Journal of Chromatography A, 1217 (2010) 7884–7890

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Accessible silanol sites – Beneficial for the RP-HPLC separation of constitutional and diastereomeric azaspirovesamicol isomers

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ARTICLE INFO

Article history:
Received 24 August 2010
Received in revised form 8 October 2010
Accepted 14 October 2010
Available online 21 October 2010

Keywords:
Azaspirovesamicol isomers
Silanol sites
Cation-exchange
Silica column
Retention mechanism
Basic analytes
VAChT

ABSTRACT

Different RP-HPLC columns (phenyl, conventional ODS, cross-linked C_{18} and special end-capped C_8 and C_{18} phases) were used to investigate the separation of four basic ionizable isomers. Using ACN/20 mM NH_4OAc aq., a separation was observed exclusively on RP columns with higher silanol activity at unusual high ACN concentration, indicating cation-exchange as main retention mechanism. Using MeOH/20 mM NH_4OAc aq., another separation at low MeOH concentrations was observed on both, RP columns with higher as well as RP columns with lower silanol activity, which is mainly based on hydrophobic interactions. The isomers were also separated on a bare silica column at higher MeOH content using NH_4OAc. Since cation-exchange governs this retention, the elution order was different compared to the RP phases. A strong retention on the silica column was observed in ACN, which could be attributed to partition processes as additional retention mechanism.

1. Introduction

The neurotransmitter acetylcholine is transported into synaptic vesicles of cholinergic neurons via the vesicular acetylcholine transporter (VAChT). In the last years, the VAChT was intensely studied as new target for molecular imaging in brain because this transporter protein is assumed to be strongly involved in neurodegenerative processes such as Alzheimer's disease (AD). The aminoalcohol vesamicol [(−)-*trans*-2-(4-phenylpiperidino)cyclohexanol] inhibits the transport of acetylcholine via binding towards VAChT. Therefore, especially ^{18}F-labeled VAChT-targeting ligands based on the vesamicol structure [1–4] are highly promising candidates for a successful quantitative imaging of cholinergic regions of the diseased brain by utilizing positron emission tomography (PET) as a powerful diagnostic tool in nuclear medicine. In 2007, we reported on the regioselective synthesis of azaspirovesamicols [5], a new class of vesamicol analogs with improved binding properties [6]. In particular, we described the regioselectivity of nucleophilic epoxide ring opening of a mixture of *syn*/*anti* epoxides with 4-phenylpiperidine, where only two out of four possible isomers could be identified. These two constitutional isomers **1a** and **1d** (Fig. 1) were isolated by fractionated crystallization and their molecular structures could be determined by crystal structure analyses [5]. The suggestion, that only two out of the four possible isomers were formed, was confirmed by ^{19}F NMR spectroscopy, TLC, and a single HPLC analysis. Using a Multospher RP18 AQ column, only two signals at very high retention times were observed, which were attributed to the two regioisomers **1a** and **1d**.

However, in the course of subsequent studies on this new class of VAChT ligands, we found evidence for the existence of another isomer. Obviously, the epoxide ring opening reaction was less regioselective than postulated and resulted in more than two isomers.

Since isomers usually possess similar physical properties, isomeric resolution is challenging, even if HPLC as separation method is used. It is almost impossible to predict the suitability of a column for such a separation. Furthermore, the azaspirovesamicol derivatives are basic analytes which pose additional difficulties and require special separation conditions regarding pH value and buffer selection [7–10]. Such experiences were already made for the chiral separation of these vesamicol analogs [11]. Therefore, we accomplished a comprehensive HPLC study to discover the actual number of azaspirovesamicol isomers, formed by this nucleophilic ring opening reaction. Several RP columns bearing different stationary phases as phenyl, conventional ODS, cross-linked C_{18} or special end-capped C_8 and C_{18} phases were tested and the existence of the four isomers **1a**–**1d** could be demonstrated reliably. However, we found interesting differences between the columns and unusual separation behaviors, encouraging us to expand these investigations. To discuss possible retention mechanisms, different eluent compositions and additives were

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doi:10.1016/j.chroma.2010.10.061

