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Author: Manuela Licata Cecilia Rustichelli Federica Palazzoli Anna Ferrari Carlo Baraldi Daniele Vandelli Patrizia Verri Filippo Marchesi Enrico Silingardi

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Hair testing in clinical setting: simultaneous determination of 50 psychoactive drugs and metabolites in headache patients by LC tandem MS✧[1]

Manuela Licata[1]✉, Cecilia Rustichelli[2], Federica Palazzoli[1], Anna Ferrari[3], Carlo Baraldi[3], Daniele Vandelli[1], Patrizia Verri[1], Filippo Marchesi[1], Enrico Silingardi[1]

[1] Department of Diagnostic Medicine, Clinical and Public Health, Division of Legal Medicine, University of Modena and Reggio Emilia, via del Pozzo 71, 41124 Modena, Italy

[2] Department of Life Sciences, University of Modena and Reggio Emilia, Via G. Campi, 103, 41125 Modena, Italy

[3] Department of Diagnostic Medicine, Clinical and Public Health, Division of Toxicology and Clinical Pharmacology, University of Modena and Reggio Emilia, via del Pozzo 71, 41124 Modena, Italy

Graphical abstract

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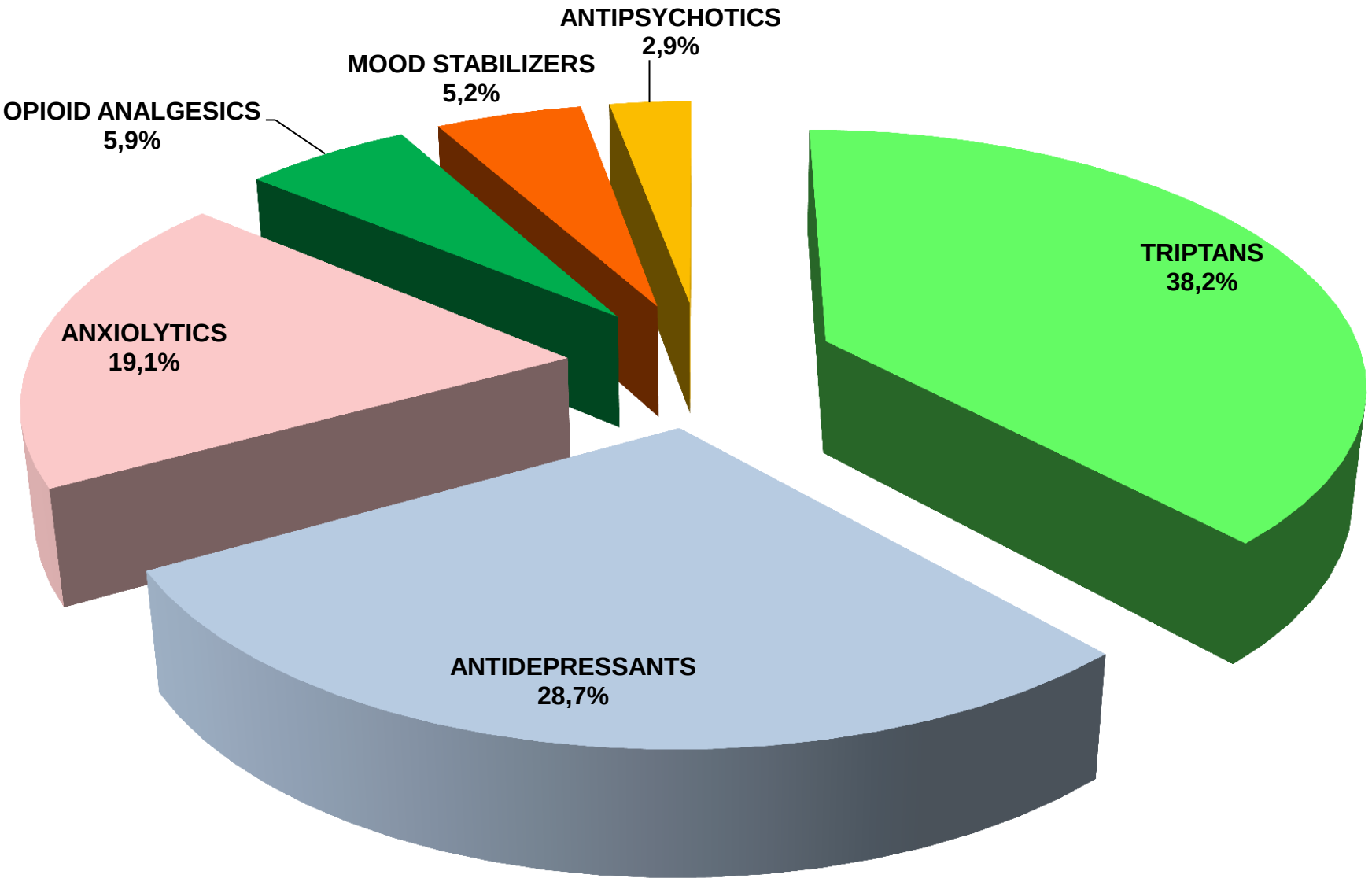
✉ Corresponding author: manuela.licata@unimore.it, Tel: +39 059 4224870; fax: +39 059 422474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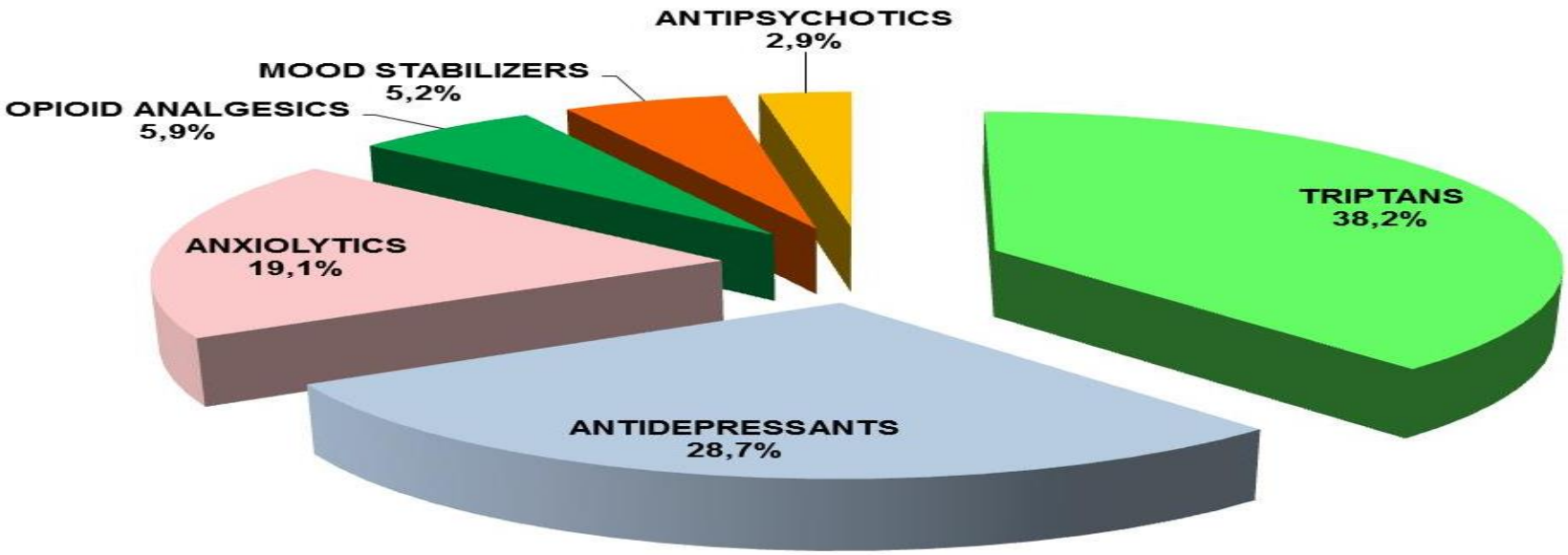
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Highlights

- A LC-MS/MS screening method for quantitative analysis of 50 psychoactive drugs in hair was validated.
- Hair extracts were purified for the first time by QuEChERS.
- The validated method was applied in 234 authentic hair samples from headache patients with known type and dosage of the taken drugs.
- The developed method could represent a helpful tool for intake monitoring.

Abstract

Headache patients suffering from recurrent attacks are a population at risk of overuse and abuse of analgesic medications. Associated with triptans, the first-line drugs recommended for the acute treatment, these patients usually take other medications such as opioids analgesics for the attack treatment, antidepressants and antiepileptics for prophylaxis treatment and benzodiazepines, non-benzodiazepine hypnotics and antipsychotics for the treatment of comorbidities. Regular and frequent use of triptans, like of any other symptomatic analgesic, can cause chronic headache and medication-overuse headache (MOH). In these circumstances, a detoxification treatment is necessary and therefore the monitoring and follow-up of the patients are crucial to the success of the treatment. In the present study, a LC tandem MS method has been developed for the identification of 50 psychoactive drugs in human hair, including triptans, benzodiazepines and metabolites, analgesics, antiepileptic, antidepressants and metabolites, a non-benzodiazepine hypnotic (z-drug), antipsychotics and metabolites. Hair samples were decontaminated, pulverized and incubated overnight in methanol; the extracts were then purified by a new and rapid QuEChERS procedure and analyzed by LC-MS/MS under gradient elution with positive ionization MRM mode. The procedure was fully validated in terms of selectivity, linearity, limit of detection and lower limit of

quantitation, precision and accuracy, carry-over, matrix effect, recovery and dilution integrity. The validated procedure has been applied to 234 real hair samples collected from headache patients with known type and dosage of the taken drugs; the obtained data could be of interest. to evaluate the xenobiotic concentrations in patients with known therapy.

Keywords: Psychoactive drugs; Hair samples; QuEChERS; HPLC-MS/MS; Headache Patients.

1. Introduction

The analysis of hair matrix has gained increasing importance for toxicologists in several fields. This alternative matrix permits a retrospective evaluation of the drug use history corresponding to several months before the actual sampling moment, depending essentially on hair length; furthermore segmental hair analysis can represent a tool to demonstrate cessation, decrease or increase of drug use or the adherence to the pharmacological treatment [1].

The choice of hair matrix to evaluate the common illicit drug exposure has been widely investigated and a wide consensus for hair data interpretation has been shown with consequent clinical and legal judgments. Today, the reliability of hair segmental analysis is well recognized, promoting the development and validation of specific and sensitive methods for therapeutic drug monitoring in hair sample [2]. In clinical setting, there is a need of screening methods suitable for a large amount of samples and with the capability to identify a wide range of analytes in a single analysis. The aim of the screening is twofold: the verification of the therapy adherence, comparing drug levels in different hair segments of the same subject, and, conversely, the possibility to prove not declared abuse/misuse of drugs.

