Journal of Chromatography, 333 (1985) 141–151
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 894

RAPID ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PORPHYRIN ESTERS ON AMINOPROPYL-BONDED SILICA

P. KOTAL*
Laboratory for the Pathophysiology of the Blood System and Liver, Prague 2 (Czechoslovakia)
B. PORSCH
Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague 6 (Czechoslovakia)
and
M. JIRSA and V. KORDAČ
Laboratory for the Pathophysiology of the Blood System and Liver, Prague 2 (Czechoslovakia)
(Received May 12th, 1985)

SUMMARY

A simple isocratic procedure for the separation of porphyrin esters according to the number of carboxyl groups using an aminopropyl-bonded silica column and a simple binary eluent is suggested. In this way, problems with the choice of a suitable unmodified silica column for isocratic separation are circumvented; entirely different aminopropyl-bonded silicas of various origins exhibit almost identical behaviour. The influence of end-capping of residual silanols and the chemical substitution of the primary amino group on the separation was studied. To achieve an isocratic separation of porphyrin esters on the bare silica used, the concentration of surface silanol groups must be decreased; suitable concentrations are tailored by means of partial trimethylsilylation. The isocratic separation of porphyrin esters can also be achieved on cyanopropyl-bonded silica. The suggested optimum procedure, using primary amino-bonded silica, gives the highest resolution and allows the baseline separation of porphyrin esters with two to eight carboxyl groups together with their Cu^{2+} complexes and synthetic tetraphenylporphyrin as the internal standard.

INTRODUCTION

Natural porphyrins can be separated in the free form according to the number of carboxyl groups by means of ion-pair gradient reversed-phase chromatography[1,2]. However, separations of esterified porphyrins are much more common because of increased stability of the esters compared with natural porphyrins, and because of simplified purification procedures[2].

Normal-phase chromatography on microparticulate silicas is generally used[2] for the separation of porphyrin esters according to the number of carboxyl groups. A number of workers have described separations of mixtures of porphyrins containing from two to eight esterified carboxyls using binary mobile phases; they stated[3–5]

0021-9673/85/$03.30

that gradient elution or at least flow programming[2,6] is necessary. Isocratic separations with binary eluents on some types of silica as Hypersil[7], Spherisorb[7] and μPorasil[8] have also been described. Four-component mobile phases were recommended[9,10] for enhancing resolution on μPorasil. In contrast, Carlson *et al.*[11] claimed that gradient elution was necessary for separating porphyrin esters with two to eight carboxyl groups if μPorasil was used. The reasons for the different behaviours of various types of silica are unclear. In this respect batch to batch variations of column packings have been mentioned[10]. It seems that the choice of a silica column suitable for the isocratic separation of porphyrin esters is complicated.

The aim of this study was to develop a simple isocratic procedure for separating porphyrin esters according to the number of carboxyl groups, to overcome problems associated with the choice of a suitable unmodified silica column and to elucidate the mechanism of separation and the role of different groups chemically bonded on the surface of the silica used. Further, we have also attempted to clarify the relationship between the concentration of silanol groups on the surface of unmodified silica and the possibility of isocratic separation, and to suggest an optimum procedure for the baseline separation of porphyrin esters with two to eight carboxyl groups together with their Cu^{2+} complexes and a suitable internal standard.

EXPERIMENTAL

Modified silicas

Separon Six NH_2 (Laboratory Instrument Works, Prague, Czechoslovakia), with a mean particle diameter, $\bar{d}_p$, of 10 μm, was end-capped with N-trimethylsilylimidazole (Fluka, Buchs, F.R.G.); this reagent does not react with primary amino groups[12]: 5 g of starting silica were shaken for 5 h at 60°C with 66 ml of a solution of N-trimethylsilylimidazole in dry toluene.

The benzaldehyde derivative of Separon Six NH_2 ($\bar{d}_p$ = 5 μm) (3 g) was prepared by reaction with benzaldehyde (5 g) for 10 h at 100°C. The dinitrofluorobenzene derivative of Separon Six NH_2 ($\bar{d}_p$ = 5 μm) (3 g) was obtained by reaction for 2 h at 37°C with a 5% solution of dinitrofluorobenzene (International Enzymes, U.K.) in ethyl acetate (20 ml) with addition of 0.1 ml of triethylamine.

