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Studying drug excretion into exhaled breath aerosol – A workflow based on an impaction sampling device and LC-HRMS/MS analysis

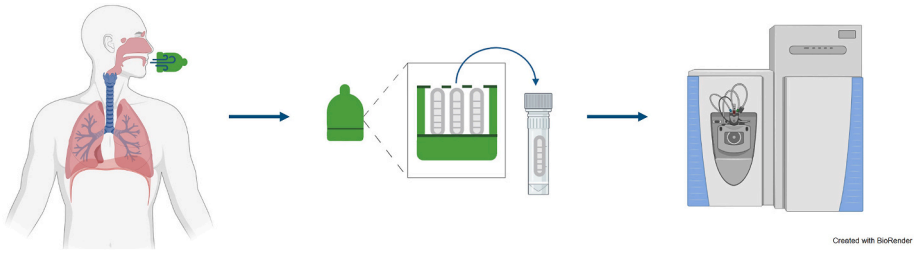
Juel Maalouli Schaar [a], Michael Kunz [b,c], Lea Wagmann [a], Olof Beck [d], Felix Mahfoud [b,c], Markus R. Meyer [a,*]

[a] Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, Center for Molecular Signaling (PZMS), Saarland University, Homburg, Germany
[b] Department of Internal Medicine III, Cardiology, Angiology and Intensive Care Medicine, University Hospital Saarland, Saarland University, Homburg, Germany
[c] Department of Cardiology, Heart Center at University Hospital Basel, Switzerland
[d] Karolinska Institute, Clinical Neuroscience, Stockholm, Sweden

HIGHLIGHTS

- Analytical evaluation of exhaled breath aerosol (EBA) as a matrix for future bioanalysis.
- Development of an analytical workflow for the detection of non-volatile drugs in EBA.
- Workflow application to human EBA samples to investigate non-volatile drug excretion into EBA.
- Demonstrated excretion of several drugs and their metabolites into human EBA.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Exhaled breath (EB) aerosol was in principle shown to be a suitable matrix for bioanalysis of volatile but also non-volatile compounds. This attracted particular interest in the field of drug analysis. However, a big gap still exists in the understanding how and which drugs and/or their metabolites are excreted into exhaled breath and could thus actually be detected. The current study aimed to develop an analytical workflow for the qualitative detection of non-volatile drugs in EB aerosol microparticles.
Results: The analyte selection covered different drug classes such as antihypertensives, anticonvulsants or opioid analgesics to investigate and understand the excretion of drugs and their metabolites into EB aerosol. A device for collecting aerosol particles from the lung through impaction was used for the non-invasive sampling procedure. Three expiration cycles per participant and device were collected. The sample preparation consisted of a collector extraction with methanol. Qualitative method development and validation were performed using reversed-phase liquid chromatography (LC) coupled to orbitrap-based high-resolution mass spectrometry (HRMS). Qualitative method validation was done according to published recommendations and international guidelines. Parameters such as selectivity, carry-over, limits of detection and identification, recovery, matrix effects, and long-term stability were evaluated. The limits of detection ranged from 100 pg/collector to 10,000 pg/collector. The procedure was finally used to analyze a total of 31 patient EB samples and demonstrated that e.g., tilidine and its metabolite nortilidine as well as tramadol and its active metabolite *O*-desmethyltramadol can be detected in EB aerosol.

* Corresponding author.
E-mail address: markus.meyer@uks.eu (M.R. Meyer).

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Significance and novelty: The work shows a comprehensive workflow for elucidating drug excretion into exhaled breath aerosol. This bioanalytical strategy and the corresponding novel data from this study are the foundation for further method development and to better understand, which drugs and their metabolites can be addressed by exhaled breath aerosol bioanalysis.

1. Introduction

Human exhaled breath (EB) analysis has recently attracted increasing attention for the detection of both volatile and non-volatile substances [1–4]. This study focuses on the non-volatile compounds, such as drugs and drugs of abuse, which are exhaled during each breath maneuver from the respiratory system, especially from the distal part of the respiratory tract, as part of microparticles from the lining fluid [5–7]. The amount of microparticles contained in the EB is significantly influenced by the type of exhalation maneuver. Exhaling to reach the residual volume, holding the breath, and then inhaling before exhaling can increase the size and number of these particles [8,9]. Thus, detection of drugs is not only influenced by the excretion into the lining fluid, but also by the sampling procedure and sampling device [10]. Especially, in older patients suffering from cardiovascular or lung diseases with reduced vital capacity, the sampling procedure can be prone to errors, as lung function and cardiovascular diseases are often closely related, and spirometric variables are reduced [11]. Compared to other biological matrices, such as blood plasma, urine, or tissues, EB is non-invasively available, the collection procedure is painless and does not interfere with patient's privacy. Additionally, EB is a matrix that can be collected as often as required and samples are easy to transport. Various sampling devices are available for the collection of human EB samples [7,12]. In general, human EB can be collected as condensate or aerosol. Non-volatile compounds, including drugs, can be collected directly and selectively in exhaled aerosol particles using adsorptive collection devices [3,6,7,10,13]. For example, the ExaBreath device (SensAbues, SensaSure Technologies) as a single barrel collection device with a fibrous filter and has allowed the detection of drugs of abuse [1–4]. This device provides only one sample and must be assembled before sampling [3]. Unlike the SensAbues device, the Breath Explor device does not consist of an electrostatic filter, but rather a unit of three parallel impaction collectors consisting of medically acceptable material such as polypropylene, polyvinylidene fluoride, and or fluorinated ethylene propylene designed to capture aerosol particles of size fractions ranging from 0.3 to 2.0 μM in diameter from the distal part of the lung [14]. Each impaction collector has a cylindrical polyhedral geometry with eight baffles arranged in a sequential order, with a radius of 4.8 mm and a length of 22 mm. The aforementioned unit consists of three collectors, is surrounded by an outer housing, a removable cap, and is portable [10]. Available study using this impaction device have also allowed the detection of some drugs of abuse, such as Δ9-tetrahydrocannabinol (THC) and methadone [6,15]. So far, no systematic evaluation on the excretion and detectability of different drug classes into the EB aerosol has been performed. Therefore, this study aimed first to develop an analytical liquid chromatography-high-resolution tandem mass spectrometry (LC-HRMS/MS) workflow for the qualitative detection of selected drugs in human EB aerosol samples. Second, to perform a proof-of-concept study with 31 patient EB samples and matched blood plasma samples to demonstrate the applicability of the method, and to allow first insight into the excretion pattern of tested drugs into EB aerosol in comparison to drugs present in plasma samples. The analytes were selected to cover different drug classes such as antihypertensives (e.g., ramipril), anticonvulsants (e.g., pregabalin), benzodiazepines (e.g., lorazepam) or opioid analgesics (e.g., tramadol) with different chemical structures and polarities to investigate and classify the excretion of these drugs and of their metabolites into human EB. The qualitative method validation was performed according to international recommendations and guidelines [16–18].

