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Determination of 74 new psychoactive substances in serum using automated in-line solid-phase extraction-liquid chromatography-tandem mass spectrometry

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ABSTRACT

A detailed description is given of the development and validation of a fully automated in-line solid-phase extraction-liquid chromatography-tandem mass spectrometry (SPE-LC–MS/MS) method capable of detecting 90 central-stimulating new psychoactive substances (NPS) and 5 conventional amphetamine-type stimulants (amphetamine, 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxy-N-ethyl-amphetamine (MDEA), methamphetamine) in serum. The aim was to apply the validated method to forensic samples. The preparation of 150 µL of serum was performed by an Instrument Top Sample Preparation (ITSP)-SPE with mixed mode cation exchanger cartridges. The extracts were directly injected into an LC–MS/MS system, using a biphenyl column and gradient elution with 2 mM ammonium formate/0.1% formic acid and acetonitrile/0.1% formic acid as mobile phases. The chromatographic run time amounts to 9.3 min (including re-equilibration). The total cycle time is 11 min, due to the interlacing between sample preparation and analysis. The method was fully validated using 69 NPS and five conventional amphetamine-type stimulants, according to the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh). The guidelines were fully achieved for 62 analytes (with a limit of detection (LOD) between 0.2 and 4 µg/L), whilst full validation was not feasible for the remaining 12 analytes. For the fully validated analytes, the method achieved linearity in the 5 µg/L (lower limit of quantification, LLOQ) to 250 µg/L range (coefficients of determination > 0.99). Recoveries for 69 of these compounds were greater than 50%, with relative standard deviations ≤ 15%. The validated method was then tested for its capability in detecting a further 21 NPS, thus totalling 95 tested substances. An LOD between 0.4 and 1.6 µg/L was obtained for these 21 additional qualitatively-measured substances. The method was subsequently successfully applied to 28 specimens from routine forensic case work, of which 7 samples were determined to be positive for NPS consumption.

1. Introduction

New psychoactive substances (NPS) is a collective term for synthetic compounds which mimic psychotropic effects on the human body in a similar fashion to conventional drugs of abuse, as defined in accordance with the UN Conventions 1961 (Single Convention on Narcotic Drugs) and 1971 (Convention on Psychotropic Substances) [1]. These substances often exhibit only minor structural modifications in comparison to conventional drugs, but can be legally traded until national restrictions are put in place. As of December 2015 the EU Early Warning System of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) was monitoring a total of 570 substances [2]. Stimulants are an important group of NPS that can be classified into synthetic cathinones, aminoindanes, phenethylamines, piperazines, pipradrols, phencyclidine-type substances and tryptamines. Typically, stimulants induce or inhibit the direct or indirect increase of neurotransmitters, e. g. noradrenalin, serotonin and/or dopamine, in the synaptic cleft. In this way, they give rise to a stimulant (pipradrols, phenethylamines, methamphetamine-like cathinones), an empathogen-entactogen (phenylpiperazines, aminoindanes, para-substituted phenethylamines, MDMA-like cathinones) or a hallucinogenic (tryptamines, hallucinogenic phenethylamines) effect. A second large group of

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NPS comprise the synthetic cannabinoid receptor agonists, which are not addressed in this work.

NPS are often sold without instruction on their use or any appropriate labelling. Despite some NPS being labelled with the drug formulation, customers cannot rely on the accuracy of the information supplied. Due to the absence of knowledge on how NPS interact with other substances, the actual active drug content being unspecified, drug impurities, dosage, and the individual drug response unknown, it is difficult for consumers to assess the risks of consuming the drug, or to calculate suitable doses. NPS often possess higher receptor-binding affinities in contrast to classical recreational drugs [3–6], thus most probably resulting in a higher potency and, very often, increased levels of undesired side effects. Adventurous experimenting and multi-drug-use are frequently observed amongst NPS users [7–9]. In summary, this results in a complicated risk assessment and a raised potential for the subjects becoming intoxicated. It is therefore unsurprising that, in the literature, a multitude of emergency room visits and occasional deaths have been ascribed to the consumption of NPS (a comprehensive summary of the literature is given in Table S1, provided as supplementary material).

