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1,3-Alternate 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel as a new type of HPLC stationary phase

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Abstract

A new *1,3-alternate* 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phase (*1,3-Alt* CalixBn) has been prepared and used for the separation of aromatic positional isomers by high-performance liquid chromatography (HPLC). The effect of organic modifier content and pH of the mobile phase on retention and selectivity of these compounds were studied. Application examples were provided for separation of purine and pyrimidine bases and non-steroidal anti-inflammatory drugs.

Keywords: High-performance liquid chromatography; Separation; *1,3-Alternate* calix[4]arene; Aromatic positional isomers; Purine and pyrimidine bases; Non-steroidal anti-inflammatory drugs

1. Introduction

The main goal of investigation of high-performance liquid chromatography (HPLC) is searching more efficient stationary phases and optimisation of the separation process. Reversed-phase chromatography (RPC) is the first choice for most regular samples. RPC is typically more convenient and rugged than other types of liquid chromatography (LC) and is more likely to result in a satisfactory final separation. Useful changes in selectivity and sample retention can be achieved by selecting appropriate type of stationary phase. There are many contemporary commercially available reversed-phase columns, e.g. RP-C_{18}, RP-C_8, RP-Phenyl and others. Recently, calixarenes also have attracted attention of many researchers because they are able to form reversible complexes with neutral as well as charged molecules [1–3]. In last years, the potential of this class of macrocyclic compounds has been shown for several applications in gas chromatography [4–7], capillary electrophoresis [8–11], solid-phase extraction [12] and overall high-performance liquid chromatography. Calix[*n*]arene-bonded ($n = 4, 5, 6, 8$) silica gel high-performance liquid chromatography stationary phases were used for separation of many inorganic and organic compounds, e.g. metal ions [13,14]; aromatic positional isomers [15–21]; uracil derivatives and estradiol epimers [17]; proline-containig peptides [22]; water soluble vitamins [23]; PAHs [18,20,24,25]; amino acid esters [25]; quinolones and sulphonamides [26]; nucleosides [16,27–29]; barbituric acids; benzoxepin and thioxanthene derivatives [25,30,31]. Several previous works have shown that calixarene-bonded stationary phases in a cone conformation are excellent in reversed-phase chromatography and exhibit promising application in HPLC. Recently, we have reported synthesis of new 25,27-dipropoxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phase in *1,3-alternate* conformation (*1,3-Alt* CalixPr) and we have confirmed that this phase possess high selectivity toward selected positional aromatic isomers [32]. In this paper, we described the synthesis of new *1,3-alternate* 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phase (*1,3-Alt* CalixBn) and its application to resolution of compounds of very similar structure (e.g. aromatic positional isomers), purine and pyrimidine bases as well as

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non-steroidal anti-inflammatory drugs. The efficiency of the column and the influence of pH of the mobile phase and organic modifier addition on retention and selectivity of chosen compounds were evaluated. The selectivity comparison of the novel phase to *1,3-Alt* CalixPr and ODS one has been investigated.

2. Experimental

2.1. Apparatus and chemicals

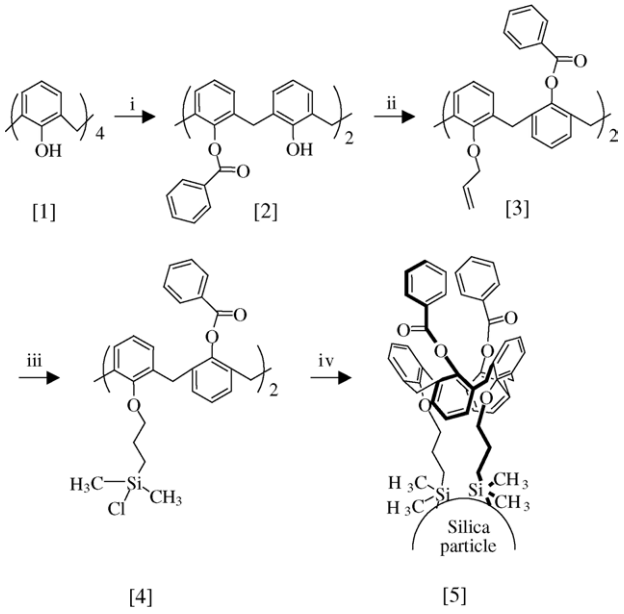
Chromatographic analyses were performed using liquid chromatograph Series 1100 (Agilent Technology Inc.) equipped with quaternary pump, autosampler, thermostated column compartment and diode-array detector. Elemental analyses were obtained on analyser Perkin-Elmer (PE 240). ^{1}H NMR spectra were recorded on Gemini 500 MHz spectrometer (Varian). Reactions were monitored by TLC on pre-coated silica gel plates (SiO_2, Merck, $60F_{254}$). Flash column chromatography was performed on silica gel 60 (SiO_2, Merck, 230–400 mesh).

