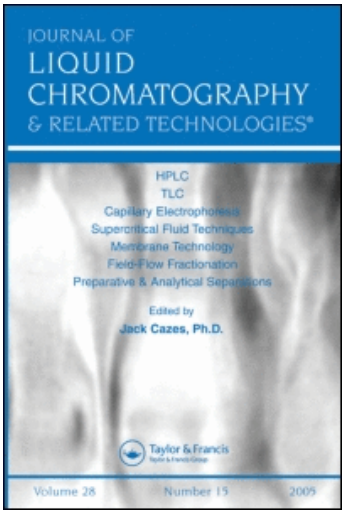

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HPLC Separation of Diazepam Conformers Coupled with Off-Line NMR Experiment

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HPLC Separation of Diazepam Conformers Coupled with Off-Line NMR Experiment

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Abstract: The HPLC analysis of diazepam conformers was carried out in the temperature range 273–313 K, increment 10 K. The pH of the mobile phase was 3.3; 5.5; 5.5 (I_c = const; 6.5; 6.5 I_c = const.). The various flow rates of 0.2; 0.5; 1.0; mL/min were used. Chiral stationary phase based on β-cyclodextrin (ChiraDex) was used for separation of diazepam. The influence of temperature, flow rate, pH of mobile phase, and ionic strength on the retention and elution profile on two peaks of diazepam conformers were studied. After that, the chiral β-cyclodextrin (0.001 mol/L; 0.003 mol/L; 0.005 mol/L) was added into the mobile phase. The increasing amount of β-cyclodextrin in the mobile phase caused the disappearance of the first peak. The complete chiral separation of diazepam was not possible because of simultaneous interconversion. The plateau between two peaks of diazepam appeared in the whole range studied, any inhibitory effects of chromatographic conditions (pH of mobile phase, temperature, flow rate, ionic strength) on interconversion was not observed, only the change of the area ratio between two peaks appeared. Off-line standard ^{1}H and COSY NMR experiments were used to study the diazepam structure in the achiral mobile phase. The results of ^{1}H and COSY NMR experiments confirmed that there is no pH dependent

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open-ring reaction of 1,4-benzodiazepine ring that could occur during the residence time of diazepam in the achiral mobile phase.

Keywords: Dynamic HPLC, Interconversion, Plateau, Diazepam, β-Cyclodextrin, Chiral additive in mobile phase, NMR

INTRODUCTION

Regulatory agencies require extensive stereochemical information about chiral drugs. The configurational stability of such drugs is in the forefront of interest.[1] The configurational stability of chiral drugs is very important because the lability of the molecule structure can lead to undesirable therapeutic effects. The problem occurs when the drug is not configurationally stable, and it should be used in enantiomeric pure form because the individual enantiomers show different therapeutically effect. Reorganization of space arrangement in the molecule of the pure enantiomer can be responsible for significantly different and ineligible interactions between drugs and the chiral receptor. The results are qualitatively different pharmacodynamical, pharmacokinetical, and therapeutical effects. It is known that the most of biochemical processes in the human body are stereochemically controlled, and because of this, chiral drugs must show the stereochemical safety.[2]

Processes connected with space rearrangement of the chiral molecule were observed in chromatography,[3–7] NMR,[8–10] and MEKC.[11] In chromatography, the presence of a chiral stationary phase (CSP) shows the influence over behaviour of configurationally unstable drugs in different ways, e.g., during separation of the racemic mixture into individual enantiomers, separated enantiomers create the racemate again on the chiral column (CSP).[12–17]

The configurationally stereo lability of chiral drugs can be characterized by different terms, each defining a specific process, e.g., enantiomerization (enantiomerization refers to the microscopic and molecule process of the reversible conversion of one enantiomer into the other $A \leftrightarrow B$[18]), racemization (racemization refers to the macroscopic and statistical process of the irreversible transformation of one of the enantiomers into the racemic mixture $A + B \rightarrow AB$[18]), isomerization, epimerization, etc. Characteristic peak profiles are obtained, if reversible interconversion occurs during the time scale of analyte separation. A typical feature of this secondary process is the plateau formed during HPLC separation. Interconversion occurring on the chiral column during the chromatographic experiment is unwanted, because it makes chromatographic separation complicated. On the other hand, it shows important information about configurational stability of drugs - very essential knowledge for correct therapeutical effects.[18]

In some cases,[13,15,19] interconversion phenomena pointed out above were observed during chiral separation of benzodiazepines (BZDs). BZDs are routinely used as racemic drugs and they are widely employed in

therapy for their anxiolitic, sedative, hypnotic, and myorelaxing properties.[20] All 1,4-benzodiazepines are chiral as a virtue of their non-planar ring, and they exist in chiral conformations.[21]

