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NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FREE ACID DICARBOXYLIC PORPHYRINS AND HEMATOPORPHYRIN DERIVATIVE ON SILICA

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SUMMARY

The isocratic separation of dicarboxylic porphyrins (hematoporphyrin, hydroxyethylvinyldeuteroporphyrin and protoporphyrin) and their isomers by normal-phase high-performance liquid chromatography is described. The stationary phase is unmodified silica and the mobile phase consists of acetone–ethyl acetate (1:1, v/v) mixtures containing water and inorganic acids. Retention (capacity factor, k') was found to decrease exponentially with the mole fraction of water (N_{H_2O}) and to increase linearly with the concentration of hydrochloric acid, following the relation $k' = A\,[HCl]\,N_{H_2O}^{-4.85}$, where A is a constant characteristic of the porphyrin. The effects of the concentration and the nature of the acid used strongly suggest that retention involves a form of the porphyrin in which the inner nitrogens are protonated. The retention is thus partly determined by the basicity of the inner nitrogens, which depends on the electron-donating power of the porphyrin side-chains. Good resolution of the various components of hematoporphyrin derivative was obtained. In comparison with reversed-phase chromatography this method shows a different retention mechanism, appears to yield results of comparable reproducibility and provides complementary information. Possible retention mechanisms based on partition or adsorption equilibria are discussed.

INTRODUCTION

The development of high-performance liquid chromatographic (HPLC) methods for the analysis of porphyrins has led to important progress in various medical fields. The diagnosis of porphyria, a disturbance of porphyrin metabolism, is aided considerably by the determination of porphyrin profiles in urine, blood and feces. These profiles are characteristic of the various forms of the disease [1–3]. The excreted porphyrins differ in the number of carboxylic acid chains, ranging from two to eight, and in isomeric forms. Various HPLC methods have been developed for the determination of porphyrins [3–17]. They have been reviewed recently

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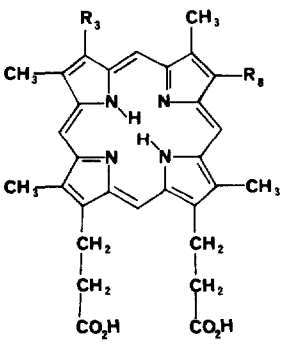

	R_3	R_8
a :	$-CH(OH)CH_3$	$-CH(OH)CH_3$
b :	$-CH(OH)CH_3$	$-CH=CH_2$
	$-CH=CH_2$	$-CH(OH)CH_3$
c :	$-CH=CH_2$	$-CH=CH_2$

Fig. 1. Structures of porphyrins: (a) hematoporphyrin; (b) hydroxyethylvinyldeuteroporphyrin; (c) protoporphyrin.

[2]. A new therapy for cancer, based on the photosensitizing properties of an empirically prepared tumor localizer [18], hematoporphyrin derivative (HpD), has also been developed [19–21]. HPLC analysis has recently revealed the complex composition of this drug [22–26], inducing intensive studies to identify the most active component of the mixture [26,27]. HpD is mainly composed of dicarboxylic porphyrins, with more or less hydrophobic side-chains, and of dimeric species.

The methyl ester derivatives of carboxylic porphyrins are more soluble in organic solvents and more easily separated than the free acid forms [5,16,25]. However, the esterification step is time-consuming and may cause unexpected reactions, especially when unknown mixtures have to be analyzed [4]. Most of the separations of porphyrin free acids have been achieved by reversed-phase chromatography using silica which is chemically modified with methyl [28], octyl [24], or octadecyl [3,4,6,7,11,15,17,22,23,26] groups. These methods generally involve gradient elution and, in some cases, ion-pairing agents [4,7,15,17,23]. Although the separation of carboxylic porphyrins on silica by thin-layer chromatography (TLC) has been known for many years [29,30], only few normal-phase HPLC methods have been developed [8–10], and the retention mechanisms have not been investigated.