Tert-butyl phenol, *di*-phenyl ether, allyl iodide, dimethylchlorosilane, benzoyl chloride, caesium carbonate, sodium carbonate and silica gels: LiChrosorb ODS, LiChrosorb Si 100 (particle size 5 μm, pore size 100 Å and specific surface area 300–400 $m^2\,g^{-1}$) were obtained from Merck (Darmstadt, Germany). Positional isomers (*ortho*, *meta* and *para*) of: nitrobenzoic acid, chlorobenzoic acid, 2-amino-chlorobenzoic acid, hydroxybenzoic acid, aminobenzhydrazide, hydroxybenzyl alcohol (HBnol), aminophenol, chlorophenol, dichlorophenol, cresol, hydroxypyridine (HPyr), pyridine methanol, dinitrobenzene, nitrophenol and nitroaniline; purine and pyrimidine bases (adenine (Ade), caffeine, thymine, cytosine (Cyt), 5-bromouracil and 5-fluorouracil) were obtained form Lancaster (Eastgate, UK). Non-steroidal anti-inflammatory drugs (Tolmetin, Fenoprofen, Ibuprofen, Diflunisal and Diclofenac) were purchased from Sigma–Aldrich (Steinheim, Germany). Reversed-phase test mixture (LA 87221) was obtained from Supelco (Deisenhofen, Germany). All solvents used as reaction media were of analytical grade and were obtained from POCh (Gliwice, Poland). Acetonitrile and methanol used as mobile phases were of HPLC grade and were purchased from Merck (Darmstadt, Germany). HPLC water was obtained by passing boiled deionized water through a Milli-Q system.

2.2. Synthesis of 1,3-alternate 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phase (1,3-Alt CalixBn)

The multistep synthesis of new *1,3-alternate* 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phase is shown in Scheme 1.

25,26,27,28-Tetrahydroxy-calix[4]arene **1** were prepared according to the reported procedures [33,34]. 25,27-



Scheme 1. Synthesis of the *1,3-alternate* 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phase [5]; (i) benzoyl chloride, ACN, K_2CO_3, reflux for 24 h; (ii) allyl iodide, Cs_2CO_3, ACN, reflux for 72 h; (iii) $(CH_3)_2SiHCl$, H_2PtCl_6, $CHCl_3$, reflux for 4 h; (iv) activated silica gel, pyridine, shaking for 4 days at room temperature.

Dibenzoiloxy-26,28-dihydroxy-calix[4]arene **2** was prepared by adding of 1 equivalent of 25,26,27,28-tetrahydroxy-calix[4]arene **1** to the 2 equivalent of benzoyl chloride and 1 equivalent of potassium carbonate in dry acetonitrile. The mixture was refluxed under an atmosphere of nitrogen for 24 h. Crude product was purified by recrystallization ($CHCl_3$/MeOH). Yield 86%, mp 260–263 °C.

^{1}H NMR ($CDCl_3$): δ 8.39–6.68 (m, 22H); 5.47 (s, 2H, O**H**); 4.02–3.51 (ABq, 8H, ArC**H_2**-Ar). 25,27-Dibenzoiloxy-26,28-diallyloxy-calix[4]arene **3** was obtained by refluxing compound **2** with excessive amount of allyl iodide (6 equivalent) in dry acetonitrile for 72 h under nitrogen, in the presence of caesium carbonate (4 equivalent) as base and a catalyst. The residue, after evaporation of the solvent, was dissolved in $CHCl_3$ and washed with 2 N HCl and water. The organic phase was separated, dried over $MgSO_4$ and evaporated. The flash chromatography (SiO_2, $CHCl_3$) of crude product yields white powder. Yield 39%, mp 240–242 °C.