From the BZDs group, the diazepam was chosen for HPLC chiral separation because of its interesting structure. As the chemical formula (Figure 1) shows, only the nitrogen atom (N^1) can be the center of chirality. However, pyramidal nitrogen is normally not configurationally stable. It converts very rapidly through a planar sp^2-hybrized transition state to a different configuration in space arrangement. It leads to existence of a mixture of two different configurations, which are easily interconvertible into one another. In the literature[22,23] these two structures are defined as M (minus) and P (plus) conformers. Conformers of diazepam are in mirror image relation, even if the compound lacks a chiral center.[23]

Stalcup et al.[22] observed the appearance of two peaks of diazepam in the enantioseparation of benzodiazepines using a chiral stationary phase (based on maltooligosacharides). Following circular dichroism detection, it was proven that the reported separation is the separation of two conformers. Sjödin et al. studied the configuration of diazepam using Hyperchem for modelling the molecular structure. They showed the existence of two conformers of diazepam. They confirmed a deviation from the planarity and non-planar ring system of diazepam molecule.

Paizs et al.[23] performed the calculation of the ring inversion barrier (Figure 2) for diazepam (17.6 kcal/mol). The resultant energy barrier of the interconversion process was too low, thus, dynamic HPLC can be used for study behaviour of diazepam in a chiral environment. The relative energies of diazepam conformers along the isomerization path were presented. The study was carried out by semi empirical ab initio restricted Hatrree-Fock, unrestricted Hatrree-Fock, and density functional methods.

The goals of our experiment were HPLC separation of diazepam conformers and study of its behaviour in different chromatographic conditions (pH, temperature, flow rate). The high stereo selectivity of a chiral environment was utilized for their separation. As it was previously shown in Figure 1, diazepam lacks a center of chirality, and the substituted 1,4-benzodiazepine

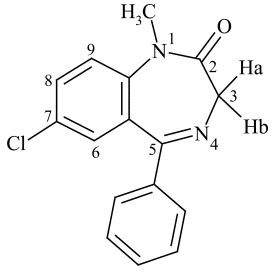
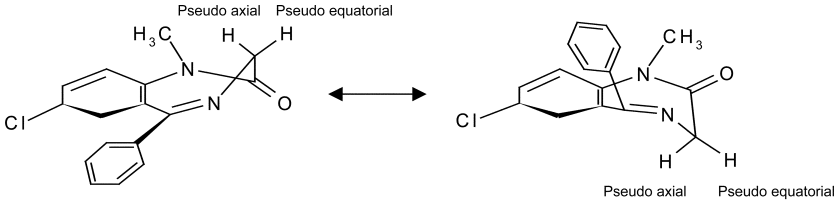


Figure 1. Chemical formula of diazepam.



M conformer P conformer

Figure 2. The conformers of diazepam (M-minus, P-plus) and inteconversion of N,N-benzodiazepine ring.[23]

ring is nonplanar and unstable. The lability of the 1,4-benzodiazepine ring predicts interconversion, thus, the diazepam can exist in different conformations. Consequently, the appearance of two peaks and the plateau formation were observed during HPLC separation of diazepam conformers.

EXPERIMENTAL

HPLC Analysis

Chiral Separation

A Chiral HPLC column, ChiraDex (250 × 4 mm I.D., particle size 5 μm) was from Merck, Germany.

Achiral mobile phases consisted of: Acetonitrile/acetate puffer 200 mM, pH = 3.3 v.v. 10/90 (A); Acetonitrile/acetate puffer 200 mM, pH = 5.5 v.v. 10/90 (B); Acetonitrile/acetate puffer 200 mM, pH = 6.5 v.v. 10/90 (C).

Acetate puffer was prepared from natrium acetate (equimolar weight) and pH was adjusted with acetic acid at value 3.3; 5.5; 6.5; ionic strength was adjusted on the constant value (with LiCl). Measurements were carried out with mobile phases prior and after adjustment of ionic strength.

Chiral mobile phases: the chiral selector (β-cyclodextrin) was added to the achiral mobile phase. The resultant concentrations were 0.001; 0.003; 0.005 mol/L.

A standard of diazepam (Slovakofarma Hlohovec, Slovakia, 99.98% purity) was dissolved in ethanol, c = 0.1 mg/mL.

Separation on a C_{18} Column

A C_{18} column Separon SGC C_{18}, from Watrex 125 × 4 mm particle size 5 μm was used.

The mobile phases were ACN/H_2O v.v. (60/40; 50/50; 40/60), MeOH/H_2O v.v (40/60; 60/40; 80/20), ACN/acetate puffer 200 mM, pH = 3.3 v.v.

(40/60; 60/40; 80/20), ACN/acetate puffer 200 mM, pH = 5.5 v.v. (40/60; 60/40; 80/20), ACN/acetate puffer 200 mM, pH = 6.5 v.v. (40/60; 60/40; 80/20).