Partially trimethylsilylated silicas were prepared by reacting Separon Six (Laboratory Instruments Works) ($\bar{d}_p$ = 5 μm) (1.6 g) with 5 ml of a 10% solution of hexamethyldisilazane (Lachema, Brno, Czechoslovakia) in dry toluene, (i) at laboratory temperature for 30 min and (ii) at 60°C for 5 h. All products were washed with toluene and methanol and dried for 3 h at 85°C. The degree of conversion was followed by elemental analysis and the results are summarized in Table I. The content of primary amino groups was determined[13] by titration with 0.1 *M* perchloric acid in anhydrous acetic acid using crystal violet as indicator.

Porphyrins

Uroporphyrin I and coproporphyrin I methyl esters were kindly provided by Prof. T. K. With (Denmark). 7-, 6- and 5-porphyrins (the numbers indicate the number of carboxyl groups) were prepared by decarboxylation of uroporphyrin ester I. The described procedure[14] was modified to yield the maximum amounts of 7-, 6- and 5-porphyrins and the minimum amount of 8- and 4-porphyrins. Uroporphyrin ester

TABLE I

ELEMENTAL ANALYSIS OF DERIVATIVES OF SEPARON SIX AND SEPARON SIX NH_2

Modifier	*Starting material*			*Derivative*		
	C (%)	*H (%)*	*N (%)*	*C (%)*	*H (%)*	*N (%)*
N-Trimethylsilyl-imidazole*	5.96	1.52	1.97	8.16	1.92	1.88
Benzaldehyde*	5.20	1.31	1.85	11.84	1.60	1.59
2,4-Dinitrofluoro-benzene*	5.20	1.31	1.85	10.68	1.46	3.92
Hexamethyldisilazane**	–§	–	–	3.42	0.94	–
Hexamethyldisilazane***	–	–	–	4.93	1.39	–

* Separon Six NH_2.
** Separon Six, 30 min, 25°C.
*** Separon Six, 5 h, 60°C.
§ No nitrogen was determined by CHN analysis.

I (10 mg) was dissolved in 0.5 ml of concentrated hydrochloric acid and diluted to 2% with distilled water. Decarboxylation in an autoclave was performed for 3 h at 140°C (the resulting mixture gave λ_{max} = 402.5 nm in 0.5 *M* H_2SO_4). This product was esterified for 1 h using H_2SO_4 in methanolic solution (1:10, v/v), extracted into chloroform, neutralized (5% solution of $NaHCO_3$) and washed with distilled water. Preparative thin-layer chromatography (20 × 20 cm DC-Fertigplatten, Kieselgel 60; Merck, Darmstadt, F.R.G.) was carried out according to Doss[15], using benzene–ethyl acetate–methanol (85:13.5:1.5) as the eluent.

Protoporphyrin was obtained by demetallization[16] of haemin using $FeSO_4$ and tetraphenylporphyrin by reaction[17] of pyrrole with benzaldehyde. Copper complexes were prepared[18] by reaction of the corresponding esters with copper acetate.

Chromatography

The liquid chromatograph consisted of a positive displacement pump (HPP 4001), a sample loop injector (LCI 30, internal sample loop 0.5 μl) or a septum injector (LCI-02), a UV–visible spectrophotometric detector (LCD 2563) (wavelength of filter, λ = 405 nm) and a potentiometric recorder (TZ 4200) with a computing integrator (CI 100). The whole equipment was produced by Laboratory Instruments Works.

Alternatively, an LC 85 B grating spectrophotometer (λ = 399 nm) of an LS-2 filter fluorimeter with excitation at 400 nm and emission at 625 nm (both from Perkin-Elmer, Norwalk, CT, U.S.A.) were used.

Pressure-resistant CGC glass columns (150 × 3.2 mm I.D.) packed with Separon Six, Separon Six CN, Separon Six NH_2 (Laboratory Instruments Works) and LiChrosorb NH_2 (Merck) were products of Laboratory Instruments Works. The modified silicas were slurry-packed into the CGC columns by means of a high-pressure membrane pump in methanol–dioxan (1:1) at 50 MPa. During chromatography a laboratory-made saturation stainless-steel column (100 × 6 mm I.D.) was always inserted between the pump and the sample injector.