It is well known that drug concentration in hair can change individually due to inter-subject variability; however, the assessment of the concentration in hair matrix of a wide cohort of individuals, whose drug intake is well known, makes possible to estimate ~~indicatively~~ the xenobiotic concentration in hair.

The LC-MS/MS procedures presented in the literature for hair analyses are often addressed to determine a limited number of well-defined analytes among drugs of abuse [3-5] or among pharmaceutical psychoactive substances and drugs of abuse [6-19]; in these cases, optimization of the sample treatment, clean-up and chromatographic conditions is focused on the physicochemical properties of the target analytes. This strategy is no more effective when a wide screening of xenobiotics has to be analyzed. Moreover, screening on large cohorts of subjects requires rapid, cheap and widescreen procedures, even to the detriment of optimal sensitivity and accuracy. For this reason, the extensive multi-target capacity of LC-MS/MS instrumentation is increasingly employed to develop protocols for drug screening after nonselective extraction procedures, which are adequate for analytes with widely different physicochemical properties [2].

Some papers in the literature described LC-MS/MS screening methods for the analysis of pharmaceuticals and drugs of abuse in hair [20-25]; nevertheless no procedure has been reported for the simultaneous analysis of the most commonly used anti-headache drugs.

Headache is a common and intense neurological disease characterized by recurrent moderate to severe headaches often in association with various nervous system symptoms. Headache treatment involves acute and preventive therapies; chronic patients with frequent attacks require both and therefore are usually treated with various drugs belonging to different pharmacological classes. Unfortunately, medication overuse frequently occurs, due to the patient's desire to treat quickly his/her headaches; this can make headache worse, leading to a Medication Overuse Headache (MOH) [26]. MOH is headache occurring on 15 or more days per month for more than 3 months, developing as a consequence of regular overuse of acute or symptomatic headache medications. Individuals with MOH typically fail to respond to acute treatments, worsening the disability associated with the syndrome; therefore the monitoring and follow-up of the patients are crucial to the success of the treatment [27-28].

The current study aims to develop an analytical procedure for the simultaneous identification in hair of 50 psychoactive drugs and metabolites belonging to different pharmaceutical classes: anxiolytics,

antidepressants, antipsychotics, mood stabilizers, opioid analgesics and triptans (Figure 1). To this purpose, analyte extraction has been performed with methanol, which has a wide spectrum of use and is compatible with testing procedures for the majority of xenobiotics; the clean-up of the obtained extracts has been achieved by QuEChERS dispersive SPE method. The purified samples have been analyzed by LC-MS/MS under gradient elution in positive scheduled MRM mode, thus monitoring the MRM transitions of the target analytes only around the expected retention time. This allows the monitoring of many MRM transitions per cycle without the need to sacrifice data quality. The procedure was fully validated in terms of selectivity, linearity, limit of detection and lower limit of quantitation, precision and accuracy, carry-over, matrix effect, recovery and dilution integrity. The validated procedure has been subsequently applied to hair samples collected from 234 patients under treatment in the Headache Centre of Modena University Hospital, for which type and dosage of taken drugs were known.

2. Material and Methods

2.1. Chemical and reagents

Standards of the target analytes (as free bases or salts) were supplied as pure substance or methanolic solution (1.0 or 0.1 mg/mL as free base) by Sigma (St. Louis, MO, USA), except Delorazepam, obtained as powder from LGC (LGC Standards S.r.l., Milano, Italy). Deuterated internal standards (ISs) were purchased as methanolic solution (0.1 mg/mL as free base) or pure substance from Sigma: Amitriptyline-D_3 hydrochloride, Citalopram-D_6 hydrobromide, Clozapine-D_4, Desalkylflurazepam-D_4, Fluoxetine-D_5 hydrochloride, α-Hydroxyalprazolam-D_5 and Morphine D_3; Pinazepam and 3,4-Methylenedioxy-N-propylamphetamine hydrochloride (MDPA HCl) were also used as internal standards and were supplied as powder by Alltech Applied Science (State College, PA, USA*).* All solvents and chemicals for LC-MS/MS were of LC-MS purity grade (Baker-VWR, Milano, Italy), while other chemicals used for sample preparation were of analytical grade (Carlo Erba, Milano, Italy). RoQ^{TM} QuEChERS dSPE kit (2.0 mL, 150 mg $MgSO_4$, 50 mg

PSA-Primary Secondary Amine, 50 mg endcapped C18) used for extract purification was supplied by Phenomenex (Torrance, CA, USA).

2.2. Study design and specimen collection

Blank hair specimens for quality control samples were collected from 10 volunteers abstinent from any drugs. Authentic hair samples were collected from headache patients participating in a study committed in collaboration with the Headache Centre of Modena University Hospital. Informed consent was received from all participants; the study was approved by the Ethical Committee of Modena and all study procedures were conducted accordingly. The type and dosages of the medications used during the three months before the hair sampling were documented for each patient in the corresponding self-report. Of a total of 300 patients, 66 samples were excluded on the basis of insufficient weight (< 50 mg) and/or length (< 4.0 cm). The selected cohort consisted of 234 white subjects (13 men and 221 women, 18 to 82 years of age, mean age: 42 years). In accordance with the Society of Hair Testing guidelines [29], hair samples were cut from the scalp of the posterior vertex of the head as close as possible to the scalp and stored at room temperature until processing. The proximal and distal ends were carefully identified for all hair samples; each hair sample was cut to obtain the proximal 3.0 cm segment, which was subjected to the sample processing. The color of the analyzed hair ranged from brown to dark brown for most samples; in some cases, evidence of cosmetic treatments was present.

2.3. Preparation of standard solutions

A stock solution was prepared and diluted with methanol to obtain working solutions at ten different concentration levels in the range 2.0–1500.0 pg/μL for all analyte; for Tramadol, 10 individual solutions were prepared in the same concentration range. An internal standard (IS) solution was also prepared by diluting a stock solution containing the deuterated analogues,

Pinazepam and MDPA with methanol up to the concentration of 500.0 pg/µL for each IS. All solutions were stored at -20°C until use.

2.4. Hair extraction and clean-up

All hair samples (3.0-cm proximal segment) were decontaminated as recommended by the Society of Hair Testing guidelines [29]. Briefly, hair samples were washed with acetone (2 x 5.0 mL) and n-hexane (2 x 5.0 mL) and dried under nitrogen at room temperature. All washed hair samples were pulverized twice by Precellys®24 (Bertin Technologies-Alphatech SpA, Genova, Italy) at a rotation frequency of 6000 rpm for 30 sec, then allowed to cool for 2 min; this treatment provided 1.0 to 2.0 mm length segments and the temperature inside the device did not exceed 40.0°C, thus avoiding any overheating of the sample. 50 mg aliquots of the pulverized hair samples were added with 2.0 mL of methanol and 20.0 µL of ISs solution and sonicated overnight at 45°C; after addition of the roQdSPE QuECheERS sorbent kit, the tubes were vortexed for 1 min and centrifuged at 5000 rpm for 4 min. 1.0 mL of each purified supernatant was transferred to a 2.0 mL plastic Eppendorf and evaporated to dryness under a stream of nitrogen. The purified extracts were reconstituted in 200.0 µL of the LC mobile phase and a 10.0 µL aliquot was subjected to LC-MS/MS analysis.

2.5. LC-MS/MS conditions

LC analyses were performed on an Agilent 1200 LC system consisting of a binary pump, an autosampler, an on-line degasser and a thermostatted column compartment (Agilent, Waldbronn, Germany).The purified hair extracts were analyzed on a Kinetex® Biphenyl column (50 × 2.1 mm; 5.0 µm particle size; pore size 100˚A) preceded by a ULTRA Biphenyl Security Guard Cartridge (2.0 x 2.0 mm) (Phenomenex, Torrance, CA, USA). The mobile phase was composed of (A) ammonium formate 1mM and 0.1% formic acid in water and (B) acetonitrile/methanol (70/30) added with ammonium formate 1 mM and 0.1% formic acid; the chromatographic separation was achieved with a gradient elution using the following program: 0-15 min, linear gradient from 5 to

95% (B); 15-17 min, isocratic at 95% (B), 17-18 min linear gradient from 95 to 5% (B). A pre-equilibration period of 12 min was used between each run. The flow-rate was 0.25 mL/min and the column temperature was 40°C. The injection volume was 10.0 μL and the injector needle was washed with methanol; the autosampler was maintained at room temperature.

The chromatographic conditions were optimized by analyzing the standard solutions and also extracts of blank hair spiked with the target analytes and the ISs.

Tandem mass spectrometry was performed using an API 4000 QTRAP mass analyzer equipped with a Turbo Ion Spray source (SCIEX Toronto, Canada) operating in ESI positive mode. The Analyst® software (version 1.5.1, SCIEX) was used for instrument control, data acquisition, qualitative and quantitative data analyses. Detection and quantitation of all analytes were accomplished using scheduled multiple reaction monitoring mode (sMRM) due to achieved high selectivity and sensibility. Optimized instrument settings were as follows: curtain gas (N2) pressure, 10 psi; CAD gas, 4 psi; nebulizer gas (GS1), 40 psi, heater gas (GS2), 50 psi, IonSpray voltage, 5500 V, Turbo Heater temperature, 550°C, declustering potential (DP), 60 V, collision cell exit potential (CXP), 10 V. The nitrogen flow was produced by a gas generation system (Nitrogen Generator model 75–72, Whatman Inc., Haverhill, MA, USA). MS/MS parameters were optimized by direct infusion of each individual analyte and IS at 100.0 pg/μL in the initial LC mobile phase at a flow of 10.0 μL/min. The validity of the chosen MRM transitions was also verified by LC-MS/MS analyses of blank hair extracts spiked with the analytes and the ISs at 100.0 pg/mg hair. The mass spectrometer was calibrated to < 2.0 mDa mass error prior to each batch analysis. All MRM transitions, collision energies and retention times for the given analytes are summarized in Table 1.

2.6. Validation

Method validation was accomplished according to the Scientific Working Group of Forensic Toxicology (SGWTOX) standard practices for method validation in forensic toxicology [30].

The following parameters were evaluated: selectivity, calibration model, limit of detection (LOD), lower limit of quantitation (LLOQ), precision, accuracy, carry-over, matrix effects, recovery and dilution integrity.

