High Performance Liquid Chromatography on Calixarene-Bonded Silica Gels. II. Separations of Regio- and Stereoisomers on *p-tert*-Butylcalix[*n*]arene Phases

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Abstract

The chromatographic behavior of new calix[*n*]arene-bonded (*n* = 4, 6, 8) silica gels are described. Cavities of different size and shape are formed depending on the number of aromatic moieties. The differences in ring size are utilized to investigate chromatographic selectivities towards analytes of various substance classes, including disubstituted aromatics, uracil derivatives, and estradiol epimers. Our results indicate that these calixarene-bonded phases show a high resolution power for regio- and stereoisomers.

Introduction

Macrocyclic compounds such as cyclodextrins (CDs) or crown ethers have been widely studied in chromatographic systems in the last decade. With calixarenes, another class of compounds of macrocyclic hosts has been introduced into analytical chemistry. In recent years, the interest in calixarenes as stationary phases in chromatography has increased more and more because of the unique opportunities to influence the specificity and selectivity of the macrocycles. The choice of calixarene ring size, conformation, and functionality allows applications of different separation principles, such as hydrophobic and ionic interaction, inclusion complexation, and charge transfer interaction.

Lin and Wu gave an overview of applications of calixarenes in analytical chemistry (1). In recent years, several applications of calixarenes were reported in liquid chromatography (2–7), gas chromatography (8–11), and capillary zone electrophoresis (12–16).

Recently, we reported the introduction of a new *p-tert*-butylcalix[4]arene-bonded silica gel (17). While other authors preferably bind calixarenes at the upper rim, we immobilized the phenolic hydroxyl groups at the lower rim to make the interior of the cavity accessible for several analyte molecules. In this paper, we present the use of larger calixarenes bonded to silica for the separation of regio- and stereoisomers and some biologically relevant compounds. We coupled *p-tert*-butylcalix[*n*]arenes (*n* = 6, 8) on silica. The chromatographic resolution power of the new [*n*]Arene phases for the selected model compounds was compared with that of RP18 and CD phases. The notation [*n*]Arene was chosen for the calixarene-bonded materials; the letter *n* in brackets represents the number of aromatic units in the calixarenes. For instance, [4]Arene phase refers to *p-tert*-butylcalix[4]arene acetic acid ester bonded onto silica gel.

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Experimental

Materials

Chemicals and reagents

Nitroanilines, xylenes, cresols, naphthols, and chloro- and diphenols were purchased from Fluka (Neu-Ulm, Germany). *p-tert*-Butylcalix[6]arene was obtained from AGROS (Gelnhausen, Germany). Serva Si 100 (5-µm particle size, 100-Å pore width), uracil, and uracil derivatives (5-hydroxymethyluracil and 3-, 5-, and 6-methyluracil) were from Serva (Heidelberg, Germany). HPLC grade methanol, NaH_2PO_4, NaOH, and H_3PO_4 were obtained from Merck (Darmstadt, Germany). 17α- and 17β-estradiol were purchased from Aldrich (Steinheim, Germany).

Preparation of p-tert-*butylcalix[n]arene (*n = *6, 8) bonded silica gels*

p-tert-Butylcalix[8]-arene was synthesized as described in the literature (18). The acetic acid esters were synthesized according to the method of Arnaud-Neu et al. (19). Subsequently, esters were hydrolyzed to the carboxylic acids with diluted hydrochloric acid. The structures were identified by elemental analysis, ^{1}H-NMR spectroscopy, mass spectrometry, and melting points.

The immobilization of the macrocycles onto silica gel with a pore width of 100 Å and a particle size of 5 µm was carried out via a short hydrophilic spacer. *p-tert*-Butylcalix[*n*]arene acetic acid esters were used as chromatographic selectors. Elemental analysis showed that the resulting materials have a surface coverage of 0.09–0.14 mmol/g silica gel (Table I). The calixarene-bonded materials were commercialized by GETEC (Halle/S.).