Mixtures of ethyl acetate with *n*-heptane (both of spectral grade) (Merck)

served as mobile phases; a water-saturated eluent was prepared by mixing dry and water-saturated mixtures in corresponding proportions. Mixtures of porphyrin esters dissolved in chloroform or mobile phase were injected, the flow-rate generally being 0.4 ml/min. To identify the individual porphyrin ester peaks, UV spectra were scanned using the stopped-flow technique with the LC 85B spectrophotometer, or a standard additions technique was used.

RESULTS AND DISCUSSION

Initial attempts to find a suitable binary composition of the mobile phase for an isocratic separation of porphyrin esters with two to eight carboxyl groups on Separon Six were unsuccessful and gradient elution proved to be necessary. Comparison of the physical constants of different bare silicas has shown that isocratic separations can be achieved on sorbents with lower specific surface areas (according to Majors[19]: Hypersil, 200 m^2/g; Spherisorb, 220 m^2/g; μPorasil, 300–350 m^2/g). However, it is well known[20] that the relative retentions, expressed as separation factors, $\alpha = k_{i+1}/k_i$ (where k_{i+1} and k_i are capacity factors of the $i + 1$ and ith peaks, respectively) of successive peaks should not depend on surface area, provided that the surface structures and the surface concentrations of silanol groups do not differ. On the other hand, it has been found that the surface acidity[21] and surface concentration of silanol groups[20] may differ widely even in silicas with very similar physical properties if the methods of preparation differ with respect to the starting raw materials. A possible explanation is that the method of preparation giving lower surface areas of the products also provides lower surface concentrations of silanol groups as well. On a bare silica with a lower concentration of surface silanol groups, relatively lower values of capacity factors of porphyrin esters with a higher number of carboxyl groups in comparison with, *e.g.*, 2-porphyrin ester, can be expected, because hydrogen bonding between silanol and ester groups[22] should be a dominant interaction.

Polar chemically bonded aminopropyl-silica is characterized by strong hydrogen bonding and, being basic, creates different selectivities[23] in comparison with bare silica. Therefore, the successful use of this sorbent for the separation of porphyrin esters would seem to be straightforward. This is corroborated in Fig. 1a, which shows the isocratic separation of porphyrin esters with two to eight carboxyl groups together with an internal standard. The chromatogram of the identical mixture in the same mobile phase on the LiChrosorb NH_2 (Fig. 1b) serves as a check of the behaviour of different types of primary amino-bonded silica. Both materials give almost the same result, although the contents of organic bonded phase and primary amino groups are completely different[24] (LiChrosorb NH_2: 3.50% C, 0.94% H, 1.04% N; 0.85 mmol/g NH_2. Separon Six NH_2: 5.5% C, 1.40% H, 2.06% N; 1.30 mmol/g NH_2). It can be concluded that in this way problems connected with the choice of a suitable type of unmodified silica can be circumvented.

Apparently, commercial aminopropyl silicas are not end-capped (the commonly used trimethylchlorosilane and hexamethyldisilazane also trimethylsilylate the amino group[12]) and therefore a considerable amount of residual silanol remains on their surface. Primary non-reacted[25] and also secondary silanols[26] originating from hydrolysis of unreacted alkoxyls of the usually used trialkoxysilane may be expected.