^{1}H NMR ($CDCl_3$): δ 7.92 (d, 4H, Bn-o, J = 8.3 Hz); 7.77 (**t**, 2H, Bn-p, J = 7.6 Hz); 7.60 (**t**, 4H, Bn-m, J = 7.6 Hz); 7.13 (d, 4H, ArH-m, J = 7.3 Hz); 6.89 (**t**, 2H, ArH-p, J = 7.6 Hz); 6.6 (d, 4H, ArH-m, J = 7.3 Hz); 6.45 (t, 2H, ArH-p, J = 7.3 Hz); 5.99–5.92 (m, 2H, –CH=, gem); 5.27 (dd, 2H, =CH cis, J = 1.46 Hz, J = 10.74 Hz); 5.15 (dd, 2H, =CH trans, J = 1.87, J = 17.33); 4.29 (d, 4H, O$\underline{CH_2}$CH, J = 4.4 Hz); 3.7 (d, 4H, $ArCH_2Ar$, J = 14.6 Hz); 3.46 (d, 4H, $ArCH_2Ar$, J = 14.6 Hz).

^{13}C NMR ($CDCl_3$): δ 37.0 (s, $ArCH_2Ar$); 71.3 (s, OCH_2); 116.3 (s, vinyl CH_2); 122.0; 124.0; 128.0; 129.0; 131.2;

131.3; 131.5; 132.6; 133.2; 133.6; 134.1; 147.4; 156.4 (13s, aromatic carbons and vinyl CH); 164.4 (s, C=O).

Expected and observed seventeen signals.

Anal. Calcd. for $C_{48}H_{40}O_6$: C 80.88 %, H 5.66 %. Found: C 80.97 %, H 5.62%.

Product **3** was dissolved in dry CH_2Cl_2, in the presence of excess of dimethylchlorosilane, and catalytic amount of H_2PtCl_6 in isopropanol was added. The mixture was refluxed under nitrogen atmosphere for 4 h. Then the excess of silane was evaporated under vacuum and dry residue (compound **4**) was used for immobilization on silica gel without purification. The *1,3-alternate* 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene stationary phase **5** was prepared by shaking activated silica gel with product **4** and catalytic amount of pyridine in dry toluene for 4 days under nitrogen atmosphere at room temperature according to the reported procedure [35,36]. The modified silica gel was filtered and washed with $CHCl_3$, MeOH and acetone. Elemental analysis gave %C 19.86, %H 2.04 (coverage density of the gel 0.344 mmol g^{-1}). Finally, unreacted silanol groups were end-capped with 1,1,1,3,3,3-hexamethyldisilazane.

Stainless steel column (250 mm × 4.6 mm i.d.) was packed with modified calix[4]arene-silica gel according to a slurry packing procedure [37]. The column efficiency determined with a commercially available test mixture was about 40,000 plates/m.

2.3. Chromatographic procedure

Analytes were dissolved in ACN/MeOH/H_2O (1:1:1, v/v/v) mixture at the concentration in range of 0.25–0.5 mg ml^{-1} and 20 μl of the solution were injected onto the chromatographic column. The retention time of the aqueous solution of potassium nitrate was used as void time marker for the calculation of the capacity factor. A mixture of ACN/MeOH (1:1, v/v) in water or 0.01 mol l^{-1} water solution of phosphate buffer was typically used as a mobile phase at flow rate of 1.0 ml min^{-1}. The pH value of this buffer was adjusted with H_3PO_4 to 2.5 or with NaOH to 6.5. Diode-array detector was operated in single wavelength mode. All analyses were carried out at 25 °C.

3. Results and discussion

3.1. Endurance of the bonded stationary phase (1,3-Alt CalixBn)

1,3-Alt CalixBn column was obtained by immobilization of *1,3-alternate* 25,27-dibenzoiloxy-26,28-diallyloxy-calix[4]arene onto silica gel in hydrosilylation process. The column stability was checked by periodic determination of *ortho*-biphenyl retention time whereas mixture of methanol and aqueous phosphate buffer of pH in range of 2.5–6.5 was used as a mobile phase. The column packing showed high chemical stability over a 6-month period of use and no loss of retention power of the prepared column was observed during that time.

