Determination of antipsychotic drugs in nails and hair by liquid chromatography tandem mass spectrometry and evaluation of their incorporation into keratinized matrices.

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ABSTRACT

Nail samples are an alternative to hair for long-term monitoring of drug use, although there are a limited number of studies about its applicability. This study presents the development and validation of a LC-MS/MS method for the determination of five antipsychotic drugs (clozapine, haloperidol, levomepromazine, olanzapine and quetiapine) in nail and in hair samples. Samples were washed with dichloromethane, pulverized with a ball mill, and incubated in water:acetonitrile (50:50 v/v) with horizontal agitation for 90 min. Then, samples were purified by solid phase extraction with OASIS MCX cartridges and analysed by LC-MS/MS. The analytical method was fully validated in nails and in hair, including: limits of detection (2.5 pg/mg and 2.5-10 pg/mg, respectively), limits of quantification (LOQ) (10 pg/mg and 10-20 pg/mg, respectively), linearity (LOQ to 10000 pg/mg), selectivity (no endogenous or exogenous interferences), accuracy (97.4-106.9% and 97.3-108.2%, respectively), imprecision (<7.9% and <8.6%, respectively), extraction efficiency (62.3% to 109.8% and 45.1% to 83.6%, respectively), matrix effect (-35.6% to 654.4% and -71.0% to -10.8%, respectively) and autosampler stability after 72 h (%loss <12.4%). Moreover, paired fingernail, toenail and hair samples from 13 patients under chronic treatment were analysed, and concentrations in the different matrices were compared. Concentrations in real samples ranged from 11.3 to 8306 pg/mg in fingernails, from 12.7 to 1755.4 pg/mg in toenails and from 17.6 to 24045.4 pg/mg in hair. Hair concentrations were generally higher than nail concentrations; however, a variable pattern was found in fingernails and toenails depending on the case. In addition, concentrations in paired hair and nail samples from a patient under chronic treatment with quetiapine at different doses were studied for 1-year.

KEYWORDS

Nails, hair, antipsychotics, LC-MS/MS

1. Introduction

Keratinized biological matrices, like hair and nails, are able to incorporate and accumulate substances over time, allowing to perform a retrospective investigation of past drug consumption. Hair has been extensively investigated for decades and nowadays is commonly used for routine analysis in Clinical and Forensic Toxicology to achieve a wider window of detection (from weeks to months) in comparison with traditional biological matrices, or when sampling cannot be performed immediately after intake [1,2]. On the contrary, nails have recently been studied as an alternative to hair for long-term substances detection in different forensic applications such as postmortem cases [3], in retrospective monitoring of abstinence [4] or chemsex [5], mainly when this matrix is not available (alopecia, during chemotherapy…) [4,6]. Nevertheless, there are still very few studies about drug incorporation into nails and nail results interpretation.

Substances are incorporated into nails through diffusion from the blood supply during nail growth. However, unlike hair, nails grow continuously and in two directions: approximately 80% of the nail is generated in the germinal matrix, while the other 20% is formed in the nail bed, contributing to the growth in nail thickness from the proximal to the distal end [7,8]. A third incorporation path, similarly to hair, is external contamination via sweat [8-10]. These incorporation pathways were observed in two different studies after the analysis of nail samples following a single dose of zolpidem [8,9]. So, in cases of chronic consumption, detected concentrations are a sum of internal and external incorporation [11].

Different analytical methods for the detection of drugs in nails have been published to date [7,12,13]. Methods for amphetamines, ketamine, cocaine and cannabinoids used mainly GC-MS [14-17]. For opioids, caffeine, nicotine and cotinine, both GC-MS and LC-MS or LC-MS/MS were employed [18,19]. For ethyl glucuronide, endogenous steroid hormones, sedative and antipsychotic drugs, LC-MS/MS was the main technique [13,20-23]. More recently, UPLC-MS/MS methods have been published for the determination of classic drugs and new psychoactive substances [4,24].

Antipsychotic drugs are used for the treatment of schizophrenia, bipolar affective disorders, unipolar depression (off-label use) and anxiety disorders [25]. Interest in the determination of these drugs in keratinized matrices is double: on the one hand to study adherence to treatment in chronic patients [26] and, on the other, in forensic cases to investigate the cause of death [25,27]. In the last two decades, several methods have been published for the analysis of antipsychotics in hair [28-36], while only 3 authors described the use of nails for the detection of these drugs [22,34,37,38]. Uematsu et al. [34] developed a method for the detection of haloperidol in hair samples and applied it to 20 nail samples. Chen et al. [22,37] published two studies comparing concentrations of clozapine in hair and nails, one in 16 psychiatric patients [22] and another comparing concentration results in hair and nails of a cadaver [37]. Finally, Kovatsi et al. [38] published a method for the detection of asenapine in hair and nail samples but did not apply the method to the analysis of real samples.

Therefore, the aim of this work was to develop and validate a method for the detection of 5 antipsychotic drugs in both nail and hair samples, and to study the viability of nail samples as an alternative to hair analysis by comparing concentrations in paired nail and hair samples.

2. Materials and methods

2.1 Chemicals and reagents

Reference standards for quetiapine, haloperidol and clozapine at 1 mg/mL in methanol, and quetiapine-d_8, haloperidol-d_4 and clozapine-d_4 at 0.1 mg/mL in methanol were purchased from Cerilliant (Round Rock, TX, USA). Olanzapine and olanzapine-d_4 at 1mg/mL and 0.1 mg/mL in acetonitrile, respectively, were also purchased from Cerilliant (Round Rock, TX, USA). Levomepromazine maleate in solid form was obtained from Sigma-Aldrich (San Luis, Missouri, USA).

LC-MS grade acetonitrile (ACN) and methanol, HPLC grade dichloromethane (DCM) and 2-propanol, and reagent grade formic acid (FA) 98–100%, were purchased from Scharlau (Sentmenat, Spain). Ammonium hydroxide (NH_4OH) 32% were 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).

