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Dedicated to Prof. Bogdan C. Simionescu on the occasion of his 75th anniversary

COMPARATIVE STUDY FOR THE RETENTION OF SOME BENZODIAZEPINES IN REVERSED-PHASE LIQUID CHROMATOGRAPHY USING C8, C18, C_6H_5 AND CN STATIONARY PHASES

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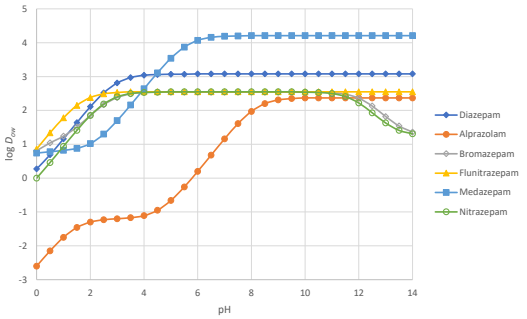
The retention behavior of six benzodiazepines (alprazolam, bromazepam, diazepam, flunitrazepam, medazepam, and nitrazepam) was studied using four different stationary phases, under reversed-phase mechanism in high-performance liquid chromatography. Four stationary phases were used for evaluating the retention of these compounds at fixed temperature. Functional dependences of the retention factor on the content of the organic modifier (methanol, or acetonitrile) in the composition of mobile phase were calculated. The extrapolated values of the retention factor for zero content of the organic modifier in mobile phase were higher for acetonitrile than for methanol for all studied compounds and for the four types of stationary phases.



INTRODUCTION

Benzodiazepines, which contain the 5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one substructure, are drugs used as hypnotics, tranquillizers, muscle relaxant and anticonvulsive[1,2]. Chemically, they are comprised of a 1,4-diazepine ring with a benzene ring fused to carbons 6 and 7 and typically a phenyl group attached to the carbon. They can be obtained by different procedures that starts with the condensation between o-phenylenediamines and various carbonyl compounds.[3] This class includes also 1,5-benzodiazepines, which can be synthetized, for example, by the condensation of o-phenylenediamines and different ketones in the presence of catalytic amount of aluminosilicate H-MCM-22 using acetonitrile as solvent at room temperature[4]. These compounds in various matrices have been investigated by liquid chromatography, with different detection techniques: UV absorption spectrometry,[5] fluorescence,[6] evaporative light scattering (ELSD),[7] or mass-spectrometry.[8,9] Their chromatographic behavior is essential for advancing an analytical method based on HPLC technique for their determination in various matrices. Therefore, a study focused on the influence of the important experimental parameters on the retention of these pharmaceutical compounds under reversed-phase (RP) mechanism is necessary. Some previous studies have been focused on their enrichment from

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liquid samples using solid-phase extraction based on sorbents of different hydrophobicities,[10,11] or on the influence of column temperature on the retention mechanism on octyl and octadecyl silica based stationary phases.[12] It is the aim of this study to extend the research on the RP-HPLC for investigating the influence of the mobile phase composition on the retention behavior of these compounds, using some stationary phases of different polarities (C8, C18, C_6H_5, and CN).

THEORETICAL BACKGROUND

Retention in RP-HPLC is described by the retention factor k experimentally obtained from the retention time t_R of the analyte and the column hold-up time t_0 by the expression (see *e.g.*[13]):

$$k = \frac{t_r - t_0}{t_o} \tag{1}$$

The value of k for a specific compound depends on two parameters, the equilibrium constant K, and phase ratio Ψ, following the formula:

$$k = K \cdot \Psi \quad (\log k = \log K + \log \Psi) \tag{2}$$

The constant K describes the equilibrium of the analyte between the stationary phase and mobile phase during the separation, and Ψ is the phase ratio for the chromatographic column (the ratio between the stationary phase volume and mobile phase volume in the chromatographic column, *i.e.* $\Psi = V_{st}/V_{mo}$).[14,15]