Recently, we reported a purification method of hematoporphyrin (HP), involving low-pressure chromatography on unmodified silica [31]. We subsequently found that acidification of the eluent led to improved separation and reproducibility. This prompted us to transpose this system to HPLC and to investigate in more detail the effects of the eluent composition on the retention of HP IX, hydroxyethylvinyldeuteroporphyrin IX (HVD) and protoporphyrin IX (PP). These dicarboxylic porphyrins are major components of HpD (see Fig. 1). This report describes the results of this study and the analysis of HpD.

EXPERIMENTAL

Materials and reagents

PP was purchased from Sigma (St. Louis, MO, U.S.A.) and used without fur-

ther purification. HP was purified as described by Vever-Bizet et al. [31], except that the low-pressure chromatography step was performed using a mixture of acetone–ethyl acetate–0.03 *M* hydrochloric acid (16:13:7, v/v/v). HVD was prepared by partial dehydratation of HP using a modified version of the procedure of Bonnett et al. [22]. Nickel and copper complexes of HVD were prepared by refluxing a dimethylformamide solution of the porphyrin containing the appropriate metal salt. HP diethyl ester (HPDEE) was purchased from Midcentury (Washington, DC, U.S.A.). HpD was prepared according to Lipson et al. [18] following the modifications introduced by Kessel and Cheng [27].

Chromatography-grade acetone and ethyl acetate were purchased from Fisons (Loughborough, U.K.). All other reagents were of the best analytical grade from Merck (Darmstadt, F.R.G.). Water was doubly distilled in quartz.

High-performance liquid chromatography

All experiments were performed isocratically. A Kratos F 400 pump was used with a Kratos Spectroflow 757 variable-wavelength detector set at 400 mm unless otherwise specified (Kratos, Ramsey, NJ, U.S.A.). The signal was monitored by a Shimadzu CR3A integrator. Samples were injected using a Rheodyne 7125 valve equipped with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.).

The separations were carried out on a 25 cm × 4.6 mm I.D. column packed with Partisil 5 (5-μm irregular silica) from Whatman (Clifton, NJ, U.S.A.). The analytical column was protected with a 7 cm long guard column packed with HC Pellosil (Whatman).

The mobile phases were acetone–ethyl acetate (1:1, v/v) containing various proportions of water (3.5–6%) and various concentrations of acid (the concentration of the acid will always refer to the total volume). The time required to equilibrate the column was about 1 h. The porphyrins were dissolved in the mobile phase before injection.

RESULTS

A typical elution profile of a mixture made of PP, HVD and HP is shown in Fig. 2. Two peaks corresponding to positional isomers are observed for HVD (see Fig. 1). They can almost be resolved to the baseline, if mobile phases which lead to higher retention as described below are used. HP consists of two diastereoisomers due to the presence of two chiral carbon atoms on the hydroxyethyl sidechains. These isomers were only partly resolved with all the eluent systems investigated.

The retention of the porphyrins was strongly dependent on the concentrations of water and acid in the mobile phase, as well as on the nature of the acid. These parameters are examined below.

Mobile phase optimization

The performances of our chromatographic system were not significantly improved by changing the ratio of acetone to ethyl acetate. Therefore this ratio was kept constant (1:1). Next, optimization was attempted by varying the water and

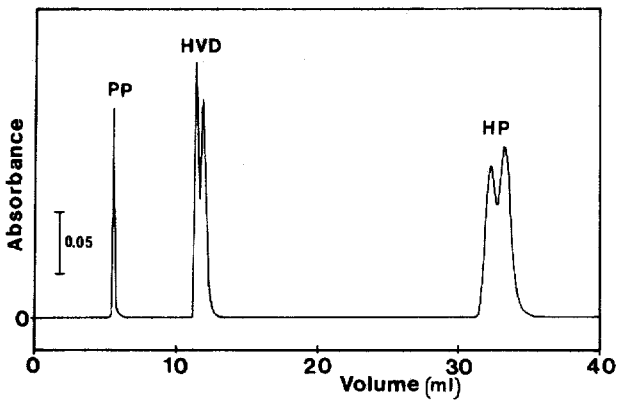

Fig. 2. Elution profile of a mixture of PP, HVD and HP. Elution was performed at 1 ml/min using a mixture of acetone–ethyl acetate (1:1, v/v) containing 4.5% water and $7.5 \cdot 10^{-4}$ *M* hydrochloric acid.