Chemicals

Chemicals and solvents were obtained from Merck, Germany.

Method

A standard solution of diazepam (c = 0, 1 mg/mL) was analysed at various temperatures, 273 K, 293 K, and 313 K and various mobile phase flow rates (0.2; 0.5; 1.0 mL/min).

Equipment

The chromatographic system consisted of a quaternary pump (Merck—Hitashi L-6000A) equipped with an injection valve (Rheodyne), DAD detector (Agilent 1100 Series). Wavelength of 230 nm was used.

NMR Experiments

The mixture with the same composition as mobile phase A (in text labelled as A_D) was prepared by the mixing of deutered solvents. The blank of A_D was measured for comparison. Spectra were accumulated thereby, better ratio signal/noise was achieved. All NMR experiments were performed on a Varian Inova 600 MHz instrument.

RESULTS AND DISCUSSION

The chiral selector was capable to distinguish both conformers of diazepam. A peak cluster of diazepam with plateau formation was observed in the whole studied range, only one chromatogram was chosen for illustration (Figure 3). The interconversion of diazepam conformers taking place during separation in presence of the ChiraDex, is evident by a plateau formation and peak broadening between 273 K–313 K. The characteristic plateau arises from molecules undergoing interconversion during the time scale of chromatographic separation.

Influence of pH of Mobile Phase, Flow Rate, Ionic Strength, and Temperature on Retention of Diazepam Conformers

The HPLC analysis of diazepam was carried out at various temperatures, various pH of mobile phase, and various flow rates (see Experimental section).

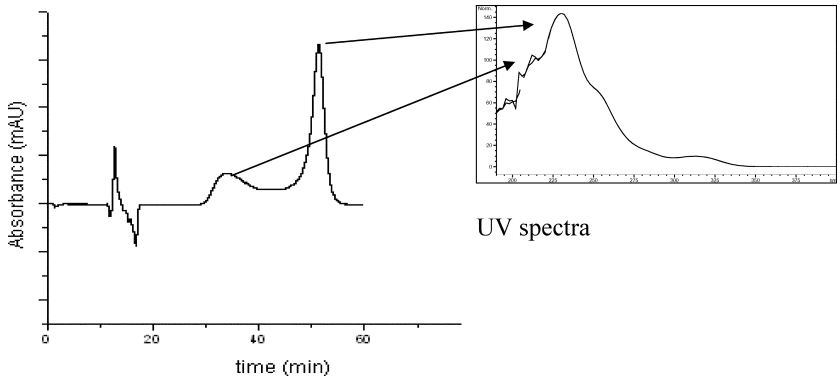


Figure 3. Peak envelope of diazepam (first tailing peak, plateau, second fronting peak). Chromatographic conditions: CSP ChiraDex, mobile phase (A), T = 273 K, Fm = 0.5 mL/min, sample solvent-ethanol, for other details see Experimental part.

A set of chromatograms were acquired, which show the behaviour of diazepam in chiral environment under different conditions.

Within the whole studied range, typical interconversion profile (peak tailing, plateau, peak fronting) was observed. Table 1 summarizes chromatographic parameters (retention factor and selectivity coefficient) for diazepam conformers under different separation conditions. Retention factor increases with the increase of pH of mobile phase. The ionic strength of mobile phase influences retention factor as can be seen in Table 1. The increase of temperature causes the decrease of k_1 and k_2 values. It should be noted, that selectivity coefficient decreases with the increase of pH. Selectivity coefficients increase with the increase of the temperature. The flow rate influences the elution profile as can be seen in Figure 4a. With the increase of flow rate, the plateau was shorter in the time scale. On the other hand, the height of the plateau increases with the increase of flow rate. Changes of the flow rate have great impact on the peak shape, as well as on the elution time of the experimental chromatograms. At higher flow rates, the relative plateau was higher. On the other hand, peak shape and selectivity were found to improve with increasing temperature as representative chromatogram (Figure 4b) shows. Increasing column temperature may decrease the apparent microheterogenity of the bonded phase, thereby improving peak shape and selectivity. Hence, the solute interacts with a very heterogeneous chiral stationary phase. At lower temperatures, this microheterogenity may be reinforced while at higher temperatures, the solute may interact with a more thermally averaged surface. The solute has access to chirally selective sites within the interior of the chiral stationary phase at elevated temperatures, which are unavailable at lower temperatures, possibly implying a phase transition.[22]

Table 1. The chromatographic parameters of diazepam conformers separated on ChiraDex at different chromatographic conditions

