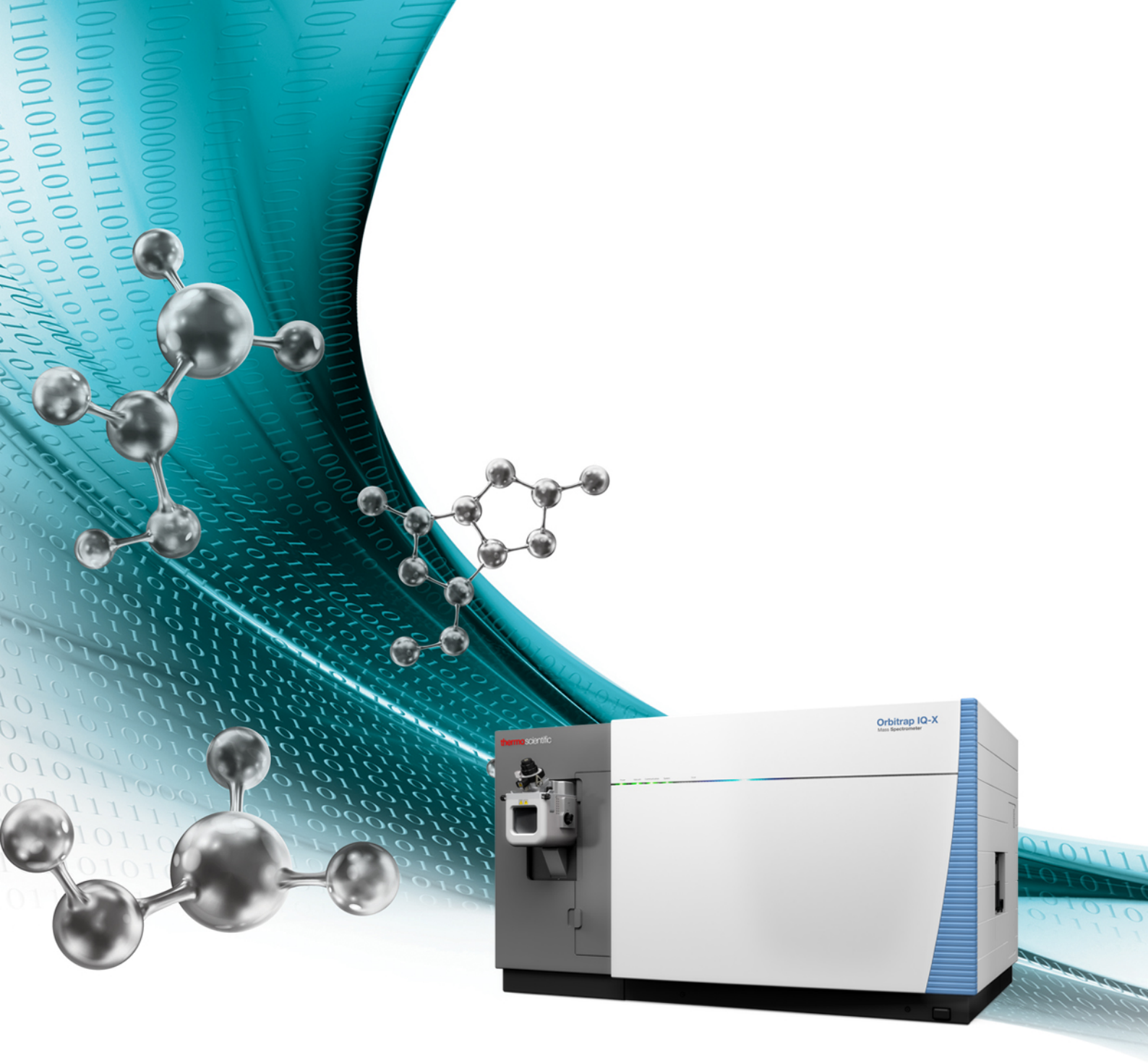

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Analysis of 4-fluoroamphetamine in cerumen after controlled oral application

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Abstract

Cerumen was found to be a promising alternative specimen for the detection of drugs. In a pilot study, drugs of abuse were identified at a higher detection rate and a longer detection window in cerumen than in urine. In this study, cerumen from subjects was analyzed after they ingested the designer stimulant 4-fluoroamphetamine (4-FA) in a controlled manner.

