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Demonstration that methadone is being present in the exhaled breath aerosol fraction

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ABSTRACT

Methadone has previously been found present in exhaled breath of methadone treated patients. This study aimed at studying if methadone is present in the aerosol fraction of exhaled breath and used different filter sampling techniques for that. Patients receiving methadone maintenance treatment were recruited for the study. Methadone was extracted from filters collecting methadone from exhaled breath using 2-propanol, methanol and ethyl acetate and measured using liquid-chromatography–tandem mass-spectrometry. The limit of quantification was 5 pg/sample and the intra-day imprecision and accuracy within 15%. The recovery of extracting methadone from filters was >90%. Two types of micro-particle filters were used in this study and were compared with the C18 silica filter (Empore) used before. The Glass fiber filter collected methadone from exhaled breath of methadone patients. The amount collected significantly exceeded the amount using the C18 Empore filter (3.6–14-fold), but the variability of amount trapped was large. The second filter type was a polymer filter. Also this filter was able to trap methadone from exhaled breath of methadone patients. The amount and variability was similar to the C18 Empore filter but smaller than the Glass fiber filter. The mean rate of methadone excretion measured with the best polymer filter was 92 pg/min with a range between 20 and 287 ($n = 5$). The polymer filter has the practical advantage of having a low flow resistance making it possible to sample without pumping assistance. The polymer filter was found to collect >90% of the exhaled methadone. The conclusion of this study was that methadone in exhaled breath is carried in the aerosol fraction known to be formed in the lung as a result of normal breathing.

1. Introduction

Following the demonstration that amphetamine and methamphetamine are detectable in human exhaled breath following intake [1], we have been studying this in more detail using methadone as a model substance due to the availability of subjects taking the drug regularly [2–4]. The aim of this line of work is to try to develop breath testing into a practical and new method for performing clinical and forensic drugs of abuse testing. Collection of a breath sample might offer a non-invasive, convenient and safe sampling procedure. A further support of this was the demonstration that also cannabis smoking can be detected using a breath sample by measuring tetrahydrocannabinol [5].

The use of two different sampling procedures and repeated samplings has indicated that methadone can be reproducibly detected in breath and that saliva contamination is not the source of this [2–4]. Recently, the aerosol fraction of breath has been further characterized [6,7]. A specially constructed sampling device based on impactor technology capable of size-fractionating breath aerosol particles was used to demonstrate that the aerosol fraction reflects the airway lining fluid of the lung. Proteins and lipids characteristic of this fluid were detected using mass spectrometry [6]. More recently the same group reported that the site of formation of the aerosol particles is the terminal bronchioles and the mechanism is the airway reopening after airway closure during normal breathing [7].

These findings triggered us to further explore the possibility that methadone is being carried in the aerosol fraction. The present study was aimed to study breath sampling using filters capable of trapping aerosol particles from air. Sampling of breath from patients undergoing methadone maintenance treatment was used as the experimental model.

2. Experimental

2.1. Chemicals and materials

Methadone and methadone-d3 were obtained as ampouled methanol solutions from Cerilliant Corporation (Round Rock,

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Texas). Methanol, acetonitrile and ethyl acetate of HPLC grade were from JT Baker (Mallinckrodt Baker BV, Deventer, Holland). 2-Propanol of "normapur" grade was from VWR International (West Chester, PA). Formic acid of analytical grade was from Merck KGaA (Darmstadt, Germany). The Milli-Q water was of ultra-pure quality (>18 MΩ/cm) and prepared in-house. The 47 mm C18 Empore disc was from Varian Inc. (Palo Alto, California). The Type A/E Glass fiber filter (1 μm pore size, 25 mm diameter) was from Pall Co., Ann Arbor, Michigan. The Technostat polymer filter (type 15, 20, 25) at different diameters was from Lindpro AB, Örebro, Sweden.

2.2. Preparation of methadone solutions

The ampouled methadone (99.9% purity, 1.000 ± 0.006 mg/ml) and methadone-d3 (no unlabelled methadone detected) 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. Study subjects

Patients undergoing methadone maintenance treatment (11 males, 4 females, aged 44–57 years) 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 140 mg. The patients were subjected to regular control of compliance to treatment and any use of illicit drugs by urine drug testing. Ethical approval was obtained from the Stockholm Regional Ethics Committee (No. 2008/1347-31).

