Journal of Chromatography B, 878 (2010) 2255–2259

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Method for determination of methadone in exhaled breath collected from subjects undergoing methadone maintenance treatment

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ARTICLE INFO

Article history:
Received 6 April 2010
Accepted 25 June 2010
Available online 3 July 2010

Keywords:
Abused drug testing
Liquid chromatography–mass spectrometry
Methadone
Human
Exhaled breath
Breath testing

ABSTRACT

At present drugs of abuse testing using exhaled breath as specimen is only possible for alcohol. However, we recently discovered that using modern liquid chromatography–mass spectrometry technique amphetamine and methamphetamine is detectable in exhaled breath following intake in drug addicts. We therefore undertook to develop a method for determination of methadone in exhaled breath from patients undergoing methadone maintenance treatment. Exhaled breath was collected from 13 patients after intake of the daily methadone dose. The compounds were trapped by filtering the air through a C18 modified silica surface. After elution of any trapped methadone the extract was analysed by a combined liquid chromatography–tandem mass spectrometry method. Recovery of trapped methadone from the filter surface was 96%, no significant matrix effect was observed, and the quantification using methadone-d3 as an internal standard was accurate (<10% bias) and precise (coefficient of variation 1.6–2.0%). Methadone was indisputably identified by means of the mass spectrometry technique in exhaled breath samples from all 13 patients. Identification was based on monitoring two product ions in selected reaction monitoring mode with correct relative ratio (±20%) and correct retention time. Excretion rates ranged from 0.39 to 78 ng/min. No methadone was detected in 10 control subjects. This finding confirms that breath testing is a new possibility for drugs of abuse testing. Collection of exhaled breath specimen is likely to be more convenient and safe as compared to other matrices presently in use.

1. Introduction

Exhaled breath is commonly being used in alcohol testing and today's technology makes it even possible to perform on-site breath testing with legally defensible results using infrared spectroscopy and to apply it for alcolocks [1,2]. Testing for other drugs of abuse traditionally requires other specimens. In traffic medicine a blood sample is needed for documenting driving under the influence, while in other testing urine is the most common specimen. Alternative specimens comprise hair, sweat and oral fluid [3,4]. In recent time oral fluid testing has been in focus both for laboratory and on-site testing [5]. This interest in oral fluid testing has demonstrated the true need for specimen alternative to blood and urine in drugs of abuse testing. While blood is invasive and need medically trained personnel and facilities, urine sampling is considered intruding on personal integrity and is not free from risk of adulteration [6].

Methadone is used in the treatment of heroin addiction and for pain control [7,8]. In methadone maintenance treatment a fixed dose is administered over long time to provide stable blood concentrations with through levels being above 250 ng/mL [9]. Compliance to the prescribed dose regimen is controlled by frequent analyses of urine and blood samples. Because of inter-individual variation in the disposition of methadone each patient receives an individual dose [9]. Blood sampling is often problematic in this patient group because of previous chronic intravenous injections and urine samples might be adulterated after relapse into side abuse [6].

Human exhaled breath is known to contain a great number of substances including non-volatile compounds [10,11]. Over 3000 analytes have been detected in human breath [12]. The anaesthetic drug propofol, which is administrated during surgery, can be measured in exhaled breath by mass spectrometry techniques with breath and blood concentrations being correlated during steady-state conditions [13]. We recently were able to demonstrate that amphetamine and methamphetamine are detectable in exhaled breath from drug addicts recovering from acute intoxications [14]. The collection of breath sample was done by passing breath air through a solid-phase extraction cartridge and subsequent analysis by liquid chromatography–tandem mass spectrometry in selected reaction monitoring (SRM) mode.

The possibility of using exhaled breath for drugs of abuse testing is attractive as it would overcome the problem of sampling difficulties and produce a sample with less risk for adulteration or

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doi:10.1016/j.jchromb.2010.06.035

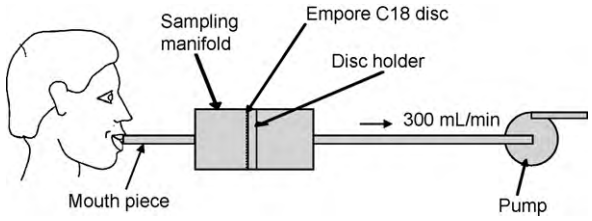

Fig. 1. Outline of the sampling device used to collect exhaled breath samples on an Empore C18 disc. The subjects were asked to breathe more deeply than normal during the 10 min sampling time.

alternate explanations. For these reasons we undertook to further investigate whether exhaled breath can be a specimen for drugs of abuse testing by investigation the possible presence of methadone in exhaled breath from patients undergoing methadone maintenance treatment.

