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Determination of delorazepam in urine by solid-phase microextraction coupled to high performance liquid chromatography

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Abstract

An SPME–HPLC–UV method for the determination of delorazepam, a representative benzodiazepine, in spiked human urine samples was developed for the first time. The performances of two commercially available fibers, a carbowax/templated resin (Carbowax/TPR-100) and a polydimethylsiloxane/divinylbenzene (PDMS/DVB), were compared, indicating the latter as the most suitable for urine samples analysis. All the aspects influencing adsorption (extraction time, pH, temperature, salt addition) and desorption (desorption and injection time, desorption solvent mixture composition) of the analyte on the fiber have been investigated. In particular, short extraction times were necessary to reach the equilibrium and very short desorption times were employed. The procedure required simple sample pre-treatment and was able to detect 5 ng/ml in spiked urine, regardless of the complexity of the matrix.
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Keywords: Delorazepam; Solid-phase microextraction; Desorption conditions

1. Introduction

Benzodiazepines are a large class of drugs commonly used as minor tranquilizers, hypnotics, muscle relaxants and anticonvulsants. They are often abused [1,2] causing profound behavioral effects and drug dependence. Moreover, there is accumulating evidence that benzodiazepines could be regarded as 'natural' drugs since they have been found in trace amount also in plants and various tissues of different animal species [3]. Thus, their determination in human body fluids could be very useful for toxicological, pharmaceutical and forensic purposes; in fact, it has been the object of several analytical investigations using different techniques such as immunoassay [4–7], radioreceptor assay [8–12] and chromatography [13–19] (HPLC, LC–MS, GC and GC–MS methods). Chromatographic techniques, usually, require complex isolation procedures such as liquid–liquid or solid-phase extraction to separate benzodiazepines from biological matrices. Solid-phase microextraction (SPME) is a new extraction

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technique introduced by Pawliszyn [20,28] for the determination of organic compounds. It allows simultaneous extraction and pre-concentration of analytes from sample matrix; furthermore SPME eliminates some disadvantages of conventional extraction techniques such as plugging of cartridges in solid-phase extraction and use of toxic solvents in liquid–liquid extraction.

SPME has been mainly applied [20–27] in combination with GC; however, SPME–GC is limited to the analysis of volatile and thermally stable compounds. In order to widen its range of application, SPME has been interfaced with HPLC. Regardless of its wide potentialities, SPME–HPLC [26–29] applications are still restricted, being limited by low reproducibility and sensitivity, that can only be increased instrumentally by using expensive hyphenated techniques, in particular LC–MS. In fact, an SPME procedure using a polyacrylate fiber recently developed [30] in conjunction with LC–ESI–MS for the determination of some benzodiazepines in human urine samples showed a LOD in the low ng/ml range. However, an extraction time of over 180 min was required to reach the equilibrium and a static desorption time of 30 min was necessary to minimize carry-over of the analytes.

In the present paper, SPME of delorazepam (7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one), a representative benzodiazepine, was optimized and interfaced with HPLC–UV. Two different fibers, a polydimethylsiloxane/divinylbenzene (PDMS/DVB) and a carbowax/templated resin (Carbowax/TPR-100) were used. Short extraction times were necessary to reach the equilibrium and very short desorption times were employed. The developed procedure was then applied to the extraction of delorazepam from urine samples.

2. Experimental

2.1. Chemicals

Delorazepam was purchased from Sigma (St. Louis, MO). Methanol stock solutions were prepared and stored in the dark at 4 °C. More dilute solutions were prepared just before use and filtered through a 0.45 μm Millex-HV type filter (Millipore).

All organic solvents used (Carlo Erba, Milan, Italy) were HPLC grade. The HPLC mobile phase was filtered through a 0.45 μm nylon membrane (Whatman Limited, Maidstone, UK) before use.

2.2. Apparatus

The SPME–HPLC apparatus consisted of a SPME device, an interface and an HPLC system.

The holder and the assembly of the SPME device for manual sampling were purchased from Supelco (Bellefonte, PA). A new fiber was conditioned before use as specified in the literature accompanying the commercial SPME products. The SPME–HPLC interface (Supelco) consists of a standard six-port HPLC Rheodyne valve with a special fiber desorption chamber (total volume: 60 μl), installed in place of the sample loop, and a bracket for bench top mounting.

