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The effect of the process variables on the HPLC separation of tricyclic neuroleptics on a calixarene-bonded stationary phase

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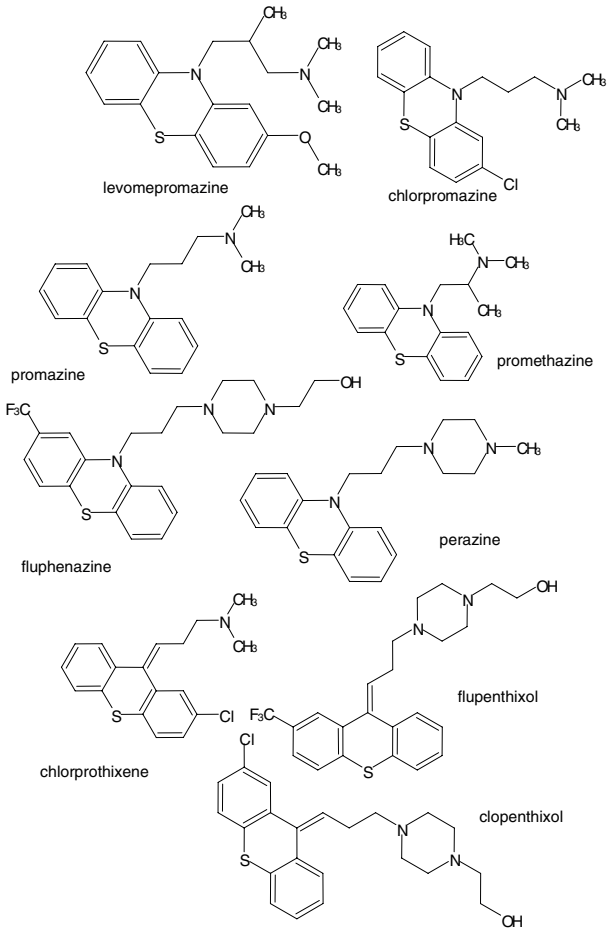
The chromatographic behavior of a new HPLC-stationary phase with supramolecular selectors on the basis of calixarenes is described for the separation of nine tricyclic neuroleptics. The effects of different chromatographic conditions (buffer system, pH-value, type and content of organic modifier, injection volume) on the separation of the analytes were studied. Additionally, the effect of structural differences of the neuroleptic analytes was studied. The chemical structure and pK_a of the neuroleptics highly influenced their separation on the calix[8]arene phase. The separation of all analytes on the investigated calixarene-bonded stationary phase was possible with a mobile phase of acetonitrile with 30 mM ammonium acetate buffer (pH 3.5) 30 : 70(v/v) using 1 ml/min flow rate.

1. Introduction

Neuroleptics which are widely used for the treatment of psychological problems are basic compounds, mainly thioxanthene and phenothiazine derivatives. Many papers describe the separation of this pharmacological group of analytes on the usual RP-stationary phases (Goldstein and Van Vunakis 1981; Kountourellis and Markopoulou 1991; Tracqui et al. 1992; Bagli et al. 1994; Karpinska and Starczewska 2002; Mizuno et al. 2002). Matsuda et al. (1979) used porous polymer resin for this separation. To our knowledge nobody has analysed neuroleptics using calixarene stationary phases up to now, except the separation of geometrical isomers of thioxanthene neuroleptics which was achieved by our group (Sokoließ et al. 2002). Furthermore, we reported about studies for evaluation of hydrophobic properties of calixarene stationary phases (Sokoließ et al. 2000).
The advances in packing materials, which have some differences than the usual RP stationary phases, may be considered as one of the main reasons causing the recent rapid development in HPLC. Calixarene stationary phase is one of these packing materials, which are macrocyclic molecules composed of phenol units linked by alkylidene groups. They belong to the class of [1 n] cyclophanes (Gutsche and Muthukrishnan 1978; Böhmer and McKervey 1991). They are able to build reversible complexes with metals and organic molecules. Nowadays, calixarene stationary phases have several applications in HPLC (Park et al. 1993; Glennon et al. 1994; Friebe et al. 1995; Brindle et al. 1996; Glennon et al. 1996; Lee et al. 1997; Gebauer et al. 1998a, 1998b; Healy et al. 1998; Kalchenko et al. 1998; Menyes et al. 1999).

