for interference with quantification of our analytes of interest. Criteria for lack of interference were acceptable analyte ion ratios (within 80–120% average of calibrator ratios) and quantification of low QC within 80–120% of target concentration.

Stability of derivatized extracts was evaluated after 24 and 48 h storage at ambient temperature on the GC/MS auto-sampler tray. Low, medium and high QC samples ($n = 6$ for each concentration) were extracted by SPE along with a set of calibration standards. Low, medium and high QC samples ($n = 3$ for each concentration) were injected onto the GC/MS–EI instrument 24 and 48 h after preparation. Concentrations of all samples were determined from the same calibration curve.

We investigated stability of stored samples, using blank oral fluid fortified at three QC concentrations. Non-deuterated analyte QC solutions (low, medium and high) were added to 400 μL aliquots of blank oral fluid in 2 mL screw top polypropylene tubes. Tubes were capped and stored at room temperature for 12 h, 4 °C for 48 h, or −20 °C with room temperature thawing for three freeze/thaw cycles. Each condition was evaluated in triplicate. Samples were fortified with internal standards immediately prior to analysis.

2.7. Clinical study

An oral fluid specimen for proof of method was obtained from an individual who self-reported MDMA use that was verified with a positive urine and/or hair test for MDMA, and who participated in a protocol designed to characterize the pharmacokinetics and pharmacodynamics of MDMA after controlled administration. The study was conducted at the Intramural Research Program, National Institute on Drug Abuse, NIH and was approved by the institutional review board (ethics committee) with participants providing written informed consent.

Table 1
Recovery and dilution integrity of sympathomimetic amines from oral fluid

	Recovery[a] (%)		Dilution integrity[b] (% undiluted)	
	Low	High	×2	×10
Amphetamine	87.5	88.8	84.9	93.0
Methamphetamine	86.5	89.5	88.2	96.6
Methylenedioxyamphetamine	85.4	88.4	91.7	99.7
HMA[c]	87.7	94.7	98.5	92.3
Methylenedioxymethamphetamine	86.0	91.3	89.3	99.6
HMMA[d]	88.0	102.6	88.8	89.8
Methylenedioxyethylamphetamine	86.9	92.6	87.0	92.8

[a] Recovery was determined by comparing peak areas of oral fluid fortified with quality control solution prior to filtration and solid phase extraction (SPE) versus blank oral fluid extracts fortified with quality control solution after SPE, $n = 6$ for each group. Low and high QC concentrations were 15 and 200 ng/mL for all analytes except for HMA and HMMA (60 and 800 ng/mL).

[b] Dilution integrity was determined by preparing a 1:2 and 1:10 dilution of high QC oral fluid samples with 0.1 M phosphate buffer ($n = 4$). Mean measured concentrations have been corrected by dilution factor (×2 or ×10) and are expressed as % of expected undiluted high QC concentration.

[c] 3-Hydroxy-4-methoxy-amphetamine.

[d] 3-Hydroxy-4-methoxy-methamphetamine.

3. Results and discussion

Calibration curves were constructed with six standards ranging from 5 to 250 ng/mL for AMP, MAMP, MDMA, MDEA and 25–1000 ng/mL for HMA and HMMA. MDA calibration curves were constructed using 7 concentrations, 5–500 ng/mL. Correlation coefficients (*R*-squared) always exceeded 0.993 for all analytes. Analyte recoveries were better than 85.4% (Table 1), with LODs of 1.0–5.0 ng/mL (Table 2 and panels A–F of Fig. 2) for a 400 μL oral fluid sample. LOQs were 5.0 ng/mL for AMP, MAMP, MDMA, MDEA, and MDA, 25 ng/mL for HMA and HMMA (Table 2). The LOQ was verified on each analytical run, as all calibrators were required to quantify within 80–120% of expected concentrations. Linear ranges are shown in Table 2.