Methods: Twelve subjects ingested placebo and 100 mg of 4-FA. Five of them were also given 150 mg of 4-FA in 150 mL Royal Club bitter lemon drink at least after 7 days. Cerumen was sampled using cotton swabs at baseline, 1 h after the ingestion of the drug and at the end of the study day (12 h). After extraction with ethyl acetate followed by solid-phase extraction, the extracts were analyzed using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS).

Results and discussion: In the cerumen of all 12 subjects, 4-FA was detected 12 h after its ingestion; in most subjects, cerumen was detected after 1 h of ingestion, ranging from 0.06 to 13.90 (median 1.52) ng per swab. The detection of 4-FA in cerumen sampled 7 days or more after the first dose suggested a long detection window of cerumen.

Conclusions: Cerumen can be successfully used to detect a single drug ingestion even immediately after the ingestion when a sufficient amount of cerumen is used.

KEYWORDS

4-fluoroamphetamine, alternative matrices, cerumen, drug abstinence, liquid chromatography–mass spectrometry

1 | INTRODUCTION

Toxicological analyses are performed for the detection of drugs, for example, in the context of abstinence monitoring, workplace drug testing, or driving under the influence of drugs. Blood, urine, saliva, and sweat are used for the detection of short- or intermediate-term intake,[1] whereas keratinized matrices such as hair[2] and nails[3] provide longer detection windows. The use of each type of specimen has its inherent limitations, such as the stability of the analytes, detection time window, possible adulteration, and interpretation of the results.[1]

Recently, cerumen (the so-called earwax) has been investigated as an entirely new type of matrix for the detection of drugs.[4,5] The first comparative study on postmortem specimens showed that the presence of various drugs except cannabis can be reliably detected in cerumen.[4] In addition, the detection window of cerumen was found to be markedly longer than that of urine. The main limitation of cerumen as an alternative specimen was its low availability.

To promote cerumen for toxicological analysis, the present pharmacokinetic study was performed after a controlled intake of 4-fluoroamphetamine (4-FA). Cerumen samples from a study on the safety profile and pharmacokinetics of 4-FA[6] were available for evaluation, whether and eventually how fast a single dose of a typical amphetamine-type drug can be detected in that matrix.

2 | EXPERIMENTAL

2.1 | Chemicals and reference standards

Reference substance of 4-FA and the deuterated internal standard 4-FA-d_5 (ISTD) were purchased from LGC Standards (Wesel, Germany). UPLC-grade water and UPLC-grade acetonitrile were purchased from VWR Chemicals (Darmstadt, Germany), ethyl acetate was from AppliChem (Darmstadt, Germany), and methanol was from Sigma-Aldrich (Steinheim, Germany). All other reagents and solvents were purchased from Merck (Darmstadt, Germany) and were of analytical or HPLC grade.

2.2 | Biological samples

A placebo-controlled, three-way crossover study was conducted in 12 healthy recreational polydrug users (7 men, 5 women; Caucasians; average age 23 years).[6] The study was approved by the Medical Ethics Committee of Maastricht University and was registered in the Dutch Trial Register (trial number: NTR6164). Written informed consent was obtained from all participants. On three occasions, each subject ingested the placebo dose (0 mg) and the 100 or 150 mg dose of 4-FA (racemic 1-(4-fluorophenyl)propan-2-amine hydrochloride from Lipomed, AG, Arlesheim, Switzerland; purity is >98.5%). The drug was dissolved in 150 mL of a bitter lemon drink (Royal Club, Vrumona B.V., Bunnik, The Netherlands) and consumed by the subjects. The minimum washout period was 7 days to avoid carryover effects.[6]

Earwax samples were obtained during each test day using Raucotupf cotton swabs size S (Lohmann-Rauscher, Neuwied, Germany) three times: before the ingestion of drug or placebo (baseline, left ear), after 1 h (left ear), and 12 h (right ear) after intake. The cerumen swabs were stored under dry and dark conditions at room temperature until further analysis. Drug-free cerumen was obtained from laboratory staff.

