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VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION METHOD FOR DETERMINATION OF DIAZEPAM IN SALIVA AND SERUM SAMPLES

VALIDACIJA METODE TEČNE HROMATOGRAFIJE SA UV SKENIRAJUĆIM DETEKTOROM ZA ODREĐIVANJE KONCENTRACIJE DIAZEPAMA U SALIVI I SERUMU

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Abstract

Like serum, saliva is a biological sample, which could be used for determination of drugs both in therapy applying and acute poisonings. Drug monitoring of benzodiazepines in saliva is an attractive alternative, because its collection is painless, non-invasive and simpler than drawing blood. The aim of this study was to develop and validate high performance liquid chromatography with photodiode array detection (HPLC-PDA) method for determination of diazepam in saliva and serum.
Saliva samples were prepared by liquid-liquid extraction with diethyleter at pH 10. Serum samples were prepaired by solid-phase extraction on Oasis HLB cartridges. Diazepam concentrations was measured in dried and in methanol redissolved extract by HPLC-UV/PDA at 230 nm. Mobile phase (pH = 3.6) composed of phosphate buffer and acetonitrile was set up using gradient flow from 1 – 1.5 mL/min. Linearity was achieved in the range of diazepam from 0.05-2 μg/mL. Retention time of diazepam was 20.400 ± 0.041 minute. Diverse range of drug concentrations could be determined with good precision and accuracy. Recoveries of spiked samples for diazepam were 93.1 ± 4.71 % and 89.96 ± 5.06 % in the human saliva and serum, respectively. The method was applied for determination of diazepam concentration in saliva and serum taken from patients after therapeutic doses and in acute poisonings.

Key words
diazepam, saliva, acute poisoning, HPLC-PDA

Ključne reči
diazepam, saliva, akutno trovanje, HPLC-PDA

1. INTRODUCTION

The use of diazepam, a tranquillizer of the benzodiazepine type, is wide-spread for the symptomatic relief of anxiety, insomnia, psychiatric disturbances, seizures and as preoperative medication. Monitoring of drug concentration is useful in patients with chronic therapy, and in cases of acute poisoning.

It is well known that salivary drug levels are useful in therapeutic drug monitoring [(1-4)].

Another important application of saliva analysis lies in 'drugs and driving' research. Diazepam use may be associated with motor vehicle accidents. Blood sampling is invasive technique and need trained stuff. Sampling of saliva is simpler than blood sampling. It

could be done with supervising of patient and prevents traumatic influences [3,5].

There are many described analytical methods for determination of benzodiazepines in biological samples [6-18]. The most frequently applied method is high performance liquid chromatography with UV detection.

The aim of this report was to develop HPLC/PDA method for determination of diazepam concentration in serum and saliva samples.

2. EXPERIMENTAL

2.1. Material

Analytical standard of diazepam D0940000, 30 mg, ID 001ZT, was obtained by Council of Europe, EDQM CS, 30026F67081, Strasbourg Cedex.

Acetonitrile, methanol and sodium dihydrogenphosphate were of HPLC purity, obtained from MERCK. Phosphoric acid and diethyleter were p.a. purity, obtained from MERCK. Water was purified by Millipore Milli-Q system.

2.2. Equipment and chromatographic condition

The development and validation work was carried out on a chromatographic system consisting of a Waters Alliance 2695 Separation Module, connected with a Waters Photo Diode Array (PDA) 2696. HPLC separation was performed at 30° using Symmetry C8 5µm, 4.6mm x 250mm analytical column equipped with a guard C18 cartridge. The autosampler was programmed with a injection volume of 50 µL. The mobile phase A consisted 6,9 g $NaH_2PO_4xH_2O$ dissolved in 1000 mL water and adjusted on pH 3,6 with 20% phosphoric acid. The mobile phase B was acetonitrile.

Ratio of mobile phases A and B and flow are in table 1.

