Fast screening of 88 pharmaceutical drugs and metabolites in whole blood by ultra-high performance liquid chromatography - tandem mass spectrometry

Abstract

Forensic investigations involving acute or lethal intoxication, drug-facilitated sexual assault, driving or workplace impairment frequently require the analysis of fresh or postmortem blood samples to check out a wide variety of pharmaceutical and illicit drugs, even after single-dose consumption. A sensitive and selective ultrahigh-performance liquid chromatography–tandem mass spectrometry UHPLC–MS/MS) screening method was developed for fast screening of 88 psychoactive drugs and metabolites in blood samples, including the ones most frequently involved in acute intoxications and forensic investigations in Italy. The new method allows short sample processing and analysis time (the whole procedure can be accomplished in less than 30 min) together with the simultaneous monitoring of a large number of pharmaceutical substances. These features represent crucial factors in the approach of acute intoxications, when the patient requires urgent and appropriate therapy. Blood sample treatment was limited to protein precipitation. Two UHPLC–MS/MS runs in positive and negative electrospray ionization modes were performed. The data were acquired at unit mass resolution in the selected reaction monitoring mode. According to international guidelines, linearity range, precision, trueness, detection and quantification limits, recovery, selectivity, specificity, carryover, and matrix effect phenomena were determined. Despite the limited sample purification and the inherent decreased chance of eliminating any potential interference, the present multiresidue screening method proved extremely effective and sensitive, allowing the detection of all tested drugs, even those belonging to structurally different classes of substances. Moreover, the developed method is easily susceptible to further expansion to encompass more drugs, either new or those becoming important for criminal investigation. This protocol was also applied to the analysis of authentic blood samples collected from victims of various crimes in routine casework, whose relevance in forensic investigations is presented in five cases.

Keywords

UHPLC–MS/MS . Whole blood . Multiresidue screening . Pharmaceutical drugs

Introduction

Several matters of forensic investigations involve the need for toxicological analysis, including lethal intoxication (suicide or homicide), abuse of pharmaceutical and/or illicit drugs, driving or workplace impairment, and drug-facilitated sexual assault. In all these cases, the analysis of biological matrices is carried out to check the possible presence of a wide variety of substances, or to exclude any hypothetical role played by drugs in the specific crime context [1].

Urine is the best specimen for comprehensive drug and poison screening, especially in the cases when the circumstantial elements are unknown [2]. Urinary excretion of the taken drug or poison may result in high concentration, making it more easily detectable than in blood specimen, for a longer period of time. However, the drug metabolites have to be identified additionally or, in some cases, exclusively. On the other hand, blood (plasma, serum, or whole blood) is the matrix of choice for drug quantification in forensic investigations because pharmacological and toxic effects can be related to its concentration in the bloodstream. Therefore, the detection of toxic substances in either fresh or postmortem blood samples offers the chance of interpreting their role in terms of acute intoxication or impairing effects at the time of blood sampling or death [3–5]. In most cases, only a limited amount of blood sample is available, and a wide range of endogenous components is present. Whenever these analytes are not known in advance or interferents are present, preliminary multi-target screening procedures proved to be necessary before accurate quantification [1]. Screening methods based on immunoassay testing are available only for few classes of drugs [6–8]—such as cannabinoids, cocaine, amphetamines, opiates, methadone, benzodiazepines, barbiturates, and tricyclic antidepressants—whereas no immunoassays are commercially available for the newest designer drugs [1] and other common substances, including anesthetics, antihistamines, and sedative–hypnotics [4]. Moreover, the use of multiple immunoassays testing requires a considerable amount of blood samples. Conversely, novel comprehensive multianalyte procedures based on hyphenated chromatography and tandem mass spectrometry are capable to detect simultaneously many drugs at once in a small sample volume, and provide systematic toxicological analysis [5, 9–14] for competent toxicological judgments and expertise in forensic investigations [13, 15]. Although gas chromatography–mass spectrometry (GC– MS) has been extensively used in the past [5, 16–18], nowadays, liquid chromatography–tandem mass spectrometry (LC–MS/MS) is replacing GC–MS as the standard technique for comprehensive screening methods in forensic laboratories [13, 14, 19–22], and in-house MS/MS libraries are successfully implemented [10, 23–25]. Increasing performances and decreasing costs of such instrumentation [5] have rapidly and deeply expanded the multi-residue capability of LC–MS/MS protocols, with significant benefit for drug screening in blood samples [8, 12, 26–30]. Moreover, ultrahigh-performance liquid chromatography (UHPLC) guarantees shorter analysis time and improved chromatographic resolution [5]. A wide variety of substances of toxicological interest

can be detected in a single run [31], provided that nonselective extraction procedures are utilized to reduce matrix effects and potential interferences. For example, protein precipitation is always required and provides good recovery also for polar analytes, unlike most SPE and LLE procedures. Protein precipitation, followed by LC–MS/MS, was utilized to achieve simple, fast, and cheap screening analyses, with high sample throughput [32].

The objective of our study was to develop and validate a fast UHPLC–MS/MS screening method for the determination of 88 psychoactive drugs and metabolites in whole blood samples, including the substances most frequently involved in forensic investigations in Italy. Drugs of abuse were not included since they are often screened separately, either with immunological tests or LC–MS/MS procedures, as in our laboratory. Our validation process included the determination of linearity range, selectivity, specificity, detection and quantification limits (LOD and LOQ), intra-assay precision, trueness, recovery, matrix, and carryover effects. The method was successfully applied to the analysis of blood samples in routine casework and proved valuable and easily adaptable in both forensic and clinical investigations; namely, it can be promptly updated to include emerging drugs or to analyze alternative biological materials.

Experimental

Reagents and materials

All 88 reference substances were purchased from either LGC Promochem SRL (Milan, Italy) or Sigma-Aldrich (Milan, Italy). Dichloromethane, methanol, formic acid, and acetonitrile were provided by Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UFPlus apparatus (Millipore, Bedford, MA, USA). Stock standard solutions were stored at −20 °C until used. Four compounds were used as the internal standards (IS), including two isotopically marked molecules (cocaine-d3 and nitrazepam-d5), together with coumachlor and althiazide, namely two substances currently not commercialized in Italy nor reported within human blood samples in the forensic and clinical literature. Three working solution mixtures were prepared by dilution in methanol at final concentrations of respectively 1 μg/mL (working solution A), 5 μg/mL (working solution B), and 10 μg/mL (working solution C). The inclusion of each analyte into the respective working solution was decided according to the upper limit of its therapeutic concentration interval [33–35], which is reported in Table 2 (<100 ng/mL, from 100 to 1,000 ng/mL, and >1,000 ng/mL, respectively for working solutions A, B, and C). Lastly, an internal standard mixture working solution was prepared in methanol, including cocaine-d3, nitrazepam-d5, and coumachlor at the final concentrations of 2 μg/mL, and althiazide at 4 μg/mL. Sample preparation Each aliquot of whole blood (0.5 mL) was fortified with 25 μL of internal standard mixture to yield a final concentration of 100 ng/mL (200 ng/mL for althiazide). One milliliter of acetonitrile/methanol 80:20 (v/v), previously stored at −20 °C, was added to the sample, which was then incubated at −20 °C

for 5 min. Afterwards, the sample was centrifuged at 4,000 rpm for 15 min, and 50 µL of the organic phase was transferred into a new vial. Finally, the vial was centrifuged once more at 14,000 rpm for 5 min, and a 1-µL aliquot was directly injected into the UHPLC–MS/MS system.

Instrumentation

All analyses were performed on a Shimadzu Nexera 30 UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray Turbo Ion source operating in the positive (ESI+) and negative (ESI–) ion modes, in two separate chromatographic runs. A Zorbax XDB-C18 column 100×2.1 mm i.d.×1.8 µm (Agilent Technologies, Italy), protected by a C18 guard column, was used for the separation of analytes. The column oven was maintained at +50 °C, and the elution solvents used were water/formic acid 5 mM (solvent A) and acetonitrile (solvent B). The mobile phase eluted under the following linear gradient conditions (a/b; v/v): from 95:5 to 37:63 in 6.0 min, then to 10:90 at 6.1 min with isocratic elution at 90 % B for 1.0 min. The flow rate was 0.5 mL/min and total time of the run was 10.0 min, so as to ensure re-equilibration at the initial conditions in between two consecutive injections. Data were recorded in the selected reaction monitoring mode (SRM). In order to establish appropriate SRM conditions, each analyte was individually infused into the ESI capillary, and the declustering potential (DP) was adjusted to maximize the intensity of the protonated molecular species [M+H]+ and [M–H]–. Conversely, the entrance potential was fixed at ±10 V (for ESI + and ESI–, respectively) for all the analytes. The collision offset voltage (CE) was adjusted to preserve approximately 10 % of the precursor ion, and the cell exit potentials (CXP) were also optimized. Each SRM transition was maintained during a time window of ±10.0 s around the expected retention time of the corresponding analyte, and the SRM target scan time (i.e., sum of dwell times for each SRM cycle) was 0.20 s, including pause times of 5 ms between consecutive SRM transitions. The best results were obtained using a source block temperature of +550 °C and an ion-spray voltage of ±3,000 V (for ESI+ and ESI–, respectively). Both Q1 and Q3 were operated at unit mass resolution. Nitrogen was employed as the collision gas at 0.005 Pa. The gas settings were as follows: curtain gas 27.0 psi, collision gas 10.0 psi, ion source gas (1) 50.0 psi, and ion source gas (2) 40.0 psi. The Analyst 1.5.2 (AB Sciex) software was used for data processing. All analytes and internal standards, their corresponding retention time, SRM transitions, and potentials are presented in Table 1.

