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Retentivity and Separability of Silica Gel Modified by Chiral Selector *N*-(3-Sulfo,3-carboxy)-propionylchitosan

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Abstract—A new sorbent based on silica gel modified by N-(3-sulfo,3-carboxy)-propionylchitosan (SCPC-modified silica gel) has been synthesized, and its chromatographic properties have been studied. The laws governing the retention and resolution of chiral compounds with the use of SCPC have been investigated. The enantioselectivity of the sorbent toward several basic compounds, including fluoxetine, chlorcyclizine, pindolol, and other medicinals, has been investigated.

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Molecules of most compounds pertaining to pharmacology or biochemistry contain one or several asymmetric centers. Synthesis of these compounds can produce enantiomer mixtures. Inasmuch as optical isomers usually differ from one another in their pharmacological and biological activities, the separation of enantiomers (enantioseparation) has become increasingly topical. High-performance liquid chromatography (HPLC) is a very popular tool for solving this problem: it provides the high efficiency and selectivity of enantioseparation, but does not require much time and is not expensive. Polysaccharides, having asymmetric centers in each structural unit, have a high potential for designing stationary chiral phases. Chitosan and its analogues are the least studied polysaccharides.

We tested *N*-(3-sulfo,3-carboxy)-propionylchitosan (SCPC) as a new stationary chiral phase for the separation of the optical isomers of several medicinals; an additional chiral center, sulfo group, and carboxy group were introduced into the amino group of SCPC (Fig. 1). The authors of [1, 2] first proposed this compound for use as a chiral selector in electrophoresis. Their data imply that SCPC has potential for use as a chiral selector in HPLC.

EXPERIMENTAL

This work was fulfilled on a Shimadzu SLC-10A (Japan) liquid chromatograph equipped with a Shimadzu SPD-10AV spectrophotometric detector and an LC-10AT Shimadzu pump. The software package CLASS-VP v.5.03 (Shimadzu) was used for chromatogram recording and processing. The loop volume was 20 μl. The detection wavelength was 230 nm. A steel column 150 × 3.2 mm was used. The column was filled with a Knauer K-1900 high-pressure pneumatic pump.

The sorbent was prepared from Silasorb S 300 silica gel (average particle size, 7.5 μm) modified with chitosan (5 kDa, 85% deacetylated) supplied by the Bioinzheneriya Center, Russian Academy of Sciences; maleic aldehyde; and sodium metasulfite (chemically grade).

The dead time of the column was 2.25 min. Mobile phases were prepared from methanol and acetonitrile

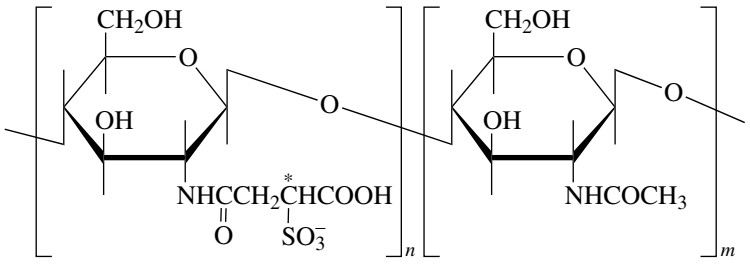

Fig. 1. Structural formula of *N*-(3-Sulfo,3-carboxy)-propionylchitosan.

