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Journal of Chromatography A, 802 (1998) 367–380

JOURNAL OF CHROMATOGRAPHY A

Capillary electrophoretic separation of cationic porphyrins

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Received 28 May 1997; received in revised form 17 November 1997; accepted 19 November 1997

Abstract

Cationic porphyrins have a wide variety of uses including those as nucleic acid binding and cleaving agents, as potential pharmacological agents, as electron donor/acceptors in intramolecular electron transfer processes and as analytical reagents. Herein, we report the separation of cationic porphyrins by capillary electrophoresis on fused silica in phosphate buffer at pH 2–5. The porphyrins studied in this work were synthesized from alkylation of the parent tetrapyridylporphyrin (TPyP) to give various pyridinium porphyrins. For example, methylation of TPyP gives a mixture of the mono-, *cis*-di-, *trans*-di-, tri- and tetramethylated porphyrins [e.g., 5,10,15,20-tetrakis(N-methyl-4-pyridiniumyl)-21H,23H-porphyrin, TMPyP(4)]. Capillary electrophoresis on a synthetic mixture showed separation of four of these compounds. Mixtures after alkylation with iodopropionic acid and bromopropylamine were also separated. The *cis*-di- and trimethylated TMPyP derivatives were separated on a small preparative scale by centrifugal partition chromatography. Capillary electrophoresis was also used to separate metallo TMPyP(4) complexes including those of cobalt, copper, iron, manganese, palladium, tin, vanadium and zinc. The conformational isomers (atropisomers) of 5,10,15,20-tetrakis(N-methyl-2-pyridiniumyl)-21H,23H-porphyrin, TMPyP(2), were also separated. Net charge, molecular mass and molecular shape all contribute to the differential retention of cationic porphyrins under capillary electrophoresis conditions. Additional factors affecting the separations, including aggregation and protonation of the porphyrins, were probed by evaluating the separation of TMPyP(4) and its butyl and octyl analogs as a function of solution conditions. Cationic porphyrins are difficult to separate using traditional chromatographic methods; capillary electrophoresis and centrifugal partition chromatography provide excellent new techniques for separation of this class of compounds. © 1998 Elsevier Science B.V.

Keywords: Porphyrins; Tetrakis(methylpyridiniumyl)porphyrins; Metalloporphyrins; Centrifugal partition chromatography; Preparative chromatography

1. Introduction

Cationic porphyrins derived from alkylation of the tetrapyridylporphyrin (TPyP) system have a wide variety of uses including that as DNA binding [1–4], RNA binding [5,6] and nucleic acid cleaving agents [7–11]; as potential chemotherapeutic [12–18], antibacterial [19,20] and antiviral [21–25] agents; as electron donor/acceptors in intramolecular electron transfer processes [26–28]; and as analytical reagents [29,30]. Many of these studies have used the methylated parent *meso*-tetrakis(4-N-methylpyridiniumyl)porphine [TMPyP(4)] macrocycle. However, increasing use is made of asymmetrically substituted members of this class in studies of the role of the number and position of the positive charges of the interaction of positively-charged porphyrins with DNA [31–35], of the cleavage of

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0021-9673/98/$19.00
PII S0021-9673(97)01172-2

nucleic acids [10,36–39], of anticancer activity [16,17], and of the design of water-soluble porphyrins bearing additional metal centers [39–41]. The expanding role of asymmetrical cationic porphyrins makes separation and characterization of these species increasingly important.

Because alkylation of the pyridyl nitrogen generally proceeds in very high yield, alkylated TPyP derivatives are often purified by precipitation/recrystallization [17,36,42,43]. Derivatives have also been purified by Dowex ion exchange [25,40,44], Bio-Rad AG1-X8 [45], Sephadex LH-20 [27,46] or G-25 chromatography [47], or, in the case of certain conjugates with net negative charge, by HPLC [38,47]. Recently, Miskelly and coworkers have separated the atropisomers of the Zn(II), Cu(II), and Ni(II) complexes of tetrakis(N-methyl-2-pyridinium-4-yl)porphyrin by preparative TLC on silica gel [48]. In this work, we have turned to centrifugal partition chromatography (CPC) to separate cationic porphyrins on a preparative scale. CPC is a countercurrent extraction technique using two immiscible liquids, one as the stationary phase and the other as the mobile phase. It is increasingly used for the preparative separations of charged molecules [49,50].

Estimation of the purity of cationic porphyrins is usually effected by elemental analysis or ^{1}H NMR, both of which use relatively large amounts of material. Elemental analysis can be indeterminate because charged porphyrins often retain significant amounts of solvent when they precipitate. NMR techniques can be complicated by the tendency of cationic porphyrins to aggregate in aqueous solution [51,52] and by pairing of counterions [53,54]. Gel electrophoresis has also been used to estimate the purity of cationic porphyrins [45,55,56], but does not readily lend itself to characterization of each of the bands. Herein we report the separation of mixtures of cationic porphyrins (Fig. 1) via capillary electrophoresis. A diode array spectrophotometer allows rapid characterization of the species of the mixture in terms of structure, protonation and aggregation. This work extends previous studies in which capillary electrophoresis has been used to separate porphyrin free acids in the natural porphyrin family [57–63] as well as some of the constituents of hematoporphyrin derivative [62,64,65].

2. Experimental

2.1. General methods

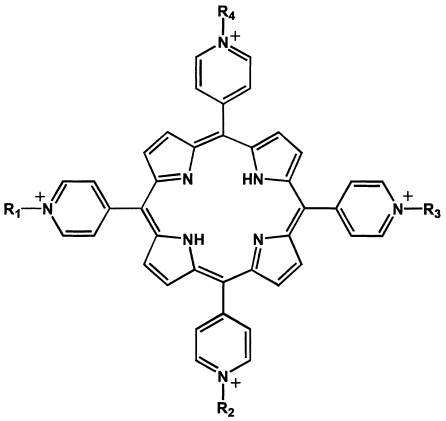
The reagents 5,10,15,20-tetra(4-pyridyl)-21H,23H-porphyrin (TPyP), 3-iodopropionic acid, 8-iodooctane, 5-iodopentyl ethyl ester, 4-iodobutane and iodomethane, were purchased from Aldrich. 5, 10, 15, 20 - tetrakis(N - methyl - 4 - pyridiniumyl) - 21H,23H-porphyrin, TMPyP(4), and the metalloporphyrins were purchased from Mid-Century (Posen, IL, USA) and Porphyrin Products (Logan, UT, USA). The purity of the metalloporphyrins was checked by CE. The TMPyP(2) isomeric mixture was purchased from Mid-Century. Sephadex LH-20 was from Pharmacia. Reactions run in sealed vessels were run in a Parr Instruments 4749 stainless steel acid digestion bomb with PTFE insert (23 ml; 1800 p.s.i.g. and 250°C maximum ratings; 1 p.s.i.= 6894.76 Pa).