Mean measured concentrations of in-house-prepared control samples ($n = 24$) were within ±12.9% of target concentrations (Table 3). Calculated CVs for the low, medium and high concentration QC samples were <8.3 and 6.8% for intra- and inter-assay precision, respectively (Table 3). Since it is expected that clinical specimens could contain oral fluid concentrations of MDMA, methamphetamine and metabolites exceeding the upper limits of quantification, it was important to verify quantification accuracy of diluted samples. Results of high concentration QC oral fluid samples diluted 50 and 90% (v/v) in phosphate buffer, investigated in quadruplicate, yielded mean measured concentrations 84.9–99.7% of target concentrations with all observations falling within 17.5% of target (Table 1). These dilution studies indicate that accurate measurement of analyte concentrations can be obtained by dilution of concentrated specimens with phosphate buffer.

To evaluate specificity, oral fluid specimens from nine non-amphetamines using volunteers were analyzed; no interfering peaks were found. Potential drug interferences at 1000 ng/mL were added to low concentration QC samples. QC concentrations were within ±20% of target and met ion ratio criteria for all analytes of interest.

Routine preventative maintenance in our laboratory consists of replacement of the GC inlet liner and clipping of approximately two inches from the head of the GC column every 150–200 sample injections. We did not encounter chromatographic problems for the analytes of interest employing this strategy for a total of approximately 2000 sample injections, after which column replacement was necessary to maintain adequate resolution between MDMA and HMMA.

3.1. Investigation of sample storage/stability

Derivatized analyte stability was evaluated with QC samples analyzed after 24 and 48 h at room temperature. All QC samples displayed a signal-to-noise ratio of at least 10:1, had Gaussian peak shapes, acceptable accuracy (concentrations within ±20% of target) and acceptable precision (less than 7.3% CV) (Table 4). Thus, specimens can be reliably analyzed for 48 h after derivatization.

Concentrations of all analytes were stable in oral fluid stored at room temperature for 12 h, 4 °C for 48 h or after three freeze-thaw cycles. Concentrations of low, medium and high QC samples, stored under these conditions were within ±20% of target (Table 5).

3.2. Proof of method

An oral fluid specimen was collected via expectoration 23 h after a participant received a single 1.0 mg/kg oral dose of

Table 2
Sympathomimetic amines in oral fluid by GC–MS–EI: limits of detection (LOD), limits of quantification (LOQ), and mean (±S.D.) slope, intercept and linear range ($n = 6$) for calibration results

Compound	Internal standard	LOD (ng/mL)	LOQ (ng/mL)	Slope	Intercept	Linear range (ng/mL)
AMP	AMP-d_{11}	2.5	5.0	0.047 (±0.002)	−0.058 (±0.026)	5–250
MAMP	MAMP-d_{14}	1.0	5.0	0.049 (±0.002)	−0.091 (±0.026)	5–250
MDA	MDA-d_5	2.5	5.0	0.089 (±0.003)	−0.134 (±0.057)	5–500
HMA	MDA-d_5	5.0	25.0	0.212 (±0.012)	−2.551 (±0.432)	25–1000
MDMA	MDMA-d_5	2.5	5.0	0.048 (±0.002)	−0.097 (±0.018)	5–250
HMMA	MDMA-d_5	2.5	25.0	0.119 (±0.005)	−1.703 (±0.173)	25–1000
MDEA	MDEA-d_6	2.5	5.0	0.053 (±0.002)	−0.072 (±0.023)	5–250

Abbreviations: amphetamine (AMP), methamphetamine (MAMP), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-amphetamine (HMA), methylenedioxymethamphetamine (MDMA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), methylenedioxyethylamphetamine (MDEA).