2. Materials and methods

2.1. Chemicals and materials

Methadone and methadone-d3 (both 1.0 mg/mL) were obtained as ampouled methanol solutions from Cerilliant Corporation (Round Rock, TX). Methanol, acetonitrile and ethyl acetate of HPLC grade were from JT Baker (Mallinckrodt Baker BV, Deventer, Holland). Formic acid and 2-propanol of HPLC grade was from Merck GmbH (Darmstadt, Germany). The Milli-Q water was of ultra-pure quality (>18 MΩ/cm) and prepared in-house. The analytical column Aquity UPLC BEH C18 (1.7 μm 1.0 mm × 100 mm) was from Waters Corporation (Milford, MA). The 47 mm C18 Empore disc was from Varian Inc. (Palo Alto, CA).

2.2. Preparation of methadone solutions

The ampouled methadone and methadone-d3 solutions were diluted to 100 μg/mL using methanol. These solutions were further diluted to suitable concentrations in 0.1% formic acid and stored at −18 °C for a maximum of 1 year.

2.3. Patients and control subjects

Thirteen patients undergoing methadone maintenance treatment (12 males, 1 female, ages 31–58) were recruited from the methadone program in Stockholm (Beroendecentrum Stockholm). The patients were in steady-state and received supervised daily doses of methadone between 70 and 155 mg. The patients were subjected to constant control of compliance to treatment by urine drug testing. As a control group ten drug-free healthy volunteers (4 males, 6 females, ages 29–66) were recruited. Ethical approval was obtained from the Stockholm Regional Ethics Committee (no. 2008/1347-31).

2.4. Sampling of exhaled breath

Compounds present in the exhaled breath were collected for 10 min by suction through a 47 mm Empore C18 disc using a membrane pump to assist the flow (about 300 mL/min). The subjects were asked to breathe more deeply than normal into a mouth piece (no. 4091148, Palmenco AB, Stockholm, Sweden) mounted in the sampling device holding the Empore disc (Fig. 1). It was estimated that all the exhaled breath was collected through the filter during the sampling period. Following sampling the Empore disc was dismantled using a tweezers and stored at −80 °C. The sampling device was carefully cleaned between uses, which takes about 15 min.

2.5. Sample preparation

Following storage the Empore disc was cut into 5 mm × 5 mm pieces using a scalpel and transferred to a 10 mL glass test-tube. A volume of 100 μL of 100 ng/mL methadone-d3 was added and mixed using a Vortex mixer, 300 μL of 2-propanol was added (to wet the surface), mixed and finally 5 mL of 20% methanol in ethyl acetate was added. This mixture was shaken for one hour in a thermostatic bath at 37 °C. Thereafter, the test-tube was centrifuged for 15 min at 3000 × *g* at 10 °C, the supernatant transferred to a new 10 mL glass test-tube, and the extraction procedure repeated using 1 mL of 20% methanol in ethyl acetate. Finally the two supernatants were combined, 10 μL of 10% aqueous formic acid added and evaporated to dryness under a stream of nitrogen at a temperature of 40 °C. The dry residue was dissolved in 100 μL of 50% methanol in ethyl acetate.

2.6. Mass spectrometry analysis

An aliquot of 3 μL was subjected to analysis by UPLC–MS/MS (Waters Quattro Premier XE). The chromatographic system was a Aquity UPLC BEH C18 column, 100 mm × 1.0 mm, particle size 1.7 μm, with a gradient system consisting of A = 0.1% formic acid and B = acetonitrile. The mobile phase was 95% A for 1.2 min, followed by a linear gradient from 5% B to 65% B to 3.0 min. The equilibration time between injections was 4.0 min (95% A). The flow rate was 0.20 mL/min.

Two product ions from the protonated molecules were monitored for methadone (*m*/*z* 310 → 265; 310 → 105) and one for methadone-d3 (*m*/*z* 313 → 268). This was done by SRM in the positive electrospray mode, with 75 ms dwell time for each channel. Other instrumental settings are provided in Table 1. The minimum detectable amount (signal to noise 3) injected on column was about ~0.2 pg.