2.3 | Cerumen sample preparation

The dry samples were essentially extracted as described previously[4] using 1 mL of ethyl acetate and 50 μL of ISTD (0.02 ng/μL of 4-FA-d_5 in acetonitrile). After sonification for 1 h, the samples were centrifuged at 13 000***g*** for 10 min, and the supernatant was transferred to silanized glass tubes and evaporated to dryness with a stream of air at room temperature.

The dry residues were redissolved in 100 μL of acetonitrile and further purified using a generic solid-phase extraction as described previously.[7] Briefly, the extracts were added to 500 μL of porcine serum and mixed with 5 mL phosphate buffer (0.1 M, pH 6.0). After conditioning of Bond Elut Certify HF columns (300 mg, 3 mL from Agilent, Waldbronn, Germany), samples were applied and the columns were washed with 1 mL of acetic acid (0.25 M) and 3 mL of methanol. The basic fractions were eluted with 3 mL of dichloromethane/isopropanol/ammonia (80:20:2, v/v/v). After evaporation to dryness, the extracts were reconstituted with 100 μL of 0.1% formic acid/acetonitrile (80:20, v/v).

2.4 | LC–MS/MS instrumentation and analytical conditions

For LC–MS/MS analysis, an Agilent 1290 infinity liquid chromatograph coupled with an Agilent G6460A Triple Quadrupole ESI LC/MS (Agilent Jet Stream technology) from Agilent Technologies (Waldbronn, Germany) was used. An amount of 2 μL of extracts (20°C autosampler temperature) was injected, and analytes were separated at 50°C on a Kinetex core-shell biphenyl column (Phenomenex, Aschaffenburg, Germany; 100 × 2.1 mm, 2.6 μm, 100 Å) equipped with a corresponding guard column. The mobile phase consisted of water containing 5 mM ammonium formate and 0.01% formic acid (A) and acetonitrile containing 0.01% formic acid (B). The elution program started with 5% B at a flow rate of 0.4 mL/min and increased linearly during 4 min to 95% kept for 2 min until reequilibration (7 min total run time). The source parameters were as follows: gas flow rate, 11 L/min (300°C); nebulizer, 55 psi; sheath gas flow rate, 12 L/min (400°C); capillary voltage, 3000 V; fragmentor voltage, 80 V. The MS/MS was operated in multiple reaction monitoring mode with the following transitions (*m/z*, collision energy in parentheses, quantifier underlined): 4-FA-d_5 159 → 142 (8); 4-FA 154 → <u>137</u> (4), 109 (16), 89 (44).

Data evaluation was performed using the Agilent MassHunter Software (version B.07.00). For identification, a deviation of ±0.1 min of the expected retention time compared to calibrators and a quantifier/qualifier ratio within 15% of the ratio measured in calibrators were required.

2.5 | Assessment of 4-FA amount in cerumen

The amount of the drug in cerumen was assessed. As weighing cerumen swabs was not practicable, only the total amount of drug per swab was assayed, and no weight-based concentrations were given. Calibrators were prepared from a series of drug-free cerumen samples of one donor by spiking with increasing volumes of a solution of 4-FA in acetonitrile (0.01 or 0.1 ng/μL) and were analyzed as described previously. The final concentrations of the calibrators were 0.01, 0.05, 0.10, 0.20, 0.25, 0.30, 0.50, 0.75, 1.0, 2.5, 5.0, and 10.0 ng/sample.