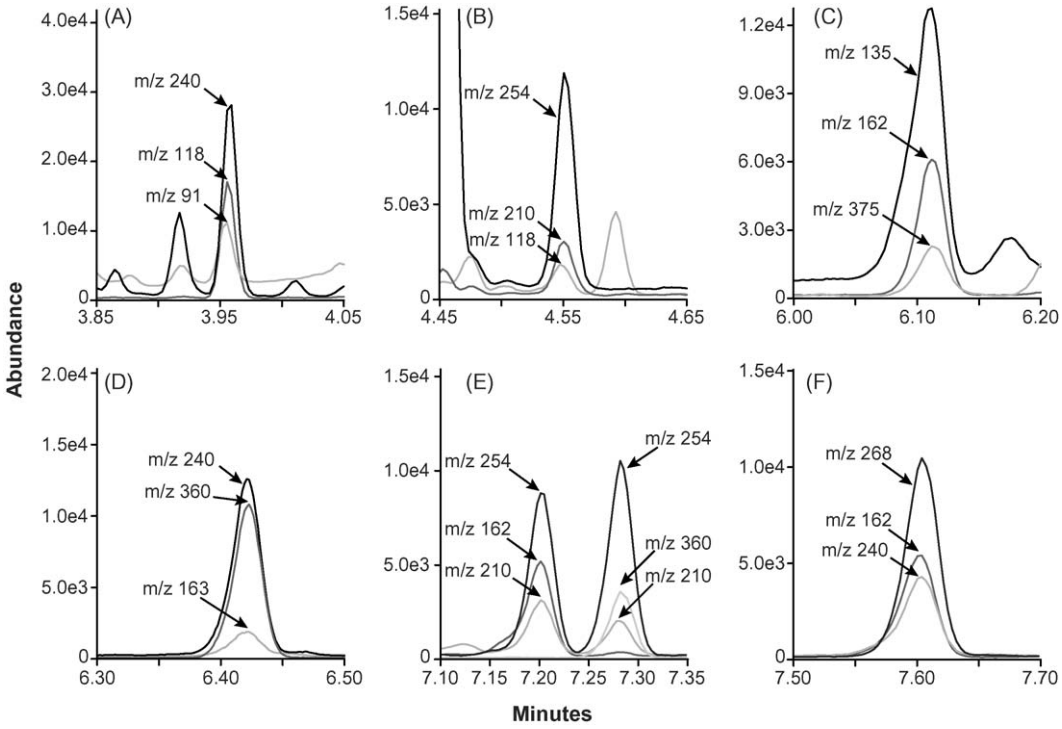

Fig. 2. Extracted ion chromatograms of blank oral fluid fortified with drug at each analytes' limit of detection containing: (A) 2.5 ng/mL amphetamine; (B) 1.0 ng/mL methamphetamine; (C) 2.5 ng/mL methylenedioxyamphetamine; (D) 5.0 ng/mL 4-hydroxy-3-methoxyamphetamine; (E) 2.5 ng/mL of methylenedioxymethamphetamine (peak at 7.19 min) and 4-hydroxy-3-methoxymethamphetamine (peak at 7.28 min); and (F) 2.5 ng/mL of methylenedioxyethylamphetamine.

Table 3
Sympathomimetic amines in oral fluid by GC/MS–EI: accuracy and precision data[a]

	Target concentration (ng/mL)	Precision		Accuracy
		Intra-assay (% CV, $n=6$)	Inter-assay (% CV, $n=24$)	(% Target, $n=24$)
AMP	15	1.5	4.2	89.4
	100	1.4	4.1	87.1
	200	1.3	3.9	88.1
MAMP	15	1.5	4.2	90.5
	100	1.3	4.0	88.9
	200	1.4	3.9	91.4
MDA	15	1.5	4.4	95.2
	100	1.6	4.1	93.2
	200	1.4	3.9	93.9
HMA	60	7.8	6.8	93.5
	400	3.0	5.8	100.7
	800	2.5	5.6	104.0
MDMA	15	1.2	4.5	92.6
	100	1.9	5.1	89.6
	200	1.9	4.7	92.1
HMMA	60	8.3	6.4	88.8
	400	2.6	5.0	94.7
	800	2.7	4.2	100.1
MDEA	15	1.7	4.5	91.6
	100	1.5	4.2	89.4
	200	1.7	3.9	90.4

Abbreviations: amphetamine (AMP), methamphetamine (MAMP), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-amphetamine (HMA), methylenedioxymethamphetamine (MDMA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), methylenedioxyethylamphetamine (MDEA).

