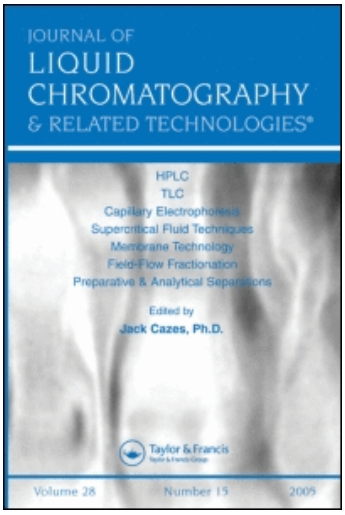

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Calix[4]pyrrole-Bonded HPLC Stationary Phase for the Separation of Phenols, Benzenecarboxylic Acids, and Medicines

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Calix[4]pyrrole-Bonded HPLC Stationary Phase for the Separation of Phenols, Benzenecarboxylic Acids, and Medicines

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Abstract: Two calix[4]pyrrole-modified silica gels were synthesized. Their separation to amino acids, phenols, benzenecarboxylic acids, and some medicines were studied. The separation process is governed mainly by interactions of hydrophobicity and hydrogen bond between stationary phase and analytes. Calix[4]pyrrole-modified HPLC columns have the potential to separate some positional isomers and medicines, which is meaningful for the further studies and applications in the fields of analytical and supramolecular chemistry.

Keywords: Calix[4]pyrroles, Macromolecules, HPLC, Phenols, Benzenecarboxylic acids

INTRODUCTION

Evolution of molecular recognition promotes advances in other scientific fields, such as chromatography. In fact, the selective recognition of host to guest molecules is closely related to the selective separation in chromatography. Host molecules, on the one hand, can be added to a mobile phase as new additives, or covalently attached to solid supports to form a new

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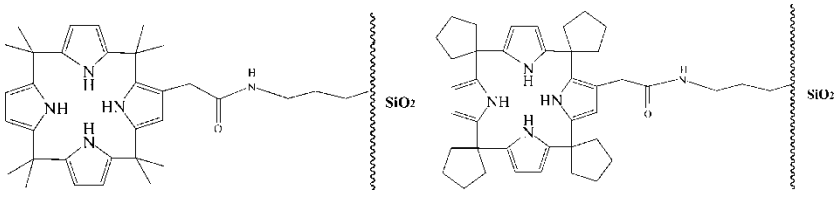
stationary phase. On the other hand, chromatography is a rapid and convenient tool to study interactions between host and guest molecules.

As representative host molecules in macrocycles, crown ether, cyclodextrin, and calixarene, have been widely used to synthesize stationary phases with new properties in chromatography. Crown ether modified stationary phases were used to separate phenols and monosaccharides,[1] isomers of cresol and xylenol.[2] Stationary phases linked with derivated cyclodextrins provided good resolution for a variety of enantiomers and positional isomers, such as *o*-, *m*-, *p*-nitroaniline,[3] phenylpropionic acid,[4] and β-carotene,[5] etc. Calixarene-bonded silica gels were used to separate alkali ions,[6] positional isomers of benzene,[7] and steroids.[8] In recent years, some newly designed stationary phases based on oligopyrrole macrocycles have been developed.[9–12] They were used to separate different inorganic and organic anions, aromatic substances, and oligonucleotides. The separation process is mainly governed by interactions of π-π stack, hydrogen bond, hydrophobicity, coordination, and coulombic force.

As one of the oligopyrrole macrocycles, calix[4]pyrroles are known as effective anion binding agents and have been applied in many aspects, such as anion recognition,[13,14] electrochemistry,[15] colorimetry,[16] and fluorescent sensor.[17] Reports on calix[4]pyrrole modified stationary phase are relatively few. The important one came from Sessler and Gale.[18] They produced calix[4]pyrrole covalently linked silica gels. Under different conditions, they realized the separation of some inorganic and organic anions, such as fluoride, chloride, Cbz-protected amino acids, phosphorylated derivatives of adenine, oligonucleotides, and some small neutral substrates. Through their work, the special separation ability traced to the interactions between calix[4]pyrroles and analytes was revealed.

In this paper, in order to explore the separation ability and mechanism of calix[4]pyrrole stationary phase, two calix[4]pyrrole modified silica gels (gel BM and BC in Figure 1) were synthesized and successfully applied to separate amino acids, phenols, benzenecarboxylic acids, and some medicines. The separation mechanism was also discussed.



Gel BM **Gel BC**

Figure 1. Sketch maps of the two silica gels.

EXPERIMENTAL

Instruments and Reagents

Instruments used in the characterization of products were: 10 DX FT-IR infrared spectrophotometer (KBr pellet method, Nicolet, USA), Vario El elemental analyzer (Elementar, Germany), Varian INOVA-400 FT-NMR (Varian, USA, $CDCl_3$ as solvent and TMS as internal standard), ZAB-HS mass spectrometer (VG, UK). Also used were LC-4A HPLC (Shimadzu, Japan) with a HW-2000 Chemstation (Nanjing, China), type 7125 six way changeover valve (Rheodyne, USA) with a sample loop (20 μL), 785A UV/Vis detector (Perkin Elmer, USA), pH meter (PHS-3B, Leici Instrument Factory, China).

