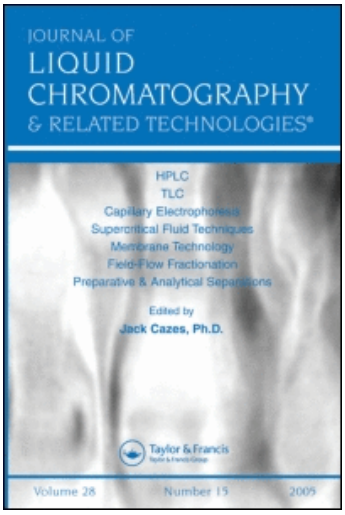

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HPLC Behaviour of Diazepam on β-Cyclodextrin Chiral Stationary Phase. Evidence of Conformational Change

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To cite this Article Fedurcová, Andrea , Májek, Pavel , Lehotay, Jozef and Čižmárik, Jozef(2006) 'HPLC Behaviour of Diazepam on β-Cyclodextrin Chiral Stationary Phase. Evidence of Conformational Change', Journal of Liquid Chromatography & Related Technologies, 29: 15, 2229 — 2244
To link to this Article: DOI: 10.1080/10826070600832913
URL: http://dx.doi.org/10.1080/10826070600832913

Journal of Liquid Chromatography & Related Technologies®, 29: 2229–2244, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600832913

HPLC Behaviour of Diazepam on β-Cyclodextrin Chiral Stationary Phase. Evidence of Conformational Change

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Abstract: The dynamic HPLC was performed with the goal to estimate the influences on the configurational stability of diazepam conformers in chiral environment. The effect of sample solvent, temperature, flow rate, and pH of mobile phase on behaviour of diazepam conformers during their separation on chiral β-cyclodextrin was studied. The separation of diazepam conformers was complicated due to simultaneous interconversion, which was the result of configurational instability of the diazepam molecule. A statistical approach was used for evaluation of the influence of individual chromatographic parameters on behaviour of diazepam in a given chiral environment. The Principal Component Analysis (PCA) and Cluster Analysis (CA) were applied for accurate interpretation of obtained results.

Keywords: Principal component analysis, Cluster analysis, Sample solvent effect, Configurational unstability

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INTRODUCTION

BDZs are widely employed in therapy for their anxiolitic, sedative, hypnotic, and myorelaxing properties. From a toxicological point of view, BDZs are considered as quite safe among the drugs acting on the central nervous system: BDZs, in fact, have only a few side effects and low incidence of dependence and tolerance, and they are used in racemic form.[1] The question remains: Why some drugs can be commonly used in racemic form? The first reason is that one enantiomeric form is not biologically active in a given chiral environment; it does not interact with receptors.

The second reason is that negative side effects of the enantiomeric form were not confirmed and proven. Regulatory agencies require extensive information on chiral drugs before they can be used to any great extent. This includes their pharmacokinetics, pharmacodynamics, uptake disposition, stability, etc. The sale of racemic drugs is not only restricted, but the demands of configurational stability, which can dramatically change resultant therapeutic effects must be accomplished.[2] For the pharmaceutical industry it is more prudent to manufacture low cost racemic drugs. The chiral environment is stereo specifically built, and it is capable to distinguish small differences in space arrangement of molecules.[3] This phenomenal feature of chiral environment was used for the separation of configurationally labile conformers of diazepam.

As is proven in this work, the chiral β-cyclodextrin was capable to partly differentiate unstable conformers of diazepam. However, diazepam reorganized spatial arrangement during its separation, with the goal to find a lower energy state or more stable confirmation in a given chiral environment. As experimental results showed, the spatial reorganization was strongly dependent on chromatographic conditions and sample solvent. This study shows the new possibilities in research of the configurational stability of some drugs.

THEORY

Multivariate analysis: a number of chromatographic and spectroscopic methods can provide analytical data of many components; from a single specimen, several variables are measured, yielding multivariate data. Due to the large extent of variables measured, it is difficult to see patterns and relationships. Thus, the aim of many multivariate analyses is data reduction without losing substantial information. For the description and better interpretation of obtained results by multivariate analysis, the following methods were used: Principal Component Analysis and Cluster Analysis.[4]

Principal component analysis reduces the dimensionality of the original data matrix while retaining the maximum amount of variability, as well as, recognizing the data structure. PCA allows visualizing the information of

the data set in a few principal components using plots of the factor loadings or factor scores where the objects are represented in the function of the first principal components (PCs). The aim of factor analysis is to transform (by calculation of a matrix rotation) the abstract factors (from principal components) into interpretable factors using varimax rotation.[5]