2.2 Nail and hair samples

Blank nail and hair samples for the preparation of calibration curves and quality control (QC) samples were voluntarily donated by the laboratory staff. Authentic specimens (fingernails, toenails and hair samples) from patients under chronic treatment with the studied antipsychotic drugs were collected at the University Hospital of Santiago de Compostela from July of 2018 to August of 2019.

Fingernail and toenail samples were obtained by cutting the overhang of the nails (nail clippings) as close as possible to the nail bed and stored separately in plastic bags at room temperature. Nail clippers were cleaned prior to use with a cleaning wipe. Hair samples were collected by cutting a hair lock as close as possible to the scalp from the *posterior vertex* region of the head. The proximal part was indicated using a string, and samples were stored at room temperature in paper envelopes. In addition, data about treatment (currently prescribed drugs and their dosage) and demographic characteristics were 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 working solutions were prepared for calibrators and for QC samples. For levomepromazine, an initial solution at 1 mg/mL was prepared by dissolving the solid standard in methanol. A working solution at 10 µg/mL containing all the analytes, except olanzapine which is unstable in methanolic solution, was prepared by dilution of the individual solutions at 1 mg/mL with methanol. This solution was diluted with methanol to obtain working solutions at 5, 1, 0.5, 0.1, 0.05 and 0.01 µg/mL. A working solution of olanzapine at 10 µg/mL was prepared separately by dilution with ACN, and further diluted to the previously mentioned concentrations with ACN to obtain the working solutions.

For the QC samples, two working solutions at 10 µg/mL were prepared, one containing olanzapine in ACN and another containing the rest of the analytes in methanol. Both were further diluted to obtain solutions at 7.5, 0.75 and 0.03 µg/mL.

Two internal standard (IStd) solutions at 1 µg/mL, one containing olanzapine-d_6 and the other the remaining deuterated analytes, were prepared in acetonitrile and methanol, respectively.

Calibrators at 10, 20, 50, 100, 500, 1000, 5000 and 10000 pg/mg were prepared by addition of 30 µL or 60 µL of the appropriate working solution to 30 mg of blank nail powder, and of 25 µL or 50 µL to 25 mg of blank hair powder. QC samples at low (30 pg/mg), medium (750 pg/mg) and high (7500 pg/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 Nails and hair decontamination, incubation and extraction

Nail and hair samples were decontaminated with 3 consecutive 2 mL of DCM washes (vortex mixing for 2 min each). The last wash solvent was dried under nitrogen stream in a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA) after the addition of 25 µL of the IStd mixtures, reconstituted in 100 µL of mobile phase, and 20 µL were injected into the LC-MS/MS.

Decontaminated samples were dried in an oven at 80°C (5-10 min), and 30 mg of nail or 25 mg of hair were pulverized with a ball mill (Precellys, Montigny le Bretonneux, France) by one cycle of 3x60s at 6500 rpm for hair and two cycles for nails. After the addition of 25 µL of the IStd mixtures, 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 with a nitrogen stream at 35°C. The sample was reconstituted in 200 µL of methanol and 2 mL of FA 2% in water, and submitted to solid phase extraction (SPE) using Oasis MCX cartridges previously conditioned with 2 mL of methanol and 2 mL of water. After loading the sample, two washing steps with 2 mL of FA 2% in water and 2 mL of water:methanol (50:50, v/v) were applied, and the cartridge was subsequently dried for 10 min. 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 with a Minispin™ Plus (Eppendorf Ibérica, San Sebastián de los Reyes, Spain), 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 x 100 mm, 3 µm) analytical column (Phenomenex, Torrance, CA, USA), with an XBridge BEH Shield RP18 (2.1x5 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 with the following gradient: starting with 20% B, increasing to 40% B over 2 min and to 50% B at 2.5 min, reaching 80% B at 6 min, and returning to initial conditions at 6.5 min. Total chromatographic run was 8 min. A divert valve was set to direct the flow to the MS from 0.5 to 6 min and the remaining time to waste.

Injection into the MS was operated in electrospray in positive mode (ESI+). 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. The MS conditions were: capillary voltage, 2 kV; source block temperature, 125°C; desolvation gas (nitrogen) temperature, 300°C; desolvation and cone gas (nitrogen) flow rate, 500 and 50 L/h, respectively. Argon was employed to promote analyte fragmentation in the collision cell.

Data acquisition was controlled with Masslynx 4.0 software and processed with Quanlynx software (Waters Corp.).

2.6 Method validation in nails and hair

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) [39]. Validated parameters and their acceptance criteria are detailed in Table 1.

2.7 Data 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. Results are presented as mean±standard deviation (SD) for normal data, and as median [interquartile range (IQR)] for non-normal data. Since one of the studied matrices (fingernails) did not follow a normal distribution, concentrations were presented as median [IQR], and correlations between concentrations in the different matrices, weight and dosage (for those patients under active treatment) were assessed using Spearman correlation. A p-value <0.05 was considered statistically significant.

3. Results

3.1 Method development and validation

An analytical method for the detection of quetiapine, haloperidol, levomepromazine, clozapine and olanzapine in nail and hair samples was developed, employing the same LC-MS/MS conditions in both cases. Chromatographic elution of all the analytes was achieved in 4.5 min (Figure 1), with a total chromatographic run of 8 min. For MS detection, the most abundant MRM transition was used for quantification, and a second transition was monitored for qualification purposes, to fulfil the European Union identification criteria [40]. MRM conditions are shown in Table 2.

For sample pre-treatment (hair or nails) different incubation solvents, including methanol, 2M sodium hydroxide, ACN, and a mixture of ACN and water in different proportions, were tested. Heating, agitation and sonication were evaluated at different time points (15, 30, 90 or 180 min). Specific tested conditions are detailed in Supplementary Table S2. Best results for both types of samples were obtained with water:ACN (50:50, v:v) and 90 min of agitation (data not shown). Finally, the sample was submitted to SPE with MCX cartridges employing the previously described method, using again the same protocol for hair or nail samples.

Once optimized, the methodology was fully validated in both matrices. The acceptance criteria were fulfilled for all parameters.