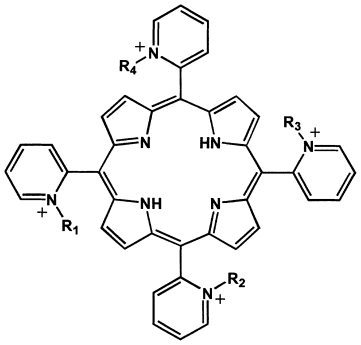
Anhydrous DMF was used as received from Aldrich. It was sealed and stored under nitrogen. Anhydrous ethyl ether (Fisher) was treated with calcium hydride overnight, then distilled and collected over molecular sieves (8–12 mesh, effective pore size 3 Å). The alkylation reactions were run under nitrogen.

NMR spectra were run on Varian VXR-400 (400 MHz) or Unity+300 (300 MHz) instruments. A small amount (1–3 mg) of the compound was dissolved into 1.5 ml [$^{2}H_{6}$]dimethyl sulfoxide (DMSO-d_6) (porphyrins) or $C^{2}HCl_3$ 1.5 ml (small molecules). To ensure complete dissolution of the cationic porphyrin product, the DMSO-d_6 solutions were heated for 10 min, or allowed to stand for 30 min at room temperature. UV–Vis spectra were run on a Shimadzu UV 3101 PC UV–Vis–near IR (NIR) scanning spectrophotometer.

2.2. Capillary electrophoresis

Capillary electrophoresis was performed on a Beckman P/ACE 5500 series with Beckman Gold chromatography software and a Beckman diode array detector. A fused silica capillary column [Beckman, 57 cm (50 cm to detector)×375 μm O.D.×75 μm





designations		R_1	R_2	R_3	R_4
5,10,15,20-tetrakis(*N*-methyl-4-pyridiniumyl)-21H,23H-porphyrin derivatives (left hand structure)					
1	TMPyP(4)	Me	Me	Me	Me
2	TBPyP(4)	Bu	Bu	Bu	Bu
3	TOPyP(4)	octyl	octyl	octyl	octyl
4		Me			
5		Me	Me		
6		Me		Me	
7		Me	Me	Me	
8		CH_2CH_2COOH			
9		$CH_2CH_2CH_2NH_2$			
10		$CH_2CH_2CH_2NH_2$	$CH_2CH_2CH_2NH_2$	$CH_2CH_2CH_2NH_2$	
5,10,15,20-tetrakis(*N*-methyl-2-pyridiniumyl)-21H,23H-porphyrin derivative (right hand structure)					
11	TMPyP(2)	Me	Me	Me	Me

Fig. 1. Cationic porphyrins in this study.

I.D.] was built in a eCAP capillary cartridge (Beckman, 100×800 μm aperture). Phosphate buffer was prepared by dissolving NaH_2PO_4 or Na_2HPO_4 in deionized water and adjusting the solution to the desired pH with phosphoric acid or sodium hydroxide. All running solutions were filtered through a 0.2 μm-membrane filter (Gelman Science, FP-200) before use.

New capillary columns were rinsed with 0.1 *M* sodium hydroxide for 30 min, then with deionized water for 30 min, then with running buffer for 1 h. The capillary column was regenerated between runs with 0.1 *M* sodium hydroxide for 10–15 min, then with deionized water for 5–10 min, then with the running buffer for 10 min. A sample solution was prepared by dissolving a small amount of cationic porphyrin (1–2 mg) into deionized water (1 ml), then filtering it through a cotton or glass wool packed in a small pipet, then storing it in the dark. About 200 μl of sample solution was diluted by addition of about 50 μl of the running buffer to be used.

Separations were performed with normal polarity from the inlet vial (anode) to the outlet vial (cathode). Pressure injections of 3 s were used. Voltages were chosen in the range 10–20 kV, de-

pending on buffer concentration (50–75 m*M*) and pH (2–7). The column temperature was maintained around 20°C.

2.3. Centrifugal partition chromatography

The CPC separations were performed using a Sanki Labs, model LNN Series 1000 centrifugal partition chromatograph, a Linear, UVIS 200 UV detector and a Beckman 110B solvent delivery pump. Porphyrin samples (5–10 mg) were dissolved in deionized water (2 ml) and filtered through glass wool. Butanol and deionized water were filtered with a 0.2-μm membrane filter (Gelman Science, FP-200). Butanol and the buffer (750 ml each) were put in a separatory funnel and mixed by shaking the funnel. This process was repeated several times, each time waiting until the layers had separated again. The saturated solvents were degassed in an ultrasonic bath (Branson, B-220) for 1 h. Methanol (filtered, degassed) was pumped into CPC to clean the cartridge channels before pumping the two solvents. Water was chosen as a mobile phase (descending mode) at a flow-rate of 4–6 ml/min and a rotor speed of 1000 rpm. The UV detection was performed at 254 nm. For each run, it was necessary to wait until the UV absorbance stabilized before the sample was injected. The sample (maximum 10 mg) was loaded only after the CPC system had equilibrated.