Both the diversity and swift development of NPS have resulted in a continual analytical challenge concerning identification and quantification. For the purposes of detecting NPS in biological matrices, selective and sensitive methods are required. Immunoassays are not adequate for the detection of most NPS due to the high structural diversity, the insufficient cut-off levels for highly potent NPS and the lack of sufficient cross-reactivity with the established conventional stimulant immunoassays [10]. At present, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is still in common use for multi-target determination of NPS. The latter technique, especially when utilized in the multiple-reaction-monitoring mode (MRM), provides high selectivity and sensitivity in combination with good precision and accuracy over a wide dynamic range, allowing the development of fast analytical methods. Some LC–MS/MS applications for the determination of a multitude of NPS in biological matrices, such as serum [11,12], blood [13–15], urine [13,16–21], oral fluid [22,23], as well as in hair [24] have been previously described. To the best of our knowledge there are only two publications which describe the determination of stimulants by LC–MS/MS in serum. In 2010, Wohlfarth et al. published a semi-quantitative method for the determination of 35 NPS (amphetamines, cathinones, tryptamines and piperazines) with LODs between 1 and 5 ng/mL [12]. In 2013 Swortwood et al. published a method for the determination of 32 NPS (cathinones, phenethylamines, piperazines and tryptamines), which fulfilled the validation requirements for 27 analytes [11].

The aim of our study was to develop and validate a sensitive, robust and high-throughput in-line SPE-LC–MS/MS method for the rapid analysis of stimulants (including NPS) in serum. The focus was set on serum as it is the dominant matrix in the author's laboratory and an important matrix through which to judge acute influence by a drug.

2. Materials and methods

2.1. Chemicals and materials

All specified substances are listed with their abbreviation, common name and substance class in Tables 1 and 2. Certified reference standard solutions (c = 1 mg/mL) of 25B-NBOMe, 2C-B-FLY, 2C-D, 2C-E, 2C-N, 2C-P, 2-DPMP, 2-FMA, amphetamine, BDB, Bromo-DragonFLY, bufotenin, BZP, cathinone, ketamine, eutylone, MBDB, mCPP, MDEA, MDMA, MDPBP, mephedrone, PCP and pentylone were obtained from Cerilliant (Round Rock, TX, USA). Internal standard solutions of BZP-d7 (c = 0.1 mg/mL), amphetamine-d5 (c = 1 mg/mL), MBDB-d5 (c = 0.1 mg/mL), MDA-d5 (c = 1 mg/mL), MDEA-d5 (c = 1 mg/mL), MDMA-d5 (c = 1 mg/mL), mephedrone-d3 (c = 0.1 mg/mL), methylone-d3 (c = 0.1 mg/mL) and methamphetamine-d11 (c = 1 mg/mL) were also purchased from Cerilliant. Standard solutions (c = 1 mg/mL) of 2,5-DMA, 2C-B, 2C-H, 2C-I, 2C-T-2, 2C-T-7, 3,4,5-TMA, 4-FA, amfepramone, bupropion, cathine, DMT, DOB, DOET, DOM, EMC, ethylone, MDPPP, methcathinone, pentedrone, PMA, PPP and PVP were purchased from Lipomed (Bad Saeckingen, Germany). Standard solutions (c = 1 mg/mL) of 2A-I, 2-FMC, 3-MeO-PCE, 3-MeO-PCP, 4-AcO-MET, 4-FBT, 4-FPP, 4-MEC, 4-MeO-PCP, 5-MAPB, 5-MeO-DALT, 6-APB, 6-EAPB, bk-MMBDB, butylone, diclofensine, dimethylcathinone, flephedrone, M-ALPHA, methylone, MDA, MDAI, MDC, methamphetamine, methoxetamine, methedrone, methylphenidate, MDPV, MPBP, naphyrone, NEB and PMEA were obtained from LGC Standards (Wesel, Germany). The hydrochloride salt of 25H-NBOMe, 4-HA, 4-MeOPP, 5-MeO-aMT, 5-MeO-DiPT, 5-MeO-DMT, 6-TMA, D2PM, DiPT, DPT, harmaline, ethylphenidate, NMT, and PMMA was obtained as a powder from Cayman Chemical Company (Ann Arbor, MI, USA). Using dimethyl sulfoxide (DMSO) as solvent, 5 mg/mL stock solutions were prepared for both D2PM and DiPT, while a 10 mg/mL solution of 4-MeOPP was produced. Stock solutions, in methanol, of 0.5 mg/mL of harmaline, 1 mg/mL of 25H-NBOMe, and 5 mg/mL of 4-HA, 5-MeO-aMT, 5-MeO-DiPT, 5-MeO-DMT, 6 TMA DPT, ethylphenidate and NMT, and 10 mg/mL of PMMA, were made up. The hydrochloride salt of MDAT was purchased from LGC Standards. MDAT was prepared at a concentration of 1 mg/mL in methanol. Pure DET was obtained from the National Measurement Institute (Sydney, Australia). Using methanol as solvent, 10 mg/mL DET stock solution was prepared. All stock solutions were stored at −20 °C.

