

Earwax as an alternative specimen for forensic analysis

Engy Shokry¹ · Jair Gonzalez Marques¹ · Paulo César Ragazzo² · Naiara Zedes Pereira¹ · Nelson Roberto Antoniosi Filho¹

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Abstract In this work, we presented, for the first time, earwax as an alternative forensic specimen for detecting 12 neuropsychotic drugs employing liquid chromatography–tandem mass spectrometry in positive and negative ion modes after straightforward extraction with methanol. The method was validated and standard curves were established by external calibration with correlation coefficients >0.99 . All precision, accuracy, matrix effects, extraction recoveries, and carryover were within acceptable limits; limits of quantification were sufficiently low to quantify almost all the samples tested. To confirm the feasibility of the study, earwax specimens were collected from actual patients treated with different combinations of the 12 drugs and analyzed by our method; the 12 drugs could be quantified from the earwax specimens of the users successfully, showing usefulness of earwax specimens, because of its noninvasive sampling and the storage of drug(s) for relatively long time together with its being relatively less contaminated by environmental impurities. This study is pioneering; many detailed studies on earwax as an alternative specimen remain to be explored.

Keywords Earwax · Cerumen · Alternative specimen · Neuropsychotic drugs · Noninvasive sampling · LC–MS/MS

✉ Engy Shokry
angeliqua26582@yahoo.com

¹ Laboratório de Métodos de Extração e Separação (LAMES), Universidade Federal de Goiás (UFG), Campus II, Samambaia, Instituto de Química (IQ), CEP 74690-900 Goiânia, GO, Brazil

² Instituto de Neurologia de Goiânia, Praça Gilson Alves de Souza, no 140, Setor Bueno, CEP 74690-970 Goiânia, GO, Brazil

Introduction

Usually, screening for drugs of abuse is the first step in clinical and forensic toxicology. The standard procedure for drug testing in toxicological analyses consists generally of an immunoassay screening performed on urine, followed by gas chromatographic–mass spectrometric confirmation [1, 2]. Blood and urine analyses have some limitations, because they lack convenience of collection in some cases and the short half-life of drugs either in urine or blood, resulting in missing administered drug(s) after a few days [3, 4]. Various biological matrices have been suggested as alternatives to urine and blood to prove the presence of illicit drugs: principally, saliva, sweat, hair, and nails [5]. The hair and nails, in spite of their expanded use, have some disadvantages including, for instance, risk of external contamination, which was considered an issue making interpretation of results a challenge [6]; in the case of a sweat patch, it must be worn for 3–7 days with a minimum of 48 h to collect adequate sweat for analysis [7].

Another biological secretion, cerumen, commonly referred to as “earwax”, that was very little exploited over decades as a matrix for biomonitoring, is introduced to this work as a matrix for detection of some psychotropic and antiepileptic drugs. Earwax sampling could be even considered more preferred as a diagnostic biological secretion, because in addition to its advantage as a noninvasive sampling technique, it is relatively less contaminated by the ambient air or by cosmetics that are the problems commonly faced in the cases of sweat and hair; this is because the ear canal is more protected from the external environment than the skin.

Until recently, earwax was looked upon as a neglected body secretion. Prokop-Prigge et al. [8] discovered that earwax conveyed important information about an

individual, including race, ethnicity, gender, diseases, food eaten, and even exposure to surrounding environmental pollutants. However, they only examined endogenous organic C₂– to –C₆ acids, but not any xenobiotic drugs. The earwax is primarily a biological fluid secreted in the external ear canal and composed of a mixture of viscous secretions from sebaceous glands and less-viscous ones from ceruminous glands, “modified apocrine sweat glands” [9]. The main components of earwax are shed layers of skin, with 60% of the earwax consisting of keratin, 12–20% saturated and unsaturated long-chain fatty acids, alcohols, squalene, and 6–9% cholesterol [9].

Given that earwax is a secretion from ceruminous gland, a specialized sweat gland, it is suggested that the drug molecules are transported into earwax, such as sweat, by passive diffusion through membranes surrounding the sweat glands. The time window when drugs are expected to arrive at the surface seems broad. For example, eccrine sweat glands transport the drug to the skin surface within hours, but sebaceous gland cells release drugs over several days to a week after they rupture. There has been evidence in the literature supporting the disposition and excretion of psychotropic and antiepileptic drugs in sweat [10, 11]. It is also worth describing that alcohol, amphetamine, cocaine, phencyclidine, and methadone have been found in sweat, often in concentrations greater than in blood [6]. On the other hand, earwax, being principally composed of keratin, is considered a keratinic matrix like hair and nails. This suggests the same mechanism of incorporation of drugs into its keratin fibers through blood circulation, which means that substances are brought into earwax as it is being secreted and accumulated in the toenails [12].

Regarding the analytical techniques applied in the area of forensic toxicology, liquid chromatography–tandem mass spectrometry (LC–MS/MS) was found to be advantageous, for screening drugs of abuse because of potentially high analytical specificity, a wide range of applicability, and high sensitivity in biological fluids with small amounts of starting material. In addition, it has overcome limitations of gas chromatography–mass spectrometry (GC–MS) due to simpler sample preparation, often without the need for derivatization [13]. Based on this, in our method, we analyzed 12 neuropsychotic drugs including antiepileptics, anxiolytics, and antipsychotics in earwax samples of users, using LC–MS/MS after a straightforward sample extraction with methanol. The drugs involved include: carbamazepine, phenytoin, levetiracetam, lamotrigine, oxcarbazepine, lacosamide, topiramate, valproic acid, phenobarbital, clobazam, clonazepam, and clozapine.