hydrochloric acid concentrations. The effect of the water content is shown in Fig. 3. For the three porphyrins studied, a decrease in the water concentration led to drastic increase of the retention, provided that some acid was present. As illustrated in Fig. 4 for HP, a plot of the logarithm of the capacity ratio versus the logarithm of the water molar fraction yielded straight lines with slopes equal to -4.8 ± 0.4. Similar observations were made with the other porphyrins. As depicted in Fig. 5, the capacity ratios were found to depend linearly on the concentration of hydrochloric acid in a range between 0.1 and 1 m*M*, regardless of the water content in the eluent mixture. At all the water concentrations used, the capacity ratios were very low in the absence of acid. Decreasing the concentration of water or increasing the concentration of acid were found to have similar effects on the resolution of HVD or HP isomers. The resolution was essentially determined by the retention times of these porphyrins.

A linear relation was found between the logarithm of the slopes of the curves depicted in Fig. 5 and the logarithm of the mole fraction of water in the mobile phase (N_{H_2O}). This relationship is shown in Fig. 6, which shows almost parallel lines for the three porphyrins studied (slope 4.85 ± 0.15). A relation between the capacity ratio (k') and the water and acid contents in the mobile phase can thus be derived.

$$k' = A\,[\mathrm{HCl}]\,N_{H_2O}{}^{-4.85} \qquad (1)$$

This equation is valid for the three porphyrins studied. The best values of A were found to be 2.75 ± 0.23, 0.75 ± 0.07 and 0.21 ± 0.04, for HP, HVD and PP, respectively.

The separation of the porphyrins requires only low acid concentrations. The activity of protons is greatly reduced in the mostly organic eluent mixture, and corrosion of the stainless-steel parts of the chromatographic system is not expected. In any case, if metal ions would have been formed and incorporated into porphyrins, the retention would have been reduced strongly (see below). Reproducible separations have been obtained for more than a year with the same column.

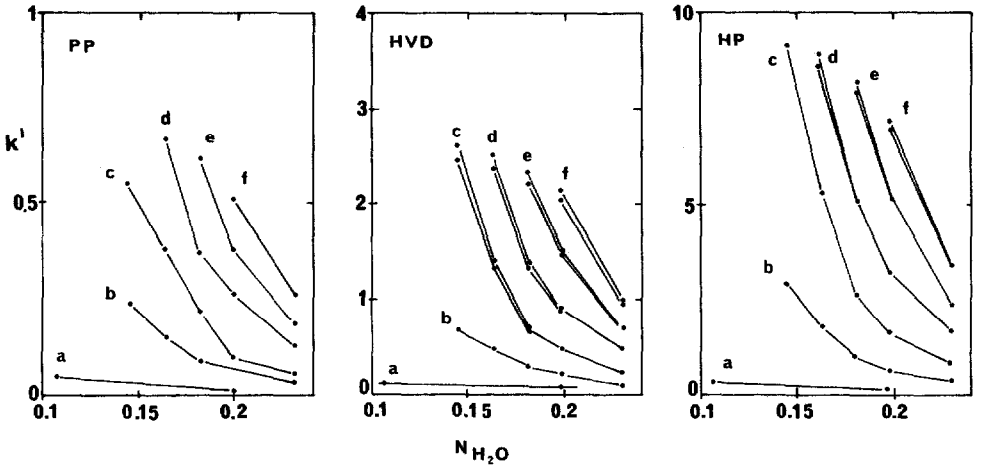

Fig. 3. Dependence of capacity ratios on the mole fraction of water in the mobile phase. Hydrochloric acid was also added to the eluent system at concentrations of: (a) 0 *M*; (b) $1 \cdot 10^{-4}$ *M*; (c) $2.5 \cdot 10^{-4}$ *M*; (d) $5 \cdot 10^{-4}$ *M*; (e) $7.5 \cdot 10^{-4}$ *M*; (f) $1 \cdot 10^{-3}$ *M*.