Table 1. Gradient table of ratio of mobile phases A and B

time (min.)	flow (mL/min)	A %	B %	curve
	1.0	85	15	
3.0	1.0	65	35	6
9.0	1.0	20	80	6
28.0	1.5	20	80	6
31.0	1.5	20	80	6
31.5	1.5	85	15	6
35.0	0.3	85	15	6

UV-VIS spectra were obtained in the range of 200.5-800 nm. Raw data were collected and processed with computer software Empower Login.

2.3. Solutions and sample preparation

Stock standard solution of diazepam was prepared by dissolving 10 mg in 10 ml methanol and stored at -4ºC. Other concentrations of diazepam were made by diluting stock standard solutions with mobile phase to achieve calibration concentrations expected to meet the levels in saliva and serum samples of patients.

Volume of 1 mL of serum sample passed through a HLB cartridge (Waters) previously conditioned with 1 mL of methanol and 1 mL of demineralized water. HLB cartridge then was washed with 1 mL of 5 % methanol and diazepam was eluted wit 1 mL of methanol. Eluat was analyzed by HPLC-PDA method.

To 2 mL saliva sample 1 mL of borate buffer pH 10 and 5 mL of diethyleter was added. The samples were mixed on mechanical shaker for 20 minutes and centrifuged on 3000 rpm for 10 min. After centrifugation organic layer was separated and evaporated in stream of air. Dry extracts were reconstituted in methanol and analyzed by HPLC-PDA method on 230 nm.

Calibration and quality control samples were prepared by adding diazepam solution in blank ("drug-free") human serum and saliva. The amounts corresponded to serum and saliva concentration of diazepam ranging from 0.05 µg/mL to 2.00 µg/mL. The calibration curves for saliva and serum spiked by diazepam were obtained by plotting diazepam peak areas for the concentrations range of 0.05, 0.10, 0.50, 1.00, and 2 µg/mL.

Concentrations of quality control samples were 0.05, 0.5 and 2 mg/L.

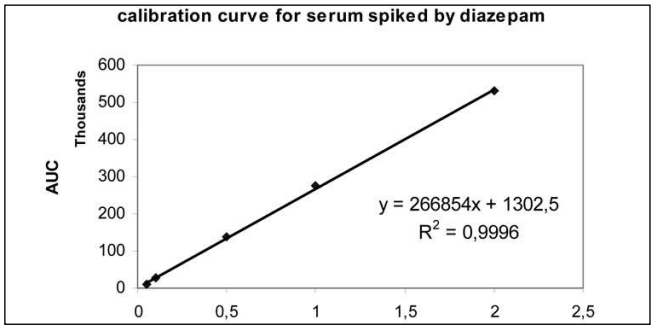
3. RESULTS

Diazepam concentrations were determined using weighed linear regression function. Table 2 shows calibration curve for diazepam in serum and saliva.

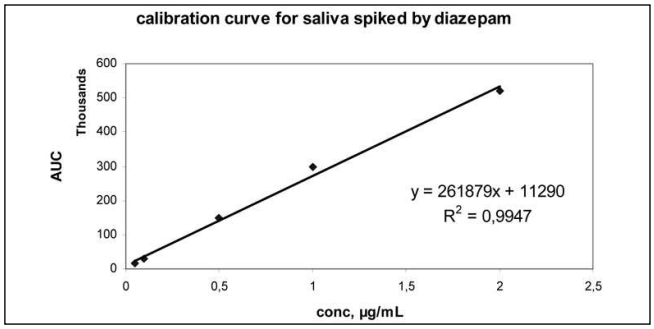
Table 2. Calibration curve for diazepam in serum and saliva

conc,µg/mL	AUC for spiked serum	AUC for spiked saliva
0.05	9839	15107
0.10	27841	29699
0.50	136964	147754
1.00	274455	297750
2.00	531429	521996

The correlation coefficients for serum and saliva spiked by diazepam were 0.9996 and 0.9947 respectively. Picture 1 and 2 shows calibration curve for spiked serum and saliva.

