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Determination of antidepressants and benzodiazepines in paired hair and nail samples

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ABSTRACT

Hair and nails are keratinized matrices that can be used in Toxicology as matrices for the long-term detection of substances. Whereas hair is an established matrix with decades of use in this field, nails have been less studied, especially including a comparison to hair samples. Specifically in the case of antidepressant and benzodiazepine drugs, very few publications analyzing these drugs in nail samples exist as of yet. For this reason, in the present study a method for the detection of 12 antidepressant and benzodiazepine drugs in hair and nail samples was developed. Samples were decontaminated with 3 washes of dichloromethane, and 25 or 30 mg of hair and nails, respectively, were pulverized. Then, the samples were incubated with 1.5 mL water:ACN (50:50, v/v) with horizontal agitation for 90 min. The supernatant was evaporated and reconstituted in 200 µL of methanol and 2 mL of 2% FA in water, submitted to solid phase extraction (SPE) using Oasis MCX cartridges and analyzed by LC-MS/MS. The method was satisfactorily validated in nail and hair samples for the following parameters: linearity, LOD (0.005–0.02 ng/mg), LOQ (0.01–0.02 ng/mg), selectivity, carryover, accuracy, imprecision, matrix effect, extraction efficiency, process efficiency and autosampler stability. Matched fingernail, toenail and hair samples were obtained from 21 patients under treatment with any of the studied drugs and analyzed with the developed method. The most frequently detected drugs were venlafaxine (n = 11), trazodone (n = 6), zolpidem (n = 5), alprazolam (n = 5) and nordiazepam (n = 5). Concentrations in hair, fingernails and toenails, respectively, were 44.31 ng/mg, 8.05–43.35 ng/mg and 7.02–22.69 ng/mg for venlafaxine; 5.40–19.08 ng/mg, 0.13–1.00 ng/mg and 0.42–1.04 ng/mg for trazodone; 13.86 ng/mg, 5.19 ng/mg and 9.11 ng/mg for fluoxetine; 7.42 ng/mg, 1.85 ng/mg and 0.03–2.81 ng/mg for sertraline; 0.40–1.42 ng/mg, 0.12 ng/mg and 0.16 ng/mg for zolpidem; and 0.02–0.11 ng/mg, 0.07–1.07 ng/mg and 0.05 ng/mg for alprazolam for the patients under active treatment. Hair concentrations were higher than nail concentrations for most drugs in patients under active treatment, with the exception of diazepam (n = 1; 0.12 ng/mg in hair and 0.41 ng/mg in fingernails). Fingernail concentrations were lower than toenail concentrations in patients under active treatment in most compared cases. Comparison of fingernails and toenails of a patient with antifungal treatment did not show an observable effect in concentrations.

1. Introduction

Hair and nails are keratinized matrices known to incorporate substances present in the blood, and store them for a long time, allowing for a retrospective determination of drug consumption. Since the first instances of hair analysis to determine exposure to toxic metal ions [1], hair analysis has progressed with the emergence of new analytical techniques for the detection of various drugs of abuse and pharmaceuticals [2,3]. Nowadays, the usefulness of hair analysis is already well established in Toxicology in different contexts such as forensic cases (fatal acute poisoning, repeated deliberate poisoning, chronic drug consumption or drug facilitated crimes (DFCs)) [4,5], workplace drug testing [6], driving license regranting [7,8], doping controls [9], clinical toxicology (to prove chronic drug exposure in withdrawal treatments [10,11] or gestational drug exposure [12,13]), or environmental pollution [14]. However, hair presents some disadvantages, such as the effect of melanin in the incorporation of some drugs [3,15], the loss of incorporated drugs

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after aggressive cosmetic treatments [16], and in some cases (chemotherapy, burn victims…) the unavailability of a hair sample. Historically, nail analysis has been used to determine exposure to different toxic metal ions [17,18], and more recently, nail samples have been proposed as an alternative to hair analysis for drugs of abuse [19]. Since both are keratinized matrices, they share common characteristics, but differ in some aspects. One of them is the growth rate, which in nails is slower (3 mm/month in fingernails and 1.5 mm/month in toenails vs. 1 cm/month in hair), continuous instead of cyclical, and on two directions (80% of the nail grows in length from the nail matrix and 20% in thickness from the nail bed) [20]. This bidirectional growth also affects substance incorporation, since drugs can be incorporated not only at the proximal side, from the nail matrix, but also all along the nail growing upwards from the nail bed. Incorporation through external contamination also differs from hair, whereas in hair contamination through smoke is more important, in fingernails the manipulation of the drug presents an important source of contamination. Sweat contamination, while also existing in hair samples, is more important in nail samples, especially in toenails, due to the larger contact surface of nails [20]. Furthermore, nails lack a cuticle layer, making them more susceptible to external contamination and unwanted extraction of the analytes during washing, and under normal circumstances, nails do not contain melanin, eliminating the bias due to pigmentation [21].

Antidepressant and benzodiazepine drugs are psychoactive substances used in the treatment of various disorders, such as depression, anxiety, neuropathic pain, fibromyalgia or attention deficit hyperactivity disorder (ADHD) [22]. The analysis of these substances in hair and nails is of interest in Toxicology in the context of drug monitoring, to study adherence to chronic treatment [23], and in cases of DFCs or poisoning, to discriminate between chronic and sporadic intake [5]. Hair analysis of benzodiazepines was first described in 1992. Sramek et al. [24] published a radioinmunoanalysis (RIA) method for the detection of diazepam, lorazepam and alprazolam. The same year, Kintz et al. [25] published a GC-MS method for the detection of various drugs, including some antidepressants and benzodiazepines. With the years, many methods for hair analysis have been developed [5], some focused on antidepressants or benzodiazepines [26–30] and others including these drugs amongst others [31–34].

The detection of benzodiazepines in nails was not described until 2007, when Irving et al. [35] published a LC-MS/MS method for the detection of 9 sedative drugs and applied it to nail and hair samples from patients under treatment with these drugs and to one DFC case. In 2013, two papers were published investigating the concentrations of zolpidem in nails over time after a single dose in order to ascertain the mechanisms of drug incorporation into nails [36,37]. Since then, only other five methods for the analysis of nails including benzodiazepines have been published. Krumbiegel et al. developed an unknown screening method for drugs in nails and hair using LC-QTOF-MS [38], and a multianalyte method for screening nail and hair samples using UHPLC-MS/MS [39]. Shu et al. [40] described a method for the analysis of various drugs, including some benzodiazepines, and Moretti et al. [41] published a method for the determination of benzodiazepines and zolpidem in nails. Most recently, Mannocchi et al. [42] published a screening method for new psychoactive substances (NPS) and classic drugs using UHPLC-MS/MS. Only one method [39] included the determination of antidepressants in nails. Four of these methods were applied to both hair and nail samples [35,38,39,42], and only two of them analyzed and compared concentrations in paired samples from the same individuals [35,39].