[a] Precision is expressed as coefficient of variation (% CV) and accuracy as percent of target concentration. Intra-assay precision was evaluated during a single run, analyzing six replicates of each quality control (QC). Intra-assay precision is presented from the run with the greatest variation. Inter-assay accuracy and precision were evaluated across four runs with each run containing six replicates of each QC.

Table 4
Stability of derivatized oral fluid extracts containing sympathomimetic amines[a]

	Target concentration (μg/L)	% Target (% CV)	
		24 h (n = 3)	48 h (n = 3)
AMP	15	98.0 (6.2)	98.3 (1.7)
	100	86.3 (2.8)	88.7 (1.2)
	200	87.3 (3.2)	89.1 (2.9)
MAMP	15	98.9 (5.8)	99.0 (1.7)
	100	88.3 (2.9)	91.0 (1.2)
	200	90.8 (2.8)	93.4 (2.9)
MDA	15	104.6 (7.2)	104.5 (2.0)
	100	91.3 (2.8)	93.4 (1.3)
	200	92.5 (3.6)	94.0 (3.1)
HMA	60	98.4 (4.4)	96.0 (2.6)
	400	100.5 (5.0)	103.3 (2.4)
	800	102.2 (6.6)	99.2 (4.8)
MDMA	15	102.5 (7.3)	102.2 (1.9)
	100	91.0 (3.0)	93.5 (1.2)
	200	94.3 (2.9)	96.6 (3.0)
HMMA	60	92.3 (3.9)	91.4 (2.6)
	400	93.4 (5.1)	96.1 (2.3)
	800	98.3 (5.3)	97.1 (4.0)
MDEA	15	101.3 (7.3)	101.3 (1.9)
	100	87.7 (2.9)	90.1 (1.1)
	200	89.6 (3.2)	91.3 (3.1)

Abbreviations: amphetamine (AMP), methamphetamine (MAMP), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-amphetamine (HMA), methylenedioxymethamphetamine (MDMA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), methylenedioxyethylamphetamine (MDEA).

[a] Accuracy, expressed as mean % target concentrations, along with precision (% CV) of quality control samples injected after ambient storage for 24 or 48 h.

Table 5
Stability of sympathomimetic amines in oral fluid[a]

	Target concentration (ng/mL)	% Target concentration (% CV)		
		RT, 12 h	4 °C, 48 h	Freeze/thaw, ×3
AMP	15	96.4 (1.3)	94.2 (3.1)	88.6 (1.8)
	100	87.1 (3.4)	94.0 (2.0)	88.4 (4.1)
	200	86.0 (1.7)	86.6 (0.4)	88.6 (2.9)
MAMP	15	98.6 (1.3)	97.1 (3.1)	88.8 (1.7)
	100	89.8 (3.1)	96.8 (2.1)	90.3 (4.1)
	200	89.7 (2.2)	90.3 (0.3)	92.0 (3.1)
MDA	15	102.3 (2.4)	100.6 (3.7)	94.1 (1.4)
	100	93.0 (3.4)	99.9 (2.0)	92.9 (4.0)
	200	91.0 (2.0)	91.1 (0.6)	94.9 (2.5)
HMA	60	93.5 (5.5)	94.2 (4.3)	89.1 (8.0)
	400	97.0 (4.7)	102.8 (5.3)	96.0 (3.9)
	800	94.5 (2.0)	96.0 (2.1)	97.5 (3.8)
MDMA	15	100.6 (1.0)	98.9 (2.9)	90.4 (1.8)
	100	92.6 (3.2)	99.6 (1.8)	92.6 (3.8)
	200	91.9 (2.0)	92.5 (0.1)	95.2 (2.6)
HMMA	60	89.1 (4.5)	89.0 (5.3)	83.2 (8.6)
	400	95.0 (4.9)	101.6 (5.6)	92.1 (3.5)
	800	97.7 (2.3)	97.8 (1.5)	99.9 (1.6)
MDEA	15	96.2 (1.4)	93.7 (2.9)	91.7 (1.8)
	100	87.4 (3.4)	94.1 (2.2)	90.0 (4.0)
	200	85.9 (2.1)	86.6 (0.1)	90.4 (2.6)

Abbreviations: amphetamine (AMP), methamphetamine (MAMP), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-amphetamine (HMA), methylenedioxymethamphetamine (MDMA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), methylenedioxyethylamphetamine (MDEA).