2. Investigations, results and discussion

The nine neuroleptics used in this study can be classified into two categories according to the chemical structure as



Structure of the analytes

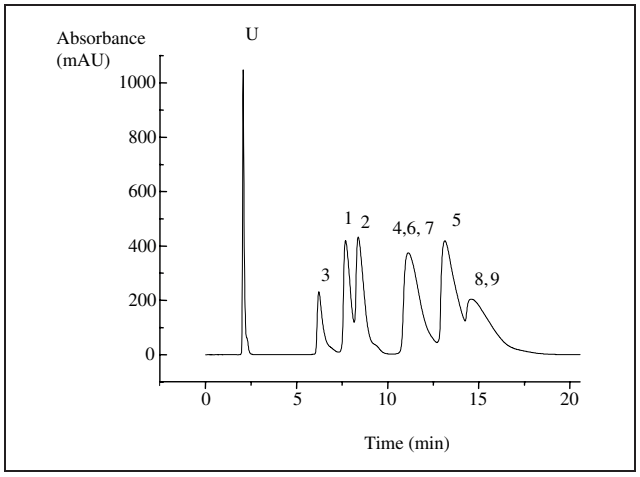


Fig. 1: Separation of neuroleptics I
Analytes: U-uracil 1-promethazine 2-promazine 3-perazine 4-levomepromazine 5-chlorpromazine 6-fluphenazine 7-clopenthixol 8-chlorprothixene 9-flupenthixol
Chromatographic conditions:
Mobile phase: ACN : 20 mM sodium dihydrogenphosphate buffer, pH 2.5, 35/65 (v/v), flow rate 1 ml/min, detection: 254 nm UV
Stationary phase: CALTREX AIII, Kromasil Si 100 (10 µ, 250 × 4 mm I.D.)

phenothiazines and thioxanthenes. Some of these phenothiazines have substitutions on the aromatic ring (fluphenazine, levomepromazine, chlorpromazine) and some have not any substitution (promethazine, promazine, perazine). These phenothiazines can be classified also structurally according to the nature of the side chain attached to the nitrogen atom of the heterocycle, some of these phenothiazines have a piperazine ring in the side chain (perazine, fluphenazine) and some have only one nitrogen atom in this side chain (promazine, promethazine, chlorpromazine, levomepromazine). The thioxanthenes clopenthixol, flupenthixol, chlorprothixene all have a substitution on the aromatic ring but only chlorprothixene has a side chain with one nitrogen atom while the other two thioxanthenes have a piperazine ring.

2.1. Influence of the pH-value on the chromatographic separation

It is well known that strong bases must be analysed in buffered mobile phases, otherwise variable ionisation of these bases can happen at different points in the peak, dependent on the local concentration, leading to peak distortion (Snyder and Kirkland 1979). The effect of pH was

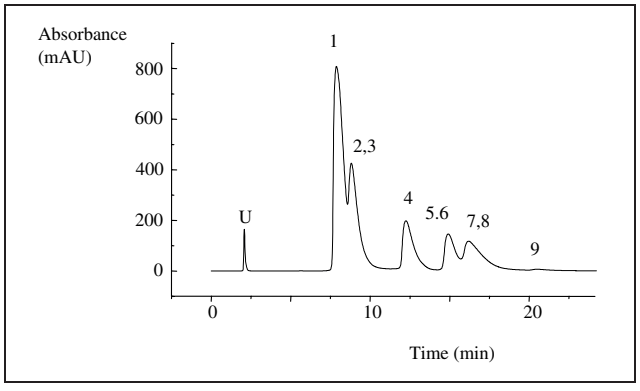


Fig. 2: Separation of neuroleptics II
Chromatographic conditions: see Fig. 1 (except pH-value: 3,0)