In the present work, an analytical method was developed for the detection of antidepressant and benzodiazepine drugs in hair and nails, and paired samples from patients under treatment with these drugs were analyzed and compared to determine the suitability of nails as an alternative to hair samples.

2. Material and methods

2.1. Chemicals and reagents

HPLC grade dichloromethane (DCM) and 2-propanol, LC-MS grade acetonitrile (ACN) and methanol (MeOH), reagent grade formic acid (FA) 98–100% and solid ammonium formate for LC-MS were purchased from Scharlau (Sentmenat, Spain). Ammonium hydroxide (NH_4OH) 32% was obtained from VWR (Radnor, Pennsylvania, USA). Water was purified with a Milli-Q water system (Millipore, Le-Mont-sur-Lausanne, Switzerland). Oasis MCX cartridges were obtained from Waters Corp. (Milford, MA, USA).

Reference standards for citalopram, fluoxetine, paroxetine, sertraline, trazodone, venlafaxine, alprazolam, diazepam, lorazepam, nordiazepam, oxazepam and zolpidem at 1 mg/mL in methanol, and citalopram-d_6, fluoxetine-d_6, paroxetine-d_6, sertraline-d_3, venlafaxine-d_3, alprazolam-d_5, diazepam-d_5, lorazepam-d_4, nordiazepam-d_5, oxazepam-d_5 and zolpidem-d_6 at 0.1 mg/mL in methanol were purchased from Cerilliant (Round Rock, TX, USA).

2.2. Nail and hair samples

Blank nail and hair samples used for the preparation of calibration curves and quality control (QC) samples were donated by the laboratory staff. All the hair and nails samples were obtained from Caucasian individuals. Hair samples were black or brown, except for the determination of the matrix effect and external interferences, where different hair colors were tested. Authentic specimens from individuals under treatment with the studied drugs were collected at the University Hospital of Santiago de Compostela from July of 2018 to June of 2020.

Nail samples were obtained by cutting the overhang of the nails using a nail clipper. Nails from all fingers were collected, and fingernails and toenails from the same person were stored in separate plastic bags. Hair samples were obtained from the posterior vertex of the head, cutting a hair lock as close as possible to the scalp. The proximal part was marked with a string, and samples were stored in paper envelopes. Data about demographic characteristics and current treatment of each participant was collected. This study was approved by the Galician Clinical Research Ethics Committee (Xunta de Galicia, Spain) (Registration code: 2018/336), and all participants signed an informed consent for their participation.

2.3. Preparation of calibration and QC working solutions

Different solutions were prepared for calibrators and for QC samples. A working solution at 10 µg/mL containing all the analytes was prepared by dilution of the commercial solutions with methanol. This solution was further diluted with methanol to obtain working solutions at 5, 1, 0.5, 0.1, 0.05 and 0.01 µg/mL. For QC samples, one solution was prepared containing diazepam and lorazepam at 10 µg/mL, and diluted to 0.75 µg/mL and 0.075 µg/mL. Another solution was prepared containing the rest of the analytes at 10 µg/mL, and diluted to 7.5 µg/mL and 0.75 µg/mL. A third solution containing all the analytes at 1 µg/mL was prepared from both of the previous solutions at 10 µg/mL, and diluted to obtain a solution at 0.03 µg/mL. An internal standard (IStd) solution at 1 µg/mL containing all the deuterated compounds was prepared in methanol.

Calibrators at 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5 and 10 ng/mg were prepared by addition of 30 or 60 µL of the appropriate working solution to 30 mg of blank nail powder, or 25 or 50 µL to 25 mg of blank hair powder. QC samples at low (0.03 ng/mg), medium (0.075 or 0.75 ng/mg) and high (0.75 or 7.50 ng/mg) concentrations were prepared by addition of 30 or 25 µL of the QC working solutions to 30 or 25 mg of blank nail or hair powder, respectively.

2.4. Decontamination, incubation and extraction

The protocol for sample pre-treatment was the same than that previously published for the determination of antipsychotic drugs in hair and nails [43]. Briefly, nail clippings and hair samples were decontaminated with 3 consecutive washes with 2 mL DCM, vortex mixing for 2 min. The last wash solvent was kept and analyzed to determine external contamination. Decontaminated samples were dried in an oven, 30 mg of nail or 25 mg of hair were weighed and pulverized with a ball mill (Precellys 24, Montigny le Bretonneux, France) by two cycles of 3 × 60 s at 6500 rpm for nails and one cycle for hair. Twenty-five microliters of the IStd mixtures were added and the powder was incubated with 1.5 mL water:ACN (50:50, v/v) with horizontal agitation for 90 min. After incubation, the sample was centrifuged, and the supernatant evaporated. The sample was reconstituted in 200 µL of methanol and 2 mL of 2% FA in water, and submitted to solid phase extraction (SPE) using Oasis MCX cartridges. Two washing steps with 2 mL of 2% FA in water and 2 mL of water:methanol (50:50, v/v) were applied, and elution was performed by addition of 3 mL of DCM:2-propanol:NH_4OH (75:24.5:0.5, v/v/v). The eluate was evaporated and reconstituted in 100 µL of ammonium formate with 0.1% FA:ACN (70:30, v/v). After centrifugation at 14,500 rpm for 10 min, 20 µL of the supernatant were injected into the LC-MS/MS.

2.5. LC-MS/MS

The HPLC system was an Alliance 2795 Separation Module with an Alliance series column heater/cooler coupled to a Quattro Micro™ API triple quadrupole (Waters Corp.). Chromatographic separation was performed using an XBridge (2.1 mm × 100 mm, 3 µm) analytical column (Phenomenex, Torrance, CA, USA), with an XBridge BEH Shield RP18 (2.1 mm × 5 mm, 3.5 µm) guard column, at 30 °C. Ammonium formate with 0.1% FA (A) and ACN (B) were used as mobile phase at a flow rate of 0.3 mL/min using the following gradient: 30% B until 0.5 min, increasing to 33% B at 5 min, to 35% B at 6.5 min, to 50% B at 9 min, reaching 80% B at 9.5 min, and returning to initial conditions at 10 min. Total chromatographic run was 14 min. A divert valve was set to direct the flow to the MS from 0.5 to 13 min and the remaining time to waste.

Optimal cone voltage, precursor-to-product ion transitions and collision energies were selected by performing a direct infusion of each individual analyte into the MS connected with a "T" valco to the LC effluent. Two MRM (Multiple Reaction Monitoring) transitions were selected per analyte, and one per IStd (Table 1). The MS was operated in electrospray in positive mode (ESI+) using the following conditions: capillary voltage 0.5 kV; source block temperature 150 °C; desolvation gas (nitrogen) temperature 400 °C; desolvation and cone gas (nitrogen) flow rate, 800 and 60 L/h, respectively. Argon was employed to promote analyte fragmentation in the collision cell.