Materials and methods

Chemicals and reagents

Reference standards for carbamazepine (purity 99%), phenytoin (certified reference material), clonazepam, lacosamide (certified reference material), phenobarbital (purity ≥ 95%), levetiracetam (purity ≥ 98%), lamotrigine (purity ≥ 98%), oxcarbazepine (purity ≥ 98%), clozapine (purity ≥ 98%), clobazam (purity ≥ 98%), valproic acid (purity ≥ 98%), and topiramate (purity ≥ 98%) were purchased from Sigma-Aldrich (Steinheim, Germany); methanol (LC–MS grade), ammonium acetate (purity ≥ 99.0%), and ammonium hydroxide solution (ACS reagent, 28.0–30.0% NH₃ basis) from Sigma-Aldrich (Riedel de Haën, Germany); acetonitrile (LC–MS grade) from J.T. Baker (Avantor Performance Materials, Corporate Parkway Center Valley, PA, USA).

Specimens

Seventeen earwax samples were obtained from 17 users of antiepileptic and anxiolytic/antipsychotic drug combinations aged ≥18 years including 10 men and seven women. The subjects enrolled in the study were treated with a single antiepileptic or different multiple antiepileptic and anxiolytic/antipsychotic drug combinations (polydrug therapy) involving the above 12 neuropsychotic drugs. The study protocol was approved by the local ethical committee at the “Universidade Federal de Goiás” (UFG), Brazil (#57880516.9.0000.5083) and the “Instituto de Neurologia de Goiânia”. Written informed consent was obtained from each participant enrolled in the study.

Sample preparation

Earwax samples (20 mg) obtained from users of single antiepileptic or multiple antiepileptic and anxiolytic/antipsychotic drug combinations were accurately weighed, mixed with 1000 µL of methanol, and subsequently vortexed (IKA MS 3 digital; IKA Japan K.K., Higashiosaka, Japan) for 10 min, then centrifuged in Eppendorf centrifuges (3000 rpm) (Rotana 460R; Hettich Instruments, Tuttlingen, Germany) for 5 min at 4 °C. The supernatant was stored at –20 °C until analysis.

For calibration samples, stock solutions of the reference standards of investigated drugs were prepared in methanol at a concentration of 1 mg/mL. Subsequent dilutions in methanol were made from the stock solutions to prepare a combined standard solution composed of 50 mg/L each of levetiracetam, lamotrigine, lacosamide, phenytoin, oxcarbazepine, carbamazepine, valproic acid, topiramate,

phenobarbital, and clozapine, 50 µg/L of clonazepam and 500 µg/L of clobazam. All stock solutions were stored in freezer at $-20\text{ }^{\circ}\text{C}$.

Drug-free earwax samples (20 mg) from healthy donors were pooled, spiked with various dilutions of the combined reference standard solution of the investigated drugs in methanol, and prepared in the same way described above to prepare an eight-point calibration curves.

Calibration curves were constructed in duplicates over the concentration range equivalent to 5–500 ng/mg earwax for the ten drugs except clonazepam and clobazam with eight concentration points. As for clonazepam and clobazam, the calibration curves were constructed over the ranges of 5–500 pg/mg earwax and 50–5000 pg/mg earwax with eight concentration points, respectively. The calibrators, used for validation purposes, were prepared in the same way at concentrations equivalent to 5, 50, and 500 ng/mg, 5, 50, and 500 pg/mg, and 50, 500, and 5000 pg/mg, for the ten drugs, clonazepam, and clobazam, respectively (low, middle, and high levels).

Equipment and conditions

Chromatographic separation was carried out using an Agilent 1290 series HPLC capillary system equipped with a quaternary pump (Agilent Technologies, Waldbronn, Germany) operated in gradient mode and coupled with thermostated autosampler and fully controlled by Analyst software (Version 1.5.2). For the chromatographic separation, preliminary trials were carried out employing commercially available C18 columns such as Zorbax[®] (XDB-C18, 150 × 4.6 mm i.d., particle size 5 µm) (Phenomenex, Torrance, CA, USA), Synergi 4µ Fusion[®] (C18, 150 × 2 mm, 4 µm) (Phenomenex), Kinetex[®] (C18, 50 × 2.1 mm, 1.3 µm) (Phenomenex) and Poroshell 120 EC-C18 (C18, 50 × 2 mm, 1.7 µm) (Agilent Technologies, Santa Clara, CA, USA), as well as a normal phase Techsphere silica column (250 × 4.6 mm, 5 µm) (HPLC Technologies, Cheshire, UK) and different mobile phases including several combinations of methanol and acetonitrile, with different concentrations of ammonium acetate and ammonium phosphate buffers with the aim of optimizing the conditions adequately to separate the drugs, to examine matrix interference, and to minimize the carryover effect.

After the preliminary tests for columns and mobile phases, the chromatographic run was finally performed by using a Poroshell 120 EC-C18 column operated at 50 °C. Mobile phase was composed of 2 mM ammonium acetate in HPLC-grade water as phase A, and HPLC-grade acetonitrile as phase B. The flow rate was maintained at 2 mL/min. The injection volume selected was 1 µL. Separation was accomplished in isocratic condition employing 75%

phase B in the first 0.5 min of the run, followed by a linear gradient from 75 to 40% phase B over the following 0.5 min, maintained in this condition for 1 min, and then returned to the initial condition from 2 to 2.2 min with phase B maintained at 75% throughout the remaining 1.8 min to equilibrate the column. The eluent from the column was directed to the mass spectrometer with splitless mode. System control and data acquisition were performed with Analyst software (AB SCIEX, Toronto, Canada) including the “Explore” option (for chromatographic and spectral interpretation) and the “Quantitate” option (for quantitative information generation). Calibration curves were constructed with the Analyst Quantitation program using a linear least-squares regression non-weighted.