Column materials

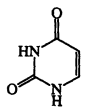
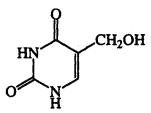
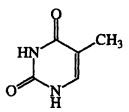
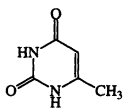
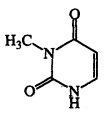
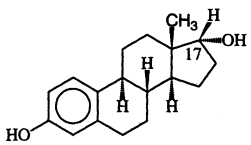
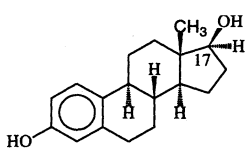
Slurries of 4-g bonded materials suspended in methanol were prepared and packed into HPLC columns (250 × 4.6-mm i.d.) to create calixarene stationary phases. The working pressure was 400 bar. An α-CD=Si 100 and a β-CD=Si 100 (5-µm particle size, 100-Å pore width, 250 × 4-mm i.d.), two γ-CD=Si 100 (10-µm particle size, 100-Å pore width, 125 × 4.6-mm i.d.), and an octadecyl=Si 100 (5-µm particle size, 100-Å pore width, 250 × 4-mm i.d.) from Serva (Heidelberg, Germany) were used to compare the results of calixarene-bonded stationary phases.

Table I. Characteristics of Calixarene-Bonded Stationary Phases

Parameter	[4]Arene*	[6]Arene*	[8]Arene*
Particle size (µm)	5	5	5
Mean pore width (Å)	100	100	100
Silica	irregular	irregular	irregular
Surface coverage (mmol/g)	0.15	0.14	0.09
pH stability	2–7	2–7	2–7

* Selector was *p-tert*-butylcalixarene acetic acid ester.

Table II. Structures of Some Analytes

Analyte	Structure
Uracil	
5-Hydroxymethyluracil	
5-Methyluracil (thymin)	
6-Methyluracil	
3-Methyluracil	
17α-Estradiol	
17β-Estradiol	

Chromatographic experiments

Apparatus

HPLC separations were performed on a MERCK-Hitachi HPLC system consisting of an L-6200 intelligent pump, a variable wavelength ultraviolet detector, and data acquisition software (Merck-Hitachi Model D-6000, HPLC-Manager Version 2).

HPLC

Chromatographic experiments were performed isocratically at room temperature. The samples were dissolved in the eluents at a concentration of 0.25 mg/mL. The injection volume was 20 µL.

Diphenols were dissolved in mobile phase containing a 0.02M NaH_2PO_4 solution (pH 3.5) with 30% methanol; nitroanilines, xylenes, and cresols with 40% methanol; and naphthols and chloro phenols with 50% methanol, respectively. Mixtures of solutes were separated on the [*n*]Arene and RP18 phases and monitored at 254 nm. The flow rate was 1 mL/min.

A mixture of uracil (U), 5-hydroxymethyluracil (5-HMU), and 3-, 5-, and 6-methyluracil (3-, 5-, and 6-MU) was injected onto the [*n*]Arene, CD, and RP18 phases using a 0.02M NaH_2PO_4 solution as a mobile phase, depending on the pH value. The absorption of analytes was monitored at 254 nm. Flow rate was 1 mL/min.

A solution of 17α- and 17β-estradiol were tested on the same columns at a flow rate of 1 mL/min. The mobile phase consisted of a 0.02M NaH_2PO_4 solution containing 60% methanol. The detection was carried out at 220 nm.

Results and Discussion

In order to study the chromatographic properties of calixarenes dependent on their ring size in HPLC, we immobilized *p-tert*-butylcalix[*n*]arene acetic acid ester derivatives (n = 4, 6, 8) onto silica gel. The calixarene-bonded phases were characterized by elemental analysis (Table I). The column materials were stable in a pH range of approximately 2–7, established by longtime experiments at these pH values and by a Tanaka method (20) using 30% buffered methanol at pH 2.7 and 7.6 for the test solutes thiourea, benzylamine, and phenol.