2.4. Synthesis of cis-di- (**5**) and trimethylated (**7**) TPyP

To a solution of TPyP in $CHCl_3$–MeOH (5:1, v/v) was added a 4- to 10-fold excess of MeI and triethylamine. The solution was heated overnight in a sealed vessel at 50–60°C. The solvents were evaporated. The crude material was dissoved in a small amount of water and purified by CPC (50 m*M* phosphate buffer, pH 8–9, mobile phase with BuOH). Two clear peaks and a trailing peak were observed. The first, sharp peak was the trimethylated derivative. The second, broader peak was the *cis*-dimethylated derivative, **5**. ^{1}H NMR *cis*-dimethyl derivative *5*, ($C^2H_3O^2H + {}^2H_2O$, 400 MHz): 9.41 (4H, pyridyl), 9.20 (2H, pyrrole), 9.10 (2H, pyrrole), 9.05 (2H, pyrrole), 8.99 (4H, pyridyl), 8.96 (4H, pyridyl), 8.87 (2H, pyrrole), 8.21 (4H, pyridyl), 4.91 (6H, CH_3). ^{1}H NMR trimethyl derivative *7*: ($C^2H_3O^2H + {}^2H_2O$, 400 MHz): 9.36 (6H, pyridyl), 9.16 and 9.08 (8H, pyrrole), 9.05 (2H, pyridyl), 8.94 (6H, pyridyl), 8.31 (2H, pyridyl), 4.85 (9H, CH_3).

2.5. Synthesis of 5,10,15-tris(4-pyridyl)-20-[N-(3-aminopropyl)-4-pyridiniumyl]-21H,23H-porphyrin (**9**)

To a solution of TPyP (62 mg, 0.10 mmol) in $CHCl_3$–MeOH (5:2, v/v) (7 ml total) was added one drop of triethylamine and bromopropylamine HBr (44 mg, 0.2 mmol). The mixture was kept in a sealed 25 ml vessel overnight at 85°C. There was no precipitation. TLC showed a substantial amount of unreacted porphyrin. The solvents were evaporated under vacuum and the residue dissolved in water–MeOH, filtered to remove unreacted TPyP(4) and the filtrate was evaporated. The residue was dissolved in a small amount of water, the pH was adjusted to 10 and the mixture was separated on the CPC (water–BuOH) to give a few mg of the desired compound. ^{1}H NMR ($C^2H_3O^2H$:^2H_2O, 5:1 400 MHz) 9.55 (2H, pyridyl), 9.39 (6H, pyridyl), 9.27 (2H, pyrrole), 9.18 (6H, pyrrole), 9.04 (2H, pyridyl), 8.97 (6H, pyridyl), 5.21 (2H, N^+CH_2), 3.46 (2H, CH_2N), 2.87 (2H, CH_2).

2.6. Synthesis of 5-(4-pyridyl)-10,15,20-tris[N-(3-aminopropyl)-4-pyridiniumyl]-21H,23H-porphyrin (**10**)

To 60 mg of the residues from the reactions above (approximately 0.07 mmol), in MeOH with one drop of triethylamine, was added bromopropylamine HBr (32 mg, 0.14 mmol). The mixture was heated in a sealed 25 ml vessel for 48 h at 80°C. The solution after reaction was filtered to give 10 mg of residue, which was the pure trialkylated compound **10** as indicated by NMR integration.

2.7. Alkylation of TPyP with 3-iodopropionic acid

TPyP (124 mg, 0.2 mmol) and 3-iodopropionic acid (400 mg, 2 mmol) were mixed in anhydrous DMF (5 ml) and the reaction mixture was stirred under nitrogen at 60°C for 48 h. The resulting warm solution was poured into a mixture of chloroform–

light petroleum (1:2, v/v, total 800 ml) with continuous stirring. A precipitate formed after 15 min. The precipitate was filtered, washed repeatedly first with chloroform and then with light petroleum and air dried, giving a purple solid (194 mg, yield 17.4%). Part of the product from propionate alkylation was dissolved in water. A small portion of this porphyrin solution (up to 3 ml) was separated on a Sephadex (LH-20) column using ethanol and water as the eluent changing the ratio gradually from 1:100 to 50:100 (v/v). The column separation was repeated about 10 times to give four fractions: first (12 mg), second (5 mg), third (2 mg), fourth (<1 mg). The first fraction gave a clear NMR spectrum. The other fractions showed impure porphyrins. The first fraction from Sephadex was **8**: ^{1}H NMR (DMSO-d_6, 400 MHz): 9.58 (8H, pyridyl), 9.21 (8H, pyrrole), 9.03 (8H, pyridyl), 5.18 (8H, N^+CH_2), 3.43 (8H, CH_2COO^-).

2.8. Synthesis of TBPyP(4) and TOPyP(4)

The tetrabutyl [66] and tetraoctyl [51] TPyP derivatives were synthesized by TPyP with 4-iodobutane or 8-iodooctane as described above except that the reaction was run at 90°C. ^{1}H NMR (DMSO-d_6, 300 MHz) of TBPyP(4): 9.57 (8H, pyridyl), 9.25 (8H, pyrrole), 9.00 (8H, pyridyl), 4.95 (8H, N^+CH_2), 2.30 (8H, CH_2), 1.65 (8H, CH_2), 1.15 (12H, CH_3). ^{1}H NMR (DMSO-d_6, 300 MHz) of TOPyP(4): 9.52 (8H, pyridyl), 8.82 (8H, pyrrole), 8.12 (8H, pyridyl), 4.95 (8H, N^+CH_2), 1.8–0.8 [60H, $(CH_2)_6CH_3$].

3. Results and discussion

3.1. Separation of TMPyP(4), TBPyP(4) and TOPyP(4)

To evaluate the ability of capillary electrophoresis to separate cationic porphyrins, we investigated a mixture of TMPyP(4) and the corresponding tetrabutyl [TBPyP(4)] and tetraoctyl [TOPyP(4)] derivatives. The separation at pH 3.0 was performed at 11 kV with 50 m*M* phosphate buffer (Fig. 2, lower panel); a similar resolution was achieved at 13 kV with 60 m*M* phosphate buffer at pH 2 (not shown).

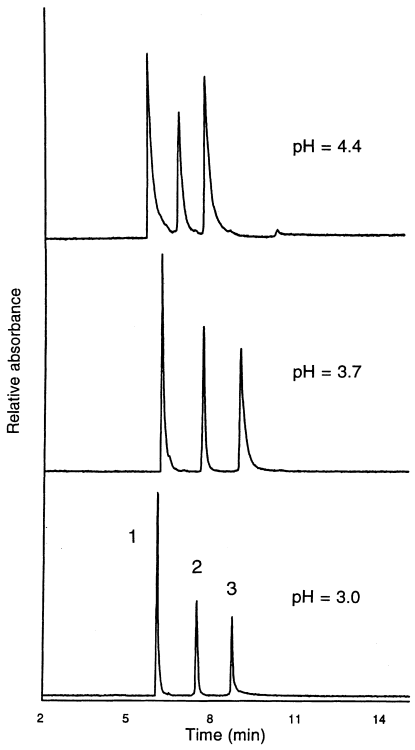


Fig. 2. Separation of a mixture of TMPyP(4) (**1**), TBPyP(4) (**2**) and TOPyP(4) (**3**) as a function of pH (50 m*M* phosphate, 11 kV).