Data acquisition was controlled with Masslynx 4.1 software and processed with Quanlynx 4.1 software (Waters Corp.).

2.6. Method validation

Validation of the method was performed separately in nail and hair samples according to the recommendations of the Scientific Working Group for Forensic Toxicology (SWGTOX) [44]. Validated parameters and their acceptance criteria are detailed in Table 2.

2.7. Statistical analysis

Statistical analysis was performed using SPSS software (24.0 version, SPSS Inc., Chicago, IL, USA). Normality of the data was tested using the Shapiro-Wilk test. Data about demographic characteristics is presented as mean ± standard deviation (SD). Since concentrations in most matrices did not follow a normal distribution, correlations between concentrations in the different matrices were assessed using Spearman correlation. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

An analytical method for the detection of 12 antidepressant and benzodiazepine drugs in nail and hair samples was developed. Chromatographic elution of all the analytes was achieved in 11 min, with a total chromatographic run of 14 min. For MS detection, the most abundant MRM transition was used for quantification, and a second transition was monitored for qualification purposes.

The decontamination method was the same for hair and nails because for these pharmaceutical drugs the external contamination due to smoke and drug manipulation is not important. For drugs of abuse, such as cannabis, these contamination pathways are more important, and a more extensive washing protocol is necessary to remove contamination in fingernails [46]. Segmentation was performed for hair when possible but not in nails because of the difficulty to segment nail clippings. Other authors performed segmentation in whole nails [39] or in nail clippings [47], but the interpretation is still unclear.

The method was fully validated in both matrices, and the acceptance criteria were fulfilled for all parameters. Linearity was verified by least square regression using $1/x$ or $1/x^2$ weighing factor in both matrices, in the ranges from the LOQ to 1 ng/mg for diazepam and lorazepam, and from the LOQ to 10 ng/mg for the rest of the analytes. The calibration model was linear for all compounds, except oxazepam for which a quadratic model was used. LOD in nails was 0.005 ng/mg for all the analytes except lorazepam, for which LOD was 0.01 ng/mg, and in hair LOD was 0.02 ng/mg for lorazepam, 0.01 ng/mg for venlafaxine, fluoxetine and oxazepam, and 0.005 ng/mg for the rest of the analytes. LOQ in nails was 0.01 ng/mg for all analytes, and in hair LOQ was 0.02 ng/mg for oxazepam, lorazepam and diazepam, and 0.01 ng/mg for the rest. At the LOQ, accuracy was 91.2–103.1% and 87.7–111.0% of the target concentration in nails and hair, respectively. Moreover, %CV was 4.8–11.5% and 1.3–9.5% in nails and hair, respectively.

Carryover was not detected for any of the analytes in nail or hair samples since the calculated concentration in blank samples (n = 3) analyzed after the injection of the upper limit of quantification point was < LOD. Despite this, two mobile phases were injected between each real sample.

No quantifiable peaks were detected in 10 different nail and hair samples, or in the blank samples fortified with common drugs of abuse and medicines at the retention time of each analyte, confirming the selectivity of the method.

Results for accuracy, intra-assay, inter-assay and total imprecision are summarized in Supplementary Table S1. Accuracy was satisfied for all the analytes, with calculated concentrations within 93.8–107.6% of the target concentration in nails, and within 96.6–106.5% in hair. Intra-assay, inter-assay and total imprecision were ≤ 9.4%, ≤ 6.9% and ≤ 11% respectively, in nail samples; and ≤ 7.6%, ≤ 4.7% and ≤ 7.6% in hair samples.

Extraction efficiency, matrix effect and process efficiency results are indicated in Table 3. Matrix effect ranged from −35.0 to 50.9% in nails and from −58.7 to 72.4% in hair, with their respective IStd showing similar effects. Matrix effect was more pronounced in hair than in nails but it could be compensated using deuterated analogues for each compound, since similar matrix effect values were detected for each compound and its corresponding IStd. Extraction efficiency was satisfactory, ranging from 60.4% to 125.3% in nail samples, and from 59.7% to 122.0% in hair samples. Process efficiency

Table 1
MRM transitions, cone voltage (CV), collision energy (Ce), retention time (Rt) and internal standard (IStd) selected for each analyte. The underlined transitions were used for quantification.

Analyte	MRM transition	CV (v)	Ce (eV)	Rt	IStd
Zolpidem	308.3- > 235.4	50	34	1.3	Zolpidem-d_6
	308.3- > 263.2	50	26		
Zolpidem-d_6	314.3- > 235.1	50	36	1.3	
Venlafaxine	278.5- > 57.4	25	17	1.4	Venlafaxine-d_3
	278.5- > 260.5	25	13		
Venlafaxine-d_3	352.9- > 316.7	25	15	1.4	
Trazodone	372.2- > 176.3	35	24	1.4	Venlafaxine-d_3
	372.2- > 148.2	35	34		
Citalopram	325.5- > 108.8	40	23	2.1	Citalopram-d_6
	325.5- > 262.4	40	19		
Citalopram-d_6	331.4- > 108.8	35	25	2.0	
Paroxetine	330.2- > 69.5	45	26	2.9	Paroxetine-d_6
	330.2- > 192.2	45	22		
Paroxetine-d_6	336.2- > 75.5	45	28	2.9	
Fluoxetine	310.2- > 43.8	25	12	4.5	Fluoxetine-d_6
	310.2- > 148.1	25	8		
Fluoxetine-d_6	316.2- > 43.5	25	10	4.4	
Sertraline	306.1- > 159.1	25	28	4.9	Sertraline-d_3
	306.1- > 275.1	25	12		
Sertraline-d_3	309.3- > 159.0	20	25	4.9	
Oxazepam	287.3- > 241.3	35	21	4.9	Oxazepam-d_5
	287.3- > 103.8	35	15		
Oxazepam-d_5	292.1- > 246.0	35	22	4.8	
Alprazolam	309.3- > 281.3	50	25	5.0	Alprazolam-d_5
	309.3- > 205.2	50	39		
Alprazolam-d_5	314.1- > 286.0	45	26	4.9	
Lorazepam	321.4- > 275.3	35	25	5.6	Lorazepam-d_4
	321.4- > 303.3	35	13		
Lorazepam-d_4	325.1- > 278.9	30	26	5.6	
Nordiazepam	271.1- > 139.8	45	26	6.2	Nordiazepam-d_5
	271.1- > 164.9	45	28		
Nordiazepam-d_5	276.1- > 140.0	50	28	6.0	
Diazepam	284.9- > 154.2	45	27	9.1	Diazepam-d_5
	284.9- > 193.2	45	33		
Diazepam-d_5	290.1- > 153.9	45	24	8.9	

ranged from 59.9% to 160.8% in nail samples, and from 38.7% to 159.2% in hair samples.

Finally, in hair samples all analytes showed to be stable after 72 h in the autosampler with a loss < 7.3%. However, in nail samples some analytes were not stable after 72 h (trazodone, nordiazepam, oxazepam and alprazolam), so stability was re-evaluated after 24 h in the autosampler, being all analytes stable with a loss < 7% (Supplementary Table S2).

