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Method validation and application of a liquid chromatography–tandem mass spectrometry method for drugs of abuse testing in exhaled breath

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ABSTRACT

A mass spectrometric method for drugs of abuse testing in exhaled breath employing a sampling device collecting aerosol particles was developed and applied in routine use. Analytes covered were amphetamine, methamphetamine, 6-acetylmorphine, morphine, cocaine, benzoylecgonine, diazepam, oxazepam and tetrahydrocannabinol. The method involved eluting drugs from the collection filter with methanol, quantification using deuterated analogs as internal standards, reversed phase chromatography with gradient elution, positive electrospray ionization and monitoring of two product ions per analyte in selected reaction monitoring mode. The measuring range was 6.0–1000 pg/filter. The intra- and inter-assay imprecision expressed as the coefficient of variation was less than 7%. Influence from matrix was noted for most compounds but was compensated for the use of co-eluting internal standards. The LLOQ was 6.0 pg/filter with intra-assay CV <5% and accuracy within 99–102% for all analytes. No chromatographic interference was observed in 20 negative control samples. The LC–MS/MS method was successfully applied for measuring drugs in unknown samples collected for the purpose of drug testing. Among the 1096 analyzed samples analytical findings were made in breath in 39 cases (3.6%). Most frequently found substances were the following: amphetamine (25 cases) methamphetamine (10 cases), THC (8 cases), cocaine (4 cases), benzoylecgonine (2 cases) and diazepam (2 cases). In conclusion, a fully validated and robust screening method suitable for the routine measurement of drugs of abuse in exhaled breath with a simple procedure for specimen collection and sample preparation was successfully developed.

1. Introduction

Testing for drugs of abuse is a common laboratory investigation with important clinical, sports doping and forensic applications. Drug testing is most commonly performed using urine samples, which is based on a long and comprehensive experience. The methodology and regulations for reliable testing using urine are well developed and can be considered as the gold standard method for drug testing. However, one problem with urine drug testing is related to the collection of valid urine specimens. This often leads to the use of procedures involving supervised urination with the consequence of inconvenience for the donor and intrusion of privacy. Interest for alternative matrices for drug testing, e.g. oral fluid, sweat, hair, has therefore emerged [1–3]. In addition, for the application of drug testing in traffic medicine where blood is the commonly demanded specimen, alternative matrices, e.g. oral fluid, are of interest because of the need for professional personnel when collecting venous blood, which is more complicated to perform at the roadside [4,5].

This situation for drug testing is quite different from that for alcohol testing, which can be done at the site of collection using breath as specimen. The breath testing can be done with technique providing evidential results if needed and has replaced the need for blood alcohol determinations in many instances [6].

Interestingly, exhaled breath has become an emerging alternative matrix also for drug testing in recent time. It was first demonstrated that amphetamine could be detected in exhaled breath [7]. Subsequently, a number of other drugs of abuse substances have been found to be detectable in collected exhaled breath [8–16]. The underlying mechanism for making this possible is believed to be the formation of aerosol particles from the airway lining fluid by the breathing process [17]. These aerosol

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particles may become contaminated with drugs present in the body, which enables drug testing using this specimen. A simple collection device is currently available which selectively collects the micrometer aerosol particles on a filter and enables further laboratory investigation of possible drug content [9].

Possible future use of drug breath testing is wide and may comprise traffic medicine [10], clinical situations where urine sampling is problematic [18,19], compliance testing in drug addiction treatment [14,19], criminal justice [15], workplace testing and investigation of accidents. Drug breath testing may serve as a viable alternative specimen whenever urine or blood cannot be collected for any reason. Interest for determination of therapeutic drugs in exhaled breath is also under development [20].

The analysis of drugs in exhaled breath is challenging because of the low pg amounts being present in the collected samples. The present report was aimed at presenting an analytical procedure using combined liquid chromatography–tandem mass spectrometry that has been developed during our exploratory work with the drug breath testing. The method is now fully validated for measurement of amphetamine, methamphetamine, 6-acetylmorphine, morphine, cocaine, benzoylecgonine, diazepam, oxazepam and tetrahydrocannabinol (THC) in exhaled breath and has been applied in routine use.

2. Materials and methods

2.1. Chemicals and materials

Amphetamine, methamphetamine, 6-acetylmorphine (6-AM), morphine, cocaine, benzoylecgonine, diazepam, oxazepam, Δ-9-tetrahydrocannabinol (THC), amphetamine-d5, 6-AM-d3, morphine-d3, cocaine-d3, diazepam-d5, oxazepam-d5, cocaine-d3 and THC-d3 were obtained as ampoulled stock solutions from LGC Standards AB (Borås Sweden). Methanol and acetonitrile of LC–MS grade were from Fisher Scientific AB (Gothenburg, Sweden). 2-Propanol of "normapur" grade was from VWR International (West Chester, PA). Ammonium formate, ammonia (25%) and formic acid of analytical grade were from Merck KGaA (Darmstadt, Germany). The Milli-Q water was of ultra-pure quality (>18 MΩ/cm) and prepared in-house. The exhaled breath sampling device was obtained from SensAbues AB (Huddinge, Sweden).

