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Modeling and prediction of the gradient retention data for ultrahigh performance liquid chromatography of benzodiazepines

Petra Valigurová, Jana Váňová, and Petr Česla*

Department of Analytical Chemistry,
The University of Pardubice, CZ−532 10 Pardubice, Czech Republic

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This paper presents a method for prediction of the gradient retention times of six samples of benzodiazepines – phenazepam, pyrazolam, flubromazepam, meclonazepam, diclazepam, and diazepam. Isocratic and gradient separation of the compounds of interest was achieved on a Luna Omega C18 column followed by RP-LC/MS detection. The results have shown good agreement between the predicted and experimental retention data; however, higher differences were obtained for fast gradients in the ultrahigh performance separation mode.

Keywords: Benzodiazepines; Liquid chromatography; Gradient elution; Prediction of retention

Introduction

Benzodiazepines (BNZ) are among the most frequent medicaments used worldwide [1] which have replaced barbiturates because of lesser danger and significantly lower risk of addiction and overdose [2]. The first discovered BNZ compound, chlordiazepine, was available in 1960 in the US market and had quickly gained popularity thanks to its safety profile. In general, BNZ are lethal at a hundred times higher dose than the usual therapeutic dose, while barbiturates are lethal at a ten-fold excess to the usual dose [1]. That is why BNZ were prescribed for long-term application for various purposes; for example, anxiety, insomnia, muscle tension, or combat neurosis.

* Corresponding author, ✉ petr.cesla@upce.cz

By the 1970s, benzodiazepines were the most common pharmaceuticals in the world [3]. BNZ were tested, controlled and, in 1990, the American Psychological Association officially confirmed that benzodiazepines would cause a dependence and thus, these medicaments were put on the list of risk-of-dependence. That is why the recommendations on the use of BNZ set down the period of application for 2–4 weeks and not longer. However, in this time, many providers continue to prescribe these preparations for months, sometimes even for years. Despite the risk of abuse and later introduction of safer BNZ, they are still one of the most prescribed classes of medicaments in the world [3]. Benzodiazepines have been used in different therapeutic areas but unfortunately, at present, one can find these drugs at the illegal street market, which leads to a lot of drug abuse, the addicted and suicides. As a result, identification and determination of BNZ in biological samples appear important for clinical and forensic analysis.

Chemically, benzodiazepines are composed of the benzene ring and a seven-membered diazepam ring, known as 1,4-benzodiazepine ring (see Fig. 1). Each benzodiazepine has different functional groups attached to the central structure that affect the binding of the molecule to the receptor of γ-aminobutyric acid A (GABAA) and thus, they modulate the pharmacological properties, or potency of the effect [4]. Also, they are related to dopamine and serotonin –neurotransmitters that are presented in a human brain, being responsible for mutual communication between the brain cells and having either tranquilizing or excitatory effects.

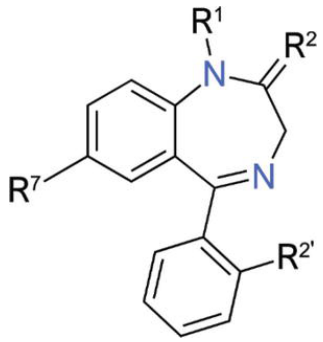

Fig. 1 General structure of benzodiazepines [5]

Among the best-known benzodiazepines is diazepam, commercially named Valium® (Hoffmann-La Roche, Switzerland). It is ordinarily used to treat less severe anxiety disorders, applicable is also in solving alcohol problems and drug addiction. Another well-known benzodiazepine is alprazolam, a.k.a. Xanax® (Upjohn-Pfizer, USA). This chemical substance is used to treat anxiety caused by depression, panic disorders, or anxiety disorders. Moreover, flunitrazepam, with a brand name Rohypnol® (Hoffmann-La Roche), is well known as well. This drug is used to treat severe insomnia and especially it helps with anesthesia, sometimes it is called sleeping pills.

Yet another drug, belonging to a group of benzodiazepines, is called bromazepam. It has similar side effects to diazepam. In our research, we have used six standards of BNZ that are equally important as other better-known benzodiazepines [6].