With respect to investigating the chromatographic selectivities and differences in steric discrimination of the [4]-, [6]- and [8]Arenes, the separation of different regio- and stereoisomers was studied. Nitroanilines, diphenols, naphthols, chloro phenols, xylenes, cresols, uracil derivatives, and 17α- and 17β-estradiol were chosen as model compounds. The structures of some investigated analytes are presented in Table II.

Regioisomers

Nitroanilines

As Figure 1 shows, all calixarene-bonded phases were capable of resolving the nitroaniline isomers, whereby best results were obtained on [4]- and [6]Arene. The elution order was found to be $m < p < o$ on all three calixarene phases. Interestingly, the [8]Arene phase shows longer retention times of the isomers in spite of having the lowest surface coverage (Table I). This effect may be caused by the formation of host–guest complexes. Additionally, on all calixarene phases, charge-transfer complexes

can play an important role because of the strong (–)I-effect of the nitro group of nitroanilines and (+)I-effect of the tert-butyl groups of calixarenes.

The different elution patterns for the nitroanilines on RP18 and β-CD (17) indicate a distinct separation mechanism. On the RP18 phase, the *p*- isomer is less retained than the *m*- and *o*- isomers, whereas on β-CD, an elution order of $m < o < p$ was obtained.

Diphenols

The separation of hydrochinone, resorcinol, and brenzcatechine on the different stationary phases is represented in Figure 2. Diphenols were optimally resolved on the [8]Arene. The elution order was generally $p < m < o$. Effects of significantly longer retention times as observed for the separation of nitroanilines

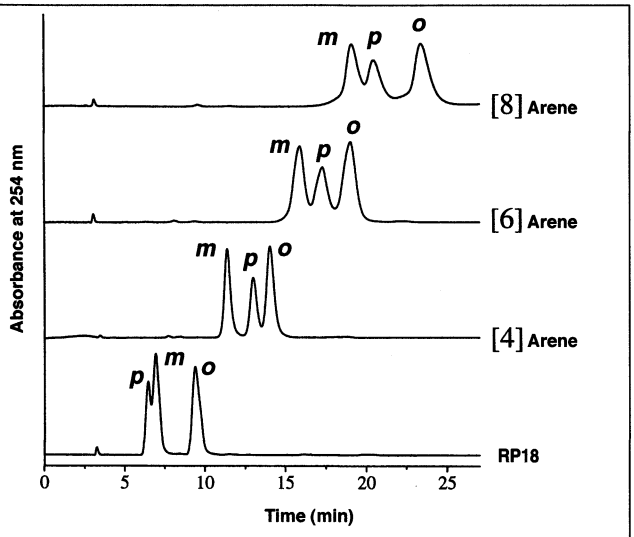


Figure 1. Isocratic separations of *o*-, *m*-, and *p*-nitroanilines on different stationary phases using 0.02M NaH_2PO_4 (pH 3.5)–methanol (60:40, v/v) as a mobile phase at a flow rate of 1 mL/min.

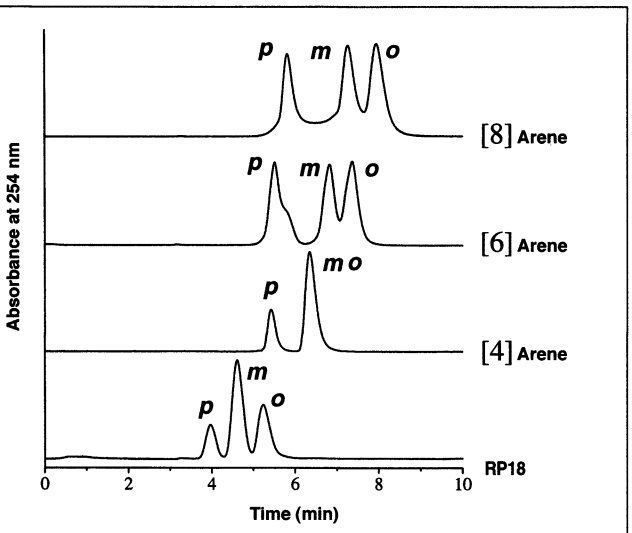


Figure 2. Isocratic separations of *o*-, *m*-, and *p*-diphenols on different stationary phases using 0.02M NaH_2PO_4 (pH 3.5)–methanol (70:30, v/v) as a mobile phase at a flow rate of 1 mL/min.

were not stated. A different solvatization, according to the position of the hydroxyls and resulting in a different accessibility of the hydrophobic surface, may be taken into account. The same elution order was found on RP18. That means that the same retention mechanism might occur.

