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Simultaneous determination of designer benzodiazepines in human serum using non-aqueous capillary electrophoresis – Tandem mass spectrometry with successive multiple ionic – Polymer layer coated capillary

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ABSTRACT

A novel non-aqueous capillary electrophoresis - tandem mass spectrometry method for the simultaneous separation, identification and quantification of nine designer benzodiazepines (bentazepam, etizolam, deschloroetizolam, diclazepam, flubromazepam, flubromazolam, nimetazepam, phenazepam, and pyrazolam) was developed. A non-aqueous running electrolyte consisting of 25 mM ammonium acetate with 100 mM trifluoroacetic acid in acetonitrile was used. The separation was carried out using a semipermanent coated capillary (successive multiple ionic-polymer coating) with a strong anodic electroosmotic flow at a negative separation voltage within twelve minutes. Electrospray ionization with a triple quadrupole mass spectrometry was utilized for the identification and quantification of selected designer benzodiazepines in a positive ionization mode. The developed method was validated and applied on the analysis of spiked serum sample following a simple liquid-liquid extraction. The LODs of the designer benzodiazepines were between 1.5 and 15.0 ng mL^{-1}.

1. Introduction

The identification and quantification of new designer drugs is an important task in the area of toxicological analysis. Designer drugs have been increasing in prevalence over the past ten years on the illegal drug market. Designer drugs are often distributed in the form of "legal highs" or "bath salts" or "not for human consumption" labelled products. Thus far, several classes of new designer drugs have been identified on the illegal drug scene. In particular, designer cathinones, phenylethylamines, synthetic cannabinoids or designer anabolic steroids are among the most frequent ones. Several reviews describing the current state of this field have been published recently [1–5].

Since the abuse of new designer drugs is becoming more and more popular, the development of new efficient, selective and sensitive analytical methods for their identification and quantification in various sample types is necessary. The routine methods used in clinical and toxicological laboratories cannot detect the presence of a new drug in analyzed samples therefore, the occurrence of false negative results and incorrect conclusions are not uncommon. In the last decade, numerous methods focusing on the analyses of different classes of new designer drugs in various matrices (e.g. herbal blends, spices, blood, urine, saliva or cerebrospinal fluid) have been reported. Several reviews were published summarizing the existing analytical methods for the determination of designer drugs in various sample types [6–11].

A novel class of designer drugs – designer benzodiazepines (DBZDs), was detected on the illegal drug scene in 2013. The first described DBZDs were diclazepam, flubromazepam and pyrazolam [12–14]. Recently, others became readily available. DBZDs have similar chemical structures to the therapeutically used benzodiazepines (BZDs). BZDs are mainly used in treating anxiety, insomnia, agitation, seizures, muscle spasms, in addition to being utilized in the treatment of drug-related withdrawal syndromes. Unlike BZDs, the pharmacological and toxicological effects of DBZDs are not well understood yet. DBZDs are not prescribed for medical purposes, and abusing DBZDs can lead to addiction, drug tolerance as well as many other serious health problems. DBZDs can also negatively impact the ability to drive a motor vehicle. Combining DBZDs with other drugs, such as ethanol, marijuana, stimulants and other psychoactive substances, can prove to be risky, since there are no available data about any possible synergic and antagonistic effects.

Abbreviations: ACN, acetonitrile; BZD, benzodiazepine; DBZD, designer benzodiazepines; MeOH, methanol; SMIL, successive multiple ionic-polymer layer; TFA, trifluoroacetic acid; TRIS, tris(hydroxymethyl)aminomethane

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Owing to the structural similarity of DBZDs to the classical BZDs, used as legal drugs in treatment, DBZDs can be positively detected by immunochemical methods. Immunochemical screening tests are based on the detection of related BZDs metabolites (namely glucuronides) and thus positive results could be obtained in a presence of DBZDs related metabolites in urine. From this point of view, positive urine samples can contain metabolites of BZDs as well as metabolites of DBZDs [15]. As a consequence, distinguishing between BZDs and DBZDs, using only immunochemical screening methods, is impossible.

In the last years, several methods for the characterization of selected DBZDs in terms of NMR spectra, MS spectra, IR spectra as well as LC-MS and/or GC-MS have been published. Moosmann et al. [13] published LC-MS/MS, GC-MS and NMR data gained from the analysis of flubromazepam in addition to its preliminary pharmacokinetics and metabolism. Other DBZDs (clonazolam, deschloroetizolam, flubromazolam and meclonazepam) were similarly characterized by Huppertz et al. [16]. A GC-MS assay of phenazepam in urine samples collected within one year period was reported by Kriikku et al. [17].

Despite the above mentioned methods, only two works dealing with the simultaneous separation and determination of several DBZDs in biological fluids have been published until now. Eleven DBZDs were simultaneously separated and detected in urine samples by a LC-MS/MS method [18]. The LOD values for pyrazolam, diclazepam, flubromazepam, meclonazepam, etizolam, phenazepam, nifoxipam, deschloroetizolam, clonazolam, flubromazolam, and flutazolam ranged from 1 to 10 ng mL^{-1}. Enzymatic hydrolysis was used for urine sample treatment prior to the analysis.