2.4. Sampling of exhaled breath on Empore and Glass fiber filter

Compounds present in the exhaled breath were collected for 1–10 min by suction through a 47 mm Empore C18 disc or a 25 mm Glass fiber filter using a membrane pump to assist the flow (pump capacity 300 ml/min). The subjects were asked to breathe more deeply than normal into an alcometer mouth piece (Palmenco AB, Stockholm, Sweden) mounted in the sampling device holding the filter [4]. The mouth was always washed with water prior to the sampling. It was estimated that all the exhaled breath was passed through the filter during the sampling period time. Following sampling the filter was dismantled using a tweezers and stored at −20 °C. The sampling device was carefully cleaned between uses with bacterial disinfectant and 70% ethanol.

Following storage the Empore filter or Glass fiber filter was cut into 5 × 5 mm pieces using a scalpel and transferred to a 10 ml glass test-tube. A volume of 25 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 1 h 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. When about 1 ml remained the solution was filtered through a 0.2 μm PTFE particle filter, which was rinsed with 2 ml of methanol, followed by evaporation. The final dry residue was dissolved in 100 μl of methanol.

Standards for quantification were prepared from fortified blank Empore or Glass fiber filters. These were prepared by using methanol solutions containing 20 or 300 ng/ml of methadone corresponding to 10–2000 pg/filter. After drying the discs were prepared for analysis as described above. Calibration curves were constructed using linear regression analysis, with weighting factor $1/x$.

2.5. Sampling of exhaled breath on polymer filter

The collection of breath samples using polymer particle filter was performed in a similar way as described above but with the following modifications. No pump assistance was needed. The extraction from filter was performed in an ultra-sound bath for 5 min at room temperature (+22 °C).

2.6. Mass spectrometry analysis system

An aliquot of 10 μl was subjected to analysis by selected reaction monitoring (SRM) LC–MS/MS (Sciex API 2000). The chromatographic system was an XTerra C18 column, 50 mm × 2.1 mm, particle size 3.5 μm, with an XTerra MS C18 10 mm × 2.1 mm, particle size 3.5 μm guard column (Waters Corporation), with mobile phase A = 0.1% formic acid and B = acetonitrile with 0.1% formic acid. The mobile phase was 85% A for 0.2 min, followed by a linear gradient from 15% B to 100% B to 2.5 min and kept at 100% B until 3.4 min. The equilibration time between injections was about 2.5 min (85% A). The flow rate was 0.425 ml/min and the column temperature was at 40 °C.

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 selected reaction monitoring (SRM) in the positive electrospray mode with a 100 ms dwell time for each channel. Other instrumental settings were: declustering potential 14, curtain gas 20 psig, collision gas (N_2) 10 psig, ion source temperature 300 °C.

2.7. Method validation

For each filter type separate calibration samples were prepared by fortifying blank filter with a methanol solution of methadone. Recovery of extracting methadone from filter was studied in the same way by comparing with a reference solution. Imprecision and accuracy in quantifications was estimated by repetitive analysis of samples prepared from fortified filters at two levels. Limit of detection (LOD, s/n = 3) and limit of quantification (LOQ, s/n = 10) was estimated from the lowest calibrator 10 pg/sample. Matrix effect was studied in an experiment where methadone was infused (1 μg methadone/ml at 10 μl/min) post-column while injection a blank matrix extract.

2.8. Statistical calculations

All calculations were made using Excel Windows Office XP.

3. Results

3.1. Method application

An initial experiment indicated that methadone was being trapped from breath on the Glass fiber filter. The LC–MS/MS results demonstrated that in all samples collected the methadone peak met criteria for identification of correct retention time relative to internal standard methadone-d3 and correct ratio (within ±20%) between the two product ions (Fig. 1). The following experiment comparing the trapped amount with the Empore filter demonstrated pronounced variability between individuals. The experiment was therefore repeated another two times in which the second used duplicate samplings using the Glass fiber filter with sampling on an Empore filter in between. The summary of these 3 experiments is given in Table 1. A paired t-test, two-sided,

Table 1
Sampling of methadone in exhaled breath using Glass fiber and Empore C18 filters.