2.6.1. Calibration and quality control samples

Blank hair specimens were decontaminated and pulverized as described above. Aliquots (50 mg) of the pulverized hair, added with 20.0 μL of the IS solution and 50.0 μL of the working solutions, were subjected to the described sample processing in order to have 10 calibration level samples in the range 2.0–1500.0 pg/mg hair for each target analyte; the concentration of the ISs was 200.0 pg/mg hair.

Quality control (QC) samples were prepared by spiking blank hair specimens (50 mg) with 20.0 μL of the ISs solution and 50.0 μL of the working solutions at three concentration levels. These QC samples were processed as described above; the final concentration in the samples was 20.0, 200.0 and 1500.0 pg/mg hair for low, medium and high levels, respectively; n=6 for each level.

Since Desvenlafaxine and Tramadol have the same retention time (5.95 min) and common fragments (58.0 and 246.2), calibration solutions and quality control samples for Tramadol method validation were prepared apart. ~~Method validation for Tramadol was accomplished separately on calibration and quality control samples containing only the individual analyte and the IS.~~

2.6.2. Selectivity

Aliquots (50 mg) of pulverized hair samples, obtained from 10 different volunteers abstinent from any drugs, were added with 70.0 μL of methanol and processed as described above. These blank samples were individually assessed for the presence of any interference across the retention window of each analyte and the ISs.

2.6.3. Calibration model

The processed calibration samples were analyzed in triplicate (injection volume: 10.0 μL). Calibration curves were generated from the peak-area ratio of each analyte quantifier transition to the assigned IS (reported in Table 1); the ratio was then plotted on the y-axis against the nominal analyte concentration to generate the standard curves by the method of least squares using a weighed (1/x) linear regression model.

2.6.4. LOD and LLOQ

The sensitivity of the developed analytical procedure was evaluated by determining the limit of detection (LOD) and the lower limit of quantitation (LLOQ) for each analyte. Sets of blank hair were fortified at 50.0, 20.0, 10.0, 5.0 and 2.0 pg/mg hair and subjected to the described sample processing. The LLOQ value represented the lowest concentration of the analyte that was capable of reproducibility providing symmetrical peaks and the minimum mass spectral identification ratios, while maintaining a bias of ± 20% and % CV < 20% [30]. The LOD value for each analyte was estimated from the standard deviation of the *y*-intercept (s_y) and the average slope (Avg_m) as: LOD = 3.3 s_y/Avg_m [30].

2.6.5. Precision and accuracy

Method precision and accuracy were determined by replicate analyses of the QC samples spiked at low, medium and high levels (n=6, each).

The intra-batch precision (repeatability) and the inter-batch precision (reproducibility) were calculated as percent relative standard deviation (RSD%) of the "found" values: RSD% = (SD/mean) x 100. Accuracy of the method was evaluated by comparing the levels found in hair samples spiked after the extraction procedure with the nominal analyte concentration; the obtained values are expressed as percent of the estimated concentration.

2.6.6. Carry-over

Carry-over effect was evaluated by injecting extracts of blank hair samples after analyses of calibration samples spiked at the upper limit of the calibration range (1500.0 pg/mg hair). For acceptance, the peak areas in the blank sample should not exceed 10% of the peak areas obtained for the lowest calibrator [30].

2.6.7. Matrix effect and recovery

According to Matuszewski et al. [31], matrix effect (ME) was evaluated for each analyte by dividing the analyte peak area in QC samples spiked after the dispersive SPE procedure (post-spiked samples) to the response for the neat standard. For post-spiked samples, 1.0 mL aliquots of blank extracts purified by QuECheRS were added with 10.0 μL of the ISs solution and 25.0 μL of the working solutions (20.0, 200.0 and 1500.0 pg/μL; n=6 for each set), dried under nitrogen flow and reconstituted in 200.0 μL of the initial LC mobile phase; for neat standard samples, 10.0 μL of the ISs solution and 25.0 μL of the working solutions (20.0, 200.0 and 1500.0 pg/μL; n=6 for each set) were dried under nitrogen flow and reconstituted in 200.0 μL of the initial LC mobile phase. Recovery values were determined by comparing the levels found in QC samples spiked before the sample processing with the levels found in the post-spiked samples, i.e. with no nominal loss of the target analytes [31].

2.6.8. Dilution integrity

During authentic sample analysis, excessively high concentrations that are above the established calibration range may be encountered. To bring the analyte concentration within the validated concentration range, the sample may be diluted, providing that accuracy and precision of the method are not significantly impacted [30]. To validate the dilution integrity, blank hair samples (50 mg) were spiked at 40 times the highest calibration sample and extracted as cited above; the extracts were then diluted 40-fold (n=6), 50-fold (n=6) and 60-fold (n=6) with purified extracts obtained from blank hair samples. 1.0 mL of the obtained samples were evaporated, reconstituted in the LC

mobile phase and analyzed against the calculated calibration curves to assess if the performance criteria were still met.

3. Results and discussion

3.1. Sample preparation

Regarding extraction phase, methanol is an universal extraction medium. The extraction mechanism provides that the hydrophilic methanol penetrates the hair matrix leading to swelling and drug liberation via diffusion; as an organic solvent, methanol dissolves neutral and lipophilic compounds [32].

The clean-up of the hair extracts is particularly important in the case of analyses based on LC-MS, because of the effects of ion-suppression/enhancement, and it is generally carried out by liquid-liquid extraction (LLE) or by solid phase extraction (SPE). SPE procedures exhibit high selectivity and the extracts may contain fewer interfering substances than LLE extraction, but are relatively laborious and time-consuming.

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method was originally developed in 2003 for extracting a wide range of pesticides in fruit and vegetables; subsequently the method was successfully applied to the analysis of other groups of compounds, including pharmaceuticals, in a wide variety of complex matrices [33]. Recently, this methodology has been applied to human whole blood for forensically relevant drugs and poisons determination [34-35].

The clean-up process by QuEChERS involves two steps: firstly, homogenized samples are extracted using an organic solvent (step 1), then, the supernatants are cleaned using a dispersive solid phase extraction (dSPE) technique (step 2). These dSPE kits contain magnesium sulfate to remove residual water and primary secondary amine (PSA) sorbent to remove sugars and fatty acids; the kits are available with or without graphitized carbon (GCB) to remove pigments and sterols and/or endcapped C18 packing to remove non-polar interferences such as lipids. Best results in terms of

recovery and clean extracts were achieved with the kit containing $MgSO_4$, PSA and endcapped C18; the method is rapid, simple and offers a selectivity similar to conventional SPE procedures.

3.2. LC-MS/MS analyses

After optimization of the chromatographic gradient, retention time reproducibility for the analytes and the ISs was estimated by analyzing 10 extracts of blank hair spiked at 100.0 pg/mg on the same day and on three different days. The repeatability of the retention times was satisfactory, with relative standard deviation (RSD%) values less than 0.4% for all analytes and ISs.

Two or three transitions were monitored for each analyte, except Eletriptan and Norfluoxetine, for which only one transition was monitored, due to the low intensity of the other transitions. For some halogen-containing analytes, we monitored as qualifier the same transition chosen as quantifier with the *m/z* values shifted according to the corresponding isotopic pattern, when the intensities of the other daughter ions were low. The analytical LC-MS/MS parameters for the target analytes are shown in Table 1; the quantifier transitions are highlighted in bold characters.

Each analyte was identified by the corresponding LC retention time, precursor ion and MRM transitions; this enabled to identify analytes with the same precursor ion and /or a MRM transition with identical *m/z* value, as occurred for Desvenlafaxine and Tramadol. These two analytes elute with similar retention time and share two common fragment ions; nevertheless they can be differentiated by the transition *m/z* 264.2 → 201.0, present as quantifier for Desvenlafaxine and absent for Tramadol. In the case of the simultaneous presence of both analytes, the quantitative determination of Tramadol should be performed by an alternative validated method previously published [36].

For the deuterated internal standards (ISs) we monitored the same quantifier transition of the target analytes with *m/z* values shifted to the corresponding mass values (Table 1). Due to the large number of analytes, only representative internal standards (deuterated and non-deuterated) were

included; the selected ISs were representative of different compound classes and had retention times spanning across the total run time (Table 1).

Figure 2 presents a chromatogram of a blank hair sample spiked at 20.0 pg/mg hair (except for Fluoxetine and Norsertraline: 50 pg/mg hair) showing the quantifier and qualifier MRM transitions for the target analytes.

3.3. Validation results

3.3.1. Selectivity

No interferences were detected at the retention times of the analytes and the internal standards; therefore the developed procedure was found to be selective for all analytes and ISs.

3.3.2. Calibration model, LOD and LLOQ

For all tested analytes the linearity was adequate in the range LLOQ–1500.0 pg/mg hair (Table 2) ~~(for Fluoxetine and Norsertraline: 50.0–1500.0 pg/mg hair)~~, with correlation coefficient values (R^2) of at least 0.990. The LOD and LLOQ values for each analyte were estimated as described above and validated by analyzing blank hair samples fortified with the analytes at the calculated LOD and LLOQ levels. The obtained data fulfilled the usually acceptance criteria, being the corresponding deviation from the expected concentration (% accuracy) less than 20%. The LLOQ values averaged 5.0–20.0 pg/mg hair, except for Fluoxetine and Norsertraline (LLOQ: 50 pg/mg hair), confirming the satisfactory sensitivity of the developed procedure; the LOD values for all analytes ranged between 2.0–10.0 pg/mg hair, except for Fluoxetine and Norsertraline (LOD: 20 pg/mg hair) ~~(Table 2).~~

3.3.3. Precision and accuracy

Intra-batch precision (repeatability), inter-batch precision (reproducibility) and accuracy were evaluated in the QC samples spiked at low, medium and high concentrations (20.0, 200.0 and

1500.0 pg/mg hair; n=6 for each level) as described above. In the case of Fluoxetine and Norsertraline, the QC samples at low level were spiked at 50.0 pg/mg hair, according to the LLOQ values estimated for these analytes. The calculated data are shown in Table 2. The intra-batch and inter-batch precision values were satisfactory, being the corresponding RSD% values lower than 20% for all analytes at all concentration levels; since the obtained data comply with the SGWTOX guidelines [30], the method was considered precise for all analytes. Accuracy values were in the range ± 20%, which are within the accepted limits for this parameter [30].

3.3.4. Carry-over

As suggested [30], the carry-over value should not exceed 10% of signal of the lowest calibrator. The observed effects were negligible for all analytes, being the peak areas in the blank sample <5% of the peak areas found for the calibrator spiked at the lowest concentration, which are within the proposed acceptance limits for this parameter.

