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Short Communication

Analysis of oxazepam in urine using solid-phase extraction and high-performance liquid chromatography with fluorescence detection by post-column derivatization

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method for oxazepam in human urine samples has been developed. The sample preparation consists of an enzymatic hydrolysis with β-glucuronidase, followed by a solid-phase extraction process using Bond-Elut C_2 cartridges. The mobile phase used was a methanol–water (60:40, v/v) mixture at a flow-rate of 0.50 ml/min. The column was a 3.5 cm × 4.6 mm I.D. C_{18} reversed-phase column. The detection system was based on a fluorescence post-column derivatization of oxazepam in mixtures of methanol and acetic acid. A linear range from 0.01 to 1 μg/ml of urine and a limit of detection of 4 ng/ml of urine were attained. Within-day recoveries and reproducibilities from urine samples spiked with 0.2 and 0.02 μg/ml oxazepam were 97.9 and 95.0 and 2.1 and 9.4%, respectively.

INTRODUCTION

Since the introduction of chlordiazepoxide in 1960, benzodiazepines have become the most used medicines in the treatment of anxiety and sleep disturbances [1,2]. Their analysis in biological samples has been accomplished using different techniques such as polarography, enzymatic assays, gas chromatography (GC), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Several reviews on this subject have been published [3–8].

The detection system usually used in the HPLC analysis of benzodiazepines is UV–visible absorption. However, other techniques such as electrochemical [9–11] and fluorescence [12] detection are available. The native fluorescence of benzodiazepines is very low and a suitable signal intensity is only reached in acidic media [13,14] or using derivatization reactions [12,15].

Oxazepam is not only a benzodiazepine widely used but also the metabolite of other benzodiazepines such as diazepam, temazepam, chlordiazepoxide, clorazepate, prazepam and medazepam, and is easily removed from the body in urine as its conjugate with glucuronic acid [1]. Inmunochemical [16], GC [17] and HPLC [9,12,18–31] analysis methods for oxazepam in biological fluids have been reported.

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Most of the HPLC methods for oxazepam are based on a preparation of the biological sample using liquid–liquid extraction (LLE) [18–23,26–30] and UV–visible detection [18–24,26–31]. However, others use the modern solid-phase extraction (SPE) for sample pretreatment [9,12,24,31] and/or a more specific detection such as electrochemical [9] or fluorescence [12]. Some of these analytical systems show a poor sensitivity [20,22–24,26]. Others require large analysis times, owing to the use of LLE or slow derivatization reactions [12].

In this work, a simple and quick analysis of oxazepam in human urine is presented. The method consist of an SPE procedure as the sample preparation step, followed by an HPLC separation with post-column derivatization and fluorescence detection. The derivatization reaction is based on the intense fluorescence that oxazepam exhibits when it is heated in acidic–alcoholic mixtures [32–35].

EXPERIMENTAL

Reagents

The benzodiazepine standards were kindly supplied by Boehringer-Ingelheim (oxazepam) and Roche (nordiazepam), and their purity was checked by HPLC. Stock solutions of 1000 μg/ml were prepared in methanol (HPLC grade) and stored at 4°C in the dark. The acetic acid was from Fluka (analytical grade). KH_2PO_4–K_2HPO_4 (pH 6.0) aqueous buffers from analytical grade reagents were prepared at two concentration levels, 10^{-2} *M* for use in the SPE and 10^{-1} *M* for buffered urine samples.

Sample preparation

Urine samples were obtained from healthy subjects and were frozen and stored at −20°C. Enzymatic hydrolysis of urine samples collected from subjects following the ingestion of oral doses of oxazepam was carried out, before the SPE, by addition of 250 U of β-glucuronidase per millilitre of urine and hydrochloric acid until the pH value was 5.0, and incubation at 37°C for 24 h [12,21,29].

Spiked urine samples were prepared by adding different amounts of oxazepam and 1 μg of nordiazepam (internal standard) per millilitre of urine and buffered to pH 6.0 with the 10^{-1} *M* buffer solution, giving a final buffer concentration of 10^{-2} *M*.