Cluster analysis detects natural grouping in data. Objects are grouped in clusters in terms of their similarity. The initial assumption is that the nearness of objects in the space is defined by variables, which reflect the similarity of their properties. There are various possibilities and rules, which are used to measure distances and how to form a linkage of individual clusters. For our purpose, we used hierarchical (agglomerative) cluster analysis; similar extent was measured by Manhattan distances and cluster aggregations are based on Ward's method.[5]

EXPERIMENTAL

Instrumentation

The HPLC system was used consisting of quaternary pump (Merck – Hitashi L-6000A) equipped with injection valve (Rheodyne) and DAD detector (Agilent 1100 Series).

Chemicals

Chemicals and solvents were obtained from Merck, Germany, a standard of diazepam (Slovakofarma Hlohovec, Slovakia, 99.98 % purity) was dissolved in ethanol, c = 0.1 mg/mL.

Sample Preparation

A standard of diazepam was dissolved in different solvents, c = 0.1 mg/mL.

HPLC

A chiral HPLC column ChiraDex (250 × 4 mm I.D., particle size 5 μm, Merck, Germany) was used. The achiral mobile phases consisted of: ACN/acetate buffer 200 mM pH = 3.3 v.v. 10/90 (1); ACN/acetate buffer 200 mM pH = 5.5 v.v. 10/90 (2); ACN/acetate buffer 200 mM pH = 6.5 v.v. 10/90 (3).

Acetate buffer was prepared from sodium acetate (equimolar weight) and pH was adjusted with acetic acid at values of 3, 3; 5, 5; 6, 5; ionic strength was

adjusted on the constant value (with LiCl). Measurements were carried out with mobile phases prior to and after adjustment of ionic strength.

Composition of Chiral Mobile Phases

ACN/acetate buffer 200 mM pH = 3.3 v.v. 10/90/0.001 mol/l β-cyclodextrin v.v 10/90 (4); ACN/acetate buffer 200 mM pH = 5.5 v.v. 10/90/0.003 mol/l β-cyclodextrin v.v 10/90 (5); ACN/acetate buffer 200 mM pH = 6.5 v.v. 10/90/0.005 mol/L β-cyclodextrin v.v 10/90 (6); Wavelength 230 nm was used. Sample loop: 20 μL. Flow rate: 0.2; 0.5; 1.0 mL/min. Temperature range: 273–313 K (increment 10 K).

Statistical Methods

Data processing: Four data matrixes (Table 1 A–D) were constructed from experimental data of qualitative and quantitative analyses of diazepam where the influence of ionic strength of mobile phase was included. The rows represent samples and columns chromatographic parameters: (temperature - T, flow rate F_m, pH of mobile phase, retention factors—k_1, k_2, selectivity coefficients—α, and peak areas—A_1, A_2 of both conformers. Auto scaling was performed to put data on a common scale (zero means and unit standard deviation).[6] Original matrixes (Table 1 A-D): dimension 27 × 6, 18 × 6, 27 × 5, 18 × 5, consist of 27 or 18 rows and 6 (5) columns - 3 columns of investigated parameters: temperature, flow rate, pH; and three (two) columns of k_1, k_2, α; (A_1, A_2)—analysed chromatographic parameters.

RESULTS AND DISCUSSION

HPLC

Not much is known about chromatographic interactions of diazepam conformers and the corresponding behaviour of these conformers during elution. Diazepam can exist in two conformations (M and P, Figure 1) that are easily interconvertible with each other. The diazepine ring adopts a shape of a boat; owing to asymmetric substitution of two such boats is possible in mirror image relation to each other.[7]

The ring inversion barrier between both conformers of diazepam is too low (17.6 kcal/mol) and interconversion (M ↔ P) is too rapid to allow the separation of conformers at room temperature.[7] However, it was shown that it is possible to partly separate both conformers. The two peaks of diazepam were observed during their separation on chiral stationary phase

Table 1. Original matrixes of investigated parameters used for statistical evaluation