Table 1
Retention (k) and separation factors (α) for aromatic positional isomers on *1,3-Alt* CalixBn stationary phase

Compounds	Retention factors (k)	Selectivity	
		($\alpha_{2/1}$)	($\alpha_{3/2}$)
Dinitrobenzene **(1a)**	2.73; 3.49; 3.49 ($p < m < o$)	1.28	1.00
Nitroaniline **(1b)**	3.80; 4.32; 5.99 ($p < m < o$)	1.14	1.38
Chlorotoluene **(1b)**	3.26; 3.28; 3.31 ($p + m < o$)	1.00	1.01
Xylene **(1b)**	5.26; 5.48; 5.55 ($p < m < o$)	1.04	1.01
Cresol **(1c)**	4.00; 4.07; 4.52 ($p < m < o$)	1.02	1.11
Hydroxybenzyl alcohol **(1d)**	1.82; 2.42; 3.29 ($p < m < o$)	1.32	1.36
Pyridylomethanol **(1e)**	1.96; 2.50; 3.09 ($p < m < o$)	1.28	1.23
Hydroxypyridine **(1e)**	0.57; 1.29; 2.75 ($p < o < m$)	2.23	2.13
Aminobenzhydrazide **(1e)**	0.48; 0.66; 1.71 ($p < m < o$)	1.40	2.57
Chlorobenzoic acid **(2a)**	1.78; 3.01; 3.01 ($o < m + p$)	1.69	1.00
2-Aminochlorobenzoic acid **(2b)**	0.37; 2.39; 3.23 ($o < m < p$)	6.37	1.35
Nitrophenol **(2c)**	2.71; 3.11; 4.52 ($p < m < o$)	1.15	1.45
Aminopheno **(2c)**	0.51; 0.76; 1.10 ($p < m < o$)	1.49	1.44
Nitrobenzoic acid **(2d)**	0.19; 1.12; 1.49 ($o < m < p$)	5.98	1.33
Hydroxybenzoic acid **(2e)**	0.53; 0.71; 3.06 ($o < m < p$)	1.33	4.31

Chromatographic conditions: flow rate 1 ml min^{-1}, detection UV at 210 or 254 or 280 nm, room temperature. Mobile phases: (1) A: ACN/MeOH (1:1, v/v) in B: water; **(1a)** 75% A; **(1b)** 50% A; **(1c)** 30% A; **(1d)** 10% A; **(1e)** 5% A; (2) A: ACN/MeOH (1:1, v/v) in B: 0.01 mol l^{-1} KH_2PO_4 at pH 6.0; **(2a)** 50% A; **(2b)** 40% A; **(2c)** 30% A; **(2d)** 20% A; **(2e)** 5% A.

3.2. Separation of aromatic positional isomers

A variety of *di*- and *tri*-substituted aromatic positional isomers, representing compound with acidic, basic and neutral character, were separated on *1,3-Alt* CalixBn. Retention capacity factors (k) and separation factors (α) of these isomers at the best, individually optimized, chromatographic conditions are given in Table 1. Most of the positional isomers were well resolved except for xylene and chlorotoluene isomers which were eluted together. The novel stationary phase exhibits strong retention power and selectivity toward analytes of very similar structure, which is comparable in many cases to the calixarene stationary phases synthesized by the other research groups [15–20]. However, aromatic isomers with polar groups (–OH, $–NH_2$, $–NO_2$, –COOH) are better resolved. The same observations were found for CABS [18] and *1,3-Alt* CalixPr [32] phases. Compounds possessing nitro substituents in the phenyl ring have higher retention times in comparison to the rest of investigated analytes, which can be explained by π-electron transfer interaction resulting from the electron-withdrawing effect of the nitro groups of analytes and π-electron system of the benzoiloxy groups of calixarene. Resolution of a number of mono and disubstituted phenol derivatives in a single run obtained on *1,3-Alt* CalixBn and ODS columns are shown in Fig. 1a and b, respectively. As it can be seen, much better separation of these solutes can be

