Achieving Proposed Federal Concentrations using Reduced Specimen Volume for the Extraction of Amphetamines from Oral Fluid

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Abstract

This article details the rapid extraction of amphetamines from oral fluid using low specimen volume, low sorbent bed mass, and fast gas chromatography with mass selective detection. The collection of a known amount of sample volume coupled with high percentage recovery of drug from the collection pad has allowed oral fluid to be increasingly employed as the specimen of choice for the detection of drug use in various applications. Additionally, low specimen volume for confirmation is required, so that adequate test volume remains for second analysis in case of batch failure or for testing at a different laboratory facility. The extracts were prepared using low bed mass sorbent, so less conditioning, washing, and elution solvent further reduced the overall cost of sample preparation. The limits of quantitation for the assay were 25 ng/mL; the intraday precision of the assays (n = 5) ranged from 0.3 to 3.99%; interday precision ranged from 0.72 to 4.6% for the amphetamine class. The percentage recovery of the drugs from the collection pads ranged from 85.4 to 89.1% (n = 6). The process lends itself to widely available automated processing instrumentation and significantly increases the efficiency of laboratories providing high-volume oral fluid analysis for drugs of abuse.

Introduction

Oral fluid is becoming increasingly popular in many areas of drug testing as a diagnostic fluid, partly due to the ease and non-invasiveness of collection (1). Applications for the detection of amphetamines in oral fluid analysis have been reported in clinical areas (2,3) as well as several drugs of abuse in epidemiological studies (4) and in roadside situations (5,6). The presence of measurable drug levels has enhanced the number of laboratories engaged in the analysis of oral fluid, and as the overall number of analyses increase, the rapid, reproducible extraction of drugs from the collection buffer is an essential component.

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However, because a limited sample volume is generally collected when oral fluid is to be tested, procedures for the use of reduced sample volume along with improved analytical capability are necessary. Most importantly for our application, the amount of specimen required for extraction is significantly reduced. Other advantages of the assays include excellent precision, accuracy, recovery from the collection pad, decreased sample preparation time, and reduced solvent volumes for conditioning, washing, and elution. The analytical procedure employed a gas chromatograph (GC) equipped with fast oven capability coupled to a mass selective detector (MS) with an inert source operating in electron impact mode.

In 2004, the Substance Abuse and Mental Health Service Administration (SAMHSA) proposed the inclusion of oral fluid as a specimen for workplace drug testing (7). The proposed confirmatory cut-off concentration for amphetamine (AMP), methamphetamine (METH), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), and methylenedioxyethylamphetamine (MDEA) was 50 ng/mL.

The objective of our study was twofold: to determine the efficiency of recovery of amphetamines from the oral fluid collection system and to achieve proposed regulatory "cut-off concentrations" for amphetamines using 62.5 µL of neat oral fluid (0.25 mL of total specimen).

Materials and Methods

Quantisal™ oral fluid collection devices were obtained from Immunalysis (Pomona, CA). Amphetamine, methamphetamine, MDMA, MDA, MDEA, and their penta-deuterated analogues were obtained from Cerilliant (Round Rock, TX). Mixed-mode solid-phase extraction columns (HCX 130 mg/1 mL capacity; Part # 902-0013-A) were obtained from Biotage (Charlottesville, VA). All reagents were of high-performance liquid chromatography (HPLC) grade, and chemicals were of ACS grade or better. The derivatizing agent, heptafluorobutyric anhydride (HFBA) was obtained from Pierce Chemical (Rockford, IL).

Calibrators and controls

Calibration standards and controls were prepared from synthetic oral fluid and diluted with Quantisal transportation buffer. Throughout the development of the assay, multiple Quantisal collection devices were selected from different lots. The device consists of a pad that is placed into the mouth for saliva collection. A blue line becomes visible when 1 mL (± 10%) of oral fluid has been collected; the pad is then placed in stabilization buffer (3 mL), capped, and the specimen sent to a laboratory for analysis. The buffer causes the amount of oral fluid in the sample to be diluted, producing approximately 4 mL of total specimen to be available for analysis.

In this experiment, the drug concentration used to fortify the synthetic oral fluid was adjusted according to the dilution factor for all calibration standards and controls. In this way, the final result obtained from the instrument did not need to be recalculated for dilution factors. For each analysis, a four-point calibration curve (25, 50, 100, and 200 ng/mL) was run with each batch; the internal standard concentration was 50 ng/mL.