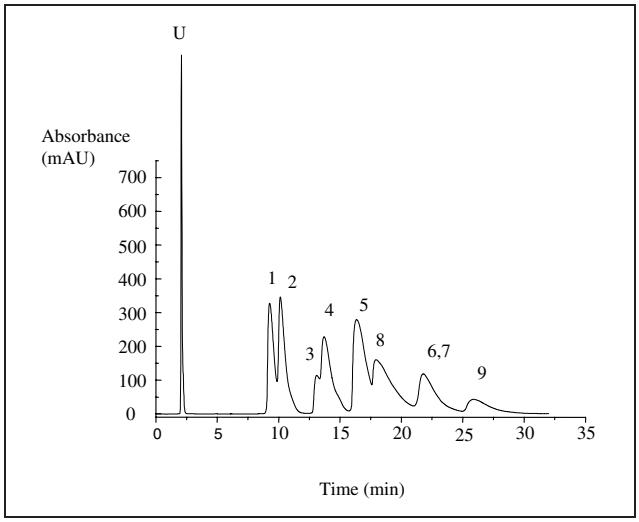


Fig. 3: Separation of neuroleptics III
Chromatographic conditions: see Fig. 1 (except pH-value: 3,5)

studied in the presence of acetonitrile or methanol as organic modifier. Using acetonitrile we have analysed the neuroleptics under 3 different pH-values: 2.5, 3.0 and 3.5 (Figs. 1, 2 and 3). Decrease in pH resulted in decreasing retention times.

At a pH-value of 3.5 all pairs could be separated except fluphenazine/clopenthixol. At pH 3.0 promazine/perazine, fluphenazine/chlorpromazine, clopenthixol/chlorprothixene could not be separated while at a pH-value 2.5 chlorprothixene/flupenthixol and levomepromazine/fluphenazine/clopenthixol could not be separated.

The order of the retention changed from one pH-value to another. At a pH-value of 3.5 in contrast to pH 2.0 and 2.5 eight analytes were separated.

In dependence on the chemical structure of the analytes we find the following order of retention: promethazine < promazine < levomepromazine < chlorpromazine. This may be due to promethazine has a ramify side chain in contrast to promazine. The side chain in levomepromazine is longer than in promethazine and the methoxy ring substituent is responsible for the more intensive interaction with the stationary phase. Levomepromazine has a methoxy group instead of the chloride ligand in chlorpromazine. It is obvious that the substitution on the phenothiazine ring system is responsible for the strength of the interaction with the cavity of the calix[8]arene (the formed temporary inclusion complex is more stable).

All compounds without a piperazine ring were retained shorter than those having a piperazine ring. The interactions of these analytes with calixarene are diminished in comparison with those of the analytes with a heterocycle in the side chain.

Perazine differs from promazine in that it has a N-methylpiperazine instead of a N,N-dimethylamino group in promazine. Perazine is sterical demanding and more basic than promazine, and so perazine will be affected by pH changes more than promazine.

At pH 3.5 promazine has been separated before perazine. At pH 3.0 both phenothiazine derivatives were eluted together, while at pH 2.5 perazine retained shorter than promazine. The higher basicity of perazine seems to be the main cause of this behavior.

Fluphenazine has a phenothiazine structure while flupenthixol is a thioxanthene derivative. Fluphenazine (pKa 7.21) is more basic than flupenthixol (pKa 6.92). At

Table 1: Influence of buffer type and pH on separation of neuroleptics

Analyte	NaH_2PO_4 pH 2.5			NaH_2PO_4 pH 3.5			NH_4Ac pH 2.5			NH_4Ac pH 3.0			NH_4Ac pH 3.5		
	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α
Promethazine	10.10	3.12		11.83	3.84		7.07	1.90		8.93	2.65		10.37	3.27	
Promazine	12.11	3.94	1.26	14.42	4.90	1.28	8.12	2.33	1.23	10.34	3.23	1.22	11.92	3.99	1.22
Levomepromazine	16.22	5.62	1.43	19.07	6.80	1.39	10.04	3.13	1.34	12.95	4.30	1.33	14.91	5.14	1.29
Chlorpromazine	20.30	7.29	1.30	24.84	9.16	1.35	11.86	3.87	1.24	15.76	5.45	1.27	18.80	6.75	1.31
Chlorprothixene	22.32	8.12	1.11	27.83	10.38	1.13	11.86	3.87	1.00	15.76	5.45	1.00	20.29	7.36	1.09
Perazine	22.32	8.12	1.00	27.83	10.38	1.00	11.86	3.87	1.00	15.76	5.45	1.00	20.29	7.36	1.00

Mobile phase: MeOH/10 mM buffer (55/45, v/v)
flow rate: 1 ml/min, detection: 254 nm UV
Stationary phase: CALTREX® Alll, Kromasil Si 100 (10 µ, 250 × 4 mm I.D.