The HPLC system used in this study includes a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA) and a Supelcosil LC-18-DB column (250 × 4.6 mm i.d., particle 5 μm, Supelco). A 5 μm Supelguard LC-18-DB precolumn (20 × 4.6 mm i.d., Supelco) was used to protect the analytical column. Mobile phase was degassed by an SCM 1000 vacuum membrane degasser (Thermo Separation Products). The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA) interfaced to an HP 85 computer equipped with an HP dual disk drive and an HP 7470A plotter. A Hewlett-Packard model HP 3395 laboratory computing integrator directly connected to the analogic output of the photodiode-array detector was also used.

2.3. Chromatographic and detection conditions

The mobile phase consisted of acetonitrile/water (65:35, v/v). The flow rate was 1 ml/min and temperature was ambient. The detection wavelength was 230 nm (4 nm band-width) and the reference signal was at 550 nm. Spectra were

acquired in the 210–400 nm range at the apex and on the ascending or descending part of each peak. Peak purity could be checked by the technique of spectra overlaying with spectra of standard, after normalisation.

2.4. Solid-phase microextraction

Two kinds of silica fibers (Supelco) coated with a 60 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) film, or a 50 μm carbowax/templated resin (Carbowax/TPR-100) film, both partially cross linked, were employed for comparative studies. The fibers were appropriate for use with most water-miscible HPLC solvents. The SPME procedure has been extensively described elsewhere [26–29]. Working solutions were prepared in 20 ml clear vials (Supelco) by dissolving various amounts of delorazepam into 15 ml phosphate buffer (0.1 I, pH 6.5). Then, the vials were sealed with hole caps and Teflon-faced silicone septa (Supelco). The extraction was carried out at room temperature for 30 min under magnetic stirring, using a cylindrical-shaped stirring bar (10×4 mm) in order to improve mass transfer from the aqueous sample into the fiber coating.

A static desorption of delorazepam from the fiber was carried out in the SPME–HPLC interface. Before transferring the fiber into the desorption chamber, the injection valve was placed in the 'load' position and a sealing needle was inserted in the HPLC interface. The sealing clamp was locked. Using a glass syringe, the chamber was flushed and filled by adding 500 μl of the desorption solvent. The fiber was then introduced into the chamber by lowering the syringe plunger. The fiber was statically desorbed for 5 min in acetonitrile. The valve was then switched to the 'injection' position, left for 60 s and then switched to the 'load' position. The fiber was removed and cleaned with water for about 1 min to eliminate carryover. The fiber was allowed to dry before accomplishing the next extraction.

In the case of urine samples, the desorption procedure was modified as follows: 3 min of static desorption using an acetonitrile/water mixture (40:60, v/v) as desorption solvent; the valve was switched to the 'load' position after only 4 s of exposition of the fiber to the moving stream of mobile phase.

2.5. Sample collection and pre-treatment

Urine samples, collected from healthy donors in the early morning, were filtered through a 0.45 μm Millex-HV type filter (Millipore). Then, 13.5 ml of each sample were added with 1.5 ml phosphate buffer (0.5 M, pH 9.7) to obtain a pH value of 6.5 and directly subjected to SPME.

3. Results and discussion

3.1. Extraction time

Preliminary experiments were performed in order to compare the extraction efficiency of PDMS/DVB and Carbowax/TPR-100 coated fibers. Fig. 1 reports the extraction time profiles established by plotting the area counts versus the extraction time for both the selected fibers. As can be seen, an equilibrium time of about 30 min was observed in both cases, while PDMS/DVB was capable of the most efficient extraction.