Validation

The analytical method was validated in accordance with the criteria and recommendations of ISO/IEC 17025:2005 international standard. The following parameters were investigated: selectivity, linearity range, LOD and LOQ, intra-assay precision, trueness, and recovery. Carryover and matrix effect phenomena were also evaluated. Whole blood combined with EDTA as a

preservative was collected from volunteers and used as the working matrix for all validation experiments. Identification criteria and repeatability of diagnostic fragment ions' relative abundances Identification criteria for the analytes were established according to national [36] and international [37–39] guidelines. Retention time is part of the acceptance criteria for chromatographic assays. In particular, deviations of 1–2 % from the calibrators or controls are acceptable for HPLC based assays. When mass spectrometry is used for the identification of an analyte, the use of at least one qualifying mass transition for each analyte, in addition to the primary fragmentation, is recommended. Variations of mass transitions intensities were considered acceptable if within ±20 %, comparatively to the corresponding control. The repeatability of relative peak intensities for the SRM transitions of each analyte was determined on five spiked fresh blood samples at three concentration levels (5, 25, and 75 ng/mL for working solution A; 25, 75, and 150 ng/mL for working solution B; and 50, 150, and 300 ng/mL for working solution C). Retention time (tR) precision at each concentration was also determined.

Selectivity and specificity

Five fresh blank blood samples from different donors were analyzed to test the selectivity of the whole analytical procedure. For each sample and all analytes, the signal to noise ratio (S/N) was measured for the corresponding mass transitions at the expected retention time windows. A ratio S/N <3 was considered satisfactory in order to verify the method specificity.

Linearity range

The linear calibration model was checked by analyzing (two replicates) whole blood samples spiked with working solutions at six final concentrations, covering different ranges according to the therapeutic interval of each analyte. More in detail, the intervals 2–75 ng/mL (2, 5, 10, 25, 50, and 75 ng/ml), 10–150 ng/mL (10, 25, 50, 75, 100, and 150 ng/ml), and 20–300 ng/mL (20, 50, 100, 150, 200, and 300 ng/ml) were investigated for the working solutions A, B, and C, respectively. The calibration was completed by internal standardization. The linear calibration parameters were obtained using the least squares regression method. The squared correlation coefficient (R2) was utilized to roughly estimate linearity.

Detection and quantification limits

The limit of detection (LOD) was calculated as the concentration of the analyte that gives a signal (peak height) equal to the average background of the blank (Sblank) plus three times its standard deviation (LOD0Sblank+3Sblank), while the LOQ was calculated as LOQ0Sblank+10Sblank. The noise was measured from ±0.05 min before the peak onset till the beginning of the peak for each analyte. The LOD values estimated from calculation were experimentally confirmed by analyzing

five blank blood samples spiked with all analytes at the concentrations approximately corresponding to their estimated LOD values.

Intra-assay precision and trueness

For all analytes, intra-assay precision (CV%) and trueness (expressed as bias percentage) were evaluated by extracting and analyzing five whole blood samples spiked at three concentration levels (5, 25, and 75 ng/mL for working solution A; 25, 75, and 150 ng/mL for working solution B; and 50, 150, and 300 ng/mL for working solution C). Although the acceptance criteria for precision and trueness are not fixed for screening methods by internationally standardized rules, it was established that intra-assay precision was satisfactory when CV% values were below 25 %. Satisfactory trueness was achieved when the experimentally determined average concentration lied within ±25 % from the expected value.

Extraction recovery and matrix effect

The extraction recoveries and matrix effects were calculated by comparing the experimental results from two sets of solutions [40, 41] at three different concentrations (5, 25, and 75 ng/mL for working solution A; 25, 75, and 150 ng/mL for working solution B; and 50, 150, and 300 ng/mL for working solution C). The analytes' recovery was calculated by the ratio between the analyte blood concentration determined after its extraction (first set) and the one determined on the spiked extract (second set). The matrix effect was calculated as the percentage ratio between the analyte chromatographic peak area detected from the second set (blood samples spiked after the extraction step) and that detected from the third set (spike after the extraction step on blank deionized water). The percentage difference highlighted matrix suppression (values below 100 %) or enhancement (values above 100 %).

Carryover effect

Carryover effect was evaluated by injecting an alternate sequence of five blank blood samples and five blank blood samples spiked with all the analytes at high concentrations (up to 75, 150, and 300 ng/mL for the working solutions A, B, and C, respectively). To ensure the absence of any carryover effect, the signal to noise ratio had to be lower than 3 for each monitored transition.

Results and discussion

UHPLC–MS/MS method

Preliminary experiments were conducted to verify that a wide range of substances with considerably different chemical structures and physical properties could simultaneously be collected after protein precipitation and determined within instrumentally compatible conditions. These experiments confirmed that the proposed sample treatment was mild enough to not extensively remove any of the studied substances, but also showed that seven of these drugs were much more effectively detected by using the negative rather than the positive ion mode. The final optimized UHPLC–MS/MS method allowed the determination of all 88 analytes and 4 internal standards using two consecutive chromatographic runs under ESI+ and ESI− conditions, respectively. Each chromatographic run was completed in 10 min, including the time required for column re-equilibration before the next injection. The list of pharmaceutical drugs included in the screening represents the molecules most frequently involved in forensic investigations in Italy, as a result of the drugs present in the Italian market and their prescription frequency (i.e., number of packaging sold). More in detail, the investigated analytes belong to various classes of drugs, as follows: 13 analgesics, 1 antitussive, 1 β-blocker agent, 9 anxiolytics, 11 antipsychotics, 1 antihistame, 9 antidepressants, 3 nonsteroidal anti-inflammatory agents, 1 antiemetic, 1 anthelminthic, 1 barbiturates, 1 antigastric agent, 3 antihypertensive drugs, 3 anticholinergic agents, 3 PDE5 inhibitors, 2 anesthetics, 2 anticonvulsants, 1 antidiarrheal opioid agonist, 1 psychostimulant, 6 benzodiazepines, zolpidem, zopiclone, methadone, scopolamine, and 8 metabolites. Their retention times ranged from 1.00 to 5.70 min for the chromatographic run conducted in ESI+, and from 2.30 to 5.90 min for the run conducted under ESI− conditions. Figure 1a–e shows the SRM chromatograms recorded from a whole blood sample, spiked with all analytes at 50 ng/mL concentration. Only one SRM transition is depicted for each analyte. Two SRM transitions were utilized to detect the target analytes, as is recommended by national [36] and international guidelines [37–39]. Although product ion spectra could be recorded on triple quadrupole instruments to better sustain drug identifications, we decided not to use this option in order not to reduce the dwell time for SRM transitions and the inherent instrumental performance.

Validation

Identification criteria and selectivity

In order to detect each analyte, two SRM transitions were selected, as summarized in Table 1. This, together with the retention time, provides sufficient identification points to achieve unequivocal recognition of the analytes [36, 37, 39]. The intra-assay precision for the retention

times measured at low, medium, and high concentrations showed random fluctuations, but always within ±1.0 %, confirming that the retention times were repeatable and not affected by the analytes' concentration. An important parameter to obtain repeatable retention times proved to be the temperature control of the UHPLC column, which was maintained at +50 °C. In a multianalyte screening method, retention time repeatability is a particularly important criterion for compounds identification. Furthermore, for each analyte the relative abundance of the two selected SRM transitions varied by less than ±20 % so proved compliant for the unambiguous identification of all analytes included in the assay. The SRM chromatograms from five blank blood samples taken from different individuals showed no interfering signals (i.e., S/N ratio minor than 3) at the expected retention time, for all the analytes, except for diltiazem. This demonstrates that the method is selective for almost all tested compounds and free from positive interferences. The only non-compliant result, observed for diltiazem, does not compromise the general applicability of the screening method on real forensic and clinical cases. In fact, this compound, usually found as a cocaine adulterant, exhibits an average S/N040±1 in blank samples. This interfering signal is negligible in comparison with the S/N074 obtained at the detection limit (0.40 ng/mL), which in turn is much lower than the therapeutic level (50–200 ng/mL).