For MS/MS analysis, an API 3200 QTRAP triple quadrupole/linear ion trap mass spectrometer (AB SCIEX) equipped with TurboIonSpray source. The instrument was operated in the electrospray ionization mode with positive/negative polarity switching and in multiple reaction monitoring (MRM) mode, whereby ion path settings were determined using the compound optimization algorithm of the Applied Biosystems/MDS Analyst 1.5.2 software. The two most intense MRM transitions, (one quantifier and one qualifier ion) were selected for each analyte with the exception of oxcarbazepine and valproic acid where only one transition (quantifier ion) was selected. Then the selected transitions were summarized to one final method using the scheduled MRM algorithm. Ion source parameters were optimized for the lower abundance compounds (curtain gas: 20 psi; ion source gas 1: 50 psi; ion source gas 2: 50 psi; temperature: 650 °C; collision gas (collision-induced dissociation): medium; interface heater: on; needle voltage: 4500 V). The parameters and the selected transitions are summarised in Table 1.

Method validation

Evaluation of method performance including limit of detection (LOD), lower limit of quantification (LLOQ), linearity, accuracy, precision, extraction recovery, carryover, and ion suppression (matrix effect) was performed according to the ICH guidelines for method validation [14].

Linearity, LOD, and LLOQ

Quantification was performed using the external calibration method. Eight standard samples at different concentrations (each containing all the investigated drugs) prepared in duplicates, were used for evaluating linearity of the calibration curves. The linear least-square regression was carried out to determine the mean intercepts, mean slope,

Table 1 Selected multiple reaction monitoring (MRM) transitions and optimized data acquisition mass spectrometric parameters

Drug	Parent ion	Detected ion	MRM transition	Dwell time (ms)	Declustering potential	Collision energy	Entrance potential	Collision entrance potential
Levetiracetam	[M + H] ⁺	Quantifier	171.4 > 126.0	150	7.0	25.0	10.0	3.0
		Qualifier	171.4 > 154.0	150	12.0	14.0	10.0	3.0
Lacosamide	[M + H] ⁺	Quantifier	251.1 > 108.1	150	21.4	10.2	10.0	3.0
		Qualifier	251.1 > 91.1	150	26.7	28.6	10.0	3.0
Lamotrigine	[M + H] ⁺	Quantifier	256.2 > 211.2	150	34.0	35.0	10.0	4.4
		Qualifier	256.2 > 145.0	150	46.0	61.0	10.0	4.4
Phenytoin	[M + H] ⁺	Quantifier	253.1 > 182.2	150	36.2	43.6	4.8	7.7
		Qualifier	253.1 > 208.1	150	18.5	29.5	6.4	4.8
Oxcarbazepine	[M + H] ⁺	Quantifier	253.0 > 180.1	150	33.0	8.0	2.4	3.8
Carbamazepine	[M + H] ⁺	Quantifier	237.3 > 194.2	150	48.0	22.0	7.9	3.2
		Qualifier	237.3 > 220.3	150	48.0	11.0	7.9	4.9
Clonazepam	[M + H] ⁺	Quantifier	316.3 > 270.0	150	49.0	37.0	10.0	3.0
		Qualifier	316.3 > 241.0	150	47.0	58.0	7.5	11.8
Clobazam	[M + H] ⁺	Quantifier	301.3 > 259.0	150	50.0	26.0	3.8	9.9
		Qualifier	301.3 > 224.0	150	44.0	45.0	3.2	4.9
Clozapine	[M + H] ⁺	Quantifier	327.3 > 270.0	150	32.0	24.0	7.0	3.6
		Qualifier	327.3 > 192.1	150	30.0	60.0	6.3	8.0
Valproic acid	[M – H] [–]	Quantifier	143.0 > 143.0	150	–35.0	–12.0	–11.0	–2.2
Phenobarbital	[M – H] [–]	Quantifier	231.1 > 42.2	150	–20.2	–34.9	–3.6	–2.2
		Qualifier	231.1 > 188.1	150	–25.9	–20.0	–3.8	–2.2
Topiramate	[M – H] [–]	Quantifier	338.0 > 78.0	150	–42.0	–61.0	–10.0	–2.2
		Qualifier	338.0 > 96.0	150	–40.0	–42.0	–10.0	–2.2

and determination coefficients (R^2) of the calibration curves.

LLOQ is considered the lowest concentration that gives a reproducible instrument response with a coefficient of variation (CV%) <20% and signal-to-noise (S/N) ratio ≥ 10 .

LOD is considered the lowest concentration that gives a reproducible instrument response with S/N ratio ≥ 3 .

Accuracy and precision

Intraday precision and accuracy were determined by analysing five replicates of the calibrators for all analytes during a single analytical run. Interday precision and accuracy were determined by analysing three replicates of samples at each level through analytical runs made on five different days.

