ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2012, *57*(7-8), 715-720

Dedicated to Professor Victor-Emanuel Sahini on the occasion of his 85th anniversary

ENRICHMENT OF SEVERAL BENZODIAZEPINES BY SOLID-PHASE EXTRACTION WITH OCTYL AND PHENYL SILICA BASED ADSORBENTS

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Received June 9, 2012

Concentration calibration curves on two silica based adsorbents (octyl and phenyl) have been investigated for six benzodiazepines (alprazolam, bromazepam, diazepam, flunitrazepam, medazepam, and nitrazepam). The chosen concentration was at μM level for studied compounds because in real samples they are present at trace levels. The analytical concentration procedure allows a minimum concentration factor of 10 within the concentration interval of 0.1-2.5 μM. The extracted benzodiazepines are analyzed by UV absorption at wavelengths specific to each studied compound. Differences in recovery were observed for both phenyl and octyl cartridge, which can be explained by the different interaction with sorbent and nature of the solvent.

INTRODUCTION

Solid-phase extraction (SPE) has become the preferred technique for the extraction and concentration of selected compounds before chromatographic analysis because of its simplicity, speed, flexibility in sorbent and solvent choice and it requires much less organic solvent that liquid-liquid extraction.[1] The compounds are transferred to the solid phase where they are retained and then are recovered by elution with a proper solvent. For organic molecules adsorbed from water on the sorbent the order of the water molecules decreases; its entropy increases which lead to negative changes in free energy making the process spontaneous.[2] One of the goal of SPE is the concentration of analytes,[3] by selecting a proper solvent that will elute the analytes completely from the solid phase using a small volume of the eluent. However, this requires that the adsorption of analyte(s) on sorbent must be reversible.

Benzodiazepines solid phase extraction protocols have been presented in the literature with the emphasis on its applicability to several biological matrices.[4] In previous studies different type of silica based sorbents such as ethyl, octyl, octadecyl, and phenyl have been used for solid-phase extraction procedures of benzodiazepines.[5-7] Determination of different benzodiazepines after SPE procedure has been accomplished by means of HPLC and GC-MS, which require a derivatization step such as acetylation.[8] Recovery obtained was between 58.8–100.55 %. Mixed phases[9] and XAD-7, Oasis HLB polymeric sorbents in which the concentration was performed by solvent evaporation under nitrogen stream[10,11] have also been used for benzodiazepines extraction. In the literature a molecular imprinted polymer solid phase extraction has been described for direct extraction and quantification of benzodiazepines in human plasma and hair samples.[12,13] Multiwalled carbon nanotubes (MWCNT) have been used for

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extraction of benzodiazepines in pork; the recovery obtained were 75 to 104%.[14] Previous studies have been focused on the different biological matrices for simultaneous clean-up and concentration of benzodiazepines and their metabolites.

The aim of this paper is to develop an analytical calibration procedure for enrichment of six benzodiazepines from aqueous solutions on two silica based sorbents. For this purpose we used two different adsorbents, octyl and phenyl silica, for extraction of these analytes and acetonitrile for re-extraction of retained compounds on SPE adsorbents. The chemical structures of benzodiazepines are characterized by a benzene ring fused on the 10- and 11 positions of the 1,4-diazepine ring. Benzodiazepines are compounds with a broad range of therapeutic effects prescribed as tranquillizers, muscle relaxants, anti-anxiety agents, and anticonvulsants. Alprazolam is a triazolobenzodiazepine; flunitrazepam and nitrazepam are nitrobenzodiazepine.[4]

EXPERIMENTAL

Materials and methods: The six compounds studied were the following benzodiazepines: alprazolam, bromazepam, diazepam, flunitrazepam, medazepam, and nitrazepam, which were kindly offered by LaborMed Pharma S.A. Their structures are presented in Fig. 1. Water (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced with a TKA Lab HP 6UV/UF instrument in the laboratory. Acetonitrile (HPLC grade) was purchased from Sigma Aldrich.

Preparation of solutions: 2 mM stock solutions for the six benzodiazepines were prepared in methanol. Five working solutions over the range 0.1 μM to 2.5 μM were further prepared by successive dilution in water. Standard solution of studied compounds in acetonitrile for calibration curves were prepared for five concentration levels ranging from 1 to 25 μM.

SPE procedure: Before sample loading, the sorbent was conditioned with 5 mL acetonitrile and 5 mL water. The volume of the individual aliquot added was 2.5 mL. The flow rate was kept constant during the experiments at 1mL/min.