Water and acetonitrile used for chromatography (LC–MS grade), as well as DMSO (analytical grade, ≥99.8%), were purchased from Roth (Karlsruhe, Germany). Water and methanol used during solid-phase extraction (HPLC grade) were also purchased from Roth. Tetrahydrofuran (analytical grade) was obtained from Merck (Darmstadt, Germany). Ammonium formate (HPLC grade), ammonium hydroxide solution 25% (LC–MS grade) and formic acid 100% (LC–MS grade) were purchased from Fluka (Steinheim, Germany). Ammonium acetate (trace metal grade) and calf serum (Iron-Fortified, USA origin, from formula-fed bovine calves, sterile-filtered, cell culture tested) were purchased from Sigma Aldrich (Munich, Germany).

Various cartridges filled with sorbent from different suppliers were tested: 10-ACER (10 mg Bond Elut Certify from Agilent, Santa Clara, CA, USA), 30-ACER (30 mg Bond Elut Certify from Agilent), 10-APCXP (10 mg Plexa PCX from Agilent), 10-JSCXP (10 mg Jordi Gel SCX from Jordi Labs, Mansfield, MA, USA) and 10-UBCXP (10 mg STYRE SCREEN BCX from United Chemical Technologies, Levittown, PA, USA). The cartridges were obtained from ITSP Solutions, Inc. (Hartwell, GA, USA). The cartridges with 10 mg sorbent have a total sorbent volume of 16 µL and a liquid in the sorbent volume of 6 µL. The total extra-column volume (primarily in the cartridge tip [not including the sorbent volume]) is 16 µL, in total 32 µL.

2.2. Instrumentation

Analyte extraction and sample cleanup were performed using an automated solid-phase extraction (SPE) sample processor "prep and load high throughput screening" (PAL HTS) from CTC Analytics (Zwingen, Switzerland), equipped with a 100 µL syringe (Hamilton; Bonaduz, Switzerland), fitted with three trays and an injection valve, kindly provided by Scientific Instruments Manufacturer (SIM) GmbH. The Instrument Top Sample Preparation (ITSP) hardware kit (including ITSP rack, needle guide) was obtained from ITSP Solutions, Inc. (Hartwell, GA, USA).

Samples were analyzed using an Agilent LC–MS/MS-system (Santa Clara, CA, USA) equipped with a 1200SL Binary Pump LC system coupled to a 6460A triple quadrupole mass spectrometer with Jet Stream technology and electrospray ionization (ESI), controlled by Mass Hunter software (Data Acquisition B.07.01, Agilent).