2.2. Standards and controls

The ampoulled stock solutions containing 100 μg/mL amphetamine, methamphetamine, 6-AM, morphine, cocaine, benzoylecgonine, diazepam, oxazepam and 1000 μg/mL THC were diluted with methanol to working solutions containing 0.25, 2.5 and 25 ng/mL of each analyte. The working solutions for preparing quality control (QC) were independently diluted. Internal standards were prepared in methanol at a concentration 25 ng/mL from the ampoulled stock solutions of 100 μg/mL.

The prepared solutions were stored at −20 °C, with a maximum storage time of 3 months.

Calibration samples were prepared by fortifying blank filters with methanol solutions of analytes. These were prepared by adding 8.0–40 μL of methanol solutions containing 0.25–25 ng/mL of analytes. After drying the devices were prepared for analysis as described below (Section 2.5). Calibration graphs were constructed using linear regression analysis, with weighting factor $1/x$. The concentrations of analytes in unknown samples were determined from the peak area ratio between analyte and internal standard by reference to the calibration graph. Internal standards were used as follows: amphetamine-d5 for both amphetamine and methamphetamine, 6-AM-d3 for 6-AM, morphine-d3 for morphine, cocaine-d3 for cocaine and benzoylecgonine, diazepam-d3 for diazepam, oxazepam-d3 for oxazepam and THC-d3 for THC. A zero standard and three calibration levels (18, 54 and 324 pg/filter) were routinely used with a lower reporting limit of 18 pg/filter applied for all compounds. Three quality control levels: QC low (20 pg/filter); QC middle (100 pg/filter) and QC high (500 pg/filter) as well as blank filter samples were used in every batch.

Table 1
Gradient system used in the LC–MS/MS method.

Time (min)	Mobile phase composition (% v/v)	
	A[a]	B[a]
0	70	30
0.6	70	30
1.5	45	55
3.1	1	99
3.6	1	99
3.61	70	30

[a] Mobile phase A consisted of 4.0 mmol/L ammonium formate with pH adjusted to 10 with 25% ammonia and mobile phase B was methanol with 4.0 mmol/L ammonium formate (99.6% methanol) and same amount of ammonia added as solvent A.

2.3. Instrumentation

The LC–MS/MS system consisted of a Thermo Fisher Scientific Dionex Ultima 3000 UHPLC with a Ultimate 2000 SRD degasser, Ultimate 3000 RS binary solvent pump system, column oven, Ultimate 3000 RS auto sampler connected to a Thermo Fisher Scientific TSQ Vantage triple quadrupole mass spectrometer. The system was operated using Chromeleon Xpress (version 3), Trace Finder Clinical Research (version 2.1) and Thermo TSQ Tune Master (version 2.3.0) softwares. The heated electrospray interface (HESI) was used with the instrument operating in the positive ion mode. Nitrogen was used as sheath, auxiliary and ion sweep gas, and argon as collision gas. The chromatography was performed using a 1.7-μm 100 mm × 2.1 mm (i.d.) Ethylene Bridged Hybrid (BEH) phenyl column (Waters Co), preceded by a 0.2 μm column filter (Waters Co). The liquid chromatography system was operated in a gradient mode with a flow rate of 650 μL/min (Table 1). Solvent A consisted of 4.0 mmol/L ammonium formate with pH adjusted to 10 with 25% ammonia and solvent B was methanol with 4.0 mmol/L ammonium formate (99.6% methanol) and same amount of ammonia added as solvent A. The injection volume was 2.0 μL and the column oven temperature 65 °C. Needle wash was performed before and after injection with 100 μL of a mixture consisting of equal volumes of methanol, acetonitrile, 2-propanol and ultra-pure water containing 0.20% ammonia. The total run time of the method was 4.0 min. The following conditions were used in the mass spectrometer: peak width 0.70 Da for Q1 and Q3, collision gas pressure 1.2 mTorr (Argon), capillary temperature 250 °C, vaporizer temperature 450 °C, sheath gas pressure 55 Arb units, ion sweep gas pressure 1.0 Arb units, aux gas pressure 18.0 Arb units, spray voltage 3000 V, DCV −4 V. The selected ions and dwell time used for each compound are presented in Table 2. Acquisition time was 0.50–3.6 min with monitoring of each analyte in a time segment of ±0.35 min of expected retention time.

2.4. Sampling of exhaled breath

A sampling procedure using a commercial sampling device was employed (SensAbues AB, Huddinge, Sweden). Micro-particles present in the exhaled breath were separately collected by asking subjects to let the exhaled breath pass through mouth piece constructed for separating saliva and larger particles from micro-particles. Micro-particles passing the mouth-piece were collected