Analytichem (Harbor City, CA, USA) Bond-Elut C_2 (100 mg) cartridges and a Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA) were used in the SPE. The cartridge was prewashed with a volume of methanol and another volume of the 10^{-2} *M* buffer. Then, the required volume of the urine sample was passed through the cartridge. The solid phase was washed with three volumes of water, 1 ml of suitable washing solvent and 1 ml of water. Finally, compounds were eluted with 1 ml of the appropriate elution solvent and 1 ml of water. The collected eluates were evaporated to dryness and reconstituted with 200 μl of mobile phase prior to their injection into the chromatographic system.

Chromatographic conditions

The chomatographic system designed was composed of two lines. An LKB 2150 HPLC pump (Barcelona, Spain), a Rheodyne (Cotati, CA, USA) 7125 injector with a 20-μl loop and a 3.5 cm × 4.6 mm I.D., 5-μm Ultrabase C_{18} column (Scharlau, Barcelona, Spain) constituted the chromatographic separation line. The other line consisted of another LKB 2150 HPLC pump for pumping the derivatization agent, acetic acid. Both lines came together using a T union in the reactor, PTFE tubing of 0.5 mm I.D. and 15 m length immersed in a water bath at 100 ± 1°C. Finally, the effluent from the reactor passed through a Shimadzu (Tokyo, Japan) RF-540 spectrofluorimeter, equipped with a Shimadzu HPLC cell with a dead volume of 12 μl and monochomators set to a λ_{ex} of 364 nm and a λ_{em} of 469 nm. The mobile phase used was methanol–water (60:40, v/v) at a flow-rate of 0.50 ml/min. The chromatographic separation was performed at room temperature. The acetic acid flow-rate in the other line was 1.10 ml/min, obtaining in the reactor a mixture of acetic acid–methanol–water (22:6:4, v/v/v). Data collection was performed us-

ing an HP 34401A digital multimeter (Madrid, Spain) and a computer with a BASIC program designed and written at home. Integration was carried out with the program NELSON version 3.5 (Perkin-Elmer, Barcelona, Spain).

RESULTS AND DISCUSSION

In a previous work [36], we examined the fluorescence reaction that oxazepam undergoes when it is heated in mixtures of methanol and acetic acid. The maximum fluorescence intensity was found using a methanol–acetic acid (7:25, v/v) mixture as the reaction medium, a heating temperature of 100°C, a reaction time of 5 min and 364 and 469 nm as the excitation and emission wavelengths, respectively. Also, the optimized reactor length found for a flow injection analysis (FIA) system was 15 m, using PTFE tubing of 0.5 mm I.D. The chromatographic system described in the Experimental section was designed with the aid of these data.

Moreover, we have studied the optimum SPE conditions for oxazepam [37]. These conditions