Table 2: Influence of buffer concentration on separation of neuroleptics

Analyte	buffer concentration (mM) 20				30				50			
	t_R (min)	k′	α	R	t_R (min)	k′	α	R	t_R (min)	k′	α	R
Promethazine	9.27	3.46			9.05	3.34			9.01	3.33		
Promazine	10.14	3.88	1.12	1.59	9.87	3.74	1.12	1.43	9.80	3.71	1.11	1.52
Perazine	13.11	5.31	1.37	5.85	12.33	4.91	1.32	3.83	12.00	4.77	1.29	3.70
Levomepromazine	13.71	5.59	1.05	0.90	13.42	5.44	1.11	1.41	13.40	5.44	1.41	1.96
Chlorpromazine	16.35	6.86	1.23	2.70	16.25	6.79	1.25	3.17	15.98	6.68	1.23	2.86
Chlorprothixene	17.94	7.63	1.11	1.26	18.00	7.63	1.12	1.60	17.89	7.60	1.14	1.76
Fluphenazine	21.78	9.48	1.24	2.65	20.73	8.94	1.17	1.98	20.32	8.77	1.15	1.89
Clopenthixol	21.78	9.48	1.00	–	20.73	8.94	1.00	–	20.32	8.77	1.00	–
Flupenthixol	25.89	11.45	1.21	2.48	24.65	10.82	1.21	2.42	24.12	10.60	1.21	2.52

Mobile phase: ACN/NaH_2PO_4 buffer, pH 3.5 (35/65, v/v)
(other conditions: see Table 1)

Table 3: Influence of buffer concentration on the separation of neuroleptics

Analyte	NaH_2PO_4 buffer (mM) 5			10			20			50		
	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α
Promethazine	9.87	2.99		10.10	3.12		12.10	3.93		12.58	4.13	
Promazine	11.93	3.82	1.28	12.11	3.94	1.26	14.71	5.00	1.27	15.16	5.18	1.25
Levomepromazine	16.26	5.57	1.46	16.22	5.62	1.43	20.19	7.23	1.45	20.68	7.43	1.44
Chlorpromazine	19.91	7.05	1.27	20.30	7.29	1.30	24.91	9.16	1.27	25.94	9.58	1.29
Chlorprothixene	21.72	7.78	1.10	22.32	8.12	1.11	27.43	10.19	1.11	28.18	10.49	1.10
Perazine	21.72	7.78	1.00	22.32	8.12	1.00	27.43	10.19	1.00	28.18	10.49	1.00

Mobile phase: MeOH/Na_2PO_4 buffer, pH 2.5 (55/45, v/v)
(other conditions see Table 1)

pH 3.5 and 3.0 flupenthixol has been retained longer. At pH 2.5 both are eluted together.

From the results it is obvious that phenothiazines have been separated before thioxanthenes (although there are exceptions). Under certain conditions chlorprothixene (thioxanthene derivative) is eluted before fluphenazine (phenothiazine derivative). This unexpected behavior is perhaps due to the absence of a piperazine ring in chlorprothixene. The results showed also that under the change in pH-values the neuroleptics which contain a piperazine ring were affected more than the others. All previous points suggest that the lone pairs of electrons of nitrogen atoms in the piperazine ring have certain effects on the formation of the temporary complex between calixarene and the tricyclic neuroleptics.

Using methanol as organic modifier, isocratic separation unfortunately gave highly broad peaks for fluphenazine, clopenthixol and flupenthixol. At low pH-values acetonitrile tends to give higher efficiency than methanol and in absence of high silanol effects (most silanol groups are unionised at low pH) acetonitrile led to a decrease in viscosity and hence improve solute diffusivity (Snyder and Kirkland 1979).

We tested the pH-values 2.5, 3.0 and 3.5 (Table 1) and the retention order is the same with all these pH-values (in contrast to acetonitrile). The separation of chlopromazine/chlorprothixene/perazine was not successful at pH 2.5 and 3.0, while at pH 3.5 chlorpromazine could be separated from the other two substances. Increase in pH resulted in increasing retention. So we used a pH-value of 3.5 for both organic modifiers for all further investigations.

