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Targeted toxicological screening for acidic, neutral and basic substances in postmortem and antemortem whole blood using simple protein precipitation and UPLC-HR-TOF-MS

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ABSTRACT

A broad targeted screening method based on broadband collision-induced dissociation (bbCID) ultra-performance liquid chromatography high-resolution time-of-flight mass spectrometry (UPLC-HR-TOF-MS) was developed and evaluated for toxicological screening of whole blood samples. The acidic, neutral and basic substances covered by the method were identified in postmortem and antemortem whole blood samples from forensic autopsy cases, clinical forensic cases and driving under the influence of drugs (DUID) cases by a reverse target database search. The screening method covered 467 substances. Validation was performed on spiked whole blood samples and authentic postmortem and antemortem whole blood samples. For most of the basic drugs, the established cut-off limits were very low, ranging from 0.25 ng/g to 50 ng/g. The established cut-off limits for most neutral and acidic drugs, were in the range from 50 ng/g to 500 ng/g. Sample preparation was performed using simple protein precipitation of 300 μL of whole blood with acetonitrile and methanol. Ten microliters of the reconstituted extract were injected and separated within a 13.5 min UPLC gradient reverse-phase run. Positive electrospray ionization (ESI) was used to generate the ions in the *m/z* range of 50–1000. Fragment ions were generated by bbCID. Identification was based on retention time, accurate mass, fragment ion(s) and isotopic pattern. A very sensitive broad toxicological screening method using positive electrospray ionization UPLC-HR-TOF-MS was achieved in one injection. This method covered basic substances, substances traditionally analyzed in negative ESI (e.g., salicylic acid), small highly polar substances such as beta- and gamma-hydroxybutyric acid (BHB and GHB, respectively) and highly non-polar substances such as amiodarone. The new method was shown to combine high sensitivity with a very broad scope that has not previously been reported in toxicological whole blood screening when using only one injection.

1. Introduction

The ability to screen for a large number of drugs and drugs of abuse is essential when handling clinical and forensic biological samples. The screening techniques used in forensic laboratories have been dominated by immunological techniques [1,2], gas chromatography–mass spectrometry (GC–MS) [3], high-performance liquid chromatography with photodiode array detection (HPLC-DAD) [4] and liquid chromatography–mass spectrometry ($LC–MS^n$ [5–9]). Over the past decade, time-of-flight mass spectrometry (TOF-MS) has been introduced and has become a powerful tool for clinical and forensic toxicology laboratories [10–25]. Immunological techniques are fast and easy to use but are limited by a lack of specificity [25]. The large commercial libraries available for GC–MS screening are very useful [3], but the growing interest in and need to detect metabolites, which by nature are often hydrophilic, limit the use of this technique. Generally, detection of polar, thermolabile and non-volatile substances is a challenge for GC–MS [7]; however, these substances are easily analyzed using $LC–MS^n$ [8]. $LC–MS^n$ techniques are powerful screening techniques as they enable the direct analysis of aqueous samples. HR-TOF-MS techniques are superior to $LC–MS^n$ because they can include more substances in a given screening. Furthermore, the higher resolving power of the HR-TOF-MS instruments results in greater specificity [13]. Another advantage of the HR-TOF-MS instruments is the potential to perform retrospective data analyses [26,27] because the data are typically acquired over a

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wide mass range in a full scan mode. Unknowns and metabolites can tentatively be identified in a sample without having reference standards [18]. Orbitrap technologies also seem to be a promising new technique in forensic drug screening [28,29].

There are several challenges when performing systematic toxicological screening analysis in whole blood samples from clinical and forensic toxicological cases. The screening method used should ideally cover all toxic substances as well as illegal and prescription drugs that may explain or contribute to the cause of death or the forensic investigation in general. A general screening method that may include all possible drugs is ideal, but the most important and obvious drugs must be included in a screening method. However, including too many drugs in the method, especially without a retention time, results in many false positive results and greatly increases the amount of time needed for a component data search. The screening method should ideally have broad dynamic ranges for all drugs and be able to identify drugs in low therapeutic concentrations as well as in the high lethal concentrations observed in suicidal cases. The span in therapeutic levels from one drug to another also has to be considered with regard to the extraction procedure. Matrix contributions can interfere with the detection of the target substance by increasing or reducing the target signal [30]. Highly polar substances can show poor retention on traditional reverse-phase analytical columns. The development of faster and more sensitive high-resolution mass spectrometers combined with UPLC makes it possible to simplify sample preparation, thus covering a wide range of different compound classes in one step. Several vendors that produce HR-TOF-MS instruments offer libraries of more than 1000 relevant toxicological substances [31–33]. This reduces the time-consuming work of purchasing and analyzing hundreds of substances to obtain retention time data and spectral information. The downside of this approach is that it is difficult to establish cut-off values or fully validate a method without having access to the reference substances included in the method. Many laboratories therefore still tend to use in-house libraries based on purchased substances [20–22]. The many new psychoactive substances (NPS) emerging in the illegal market [34] are frequently not covered by a commercially available library unless continuously updated by the supplier. Drift in the retention time of components on the analytical column due to general use and interfering components in the sample matrix is difficult to discover without access to the reference substances.