Linearity and evaluation of LOD and LOQ

All the calibration plots, built from spiked blank blood samples at five or six concentration levels and extending for more than one order of magnitude, showed good linearity. Table 2 reports the resulting R2 values that range from 0.985 and 0.999 and roughly indicate good fit and linearity of the calibration curves, with respect to the screening purpose of the method. Table 2 also reports LOD and LOQ values, calculated from the analysis of five blank samples and confirmed experimentally by the analysis of blank samples spiked with the analytes at concentrations approximating LOD values. In these experiments, all analytes were clearly detected. LOD values lay between 0.04 (sufentanil) and 15.00 ng/mL (barbital), LOQ values between 0.13 and 50.00 ng/mL (sufentanil and barbital, respectively). For all analytes, both LOD and LOQ values are significantly lower than the corresponding therapeutic levels, confirming that the present method is highly reliable for screening purposes and scarcely susceptible of yielding false negative results, possibly even after a single-dose consumption.

Intra-assay precision and trueness

Intra-assay data on precision and trueness are reported in Table 3. The results demonstrated satisfactory intra-assay precision, at least at "medium" and "high" concentration levels, as the percent variation coefficient (CV%) is lower than 25 % for almost all analytes, from 25 to 300 ng/mL, for working solutions B and C. Only norfentanyl, norbuprenorphine, and paroxetine presented an unsatisfactory CV% value (>25 %) at more than one concentration level. Almost all

CV% values exceeding 25 % are relative to samples spiked at 5 ng/mL (or “low level”), namely a concentration largely below the therapeutic intervals and rarely observed in real samples of forensic interest. At the low level concentration, 66 out of 88 drugs proved compliant with intra-assay precision requirements (67 %), whereas 88/88 (100 %) and 85/88 (96 %) drugs were compliant at medium and high concentrations, respectively. Intra-assay precision apparently improves with the absolute analytes’ concentration: in fact, 100 % compliant values are observed at 150 and to 300 ng/mL concentrations. The trueness, expressed as percent bias, varied from excellent for some analytes to unsatisfactory for other compounds (Table 3). For example, clonazepam and levamisole exhibited a brilliant +0.1 % bias, at 25 and 150 ng/mL concentrations, respectively. On the other side, positive and negative bias as high as +397.2 % and −38.1 % were observed for ibuprofen at 50 ng/mL and alfentanil at 5 ng/mL, respectively. Overall, 45 out of 88 drugs showed compliant trueness at all concentration levels, while the rest of them (43 out of 88) proved not compliant at least at one concentration level. These results demonstrate that an additional semi-quantitative information can be achieved only for the analytes with satisfactory precision and trueness, despite the present method is mainly intended for screening purposes, and rough estimation of blood concentration can be obtained for all the drugs included in the present study. On the whole, the majority of experimental biases were within the acceptable limit of 25 % at all concentrations, ranging from 5 to 300 ng/mL. The reasons for observing a few unsatisfactory trueness values are likely to be found in both the unselective sample treatment, possibly leaving co-eluting extraneous substances capable of signal suppression or enhancement, and the use of a limited choice of internal standards for quantification. Nevertheless, overestimated and underestimated results do not compromise the general applicability of this screening method since all positive samples have to be newly processed with more accurate and exact confirmation analyses.

Extraction recovery and matrix effect

For each analyte, extraction recovery and matrix effect results are shown in Table 3. The average extraction recovery is around 60 %, with the minimum observed value of 22.1 % for norbuprenorphine at 25 ng/mL, and the maximum value equal to 86.5 % for lidocaine at 300 ng/mL. Low recovery values are likely to be due to the generic conditions adopted in the extraction procedure, possibly yielding partial adsorption and co-precipitation of the analytes within the proteic matter. However, none of the recovery values is too low to prevent the detection of the corresponding analyte, so that the wide screening capability of the present method turns out not to be affected. Moreover, the low concentration levels under investigation are considerably lower than those expected in real samples, around the therapeutic range. In the practical situations, the observed extraction recoveries are sufficiently large and repeatable to allow the unequivocal identification of all target substances. For almost all analytes, the matrix effect is negative, i.e.,

signal suppression is observed. The highest negative effect is seen for norbuprenorphine at 25 ng/mL (−88.3 %), while the largest positive value is +22.2 % for scopolamine at 75 ng/mL. On average, the matrix effect is estimated around −33 %. Ion suppression is quite common in ESI, whenever complex mixtures are studied, since the co-elution of analytes and extraneous substances makes the competition for the charge dependent on their relative chemical and physical properties. In the present case, the modest blood sample cleanup and the co-elution of some analytes, due to the short chromatographic run, are most likely to produce the observed matrix effect [42]. In particular, protein precipitation does not completely remove the endogenous substances, such as lipids, phospholipids, and fatty acids, that may affect the ESI droplet desolvation process [43]. However, signal suppression does not affect significantly the detection capability in this method since LOD values for all analytes are still lower than the expected concentrations in real blood samples. Some analytical parameters such as intra-assay precision and trueness are likely to be affected by the use of a limited number of internal standards, in combination with differential matrix effects. Nevertheless, the use of a huge number of internal standards increases the number of monitored analytes and the cost of this screening method. Therefore, semi-quantitative results obtained from the present screening method are then corrected by running confirmatory procedures with appropriate and carefully selected, i.e., isotopically labeled, internal standards.

Carryover effect

No carryover effect was observed under the conditions described in the “Experimental” section. Blank blood samples, alternatively analyzed with samples spiked at high concentration (up to 300 ng/mL), showed S/N values always lower than 3 at the retention times of the tested analytes. As for selectivity testing, diltiazem was the only analyte showing S/N>3 (45.2±2.3). However, the recorded S/N value is substantially identical to that observed in the analysis of consecutive blank samples (see above), demonstrating once more that no carryover effect is present.

Valproic acid

Valproic acid is a pharmaceutical compound mostly used as an antiepileptic. The therapeutic range reported in the scientific literature ranges from 40 to 100 µg/mL (Table 2). These concentrations are extremely high in comparison with the range investigated in this study. On the other hand, the addition of many substances in high concentration may create unrealistic perturbing effects on the method performances. Therefore, excessive analyte concentrations were avoided and, for valproic acid, only selectivity and carryover effects were evaluated. In both cases, the S/N value was lower than 3, successfully satisfying the acceptance requisite. In general, the present screening method proved reliable for determining the presence or absence of valproic acid in real blood samples, but for quantitation, a specific method was developed and validated [44].

Case reports

Our laboratory is continuously using the present screening method for the analysis of acute intoxications and postmortem blood samples. In most cases, one or more of the molecules included in this screening are identified. Afterwards, a confirmation analysis is usually performed. Some interesting cases of multiple positive identifications are reported in the following examples, in order to demonstrate the practical usefulness and general applicability of this comprehensive screening method.

Case 1

A 16-year-old girl attempted suicide by throwing herself from the seventh floor of the building where she lived. She was immediately rescued and hospitalized at an intensive care unit (ICU), but after approximately 3 h she was declared dead. Screening analyses revealed the presence of fentanyl, lidocaine, and dihydrocodeine. No confirmation analysis was performed since all three drugs were part of the pharmacological treatment executed at the ICU. The SRM chromatogram obtained from the UHPLC–MS/MS experiment is shown in Fig. 2a.

Case 2

When arriving back at home after a night out, a man with previous episodes of drug addiction realized that he had lost the keys of his apartment. Therefore, he persuaded his neighbor to let him climb his balcony, but he fell down from the third floor and immediately died. His blood sample was found positive to clonazepam, 7-aminoclonazepam, buprenorphine, norbuprenorphine, dextromethorphan, diltiazem, paracetamol, and lidocaine. The corresponding SRM chromatogram is shown in Fig. 2b. The results of confirmation analyses were consistent with the screening, proving a state of acute intoxication due to the presence of clonazepam and buprenorphine. Furthermore, high levels of morphine (from abuse of heroin) were also detected with a specific procedure for drugs of abuse, in coherence with the presence of dextromethorphan and paracetamol, commonly used as heroin adulterants.

Case 3

A 54-year-old man was found dead in his apartment. Due to his previous poor health conditions, the death was initially attributed to natural causes. After a few days, two men and one woman were arrested for having used the credit card of the deceased man the same night of his death. The prosecution also demonstrated that two of them took the victim out for dinner that night. Our laboratory was asked to verify if the blood of the deceased man contained any drug and, in case, if the concentration was allegedly lethal. Delorazepam, bromazepam, diazepam, and lormetazepam were found in the blood samples, as shown in Fig. 2c. The quantitative results from confirmation analyses suggested a past exposure to benzodiazepines and a recent administration of

lormetazepam, found at 9 ng/mL concentration. Afterwards, the arrested did not challenge the results and admitted to have given a unspecified sleeping drug to the victim.