3.3.5. Matrix effect and recovery

Concerning the matrix effect, there are often endogenous matrix components that may co-elute with the target analytes, which are often invisible to the MS detector at the monitored masses but that may significantly affect the efficiency and reproducibility of the ionization process. This may cause an erroneous decrease/enhancement (ionization suppression/enhancement) in the signal response, which is termed "matrix effect"; the matrix effect had to be considered when quantifying a compound. The matrix effect was evaluated according to the model proposed by Matuszewski [32] at low (20.0 pg/mg hair, except for Fluoxetine and Norsertraline: 50 pg/mg hair), medium (200.0 pg/mg hair) and high (1500.0 pg/mg hair) levels. The suggested values for matrix effect [30] should be lower than 25% for both suppression and enhancement; no marked matrix effect was observed in the QC samples for most analytes, being the corresponding values within the prescribed range (Table 2). A considerable matrix effect in terms of ionization enhancement (≥ +75%) was found for

Eletriptan, Zolmitriptan, Frovatriptan, Almotriptan, Rizatriptan, and also for Levomepromazine. Nortriptyline, Amitriptyline, Duloxetine and Sertraline showed effects of ion suppression ranging from –59% to –71%, whereas for Lorazepam, Lormetazepam, Citalopram and Clonazepam the effect of ion suppression ranged from –30 to –35%. This fact indicates the presence in the final extracts of residual matrix components affecting markedly the ionization process only for the cited analytes; for quantitative analyses, the matrix effect has been compensated by the use of an ~~adequate~~ Internal Standard. The addition of ISs characterized by similar retention time and ionization behavior of the target analytes, such as isotopically labelled ISs, can efficiently compensate the matrix effect. As a matter of fact their use is rather expensive, especially in multideterminative methods, where an IS for each analyte is theoretically required [37]; for this reason, we selected only some adequate ISs. In this way, the matrix effect can be minimized and the variability (RSD%) of the matrix effect measured in different QC samples was less than 20%. Recovery was calculated at low, medium and high concentrations (Table 2). The recovery values were higher than 84% for most analytes, suggesting that the extraction efficiency was adequate at all the tested concentrations; for 9 analytes the recovery averaged 51.4–75.1 % and only 4 analytes presented recovery values in the range 32.4–47.6%.

The low recovery values observed for some analytes can be explained on the basis of the choice of methanol as extraction solvent. Methanol does not fit as the best QuEChERS solvent, resulting in a lack of analyte partitioning [38]; nevertheless we chose methanol because it can extract almost all drugs (neutral, hydrophilic and moderately lipophilic compounds): due to its hydrophilic character, it penetrates the hair, producing swelling of the matrix and the liberation of the drugs. Despite the low recovery values found for some analytes, the developed method can afford LOD and LLOQ values that are adequate to monitor drug use pattern. As a matter of fact, the purpose of this study was the development of a simple and fast method for the analysis of a wide range of analytes in the hair matrix (multideterminative screening method).

3.3.6. Dilution Integrity

In the dilution integrity studies, the obtained values fulfilled the suggested acceptance criteria: RSD% values for precision were < 20% and accuracy values were in the range ± 20%. Since the dilution integrity was satisfied, extracts of authentic hair samples with analyte concentrations exceeding the calibration range were diluted and reanalyzed until the concentration was within the calibration range.

3.4. Authentic hair analysis results

The validated analytical method was applied to authentic hair samples from 234 headache patients. Among the analyzed samples, 58.1% (n = 136) confirmed positive for at least one of the target analytes and 30.3% (n = 71) for at least two drugs. Triptans were the most detected analytes in the positive headache patients (38.2%; n = 52), followed by antidepressants (28.7%; n = 39) and anxiolytics (19.1%; n = 26); opioid analgesics, mood stabilizers and antipsychotics were found less frequently in the examined cases (2.9 – 5.9%; n = 19 total), as shown in Figure 3.

The negative hair samples were distributed as follows: 81 cases (34.6%) were negative expected (none of the 50 analytes declared in the self reports), 10 cases (4.3%) reported a triptan intake in the last 3 months below 4 DDD (defined daily dose = Almotriptan < 50 mg, Frovatriptan < 10 mg, Sumatriptan < 200 mg, Zolmitriptan < 10 mg), 2 cases described the intake of Etizolam (2.5 mg in 3 months) and Alprazolam (used twice in the last week), 2 cases declared the intake of Levosulpiride (used in an episode of headache and vomit), 2 cases reported an occasional Citalopram intake only in the last month, 1 case described a Paroxetine intake but the patient showed a poor adherence to the pharmacological treatment.

Figure 4 shows the MRM chromatogram of a hair sample taken from a female patient declaring a regular (daily) oral use of Amitriptyline (50.0 mg/day) and Diazepam (4.0 mg/day) during the last three months; Diazepam was also assumed intravenous for 30 days (20.0 mg/day). Moreover the patient reported the oral intake of Topiramate (100.0 mg/day for 7 days), Rizatriptan (10.0 mg/day

for 20 days) and the intravenous use of Tramadol (100.0 mg/day for 6 days). The developed analytical procedure enabled the identification and the quantitation in the hair sample of the cited substances: Amitriptyline (2053.0 pg/mg), Diazepam (434.0 pg/mg), Topiramate (3646.0 pg/mg), Rizatriptan (11.0 pg/mg), Tramadol (789.0 pg/mg) and also of Nordazepam (Diazepam metabolite; 129.0 pg/mg), Nortriptyline (Amitriptyline metabolite; 491.0 pg/mg) and O-Desmethyltramadol (Tramadol metabolite; 81.2 pg/mg), while N-desmethyltramadol level was < LOD.

Figure 5 shows the MRM chromatogram of a hair sample taken from a male patient known to be an Eletriptan overuser (oral intake: 40.0 mg daily for 90 days). The patient reported also an intravenous therapy with Clomipramine and Delorazepam (12.0 mg/day and 0.5 mg/day for ten days, respectively) and the oral intake of Amitriptyline (10.0 mg/day for 19 days). Sumatriptan was administered only once (6.0 mg/day iv) in the examined period. The patient fulfilled the diagnostic criteria for MOH, being the taken doses of Eletriptan above the threshold value established by the guidelines [26]. The hair analysis confirmed the presence of the declared substances: Eletriptan (4375.0 pg/mg), Clomipramine (350.0 pg/mg), Delorazepam (17.0 pg/mg), Amitriptyline (221.0 pg/mg) and also of Nortriptyline (139.0 pg/mg); Lorazepam was found in traces (< LLOQ), whereas Sumatriptan level was below the corresponding LOD value. The obtained data confirmed the ability of the developed method to monitor occasional intakes (10 doses in 3 months for Clomipramine and Delorazepam), although the single administration of Sumatriptan was not detected.

Table 3 reports in details the obtained results, showing for each analyte the total amount declared by the patients during the 3 months before the hair collection and the range concentration (pg/mg hair) found in the hair of the positive samples. None of the analyzed real samples was simultaneously positive to both Desvenlafaxine and Tramadol.

In the case of metabolites subjected to quantitative determination, we calculated the parent drug to metabolite concentration ratio in the analyzed hair samples (Table 3).

For Citalopram, the parent drug exhibited higher concentrations compared to its metabolite N-Desmethylcitalopram, with concentration ratios always greater than 1.0, as reported also in the literature [22]; also Delorazepam levels were always higher compared to those of its metabolite Lorazepam.

The calculated values for Amitriptyline/Nortriptyline concentration ratio were similar to those reported by other authors [11, 22-23]; the samples showing higher levels of Nortriptyline, i.e. leading to parent drug/metabolite ratio values < 1.0, belonged to patients with gray hair or subjected to cosmetic treatments.

In the hair samples positive to Venlafaxine, the concentration of Desvenlafaxine was always higher compared to the parent drug (ratio < 1.0), confirming the preferential incorporation of the metabolite into the keratin matrix.

The metabolite O-Desmethyltramadol was detected only in 6 hair samples, with concentration levels always lower than those of the parent drug, confirming the major incorporation of Tramadol into the hair matrix, as pointed also in our previous paper [36].

For other analytes the number of positive hair samples was too scarce for a meaningful evaluation of the parent drug to metabolite concentration ratio.

4. Conclusions

A sensitive, reproducible and rapid LC-MS/MS screening method has been developed for the detection of 50 psychoactive drugs and metabolites; the method was fully validated according to international guidelines. For the first time a modified QuEChERS dSPE method was used in the cleanup of the methanolic hair extracts; the proposed sample processing is rapid, simple and offers adequate recoveries for most analytes. The LC-MS chromatographic profile was good, well reproducible and no interfering peaks were observed in quality control samples.

The applicability of the screening method has been demonstrated by analyzing 234 authentic hair samples from headache patients; among the analyzed samples, 136 (58.1%) confirmed positive for

at least one of the target analytes and the negative cases were negative expected or were related to patients with poor adherence to treatments or with random drug use in the three studied months. On the basis of the obtained data, the present study provides the quantitative measurements of the target xenobiotics in hair of patients with known intake and represents also a strategy for therapeutic drug monitoring in the clinical field in order to evaluate the adherence to the prescribed therapy in polypharmacy patients and also to assess a potential drug misuse/abuse not declared in the self-report.

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Fig. 1

List of the target analytes with their different therapeutic classes.

