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Determination of low levels of benzodiazepines and their metabolites in urine by hollow-fiber liquid-phase microextraction (LPME) and gas chromatography–mass spectrometry (GC–MS)

André Valle de Bairros[a,b,*], Rafael Menck de Almeida[a], Lorena Pantaleão[a], Thiago Barcellos[c], Sidnei Moura e Silva[c], Mauricio Yonamine[a]

[a] Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil
[b] Center of Chemical, Pharmaceutical and Food Sciences, Federal University of Pelotas, Brazil
[c] Institute of Biotechnology, University of Caxias do Sul, Brazil

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ABSTRACT

In this study, it is shown a method for the determination of benzodiazepines and their main metabolites in urine samples by hollow-fiber liquid-phase microextraction (LPME) in the three-phase mode. Initially, the hydrolysis step was performed using 100 μL of sodium acetate 2.0 mol/L buffer solution (pH 4.5), 25 μL of β-glucuronidase enzyme and incubation for 90 min at 55 °C. In parallel with hydrolysis, the LPME fiber (9 cm) was prepared. Its pores were filled with a mixture of dihexyl ether: 1-nonanol (9:1). Afterwards, a solution of 3.0 mol/L of HCl was introduced into the lumen of the fiber (acceptor phase). After hydrolysis, the fiber was submersed in the alkalinized urine (pH 10) containing 10% NaCl. Samples were then submitted to orbital shaking (2400 rpm) for 90 min. The acceptor phase was later withdrawn from the fiber, dried and the residue derivatized with trifluoroacetic anhydride (TFAA) for 10 min at 60 °C with further addition of *N*-methyl-*N*-tert-butyldimethylsilyltrifluoroacetamide containing 1% *tert*-butyldimethylchlorosilane (MTBSTFA) for 45 min at 90 °C followed by determination by gas chromatography–mass spectrometry (GC–MS). The calibration curves obtained showed linearity over the specified range, with a similar sensitivity to traditional techniques and a higher detection capability compared to most of the miniaturized methods described in the literature. The method has been developed and successfully validated and applied to urine samples from real cases of benzodiazepines intake.

1. Introduction

Benzodiazepines are central nervous system (CNS) depressant drugs often prescribed for the treatment of insomnia, anxiety, and epilepsy. However, this class of drugs can promote both tolerance and dependence and may also have an impact on the performance of given situations such as driving a vehicle or the ability to remain focused at work [1,2]. Despite the distribution control of benzodiazepines in many countries, this class of drugs has been used in suicide attempts [3,4]. Because of its pharmacological effects, high commercialization and relative ease in obtaining such substances, they are also used in many countries in drug-facilitated crimes (DFC). Diazepam, clonazepam and flunitrazepam were the most frequently benzodiazepines found in biological samples of DFC victims [5–8]. In these cases, benzodiazepines and their metabolites are the target analytes according to the Society of Forensic Toxicologists (SOFT) and United National Office on Drugs and Crime (UNODC) [9,10]. One of the analytical features required by these organizations is the sensitivity to determine very low concentrations of benzodiazepines and its metabolites in biological fluids when DFC is suspected.

A number of studies have been reported in the literature on the determination of benzodiazepines and their metabolites in biological fluids. Liquid and gas chromatography (LC and GC, respectively) are the main techniques to determinate these molecules, while liquid–liquid extraction (LLE) and solid phase extraction (SPE) are the most frequently used sample preparation techniques [11–15]. However, LLE and SPE require costly consumables such as solvents and cartridges. Having this in mind, miniaturized methods such as hollow-fiber liquid-phase microextraction (LPME) could be an interesting alternative for the extraction of benzodiazepines.

* Corresponding author at: Av. Professor Lineu Prestes, 580, bloco 13B CEP: 05508-900 São Paulo, SP, Brazil. Tel.: +55 11 3091 2194; fax: +55 11 3031 9055.
E-mail address: andrebairros@usp.br (A.V. de Bairros).

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However, this technique has been scarcely explored and is still seen as a challenge [16–18].

Hollow-fiber liquid-phase microextraction (LPME) is a relatively new technique and can be performed in a two-phase or three-phase mode. In a two-phase extraction system, the hydrophobic solvent is immobilized as a thin supported liquid membrane (SLM) into the pores of a porous hollow fiber. The lumen of the fiber is also filled with hydrophobic solvent (acceptor phase) and the system is placed in contact with the sample (donor phase). The analytes are extracted from the sample (aqueous phase), through the SLM (organic phase) and finally into the acceptor phase. In the case of a three-phase extraction system, the analytes must be ionized with an aqueous solution (acceptor phase) inside the lumen of the hollow fiber. Consequently, the analytes will get trapped in the acceptor phase. Due to the high sample-to-acceptor volume ratio, very high enrichments can be obtained by using LPME, especially in the three-phase mode. This technique provides a better recovery when compared with the two-phase described previously. It has also an easy drying process and subsequent derivatization of the analytes plus a chemical reaction that increases the selectivity and stability of the compounds. Therefore, an excellent clean-up has been reported from complex biological matrices such as urine samples due to the size of the pores as they provide microfiltration of macromolecules [19–22].