Linearity was verified by least square regression using 1/x-weighing factor in both matrices, showing a linear dynamic range from the LOQ to 10000 pg/mg for all analytes. LOD in nails was 2.5 pg/mg for all the analytes, and in hair 2.5 pg/mg for haloperidol and clozapine, 5 pg/mg for quetiapine and olanzapine, and 10 pg/mg for levomepromazine. LOQ in nails was 10 pg/mg for all the analytes, and in hair 10 pg/mg for all the analytes except for levomepromazine, for which the LOQ was 20 pg/mg. At the LOQ, accuracy was 97.0%-111.7% and 98.5%-108.0% of the nominal concentration in nails and hair, respectively. Moreover, %CV was 4.4%-8.4% and 3.7%-12.4% in nails and hair, respectively. A representative chromatogram of the quantifier transition for each analyte at the LOQ in hair (1A) and nail (1B) samples is shown in Figure 1.

Selectivity was confirmed because no quantifiable peaks were detected in 10 different nail and 10 different hair samples, nor in the blank samples fortified with common drugs of abuse and medicines at the retention time of each analyte. Moreover, there was no evidence of carryover since the blank samples (n=3) analysed after the injection of the upper limit of quantification point calculated a concentration <LOD.

Results for accuracy, intra-assay, inter-assay and total imprecision are summarized in Table 3. Accuracy was satisfied for all the analytes, with calculated concentrations within 97.4-106.9% of the nominal concentration in nails, and within 97.3-108.2% in hair. Intra-assay, inter-assay and total imprecision were <7.3%, <5% and <7.9% respectively, in nail samples; and <5.9%, <8% and <8.6% in hair samples.

Matrix effect, extraction efficiency and process efficiency results are indicated in Table 4. Matrix effect ranged from -35.6% to 654.4% in nails and from -71.0% to -10.8% in hair, with their respective IStd showing similar effects. Extraction recovery was satisfactory, ranging from 62.3% to 109.8% in nail samples, and from 45.1% to 83.6% in hair samples. Process efficiency ranged from 47.9% to 620.1% in nail samples, and from 17.7% to 74.1% in hair samples.

Finally, all the analytes showed to be stable after 72 h in the autosampler, with a %loss <12.4% and <11% in nails and hair, respectively (Supplementary Table S3).

3.2 Application to real samples

Nail and hair specimens from 13 patients under treatment with one or more of the studied antipsychotic drugs were analysed with the described method. Paired fingernail, toenail and hair samples for each patient were obtained at the same time. Ten participants provided all three samples, while the other 3 provided only one or two samples. For hair samples the proximal 2 cm segment was analysed, and in cases where the length was sufficient (n=4), the next 2 cm were also analysed (distal segment). In addition, one of the participants provided samples for one year, at different timepoints (6 fingernail samples, 4 toenail samples and 7 hair samples), allowing to study the evolution of concentrations over time.

Of the 13 patients, 4 were female and 9 were male, with ages ranging from 25 to 77 years old (Mean±SD= 52.5±18.5), and weights between 44 and 100 kg (Mean±SD= 78±17). Poly-medication was common in these patients, 4 of them received two antipsychotic drugs and

one received four. Administrated dosages are indicated in Table 5, showing a wide range of values, depending on the drug and the presence of poly-medication.

The most frequently detected drug was quetiapine (n=11), followed by haloperidol (n=5), olanzapine (n=5), levomepromazine (n=4) and clozapine (n=3). Detailed results for each drug are presented in Table 5. No external contamination was detected for any sample.

3.2.1 Nail and hair concentrations for each drug

Quetiapine

Eleven cases were positive for quetiapine, although only 8 were under active treatment at the moment of sampling. All of them tested positive in the three matrices, when available, and the median highest concentrations were observed in hair (2305.4 [3635.8] pg/mg), followed by toenails (928.4 [639.7] pg/mg) and fingernails (845.3 [1714.5] pg/mg). Segmental hair analysis (n=2) showed similar concentrations in both segments for one case and concentration in the distal segment five times lower than in the proximal segment, although the distal segment was dyed red.

Of the 3 patients that were not under active treatment with quetiapine at the moment of sampling, 2 donated hair, and both cases tested negative. Fingernails were available in the 3 cases, but only 1 specimen tested positive (37.9 pg/mg). Finally, the 2 samples that tested negative in fingernails tested positive in toenails (41.4 [35.5] pg/mg).

Correlations between concentrations in the three different matrices, between concentrations in each matrix and quetiapine dose, and between concentrations in each matrix and patients' weight were investigated for those patients under active treatment. A statistically significant correlation was found between fingernail and toenail concentrations (Spearman ρ= 0. 857), and between quetiapine fingernail or hair concentrations and patient weight (Spearman ρ= 0.865 and ρ= 0.886, respectively).

Haloperidol

Five cases were positive for haloperidol, and all of them tested positive in hair samples (22.6-24045.4 pg/mg). However, nail samples were only positive in those cases under active treatment at the time of sample collection (n=3). In paired samples, the highest concentrations were found in hair (>20 times higher, 1877.7-24045.4 pg/mg), while similar or higher concentrations were observed in toenails (351.1-615 pg/mg) than in fingernails (283.5-340.5 pg/mg).

Olanzapine

Olanzapine was detected in 5 cases, all of them under active treatment. In the 3 cases where all the samples were available similar concentrations were found in hair and fingernails, while toenail concentrations varied, showing much higher concentrations in toenails in two cases and a lower concentration in one. In addition, one patient who had started treatment two weeks before sampling (no hair sample available) tested positive in toenails and in fingernails,

but at low concentrations (29.9 pg/mg and <LOQ, respectively). In another case the toenail sample was not available, and concentrations in fingernails (448.3 pg/mg) were higher than in hair (56.4 pg/mg). Finally, only in one case hair was segmented, with higher concentrations in the proximal segment (132.4 pg/mg) than the distal (17.6 pg/mg).

Levomepromazine

Four cases were positive for levomepromazine, half of them under active treatment. These 2 cases showed much higher concentrations in hair than in nails (4.5-8 times higher); and the concentration in fingernails was similar to that observed in toenails in one case, and 2.6 times lower than the concentration in toenails in the other. Of the two cases that were not under active treatment, one was negative in hair and positive in fingernails (toenail not available), but the other had much higher concentration in hair than in nails (13-16 times higher). For the only case with proximal and distal hair samples, the concentrations in both segments were very similar.