2.7. Quantification

Standards for quantification were prepared from fortified blank Empore discs.

These were prepared by adding 10, 25, 50, 100 and 200 μL (corresponds to 3.0, 7.5, 15, 30 and 60 ng on the surface) of a solution containing 300 ng/mL of methadone. After drying the discs were prepared for analysis as described above. Calibration curves were constructed using linear regression analysis, with weighting factor 1/*x*.

2.8. Method validation

Five replications of the calibration curve were analysed on different occasions. Limit of detection (LOD) and lower limit of quantification (LLOQ) was assessed by applying 10 pg of methadone onto a blank Empore disc and subject it for analysis. Imprecision and accuracy were estimated by analysis of six replicates of methadone applied on blank Empore discs at three levels (3.0, 15, 45 ng/disc). Recovery of extracting methadone from the Empore disc was estimated by comparison with a reference sample prepared directly in the final extract solvent. Matrix effects were estimated by extracting blank filter and filter from healthy volunteer and fortify with methadone in the final extract. This was compared with a reference sample without matrix. In addition, an infusion experiment was performed where injection of a control breath extract was injected while infusing methadone post-column and compared with injection of mobile phase A. The infusion rate was 10 μL/min and the infused methadone solution was 0.5 μg/mL in 0.1% formic acid in 50% methanol.

Table 1
Instrumental settings of the mass spectrometer.

Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
Methadone	310	265	25	16
Methadone	310	105	25	30
Methadone-d3	313	268	24	14

Source block temperature, 130 °C; desolvation gas temperature, 350 °C; desolvation gas flow, 950 L/h; cone gas flow, 50 L/h; capillary voltage, 2.0 kV; collision gas flow, 0.30 mL/min; multiplier voltage, 700 V.

Table 2
Summary of data obtained for methadone sampled in exhaled breath from 13 methadone maintenance patients.

Case no.	Methadone dose (mg/d)	Number of breaths	Sampling time after dose intake (min)	Mouth wash prior to sampling	Methadone excretion (ng/min)
1	90	41	13	No	1.0
2	100	59	44	Yes	0.39
3	100	127	27	No	1.9
4	140	91	10	Yes	5.8
5	80	94	25	Yes	1.2
6	155	45	10	Yes	0.87
7	100	42	60	Yes	3.5
8	100	56	13	Yes	1.5
9	120	35	>10[a]	No	1.4
10	70	46	12	Yes	0.90
11	100	66	13	Yes	0.93
12	100	90	18	Yes	>6.0[b]
13	120	59	8	Yes	2.6

[a] Not noted.
[b] Extrapoled value from 180 ng/disc standard was 78.

3. Results

3.1. Method validation

The peak area ratio of methadone to methadone-d3 was linear between 3 and 60 ng per sample corresponding to 0.3 and 6.0 ng methadone exhaled in breath per min. The correlation coefficients (r^2) of the calibration curves were between 0.991 and 0.999 (mean 0.996, $n = 5$). LOD (signal to noise 3) was estimated to 4 pg/sample (~0.4 pg in breath/min) and LLOQ (signal to noise 10) was estimated to 15 pg/sample, while the calibrated measuring range was 3.0–60 ng/sample.

Imprecision (coefficient of variation, CV) was estimated within-series to 1.6%, 1.9% and 2.0% at levels 3.0, 15, and 45 ng/sample ($n = 6$). The accuracy was 104%, 109% and 104%, respectively. The extraction recovery of methadone from the Empore disc surface was measured in duplicate using samples at the 15 ng/sample level and was 96.6% ($n = 4$). Matrix effects were estimated by addition of methadone (15 ng/sample) to extracts prepared from blank Empore discs and from Empore discs used for collection of exhaled breath from a healthy volunteer. The methadone peak area was compared with the reference sample containing no matrix. The matrix effect for blank Empore discs was 109% (SD 9, $n = 8$) and for breath sample discs 108% (SD 40, $n = 8$). The response of infused methadone following injection of matrix is shown graphically in Fig. 2. Although a drop in response was seen shortly following the void volume no matrix effect was observed at the retention time of methadone. The increase in response followed the increasing acetonitrile content.