2. Experimental

2.1. Chemicals and other materials

Bisoprolol-d_5, carbamazepine, diazepam-d_5, pregabalin, ramipril, ramipril-d_5, ramiprilat, ramiprilat-d_5 and tramadol-$^{13}cd_3$ were purchased from LGC (Luckenwalde, Germany). Bisoprolol, bromazepam, bromazepam-d_4, carbamazepine-d_{10}, *O*-desmethyltramadol (ODMT), *O*-desmethyltramadol-d_6 hydrochloride, diazepam, lorazepam, lorazepam-d_4, metoprolol tartrate, metoprolol-d_7 tartrate, nordazepam, nordazepam-d_5, nortilidine hydrochloride, nortilidine-d_3 hydrochloride, pregabalin-d_6, tapentadol hydrochloride, tapentadol-d_3 hydrochloride, tilidine hydrochloride hemihydrate, tilidine-d_6 hydrochloride and tramadol hydrochloride were obtained from Merck (Darmstadt, Germany). Acetonitrile (ACN), ammonium formate, formic acid, methanol, and all other chemicals (LC-MS or analytical grade) were purchased from VWR (Darmstadt, Germany). The water was purified in-house with a Millipore filtration unit (18.2 Ω x cm water resistance). Breath Explor sampling devices were from Munkplast AB (www.munkplast.se, Uppsala, Sweden).

2.2. EB aerosol collection and proof-of-concept samples

Sample collection was conducted using the Breath Explor sampling device containing three collectors. Microparticles from EB were collected by impaction on the collectors [6]. Three expiration cycles into one sampling device were collected from each patient. The cycles consisted of full exhalation until reaching the residual volume, holding the breath, inhaling the vital capacity, placing the device into the mouth, and then exhaling until the individual residual volume. For method validation, blank matrix free of compounds of interest was collected from ten volunteers. For proof-of-concept testing, EB loaded sampling devices and blood samples were collected from patients at Saarland University Hospital, Internal medicine III, Homburg, Germany. EDTA blood samples and EB samples were collected simultaneously and were stored at −20 °C, with a maximum storage time of four weeks after collection. Ethical approval was obtained from the local ethic committee (AT-HOM, No. 257/22) and all patients and volunteers provided written informed consent. EB samples of 31 patients (24 males, seven females, mean age 70, aged 27–87 years) were included in this study. Medication plans were provided as shown in Table 1.

2.3. Sample preparation

EB samples were prepared based on previously published procedures [1,3] as follows: For method development and validation, the EB carrying collectors were fortified with the respective compounds. The three collectors were removed from the device and placed into 5 mL tubes. Afterwards, 3 mL of methanol containing all internal standards in a concentration of 100 ng mL^{-1} except for lorazepam-d_4 where the concentration was 200 ng mL^{-1} were added. After 5 min, the mixture was shaken for 2 min at 1400 rpm using a Cell Media Thermo Shaker (CellMedia GmbH, Zeitz, Germany) and centrifugated for 3 × 2 min at 3000 rcf with a Hettich Rotofix 32a centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Samples were finally evaporated to dryness under nitrogen gas (70 °C) and reconstituted in 100 μL eluent A and eluent B (1:1, v/v, see LC-HRMS/MS conditions). Following centrifugation for 2 min at 18,407 rcf with Eppendorf centrifuge 5424 (Eppendorf AG, Hamburg, Germany) 10 μL of the supernatant was

Table 1
Patient samples and prescribed medication and mode of intake provided by medication plans.