The order of elution is that expected for simple dependence of the hydrodynamic flows on the size of the molecule, with the smallest (methyl substituents) coming off the column first and the largest (octyl substituents) coming off the column last. However, the excellent separation may also arise from other factors. To probe this, we explored two possibilities: differences in the extent of aggregation of the porphyrins in water and differences in the pK_a values of the center nitrogen atoms.

Differential aggregation might contribute to the separation of these three porphyrin derivatives [67]. The question of whether TMPyP(4) is a monomer or dimer at optical concentration has been a matter of debate, though most current analysis favors the monomer [1,34,51,53,68–74]. However, salt titrations show that TOPyP(4) has a greater tendency to aggregate in aqueous solution [51] than does TBPyP(4) [67], which in turn dimerizes more readily than TMPyP(4) [31,51]. Aggregation of TOPyP(4) upon the addition of salt results in a red shift of the

Soret by about 20 nm and a decrease in intensity of approximately 60% [51]. The optical spectra of TMPyP(4), TBPyP(4) and TOPyP(4) at pH 3 (Fig. 3) show red shifts of the Soret maxima in the order methyl (422 nm)<butyl (425 nm)<octyl (427 nm). This red shift of the Soret as a function of increasing size of the side chain is consistent with more aggregation of the more hydrophobic porphyrins. To evaluate the possibility of porphyrin aggregation under the separation conditions, TOPyP(4) was injected at three different concentrations: $1.52 \cdot 10^{-4}$, $6.08 \cdot 10^{-6}$ (25-fold dilution) and $3.04 \cdot 10^{-6}$ *M* (50-fold dilution). The Soret maxima of TOPyP(4) at these three concentrations were all the same (data not shown). This result indicates that little or no self-aggregation occurred under the conditions of the experiment.

Differential protonation of the center nitrogens might also contribute to the separation. At neutral pH, only two of the center nitrogens are protonated but all four center nitrogens of porphyrins can be protonated at low pH. Protonation results in a red shift of the Soret [75]. The pK_a values of the center nitrogens are sensitive to both the ionic strength and nature of the ions in solution. For TMPyP(4), approximate values of pK_{a3} (protonation of the third central nitrogen) and pK_{a4} (protonation of the fourth central nitrogen) are 2 and 1, respectively [76,77]. Because the separation was run under the conditions

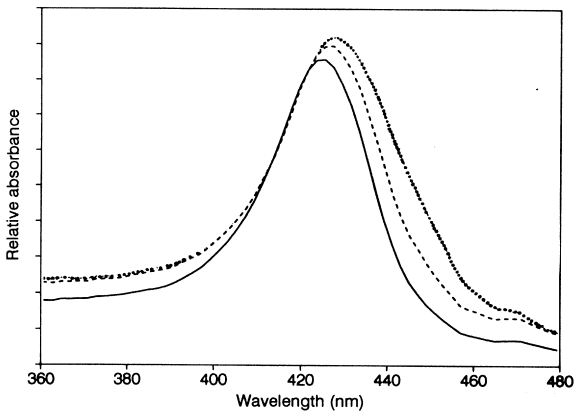


Fig. 3. The UV–Vis spectra of TMPyP(4), TBPyP(4) and TOPyP(4) separated by capillary electrophoresis (pH 3.0, 50 m*M* phosphate, 13 kV): (———) TMPyP(4); (– – –) TBPyP(4); (· · ·) TOPyP(4).

where the pH is approximately equal to the pK_a values of the center nitrogen atoms, differences in the values of pK_{a3} and pK_{a4} would result in different average net charges for these porphyrins which would in turn result in different mobilities. Although protonation of the inner nitrogens is slow on the NMR time scale [78], it is likely to be fast on the CE time scale (20 min).

To investigate the possibility of protonation of the center nitrogen atoms, the optical spectrum of each component [TMPyP(4), TBPyP(4), and TOPyP(4)] was recorded from the electropherograms at different pH values. The spectra of TMPyP(4) and TBPyP(4) were independent of pH between pH 2 and 3.7 (data not shown), indicating that the pK_a of the center nitrogens of TMPyP(4) was lower than 2.0 under the buffer conditions used in the electrophoresis. This pK_a value is lower than that reported in the literature [76,77]. However, the values of pK_{a3} and pK_{a4} are known to be sensitive to buffer strength and composition. For example, the pK_{a3} value for TMPyP(4) is 1.4 in 0.2 *M* $NaNO_3$ and 2.2 in 2.0 *M* $NaNO_3$ [76]. The current studies were run at lower ionic strengths (<0.1 *M*) than those used in the literature studies; this is expected to result in lower observed pK_a values. The spectrum of TOPyP(4) was similar to that of TMPyP(4) and TBPyP(4) at pH 3.0 and 3.7, but shifted 18 nm to the red at pH 2.1. This red shift is appropriate for the protonated porphyrin [79]. This suggests that the pK_{a3} and pK_{a4} of TOPyP(4) are higher than those of TMPyP(4) and TBPyP(4) under these buffer conditions.

Although the optical studies as a function of pH indicate a role for protonation of the center nitrogen atoms in the separation, this effect does not result in substantial changes in the electropherogram as a function of pH, as seen in Fig. 2. pH changes from 2–4 have very little effect on the retention times of the porphyrins. As the pH increased, the resolution decreased (Fig. 2); separation could not be achieved at pH values≥5 presumably because the porphyrins interact with the capillary walls at higher pH values.