Extraction procedure

For calibrators and controls, an aliquot (0.25 mL) of drug free Quantisal buffer was used. For the calibration curve, unlabelled drugs were added at concentrations of 25, 50, 100, and 200 ng/mL (6.25, 12.5, 25, and 50 µL, respectively, of 250 ng/mL stock solution). Deuterated amphetamine-d_5, methamphetamine-d_5, MDMA-d_5, MDA-d_5, and MDEA-d_5 were added (12.5 µL of 250 ng/mL = 50 ng/mL) to each specimen, calibrator, or control (Table I).

Potassium phosphate buffer (0.1M, pH 6.0, 0.5 mL) was added to each specimen. The solid phase cartridges were placed onto the positive pressure manifold and conditioned with methanol (1 mL), deionized water (1 mL), and 0.1M phosphate buffer (pH 6.0; 1 mL). The specimen was then allowed to drain through the column. The columns were washed with deionized water (1 mL), 0.1M hydrochloric acid (1 mL), and methanol (1 mL). The columns were allowed to dry. Glass collection tubes were placed into the collection rack, and the drugs were eluted with methanol/ammonium hydroxide (98:2, v/v; 1 mL). Ten microliters of 0.35M sulfuric acid/acetone (25:75, v/v) was added as a keeper solvent, and the extracts were evaporated to dryness. Ethyl acetate (50 µL) and heptafluorobutyric anhydride (HFBA, 20 µL) were added, and the samples were heated at 60°C for 20 min. The derivatized extract was evaporated to dryness and reconstituted in ethyl acetate (40 µL). The extracts were transferred to autosampler vials for analysis using GC–MS.

Table I. Fortification of Quantisal Buffer for the Preparation of Calibration Standards

Calibration	Standard Internal (250 ng/mL)	Drug Added (250 ng/mL)	Equivalent Neat Oral Fluid Concentration (ng/mL)
Negative	12.5 µL	0	0
25 ng/mL	12.5 µL	6.25 µL	25
50 ng/mL	12.5 µL	12.5 µL	50
100 ng/mL	12.5 µL	25 µL	100
200 ng/mL	12.5 µL	50 µL	200

Analytical procedure

An Agilent Technologies 6890 GC coupled to a 5975 mass selective detector (MSD) with fast GC capability and an inert source, operating in electron impact mode was used for analysis (GC–MS). The injection mode was splitless for all specimens, and helium was the carrier gas. The gas chromatographic column was 5% phenyl-95% methyl silicone DB-5 (15 m x 0.25-mm i.d., 0.25-µm film thickness, J&W Scientific, Palo Alto, CA). The transfer line was held at 280°C, the MS source was operated at 230°C, and the quadrupole was kept at 150°C.

The injection temperature was 150°C; the injection volume was 1 µL. The oven was programmed from 60°C for 1 min, ramped to 140°C at 25°C/min, then ramped to 200°C at 20°C/min, and finally to 240°C at 80°C/min.

The monitored ions were as follows (quantitative ions in bold type): amphetamine **240**, 118, 91 (d_5 **244**, 123); methamphetamine **254**, 210, 118 (d_5 **258**, 213); MDMA **254**, 210, 162 (d_5 **258**, 213); MDA **135**, 240, 375 (d_5 **136**, 244); MDEA **268**, 240 (d_5 **273**, 241).

Method validation

Linearity and selectivity. Calibration using corresponding deuterated internal standards was calculated using linear regression analysis over a concentration range of 25–200 ng/mL for the amphetamine class. Peak-area ratios of target analytes and their respective deuterated standards were calculated using Agilent DrugQuant ChemStation software. The data were fit to a linear least-squares regression curve forced through the origin. The linearity of the assays was established with four calibration points, excluding the drug-free matrix. The sensitivity of the method was determined by establishing the limit of quantitation (LOQ) defined as the lowest concentration detectable with a signal-to-noise (S/N) ratio of at least 10 and retention time within 0.2 min of the calibration standard. Because all values are quantitated, the limit of detection was not determined.

Drug-free oral fluid specimens were obtained from volunteers and extracted and analyzed according to the described procedures in order to assess interference from the collection buffer with the assays. In addition, potential interferences from commonly encountered drugs were added to the drug-free oral fluid specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed using the described procedures at a concentration of 200 ng/mL: morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, cocaine, norcocaine, cocaethylene, benzoylecgonine, tetrahydrocannabinol (THC), 9-carboxy-THC, pseudoephedrine, phentermine, fluoxetine, sertraline, zolpidem, carisoprodol, methylphenidate, norbuprenorphine, cotinine, methadone, phencyclidine, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, nitrazepam, triazolam, α-hydroxytriazolam, secobarbital, pentobarbital, butalbital, amobarbital, butabarbital, and phenobarbital.