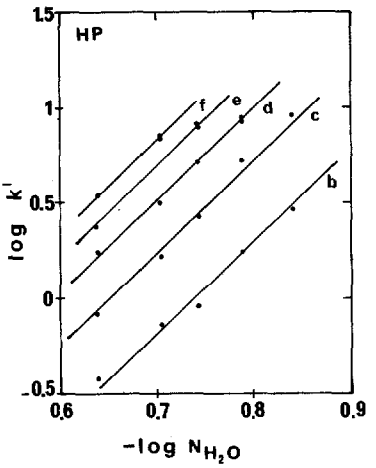

Fig. 4. Bi-logarithmic representation of the variation of capacity ratio of hematoporphyrin with the mole fraction of water in the mobile phase. Hydrochloric acid was also added to the mobile phase at concentrations of: (b) $1 \cdot 10^{-4}$ *M*; (c) $2.5 \cdot 10^{-4}$ *M*; (d) $5 \cdot 10^{-4}$ *M*; (e) $7.5 \cdot 10^{-4}$ *M*; (f) $1 \cdot 10^{-3}$ *M*.

Dependence of retention on the nature of the acid

As shown in Fig. 7, the retention of the three porphyrins strongly depends on the nature of the acid used, although in all cases a nearly linear relationship between the capacity ratio and the concentration of acid was observed. Perchloric acid was found to have a negligible effect, whereas the effect of hydrochloric acid was the largest. Intermediate effects were found for hydrobromic acid and sulfuric acid. To allow comparison, acid rather than proton concentrations are used in Fig. 7. In terms of the proton concentration, sulfuric acid appears to have a smaller effect than hydrobromic acid. The resolution of isomers was not affected by the nature of the acid.

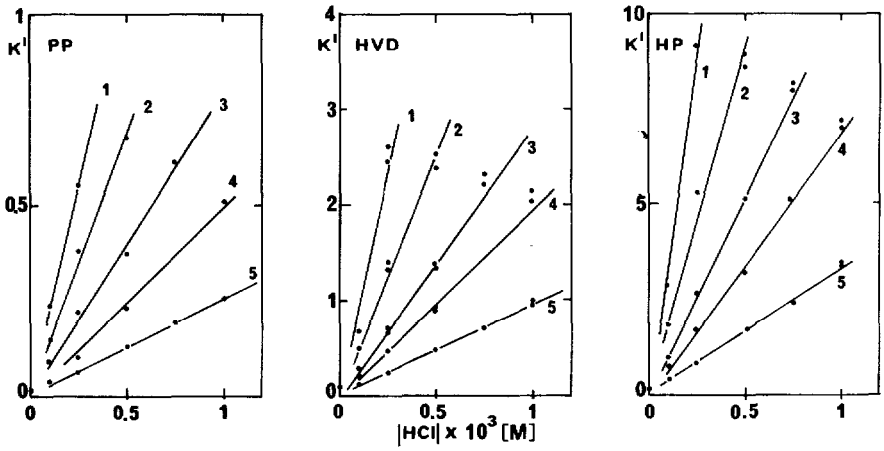

Fig. 5. Dependence of capacity ratios on the concentration of hydrochloric acid in the mobile phase. The eluent system also contained water: (1) 3.5%; (2) 4%; (3) 4.5%; (4) 5%; (5) 6%.