Patient Sample No.	Medication	Mode of intake
1	Bisoprolol 2.5 mg	1-0-1
	Ramipril 5 mg	1-0-0
2	Bisoprolol 2.5 mg	1-1-1
3	Metoprolol retard 95 mg	1-0-1
4	Bisoprolol 5 mg	1-0-1
5	Bisoprolol 5 mg	1-0-1
6	Bisoprolol 2.5 mg	1-1-1
7	Pregabalin 25 mg	1-0-1
8	Bisoprolol 2.5 mg	1-0-0
	Ramipril 2.5 mg	1-0-0
9	Bisoprolol 2.5 mg	1-0-0
10	Ramipril 10 mg	1-0-0
11	Bisoprolol 2.5 mg	1-0-0
12	Bisoprolol 5 mg	1-0-0
13	Ramipril 10 mg	1-0-0
14	Bisoprolol 5 mg	1-0-1
15	Metoprolol retard 95 mg	½-0-0
	Ramipril 2.5 mg	1-0-1
16	Tilidine retard 200 mg	1-0-1
17	Bisoprolol 2.5 mg	1-0-0
	Pregabalin 50 mg	½-0-½
18	Tapentadol retard 100 mg	1-1-1
	Tilidine retard 50 mg	1-0-0
19	Bisoprolol 1.25 mg	1-0-0
	Tilidine retard 200 mg	1-0-1
20	Bisoprolol 5 mg	1-0-0
21	Bisoprolol 5 mg	½-0-½
22	Metoprolol retard 95 mg	½-0-½
	Ramipril 10 mg	½-0-0
	Tilidine retard 50 mg	1-0-0
23	Ramipril 5 mg	1-0-0
	Tapentadol retard 100 mg	1-0-1
24	Lorazepam 1 mg	0-0-1
25	Bisoprolol 2.5 mg	1-0-1
26	Bromazepam 3 mg	0-0-1
	Metoprolol retard 95 mg	1-0-0
27	Bisoprolol 1.25 mg	1-0-0
	Bromazepam 6 mg	½-0-0
28	Tramadol retard 100 mg	1-0-1
29	Bisoprolol 5 mg	1-0-0
	Ramipril 5 mg	1-0-0
30	Lorazepam 1 mg	0-0-1
31	Bisoprolol 2.5 mg	1-0-1
	Tramadol 50 mg	1-0-0
	Metoprolol retard 95 mg	1-0-0

injected onto LC-HRMS/MS system. Human plasma samples (100 μL) were precipitated with 200 μL of ACN (0.1 % formic acid) and analyzed both according to Helfer et al. [19]. Ten microliters of analytes corresponding internal standards at a final plasma concentration of 10 ng mL^{-1} except for lorazepam-d_4 where the concentration was 20 ng mL^{-1} were added. After shaking and centrifugation (15,000×*g*, 30 min), the supernatant was transferred into an LC vial and 10 μL were injected onto the LC-HRMS/MS system.

2.4. LC-HRMS/MS conditions

A Thermo Fisher Scientifc (TF, Dreieich, Germany) Dionex UltiMate 3000 RS pump consisting of a degasser, a quaternary pump, a DL W2 wash system and an HTC PAL autosampler (CTC Analytics AG, Zwinger, Switzerland), coupled to a TF Q Exactive Orbitrap high-resolution mass spectrometry equipped with a heated electrospray ionization (HESI)-II source was used. Gradient reversed-phase phase elution was performed on a Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm) for EB samples and for human plasma samples. The mobile phases consisted of 2 mM aqueous ammonium formate containing acetonitrile (1 %, v/v) and formic acid (0.1 %, v/v, pH 3, eluent A), as well as 2 mM ammonium formate solution with acetonitrile:methanol (1:1, v/v) containing water (1 %, v/v) and formic acid (0.1 %, v/v, eluent B). The gradient using a flow rate of 0.5 mL min^{-1} was programmed as follows: 0.0–1.0 min hold 5 % B, 1.0–7.0 min 75 % B, 7.0–9.5 min hold 75 % B, and 9.51–10 min hold 5 % B. The injection volume was set to 10 μL and the column oven temperature was 40 °C. Mass spectrometric analysis was performed in positive full-scan mode and data-dependent MS2 (dd-MS2) with priority to mass-to-charge ratios (*m/z*) of the compounds (inclusion list) and scan segments. HESI-II source conditions were as follows: ionization mode, positive, sheath gas, 60 AU; auxiliary gas, 10 AU; sweep gas, 3 AU; spray voltage, 3.5 kV in positive; heater temperature 320 °C; ion transfer capillary temperature, 320 °C; and S-lens RF level, 60.0. The settings for full-scan data acquisition were as follows: polarity, positive resolution, 35,000; scan range, *m/z* 130–500; automatic gain control (AGC) target, 1e6; maximum injection time, 120 ms; microscans, 1; spectrum data type, profile, dd-MS2, discovery; resolution, 17,500; isolation window, 1.0 *m/z*; AGC target, 2e5; maximum injection time, 250 ms; high-collision dissociation cell with stepped normalized collision energy, 17.5, 35.0, 52.5; loop count, 5; minimum AGC target, 1.e3 (corresponds to a signal intensity threshold of 4e3); exclude isotopes, on; and spectrum data type, profile. A comprehensive overview of the instrument parameters described can be found in Table S1 in the Supplemental. Mass calibration was performed prior to analysis according to the manufacturer's recommendations using external mass calibration. In addition, the instrument performance was evaluated by analyzing a mixture of all compounds included in the method at the limit of detection (LOD) concentration prior to each analysis. For identification of the analytes and examination of the chromatographic separation TF Xcalibur Qual Browser software version 4.1 was used.