The dependence of retention factor k on the mobile phase composition ϕ is typically expressed by the following expression:[16]

$$\log k = a + b_1 \cdot \phi + b_2 \cdot \phi^2 \tag{3}$$

In expression (3), ϕ is defined as the volume fraction of the organic component in the mobile phase obtained based on the formula:

$$\phi = \frac{V_{org}}{V_{org} + V_w} \tag{4}$$

In theory, for $V_{org} = 0$ (water as a mobile phase) the value of $\log k$ in expression (3) will be a constant $\log k_w$ indicated as the retention factor for water. The parameter k_w is a useful descriptor of hydrophobic character of solutes and of stationary phases (for a test compound), it is utilized as a lipophilicity index,[17-19] and expression (3) is even utilized in computer assisted programs for HPLC method optimization.[20-22]

The value of $\log k_w$ in expression (3) is typically obtained by extrapolating to $\phi = 0$ of the expressions (3) of dependencies that fit the experimental data of the dependence of $\log k$ on ϕ with values of ϕ higher than zero (frequently in the range 0.3–0.7). For most columns and compounds the use of pure water as a mobile phase will generate unreasonably long retention times, besides possible problems with the de-wetting of the RP-HPLC column.[13] However, such procedure for obtaining $\log k_w$ values may be not adequate as further indicated.

A simple deduction of expression (3) can be obtained using Hildebrand solubility parameter δ for describing the value of K.[23] Based on Hildebrand solubility parameter, the value of K (for a specific compound X) is given by the expression:[13]

$$\log K\,(X) = \frac{V_x}{2.303RT} \cdot [(\delta_X - \delta_{mo})^2 - -(\delta_X - \delta_{st})^2] \tag{5}$$

In expression (5) V_x is the molar volume of the analyte X and δ_{mo}, δ_{st}, δ_X are the Hildebrand solubility parameters for the mobile phase, stationary phase and analyte X, respectively (R is the gas constant and T the absolute temperature). A quadratic expression in δ is obtained for $\log K(X)$ by substituting the value of δ_{mo} in expression (5) with the formula:

$$\delta_{mo} = (1 - \phi) \cdot \delta_w + \phi \cdot \delta_o \tag{6}$$

where δ_w and δ_o are the Hildebrand solubility parameters for water and the organic solvent, respectively. The expression for $\log K(X)$ has in this case the following form:

$$\log K\,(X) = \frac{V_X}{2.303RT}\{(1 - \phi)^2\delta_w + \phi^2\delta_o + +2\phi(1 - \phi)\delta_w\delta_o - \delta_{st}^2 + +2\delta_X[(1 - \phi)\delta_w + \phi\delta_o - \delta_{st}]\} \tag{7}$$

Formula (7) clearly indicates a quadratic dependence of $\log K(X)$ on ϕ. From here, for obtaining the formula (3) for $\log k$ it was assumed that δ_w, δ_o, δ_{st} and δ_X as well as Ψ are independent of ϕ, and can be captured in the constants $a = \log k_w$, b_1, and b_2 ($\log \Psi$ being included in parameter a). The assumption that δ_{st} as well as Ψ are independent

of ϕ ignores however that the organic solvent in RP-HPLC modifies the nature of stationary phase by adsorption. In reality, different organic solvents, besides their different chemical nature, occupy different surface areas on the stationary phase and generate adsorbed layers of different thicknesses.[24-26] This process will affect the values of δ_t and of Ψ and as a result equation (3) must be modified. Some simple calculations will lead to a more complicated formula for log *k* of the form:

$$\log k = a' + b'_1 \cdot \phi + b'_2 \cdot \phi^2 \qquad (8)$$

where:

$$a' = \frac{V_X}{2.303RT}\{\delta_w^2 - \delta_{st}^2(\phi) - \\ -2\delta_X[\delta_w - \delta_{st}(\phi)] + \log\frac{V_{st} + V_{ads}(\phi)}{V_{mo}}\} \qquad (8a)$$

$$b'_1 = \frac{-2V_X}{2.303RT}[\delta_w + \delta_w\delta_o - \delta_X(\delta_w + \delta_o)] \qquad (8b)$$

$$b'_2 = \frac{2V_X}{2.303RT}(\delta_w - \delta_o)^2 \qquad (8c)$$

Formula (8) indicates that the value of $\log k_w = a'$ as obtained by taking $\phi = 0$ in expression (3) may in fact be dependent on the nature of the organic solvent utilized for generating parameters a', b'_1 and b'_2. This hypothesis is further verified in this study for a series of benzodiazepines.