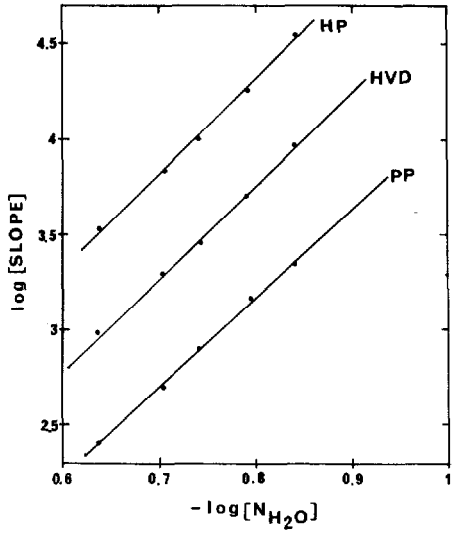

Fig. 6. Logarithms of the slopes of the straight lines shown in Fig. 5 versus the logarithm of the mole fraction of water in the mobile phase.

Chromatographic behavior of porphyrin ester and metalloporphyrins

To allow comparison, the retention of HPDEE and the nickel and copper complexes of HVD was examined. Using a mixture of acetone–ethyl acetate (1:1, v/v) containing 5% water and $5 \cdot 10^{-4}$ *M* hydrochloric acid as the mobile phase, the capacity ratio of HPDEE was found to be 2.08. This indicates that esterification has a relatively small effect on retention. Conversely, metallation has a drastic effect. The nickel and copper complexes of HVD were only weakly retained with capacity factors below $5 \cdot 10^{-2}$.

Analysis of HpD

HpD has been reported to be a mixture of monomeric porphyrins and dimers linked by ether [32] or ester [33] bonds. Dimerization is generally associated

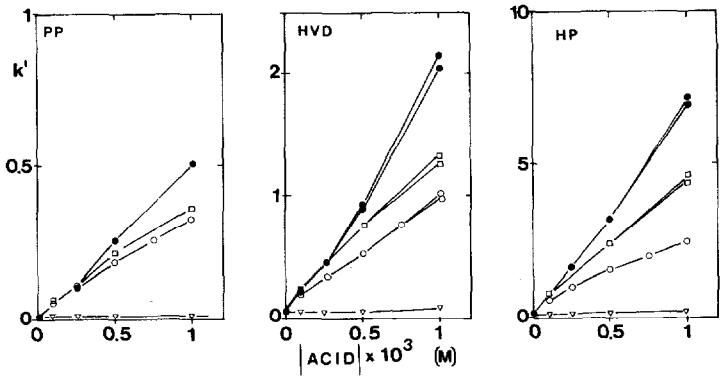

Fig. 7. Dependence of the capacity ratios on the nature and concentration of acids in the mobile phase. The eluent mixture contained 5% water. $\nabla = HClO_4$, $\square = H_2SO_4$, $\circ = HBr$, $\bullet = HCl$.

with a blue shift of the Soret absorption band. HpD analysis was thus performed with detection at 400 and 370 nm, with the peak-area ratio (S_{400}/S_{370}) being computed for each peak. A typical chromatogram recorded at 370 nm is shown in Fig. 8. In addition to the positively identified peaks of PP, HVD and HP, about 25 additional peaks can be seen. These peaks can be classified into four groups according to their S_{400}/S_{370} ratios. A first group (I) with $S_{400}/S_{370} \geqq 2.5$ includes PP, HVD, HP and components eluting before HVD. The high S_{400}/S_{370} ratios can be assigned to monomeric porphyrins. The second group (II) has an S_{400}/S_{370}

