

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DIRECT DETERMINATION OF DIAZEPAM IN WHOLE BLOOD AND SERUM – OPTIMIZATION OF SOLID-PHASE EXTRACTION METHOD

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ABSTRACT

Herein, we present a simple and rapid high performance liquid chromatographic (HPLC) method with UV-detection for the direct determination of diazepam in whole blood and serum that can be used for monitoring diazepam levels in clinical samples analysis. The isolation of diazepam and the internal standard bromazepam from serum and whole blood samples was performed using solid phase extraction method with RP select B cartridges. The analytes were separated employing a reversed phase C8 column with a mobile phase composed of 0.1 % (V/V) triethylamine in water (pH 3.5) and acetonitrile (63:37, V/V). UV detection was carried out at 240 nm. Linearity was achieved in the range from 10.0-1000.0 ng/ml for serum and whole blood. The method was applied to spiked and real biological samples after an oral administration of 10 mg diazepam. In conclusion, the proposed method is simple, rapid and provides efficient clean-up of the complex biological matrix and high recovery of diazepam.

Keywords: diazepam, solid-phase extraction, HPLC, whole blood, serum

INTRODUCTION

Diazepam (7-chloro-1, 3-dihydro-1-methyl-5-phenyl-2H-1, 4-benzodiazepin-2-one) is a benzodiazepine generally used as hypnotic, anxiolytic and muscle relaxant. Benzodiazepines are considered one of the major drug abuse groups and they belong to the most common class of drugs in the clinical toxicology practice. Diazepam is routinely prescribed as the standard first-line treatment for acute convulsions and prolonged status epilepticus [1]. It is a lipophilic drug and can readily pass through the blood-brain barrier (BBB) and some other lipophilic tissues. However,

due to its lipophilicity, it is rapidly redistributed out of the brain. Due to fast distribution, serum levels of diazepam fall down quickly in the brain leading to repeated dosing, accumulation in the body, and serious complications [2]. The half-life of diazepam after oral administration in normal volunteers and psychiatric patients, particularly after prolonged therapy was estimated to be in the range from 9 to 35 h [3].

Several high-performance liquid chromatographic (HPLC) methods have also been reported for the determination of diazepam alone [4-8]

or in combination with other drugs [8-12] from pharmaceutical dosage forms, as well as in the forensic medicine in biological samples. Reported extraction include extraction mode with a polydimethylsiloxane [12, 13] and solvent-modified solid-phase microextraction by capillary gas chromatography [14].

The aim of this study is to develop a new HPLC method suitable for determination of diazepam in biological fluids employing solid phase extraction for sample preparation, which enables simple and rapid isolation and concentration of the analysed drug. For minimizing the variability caused by sample pre-treatment, we present a method of internal standardization for the quantification of diazepam.

EXPERIMENTAL

2.1. Materials

Diazepam working standard was supplied by Select Chemie, Switzerland and the internal standard bromazepam was obtained from Sigma-Aldrich, Belgium. HPLC grade acetonitrile and methanol were purchased from Across Organics, Belgium. Triethylamine, o phosphoric acid, sodium carbonate and columns for solid phase extraction were obtained from Merck, Germany.

2.2. Instrument and chromatographic conditions

The development and validation work was carried out on a chromatographic system consisting of Perkin Elmer LC series 200 pump, ultraviolet diode array detector (Perkin Elmer LC 235 C) and autosampler Perkin Elmer LC ISS series 200. The chromatographic system was controlled by the software package Turbochrom Version 4.1. plus and UV-spectrometric data were produced by TurboScan Version 2.0. A reverse phase Supelco-syl LC-8-DB, 250 x 4.6 mm I.D. (5 μ m, particle size), protected by a guard column SupelguardTM LC-8-DB (2 cm) was used for separation. The mobile phase was consisted of 0.1 % (V/V) triethylamine in water with pH=3.5 and acetonitrile (63:37, V/V).

In order to achieve a good shape and location of diazepam peaks and the internal standard in the corresponding chromatograms, a series of parameters including composition and

pH of mobile phase, column packing, flow rate and detection wavelength were tested. The final choice of the stationary phase giving satisfying resolution and run time was LC-8-DB. Triethylamine solution was prepared by adding 100 μ L triethylamine in 100 mL H₂O with pH adjusted to 3.5 with concentrated o-phosphoric acid. The mobile phase was filtered and degassed with helium. Chromatographic separations were performed at 37° C, with mobile phase flow rate of 1.3 mL/min and ultraviolet detection at 240 nm. The injection volume was 100 μ L.