The validation of the quantitative assay of 4-FA was performed according to the current guidelines of the Society of Toxicological and Forensic Chemistry.[8] However, the problem in obtaining a homogeneous pool of a blank cerumen matrix complicated validation. For statistical evaluation, Valistat 2.0 software (Arvecon GmbH, Walldorf, Germany) was used. Selectivity was assessed with drug-free human cerumen samples from 10 donors with and without the addition of ISTD (blank and zero samples). The matrix effects were determined by comparing the results from the analyses of solutions of reference substances in solvent and blank matrix extracts at two levels (0.5 and 5.0 ng/sample) with five repetitions each. Sensitivity was evaluated in terms of limit of detection (LOD) and lower limit of quantification (LLOQ) using the data measured with six calibrators in a lower range (0.03–0.09 ng/sample) as described in the guideline of the German Institute for Standardization (DIN 32645). Precision and accuracy were assessed by

TABLE 1 Dosage and results of 4-fluoroamphetamine (4-FA) analysis in the cerumen sampled prior (0 h) and after the drug or placebo consumption (1 and 12 h) of the 12 subjects, shown as negative (–) or amount per sample

Subject	Sampling time (h)	Sequence of dosage (mg)			4-FA (ng/cerumen sample)		
1	0	0	100	150	–	–	6.00
	1				–	0.23	4.51
	12				–	1.30	13.90
2	0	0	100	150	–	–	–
	1				–	–	–
	12				–	3.50	2.70
3	0	100	0	150	–	–	–
	1				0.16	–	1.52
	12				0.42	–	8.20
4	0	100	0	150	–	–	–
	1				–	–	0.11
	12				1.50	–	2.60
5	0	100	0	n.a.	–	–	
	1				1.93	–	
	12				1.10	–	
6	0	100	150	0	–	–	0.22
	1				–	0.60	–
	12				2.90	8.50	0.29
7	0	0	100	n.a.	–	–	
	1				–	–	
	12				–	0.25	
8	0	0	100	n.a.	–	–	
	1				–	0.06	
	12				–	5.90	
9	0	100	0	n.a.	–	–	
	1				0.19	–	
	12				2.90	–	
10	0	100	0	n.a.	–	0.36	
	1				0.51	0.07	
	12				5.00	1.10	
11	0	0	100	n.a.	–	–	
	1				–	0.89	
	12				–	7.10	
12	0	100	0	n.a.	–	0.10	
	1				0.48	–	
	12				7.00	0.11	

Note. Some samples were not available (n.a.) due to dropouts in the 150 mg dose condition.

analyzing cerumen spiked with two amounts (0.2 and 1.0 ng/sample) in duplicate at 5 days. The stability of the analytes in the extracts stored in the autosampler (20°C) was evaluated by the repeated analysis of the two samples (0.2 and 0.75 ng/sample) every 12 h.

2.6 | Statistical evaluation

The amounts of 4-FA determined in cerumen was compared with the pharmacokinetic data in serum. Because of the inhomogeneity of the cerumen data, nonparametrical comparisons were performed using the Mann–Whitney U test or Spearman's rank–order correlation (SPSS version 25, IBM, Ehningen, Germany).

3 | RESULTS

3.1 | Analytical method

A relevant ion suppression was not noted (110.6% ± 9.5% and 90.8% ± 10.7% for 0.2 and 0.75 ng of 4-FA/sample, respectively). The LOD and LLOQ were estimated as 0.025 ng/sample and 0.05 ng/sample, respectively. Linearity was established for the calibration range up to 15.0 ng/sample. The relative standard deviations as a dimension of precision were less than 11% for both tested amounts (0.2 and 1.0 ng/sample). The processed samples containing 4-FA were stable, exhibiting deviations after repeated analyses for 5 days of less than 15%.

3.2 | Cerumen analysis results

The amounts of 4-FA determined by the analysis of all cerumen samples are shown in Table 1. The 4-FA values were in the range of 0.06–13.9 ng/sample. One 4-FA amount exceeded the highest calibrator (10 ng/sample).