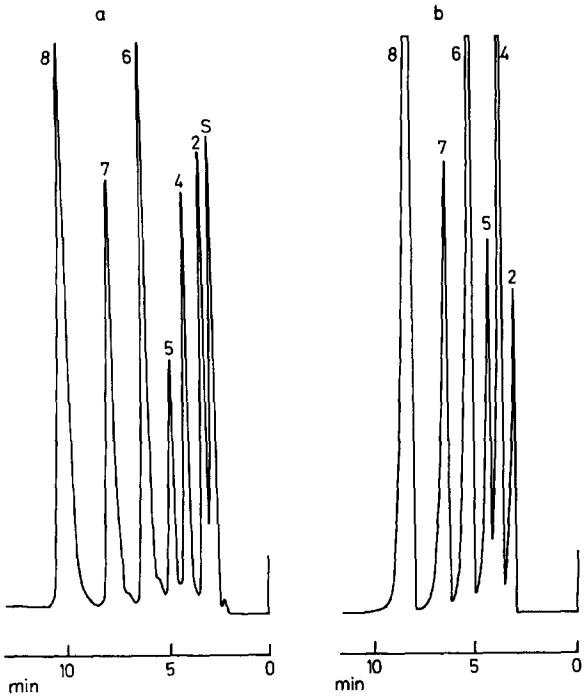

Fig. 1. Separation of porphyrin esters on a CGC column packed with (a) Separon Six NH_2 and (b) LiChrosorb NH_2. Sample: 10 μl of solution in chloroform. Mobile phase: 57% (v/v) ethyl acetate in *n*-heptane. Flow-rate: 0.4 ml/min. Di- (proto-), tetra- (copro-), penta-, hexa-, hepta- and octa(uro)-carboxylic porphyrin esters are numbered as 2, 4, 5, 6, 7, 8, respectively. Internal standard: synthetic tetraphenylporphyrin (S). Spectrophotometer: LC-85 B, λ = 399 nm (0.16 a.u.f.s.).

To elucidate the role and/or cooperation of primary amino groups and silanols, benzaldehyde and dinitrofluorobenzene derivatives of the amino phase, together with its end-capped modification, were prepared. A chromatographic comparison using the same composition of mobile phase is shown in Fig. 2. Capacity factors and separation factors, α, corresponding to the original Separon Six NH_2, to all three modifications and to LiChrosorb NH_2, are summarized in Table II. It can be seen that the capacity factors decrease after end-capping but the selectivity remains almost unchanged. In contrast, chemical derivatization of primary amino groups considerably decreases α to approximately the same level. Lowering of the capacity factors (k') with the benzaldehyde derivative probably reflects the decrease in its polarity, whereas an increase in k' with the dinitrofluorobenzene derivative may be due to a charge-transfer complex with the tetrapyrrole ring.

It can be concluded that combined interactions of silanols and primary amino groups with porphyrin esters play an important role; the primary amino group determines mainly the selectivity and residual silanols largely influence k'. With LiChrosorb NH_2, approximately the same values of α are found; the slightly lower capacity factors may be due to the structural differences in the starting silicas, *e.g.*, owing to the different surface areas[25] (similar behaviour is also found[24] in the chromatography of chloronitrobenzene isomers on these sorbents in *n*-heptane).

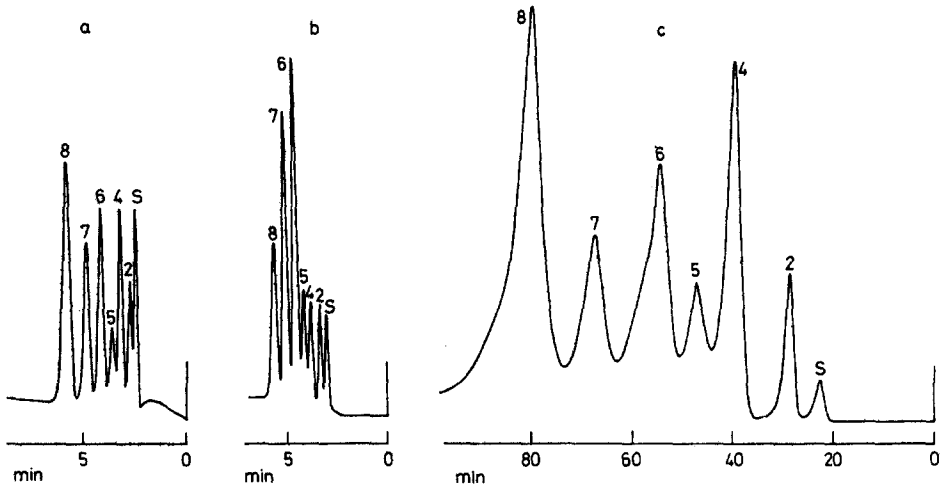

Fig. 2. Separation of porphyrin esters on Separon Six NH_2 modified with (a) N-trimethylsilylimidazole, (b) benzaldehyde and (c) 2,4-dinitrofluorobenzene. Other conditions as in Fig. 1.

TABLE II

CAPACITY FACTORS AND SEPARATION FACTORS OF PORPHYRIN ESTERS ON PRIMARY AMINO-BONDED SILICAS AND THEIR CHEMICAL DERIVATIVES IN 57% (v/v) ETHYL ACETATE–*n*-HEPTANE

Column I, Separon Six NH_2 (A); column II, trimethylsilylated A; column III, benzaldehyde derivative of A; column IV, dinitrofluorobenzene derivative of A; column V, LiChrosorb NH_2.