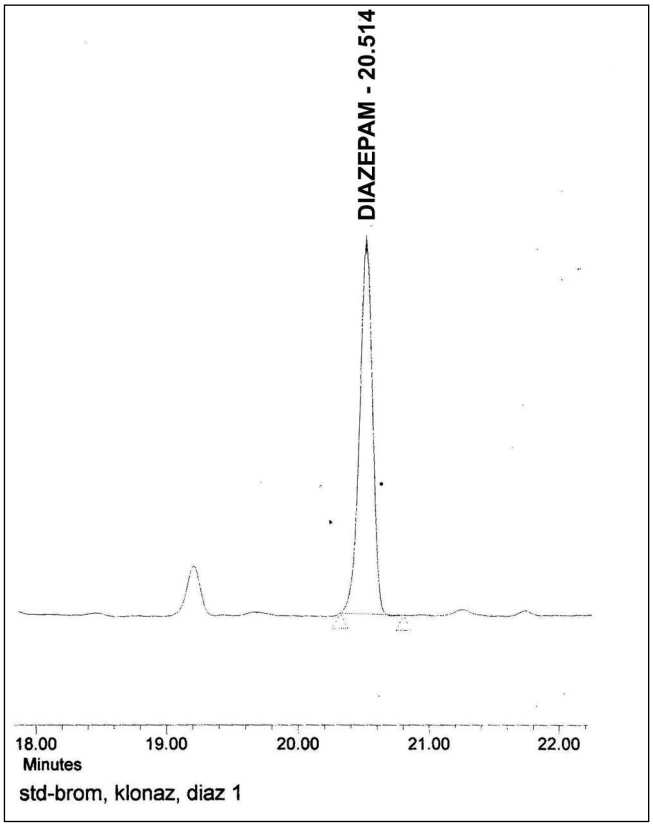


Picture 1. Calibration curve for spiked serum



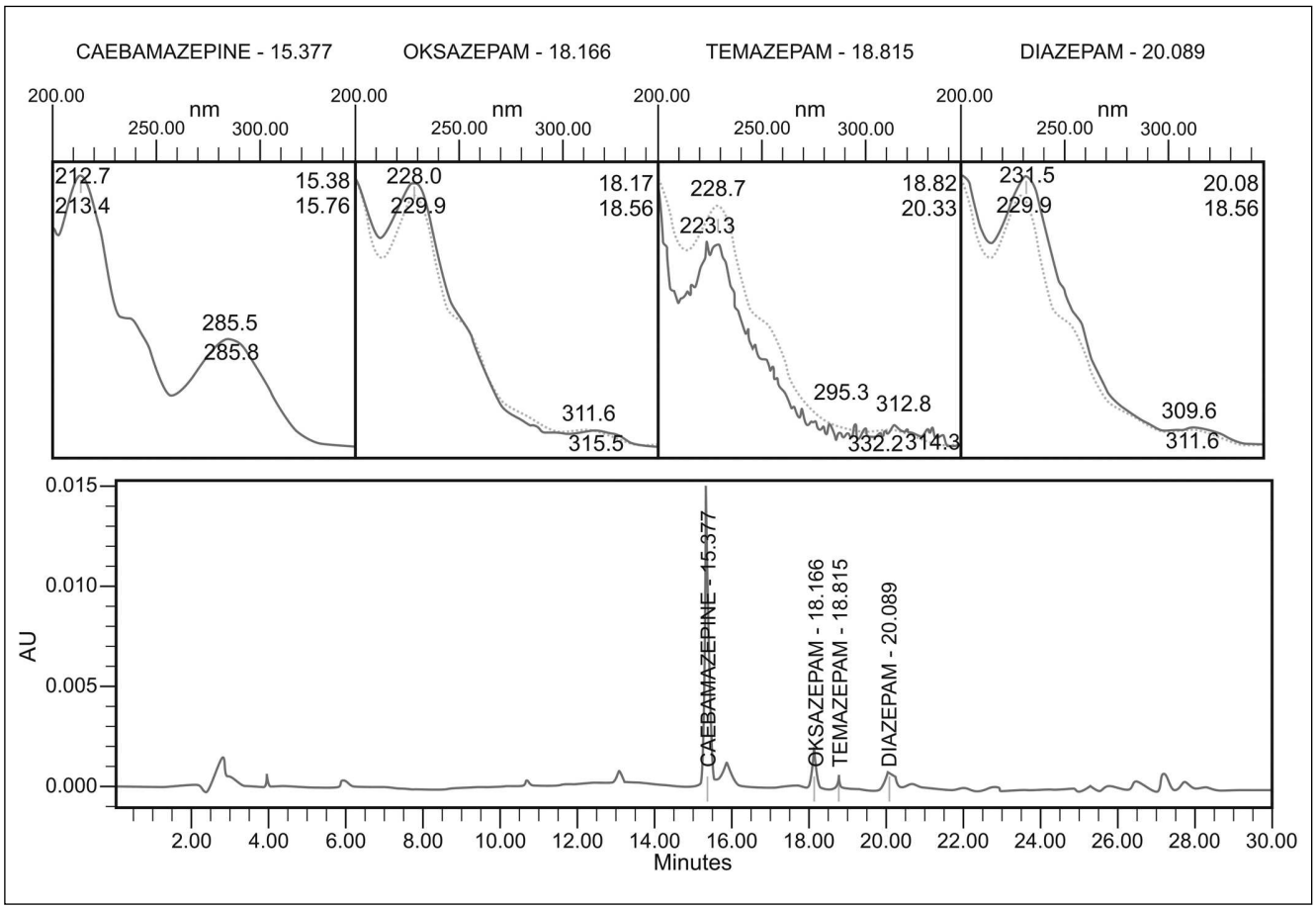
Picture 2. Calibration curve for spiked saliva