Precision. Inter- and intraday assay precision of the assays were determined at the cut-off calibration point of 50 ng/mL. Intraday data were obtained from five analyses performed on one day; interday data were obtained by analyzing one specimen per day over five days (n = 5).

Extraction efficiency from the pad. One of the issues associated with oral fluid analysis is recovery of drug from a collection pad if a device is used. Extraction efficiency for these drugs was determined.

A synthetic oral fluid matrix, which matched the immunoassay responses of three human negative oral fluid samples, was prepared, comprising 25mM phosphate buffered saline (pH 7.0), 30mM sodium bicarbonate, 0.1% albumin, amylase, and 0.1% Proclin 300 as a preservative.

Synthetic oral fluid was used as opposed to authentic drug-free saliva primarily because of the amount required in order to carry out all the experiments. The effect of real oral fluid on the drugs compared to the effect in synthetic material is minimized during the procedure because of several dilution factors. Firstly, the oral fluid collected is diluted fourfold with transportation buffer; secondly, only 250 µL is then used for analysis (62.5 µL of neat oral fluid). Synthetic oral fluid was fortified with the amphetamines under investigation at the proposed cut-off concentration of 50 ng/mL, and then the pad was dipped into the oral fluid until the volume adequacy indicator turned blue showing that 1 mL (± 10%) of oral fluid had been absorbed. The pad was then removed and placed into the extraction buffer (3 mL) where it remained at room temperature overnight to simulate shipping to an analytical laboratory. The following day, a specimen volume of 0.25 mL (62.5 µL of neat oral fluid) was used for analysis. The procedure was repeated using six different collection devices.

Fortification of oral fluid

Although it is true that very high concentrations of amphetamines can be detected immediately after smoking, the concentrations were selected for our study since they approximate the levels suggested by the proposed federal guidelines for drugs analysis in oral fluid.

Results and Discussion

The overall assay was accurate, precise and reproducible, and was able to detect the amphetamines in oral fluid at the proposed Federal cut-off concentration. An example of the chromatographic data at a concentration of 50 ng/mL of neat oral fluid is shown in Figure 1.

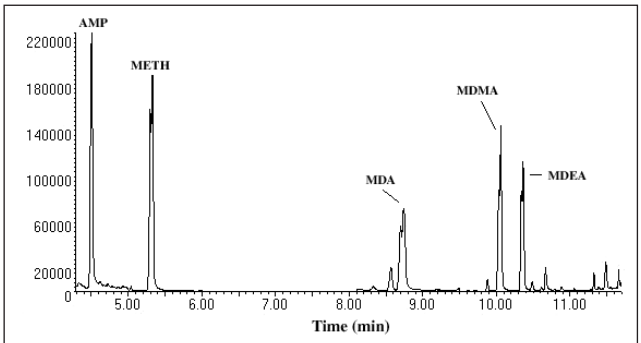

Figure 1. Total ion chromatogram of amphetamines extracted from oral fluid at a concentration of 50 ng/mL using 0.25 mL specimen volume.

Method validation

The limits of quantitation and linearity and calibration curve coefficients are shown in Table II. For all compounds, the correlation coefficient (r^2) over the range 25–200 ng/mL was greater than 0.99; and the limit of quantitation was 25 ng/mL. Because all specimens are quantitated, the absolute limit of detection was not determined. Intraday coefficient of variation was less than 4% for all drugs and less than 5% for interday precision (Table III).

Placing the pad in the Quantisal buffer overnight and analyzing the samples the following day allowed the recovery of

Table II. Limits of Quantitation, Linearity, and Calibration Curve Equations, Forced Through the Origin, for Amphetamines in Oral Fluid

Analyte	Limit of quantitation (ng/mL)	Equation (mean SD)	Correlation (r^2)	Linearity range (ng/mL)
Amphetamine	25	y = 0.0204x	0.999	25–200
Methamphetamine	25	y = 0.0199x	0.997	25–200
MDA	25	y = 0.0201x	0.999	25–200
MDMA	25	y = 0.0186x	0.999	25–200
MDEA	25	y = 0.0258x	0.998	25–200

Table III. Inter- and Intraday Precision for the Amphetamine Assay Determined at the Cut-Off Concentration (50 ng/mL)