1-Hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIC), (1-benzotriazolyl)oxy tris(dimethylamino) phosphonium hexafluorophosphate (BOPPF6), and 4-dimethylaminopyridine were purchased from J&KChemica. Dichloromethane, methanol, tetrahydrofuran, hexane, dimethyl formamide, triethylamine, acetyl chloride, pyridine, and n-butylamine were of AR grade. Acetonitrile was chromatographic grade. Meso-octamethylcalix[4]pyrrole and meso-tetraspirocyclopentylcalix[4]pyrrole were synthesized in our laboratory according to literatures.[19–21]

The starting silica gel (particle diameter 5 μm, mean pore size 10 nm, surface area 300 m^2/g) was prepared in our laboratory.

Synthesis of Gel BM and BC

The chemical properties of meso-tetraspirocyclopentylcalix[4]pyrrole are similar to those of meso-octamethylcalix[4]pyrrole, so the synthetic procedures of its derivatives are also the same as those of meso-octamethylcalix[4]-pyrrole.[18,22,23] The chemical structures of calix[4]pyrrole derivatives are shown in Figure 2, which were used in the synthesis of calix[4]pyrrole-bonded stationary phase. Here we only give the data of ester **2** and acid **4**.

β-Ethylcarbonylethyl-meso-tetraspirocyclopentylcalix[4]pyrrole 2

Yield was 21% (121 mg of monoester from 497 mg of raw material). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 8.62$ (s, 1H, NH), 7.13 (s, 1H, NH), 7.00 (s, 1H, NH), 6.98 (s, 1H, NH), 5.87–5.80 (m, 5H, pyrroleCH), 5.73 (s, 1H, pyrroleCH), 5.65(s, 1H, pyrrole CH), 4.20 (q, 2H, $J = 7.2$ Hz, ethylCH_2), 3.58 (s, 2H, CCH_2), 2.11–1.97 (m, 16H, CH_2), 1.70–1.55 (m, 16H, CH_2), 1.32 (t, 3H, $J = 7.2$ Hz, ethyl CH_3); IR,ν/cm^{-1}(KBr pellet) 3418(s), 3342(w), 3105(w), 2957 (s), 2871(m), 1725(s), 1578(m), 1454(s), 1182(s), 1041(s), 764(s); Anal. Calcd for $C_{40}H_{50}N_4O_2$: C,

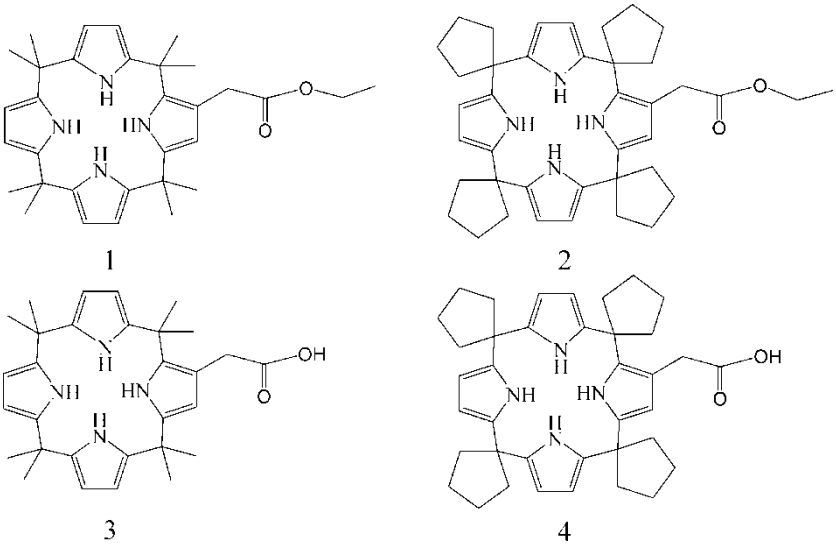


Figure 2. Chemical structures of calix[4]pyrrole derivatives.

77.63%; H, 8.14%; N, 9.05%. Found: C, 77.57%; H, 8.19%; N, 9.11%; MS (FAB+, m/z): 618(M^+, 100).

β-Hydroxycarbonylethyl-meso-tetraspirocyclopentylcalix[4]pyrrole 4

Yield was 82% (429 mg of monoester from 524 mg of raw material). ^{1}H NMR(400 MHz, $CDCl_3$) $\delta = 8.05$ (s, 1H, NH), 7.08 (s, 1H, NH), 7.03 (s, 1H, NH), 6.98 (s, 1H, NH), 5.87-5.81 (m, 5H, pyrroleCH), 5.76 (s, 1H, pyrroleCH), 5.72 (s, 1H, pyrroleCH), 3.65 (s, 2H, CCH_2), 2.16–1.99 (m, 16H, CH_2), 1.68 (s, 16H, CH_2); IR,ν/cm^{-1}(KBr pellet) 3422(s), 3107(w), 2956(s), 2862(s), 1722(s), 1577(m), 1450(s), 1185(s), 764(s); Anal. Calcd for $C_{38}H_{46}N_4O_2$: C, 77.25%; H, 7.85%; N, 9.48%. Found: C, 77.18%; H, 7.76%; N, 9.42%; MS (FAB+, m/z): 590(M^+, 100), 545(M-COOH, 85).