*Ester**	*Column I*		*Column II*		*Column III*		*Column IV*		*Column V*	
	k'	α	*k'*	α	*k'*	α	*k'*	α	*k'*	α
8	3.05		1.39		1.19		26.8		2.33	
		1.43		1.42		1.20		1.20		1.46
7	2.13		0.98		0.99		22.4		1.59	
		1.44		1.40		1.25		1.25		1.44
6	1.47		0.70		0.79		17.9		1.10	
		1.63		1.55		1.32		1.17		1.51
5	0.90		0.45		0.60		15.3		0.73	
		1.39		1.41		1.28		1.20		1.43
4	0.65		0.32		0.47		12.7		0.51	
		2.24		2.7		1.68		1.43		2.04
2	0.29		0.12		0.28		8.9		0.25	
		2.23		–		1.87		1.31		
S**	0.13		0.02		0.15		6.8		–	

* Numbers represent the number of carboxyl groups.
** Synthetic tetraphenylporphyrin.

If the content of ethyl acetate in the eluent is reduced, the resolution of porphyrin esters on the original Separon Six NH_2 and all three surface modifications is enhanced; corresponding values of k' and α are summarized in Table III. A change in the composition of the mobile phase results in an increase in the selectivity of separation, indicating a secondary effect of the mobile phase[22]. At identical values of k', a decrease in resolution occurs with the benzaldehyde derivative in comparison with the original amino phase (as in Table II); however, this resolution of the benzaldehyde derivative and also the time of analysis are acceptable.

Interesting effects on the separation of porphyrin esters are obtained if water-

TABLE III

CAPACITY FACTORS AND SEPARATION FACTORS OF PORPHYRIN ESTERS ON PRIMARY AMINO-BONDED SILICAS AND THEIR CHEMICAL DERIVATIVES IN 40% (v/v) ETHYL ACETATE–*n*-HEPTANE

Column I, Separon Six NH_2 (A); column II, trimethylsilylated A; column III, benzaldehyde derivative of A.

*Ester**	*Column I*		*Column II*		*Column III*	
	k'	α	*k'*	α	*k'*	α
8	10.75	1.60	4.77	1.51	4.80	1.41
7	6.72	1.61	3.15	1.55	3.40	1.43
6	4.17	1.63	2.03	1.55	2.37	1.40
5	2.55	1,60	1.31	1.58	1.48	1.37
4	1.59	2.53	0.83	2.51	1.08	2.77
2	0.63		0.33		0.39	

* See Table II.

saturated (25% and 100%) mobile phases are used, as illustrated in Fig. 3 and expressed in terms of *k'* and α in Table IV. Water enrichment of the mobile phase decreased[25] *k'* in normal-phase chromatography on bare silica; here, the opposite is true. In comparison with water-free conditions, the separation time doubles at 100%