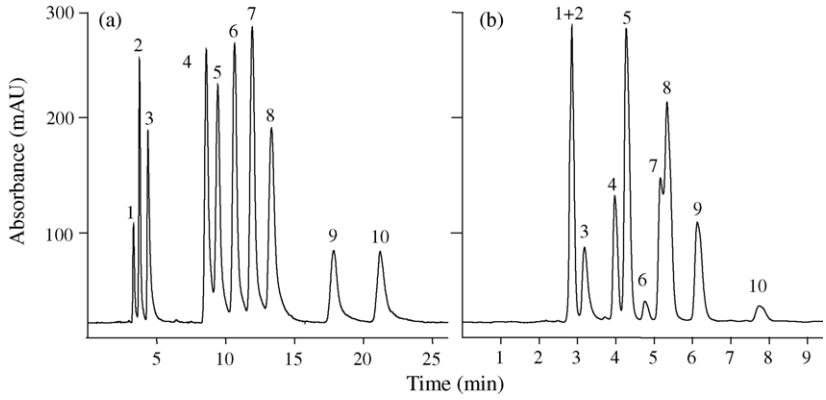


Fig. 1. The chromatograms of mono and disubstituted phenol derivatives on *1,3-Alt* CalixBn (a) and ODS (b). Mobile phase: 30% ACN/MeOH (1:1, v/v) in 0.01 mol l^{-1} KH_2PO_4 water solution, pH 6.0; flow rate 1 ml min^{-1}; UV 280 nm, room temperature. Analytes: (1) *p*-aminophenol; (2) *m*-aminophenol; (3) *o*-aminophenol; (4) *p*-nitrophenol; (5) *m*-nitrophenol; (6) *p*-chlorophenol; (7) *o*-chlorophenol; (8) *o*-nitrophenol; (9) 2,6-dichlorophenol; (10) 2,5-dichlorophenol.

achieved on novel calixarene column than on the ODS one. Ten phenol derivatives were successfully resolved on *1,3-Alt* CalixBn in isocratic mode. Moreover, the retention values of the analytes were greater than on ODS.

The elution order of positional isomers of the investigated compounds on *1,3-Alt* CalixBn column strongly depends on their chemical nature. Polar neutral, basic and weak acidic compounds (for example, dinitrobenzene, nitroaniline and nitrophenol) were eluted in order: *para* < *meta* < *ortho*, which may be associated to guest-host interactions of the calixarene cavity and the analyte molecule. Retention order of hydroxypyridine isomers was the only exception in above described chromatographic behaviour. Pryridine-2-ol and pyridine-4-ol can exist in tautomeric form of pyridin-2(1*H*)-one and pyridin-4(1*H*)-one, respectively. This may influence acidic and steric properties of the isomers although the observed elution order of hydroxypyridine: *para* < *ortho* < *meta* is difficult to rationalize on this phenomenon alone. The order of elution of substituted benzoic acid positional isomers was: *ortho* < *meta* < *para*, and roughly reflects the decreasing acid strength. This will be discussed in the following section in correlation to influence of acidity of the mobile phase on the analytes retention.

3.3. Effect of pH of the mobile phase

Retention in reversed-phase chromatography increases for more hydrophobic compounds. An acid or base undergoes ionization and its retention is reduced when it becomes less hydrophobic. The influence of variation in pH of the mobile phase on retention and peak symmetry was investigated in the range of pH 2.5–6.5 for nitrobenzoic acid positional isomers (Fig. 2a) and for hydroxypyridine positional isomers (Fig. 2b).

The retention of *ortho*-, *meta*- and *para*-nitrobenzoic acid isomers strongly depends on pH of the mobile phase. The pK_a values of these acids are in range of 2.17–3.54 and its retention is reduced by almost 5-fold when they lose a proton as pH increases. This is consistent with reversed-phase retention mechanism in which ionic species are merely retained. Retention of *para*-hydroxypyridine (pK_a 4.82) and *ortho*-hydroxypyridine (pK_a 11.62) does not change much with changes in pH of the mobile phase (Fig. 2b). Both the isomers may exist in tautomeric form of pyridin-(1*H*)-one, which ionize in the much lower degree. *meta*-Hydroxypyridine (pK_a 4.92) does not undergo tautomerization and therefore it is sensible to changes in pH of mobile phase. The