2.5. Method validation

Qualitative method validation for selected parameters was done according to international recommendations and guidelines [16,18,20,21]. Selectivity was tested by collecting ten EB samples free of the compounds of interest and preparing them as described in 2.3. Blank samples were analyzed for interfering signals at the corresponding retention times (RT) of the analytes. Selectivity of the analytical procedure is demonstrated by the absence of interferences [16,22]. For carry-over testing, two blank EB samples were prepared and injected after EB samples that were spiked with all analytes in a concentration three times higher than methods LOD and prepared (n = 3). No carry-over in blank samples injected after these spiked samples should be observed [17]. The validation of the range limits, LODs and limits of identification (LOIs) was conducted based on the signal-to-noise ratio. LODs were tested using breath carrying collectors spiked with decreasing analytes concentrations (10,000–10 pg $collector^{-1}$), prepared as described in 2.3, and measured in triplicate for at least ten runs on different days to simultaneously assess the repeatability of the method. The LOD was determined at the concentration where the response was always higher than three times the noise level of the background signal [17,20,22]. LOIs determination was performed using the same procedure for the LOD and the reference library for MS2 data identification [23]. Recoveries (RE) and matrix effects (ME) were analyzed using the corresponding LOD concentrations and three different sample sets in six replicates according to Matuszewski et al. [21]. Regarding the recommendations, ME should be <25 % and the coefficient of variation (CV) should not be greater than 15 % for RE and ME [17,21]. Long-term stability of the compounds in matrix was evaluated for 42 days by fortifying breath carrying collectors with the respective LOD concentrations (n = 3) and stored in the freezer under the same storage conditions as the authentic patient samples (six weeks, −20 °C). Stability results should be within ±15 % of the nominal concentrations according to the ICH M10 guideline on bioanalytical method validation [18]. A summary of the validation workflow is shown in Fig. S1 and Table S3.

3. Results and discussion

3.1. Method development

The analyte selection comprised different drug classes, different chemical properties, and metabolites to investigate their excretion into EB. Detailed information, such as chemical structures of the compounds considered, can be found in Tables S2 and S4 and Fig. S2 in the Supplemental. ODMT, nordiazepam, and ramiprilat were included in the method as these are the active metabolites [24]. Additionally, diazepam, ODMT and tramadol were included for comparison purposes, as they were already successfully detected in EB in previous studies [2,3]. During method development different columns were evaluated, including a SeQuant ZIC HILIC column (150 mm × 2.1 mm, 3.5 μm), a TF Accucore Phenyl-Hexyl column (100 mm × 2.1 mm, 2.6 μm), and a Waters Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm). The peak characterization using reversed-phase chromatography on the C18 column was not ideal for pregabalin and particularly ramiprilat. The addition of a higher amount of formic acid to the reconstitution solvent did not improve the peak shape of either. For the initial evaluation, the C18 column was chosen as a compromise to detect all analytes in one method. However, it should be noted that using a hydrophilic column could significantly improve the detection of these zwitterionic structures, whereas the retention of analytes such as diazepam, ODMT, nordazepam, nortilidine, tilidine, and tramadol will be compromised. Fig. 1 shows the extracted ion chromatograms (EIC) for the investigated analytes and metabolites using the C18 column at the corresponding LOD concentration. Peaks were typically described by 15–25 scan points, which should be sufficient for reproducible detection [25,26].

3.2. Method validation

Determined LODs (25–10,000 pg collector^{-1}) and LOIs (25–10,000 pg collector^{-1}) are given in Table 2. Carbamazepine, nordazepam, ramipril, and tapentadol showed the lowest LODs (10 pg collector^{-1}) and pregabalin the highest at 10,000 pg collector^{-1}. Comparing the reported limits for diazepam to the study by Beck et al. [3], the LOD (1 pg collector^{-1}) and LOI (29 pg collector^{-1}) for diazepam were higher in the current study (LOD and LOI both 50 pg collector^{-1}). However, there are differences between both studies. First, sampling collection was performed using the SensAbues device with a single thin fibrous filter. Second, analysis was done with a TF TSQ Vantage triple quadrupole mass spectrometer [3]. Meyer et al. reported the detection of ODMT and tramadol in EB with LOIs of 10 pg filter^{-1} using also the SensAbues device and a TF TSQ Quantiva triple quadrupole mass spectrometer [2]. In this study, they achieved concentrations down to 100 pg collector^{-1} for ODMT and tramadol. Nevertheless, the reported concentrations for diazepam in patient samples ranged from 2 to 145 pg filter^{-1} and for tramadol from 58 to 1090 pg filter $^{-1}$ [2,3]. Thus, qualitative detection can be expected in most cases using the current procedure.

Table 2
Determined limits of detection (LODs) and limits of identification (LOIs) given in pg collector^{-1} and pg × g collector^{-1}. ODMT= *O*-desmethyltramadol.

Compound	LOD pg collector^{-1}	LOD pg × g collector^{-1}	LOI pg collector^{-1}	LOI pg × g collector^{-1}
Bisoprolol	25	29	50	59
Bromazepam	25	29	25	29
Carbamazepine	10	12	10	12
ODMT	100	118	100	118
Diazepam	50	59	50	59
Lorazepam	100	118	100	118
Metoprolol	25	29	25	29
Nordazepam	10	12	10	12
Nortilidine	100	118	100	118
Pregabalin	10,000	11,765	10,000	11,765
Ramipril	10	12	20	24
Ramiprilat	500	588	1000	1176
Tapentadol	10	12	10	12
Tilidine	100	118	100	118
Tramadol	100	118	100	118

