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Systematic forensic toxicological analysis by liquid-chromatography -quadrupole-time-of-flight mass spectrometry in serum and comparison to gas chromatography-mass spectrometry

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ABSTRACT

Comprehensive screening procedures for psychoactive agents in body fluids are an essential task in clinical and forensic toxicology. With the continuous emergence and adaption of new psychoactive substances (NPS) keeping a screening method up to date is challenging. To meet these demands, hyphenated high-resolution mass spectrometry has gained interest as extensive and expandable screening approach. Here we present a comprehensive method for systematic toxicological analysis of serum by liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) with data independent acquisition. The potential of this method was demonstrated by analysis of 247 authentic serum- and 12 post-mortem femoral blood samples. Thus 950 compounds, comprising 185 different drugs and metabolites could be identified. For the detected substances, including pharmaceutical substances, illicit drugs as well as NPS, serum concentrations were confirmed ranging from traces to toxic values indicating the capability for forensic toxicological requirements. Positive identification of drugs was achieved by accurate mass measurement (±5 ppm for $[M+H]^+$; ±10 ppm for $[M-H]^-$), retention time (±0.35 min), isotopic pattern match (less than 10 *m/z* RMS [ppm]), isotope match intensity (less than 20% RMS) and the presence of at least two fragment ions. The LC-QTOF-MS procedure was shown to be superior to serum screening by GC–MS, since 240% (335 versus 141) more drugs were identified in serum samples compared to GC–MS.

1. Introduction

Non-targeted screening of body fluids for psychoactive agents is an essential task in clinical and forensic toxicology before confirmation and quantification of the uncovered substances can be conducted. In contrast to clinical toxicology, where urine is most widely used for non-targeted screening, in forensic toxicology, screening is often performed in serum since frequently only blood samples are submitted to the laboratory for analysis. Screening procedures often comprise a combination of immunoassays, gas chromatography–mass spectrometry (GC–MS), liquid chromatography with diode array detection (LC-DAD), and/or hyphenated mass spectrometry [1–3]. In the last decades liquid chromatography–mass spectrometry (LC–MS) has become increasingly important, using multi-target screening approaches with a defined number of compounds [4–7]. However, systematic toxicological analysis (STA) by GC–MS in urine or serum has still its place in comprehensive drug screening thanks to large reference libraries e.g. the Maurer/Pfleger/Weber GC–MS libraries [8,9] and the possibility to use deconvolution algorithms like the freeware program AMDIS (Automated Mass Spectral Deconvolution and Identification System) combined with the appropriate target library for STA [10–12].

More recently, liquid chromatography combined with time-of-flight mass spectrometry (LC-TOF-MS) has gained more attention in clinical and forensic toxicology [13]. For example, elucidation of *in vitro* and *in vivo* drug metabolism [14–17], but also comprehensive screening procedures for the detection of pharmaceuticals, drugs of abuse and other toxins mostly in urine have been described [18,19]. The identification criteria for compounds were based on accurate mass, retention time (RT), isotopic pattern and relative abundance of the isotopic peaks. Additionally, liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) allows for the generation of fragments obtained from collision-induced dissociation (CID). Hence, additional

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identification criteria are available for the unequivocal identification of a compound using one or more fragment ions with their appropriate exact mass or by comparison with library spectra. This approach has been applied for some toxicological screening procedures comprising urine, post mortem blood and hair [20–25] but also for the simultaneous screening and quantification of a defined number of drugs in blood [26]. Pedersen et al. [27] describe a forensic screening method for illicit and medicinal drugs in whole blood samples that covered 256 compounds using LC-QTOF-MS with data independent acquisition. The same technique was used for a combined targeted and non-targeted screening approach to 44 authentic forensic whole blood samples, describing the additional identification of compounds not included in a targeted screening approach [28].

Herein, we present a comprehensive method for STA by LC-QTOF-MS in serum comprising a broad spectrum of forensic-toxicological relevant drug classes as well as illicit drugs and the group of new psychoactive substances (NPS) with the possibility for retrospective data analysis. Identification criteria are examined to differentiate between true hits and false positive findings. The applicability will be demonstrated using authentic serum samples from forensic casework and positive findings are correlated with the corresponding serum concentrations. Since such modern screening approaches are rarely compared to more traditional drug screening techniques [23,29], the acquired data is compared with our routinely used STA procedure using GC–MS.

2. Materials and methods

2.1. Chemicals and reagents

Diethyl ether, ethyl acetate, methanol, sodium hydroxide, sodium sulphate (Na_2SO_4) and acetic anhydride were obtained from VWR (Darmstadt, Germany). Pyridine and ammonium formate were obtained from Sigma-Aldrich (Steinheim, Germany). UHPLC-MS grade acetonitrile was from Chemsolute (Renningen, Germany) and formic acid from Biosolve (Dieuze, France). Trimipramine-D_3 maleate solution and pentobarbital-D_5 were purchased from Cerilliant (Round Rock, TX, USA). All other standard substances used were from Cerilliant or from LGC standards (Wesel, Germany). Reference serum containing drugs of abuse was purchased from Medichem Diagnostica (Steinenbronn, Germany). All chemicals were of LC–MS or GC–MS grade.