Unknown concentrations of diazepam in serum and saliva samples of patients were calculated using the corresponding factor from calibration curve. The factor is calculated from the mean value peak-area of diazepam for each mentioned concentration. Recovery was determined, for each concentration, as the mean value of three samples by comparing the peak areas of the extracted and non-extracted samples.

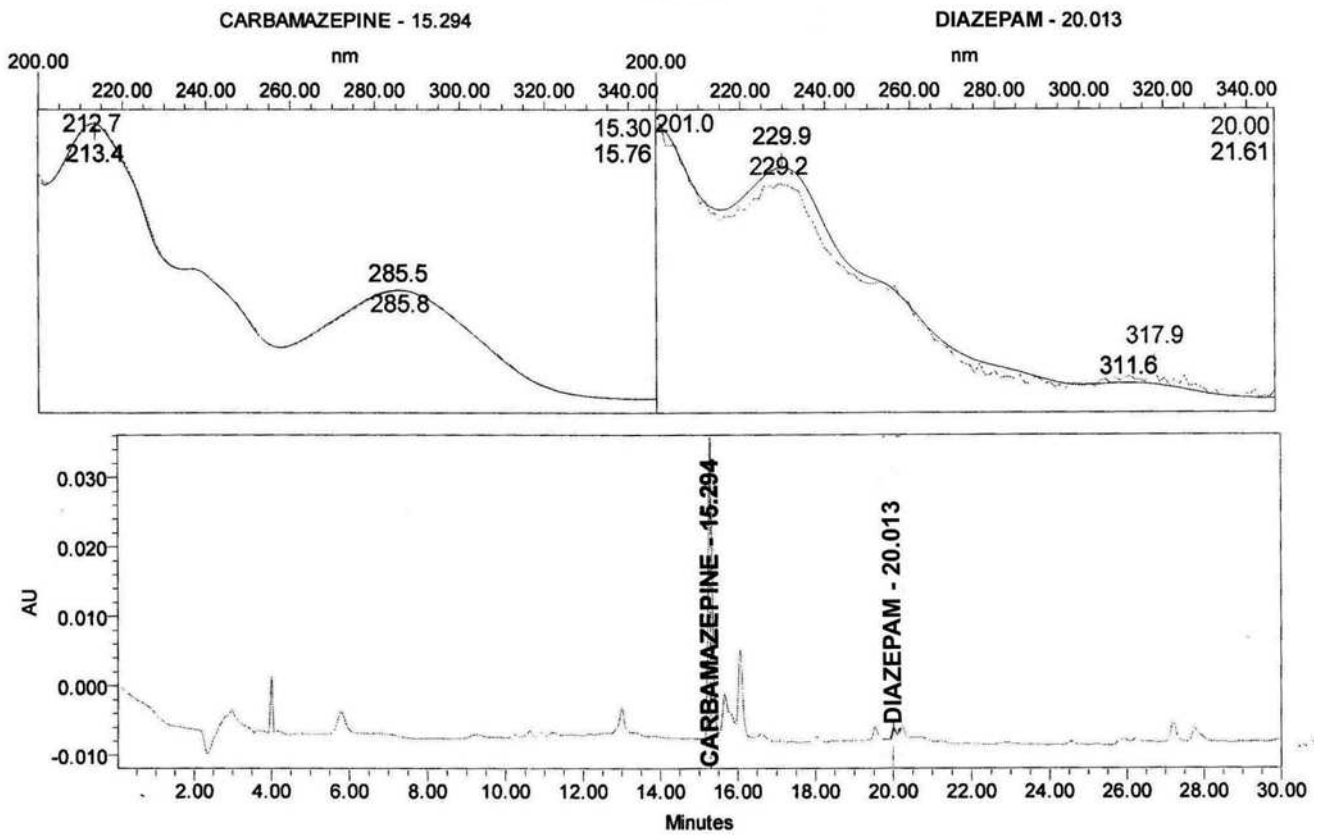


Picture 3. Chromatogram of saliva spiked with diazepam, c=0.5 µg/mL

Analytical recovery for saliva and serum spiked by diazepam were 93,1 ± 4,71 % and 89,96 ± 5,06 %, respectively.



Picture 4. Chromatogram of serum sample from patient acutely poisoned by drugs



Picture 5. Chromatogram of saliva sample from patient acutely poisoned by drugs

Calculation of diazepam concentration was done on the basis of calibration curve which was created after analysis of saliva and serum spiked by carbamazepine standard solution ranged from 0.05 to 2 mg/L. Linear regression for spiked saliva and serum were Y= 261879*X + 11290 and Y= 266854*X + 1302.5, respectively.

Pictures 3-5 show chromatograms of saliva spiked by diazepam and patients serum and saliva samples.

In Table 4. limit of detection (LOD), limit of quantitation (LOQ), standard deviation (SD) and coefficient of correlation (CV) for spiked saliva and serum were presented.

Limits of quantification in saliva and serum samples were 0.013 and 0.024 μg/mL, respectively.