Selectivity of the method was given since no interfering signals and false positive results for the compounds were detected at the RT and the corresponding *m/z* of analytes. Furthermore, no analyte carry-over was observed in the extracted blank breath samples injected after the spiked sample. Matrix effects and recoveries with corresponding CVs are summarized in Figs. 2 and 3. Matrix effects ranged from 82 to 124 % with CVs between 3 and 34 % at the LOD. The reported low matrix effects are in accordance with previous findings for human EB, being obviously a matrix with low matrix effects in comparison to other biological matrices [27]. Only bromazepam (CV 29 %), ODMT (CV 26 %), diazepam (CV 23 %), ramipril (CV 34 %), and ramiprilat (CV 17 %) showed CVs above 15 % at low concentrations. Considering the recovery at the LOD concentrations, the CVs are the crucial factor for a reproducible detection. As shown in Fig. 3, bromazepam (CV 30 %), carbamazepine (CV 17 %), lorazepam (CV 19 %), metoprolol (CV 24 %), nordazepam (CV 39 %), ramipril (CV 40 %), ramiprilat (CV 47 %), and

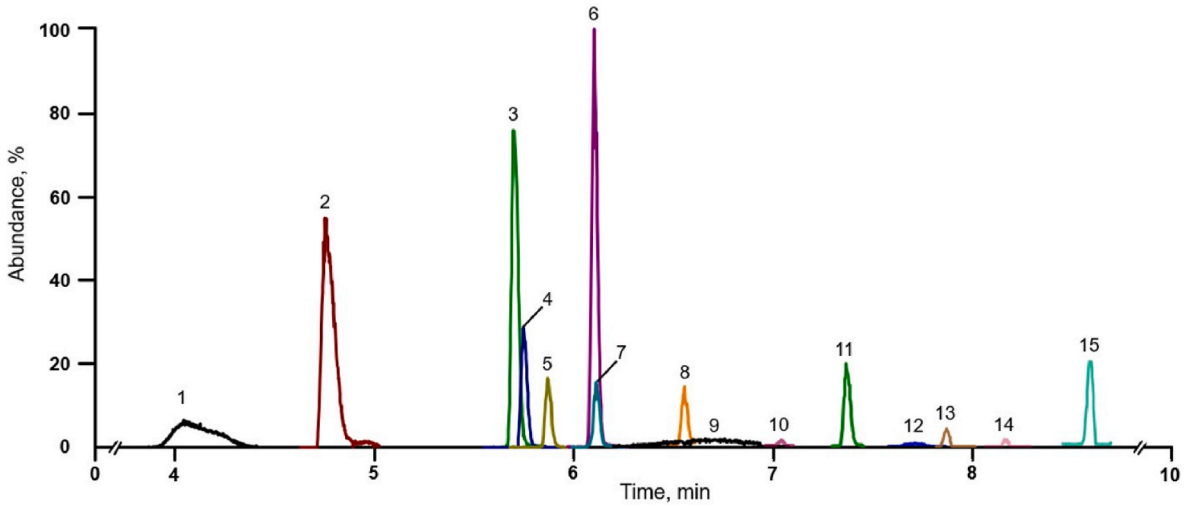

Fig. 1. Extracted ion chromatograms of pregabalin (1), *O*-desmethyltramadol (2), tramadol (3), metoprolol (4), tapentadol (5), nortilidine (6), tilidine (7), bisoprolol (8), ramiprilat (9), bromazepam (10), carbamazepine (11), ramipril (12), lorazepam (13), nordazepam (14), and diazepam (15) in the corresponding LOD concentration after LC-HRMS/MS analysis.

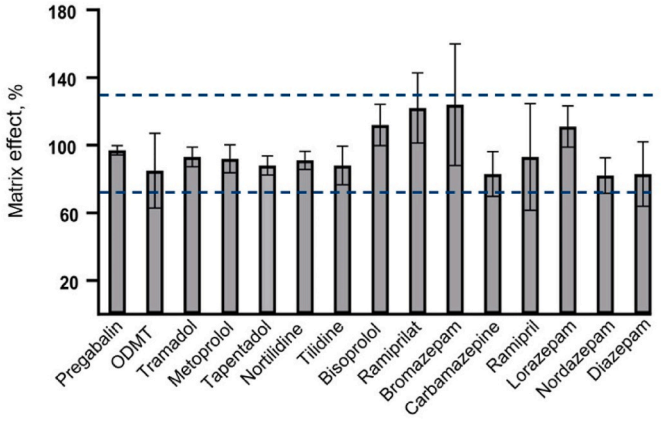

Fig. 2. Matrix effect of the 15 compounds, %. Acceptable values were marked from 75 to 125 %. ODMT = *O*-desmethyltramadol.

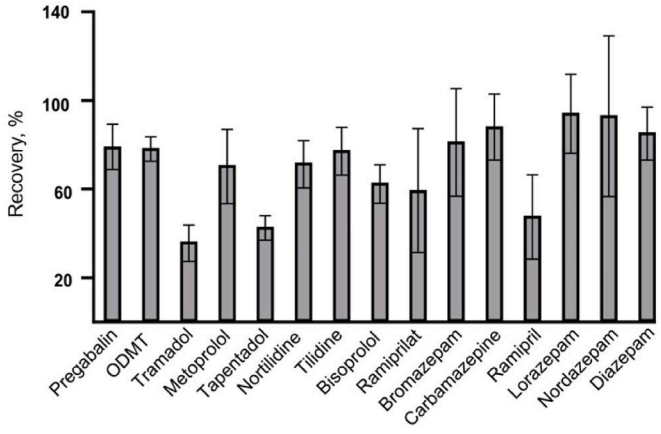

Fig. 3. Recovery of the 15 compounds, %. ODMT = *O*-desmethyltramadol.

tramadol (CV 23 %) are not within the recommended acceptance criteria [17,21]. Therefore, these analytes can only be detected using this method with limitations at low concentrations. The long-term stability in matrix was tested, covering the maximum storage time of six weeks of the proof-of-concept samples, which were stored for maximum of four weeks at −20 °C before analysis. All analytes, except bisoprolol (−18 %), ramipril (−17 %), and ramiprilat (−19 %) showed sufficient long-term stability in matrix using the internal standard (IS)-normalized peak areas given in Table 3. Consequently, all other compounds can be stored under the mentioned conditions for at least six weeks.