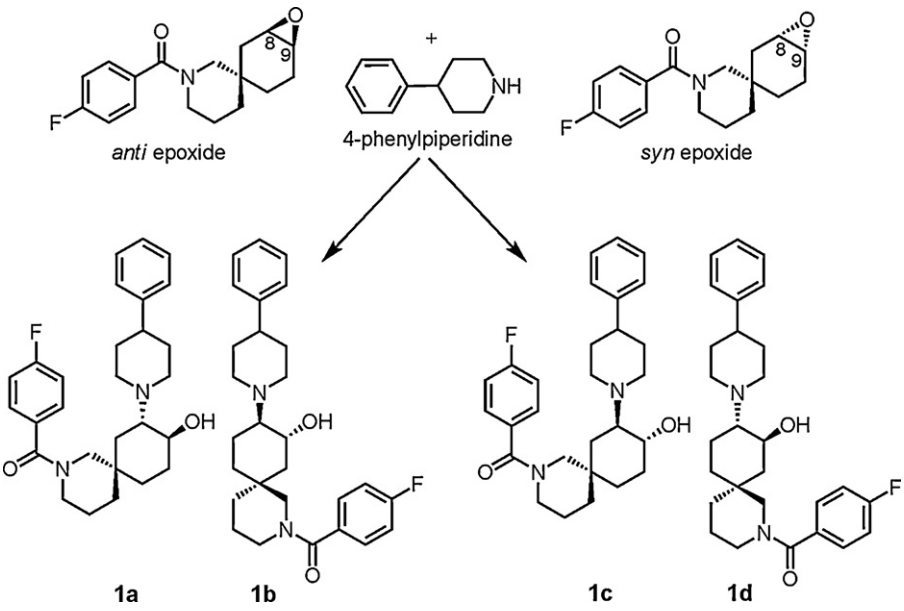

Fig. 1. The four possible isomers **1a**–**1d** of 4-fluorobenzoyl azaspirovesamicol derivatives. **1a**/**1b** and **1a**/**1d** as well as **1c**/**1b** and **1c**/**1d** are pairs of regioisomers or constitutional isomers, respectively **1a**/**1c** and **1b**/**1d** are two pairs of diastereomers.

tested and the investigations were extended using a bare silica column.

2. Experimental

2.1. Instrumentation and chemicals

Analytical chromatographic separations were performed on a JASCO LC-2000 system, incorporating a PU-2080*Plus* pump, AS-2055*Plus* auto injector (100 μL sample loop), and a UV-2075*Plus* UV detector (monitoring at 254 nm). The following columns were used; LiChrospher 60 RP Select B – 5 μm (VWR International, Germany), Multospher 100 RP18 – 5 μm, Multospher 100 RP18 FBS – 5 μm, Multospher 100 Si – 5 μm (CS-Chromatographie Service, Germany) (250 mm × 4.0 mm), and Nucleosil 100 RP18 AB – 5 μm, Nucleodur Sphinx RP – 5 μm (Machery-Nagel, Germany), Reprosil-Gold 120 C18 – 5 μm, Reprosil-Pur Basic-C18-HD – 5 μm (Dr. Maisch-GmbH, Germany) (250 mm × 4.6 mm).

The semipreparative separation of the isomers **1a**–**1d** was performed on a Merck-Hitachi system D-7000 with an L-7100 pump, a Rheodyne injection valve with a 500 μL sample loop and a L-7400 UV detector by using a 250 mm × 10.0 mm Nucleodur Sphinx RP – 5 μm column.

The retention factors (k), separation factor (α) and resolution (R_S) were calculated according to international recommendations [12,13]. The asymmetry factor (A_S) was calculated at 10% of the total peak height. The column hold-up times (t_M) for each column were estimated by injection of water. The pH value stated (6.8 for NH_4OAc) was measured in the aqueous buffer before dilution in the final eluent using a pH meter (inoLab pH Level 1) with a Sentix 60 Plus electrode (WTW-GmbH, Weilheim, Germany). The ammonium acetate (NH_4OAc) concentration stated as 20 mM NH_4OAc aq. represents the concentration in the aqueous component of an eluent mixture. When it was stated as 1 and 5 mM NH_4OAc ov., the overall concentration in the mobile phase is represented. The latter also applies for 0.05% (v/v) trifluoroacetic acid (TFA) designation.

Mass spectra were recorded on a Mariner Biospectrometer Workstation (Applied Biosystems) using ESI.

Acetonitrile (ACN, VWR International, Germany) and methanol (MeOH, Acros Organics, Belgium) were of HPLC gradient grade and NH_4OAc (Sigma–Aldrich, Germany) and TFA of HPLC grade.

2.2. Sample preparation and analyses

The isomeric mixture **1a**–**1d** was synthesized as described earlier [5]. For HPLC studies the crude product was pre-purified by flash chromatography (silica gel, ethyl acetate/MeOH 2:1) to obtain a nearly colorless oil. Washing with small amounts of *n*-hexane and evaporation of the residual solvent yielded a slight yellow solid. Sample solutions for analytical HPLC separations were prepared by dissolving about 1 mg solid in 1 mL of an appropriate eluent mixture. The injection volume was 5–10 μL.

The determination of the molecular structures of **1a** and **1d** via X-ray structure analysis was already described earlier [5], therefore they could be reliably attributed to the signals in the chromatograms. Since the ring opening reaction allows the formation of only that four isomers shown in Fig. 1, **1b** and **1c** have to be attributed to the two remaining molecular structures. The reaction of pure *syn* epoxide would result in isomers **1c** and **1d**, with **1d** already structurally described. Therefore, we determined indirectly the molecular structure of **1c** and finally also of **1b**. Since the *syn* and *anti* epoxides could not be separated by flash chromatography, 35 mg of *syn* epoxide was separated via preparative TLC (silica gel plates 20 mm × 20 mm × 1 mm, 100% methyl *tert*-butyl ether). Subsequent reaction of the *syn* epoxide with 4-phenylpiperidine provided the isomers **1c** and **1d**, which were analyzed by HPLC using the Nucleodur Sphinx column (95% ACN/20 mM NH_4OAc, 1.0 mL/min). Thus, all isomers could be attributed to their molecular structures as shown in Fig. 1 as well as to their signals in the chromatograms. According to our HPLC studies, the nucleophilic ring opening reaction provides 53% **1a**, 23% **1b**, 19% **1c**, and 5% **1d** (±1%, reaction conditions as described in [5]).