2.3. Solutions, sample preparation and calibration curves

Stock solutions of 1 mg/mL of diazepam and bromazepam were prepared monthly in methanol and stored at +4°C. No change in stability over the period of 1 month was observed. The working solutions were prepared by diluting appropriate portions of these solutions with distilled water.

Human serum was prepared from heparinized whole blood samples. Blood samples were collected from healthy volunteers and stored at -20° C. After thawing, samples were spiked daily with stock solutions of diazepam and internal standard.

A solid-phase extraction vacuum manifold (Merck) was used for sample preparation. Spiked sample (total volume 1 mL) was introduced into the extraction cartridge with RP select B solid phase (200 mg) under vacuum at 5 psi. The cartridge was conditioned sequentially by 2 mL methanol, 2 mL water, followed by 0.1 mL of 0.1 mol/L sodium carbonate solution. For blood samples conditioning of the columns was finished with additional 0.05 mL of heparin (1000 units/mL). Water (2 mL) was used to rinse the cartridge. The clean up was accomplished with additional 0.05 mL of methanol through the cartridge. Elution was then performed in two steps: the first one with 0.2 mL of methanol and this eluate was collected in a clean tube without vacuum, and the second step with additional 0.1 mL methanol. The tube with eluate and cartridge were centrifuged at 3500 rpm for 5 min. After centrifugation, the sample was filtered using filter, pore size of 0.45 μ m, and 100 μ L volume was injected into the HPLC system.

Seven-point calibration curves were obtained by spiking blank whole blood and serum samples with increasing amounts of diazepam at 20, 50, 100, 250, 500, 750 and 1000 ng/mL and

200 ng/ml for internal standard. The standard samples were prepared according to the procedure as unknown samples. The calibration curves were obtained by plotting the peak area ratio of diazepam to internal standard versus concentration of diazepam in ng/ml. The regression equations were calculated by the least-squares method. The robustness of the method was determined by samples analysis under a variety of conditions such as small changes in the percentage of mobile phase acetonitrile, in the pH, in the mobile phase flow rate and in the temperature.

RESULTS AND DISCUSSION

3.1. Method development

A series of studies were conducted in order to develop a convenient and easy-to-use method for quantitative analysis of diazepam in whole

most satisfactory when mobile phase consisted of 0.1 % (V/V) triethylamine in water with pH 3.5 and acetonitrile in volume fractions 63:37. A set of column packing including C8, C18 and LC-8-DB with different lengths and particle sizes were tested and the LC-8-DB packing showed the best separation. Among several flow-rates tested (0.8-2 mL/min) the rate of 1.3 mL/min was the best with respect to the location and resolution of the diazepam peaks and internal standard from the interfering peaks. The elution was monitored in the whole UV region and the wavelength of 240 nm exhibited the best detection.

A typical chromatogram of standard solutions of diazepam and internal standard (1000 ng/ml) produced by the developed HPLC method is shown in Figure 1. Retention time of internal standard and diazepam are 7.3 min and 13.9 min, respectively.

In order to obtain satisfactory values for recovery of investigated drugs, different types of

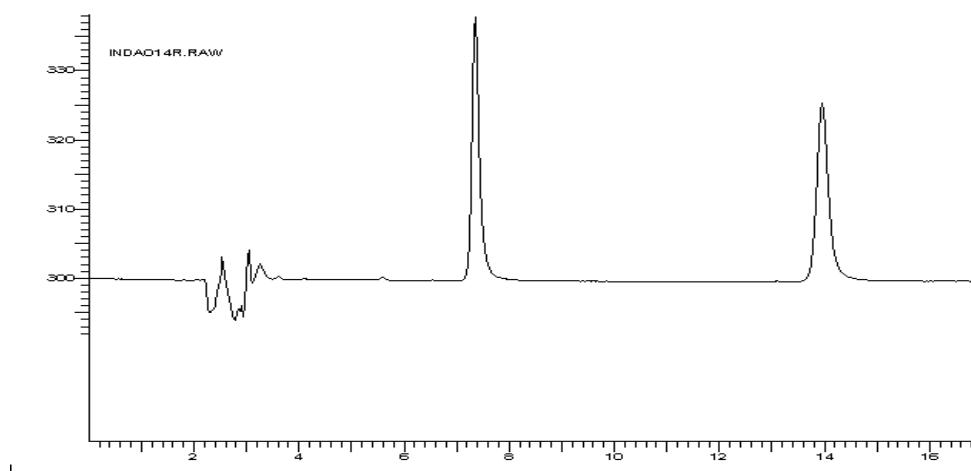


Figure 1. Chromatogram of standard solutions of internal standard (1) and diazepam (2)

blood and serum samples. Several HPLC method variables with respect to their effect on the separation of diazepam and the internal standard (bromazepam) from the matrix were investigated. In addition, the internal standard method can be beneficial when transfers, evaporations and solid phase extraction, or other experimental operations that can cause losses are used since the ration of analyte to internal standard remains constant.