[a] *Bioinzheneriya Center, Russian Academy of Sciences*

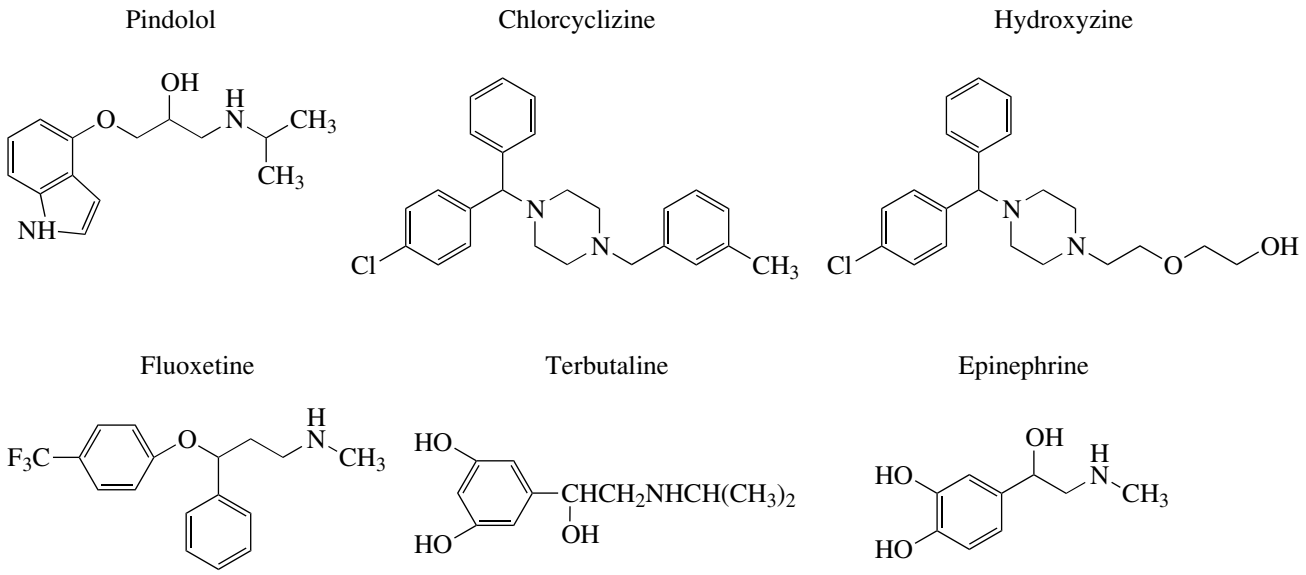

Fig. 2. Structural formulas of model compounds used for investigating the enantioselectivity of SCPC-modified silica gel.

(from Cryochrom, Russia), acetic acid, and triethylamine (chromatographic grade). We also used 0.1–1.0 mg/ml solutions of nicotinamide (chemically pure grade) and racemic mixtures of the test compounds (from Merck, Germany), which were prepared by dissolving the specified compounds in the mobile phase.

RESULTS AND DISCUSSION

In this work, we attempted to design a new chiral stationary phase for HPLC using SCPC.

The efficiency of chiral sorbents is determined by the difference between the formation energy of diastereomeric complexes of the substrate ligand. Apart from adequately choosing chiral ligands, it is important how chiral sorbents are prepared. The synthesis should ensure the preservation of the chiral configuration of grafted ligands and the reactivities of functional groups. The chiral molecule should be fixed in the strict positions in order to avoid steric hindrances. In practice, this is achieved by using a long, flexible leg or by diluting grafted chiral ligands on the substrate surface to decrease their surface concentration.

The underlying idea of most approaches to designing chiral stationary phases is the chemical modification of mineral substrates, above all silica gels. The advantages of silica gels are as follows: nonswelling in various solvents, mechanical strength, thermostability, high mass-transfer rates, separability to narrow fractions, insignificant intrinsic catalytic activity, availability, and relatively low costs [3].

The efficiency of a chromatographic column packed with the sorbent was evaluated for nicotinamide with the methanol–0.1% triethylamine acetate (TEAA) mixture (50 : 50) as a mobile phase. The column efficiency was 16 380 theoretical plates per meter.

The retention laws, efficiency, and enantioselectivity of SCPC-modified silica gel in reversed-phase and polar-organic liquid chromatography (RP-HPLC and PO-HPLC, respectively) were studied for several optically active compounds, whose structural formulas are displayed in Fig. 2.