Silica gel BM: Detailed description of its synthetic procedures was published elsewhere.[18] Trimethylsilyl protected aminopropyl silica gel used was 3.5 g. Compound **3** used was 194 mg (0.40 mmol). After being reacted, washed, and dried, the stationary phase was packed into a 150 mm × 4.6 mm I.D. stainless steel column by conventional slurry packing procedures. Elemental analysis: trimethylsilyl-protected silica gel: C 5.31%, H 1.79%, N 1.56%; calix[4]pyrrole silica gel: C 6.37%, H 2.03%, N 1.94%. That corresponded with calculated surface coverage $\sim 0.82\ \mu$mol m^{-2}.

Silica gel BC: Compound **4** used was 210 mg (0.36 mmol). Elemental analysis: trimethylsilyl-protected silica gel, C 5.31%, H 1.79%, N 1.56%;

calix[4]pyrrole silica gel: C 6.72%, H 2.16%, N 2.01%. That corresponded with calculated surface coverage $\sim 0.84\ \mu mol \cdot m^{-2}$.

RESULTS AND DISCUSSION

In this paper, gel BM and BC were derived from aminopropyl silica gel. In order to study the separation mechanism of calix[4]pyrrole stationary phase, all experiments were carried out on both a calix[4]pyrrole-modified silica gel column and aminopropyl silica gel column as contrast.

IR Characterization of Synthesized Silica Gel

IR spectra of bare silica gel, end capped aminopropyl silica gel, and gel BC is shown in Figure 3. As initial fundus (a in Figure 3), the wide absorbing peak between 3500 and 3600 cm^{-1} corresponds to the hydroxyl unit on the surface of silica gel. After silica gel was modified by aminopropyl silicane and end capped, the absorbing peak at 3470 cm^{-1} of amino unit and that of C-H bond around 2900 cm^{-1} become obvious. To gel BC (or BM), the appearance of absorbing peaks at 2944 and 2862 cm^{-1} of C-H bond, 3016 cm^{-1} of C-H bond on pyrrole ring, 1562 cm^{-1} of C═C bond on pyrrole ring, and obvious 1698 cm^{-1} of C═O bond prove the successful linkage of calix[4]pyrroles on silica gel.

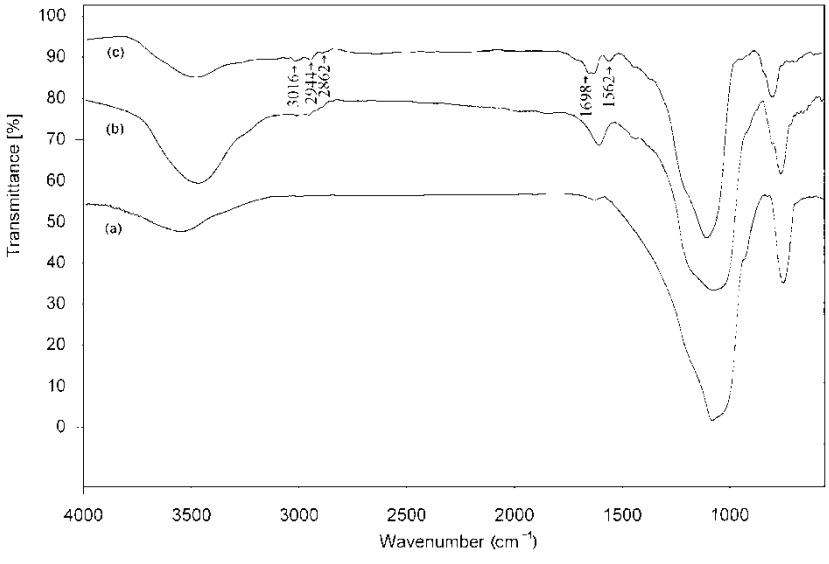


Figure 3. FTIR spectra of silica gel (a), end capped aminopropyl silica gel (b) and gel BC (c).

Initial Experiments to the Two Kinds of Stationary Phases

Separation of Inorganic Anions on Calix[4]pyrrole Columns

The retention of anions can clearly reflect the selectivity of the columns. Here F^-, Cl^-, Br^-, and I^- were chosen as analytes to compare gel BM and BC columns with end capped aminopropyl silica gel columns.

In Table 1, it is very clear that gel BM and BC show obvious selectivities to anions. The elution order of F^-, Cl^-, Br^-, and I^- is consistent with the strength that calix[4]pyrroles bind them. But on aminopropyl silica gel, anions are eluted at nearly the same time.

Separation of Amino Acids on Calix[4]pyrrole Columns

It is very important to analyze amino acids in the fields of biochemistry, medicine, food, and life science. Precolumn derivatization and RP-HPLC with UV detection are commonly used. Derivatization reagents mainly include phenylthiohydantoin,[24] dansyl chloride,[25] *o*-phthalaldehyde,[26] and 2, 4- dinitrofluorobenzene.[27] In our initial tests, we used another reagent, 2, 4- dinitrochlorobenzene (CDNB), to derivate amino acids and separate them on gel BM and BC columns.[28–30] The results are illustrated by Figure 4.

Phosphate buffer (20 mM) with different pH values were used as mobile phases to study the effect of pH value on the separation of the derivated amino acids. The pH value of the electrolyte was varied between 5.0 and 8.0 (Figure 5).