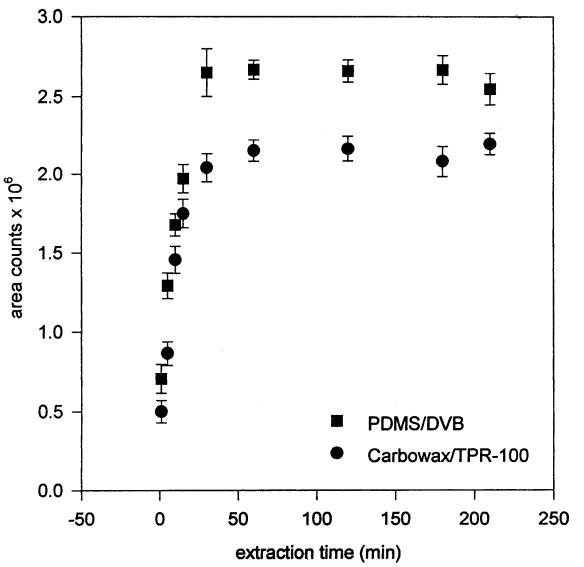

Fig. 1. Extraction time profiles obtained with both the selected fibers. Delorazepam concentration: 0.5 μg/ml.

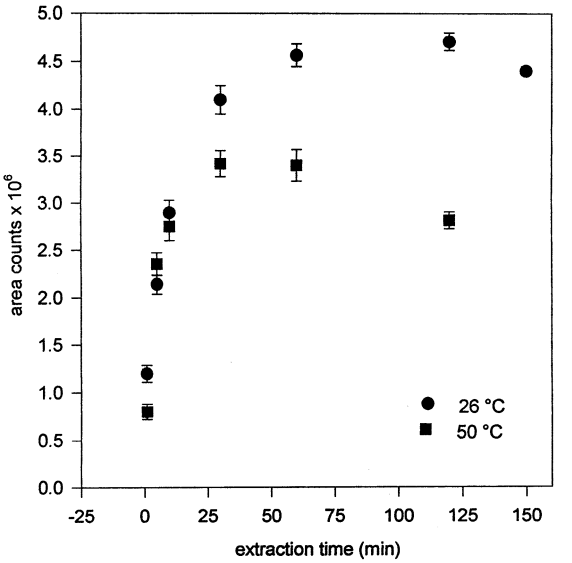

Fig. 2. Extraction time profiles obtained with the Carbowax/TPR-100 fiber at room temperature and at 50 °C, respectively. Delorazepam concentration: 1 μg/ml.

In any case, it is possible to obtain good extraction yields and reliable analysis also in non-equilibrium conditions. In fact [11], the amount of the analyte adsorbed on the fiber is proportional to the initial concentration in the sample matrix, once the agitation conditions and the sampling time are held constant, and hence, SPME quantitation is feasible even before adsorption equilibrium is reached. Thus, in the case of routine analysis, very short extraction times can be used in order to obtain a considerable gain of time.

3.2. Extraction temperature

Fig. 2 reports the extraction time profiles obtained using the Carbowax/TPR-100 fiber at room temperature and at 50 °C, respectively. As apparent, equilibration times of about 30 min were obtained in both cases; however, at 50 °C the absolute responses were lower, probably due to the fact that adsorption is an exothermic process. It is also apparent that a marked decrease of response occurs after a prolonged exposition of delorazepam at 50 °C, probably caused by its decomposition. A similar behavior was observed using the PDMS/DVB fiber and thus room temperature was chosen for further experiments.

3.3. Ionic strength and pH

The distribution constant and thus the extracted amount, strongly depend on the characteristics of the matrix such as ionic strength and pH.

Generally speaking, salt addition often improves the recovery, especially in the case of polar (hydrophilic) compounds that are difficult to extract. Thus, experiments were performed by increasing progressively the ionic strength of the extraction solutions. However, no significant effects were observed using the Carbowax/TPR-100 while a response decrease was observed in the case of the PDMS/DVB.

Since analytes in the neutral forms are more efficiently extracted by the non-ionic polymeric coatings, the effect of the pH on the extraction efficiency of the drug with both the fibers was

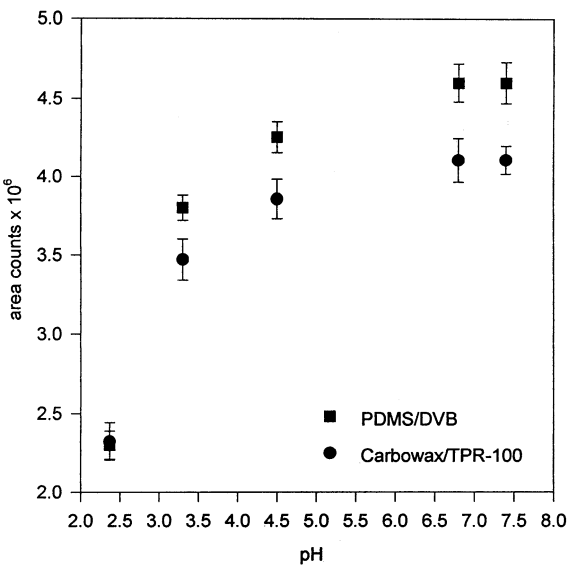

Fig. 3. Effect of the pH on the extraction efficiency obtained with both the selected fibers. Delorazepam concentration: 1 μg/ml. Extraction time: 30 min.