Clozapine

Three cases were positive for clozapine. Only one was under active treatment, and tested positive in all matrices, with the highest concentration in the distal hair segment (6327.3 pg/mg), followed by the proximal hair segment (4313.5 pg/mg), toenails (1220.2 pg/mg) and fingernails (618.8 pg/mg). In the other two cases, concentrations close to the LOQ or negative were found.

3.2.2 Evolution of quetiapine concentrations over time

One patient provided nail and hair samples over a year period. Evolution of quetiapine concentrations and doses over time is shown in Figure 2. The patient started treatment with 100 mg/day of quetiapine three weeks before the first sample was collected, and increased to 200 mg/day before the second sample was taken (7 months after starting treatment). After 10 months of treatment (May-19), the dose decreased again to 100 mg/day. Seven hair, 6 fingernail and 4 toenail samples were collected during this period.

Concentrations were always higher in hair than in nails, and similar between fingernail and toenail samples, although with a different pattern over time. Three weeks after starting treatment, quetiapine was detected in all matrices, but at low concentrations (263.4 pg/mg in hair, 58.7 pg/mg in fingernails and 88.5 in toenails). Concentrations increased notably in samples collected 7 months later (4623.07 pg/mg in hair and 1706.95 pg/mg in toenails), one week after doubling the dose (Feb-19). Although the dose remained unchanged for the next 3 months, hair concentrations detected in the third and fourth samples (Apr-19 and May-19) were lower than those found in the second sample (1.4-1.8 times lower). Nevertheless, hair concentrations increased over time until reaching again the highest concentration in sample 5 (Jun-19) (4744.5 pg/mg in hair, 2338.4 pg/mg in fingernails and 928.4 in toenails). In addition, in samples collected in Jun-19, the concentrations in fingernails and toenails were not similar as in the previous paired samples (2.5 times higher in fingernails). Finally, two months after decreasing the dose, a descending trend can be seen in hair, while the last fingernail sample showed an unexplained increase in concentration.

4. Discussion

A LC-MS/MS method for the detection of 5 antipsychotic drugs in nail and hair samples was developed and fully validated. Analytes showed a higher degree of ion suppression in hair than in nails. Moreover, matrix effect for olanzapine in nails presented an extremely high ion enhancement at the low QC but not at the high QC. This effect can be compensated with the use of the deuterated analogue (olanzapine-d_8) as IS since it shows the same behaviour as olanzapine. In addition, matrix effect experiments showed high %CV for some of the compounds. However, the same pattern was observed for the corresponding deuterated IS. High values of %CV indicate a high inter-sample variability and highlight the importance of using the deuterated analogue as IS. Finally, a low process efficiency in hair samples was observed for most of the compounds, but it was enough to reach an LOQ of 10-20 pg/mg. Despite these issues, all validation parameters showed satisfactory results.

Only four methods have been previously published for the determination of antipsychotics in nails samples [22,34,37,38], all of them focused on the determination of a single analyte. The LOQ achieved by Chen et al. [37] for clozapine was similar to ours (10 pg/mg); however, much higher LOQs were reported in the other 2 published methods [500 pg/mg and 625 pg/mL (immunoassay method) for clozapine and haloperidol, respectively] [22,34].

The detection of antipsychotics in hair samples has been more extensively studied [30,31,33,35,36]. Some previously published methods detected only one or two antipsychotic drugs simultaneously, with higher LOQ values compared to ours. Specifically, LOQs of 90 pg/mg for quetiapine [33], and 100 pg/mg for clozapine [35] and haloperidol [30] were reported in methodologies for the determination of a single drug; while Weinmann et al. [36] detected clozapine and haloperidol at LOQs of 51 pg/mg and 39 pg/mg, respectively. However, Fisichella et al. [31] analysed 87 psychoactive drugs, including the five antipsychotics detected in the present study, achieving LOQs of 6.1 pg/mg for clozapine, 1.6 pg/mg for haloperidol, 11.7 pg/mg for levomepromazine, 10.5 pg/mg for olanzapine and 31.1 pg/mg for quetiapine. Concentrations of antipsychotic drugs in hair samples in these papers were similar to those found in the present study.

Our analytical method was successfully applied to the analysis of samples from 13 patients under antipsychotic treatment. For haloperidol, levomepromazine and clozapine, the highest concentrations in patients under active treatment were detected in hair, followed by toenails, and the lowest concentrations were observed in fingernails. Quetiapine concentrations were also higher in hair than in nails but fingernail/toenail ratio varied case-by-case. Nevertheless, when only samples from patients under active treatment were compared, a correlation was found between concentrations in fingernails and toenails (Spearman ρ= 0.857, n=7). However, this correlation should be considered with caution and further evaluated due to the limited number of studied cases. On the other hand, olanzapine concentrations did not follow this distribution, with varying concentrations in each matrix depending on the patient. The analysis of a higher number of samples for this compound would help to determine the distribution pattern for this drug. Moreover, since extraction recovery for olanzapine in our method was very different between nail and hair samples, no definite conclusion can be reached about the

correlation of this analyte in both matrices. Similarly, other authors also found a higher concentration in hair samples compared with nails for cocaine, codeine and morphine [16,41,42], while the opposite was found for amphetamines and methadone [42]. For antipsychotics, the first studies were from Uematsu et al. [34,43], who analysed hair and nail samples from patients under haloperidol treatment. Nail concentrations in patients with a fixed daily dose for more than four months were higher than those found in our study (670 to 16890 pg/mg vs 107.3 to 615.0 pg/mg), although similar doses were administrated in both studies. They also found higher concentrations of haloperidol in hair than in nails (2330-245000 pg/mg vs. 670-16890 pg/mg) [34]. In a posterior study, Uematsu et al. [43] compared levels of haloperidol in grizzled hair and nails from 7 individuals. They observed that, in the same individual, concentrations in black hair were ten times higher than those in white hair, and fingernail concentrations were 3.4% of those found in black hair. The most likely explanation, under the authors, is that since antipsychotics are alkaline molecules, they bind to the melanin present in hair and accumulate in larger concentrations in this matrix compared to nails. In our study, where all the specimens were from patients with dark hair, higher concentrations were also found in hair compared to nails for all antipsychotics except for olanzapine.