For further elucidation, the four isomers **1a**–**1d** were separated by semipreparative HPLC using a Nucleodur Sphinx column (250 mm × 10.0 mm). The pre-purified solid (0.85 mg) was dissolved in 500 μL ACN and injected in one step. The isomeric purity of the four collected fractions were controlled by analytical HPLC. Evaporation of the solvent and dissolving in 80% ACN/H_2O pro-

Table 1
Separation parameters of the isomers **1a**–**1d** obtained on three different RP columns using ACN or MeOH as organic modifiers. A: ACN/20 mM NH_4OAc aq., B: MeOH/20 mM NH_4OAc aq.

	Nucleodur Sphinx				LiChrospher Select B				Multospher RP18			
	k	α	R_S	A_S	k	α	R_S	A_S	k	α	R_S	A_S
					95% A; 1 mL/min							
1a	3.33	0.00	0.00	2.45	4.43	0.00	0.00	3.11	3.09	0.00	0.00	3.61
1d	4.30	1.29	3.52	1.76	5.58	1.26	2.84	2.46	3.97	1.29	2.44	1.43
1b	4.81	1.12	1.68	2.09	6.07	1.09	1.10	2.41	4.41	1.11	1.29	0.85
1c	5.43	1.13	1.90	1.47	7.00	1.15	1.90	2.28	4.73	1.07	1.24	1.52
					48% B; 0.6 mL/min				50% B; 0.75 mL/min			
1d					11.00	0.00	0.00	2.95	13.84	0.00	0.00	2.97
1a					13.15	1.19	3.28	2.68	15.14	1.09	2.00	3.26
1b					14.77	1.12	1.94	4.21	18.09	1.19	3.18	4.99
1c					17.12	1.16	2.62	2.51	20.27	1.12	1.92	3.50

vided samples used for ESI-MS. For each isomer a molecular mass of 451.27 m/z was observed.

3. Results and discussion

Since each of the four possible isomers **1a**–**1d** consists of two aromatic rings as well as one C=O group enabling π–π interactions, we tested a bifunctional phenyl type column (Nucleodur Sphinx), consisting of propylphenyl and C_{18} spacers [14]. Because of the basic character of the analytes (tertiary amines) several columns were selected (LiChrospher Select B, Multospher FBS, Reprosil-Gold, Reprosil-Pur Basic, and Nucleosil AB) which are specially designated for the separation of basic analytes, due to their potential to reduce peak tailing. The LiChrospher Select B phase consists of C_8 alkyl chains, whereas free silanol groups are bidentate end-capped [14]. The Multospher FBS column is based on polymeric bound C_{18} chains resulting in completely covering of the silica surface, according to the provider [14]. Similar to this column, the Nucleosil AB column as a cross-linked type of polymer-coated C_{18} phases with 25% carbon content was used, giving increased shielding of the silica matrix and additionally demonstrating a distinct steric selectivity. This column is especially recommended for the separation of isomers [14]. The Reprosil-Gold and Reprosil-Pur Basic phases are base-deactivated by double bound end-capping resulting in carbon contents of 20 and 25%, respectively [14]. In order to compare the results obtained with these special columns, also a common end-capped ODS column (Multospher RP18) and a bare silica column (Multospher Si 100) were used.

In general, aqueous ACN and MeOH mixtures with NH_4OAc were tested as eluents.

3.1. RP columns

Based on this HPLC study the formation of all four possible azaspirovesamicol isomers **1a**–**1d** could be demonstrated. The separation was successful with three of the RP columns tested: (i) Nucleodur Sphinx, (ii) LiChrospher Select B, and (iii) Multospher RP18. Interestingly, best separations were achieved using an unusual high ACN concentration of 95% ACN/20 mM NH_4OAc aq. For all three columns almost the same resolution pattern with an elution order of **1a**–**1d**–**1b**–**1c** was observed (Table 1 and Fig. 2), although these columns belong to different classes of stationary RP-phases (phenyl-, base-deactivated and conventionally end-capped phase). Obviously, π–π interactions [15,16] at the Nucleodur Sphinx phase do not play a significant role in the separation under these conditions. With decreasing ACN content the resolution initially deteriorated, however at a certain ACN concentration a separation of the signals could be observed again. Interestingly, compared to the separation at high ACN content the elution order changed from **1a**–**1d**–**1b**–**1c** to **1d**–**1a**–**1b**–**1c** (see exemplarily the separation patterns on Multospher RP18 in Fig. 2). By contrast, no separation and decreased retention were observed when high concentrations of MeOH as organic modifier were used. However, reducing the MeOH content also resulted in a separation of the isomers showing a similar resolution pattern as observed at low ACN concentrations. Compared to ACN the resolution and selectivity of this separation is improved when MeOH was used, despite an increased peak tailing (see Fig. 2), which remained rather unaffected by increasing the buffer concentration.