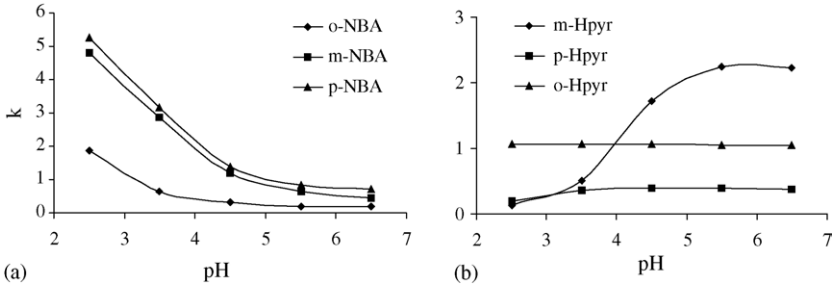


Fig. 2. Influence of the pH of mobile phase on the capacity factors of nitrobenzoic acid NBA (a) and hydroxypyridine Hpyr (b) positional isomers. Chromatographic conditions: (a) 40% ACN/MeOH (1:1, v/v); (b) 5% ACN/MeOH (1:1, v/v) in 0.01 mol l^{-1} KH_2PO_4 water solution; flow 1 ml min^{-1}, UV at 254 nm, room temperature.

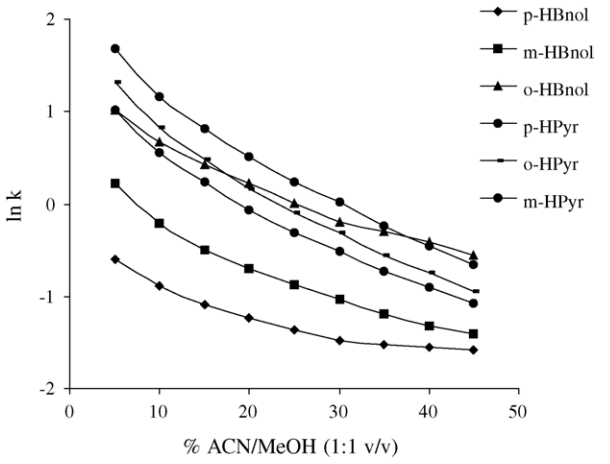


Fig. 3. Influence of the acetonitrile/methanol (1:1, v/v) content of the mobile phase on logarithmic capacity factors of hydroxybenzyl alcohol (HBnol) and hydroxypyridine (HPyr) positional isomers. Chromatographic conditions: ACN/MeOH (1:1, v/v) in 0.01 mol l^{-1} KH_2PO_4 water solution, pH 6.0; flow 1 ml min^{-1}, UV at 254 nm, room temperature.

dependence of retention factor of this isomer on pH of the mobile phase exhibit characteristic S-shape with inflection point at pH of about 4.5. In due course, this leads to alter elution order of hydroxypyridine isomers in different pH ranges. The retention order of hydroxypyridine isomers was *meta* < *para* < *ortho* in lower pH range (2.5–3.0) whereas *meta* isomer was eluted after *ortho* isomer when pH of the mobile phase was increased from 4.0 to 6.5.

3.4. Influence of organic modifier content

The employed mobile phase was triple component mixture of acetonitrile, methanol and water. Therefore, it is not a surprise that the influence of organic modifier on retention of the investigated compounds does not follow linear solvent strength dependence. The plots of logarithmic capacity factor of hydroxybenzyl alcohol and hydroxypyridine isomers against the volume percentage of acetonitrile/methanol (1:1, v/v) in mobile phase are given in Fig. 3. The experimental data may be well fitted to Schoenmaker's equation [38]:

$$\ln k' = A\Phi^2 + B\Phi + C$$

The peak symmetry was improved on addition of organic modifier, but the resolution of isomers was gradually lost as the capacity factors were decreased. In general, increase in organic modifier content in mobile phase leads to decrease in the retention of the investigated compounds, which suggests that *1,3-Alt* CalixBn phase behaves as a reversed-phase material, and hydrophobic interaction is one of the important factors playing role in the retention of the analytes.

3.5. Application examples

3.5.1. Separation of purine and pyrimidine bases

Reversed-phase liquid chromatography is widely used for separation and quantitative analysis of purine and pyrimidine bases. The retention behaviour of these compounds on the ODS stationary phase had been well established [39–42]. Recently, *p-tert*-butyl-calix[*n*]arene-bonded stationary phases ($n = 6, 8$) were also used for separation of these compounds [28,29]. Fig. 4a and b illustrate separation of some purine and pyrimidine bases in the single run obtained on novel *1,3-Alt* CalixBn phase and on ODS under the same chromatographic conditions. Solutes show sharp and symmetrical peaks with reasonable retention times. The elution order of the analytes on both *1,3-Alt* CalixBn and ODS was analogous. However, *1,3-Alt* CalixBn phase exhibited higher retention power and selectivity toward purine and pyrimidine bases than ODS. Adenine and uracil were eluted together on octadecyl-silica phase and separation of 5-fluorouracil (5-F-Ura) was not complete. The retention of the bases with pK_a well above or below the pH range of the mobile phase was stable in range of pH 2.5–6.5. Retention of Ade (pK_{a1} 4.5) and cytosine (pK_a 4.45) with pK_a values within the pH range being studied changed with pH of the mobile phase in the manner characteristic for transition of ionization state (Fig. 5).