Experiment no.	Glass fiber methadone (pg/min) mean ± SD	Range	Empore C18 methadone (pg/min) mean ± SD	Range	*n*
1	1045 ± 1905	23–3900	74.3 ± 61.6	29–162	4
2	636 ± 806	20–1703	107 ± 104	11–285	5
3[a]	932 ± 1301[b]	77–3933	256 ± 251	31–573	5

[a] Some samplings were done after dose intake.
[b] Mean of 2 samplings in 4 of the cases.

Table 2
Sampling of methadone from exhaled breath using a polymer filter (type 20).

Diameter of filter (mm)	Sampling time (min)	Methadone (pg/min) mean ± SD	Range	*n*
47	3	86 ± 52	18–168	6
32	3	112 ± 89	47–266	5
32	3	92 ± 62	28–199	6
32	1	204 ± 116	21–326	6

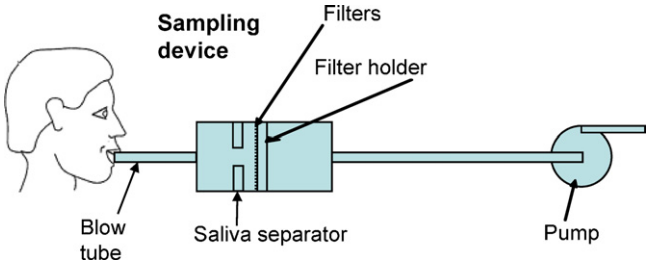

Fig. 1. Schematic presentation of the filter holder manifold used for exhaled breath sampling.

Table 3
Sampling of methadone in breath using variable densities of the polymer particle filter.

Type	Methadone (pg/min) mean ± SD	Range	*n*
15	74.4 ± 80.4	24–216	5
20	71.0 ± 90.1	19–231	5
25	91.8 ± 110	20–287	5

documented that the Glass fiber filter collected significantly more methadone than the Empore filter ($p = 0.032$).

Another line of experiment was using a polymer particle filter that did not require pumping to assist the sampling. A first experiment studied two diameters of the filter, two sampling times and variability between two consecutive samplings (Table 2). The LC–MS/MS results demonstrated that in all samples collected the methadone peak met criteria for identification of correct retention time relative to internal standard methadone-d3 and correct ratio (within ±20%) between the two product ions (Fig. 2). The results indicated that both the filter diameter and sampling time could be reduced. The reproducibility was much better than with the Glass fiber filter and the results were in accordance with those obtained with Empore disc (see Table 1). Experiments with even smaller diameters (22 and 13 mm, type 25) demonstrated comparable results as for 47 and 32 mm (see Table 1), 155 ± 55 pg/min for 22 mm and 122 ± 50 pg/min for 13 mm ($n = 5$). However, at the smallest diameter the study subjects complained about inconvenient back-pressure (Figs. 3–5).

The polymer particle filter is available in different densities (i.e. thickness). When testing three of these (15, 20, 25) the highest mean value was obtained for type 25 (Table 3), which is the most dense quality. However, the variability of the data did not allow for statistical support of any firm conclusion.

Finally an experiment was performed by placing two polymer filters (type 25) on top of each other. The mean value of the first filters was 124 ± 45 pg methadone per min and for the second filters 12 ± 6 pg/min (8.8% of total).

3.2. Method validation

The extraction recovery of methadone from fortified filters were 94 ± 6% ($n = 8$) for the polymer filter and 92% for the Glass filter ($n = 2$). The LOD was estimated by injecting an extract from the lowest calibrator and was found to be 1.5 pg/sample (0.15 pg on column). The calculated limit of quantification (LOQ) was 5 pg/sample (s/n = 10). The calibration curves were linear within the measuring range 10–2000 pg/sample, with correlation coefficients between 0.996 and 0.999 ($n = 7$). The imprecision and accuracy in quantification were 10.7% and 97.5% at level 40 pg/sample, and 7.2% and 103% at level 300 pg/sample ($n = 7$).

A transient drop (~10 s) in response for infused methadone was seen after the elution of the void volume in the matrix experiment. No matrix effect could be observed near the retention time of methadone.