3.2. Application of the method

Methadone was detected in the sampled exhaled breath from all 13 studied patients, which was in accordance with the daily observed dose intake of methadone (Fig. 3(a), Table 2). In all cases this was also supported by compliance to treatment as controlled by routine analysis of urine and by supervised dose intake. None of the 10 control subjects had detectable levels of methadone (<0.005 ng/min) in the exhaled breath samples (Fig. 3(b)). The detection level was set by the contribution of methadone-d3 to the two methadone channels.

Identification of detected methadone was based on a correct relative (to methadone-d3) retention time (±0.5%) and correct (<±20%) relative ion intensity ratio between the two product ions (see Fig. 3(a) and (c)). The amount of methadone collected from breath was high enough to produce strong analytical response. This makes the identification secure and methadone was identified according to these criteria in samples from all methadone patients. The amount of methadone ranged >15-fold from 0.39 to >6.0 (78) ng/min. The highest value obtained was outside the measuring range and appeared to be an outlier. Table 2 summarizes the results and collected data for the 13 patient samples. No difference in results could be observed between subjects sampled with or without mouth wash prior to sampling (Table 2). No significant correlation of excretion rate with methadone dose was observed.

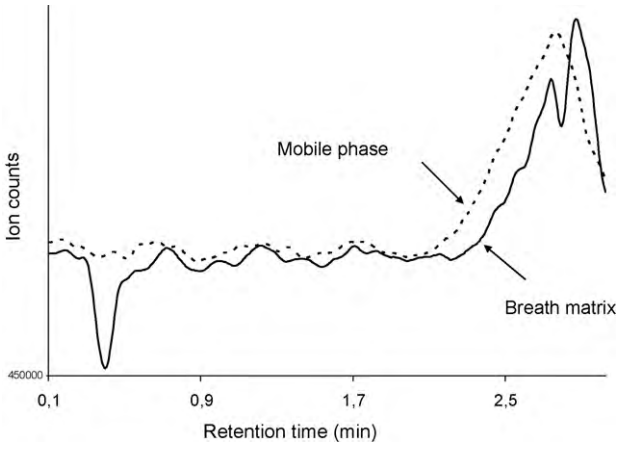

Fig. 2. The matrix effect on methadone ionisation was studied at the *m*/*z* 310/265 transition. Methadone was infused post-column (infusion rate 10 μL/min of a methadone solution containing 0.5 μg/mL in 0.1% formic acid in 50% methanol) and a control breath extract was injected (breath matrix trace). For comparison, control mobile phase A was injected.