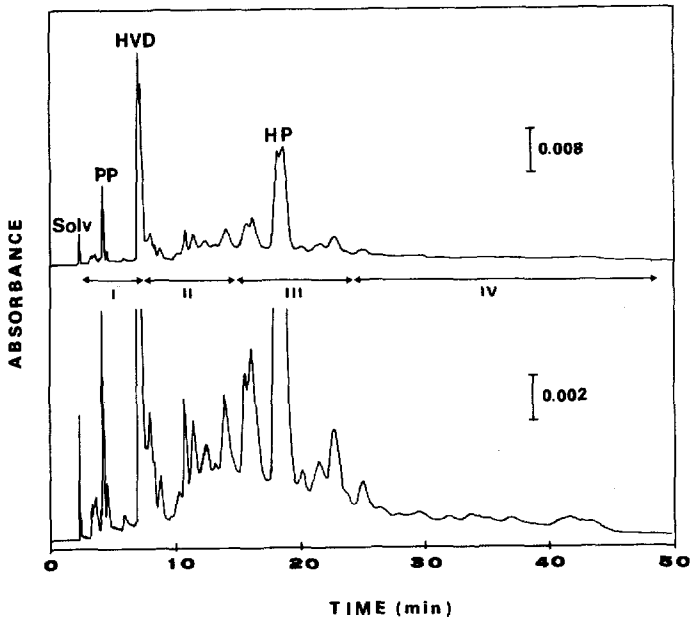

Fig. 8. Elution profile of HpD (normal and expanded scale). Peaks are separated into groups as described in the text. Elution was performed at 1 ml/min using a mixture of acetone–ethyl acetate (1:1, v/v) containing 5% water and $6 \cdot 10^{-4}$ *M* hydrochloric acid.

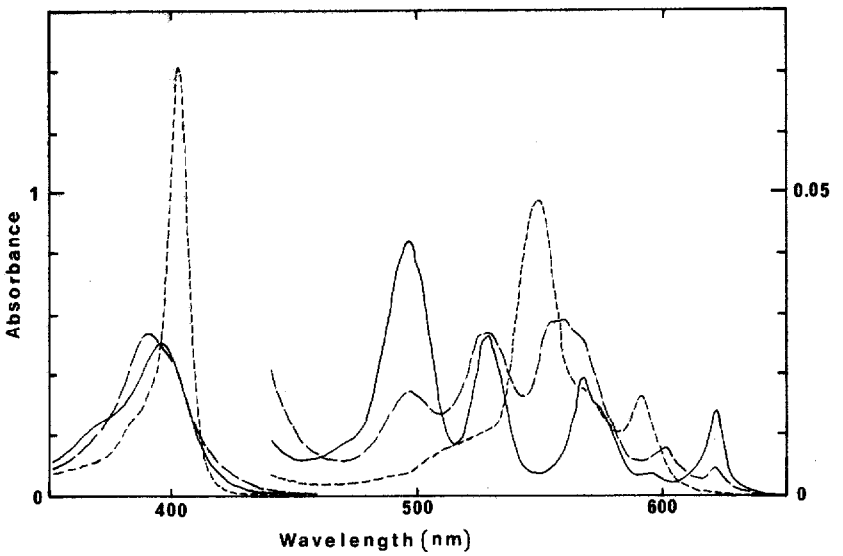

Fig. 9. Absorption spectra of hematoporphyrin ($3 \cdot 10^{-6}$ *M*) in acetone–ethyl acetate mixtures containing 5% water and perchloric acid. Acid concentrations were: ——, 0 (100% of the PH_2 form); ---, $1 \cdot 10^{-3}$ *M* (67% of the PH_3^+ form); ---, $5 \cdot 10^{-2}$ *M* (72% of the PH_4^{2+} form). Optical path length: 1 cm.

ratio between 1.6 and 1. The third group (III) has an S_{400}/S_{370} ratio below 0.9. All these species with enhanced absorption at 370 nm can be identified as dimers (see Discussion). The fourth group (IV) corresponding to the last eluted component has an S_{400}/S_{370} ratio higher than 1. The different groups are identified in Fig. 8.

Protonation equilibria of porphyrins

The presence of acid in the mobile phase may lead to protonation of the inner nitrogens atoms [34,35] of the porphyrins according to the following equilibria:

$$PH_3^+ + H_2O \overset{K_{N1}}{\rightleftharpoons} PH_2 + H_3O^+ \tag{2}$$

$$PH_4^{2+} + H_2O \overset{K_{N2}}{\rightleftharpoons} PH_3^+ + H_3O^+ \tag{3}$$

with

$$K_{N1} = \frac{[PH_2]\,[H_3O^+]}{[PH_3^+]\,[H_2O]} \tag{4}$$

and

$$K_{N2} = \frac{[PH_3^+]\,[H_3O^+]}{[PH_4^{2+}]\,[H_2O]} \tag{5}$$

As shown in Fig. 9 for HP, the neutral (PH_2), monoprotonated (PH_3^+) and diprotonated (PH_4^{2+}) forms exhibit distinctly different spectra, characterized in the visible range by four, three and two peaks, respectively. It can be seen that the disappearance of the neutral form can be easily followed by a decrease of the