2.2. Serum samples

Serum samples were routinely submitted to the authors' laboratory by the police of Lower Saxony with the request for drug analysis. The standard procedure comprises immunochemical screening for standard drugs of abuse and non-targeted screening by GC–MS of serum samples as described previously [12]. Positive findings were subsequently confirmed and quantified by specific targeted analysis comprising several validated GC–MS or LC–MS methods used in routine laboratory work. Prior to analysis by LC-QTOF-MS, aliquots of serum samples and post-mortem blood were stored without preservatives at −20 °C for not more than two years.

2.3. Sample preparation for systematic toxicological analysis by GC–MS

Sample preparation was carried out as described previously [30]. Briefly, 1 mL of serum mixed with 50 μL internal standard trimipramine-D_3 (10 μg/mL in methanol) were extracted with 5 mL diethyl ether/ethyl acetate (1:1 vol/vol) after 1 mL of saturated sodium sulphate solution was added to the sample. Then, the mixture was centrifuged for phase separation and the organic phase was transferred and saved in a pear shaped flask. The aqueous phase was adjusted to pH 8–9 with 1 mL of aqueous sodium hydroxide (1 mol/L). The mixture was extracted again with 5 mL diethyl ether/ethyl acetate. Both, neutral and alkaline organic layers were then pooled into the same pear shaped flask and evaporated to dryness at 40 °C under reduced pressure. The residue was dissolved in 100 μL of methanol and 1 μL was injected into the GC–MS system. A portion of the extract was derivatized in glass vials with conical bottom by adding 20 μL of pyridine and 30 μL of acetic anhydride and incubation in a microwave oven for 5 min at 450 W. Afterwards, the reaction mixture was evaporated to dryness under nitrogen flow at 40 °C and the residue was dissolved in 30 μL of methanol. 1 μL was injected into the GC–MS system.

2.4. GC–MS settings

The GC–MS instrument was an Agilent Technologies (AT, Waldbronn, Germany) 7890A gas chromatograph combined with a 5975C MSD mass spectrometer. The GC conditions for the screening procedures were as follows: splitless injection mode; column, Macherey-Nagel Optima 5MS Accent (30 m × 0.25 mm i.d.); injection port temperature 270 °C; helium carrier gas flow 1 mL/min; column temperature programmed from 100 to 340 °C at 30 °C/min, initial time 2 min, final time 6 min. The MS conditions were as follows: electron ionization mode, ionization energy 70 eV, ion source temperature 300 °C, capillary directed interface 300 °C; full scan mode *m/z* 44–600, 1 scan/s. The GC–MS screening method is checked routinely by injecting a standard mixture of substances with different chromatographic characteristics, following the guidelines for quality assurance of the Society of Toxicological and Forensic Chemistry (GTFCh).

2.5. GC–MS data analysis

The full scan data files acquired by the GS–MS system were evaluated semi-automatically using the AMDIS software in simple mode (Automated Mass Spectral Deconvolution and Identification System; http://www.amdis.net/) as described previously [12]. The used target library was an AMDIS-readable version of the Maurer/Pfleger/Weber MPW_2011 library [8]. The AMDIS settings for deconvolution and identification were as follows: width, 12; adjacent peak subtraction, 2; sensitivity, high; resolution, medium; shape requirement, medium; minimum match factor (MMF), 50.

2.6. Sample preparation for systematic toxicological analysis by LC-QTOF-MS

Sample preparation for the LC-QTOF-MS procedure was in accordance to the well established neutral and alkaline liquid–liquid extraction described above for STA in serum by GC–MS. In brief, to 0.2 mL of serum 10 μL internal standard trimipramine-D_3 (10 μg/mL in methanol) and pentobarbital-D_5 (20 μg/mL) were added. Extraction was done with 1 mL diethyl ether/ethyl acetate (1:1 vol/vol) after 0.2 mL of saturated sodium sulphate solution was added to the sample. Then, the mixture was centrifuged for phase separation and the organic phase was transferred and saved in an eppendorf reaction tube. The aqueous phase was adjusted to pH 8–9 with 0.2 mL of aqueous sodium hydroxide (1 mol/L). The mixture was extracted again with 1 mL diethyl ether/ethyl acetate. Both, neutral and alkaline organic layers were then pooled and evaporated to dryness at 40 °C under a nitrogen flow. The residue was dissolved in 100 μL of 50% mobile phase A + mobile phase B and 1 μL was injected into the LC system.