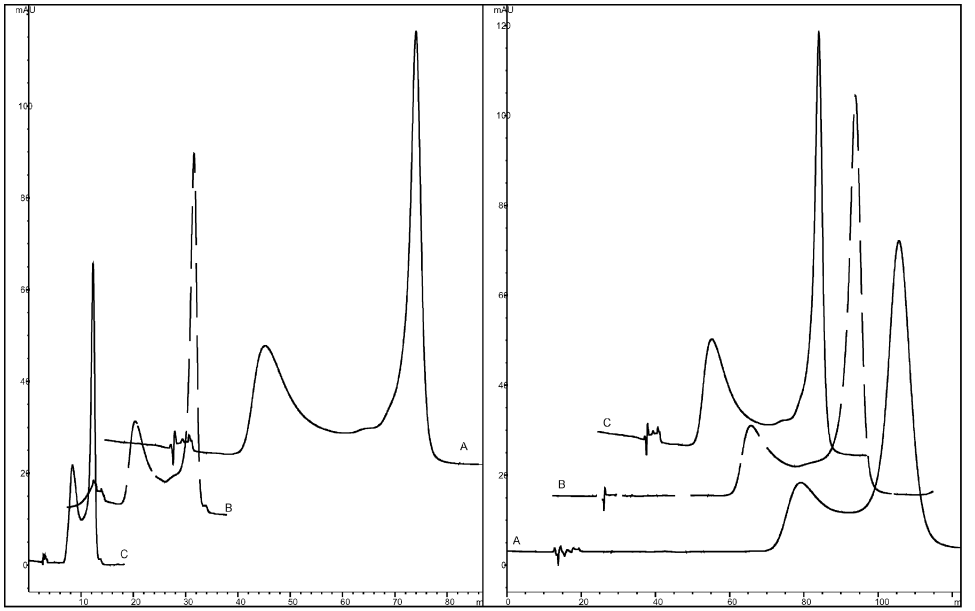
	T = 273 K			T = 293 K			T = 313 K		
pH	k_1	k_2	α	k_1	k_2	α	k_1	k_2	α
Fm = 0.2 mL/min									
3.3	2.74	4.06	1.48	1.53	2.83	1.84	0.70	2.17	3.10
5.5	3.91	5.11	1.31	2.07	3.57	1.72	1.46	2.79	1.91
5.5 I_c = konst	4.24	6.06	1.43	2.91	4.46	1.53	1.49	3.12	2.10
6.5	5.45	7.20	1.32	3.40	4.91	1.45	2.01	3.55	1.76
6.5 I_c = konst	4.89	6.88	1.40	3.39	5.09	1.50	2.16	3.44	1.59
Fm = 0.5 mL/min									
3.3	2.65	3.58	1.35	1.46	2.75	1.88	0.90	2.07	2.29
5.5	3.68	4.78	1.30	2.49	3.53	1.42	1.31	2.64	2.02
5.5 I_c = konst	3.40	4.36	1.28	2.34	3.72	1.59	1.63	3.18	1.95
6.5	5.23	6.48	1.24	3.80	4.87	1.28	2.22	3.67	1.65
6.5 I_c = konst	5.25	6.52	1.24	3.66	4.86	1.32	2.41	3.48	1.44
Fm = 1.0 mL/min									
3.3	2.12	3.10	1.47	1.10	2.51	2.27	0.55	2.10	3.81
5.5	3.44	4.27	1.24	2.31	3.37	1.46	1.71	2.74	1.61
5.5 I_c = konst	3.54	5.01	1.42	2.81	4.24	1.51	2.08	3.29	1.58
6.5	5.05	5.71	1.13	3.55	4.69	1.32	2.58	3.67	1.42
6.5 I_c = konst	5.03	5.82	1.16	3.27	4.55	1.39	2.31	3.41	1.47

$k_1 \pm 0.03$, $k_2 \pm 0.01$, $\alpha \pm 0.03$.

Influence of Flow Rate, Temperature, pH of Mobile Phase, and Ionic Strength on On-Column Interconversion of Diazepam

During the HPLC experiment, the chromatographic column was a chemical reactor (interconversion) and separation device (separation of diazepam). During diazepam separation, it reorganized spatial arrangement with the goal to find a lower energy state and/or more stable confirmation in a given chiral environment. It should be noted, that the interconversion is too rapid and it is a concurrence process of conformers separation. As a result of this phenomenon, the complete separation of diazepam conformers was not possible.