Matrix effect

The matrix effect was calculated using the mixed data of the matrix factors (MF), obtained from two concentration levels (both low and high calibrators), each in triplicate (i.e., $n = 6$ in total). The matrix factor (MF) is defined as the analyte peak area ratio of blank earwax sample extract spiked with

an analyte after extraction to the reference standard in methanol containing equivalent amount of the analyte neat sample. If MF is equal to 1, this means that no matrix effects are present; if $MF < 1$, this means that there is ionization suppression, whereas $MF > 1$ may be due to ionization enhancement and/or analyte loss in the absence of matrix.

Extraction recovery

The extraction recovery was calculated in terms of recovery $C/B \times 100$, where C is the analyte peak area of blank earwax spiked with a reference standard before the extraction, and B is the analyte peak area of earwax spiked with the reference standard after the extraction. The extraction recovery was calculated using the mixed data, obtained from two concentrations levels, each in triplicate (both low and high levels; $n = 6$ in total; for the concentrations, see “Sample preparation” section).

Carryover effect

Carryover was investigated by injecting 1 μL of blank sample in triplicate immediately after the highest calibration standard and the response was observed at the retention time of the investigated drugs. It should not be greater

than 20% of the LLOQ response. The carryover effect was calculated using the mixed data, obtained from two concentration levels (both low and high calibrators), each in triplicate (i.e., $n = 6$).

Results and discussion

There are different types of fluids secreted by the body that are also present within the body at any given time. These fluids may be useful in helping forensic scientists to detect drugs involved in crimes such as psychotropic agents including the neuropsychotic drugs. In addition, abuse of neuropsychotic drug(s) is also common due to wide clinical application and easy availability. In other words, these drugs are regarded as mental depressants that can be related to abuses, crimes, and suicides, because they depress the central nervous system and their overdosing may be potentially life-threatening. Then, in this work earwax was introduced as a potential alternative for other classically used biological fluids for detection of selected psychotropic agents employing LC–MS/MS as the applied analytical technique.

Regarding the chromatography, as mentioned before, all the tested columns were not able to resolve the analytes sufficiently, and the best column that presented short analysis time, relatively low analytical pressure, and a good resolution not only between the investigated drugs, but also from the encountered matrix interference was found to be Poroshell® 120 EC-C18 (2×50 mm, $1.7 \mu\text{m}$). It contains totally porous particles which are ideal for fast high resolution separations at low pressures.

As for the MS, two MRM transitions were optimized for each drug; one was used as the quantifier ion and the other as the qualifier ion with the exception of oxcarbazepine and valproic acid as mentioned earlier, for which just one MRM transition was available for each of the two analytes. Figure 1 shows MRM chromatograms of the investigated drugs and their retention times in blank earwax samples, spiked with reference standard drugs, while Fig. 2 represents MRM chromatograms of selected authentic patient samples in the study group (drug users), overall showing peaks of all the investigated drugs.

The qualifier ion was found important for confirmation of the peak identity of specifically one of the investigated drugs namely phenobarbital, for which an interfering peak with the same MRM transition as the quantifier ion of phenobarbital ($231.1 > 42.2$) and close to its retention time was detected in the chromatogram of the sample obtained from subject #15 (Fig. 2b); nevertheless, in addition to the retention time matching, the phenobarbital peak identity was further confirmed by phenobarbital qualifier ion peak ($231.1 > 188.1$) appearing at the same retention time.

Calibration curves were generated by plotting the peak area versus the spiked analyte concentrations. The validation results showed that the calibration standards were proportional to the nominal concentrations over the range of 5–500 ng/mg for all the investigated drugs with the exception of clonazepam and clobazam, which were found to be linear over the range of 5–500 pg/mg and 50–5000 pg/mg, respectively. The devised method was found to be linear over the dynamic ranges for all analytes with R^2 values >0.99 (Table 2).

The LLOQ was 5 pg/mg and 50 pg/mg for clonazepam and clobazam, respectively, and 5 ng/mg for the other analytes. These LLOQ were arbitrarily established to be the lowest concentrations on the corresponding calibration curves. As for LOD, it was 1.5 and 15 pg/mg for clonazepam and clobazam, respectively, and 1.5 ng/mg for the other analytes.

The results of the mixed data ($n = 6$ each) of MF, extraction recoveries, and carryover effects, obtained from both low and high concentration levels are shown in Table 2. The MF values were all found within the acceptable limit and the carryover effect was negligible ($<5\%$ of the LLOQ response) for all the investigated drugs as shown in Table 2.

Sample extraction was optimized by adjusting some variables in the extraction process such as extraction solvent, volume, and time in vortex. Methanol was selected as the best extraction solvent. Alternate solvents or combinations (methanol, acetonitrile, and/or water) were attempted and compared with the objective of choosing the best extraction mixture, but resulted in lower extraction recoveries or higher background peaks. The optimum extraction time and volume of extraction solvent were found to be 15 min and 1 mL, respectively. The extraction recoveries of the investigated drugs expressed as percent ratios are shown in Table 2. A second extraction under the same conditions was also performed on the exhausted earwax samples and produced less than 18% compared to the first one for all analytes.

Precision and accuracy of the present method were determined by analysing quality control samples at three different concentration levels (low, medium and high) for individual analytes. A summary of the accuracy (expressed as %) and precision (expressed as % relative standard deviation) data of the individual quality control samples for the investigated drugs is shown in Table 3.