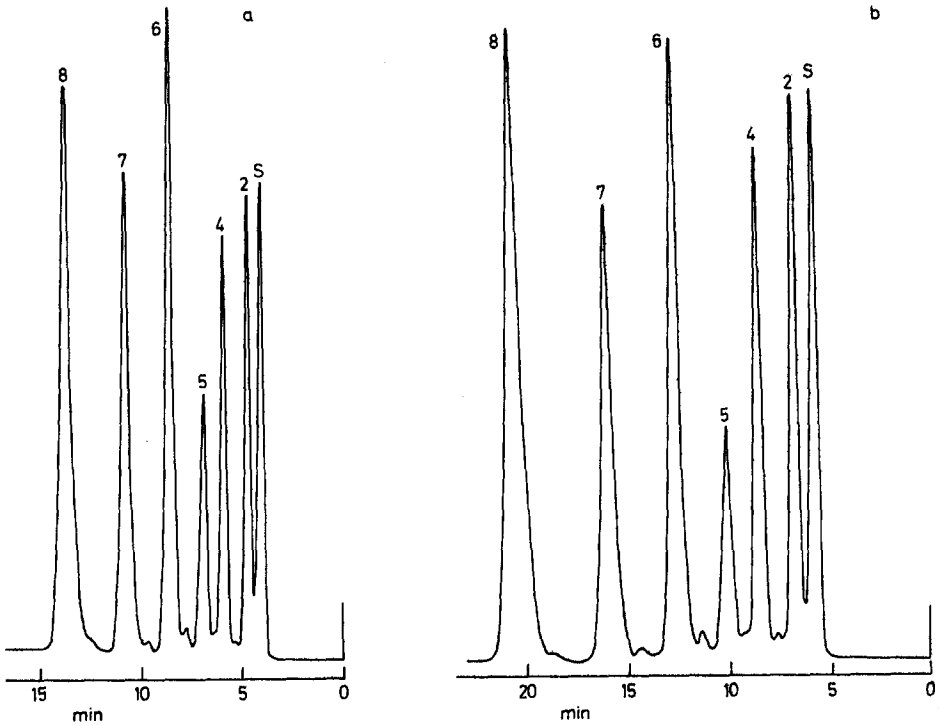

Fig. 3. Separation of porphyrin esters on a CGC column packed with Separon Six NH_2 in water-containing mobile phase: (a) 25% saturation and (b) 100% saturation. Other conditions as in Fig. 1.

TABLE IV

CAPACITY FACTORS AND SEPARATION FACTORS OF PORPHYRIN ESTERS ON PRIMARY AMINO-BONDED SILICAS IN A WATER-SATURATED MOBILE PHASE, ON LESS POLAR CYANOETHYLSILICA AND ON BARE SILICA WITH THE SURFACE CONCENTRATION OF SILANOL GROUPS DECREASED BY TRIMETHYLSILYLATION

Column I, Separon Six NH_2; column VI, Separon Six CN; column VII, gently silanized Separon Six (65% surface coverage).

*Ester**	*Column I****		*Column I*§*		*Column VI*§§*		*Column VII*§§*	
	k'	α	*k'*	α	*k'*	α	*k'*	α
8	4.39	1.37	7.14	1.35	1.72	1.44	2.53	1.44
7	3.21	1.38	5.29	1.33	1.19	1.43	1.75	1.46
6	2.33	1.38	3.98	1.34	0.83	1.45	1.20	1.41
5	1.69	1.31	2.97	1.27	0.57	1.46	0.85	1.39
4	1.29	1.61	2.34	1.51	0.39	2.05	0.61	2.18
2	0.80	1.45	1.55	1.35	0.19	–	0.28	
S**	0.55		1.15		–			

* See Table II.
** Synthetic tetraphenylporphyrin.
*** Mobile phase 57% (v/v) ethyl acetate–*n*-heptane, 25% water saturation.
§ Mobile phase 57% (v/v) ethyl acetate–*n*-heptane, 100% saturation.
§§ Mobile phase 30% (v/v) ethyl acetate–*n*-heptane.

saturation. Protonation of the primary amino group may be suggested as a reasonable explanation. An observed slight decrease in α again seems to originate in secondary solvent effects[22]; the influence of hydrophobic interactions, as described for diol bonded phase[27], is not very likely. These effects provide additional evidence of the key-role of the primary amino group in this separation.

Assuming that the surface concentration and/or activity of silanol groups play the decisive role in the possibility of isocratic separation of porphyrin esters on bare Separon Six, it should be possible to achieve isocratic conditions by decreasing the amount of surface silanols. The trimethylsilyl group can be expected to be non-interacting in the mobile phase used and, therefore, was applied to decrease the number of surface silanols. A chromatogram of the mixture of porphyrin esters on the gently trimethylsilylated Separon Six is shown in Fig. 4a; it can be seen that the surface properties can be tailored according to a particular elution problem. The separation achieved in a mobile phase containing 30% ethyl acetate is roughly comparable to partition on Separon Six NH_2 in *n*-heptane with 57% ethyl acetate, but it gives lower values of *k'* and α (Table IV).

The reduction in the concentration of surface silanols after trimethylsilylation can be calculated on the basis of elemental analysis; fully trimethylsilylated Separon Six (specific surface area 450 m^2/g) with a fully capped surface[25] gives[24] a content of 5.3% C and 1.5% H; 65% capping of surface silanols may be expected in the case of gently treated material. Vigorously silanized Separon Six does not give a reasonable separation, even with a further decrease in the content of ethyl acetate in the eluent (Fig. 4b).