More recently, Chen et al. [22] analysed 16 paired nail and hair samples from patients under treatment with clozapine, and found higher concentrations in hair (16700-59200 pg/mg) than in nails (1600-14140 pg/mg), with a statistically significant correlation between both matrices. Moreover, in a different study, Chen et al. [37] analysed fingernails, toenails and hair (six segments) of a bloated cadaver and, again, they found the highest clozapine concentrations in hair (145.4-686.3 pg/mg); however, in contrast to what we found in our samples, clozapine concentrations in fingernails (89.0-538.5 pg/mg) were higher than those observed in toenails (64.6-130.1 pg/mg). In this second study [37], clozapine hair and nail concentrations were much lower than those reported previously [22], which were clearly affected for the immersion of the cadaver in a river during a long time. The soaking in water obviously reduced clozapine concentrations in these samples.

Segmental hair analysis could be performed for cases AP5, AP6, AP10 and AP11. In 4 out of 8 of the analytes detected in those cases (quetiapine in AP6, clozapine in AP10, and haloperidol and levomepromazine in AP11), similar concentrations were observed in both hair segments, and subjects were under treatment with these drugs. On the other hand, in case AP6 lower concentrations of haloperidol and higher concentrations of olanzapine were found in the proximal segment, pointing to a possible change in medication in the last months. Lastly, in case AP5, where the distal segment was red dyed, haloperidol and quetiapine concentrations in the distal segment were lower than what would be expected, probably due to the cosmetic treatment.

Correlations between dose and antipsychotic concentrations in the three biological matrices were studied only for quetiapine due to the higher number of positive samples for this drug. No significant correlation between current dose and quetiapine concentration was found in any matrix. This could be due to a change in the medication history, since only information about current dosage was available, and keratinized matrices reflect consumption in the past 1-6 months. On the contrary, Uematsu et al. [34] found that both nail and hair concentrations

significantly correlated with the administered dose, despite not being significantly correlated between them. In patients who were no longer under treatment with any drug, positive results were still obtained in one or two of the matrices, proving the wide window of detection in these matrices. Nevertheless, low analyte concentrations were detected in these cases. Weinmann et al. [36] did not find a correlation between hair concentration, dosage nor melanin content, and hypothesized that body-fat content could be the cause for this finding. BMI data of our participants was not available, but a significant correlation between quetiapine concentrations in fingernails and hair and patient weight was found, indicating that this could be the case.

To the best of our knowledge, the evolution of drug concentrations over time in paired hair and nail samples from a patient under chronic treatment has not been previously studied. In our study, samples from the same individual under chronic treatment with quetiapine were collected from the beginning of the treatment and over a year period (Figure 2). The presence of quetiapine in the keratinized matrices was detected 3 weeks after the beginning of the treatment. Although it is common to assume a hair growth rate of 1 cm/month, scalp hair growth ranges between 0.6 to 1.4 cm/month [2], so the detection of quetiapine in the first hair sample could be explained by a fast growth rate in this individual as well as some incorporation via sweat contamination. Meanwhile, nail growth is slower than hair growth (an average of 3 mm/month for fingernails and 1 mm/month for toenails is generally accepted [12]), so incorporation through the nail matrix would not be detected until 10 to 18 weeks after the beginning of the treatment [6]. However, in addition to the longitudinal growth, the nail also grows in thickness from the nail bed. In fact, previous studies have reported that incorporation into the nail bed could be detected 2 to 3 weeks after intake. Moreover, incorporation via sweat contamination is especially significant in nail samples, being possible to detect drugs in nail clippings even 24 h after intake [8]. Therefore, the detection of quetiapine in nail samples three weeks after the beginning of the treatment could be explained by the combination of the incorporation of this drug through the nail bed as well as sweat contamination. The following hair and nail samples collected from this patient showed a fluctuation in quetiapine concentrations over time, even when the same dosage was administrated for more than 6 months. In addition, when the dosage was halved, a decrease was detected in hair concentrations after 3 months but not in fingernails, probably for a delay in the window of detection of fingernails compared with hair. There is not a clear explanation for these fluctuations in quetiapine concentrations, although intraindividual variations (comorbidities, concomitant drugs…) could be the reason for these differences.

The main limitations of this study were the scarce real cases available to compare drug concentrations and distribution in the different matrices, especially for some of the analytes, and the lack of detailed information about previous dosage administrated before sample collection. However, this is the first study to deepen in the knowledge about antipsychotic drugs in keratinized matrices, and to compare quetiapine concentrations over time in the three keratinized samples.

5. Conclusion

The present work describes a LC-MS/MS method for the simultaneous determination of the most commonly prescribed antipsychotic drugs in Spain in hair and, to the best of our knowledge, for the first time in nails. The method was successfully validated and applied to nail and hair specimens from 13 patients under antipsychotic treatment. Drug distribution in paired samples was studied, detecting usually higher concentrations in hair than in nails, and higher concentrations in toenails than in fingernails. In addition, drug concentrations over time in paired nail and hair samples from a patient under chronic treatment were described for the first time. We conclude that nail samples could be used as an alternative to hair for antipsychotic drugs when long-term exposure detection is needed.