The established developed method was successfully applied to detection and quantitation of the drugs in earwax samples of patients treated with single/multiple antiepileptic, anxiolytic, and antipsychotic drug combinations, confirming the secretion of these drugs into earwax of individuals using them, which matches previous reports in the literature about their presence in sweat [10, 11] and

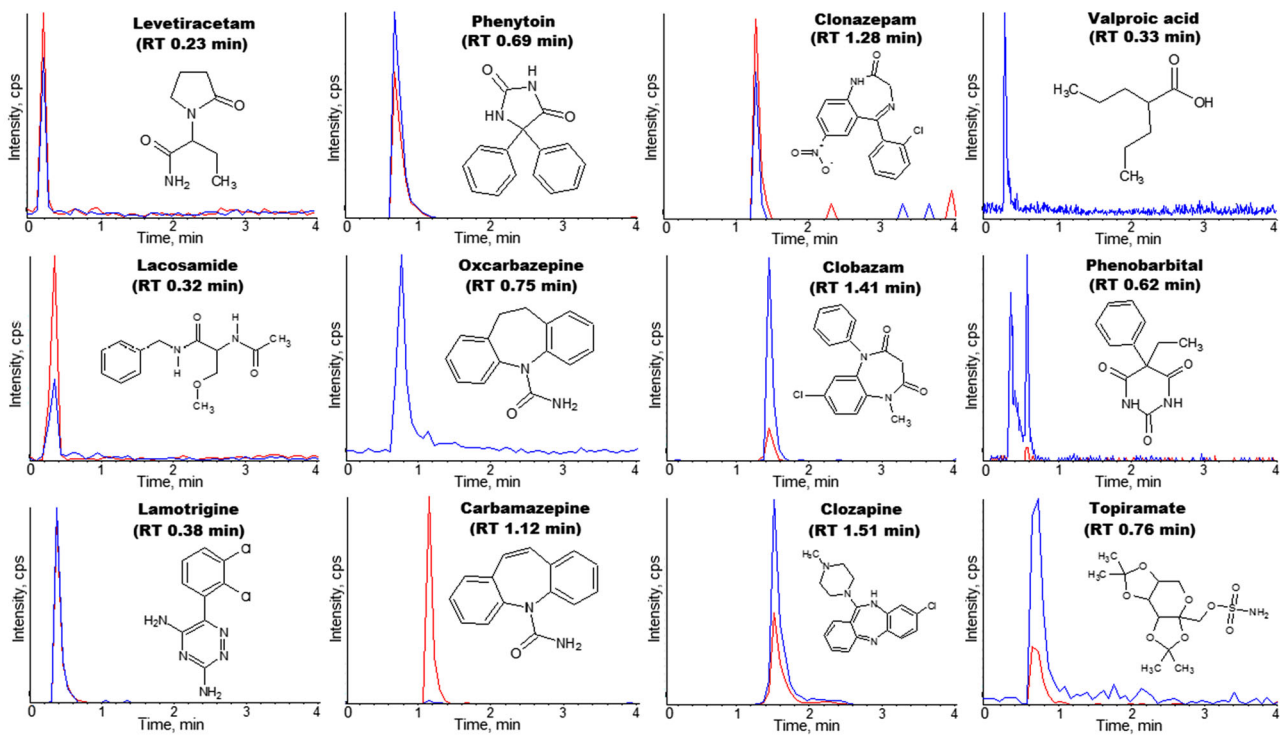


Fig. 1 Multiple reaction monitoring (MRM) chromatograms of an earwax sample spiked with the investigated drugs. The blue lines show quantifier ion tracing; the red lines show qualifier ion tracing; RT retention time