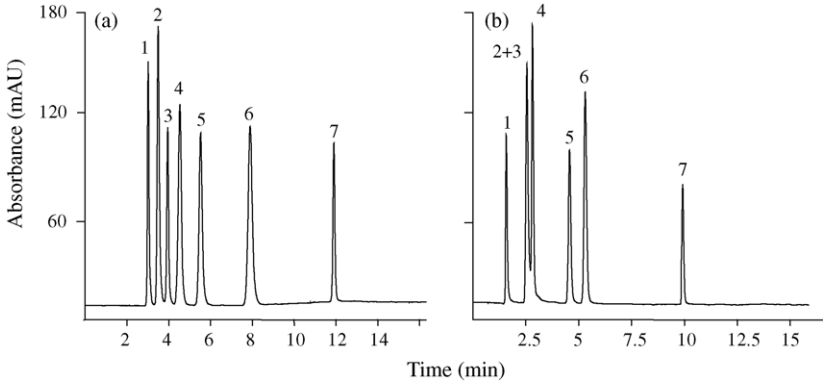


Fig. 4. The chromatograms of purine and pyrimidine bases on *1,3-Alt* CalixBn (a) and ODS (b). Mobile phases: A: ACN/MeOH (1:1, v/v) in B: 0.01 mol l^{-1} KH_2PO_4 at pH 2.5; gradient from 5 to 30% A within 8 min and then 35% A isocratic for 10 min; flow rate 1 ml min^{-1}; UV 254 nm, room temperature. Analytes: (1) cytosine; (2) adenine; (3) uracil; (4) 5-fluorouracil; (5) thymine; (6) 5-bromouracil; (7) caffeine.

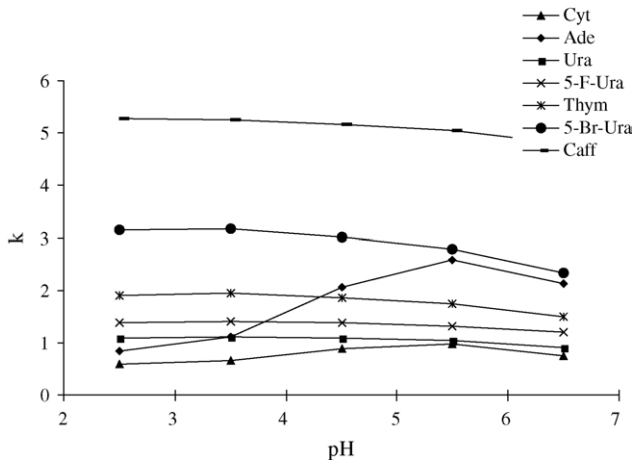


Fig. 5. Influence of the pH of mobile phase on the capacity factors of purine and pyrimidine bases. Mobile phases: A: ACN/MeOH (1:1, v/v) in B: 0.01 mol l^{-1} KH_2PO_4; gradient from 5 to 30% A within 8 min and then 35% A isocratic for 15 min; flow rate 1 ml min^{-1}; UV 254 nm, room temperature.

3.5.2. Separation of non-steroidal anti-inflammatory drugs

Gas chromatography [43–45] and high-performance liquid chromatography [46–51] techniques are widely used for determination of non-steroidal anti-inflammatory drugs (NSAIDs) in pharmaceutical formulations and biological fluids. NSAIDs are acidic molecules possessing diverse structural features. Therefore, they typically give rise to problems in RP–HPLC analysis and demand low pH of mobile phase. *1,3-Alt* CalixBn column was used for separation of five NSAIDs with structures represented in Fig. 6. The separation conditions were optimized by changing pH of the aqueous buffer (2.5–6.5), the nature of organic modifier (methanol, acetonitrile), column temperature (20–40 °C) and the flow rate of the mobile phase (0.8–1.2 ml min^{-1}). The peak resolution, peak symmetry and analysis times were chosen as optimization criteria. The best resolution and the shortest analysis time was obtained for 0.01 mol l^{-1} phosphate buffer at pH 6.5 in gradient elution (15–40%, (v/v) of acetonitrile/methanol mixture (1:1, v/v) in 25 min). Resolu-