The emergency of new compounds in this class continues and benzodiazepines reach the drug black market when used for recreational purposes. The abuse of these substances has then led to many crimes and even deaths [7]. That is why the development of new techniques and methods that allow rapid detection and identification of these compounds in laboratories is needed. Benzodiazepines are usually tested in blood, plasma, or urine [8]. A wide range of methods is used for analyses of benzodiazepines, including electrochemical and chromatographic methods; the latter, however, being used more often. The most common columns for separation of benzodiazepines are C8 or C18. As mobile phases, usually acetonitrile/water mixture, methanol, or a combination of buffer – 5 mM ammonium formate with 0.01% ammonium hydroxide (40%) and methanol (60%) are being selected [9].

Banaszkiewicz et al. used LC-MS/MS in 2017–2019 for the analysis of benzodiazepines in 145 blood samples. From the obtained data, they found that the most used benzodiazepine is nordazepam, diazepam, temazepam, oxazepam, and midazolam. Liquid-liquid extraction was used for a sample preparation step [7]. Capillary electrophoresis coupled with mass spectrometry for analysis of blood samples was also reported in combination with microwave-assisted extraction. This combination is consistent with green-chemistry principles as it requires small volumes of reagents [10]. For quantification of seven benzodiazepines from urine, high-performance thin-layer chromatography was used in connection with the mass spectrometry. A mixture of chloroform with glacial acetic acid (9:2, v/v) was the mobile phase of choice [11].

In the present work, we have compared prediction ability of the most common retention models with the data experimentally obtained in the fast gradient separation of benzodiazepines using ultrahigh performance liquid chromatography. The influence of the gradient profiles on prediction errors is evaluated and discussed.

Materials and methods

Reagents and materials

Six standards of benzodiazepines were used: phenazepam, pyrazolam, flubromazepam, meclonazepam D4, diclazepam, and diazepam D5; all as methanolic solutions with the same concentration of 1 mg/mL (Sigma-Aldrich, St. Louis, MO, USA), when the basic characteristics of the samples are surveyed in Table 1. Acetonitrile and methanol (both Chromasolv, LC/MS grade) were also

from Sigma-Aldrich, thiourea and formic acid from LachNer (Nerativice, Czech Republic). Deionized water was prepared using Milli-Q purification unit (Merck Millipore, Billerica, MA, USA), with resistivity of 18.2 MΩ/cm.

Instrumentation

Chromatographic analyses were performed using an HPLC system Agilent 1260 Infiniti II PRIME (Agilent, Palo Alto, CA, USA), equipped with quaternary ultra-high-pressure pump with 80 MPa pressure limit, autosampler with integrated column oven and diode-array detector. The system was coupled with quadrupole-type mass spectrometer Agilent iQ MSD detector (Agilent).

Separations were performed using a Luna Omega C18 column (length: 100 mm, i.d.: 2.1 mm, packed with 1.6 μm fully porous particles Phenomenex; Torrance, CA, USA). For analysis, 1 μL of benzodiazepine sample was injected into the chromatographic system. For isocratic elution, mobile phase containing acetonitrile + water with a volume ratio of acetonitrile $\varphi_{ACN} = 0.45–0.75$ was used.

Table 1 Physical and chemical characteristics of benzodiazepines

Compound	Molar mass [g/mol]	pK_a		log P	λ_{max} [nm]
		Acidic	Basic		
Phenazepam	349.61	11.58	2.18	3.371	250
Pyrazolam	354.21	–	2.18	0.902	245
Flubromazepam	333.16	11.55	2.32	3.026	240
Meclonazepam	329.74	11.24	1.70	3.057	255
Diclazepam	319.19	–	1.75	2.967	250
Diazepam	284.74	–	3.40	2.801	240

pK_a and log P values were calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2021 ACD/Labs)