Table 3
Results of long-term stability of the compounds in matrix, peak area deviation of the nominal concentration between time point t_0 and t_1 IS- normalized, %. ODMT= *O*-desmethyltramadol.

Compound	Corresponding IS	Long-term stability (6 weeks, −20 °C), %
Bisoprolol	Bisoprolol-d_5	3
Bromazepam	Bromazepam-d_4	−18
Carbamazepine	Carbamazepine-d_{10}	−5
ODMT	ODMT-d_6	7
Diazepam	Diazepam-d_5	−9
Lorazepam	Lorazepam-d_4	−13
Metoprolol	Metoprolol-d_7	3
Nordazepam	Nordazepam-d_5	−7
Nortilidine	Nortilidine-d_3	−1
Pregabalin	Pregabalin-d_6	−17
Ramipril	Ramipril-d_3	−19
Ramiprilat	Ramiprilat-d_5	4
Tapentadol	Tapentadol-d_3	4
Tilidine	Tilidine-d_6	−2
Tramadol	Tramadol-$^{13}cd_3$	−6

3.3. Proof-of-concept testing

The method was used to analyze a total of 31 EB samples. Details on the medication can be found in Table 1. The concentrations of drugs in matched plasma samples are shown in Table 4. Most samples were expected to contain bisoprolol (18), followed by metoprolol (5) and tilidine (4). Samples expected to contain diazepam, its active metabolite nordazepam, and carbamazepine could not be acquired during the ten-month evaluation period. Tramadol was expected to be contained in two samples but was detected only in one sample with a matched plasma concentration of 169 ng mL^{-1}. The active metabolite ODMT was found in the same plasma sample at a concentration of 31 ng mL^{-1} but not detectable in EB. However, ODMT was detected in the second EB sample and a matched plasma concentration of 3 ng mL^{-1} was determined, while tramadol (plasma concentration of 2 ng mL^{-1}) was not detected. As shown in Table 4, tilidine was detected in EB in two out of four samples and nortilidine in three out of four samples. Tilidine and its active metabolite were simultaneously detectable in two patient EB samples where the matched plasma concentrations were 14 ng mL^{-1} and 20 ng mL^{-1} for tilidine and 98 ng mL^{-1} and 131 ng mL^{-1} for nortilidine. Nortilidine was detected in another EB sample with a matched plasma concentration of 20 ng mL^{-1}, while tilidine (plasma concentration 1 ng mL^{-1}) was not detected. The corresponding plasma concentrations for tilidine (1 ng mL^{-1}) and nortildine (6 ng mL^{-1}) were also lower in the sample where neither tilidine nor nortilidine were detected in EB. Previous studies using the Breath Explor device focused on the detection of drugs abuse such as THC, amphetamine, cocaine as well as the detection of methadone [6,15,28]. These include the study by Feltmann et al. on the prevalence of illicit drug use among nightlife attendees, which allowed the detection of bromazepam, lorazepam, and pregabalin in EB aerosol and the study of Sinapour et al., which demonstrated a detection of pregabalin in two cases [28,29]. The detection limit was 1 pg collector^{-1} and the sample collection consisted of ten expiration cycles through the device per participant in both studies [28,29]. These three

Table 4
Number of obtained exhaled breath (EB) samples, concentrations in the matched plasma samples, ng/mL, and number of detected analytes in the obtained EB samples. ODMT = *O*-desmethyltramadol, n.d. = not detected.

Compound	Obtained EB samples	Concentrations in matched plasma samples, ng mL^{-1}	Detected in obtained EB samples
Bisoprolol	18	1–70	n.d.
Bromazepam	2	16, 41	n.d.
Carbamazepine	0	–	–
ODMT	2	3	detected
		31	n.d.
Diazepam	0	–	–
Lorazepam	2	2, 199	n.d.
Metoprolol	5	4–71	n.d.
Nordazepam	0	–	–
Nortilidine	4	6,	n.d.
		20	detected
		20	detected
		98	detected
		131	detected
Pregabalin	2	1108, 1130	n.d.
Ramipril	2	3	n.d.
Ramiprilat	1	34	n.d.
Tapentadol	1	209	n.d.
Tilidine	4	1,	n.d.
		1	n.d.
		14	detected
		20	detected
Tramadol	2	2,	n.d.
		169	detected

analytes were not detected in the present method. However, there were only two samples available for each of these analytes and the LODs were higher, especially for pregabalin (LOD 10,000 pg collector^{-1}) but also for bromazepam (LOD 25 pg collector^{-1}) and lorazepam (100 pg collector^{-1}). One possible explanation for the non-detection of the other compounds, e.g. ramipril (plasma concentration 3 ng mL^{-1}) or bisoprolol (plasma concentration 1–70 ng mL^{-1}) in patients EB aerosol may be the corresponding low plasma concentrations and consequently possibly low concentrations in EB, not exceeding the method LOD. Another explanation could be that the selected compounds are actually not excreted into EB. This would explain the non-excretion of pregabalin in the few samples that have been examined so far, despite significantly higher plasma concentrations (1108 and 1130 ng mL^{-1}). A comparison of the chemical structures of tramadol, ODMT, tilidine, which were both detected in EB, reveals a similarity (see Fig. S2). However, even in comparison with previously published studies, no clear trend between structure and excretion can yet be postulated. Comparing human breath condensate and EB, at least methadone could be detected in both matrices [28,30]. Therefore, further authentic samples are of vital importance. Nevertheless, this is a report about the detection and thus excretion of tilidine and nortilidine into human EB. Further studies of their EB kinetics should be encouraged.