Table 4: Influence of buffer concentration on separation of neuroleptics

Analyte	NH_4Ac buffer (mM) 10			30			50		
	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α
Promethazine	10.46	3.27		11.63	3.69		10.87	3.39	
Promazine	12.02	3.91	1.20	13.59	4.48	1.21	12.57	4.08	1.20
Levomepromazine	15.14	5.18	1.33	17.48	6.05	1.35	15.96	5.44	1.34
Chlorpromazine	18.91	6.72	1.30	22.23	7.96	1.32	20.23	7.17	1.32
Chlorprothixene	20.62	7.42	1.10	24.42	8.85	1.11	22.49	8.08	1.13
Perazine	20.62	7.42	1.00	24.42	8.85	1.00	22.49	8.08	1.00
Fluphenazine	25.99	9.62	1.30	26.06	9.51	1.08	26.04	9.51	1.18
Flupenthixol	27.11	10.07	1.05	28.56	10.52	1.11	27.63	10.15	1.07
Clopenthixol	28.24	10.54	1.05	29.92	11.06	1.05	28.97	10.70	1.05

Mobile phase: Gradient A–MeOH B–NH_4Ac buffer, ph 3.5

time/min	A%	B%
0–23	55	45
> 23	70	30

(other conditions: see Table 1)

2.2. Buffer concentration

It is well known that increasing the buffer concentration decreases retention time due to the competitive interaction of the buffer cation with the residual silanols (Snyder et al. 1997 b).

Using acetonitrile as organic modifier we performed the investigations with sodium dihydrogenphosphate buffer (pH 3.5) at different concentrations (20, 30, 50 mM) (Table 2). The change of the buffer concentration led to weak changes in the retention times of the analytes. This may be due to endcapped manner of the used column.

For all pairs of analytes an increase of the buffer concentration led to decrease in α values except for perazine/levomepromazine which had α values of 1.05, 1.11 and 1.41 at 20, 30 and 50 mM buffer, respectively.

With methanol we analyzed six analytes using isocratic conditions with sodium dihydrogenphosphate buffer at concentrations of 5, 10, 20 and 50 mM (Table 3). The results are opposite to the expected, increase of the buffer concentration resulted in increase in retention times. Chlorprothixene and perazine could not be separated under all buffer concentrations used and with methanol.

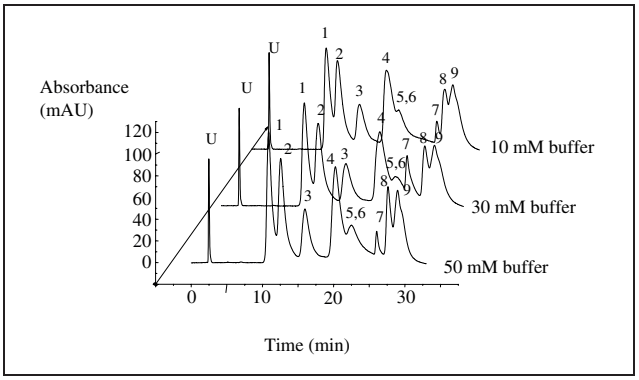


Fig. 4: Separation of neuroleptics IV
Analytes: U-uracil 1-promethazine 2-promazine 3-levomepromazine 4-chlorpromazine 5-chlorprothixene 6-perazine 7-fluphenazine 8-flupenthixol 9-clopenthixol
Chromatographic conditions: see Fig. 1 except mobile phase:
Gradient:

Time (min)	Methanol (%)	10, 30 or 50 mm NH_4AC buffer (pH 3.5) (%)
0–23	55	45
23	70	30

Under gradient conditions (with methanol) the retention times increased with the increase of ammonium acetate concentration from 10 mm to 30 mM, then decreased when the concentration increased from 30 mM to 50 mM.

Also at all these concentrations using gradient elution we could not achieve a separation between chlorprothixene and perazine. But we could separate the peaks of fluphenazine, flupenthixol and clopenthixol with a good selectivity (α 1.05–1.30) in contrast to the broad peaks under isocratic conditions. With a 30 mM ammonium acetate buffer and methanol the best separation of fluphenazine and flupenthixol resulted (Fig. 4, Table 5).

2.3. Buffer type

Canals et al. (2001) and Espinosa et al. (2002) found that the nature of the buffer can give rise to important changes in retention and peak shape. Some of these changes can be caused by difference in the effective pH which occurs when different buffers of the same pH are mixed in the same proportion with a given organic modifier.