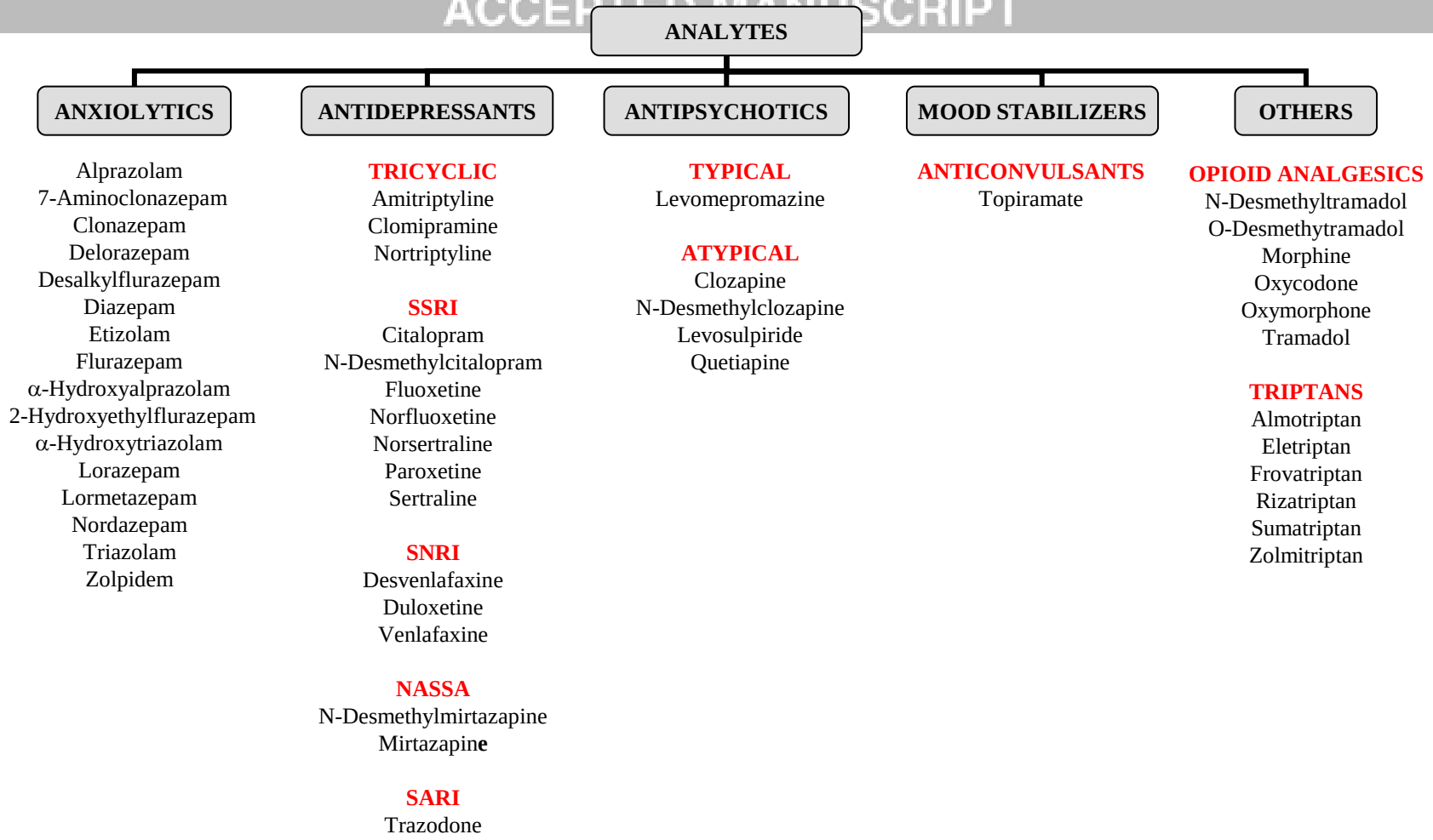
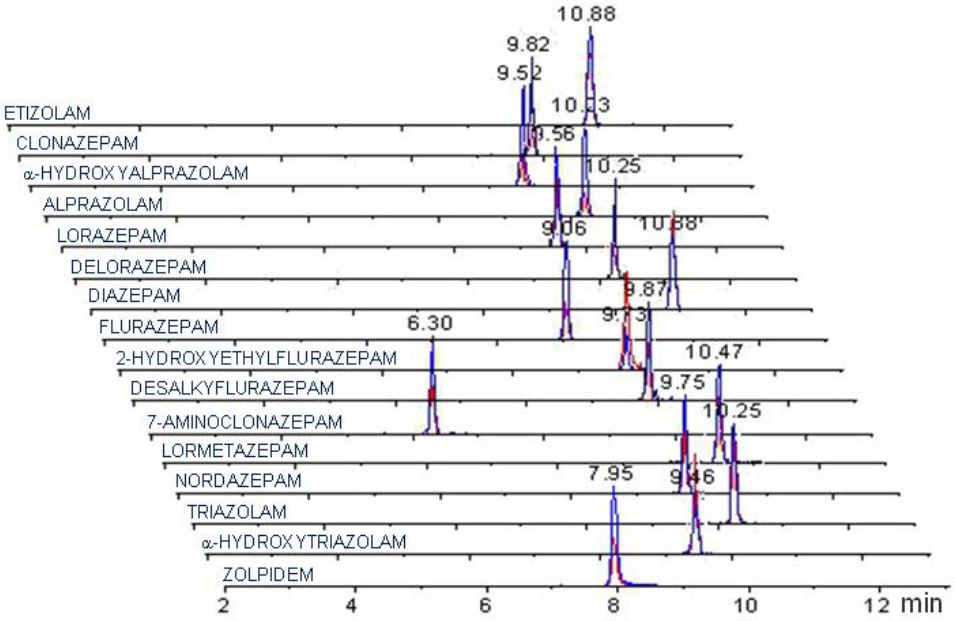
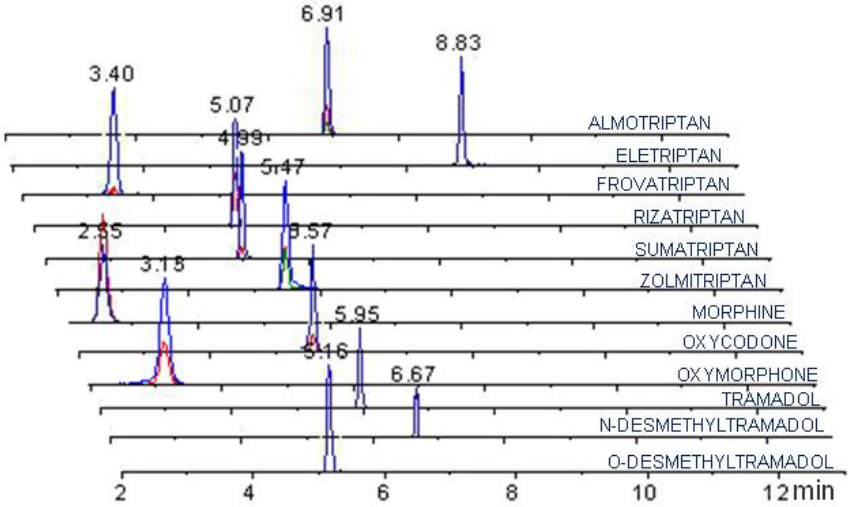
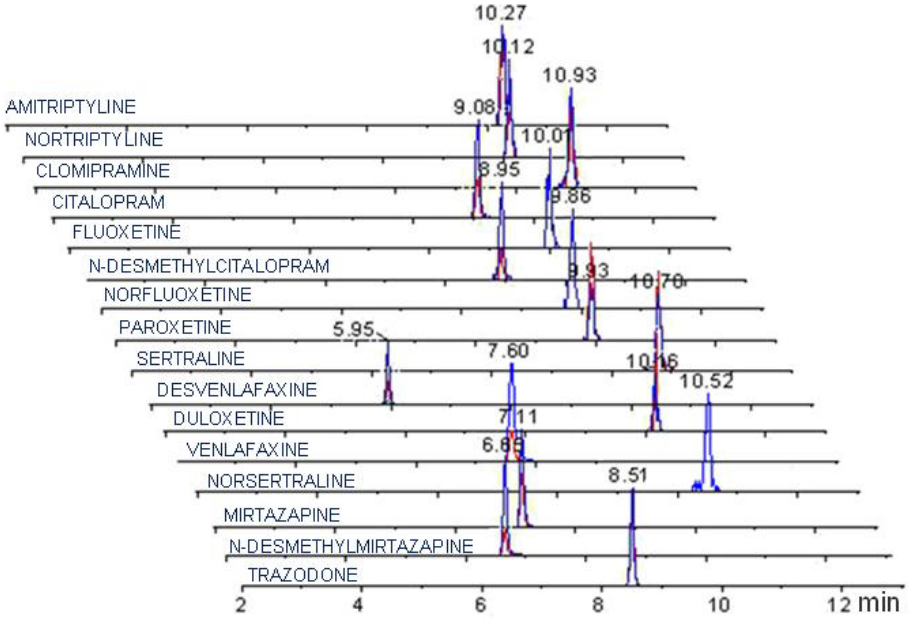


Fig. 2

Representative chromatograms showing the selected quantifier and qualifier MRM transitions for the 50 analytes in a blank hair sample spiked at 20.0 pg/mg (except for Fluoxetine and Norsertraline: 50 pg/mg).







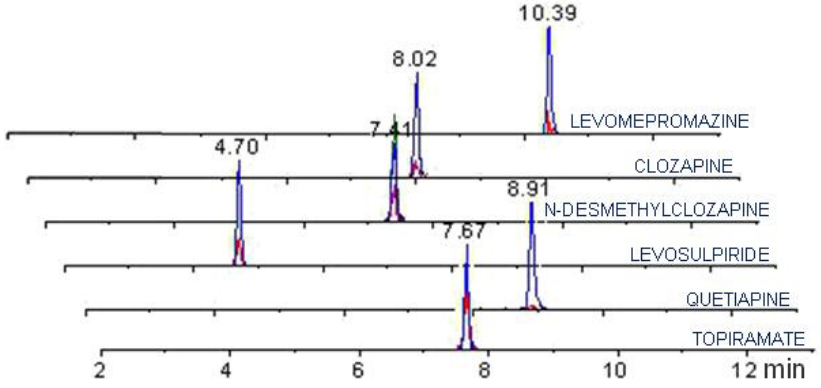


Fig. 3

Pie chart summarizing the drug classes found in the analyzed authentic hair samples.