The precision of the method was assessed by calculating coefficient of variation (CV) for measured parameter of the method (peak area of diazepam) and determined on the same day (n=7). The inter- and intra-day coefficients of variation (CV) for spiked serum and saliva was shown in Table 3.

The spiked saliva and serum extracts were stable when kept in the refrigerator for 48 hours. Mean value was 95.61 % (from 93.54 to 102.02 %).

Saliva and serum constituents did not interfere in diazepam assay.

The method was applied to determine the concentration of diazepam in patient's serum or saliva samples after therapeutic doses and in acute poisonings.

Table 3. Inter-day and intra-day coefficients of variation (CV) for spiked serum and saliva

Conc. (μg/mL)	Intra day CV(%) serum	Intra day CV(%) saliva	Inter day CV(%) serum	Inter day CV(%) saliva
0.05	4.12	4.96	4.36	5.28
0.5	3.18	4.03	4.79	3.98
2	3.96	3.37	4.04	4.71

Table 4. Limits of detection (LOD), limits of quantitation (LOQ), standard deviations (SD) and coefficients of variation (CV), for spiked saliva and serum

	Sd	KV (%)	LOD (mg/L)	LOQ (mg/L)
Standard solution	0,001 (701,2)	2,85	0,003	0,013
spiked serum	0,001 (517,1)	5,11	0,002	0,008
spiked saliva	0,002 (1136)	7,52	0,007	0,024

4. DISSCUSION

Extraction procedure of diazepam from biological samples uses both liquid-liquid [7,8] and solid-phase methods [9, 10,13,18].