3.1. Application to real samples

Paired fingernail, toenail and hair specimens were obtained simultaneously from 12 patients. Other 9 patients provided only one or two samples. In total, 16 fingernail samples, 17 toenail samples and 16 hair samples were collected. For hair samples the proximal 2 cm segment was analyzed, and in cases where the length was sufficient (n = 8), the next 2 cm were also analyzed (distal segment). One participant had started treatment with zolpidem a month and a half before collection of the samples (fingernails, toenails and hair), and donated other 4 fingernail and 3 toenail samples over the next five months.

Of the 21 patients, 13 were female, ages ranged from 25 to 83 years old (mean ± SD= 54.7 ± 17.6), and weight between 44 and 112 kg (mean ± SD= 75.2 ± 18.8). Cosmetic treatment (hair dye) was only reported for three participants (cases 4: in distal segment, 13, 14), and presence of onychomycosis in nails was reported in other two cases (17: in toenails, 18: in fingernails and toenails). Only patient 17 was using an antifungal treatment. Poly-medication was not common, as only 3 patients were under simultaneous treatment with an antidepressant and a benzodiazepine and 2 patients were under treatment with three of the target drugs.

The most frequently detected drugs were venlafaxine (n = 11), trazodone (n = 6), zolpidem (n = 5), alprazolam (n = 5) and nordiazepam (n = 5). For some of the analytes only one sample was positive. Detailed results for each drug, including current dosage and concentrations detected in each matrix are presented in Table 4. No external contamination was detected for any sample.

3.2. Venlafaxine

Twelve cases were positive for venlafaxine, although only three of the patients were under treatment with this drug at the time of sampling (cases 1, 19 and 21). In those cases that were not under treatment, concentrations varied in a wide range: in some cases (6, 14, 17, 18) low concentrations (0.01–0.05 ng/mg) were detected in some matrices while others were negative; but in other cases (4, 5, 9, 15, 20) concentrations were much higher (in the range of 0.42–43.58 ng/mg). In addition, concentrations were in some cases higher in toenails (cases 5 and 20, concentrations were 5 and 3 times higher, respectively), while in other cases hair concentrations were higher (cases 4 and 9; 7 and 2.5 times higher, respectively).

In the cases under treatment (1, 19 and 21) concentrations were higher than those described above (with the exception of case 9, which had very high concentrations despite not being listed as under treatment, possibly due to a recent change in the patient's medication, unknown to the authors). Concentrations in these cases were in the range of 7.02–44.31 ng/mg in nails, with similar values between fingernails and toenails. Moreover, in case 19, the only one with a hair sample, concentration in hair was 2 times higher than in nails.

Table 2
Summary of the validated parameters and acceptance criteria.

Parameter	Procedure	Acceptance criteria
Linearity	5 calibration curves in 5 different days	$r^2 \geq 0.99$ Calibrators residuals ± 20%
LOD	Analysis of blank nail/hair samples at decreasing concentrations in 2 different days (n = 6)	Two MRM transitions with a signal to noise ratio > 3 and adequate ion ratio[45]
LOQ	Analysis of blank samples at the lowest calibrator concentration in 2 different days (n = 6)	Two MRM transitions with a signal to noise ratio > 10, 80%–120% of target concentration and %CV < 20%
Carryover	Analysis of a blank matrix sample immediately after the upper limit of quantification point (n = 3)	Calculated concentration < LOD
Selectivity	Endogenous interferences: 10 blank samples from different sources spiked with IStd Exogenous interferences: Blank samples fortified with 36 common drugs of abuse and medicines at 25 ng/mg.[a]	No interferences detected
Accuracy	Analysis of 3 replicates at low, medium and high QC concentrations in 5 different days (n = 15)	80%–120% of target concentration
Intra-assay, inter-assay and total imprecision	Analysis of 3 replicates at low, medium and high QC concentrations in 5 different days (n = 15)	%CV < 20%
Matrix effect	At low and high QC concentrations, comparing mean peak areas in blank samples (n = 10) fortified after extraction with mean peak areas of the analytes fortified in the mobile phase (n = 10)	–
Extraction efficiency	At low and high QC concentrations, comparing mean peak areas in blank samples fortified before extraction (n = 10) with blank samples fortified after extraction (n = 10)	–
Process efficiency	At low and high QC concentrations, comparing mean peak areas in blank samples fortified before extraction (n = 10) with mean peak areas of the analytes fortified in the mobile phase (n = 10)	–
Autosampler stability	At low, medium and high QC concentrations (n = 3 each), comparing freshly prepared QC samples and after 24/72 h in the autosampler at 6 °C	Re-injected samples quantified within ± 20% of freshly prepared samples

LOD: limit of detection; LOQ: limit of quantification;
[a] Exogenous interferences: Codeine, methadone, morphine, 6-acetylmorphine, fentanyl, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpirrolidine, amphetamine, methamphetamine, methylendioxyanfetamine, methylendioximethamphetamine, methylendioxyethylamphetamine, cocaine, cocaethylene, benzoylecgonine, ecgonine methyl ester, lysergic acid, ketamine, norketamine, gamma hydroxybutyrate, nicotine and cotinine, amitriptyline, temazepam, lormetazepam, flunitrazepam, 7-aminoflunitrazepam, clonazepam, triazolam, nitrazepam, bromazepam, clozapine, haloperidol, levomepromazine, olanzapine, quetiapine, ibuprofen, acetaminophen, diclofenac, naproxen, zopiclone and omeprazole.

Spearman correlations between concentrations of venlafaxine in the different matrices were statistically significant for fingernails vs. toenails ($\rho = 0.847$), fingernails vs. proximal hair ($\rho = 0.868$), toenails vs. proximal hair ($\rho = 0.898$), toenails vs. distal hair ($\rho = 0.941$) and proximal vs. distal hair ($\rho = 0.893$).

3.3. Trazodone

Six cases were positive for trazodone. In two cases (4 and 7), patients were not under treatment with trazodone at the moment of collection. Case 4 was only positive in toenails (0.02 ng/mg), while case 7 was positive in all samples at high concentrations (0.13 ng/mg, 1.61 ng/mg and 8.57 ng/mg in fingernails, toenails and proximal hair, respectively). The four cases under treatment were positive in all the matrices provided; in case 15 only toenails were available (0.56 ng/mg) and in the remaining three (8, 14, 19) concentrations in hair were 8–45 times higher than in toenails and 16–56 times higher than in fingernails.

Spearman correlations between concentrations of trazodone in the different matrices were not statistically significant.

3.4. Sertraline

For this drug (cases 5, 6, 17, 18) hair concentrations were higher than nail concentrations, and concentrations in toenails higher than those in fingernails. Cases 5 and 6 were not under active treatment but were positive in all analyzed matrices. Patients 17 and 18 had sertraline prescribed at the moment of sample collection, but toenails from patient 18 showed a lower concentration (0.03 ng/mg) than those of cases 5 and 6.