Among other regioisomers tested, only the isomers of α- and β-naphthol were baseline separated. β-Naphthol eluted first on both [*n*]Arene and RP18. Capacity factors yielded on the [6]Arene phases are significantly higher than those on [8]- and [4]Arene, as well as on RP18. Chloro phenols, xylenes, and cresols were only partially resolved. In all cases, isomers of *meta* and *para* eluted together (elution order: chloro phenols, $o < m \approx p$; xylenes, $o < m \approx p$; cresols, $m \approx p < o$).

Uracil derivatives

U, 5-HMU, and the positional isomers 3-, 5-, and 6-MU were studied. The substances were chromatographed on [*n*]Arene, CD (α, β, and γ) and RP18 phases. As represented in Figure 3, the best results were obtained at pH 3. The compounds showed reasonable retention times with sharp and symmetrical peak shapes. The elution order 5-HMU < U < 6-MU < 5-MU < 3-MU on [*n*]Arene columns strictly follows the polarity properties of the uracils. Because of its hydroxyl group, 5-HMU is the most polar molecule and eluted first. Substitution of the hydroxymethyl group by a methyl group such as 5-MU and 6-MU resulted in a less polar structure. 3-MU shows the highest hydrophobicity because of the substitution of hydrogen at position 3 of the pyrimidine moiety by a methyl group, and it was the most strongly retained compound. Baseline separations of positional isomers of 5-MU and 6-MU were not achieved, but interestingly, resolution was improved on [8]Arene. A resolution factor of 1.1 was calculated. The results indicate that improved selectivity, depending on ring size, may be caused by hydrophobic interaction and additionally by host–guest complexation. Under the same chromatographic conditions, uracil derivatives were chromatographed on CD and RP18 columns. In both cases, 5-MU and 6-MU were not separated. As shown in Figure 3, CD and RP18 show significantly lower selectivities and less satisfactory separations in comparison with the calixarene bonded phases. The retention order on RP18 was the same as that found on [*n*]Arenes. A different elution order was obtained on CD phases (Figure 3). With increasing cavity size, only a marginal improvement is achieved. It should be observed that 3-MU elutes on the γ-CD column in front of 5-MU and 6-MU. Hence, it can be concluded that a different retention mechanism takes place.

Stereoisomers—Estradiol

The chromatographic behavior of 17α- and 17β-estradiol is shown in Figure 4. On all calixarene phases, a separation was achieved whereby the 17α-estradiol (*trans*) was the first eluting isomer. On RP18 and β-CD phases under the same conditions, the isomers appeared as single co-eluting peaks. However, Zarzycki et al. resolved the epimers on octadecyl using β-CDs as additives in mobile phase (21). The eluent consisted of acetonitrile–water (30:70). With and without β-CD, 17β-estradiol was less retained than 17α-estradiol. With increasing ring size of calixarenes, a significant increase in retention time can be observed in connection with slightly higher resolution factors of the epimers.