Under identical conditions, derivated amino acids were eluted at nearly the same time on aminopropyl silica gel columns. But on calix[4]pyrrole columns, eight amino acids were separated and the elution order is meaningful.

Table 1. Retention time of inorganic anions using gel BM, gel BC and aminopropyl silica gel[a]

	Elution time [min]		
Anion	Aminopropyl silica gel	Silica gel BM	Silica gel BC
F^-	3.176	14.284	13.710
Cl^-	3.172	13.656	13.238
Br^-	3.173	13.441	12.893
I^-	3.170	13.372	12.765

[a]Time given is the individual elution of tetrabutylammonium anions. Mobile phase CH_3CN + 0.75 mM o-phthalic acid, flow rate 0.5 mL min^{-1}, indirect UV detection at 254 nm, column temperature 25°C.

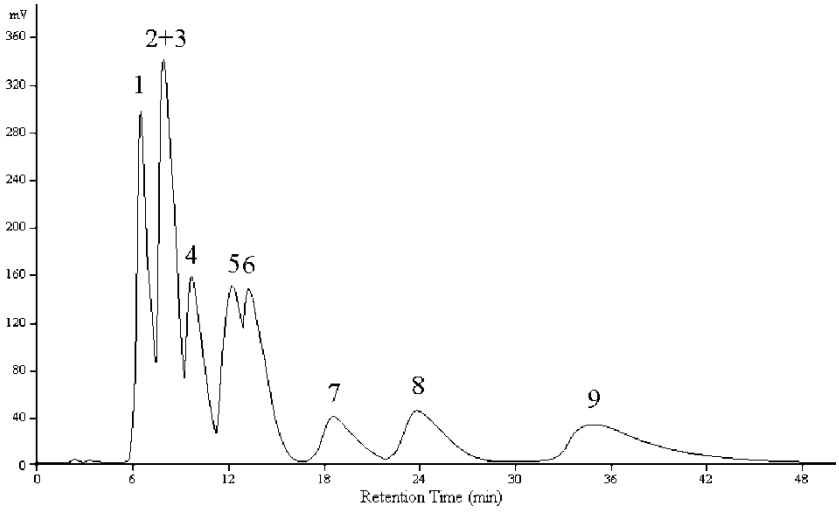


Figure 4. HPLC separation of amino acids on gel BC column. Chromatographic conditions: Mobile phase $CH_3CN/20$ mM phosphate buffer (30/70, v/v) at PH 6.0, flow rate 0.5 mL min^{-1}, UV detection at 360 nm, injection volume, 20 μL. Peaks: 1: DNP-serine; 2: DNP-glycine; 3. DNP-alanine; 4: DNP-valine; 5: DNP-leucine; 6: DNP-OH; 7: DNP-tyrosine; 8: DNP-phenylalanine; 9: DNP-glutamic acid.

Because of strong interaction of the hydrogen bond between two carboxylic anions of dinitrophenyl (DNP)-glutamic acid and calix[4]pyrrole subunits present on the column, it was eluted at last. Along with the increase of hydrophobicity of R-substituted units, the retention time of DNP derivatives of glycine, alanine, valine, leucine, and phenylalanine were, in turn, prolonged. Owing to the hydroxyl in R-substituted units, hydrophilicity of serine is stronger than that of alanine, despite their molecular structures being similar. The situation between tyrosine and phenylalanine is the same. Therefore, DNP-serine and DNP-tyrosine were eluted before DNP-alanine and DNP-phenylalanine, respectively, which implied again that the interaction of hydrophobicity coming from large amounts of C-C and C-H bond in calix[4]pyrrole subunits was another separation mechanism besides the hydrogen bond for calix[4]pyrrole columns. The fact that the retention time of eight compounds decreased along with the increase of pH value, also exposed the combined separation mechanism of the calix[4]pyrrole columns.

Separation of Phenols on Calix[4]pyrrole Columns

In nature, owing to the pollution of industrial waste water and pesticide, phenols widely exist as toxic organic compounds. Scientists in the field of environmental protection always give much attention to the monitoring of phenols. Till now, many methods have been developed to analyze them,

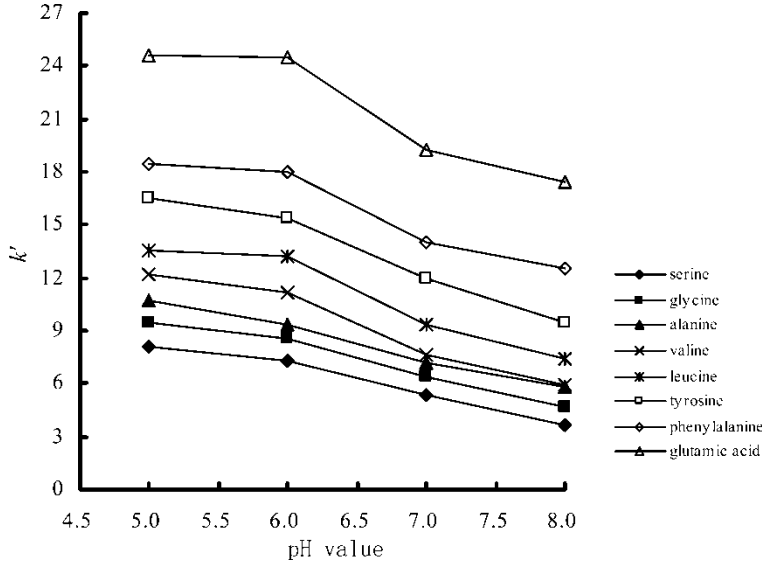


Figure 5. Effect of pH value of the mobile phase on the retention of amino acids on gel BC Column. Chromatographic conditions: Mobile phase 20 mM acetate buffer, flow rate 0.5 mL min^{-1}, UV detection at 360 nm. In the Figure, $k' = (t_R - t_0)/t_0$, where t_R is the retention time and t_0 is the dead time of column.