Results and discussion

Benzodiazepines analyzed in the work are relatively lipophilic compounds, with similar logarithms of octanol/water partition coefficients, log P, in the narrow range of 2.8 to 3.37, with only a slightly lower value for pyrazolam (see Table 1). The log P parameter of the compounds can be related to both the pharmacokinetic behavior of a drug in the organism and the chromatographic properties during the HPLC analysis [12]. Under physiologic conditions, the compounds are typically neutral. In reversed-phase system, the retention of benzodiazepines depends on the composition of the mobile phase. It was shown by Barbosa et al. [13,14] that the retention factors can be linearly correlated with Reichardt's normalization

solvatochromic parameter E_T^N in mobile phases containing different percentages of acetonitrile. The retention mechanism of benzodiazepines is the same for a wide range of acetonitrile/water mixtures, as indicated by the entropy-enthalpy compensation, and opposite to the methanol/water mixture dominated by the competitive hydrogen bonding to the solvation process [15]. With respect to these studies, the acetonitrile/water-based mobile phases should provide a suitable environment for the development of a HPLC separation method in the reversed-phase system based on simple retention modeling. To verify this assumption, we have investigated the retention behavior of benzodiazepines under isocratic conditions in the mobile phase containing 45–75 % (v/v) of acetonitrile in water (φ_{ACN} = 0.45–0.75).

For the proper separation, we have tested a series of stationary phases including three types based on C18, i.e. Ascentis Express (Supelco, Bellefonte, PA, USA), Kinetex and Luna Omega columns (Phenomenex, Torrance, CA, USA), phenyl phase (Ascentis Express PH column) and mixed C18-pentafluorophenyl phase ACE C18-PFP column (ACE, Aberdeen, Scotland, UK). Among the columns used, the best separation of benzodiazepines was achieved with the C18 column Luna Omega; particularly, due to the presence of fully porous particles. Other columns were packed with superficially porous particles (data not shown).

The composition of the mobile phase was optimized for mixtures of acetonitrile and methanol mixed with water and containing additives (acetic acid and phosphate buffer). The standards were separated by reversed-phase chromatography with UV detection, and identification of the compounds of interest was verified using MS detection. In Table 2, the retention data — herein, the retention factors k —, of the benzodiazepines are shown for the mobile phases containing acetonitrile-and-water mixtures.

Table 2 Retention factors, k, of benzodiazepines on Luna Omega C18 column; average values (n = 3)

Compound	φ_{ACN}						
	0.45	0.50	0.55	0.60	0.65	0.70	0.75
Meclonazepam	2.967	2.025	1.425	1.057	0.800	0.601	0.466
Pyrazolam	1.079	0.956	0.840	0.741	0.651	0.550	0.472
Flubromazepam	2.623	1.820	1.306	0.996	0.778	0.604	0.488
Diclazepam	4.875	3.291	2.311	1.724	1.321	1.016	0.808
Diazepam	4.313	3.004	2.139	1.617	1.255	0.929	0.789
Phenazepam	3.161	2.159	1.533	1.154	0.894	0.691	0.555

The retention factors were calculated using the isocratic retention times of the compound(s) given and hold-up time, measured using thiourea. Fig. 2 shows dependencies of the retention factors on the volume fraction of acetonitrile in the mobile phase plotted in the arithmetic scale (i.e. k vs. φ_{ACN}) and semilogarithmical scale (i.e. ln k vs. φ_{ACN}). Typically, the semilogarithmical dependency depicts a linear shape in the reversed-phase system, which can be described using Eq. (1):

$$\ln k = \ln k_w - S \cdot \varphi_{ACN} \tag{1}$$

where k_w represents the extrapolated value of the retention factor of the given compound in pure water, where S is the solvent strength parameter [16]. Transferred to the arithmetic scale, Eq.(1) can be rewritten as:

$$k = k_w \cdot e^{-S \cdot \varphi_{ACN}} \tag{2}$$

By plotting the experimental retention factors k and ln k, we have observed a slightly convex behavior for most of the studied benzodiazepines in the range of mobile phases except for pyrazolam which was retained on the stationary phase only very weakly (see again Fig. 2). Bias from nonlinearity can sometimes be observed in reversed-phase LC, especially for the high range of mobile phases [17].