All cerumen samples obtained before the first consumption of 4-FA were negative. In eight subjects, the cerumen samples obtained 1 h after the drug ingestion were already positive (0.06–1.90, median 0.36 ng/sample). At the end of the study day (12 h), 4-FA was detected in all samples (0.25–7.10, median 2.90 ng/sample). The results of the drug analysis were similar in the five subjects who consumed a 150 mg dose. 4-FA was detectable in the cerumen of four subjects (0.11–4.50, median 1.05 ng/sample) and all five subjects (2.60–13.90, median 8.20 ng/sample) after 1 and 12 h, respectively. Unexpectedly, one cerumen sample was already positive for 4-FA at baseline (6.00 ng/sample). This was also

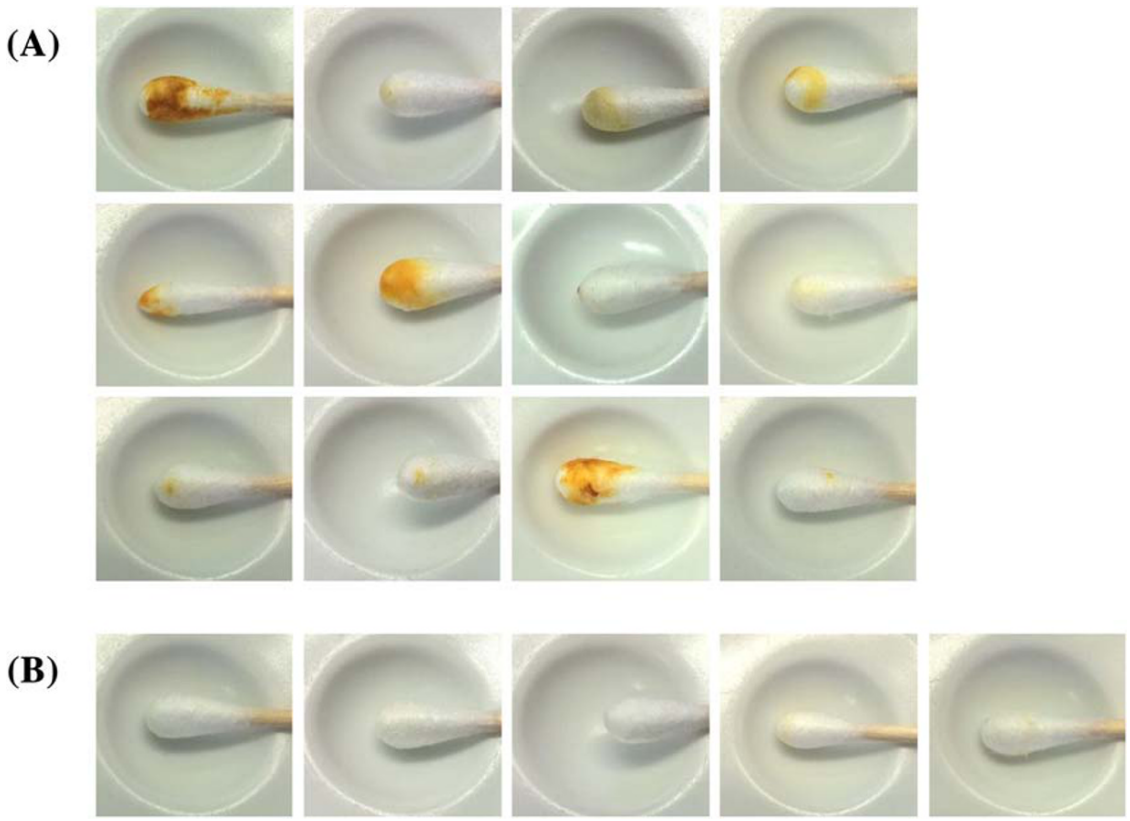

FIGURE 1 Photo documentation of the 17 cerumen swabs taken 1 h after the ingestion of 100 (n = 12) or 150 mg (n = 5) of 4-FA. A, The 12 samples are with drug-positive findings, and B, the samples are the drug-negative samples ($2_{100\ mg}$, $2_{150\ mg}$, $4_{150\ mg}$, $6_{100\ mg}$, $7_{100\ mg}$, cf, Table 1) [Colour figure can be viewed at wileyonlinelibrary.com]

observed in the placebo condition in three out of seven subjects (0.10–0.36, median 0.22 ng/sample) if an experiment of 100 or 150 mg drug consumption preceded (cf, sequences of conditions are given in Table 1 and examples of chromatographic data are shown in Figure 2).