3.2. Separation of atropisomers of TMPyP(2)

Porphyrins with substituents in the 2-position on the phenyl ring have substantial hindrance to rotation

of the ring due to steric interference between the 2-substituent and the porphyrin macrocycle. For 2-substituted porphyrins in which each ring has identical substituents, four isomers, termed atropisomers, are possible: αααα, ααaβ, ααββ and αβαβ. In general, cationic atropisomers are synthesized via separation of the neutral precursors followed by alkylation. This route was followed by Dabrowiak and coworkers, who synthesized isomers of alkylated pyridyl porphyrins by separating the neutral precursors by column chromatography and then alkylating [80]. Very recently, Miskelly and coworkers have separated the atropisomers of the Zn(II), Cu(II) and Ni(II) complexes of TMPyP(2) via thin-layer chromatography; the parent free base porphyrin did not separate under these conditions, however [48].

Capillary electrophoresis has proved to be an excellent analytical technique for separating cationic porphyrin atropisomers. A mixture of the TMPyP(2) atropisomers was separated using 60 m*M* phosphate at pH 2 (Fig. 4, lower panel). The separation was not significantly affected by the voltage chosen; voltages of 10, 11 or 13 kV gave only slightly different resolutions. Atropisomers could be separated well in 50, 60 or 75 m*M* phosphate buffer but not at ionic strengths lower than 50 m*M*.

Statistical formation of the atropisomers would result in a 1:4:2:1 mixture of the αααα, ααaβ, ααββ and αβαβ forms [81]. However, the four atropisomers are not observed at these ratios. This may be because the isomers are not formed in a statistical mixture or because the isomeric composition changed during the isolation and purification [82].

The excellent separation is not due to different aggregation of atropisomers, as indicated by the fact that the UV–Vis spectra of all the atropisomers were the same (data not shown). To investigate differential protonation of the central nitrogens, the separation was conducted at different pH values (Fig. 4); the separation was independent of the buffer pH. A good separation can still be obtained at pH 4, where the center nitrogens are not protonated. Therefore, differential protonation of the center nitrogens is not a major factor contributing to the separation. We conclude that the separation is due to different electrophoretic mobilities arising from the different shapes and dipole moments of the four atropisomers.

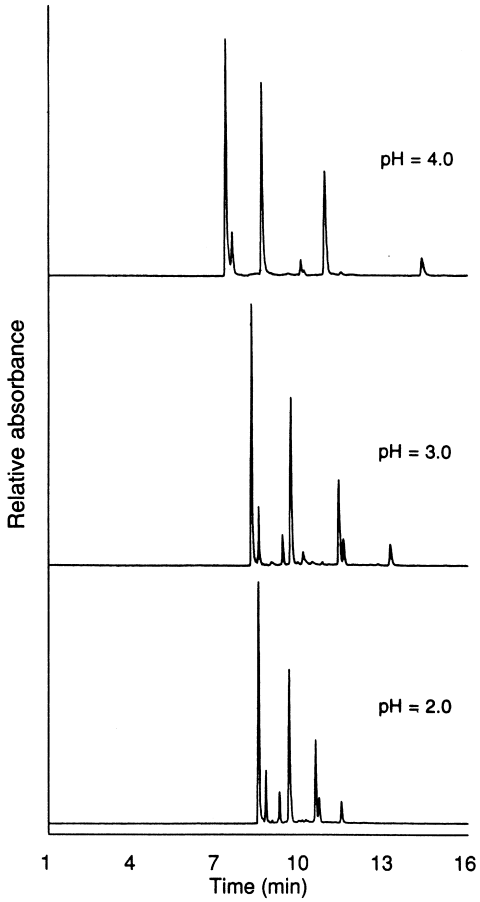


Fig. 4. The separation of the atropisomers of TMPyP(2) as a function of pH (60 m*M* phosphate, 15 kV).

3.3. Partially methylated TPyP derivatives

Alkylated TPyP derivatives can be synthesized by alkylation of the parent tetrapyridyl porphyrin with an alkyl halide, commonly an alkyl iodide or tosylate. These reactions generally proceed in high yield. For full alkylation, it is necessary to use a solvent in which both starting neutral and product cationic porphyrin are soluble; dimethyl formamide is the solvent of choice [68]. Partial alkylation of TPyP results in mixtures of porphyrins with one, two, three and four side chains extending off the pyridinium rings. If these materials could be isolated in quantity, and their purity established, they might be useful in the synthesis of more complex cationic

porphyrins. To this end we have investigated the separation of mixtures of partially alkylated porphyrins.

Initial studies were performed in the methyl series. The monomethyl tetrapyridylporphyrin **4** was synthesized by alkylation of TPyP with MeI in chloroform. The monomethyl product **4** is only sparingly soluble in this solvent; NMR showed **4** to be the major product of the precipitate. Alkylation of TPyP with a 4- to 10-fold excess of MeI and triethylamine in $CHCl_3$–MeOH (5:1, v/v) (heated overnight in a sealed vessel at 50–60°C) gave a mixture containing substantial amounts of di- and trimethylated derivatives. To separate the components of the mixture on a preparative scale, the crude product from this reaction mixture was introduced into the CPC (50 m*M* phosphate buffer pH 8–9 mobile phase with BuOH or water–BuOH as the stationary phase). Two clear peaks were observed (Fig. 5). The first, sharp, peak was the trimethylated derivative **7**, as shown by integration of the NMR (see Section 2). The second, broader peak was the *cis*-dimethylated derivative **5**, as shown by an analysis of the coupling patterns in the aromatic region of the NMR (see Section 2). Substantially less resolution was seen at lower pH values, presumably because the peripheral pyridine nitrogen atoms were protonated, resulting in smaller charge differences among the components of the mixture.

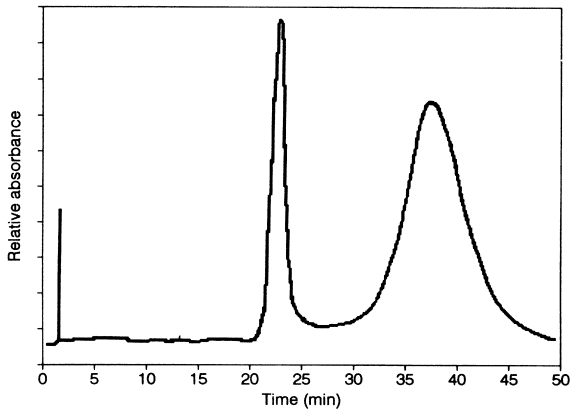


Fig. 5. CPC of a mixture of the trimethylated **7** (23 min) and *cis*-dimethylated **5** (37 min) TPyP. Water (pH 5) as the mobile phase, butanol as the stationary phase; descending mode; 1000 rpm; 3 ml/min.