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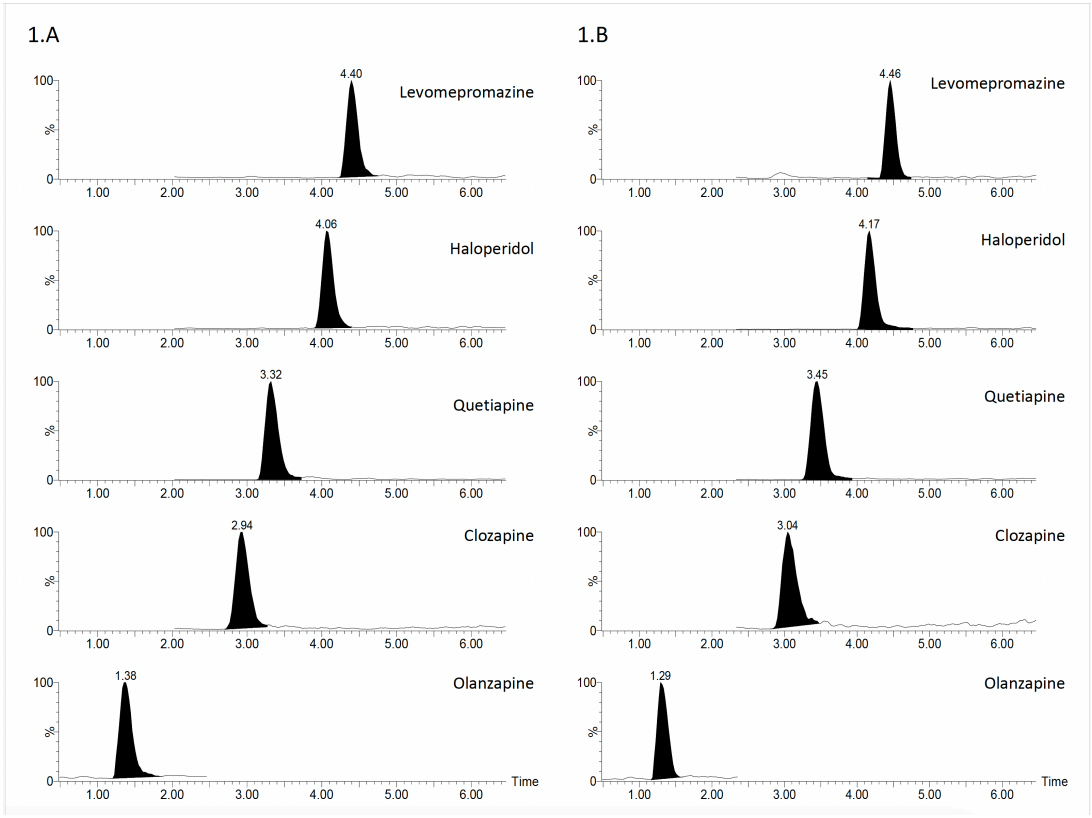
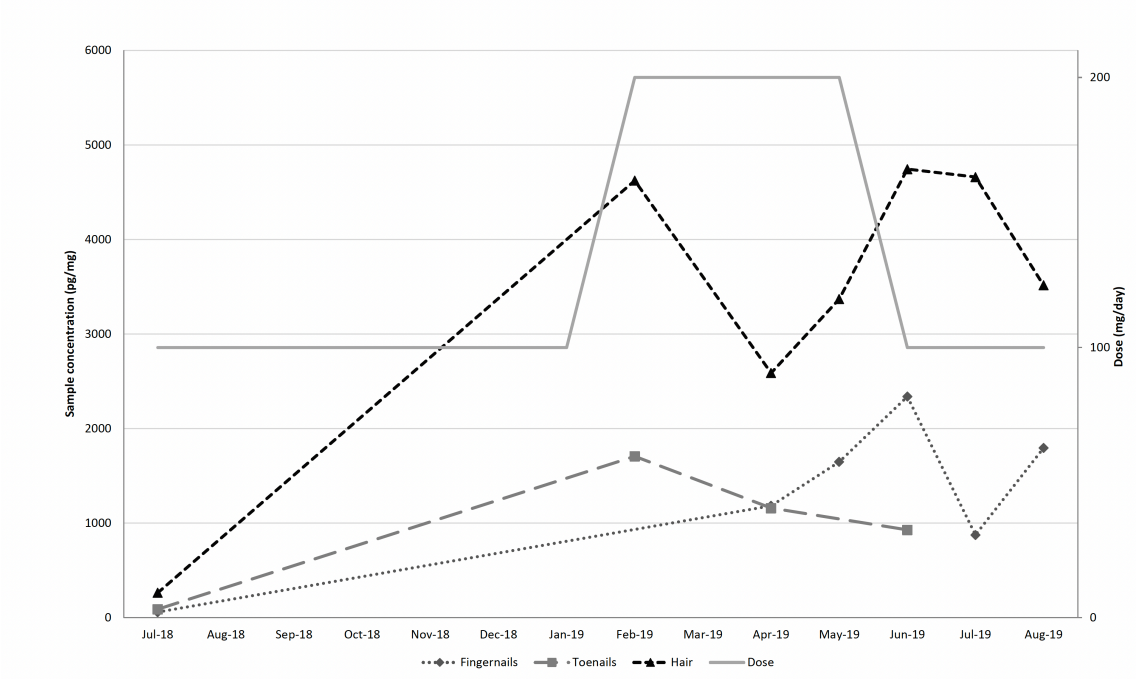
628 **Figure 1.** MRM chromatograms of the quantifier transition of quetiapine, haloperidol,
629 levomepromazine, clozapine and olanzapine in a blank hair (1A) and nail (1B) samples fortified
630 at the LOQ.



631

632

633 **Figure 2**. Quetiapine dose and fingernails, toenails and hair concentrations over 1-year period.



634

635 **Table 1**. 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 [33]
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 nominal concentration and %CV<20%
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.*	No interferences detected
Carryover	Analysis of a blank matrix sample immediately after the upper limit of quantification point (n=3)	Calculated concentration <LOD
Accuracy	Analysis of 3 replicates at low, medium and high QC concentrations in 5 different days (n=15)	80%-120% of the nominal 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 72 hours in the autosampler at 6°C	Re-injected samples quantified within ±20% of freshly prepared samples
*The list of exogenous interferences is detailed in Supplementary Table S1		

Table 2. MRM transitions, cone voltage (CV), collision energy (CE), retention time (Rt) and internal standard (IStd) selected for each analyte.