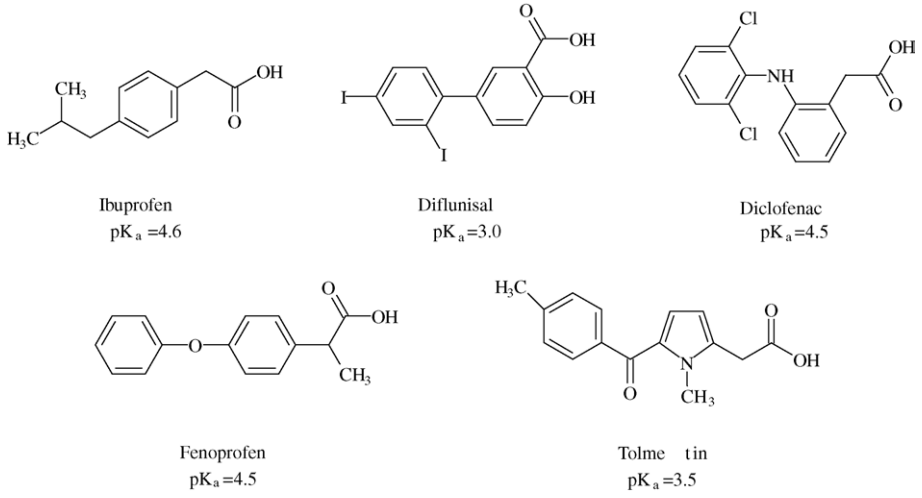


Fig. 6. Structures and pK_a values of studied NSAIDs.

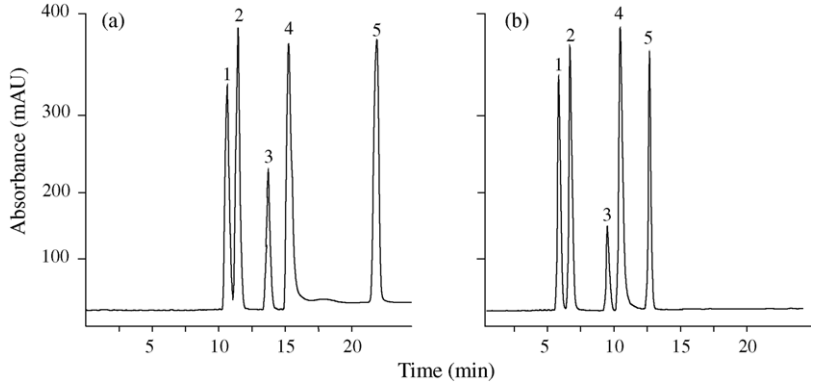


Fig. 7. The chromatograms of NSAIDs on *1,3-Alt* CalixBn (a) and ODS (b). Mobile phases: A: ACN/MeOH (1:1, v/v) in B: 0.01 mol l^{-1} KH_2PO_4 at pH 6.5; gradient from 15 to 40% A within 25 min; flow rate 1 ml min^{-1}; UV 275 nm, T = 30 °C. Analytes: (1) tolmetin; (2) fenoprofen; (3) ibuprofen; (4) diflunisal; (5) diclofenac.

tion factors of the analytes separated on *1,3-Alt* CalixBn and ODS columns were comparable (Fig. 7a and b). However, retention of NSAIDs on *1,3-Alt* CalixBn phase was higher than on ODS under the same chromatographic condition.

4. Conclusions

Screening evaluation of *1,3-alternate* 25,27-dibenzoiloxy-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phase (*1,3-Alt* CalixBn) revealed that a new stationary phase is chemically stable and can be successfully used for separation of positional isomers of aromatic compounds. The applicability of the novel chromatographic column to analysis of selected biologically vital compounds and drugs was also demonstrated.

Surface properties of *1,3-Alt* CalixBn stationary phase and mechanism of retention are going to be investigated soon.

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