The stability of transient complexes of diazepam conformers with chiral stationary phase was not the same (principle of microscopic reversibility). As can be seen in Figures 3–5, the second eluted conformer was in excess over the first eluted conformer. According to the shape of peaks (Figures 3–5) it can be assumed that the rate of interconversion reaction is higher in mobile phase than in the stationary phase. However, the first conformer created a less stable complex with the chiral stationary phase, it spends more time in



a) b)

Figure 4. a) The influence of flow rate on elution profile of diazepam. Temperature 313 K, mobile phase composition: ACN/acetate buffer pH = 6.5 with constant ionic strength, flow rate A = 0.2 mL/min, B = 0.5 mL/min, C = 1.0 mL/min, for detailed chromatographic condition, see Experimental part. b) The influence of temperature on elution profile of diazepam. Flow rate 0.2 mL/min, mobile phase composition: ACN/acetate buffer pH = 6.5 with constant ionic strength, flow rate A = 273 K, B = 293 K, C = 313 K, for detailed chromatographic condition, see Experimental part.

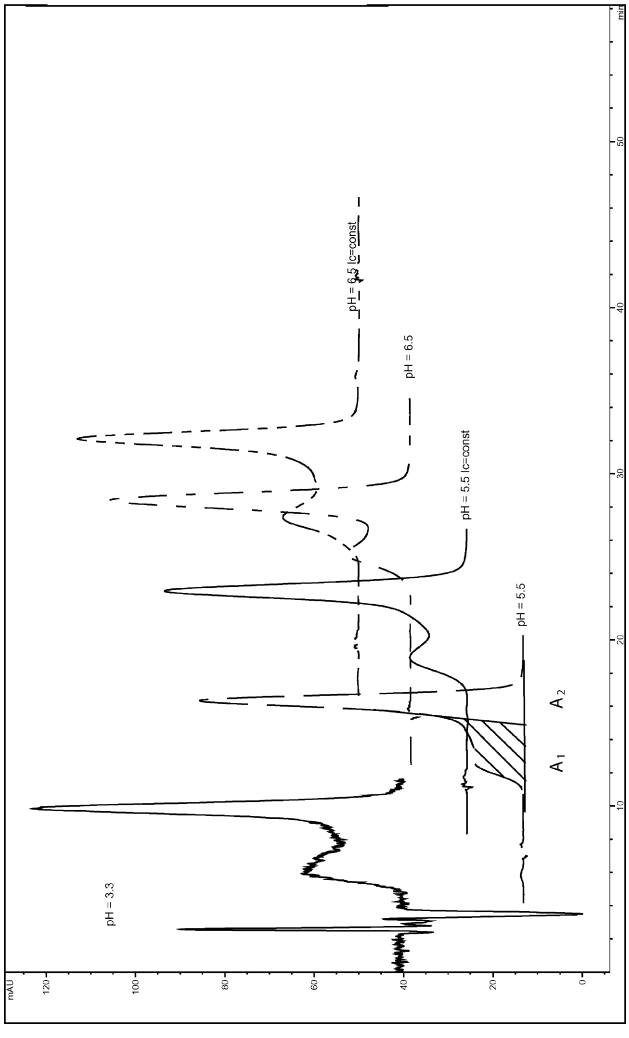


Figure 5. The influence of pH mobile phase and ionic strength on elution profile of diazepam. Mobile phase composition: ACN/acetate buffer 200 mmol/L (10/90), T = 293 K, Fm = 1.0 mL/min, for detailed chromatographic condition, see Experimental part.

mobile phase and, thus, it should undergo interconversion more rapidly than the second one. But, as experimental results showed, the interconversion reaction was strongly influenced by chromatographic conditions, as well as temperature and flow rate. The diazepam conformers underwent interconversion according to chromatographic conditions. The increasing flow rate caused an increase in area of the second eluted conformer. The opposite phenomenon was observed at increasing temperature. The area of the second eluted conformer was decreased with an increase of temperature.

The influence of chromatographic conditions (temperature, flow rate, pH of mobile phase, and ionic strength) on the area of the first and the second eluted conformers (A_1 and A_2, Figure 5) is summarized in Table 2.

Influence of Flow Rate on Interconversion

At a temperature of 273 K and mobile phase composition A (see Experimental part) the area ratio between the first and the second eluted peak did not change with the increase of the flow rate. Probably, the interconversion process was very slow at given chromatographic conditions and, thus, the influence of flow rate on peak areas (A_1 and A_2) was not observed.

In turn, in all the other cases, the A_1 and A_2 were varied with the increasing flow rate. The increase of the flow rate caused the decrease of A_1 and simultaneously, the increase of A_2 occurred. At the higher flow rate, the residence time of diazepam in chromatographic column was shorter than at lower flow rate. This observation can be explained by the shorter time allowed for interconversion and, consequently, the smaller amount of molecules that can be interconverted at a lower chromatographic time scale. If the flow rate decreased, the interconversion time, as well as the retention time is increased. Therefore, a larger number of molecules interconverted giving rise to a higher plateau.

Influence of Temperature on Interconversion

The increasing temperature had a different influence on interconversion as did the increasing flow rate. It means that the decrease of temperature caused the decrease of A_2 (increase of A_1).