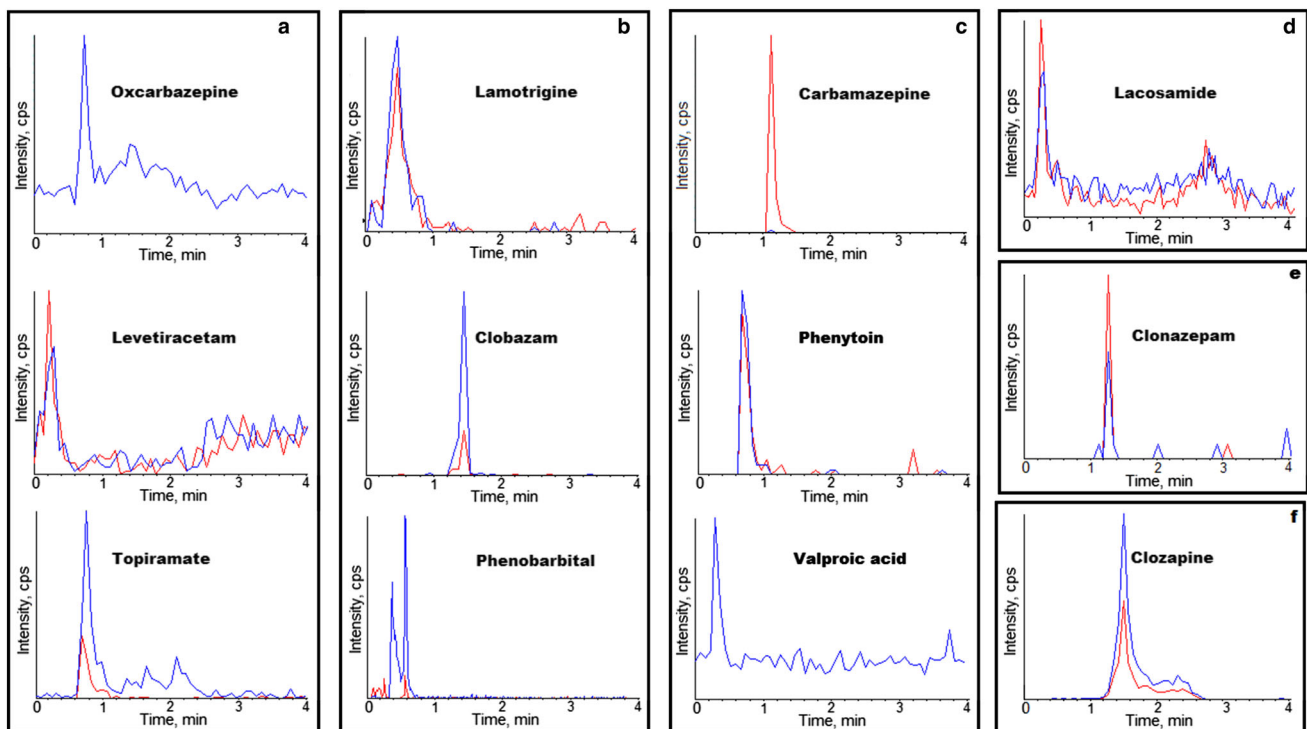


Fig. 2 MRM chromatograms of actual earwax samples of **a** subject #1 showing peaks of oxcarbazepine, levetiracetam, and topiramate; **b** subject #15 showing peaks of lamotrigine, clobazam, and phenobarbital; **c** subject #4 showing peaks of carbamazepine,

phenytoin, and valproic acid; **d** subject #5 showing peaks of lacosamide; **e** subject #16 showing peak of clonazepam; **f** subject #17 showing peak of clozapine

Table 2 Determination coefficients, matrix effects, extraction recoveries, and carryover effects

Drug	Determination coefficient (R^2)	Matrix factor ^a (mean \pm SD)	Extraction recovery ^a (mean \pm SD, %)	Carryover effect ^a (%)
Levetiracetam	0.9958	0.99 \pm 0.07	93.5 \pm 1.3	4.4
Lacosamide	0.9921	1.01 \pm 0.05	96.6 \pm 4.2	3.0
Lamotrigine	0.9987	1.00 \pm 0.07	85.2 \pm 1.3	2.2
Phenytoin	0.9967	1.10 \pm 0.15	90.0 \pm 4.4	3.2
Oxcarbazepine	0.9972	0.93 \pm 0.13	96.3 \pm 1.4	3.9
Carbamazepine	0.9948	0.99 \pm 0.05	94.2 \pm 2.3	0.8
Clonazepam	0.9925	1.01 \pm 0.14	86.3 \pm 4.4	3.1
Clobazam	0.9940	1.11 \pm 0.21	97.7 \pm 2.4	2.8
Clozapine	0.9984	0.96 \pm 0.14	92.0 \pm 11.1	2.4
Valproic acid	0.9945	1.12 \pm 0.18	82.6 \pm 0.82	0.8
Phenobarbital	0.9981	0.95 \pm 0.14	95.2 \pm 1.1	0.8
Topiramate	0.9966	1.03 \pm 0.12	99.5 \pm 0.64	1.1

SD standard deviation

^a Mixed data were obtained at both 5 and 500 pg/mg ($n = 3$ each, and 6 in total) for clonazepam, at both 50 and 5000 pg/mg ($n = 3$ each, and 6 in total) for clobazam, and at both 5 and 500 ng/mg ($n = 3$ each, and 6 in total) for the other ten drugs

hair [15–17]; however, earwax can be even more preferred than sweat and hair because of noninvasive sample collection, minimum sample pretreatment, and less external contamination. Our data strongly support the potential to detect these drugs in earwax specimens of drug abusers who use these drugs for recreational purposes [18–20] or of victims of crimes and of suicides by drug intoxication [21, 22], as well as of drug-facilitated crimes and sexual assaults [23, 24].

We measured the concentrations of the drugs encountered in earwax samples obtained from 17 patients (10 men and seven women), 18–46 years of age, and the quantitation results of the drugs versus the administered daily doses are shown in Table 4. Graphical representation of the concentrations encountered for each of the studied analytes is displayed in Fig. 3. As shown in Fig. 3, levetiracetam at a concentration of 52.0 ng/mg was detected in an earwax sample collected from subject #1 receiving a daily dose of 500 mg. Lacosamide was administered to subjects #5 and #7 at a daily dose of 200 mg. In our results, only trace (below LLOQ) of this drug was detected in earwax sample obtained from subject #7, while 13.2 ng/mg of lacosamide was detected in the earwax sample obtained from subject #5. Lamotrigine was detected in measurable quantities of 8.5–115 ng/mg in samples from subjects # 3, 7, 9, 11, and 15 receiving doses in the range of 100–200 mg/day. Phenytoin was successfully detected in concentrations between 8.7 and 243 ng/mg in three samples from three subjects receiving an equal dose of 300 mg/day, one of which discontinued