examined by using different pH buffers and the relevant results are shown in Fig. 3. Most benzodiazepines are in fact weak bases and are present in their undissociated form at neutral or alkaline pH. As expected, a response increase was observed by varying pH from 2 to 7.

3.4. Desorption conditions and 'carryover'

Desorption of the analyte from the fiber in the SPME–HPLC interface can be dynamic or static. The dynamic desorption mode, potentially able to quickly desorb delorazepam, produced quantitative recoveries but very broad chromatographic peaks. The static desorption technique was then used for further experiments. The first step of optimization was the choice of the most efficient desorption solvent; thus the fiber was soaked in various acetonitrile/water solvent mixtures in the interface before the injection, using 3 min of desorption time and switching the valve in the load position 4 s after the injection. In order to evaluate carryover and percentage desorption, the fiber was left in the chamber after each experiment and a second chromatographic run was performed leaving the interface valve in the inject position. The relevant results are shown in Table 1. The best desorption conditions were obtained using an acetonitrile/water mixture (90:10, v/v) for the Carbowax/TPR-100 and only acetonitrile for the PDMS/DVB fibers (more than 90% of desorption).

Further experiments were oriented to investigate the effect of the static desorption time and of the exposition time of the fiber to the moving stream of mobile phase, that are known to be critical steps for the optimization of the desorption yield and of the chromatographic efficiency, respectively. The relevant results are also reported in Table 1. Using 5 min of static desorption in the best desorption solvent already optimised (see above) for each fiber, and switching the valve in the load position 1 min after the injection, a quantitative desorption for the PDMS/DVB and a desorption of 82% for the Carbowax/TPR-100 were obtained. An almost quantitative desorption was found for the latter fiber using the same conditions but 30 min of desorption time.

3.5. Linear range, detection limits and precision

The dynamic range of the developed SPME–HPLC–UV procedure resulted linear for both the fibers from the LOQ values for two concentration decades, with correlation coefficients better than 0.996 and intercepts not significantly different from zero at 95% confidence level.

The estimated LOD obtained in this study on standard solutions were 1 and 5 ng/ml for the PDMS/DVB and the Carbowax/TPR-100, respectively; the estimated LOQ were 6 and 18 ng/ml for the PDMS/DVB and the Carbowax/TPR-100, respectively; LOD and LOQ were both calculated as three and ten fold the standard deviation of the intercept of the calibration curves (according to IUPAC [31]).

The following repeatability data were obtained in the concentration range 0.01–0.5 μg/ml: $4.9 \pm 0.5\%$ and $4.7 \pm 0.3\%$ for the PDMS/DVB and the Carbowax/TPR-100, respectively.

3.6. Urine samples analysis

Once the study on extraction and desorption conditions was completed, the procedure was applied to urine samples. An excessive desorption of interfering substances arising from the matrix, especially in the case of the Carbowax/TPR-100, was observed using the desorption conditions optimized for standard solutions of delorazepam. Further experiments were then performed only with the PDMS/DVB fiber. A first attempt to minimize matrix interferents was made by adding phosphate buffer (0.1 I, pH 9.7) to urine samples; however, a strong loss of sensitivity was observed in this case. Then, in order to reduce the desorption of matrix components adsorbed on the fiber and to improve the chromatographic efficiency, desorption conditions were modified paying attention to preserve a high desorption yield of delorazepam. A good compromise was found using 3 min of desorption time in an acetonitrile/water (40:60, v/v) mixture and 4 s of exposure time of the fiber to the moving stream of mobile phase. A desorption percentage of 55% (and, consequently, a carryover of 45%) was observed in this case. It is worth noting that these data are equal to those