3.5. Zolpidem

This drug was detected in five cases. Those cases that were not under treatment with the drug (2, 4 and 6) tested negative in nails and positive in hair, but with concentrations very close to the LOQ (case 4 had higher concentrations in the distal segment, consistent with a past treatment).

Two cases were under active treatment (13 and 17). Case 17 was positive in all matrices, with hair concentrations 10 times higher than nail concentrations, and similar figures between fingernails and toenails (0.12 and 0.16 ng/mg, respectively) and between proximal and distal hair segments (1.42 and 1.46 ng/mg in, respectively). Patient 13, under chronic treatment with 5 mg/day of zolpidem, provided the first sample set (fingernails, toenails and hair) seven weeks after starting treatment. In nail samples the observed chromatographic peaks did not meet the LOD criteria for a positive result; however, the hair sample was positive despite having been dyed before sample collection. Specifically, hair concentrations were almost 0.40 ng/mg in the proximal segment (2 cm corresponding to the previous 2–3 months, showing the incorporation of the drug since the start of the treatment) and 0.02 ng/mg in the distal segment (it could be attributable to incorporation of the drug through sweat or sebum during these months and the effect of the dye in the hair integrity). Four fingernail samples and three toenail samples were collected on the following five months; in fingernails only sample 3 was positive (above LOD), and in toenails concentrations were close to the LOQ and increased from > LOD in sample 2–0.02 ng/mg in sample 5, after almost six months of treatment (Table 5).

Two authors studied the incorporation of zolpidem in nail samples after the consumption of a single dose. Hang et al. [36] found concentrations in the range of 0.06–1.74 pg/mg in fingernails and

Table 3
Matrix effect, extraction recovery and process efficiency at low (0.03 ng/mg) and high (0.75 or 7.50 ng/mg) QC concentrations in nail and hair samples.

Analyte	QC	Nail samples			Hair samples		
		Extraction efficiency (%) (n = 10)	Matrix effect [% (%CV)] (n = 10)	Process efficiency (%) (n = 10)	Extraction efficiency (%) (n = 10)	Matrix effect [% (%CV)] (n = 10)	Process efficiency (%) (n = 10)
Venlafaxine	LOW	90.7	-10.7 (6.3)	81.0	95.3	-45.4 (24.9)	52.1
	HIGH	100.3	7.7 (4.5)	107.9	72.9	-22.0 (6.6)	56.9
Venlafaxine-d_3	LOW	88.4	-17.5 (7.2)	72.9	105.0	-39.2 (9.5)	63.8
	HIGH	99.4	2.3 (5.5)	101.7	74.2	-22.0 (6.9)	57.9
Trazodone	LOW	92.1	-35.0 (5.4)	59.9	93.7	-58.7 (12.2)	38.7
	HIGH	104.7	-14.0 (4.4)	90.0	72.9	-44.0 (11.7)	40.9
Citalopram	LOW	82.9	-2.5 (4.6)	80.8	87.1	-42.6 (16.5)	50.1
	HIGH	88.3	-4.7 (3.0)	84.1	72.8	-29.4 (7.4)	51.4
Citalopram-d_6	LOW	80.4	-3.4 (3.9)	77.7	91.7	-35.6 (15.3)	59.0
	HIGH	88.7	-7.5 (4.1)	82.1	73.8	-30.4 (7.8)	51.4
Paroxetine	LOW	60.4	21.2 (12.6)	73.2	93.9	-29.9 (31.5)	65.6
	HIGH	67.5	-3.3 (9.6)	69.8	59.7	-21.3 (10.4)	47.0
Paroxetine-d_6	LOW	54.0	13.2 (8.2)	61.1	77.6	-20.2 (22.5)	61.9
	HIGH	62.4	-2.9 (10.1)	60.6	59.7	-24.0 (10.5)	41.7
Fluoxetine	LOW	71.3	13.3 (11.5)	80.8	100.6	-25.6 (32.1)	74.9
	HIGH	85.2	6.3 (5.5)	90.5	65.7	-2.9 (12.6)	63.8
Fluoxetine-d_6	LOW	69.1	10.4 (9.0)	76.2	91.7	-13.7 (26.4)	79.1
	HIGH	79.1	0.9 (6.0)	79.9	65.2	-6.4 (11.5)	61.0
Sertraline	LOW	75.9	-14.9 (17.5)	64.6	93.5	-43.2 (43.2)	53.1
	HIGH	76.7	-14.5 (7.2)	65.6	65.3	-31.1 (21.7)	45.0
Sertraline-d_3	LOW	70.4	-22.5 (17.2)	54.6	71.0	-49.2 (32.5)	36.1
	HIGH	76.0	-21.2 (9.6)	59.9	64.8	-39.9 (18.6)	38.9
Zolpidem	LOW	88.6	-21.2 (4.4)	69.8	100.5	-41.5 (6.2)	58.8
	HIGH	92.3	-12.4 (5.0)	80.8	71.9	-27.4 (3.1)	52.2
Zolpidem-d_6	LOW	90.3	-26.5 (11.5)	65.4	101.5	-42.1 (9.5)	58.8
	HIGH	94.9	-17.8 (11.4)	78.0	80.0	-21.3 (5.0)	40.0
Oxazepam	LOW	78.2	27.2 (7.9)	99.5	101.1	55.8 (19.9)	159.2
	HIGH	83.3	15.0 (4.2)	95.8	67.7	19.2 (5.4)	80.7
Oxazepam-d_5	LOW	80.5	21.7 (13.1)	98.0	95.8	60.5 (6.8)	153.8
	HIGH	82.2	11.5 (11.3)	91.7	66.7	16.7 (5.7)	77.8
Alprazolam	LOW	71.8	16.7 (7.5)	83.9	82.9	-1.5 (8.9)	81.7
	HIGH	78.5	6.7 (2.8)	83.7	64.6	-3.5 (3.2)	62.3
Alprazolam-d_5	LOW	76.5	11.1 (13.9)	85.0	91.7	2.7 (9.0)	94.2
	HIGH	77.8	4.5 (13.0)	81.4	64.4	-3.7 (4.0)	62.1
Lorazepam	LOW	74.9	46.0 (12.2)	109.3	91.8	72.4 (23.3)	158.2
	HIGH	77.9	50.9 (14.7)	117.5	61.7	53.0 (6.5)	94.4
Lorazepam-d_4	LOW	70.0	50.0 (14.5)	105.1	87.5	49.4 (5.7)	130.7
	HIGH	73.3	49.4 (17.1)	109.5	60.3	51.7 (6.4)	91.5
Nordiazepam	LOW	84.0	27.0 (9.0)	106.8	94.9	-11.5 (23.5)	84.0
	HIGH	81.8	18.8 (5.8)	97.2	71.1	-10.9 (14.5)	63.4
Nordiazepam-d_5	LOW	78.5	12.6 (3.4)	88.4	93.7	-9.4 (25.5)	84.8
	HIGH	81.4	9.8 (5.0)	89.4	69.9	-22.4 (32.2)	54.3
Diazepam	LOW	125.3	28.3 (17.0)	160.8	122.0	-23.1 (12.6)	93.8
	HIGH	85.5	12.6 (3.1)	96.2	72.3	-34.0 (12.5)	47.7
Diazepam-d_5	LOW	83.8	3.2 (5.0)	86.5	108.0	-23.3 (12.1)	82.9
	HIGH	86.1	5.0 (4.4)	90.4	71.5	-27.4 (9.3)	51.9