With acetonitrile as organic modifier we performed the experiments with 60% NaH_2PO_4, NH_4Ac and KH_2PO_4 buffers of 30 mM concentration at pH 3.5 (Table 5). Also we used unbuffered mobile phase at pH 3.0 (H_3PO_4) and compared with the buffered one under the same conditions (Table 6).

All pairs of analytes were separated with a sodium dihydrogenphosphate buffer except fluphenazine/clopenthixol. We could not achieve a separation of fluphenazine/clopenthixol and perazine/levomepromazine with ammonium acetate. With potassium dihydrogenphosphate buffer the analytes fluphenazine, clopenthixol and chlorprothixene eluted together. This shows that the choice of the buffer type for neuroleptic separation on a silica-bonded calix[8]arene stationary phase is very important. The retention times for the analytes increased in the order of KH_2PO_4 buffer < NaH_2PO_4 buffer < NH_4Ac buffer. With unbuffered mobile phase, the retention times highly decreased but α values did not highly change except for chlorprothixene/flupenthixol (α is 1.30 with buffered mobile phase and 1.15 with unbuffered mobile phase). Separation of the nine analytes was also achieved with 70% 30 mM ammonium acetate buffer at pH 3.5 (Fig. 5).

Sodium dihydrogenphosphate 10 mM and ammonium acetate buffer were used at pH 2.5 and 3.5 (with methanol). At pH 2.5 sodium dihydrogenphosphate gave higher

Table 5: Influence of buffer type and injection volume on the separation of neuroleptics

Analyte	buffer type NH_4Ac*			NaH_2PO_4*			KH_2PO_4*			KH_2PO_4**		
	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α
Promethazine	6.81	2.30		6.40	2.13		6.27	2.07		6.41	2.16	
Promazine	7.30	2.54	1.10	6.81	2.33	1.09	6.61	2.24	1.08	6.76	2.34	1.08
Perazine	8.98	3.36	1.32	8.21	2.98	1.28	7.86	2.85	1.27	8.07	2.98	1.28
Levomepromazine	8.98	3.36	1.00	8.67	3.24	1.09	8.37	3.10	1.09	8.61	3.25	1.09
Chlorpromazine	10.79	4.24	1.26	9.96	3.87	1.20	10.01	3.91	1.26	10.29	4.07	1.26
Chlorprothixene	11.67	4.66	1.10	10.73	4.25	1.10	11.08	4.43	1.13	11.39	4.62	1.13
Clopenthixol	12.48	5.05	1.08	11.29	4.52	1.06	11.08	4.43	1.00	11.39	4.62	1.00
Fluphenazine	12.48	5.05	1.00	11.29	4.52	1.00	11.08	4.43	1.00	11.39	4.62	1.00
Flupenthixol	14.40	5.99	1.18	13.42	5.57	1.23	12.84	5.29	1.20	13.13	5.48	1.19

Mobile phase: ACN/30 nM buffer, pH 3.5 (60/40, v/v)
Injection volume: *: 10 µl and **: 5 µl
(other conditions: see Table 1)

retention times than ammonium acetate (Table 1) and the former could separate chlorpromazine from chlorprothixene and perazine while this was not possible using ammonium acetate buffer. At pH 3.5 sodium dihydrogenphosphate also gave higher retention times. But with both buffers we achieved a separation of chlorpromazine, chlorprothixene and perazine. Under both pH values the use of sodium dihydrogenphosphate led to higher separation factors than ammonium acetate.

2.4. Type and content of the organic modifier

We investigated different concentrations of acetonitrile (30, 35, 40%) (Table 7) and of methanol (55, 60, 65%) (Table 8). As expected increase in content of the organic modifier was followed by decreasing retention and this was also the case for α values. This result indicates that the used stationary phase behaves as a reversed phase packing and the hydrophobic interaction is one of the factors having a role in the retention of neuroleptics. With 60% and 65% methanol in the mobile phase chlorpromazine, chlorprothixene and perazine were eluted together, while with 55% methanol we found two peaks (chlorpromazine was separated from both the other compounds).