Table 1
Percentage of desorption and carryover obtained for delorazepam by varying the composition of the desorption solvent mixture, the desorption time and the injection time

Desorption solvent mixtures	Desorption time (min)	Injection time (s)	Desorption percentage		Carryover[a]	
			PDMS/DVB (%)	Carbowax/TPR-100 (%)	PDMS/DVB (%)	Carbowax/TPR-100 (%)
Water	3	4	12	10	88	90
Acetonitrile:water (40:60, v/v)	3	4	55	50	45	50
Acetonitrile:water (80:20, v/v)	3	4	60	58	40	42
Acetonitrile:water (90:10, v/v)	3	4	67	72	33	28
Acetonitrile	3	4	91	60	9	40
Acetonitrile	5	60	96	70	4	30
Acetonitrile:water (90:10, v/v)	5	60	79	82	21	18

Data obtained from aqueous solutions.
[a] See text for experimental conditions.

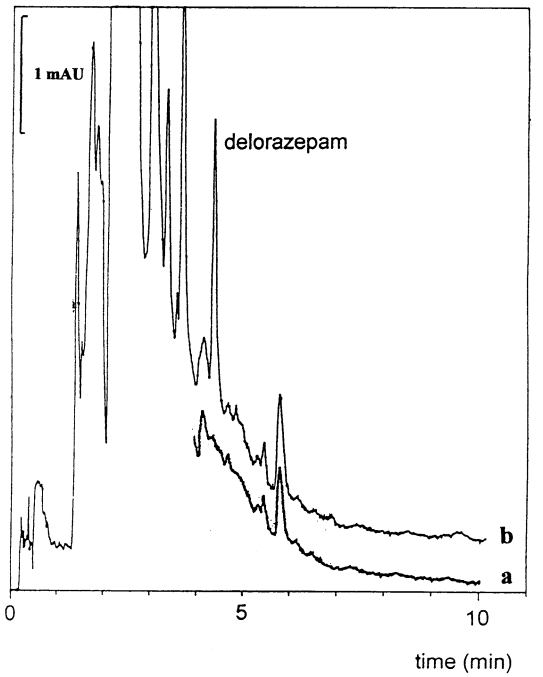

Fig. 4. SPME (PDMS/DVB)–LC–UV chromatograms obtained from (a) a blank urine sample and (b) a urine sample spiked with 5 ng/ml of delorazepam.

reported in Table 1, clearly indicating that desorption percentage and carryover were not influenced by passing from aqueous solutions to urine samples. In the present case, to eliminate carryover, the fiber was removed and cleaned with the desorption mixture for about 1 min.

Obviously, a loss of sensitivity was observed during the analysis of urine samples due to both the slightly modified desorption conditions and to a considerable matrix effect. Thus, a calibration curve in urine was constructed; it resulted linear from the LOQ value for two concentration decades, with correlation coefficient better than 0.993 and intercept not significantly different from zero at 95% confidence level.

The estimated LOD and LOQ were 5 and 27 ng/ml, respectively, calculated according to IUPAC (see above) as three and ten fold the standard deviation of the intercept of the calibration curve. The repeatability obtained in urine, in the concentration range 0.05–0.5 μg/ml, was 6.9 ± 0.5%.

Fig. 4 reports the SPME–LC–UV chromatograms obtained from (a) a blank urine sample and (b) a urine sample spiked with 5 ng/ml of delorazepam. As apparent, delorazepam was clearly detected at this concentration level and is well resolved from matrix components. The separation was performed under simple isocratic elution conditions in less then 10 min.

4. Conclusions

An SPME–HPLC–UV method for the determination of delorazepam, a representative benzodiazepine, in human urine samples was developed for the first time. The procedure requires simple sample pre-treatment and, though the desorption conditions optimized for standard solutions needed to be modified for the analysis of urine samples, due to complexity of the matrix, it was able to detect 5 ng/ml in spiked urine, when the PDMS/DVB fiber was utilized. The results obtained were comparable to those obtained by LC–UV methods previously developed for benzodiazepine analysis [13–15] employing traditional extraction techniques. The present method has also a comparable sensitivity with respect to an existing [30] SPME–LC–MS method for the determination of benzodiazepines, with the great advantage of a routine and less expensive instrumentation.

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