Equipments: An automated SPE HT400E system was used in this study. Two types of sorbents containing octyl and phenyl silica bonded phases have been used; with the characteristics presented in Table 1.

The absorption spectra were recorded with a Jasco V-530 double beam spectrometer, in 1 cm quartz cells. The absorbance of studied compounds were measured at λ_{max} = 220 nm for alprazolam, at λ_{max} = 226 nm for bromazepam, and diazepam and at λ_{max} = 254 nm for flunitrazepam, medazepam, and nitrazepam. All experiments were performed at room temperature.

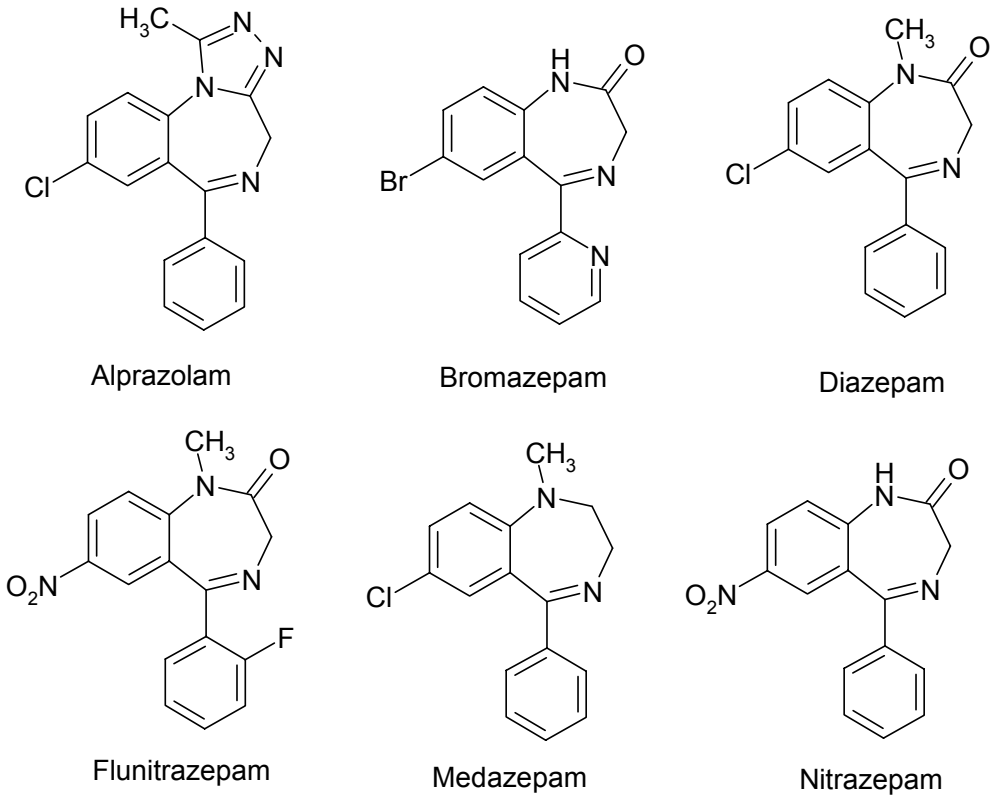

Fig. 1 – Chemical structures of benzodiazepines.

Table 1

Characteristics of silica based sorbents used

Cartridge type	**Sorbent mass** mg	**Sample loading** mL	**Surface area** m^2/g	**Particle size** μm	**Average pore size** A	**Carbon loading** %
SampliQ C8	200	4	571	45	60	15.6
SampliQ Phenyl	200	4	562	45	60	12.6

RESULTS AND DISCUSSION

The studied compounds are characterized by a moderate hydrophobic character, given by their theoretical octanol/water partition constant (log K_{ow}). The log K_{ow} values are given in Table 2. Four benzodiazepines have relative similar log K_{ow} values and we can expect to have similar retention behavior, although they have different polarity. 1,4-Benzodiazepines have a basic character due to the nitrogen atom in position 4 and the other nitrogen atoms which can be protonated. Compounds are partitioning between the sorbent and water according to the partial distribution coefficients.