TABLE I

PROTONATION EQUILIBRIA OF PORPHYRINS WITH VARIOUS ACIDS

Porphyrin	Acid	K_{N1} ($\times 10^{-4}$)	K^*_{N2}* ($\times 10^{-4}$)	λ** (nm)	ϵ ($M^{-1}\,cm^{-1}$)
HP	$HClO_4$	1.62 ± 0.36	68.4 ± 7.2	402	$6.2 \cdot 10^5$
	H_2SO_4	1.82 ± 0.20	33.7 ± 3.4	404	$5.1 \cdot 10^5$
	HBr	1.66 ± 0.36	6.12 ± 0.36	405	$2.1 \cdot 10^5$
	HCl	1.62 ± 0.36	7.74 ± 0.54	409	$2.55 \cdot 10^5$
HVD	HCl	2.02 ± 0.22	16.6 ± 1.1	412	
PP	HCl	7.52 ± 0.50	35.7 ± 1.4	415	

*The value of K_{N2} may include a contribution of equilibria 6 and 7.
**Position of the Soret band and extraction coefficient.

absorption at 497 and 621 nm, where the absorption coefficients of the other forms are small. In the near UV, the diprotonated form exhibits a sharp and intense Soret peak, well clear from the bands of PH_2 and PH_3^+. Thus, even when the two protonation steps overlap somewhat, it is possible to derive the equilibrium constants K_{N1} and K_{N2} independently, using standard spectrophotometric methods. Although the water concentration in our solvent mixtures was low, it was sufficient to ensure proton solvation. The proportion of protonated species was found to depend on the water content. Therefore, water is explicitly taken into account in equilibria 2 and 3. K_{N1} and K_{N2} are expressed as dimensionless constants. Whereas K_{N1} values were not significantly affected by the nature of the acid, K_{N2} values were found to vary by about one order of magnitude for different acids. The values of K_{N1} and K_{N2} for various acids and porphyrins are given in Table I. The spectra of the diprotonated species were also found to depend on the nature of the acid. An increasing red shift, hypochromism and broadening of the Soret peak were observed for sulphuric acid, hydrobromic acid and hydrochloric acid as compared to perchloric acid. The position of the Soret band is given in Table I.

DISCUSSION

Although the chromatographic system described above employs isocratic elution only, the separation of three dicarboxylic porphyrins with various hydrophobicities is readily achieved. Good resolution of isomers is obtained, which might help in the identification of unknown porphyrins. The analysis time can be adjusted by varying the concentrations of water and/or acid in the mobile phase. With mixtures containing the three porphyrins, a good compromise between isomer resolution and retention is obtained with analysis times not exceeding 30 min. The combination of acetone, ethyl acetate and water provides a reasonable solubility of the three porphyrins.

Silicas from various commercial sources have been reported to differ greatly in their surface acidity [36]. When polar and/or aqueous solvents are used without adding acid, exchange between the stationary and the mobile phases causes us to

expect that the surface acidity of the silica will change with time, leading to changes in the chromatogram and to poor reproducibility. This was observed to some extent in low-pressure chromatography of HP on silica using acetone–ethyl acetate–water mixtures [31]. As mentioned above, the addition of hydrochloric acid to the mobile phase led to improved separation and reproducibility. It is clear, however, that the addition of acids not only makes it possible to standardize the silica, but also plays a fundamental role in the retention of porphyrins, suggesting ionizable groups to be involved in the mechanism. In neutral or acidic solutions, the carboxylic chains of the porphyrin remain fully protonated. Esterification of these acidic groups results in minor decreases in the retention times, which is believed to reflect the overall increase in hydrophobicity of the molecule. Thus, the propionic acid chains do not appear to play a major role in the retention mechanism.