Reversed-Phase HPLC on SCPC-Modified Silica Gel

When choosing the enantiorecognition conditions in RP-HPLC, we studied the retention, selectivity, and efficiency as functions of the following factors:

(1) the nature of the organic modifier and its concentration in the mobile phase;

(2) the pH and composition of the aqueous component of the mobile phase.

Table 1 displays the results obtained from the investigation of the chromatographic properties of the test sorbent in the RP-HPLC mode. The organic modifier used was methanol or acetonitrile. The electrolytes in the mobile phase were triethylammonium acetate (TEAA), ammonium chloride, or glacial acetic acid; all these compounds contain a cation that can compete with the basic groups of the sorbate for the interaction with the carboxy group and sulfo groups of SCPC.

With the use of acetonitrile-containing mobile phases, the retention times of the test model compounds were comparable with the dead time of the column; therefore, the experiments were carried out in the presence of methanol.

With the use of aqueous methanol as the mobile phase, high retention times and the absence of enantio-

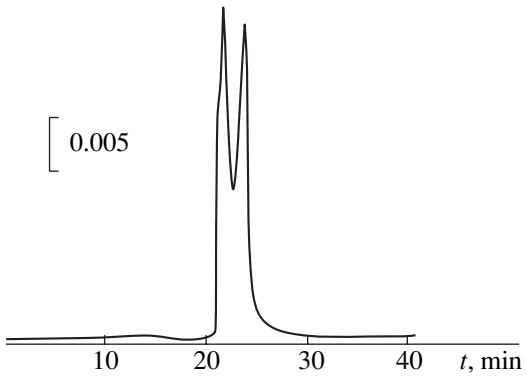

Fig. 3. Chromatogram for fluoxetine enantioseparation (mobile phase, methanol : 0.05% TEAA (50 : 50); pH 4.6; flow rate, 1 ml/min; λ = 230 nm).

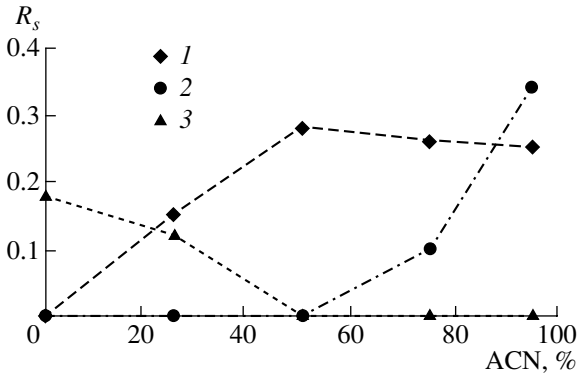

Fig. 4. Resolution of enantiomers R_S for (*1*) hydroxyzine, (*2*) fluoxetine, and (*3*) epinephrine vs. acetonitrile proportion in the mobile phase (acetonitrile : methanol, 0.1% TEAA).

selectivity were observed. The retention time rapidly drops when an electrolyte (0.05%) whose cation is capable of the competitive interaction with the sulfo groups and carboxy group of SCPC is added to the aqueous-methanol mobile phase. The increase in the electrolyte concentration from 0.1 to 1.0% induces only small changes in the retentivity. The retention times increase with increasing methanol proportion in the mobile phase, but the enantioselectivity decreases. Enantioseparation does not occur when the methanol proportion in the mobile phase is 70%.

Acetic acid is an unsuccessful modifier of the aqueous component of the mobile phase: with 0.05% acetic acid, the retention times are comparable with the dead time of the column. Enantiorecognition is observed when TEAA (0.05 or 0.1%) or NH_4Cl (10^{-3} mol/l) is added to the mobile phase. The resolution R_S decreases to zero when the TEAA concentration increases from 0.05 to 1% or the NH_4Cl concentration increases from 10^{-3} to 5×10^{-3} mol/l.