3.4. Limitations and outlook

The prescribed sampling procedure provides can lead to limited amount of EB, as the patients had observable difficulties in performing more than three cycles through the Breath Explor device. Our study enrolled predominantly older multimorbid patients with various comorbidities who did not have sufficient lung capacity, thus the expiration cycles were reduced to three per sample in contrast to the instructions for use of twelve breath maneuvers. Consequently, a lower amount of EB and exhaled microparticles can be assumed. Nevertheless, the results cannot be generalized, as a specific group of patients was primarily considered. Another crucial point is the lack of standardization in the collection of patients EB. There was no control over the total amount of EB passing through the device and therefore no information on the actual amount of microparticles retained by impaction on the collectors. One potential approach for standardizing the sampling procedure would be to utilize a sampling application that assist patients in adhering to the expiration cycles. This approach could potentially compensate for minor variations in sample collection. Especially regarding the possibility of home-sampling. Another strategy would be to couple a spirometer to the sampling device and at least record the spirometric parameters for monitoring individual differences in breathing through the device. However, there would be no clear quantitative indication of the exhaled amount. Therefore, the optimal approach would be the identification of an endogenous non-volatile biomarker that can be collected and detected simultaneously. This endogenous biomarker could serve as a reference for the EB amount from each individual, thus directly relating to the amount of drug exhaled through the device. The significance of this matter and a solution to standardize the sample collection is crucial for further investigation, particularly for the quantification of analytes that have already been qualitatively detected in EB. However, a final evaluation of drug excretion into EB and drug monitoring requires further patient samples. In particular, regarding the limitations of this alternative sample matrix.

4. Conclusions

The present study reports a workflow for the qualitative detection of eleven selected drugs and four metabolites in EB. The sample preparation and the analytical part are straightforward and allow the inclusion of additional compounds., The excretion of tilidine and its metabolite nortilidine into EB was successfully demonstrated. Furthermore, tramadol and ODMT were detected in EB. Nonetheless, further investigations are required. On the one hand, additional samples are required to further investigate the actual excretion of drugs and metabolites into EB, particularly for analytes for which no current data are available. On the other hand, a standardization is necessary to determine the actual amount of EB sampled. The results confirm the potential of using EB as an additional matrix setting such as drug monitoring programs.

CRediT authorship contribution statement

Juel Maalouli Schaar: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Michael Kunz:** Writing – review & editing, Investigation. **Lea Wagmann:** Writing – review & editing, Conceptualization. **Olof Beck:** Writing – review & editing. **Felix Mahfoud:** Writing – review & editing, Resources. **Markus R. Meyer:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Felix Mahfoud reports financial support was provided by German Research Foundation. Felix Mahfoud reports financial support was provided by German Cardiac Society. Felix Mahfoud reports financial support was provided by German Heart Foundation. Felix Mahfoud reports a relationship with Ablative Solutions, Medtronic, ReCor Medical, Amgen, Astra-Zeneca, Bayer, Boehringer Ingelheim, Inari, Merck, Servier, and Terumo that includes: consulting or advisory and speaking and lecture fees. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

[1] N. Stephanson, S. Sandqvist, M.S. Lambert, O. Beck, Method validation and application of a liquid chromatography-tandem mass spectrometry method for drugs of abuse testing in exhaled breath, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 985 (2015) 189–196.
[2] M.R. Meyer, S. Rosenborg, M. Stenberg, O. Beck, First report on the pharmacokinetics of tramadol and O-desmethyltramadol in exhaled breath compared to plasma and oral fluid after a single oral dose, Biochem. Pharmacol. 98 (2015) 502–510.
[3] O. Beck, N. Stephanson, S. Sandqvist, J. Franck, Detection of drugs of abuse in exhaled breath using a device for rapid collection: comparison with plasma, urine and self-reporting in 47 drug users, J. Breath Res. 7 (2013) 026006.
[4] S. Ullah, S. Sandqvist, O. Beck, A liquid chromatography and tandem mass spectrometry method to determine 28 non-volatile drugs of abuse in exhaled breath, J. Pharm. Biomed. Anal. 148 (2018) 251–258.
[5] P. Kuban, F. Foret, Exhaled breath condensate: determination of non-volatile compounds and their potential for clinical diagnosis and monitoring. A review, Anal. Chim. Acta 805 (2013) 1–18.
[6] S. Seferaj, S. Ullah, A. Tinglev, S. Carlsson, J. Winberg, P. Stambeck, O. Beck, Evaluation of a new simple collection device for sampling of microparticles in exhaled breath, J. Breath Res. 12 (2018) 036005.