EXPERIMENTAL

The six studied benzodiazepines studied were kindly offered by LaborMed Pharma Company. Acetonitrile (ACN) and methanol (MeOH), chromatographic grade, were purchased from Sigma Aldrich. Ultrapurified water was prepared in the laboratory with a TKA Lab HP 6UV/UF instrument. All experiments were performed on a liquid chromatographic system (Agilent 1100 Series) consisting from the following modules: degasser (G1322A), binary pump (G1312A), auto sampler (G1329A), column thermostat (G1316A) and variable wavelength detector (G1314A). Chromatographic data were acquired by Agilent Chemstation software revision B.03.02.

The studies were performed on four silica based columns with the following properties:

i) Zorbax Eclipse XDB-C18: length 150 mm, internal diameter 4.6 mm, particle size 5 μm;

ii) Zorbax Eclipse XDB-C8: length 150 mm, internal diameter 4.6 mm, particle size 5 μm;

iii) Zorbax Eclipse XDB-Phenyl: length 150 mm, internal diameter 4.6 mm, particle size 5 μm;

iv) Luna CN: length 150 mm, internal diameter 4.6 mm, particle size 3 μm.

All runs were performed in isocratic mode at a temperature of 25^{0}C. Injection volume was 1 μL, the flow rate was 1 ml/min, and UV detection at 254 nm for all compounds. Acetonitrile and methanol were used as organic modifiers of the mobile phase. Different mobile phase compositions were generated using the pumping capability of the HPLC instrument. The mobile phase composition was modified in steps of 0.05 units for ϕ in the range from 0.35 to 0.7 (corresponding to an organic phase content C_o between 35% and 70%). The retention factor was calculated based on equation (1). A solution of 300 μg/ml uracil was used to measure t_0 values.[27,28]

RESULTS AND DISCUSSION

Various experimental results are indicated in Tables 1 to 4 for the four chromatographic columns evaluated in this study, for each of the six benzodiazepines, and for the two organic solvents. The results were obtained by fitting first the experimental data of log *k* as a function of ϕ for equation (3). An example of a group of such dependences obtained by using C_6H_5-silica based stationary phase and acetonitrile as organic solvent in mobile phase is represented in Fig. 1. In Fig. 1, the value of ϕ was replaced by the concentration of organic phase C_o in the mobile phase ($C_o = 100\phi$).

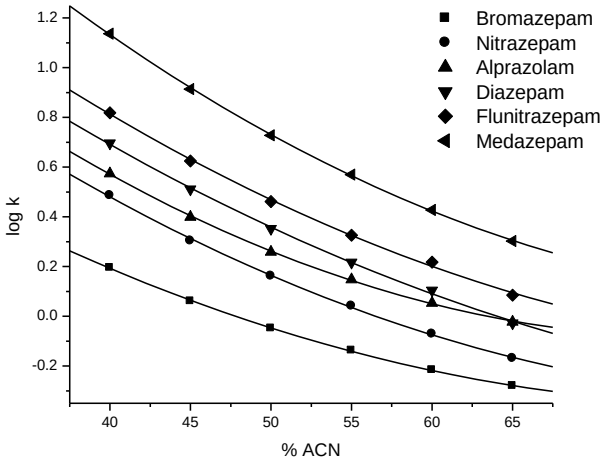

Fig. 1 – The second order polynomial fitting applied to the retention data of studied benzodiazepines on phenyl silica stationary phase.

The polynomial regressions applied to the experimental data sets {log *k*; ϕ} for each studied benzodiazepine resulted in the values of empirical parameters $a = \log k_w$, b_1 and b_2, which are listed for each used HPLC column in Tables 1–4. All regressions are characterized by high values of the correlation coefficients ($R > 0.9900$).