The different properties of starting silicas would probably also influence the

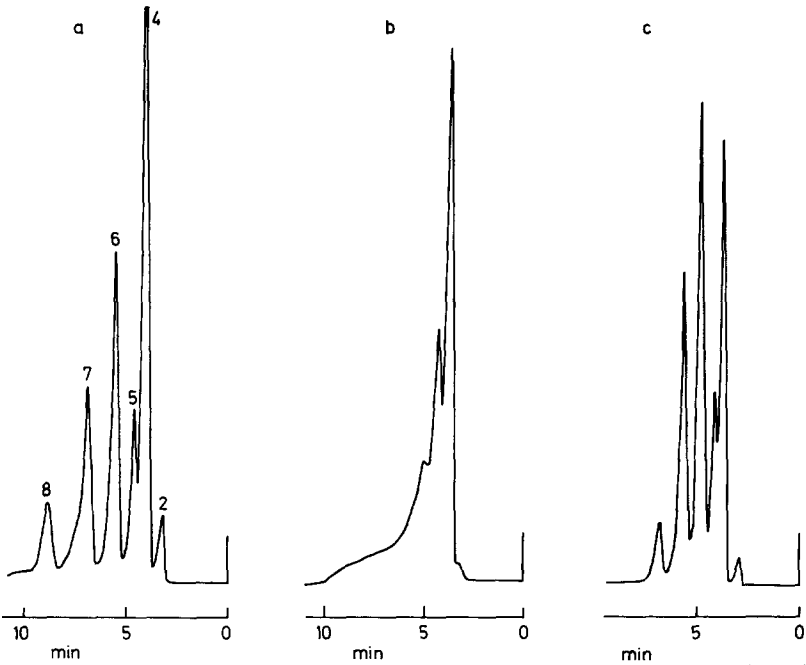

Fig. 4. Separation of porphyrin esters on (a) gently and (b) vigorously trimethylsilylated Separon Six and (c) on Separon Six CN. Mobile phase: (a) 30% (v/v), (b) 20% (v/v) and (c) 30% (v/v) ethyl acetate in *n*-heptane. Spectrofluorimeter: LS-2. Other conditions as in Fig. 1.

behaviour of mildly trimethylsilylated materials in proportion to the degree of silanization and, as a further drawback, lower solubilities of porphyrin esters in the mobile phase may be expected.

To supplement the possibilities of the isocratic separation of porphyrin esters, a chromatogram obtained using an eluent containing 30% ethyl acetate on Separon Six CN is shown (Fig. 4c). This material also gives reasonable separations, similarly[28] to Micropak CN; however, the lower polarity of the cyanoethyl group in comparison with the aminopropyl ligand would give a lower resolution at the same composition of the mobile phase, *e.g.*, at 57% ethyl acetate as used with aminopropyl-bonded silica.

In summary, the isocratic separation of porphyrin esters according to the number of carboxyl groups can be achieved on silicas that normally would not allow such a separation by tailoring the concentration of surface silanols to a suitable level using trimethylsilylation. Alternatively, cyanoethyl-bonded silicas can be used. The best results are obtained using aminopropyl-bonded silica; the highest resolution is attained with a mobile phase rich in ethyl acetate. This method permits the baseline separation of all porphyrin esters with two to eight carboxyl groups together with their Cu^{2+} complexes and tetraphenylporphyrin as the internal standard if two CGC columns in series (equivalent to a standard column of 250 × 4 mm I.D.) are used (Fig. 5). Finally, the chromatogram in Fig. 6a serves as an example of a clinical application, showing a typical spectrum of porphyrins in the urine of a patient with