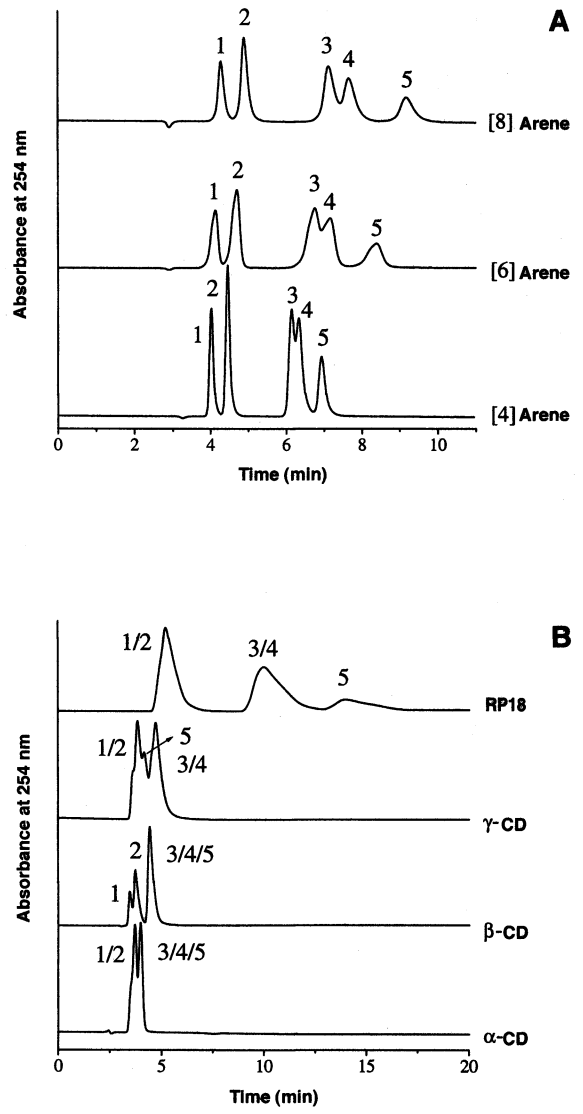


Figure 3. Isocratic separations of uracil and uracil derivatives on [*n*]Arene stationary phases (A); and CDs and RP_{18} (B). The eluent was 0.02M phosphate solution (pH 3) at a flow rate of 1 mL/min, and the solutes were 5-hydroxymethyluracil (1), uracil (2), 5-methyluracil (3), 6-methylurail (4), and 3-methyluracil (5).

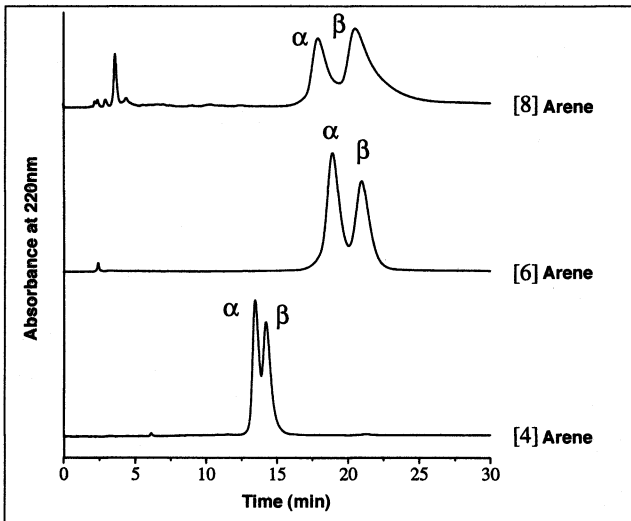


Figure 4. Isocratic separations of 17α- and 17β-estradiol on different calixarene stationary phases using 0.02M phosphate solution–methanol (40:60, v/v) as a mobile phase at a flow rate of 1 mL/min.

Conclusion

The calixarene-bonded phases described in this paper show, in contrast to the bonded CDs, highly hydrophobic properties becausse of the aromatic moieties and *tert*-butyl groups. Despite the relatively simple structure of these calixarenes, considerable chromatographic selectivities were observed in comparison with the RP18 separations. Based on several applications, we demonstrated that the resolution power of calix[*n*]arenes, especially for regio- and stereoisomers, depends on the shape and size of cavities. Furthermore, depending on the nature of the solutes, different mechanisms seem to be responsible for the separation, ranging from charge-transfer to inclusion complexes.

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