Journal of Chromatography, 636 (1993) 171–178
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 876

Capillary electrophoresis analysis of polyhaematoporphyrin, a photosensitizer used in photodynamic therapy

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ABSTRACT

Free solution capillary electrophoresis and micellar electrokinetic capillary chromatography are used to separate components in polyhaematoporphyrin (PHP), a preparation similar to Photofrin which is used as a photosensitizing agent in photodynamic therapy. PHP is known to contain haematoporphyrin (HP), protoporphyrin (PP), hydroxyethylvinyldeuteroporphyrin (HVD) and a mixture of ester, ether and carbon–carbon linked oligomers. The use of reference samples of HP and PP, together with partially dehydrated HP, has allowed assignment of HP and HVD peaks in the electropherograms of PHP in 20 m*M* phosphate pH 6.9 and 20 m*M* disodium tetraborate pH 9.2 buffers using sodium dodecyl sulphate in the range 0–100 m*M*. Changes in the pattern and areas of monomer peaks and oligomer distribution following base hydrolysis (which cleaves ester linkages) and acid hydrolysis (which cleaves ester and ether linkages) yields information consistent with that from HPLC studies.

INTRODUCTION

Photodynamic therapy (PDT) is one of the most recent breakthroughs in the treatment of cancer. It is an experimental treatment which selectively destroys cancer cells by an interaction between absorbed light and a retained photosensitizing agent [1]. There is strong evidence that the mechanism involves incident laser light activating the photosensitizer, which in turn converts triplet O_2 to the excited singlet O_2 [2,3]. It is believed that these reactive but short-lived singlet O_2 attack certain lipids in cell membranes [4] and reactive side chains in protein [5], causing membranes to rupture, thus killing the cell.

PDT is best used for the treatment of thin and superficial tumours, especially in skin cancer. Nevertheless, with the advances in laser and fibre optics, endoscopic treatments are also possible. Trials have shown other cancers such as lung and gastric have also been successfully treated.

One of the photosensitizers currently under trial is polyhaematoporphyrin (PHP), a preparation similar to Photofrin [6]. It is made from haematoporphyrin (HP) by treating first with a mixture of sulphuric and acetic acids and then a mild base. This produces haematoporphyrin derivative (HPD), which contains HP, its dehydration products protoporphyrin (PP) and hydroxyethylvinyldeuteroporphyrin (HVD), and a mixture of porphyrin oligomers [6,7]. PHP, like Photofrin, is obtained by selective depletion of the monomeric porphyrins in HPD so as to enrich the tumour-localising oligomeric fraction which is responsible for both the retention and photosensitizing properties *in vivo* [8]. Fig. 1 shows structures of HP, PP and HVD.

The precise composition of the oligomeric fraction and the structures of its components are not yet determined. This information is essential in providing better understanding of the mechanisms involved and to assist in the search for the critical photosensitizing species. So far, analytical techniques used have included HPLC [6–13], mass spectrometry (MS) [6,7,10,14], TLC [15] and gel chromatography

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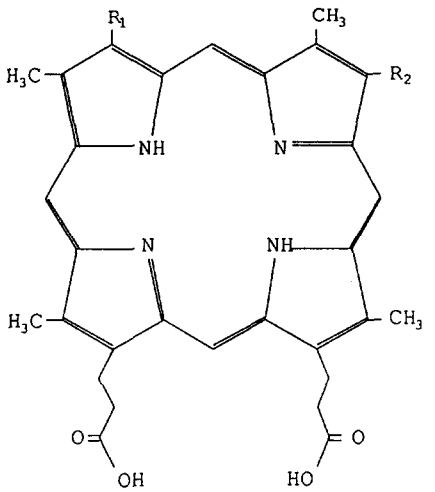

HP : R_1 = R_2 = $CHOHCH_3$
PP : R_1 = R_2 = $CH=CH_2$
HVD : R_1 = $CH=CH_2$; R_2 = $CHOHCH_3$

Fig. 1. Structures of haematoporphyrin monomers.