Capillary electrophoresis was used to separate a synthetic mixture of authentic samples of the monomethylated **4**, *cis*-dimethylated **5**, trimethylated **6** and tetramethylated **1** TMPyP(4); excellent separation was achieved in 75 m*M* phosphate buffer at pH 3 (Fig. 6). The separation was sensitive to the pH and ionic strength of the buffer as well as the applied voltage. At pH 2, only three peaks were observed (Fig. 6). When the pH was higher than 5, no clear separation was observed. The best separation of this mixture was observed at pH values from 3 to 4, where the unalkylated pyridyl rings are presumably protonated. The ionic strength of buffer was also

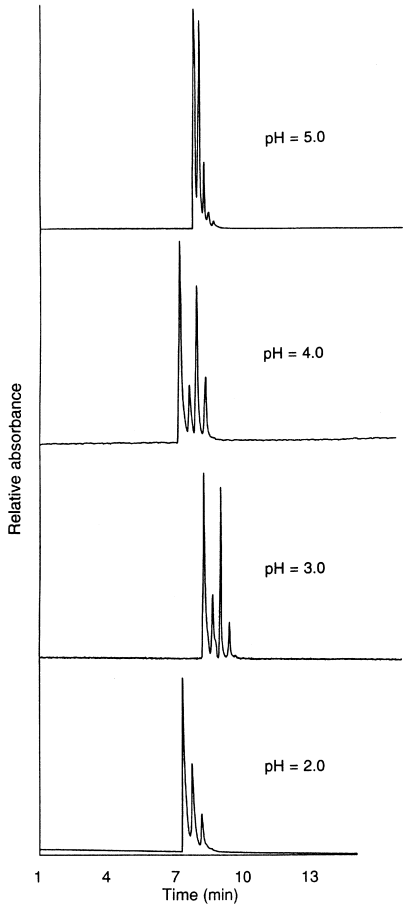


Fig. 6. CE separation of a mixture of methylated TPyP derivative porphyrins as a function of pH (75 m*M* phosphate, 10 kV). The order of elution at pH 3 is: mono- (**4**), *cis*-di- (**5**), tri- (**7**) and tetra- (**1**) alkylated TPyP.

important in this separation. If the buffer concentration was below 50 m*M*, only a single peak was observed. At a buffer concentration of 100 m*M*, only a diffuse and broad peak was observed. When the voltage was higher than 10 kV, the separation time was shorter but the resolution was poorer. At voltages lower than 10 kV, peak broadening occurred. At pH values where the pyridyl nitrogens are not protonated (pH>5), good separations were not observed, presumably because the cationic porphyrins interact with the partial negative charges of the silica on the capillary walls. Also, the four components were not separated completely at low pH (pH 2).

The UV–Vis spectra of TMPyP components separated by capillary electrophoresis are shown in Fig. 7. The absorbance maximum gradually shifts to the blue as the number of methyl groups (quaternary centers) increases. The absorbance of the monomethyl derivative exhibits a red shift of about 8 nm compared with that of TMPyP(4). The slight differences in absorbance maxima may indicate that decreasing the charge on TPyP derivatives results in either higher pK_a values for the center nitrogens or increasing aggregation. Studies with TMPyP(4), TBPyP(4) and TOPyP(4) (above) indicate that differential protonation may be more important, but both factors would result in similar spectral shifts.

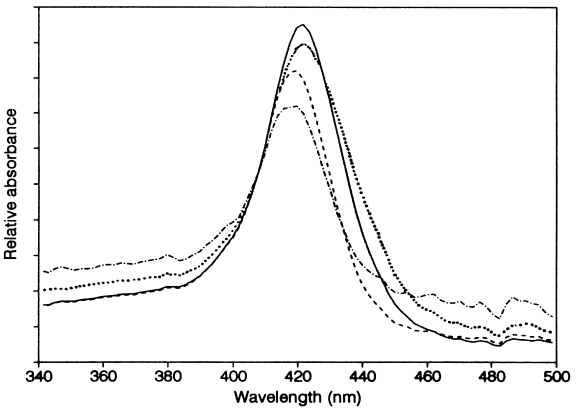


Fig. 7. The UV–Vis spectra of methylated TPyP derivatives separated by capillary electrophoresis (pH 3.0, 75 m*M* phosphate, 11 kV): mono- (**4**, ———), *cis*-di- (**5**, · · ·), tri- (**7**, – – –) and tetra- (**1**, · – · – ·) alkylated TPyP.

3.4. Separation of TPyP derivatives bearing additional charges

Alkylation of TPyP with ω-halo acids gives porphyrins which are expected to be zwitterions with net charges of zero at neutral pH. These porphyrin derivatives, if they could be separated in quantity, would be potential starting materials for asymmetric cationic porphyrin derivatives. Alkylation of TPyP with a 10-fold excess of 3-iodopropionic acid in anhydrous DMF at 60°C for 48 h gave a mixture of products. The monoalkylated derivative **8** could be separated by chromatography on Sephadex LH-20 using ethanol and water as eluents.

Optimal analytical separation of the propionate TPyP derivatives by capillary electrophoresis was achieved at 13 kV in 50 m*M* phosphate buffer at pH 2.45 (Fig. 8). Voltages higher or lower than 13 kV gave different resolutions, but did not affect the separation significantly. Relatively low pH values (<3) were needed to achieve this separation. No separation was observed at pH 5, where these propionate TPyP derivatives are presumably zwitterions with a net charge of zero, and hence have no electrophoretic mobility.

The UV–Vis spectra of each component of the mixture was recorded during the CE separation (data not shown). Similar to the mixture of methylated TMPyP derivatives, components of the propionate mixture with longer migration times had increasingly red-shifted Soret bands. As above, this may be explained in terms of either increasing protonation of the center nitrogens or increasing aggregation as the porphyrin becomes more highly substituted.