Since two different resolution patterns are observed depending on the concentration of organic modifier, we assume that at least two separation mechanisms are involved in the retention process on these three stationary phases. Operating at low content of organic modifier, hydrophobic interactions between the analytes and the alkyl chains of the stationary phase mainly control the retention. However, at 95% ACN hydrophobic interactions should be rather low. Additionally to bound alkyl chains, silica provides also free silanol groups despite end-capping, which are able to contribute to the retention process of basic analytes [7–9,17,18]. The

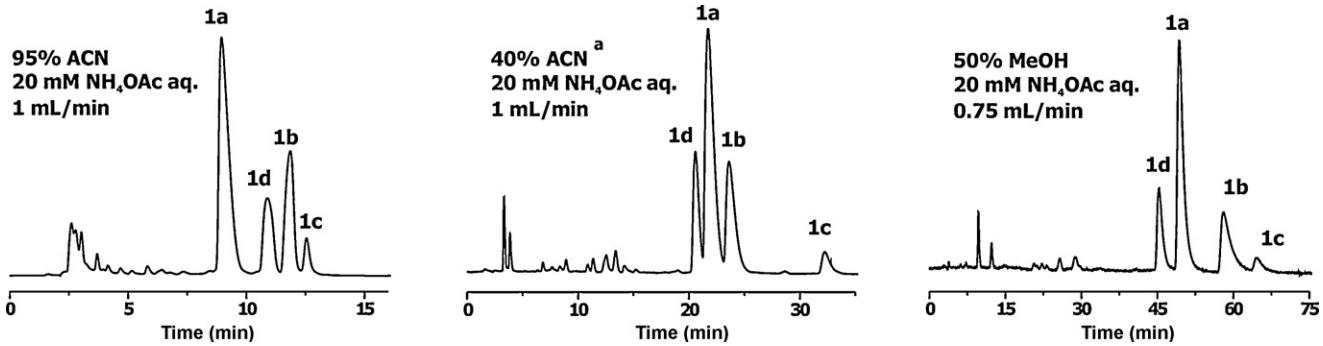

Fig. 2. Separation patterns of isomers **1a**–**1d** on Multospher RP18 column using different eluents. [a]Further reducing of ACN concentration resulted in deterioration of the resolution.

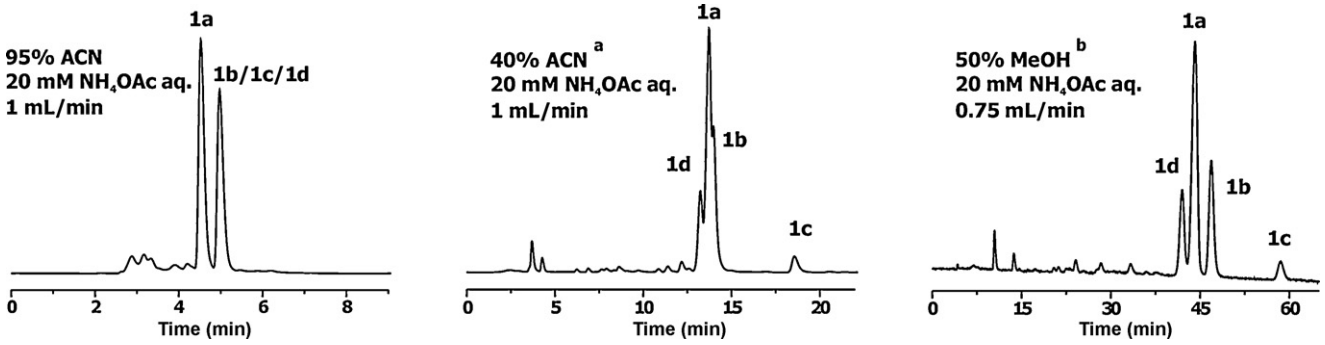

Fig. 3. Separation patterns of the isomers **1a**–**1d** on Reprosil-Gold column at different eluents. [a]Further reducing of ACN concentration resulted in complete overlay of **1d**, **1a**, and **1b**. [b]Further reducing of MeOH concentration resulted in overlay of **1d** and **1a**.

quantity of unbound silanol groups strongly depends on the particular process of end-capping and the alkyl binding technique. It is well known that silica acts as cation-exchanger depending on the pH value and the acidity of the silanol sites [9,19]. Since we used aqueous NH_4OAc with a pH value of 6.8 silanol groups are mostly ionized [20] as well as the analytes **1a**–**1d** (pK_a value for protonated vesamicol is 9.0 [21]). Therefore, cation-exchange interactions can occur between protonated analytes and ionized silanols, which are occupied by buffer NH_4^+ ions. Even if the pH value of the eluent is slightly higher due to the organic modifier, these interactions obviously determine the successful separation of the isomers at high ACN content. Working without buffer, the analytes strongly retain on the stationary phases by ionic binding to SiO^- groups. Thus, the retention of all isomers increases by a factor of 6–8 using 95% ACN/water and a flow rate of 1 mL/min, while maintaining the elution order of **1a**–**1d**–**1b**–**1c**.