Case 4

A 57-year-old man died after a sexual intercourse with a prostitute. The analysis of his blood revealed the presence of sildenafil. The confirmation analysis determined the drug at a concentration of 29 ng/mL. The corresponding SRM chromatogram is shown in Fig. 2d.

Case 5

Blood screening analysis of a deceased old woman was requested to our laboratory because the nurse who assisted the woman was suspected of having caused her death by giving a wrong therapy or dosage. Our screening revealed the presence in the blood of quetiapine, paracetamol, mirtazapine, bromazepam, lormetazepam, and haloperidol, but the confirmation analyses indicated that blood concentrations were within the therapeutic range for all drugs. The corresponding SRM chromatogram is shown in Fig. 2e.

Conclusion

The development of UHPLC technology and fast MS/MS electronics has recently expanded the differentiation between screening procedures and confirmation methods. While the latter should achieve as much specificity and accuracy as possible, screening procedures are currently addressed to the accomplishment of high efficiency and high throughput objectives, even to the detriment of some sensitivity, trueness, and selectivity. Accordingly, the present study has been focused on the development of a fit-for-purpose analytical method, in order to detect in blood a large set of pharmaceutical substances selected among the ones most frequently found in acute intoxications and authoptic reports in Italy. The choice of a very simple and unselective preliminary sample treatment was coherent with the needs of both providing fast processing and reporting in the cases of acute intoxication, when the adoption of prompt and correct medical treatments may save the patient's life, and expanding as much as possible the range of drugs included in a single screening. On the other hand, the most common drugs of abuse were not included in our analytical procedure since rapid screening is commonly provided by immunochemical tests, widely available in most clinical laboratories and emergency rooms, or by comprehensive LC–MS/MS procedures, recently introduced in several forensic laboratories, including ours, where all the most common drugs of abuse are screened on 0.1-ml blood sample. The effective use of the present method in our daily laboratory practice, some examples of which are reported in this study, demonstrates that the posed objectives were fully accomplished since (1) the entire procedure, from blood sampling to completion of the analysis, can be performed in less than 30 min; this is the key issue of the

present method that proved to be of crucial importance in the cases of acute intoxication and urgent need of appropriate therapy setting; (2) multiple positive identification of active drugs are frequently met; (3) the therapeutic concentrations of all screened drugs largely exceed the LOD and LOQ experimental values of the method, while in most cases of acute intoxication the blood concentrations are even higher; (4) for the majority of screened drugs, the precision of quantitative determinations is satisfactory, assuring good repeatability on different blood samples, while the scarce accuracy recorded for some analytes is corrected by the subsequent confirmation analyses; and (5) the method is flexible and easily susceptible of further expansion to encompass more drugs, either new or becoming important in the forensic investigations.

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	Compound	t_R (min)	Precursor Ion		DP (V)	Target			Qualifier		
			$[M-H]^-$	$[M+H]^+$		Fragment	CE (V)	CXP (V)	Fragment	CE (V)	CXP (V)
1	Alfentanil	3.4		417.0	+52	164.9	47	16	268.3	24	8
2	Alprazolam	4.7		309.0	+77	205.1	56	9	280.9	36	12
3	Amisulpride	2.1		370.0	+52	195.9	55	8	112.0	33	13
4	Amitriptyline	4.2		278.0	+82	233.1	24	10	90.9	32	10
5	Amobarbital	4.0	225.0		–42	42.1	50	11	182.1	17	8
6	Aripiprazole	4.1		447.9	+137	284.9	36	13			
				449.9	+61				287.1	34	12
7	Atenolol	1.3		267.1	+74	145.0	33	13	190.3	26	9
8	Barbital	2.3	183.2		–70	42.1	43	15	140.1	16	6
9	Biperiden	3.9		312.0	+55	98.1	31	47	70.3	68	13
10	Bromazepam	3.9		315.9	+88	182.2	42	8			
				318.0	+85				182.0	43	16
11	Buprenorphine	3.7		468.0	+54	55.1	91	10	414.3	48	10
12	Bupropion	3.0		240.0	+63	184.1	17	14	130.9	37	11
13	Buspiron	3.2		386.0	+50	95.1	70	8	150.1	40	11
14	Carbamazepine	4.3		237.0	+190	194.1	26	17	193.1	46	9
15	Chlorpromazine	4.3		319.0	+40	86.1	25	13	58.0	63	8
16	Citalopram	3.5		325.0	+88	262.0	26	12	109.1	34	10
17	Clonazepam	4.7		316.0	+91	214.2	51	17	269.7	34	10
18	Clotiazepam	5.7		319.0	+70	154.0	38	14	291.0	30	14
19	Clozapine	3.3		326.9	+72	270.0	31	12	192.1	57	9
20	Delorazepam	5.2		304.9	+27	139.9	39	21	242.1	37	11
21	Demoxepam	3.8	285.1		–80	241.1	20	10	152.1	24	11
22	Desalkylflurazepam	4.9		289.0	+79	139.9	38	12	226.0	38	16
23	Dextromethorphan	3.4		272.2	+46	171.1	50	15	215.1	34	10
24	Diazepam	5.6		285.0	+51	154.0	36	7	193.1	43	15
25	Dihydrocodeine	1.6		302.2	+70	199.1	42	9	128.1	75	11
26	Diltiazem	3.7		415.0	+14	178.0	32	15	150.0	58	16
27	Diphenhydramine	3.5		256.1	+80	165.1	54	14	167.0	20	8
28	Embutramide	4.8		294.0	+57	121.0	34	11	191.0	22	17
29	Fentanyl	3.5		337.0	+175	188.2	31	9	105.1	50	10
30	Flunitrazepam	5.0		314.0	+35	268.2	36	11	239.1	47	11
31	Fluoxetine	4.3		310.1	+36	44.1	48	5	148.0	12	14
32	Flurazepam	3.6		390.0	+30	316.9	34	13			
				388.1	+33				316.9	26	12
33	Haloperidol	3.7		376.0	+45	123.0	53	16	164.9	33	13
34	Ibuprofen	5.9	205.0		–42	161.1	10	7	159.0	9	13
35	Ketamine	2.3		238.0	+100	125.0	38	11	207.1	20	9
36	Ketoprofen	5.2		255.0	+38	209.1	19	9	104.9	32	11
37	Ketorolac	4.5		256.2	+42	105.0	25	12	77.0	62	11
38	Levamisole	1.7		204.9	+52	178.1	30	8	123.0	39	16
39	Levomepromazine	4.2		329.0	+22	100.0	25	18	58.1	53	9
40	Lidocaine	2.2		235.1	+230	86.1	27	11	58.0	47	14
41	Loperamide	4.7		477.0	+12	266.1	34	14	210.1	64	14
42	Lorazepam	4.6		323.0	+27	277.0	34	12			
				321.0	+23				303.0	22	13
43	Lormetazepam	5.2		335.0	+55	288.9	30	13			
				337.0	+75				291.0	28	12
44	Methadone	4.2		310.0	+240	265.1	20	10	105.0	33	9
45	Methylphenidate	2.6		234.2	+66	84.3	24	6	56.1	63	7