A different study involved alkylation of TPyP with 3-bromopropyl amine and gave derivatives with aminopropyl side chains. Via a combination of partial alkylation and centrifugal partition chromatography both the mono- (**9**) and trisubstituted (**10**) compounds were isolated (Fig. 9 and Section 2). The aromatic regions of the ^{1}H NMR spectra of these porphyrins are shown in Fig. 10.

4. Separation of metalloporphyrins

A mixture of metalloporphyrins was investigated

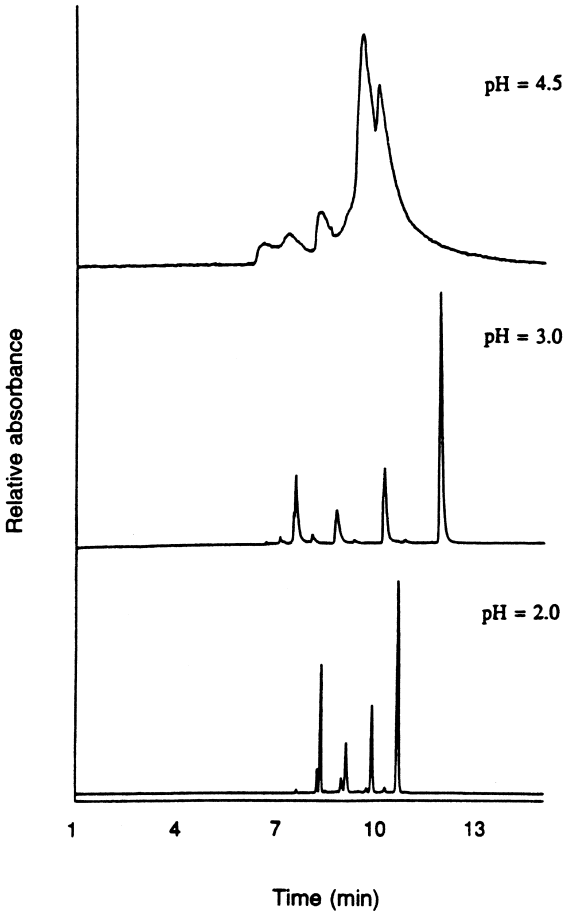


Fig. 8. CE separation of the reaction mixture of TPyP and ICH_2CH_2COOH as a function of pH (50 m*M* phosphate, 13 kV).

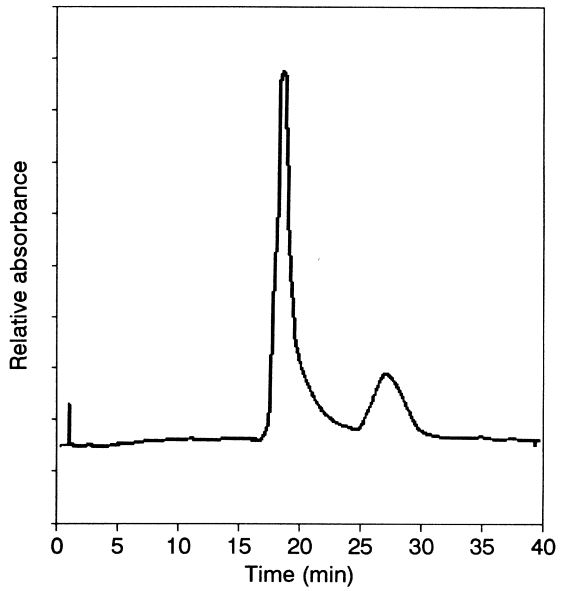


Fig. 9. CPC of the reaction mixture of $BrCH_2CH_2CH_2NH_2$ and TPyP. The sharp peak at 18 min is the monoalkylated porphyrin **9**. Water (pH 10) as the mobile phase, butanol as the stationary phase; descending mode; 1000 rpm; 5 ml/min.

to study the role of molecular mass, net charge and shape on the electrophoretic mobility of the TMPyP(4) derivatives. Eight metal chelates were chosen: Cu, Pd, Zn, VO, Fe, Mn, Co and Sn. The mixture was separated using 50 m*M* phosphate buffer at pH 2.1 with an applied voltage of 9 kV (Fig. 11). This separation was sensitive to the applied voltage; voltages of either 8 or 10 kV gave poorer separations (data not shown). The order of elution was determined by injecting each compound separately and mixtures as necessary to determine the relative migration times. Assignments were checked by recording the optical spectrum of each peak using the diode array spectrometer. Under the conditions employed, all of the metalloporphyrins separated except the Fe(III) and Mn(III) chelates.

Two of the chelates, the Cu(II) and Pd(II) species, are four-coordinate in aqueous solution because neither binds significantly to water [83–85]. Both of these chelates have a net charge of 4+ (four pyridiniumyl nitrogen atoms at the periphery, 2+ for the metal and 2− for the central N^- atoms). Zn(II)TMPyP(4) is five-coordinate, with a water molecule as the axial ligand in aqueous solution [86]; the net charge is 4+. This chelate has been reported to lose zinc at low pH (<2) [30]; loss of zinc was not observed under the capillary electrophoresis conditions.

The VO, Fe(III), Co(III) and Mn(III) chelates have relatively complicated axial equilibria. Vanadyl TMPyP(4) exists as a mixture of five-coordinate and six-coordinate species in nonaqueous solvents, but is found only as the six-coordinate aquo complex in water in the absence of other coordinated counterions