Analyte	MRM transition	CV (v)	CE (eV)	Rt	IStd
Olanzapine	313.23->256.2 313.23->84.1	35 35	42 22	1.2	Olanzapine-d_8
Olanzapine-d_8	321.37->261.3	40	25	1.3	
Clozapine	327.16->192.2 327.16->270.1	35 35	38 22	3	Clozapine-d_4
Clozapine-d_4	331.34->272.5	40	23	2.9	
Quetiapine	384.22->253.2 384.22->279.2	35 35	22 26	3.4	Quetiapine-d_8
Quetiapine-d_8	392.36->258.3	35	23	3.3	
Haloperidol	376.24->165.1 376.24->123.1	35 35	24 38	4.12	Haloperidol-d_4
Haloperidol-d_4	380.35->169.3	40	23	4.12	
Levomepromazine	329.24->100.1 329.24->167.1	30 30	18 50	4.4	Quetiapine-d_8

Table 3. Imprecision and accuracy in nail and hair samples at low (30 pg/mg), medium (750 pg/mg) and high (7500 pg/mg) QC concentrations.

	Analyte	Intra-assay imprecision (%CV)			Inter-assay imprecision (%CV)			Total imprecision (%CV)			Accuracy (n=15; %)		
		LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH	LOW	MEDIUM	HIGH
Nail samples	Quetiapine	3.1	2.8	2.5	1.2	3.1	1.5	3.3	4.2	2.9	98.4	106.9	97.4
	Haloperidol	3.8	2.6	2.0	0.0	3.5	3.1	3.8	4.4	3.7	102.0	104.8	102.0
	Levomepromazine	7.0	7.3	3.9	0.0	3.2	4.9	7.0	7.9	6.3	96.7	98.4	99.0
	Clozapine	3.1	1.9	2.9	1.7	1.3	5.0	3.5	2.3	5.8	99.1	106.6	98.1
	Olanzapine	4.9	2.3	4.5	0.0	3.4	3.4	4.9	4.1	5.6	98.3	97.9	99.0
Hair samples	Quetiapine	3.4	3.2	4.8	1.7	2.4	0.0	3.8	4.0	4.8	97.3	104.3	97.3
	Haloperidol	2.7	2.0	4.0	2.3	1.5	1.9	3.5	2.5	4.4	101.5	106.7	102.9
	Levomepromazine	4.7	5.9	3.2	6.2	3.1	8.0	7.7	6.7	8.6	100.7	108.2	103.1
	Clozapine	3.7	2.8	4.0	2.7	1.6	3.4	4.6	3.2	5.2	99.5	105.1	101.9
	Olanzapine	3.6	4.3	1.9	4.0	0.0	2.3	5.3	4.3	3.0	100.4	99.8	101.9

Table 4. Matrix effect, extraction recovery and process efficiency at low (30 pg/mg) and high (7500 pg/mg) QC concentrations in nail and hair samples.

Analyte	Concentration	Nail samples			Hair samples		
		Extraction recovery [% (%CV)] (n=10)	Matrix effect [% (%CV)] (n=10)	Process efficiency [% (%CV)] (n=10)	Extraction recovery [% (%CV)] (n=10)	Matrix effect [% (%CV)] (n=10)	Process efficiency [% (%CV)] (n=10)
Quetiapine	LOW	79.7 (20.8)	25.5 (10.9)	100.0 (12.2)	66.9 (30.1)	-38.1 (20.9)	41.4 (4.3)
	HIGH	77.7 (5.8)	-9.5 (6.1)	70.3 (5.1)	83.6 (6.3)	-12.7 (5.5)	73.1 (4.7)
Quetiapine-d_8	LOW	75.4 (20.3)	20.5 (9.3)	90.8 (14.1)	67.9 (28.7)	-36.9 (21.0)	42.8 (2.4)
	HIGH	74.7 (8.3)	-10.9 (6.5)	66.5 (4.7)	83.1 (5.1)	-10.8 (6.1)	74.1 (2.1)
Haloperidol	LOW	85.6 (20.0)	-7.5 (15.9)	79.1 (4.1)	63.7 (32.8)	-56.8 (24.2)	27.5 (4.5)
	HIGH	73.2 (11.9)	-24.3 (8.3)	55.4 (3.9)	75.8 (21.3)	-38.5 (23.6)	46.6 (5.5)
Haloperidol-d_4	LOW	76.6 (20.9)	-12.6 (15.2)	67.0 (2.8)	63.5 (34.7)	-55.1 (24.5)	28.5 (3.2)
	HIGH	71.2 (11.4)	-26.8 (8.8)	52.1 (2.8)	74.5 (20.2)	-37.8 (23.3)	46.4 (2.3)
LMZ	LOW	76.1 (12.8)	36.6 (12.9)	103.9 (22.8)	45.1 (28.1)	-32.6 (21.0)	30.4 (6.6)
	HIGH	62.3 (6.9)	-6.9 (5.0)	58.0 (5.7)	70.5 (11.1)	-14.4 (12.9)	60.3 (5.4)
Clozapine	LOW	91.7 (44.7)	17.7 (32.7)	108.0 (14.5)	64.8 (42.8)	-47.2 (36.3)	34.2 (5.3)
	HIGH	73.5 (16.3)	-14.4 (9.4)	62.9 (5.8)	82.9 (11.2)	-15.1 (10.7)	70.4 (4.9)
Clozapine-d_4	LOW	71.0 (48.6)	5.0 (31.5)	74.6 (14.5)	66.0 (39.8)	-40.3 (38.9)	39.4 (5.3)
	HIGH	69.2 (18.3)	-16.7 (10.4)	57.7 (5.0)	78.5 (9.6)	-11.5 (12.2)	69.5 (1.3)
Olanzapine	LOW	109.8 (30.2)	268.6 (26.5)	404.6 (56.8)	52.1 (35.3)	-59.4 (29.3)	21.1 (9.0)
	HIGH	74.4 (25.5)	-35.6 (20.4)	47.9 (12.8)	64.2 (19.2)	-70.8 (15.7)	18.8 (2.3)
Olanzapine-d_8	LOW	82.2 (48.2)	654.4 (32.7)	620.1 (157.1)	45.3 (36.5)	-60.9 (31.9)	17.7 (8.9)
	HIGH	91.1 (25.5)	-16.2 (17.5)	76.4 (58.5)	61.7 (19.2)	-71.0 (16.9)	17.9 (3.0)

LMZ: levomepromazine

655 **Table 5**. Fingernail, toenail and proximal and distal hair concentrations (pg/mg) of haloperidol,
656 levomepromazine, clozapine, olanzapine and quetiapine in real samples.