46	Metoclopramide	2.3		299.9	+23	184.0	41	18	227.0	26	19
47	Mianserin	3.5		265.0	+122	208.1	27	14	58.1	45	14
48	Midazolam	3.5		325.9	+14	291.0	38	12	249.1	50	11
49	Mirtazapine	2.6		266.0	+28	195.0	36	9	72.0	26	11
50	Nitrazepam	4.5		282.1	+30	236.0	32	11	180.0	50	9
51	Norbuprenorphine	3.1		414.2	+47	187.0	50	15	101.3	46	8
52	Nordiazepam	4.9		271.0	+71	140.0	37	13	208.0	38	18
53	Norfentanyl	2.3		233.2	+77	84.3	23	7	55.0	49	10
54	Norketamine	2.2		224.0	+28	125.0	37	10	207.0	17	20
55	Olanzapine	1.8		313.0	+58	256.1	33	11	198.0	52	9
56	Omeprazole	3.3		345.9	+50	198.0	15	8	136.0	49	11
57	Oxcarbamazepine	3.7		252.9	+50	180.1	40	15	208.0	28	9
58	Oxybutynin	4.3		358.0	+20	124.0	27	5	72.1	52	7
59	Oxycodone	1.8		316.0	+18	241.0	38	10	256.1	35	11
60	Oxymorphone	1.0		302.2	+80	284.0	28	12	227.2	38	10
61	Paracetamol	1.5		152.0	+32	110.0	22	15	65.0	39	11
62	Paroxetine	3.8		329.9	+19	192.0	29	16	123.1	33	14
63	Pentazocine	3.1		286.2	+30	175.2	35	6	173.0	40	8
64	Pericyazine	3.7		366.0	+14	142.1	30	11	114.0	37	14
65	Phenacetin	3.5		180.0	+77	110.1	29	15	138.0	21	16
66	Phenobarbital	3.2	231.2		–70	42.0	43	13	188.0	14	8
67	Promazine	3.9		285.1	+12	86.1	24	13	58.1	57	7
68	Quetiapine	3.5		384.1	+70	221.1	49	9	253.1	31	11
69	Ramipril	4.1		417.2	+20	234.1	29	10	91.1	86	14
70	Remifentanil	2.9		377.1	+43	112.9	39	12	317.2	22	14
71	Risperidone	3.1		411.1	+18	191.0	40	8	110.0	66	19
72	Scopolamine	1.8		304.1	+30	138.1	27	13	156.1	22	9
73	Secobarbital	4.3	237.2		–19	41.9	45	10	194.2	17	8
74	Sildenafil	3.6		475.2	+59	58.1	74	10	99.9	36	14
75	Sufentanil	4.0		387.2	+26	238.1	27	8	355.2	27	16
76	Tadalafil	4.8		390.0	+59	268.0	17	11	169.1	47	15
77	Telmisartan	4.7		512.2	+60	497.0	47	20	276.0	62	11
78	Tramadol	2.6		264.1	+35	58.1	46	11	246.1	15	14
79	Trazodone	3.2		372.1	+125	176.0	33	16	148.0	43	7
80	Triazolam	4.8		343.0	+36	308.0	37	13	314.9	39	13
81	Valproic Acid	4.8	142.9		–51	142.9	30	10			
82	Vardenafil	3.3		489.2	+27	151.0	52	7	312.0	52	11
83	Venlafaxine	3.1		278.1	+55	58.0	22	10	260.2	17	11
84	Zolpidem	2.9		308.1	+50	92.0	63	12	220.1	60	9
85	Zopiclone	2.5		389.0 391.1	+71 +57	245.0	25	10	246.9	25	11
86	7-aminoclonazepam	2.7		286.1	+111	121.1	39	8	222.2	35	19
87	7-aminoflunitrazepam	3.1		284.0	+74	227.0	35	10	135.2	35	12
88	7-aminonitrazepam	1.8		252.0	+91	120.9	35	18	94.0	48	15
IS	Althiazide (ALT)	3.8	382.0		–78	269.0	36	8	260.0	30	11
IS	Nitrazepam-d_5 (NIT-d_5)	4.5		287.2	+80	185.1	42	14	212.2	47	14
IS	Cocaine-d_3 (COC-d_3)	2.8		307.2	+80	185.1	28	8	85.1	47	14
IS	Coumachlor (COU)	6.3		343.0	+37	163.0	20	8	285.0	30	10

Table 1 SRM transitions and experimental conditions for all compounds and internal standards detection

	Compound	Working solution	IS	Linearity Range (ng/mL)	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Therapeutic Level (ng/mL)
1	Alfentanil	A	COC-d_3	2 – 75	0.994	0.13	0.43	9 – 10
2	Alprazolam	A	NIT-d_5	2 – 75	0.999	0.25	0.82	5 – 50
3	Amisulpride	B	COC-d_3	10 – 150	0.997	0.13	0.45	50 – 400
4	Amitriptyline	B	COC-d_3	10 – 150	0.994	0.24	0.81	80 – 200
5	Amobarbital	C	ALT	20 – 300	0.997	6.00	20.0	2 – 12 (µg/mL)
6	Aripiprazole	B	CCL	10 – 150	0.992	0.68	2.25	50 – 350
7	Atenolol	B	COC-d_3	10 – 150	0.991	0.49	1.65	120 – 870
8	Barbital	C	ALT	50 – 300	0.997	15.0	50.0	5 – 30 (µg/mL)
9	Biperiden	A	CCL	2 – 75	0.995	0.25	0.83	4 – 6
10	Bromazepam	B	NIT-d_5	10 – 150	0.994	1.00	3.33	50 – 170
11	Buprenorphine	A	COC-d_3	2 – 75	0.991	0.60	2.00	0.2 – 0.7
12	Bupropion	A	CCL	2 – 75	0.998	0.13	0.42	25 – 100
13	Buspiron	C	COC-d_3	20 – 300	0.992	0.81	2.68	0.9 – 5 (µg/mL)
14	Carbamazepine	C	NIT-d_5	20 – 300	0.993	0.96	3.19	4 – 12 (µg/mL)
15	Chlorpromazine	B	CCL	10 – 150	0.994	0.59	1.96	2 – 122
16	Citalopram	B	COC-d_3	10 – 150	0.996	0.28	0.93	20 – 200
17	Clonazepam	A	NIT-d_5	2 – 75	0.995	0.33	1.09	20 – 80
18	Clotiazepam	B	NIT-d_5	10 – 150	0.991	0.16	0.54	10 – 700
19	Clozapine	B	NIT-d_5	10 – 150	0.986	0.76	2.53	350 – 450
20	Delorazepam	A	NIT-d_5	2 – 75	0.997	0.13	0.44	12 – 14.5
21	Demoxepam	C	ALT	20 – 300	0.993	3.33	11.1	0.4 – 4 (µg/mL)
22	Desalkylflurazepam	A	NIT-d_5	5 – 75	0.996	0.87	2.90	40 – 60
23	Dextromethorphan	A	COC-d_3	2 – 75	0.997	0.13	0.43	1 – 8
24	Diazepam	B	NIT-d_5	10 – 150	0.990	0.17	0.57	100 – 1000
25	Dihydrocodeine	B	COC-d_3	10 – 150	0.985	0.50	1.65	72 – 146
26	Diltiazem	B	COC-d_3	10 – 150	0.997	0.40	1.35	50 – 200
27	Diphenhydramine	B	COC-d_3	10 – 150	0.994	0.50	1.67	100 – 1000
28	Embutramide	C	CCL	20 – 300	0.991	0.92	3.07	3 – 12.1 (µg/mL)
29	Fentanyl	A	COC-d_3	2 – 75	0.993	0.19	0.64	0.1 – 5
30	Flunitrazepam	A	NIT-d_5	2 – 75	0.997	0.38	1.27	1.5 – 20
31	Fluoxetine	B	COC-d_3	10 – 150	0.988	0.67	2.24	150 – 500
32	Flurazepam	A	NIT-d_5	2 – 75	0.998	0.18	0.60	0.5 – 30
33	Haloperidol	A	CCL	2 – 75	0.999	0.12	0.39	5 – 40
34	Ibuprofen	C	ALT	50 – 300	0.994	13.9	46.5	20 – 30 (µg/mL)
35	Ketamine	C	CCL	20 – 300	0.998	0.24	0.80	0.64 – 2.2 (µg/mL)
36	Ketoprofen	C	CCL	20 – 300	0.991	0.71	2.35	6 – 15 (µg/mL)
37	Ketorolac	C	CCL	50 – 300	0.989	6.74	22.5	0.22 – 3.5 (µg/mL)
38	Levamisole	C	NIT-d_5	20 – 300	0.998	0.25	0.84	0.7 – 1.5 (µg/mL)
39	Levomepromazine	B	COC-d_3	10 – 150	0.994	0.37	1.23	15 – 140
40	Lidocaine	C	COC-d_3	20 – 300	0.992	4.80	16.0	2 – 5 (µg/mL)
41	Loperamide	A	CCL	2 – 75	0.996	0.26	0.88	2 – 3.98
42	Lorazepam	B	NIT-d_5	10 – 150	0.993	0.50	1.68	5 – 240
43	Lormetazepam	A	NIT-d_5	2 – 75	0.998	0.39	1.29	6 – 16
44	Methadone	B	COC-d_3	10 – 150	0.997	2.70	9.01	50 – 1000
45	Methylphenidate	A	COC-d_3	2 – 75	0.994	0.68	2.60	8 – 58
46	Metoclopramide	B	COC-d_3	10 – 150	0.994	0.10	0.34	40 – 130
47	Mianserin	A	NIT-d_5	5 – 75	0.998	1.33	4.44	15 – 70
48	Midazolam	B	NIT-d_5	10 – 150	0.994	0.45	1.49	10 – 147
49	Mirtazapine	A	NIT-d_5	2 – 75	0.998	0.09	0.31	20 – 100
50	Nitrazepam	A	NIT-d_5	2 – 75	0.998	0.38	1.26	30 – 70
51	Norbuprenorphine	B	COC-d_3	25 – 150	0.987	5.47	18.2	1 – 2.6
52	Nordiazepam	C	NIT-d_5	20 – 300	0.999	0.17	0.57	0.17 – 1.84 (µg/mL)