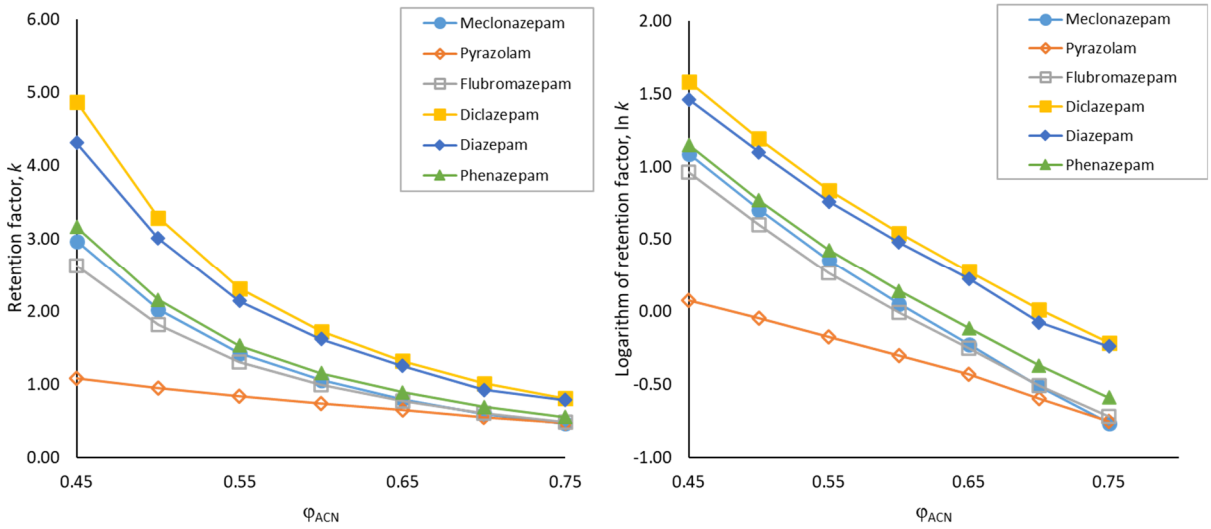

Fig. 2 Dependencies of retention of benzodiazepines on the composition of mobile phase
Left plot – arithmetical scale of k versus concentration of acetonitrile, φ_{ACN};
right – semilogarithmical scale of ln k versus φ_{ACN}

The dependencies shown in Figure 2 were fitted with the linear solvent strength model (Eq. (1)) and regression parameters were calculated (for the arithmetical scale using nonlinear regression and for semilogarithmical dependency using linear regression). The regression parameters with relative standard deviations and adjusted coefficient of determination are shown in Tables

3 and 4. Both retention models show high goodness of fit of the experimental data, as the adjusted *R*-squared values are in all cases higher than 0.98. It can be seen from the comparison of the results that the application of the model in arithmetical form produces a slightly better fit of the experimental data.

Table 3 Regression parameters of the dependency of logarithm of retention factor, ln *k*, of benzodiazepines on concentration of acetonitrile, φ_{ACN}, fitted using Eq. (1) in semilogarithmical scale (linear regression)

Compound	ln k_w	RSD	S	RSD	Adj-R^2
Meclonazepam	3.771	3.08 %	6.117	3.11 %	0.9942
Pyrazolam	1.330	3.31 %	2.745	2.62 %	0.9959
Flubromazepam	3.380	4.08 %	5.549	4.09 %	0.9901
Diclazepam	4.163	3.66 %	5.929	4.22 %	0.9895
Diazepam	3.950	3.72 %	5.698	4.23 %	0.9893
Phenazepam	3.647	4.06 %	5.741	4.23 %	0.9894

Table 4 Regression parameters of the dependency of retention factor, *k*, of benzodiazepines on the concentration of acetonitrile, φ_{ACN}, fitted using Eq. (2) in arithmetical scale (nonlinear regression)

Compound	k_w	RSD	S_1	RSD	Adj-R^2
Meclonazepam	57.764	11.78 %	6.642	3.49 %	0.9949
Pyrazolam	3.637	3.68 %	2.678	2.46 %	0.9967
Flubromazepam	40.071	13.85 %	6.117	4.41 %	0.9914
Diclazepam	92.732	15.04 %	6.603	4.48 %	0.9915
Diazepam	70.207	12.69 %	6.249	3.97 %	0.9931
Phenazepam	54.131	14.67 %	6.371	4.50 %	0.9912