In our extensive preliminary experiments, a series of aqueous mobile phases containing buffer solutions with different pH values in combination with different modifiers including acetonitrile, 2-propanol and triethylamine with different volume fractions were tested. The results were

solid phase extraction cartridges (C18, TSC and RP-select B) were tested.

The extraction recoveries were calculated by comparing the peak height of diazepam obtained for low, medium and high level quality control samples (n=3, for each level for diazepam; n=9, for internal standard) and those resulting from the direct injection (n=3, working solutions) of the theoretical amount of either diazepam or internal standard (=100 % recovery). These results are presented in Table 1.

As can be seen, the satisfactory values for recovery of diazepam and internal standard were obtained when solid phase extraction was performed on RP-select B cartridges. These cartridge-

Table 1. Absolute recoveries of diazepam and internal standard from spiked serum and blood samples

	Mean recovery (%)		
	TSC	C18	RP select B
serum			
$\gamma(\text{diazepam})/(\text{ng/mL})$			
25.0	26.4	80.3	99.7
300.0	27.3	81.1	98.1
800.0	25.6	84.2	100.1
$\gamma(\text{internal standard})/(\text{ng/mL})$			
200.0	22.7	82.1	99.8
whole blood			
$\gamma(\text{diazepam})/(\text{ng/mL})$			
25.0	34.1	59.2	80.3
300.0	36.8	52.4	80.1
800.0	35.4	51.3	82.7
$\gamma(\text{internal standard})/(\text{ng/mL})$			
200.0	35.7	52.7	79.5

es are thus more suitable for enrichment of the somewhat stronger polar compounds, whereas C18 cartridges are more suitable for nonpolar molecules using the same matrix.

Before introducing the spiked samples, cartridges for solid phase extraction were conditioned with 0.1 mol/L sodium carbonate solution. In our studies, the buffer for precondition of columns a pH 10 was found to be most appropriate for diazepam and internal standard adsorption. During the initial development of the procedure, a total of 0.5 mL of methanol was used to elute diazepam in two 0.25 mL aliquots. Lower eluent volume was advantageous to avoid the need for evaporation at low serum and blood drug levels. It was found that the first 0.2 mL eluent removed 60 to 65 % of diazepam from the column, and the additional 0.1 mL of methanol was sufficient to achieve good recovery. The minimum volume required to achieve recovery higher than 80 % was 0.3 mL. When elution of diazepam is carried out in one-step with 0.3 mL methanol, unsatisfactory values for recovery were obtained. The investigations show that the best results for recovery of diazepam were obtained with the addition of 0.2 mL of methanol followed by another portion of 0.1 mL.

Under the chromatographic conditions described, diazepam and the internal standard peaks were well resolved and endogenous serum and

blood components did not give any interfering peaks. Typical chromatograms of blank serum and blood in comparison to spiked samples are shown in Figure 2.

The developed HPLC method was used for analysis of patient plasma samples after oral administration of diazepam.

3.2. Method validation

3.2.1. Linearity, limit of detection and quantification

Linear detector response for the peak-height ratios of diazepam to internal standard was observed in concentration range between 20.0 and 1000.0 ng/mL with correlation coefficients of 0.9983 and 0.9978 for serum and blood samples, respectively. Respective regression equations for diazepam obtained after preparation of samples using solid-phase extraction were, $y = 0.0153 \cdot \gamma + 0.024$ for serum and $y = 0.0149 \cdot \gamma + 0.015$ for blood samples.

Under the experimental conditions used, the lower limit of detection was 10 ng/mL at a signal-to-noise ratio of 3. The limit of quantification was defined as the lowest amount detectable with a precision of less than 15 % ($n=5$) and an accuracy of ± 15 % ($n=5$). The limits of quantification were 20 ng/mL for serum and blood samples.