A SPE procedure includes four steps: activation and conditioning of sorbent, sample loading, removal of interferences (to simplify the original matrix - optional step), and elution of the adsorbed analytes.[2]

Solid phase extraction analytical procedures for enrichment of benzodiazepines from aqueous solutions on SampliQ cartridges were studied according to the diagram given in Fig 2. After conditioning, the SPE cartridge was loaded with 25 mL sample, the compound was then eluted with 2.5 mL acetonitrile, followed by UV absorbance measurement. Before desorption, the sorbent was dried for five minutes for removing the trapped water. The presence of water in the cartridges can influence the capacity of solvent to extract the analyte either by preventing entering the pores of adsorbents in the case of immiscible solvent or by mixing with water for miscible solvents reducing their elution strength.[16,17]

Hydrophobic interactions are responsible for the extraction and elution of the benzodiazepines compounds on octylsilica based adsorbent; for phenyl-silica beside the hydrophobic interactions, π-π interactions can be established. Nevertheless, two other interactions are likely to occur between analyte molecule and adsorbent surface (polar interactions with silanols, and electrostatic interactions with traces of metallic ions).[18] At this level of concentration no overloading of the sorbent can be produced, the only process that influences the extraction being the retention. The conformation of molecules has a role in the interaction between the compound and adsorbents. Phenyl group attached to the silica sorbent has a planar geometry, benzodiazepines have geometry in which phenyl ring attached to the 1,4-benzodiazepines moieties is perpendicular to it. Interaction between phenyl moieties and benzodiazepines is expected to be strong. The orientation of the molecule at the surface of sorbent has an influence on retention.

Table 2

Octanol/water partition constant of the studied compounds

Compound	**Molecular weight**	**log K_{ow}*** **(calculated)**
Flunitrazepam	313.28	1.91
Bromazepam	316.15	1.93
Nitrazepam	281.26	2.45
Diazepam	284.74	2.70
Alprazolam	308.76	3.87
Medazepam	270.75	4.43

* Calculated with EpiSuite Software (KOWWIN)[15]

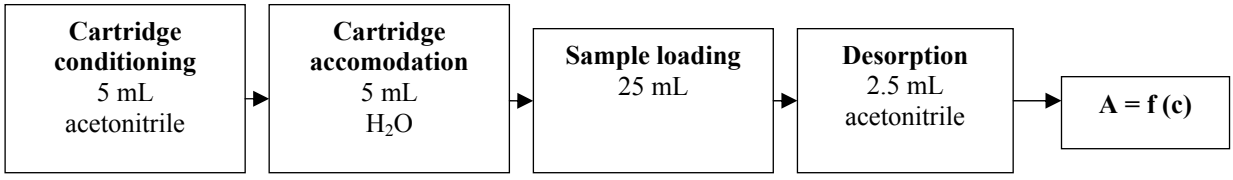

Fig. 2 – Schematic diagram of the SPE calibration procedure.

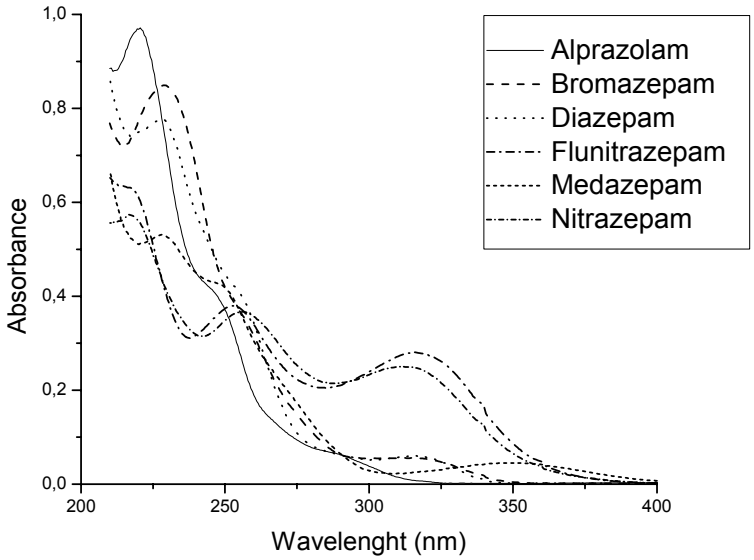

Fig. 3 – UV absorption spectra of studied benzodiazepines in acetonitrile.

The UV absorption spectra for the six benzodiazepines in acetonitrile are presented in Fig. 3.

The calibration curves of benzodiazepines presented in Fig. 4 were constructed by plotting absorbance versus concentration over the range 1-25 μM, using acetonitrile as sample solvent. The recovery is the ratio between the amount of analyte extracted and the amount of sample applied to the cartridge.[19] The variables that affect the recovery of a solid phase extraction procedure are the conditioning solvent, flow rate, sample properties, drying time, eluting solvent.[20] The eluting solvent must be able to displace all the analyte from the sorbent with minimum volume.