the medication almost 2 months before the sample collection (subject #4). Oxcarbazepine was detected at concentrations 5.0–326 ng/mg for samples from subjects receiving doses of 600–1200 mg/day. Carbamazepine administered to eight subjects at a dosage range of 200–1200 mg/day was detected in all the samples at concentrations ranging from 13.2 to 260 ng/mg. Two subjects receiving clonazepam at doses of 1 and 2 mg/day showed concentrations of 5.6 and 8.4 pg/mg, respectively, in earwax samples of the subjects. Clobazam, valproic acid, and topiramate showed concentrations in the ranges of 187–4850 pg/mg, 9.8–176 ng/mg, and 8.0–35.3 ng/mg, respectively, in samples from subjects receiving doses in ranges of 10–20 mg/day, 500–1000 mg/day, and 100–200 mg/day, respectively. A small difference was found between the concentrations of phenobarbital detected in two subjects' samples receiving the same dose of 50 mg/day as shown in Fig. 3. Finally, clozapine detected in a single sample obtained from subject #17 receiving a dose of 300 mg/day, showed a concentration of 31.7 ng/mg. As seen, the variability in the concentrations of some of the drugs detected in earwax samples of subjects involved in the study was remarkable. Interindividual variability could partly explain the variability in earwax drug concentrations in subjects even receiving the same dose, which matches results reported for the same drugs in hair and sweat [16, 25, 26]. Studying the impact of interindividual variations including age, gender, health status, racial differences, etc., along with the time course of the investigated drugs in earwax is noteworthy, to be

Table 3 Intraday and interday accuracy and precision results of the assay

Drug	Nominal concentration (ng/mg)	Intraday (5 replicates)		Interday (triplicate for 5 days)	
		Accuracy (%)	Precision (% RSD)	Accuracy (%)	Precision (% RSD)
Levetiracetam	5	91.2	10.5	97.2	20.2
	50	90.6	15.9	101	9.25
	500	100	1.91	99.3	1.82
Lacosamide	5	97.4	6.57	104	12.3
	50	100	5.14	102	6.62
	500	94.4	4.41	95.3	0.87
Lamotrigine	5	100	5.38	102	8.64
	50	87.4	5.01	96.3	8.87
	500	97.6	2.36	99.0	2.80
Phenytoin	5	80.0	2.25	103	19.5
	50	97.2	4.47	106	11.3
	500	103	2.80	104	8.34
Oxcarbazepine	5	80.6	4.22	95.4	14.1
	50	92.7	4.53	107	12.4
	500	95.7	2.18	98.0	5.22
Carbamazepine	5	98.2	2.85	104	4.63
	50	103	4.60	110	8.86
	500	97.0	1.37	101	3.56
Clonazepam	5 (pg/mg)	85.7	7.93	97.2	10.3
	50	93.9	10.4	101	8.57
	500	108	15.3	99.5	1.27
Clobazam	50 (pg/mg)	80.3	1.82	101	18.8
	500	89.9	4.18	103	13.2
	5000	98.4	6.63	104	13.1
Clozapine	5	121	2.32	116	3.81
	50	87.0	10.3	102	13.1
	500	98.9	6.24	102	2.97
Valproic acid	5	118	5.77	106	9.21
	50	86.3	1.97	91.7	7.44
	500	105	2.59	101	4.40
Phenobarbital	5	111	5.03	110	8.01
	50	91.1	2.83	92.0	6.67
	500	90.0	3.02	102	10.4
Topiramate	5	101	11.7	101	1.18
	50	89.8	2.65	101	13.8
	500	106	6.02	96.2	9.64

RSD relative standard deviation

addressed in future studies by the authors. All the drugs investigated were successfully quantified in all samples with the exception of lacosamide which was found in 1 out of 2 samples (Fig. 3), meanwhile phenytoin was even detected and quantified in an earwax sample of a subject who stopped treatment with phenytoin almost 2 months before the sample collection (subject #4). This means that

earwax sampling is not only effective for detecting recent use, but also for past drug use (probably a few months back), unlike hair that can be used to monitor drug use for weeks to months back in time, but appears not to be suitable for shorter periods. The results obtained in this work are very promising regarding the potential of earwax for detecting the use of the drugs investigated.

Table 4 Patient information, together with their drug administration regimen versus the corresponding detected drug concentrations in earwax samples

Subject #	Age (years)	Sex	Drugs involved	Treatment dose (mg/day)	Drug concentration in earwax (ng/mg)
1	18	M	Oxcarbazepine	600	5.0
			Topiramate	200	35.3
			Levitracetam	500	52.0
2	25	F	Carbamazepine	200	13.2
			Clobazam	10	2770 (pg/mg)
			Clonazepam	2	8.4 (pg/mg)
3	40	M	Carbamazepine	400	103
			Lamotrigine	100	27.2
4	21	M	Clobazam	10	187 (pg/mg)
			Carbamazepine	1200	260
			Valproic acid	1000	176
			Phenytoin ^a	300	8.7
5	35	F	Lacosamide	200	13.2
6	20	F	Phenytoin	300	243
7	46	F	Lamotrigine	200	47.9
			Oxcarbazepine	1200	326
			Lacosamide	200	<LLOQ
8	30	F	Topiramate	100	8.0
			Oxcarbazepine	900	189
9	37	F	Carbamazepine	400	127
			Lamotrigine	100	8.5
10	19	M	Clobazam	20	3800 (pg/mg)
			Carbamazepine	200	48.8
11	20	M	Lamotrigine	200	115
			Clobazam	20	4850 (pg/mg)
			Carbamazepine	400	109
			Phenytoin	300	119
12	18	M	Topiramate	200	31.1
			Phenobarbital	50	5.6
			Valproic acid	500	13.6
13	19	M	Carbamazepine	400	136
14	23	M	Clobazam	20	4810 (pg/mg)
			Valproic acid	1000	122
15	25	M	Lamotrigine	100	14.7
			Clobazam	10	838 (pg/mg)
			Phenobarbital	50	6.3
16	20	M	Clonazepam	1	5.6 (pg/mg)
			Carbamazepine	400	100
17	45	F	Clozapine	300	31.7
			Valproic acid	1000	9.8