0.05–3.29 pg/mg in toenails. Madry et al. [37] found concentrations of 0.15–15.1 pg/mg in nails and 1.1–10.8 pg/mg in hair. Both observed a peak of concentrations at 10–18 weeks, when incorporation through the nail matrix reaches the free edge. Taking into account that patient 13 was taking a daily dose of zolpidem, after 10 weeks of treatment, concentrations should be high enough to be detected in nails, but only toenails were positive after 16 weeks, and at concentrations close to those reported for a single dose. Moreover, both authors observed incorporation into nails via sweat contamination one day (0.8–15.1 pg/mg) [37] or a week (0.05–1.74 pg/mg) [36] after the consumption of the drug, but in both cases the LOQ was lower than ours (0.05 pg/mg [36] and 0.1 pg/mg [37] vs. 10–20 pg/mg in this method); therefore even if sweat incorporation was present after the first days, the concentrations incorporated were too low to be detected with our method.

3.6. Alprazolam

Five cases were positive for alprazolam, all under active treatment with the drug. When all the samples were available (cases 7, 12 and 19), concentrations in hair were higher than in nails. Moreover, case 15 was positive in toenails (0.05 ng/mg) but negative in fingernails, and case 16 was positive in fingernails (1.07 ng/mg), the only matrix available.

3.7. Nordiazepam

Five cases were positive for nordiazepam, but none of the patients were under active treatment with this drug or any of its parent compounds except for case 3, which had diazepam prescribed. In case 3, nordiazepam concentrations in hair were double those in nails (0.20 vs. 0.10 ng/mg). In the other four cases, positive results in nails (cases 10 and 19) and in hair (case 17) were observed at low concentrations (0.02–0.04 ng/mg), except for case 5 (0.84 ng/mg in proximal hair, 0.69 ng/mg in toenails and 1.02 ng/mg in fingernails).

Spearman correlations between concentrations of nordiazepam in the different matrices were only significant between fingernails and proximal hair (ρ = 0.975).

Table 4
Fingernail, toenail and hair concentrations (ng/mg) of each drug detected in real samples.

Case	Age	Sex	Dose (mg/day)	Fingernails (ng/mg)	Toenails (ng/mg)	Proximal Hair (ng/mg)	Distal Hair (ng/mg)	Observations
Venlafaxine								
4	66	F	NT	4.76	2.35	16.60	1.85	Distal segment dyed
5	27	F	NT	NEG	3.03	0.61	2.88	
6	31	F	NT	N/A	NEG	0.05	N/A	Proximal hair: 3 cm
9	56	M	NT	10.64	15.61	40.01	43.58	
14	83	F	NT	NEG	0.01	NEG	NEG	Hair dyed
15	74	F	NT	N/A	0.57	N/A	N/A	
17	44	F	NT	0.05	0.03	NEG	NEG	Toenails: onychomycosis + treatment
18	35	F	NT	N/A	0.01	N/A	N/A	Nails: onychomycosis
20	47	M	NT	N/A	1.30	0.42	N/A	Proximal hair: 1 cm
1	74	M	150	8.05	7.02	N/A	N/A	
21	53	F	225	43.35	N/A	N/A	N/A	
19	51	F	300	20.11	22.69	44.31	N/A	Proximal hair: 5 cm
Trazodone								
4	66	F	NT	NEG	0.02	NEG	NEG	Distal segment dyed
7	83	M	NT	0.13	1.61	8.57	N/A	Proximal hair: 3 cm
14	83	F	50	0.13	0.63	5.40	4.66	Hair dyed
8	39	M	100	0.34	0.42	19.08	N/A	
15	74	F	100	N/A	0.56	N/A	N/A	
19	51	F	200	1.00	1.04	16.81	N/A	Proximal hair: 5 cm
Citalopram								
15	74	F	20	N/A	2.59	N/A	N/A	
Paroxetine								
20	47	M	NT	N/A	0.03	0.09	N/A	Proximal hair: 1 cm
Fluoxetine								
5	27	F	60	5.19	9.11	13.86	8.22	
Sertraline								
5	27	F	NT	0.89	1.00	1.48	0.75	
6	31	F	NT	N/A	0.06	0.30	N/A	Proximal hair: 3 cm
18	35	F	50	N/A	0.03	N/A	N/A	Nails: onychomycosis
17	44	F	150	1.85	2.81	7.42	8.97	Toenails: onychomycosis + treatment
Zolpidem								
2	77	F	NT	NEG	NEG	0.01	N/A	Proximal hair: 5.5 cm
4	66	F	NT	NEG	NEG	0.01	0.08	Distal segment dyed
6	31	F	NT	N/A	NEG	0.01	N/A	Proximal hair: 3 cm
13	54	F	5	NEG	NEG	0.40	0.02	Hair dyed
17	44	F	10	0.12	0.16	1.42	1.46	Toenails: onychomycosis + treatment
Oxazepam								
5	27	F	NT	NEG	NEG	0.03	NEG	
Alprazolam								
7	83	M	0.25	NEG	NEG	0.02	N/A	Proximal hair: 3 cm
12	63	F	0.5	NEG	N/A	0.02	0.02	
15	74	F	1	NEG	0.05	N/A	N/A	
16	64	F	2	1.07	N/A	N/A	N/A	
19	51	F	3	0.07	0.05	0.11	N/A	Proximal hair: 5 cm
Lorazepam								
5	27	F	1	NEG	NEG	0.02	NEG	
Nordiazepam								
3	51	M	NT	0.10	N/A	0.20	N/A	
5	27	F	NT	1.02	0.69	0.84	0.74	
10	25	M	NT	NEG	0.03	NEG	N/A	Proximal hair: 3 cm
17	44	F	NT	0.02	0.02	0.04	0.04	Toenails: onychomycosis + treatment
19	51	F	NT	0.02	0.02	NEG	N/A	Proximal hair: 5 cm
Diazepam								
3	51	M	30	0.41	N/A	0.12	N/A	

NEG = Negative sample
N/A = Sample not available
NT = Not currently under treatment with the indicated drug

Table 5
Zolpidem concentrations (ng/mg) in the fingernails, toenails and hair samples collected from case 13 during a 5-month period.