such as RP-HPLC,[31,32] HPCE,[33] and HPGC,[34] etc. Figure 6 shows the analytical results of six phenols on a calix[4]pyrrole column (gel BM) and on an aminopropyl silica gel column. The effect of buffer concentration on the retention of six phenols was illustrated with Figure 7. In fact, the result of the phenols on silica gel BC is similar.

On calix[4]pyrrole columns, six phenols could be separated while under identical conditions; they were separated poorly on aminopropyl silica gel columns. On gel BM and BC columns, the retention time of the phenols was prolonged but the resolutions were obviously improved. Moreover, compared with other methods for analyzing phenols, the effective separation on calix[4]pyrrole columns was procured under aqueous mobile phase.

Because of weak ionization (Table 2) in mobile phase, six phenols could not be retained effectively on aminopropyl silica gel columns (Figure 6b), but on calix[4]pyrrole silica gel columns, the multiple interactions, such as hydrophobicity and hydrogen bond, led to good separation. In water, the existence of an NH_2 unit on the phenol skeleton (positively charged) led to the interaction between aminophenol and calix[4]pyrrole stationary phase, which caused the first elution of m-aminophenol. In fact, the peaks of o-, m- and p- aminophenol were overlapped on calix[4]pyrrole columns (data not shown). The elution order of diphenols and phloroglucinol was associated with their ionized degree in mobile phase. The solute with a smaller

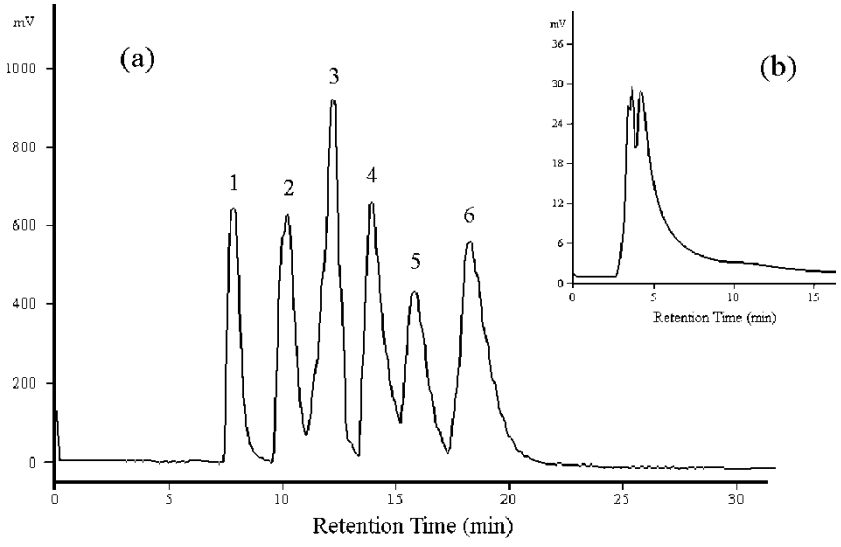


Figure 6. HPLC separation of phenols on gel BM column and aminopropyl silica gel column. Chromatographic conditions: Mobile phase water, flow rate 0.7 mL min^{-1}, UV detection at 254 nm, injection volume 20 μL. In the Figure, (a) gel BM column; (b) aminopropyl silica gel column. Peaks: 1: m-aminophenol; 2: hydroquinone; 3: catechol; 4: resorcinol; 5: phenol; 6: phloroglucinol.

dissociation constant would be ionized more easily and interacted more strongly with stationary phase, its retention time was also longer. The reason why catechol was eluted before resorcinol was probably caused by the intramolecular hydrogen bond, which weakened the interaction between catechol anion and stationary phase. As we know, hydrophobicity of phenol is the greatest in six phenols and on an ODS column, phenol was eluted last. On calix[4]pyrrole columns, because of weak ionization, interaction of hydrophobicity was still the main factor of phenol that controlled its retention and made it elute behind. The increase of buffer concentration slightly weakened the retention of the phenols (Figure 7), which revealed that the separation mechanism of calix[4]pyrrole columns was similar to that of ion exchange chromatography. From the separation of diphenols on calix[4]pyrrole columns, it is predicted that silica gel BM and BC should have the potential to separate some positional isomers.