LLOQ lower limit of quantification

^a Drug administration discontinued almost 2 months before sample collection

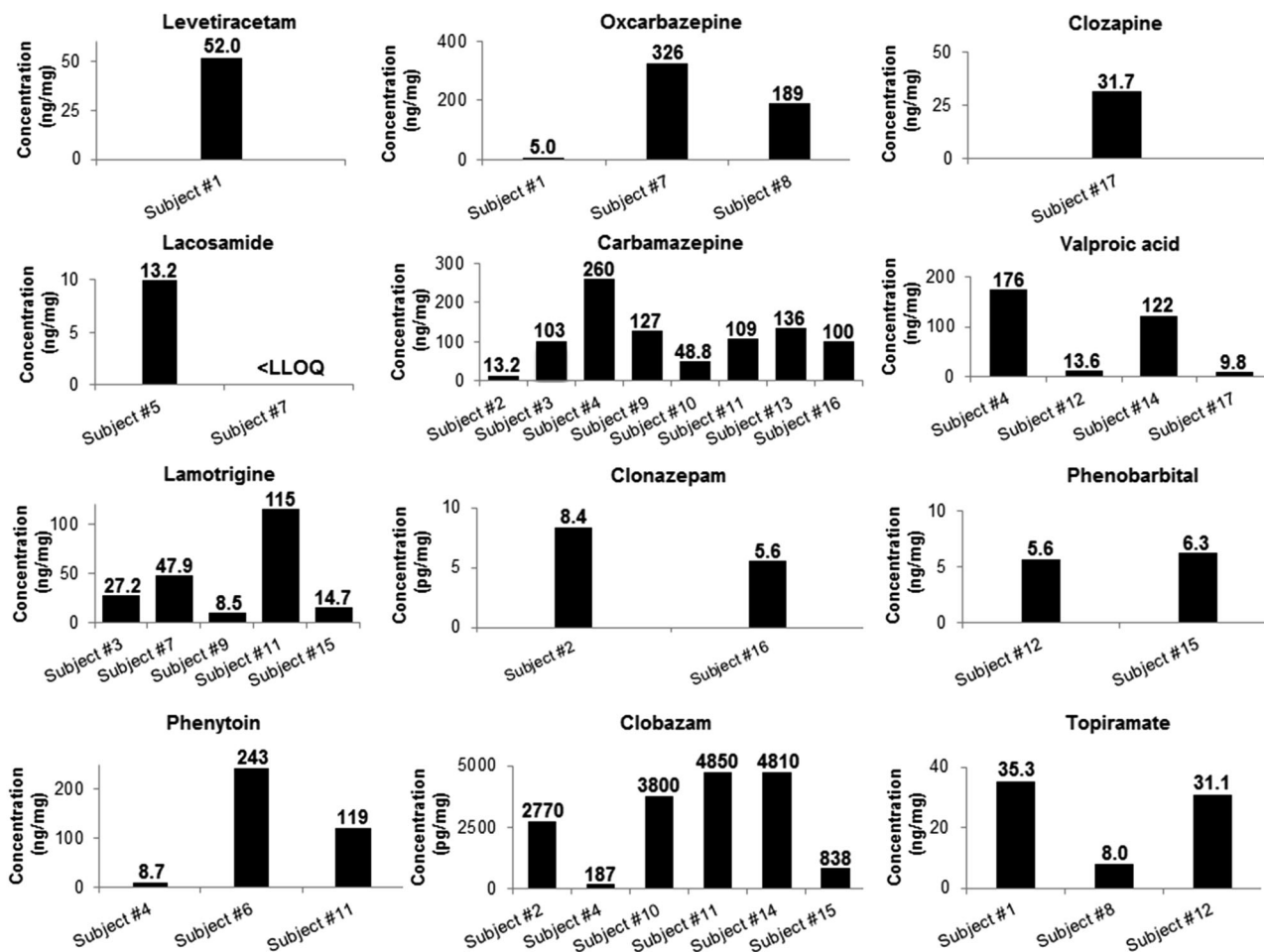


Fig. 3 Concentration profiles of each of the investigated neuropsychotic drugs in authentic earwax samples of 17 subjects enrolled in the study

Conclusions

Antiepileptics, hypnotics, anxiolytics, and antipsychotics are examples of drugs with psychotropic effects, which makes them potential candidates for abuse and drug-facilitated crimes, making it necessary to develop the methods to determine these drugs with high certainty. In this sense, an LC–MS/MS method was developed to detect the use of some of these neuropsychotic drugs using earwax samples of users as a new alternative specimen. The developed method represents an attractive combination of noninvasive sampling, simple sample preparation (15 min), short run time (4 min), and high sensitivity. It also overcomes the limitations faced in detection of drugs of abuse in other human specimens such as hair and sweat because of ease of sample collection, minimum sample pretreatment, and relatively less external contamination. Additionally, using earwax as a matrix for the detection of such drugs, it was possible to detect the analytes recently administered (within a week), as well as drugs administered some

months before. In conclusion, our approach presents earwax as a potential alternative forensic specimen for detecting use of drugs related to abuse, drug facilitated crimes, poisonings, homicides, and suicides. To our knowledge, this is the first study to propose the earwax as a forensic analysis specimen.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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