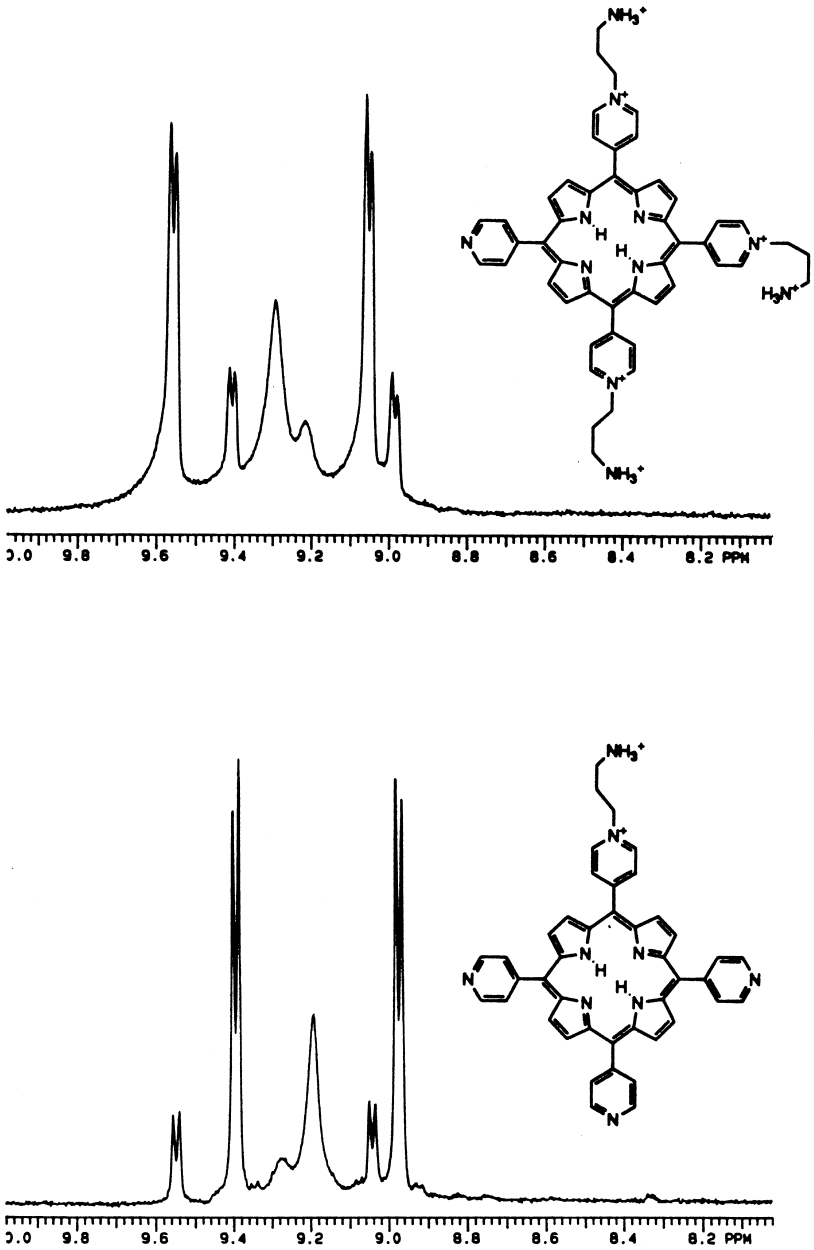


Fig. 10. The aromatic regions of the ^{1}H NMR spectra (400 MHz) of the mono- (**9**) and tri- (**10**) derivatives of TPyP with $CH_2CH_2CH_2NH_2$ side chains in $C^2H_3O^2H$–^2H_2O (5:1, v/v), 60°C.

[87]. The complex has a net charge of 4+. The forms of the Fe(III)TMPyP(4) in aqueous solution have been studied by a number of groups [88]. In aqueous solution at pH 2, magnetic moment studies indicate that the iron chelate is a high spin Fe(III) complex, which is presumably the aquo form

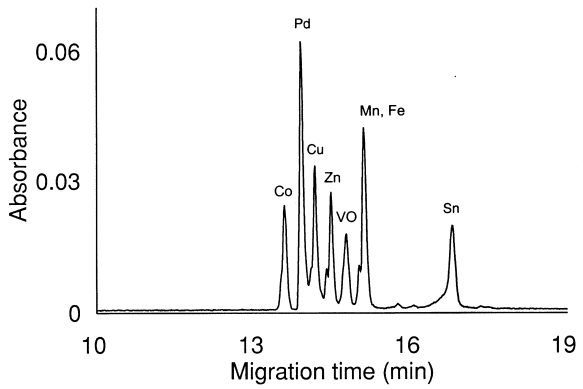


Fig. 11. Separation of the indicated metallo TMPyP(4) derivatives. Conditions: 50 m*M* phosphate; pH 2.1; 9 kV; 57 cm capillary (40 cm to detector).

Fe(III)TMPyP(4)$(H_2O)^{5+}$ [89]. Titration reveals a pK_a of approximately 5.5 which has been ascribed to the equilibrium [88]:

$$FeTMPyP(4)(H_2O)^{5+} + H_2O \rightleftharpoons FeTMPyP(4)(OH)(H_2O)^{4+} + H^+$$

The monomer is in equilibrium with the μ-oxo complex which has two Fe(III)TMPyP(4) molecules with an oxo bridge; the equilibrium constant for formation of the μ-oxo complex is approximately $2 \cdot 10^3\ M^{-1}$ [88]. For the purposes of our experiments, the Fe(III) complex is best characterized as monomeric and five-coordinate with a net charge of 5+. The axial ligation of Co(III)TMPyP(4) is complicated, being a function of counterion and solvent. However, a recent study has shown that the 5+ diaquo species predominates in aqueous solution, even with Cl^- or NO_3^- counterions [90]. The Mn(III) chelate has very similar properties to the cobalt derivative. An observed pK_a of 8.0 has been assigned to loss of a proton from Mn(III)TMPyP(4)$(H_2O)_2^{5+}$ to give Mn(III)TMPyP(4)$(H_2O)(OH)^{4+}$. Thus, a net charge of 5+ is expected for this complex under the conditions of the experiment [91].

Less is known about the axial ligand equilibria of SnTMPyP(4). Because tin is found in the Sn(IV) oxidation state, the metalloporphyrin itself has a net charge of 6+. The species is six-coordinate [86]. Insofar as we are aware, the pK_a values of the axial water molecules have not been measured. It is possible that they are deprotonated (e.g., hydroxide) even at pH 2 due to the high net positive charge of the molecule.

Net charge, molecular mass and the shape of the molecule all control the relative migration times in capillary electrophoresis. A linear regression to net charge, the number of axial ligands (to account for the deviation of the shape from planarity) and molecular mass showed that molecular mass was the dominating controlling factor, with higher-molecular-mass metalloporphyrins having longer retention times. The correlation, however, is relatively poor, indicating that additional factors also control the separation.

Acknowledgements

This work was supported in part by the National Institutes of Health (AI127196). NMR facilities were funded by the National Science Foundation (BIR-9214443) and the Georgia Research Alliance. We thank Dr. Peter Hambright for useful discussions and Dr. Raymond Bae for assistance.

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