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Rapid and simple procedure for the determination of cathinones, amphetamine-like stimulants and other new psychoactive substances in blood and urine by GC–MS

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ABSTRACT

In the last few years an increasing number of new psychoactive substances (NPS), with different chemical structures (of which 37% are stimulants), have been released into the illicit drug market. Their detection and identification in biological samples is hence of great concern.

The aim of this work was to develop a high-throughput and rapid method for the determination of different classes of stimulants (amphetamine-type stimulants, cathinones, phenethylamines and ketamine analogues) from blood and urine samples using GC–MS.

The proposed method allows the almost simultaneous derivatization and extraction of analytes from biological samples in a very short time, by using hexyl chloroformate as derivatization agent. The extraction of analytes was performed by Dispersive Liquid Liquid Microextraction (DLLME), a very rapid, cheap and efficient extraction technique that employs microliter amounts of organic solvents.

The chromatographic method allowed for the separation of 26 stimulants including positional isomers (3-MMC and 4-MMC). The method was validated on urine and blood samples with the ability to detect and quantify all analytes with satisfactory limits of detection (LODs) ranging between 1 and 10 ng/mL, limits of quantification (LOQs) between 2 and 50 ng/mL, selectivity and linearity (5–1000 ng/mL).

The method was then applied to real samples from forensic cases, demonstrating its suitability for the screening of a wide number of stimulants in biological specimens.

1. Introduction

In the last few years an increasing number of new psychoactive substances (NPS), with different chemical structures, have been released into the illicit drug market. With an average emergence rate of more than one substance per week, there is an unprecedented influx of NPS in the illicit drug market worldwide [1–3]. These substances, synthesized as analogues of existing drugs to bypass laws and regulations, generally have the same or even higher effects than the compounds they derive from. Stimulants that share a phenylethylamine chemical structure are one of the major classes stimulants on the recreational drug market. These include both scheduled drugs like amphetamine-type stimulants (ATS) as well as of NPS such as phenethylamines and cathinones, that are included in a *per se* class. The latter two classes account for 37% of emerging substances [1]. Cathinones are derivatives of an active stimulant found in *Catha edulis* (khat), from which a diverse range of β-keto-amphetamines have been synthesized and sold as a 'legal' alternative to ATS.

In addition, amphetamine-type stimulants, in particular methamphetamine and MDMA have shown an increase in seizures of 21% and 122%, respectively [1].

The number of reported adverse events, toxicity and fatalities associated to NPSs abuse are ever increasing. Several fatalities and intoxications related to NPS use have been reported [4–12]. The latter dangers are augmented by polydrug use that is a common pattern of NPS use [13].

Therefore, the identification of NPS and amphetamine-type stimulants in biological samples is of great importance for forensic and clinical toxicologists, in order to evaluate the spread of NPS among population, and to diagnose intoxications and impairment due to the use of these substances.

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Several challenges are related to the identification of these compounds in biological samples, such as the large number of potential structures and the constant introduction of novel compounds.

Additionally, NPS are hardly detected under common immunoassay screening used for routine drug screening for their cost-effectiveness and rapid reporting. Furthermore, in case of a positive result, the identification of the actual substance that can cross-react with the immunoassay must be carried out by a chromatographic/mass spectrometric technique, able to distinguish among similar structures and isomers.

To overcome this problem some screening methods based on chromatography coupled with mass spectrometry have been proposed [14–25]. The use of hyphenated techniques such as LC–MS/MS or GC/MS requires a clean-up step before the instrument analysis, especially when it is performed on highly complex matrices like blood. Sample pre-treatment is in fact necessary to render the sample analysable by an effective purification from undesired matrix components, to reduce the matrix effect and therefore to enhance sensitivity.

Dispersive Liquid Liquid Microextraction (DLLME), is a liquid–liquid extraction with a ternary solvent system which takes advantage of the miscibility of the aqueous phase, a dispersive solvent and a high density extractant.

Initially developed for the analysis of polycyclic aromatic hydrocarbons (PAHs) in water by GC, and subsequently for the determination of organophosphoric pesticides by GC–MS [26,27], it has then been applied also for the analysis of drugs in biological samples [28–30]. DLLME is performed by the rapid injection of a mixture of low amounts of an organic solvent immiscible with water as the extractant, and a disperser solvent miscible with water, into the aqueous sample. The turbid mixture produced causes the formation of fine droplets (cloudy solution), which are dispersed through the aqueous sample creating a high exchange surface, allowing an effective and rapid extraction.