Toxicological screening methods using TOF-MS instruments to detect over-the-counter drugs, prescription drugs and illegal drugs in whole blood have been described previously [12,14,16,19–21,35]. These methods are based on different extraction methodologies for different drug classes and the use of both positive and negative ionization, which results in two injections because positive/negative switching is not optimal on the currently availably HR-TOF-MS instruments. The objective of this work was to develop a broad toxicological screening method that covers acidic and neutral drugs, such as mild pain relievers, anti-inflammatory drugs, anticonvulsants, antiepileptic drugs, skeletal muscle relaxants and sedatives, together with a large group of basic substances in a single injection. The sensitivity of this method should approximately equal the confirmation methods used in our laboratory [36–44] as well as the sensitivity needed for the specific drug (e.g., the lower therapeutic level [45] and/or legal limits for DUID cases in Denmark [46]).

To the best of our knowledge, no previous work has been presented that describes a targeted UPLC-HR-TOF-MS screening method in whole blood using only a single injection that covers this broad range of substances of different physical and chemical properties.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (Fluka LC-MS Chromasolv) was purchased from Sigma-Aldrich Chemie GmBh (Steinheim, Germany). Acetonitrile (LiChrosolv® hypergrade for LC–MS) was purchased from VWR International (Radnor, Pennsylvania, USA). Hydrochloric acid (fuming 37% for analysis), formic acid (98–100%), 2-propanol (LiChrosolv gradient grade for LC), acetic acid (glacial, 100% analytical grade) and sodium hydroxide monohydrate (99.99% pure) were purchased from Merck (Darmstadt, Germany). Purified water was prepared by a Direct-Q 3 apparatus (Millipore, Bedford, MA). Nitrogen 99.999% was purchased from Air Liquide Danmark A/S (Horsens, Denmark).

2.2. Reference substances

The reference substances and solutions were obtained from a variety of sources: Sigma-Aldrich (Schnelldorf, Germany), Cerilliant (Round Rock, TX, USA), Toronto Research Chemicals Inc. (TRC) (North York, Canada), Lipomed AG (Arlesheim, Switzerland), Chiron (Trondheim, Norway), Temmler Pharma GmbH & Co. KG (Marburg, Germany), Australian Government National Measurement Institute (Sydney, Australia), Unikem (Copenhagen, Denmark), Roche (Basel, Switzerland), Cambrex Profarmaco (Milano, Italy), Pfizer (Ballerup, Denmark), Wyeth Pharmaceuticals (NY, USA), Synthon (Sante Fe, Argentina), Mundipharma Research (Cambridge, UK), Lundbeck (Copenhagen, Denmark), AstraZeneca (Cheshire, UK), Ethypharm (Houdan, France), and Jucker Pharma (Stockholm, Sweden). The identity and sufficient purity of the substances was always monitored by mass spectrometry. Some substances were seized drugs obtained from the Danish Police, and their identities were measured using different spectrometric techniques (e.g., GC–MS, HR-TOF-MS, nuclear magnetic resonance spectroscopy (NMR) [47]). Several of the new psychoactive substances (NPS) were obtained from the European Project RESPONSE, a project co-funded by the European Union Program, "Prevention of and Fight against Crime."

2.3. Biological materials

Whole blood samples from healthy volunteers who were free of any xenobiotics were obtained from the local blood bank at Aarhus University Hospital (Skejby, Denmark) and were used as blank matrix and for preparation of calibrators and quality control (QC) samples. The samples were analyzed using the described UPLC-HR-TOF-MS method and a quantitative method for THC and THC-COOH. Antemortem whole blood samples (DUID and clinical forensic samples) submitted to our laboratory for routine toxicological analysis and postmortem whole blood samples collected during medico-legal autopsies were also included. Antemortem whole blood from forensic traffic cases was collected and preserved in Venosafe VF-053SF32 tubes containing 6.8 mg of NaF and 15.7 mg of citrate–EDTA buffer ingredients (FS mixture). All postmortem samples were mixed with NaF (5 mg/g). All samples were stored at −18 °C until use.

2.4. Quality control samples

2.4.1. QC-RT

A solution containing 325 substances, used to verify that the retention time was within the detection window, was prepared in methanol: Milli-Q water (15:85) with 0.1% formic acid. For most basic substances, the concentrations were 30 ng/mL. For neutral