Greater separation numbers and the best enantioselectivities and resolutions were obtained with the use of TEAA as an electrolyte. Figure 3 exemplifies the chromatogram of fluoxetine enantioseparation.

Thus, the enantioselectivity is affected by the nature of the electrolyte cation and its concentration. Probably, the electrolyte cation effects the electrostatic interactions between sorbate and SCPC molecules, which is in turn responsible for the chiral selectivity of the sorbent. As the pH of the aqueous component of the mobile phase increases from 3.5 to 5.4, the retention times of the model compounds increase. The optimal pH of fluoxetine, hydroxyzine, and epinephrine enantioseparation is 4.6. Likely, the carboxy group of SCPC, whose pK_a is 5.8 [2], is only insignificantly dissociated under these conditions, which favors hydrogen bonding with the amino or hydroxy groups of the racemates.

Polar Organic HPLC on SCPC-Modified Silica Gel

It is known from the related literature [4] that for many chiral selectors, enantiorecognition can be increased by the use of mobile phases with high proportions of an organic modifier, i.e., in PO-HPLC. In our PO-HPLC experiments, the mobile phase used was methanol or acetonitrile plus methanol with TEAA or acetic acid additives.

Table 2 displays the results of our investigation of the retention and enantioseparation as a function of the following factors: the acetonitrile and methanol proportion in the mobile phase and the nature and concentration of the electrolyte.

The effect of the acetonitrile and methanol proportion on the retention and enantioseparation was studied in racemic mixtures of hydroxyzine, fluoxetine, and epinephrine. The retention decreases with increasing acetonitrile proportion in the mobile phase. This effect is above all caused by the higher polarity of acetonitrile.

Figure 4 displays the resolution R_S as a function of acetonitrile proportion in the mobile phase. At high acetonitrile concentrations and lower retentions, enantioseparation is better for fluoxetine and hydroxyzine, while the epinephrine enantiomers are only partially separated at high methanol concentrations. This differentiated effect can be caused by different characters of the interaction of the sorbates with the chiral centers of the selector. For the bulky fluoxetine and hydroxyzine molecules, hydrophobic interactions and steric discrimination are significant, while for the epinephrine molecules with their three OH groups, hydrogen bonds are significant. Therefore, this differentiated effect can be due to the interplay of hydrophobic interactions, hydrogen bonding, steric hindrances, and competitive interactions of TEAA cations with the selector. Thus, the sorbate structure is the key factor governing the differentiated effect of the nature of the organic solvent and its proportion in the mobile phase on the retention and separation of racemic mixtures.

Table 1. Effect of the composition, concentration, and pH of the aqueous component of the mobile phase (MP) and of the modifier proportion on the chromatographic properties of SCPC-modified silica gel (flow rate, 1.0 ml/min; λ = 230 nm)