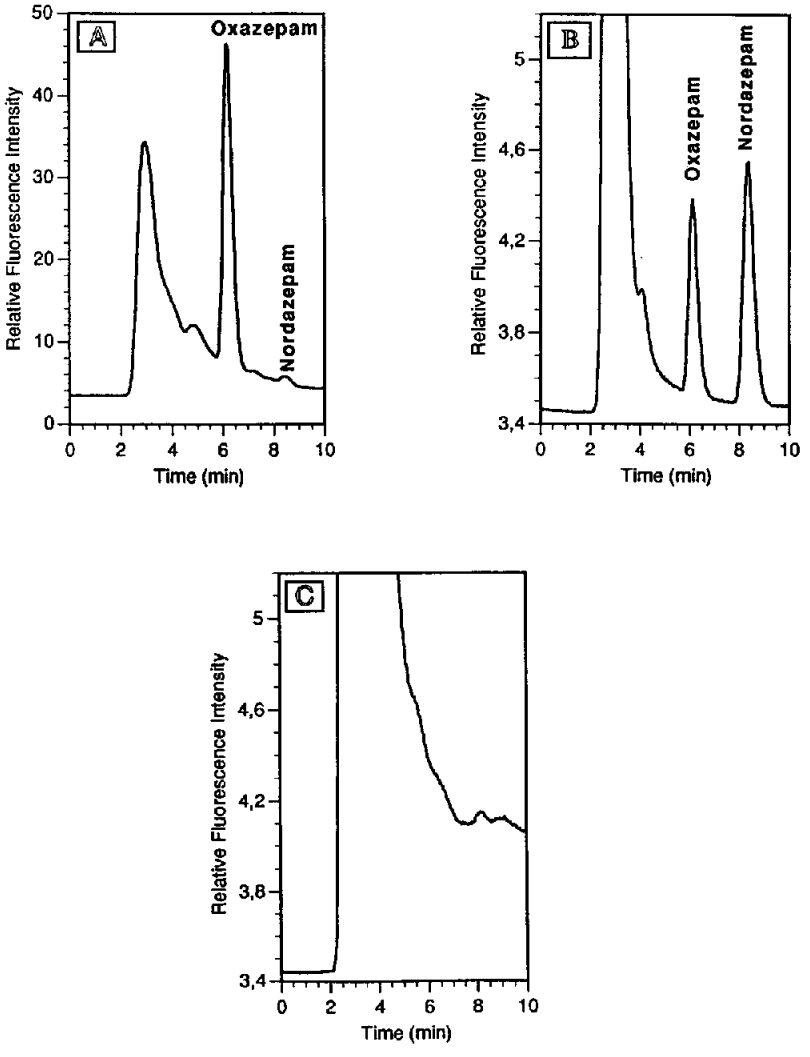

Fig. 1. Chromatograms (A) from a urine sample collected during the 8 h after an oral ingestion of 10 mg of oxazepam and submitted to enzymatic hydrolysis, (B) corresponding to a urine sample spiked with 2 μg/ml oxazepam and 1 μg/ml nordiazepam and (C) from a blank of urine without oxazepam or nordiazepam.

were a sample pH of 6.0, 1 ml of methanol–water (20:80, v/v) as the washing solvent and 1 ml of methanol–water (60:40, v/v) as the elution solvent, using Bond-Elut C_2 cartridges. The composition of the washing solvent was more carefully revised in this work. In this sense, the SPE of urine samples spiked with 1 μg/ml oxazepam was performed using different compositions of the washing solvent (methanol–water: 20:80, 25:75, 30:70 or 35:65). The washing solvent that produces the best resolution of the oxazepam peak with the highest area is that of composition 25:75.

Taking into account the fact that usually there is no restriction on the sample urine volume available for the analysis of these kinds of samples, five different volumes of urine were assayed (1, 2, 3, 4 and 5 ml). The results showed that is possible to use a sample volume of 5 ml without a significant loss in resolution in the chromatogram, obtaining a higher preconcentration factor and the corresponding improvement in sensitivity. Thus, the sample volume was set to 5 ml. Volumes larger than 5 ml produce an undesirable increase in the time required to carry out the SPE.

A calibration curve was performed using aqueous samples, prepared by dilution with water of different amounts of oxazepam and 25 μg/ml nordiazepam. A linear range from 0.25 to 25 μg/ml [$A_{oxa}/A_{nor} = (-0.0094 \pm 0.0042) + (0.13896 \pm 0.00044)\, C_{oxa}$ (μg/ml), $r = 0.99996$] and a limit of detection of 0.1 μg/ml were attained. Using the preconcentration factor obtained in the SPE and evaporation–reconstitution steps (× 25, 5 ml of urine sample added to the cartridge *versus* 200 μl of mobile phase), these values become: a linear range from 0.01 to 1 μg/ml of urine and a limit of detection of 4 ng/ml of urine. The limit of detection was calculated as the oxazepam concentration that produces a chromatographic peak whose height is three times the baseline noise.

Within-day recoveries and reproducibilities (as relative standard deviation) from urine samples spiked with 0.2 and 0.02 μg/ml oxazepam and 1 μg/ml nordiazepam and buffered to pH 6.0 were 97.9 and 95.0 % and 2.1 and 9.4%, respectively, for four determinations.

Finally, the method was applied to urine samples from subjects following the intake of oral doses of oxazepam. Fig. 1 shows the chromatograms obtained from (A) a urine sample collected during the 8 h after an oral ingestion of 10 mg of oxazepam and submitted to enzymatic hydrolysis, (B) a urine sample spiked with 2 μg/ml oxazepam and 1 μg/ml nordiazepam and (C) a blank of urine. Chromatogram A shows an oxazepam peak corresponding to 14 μg/ml.

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