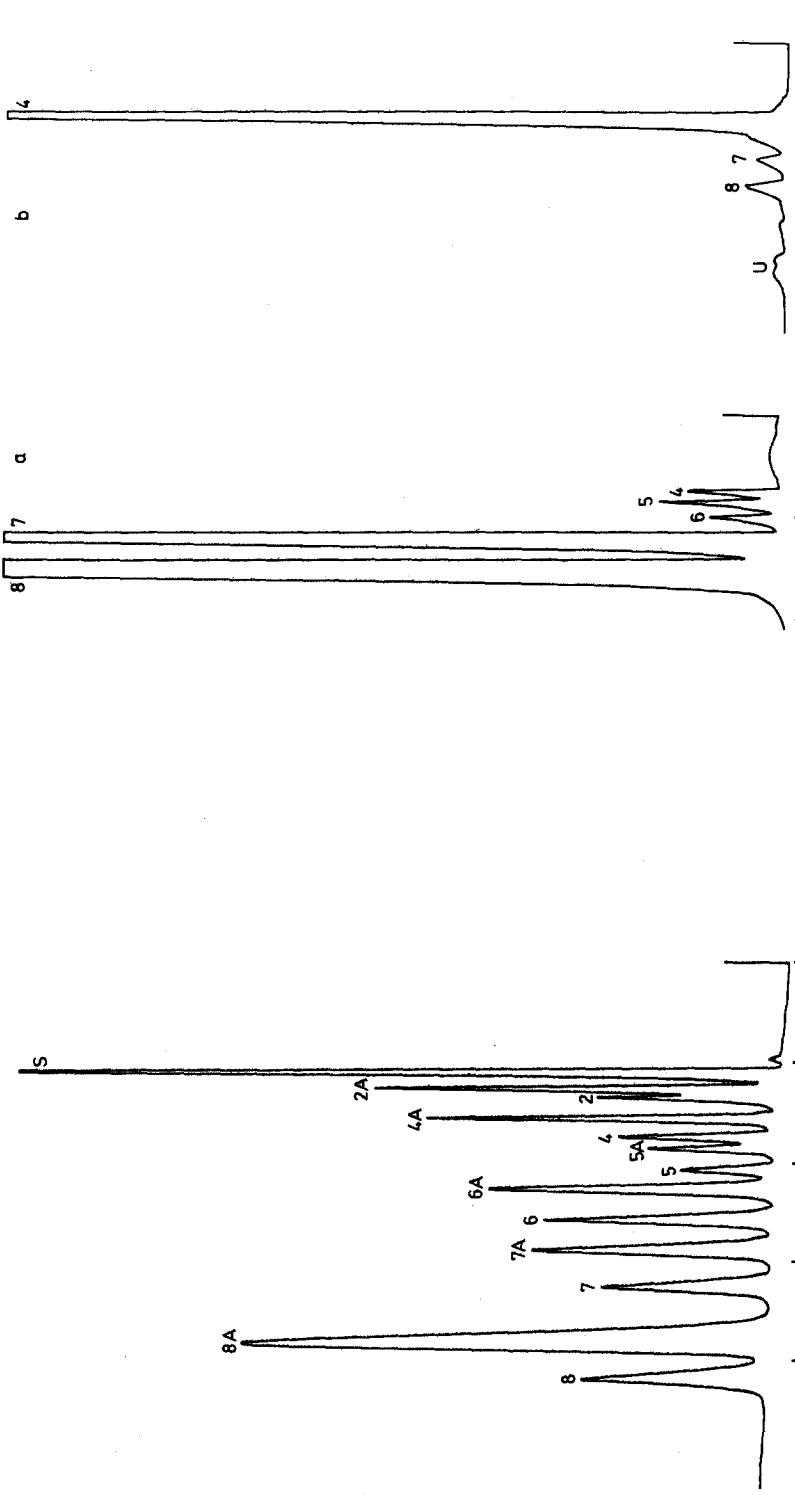

Fig. 5. Separation of porphyrin esters together with their Cu^{2+} complexes and an internal standard on two CGC columns in series packed with Separon Six NH_2. Cu^{2+} complexes of corresponding porphyrin esters are numbered 2A, 4A, 5A, 6A, 7A, 8A. Other conditions as in Fig. 1.

Fig. 6. Separation of (a) esters of a mixture of porphyrins isolated from the urine of a patient with porphyria cutanea tarda and (b) esterified porphyrins produced by *Saccharomyces cerevisiae* (CCY-RIBM 75, Hansen) on a CGC column packed with Separon Six NH_2. *Flow-rate*: 0.6 *ml/min*. Spectrophotometer: LCD 2563, λ = 405 nm. Other conditions as in Fig. 1.

porphyria cutanea tarda. Fig. 6b shows the identification of the porphyrins produced by the semi-anaerobically cultivated yeast *Saccharomyces cerevisiae*.

ACKNOWLEDGEMENTS

The authors thank Dr. S. Vozka of Laboratory Instruments works, Prague, and Dr. I. Rosenberg of the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, for valuable discussions. The supply of empty CGC columns by Dr. S. Vozka is gratefully acknowledged.

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