Sample	pH	F_m [mL/min]	T [K]	k_1	k_2	α
A						
1	3.3	0.2	273	2.74	4.06	1.48
2	3.3	0.2	293	1.53	2.83	1.84
3	3.3	0.2	313	0.7	2.17	3.1
4	3.3	0.5	273	2.65	3.58	1.35
5	3.3	0.5	293	1.46	2.75	1.88
6	3.3	0.5	313	0.9	2.07	2.29
7	3.3	1.0	273	2.12	3.1	1.47
8	3.3	1.0	293	1.1	2.51	2.27
9	3.3	1.0	313	0.55	2.1	3.81
10	5.5	0.2	273	3.91	5.11	1.31
11	5.5	0.2	293	2.07	3.57	1.72
12	5.5	0.2	313	1.46	2.79	1.91
13	5.5	0.5	273	3.68	4.78	1.3
14	5.5	0.5	293	2.49	3.53	1.42
15	5.5	0.5	313	1.31	2.64	2.02
16	5.5	1.0	273	3.44	4.27	1.24
17	5.5	1.0	293	2.31	3.37	1.46
18	5.5	1.0	313	1.71	2.74	1.61
19	6.5	0.2	273	5.45	7.2	1.32
20	6.5	0.2	293	3.4	4.91	1.45
21	6.5	0.2	313	2.01	3.55	1.76
22	6.5	0.5	273	5.23	6.48	1.24
23	6.5	0.5	293	3.8	4.87	1.28
24	6.5	0.5	313	2.22	3.67	1.65
25	6.5	1.0	273	5.05	5.71	1.13
26	6.5	1.0	293	3.55	4.69	1.32
27	6.5	1.0	313	2.58	3.67	1.42
B						
1	5.5	0.2	273	4.24	6.06	1.43
2	5.5	0.2	293	2.91	4.46	1.53
3	5.5	0.2	313	1.49	3.12	2.1
4	5.5	0.5	273	3.4	4.36	1.28
5	5.5	0.5	293	2.34	3.72	1.59
6	5.5	0.5	313	1.63	3.18	1.95
7	5.5	1.0	273	3.54	5.01	1.42
8	5.5	1.0	293	2.81	4.24	1.51
9	5.5	1.0	313	2.08	3.29	1.58
10	6.5	0.2	273	4.89	6.88	1.4
11	6.5	0.2	293	3.39	5.09	1.5
12	6.5	0.2	313	2.16	3.44	1.59
13	6.5	0.5	273	5.25	6.52	1.24

(*continued*)

Table 1. Continued

Sample	pH	F_m [mL/min]	T [K]	k_1	k_2	α
14	6.5	0.5	293	3.66	4.86	1.32
15	6.5	0.5	313	2.41	3.48	1.44
16	6.5	1.0	273	5.03	5.82	1.16
17	6.5	1.0	293	3.27	4.55	1.39
18	6.5	1.0	313	2.31	3.41	1.47

Sample	pH	F_m [mL/min]	T [K]	A_1 (%)	A_2 (%)
C					
1	3.3	0.20	273	30	70
2	3.3	0.20	293	49	51
3	3.3	0.20	313	66	34
4	3.3	0.50	273	30	70
5	3.3	0.50	293	42	58
6	3.3	0.50	313	55	45
7	3.3	1.00	273	30	70
8	3.3	1.00	293	39	61
9	3.3	1.00	313	41	59
10	5.5	0.20	273	40	60
11	5.5	0.20	293	57	43
12	5.5	0.20	313	60	40
13	5.5	0.50	273	40	60
14	5.5	0.50	293	50	50
15	5.5	0.50	313	59	41
16	5.5	1.00	273	38	62
17	5.5	1.00	293	40	60
18	5.5	1.00	313	51	49
19	6.5	0.20	273	40	60
20	6.5	0.20	293	52	48
21	6.5	0.20	313	53	47
22	6.5	0.50	273	29	71
23	6.5	0.50	293	45	55
24	6.5	0.50	313	50	50
25	6.5	1.00	273	29	71
26	6.5	1.00	293	33	67
27	6.5	1.00	313	49	51
D					
1	5.5	0.20	273	40	60
2	5.5	0.20	293	45	55
3	5.5	0.20	313	60	40
4	5.5	0.50	273	37	63
5	5.5	0.50	293	45	55
6	5.5	0.50	313	50	50

(*continued*)