Besides the extrapolation of the data to $\phi = 0$

that leads to the values of log k_w, the values for log k were also calculated for $\phi = 1$. Those results are indicated as log k_o and are also given in Tables 1 to 4. One more characteristic of polynomial dependence (3) is that this admits a minimum point, calculated for ϕ as being $\phi_{min} = -b_1/2b_2$. The calculated values for ϕ_{min} expressed in concentration of organic phase as $(C_o)_{min}$ are also given in Tables 1–4.

Table 1

Regression parameters of studied benzodiazepines for C8 silica column

Organic modifier	Benzodiazepine	log k_w	b_1	b_2	log k_o	$(C_o)_{min}$
Acetonitrile	Alprazolam	3.8259	–0.12475	0.000976	1,1109	63.91
	Bromazepam	2.7263	–0.08787	0.000580	–0.2607	75.75
	Diazepam	4.1879	–0.12229	0.000951	1,4689	64.29
	Flunitrazepam	3.5228	–0.10336	0.000747	0.6568	69.16
	Medazepam	4.4372	–0.10689	0.000701	0.7582	76.24
	Nitrazepam	3.1617	–0.09650	0.000671	0.2217	71.91
Methanol	Alprazolam	4.2796	–0.08430	0.000334	–0.8104	126.2
	Bromazepam	3.4001	–0.07056	0.000270	–0.9559	130.7
	Diazepam	4.7785	–0.08737	0.000326	–0.6985	161.8
	Flunitrazepam	3.9925	–0.07984	0.000298	–1.0115	133.9
	Medazepam	5.6472	–0.08897	0.000268	–0.5698	165.9
	Nitrazepam	3.5949	–0.06931	0.000230	–1.0361	150.6

Table 2

Regression parameters for studied benzodiazepines for C18 silica column

Organic modifier	Benzodiazepine	log k_w	b_1	b_2	log k_o	$(C_o)_{min}$
Acetonitrile	Alprazolam	3.5192	–0.11202	0.000831	0.6272	67.40
	Bromazepam	2.7161	–0.09703	0.000772	0.7331	62.85
	Diazepam	4.1614	–0.11728	0.000886	1.2934	66.18
	Flunitrazepam	3.5236	–0.10162	0.000724	0.6016	70.17
	Medazepam	4.5378	–0.10509	0.000685	0.8788	76.71
	Nitrazepam	3.8532	–0.12812	0.000804	–0.9188	79.67
Methanol	Alprazolam	4.3156	–0.0839	0.000332	–0.7544	126.3
	Bromazepam	3.5558	–0.0734	0.000292	–0.8642	125.6
	Diazepam	4.8102	–0.0868	0.000330	–0.5698	131.5
	Flunitrazepam	3.9964	–0.0786	0.000291	–0.9536	135.1
	Medazepam	5.5122	–0.0823	0.000234	–0.3778	175.8
	Nitrazepam	3.7287	–0.0719	0.000256	–0.9013	140.4

Table 3

Regression parameters of studied benzodiazepines for C_6H_5 silica column

Organic modifier	Benzodiazepine	log k_w	b_1	b_2	log k_o	$(C_o)_{min}$
Acetonitrile	Alprazolam	2.7781	–0.0746	0.000485	0.1681	76.91
	Bromazepam	1.8326	–0.0546	0.000340	–0.2274	80.29
	Diazepam	2.6696	–0.0624	0.000323	–0.3404	96.59
	Flunitrazepam	2.9385	–0.0682	0.000375	–0.1315	90.93
	Medazepam	3.6881	–0.0828	0.000473	0.1381	87.52
	Nitrazepam	2.5168	–0.0664	0.000386	–0.2632	86.01
Methanol	Alprazolam	3.7613	–0.0586	0.000087	–1.2287	336.8
	Bromazepam	1.9676	–0.0136	–0.000268	–2.0724	–25.37
	Diazepam	3.3804	–0.0469	–0.000031	–1.6196	–756.5
	Flunitrazepam	4.5304	–0.0730	0.000173	–1.0396	210.9
	Medazepam	5.4439	–0.0850	0.000218	–0.8761	194.9
	Nitrazepam	2.4004	–0.0203	–0.000236	–1.9896	–43.01