53	Norfentanyl	A	COC-d_3	2 – 75	0.991	0.42	1.41	0.1 – 5
54	Norketamine	C	CCL	20 – 300	0.996	0.30	1.00	0.64 – 2.2 (µg/mL)
55	Olanzapine	A	NIT-d_5	5 – 75	0.996	0.97	3.23	10 – 75
56	Omeprazole	A	COC-d_3	2 – 75	0.998	0.09	0.29	0.23 – 1.66
57	Oxcarbamazepine	C	NIT-d_5	20 – 300	0.995	0.57	1.90	12 – 30 (µg/mL)
58	Oxybutynin	A	CCL	2 – 75	0.993	0.22	0.74	10 – 20
59	Oxycodone	A	COC-d_3	5 – 75	0.995	0.95	3.17	20 – 50
60	Oxymorphone	B	COC-d_3	10 – 150	0.993	0.54	1.80	100 – 700
61	Paracetamol	C	CCL	20 – 300	0.997	1.73	5.76	10 – 20 (µg/mL)
62	Paroxetine	A	CCL	5 – 75	0.995	1.03	3.45	10 – 75
63	Pentazocine	B	NIT-d_5	10 – 150	0.991	0.30	1.00	100 – 300
64	Pericyazine	A	NIT-d_5	2 – 75	0.994	0.22	0.73	5 – 30
65	Phenacetin	C	COC-d_3	20 – 300	0.997	0.48	1.60	0.2 – 7 (µg/mL)
66	Phenobarbital	C	ALT	20 – 300	0.997	3.45	1.49	2 – 30 (µg/mL)
67	Promazine	B	COC-d_3	10 – 150	0.995	0.60	2.00	10 – 400
68	Quetiapine	B	NIT-d_5	10 – 150	0.993	0.52	1.72	50 – 500
69	Ramipril	B	COC-d_3	10 – 150	0.996	0.71	2.38	6 – 260
70	Remifentanil	A	COC-d_3	2 – 75	0.997	0.06	0.21	1 – 40
71	Risperidone	A	NIT-d_5	2 – 75	0.998	0.13	0.44	10 – 90
72	Scopolamine	A	COC-d_3	2 – 75	0.997	0.25	0.82	0.3 – 2
73	Secobarbital	C	ALT	20 – 300	0.998	9.68	2.26	2 – 10 (µg/mL)
74	Sildenafil	B	NIT-d_5	10 – 150	0.995	1.33	4.44	280 – 450
75	Sufentanil	A	COC-d_3	2 – 75	0.994	0.04	0.13	0.5 – 11
76	Tadalafil	A	NIT-d_5	5 – 75	0.996	1.44	4.81	4.9 – 13.7
77	Telmisartan	C	NIT-d_5	20 – 300	0.998	2.74	9.13	0.51 – 3.28 (µg/mL)
78	Tramadol	B	COC-d_3	10 – 150	0.998	2.21	7.35	100 – 800
79	Trazodone	C	NIT-d_5	20 – 300	0.997	0.15	0.50	0.65 – 1.5 (µg/mL)
80	Triazolam	A	NIT-d_5	2 – 75	0.998	0.24	0.80	6 – 17
81	Valproic Acid	C	-	-	-	-	-	40 – 100 (µg/mL)
82	Vardenafil	A	NIT-d_5	5 – 75	0.993	1.43	4.76	2.8 – 17
83	Venlafaxine	B	COC-d_3	10 – 150	0.997	0.13	0.43	250 – 750
84	Zolpidem	B	NIT-d_5	10 – 150	0.994	0.17	0.55	80 – 150
85	Zopiclone	A	NIT-d_5	2 – 75	0.999	0.52	1.72	10 – 50
86	7-aminoclonazepam	A	NIT-d_5	2 – 75	0.998	0.12	0.41	20 – 70
87	7-aminoflunitrazepam	B	NIT-d_5	10 – 150	0.997	0.47	1.55	20 – 500
88	7-aminonitrazepam	A	NIT-d_5	2 – 75	0.999	0.33	1.09	18 – 53

Table 2 For each compound, the corresponding working solution and internal standard, linearity range, squared correlation coefficient, LOD and LOQ values, and therapeutic level are reported

		Low level					Medium level					High Level				
	Compound	Conc. (ng/mL)	PR[a] (CV%)	TR[b] (bias%)	RE[c] (%)	ME[d] (%)	Conc. (ng/mL)	PR (CV%)	TR (bias%)	RE (%)	ME (%)	Conc. (ng/mL)	PR (CV%)	TR (bias%)	RE (%)	ME (%)
1	Alfentanil	5	40.0	–38.1	58.7	–58.7	25	12.1	+10.6	51.8	–61.8	75	3.4	+6.5	71.8	–51.1
2	Alprazolam	5	23.8	+11.8	54.8	–42.6	25	14.5	+8.8	49.7	–44.1	75	9.9	+1.7	66.7	–32.0
3	Amisulpride	25	23.3	+3.3	57.4	–22.1	75	4.5	–19.0	66.5	–14.3	150	2.9	–10.5	74.4	–12.8
4	Amitriptyline	25	16.0	+28.6	52.5	–42.6	75	5.6	–20.5	57.9	–40.3	150	6.6	–2.7	67.3	–30.3
5	Amobarbital	50	24.5	+137.0	62.2	–37.4	150	12.7	+136.8	63.2	–43.7	300	9.9	+167.3	72.2	–43.4
6	Aripiprazole	25	16.1	+29.6	53.4	–42.6	75	9.0	–15.1	59.1	–40.3	150	4.9	+3.3	67.3	–30.3
7	Atenolol	25	14.7	+138.7	47.6	–26.7	75	7.4	+74.7	56.1	–23.7	150	6.5	+105.5	69.7	–23.5
8	Barbital	50	19.7	+92.4	59.8	+17.6	150	5.3	+102.8	60.7	–3.4	300	8.2	+114.5	71.9	–2.3
9	Biperiden	5	34.6	–33.5	53.9	–24.3	25	11.3	–39.9	46.3	–38.1	75	5.2	–29.8	63.9	–21.0
10	Bromazepam	25	12.3	+36.3	50.6	–14.9	75	7.2	+27.8	57.4	–3.8	150	3.5	+29.3	68.8	+3.3
11	Buprenorphine	5	35.8	+23.3	55.9	–46.5	25	15.9	+13.9	43.2	–48.2	75	7.5	+42.4	56.0	–47.3
12	Bupropion	5	15.2	+3.0	58.5	–30.1	25	12.9	–20.8	48.9	–39.6	75	4.8	–8.2	70.9	–20.2
13	Buspiron	50	21.7	–8.2	56.2	–30.6	150	5.9	+4.7	58.8	–30.1	300	6.7	–1.5	69.3	–22.5
14	Carbamazepine	50	28.0	–51.2	39.9	–62.4	150	8.5	+8.5	41.9	–63.9	300	15.4	+16.7	46.9	–61.1
15	Chlorpromazine	25	27.5	+10.6	59.4	–51.3	75	10.9	–29.3	59.4	–53.7	150	8.3	21.7	70.6	–47.8
16	Citalopram	25	15.2	+19.3	57.9	–29.6	75	6.2	–5.5	63.7	–30.2	150	3.8	+7.1	70.8	–30.2
17	Clonazepam	5	22.7	+15.2	56.9	–40.6	25	14.0	+0.1	43.0	–39.2	75	9.1	–5.6	73.0	–26.3
18	Clotiazepam	25	18.2	+23.6	55.9	–52.6	75	8.8	+3.0	63.9	–50.3	150	5.9	–2.8	73.8	–43.7
19	Clozapine	25	20.4	+25.4	52.9	–42.3	75	11.6	+8.4	58.7	–36.5	150	8.1	+1.5	64.8	–39.5
20	Delorazepam	5	21.7	+22.1	53.2	–34.4	25	14.6	+1.7	48.9	–36.3	75	10.8	+2.6	66.5	–21.1
21	Demoxepam	50	20.7	–21.2	59.3	–6.9	150	8.2	–9.2	59.1	–23.2	300	8.7	+7.5	70.3	–20.6
22	Desalkylflurazepam	5	16.7	+26.7	55.1	–37.1	25	13.7	+3.4	48.9	–40.8	75	10.8	–0.4	69.7	–24.3
23	Dextromethorphan	5	29.2	–16.2	56.5	–32.5	25	15.5	–14.9	45.7	–41.5	75	4.3	+8.3	65.0	–31.5
24	Diazepam	25	17.2	+34.0	53.6	–47.5	75	8.4	+9.4	61.2	–44.4	150	6.0	+8.0	70.4	–36.1
25	Dihydrocodeine	25	19.4	+33.8	50.4	–20.1	75	3.7	+15.5	59.5	–2.6	150	1.8	+27.2	69.7	–5.6
26	Diltiazem	25	14.5	+15.3	54.2	–36.9	75	8.0	–1.0	60.5	–34.3	150	2.6	+6.4	68.3	–32.2
27	Diphenhydramine	25	19.4	+18.2	56.9	–18.3	75	3.7	–22.9	60.3	–26.3	150	4.5	–2.9	69.7	–25.6
28	Embutramide	50	21.2	–20.8	60.9	–46.5	150	4.8	–8.4	62.2	–48.1	300	6.2	–7.7	76.2	–39.6
29	Fentanyl	5	31.3	–8.2	53.4	–36.6	25	12.2	–0.3	50.2	–34.2	75	8.2	+9.0	68.2	–29.0
30	Flunitrazepam	5	19.5	+25.3	59.4	–27.0	25	13.8	+14.4	51.2	–30.7	75	10.2	+5.7	69.8	–16.0
31	Fluoxetine	25	24.2	–29.1	42.8	–62.1	75	8.8	+5.3	47.9	–60.4	150	22.5	–42.1	54.4	–56.3
32	Flurazepam	5	29.1	+0.2	55.6	–41.7	25	15.9	+9.4	56.6	–41.0	75	11.5	–0.3	65.4	–38.5
33	Haloperidol	5	13.3	–3.6	51.6	–27.5	25	13.0	–23.6	43.1	–30.8	75	5.3	–14.2	61.7	–16.7
34	Ibuprofen	50	16.5	+397.2	68.9	–19.3	150	10.0	+354.5	65.2	–42.7	300	8.4	+364.0	72.5	–39.8