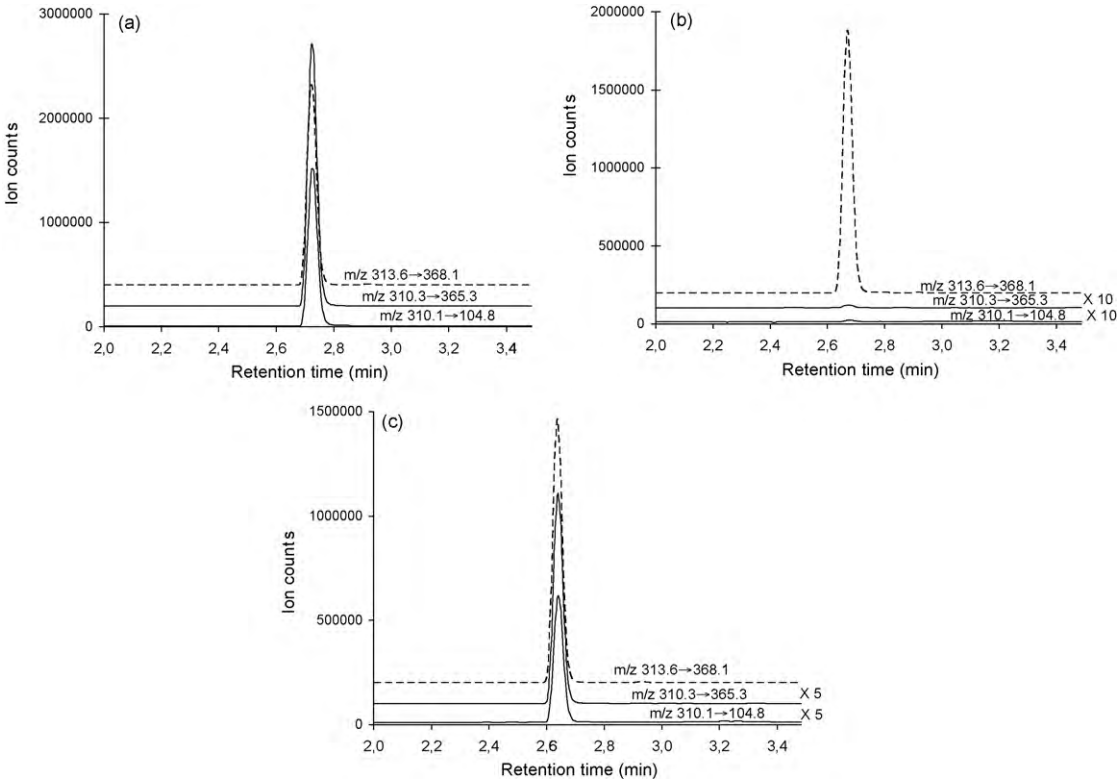

Fig. 3. Chromatograms from the identification of methadone in exhaled breath from (a) a methadone patient (subject 11 of Table 2), (b) a control subject with no detectable methadone, and (c) a standard prepared from a blank filter with added methadone (3.0 ng). Identification using LC–MS/MS was based on the presence of compound with correct retention time and with correct relative abundance of the two product ions. The markings ×10 and ×5 in the figure means that the response was multiplied this much relative to the internal standard channel.

Each subject was breathing at own chosen pace. The number of breaths during the 10 min sampling time was therefore recorded (Table 2). Table 2 also reports the actual sampling time after dose intake. Due to practical reasons this time interval could not be the same for all subjects but varied between 8 and 60 min.

4. Discussion

This study reports the original observation that methadone can be detected in exhaled breath collected from patients undergoing methadone maintenance treatment. This observation opens up a new possible specimen for monitoring compliance in methadone treatment.

The identification of methadone by mass spectrometry followed commonly accepted criteria being successfully applied in urine drug testing [15]. The fact that all 13 patients and no control subject had methadone detectable in breath makes the results convincing. This was also the case with our previous finding on amphetamine and methamphetamine [14]. The excretion rate of methadone (0.39–78 ng/min) was much higher than was seen before for amphetamine and methamphetamine (0.2–139 pg/min). This might be caused by several reasons one being that the methadone patients were in steady-state while the amphetamine intoxication cases were in recovery phase. Other pharmacokinetic features might also differ between compounds possibly making methadone more available for breath transport. However, blood levels of amphetamine and methamphetamine measured in these patients at the time of breath sampling were in the range of methadone under steady-state conditions. Nevertheless, the sampling of methadone patients was performed following the daily dose intake of methadone. Another reason for different measured excretion rates might be that the sampling procedure was different in the two experiments. In the present study we used a wider sampling disc providing more optimal flow rate [16], and there was also a shorter distance between the subject and the filter. In three subjects no mouth wash was performed before sampling indicating that material present in mouth did not contribute to the measured methadone as the results were similar as for the other subjects.

Propofol was measured in the gas phase with calibrators prepared using a gas generator [13]. Apart from the gas phase human breath also contains components carried in particles and droplets, which can be collected as exhaled breath condensate [17]. It was shown that this fraction derives mainly from the central airways in addition to the airway lining fluid [17]. The mechanism for excretion of methadone in exhaled breath remains to be determined.

In conclusion, this observation of methadone being present and measurable in exhaled breath demonstrates that drug testing using breath is feasible and deserves further investigation. Exhaled breath might be developed into a much more easily accessible and safer specimen than other matrices presently used for drug testing. Our results so far demonstrate that current bioanalytical technology enables the measurement of methadone, amphetamine and methamphetamine and possibly other drugs of abuse in exhaled breath. Since methadone is chronically administered in methadone maintenance therapy it might be used as a study analyte for more detailed studies on the excretion mechanism and development of sampling techniques. One practical limitation at the moment is the relatively long sampling time of 10 min.

Acknowledgements

We thank Inger Engman-Borg for assistance in the clinical part if this work. This work was supported by grants from the

Swedish Governmental Agency for Innovation Systems, Vinnova, the Swedish Research Council and the Stockholm County Council.

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