Compound	k	N*	α	R_S
MP: (50 : 50) *Me*OH** : H_2O				
Pindolol	11.72	840	1.0	–
Fluoxetine	***	–	–	–
Hydroxyzine	***	–	–	–
Epinephrine	20.65	600	1.0	–
MP: (50 : 50) *Me*OH : 0.05% TEAA pH 4.6				
Pindolol	2.51	2100	1.0	–
Fluoxetine	9.94	15200	1.09	0.94
Hydroxyzine	12.03	1670	1.2	0.61
Epinephrine	13.05	2030	1.17	0.25
MP: (50 : 50) *Me*OH : 0.1% TEAA pH 4.6				
Pindolol	1.30	1000	1.0	–
Fluoxetine	0.90	1000	1.36	0.44
Hydroxyzine	0.92	1030	1.16	0.22
Epinephrine	1.03	730	1.31	0.34
MP: (50 : 50) *Me*OH : 1.0% TEAA pH 4.6				
Pindolol	0.67	1020	1.0	–
Fluoxetine	0.49	1870	1.0	–
Hydroxyzine	0.48	1330	1.0	–
Epinephrine	0.56	1800	1.0	–
MP: (50 : 50) *Me*OH : 10^{-3} M NH_4Cl pH 4.8				
Fluoxetine	23.97	5970	1.05	0.34
Hydroxyzine	12.62	1510	1.0	–
Epinephrine	16.13	30	1.26	<0.1
MP: (50 : 50) *Me*OH : 5×10^{-3} M NH_4Cl pH 4.5				
Fluoxetine	7.46	1550	1.0	–
Hydroxyzine	4.44	1540	1.0	–
Epinephrine	8.47	720	1.0	–
MP: (50 : 50) *Me*OH : 0.1% TEAA pH 3.5				
Fluoxetine	0.25	750	1.0	–
Hydroxyzine	0.17	480	1.0	–
Epinephrine	0.10	550	1.0	–
MP: (50 : 50) *Me*OH : 0.1% TEAA pH 5.4				
Pindolol	4.78	560	1.0	–
Fluoxetine	3.92	530	1.0	–
Hydroxyzine	3.70	810	1.0	–
Epinephrine	3.37	1360	1.0	–
MP: (70 : 30) *Me*OH : 0.1% TEAA pH 4.6				
Pindolol	2.09	1610	1.0	–
Fluoxetine	1.36	1090	1.0	–
Hydroxyzine	1.2	1940	1.0	–
Epinephrine	3.02	1920	1.0	–

* The number of theoretical plates per meter of the column.
** Methanol.
*** Not eluted.

Table 2. Effect of the composition of the mobile phase (MP) and of the electrolyte proportion on the chromatographic properties of SCPC-modified silica gel (flow rate, 1.0 ml/min; λ = 230 nm)

Compound	k	N*	α	R_S
MP: (95 : 5) CAN** : *Me*OH, 0.1% TEAA				
Fluoxetine	0.40	1750	1.14	0.34
Chlorcyclizine	0.33	960	1.0	–
Terbutaline	0.36	1070	1.0	–
Hydroxyzine	0.39	3360	1.25	0.25
Epinephrine	0.32	590	1.0	–
MP: (50 : 50) ACN : *Me*OH, 0.1% TEAA				
Fluoxetine	21.69	1250	1.0	–
Chlorcyclizine	3.98	650	1.0	–
Terbutaline	9.76	210	1.0	–
Hydroxyzine	1.65	1330	1.14	0.28
Epinephrine	10.20	610	1.0	–
MP: (50 : 50) ACN : *Me*OH, 0.2% TEAA				
Fluoxetine	10.86	1020	1.0	–
Chlorcyclizine	2.40	810	1.17	0.30
Terbutaline	2.89	430	1.0	–
Hydroxyzine	0.86	680	1.20	0.23
Epinephrine	7.65	290	1.0	–
MP: (50 : 50) ACN : *Me*OH, 0.5% TEAA				
Fluoxetine	5.08	1090	1.0	–
Chlorcyclizine	1.74	1320	1.14	0.39
Terbutaline	1.25	500	1.0	–
Hydroxyzine	0.85	350	1.21	<0.1
Epinephrine	5.73	30	1.02	<0.1
MP: *Me*OH, 0.1% TEAA				
Fluoxetine	13.42	3570	1.0	–
Hydroxyzine	3.23	1530	1.0	–
Epinephrine	12.48	690	1.07	0.18
MP: *Me*OH, 0.5% TEAA				
Fluoxetine	3.87	1610	1.0	–
Hydroxyzine	1.27	830	1.0	–
Epinephrine	2.19	1440	1.0	–
MP: *Me*OH, 0.25% AcOH***				
Fluoxetine	0.72	1050	1.73	0.74
Chlorcyclizine	1.47	240	1.0	–
Terbutaline	0.56	850	1.0	–
Hydroxyzine	0.50	2490	1.16	0.43
Epinephrine	2.65	170	1.0	–
MP: *Me*OH, 0.5% AcOH				
Fluoxetine	0.72	220	1.45	<0.1
Chlorcyclizine	0.82	230	1.51	<0.1
Terbutaline	0.50	930	1.17	<0.1
Hydroxyzine	0.45	2430	1.19	0.27
Epinephrine	2.52	200	1.0	–
MP: *Me*OH, 1.0% AcOH				
Fluoxetine	0.22	390	1.0	–
Hydroxyzine	0.46	1150	1.20	<0.1
Epinephrine	2.22	630	1.0	–

* The number of theoretical plates per meter of the column.
** Acetonitrile.
*** Acetic acid.