35	Ketamine	50	32.4	–53.2	60.6	–20.1	150	7.8	–35.2	62.2	–21.7	300	8.0	–26.8	80.0	–16.5
36	Ketoprofen	50	17.1	–26.0	52.6	–12.9	150	7.5	–3.1	56.1	–24.8	300	6.6	+15.2	70.9	–18.5
37	Ketorolac	50	17.3	–29.2	52.9	–24.5	150	5.5	+22.7	54.2	–25.6	300	5.4	+47.3	68.8	–19.7
38	Levamisole	50	30.0	–35.9	61.0	–14.2	150	12.2	+0.1	62.1	–19.5	300	12.1	–14.5	79.3	–16.8
39	Levomepromazine	25	22.6	+20.7	43.0	–42.2	75	4.7	–9.2	48.4	–40.3	150	9.3	–16.9	57.1	–40.6
40	Lidocaine	50	26.5	–11.7	65.6	–11.4	150	9.5	–6.5	58.6	–22.4	300	10.3	+3.6	86.5	–9.8
41	Loperamide	5	26.7	–16.2	57.2	–38.2	25	15.1	–31.1	49.8	–39.9	75	6.8	–14.9	68.3	–22.8
42	Lorazepam	25	12.9	+25.3	49.4	–29.7	75	8.3	+15.9	57.6	–29.1	150	2.2	+24.4	66.9	–22.2
43	Lormetazepam	5	21.4	+16.1	53.2	–50.3	25	14.8	+5.0	48.6	–49.5	75	9.6	+6.7	65.5	–37.3
44	Methadone	25	23.3	+37.3	55.3	–13.5	75	4.9	+13.0	69.5	–16.9	150	8.8	+25.1	81.4	–4.6
45	Methylphenidate	5	35.5	–40.6	56.9	–34.3	25	11.9	–30.4	48.6	–41.4	75	4.1	–28.5	68.5	–27.1
46	Metoclopramide	25	13.5	+19.3	58.3	–10.1	75	5.4	–10.0	66.7	–6.4	150	2.2	–2.0	77.4	–4.3
47	Mianserin	5	13.9	–7.5	62.0	–36.7	25	18.2	–21.5	57.3	–38.5	75	9.5	–24.6	64.9	–35.6
48	Midazolam	25	13.9	+33.1	53.7	–46.0	75	9.2	+18.1	59.5	–46.9	150	7.7	+3.6	69.5	–41.5
49	Mirtazapine	5	19.2	–1.7	58.7	–25.5	25	13.8	+1.6	51.2	–39.7	75	8.1	–1.6	69.9	–30.4
50	Nitrazepam	5	17.3	+35.5	55.6	–29.1	25	12.9	+17.6	50.1	–37.2	75	7.1	+11.1	69.9	–19.7
51	Norbuprenorphine	-					25	21.9	+23.5	22.1	–88.3	75	53.2	–82.4	25.9	–86.1
52	Nordiazepam	50	24.6	+11.2	53.8	–50.0	150	7.7	+46.1	55.0	–52.1	300	10.4	+35.9	68.9	–43.1
53	Norfentanyl	5	43.8	–47.6	40.6	–66.5	25	18.1	+21.7	35.8	–66.9	75	26.9	–43.5	46.2	–56.8
54	Norketamine	50	24.9	–37.8	57.9	–20.5	150	9.4	–30.1	60.3	–24.1	300	8.6	–25.3	79.4	–13.7
55	Olanzapine	5	16.0	–23.8	38.0	+9.9	25	15.9	–26.5	32.5	–67.5	75	13.9	–31.5	42.5	–20.5
56	Omeprazole	5	21.6	+37.4	54.1	–28.6	25	15.1	+46.9	49.4	–36.0	75	5.1	+48.0	69.8	–12.5
57	Oxcarbazepine	50	22.5	+9.4	47.8	–39.8	150	9.0	+35.6	49.6	–44.0	300	12.4	+21.9	64.3	–34.4
58	Oxybutynin	5	20.2	–4.6	60.9	–41.4	25	13.8	–31.0	53.7	–45.9	75	9.2	–13.9	68.9	–34.5
59	Oxycodone	5	17.1	+23.2	55.8	–19.5	25	8.5	+23.8	50.0	–20.1	75	4.9	+23.5	68.7	+8.1
60	Oxymorphone	25	21.3	+25.6	43.6	–37.6	75	4.6	–5.6	62.4	–32.8	150	6.4	+20.5	65.1	–23.8
61	Paracetamol	50	22.2	–33.4	56.4	–28.1	150	6.1	–34.5	59.5	–32.6	300	6.4	+0.5	77.0	–21.9
62	Paroxetine	5	38.3	–67.2	41.5	–71.0	25	22.9	–27.7	35.0	–75.8	75	36.5	–69.5	42.9	–70.8
63	Pentazocine	25	19.9	+23.7	55.2	–13.9	75	11.1	+4.6	58.5	–21.0	150	4.0	–6.5	65.6	–20.9
64	Pericyazine	5	30.5	+63.8	42.8	–16.0	25	16.4	+81.3	43.0	–15.0	75	6.1	+23.4	48.3	–12.2
65	Phenacetin	50	19.5	–23.9	59.1	–36.8	150	3.6	–5.6	60.7	–32.8	300	8.0	–6.9	72.7	–28.6
66	Phenobarbital	50	22.5	+59.8	60.2	–20.2	150	10.0	+71.3	59.9	–33.9	300	8.4	+95.7	70.7	–32.2
67	Promazine	25	32.5	+40.3	41.3	–48.5	75	3.6	+2.1	41.8	–51.8	150	11.8	–2.8	47.5	–50.8
68	Quetiapine	25	8.5	+20.9	53.9	–40.3	75	12.8	+10.9	63.9	–38.1	150	6.3	–9.5	69.5	–32.8
69	Ramipril	25	16.2	+29.0	50.8	–44.6	75	5.3	+10.8	58.8	–45.8	150	4.3	+24.0	67.6	–40.5
70	Remifentanil	5	22.3	–7.9	56.0	–47.7	25	12.5	–8.3	48.8	–49.8	75	4.4	–0.7	67.3	–34.0

71	Risperidone	5	25.4	–15.3	61.1	–25.9	25	18.7	–14.9	55.6	–33.3	75	10.3	–28.7	64.4	–60.5
72	Scopolamine	5	22.4	+29.8	59.6	–1.6	25	9.4	+26.4	51.5	–12.9	75	2.7	+37.7	71.4	+22.2
73	Secobarbital	50	21.2	+62.9	65.0	–35.0	150	10.1	+62.7	62.8	–45.4	300	8.5	+91.3	70.1	–44.2
74	Sildenafil	25	7.7	+76.1	56.2	–18.8	75	10.3	+65.1	64.9	–4.2	150	8.4	+49.7	73.9	–1.4
75	Sufentanil	5	25.9	–8.4	56.4	–35.2	25	14.4	–18.6	49.6	–39.3	75	5.4	–6.8	68.2	–27.2
76	Tadalafil	5	19.0	+43.0	55.9	–21.9	25	8.5	+27.0	52.4	–23.9	75	11.1	+25.2	70.3	–2.7
77	Telmisartan	50	31.1	+106.0	66.6	–73.1	150	2.5	+172.8	53.3	–82.5	300	10.8	+80.3	54.9	–82.9
78	Tramadol	25	21.0	+9.9	55.9	–15.7	75	5.1	–13.8	64.1	–16.6	150	7.4	–8.3	74.9	–14.0
79	Trazodone	50	19.5	+0.1	58.5	–46.2	150	12.8	+22.1	62.5	–43.5	300	11.0	–3.9	73.9	–34.3
80	Triazolam	5	19.8	+9.5	58.3	–44.4	25	13.1	+7.3	49.7	–46.6	75	12.6	+2.3	65.1	–33.8
82	Vardenafil	5	22.0	+2.0	60.3	–70.8	25	18.0	+58.1	51.5	–67.2	75	12.5	+11.6	63.8	–65.2
83	Venlafaxine	25	22.6	+19.4	56.3	–27.8	75	5.2	–8.1	64.1	–28.1	150	5.6	–7.1	72.9	–26.6
84	Zolpidem	25	3.2	+3.8	60.0	–14.3	75	9.5	–7.4	68.7	–15.1	150	5.4	–15.9	72.3	–17.5
85	Zopiclone	5	16.4	+49.3	58.1	–1.2	25	16.8	+52.6	53.9	–6.0	75	8.7	+25.0	68.5	+15.3
86	7- aminoclonazepam	5	28.7	–22.2	53.1	–66.0	25	15.0	+13.3	46.8	–69.2	75	10.0	–4.8	63.7	–59.4
87	7-aminoflunitrazepam	25	19.9	+28.7	60.7	–51.0	75	12.2	+21.1	65.2	–55.7	150	7.8	+1.5	70.8	–54.7
88	7-aminonitrazepam	5	28.8	–13.6	51.8	–36.2	25	15.5	+17.3	46.5	–58.5	75	8.5	–5.3	67.1	–42.1