4 | DISCUSSION

Methods of testing for drugs are of major importance in various contexts. The continuous improvement in analytical techniques and procedures allows analyses with high sensitivity and increasing reliability of the results. Specimens like hair or sweat used in the routine analysis are of interest, representing alternatives to blood, urine, or saliva.[9] In the search for specimens that provide a longer detection window than urine and avoid the problem of cosmetic manipulation of hair, cerumen as an easily accessible matrix was recently evaluated and found to be a promising alternative.[4] Cerumen is secreted by glands located in the external auditory canal[22] and a mixture of several types of lipids and is slightly acidic.[26] Along with hair and nail, it can be considered as a keratinized matrix.[24] It was assumed that drugs excreted in sebum or sweat may also be detectable in cerumen which is a mixture of both.[22] However, the low sample amount of cerumen was considered to be its main limiting factor. The maximum amount of a cerumen sample was less than 10 mg, whereas easily 20 mg or more of hair samples can be used for the analysis. However, in 38 deceased drug users, the presence of amphetamine, cocaine, opiates, and methadone was reliably detected,[4] assuming recent and, also to a certain extent, past use. To further characterize cerumen for forensic toxicological analysis, controlled studies are essential.

In this study, cerumen of 12 subjects who had consumed the designer stimulant 4-FA, which gained popularity especially in the Netherlands,[10] was analyzed. After the oral administration of 100 mg of 4-FA to all subjects and 150 mg to five of them, not only cerumen but also blood and urine samples were collected during the 12 h study (results are given by de Sousa Fernandes Perna et al[6] and Toennes et al[11]). Cerumen was sampled three times after the drug consumption: at baseline and after 1 h (both from the left ear) and after 12 h (from the right ear). A serial sampling at short time intervals was not performed because cerumen secretion yielded not enough material during that time frame. A baseline sample was considered mandatory to compare the analytical findings after the drug consumption with sampling at the end of the study where cerumen was expected to be positive for 4-FA. Therefore, these two key samples were collected from the left and the right ear. In addition, a resampling of the left ear