Table 4

Regression parameters of studied benzodiazepines for CN silica column

Organic modifier	Benzodiazepine	log k_w	b_1	b_2	log k_o	$(C_o)_{min}$
Acetonitrile	Alprazolam	1.2980	–0.03092	0.000187	0.0760	82.67
	Bromazepam	0.5879	–0.04259	0.000348	–0.1911	61.19
	Diazepam	1.3368	–0.02852	0.000141	–0.1052	101.1
	Flunitrazepam	1.2267	–0.02645	0.000120	–0.2183	110.2
	Medazepam	1.1949	–0.05615	0.000481	0.3899	58.37
	Nitrazepam	1.1484	–0.02671	0.000139	–0.1326	96.08
Methanol	Alprazolam	2.5180	–0.05535	0.000330	0.2830	83.86
	Bromazepam	0.7007	–0.02611	0.000065	–1.2603	200.8
	Diazepam	2.7120	–0.06036	0.000355	0.2260	85.01
	Flunitrazepam	2.2841	–0.04961	0.000289	0.2131	85.83
	Medazepam	1.3903	–0.04302	0.000199	–0.9217	108.1
	Nitrazepam	2.1303	–0.04629	0.000270	0.2013	85.72

In agreement with the hypothesis developed in the theoretical part, the regression parameters listed in Tables 1–4 refer to the significant differences for the log k_w values between MeOH and ACN. Not considering the adsorption of organic phase on the stationary phase that modifies the nature of the stationary phase as the organic composition changes, these values should be the same or very close owing to the basic significance as corresponding to the analyte partition between water as mobile phase and stationary phase. Such differences have been observed in other investigations reported by the literature.[29,30]

The complexity of the retention mechanism in RP-HPLC on the four stationary phases, as well as the complexity of the studied molecules, is expected not to lead to good correlations between experimental outcome and molecular characteristics of species involved into chromatographic process. On the other hand, these studied molecules may be involved in molecular structure changes,[12] which are dependent on the polarity of the environmental solvent (i.e. mobile phase).[31] However, a study of the variation of octanol/water partition coefficient logD_{ow} for the evaluated analytes indicated that in the interval of pH for the mobile phase used in this work, this value may change very little. This is indicated in Fig. 2 that shows the changes of logD_{ow} for the evaluated benzodiazepines in the pH interval between 0 and 14 (evaluated with the aid of computing MarvinSketch package[32]).