Separation of Benzenecarboxylic Acids on Calix[4]pyrrole Columns

Benzenecarboxylic acids are important raw materials for chemical and pharmaceutical industries. They are also widely spread pollutants which mainly come from petroleum, coal, and plants.[36,37] The recent progress in analyzing benzenecarboxylic acids includes IEC,[38] HPCE,[39] and HPGC.[40] Here eight

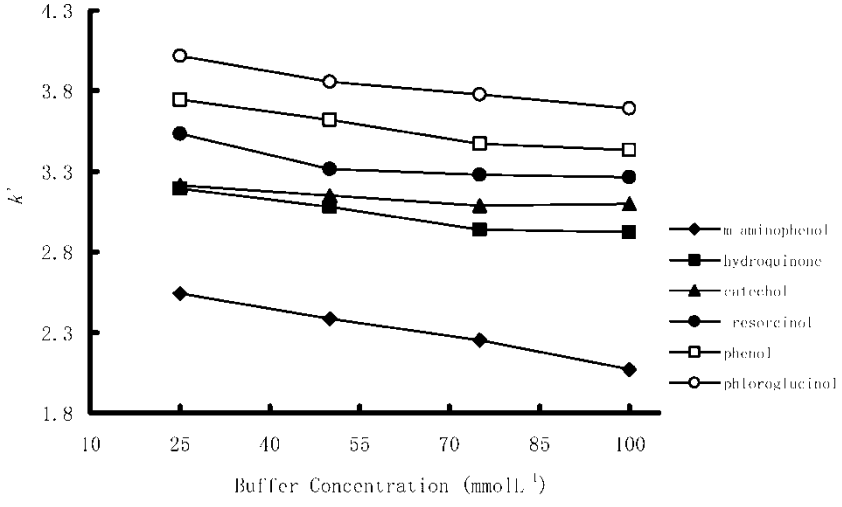


Figure 7. Effect of buffer concentration on the retention of the phenols on gel BM column. Chromatographic conditions: Mobile phase phosphate buffer, pH = 6.0, flow rate 0.7 mL min^{-1}, UV detection at 254 nm.

benzenecarboxylic acids were analyzed by calix[4]pyrrole columns. Under the same conditions, the analysis of them on aminopropyl silica gel column was also given for comparison (Figure 8). The effect of content of methanol on the retention of the carboxylic acids was displayed in Figure 9.

These data reveal again that calix[4]pyrrole columns can separate some isomers. Interestingly, the separation results expose another important fact, namely, in the process of molecular recognition, interactions can work at multiple sites at the same time. Though the ionized degree of dicarboxylic acids was weaker than that of nitrobenzoic acid in mobile phase (Table 3), the cooperation of two carboxyl anions in dicarboxylic acid molecules and calix[4]pyrrole subunits caused their longer retention on gel BM and BC columns than nitrobenzoic acids.

Separation of Medicines on Calix[4]pyrrole Columns

It is well known that organic acids and their derivatives (such as salts) have been widely used in the pharmaceutical and food industry. Chromatography,

Table 2. Dissociation constants of six phenols[35]

	m-Aminophenol	Hydroquinone	Catechol	Resorcinol	Phenol	Phloroglucinol
pKa_1	9.87	10.35	9.40	9.40	9.89	8.0

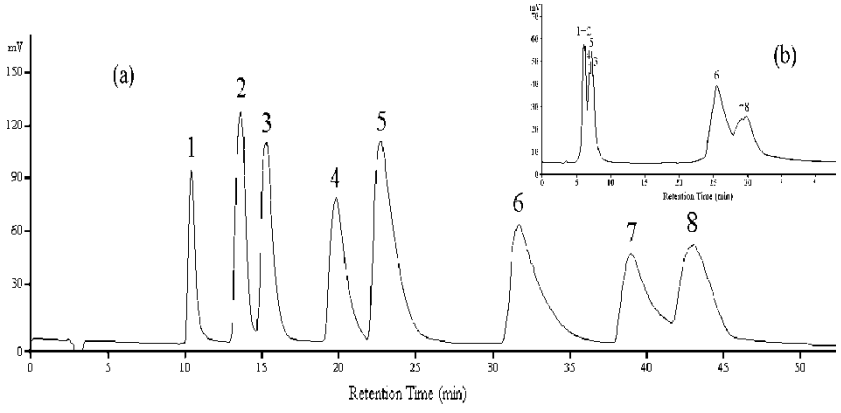


Figure 8. HPLC separation of benzenecarboxylic acids on calix[4]pyrrole column (gel BC). Chromatographic conditions: Mobile phase 30/70, methanol/10 mM phosphate buffer at PH 6.0, flow rate 0.6 mL min^{-1}, UV detection at 254 nm, injection volume 20 μL. Peaks: 1: *p*-aminobenzoic acid; 2: *p*-hydroxybenzoic acid; 3. Benzoic acid; 4: o-nitrobenzoic acid; 5: m-nitrobenzoic acid; 6: phthalic acid; 7: terephthalic acid; 8: isophthalic acid.

such as HPLC, IC, and CE are rapid and accurate methods for their analysis. As a new stationary phase with complex mechanisms of hydrophobicity and hydrogen bonds, it was rational to deduce that gel BM and BC could be used to separate some medicines.