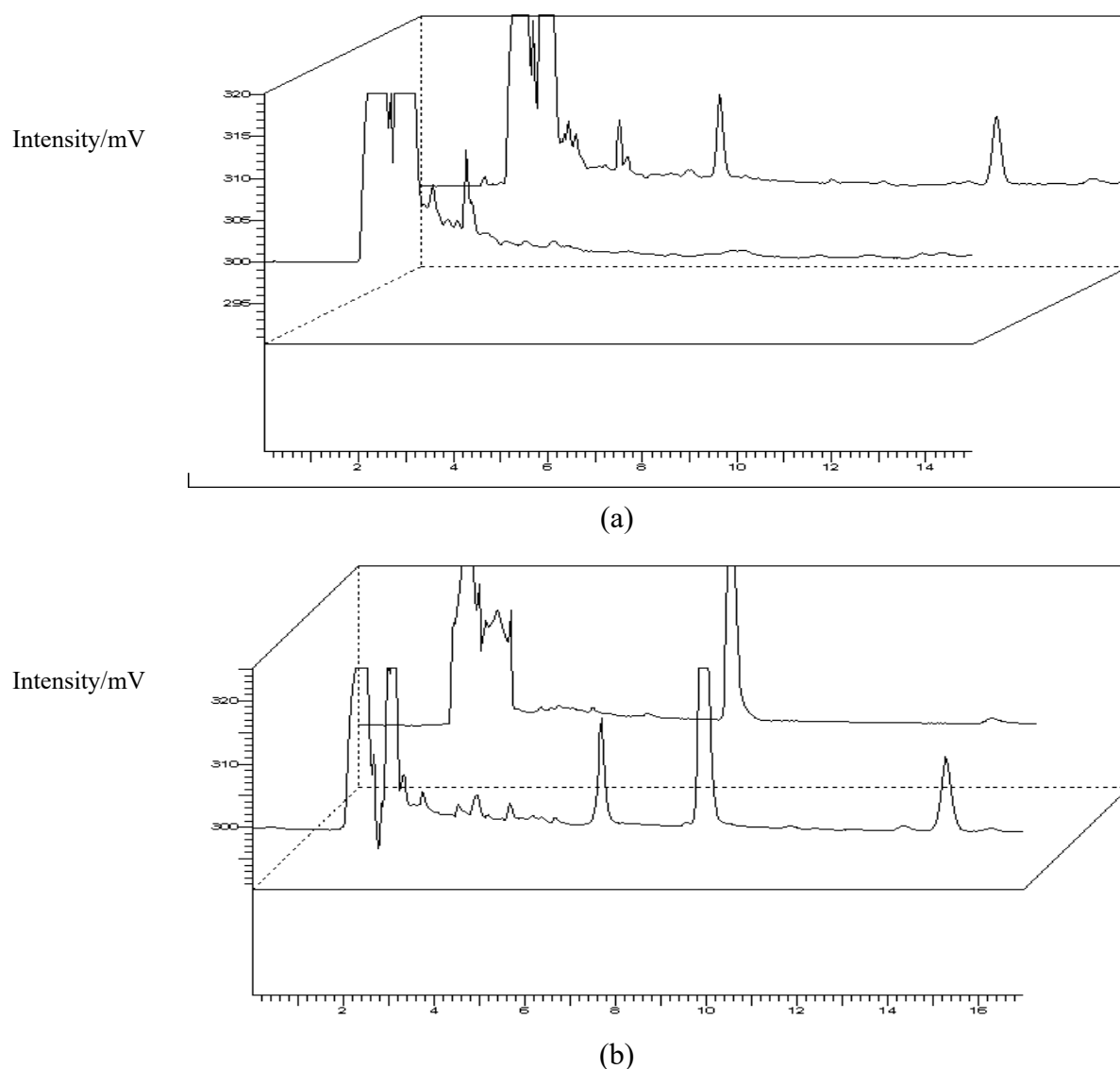


Figure 2. Chromatograms of blank (1) and spiked (2) serum (a) and blood (b) samples.

3.2.2. Precision and accuracy

In one day and on three different days, spiked samples from each concentration used for construction of calibration curves were prepared in triplicate and analyzed by the proposed HPLC method. Then, the corresponding coefficients of variation were calculated. The intra- and inter-day variations of the method throughout the linear range of concentrations are shown in Table 2. The RSD for intra-day precision ranged from 2.8 to 6.2 % for serum samples and from 4.7 to 7.1 % for blood samples. For inter-day precision, RSDs ranged from 3.0 to 6.1 % for serum samples and from 3.3 to 7.9 % for blood samples. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs.

Intra- and inter-day accuracy was determined by measuring blood and serum quality control samples at low, middle and high concentration levels. An indication of accuracy was based on the calculation of the relative error of the mean observed concentration as compared to the nominal concentration. Accuracy data are presented in Table 2. Relative errors at all three concentrations studied for serum and blood samples are less than 4.4 % and it is obvious that the method is remarkably accurate which ensures obtaining of reliable results.