Influence of pH of Mobile Phase and Ionic Strength on Interconversion

The influence of both parameters on interconversion cannot be strictly explained from the obtained results. The dependence of elution profile and plateau formation was not monotonous and, in some cases, appearance of three peaks was observed (Figure 6).

Table 2. The peak areas of first and second eluted conformers. The areas A_1 (%) and A_2 (%) corresponding with Figure 5

Temperature (K)	273		293		313	
Flow rate (mL/min)	Area (mAU^2)					
	A_1 (%)	A_2 (%)	A_1 (%)	A_2 (%)	A_1 (%)	A_2 (%)
Mobile phase: ACN/acetate buffer 200 mM, pH = 3.3 (10/90)						
0.2	30	70	49	51	66	34
0.5	30	70	42	58	55	45
1.0	30	70	39	61	41	59
Mobile phase: ACN/acetate buffer 200 mM, pH = 5.5 (1090)						
0.2	40	60	57	43	60	40
0.5	40	60	50	50	59	41
1.0	38	62	40	60	51	49
Mobile phase: ACN/acetate buffer 200 mM, pH = 5.5, I_c = konst (1090)						
0.2	40	60	45	55	60	40
0.5	37	63	45	55	50	50
1.0	33	67	40	60	45	55
Mobile phase: ACN/acetate buffer 200 mM, pH = 6.5 (1090)						
0.2	40	60	52	48	53	47
0.5	29	71	45	55	50	50
1.0	29	71	33	67	49	51
Mobile phase: ACN/acetate buffer 200 mM, pH = 6.5, I_c = konst (1090)						
0.2	40	60	50	50	63	37
0.5	33	67	49	51	54	45
1.0	28	72	49	51	54	45

$A_1 \pm 4\%$, $A_2 \pm 2\%$.

Influence of Chiral Mobile Phase on Separation

Marked differences were found when β-cyclodextrin was added to the mobile phase. Figures 7 B-D show the chromatograms of diazepam conformer separations, with the chiral selector present in the mobile phase at three different concentrations of β-cyclodextrin (0.001, 0.003, 0.005).

The increase of additional β-cyclodextrin to the mobile phase leads to the presence of one peak in the chromatogram (Figure 7). It is evident, that the increasing concentration of chiral additive in mobile phase results in the disappearance of the first peak (Figure 7 D). Only one peak was observed in the chromatograms at $c_{\beta\text{-cyclodextrin}} = 0.005\,\text{mol/L}$.

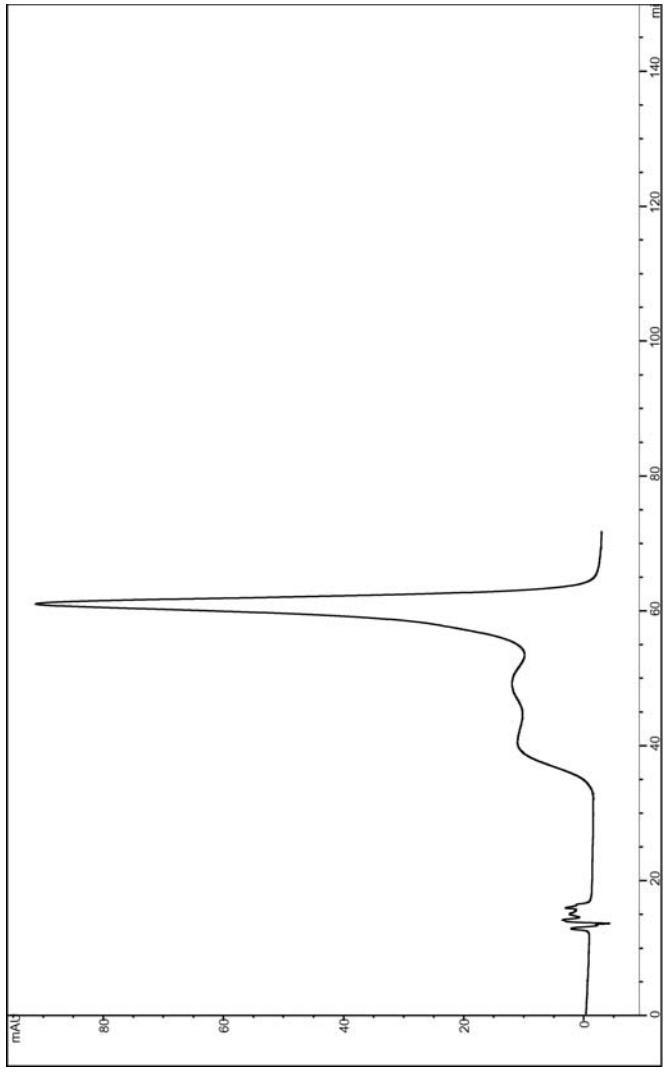


Figure 6. Chromatogram of diazepam conformers, temperature 313 K, Fm = 0.2 mL/min, mobile phase composition: ACN/acetate buffer pH = 6.5, 200 mmol/L, (10/90), for detailed chromatographic condition, see Experimental part.