Sample number	Days after start of treatment	Concentrations			
		Fingernails	Toenails	Proximal hair	Distal hair
1	53 days	NEG	NEG	0.40	0.02
2	91 days	NEG	> LOD	N/A	N/A
3	125 days	> LOD	0.01	N/A	N/A
4	146 days	NEG	N/A	N/A	N/A
5	171 days	NEG	0.02	N/A	N/A

N/A: No sample was collected; NEG: Negative sample

3.8. Other drugs

Citalopram, lorazepam, fluoxetine and diazepam were detected in one case each in patients under treatment with the drugs (Table 4), while paroxetine and oxazepam were also detected in one case each, but in patients that were not under treatment with the drugs at the time of sampling. When both types of matrices were available, concentrations were higher in hair than in nails for all compounds, except for diazepam, for which fingernail concentrations were twice those in hair.

In general, for drugs with low therapeutic doses (zolpidem, alprazolam, lorazepam, paroxetine oxazepam) concentrations were higher in hair than in nail. Even in those patients that were not

under treatment with the drug, low concentrations could be detected, usually in hair samples, presumably because the patients had been taking the medication until sometime before sample collection. One case was positive for both diazepam and its metabolite nordiazepam, and while concentrations of the metabolite were higher in the hair than in the nail samples, concentrations of the parent compound were higher in the nails. For trazodone, fluoxetine and sertraline, with higher therapeutic doses (50–200 mg/day), hair showed higher concentrations than nails in the majority of the studied cases. For venlafaxine, with doses between 150 and 300 mg/day, hair concentrations were higher than nail concentrations in four cases and lower in other four. Nevertheless, these last four cases were not under active treatment at the time, so the difference in concentrations may be due to the different growth rates of nails (3.47 mm/month in fingernails and 1.62 mm/month in toenails) and hair (1 cm/month). In the rest of the patients, concentrations in the different matrices varied case-by-case, but since the drug consumption history was unknown for these patients, no conclusions could be reached about hair and nail incorporation for this analyte.

Overall, most analytes incorporated to hair in higher concentrations than to nails. It is well-known that some drugs have a high affinity for melanin and are incorporated more into dark-colored hair [26,48]. As a matter of fact, all hair samples analyzed in this study had colors ranging from light brown to black, which would explain this higher incorporation into hair. On the contrary, melanin is not usually present in nails in healthy circumstances [21].

We only found data about concentrations in nail samples for diazepam, nordiazepam, oxazepam, alprazolam, lorazepam and zolpidem. Concentrations of these analytes were similar to those found in this study in nails of patients under treatment with the drugs (0.01–0.50 ng/mg) [35]. Shu et al. [40] found concentrations ranging from similar to higher than those reported in this study (0.04–5.30 ng/mg) in high-risk populations. In postmortem cases, Mannocchi et al. [42] found higher concentrations of lorazepam (0.12–0.22 ng/mg) and Krumbiegel et al. [39] also found higher concentrations for several benzodiazepines (0.01–1.68 ng/mg). Moretti et al. [41] analyzed samples from occasional users and autopsies, and found concentrations lower for most benzodiazepines (0.004–0.12 ng/mg) and higher for lorazepam (0.62 ng/mg) than the ones reported in this study.

For these drugs, only three authors analyzed paired hair and nail samples [36,37,39]. Two of them focused on zolpidem after a single dose [36,37]. Madry et al. [37] investigated concentrations in paired hair and fingernail samples from 9 volunteers after the administration of a single dose of zolpidem. Comparing the concentrations of the segments corresponding to the incorporation at the moment of the administration, they found concentrations 2–10 times higher in hair than in nails. Hang et al. [36] studied zolpidem concentrations in fingernails and toenails of 7 subjects after the administration of a single dose. They compared concentrations in fingernails and hair samples from the same subjects and found that hair concentrations were around 1000 times higher than in nails. Krumbiegel et al. [39] investigated 76 substances, including some benzodiazepines, in hair and nails from 7 postmortem cases although no comparison was made by the authors between both types of matrices because nail samples were segmented longitudinally, and concentrations were different between segments, making it difficult to compare results. Nonetheless, detected concentrations were higher in hair than in nails for diazepam (0.20–3.18 vs. 0.10–1.68 ng/mg in hair and nails, respectively), nordiazepam (0.10–4.09 vs. 0.09–2.63 ng/mg), and zolpidem (0.08–0.14 vs. 0.01–0.05 ng/mg); but higher in nails than in hair for oxazepam (0.04–2.33 vs. 0.01–0.56 ng/mg). These results are similar to those found in this study for nordiazepam and zolpidem, although for diazepam it was the opposite, as case 3 had higher concentrations in nails than in hair.

Nevertheless, there are many studies comparing nail and hair concentrations, with different results. For example, for antipsychotic drugs (quetiapine, haloperidol, levomepromazine and clozapine) all studies found higher concentrations in hair than in nails [43,48–51]. In the case of drugs of abuse, the results were quite variable. For cannabinoid compounds, concentrations were higher in fingernails than in hair for THCCOOH [52] and higher in fingernails, but lower in toenails, for THC, CBN and CBD [46]. Cocaine concentrations were reported to be higher in hair than in nails in three publications [21,53,54], and lower in one [55]. The metabolites benzoylecgonine (BE), norcocaine and cocaethylene were found in higher concentrations in hair in one case [21] and BE and ecgonine methyl ester (EME) were higher in nails in another [54]. While morphine was always detected at higher concentrations in nails [54–56], codeine was detected in higher [53,56] or lower [54] concentrations, and 6-acetylmorphine (6-AM) was detected in similar [55], higher [56] or lower [54] concentrations in hair. Buprenorphine was found at higher concentrations in nails, but the metabolite norbuprenorphine was found at similar concentrations in both matrices [57]. Ethylglucuronide (EtG) concentrations were always higher in nails [58–61], while terbinafine [62] cotinine and hydroxycotinine [63] were found in higher concentrations in hair samples. Finally, for amphetamine-type stimulants, the matrix with higher concentrations varied case-by-case [64–66], was higher in nails [54,67] or higher in hair [68], depending on the study.