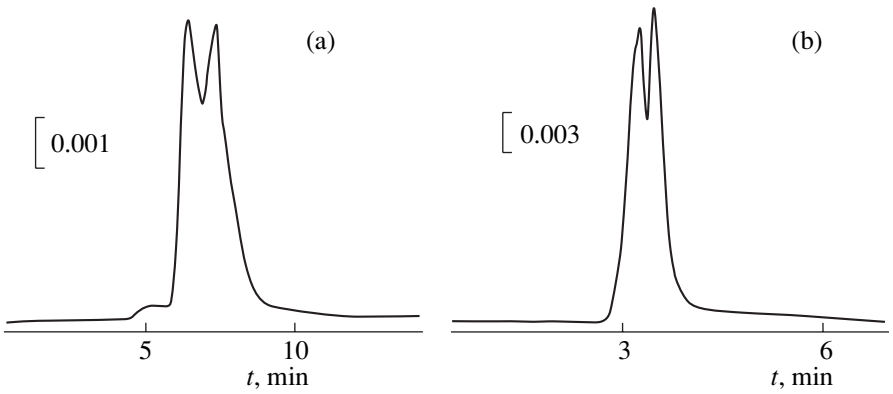

Fig. 5. Chromatogram for (a) chlorcyclizine enantioseparation (mobile phase: acetonitrile : methanol (50 : 50), 0.5% TEAA) and (b) hydroxyzine enantioseparation (mobile phase: methanol, 0.25% acetic acid); flow rate, 1 ml/min; λ = 230 nm.

We studied the effect of the nature of an electrolyte, which was TEAA or acetic acid, and its proportion on the retention and separation of various compounds. An increase in the proportion of TEAA or acetic acid in the mobile phase decreases the retention times; TEAA has a far stronger effect. This can be explained by the fact that in the presence of $N^+(C_2H_5)_3$ cations, the competitive interaction with the acid groups of the selector in PO-HPLC is more noticeable.

An increase in the TEAA concentration from 0.1 to 0.5% in the acetonitrile–methanol (50 : 50) mobile phase differently effects the enantioseparation: for epinephrine, the separation deteriorates; for chlorcyclizine, it improves. Therefore, the effect of TEAA is also determined by the sorbate structure. An increase in the acetic acid concentration to 1% decreases the resolution of enantiomer peaks.

The nature of the additive also determines the range of separable compounds. For example, in the presence of acetic acid, epinephrine enantioseparation does not occur; instead, chlorcyclizine and terbutaline are added to the list of partially separable racemates.

In summary, SCPC-modified silica gel provides a partial enantioseparation of fluoxetine, hydroxyzine, chlorcyclizine, epinephrine, and terbutaline. The selectivity in PO-HPLC is in general better than in RP-HPLC. Figures 5a and 5b display the PO-HPLC chromatograms for the enantioseparation of chlorcyclizine and hydroxyzine. The low resolutions are evidently on account of the low performance of the column. In this work, we have demonstrated that *N*-(3-sulfo,3-carboxy)propionylchitosan chemically immobilized on silica gel shows enantioselectivity toward several basic medicinals. The need for improving the separation efficiency and selectivity makes it necessary to investigate the parameters of silica gel modification and the properties of the matrix. From our standpoint, silica gels with smaller particle sizes and larger, homogeneous pores have perspectives.

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