Recently, we have published another validated LC-TOF-MS method for the simultaneous separation and quantification of nine DBZDs in serum (namely pyrazolam, phenazepam, etizolam, flubromazepam, diclazepam, deschloroetizolam, bentazepam, nimetazepam, and flubromazolam) [19]. A liquid-liquid extraction (LLE) was employed for sample clean-up as well as preconcentration. The LOD values ranged from 0.10 ng mL^{-1} to 0.15 ng mL^{-1}.

Capillary electrophoresis hyphenated with mass spectrometry (CE-MS) is recognized as a complementary technique to LC-MS methods. CE has several benefits which can be utilized in toxicological analyses. The main advantages include low sample and reagent consumption, high peak efficiency and availability of different separation mechanisms (i.e. capillary zone electrophoresis, micellar electrokinetic chromatography, capillary gel electrophoresis and capillary isotachophoresis). To overcome the main drawback of CE consisting of relatively high LODs in case of a spectrophotometric detection, the hyphenation of CE with MS with electrospray ionization (ESI) is often used. The general applicability of CE and CE-MS for toxicological analyses was summarized in reviews [11,20–22]. In addition, CE separation of target analytes can be done in a non-aqueous environment by so-called non-aqueous capillary electrophoresis (NACE). Non-aqueous background electrolytes (BGEs) are employed for the separations of analytes with either poor or no solubility in aqueous environments, and also for the fine tuning of selectivity of sample components with problematic separation (i.e. in case of analytes that are difficult to ionize in the aqueous environments) [23,24]. Moreover, the components of non-aqueous BGEs are usually volatile that is necessary for hyphenation of NACE with ESI-MS. NACE-ESI-MS could be used as an alternative method for the separation and detection of analytes of toxicological interests.

The aim of this work was to develop a novel NACE-ESI-MS/MS method for the simultaneous analysis of nine DBZDs in serum samples. Chemical structures of the studied DBZDs are shown in Fig. 1. The developed method was validated in accordance with the common forensic requirements [25] and successfully applied on spiked serum samples.

2. Material and methods

2.1. Chemicals and reagents

Acetic acid (≥ 99%, w/v), acetonitrile (LC-MS grade) ammonium acetate (≥ 99.9%), dextran sulfate sodium salt (Mr ~ 40,000), hexadimethrine bromide (Polybrene), hydrochloric acid (37%, *w/v*), 1-chlorobutane, methanol (LC-MS grade), sodium hydroxide, trifluoroacetic acid (TFA, ≥ 99%, *w/v*), tris(hydroxymethyl)aminomethane (TRIS) and human drug-free serum (sterile-filtered)were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of bentazepam, diclazepam, etizolam, deschloroetizolam, flubromazepam, flubromazolam, nimetazepam, phenazepam, and pyrazolam were purchased from Chiron AS (Trondheim, Norway). Deuterated analogues of etizolam-d5, phenazepam-d4, and diazepam-d5 (internal standards, ISs) were also provided by Sigma-Aldrich. All standards were purchased in a quality of certified reference materials in methanol with a concentration of 1.0 mg mL^{-1}. Other chemicals listed above were of analytical grade purity if not stated otherwise. Deionized water (18 MΩ/cm) was prepared using a Millipore purification system (Millipore, Molsheim, France).

2.2. Instrumentation

Capillary electrophoresis Agilent 7100 equipped with an Agilent 6460 triple quadrupole mass spectrometry detector (Agilent, Waldbronn, Germany) was used for the CE-MS analyses. Optimization of the background electrolyte (BGE) composition was carried out using the same instrument equipped with a diode array detector (DAD). During the CE-DAD analyses the MS detector was disconnected. The bare fused silica capillary (50 μm id, 365 μm od, MicroSolv, Eatontown, NJ, USA) was activated by rinsing with 1 M sodium hydroxide solution for 30 min followed by deionized water for 30 min. The successive multiple ionic-polymer layer (SMIL) coating was performed based on previously published methods [26]. The coating procedure can be briefly summarized by the following steps: (i) the activated capillary was rinsed with 5% (*w/v*) hexadimethrine bromide for 30 min and allowed to react for 30 min; (ii) a 3% (*w/v*) solution of dextran sulfate was flushed through the capillary for 15 min and allowed to react for another 30 min; (iii) finally, the capillary was rinsed with the hexadimethrine bromide solution for 30 min followed by flushing with water for 30 min. The coating was carried out using a micropump (New Era Pump System, Farmingdale, NY, USA) at a flow rate of 20 μL min^{-1}. The stability of the SMIL coating using non-aqueous BGE was extremely high (> 1 000 analyses without damage of coating and without a significant shift of the electroosmotic mobility).

2.2.1. CE-DAD conditions

When using the DAD detector only, the used uncoated fused silica and SMIL capillary were of 48.5 cm (40 cm effective length) length and 50 μm i.d. The separation voltage was + 20 kV and − 20 kV for uncoated fused silica and SMIL capillary, respectively. A sample injection was performed hydrodynamically by 50 mbar for 5 s. The detection wavelengths were set up at 222 nm. The capillary was thermostated to 25 °C. Before the first use, the capillary was rinsed with 1 M NaOH, deionized water for 15 min and then with the BGE for 30 min in case of fused silica capillary. For the SMIL capillary, rinsing by deionized water and BGE were used only. Between the individual analyses, the capillary was flushed with a deionized water for 5 min and with the BGE for 5 min. At the beginning of each working day the capillary was rinsed with the deionized water and with the freshly prepared running electrolyte, each for 15 min. All of the measurements were performed five times unless stated otherwise.