Based on the regression parameters, the gradient retention data of benzodiazepines were predicted. Then, the corresponding differential equation describing migration of the solute within a chromatographic column can be solved with the aid of numerical integration:

$$\int_0^{t_R - t_0 - t_D} \frac{\mathrm{d}\, t}{t_0 k} = 1 - \frac{t_D}{t_0 k(\varphi_{\mathrm{in}})} \tag{3}$$

where t_R and t_0 represent the retention and dead time, $k(\varphi_{\mathrm{in}})$ denotes the retention factor at the initial gradient conditions φ_{in}, and t_D is the gradient dwell volume of the system used for separation.

By integrating Eq. (3) for the retention of benzodiazepines described by the afore-mentioned LSS models using Riemann sum, we have predicted and experimentally verified the gradient retention times for linear gradients with three different profiles. The gradient profiles are described by means of the initial concentration of acetonitrile, φ_{in}, and gradient steepness, B:

$$B = \frac{\varphi_G - \varphi_{\text{in}}}{t_G \cdot F_m} \tag{4}$$

where φ_G is the final gradient concentration of acetonitrile in the mobile phase, t_G the gradient time, and F_m is flow rate of the mobile phase. The values of predicted retention times in the gradients with the values of prediction errors are presented in Table 5. The prediction errors were calculated as

$$\text{PE}\,[\%] = \frac{t_{R,\text{exp}} - t_{R,\text{pred}}}{t_{R,\text{exp}}} \cdot 100 \tag{5}$$

where $t_{R,\text{exp}}$ is the experimentally determined gradient retention time and $t_{R,\text{pred}}$ denotes the predicted gradient retention time, respectively.

Table 5 Predicted values of gradient retention times, *t*-pred, and prediction errors, PE, for benzodiazepines calculated using the parameters calculated by nonlinear regression (arithmetical scale retention model, Eq.(2))

Gradient No.	1		2		3	
φ_{in}	0.5		0.6		0.7	
φ_G	1.0		1.0		1.0	
B (mL^{-1})	0.208		0.167		0.125	
Name	*t*-pred	PE [%]	*t*-pred	PE [%]	*t*-pred	PE [%]
Meclonazepam	2.928	−15.5	2.206	7.8	1.748	3.5
Flubromazepam	2.803	−2.9	2.166	9.3	1.753	−9.4
Diclazepam	3.743	−4.4	2.786	9.9	2.108	−5.2
Diazepam	3.593	−4.2	2.711	9.3	2.098	−5.2
Phenazepam	3.048	−18.9	2.316	7.3	1.823	−4.8

Gradient time t_G = 12 min, flow-rate F_m = 0.2 mL/min.

By comparing the predicted retention times for the three gradient profiles, better values of prediction errors were achieved with the regression parameters presented in Table 4; i.e., when using the retention model defined by Eq. (2). We have compared the experimental retention times obtained for three gradients differing in the steepness with the predicted values of retention times using the

arithmetical scale retention model presented in Table 5. Lower prediction errors of retention times for the arithmetical scale model given by Eq. (2) are probably due to a slightly better accuracy of fitting the isocratic retention model data (see Table 3 *vs.* Table 4). The semilogarithmical scale model according to Eq. (1) can also produce higher residuals for mobile phases with low retention factors of the compounds, which can be important especially for early eluting compounds.

Finally, we have tested the retention models for the prediction of fast gradients under conditions in the regime of ultrahigh performance. Thus, we separated the selected benzodiazepines using three gradients with a steepness of 0.75 mL^{-1} and flow rates from 0.2 to 0.4 mL/min. The experimentally achieved times of analyses (corresponding to the last eluting peaks) were in the interval of 5.8 min to 2.4 min for the highest flow rate of the mobile phase. Comparison of experimentally determined vs. predicted gradient chromatograms is presented in Fig. 3. The results suggest us that although the precise prediction of retention time is slightly less accurate in case of fast gradients, the position of peaks in the chromatogram — i.e., the order of elution — is maintained for the separation.