The regression parameters (slopes and intercepts) for the calibration curves of the six benzodiazepines in acetonitrile are given in Table 3. As can be observed the correlations were very good (r being higher than 0.9900 for all six benzodiazepine).

The six graphs for concentration analytical procedures on C8 and Phenyl cartridges for benzodiazepines are depicted in Fig. 5. When the UV absorbance versus concentration is plotted for studied benzodiazepines it can be observed a linear relationship with high correlation coefficient (between 0.91-0.99).

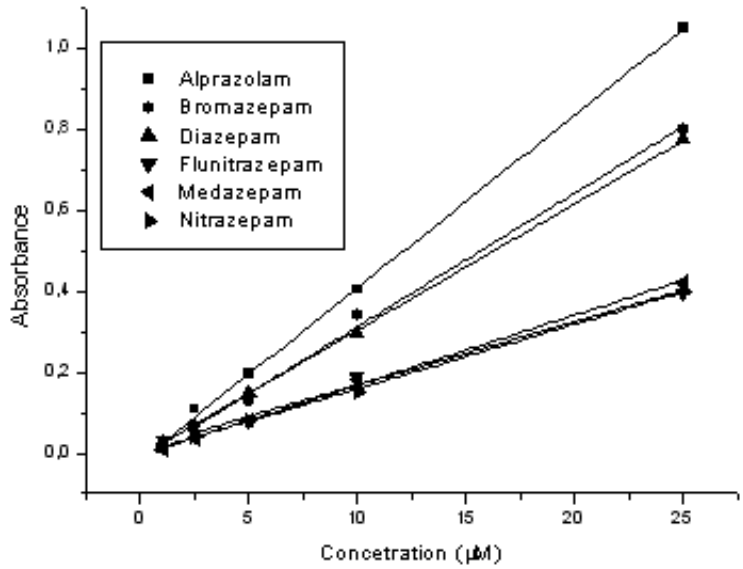

Fig. 4 – Calibration curves for the six benzodiazepines in acetonitrile.

Table 3
Calibration data for benzodiazepines in acetonitrile
(A – absorbance; C – concentration; r – correlation coefficient)

Compound	Equation	r
Alprazolam	A = 0.0426 C – 0.0172	0.9988
Bromazepam	A = 0.0329 C – 0.0177	0.9941
Diazepam	A = 0.0311 C – 0.0077	0.9994
Flunitrazepam	A = 0.0155 C + 0.0129	0.9924
Medazepam	A = 0.0172 C – 0.0048	0.9969
Nitrazepam	A = 0.0159 C – 0.0005	0.9990

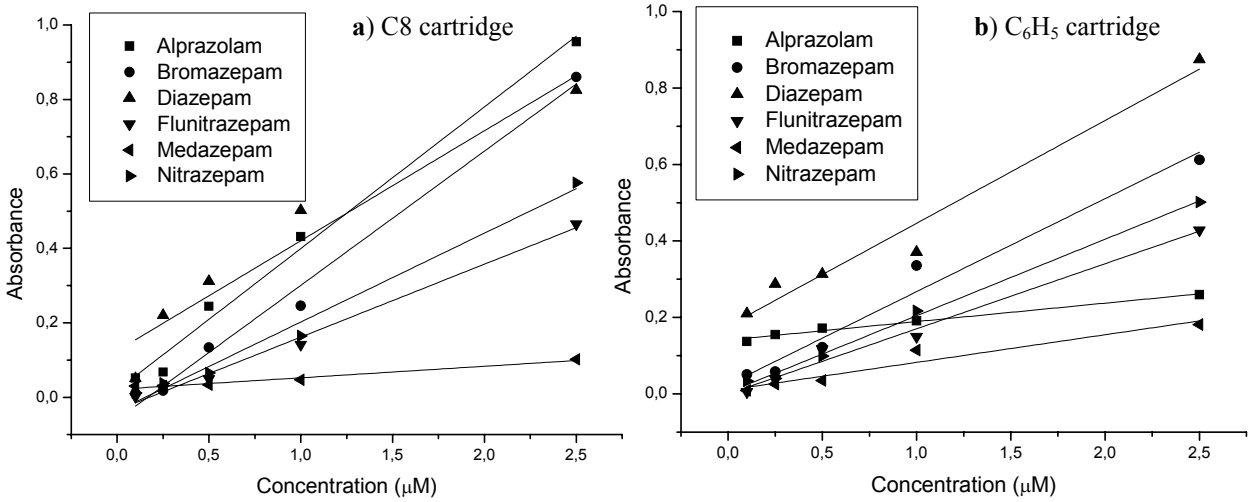

Fig. 5 – Calibration curves for the six benzodiazepines based on 10-fold enrichment SPE procedure.