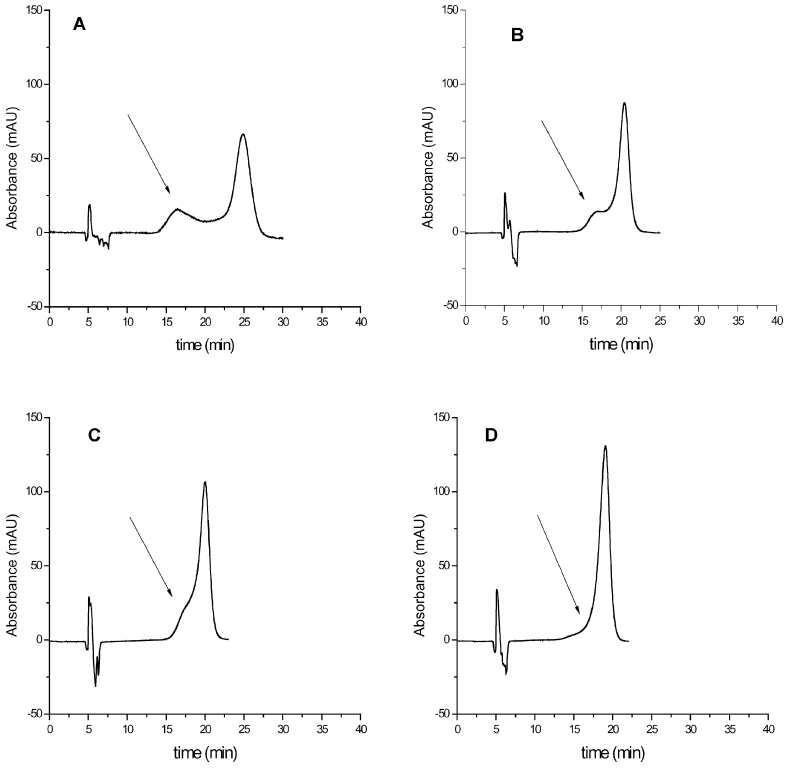


Figure 7. Influence of β-cyclodextrin addition in mobile phase on separation. A-mobile phase without β-cyclodextrin, B-C mobile phase with chiral selector. Concentration of chiral additive: B-0.001 mol/L, C-0.003 mol/L, D-0.005 mol/L. Mobile phase composition: ACN/acetate buffer pH = 3.3; v.v 10/90, Fm = 0.5 mL/min, temperature 293 K detailed chromatographic condition, see Experimental part.

NMR Experiments

The goal of the NMR experiments was to check if, under the same conditions as in the mobile phase (A), there is pH dependent open-ring reaction of diazepam (Figure 8).

It is well known from the literature,[24] that in the case of cyclic imines such as BZDs, at low pH (pH $\leq$4, 2) reversible open ring reaction can occur. Stalcup et al.[22] experimentally proved, that off-line heating of diazepam during 1 h in 6 M HCl is required for the noticeable changes on the chromatogram which refers to acid hydrolysis of diazepam.

In our case, ^{1}H NMR experiments were carried out to verify the diazepam structure in the presence of the achiral mobile phase. On-column reaction (resulting from composition of mobile phase) was necessary for the estimation.

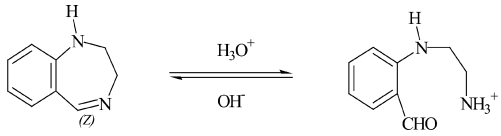


Figure 8. Ring opening reaction of cyclic imines.[24]

A standard of diazepam was dissolved in mixtures A_D (see Experimental part, NMR experiment). As is shown in Figures 1 and 2, there are two hydrogens occupying axial and/or equatorial position on the 3rd carbon atom.

NMR unambiguously proved this; it means that the rate of reversible ring opening reaction is slow in the NMR time scale. The observation of two doublet signals: δ (3-H_a) = 3.92 ppm; δ (3-H_b) = 4.58 ppm corresponded to the closed-ring structure of diazepam (Figure 9). The observed geminal conplay constant between the two protons was 11.4 Hz, and their chemical shift was in an expected region. The COSY spectrum (Figure 10) confirmed the close through bond connectivity of the two hydrogens.