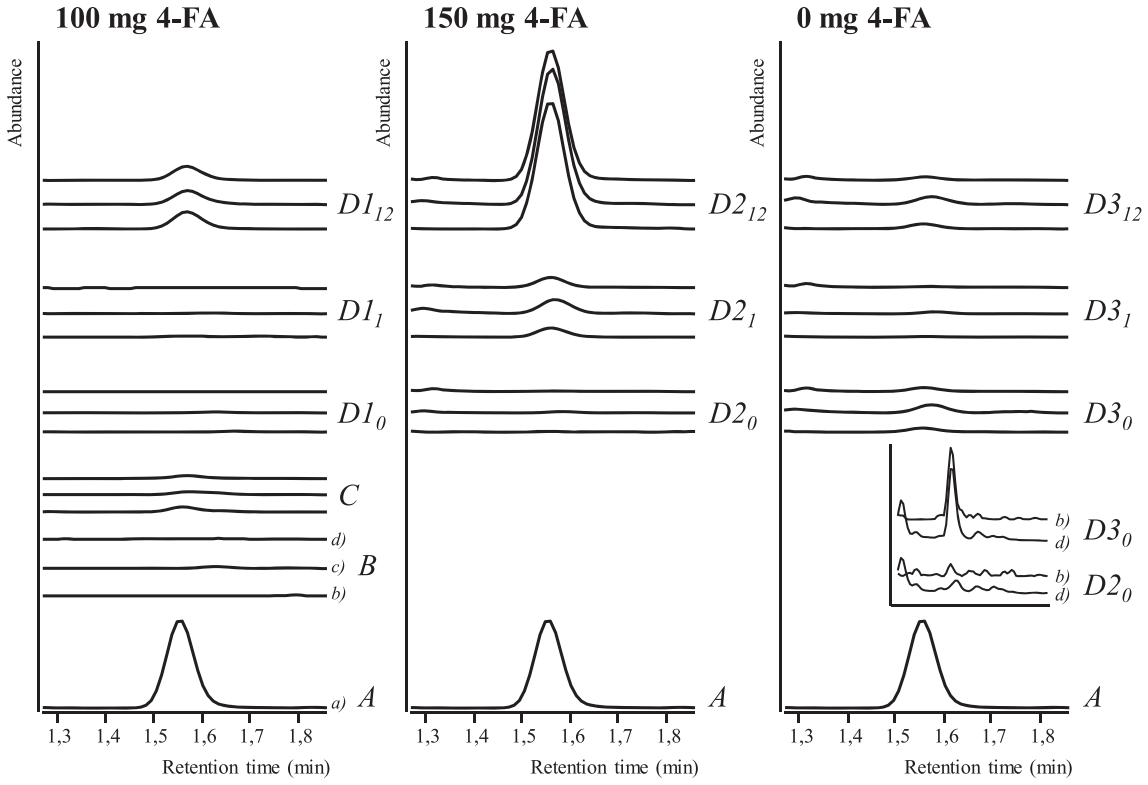

FIGURE 2 Extracted ion chromatograms of deuterated internal standard 4-FA-d_5 (*A*, *a*: 159 → 142) and 4-FA (quantifier and two qualifiers; *b*: 154 → 89, *c*: 154 → 137, *d*: 154 → 109) in a blank sample (*B*), a calibrator (*C*, 0.25 ng) all cerumen samples from subject 6 (cf, Table 1 for estimated amounts; sequence of conditions: 100 mg *D*1, 150 mg *D*2, 0 mg *D*3). These are representative for a negative result in baseline cerumen ($D1_0$) and positive results in samples 1 and 12 h after the drug ingestion (subscripts 1 and 12 of *D*1 and *D*2) and in the two ears in the placebo condition ($D3_0$ and $D3_{12}$). All chromatograms are in equal scale. In the inset, a positive ($D3_0$) and a negative ($D2_0$, baseline) sample are contrasted

1 h after the consumption of the drug was included to detect drug excretion at the early stage of the experiment.

4.1 | Excretion of 4-FA into cerumen

Although baseline samples were consistently negative before the first consumption of 4-FA, the resampling of the same ear after 1 h exhibited positive results in 8 out of 12 (66.6%) and 4 out of 5 subjects (80%) after the intake of the 100 and 150 mg dose, respectively. In the sample from the other ear 12 h after the intake of 4-FA, cerumen samples of all subjects exhibited positive results. This shows one key finding of this study: a single consumption of a typical 4-FA dose[12] can be reliably detected in cerumen (Figure 2), which may be possible already 1 h after the intake. However, the lower rate of 4-FA detection at this time may be due to the limited amount of cerumen left after the baseline sampling from the same ear 1 h before. This can be illustrated by a visual comparison of the analyzed cerumen samples. The visual inspection of the cotton swabs of the positive samples compared with those that were negative (Figure 1) supports the assumption that the latter samples did not contain a sufficient amount of cerumen leading to negative results.

The amount of excreted drug was in the range of 0.06–13.9 (median 1.7) ng/sample. Assuming that the sample quantity of cerumen is less than 10 mg,[4] the resulting concentrations might be higher than those expected in hair after a single drug ingestion.[13] The fast detectability of a drug in cerumen may be explained by the fact that cerumen partly consists of sweat,[14] and various sympathomimetic amines have been detected even after single doses in sweat.[15]

In the baseline samples of cerumen from four subjects, 4-FA could be detected (exemplified for subject 6 in the inset of Figure 2), which was unexpected at first, but in all these cases, a study condition with the drug had preceded 7 days (referenced as subjects $6_{0\ mg}$, $10_{0\ mg}$, and $12_{0\ mg}$ in Table 1) or 91 days (subject $1_{150\ mg}$). In other cases with negative baseline samples (Table 1, subjects $2_{150\ mg}$, $3_{0\ mg}$, $4_{0\ mg}$, $5_{0\ mg}$, $6_{150\ mg}$, $9_{0\ mg}$), these followed experimental days with drug ingestion with a longer delay of 21–70 days (median 35 days). From our first study,[4] we concluded that cerumen has a much longer detection window than urine, which may be in the range of 1–2 months. Therefore, the positive findings in baseline samples are explained by preceding drug consumption and confirm a detection window of at least 7 days.