To verify the assumption, that mainly cation-exchange is responsible for the separation of the isomers at 95% ACN on these three columns, we decreased the eluent pH by utilizing 0.05% (v/v) TFA instead of NH_4OAc. Under these acidic conditions the silanol groups are protonated and non-ionized, therefore, ionic interactions to the protonated, ionized analytes are impossible. As expected, at high ACN concentration no separation was observed, and the analytes passed through the columns almost without retention. Reducing the concentration of ACN to 30% caused a retention on the stationary phase even under acidic conditions, which obviously results from hydrophobic interactions. However, compared to the separation observed under neutral conditions (NH_4OAc), the isomers are poorly resolved. This could be an indicator for the contribution of cation-exchange to the separation process also at low ACN content under neutral conditions albeit to a lesser extent.

If cation-exchange interactions are responsible for the unusual retention and separation at high ACN concentration, why was this separation not observed by using MeOH? Compared to ACN, significantly decreased retention values were obtained. In contrast to ACN, MeOH is able to form hydrogen bonds. It is conceivable, that due to the formation of hydrogen bonds between MeOH and the silyl ether functionalities (generated by the coupling of the ODS or TMS groups to the silica), the analytes are hampered to bind on free silanol groups because of sterical effects. Thus, cation-exchange is almost suppressed and cannot contribute to the retention. Furthermore, at high concentrations of the organic modifier, aqueous MeOH is a stronger eluenting solvent than ACN, because when the water content decreases to below 28%, the surface tension of MeOH is lower than of ACN [22,23]. Therefore, in solutions with high MeOH content the analytes remain preferably within the mobile phase, whereas in solutions with high ACN content the analytes are forced to the stationary phase, where they can interact with the silanol sites. Additionally, it is known, that ACN solvates C_{18} alkyl chains rather well and therefore facilitates the access of analytes to residual silanol sites [24].

Our assumption, that cation-exchange mainly contributes to the separation process at high ACN content and does not occur using MeOH, explains also an interesting experimental finding. To reproduce separations obtained in ACN/20 mM NH_4OAc aq. we observed unusual long equilibration times of the phases, which was rather independence of the concentration of organic modifier. When the equilibration time was not sufficient, retention times increased considerably. This “slow column equilibration” is known to occur when analyzing ionizable compounds [25]. Using MeOH/20 mM NH_4OAc aq. the equilibration time was much shorter and the retention times were reproducible. Obviously, equilibration of SiO^- and NH_4^+ is quite low, which does not play an important role in MeOH since the silanol groups do not contribute to the separation process.

Although, the LiChrospher Select B column is recommended as a silanol-deactivated phase by the manufacturer, we assume that this stationary phase provides a considerable amount of residual silanol sites able to contribute to the separation process of ionizable analytes. This column may be indeed appropriate for the separation of certain basic analytes such as used in the Engelhardt test [26,27]. However, several reports confirmed our assumption and described the accessibility of residual silanol sites on this stationary phase [28–30]. According to the YMC Phase Selection Guide (YMC Europe GmbH), the LiChrospher Select B phase can be classified as a phase of middle silanol activity and low hydrophobicity, because of the C_8 alkyl chains.

Compared to the three columns discussed so far, the separation of the isomers was significantly inferior using the columns: Nucleosil AB, Multospher FBS, Reprosil-Gold, and Reprosil-Pur Basic. These stationary phases are characterized by high hydrophobicity (carbon load 20–25%) generated by strong covering of free silanol groups. Therefore, cation-exchange processes should be mainly suppressed. As a result, at 95% ACN only two signals and a decreased retention were observed. Isomer **1a** eluted in front of **1b**, **1c**, and **1d**, which were not separated (Fig. 3). At low ACN concentration an initiating separation was observed comparable to the first three columns indicating the dominance of hydrophobic interactions. Testing MeOH as organic modifier, almost the same resolution as for the columns discussed above could be obtained with a resolution pattern of **1d**–**1a**–**1b**–**1c**, which is also mainly based on hydrophobic interactions (see Fig. 3). A clear outlier of this series was the Multospher FBS column, generating separations which were characterized by unusual strong peak broadening which prevents a resolution both in ACN and in MeOH.