4. Discussion

This study further confirms that methadone is present in exhaled breath from patients undergoing methadone maintenance treatment and demonstrates for the first time that the exhaled methadone can be trapped using micro-particle filter techniques known to capture aerosol particles. Procedures for sampling exhaled breath using these filters as well as procedures for chemical analysis of trapped methadone were developed and validated.

Human breath contains both volatile and non-volative substances [8–10]. The non-volatile fraction can be trapped as exhaled breath condensate [9] that contains biomolecules including leuokotriene B4 and 8-isoprostane that have been measured using high performance liquid chromatography [11–16] and this has been demonstrated also for methadone [3]. One part of the condensate fraction is the droplets making up a micro-particle aerosol fraction which is a normal part of human exhaled breath [17]. This bio-aerosol fraction has been extensively studied and characterized in relation to health issues concerning infectious diseases, toxicity and allergens [18]. Based on the recent demonstration that the human breath aerosol fraction is formed from the respiratory tract lining fluid [7] we wanted to specifically study the presence of methadone in this fraction of exhaled breath.

An alternative and more selective way of sampling the aerosol fraction of breath than using condensate is to use micro-particle filters. These filters are being used for filtering of in-door air. One type is Glass fiber filters that are being used both for air and fluid micro-particle filtration. For example, Glass fiber filters have been

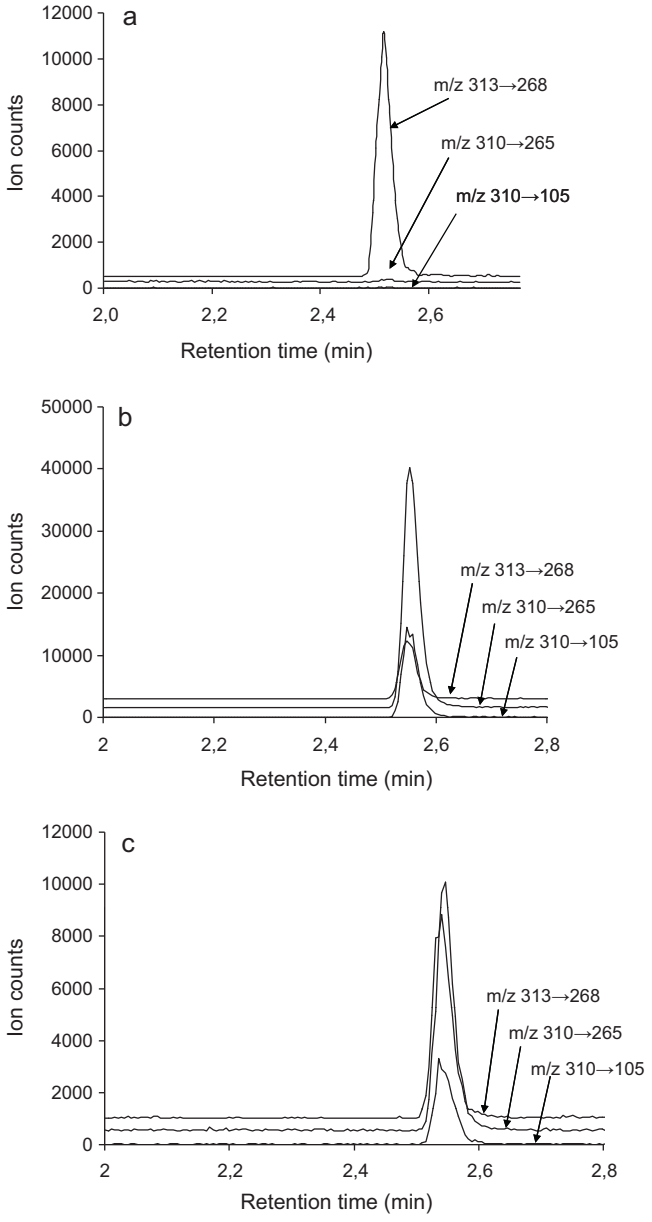

Fig. 2. Chromatograms obtained from the analysis of methadone in extracts from Glass fiber filters; methadone is monitored at transitions m/z 310 → 265 and m/z 310 → 105 and internal standard at 313 → 268. No interfering peaks were observed in extracts from blank filter (a). The chromatograms from calibrators prepared from fortified filters were also free from matrix peaks (b). The calibrator shown in (b) contains 1000 pg/sample. The extracts prepared from filters used for sampling of exhaled breath were also free from chromatographic interferences (c). The example extract was from a subject found to exhale 77 pg methadone/min. Identification of methadone 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.