	AMP*	METH	MDA	MDMA	MDEA
Intraday					
Replicate 1	49.5	48.9	50.3	48.4	48.9
Replicate 2	48.7	48.2	50.4	47.4	53
Replicate 3	49.3	48.5	50.4	47.9	49.2
Replicate 4	48.9	48.2	50.1	47.2	52.8
Replicate 5	48.7	48.2	50.1	47.3	49.6
Mean	49.02	48.4	50.26	47.64	50.7
SD	0.36	0.31	0.15	0.5	2.02
CV (%)	0.74	0.64	0.3	1.06	3.99
Interday					
Day 1	49.5	48.9	50.3	48.4	48.9
Day 2	49.6	49.7	50.2	49.2	53.3
Day 3	49.9	49.8	50.9	49.7	49.6
Day 4	49.9	50.3	50.8	50	50.7
Day 5	50.6	48.8	51	49	47.1
Mean	49.9	49.5	50.64	49.26	49.92
SD	0.43	0.64	0.36	0.62	2.3
CV (%)	0.86	1.29	0.72	1.26	4.6

* Abbreviations: AMP, amphetamine; METH, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine; and MDEA, methylenedioxyethylamphetamine.

Table IV. Oral Fluid Fortified at 50 ng/mL, Incorporated onto the Collection Pad, and Allowed to Remain in the Transportation Buffer Overnight at Room Temperature*

Device #	AMP†	METH	MDA	MDMA	MDEA
1		44.4	45.8	45.7	44.5	46.4
2		46.3	47.3	47.3	45.8	47.3
3		42.5	42.4	42.5	41.0	42.3
4		43.4	44.4	44.5	42.7	44.8
5		41.5	41.5	41.7	39.9	41.6
6		42.9	44.1	44.2	42.4	45.0
Mean		**43.50**	**44.25**	**44.32**	**42.72**	**44.57**
SD		**1.67**	**2.38**	**2.29**	**2.43**	**2.49**
CV (%)		**3.85**	**5.38**	**5.17**	**5.68**	**5.59**
Recovery (%)		**87.00**	**88.50**	**88.63**	**85.43**	**89.13**

* 0.25 mL of specimen extracted and analyzed.
† Abbreviations: AMP, amphetamine; METH, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine; and MDEA, methylenedioxyethylamphetamine.

amphetamines from the collection pad to be determined. In this way, losses occurring during overnight transportation to a laboratory were assessed. Six replicate analyses at the cutoff value using different devices, were analyzed to assess reproducibility of the extraction efficiency, and a full calibration curve was included with each batch. The recovery of amphetamines from the pad and the transportation buffer was greater than 85% for all drugs (Table IV).

The ability to use a low volume of collected specimen in order to achieve relevant cut-off concentrations is an important parameter in oral fluid analysis. The specimen volume is so often limited that multiple confirmatory procedures cannot be carried out, and sufficient donor sample for a second laboratory testing may also be an issue. If methods that use less sample, coupled with highly efficiency extraction and robust analytical methods can be developed, the oral fluid drug test panel can be broadened and more easily implemented into workplace testing.

The collection of oral fluid has been described as the most overlooked variable in oral fluid testing. To date, commercially available collection devices have either been highly inefficient in their drug recovery (8) or are unable to determine how much oral fluid has been collected for analysis, thereby calling into question any quantitative result (9). The efficiency of drug removal from any collection pad or swab is a critical parameter in determining the quantitative value of drug present in a sample, and must be included in any experimental protocol.

We have previously reported the efficient recovery and stability of THC and its metabolite THCA from the Quantisal device (10,11), as well as meperidine, tramadol, oxycodone, and propoxyphene (12,13). Other authors have assessed the efficacy of the Quantisal device for various drugs, and reported the recoveries of amphetamine and methamphetamine to be 94.3% and 103.8%, respectively (14).

Recovery

The determination of drug recovery from oral fluid collection devices is an important issue, since the retention of drugs on a pad or other material will affect the amount, which can be detected. The drawback to less than 100% recovery is that a specimen containing drug at or just above the cut-off value may be reported as negative because of losses in recovery. However, the bias towards negativity for specimens close to the cut-off is acceptable in a program intended for the deterrence of drug use. In our work, we do not correct for the efficiency in order to report a quantitative value.

Conclusions

In summary, amphetamines are extracted from the collection pad and buffer with an average efficiency over 85%. The use of compact solid-phase extraction columns (130 mg/1 mL capacity) considerably shortened the time required for sample preparation and used 50% less conditioning and elution volumes than previous procedures, saving both time and money. The overall sample processing was rapid, efficient, and reproducible with minimal specimen volume required and low solvent usage.

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