Table 1. Continued

Sample	pH	F_m [mL/min]	T [K]	A_1 (%)	A_2 (%)
7	5.5	1.00	273	33	67
8	5.5	1.00	293	40	60
9	5.5	1.00	313	45	55
10	6.5	0.20	273	40	60
11	6.5	0.20	293	50	50
12	6.5	0.20	313	63	37
13	6.5	0.50	273	33	67
14	6.5	0.50	293	49	51
15	6.5	0.50	313	54	45
16	6.5	1.00	273	28	72
17	6.5	1.00	293	49	51
18	6.5	1.00	313	54	45

Ionic strength is included: A. Sample 1–27: standard of diazepam dissolved in EtOH (c = 0.1 mg/mL); B. Sample 1–18: standard of diazepam dissolved in EtOH (c = 0.1 mg/mL); C. Sample 1–27: standard of diazepam dissolved in EtOH (c = 0.1 mg/mL); D. Sample 1–18: standard of diazepam dissolved in EtOH (c = 0.1 mg/mL).

(based on maltooligosacharides). The following circular dichroism detection bears out that the reported separation is the separation of conformers.[8]

The Goals of our Study

- Find appropriate chromatographic conditions for separation of diazepam conformers.

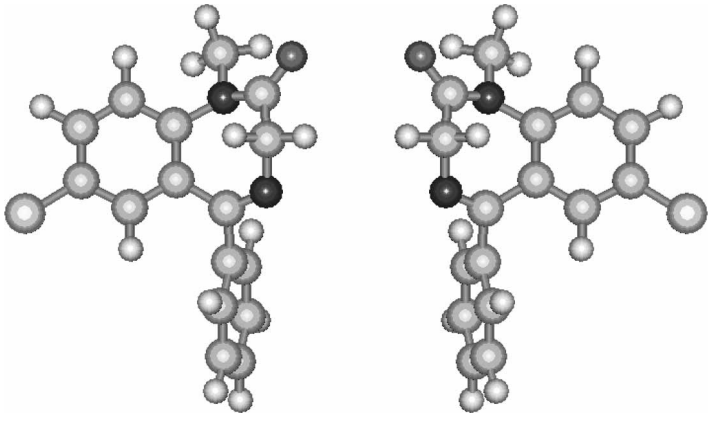

Figure 1. Optimised geometry of diazepam in PM3 method for M and conformation P.

- Study the behaviour of diazepam conformers under different chromatographic conditions.
- Evaluate the influence of sample solvent, temperature, flow rate and pH of mobile phase on separation and interconversion of diazepam conformers.

The chiral stationary phase based on β-cyclodextrin was chosen for separation of diazepam conformers. The β-cyclodextrin is well known chiral stationary phase and several separations of benzodiazepines were performed on this stationary phase.

At first it was necessary to find the appropriate mobile phase. Several mobile phases with different compositions were used, but the best separation was achieved with ACN/acetate buffer 200 mmol/L pH = 3.3 v.v. 10/90. As follows, the mobile phases with different pH (5.5, 6.5) were employed (see Experimental part—Chromatographic conditions). The measurements were carried out prior to and after adjustment of ionic strength, with the goal to evaluate the influence of ionic strength on the separation and the interconversion of diazepam conformers. After that, the effect of flow rate, the column temperature, and the sample solvent on the behaviour of diazepam in given chiral environment was investigated. The temperature range 273 K–313 K (increment 20 K) and the flow rates 0.2, 0.5, 1.0 mL/min were used.

As previously shown, the diazepine ring is unstable and undergoes interconversion with a low energy barrier. According to these facts, typical interconversion profile was observed—tailing peak, plateau, fronting peak (Figure 2).

Influence of β-Cyclodextrin in Mobile Phase

Chiral media such as β-cyclodextrin can affect interconversion reactions. In order to determine the influence of the chiral selector on the on—column processes (separation and interconversion) the chiral β-cyclodextrin was added to the mobile phase. The increasing concentration of β-cyclodextrin leads to the disappearance of the first peak. Only one conformer was observed on chromatograms at c (β-cyclodextrin) = 0.005 mol/L.