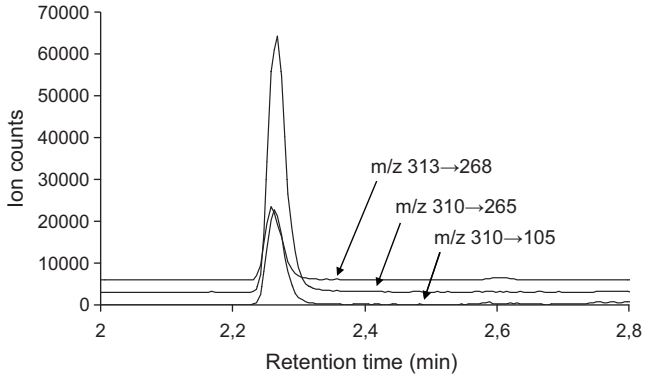

Fig. 3. Chromatogram from the determination of methadone in exhaled breath using the polymer micro-particle filter; methadone is monitored at transitions m/z 310 → 265 and m/z 310 → 105 and internal standard at 313 → 268. The subject was found to exhale 271 pg methadone/min. Identification of methadone 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.

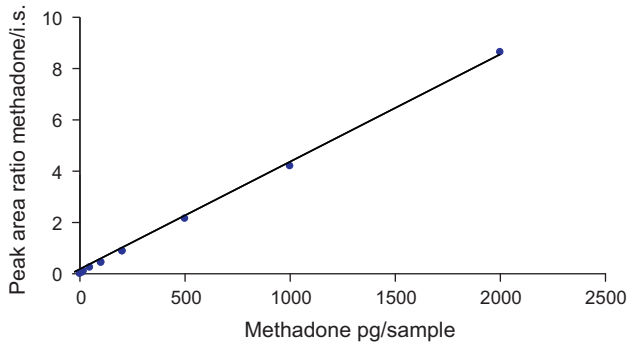

Fig. 4. A calibration curve extracted from fortified polymer filters. Amounts of methadone were 0, 10, 20, 50, 100, 200, 500, 1000, 2000 pg/sample.

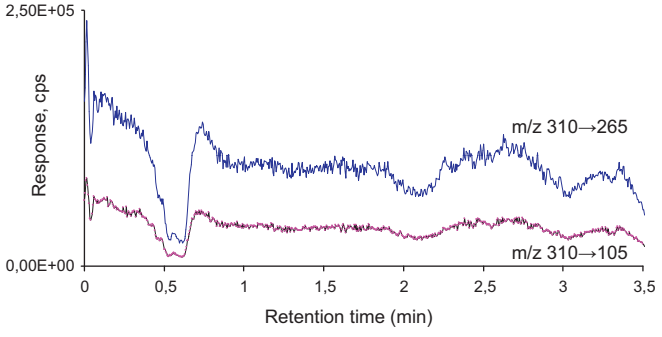

Fig. 5. The graph shows the effect of injecting a blank Glass fiber filter extract while infusing methadone post column.

used for trapping insecticides that are aerosol carried in high yield [19,20]. In addition, polymer particle filters are available for indoor air filtration and for respiratory protection devices [21]. In our study we explored both these type of filters for collecting the aerosol fraction of breath. We found that both filtering techniques are capable of trapping the methadone carried in exhaled breath. The amount of methadone trapped with the micro-particle filters and reference Empore filter indicates that almost all methadone is carried in the aerosol fraction and very little (if any) in the vapour phase. This finding is consistent with the fact that methadone is a non-volatile compound. This demonstration that methadone is exhaled in the aerosol fraction might be used for developing a better normalisation than is obtained by referring to sampling time. The expression of exhaled amount of methadone per minute might be a reason for the observed variability between samplings but has been used until now in our work [1–5].

In conclusion, the result obtained in this study suggests that methadone is being carried from the lung by the aerosol that is formed from the respiratory tract lining fluid during normal breathing. This in turn makes it possible to hypothesize that also other abused substances may be carried this way, which supports the proposal that exhaled breath is a new possible matrix in clinical and forensic toxicology.

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