To study the retention behavior of azaspirovesamicol isomers on the different RP phases in dependence of eluent composition, $\log k$ values of isomer **1a** (representative for all isomers) were plotted as a function of the concentration of both ACN and MeOH (Fig. 4). Comparing the two organic modifiers, the $\log k$ val-

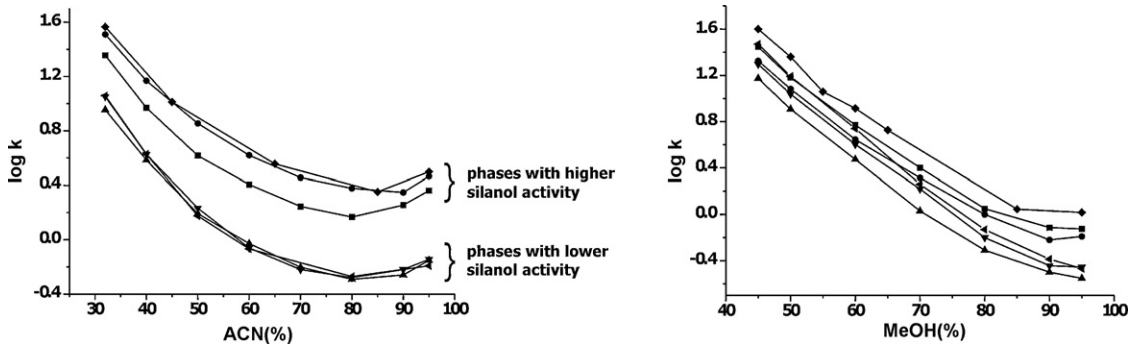

Fig. 4. Log k as a function of eluent composition on seven different phases; left: ACN/20 mM NH_4OAc aq., right: MeOH/20 mM NH_4OAC aq.; (♦) Nucleodur Sphinx, (●) LiChrospher Select B, (■) Multospher RP18, (▲) Nucleosil AB, (▼) Reprosil-Gold, (◄) Reprosil-Pur Basic.

ues decrease almost linear with increasing MeOH concentration, whereas a non-linear curve is observed for ACN. The latter one is an expression for a dual retention mechanism based on silanophilic and hydrophobic interactions. The U-shape of the curve is mainly caused by cation-exchange processes which increase with decreasing buffer concentration (with decreasing aqueous component the buffer concentration of the eluent decreases at this method of eluent preparation, see also experimental part and Section 3.2). This combined effect of cation-exchange interaction and reversed phase mechanism has been described in literature [9,19,31–34]. By contrast, the almost linear behavior observed in MeOH is mainly generated by a single retention mechanism based on hydrophobic interactions, since cation-exchange is almost suppressed.

Considering the strength of retention in ACN, significant differences between the three types of phases can be observed. Phases with higher silanol activity (Multospher RP18, Nucleodur Sphinx, and LiChrospher Select B) show considerably higher log k values than phases with lower silanol activity (Nucleosil AB, Reprosil-Gold, and Reprosil-Pur Basic), especially at high ACN content. With increasing water concentration the retention also increased, however, the differences between the log k values of the columns are diminished, because the silanol activity decreases whereas the influence of hydrophobic interactions increases. Using MeOH as organic modifier the differences between the column types are less pronounced, because cation-exchange processes are almost suppressed and therefore contribute only marginal to the retention.

3.2. Bare silica column

To further demonstrate the positive contribution of silanol site interactions to the separation of the azaspirovesamicol isomers **1a**–**1d**, bare silica as adsorbent (Multospher 100 Si column) was tested using reversed phase conditions. Several reports described this approach to be very efficient especially for the separation of organic amines [35–38]. Different retention mechanisms are discussed in the literature, such as cation-exchange, dipole interactions, hydrogen bonding, and adsorption due to hydrophobic interactions [33,39]. However, it is currently general opinion that the predominant retention mechanism of such analytes in buffered aqueous organic eluents is cation-exchange [9,39,40]. When typical HILIC conditions with ACN as organic modifier at concentrations of 97.5–70% are used [41], the formation of a water enriched layer on the silica surface is suggested [41–43]. This enables partitioning between the polar water phase and the hydrophobic mobile phase as additional retention mechanism.

Recently, McCalley [33] described the dependence of the contribution of cation-exchange on the buffer concentration (using ammonium formate as buffer and HILIC conditions of 90% ACN). Accordingly, with increasing salt concentration the retention decreased, because of the higher concentration of NH_4^+ ions competing with ionized analytes. To study this influence in the ammonium acetate system, we used two different methods of buffer preparation: Method A: The eluent was mixed from reservoir A containing 95% ACN and 5% aqueous 20 mM NH_4OAc and reservoir B containing 25% ACN and 75% aqueous 20 mM NH_4OAc. Method B: The eluent was mixed from reservoir A containing 1 mM (or 5 mM) NH_4OAc dissolved in 95:5 (v/v) ACN/water and reservoir B containing 1 mM (or 5 mM) NH_4OAc dissolved in 25:75 (v/v) ACN/water. Using the conventional method A, the buffer concentration in the mobile phase increase with increasing water content of the eluent. By contrast, a constant buffer concentration is achieved when method B is used.

Starting with method A at 95% ACN/20 mM NH_4OAc aq. (equates to 1 mM overall buffer concentration) a partial separation was observed similar to the RP phases (Fig. 5). Isomer **1a** (k = 8.23) could be separated well and eluted in front of the other isomers, whose elution order is slightly different compared to the RP phases (**1a**/**1b**/**1c** + **1d** versus **1a**/**1d**/**1b**/**1c**).