Influence of Sample Solvent

To check all aspects of analysis, it was necessary to investigate the influence of sample solvent on the separation of diazepam conformers. Surprisingly, the appearance of two peaks of diazepam was observed when diazepam was dissolved in less polar solvents such as EtOH, IPA, PrOH. In turn, only one peak arises on chromatograms when polar solvents (H_2O, MeOH), ACN and non-polar Hex were used. Another approach to solve this problem, was to dissolve diazepam in mobile phases (for details see experimental), but

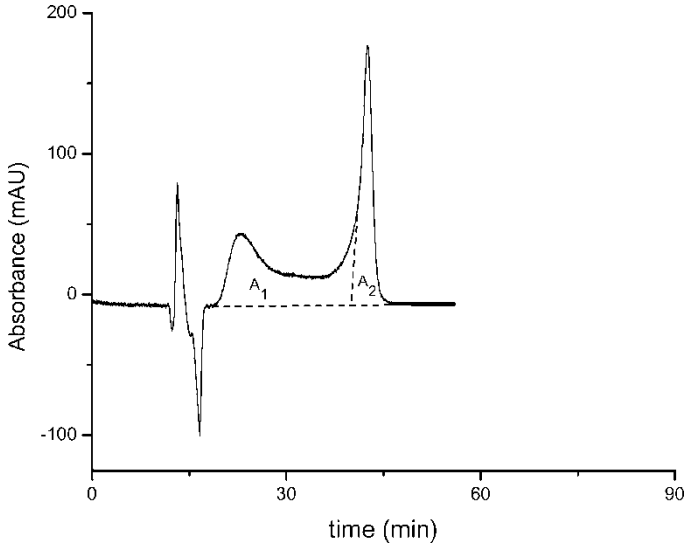

Figure 2. The typical elution profile of diazepam conformers that shows interconversion (first tailing peak, plateau, fronting peak). Chromatographic conditions: $F_m = 0.2$ mL/min, T = 313 K, mobile phase (1), sample solvent: ethanol, for details see experimental part.

only one peak was observed, too. As follows, MeOH, EtOH, PrOH, IPA, and Hex substituted the ACN in mobile phases with the goal to evaluate the importance of individual solvents on interconversion of diazepam conformers. However, the results were similar with those obtained in the case of polar and non-polar solvents, only one peak was observed. The examples of obtained chromatograms are depicted in Figure 3. The problem seems to be more complex, and as the results showed, the sample solvent also plays an important role.

Statistical Evaluation

For the evaluation of the acquired set of results *(*Table 1 A–D*)* describing the behaviour of diazepam conformers in given chiral environment, we used PCA and CA. The statistical treatment of experimentally obtained data enables better interpretation of original matrixes. Some statements described below were concluded from original matrices, but the statistical method allowed the determination of whether the studied parameters have important influence on k_1, k_2, α (A_1 and A_2), or not. Before statistical analysis, the obtained chromatographic results were divided into two groups.