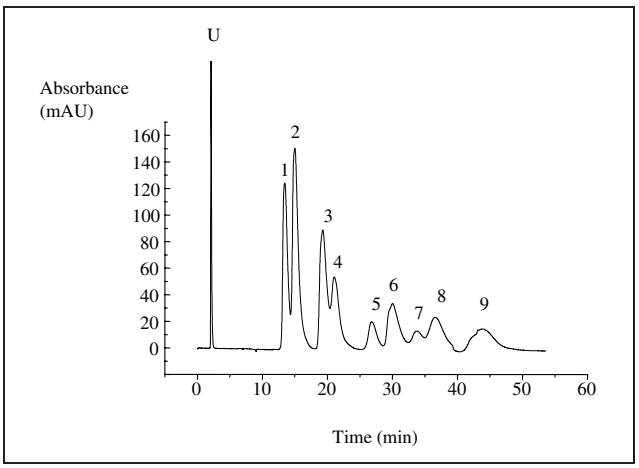


Fig. 5: Analytes: U-uracil 1-promethazine 2-promazine 3-perazine 4-levomepromazine 5-chlorpromazine 6-chlorprothixene 7-clopenthixol 8-fluphenazine 9-flupenthixol
Chromatographic conditions:
Mobile phase: ACN : 30 mM NH_4AC buffer, pH 3.5 (30/70, v/v), flow rate: 1 ml/min
Other conditions: see Fig. 1

Our results indicate that there is a difference between acetonitrile and methanol efficiencies as organic modifiers for neuroleptic separation on the calix[8]arene-bonded phase. At low pH acetonitrile gives better results than methanol as mentioned before (Snyder and Kirkland 1979). Bliesner and Sentell (1993) have suggested that different modifiers solvate the alkyl ligands of the stationary phase to different extents, leading to variations in the degree of penetration of the phase.
Kunsági-Máté et al. (2002) suggested that the solvent alters the stability of the complex between calixarene and drug. Since the solvation energies are proportional to the solvent permittivities, a solvent of high permittivity leads to a decreased stability of the host-guest complexes.

2.5. Injection volume

5 µl and 10 µl of the sample were injected (Table 5). The results show that decrease the injection volume resulted in increased retention time but α values did not significantly change. It was suggested by Cox and Snyder (1989) and Snyder et al. (1997a) that the initially adsorbed charged molecules discourage further adsorption of sample molecules of the same charge (mutal repulsion effect).

2.6. Flow rate

We investigated the use of 30% acetonitrile and 70% 30 mM NH_4Ac buffer (pH 3.5) under two different flow

Table 6: Influence of buffer presence or absence on separation of neuroleptics

Analyte	mobile phase (pH 3,0): ACN/20 mM NaH_2PO_4			ACN/H_3PO_4		
	t_R (min)	k′	α	t_R (min)	k′	α
Promethazine	7.88	2.78		5.39	1.59	
Promazine	8.82	3.23	1.16	5.99	1.87	1.18
Perazine	8.82	3.23	1.00	5.99	1.87	1.00
Levomepromazine	12.26	4.88	1.51	7.81	2.74	1.46
Chlorpromazine	14.91	6.16	1.26	9.25	3.44	1.25
Fluphenazine	14.91	6.16	1.00	9.25	3.44	1.00
Clopenthixol	16.20	6.78	1.10	10.43	4.00	1.17
Chlorprothixene	16.20	6.78	1.00	10.43	4.00	1.00
Flupenthixol	20.50	8.84	1.30	11.69	4.60	1.15

Mobile phase: ACN/buffered or unbuffered aqueous phase, pH 3.0 (35/65, v/v)
(other conditions: see Table 1)

Table 7: Influence of ACN content in mobile phase on separation of neuroleptics

Analyte	ACN (%) 30				35				40			
	t_R (min)	k′	α	R	t_R (min)	k′	α	R	t_R (min)	k′	α	R
Promethazine	15.04	6.02			9.05	3.34			6.40	2.13		
Promazine	17.01	6.94	1.15	2.29	9.87	3.74	1.12	1.43	6.81	2.33	1.09	1.20
Perazine	22.50	9.50	1.37	5.28	12.33	4.91	1.32	3.83	8.21	3.02	1.29	4.06
Levomepromazine	24.90	10.62	1.12	1.83	13.42	5.44	1.11	1.41	8.67	3.24	1.08	1.16
Chlorpromazine	31.19	13.55	1.28	3.98	16.25	6.79	1.25	3.17	9.96	3.87	1.19	2.38
Chlorprothixene	35.16	15.40	1.14	1.99	18.00	7.63	1.12	1.60	10.73	4.25	1.10	1.60
Fluphenazine	46.43	20.66	1.34	4.37	20.73	8.94	1.17	1.98	11.29	4.52	1.06	0.79
Clopenthixol	46.43	20.66	1.00	–	20.73	8.94	1.00	–	11.29	4.52	1.00	–
Flupenthixol	58.51	26.30	1.27	4.25	24.65	10.82	1.21	2.42	13.42	5.56	1.23	2.19