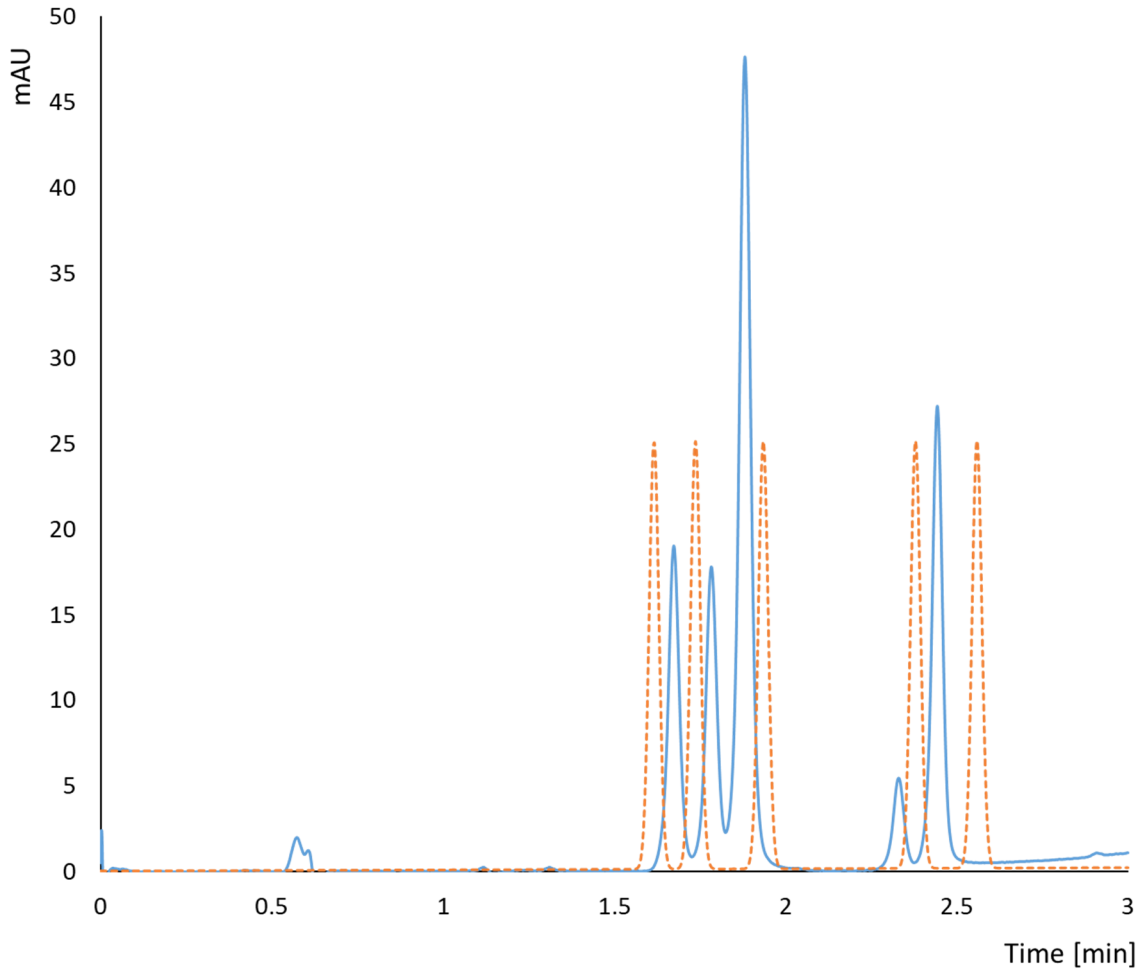

Fig. 3 Fast gradient separation benzodiazepines – comparison of experimental (full line) and predicted (dotted) chromatograms

Conclusions

In the present work, we have shown that the prediction of gradient retention times of benzodiazepines in reversed-phase liquid chromatography is possible by solving of the gradient equation with the aid of numerical integration.

The parameters of the retention models were obtained from isocratic scouting runs with relatively long gradients and further applied in fast steeper gradients. The prediction errors were lower for application of the arithmetical scale model which was further applied to ultrahigh-pressure separation of benzodiazepines.

Acknowledgments

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