Separation of Tranexamic Acid and Aminomethylbenzoic Acid

Tranexamic acid and aminomethylbenzoic acid are all hemostatic, which inhibit activation of plasmin. At present, methods for the determination of

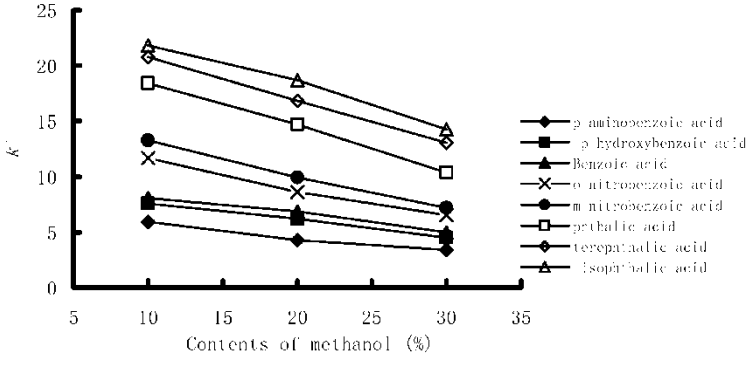


Figure 9. Effect of contents of methanol on the retention of the carboxylic acids on gel BC column. Chromatographic conditions: Mobile phase phosphate buffer, pH = 6.0, flow rate 0.6 mL min^{-1}, UV detection at 254 nm.

Table 3. Dissociation constants of eight benzenecarboxylic acids[35]

	pKa_1	pKa_2
p-Aminobenzoic acid	4.92	/
p-Hydroxybenzoic acid	4.58	/
Benzoic acid	4.21	/
o-Nitrobenzoic acid	2.17	/
p-Nitrobenzoic acid	3.44	/
Phthalic acid	2.98	5.28
Terephthalic acid	3.51	4.82
Isophthalic acid	3.46	4.46

them are mainly titration,[41] spectrophotometry,[42] HPLC,[43] and flow injection.[44] Figure 10 illustrates the separation of the acids on a silica gel BM column without derivatization. Remarkably, for the sake of the NH_2 unit, the retention of the column to two acids was not strong, but they were basically separated. While on an aminopropyl silica gel column, both acids were eluted together as a narrow peak. The order of elution on calix[4]pyrrole columns was also opposite to that obtained on RP-HPLC used for their separation. This validated again that the degree of anionization, which is the pKa of

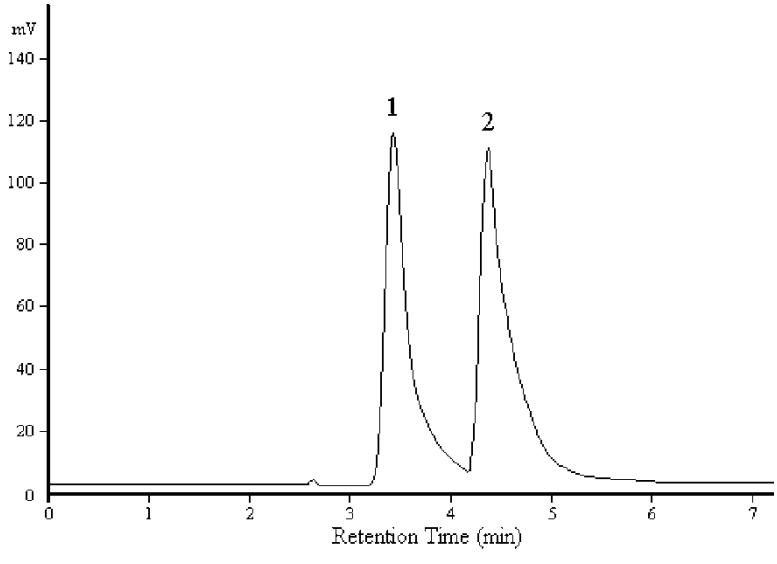


Figure 10. HPLC separation of tranexamic acid and aminomethylbenzoic acid on calix[4]pyrrole column (gel BM). Chromatographic conditions: Mobile phase 20 mM phosphate buffer at PH 6.0, flow rate 0.5 mL min^{-1}, UV detection at 205 nm, injection volume 20 μL. Peaks: 1: tranexamic acid; 2: aminomethylbenzoic acid.