Mobile phase: ACN/30 mM Na_2PO_4 buffer, pH 3.5
(other conditions: see Table 1)

Table 8: Influence of MeOH content in mobile phase on separation of neuroleptics

Analyte	MeOH content (%) 55			60			65		
	t_R (min)	k′	α	t_R (min)	k′	α	t_R (min)	k′	α
Promethazine	11.83	3.84		8.48	2.50		6.59	1.73	
Promazine	14.42	4.90	1.28	10.02	3.13	1.25	7.56	2.14	1.23
Levomepromazine	19.07	6.80	1.39	12.68	4.23	1.35	9.05	2.76	1.29
Chlorpromazine	24.84	9.16	1.35	15.62	5.44	1.29	10.86	3.51	1.27
Chlorprothixene	27.83	10.38	1.13	15.62	5.44	1.00	10.86	3.51	1.00
Perazine	27.83	10.38	1.00	15.62	5.44	1.00	10.86	3.51	1.00

Mobile phase: MeOH/10 mM NaH_2PO_4 buffer, pH 3.5
(other conditions: see Table 1)

rates (1 ml/min and 2 ml/min). The results indicated that retention decreased while k′ and α values were not considerably affected (Table 9).

3. Experimental

3.1. Chemicals

Acetonitrile (ACN) was HPLC grade and purchased from LGC Promochem (Wesel, Germany). HPLC grade methanol (MeOH) was purchased from Mallinckrodt Baker B.V. (Deventer, Netherland). Sodium hydroxide, acetic acid, phosphoric acid, potassium hydroxide, ammonium hydroxide, sodium dihydrogenphosphate, potassium dihydrogenphosphate and ammonium acetate were purchased from Merck KgaA (Darmstadt, Germany).

Table 9: Effect of flow rate on separation of neuroleptics

Analyte	flow rate 1 ml/min			2 ml/min		
	t_R (min)	k′	α	t_R (min)	k′	α
Promethazine	13.46	5.37		6.65	5.19	
Promazine	15.01	6.11	1.14	7.42	5.91	1.14
Perazine	19.30	8.14	1.33	9.58	7.92	1.34
Levomepromazine	21.08	8.98	1.10	10.48	8.76	1.11
Chlorpromazine	26.81	11.70	1.30	13.31	11.39	1.30
Chlorprothixene	30.02	13.21	1.13	14.76	12.75	1.12
Clopenthixol	33.74	14.98	1.13	16.84	14.68	1.15
Fluphenazine	36.61	16.33	1.09	18.23	15.97	1.09
Flupenthixol	43.79	19.74	1.21	21.90	19.39	1.21

Mobile phase: ACN/30 mM NH_4Ac buffer, pH 3.5 (30/70, v/v)
(flow rate: 1 or 2 ml/min; other conditions: see Table 1)

Analytes: promethazine, promazine, perazine, levomepromazine, chlorpromazine, chlorprothixene, clopenthixol, flupenthixol, fluphenazine were kindly supplied by Tropon (Cologne, Germany).

3.2. Equipment

HPLC: HP1090II model equipped with a diode array detection (DAD) system (Hewlett Packard, Waldbronn, Germany). pH-Meter: Knick Elektronische Messgeräte GmbH & Co. (Berlin, Germany).

3.3. Column

CALTREX® AIII column (250 × 4 mm I.D.) was kindly supplied by Synaptec GmbH (Greifswald, Germany). The calixarene stationary phase contains silica-bonded calix[8]arene (basic silica: endcapped Kromasil Si 100 A° pore diameter, 10 μm particles; manufacturer: EKA Chemicals (Bohus, Sweden).

3.4. Chromatography

The experiments were performed with isocratic (except one run which was gradient) elution. The binary mobile phase consisted of different proportions of ACN or MeOH in the buffered solution (see figures and tables). The eluents were degassed before running. In all cases, the column temperature was set at 40 °C. The hold-up times (t_0) were determined according to Sokoließ et al. (2003) from injections of uracil with UV detection at 254 nm in the mobile phase of each investigation.

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