Analytical recovery for benzodiazepines after alkaline extraction with diethyleter were 70.3-86.9% and 63.9-77.2% for plasma and saliva respectively [8].

We used neutral solid-phase extraction procedure for serum samples, which is fast and simple. Analytical recovery after solid phase extraction of diazepam was very satisfactory (89.96 %). Moreover, this kind of sample preparation is suitable for toxicological screening, because it enables identification not only diazepam, but many other drugs which could be involved in intoxication.

Saliva samples were prepared by liquid-liquid extraction because of its high viscosity. Analytical recovery was better then after solid phase extraction. This is understandable, because extraction condition was adjusted for diazepam extraction, and not for screening.

The proposed HPLC-PDA method enables quantification of diazepam in saliva and serum samples. Retention time of diazepam was 20.400 ± 0.041 minutes. No matrix interference was observed at retention time of the analyte under condition performed in this method.

The identification of diazepam was based on the retention time and UV spectrum from computer library and the same parameters for peak in serum and saliva samples spiked by working standard solution of diazepam.

Due to computer library, the same chromatographic condition of described HPLC/PDA method enables identification of about 1000 other drugs and their metabolites. It has importance in toxicological screening, especially when patient is unconscious and when the cause of intoxication is not known.

HPLC-PDA method in comparison to LC-MS [7] is less sensitive, but it offer advantage because of low costs of analysis. Moreover, this method has acceptable sensitivity in monitoring of diazepam concentration in both therapy applying and in acute poisonings.

The HPLC-PDA method was applied to determine the concentration of diazepam in patients' serum and saliva samples.

5. CONCLUSION

Described HPLC-PDA method is sensitive and simple, and could be applied for determination of salivary and serum concentration of diazepam in both treated and acutely poisoned patients. The HPLC-PDA method could be applied for monitoring subjects driving under the influence of diazepam.

Apstrakt

Kao i serum, saliva je biološki materijal koji se može koristiti za određivanje koncentracije lekova nakon terapijske primene i u akutnim trovanjima. Praćenje lekova iz grupe benzodiazepina u salivi je dobra alternativa, zbog toga što je uzorkovanje bezbolno, neinvazivno i jednostavnije u odnosu na uzorkovanje krvi. Cilj ovog rada bio je da se razvije i validira metoda tečne hromatografije sa UV skenirajućim detektorom (HPLC-PDA) za određivanje diazepama u salivi i serumu.

Uzorci salive pripremani su tečno-tečnom ekstrakcijom dietiletrom pri pH vrednosti 10. Uzorci seruma pripremani su čvrsto-tečnom ekstracijom na Oasis HLB kertridžima. Određivanje koncentracije diazepama je vršeno nakon rekonstitucije suvog ekstrakta u metanolu HPLC-UV/PDA metodom na 230 nm. Protok mobilne faze (pH = 3,6), koja se sastojala od fosfatnog pufera i acetonitrila, bio je podešen gradijent programom i iznosio je od 1 – 1,5 mL/min. Linearnost je postignuta u opsegu koncentracija od 0,05-2 μg/mL. Retenciono vreme diazepama iznosilo je 20,400 ± 0,041 minuta. Širok opseg koncentracija leka može se odrediti sa dobrom preciznošću i tačnošću. Prinos ekstrakcije za diazepam u serumu iznosio je 89,96 ± 5,06 % a za salivu 93,1 ± 4,71 %. Metoda je primenjena u određivanju koncentracije diazepama u uzorcima salive i seruma dobijenih od bolesnika nakon terapijske primene i u akutnim trovanjima ovim lekom.

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