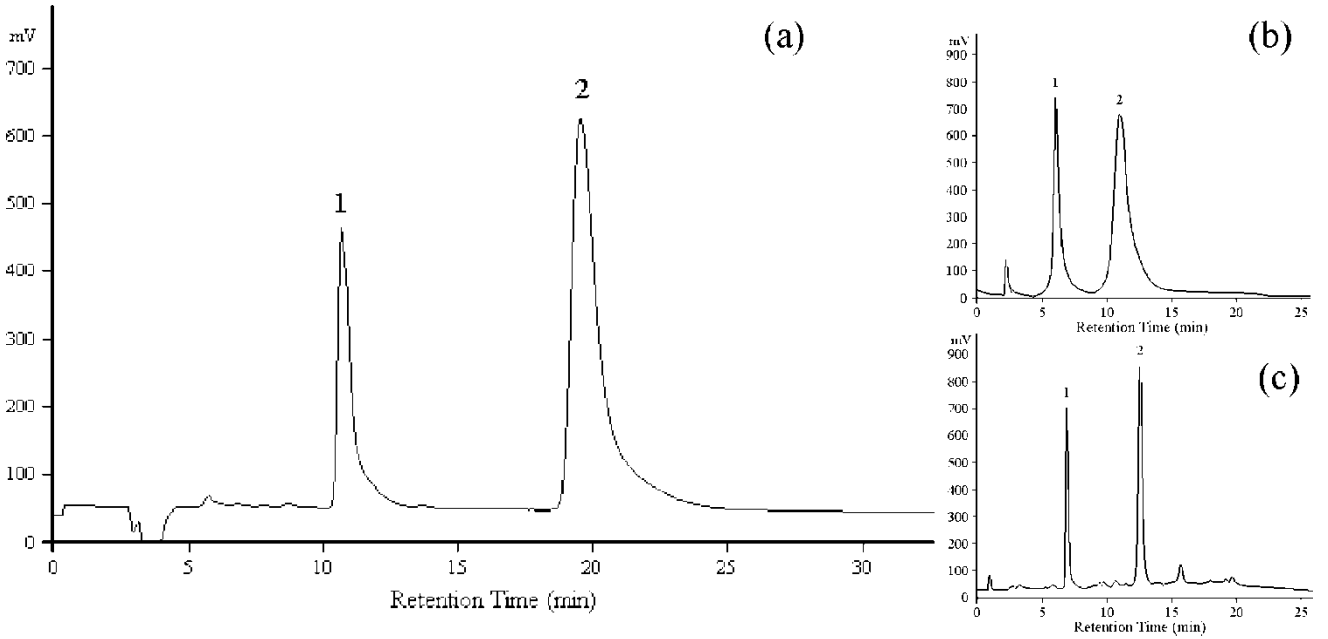


Figure 11. HPLC separation of cefoperazone sodium and sulbactam sodium on three kinds of columns. Chromatographic conditions: Mobile phase 40/60, acetonitrile/10 mM phosphate buffer at PH 5.0, flow rate 0.6 mL min^{-1}, UV detection at 210 nm, injection volume 20 μL. Peaks: 1: SBT; 2:CPZ. (a) Gel BM column; (b) aminopropyl silica gel column; (c) C_{18} column, flow rate 1.0 mL min^{-1}.

aminomethylbenzoic acid, was much greater than that of tranexamic acid, which influenced the separation.

Separation of Cefoperazone Sodium (CPZ) and Sulbactam Sodium (SBT) for Injection

CPZ and SBT is a combination drug of antibiotic and enzyme inhibitor. They have prominent synergistic action when used together and significant curative effect on bacterial infections. The routine detection method for them is RP-HPLC.[41] Here, we tried to separate them for injection directly with calix[4]-pyrrole columns and acquired good results. The representative chromatogram is as Figure 11.

In Figure 11, it is obvious that for CPZ and SBT, the separation effectiveness of a C_{18} column is the best. On an aminopropyl silica gel column, because of the strong coulombic force but weaker hydrophobicity, the elution of the medicines is faster but their peaks broaden. The separation results on calix[4]-pyrrole columns is intermediate. This example exposes, that for many salified medicines, calix[4]pyrrole columns are one of a new and effective choice for their detection.

Separation of Phenylacetic Acid (PAA) and Benzylpenicillin Potassium (KPen G)

Phenylacetic acid is not only the important precursor for the synthesis of penicillin G (Pen G) but also the main product in the hydrolysis of it. In the course of production of penicillin, it is useful to detect PAA and Pen G at the same

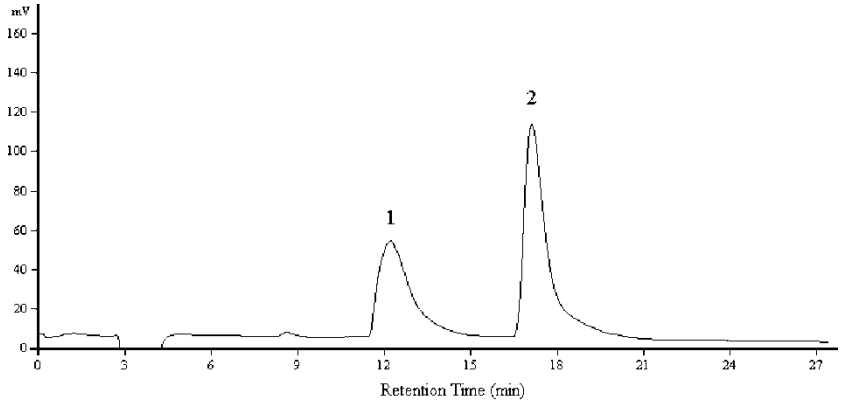


Figure 12. HPLC separation of phenylacetic acid and benzylpenicillin potassium on calix[4]pyrrole column (gel BC). Chromatographic conditions: Mobile phase 30/70, acetonitrile/20 mM phosphate buffer at PH 5.0, flow rate 0.5 mL min^{-1}, UV detection at 230 nm, injection volume 20 μL. Peaks: 1: PAA; 2: KPen G.

time to control the yield of PAA. Detection of PAA and KPen G is commonly by RP-HPLC.[45,46] Figure 12 shows the separation of PAA and KPen G on a calix[4]pyrrole column. The good results proved again that calix[4] pyrrole columns could be used to separate anionic substances, which were effective parts of medicines.

CONCLUSIONS

On calix[4]pyrrole modified silica gel columns, the separation of inorganic anions, amino acids, phenols, benzenecarboxylic acids, and some medicines were obtained. Interactions of the hydrogen bond and hydrophobicity influenced the separation results, which makes calix[4]pyrrole columns present some properties combining RP-HPLC and IEC. Calix[4]pyrrole columns showed the ability to separate some positional isomers and medicines. That will enlighten the further studies on the mechanism of molecular recognition and extend the applications of calix[4]pyrroles in the field of separation and analysis.

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