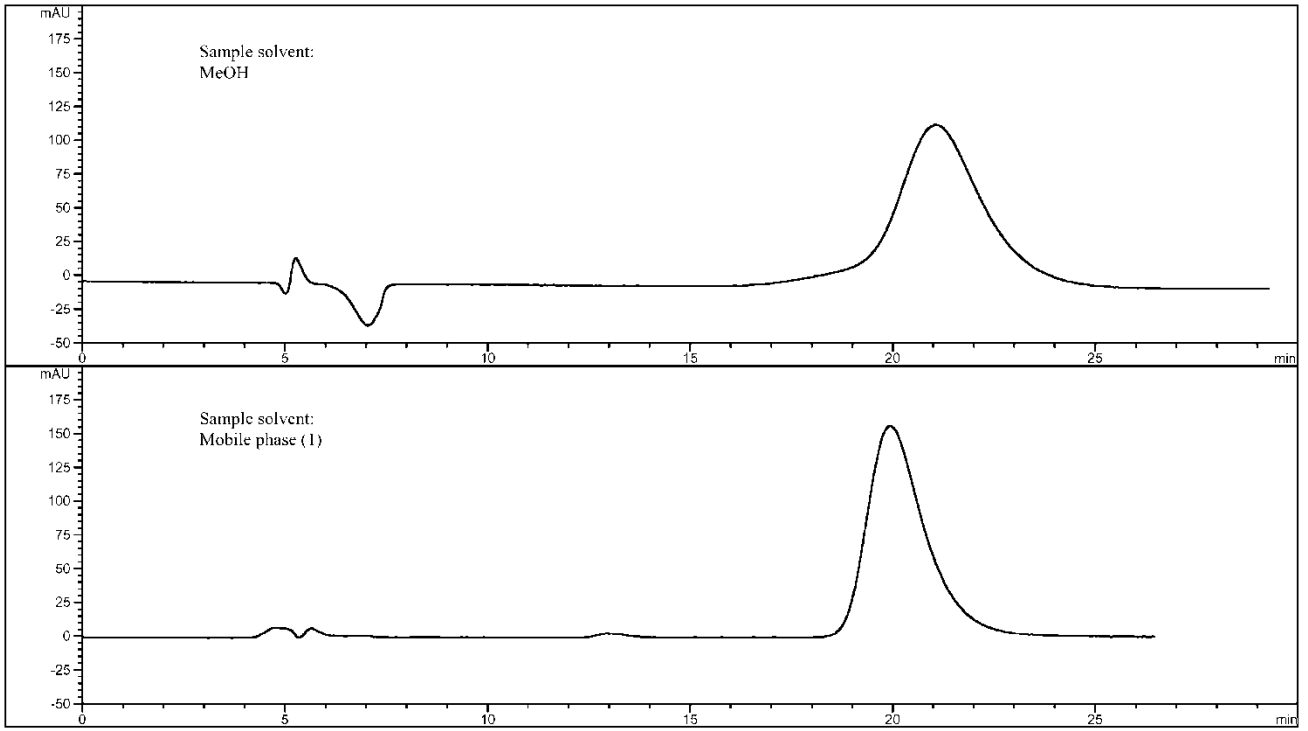

Figure 3. Chromatograms of diazepam conformers. The standard of diazepam was dissolved in different sample solvents. Experimental conditions: Fm = 0.5 mL/min, T = 273 K, mobile phase (1), for other details see experimental part.

The First Group

Retention factors (k_1, k_2) and selectivity coefficients (α) – described the behaviour of diazepam conformers in given chiral environments from a qualitative point of view. Retention factors showed how long were the individual conformers hold on CSP, and the selectivity coefficient describes the possibility of CSP to distinguish both conformers and consecutively discriminate them.

The Second Group

Chromatographic results comprised the peak areas of individual conformers: A_1—first eluted conformer with unresolved zone (plateau), and A_2—the second eluted conformer. This group describes quantitative changes in the amount of both conformers during experiment. The example of individual peak areas is shown in Figure 2.

It should be noted that each studied parameter (T, F_m, pH) influenced the stability of individual conformers. The goal was to determine the statistical importance of individual parameters.

Table 2 shows that for 6 analysed variables (pH, F_m, T, α, k_1, k_2), 99.21 % of the total variance is extracted by the four principal components (PC). The first latent variable PC1 with total variance 59.45 % describes diazepam conformers in a given chiral environment from a qualitative point of view. The second one PC2 with total variance 16.88% reflects the influence of flow rate on processes which occurred on the chromatographic column, the third PC3 with the total variance 16.67 % is created by the pH variable, and the fourth PC4 with the total variance 6.21 % shows the possibility of CSP to distinguish both conformers and discriminate them.

From the dendrogram in Figure 4 two main clusters can be observed. The first cluster consists of the following parameters: selectivity coefficient, flow rate, and temperature. The second cluster is formed by retention factors and pH. As can be seen, the retention factors were influenced by pH and selectivity coefficient was influenced by temperature. The selectivity of a given chromatographic system was being improved with increasing temperature.

Table 2. PCA extraction, eigenvalues that describe behaviour of diazepam conformers in given chiral environment from qualitative point of view where ionic strength was not included

Value	Eigenvalue	Total variance (%)	Cumulative eigenvalue	Cumulative %
1	3.567	59.45	3.567	59.45
2	1.013	16.88	4.580	76.33
3	1.000	16.67	5.580	93.00
4	0.372	6.21	5.952	99.21