From Fig. 5 one can be seen that sensitivity of calibration function for five benzodiazepines (alprazolam, bromazepam, diazepam, flunitrazepam, and nitrazepam) are much higher then for medazepam and acetonitrile could be a suitable solvent for desorption. Acetonitrile is an aprotic, hydrogen bond acceptor solvent chosen because it has an elutropic power greater than methanol. Medazepam has the highest hydrophobic character (log K_{ow} = 4.43) of the benzodiazepines studied and as can been observed from the Fig. 5.a the retention on the C8 cartridges is strong, desorption is slower, which influence the recovery value of this analyte. Therefore, medazepam is not completely removed from the sorbent due to strong retention. Experimental recoveries are obtained between 24 to 82%. A possible explanation may be the slow mass transfer, due to a slow kinetics, but also to possible secondary interactions with silanol groups on C8 cartridge. The same behavior is present on phenyl type cartridge. For alprazolam the recovery obtained was between 79-91%, for bromazepam 43-99 %, for flunitrazepam 70-94 %, and for nitrazepam 77-96 %.

In Fig. 5.b one can be observed that for medazepam and alprazolam interaction with the phenyl cartridge is strong, which has an influence on the recovery value. For alprazolam the recovery was between 35-88 %, for medazepam between 43-79 %. The low recovery values obtained for these two compounds can be explained by the strong interactions with the sorbent according to their high hydrophobicity (see Table 2), which are not favorable to their desorption from the adsorbent surface. The highest sensitivity was obtained for diazepam and bromazepam. In the case of bromazepam the recovery obtained was 73-99%, for flunitrazepam 95-99 %. The sensitivity of benzodiazepines on phenyl cartridges is lower than for C8 cartridge. This can be observed from the slope values of the calibration curves for the two types of adsorbents used in this study. The main regression parameters for benzodiazepine used in this study are given in Table 4.

Table 4

Calibration equations for benzodiazepine after enrichment procedure on C8 and Phenyl cartridge
(A – absorbance; C – concentration; r – correlation coefficient)

Cartridge	**C8**		**Phenyl**	
Compound	**Equation**	**r**	**Equation**	**r**
Alprazolam	A = 0.3801 C + 0.0195	0.9957	A = 0.0483 C + 0.1408	0.9810
Bromazepam	A = 0.3604 C - 0.0596	0.9951	A = 0.2432 C + 0.0242	0.9634
Diazepam	A = 0.2953 C + 0.1251	0.9203	A = 0.2687 C + 0.1774	0.9609
Flunitrazepam	A = 0.1987 C + 0.0311	0.9943	A = 0.1702 C + 0.0001	0.9805
Medazepam	A = 0.0310 C + 0.0214	0.9693	A = 0.0723 C + 0.0099	0.9159
Nitrazepam	A = 0.2385 C - 0.0363	0.9831	A = 0.2003 C + 0.0039	0.9957

In SPE, the recovery depends on the sample volume and on the breakthrough volume, related to the amount and the nature of the sorbent.[21,22] Because of the strong interaction of benzodiazepines with both sorbents it can be expected that the value of breakthrough volumes could be high. Mercolini *et al.* used a metyl silica based sorbents for the extraction of 15 benzodiazepines in human plasma with an extraction yield of 97%.[23] Several papers have been published regarding the use of solid phase extraction procedure for different matrices (plasma, serum, urine) using as sorbent C18 or hydrophilic-lipophilic polymer.[24,25]

CONCLUSIONS

This SPE study performed on C8 and phenyl silica based cartridges have shown that is possible to establish concentration curves with good linearity for benzodiazepines from aqueous samples. The enrichment of these compounds on C8 is however preferable when applied to aqueous samples, which allows the determination of benzodiazepines at lower concentration levels than determined by using phenyl silica based adsorbent. Both adsorbents have similar characteristics, excepting the carbon content, which is higher for octyl silica adsorbent and thus explaining its higher affinity to benzodiazepines.

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