[7] H. Huang, J. Yang, C. Tao, L. Hu, T. Huan, W. Zhang, K. Zhang, X. Li, Exhaled breath analysis of non-volatile drugs: towards clinical applications, TrAC, Trends Anal. Chem. 171 (2024) 117541.
[8] P. Larsson, B. Bake, A. Wallin, O. Hammar, A.C. Almstrand, M. Larstad, E. Ljungstrom, E. Mirgorodskaya, A.C. Olin, The effect of exhalation flow on endogenous particle emission and phospholipid composition, Respir. Physiol. Neurobiol. 243 (2017) 39–46.
[9] B. Bake, P. Larsson, G. Ljungkvist, E. Ljungstrom, A.C. Olin, Exhaled particles and small airways, Respir. Res. 20 (2019) 8.
[10] V. Malave, K. Jeerage, E. Garboczi, T. Lovestead, 3D computational fluid and particle dynamics simulations: metrics of aerosol capture by impaction filters, J. Breath Res. 18 (2023).
[11] S.H.R. Ramalho, A.M. Shah, Lung function and cardiovascular disease: a link, Trends Cardiovasc. Med. 31 (2021) 93–98.
[12] F. Xu, J. Zhou, H. Yang, L. Chen, J. Zhong, Y. Peng, K. Wu, Y. Wang, H. Fan, X. Yang, Y. Zhao, Recent advances in exhaled breath sample preparation technologies for drug of abuse detection, TrAC, Trends Anal. Chem. 157 (2022) 116828.
[13] A.D. Tinglev, S. Ullah, G. Ljungkvist, E. Viklund, A.C. Olin, O. Beck, Characterization of exhaled breath particles collected by an electret filter technique, J. Breath Res. 10 (2016) 026001.
[14] A.C. Almstrand, E. Ljungstrom, J. Lausmaa, B. Bake, P. Sjovall, A.C. Olin, Airway monitoring by collection and mass spectrometric analysis of exhaled particles, Anal. Chem. 81 (2009) 662–668.
[15] K.M. Jeerage, C.N. Beuning, A.J. Friss, L.C. Bidwell, T.M. Lovestead, THC in breath aerosols collected with an impaction filter device before and after legal-market product inhalation-a pilot study, J. Breath Res. 17 (2023).
[16] S.M.R. Wille, W. Coucke, T. De Baere, F.T. Peters, Update of standard practices for new method validation in forensic toxicology, Curr. Pharmaceut. Des. 23 (2017) 5442–5454.
[17] T. Scientific Working Group for Forensic, Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology, J. Anal. Toxicol. 37 (2013) 452–474.
[18] EMA, ICH, Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis, European Medicines Agency, Amsterdam, The Netherlands, 2022.
[19] A.G. Helfer, J.A. Michely, A.A. Weber, M.R. Meyer, H.H. Maurer, Liquid chromatography-high resolution-tandem mass spectrometry using Orbitrap technology for comprehensive screening to detect drugs and their metabolites in blood plasma, Anal. Chim. Acta 965 (2017) 83–95.
[20] F.T. Peters, O.H. Drummer, F. Musshoff, Validation of new methods, Forensic Sci. Int. 165 (2007) 216–224.
[21] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[22] EMA, ICH, Q2(R2) Guideline on Validation of Analytical Procedures, European Medicines Agency, Amsterdam, The Netherlands, 2023.
[23] L.H.J. Richter, C.M. Jacobs, F. Mahfoud, I. Kindermann, M. Bohm, M.R. Meyer, Development and application of a LC-HRMS/MS method for analyzing antihypertensive drugs in oral fluid for monitoring drug adherence, Anal. Chim. Acta 1070 (2019) 69–79.
[24] R.C. Baselt, Disposition of Toxic Drugs and Chemicals in Man, eleventh ed., Biomedical Publications, Seal Beach, CA, 2017.
[25] S.K. Manier, A. Keller, M.R. Meyer, Automated optimization of XCMS parameters for improved peak picking of liquid chromatography-mass spectrometry data using the coefficient of variation and parameter sweeping for untargeted metabolomics, Drug Test. Anal. 11 (2019) 752–761.
[26] S. Barnes, H.P. Benton, K. Casazza, S.J. Cooper, X. Cui, X. Du, J. Engler, J. H. Kabarowski, S. Li, W. Pathmasiri, J.K. Prasain, M.B. Renfrow, H.K. Tiwari, Training in metabolomics research. I. Designing the experiment, collecting and extracting samples and generating metabolomics data, J. Mass Spectrom. 51 (2016) 461–475.
[27] M. Khoubnasabjafari, E. Rahimpour, A. Jouyban, Exhaled breath condensate as an alternative sample for drug monitoring, Bioanalysis 10 (2018) 61–64.
[28] H. Sinapour, J. Guterstam, S. Grosse, J. Astorga-Wells, P. Stambeck, M. Stambeck, J. Winberg, S. Hermansson, O. Beck, Validation and application of an automated multitarget LC-MS/MS method for drugs of abuse testing using exhaled breath as specimen, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 1240 (2024) 124142.
[29] K. Feltmann, T.H. Elgan, M. Bottcher, S. Lierheimer, S. Hermansson, O. Beck, J. Gripenberg, Feasibility of using breath sampling of non-volatiles to estimate the prevalence of illicit drug use among nightlife attendees, Sci. Rep. 12 (2022) 20283.
[30] O. Beck, S. Sandqvist, P. Eriksen, J. Franck, G. Palmskog, Determination of methadone in exhaled breath condensate by liquid chromatography-tandem mass spectrometry, J. Anal. Toxicol. 35 (2011) 129–133.