4.2 | Correlation of drug amounts in cerumen and serum

The data from this study can be used to evaluate factors influencing the excretion or detection of drugs in cerumen. The different doses of a drug and its concentrations in serum may influence its secretion or detection in cerumen. It is most probable that the ingestion of increasing drug doses or a higher frequency of use may result in a higher accumulation of drugs in cerumen.

In this study, single doses of 100 and 150 mg of 4-FA led to amounts of 0.25–7.10 ng/sample (median 2.90, n = 12) and 2.60–-13.90 ng/sample (median 8.20, n = 5), respectively. The large variation showed that the correlation of dose and disposition in cerumen is not predictable.

TABLE 2 4-FA serum concentrations (maximum, C_{max}, and 1 h after ingestion, $C_{1\ h}$) and areas under the time curves of serum concentrations (until 1 h, $AUC_{0 \rightarrow 1\ h}$ and until the end of the study day, $AUC_{0 \rightarrow 12\ h}$) for the subjects and the dosages[11]

Subject	Dosage (mg)	C_{max} (ng/mL)	$AUC_{0 \rightarrow 12\ h}$ (ng/mL h)	$C_{1\ h}$ (ng/mL)	$AUC_{0 \rightarrow 1\ h}$ (ng/mL h)
1	100	155	1329	126	48
2	100	186	1498	155	94
3	100	192	1608	179	130
4	100	260	1919	168	68
5	100	276	2049	276	149
6	100	171	1439	125	56
7	100	221	1401	176	62
8	100	203	1497	188	125
9	100	198	1201	102	35
10	100	189	1651	90	34
11	100	316	2884	236	104
12	100	186	1525	186	97
1	150	278	2114	229	132
2	150	252	1967	252	138
3	150	319	2088	319	216
4	150	500	2767	500	205
6	150	322	2556	229	125

Cerumen is produced by secretory glands,[14] and a transfer of drugs and metabolites from the blood into cerumen is expected. The concentrations in cerumen may, therefore, be proportional to that in the blood. The correlation of the drug amounts in cerumen was tested using the data from the corresponding serum samples (the 4-FA data from the study described by Toennes et al [11] are given in Table 2). This includes serum concentrations at the time of the first cerumen sampling ($C_{1\ h}$), the highest serum concentrations reached (C_{max}), and the areas under the curve until 1 and 12 h ($AUC_{0 \rightarrow 1\ h}$, $AUC_{0 \rightarrow 12\ h}$). Spearman's rank correlation tests were not significant for any of the comparisons ($P > 0.05$). This is not surprising when compared with the study results obtained with another secretion product, saliva. The concentrations in the saliva were also unpredictable, exhibiting a wide variation of ratios with serum concentrations.[11]

No weight-based concentrations were evaluated, but instead the total amount of the drug as extracted from the sampled cerumen was shown. The extent of sampling using the swabs and the inevitable lack of standardization of this procedure most probably determine the yield of cerumen and that of the secreted drug which may be lower than the true total amount of the excreted drug.

5 | CONCLUSION

Cerumen is a promising alternative specimen for the detection of 4-FA. In this study, a single oral intake of 4-FA was confirmed in all 12 subjects after the consumption of 100 or 150 mg of the drug. The detection of 4-FA in cerumen sampled 7 days or more after the first dose supports a long detection window in that matrix. The drug detection in cerumen is mainly determined by the amount of cerumen sampled rather than the blood concentration.

As a perspective, cerumen may be a useful specimen in cases where it is too late for urine tests, for example, in cases of drug-assisted assaults or where hair samples may not be reliable (by coloration). However, a number of issues, such as potential external contamination or ways of adulteration, need to be investigated.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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