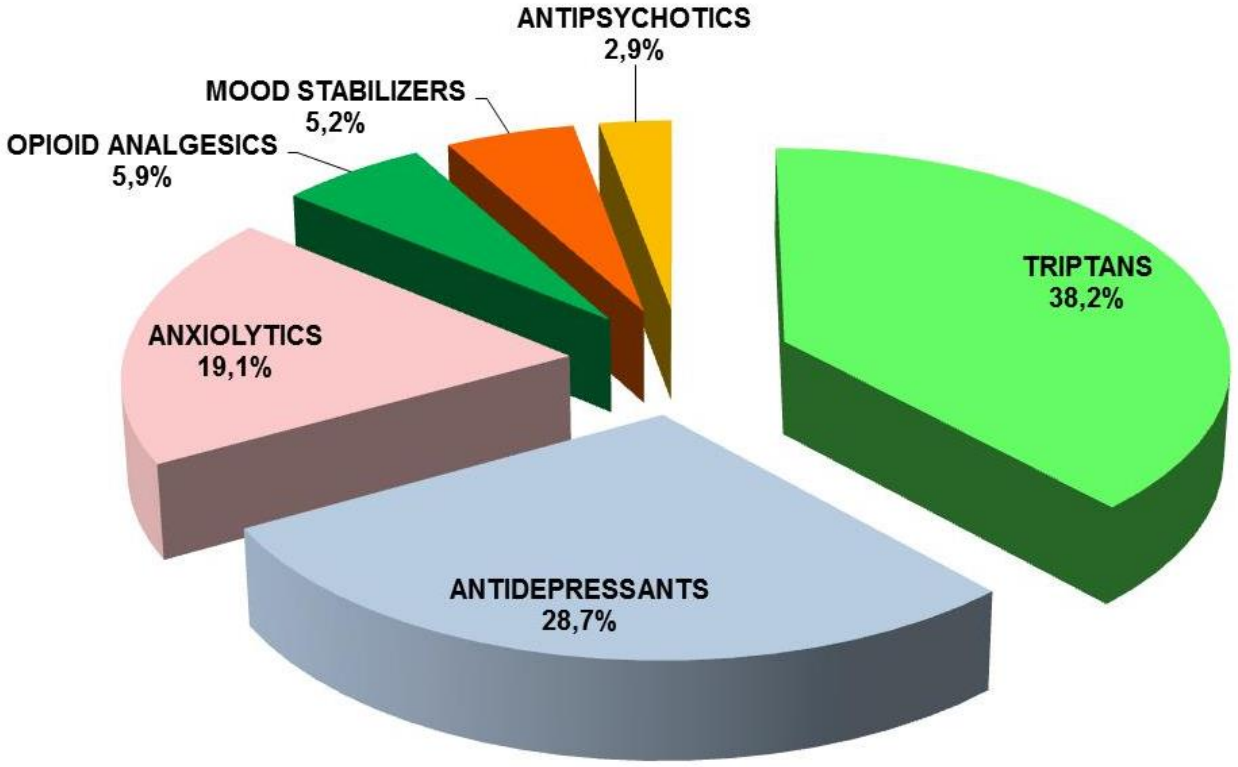

ANTIPSYCHOTICS
2,9%
MOOD STABILIZERS
5,2%
OPIOID ANALGESICS
5,9%
TRIPTANS
38,2%
ANXIOLYTICS
19,1%
ANTIDEPRESSANTS
28,7%

Fig 4

MRM chromatogram of a real hair sample positive for: Diazepam (434.0 pg/mg), Nordazepam (129.0 pg/mg), Amitriptyline (2053.0 pg/mg), Nortriptyline (491.0 pg/mg), Topiramate (3646.0 pg/mg), Rizatriptan (11.0 pg/mg), Tramadol (789.0 pg/mg) and O-Desmethyltramadol (81.2 pg/mg). N-desmethyltramadol level was < LOD.

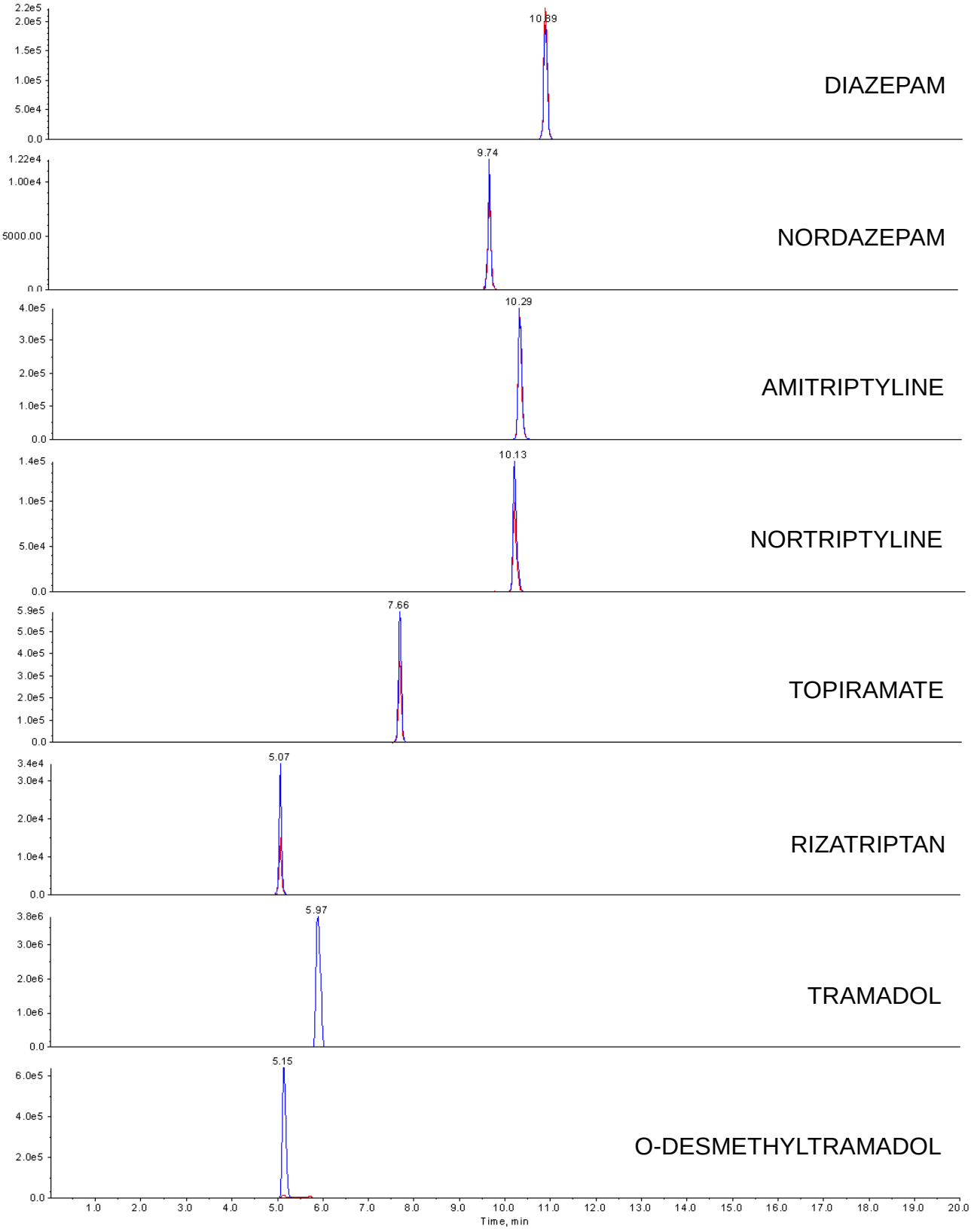


Fig. 5

MRM chromatogram of a real hair sample positive for: Clomipramine (350.0 pg/mg), Delorazepam (17.0 pg/mg), Eletriptan (4375.0 pg/mg), Amitriptyline (221.0 pg/mg), Nortriptyline (139.0 pg/mg). Lorazepam level was < LLOQ and Sumatriptan level was < LOD.

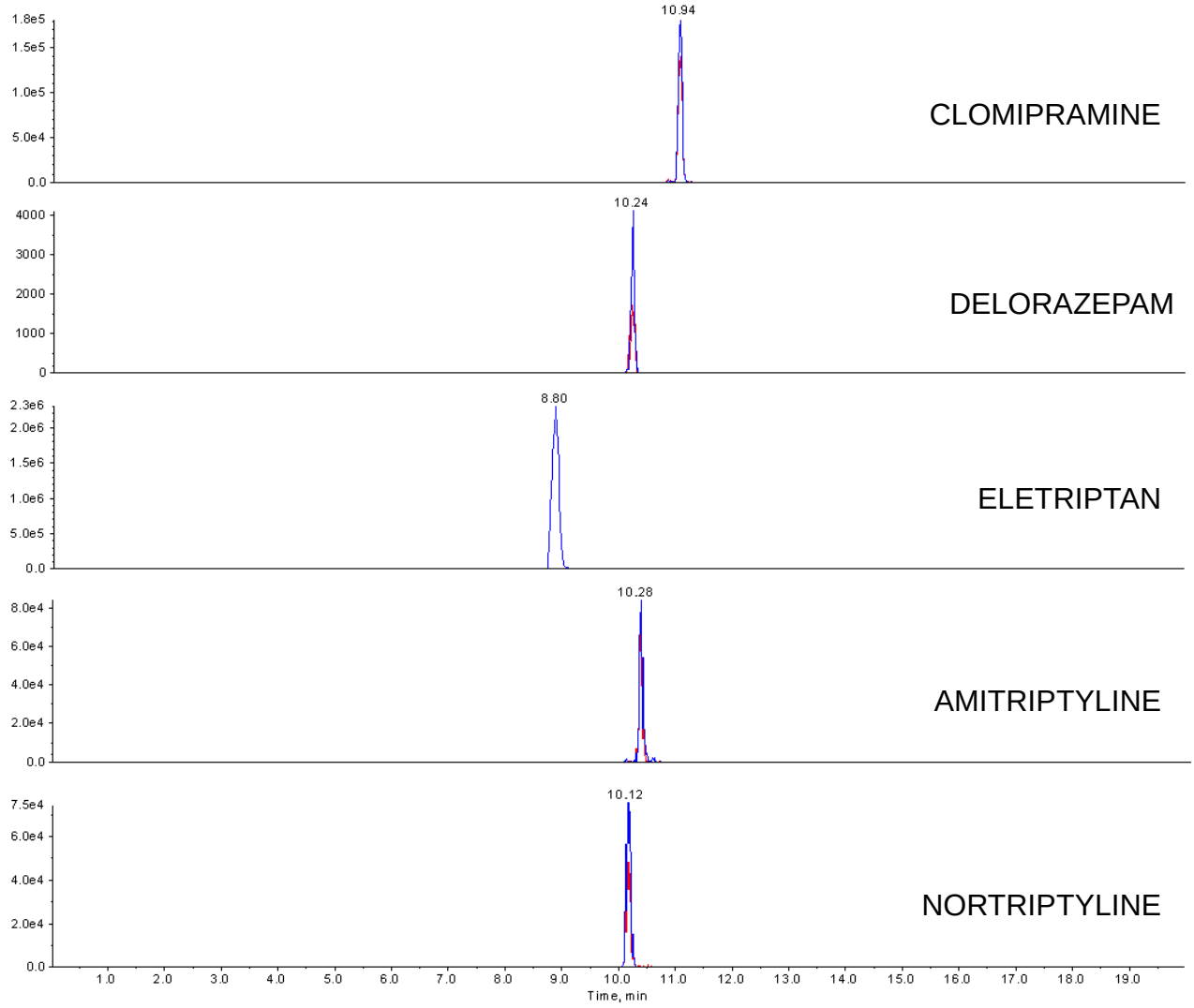


Table 1 LC-MS/MS parameters for the target anaytes and the Internal Standards (quantifier transitions are highlighted in bold characters); CE: collision energy (Hz).