[a]Intra-assay precision
[b]Trueness
[c]Recovery
[d]Matrix effect

Table 3 For each compound, intra-assay precision, trueness, recovery and matrix effect at low, medium and high concentration are reported

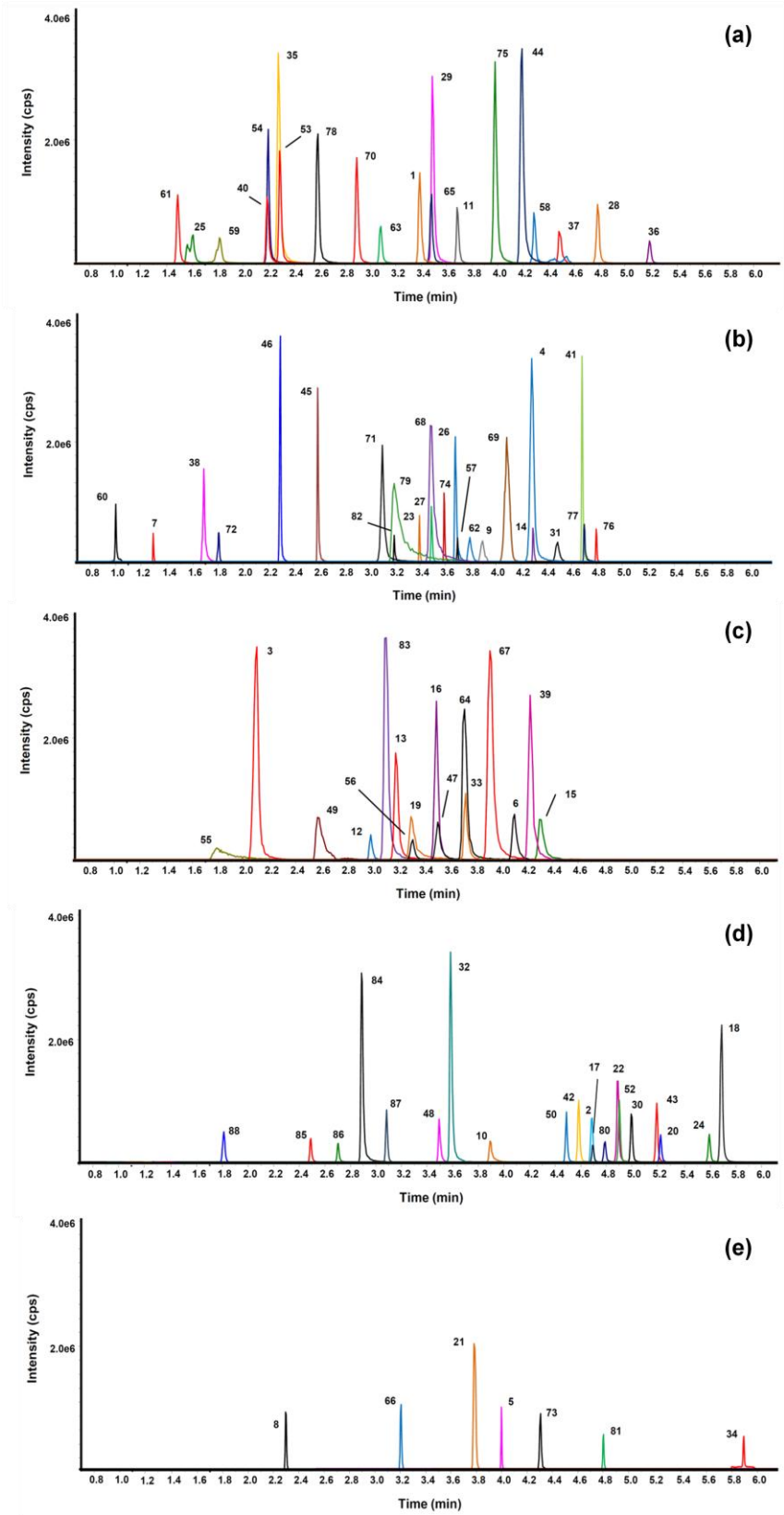

Fig. 1 SRM chromatograms recorded from a whole blood sample spiked with all analytes at 50 ng/mL concentration. For each analyte, labeled by the progressive number assigned in Table 1, only the target ion is shown. a–d SRM chromatograms for ESI+ ion mode. e SRM chromatogram for ESI− ion mode

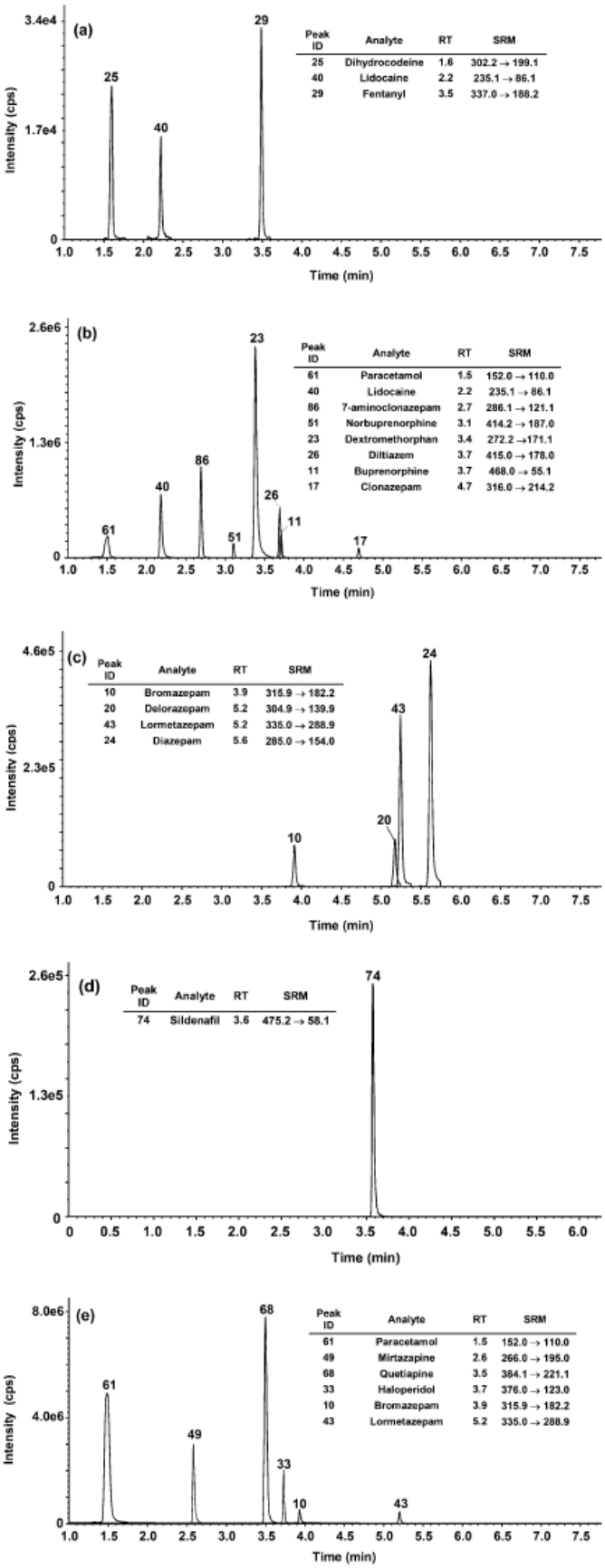

Fig. 2 SRM chromatograms obtained from UHPLC–MS/MS experiments. a Case report 1. b Case report 2. c Case report 3. d Case report 4. e Case report 5