Quetiapine					
Case	Dose (mg/day)	Fingernails	Toenails	Proximal Hair	Distal Hair
AP4	NT	37.9	N/A	NEG	N/A
AP12	NT	NEG	59.1	NEG	N/A
AP3	NT	NEG	23.6	N/A	N/A
AP5	100	80.3	<LOQ	191.4	38.6
AP8	100	1794.8	928.4	3515.8	N/A
AP13	150	8306.3	1147.5	4090.3	N/A
AP6	200	77.5	359.4	454.4	490.5
AP2	250‡	1150.2	1316.2	2305.4	N/A
AP1	300	562.8	676.5	N/A	N/A
AP7	600	N/A	1755.4	1862.8	N/A
AP9	1800	845.3	813.5	4328.6	N/A
Haloperidol					
Case	Dose (mg/day)	Fingernails	Toenails	Proximal Hair	Distal Hair
AP5	NT	NEG	NEG	1144.0	351.9
AP6	NT	NEG	NEG	22.6	80.1
AP11	10	283.5	615.0	21797.7	24045.4
AP9	30	340.5	351.1	18424.8	N/A
AP7	100 mg/month*	N/A	107.3	1877.7	N/A
Olanzapine					
Case	Dose (mg/day)	Fingernails	Toenails	Proximal Hair	Distal Hair
AP6	10	106.9	553.9	132.4	17.6
AP3	15	<LOQ	29.9	N/A	N/A
AP4	30†	448.3	N/A	56.4	N/A
AP9	60	145.2	92.7	266.2	N/A
AP12	450 mg/month*	102.2	1034.9	85.3	N/A
Levomepromazine					
Case	Dose (mg/day)	Fingernails	Toenails	Proximal Hair	Distal Hair
AP4	NT	86.5	N/A	NEG	N/A
AP12	NT	<LOQ	12.7	164.8	N/A
AP9	100	227.8	379.1	3072.5	N/A
AP11	200	608.5	1562.2	7216.0	7562.2
Clozapine					
Case	Dose (mg/day)	Fingernails	Toenails	Proximal Hair	Distal Hair
AP9	NT	<LOQ	14.1	<LOQ	N/A
AP12	NT	11.3	NEG	<LOQ	N/A
AP10	400	618.8	1220.2	4313.5	6327.3

NEG= Negative sample
N/A = Sample not available
NT = Not currently under treatment with the indicated drug
†Changed from 5 mg/day ten days before sampling
‡Changed from 200 mg/day one month before recollection
*Dose administered monthly via intramuscular injection

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Supplementary Table 1. List of common drugs of abuse and medicines included in the study o exogenous interferences at 25 ng/mg

Group	Compounds
Opioids	Codeine, methadone, morphine, 6-acetylmorphine, fentanyl
Methadone	Methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpirrolidine
Amphetamines	Amphetamine, methamphetamine, 3,4-methylendioxiamphetamine (MDA), 3,4-methylendioximethamphetamine (MDMA) and 3,4-methylendioxiethylamphetamine (MDEA)
Cocaine	Cocaine, cocaethylene, benzoylecgonine and ecgonine methyl esther
Other drugs of abuse	Lysergic acid diethylamide (LSD), ketamine, norketamine and gamma-hydroxybutyric acid (GHB)
Nicotine	Nicotine and cotinine
Antidepressants	Amitriptyline and paroxetine
Benzodiazepines	Alprazolam, temazepam, lormetazepam, lorazepam, flunitrazepam, 7-aminoflunitrazepam, clonazepam, diazepam, nordiazepam, oxazepam, triazolam, nitrazepam and bromazepam
Nonsteroidal anti-inflammatory drugs	Ibuprofen, acetaminophen, diclofenac and naproxen
Other medicines	Zolpidem, zopiclone and omeprazol

Supplementary Table 2. Conditions tested during sample pre-treatment optimization.

INCUBATION	NaOH		Heating at 40ºC	90 min
	MeOH		Sonication	30 min
			Agitation	90 min
	ACN		Agitation	90 min
	ACN:Water	70:30	Agitation	90 min
		50:50	Agitation	90 min
		50:50	Sonication	15 min
		50:50	Sonication	30 min
		50:50	Sonication	60 min
		50:50	Agitation	180 min
		50:50	Agitation + Incubation at 25ºC	180 min + 15 h
		50:50	Agitation + Incubation at 50ºC	180 min + 15 h
EXTRACTION	SPE with OASIS MAX (60 mg)		W1: 5% NH_4OH in MeOH W2: MeOH E: FA 2% in MeOH	
	SPE with OASIS HLB (60 mg)		W1: FA 2% in water W2: MeOH E: 5% NH_4OH in MeOH	
	SPE with OASIS MCX (60 mg)		W1: FA 2% in water W2: MeOH E: 5% NH_4OH in MeOH	
	SPE with OASIS MCX (60 mg)		W1: FA 2% in water W2: MeOH:Water 50:50 E: DCM:IPA:NH_4OH 75:24.5:0.5	
SPE: Solid phase extraction; W1: First wash solvent; W2: Second wash solvent; E: Elution solvent				

Supplementary Table 3. Autosampler stability (%loss) in nail and hair samples at low (30 pg/mg), medium (750 pg/mg) and high (7500 pg/mg) QC concentrations.

Analyte	Concentration	%Loss	
		Nail samples	Hair samples
Quetiapine	LOW	1.60	2.16
	MEDIUM	2.43	5.43
	HIGH	0.69	0.84
Haloperidol	LOW	-9.09	10.65
	MEDIUM	-1.76	9.54
	HIGH	-3.77	11.26
Levomepromazine	LOW	-8.20	-0.83
	MEDIUM	-12.31	-8.27
	HIGH	-12.03	-0.68
Clozapine	LOW	-9.29	7.82
	MEDIUM	-3.67	8.75
	HIGH	-8.11	7.51
Olanzapine	LOW	-0.62	-10.72
	MEDIUM	-6.87	4.21
	HIGH	-3.33	3.99