The main advantage of the DLLME technique respect to the usual liquid/liquid extraction or solid-phase extraction (SPE), generally used for the sample purification, is the use of very low amounts of organic solvents, that renders the technique very cheap and without the need of commercial cartridges.

GC–MS is the most established technique in forensic and clinical toxicology labs. This technology is a requirement for any lab performing toxicological analysis and is less economically demanding than LC–MS/MS. Therefore, methods developed for GC–MS inherently have a wider range of applicability in toxicology laboratories.

The aim of this work was to develop a GC–MS method for the analysis of more than 25 stimulants of different classes including amphetamine-type stimulants, synthetic cathinones, phenethylamines, ketamine and analogues, benzofurans and tryptamines in blood and urine using ultra-rapid DLLME and simultaneous derivatization for sample pre-treatment.

2. Materials and methods

2.1. Chemicals and reagents

Amphetamine, methamphetamine, 4-fluoromethcathinone (FMC), 4-methylamphetamine (4-MA), cathinone, N-methyl-2-aminoindane (NM2AI), 3-methylmethcathinone (3-MMC), pentedrone, methedrone, methylbenzodioxolylbutanamine (MBDB), 4-methylthioamphetamine (4-MTA), methylone, ethylone, butylone, norketamine, pentylone, α-pyrrolidinopentiophenone (α-PVP), ketamine, 4-bromo-2,5-dimethoxyphenethylamine (2-CB), and methoxetamine were supplied from LGC standards (Milan, Italy). 3,4-methylenedioxymethamphetamine (3,4-MDMA), 3,4-methylenedioxyamphetamine (3,4-MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), 4-methylethcathinone (4-MEC), methylenedioxypyrovalerone (MDPV), mephedrone (4-MMC), mephedrone-D3 were purchased from CHEBIOS Ltd. (Rome, Italy).

Water, chloroform, sodium hydroxide, sodium chloride and methanol were purchased from 3V-Chemicals (Rome, Italy); hexyl chloroformate was purchased from Sigma-Aldrich (Milano, Italy). All reagents and solvents were of analytical grade.

Standard compounds were stored according to supplier recommendations until their use.

2.2. Preparation of working solutions

Individual methanolic stock solutions containing 1 mg/mL of each of the listed standards were used to prepare a working mixture of standards at 10 μg/mL (MIX STIMULANTS).

An internal standard stock solution of mephedrone-D3 was prepared at a concentration of 10 μg/mL.

Stock and working solutions were stored at −20 °C until use.

2.3. GC–MS equipment and method

The GC–MS system used was an Agilent 7890 gas chromatograph coupled to an Agilent 5975C quadrupole mass detector (Agilent Technologies Italia, Milan, Italy) operating at 70 eV in electron ionisation mode. The apparatus was equipped with a J&W 5% phenyl-methylsilicone capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, CPS Analitica, Mi, Italy). Helium was used as carrier gas at a constant flow of 1 mL/min.

The chromatographic conditions were set as follows: The oven temperature was held at 130 °C for 2 min, increased to 270 °C at 15 °C/min, and a final temperature ramp at 50 °C/min, to 310 °C (held for 4 min). The injection port was set at 270 °C in splitless mode. The mass detector was operated in scan mode (scan range from *m*/*z* 50–390).

2.4. Sample preparation

Urine samples: 2.0 mL samples were spiked with 10 μL of deuterated internal standard, to obtain a final concentration of 50 ng/mL. This was followed by 200 μL of 0.2 M sodium hydroxide (containing 20 mg/mL of sodium chloride) and 500 μL of methanol.

Blood samples: 2.0 mL were also spiked with the deuterated internal standard solution at 50 ng/mL. The samples were then deproteinized with 2.0 mL of methanol and centrifuged at 6000 rpm for 10 min. The clear supernatant was transferred into a 15-mL conical tube containing 1 mL of water, and then 1 mL of 0.2 M sodium hydroxide containing 10 mg/mL of sodium chloride was added, in order to reach pH over 9.

The resulting solutions were rapidly derivatized by the addition of 20 μL of hexyl chloroformate and 30 s of manual shaking.

To obtain the formation of the cloudy solution, 350 μL of a 1:2.5 mixture of chloroform/methanol, were rapidly added to urine samples (700 μL for deproteinized blood samples). The samples were then centrifuged at 4400 rpm for 4 min to deposit the fine droplets of the extractant phase at the bottom of the tube. The infranatant phase (about 50 ± 5 μL) was transferred into a vial and 1.0 μL was directly injected in the GC–MS system.

The authentic samples with concentrations above calibration curves were diluted ten times with control blood/urine, so that the results of determinations were within the ranges of the calibration curves.