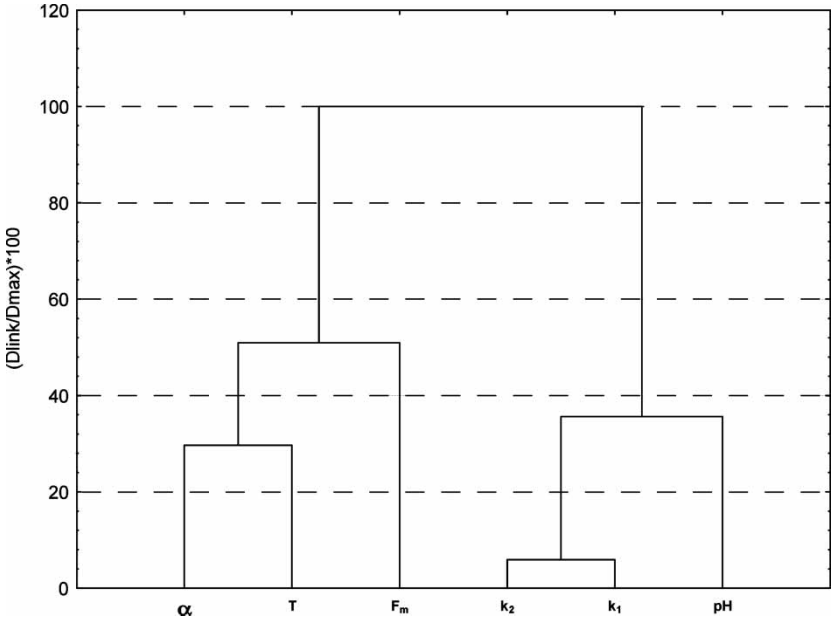

Figure 4. Dendrogram of investigated parameters (T, F_m, pH) and chromatographic parameters k_1, k_2, α of diazepam conformers during their residence on CSP, ionic strength was not included.

From the PCA of the data set where ionic strength was included we obtained the same results: total variance for three components was 92.90 %, only PC1 (59.44 %), PC2 (16.78 %), PC3 (16.68 %), and from the CA, we realized the same conclusion.

Table 4 shows that for 5 analysed variables (pH, F_m, T A_1, and A_2) 97.95% of the total variance of data is extracted by the three principal components. The first latent variable PC1 with the total variance 57.31% describes quantitative changes in the amount of both conformers during the experiment, the second one, PC2, with the total variance 21.07% includes the flow rate on chromatographic columns, and the third, PC3, with the total variance 19.58% is created by the pH variable.

In the dendrogram (Figure 5) two main clusters can be distinguished. They show the influence of studied parameters (T, F_m, pH of mobile phase) on the stability of individual conformers. The flow rate affected the stability of the second eluted conformers. The increase of the flow rate caused the increase of A_2. According to this finding, it can be summed up that the second eluted conformer was formed from the first eluted conformer at a higher flow rate. The flow rate influenced the residence time of diazepam conformers on the chromatographic column. Thus, the time for interconversion was reduced at the higher flow rate. The shorter

Table 3. Factor loadings after varimax rotation—PCA extraction; bold marked loadings have the main contribution in the new latent variable

Variable	Factor 1	Factor 2	Factor 3	Factor 4
pH	−0.041	0.009	**− 0.972**	−0.231
F_m	0.019	**1.000**	0.009	−0.002
T	**0.948**	−0.016	−0.108	0.283
k_1	**− 0.756**	−0.014	−0.566	−0.258
k_2	**− 0.740**	−0.121	−0.602	−0.167
α	0.425	−0.004	0.362	**0.830**
Expl. Var	2.201	1.015	1.770	0.916

time in the chromatographic scale allowed the interconversion only of a small amount of molecules. If the flow rate decreased, the interconversion time, as well as, the residence time of individual conformers on CSP increased. A larger number of molecules were interconverted, thus, the changes in peak areas A_1 and A_2 were observable.

On the other hand, the T and pH of the mobile phase influenced the stability of the first eluted conformer. The stability of the first eluted conformer was improved at a lower temperature when the increase of A_1 was observed.

From the PCA of the data set, where ionic strength was not included, we obtained the same results: total variance for three of the four components were 97.15%, only PC1 (57.15 %), PC2 (20.00 %), PC3 (20.00 %) and from the CA is the same conclusion.

According to the calculated results of PCA and CA, the flow rate and pH are in reversed relationship (Table 3 and 5). This means that the increasing flow rate had the opposite influence on behaviour of diazepam conformers as the increasing pH of mobile phase.

Table 4. PCA extraction, eigenvalues, which describe quantitative changes in the amount of both conformers during, experiment, ionic strength is included

Value	Eigenvalue	Total variance (%)	Cumulative eigenvalue	Cumulative %
1	2.866	57.31	2.865	57.31
2	1.053	21.07	3.918	78.37
3	0.978	19.58	4.897	97.95
4	0.102	2.05	4.999	99.99
5	0.000	0.005	5.000	100.00