Analyte	**MRM transitions**	**CE**	**Tr (min)**	**IS**
Almotriptan	336.1 → **58.0**, 201.2, 291.2	**52**, 23, 29	6.91	Clozapine-D_4
Alprazolam	309.2 → **281.0**, 274.0	**38**, 36	10.23	Pinazepam
7-Aminoclonazepam	286.1 → **121.0**, 222.1	**44**, 36	6.30	Desalkylflurazepam-D_4
Amitriptyline	278.3 → **233.2**, 117.1	**25**, 34	10.27	Amitriptyline-D_3
Citalopram	325.2 → **109.0**, 262.2	**35**, 28	9.08	Citalopram-D_6
Clomipramine	315.3 → **86.0**, 58.0	**26**, 62	10.93	Citalopram-D_6
Clonazepam	316.1 → **270.1**	**36**	9.82	Desalkylflurazepam-D_4
	318.1 → 272.0	37		
Clozapine	327.2 → **270.2**, 227.0	**32**, 44	8.02	Clozapine-D_4
Delorazepam	305.1 → **140.1**	**44**	10.25	Desalkylflurazepam-D_4
	307.0 → 142.0	43		
Desalkylflurazepam	289.0 → **140.1**, 226.1	**43**, 41	9.87	Desalkylflurazepam-D_4
N-Desmethylcitalopram	311.6 → **109.0**, 262.1	**31**, 28	8.95	Citalopram-D_6
N-Desmethylclozapine	313.2 → **270.2**, 227.0, 191.9	**34**, 37, 53	7.41	Clozapine-D_4
N-Desmethylmirtazapine	252.2 → **195.0**, 209.1	**32**, 32	6.65	Clozapine-D_4
N-Desmethyltramadol	250.9 → **44.0**, 233.2	**37**, 12	6.67	MDPA
O-Desmethytramadol	250.9 → **58.1**, 233.0	**40**, 17	5.16	MDPA
Desvenlafaxine	264.2 → **201.0**, 58.0, 246.2	**24,** 39, 18	5.95	Desalkylflurazepam-D_4
Diazepam	285.1 → **193.1**, 154.0	**46**, 39	10.88	Desalkylflurazepam-D_4
Duloxetine	298.1 → **154.1**, 44.1	**9**, 32	10.16	Amitriptyline-D_3
Eletriptan	384.0 → **84.0**	**50**	8.83	Clozapine-D_4
Etizolam	343.3 → **314.2**, 138.0	**25**, 48	10.88	Desalkylflurazepam-D_4
Fluoxetine	310.3 → **148.2**	**13**	10.01	Fluoxetine-D_5
	311.2 → 149.2	13		
Flurazepam	388.2 → **315.0**	**33**	9.06	Desalkylflurazepam-D_4
	390.2 → 317.0	34		
Frovatriptan	245.0 → **213.8**, 188.0	**20**, 20	3.40	Morphine-D_3
α-Hydroxyalprazolam	325.3 → **297.1**	**37**	9.52	α-Hydroxyalprazolam-D_5
	327.1 → 299.1	34		
2-Hydroxyethylflurazepam	333.2 → **246.1**, 109.1	**37**, 40	9.73	α-Hydroxyalprazolam-D_5
α-Hydroxytriazolam	359.1 → **331.1**, 175.9	**40**, 40	9.46	α-Hydroxyalprazolam-D_5
Levomepromazine	329.2 → **99.9**, 242.1	**28**, 34	10.39	Clozapine-D_4
Levosulpiride	342.3 → **112.1**, 214.1	**36**, 46	4.70	α-Hydroxyalprazolam-D_5
Lorazepam	321.1 → **275.1**	**32**	9.56	Desalkylflurazepam-D_4
	323.1 → 277.2	33		
Lormetazepam	335.1 → **289.1**	**30**	10.47	Desalkylflurazepam-D_4
	337.1 → 291.0	30		
Mirtazapine	266.3 → **195.0**, 72.0	**35**, 31	7.11	Clozapine-D_4
Morphine	286.2 → **181.0**, 165.0	**48**, 54	2.55	Morphine-D_3
Nordazepam	271.1 → **165.1**, 208.1	**40**, 38	9.75	Desalkylflurazepam-D_4
Norfluoxetine	296.0 → **134.0**	**10**	9.86	α-Hydroxyalprazolam-D_5
Norsertraline	292.1 → **159.0**, 129.0	**29**, 28	10.52	Citalopram-D_6
Nortriptyline	264.3 → **233.2**, 117.1	**22**, 31	10.12	Amitriptyline-D_3
Oxycodone	316.3 → **298.2**, 240.9	**27**, 40	5.57	Citalopram-D_6
Oxymorphone	302.0 → **284.0**, 227.1	**27**, 40	3.13	Citalopram-D_6

Paroxetine	331.2 → **193.3**, 70.0	**30**, 51	9.93	Fluoxetine-D_5
Quetiapine	384.2 → **253.1**, 279.2	**32**, 53	8.91	Citalopram-D_6
Rizatriptan	270.4 → **201.0**, 157.9	**18**, 29	5.07	Clozapine-D_4
Sertraline	306.2 → **274.9**, 158.8	**18**, 36	10.70	Amitriptyline-D_3

Table 1 (continued)

Analyte	**MRM transitions**	**CE**	**Tr (min)**	**IS**
Sumatriptan	296.4 → **58.0**, 201.2	**45**, 22	4.99	Clozapine-D_4
Topiramate	340.2 → **263.8**, 184.1	**12**, 19	7.67	α-Hydroxyalprazolam-D_5
Tramadol	264.9 → **58.0**, 246.0	**43**, 16	5.95	MDPA
Trazodone	372.2 → **176.0**, 147.9	**35**, 49	8.51	Citalopram-D_6
Triazolam	343.1 → **308.1**, 314.9	**38**, 39	10.25	Pinazepam
Venlafaxine	278.3 → **260.3**, 215.0	**18**, 24	7.60	Citalopram-D_6
Zolmitriptan	288.0 → **58.0**, 182.1, 243.2	**46**, 37, 26	5.47	Clozapine-D_4
Zolpidem	309.3 → **236.2**, 264.2	**48**, 37	7.95	α-Hydroxyalprazolam-D_5
Internal standard	**MRM transitions**	**CE**	**Tr (min)**	
Amitriptyline-D_3	281. 8 → **233.7**	**26**	10.26	
Citalopram-D_6	331.8 → **109.1**	**35**	9.10	
Clozapine-D_4	331.2 → **272.2**	**34**	8.00	
Desalkylflurazepam-D_4	293.2 → **139.9**	**43**	9.85	
Fluoxetine-D_5	315. 8 → **153.9**	**13**	10.03	
α-Hydroxyalprazolam-D_5	330.3 → **302.1**	**37**	9.50	
MDPA	223.2 → **164.0**	**22**	6.60	
Morphine-D_3	289.3 → **181.0**	**54**	2.54	
Pinazepam	309.1 → **241.1**	**48**	11.60	

Table 2 LOD and LLOQ values expressed as pg/mg hair and method validation results at low (L, 20.0 pg/mg hair, except for Fluoxetine and Norsertraline: 50.0 pg/mg hair), medium (M, 200.0 pg/mg hair) and high (H, 1500.0 pg/mg hair) concentrations.

Analyte	LOD	LLOQ	Intra-batch precision			Inter-batch precision			Accuracy %			Recovery%			Matrix Effect %		
				RSD%			RSD%										
			L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
Almotriptan	2.0	10.0	4.2	3.9	4.8	5.5	4.9	5.1	10.6	12.3	8.2	97.4	97.9	98.5	78.2	48.8	32.2
Alprazolam	5.0	10.0	9.4	8.5	2.7	11.4	9.2	2.9	9.6	8.3	0.7	93.0	93.8	94.3	-23.3	-18.6	-3.2
7-Aminoclonazepam	2.0	10.0	8.9	6.4	4.1	9.3	6.8	4.2	- 7.3	- 6.2	- 1.4	95.0	95.7	97.0	-20.2	-17.5	-11.1
Amitriptyline	2.0	10.0	7.9	6.9	6.3	10.8	9.5	8.9	9.2	8.3	1.5	74.1	74.6	75.1	-70.6	-50.2	-24.1
Citalopram	5.0	10.0	4.0	3.1	2.9	7.3	8.3	3.1	6.8	8.6	4.0	95.2	96.0	96.8	-32.2	-16.8	-10.1
Clomipramine	10.0	20.0	8.3	6.1	2.1	3.2	12.1	6.9	- 10.8	- 9.7	- 2.3	46.8	47.3	47.6	-18.2	-11.0	-9.2
Clonazepam	5.0	10.0	8.1	5.9	2.1	9.2	6.2	2.3	8.0	7.7	0.4	90.4	91.1	91.9	-30.2	-19.9	-10.2
Clozapine	10.0	20.0	3.0	1.7	1.5	3.3	3.7	4.0	9.6	7.8	1.6	98.2	98.9	99.2	18.2	15.3	10.3
Delorazepam	5.0	10.0	8.7	5.6	3.4	9.6	6.4	3.9	7.1	8.5	7.6	95.9	97.0	97.9	-23.2	-14.1	-9.2
Desalkylflurazepam	10.0	20.0	4.3	2.0	1.7	7.1	7.9	2.5	12.0	10.9	10.7	96.3	96.8	97.5	-24.2	-15.2	-10.2
N-Desmethylcitalopram	5.0	10.0	8.2	5.1	3.3	9.5	6.9	3.9	2.2	4.7	1.1	96.8	97.4	98.2	-22.5	-16.1	-13.3
N-Desmethylclozapine	5.0	10.0	9.4	5.6	3.1	10.1	5.9	6.0	- 11.3	- 10.2	- 4.7	96.6	97.6	98.3	15.2	9.6	8.3
N-Desmethylmirtazapine	2.0	10.0	8.3	7.4	4.9	10.2	9.4	5.9	- 11.7	- 9.9	- 10.4	93.9	94.3	95.0	19.6	10.8	2.1
N-Desmethyltramadol	10.0	20.0	8.9	7.6	3.7	12.7	10.9	4.2	- 9.2	- 9.5	- 4.0	91.2	92.0	92.7	-22.2	-13.0	-2.2
O-Desmethytramadol	2.0	10.0	8.9	7.4	3.6	10.5	9.4	3.7	- 12.0	- 11.7	- 4.2	98.3	98.9	99.1	-24.4	-14.4	-2.5
Desvenlafaxine	5.0	10.0	8.5	3.2	1.7	9.7	4.2	3.9	- 5.6	- 2.4	- 4.5	97.1	97.7	98.4	-18.2	-8.6	-6.2
Diazepam	5.0	10.0	9.6	3.8	2.7	10.3	4.5	5.4	3.8	6.7	4.7	84.4	85.8	86.3	-15.2	-10.6	-6.2
Duloxetine	10.0	20.0	10.1	9.6	9.3	11.4	10.5	10.7	10.7	9.6	5.1	52.1	52.7	58.3	-71.2	-49.2	-18.3
Eletriptan	2.0	10.0	6.2	3.3	2.1	6.6	3.7	4.2	11.5	9.1	1.6	93.9	94.4	95.1	85.2	67.5	38.2
Etizolam	2.0	10.0	8.8	6.6	5.1	9.4	6.7	6.3	11.6	10.7	2.4	97.3	97.7	98.5	-21.2	-13.5	-8.3
Fluoxetine	20.0	50.0	7.7	2.4	8.4	9.2	3.7	12.8	10.9	10.1	4.9	58.8	59.3	60.0	-19.9	-15.7	-5.1
Flurazepam	2.0	10.0	7.9	6.4	2.7	9.4	8.1	4.0	- 10.3	- 9.6	- 2.2	51.4	52.0	52.6	-24.7	-14.4	-4.2
Frovatriptan	2.0	5.0	3.3	8.9	2.6	5.9	9.2	5.0	12.1	11.2	14.5	92.4	92.9	93.3	80.2	59.8	35.2
α-Hydroxyalprazolam	5.0	10.0	6.4	5.8	3.1	8.7	8.2	3.3	10.8	6.4	0.5	94.8	95.4	96.0	8.2	4.2	2.2
2-Hydroxyethylflurazepam	5.0	10.0	8.8	9.2	3.3	10.4	9.9	3.5	11.0	9.5	3.1	90.7	91.1	91.8	4.6	3.6	2.4
α-Hydroxytriazolam	5.0	10.0	7.4	2.0	3.9	9.5	2.3	4.3	11.8	10.2	1.6	92.6	93.1	93.9	7.8	5.2	3.8
Levomepromazine	10.0	20.0	7.5	4.6	9.0	10.1	9.6	11.4	12.6	11.7	6.1	88.2	88.7	89.5	82.1	67.6	50.1
Levosulpiride	2.0	10.0	5.8	3.0	3.1	8.2	3.3	3.6	- 11.5	- 10.5	- 6.0	97.1	97.6	98.2	22.2	10.5	2.3
Lorazepam	10.0	20.0	9.9	6.3	3.9	13.7	6.7	4.1	13.0	12.6	2.7	40.6	41.1	42.0	-35.0	-24.0	-15.2