Compared to the RP phases, the retention on bare silica was considerably increased at high ACN concentrations, which can be attributed to partition processes as additional retention mechanism known to occur under such HILIC conditions. Interestingly, this retention decreased drastically with increasing water concentration, thus already at 75% ACN/20 mM NH_4OAc aq. (equates to 5 mM overall buffer concentration) the separation deteriorated ($k_{(\mathbf{1a}–\mathbf{1d})}$ = 1.8–2.0). This retention behavior is typically for HILIC separations, however the extent is rather unusual. At 75% ACN/20 mM NH_4OAc aq. a significant retention would be expected because of cation-exchange, which is, however, obviously considerably suppressed at this mobile phase composition. It is conceivable, that this unusual behavior is caused by the alteration of the buffer concentration, which changes from 1 mM at 95% ACN to 5 mM at 75% ACN/20 mM NH_4OAc aq. resulting in the reduction of retention observed. Testing method B with an overall buffer concentration of 1 and 5 mM confirmed this assumption, because at 1 mM NH_4OAc ov. 95:5 ACN/water and 5 mM NH_4OAc ov. 75:25 ACN/water almost the same chromatograms were obtained as with the corresponding method A mixtures of 95% ACN/20 mM NH_4OAc aq. and 75% ACN/20 mM NH_4OAc aq., respectively. Fig. 6 shows the dependence of retention on the buffer concentration. At 1 mM overall buffer concentration log k values are considerably higher than at 5 mM, independent of the ACN content. By contrast, log k values obtained with method A (20 mM NH_4OAc aq.) are highly dependent on the eluent composition, because with increasing ACN the buffer concentration decreased and the retention increased. The corresponding curve (▲) draws through the 1 (■) and 5 mM (●) concentration curves almost exactly at these ACN contents in which the buffer concentrations are equal.

Compared to ACN, the retention was significantly decreased using 95% MeOH/20 mM NH_4OAc aq. (method A) and slight dif-

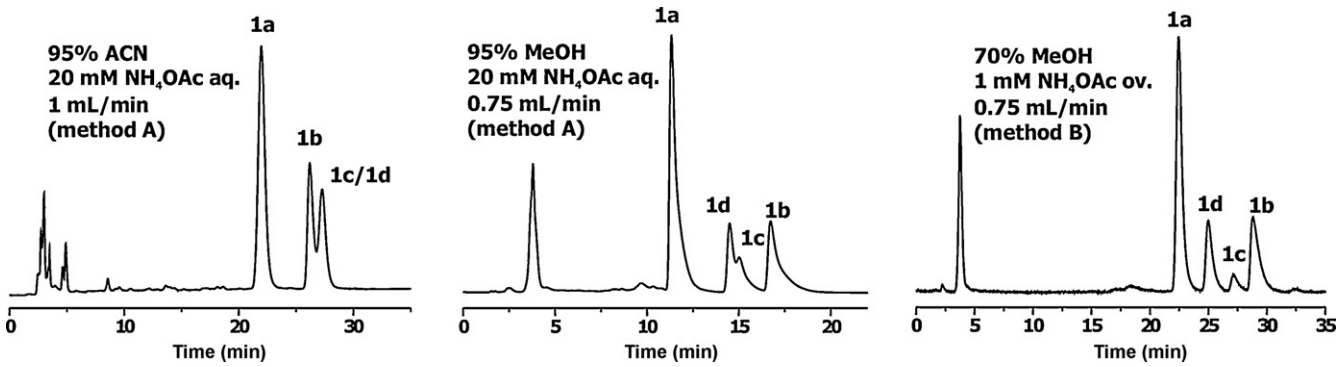

Fig. 5. Separation patterns of the isomers **1a**–**1d** on the bare silica column Multospher 100 Si using differently buffered ACN and MeOH as organic modifier.

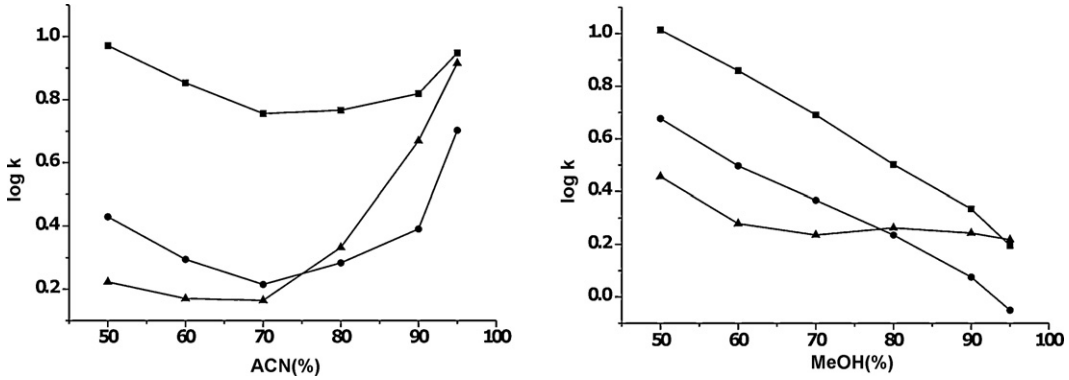

Fig. 6. Log k as a function of eluent composition on the bare silica column Multospher 100 Si; (■) 1 mM NH_4OAc ov., (●) 5 mM NH_4OAc ov. (method B, constant buffer concentration); (▲) 20 mM NH_4OAc aq. (method A, varying buffer concentration).