[a] Quality control samples fortified with drug in blank oral fluid stored 12 h at room temperature, 48 h at 4 °C or that underwent 3 cycles of freezing at −20 °C and thawing at room temperature prior to analysis. Measured analyte concentrations are expressed as mean percentage of target quality control concentrations (% CV), n = 3 for each condition.

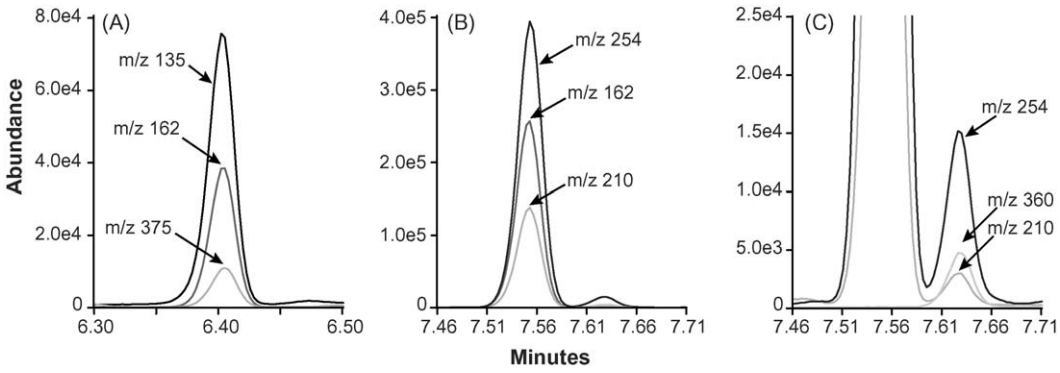

Fig. 3. Extracted ion chromatograms of oral fluid collected via expectoration from a participant 23 h after an oral dose of 1.0 mg/kg methylenedioxymethamphetamine (MDMA). Sample contains: (A) 16.2 ng/mL methylenedioxyamphetamine (MDA); (B) 111.3 ng/mL MDMA (peak at 7.55 min); and (C) trace amount of 4-hydroxy-3-methoxymethamphetamine (HMMA, peak at 7.62 min). Note: this analysis was performed after replacement of gas chromatographic column, therefore, retention times are different for MDA, MDMA and HMMA than are observed in Fig. 2.

MDMA. Chromatographic data are presented in panels A, B and C of Fig. 3. The oral fluid specimen contained 111.3 ng/mL MDMA, and 16.2 ng/mL MDA. There was a trace concentration of HMMA, fulfilling LOD requirements (2.5 ng/mL) but less than the LOQ (25 ng/mL) 23 h after the low dose. The concentration of HMA did not exceed the 5.0 ng/mL LOD.

In conclusion, this report details the validation of a sensitive and specific GC/MS–EI assay for measuring sympathomimetic amines in oral fluid specimens. This is the first oral fluid assay for sympathomimetic amines that includes quantification of HMA. This method requires a specimen volume of 400 μL and achieved LOQs of 5.0 ng/mL for MDMA, MDEA, MDA, AMP and MAMP and 25 ng/mL for HMMA and HMA. The Substance Abuse and Mental Health Services Administration (SAMHSA) guidelines proposed for confirmatory oral fluid drug testing require an assay cut-off of 50 ng/mL for AMP, MAMP, MDMA, MDA and MDEA [29]. The LOQs achieved with this assay exceed cut-offs proposed by SAMHSA guidelines for amphetamines oral fluid testing. Inter-assay precision, expressed as coefficient of variation, was less than 6.8% for all analytes. It is difficult to obtain sufficient oral fluid from subjects experiencing acute effects of sympathomimetic amines, making analytical sensitivity an important priority. This method enables characterization of MAMP, MDMA and metabolites pharmacokinetics in oral fluid following controlled drug administration, as demonstrated by preliminary data from a subject 23 h after receiving a single 1.0 mg/kg oral MDMA dose.

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