A factor that can influence the comparison between nail and hair samples is the selected nail sample (fingernails or toenails) by each author. Since fingernail and toenail samples differ in growth rates and possible contamination pathways, different concentrations can be found in both samples, and it should be taken into account when choosing the type of nail sample to analyze. Moreover, differences in personal hygiene can contribute to the variability of the results (for example, a wash-out effect in fingernails due to frequent hand washing). In the present work, paired fingernail and toenail samples were analyzed to investigate possible differences in the concentrations of the analytes. For most drugs, concentrations were slightly higher in toenail samples in patients under active treatment. For example, for venlafaxine (n = 1), trazodone (n = 2), fluoxetine (n = 1), sertraline (n = 1), zolpidem (n = 1) and alprazolam (n = 1), concentrations in toenails were 1.24–2.2 times higher than in fingernails. Nevertheless, some exceptions were observed, and in one case trazodone concentrations were almost the same in both samples and in another, venlafaxine concentrations were 1.15 times higher in fingernails. In patients that were not under active treatment, this trend was not observed, but since we do not have data about the previous treatment, these differences could be attributed to the aforementioned differences in growth rates and drug incorporation. Other authors found zolpidem at higher concentrations in toenails (up to 3 times) [36] and some benzodiazepines at similar median concentrations in both nail samples [40]. For antipsychotics, similar or higher concentrations were found in toenails of psychiatric patients [43] but lower concentrations in toenails of a bloated cadaver [50]. In the case of drugs of abuse, the fingernails were the sample with higher concentrations in most cases. Specifically, cannabinoids were found in much higher concentrations in fingernails (8–29 times higher) [46], or in similar median concentrations [40]. Cocaine was found at higher concentrations in fingernails in all studies [40,54,69,70], while the metabolites BE, EME, norcocaine and cocaethylene were higher in fingernails in most cases [40,54,69,70] and similar in one [69]. Morphine, 6-AM and EDDP were also higher in fingernails [54,70], codeine was similar [70] or higher in toenails [54] and methadone was also higher in toenails [54]. Concentrations of amphetamines were similar in both nail samples [40,66] or higher in toenails [40,54,64]. Lastly, median concentrations were also higher in fingernail samples for EtG [40] and phencyclidine (PCP) [71].

Another factor that influences concentrations found in the different matrices is the growth rate. According to the mean growth

Table 6
Cut-off concentrations proposed for different drugs in nail samples.

Reference	Analyte	Concentrations	Cut-off	Hair (pg/mg)	Fingernail (pg/mg)	Toenail (pg/mg)
Berger 2014 [72]	EtG	Toenail > Fingernail > Hair	Excessive consumption	> 30	> 123	–
			Abstinence	< 7	< 59	–
Cappelle 2017 [58]	EtG	Fingernail > Hair	Excessive consumption	> 30	> 56	–
Cappelle 2018 [54]	COC	Hair > Fingernail > Toenail	Chronic use	500	440	150
	BE	Fingernail > Toenail > Hair	Chronic use	50	175	105
	EME	Fingernail > Hair > Toenail	Chronic use	50	80	40
	AMP	Toenail > Fingernail > Hair	Chronic use	200	485	505
Cobo-Golpe 2020 [46]	THC	Fingernail > Hair > Toenail	Chronic use	50	–	16.5

AMP: Amphetamine; BE: Benzoylecgonine; COC: Cocaine; EME: Ecgonine methyl ester; EtG: Ethyl glucuronide; THC: Tetrahydrocannabinol

rates of hair (1 cm/month), fingernails (3 mm/month) and toenails (1.5 mm/month), the detection windows in simultaneously obtained samples are quite different. In addition, in nail samples there is also a bidirectional drug incorporation. Moreover, the differing length of nails between individuals increases this variability and makes interpretation in nails even more difficult. In any case, considering a mean nail length of 1 cm, the clipping of a fingernail would inform of a consumption done 3–4 months before (6–7 months in the case of toenails), while the first 2 cm of hair samples would inform about the 2–3 months previous to the sample collection. Finally, since we only had data about consumption at the time of sampling, but the detected concentration in nail samples corresponded to 3–4 months (fingernails) or 6–7months (toenails) before, it was difficult to compare dosage with concentrations. Nevertheless, the objective of this work was the study of chronic consumers, and comparisons were made supposing that with a constant consumption there would be a constant incorporation and the concentrations would be homogeneous along the length of the samples.

An important consequence of the observed differences in concentrations between hair, fingernails, and toenails is that cut-off concentrations established for hair are not valid to interpret concentrations measured in nails. Some authors have proposed preliminary cut-off concentrations for nail samples (Table 6). In the case of EtG, the marker of alcohol use, fingernail concentrations are higher than hair concentrations, and using the cut-off concentration in hair for the determination of excessive alcohol consumption (> 30 pg/mg), two authors have proposed preliminary cut-offs in fingernails of 123 pg/mg [72] and 56 pg/mg [58]. Another author [59] also detected higher EtG concentrations in fingernails than hair, and proposed that the cut-off in fingernails should be higher than the one in hair, but did not have enough samples to calculate a cut-off. There are proposed cut-offs for other drugs of abuse, including THC in toenails [46], and amphetamine, cocaine, benzoylecgonine and ecgonine methyl ester in both fingernails and toenails [54] (Table 6). To establish a cut-off in nails a significant number of samples should be analyzed. Unfortunately, in this study the number of positive cases was not enough to allow the calculation of a cut-off for benzodiazepines in nails, although seeing that hair concentrations are higher than nail concentrations for most drugs, the cut-off in nails should be lower than the 50 pg/mg established for hair samples [73].

The effect of onychomycosis was investigated in two cases: patient 17, under treatment with sertraline and zolpidem, reported onychomycosis in toenails, treated with an antifungal cream; concentrations in toenails were 1.5 times higher than in fingernails (sertraline) or slightly higher (zolpidem), showing a similar behavior to other positive cases; the samples were also positive for venlafaxine and nordiazepam, with lower concentrations in toenails than in fingernails. Patient 18, under treatment with sertraline, reported onychomycosis in fingernails and toenails, untreated; the toenail sample was positive, but since no other matrices were available for analysis, no conclusion could be reached. So, nail treatments do not seem relevant in patients undergoing treatment, but more cases should be analyzed in order to reach a definite conclusion.

4. Conclusion

The present work describes a LC-MS/MS method for the simultaneous determination of twelve antidepressant and benzodiazepine drugs in nail and hair samples. The method was successfully validated and applied to paired fingernail, toenail and hair samples from 21 patients under treatment with these drugs. Drug distribution in paired samples was studied, detecting higher concentrations in hair than in nails in most cases, probably due to these drugs binding to hair melanin. The similar or higher concentrations found in toenails than in fingernails could be due to the slower growth of toenails, which allows for an accumulation of higher concentrations over time, a higher incorporation of substances via sweat contamination and/or a wash-out effect in fingernails due to frequent hand-washing. Antidepressant drugs and some benzodiazepines were compared for the first time in nail and hair samples. The effect of nail antifungal treatment in one case was assessed and no relevant effect was observed. Nail samples seem to be a promising alternative to hair samples for the detection of long-term consumption of antidepressant and benzodiazepine drugs.

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CRediT authorship contribution statement

María Cobo-Golpe: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Ana de-Castro-Ríos:** Writing – review & editing. **Angelines Cruz**: Conceptualization, Writing – review & editing, Supervision. **Mario Páramo**: Conceptualization, Resources. **Manuel López-Rivadulla**: Resources, Project administration. **Elena Lendoiro**: Conceptualization, Writing – review & editing, Supervision, Project administration.

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Conflict of interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2021.110935.

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