Separation of Diazepam on C_{18} Column

Only one peak of diazepam was observed during the achiral separation on a C_{18} column. Separation of the diazepam conformer was not possible on the

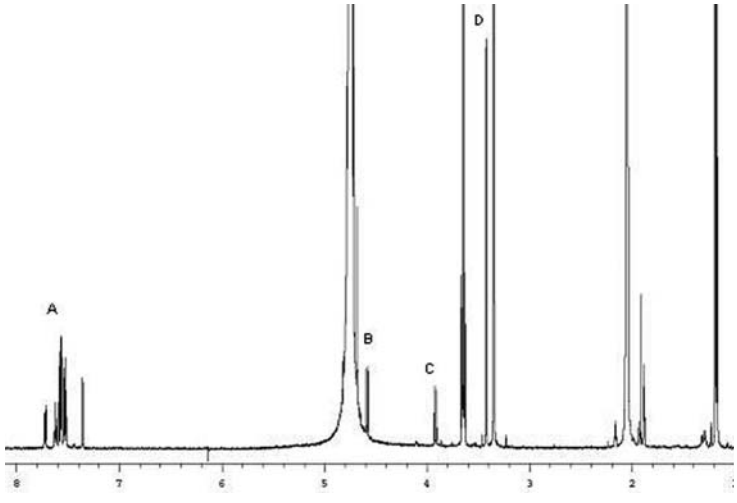


Figure 9. ^{1}H NMR spectrum measured in deutered solvents with the same ratio as mobile phase (A) see Experimental part, in figure section A are hydrogens signals on aromatic rings; B, C are doublet signals of 3-H_a and 3-H_b, D (singlet) is signal of 1-N-methylated group.

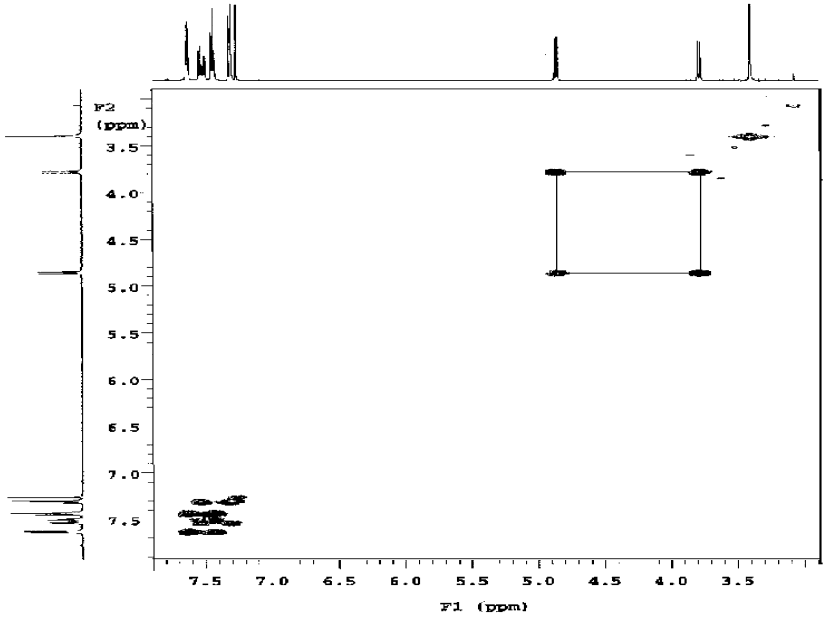


Figure 10. 2D COSY experiment with responding correlation (denoted as square in the right top) for closed form of 3-H substituted diazepam.

achiral stationary phase. Probably, the high stereo selectivity of the chiral environment was necessary for separation of diazepam conformers (two peaks appeared during chiral separation on β-cyclodextrin).

The achiral separation was used to eliminate the presence of impurities, which can be latent in the peak envelope of diazepam during chiral separation. The experiments did not confirm the presence of other compounds which can coelute with diazepam. At all separation conditions (See Experimental part for details) only one peak was observed.

CONCLUSION

The complete separation of diazepam conformers was not possible because of interconversion, which arises from stereo lability of a diazepam molecule. The interconversion was determined even at a lower temperature (273 K). The interconversion and separation of diazepam conformers was the same in the time scale, thus plateau formation was observed in the whole studied range. During the separation of diazepam conformers, the interconversion was observed depending on flow rate, temperature, pH ionic strength of mobile phase.

The influence of chromatographic parameters on interconversion can be summarized in following way:

1. the influence of the flow rate was observed;
2. the change of the pH of the mobile phase and temperature of the separation were important;
3. the stability of individual diazepam conformers in a given chiral environment strongly depended on chromatographic conditions, as it was shown in the experimental results;
4. the addition of β-cyclodextrin in the mobile phase caused the formation of only one conformer at $c_{\beta\text{-cyclodextrin}} = 0.005\,\text{mol/L}$;
5. the closed ring structure of diazepam in the presence of the achiral mobile phase was confirmed by the standard ^{1}H and COSY NMR measurement of diazepam in deutered solvents.

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