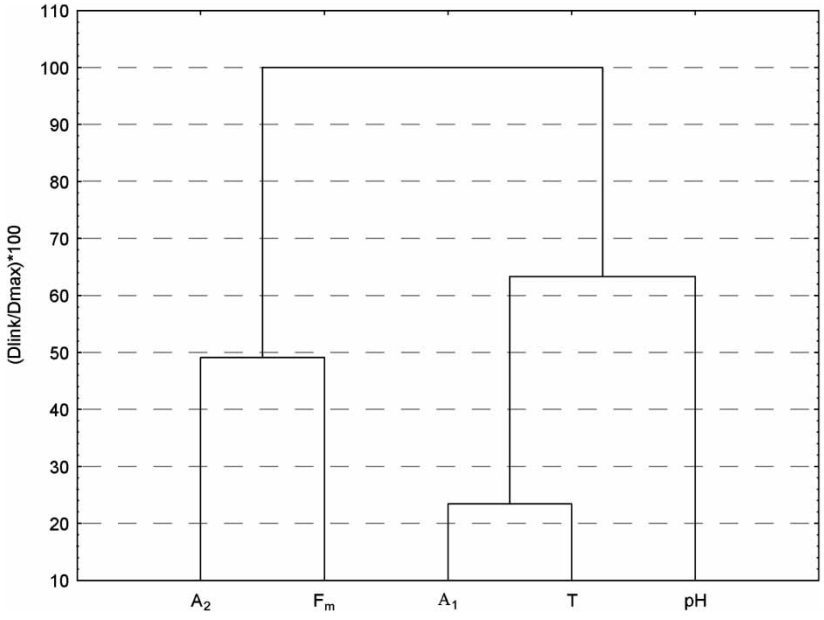

Figure 5. Dendrogram of investigated parameters (T, F_m, pH) and peak area of individual conformers (A_1 and A_2) during their residence on CSP, ionic strength is included.

CONCLUSION

Experimentally obtained data and their statistical analysis reported here were the necessary steps for characterization and interpretation of behaviour of diazepam conformers in given chiral environments. It is now well documented that the sample solvent, temperature, flow rate, pH of mobile phases, chiral additive in mobile phase affected the conformational stability of diazepam conformers in the following way.

Table 5. Factor loadings after varimax rotation—PCA extraction; bold marked loadings have the main contribution in the new latent variable

Variable	Factor 1	Factor 2	Factor 3
pH	0.066	−0.025	**0.997**
F_m	−0.117	**− 0.992**	0.027
T	**0.825**	−0.143	−0.061
A_1	**0.970**	0.201	0.099
A_2	**− 0.971**	−0.195	−0.106
Expl. Var	2.764	1.085	1.020

- The two conformers of diazepam were observed during HPLC depending on used sample solvents.
- The second eluted conformer was the most stable in given chiral environments (it was in excess over the first eluted conformer).

The conformation of the first eluted conformer was preferred at a lower temperature; the second eluted conformer was formed from the first eluted conformer at a higher flow rate.

The statistically important influence on the retention of diazepam conformers on CSP was the pH; the statistical evaluation showed that the selectivity coefficients were most influenced by temperature (selectivity of chromatographic system was improved at higher temperature). According to the PCA and CA analysis, it can be concluded that ionic strength had no influence on behaviour of diazepam conformers in a given chiral environment and the F_m and pH of mobile phase are in reversed relationship.

The role of the chiral additive in mobile phase (β-cyclodextrin) in configurational stability of diazepam conformers must be stressed. The increasing addition of β-cyclodextrin caused the successive disappearance of the first eluted conformer. Only one conformer was observed at c (β-cyclodextrin) = 0.005 mol/L.

As results have shown, the stability of diazepam conformers was conditioned to the chromatographic conditions, as well as, sample solvent and chiral additive in the mobile phase. To prove configurational stability or unstability of some drugs in the presence of the chiral environment is very important because of the safety of the treatment. Configurational stability of drugs is one of the most important parameters for achievement of the required therapeutic effect.

ABBREVIATIONS

CAN – Acetonitrile, BDZs – Benzodiazepines, CA – Cluster analysis, CSP – Chiral stationary phase, EtOH – Ethanol, H_2O – Water, Hex – Hexane, IPA – Isopranol, M – Minus, MeOH – Methanol, P – Plus, PCA – Principal component analysis, PCx – xth principal component, PrOH – Propanol.

ACKNOWLEDGMENTS

The authors acknowledge the support of the Grant Agency of Slovak Republic (VEGA 1/2460/05 and VEGA 1/1186/04) and the Agency for International Science and Technology Cooperation in Slovakia (Grant No. 035/2001, USA-SK).

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Received February 24, 2006
Accepted March 24, 2006
Manuscript 6845