Urine samples continue to be widely used as a biological matrix for the analysis of psychoactive substances in forensic cases because of the large volume of sample available for analysis, the relative simplicity of sample preparation and also its broad drug detection window compared to blood [9,10,23]. During the metabolism process of benzodiazepines up to their excretion, these molecules undergo reactions of phase I followed by a second stage where there is an addition of a glucuronide group. The glucuronidation binds hydroxy-benzodiazepines, transforming these compounds into more polar products and thus excreted by this route. Therefore, it is necessary to perform an hydrolysis step for drug extraction procedure for GC analysis [12,24]. In this case, enzymatic hydrolysis is preferable compared to chemical hydrolysis, avoiding benzodiazepines degradation which can generate benzophenones [12,24,25]. Although enzymatic hydrolysis allows free drug/metabolite in the urine sample, benzodiazepines metabolites present in this biological matrix are more polar than the parent drug, requiring a derivatization step when determined by GC–MS [26,27].

Silylation, acylation and alkylation derivatizing agents are the most commonly used in the determinations of benzodiazepines and its metabolites by GC–MS [11,13,27,28]. In some circumstances, double derivatization (two steps) is used to reach the analytical purpose [13,29]. According to other studies, this procedure is the key to improve GC–MS selectivity and sensitivity over single-step procedures studies. This strategy is useful to avoid artifact formation, tailing peaks and to produce a better repeatability without the need for different procedures in sample preparation. Furthermore, there is an increase of sensitivity and a wider range of evaluated analytes [13,29–31].

The aim of the present study was to develop a sensible method for the determination of 11 benzodiazepines and their main metabolites (medazepam, chlordiazepoxide, diazepam, nordiazepam, oxazepam, lorazepam, nitrazepam, flunitrazepam, clonazepam, 7-aminoflunitrazepam and 7-aminoclonazepam) in urine samples using hollow-fiber liquid phase microextraction (LPME) in three-phase mode and double derivatization for further detection by gas chromatography-mass spectrometry (GC–MS). The method has been fully validated and it was successfully applied in urine samples from real cases involved with benzodiazepine exposure.

2. Experimental

2.1. Chemicals

Medazepam, diazepam, nordiazepam, flunitrazepam, chlordiazepoxide, oxazepam, nitrazepam, 7-aminoflunitrazepam, lorazepam, clonazepam and 7-aminoclonazepam solutions (1.0 mg/mL) and internal standards diazepam-D5, oxazepam-D5, 7-aminoflunitrazepam-D7, clonazepam-D4 and 7-aminoclonazepam -D4 were purchase from Cerilliant Analytical Reference Standards® (Round Rock, TX, USA). Dihexyl ether, 1-nonanol, undecane, decanol, 1-octanol, xilol, β-glucuronidase from *Helix pomatia* type 2 in aqueous solution 100,000 units/mL, *N*-methyl-*N*-*tert* -butyldimethylsilyltrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane (MTBSTFA), trifluoroacetic anhydride (TFAA) and ethyl acetate were purchased from Sigma-Aldrich® (MO, USA), while sodium hydroxide, sodium acetate and hydrochloric acid were purchased from Merck® (Darmstadt, Germany).

2.2. Instrumentation

Hollow-fiber Q3/2 Accurel KM polypropylene (600 μm i.d., 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana® (Wuppertal, Germany). Gel-loading pippete tips Round CC 4853 (0.5 mm; 1–200 μL) were purchased from Costar® (Corning, NY, USA). Extraction was performed using a multi-tube vortexer® model VWR VX-2500 (Thorofare, NJ, USA). The analyses was performed using an Agilent 6850 Network GC System gas chromatograph coupled with an Agilent® 5975 Series quadrupole mass seletive detector (MSD) (Wilmington, DE, USA). Samples were injected into the GC–MS by means of an autosampler (Agilent 7693). Injections were made using splitless mode (2 min and afterwards split vent was turned on in a ratio of 1:50). Chromatographic separation was achieved on a HP-5MS fused-silica capillary column (30 m × 0.25 mm × 0.1 μm film thickness) using helium as the carrier gas with 1.0 mL/min at a constant flow rate mode. The column oven temperature program was as follows: first held at 150 °C (hold 1 min), then programed at 30 °C/min to 220 °C (hold 1 min); 20 °C/min at 300 °C (hold 3 min). The total analytical time was 11.33 min. Injection port and transfer line were set at 260 °C and 280 °C respectively. The MS was operated by electron ionization (70 eV) in selected ion monitoring (SIM) mode.

2.3. Optimization of the method

The study of method optimization was performed taking into consideration the choice of supported liquid membrane, pH of donor phase, influence of acceptor phase, time of extraction, shaking speed, salt addition on the extraction yield and two steps derivatization strategy. Fortified urine samples at a concentration of 50 ng/mL for each analyte were submitted to the previous described method. The efficiency of extraction was evaluated by the absolute area produced by each analyte in all tested conditions. The following parameters were studied: supported liquid membrane (dihexyl ether, xilol, 1-nonanol, decanol, 1-octanol and different proportions of the mixture between dihexyl ether and 1-nonanol); pH of donor phase (pH 8, 9, 10, 11, 12 and 13); acceptor phase (0.05, 0.1, 1.0, 3.0 and 5.0 mol/L HCl); time of extraction (15, 30, 60, 90 and 120 min) and agitation (1200, 1560, 1800, 2040 and 2400 rpm). The salting out effect was also tested by adding 0, 5, 10 and 20% of NaCl (m/v) in the sample before extraction. All remaining parameters were fixed at a certain rate while changing a given variable of those described in Section 2.5. For the study of double derivatization process, it was verified the influence of TFAA and MTBSTFA at different temperatures (TFAA – 60, 70 and 80 °C; MTBSTFA – 70, 80 and 90 °C)