ferences in the elution order were observed (see chromatogram in Fig. 5). These differences especially at high concentrations of organic modifier were expected, because of the ability of MeOH to form hydrogen bonds preventing the formation of a water enriched layer as discussed for ACN. Thus, partitioning as additional retention mechanism is not possible.

In contrast to the RP phases, which were not suitable to separate the isomers at higher MeOH concentrations, with the silica column we observed a baseline separation at 70% MeOH/1 mM NH_4OAc ov. (method B) with different elution order compared to the RP columns (Fig. 5). Obviously, this change in elution order is caused by cation-exchange processes and confirms our assumption, that on RP phases MeOH hampers the accessibility of silanol sites by its binding to silyl ether functionalities via hydrogen bonding. These silyl ether groups are not available on the bare silica column, resulting in the separation of **1a**–**1d**.

Testing this phase without NH_4OAc resulted in very strong retention of the analytes. A more than tenfold increase of k was observed using 90% ACN or MeOH/water, which indicates the strength of interactions with the silanol groups. Expectedly, the analytes passed the column almost without retention and separation when the pH value of the eluent was decreased using 0.05% (v/v) TFA.

The diagrams in Fig. 6 illustrate not only the dependence of retention on the buffer concentration but also quite impressive on the kind of organic modifier. The curves of 1 and 5 mM overall buffer concentration are non-linear when ACN was used, probably caused by the contribution of opposite interaction mechanism. Starting at 50% ACN the log k values decrease linearly at increasing ACN content and are comparable to the MeOH curves. For both organic modifiers decreasing hydrophobic and constant cation-exchange interactions contribute to the retention. However, in the range of 70–95% ACN the log k values increase considerably. This can be attributed to partitioning processes as additional interaction mechanism which obviously increases with increasing ACN content. Thus, at least three interaction mechanisms contribute to the retention: (i) constant cation-exchange, (ii) hydrophobic interactions which decrease with increasing ACN content, and (iii) partitioning processes starting at ~70% ACN. By contrast, in MeOH the log k values decrease linearly at increasing MeOH content, because only constant cation-exchange and decreasing hydrophobic interactions contribute to the retention.

4. Summary and conclusion

The separation of four basic azaspirovesamicol isomers (**1a**–**1d**) was achieved on different stationary RP phases and two separation patterns were observed. Using ACN/20 mM NH_4OAc aq. a separation with an elution order of **1a**–**1d**–**1b**–**1c** was obtained exclusively on columns with higher silanol activity at unusual high concentration of organic modifier. Cation-exchange is supposed as main retention mechanism for this separation. By contrast, using MeOH as organic modifier a separation with an elution order of **1d**–**1a**–**1b**–**1c** was observed on both, columns with higher and columns with lower silanol activity. This separation is mainly based on hydrophobic interactions.

Additionally, bare silica as stationary phase was tested under RP conditions, providing cation-exchange as main retention mechanism. For the first time, the isomers were separated also at higher MeOH content and aqueous NH_4OAc, resulting in an elution order different compared to the RP phases. A strong retention was observed with ACN as organic modifier, which could be attributed to partition processes as additional retention mechanism. Further-

more, the dependence of the retention of **1a**–**1d** on the buffer concentration was demonstrated on the bare silica column. Utilizing method A, the buffer concentration increases with decreasing content of organic modifier, since the buffer was added only to the water part of the mobile phase. This conventional method can be beneficial, when in addition to cation-exchange also strong hydrophobic interactions contribute to the retention process (e.g. basic analytes on RP phases). Thus, at high content of organic modifier, cation-exchange governs the retention; whereas at low content of organic modifier hydrophobic interactions predominate. Due to these two opposite effects, quite balanced retention times can be achieved over the entire area of eluent composition. By contrast, utilizing method B with an overall constant buffer concentration the retention time can increase drastically, when the buffer concentration is low, which is, however, often necessary to achieve appropriate separations of ionizable analytes. Method B can be beneficial for studying retention mechanisms of ionizable analytes, because undesirable effects based on changes of buffer concentration cannot influence the retention.

In conclusion, we found that accessible silanol sites enabled the successful separation of four azaspirovesamicol isomers on RP columns in ACN, resulting in separation parameters superior compared to the hydrophobic separation observed in MeOH. Therefore, silanol groups should not only be considered as negative side effect but also as advantage for the separation of ionizable analytes.

Acknowledgements

We thank Dr. A. Hiller for providing the MS-data. This project was supported by a grant from the German Research Foundation (WE 2927/1-2).

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