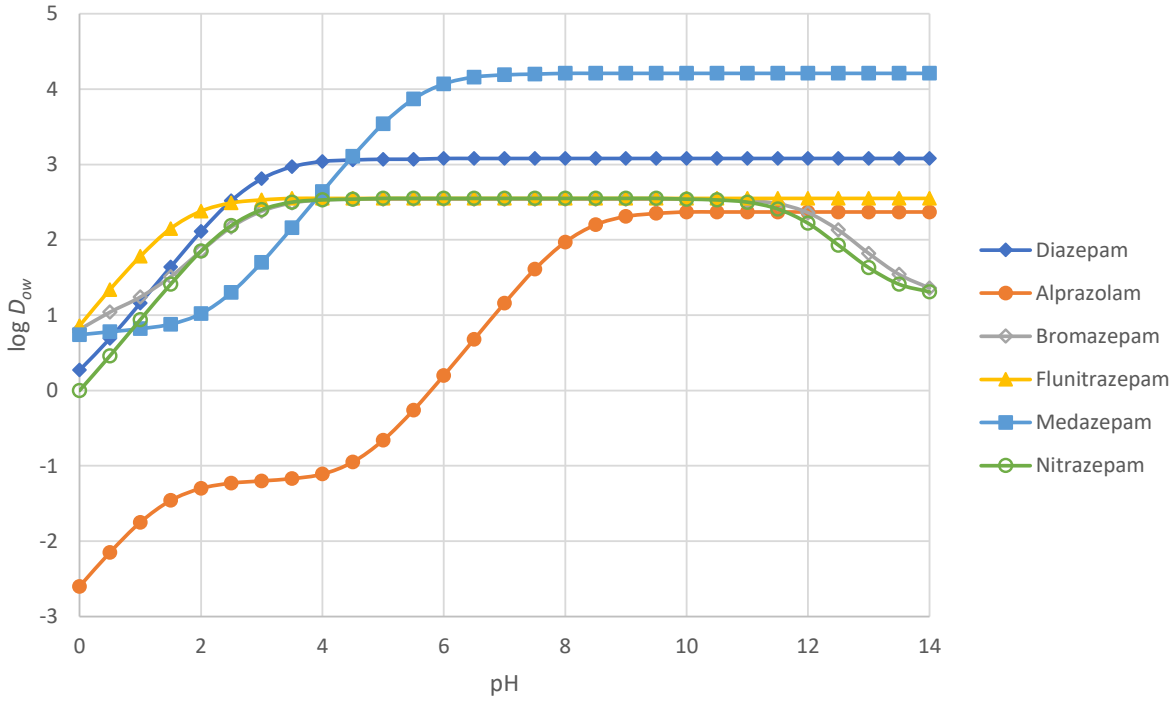

Fig. 2 – Changes of log D_{ow} for the six benzodiazepines in the pH interval between 0 and 14.

The graphs from Fig. 2 indicate that in the pH range around 7.0 (the expected pH of the mobile phase) all analytes do not have a change in the value of log D_{ow} and a change in structure is not likely to occur. On the other hand, such a change for alprazolam may take place. No difference in the shape of dependence of log k *vs.* ϕ was however noticed for this compound as compared to the other benzodiazepines.

Differences between retention behavior of the studied compounds observed for C_6H_5 and CN compared to C8 and C18 stationary phases could be a result of additional interactions. Generally, π-π and dipole-dipole intermolecular forces are relevant for C_6H_5 and CN stationary phases,[13,33] while van der Waals forces are dominant for C8 and C18, and all these interactions could change the order of elution of eluted compounds.[34]

Some of the main conclusions resulted from the interpretation of data included in Tables 1–4 can be pointed out as following:

– the behaviors of studied benzodiazepines on C8 and C18 are clearly grouped according to the organic solvent used in the mobile phase: thus, a trend of behavioral modification of the retention for a content of acetonitrile $(C_o)_{min}$ around 65 – 75% was noticed for all six benzodiazepines; also, for acetonitrile the values of log k_o were positive excepting two cases, and negative in case of using methanol;

– an approximative value for logk_o = –1 (the most situations for methanol) on C8 and C18 supposes, by taking into consideration the definition of k from eq. 1, a value for the retention time t_r of studied analyte equal to 1.1 × t_0, *i.e.* the analyte elutes very close to the dead time of the separations;

– in case of acetonitrile and C8, C18 stationary phases, an average value of 0.75 for logk_o means a retention time for analytes for 100% acetonitrile in mobile phase equal to about 6.5 × t_0, showing that the analytes still have a significant retention on both stationary phases;

– this trend can not be distinctly observed for CN stationary phase, where the resulted data for logk_o and $(C_o)_{min}$ are rather erratic, with no clear tendency for one of the organic solvents;

– less erratic behavior was observed for C_6H_5 compared to CN stationary phase, although in this case the values of $(C_o)_{min}$ were all situated in the interval [0; 100] for acetonitrile and different to this interval for methanol, which means that the two organic solvents have different effects on the studied compounds during the elution process.

CONCLUSIONS

This study showed that the chromatographic behavior of six benzodiazepines on four different stationary phases is different for the two organic solvents used in the composition of mobile phase. The main chromatographic descriptors calculated for these analytes showed that there are significant differences of trends observed for the four stationary phases and for the two organic solvents used in the composition of the mobile phase. Similarities in behavior of analytes were expected to be observed for all four stationary phases, which however did not result from these experiments. This remark is in disagreement with the solvophobic theory,[35] which in principle does not account the nature of the stationary phase and it is mainly based on interactions occurring in the bulk of mobile phase. Another explanation could be based on the complex molecular structures of the studied compounds; they could be involved in changes when the polarity of the mobile phase is changed by modification of the mobile phase composition, which may differ from molecule to molecule in this group of chosen benzodiazepines.

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