Conversely, complexation of the porphyrin with nickel or copper results in a drastic decrease of the retention, indicating that the inner nitrogens of the ring are directly involved in the interaction with silica. These nitrogens can each accept one proton, leading to monocationic (PH_3^+) and dicationic (PH_4^{2+}) species (see eqns. 2 and 3). As shown in Table I, the basicity of the nitrogens depends on the nature of the porphyrin side-chains. Vinyl groups are more electron-withdrawing than alcoholic chains. The basicity of the inner nitrogens increases in the order: PP < HVD < HP.

As can be seen for hematoporphyrin in Table I, the experimental values of K_{N2} and the position of the Soret band of the diprotonated species are dependent on the nature of the acid used. This strongly suggests that the diprotonated species may bind one or two anions in solution according to:

$$PH_4^{2+} + X^- \rightleftarrows PH_4X^- \quad (6)$$

$$PH_4X^- + X^- \rightleftarrows PH_4X^- \quad (7)$$

Such complexes have been reported in crystals [37]. The values of K_{N2}* reported in Table I are apparent equilibrium constants, which reflect contribution of equilibria 6 and 7 to the overall process of forming the diprotonated porphyrin. The affinity of anions for the diprotonated species in solution was concluded to be

$$ClO_4^- < SO_4^{2-} < Br^- \approx Cl^- \quad (8)$$

This order also describes the importance of the red shift and the broadening of the Soret band, which is assumed to be related to the strength of the bond between anions and diprotonated forms.

The above results strongly suggest that the retention of porphyrins on silica involves a protonated species, in particular the diprotonated form. Indeed, retention is strongly dependent on the addition of acid, and the order of elution of porphyrins appears to be determined by basicity of the inner nitrogens. Furthermore, if different acids are compared, the stabilization of the diprotonated form by associated anions results in a higher retention.

In our system, silica may be fully hydrated, and the retention may be mainly determined by partition of the porphyrin between the organic mobile phase and

a polar stationary phase consisting of several layers of water absorbed on the support [38]. The more polar diprotonated form may be preferentially retained on the stationary phase. The effect of decreasing the water concentration on the retention may be partly explained by a shift of equilibria 2 and 3 towards the formation of the diprotonated form. It is worth noting, however, that the logarithm of the capacity ratio is a linear function of the mole fraction of water (see eqn. 1 and Figs. 4 and 6). This may suggest that retention of the porphyrin on silica obeys the Snyder–Soczewinski model [39], one porphyrin molecule interacting directly with silica and covering about five silanol groups. This value would be consistent with the surface concentration of silanols assumed [39] to be $8 \cdot 10^{-6}$ mol/m^2 and a surface area of the porphyrin molecule of about 100 Å^2 [40]. Anions associated to the diprotonated form may serve as a bridge between the porphyrin and silica.

The performance of our chromatographic system is clearly demonstrated by the analysis of HpD. As mentioned before, this drug mainly contains monomeric species and dimers. Numerous structural isomers are expected to result from coupling two HP molecules by ether or ester bonds. In addition, cross-coupling between HP and HVD may occur. The peaks in groups II and III are likely to represent well resolved isomers. In comparison, reversed-phase systems generally show poor resolution of the dimers, which elute as a single broad band [33].

Our system, which involves simple isocratic elution, may be useful for controlling and standardizing HpD, which has been reported to show some variability in its biological efficiency [41].

In conclusion, the separation of dicarboxylic porphyrins on unmodified silica can be readily achieved in a reproducible way by controlling the concentrations of water and acid in the mobile phase. The separation mechanism involves a protonated form of the porphyrin and is determined mainly by differences in the basicity of the inner nitrogens, which in turn, is determined by the electron donating properties of the side-chains of the tetrapyrrolic nucleus. The retention mechanism of this chromatographic system is thus totally different from that of reversed-phase chromatography. In this latter case, retention depends on the hydrophobicity of the side-chains. Thus, both methods appear to be complementary.

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