Lormetazepam	5.0	10.0	7.3	2.5	3.9	7.5	3.2	4.2	11.5	10.6	3.3	56.4	56.9	57.5	-32.2	-24.1	-13.2
Mirtazapine	2.0	10.0	9.6	7.4	3.9	11.7	9.9	4.7	7.7	5.9	5.4	98.8	99.3	99.5	18.2	3.5	2.7

Table 2 (continued)

Analyte	**LOD**	**LLOQ**	**Intra-batch precision**			**Inter-batch precision**			**Accuracy %**			**Recovery%**			**Matrix Effect %**		
				RSD%			**RSD%**										
			L	**M**	**H**	**L**	**M**	**H**	**L**	**M**	**H**	**L**	**M**	**H**	**L**	**M**	**H**
Morphine	5.0	10.0	6.4	2.8	5.2	6.5	3.9	5.5	9.3	9.7	2.2	91.7	92.0	92.6	17.2	10.8	8.2
Nordazepam	10.0	20.0	6.5	5.7	1.4	7.8	5.9	1.8	7.1	5.5	3.0	73.8	74.2	74.8	-17.2	-11.3	-6.2
Norfluoxetine	10.0	20.0	7.2	9.5	8.8	9.2	11.0	10.7	8.6	6.4	5.6	32.4	32.9	33.3	7.2	3.2	2.3
Norsertraline	20.0	50.0	2.7	7.2	5.4	7.2	8.4	9.2	5.7	3.3	2.9	65.6	66.0	66.5	-22.1	-14.2	-4.5
Nortriptyline	5.0	10.0	9.3	4.8	7.7	10.9	6.0	10.7	- 4.1	- 3.8	- 3.2	60.2	60.5	60.8	-71.2	-51.4	-23.2
Oxycodone	2.0	10.0	2.8	7.2	9.3	5.0	7.3	11.3	6.4	3.9	0.1	95.3	95.7	96.3	-22.3	-14.1	-8.2
Oxymorphone	2.0	10.0	8.0	1.3	4.1	9.7	3.2	4.2	7.3	5.2	1.7	97.2	97.8	98.1	-20.2	-18.0	-9.2
Paroxetine	2.0	10.0	9.1	9.5	7.5	12.2	11.0	10.6	8.3	7.7	1.1	54.5	54.9	55.3	-14.0	-11.5	-8.3
Quetiapine	2.0	10.0	8.2	4.7	3.5	9.1	4.8	4.3	- 12.6	- 11.3	- 7.4	90.0	90.6	91.4	-22.1	-18.2	-15.2
Rizatriptan	2.0	10.0	8.8	3.3	3.5	9.8	4.4	4.2	- 7.9	- 4.2	- 4.6	97.8	98.3	98.6	75.0	39.2	27.2
Sertraline	2.0	10.0	9.3	5.1	6.9	10.7	7.2	9.5	10.5	9.6	7.8	37.4	37.9	38.3	-59.1	-30.7	-12.1
Sumatriptan	2.0	10.0	5.7	4.2	2.3	7.0	4.8	2.4	- 8.5	- 7.3	- 6.2	95.3	95.8	96.2	24.0	15.2	10.2
Topiramate	5.0	10.0	3.9	6.1	4.1	7.2	7.2	5.0	- 10.0	- 5.9	- 6.2	98.2	98.6	99.0	18.1	8.2	1.2
Tramadol	2.0	10.0	9.8	5.0	3.5	9.9	6.9	3.8	- 11.4	- 7.8	- 6.9	97.2	97.7	98.1	-21.2	-11.2	-8.2
Trazodone	2.0	10.0	6.9	3.7	2.2	10.2	4.4	2.3	14.4	13.2	11.7	89.9	90.2	90.9	-23.3	-19.8	-15.1
Triazolam	5.0	10.0	8.7	7.0	4.4	9.0	7.8	4.6	- 2.1	- 5.4	- 1.1	95.9	96.4	96.9	-22.3	-14.9	-10.2
Venlafaxine	2.0	10.0	8.6	9.7	3.1	9.6	10.6	3.1	- 10.2	- 2.8	- 5.7	97.3	97.9	98.5	-23.4	-15.3	-12.7
Zolmitriptan	2.0	10.0	4.3	3.5	3.8	4.7	4.3	4.0	3.8	7.2	10.4	98.6	99.1	99.5	80.1	63.7	40.2
Zolpidem	2.0	10.0	8.4	9.1	5.8	10.9	9.9	7.1	- 10.7	- 11.7	- 12.1	98.2	98.7	99.3	18.7	8.7	3.2

Table 3 Concentration range of the target analytes in the positive hair samples, oral intake declared by the patients in the self-report and parent drug to metabolite concentration ratio (n.d. = not determinable).

Pharmacological class	Analyte	Number of positive hair samples	Total intake (mg/3 months)	Concentration range in the analyzed samples (pg/mg hair)	Parent drug to metabolite concentration ratio
ANXIOLYTICS	Alprazolam α-Hydroxyalprazolam	7	0.25 – 135.0	12.3 – 184.0 < LLOQ	n.d.
	Clonazepam Aminoclonazepam	1	90.0	32.0 117.0	0.27
	Delorazepam Lorazepam	23	0.8 – 1350.0	11.3 – 1568.0 < LLOQ – 382.0	n.d. – 11.12
	Diazepam Nordazepam	3	160.0 – 9000.0	162.0 – 2504.0 129.0 – 5476.0	0.46 – 3.36
	Flurazepam 2-Hydroxyethylflurazepam Desalkylflurazepam	1	1350.0	359.0 12.0 868.0	29.92 0.41
	Lorazepam	7	67.5 – 1900.0	73.0 – 227.0	
	Lormetazepam Lorazepam	1	6750.0	1207 503	2.40
	Triazolam α-Hydroxytriazolam	1	23.0	16.0 < LOD	n.d.
	Zolpidem	2	450.0 – 4500.0	480.0 – 2956.0	
ANTIDEPRESSANT	Amitriptyline Nortriptyline	24	180.0 – 9000.0	52.0 – 13656.0 38.3 – 8148.0	0.31 – 4.18
	Clomipramine	3	120.0 – 20250.0	79.0 – 10761.0	
	Citalopram N-Desmethylcitalopram	16	270.0 – 3600.0	158.0 – 7156.0 103.0 – 4757.0	1.14 – 2.87
	Fluoxetine Norfluoxetine	4	1800.0 – 5400.0	4885.0 – 54234.0 13330.0 – 50494.0	0.37 – 1.07
	Paroxetine	4	900.0 – 1800.0	717.0 – 5857.0	
	Sertraline Norsertraline	4	750.0 – 9000.0	437.0 – 55931.0 899.0 – 59357	0.49 – 1.27
	Duloxetine	16	1200.0 – 5400.0	119.0 – 27470.0	
	Venlafaxine Desvenlafaxine	6	3375.0 – 20250.0	465.0 – 5801.0 1877.0 – 7170.0	0.21 – 0.86
	Mirtazapine N-Desmethylmirtazapine	2	1350.0 – 4050.0	426.0 – 747.0 692.0 – 1673.0	0.45 – 0.62
	Trazodone	1	9000.0	6379.0	
ANTI PSYCHOTICS	Levomepromazine	1	2250.0	1037.0	
	Levosulpiride	4	250.0 – 6750.0	46.0 – 418.0	
	Quetiapine	2	562.5 – 4500.0	491.0 – 2188.0	
MOOD STABILIZERS	Topiramate	12	700 – 9000.0	287.0 – 5698.0	
OPIOID ANALGESICS	Morphine	1	45000.0	4624.0	
	Tramadol N-Desmethyltramadol O-Desmethyltramadol	14	37.5 – 7000.0	36.0 – 2330.0 < LOD < LOD – 341.0	n.d. – 20.08
	Oxycodone Oxymorphone	2	40.0 – 7200.0	26.0 – 5700.0 <LLOQ – 195.0	n.d. – 29.23
TRIPTANS	Almotriptan	24	12.5 – 2250.0	10.0 – 1845.0	
	Eletriptan	26	200.0 – 7200.0	23.0 – 4375.0	
	Frovatriptan	4	40.0 – 180.0	5.1 – 11.0	
	Rizatriptan	19	10.0 – 3000.0	10.2 – 1656.0	
	Sumatriptan	24	126.0 – 27000	10.7 – 982.0	
	Zolmitriptan	5	7.5 – 1350.0	18.0 – 75.0	