3.2.3. Stability of diazepam in serum and blood samples

Stability of diazepam in serum and blood was investigated using spiked samples at two dif-

Table 2. *Intra-and inter-day precision and accuracy data*

Diazepam nominal concentration (ng/mL)	Intra-day		Inter-day	
	Mean (n=3) observed concentration (ng/mL)	Relative standard deviation (%)	Mean (n=9) observed concentration (ng/mL)	Relative standard deviation (%)
Precision				
Serum				
20.0	19.8	6.2	19.3	6.1
50.0	49.2	5.4	50.1	4.6
100.0	100.3	2.8	101.4	3.9
250.0	249.1	3.4	249.3	3.0
500.0	498.4	4.2	498.9	3.2
750.0	750.2	4.0	748.2	3.1
1000.0	997.5	4.1	998.5	3.4
Whole blood				
20.0	19.4	7.1	19.5	7.9
50.0	50.2	6.8	50.4	7.2
100.0	99.6	6.4	101.2	6.9
250.0	248.6	6.0	248.7	5.6
500.0	495.9	5.7	496.3	4.9
750.0	748.5	5.5	747.2	4.6
1000.0	998.1	4.7	998.5	3.3
Accuracy		Relative error (%)	Relative error (%)	
Serum				
25.0	25.2	-0.8	25.3	-1.2
300.0	298.4	0.5	297.6	0.8
800.0	798.3	0.2	797.4	0.3
Whole blood				
25.0	24.6	1.6	26.1	-4.4
300.0	297.5	0.8	298.7	0.4
800.0	797.4	0.3	797.4	0.3

ferent concentration levels i.e., 20 ng/mL and 1000 ng/mL and were prepared in duplicate. Spiked samples were analysed after different storage conditions: immediately, after staying in an autosampler for 2, 12 and 24 hours, after one and three freeze/thaw cycles and after 1 month stored at -20°C . The results from the stability studies show that relative errors at two different concentrations studied were in range from 0.5 to 7.3 % and it is obvious that diazepam added to plasma are stable in the different storage conditions.

3.2.4. Robustness of the method

To assess the robustness of the quantitative method, deliberate changes in flow rate, percentage of acetonitrile, pH and temperatures were performed. These alterations did not show any significant changes on retention time and peak parameters for both diazepam and the internal standard. According to the system suitability pa-

rameters under the modified conditions and the specificity of the method, we can conclude that the method conditions are robust.

CONCLUSION

The developed HPLC method employing solid-phase extraction for sample preparation is simple, convenient and economical for the determination of diazepam in serum and blood samples. This method provides a linear response across a wide range of concentrations and it utilizes a mobile phase which can be easily prepared and diluent is economic, readily available. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. Diazepam and the internal standard bromazepam have been successfully separated.

The proposed method is simple, rapid and provides efficient clean-up of the complex biological matrix and high recovery of diazepam. Finally, the method has been implemented to monitor diazepam levels in clinical samples.

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Резиме**ВИСОКО-ЕФИКАСНА ТЕЧНА ХРОМАТОГРАФСКА МЕТОДА
ЗА ДИРЕКТНО ОДРЕДУВАЊЕ НА ДИЈАЗЕПАМ ВО ПОЛНА КРВ
И СЕРУМ-ОПТИМИЗАЦИЈА НА ЦВРСТОФАЗЕН ЕКСТРАКЦИСКИ МЕТОД**

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Целта на оваа студија беше да се развие едноставен и брз хроматографски метод преку УВ-детекција за директно одредување на дијазепам во полна крв и серум за следење на неговите концентрации во клиничката средина. Изолацијата на дијазепам и на интерниот стандард бромазепам од серум и полна крв беше изведено преку метод на екстракција преку цврста фаза и користење на RP селект В кертриџи. Аналитите беа издвоени преку користење на реверзнофазна C8 колона со мобилна фаза составена од 0,1 % (V/V) триетиламин во вода (pH 3.5) и ацетонитрил (63:37, V/V), а УВ-детекцијата беше изведена на 240 nm. Линеарноста на методот беше достигната во концентрациски ранг од 10 до 1000 ng/ml за серум и полна крв. Апликацијата на развиениот метод беше изведена на спајкувани и вистински биолошки примероци по перорална администрација на 10 mg дијазепам. Предложениот метод обезбедува едноставно, брзо и ефикасно чистење на комплексниот биолошки матрикс со висок степен на добивка на дијазепам.

Клучни зборови: дијазепам, екстракција преку цврста фаза, полна крв, серум