[16]. Three types of linkages between separate porphyrin monomers have been identified. Ester linkages, formed by condensation of hydroxyethyl and propionic acid groups, may be characterised by their lability to base hydrolysis or $LiAlH_4$ reduction [7]. Ether linkages resulting from reduction of hydroxyethyl groups are base-stable but acid-labile [6,9]. Carbon–carbon linkages are also produced by reaction of hydroxyethyl groups and are resistant to both acid and base hydrolysis [10]. Typical Photofrin II samples consist of monomers (15%), ester-linked (10–20%), ether-linked (20–30%) and acid resistant (35–50%) oligomers [10]. Structural information on oligomers up to degree of polymerization = 3 is available from MS [10]. Oligomers may contain up to 15 HP or HVD units [17].

In the present work, the separation of components of PHP has been studied using capillary electrophoresis (CE). CE is a recently developed electroseparation technique capable of separating complex mixtures of both ionic and neutral water-soluble materials [18–21]. Two modes of CE, free solution capillary electrophoresis (FSCE) and micellar electrokinetic capillary chromatography (MECC), in which micellar reagents are added to the background electrolyte, have been used. The high resolution of CE together with its complementarity to HPLC (HPLC and MECC separate analytes on the basis of hydrophobicity while FSCE separates on the basis of charge-to-size ratio) makes CE an ideal technique to investigate PHP. The effects of altering buffer pH and the type and concentration of micellar additives have been studied to establish suitable conditions for identification of monomers and oligomers in PHP and its hydrolysis products.

EXPERIMENTAL

Materials

Samples of polyhaematoporphyrin (PHP) of 5 mg cm^{-3} were supplied by the Department of Biochemistry, University of Leeds, as a sterile solution in phosphate-buffered saline pH 7. Acid-hydrolysed samples were prepared by treating the PHP solution (3 cm^3) with 1 *M* HCl (6 cm^3) in a boiling water bath for 30 min. Base-hydrolysed samples were prepared by reacting PHP (3 cm^3) with 1 *M* NaOH (6 cm^3) at room temperature in the dark for 24 h. Both hydrolysed samples were neutralized (6 cm^3 of 1 *M* base or acid) at the end of the reaction period.

Stock solutions of 5 mg cm^{-3} haematoporphyrin (HP) dihydrochloride and protoporphyrin (PP) disodium salt (Sigma, Poole, UK) were prepared in dimethyl sulphoxide (DMSO). DMSO and all buffer components were supplied by Fisons (Loughborough, UK). Dehydration of HP was carried out by heating the stock solution of 5 mg cm^{-3} HP in DMSO in a thermostatted water bath at 60°C for 5 h or 24 h.

Buffers used were (i) phosphate pH 6.9 (10 m*M* K_2HPO_4, 10 m*M* KH_2PO_4, ionic strength I = 0.040 mol kg^{-1}), (ii) borate pH 9.2 (20 m*M* $Na_2B_4O_7 \cdot 10H_2O$, I = 0.040 mol kg^{-1}). Micellar additives were sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) (Sigma, Poole, UK).

Methods

For CE separations, porphyrin samples were diluted 1:20 with deionized water (Elgastat UHQ) or 100 m*M* SDS, since detergents are known to disaggregate porphyrin esters [22]. Samples and background electrolytes were filtered (0.22-μm Millipore) before injection and separations in the CE instru-

ment (PACE 2100, Beckman, High Wycombe, UK). The fused-silica capillary used was 57 cm long with an internal diameter of 75 μm, 50 cm to the detector and thermostatted at 25°C. The samples were injected hydrodynamically for 2 s (12 nl), separated using a voltage of +15 kV or +20 kV and detected by absorbance at 340 nm.

RESULTS AND DISCUSSION

Monomeric species in polyhaematoporphyrin

The polyhaematoporphyrin investigated in this study has been shown, using fluorescence detection, to consist of oligomers and monomers in relative proportions 83% and 17% (w/w), respectively [23].

Electropherograms of PP and HP in borate–SDS buffers are given in Fig. 2. PP gives a single sharp peak whilst diastereoisomers of HP show a partially resolved doublet with impurity peaks also present. The same pattern of peaks was observed in phosphate–SDS buffers.

Mobilities, μ, were determined using the equation [18],

$$\mu = \left(\frac{1}{t} - \frac{1}{t_0}\right)\frac{lL}{V}$$

with t the analyte migration time, t_0 the migration time for neutral marker, l the length of capillary to detector, L the total length of the capillary and V the applied voltage. In the actual electropherograms a neutral marker was always observed due to the presence of DMSO in the injected solutions, which causes a refractive index difference between sample and background electrolyte solutions. The migra-

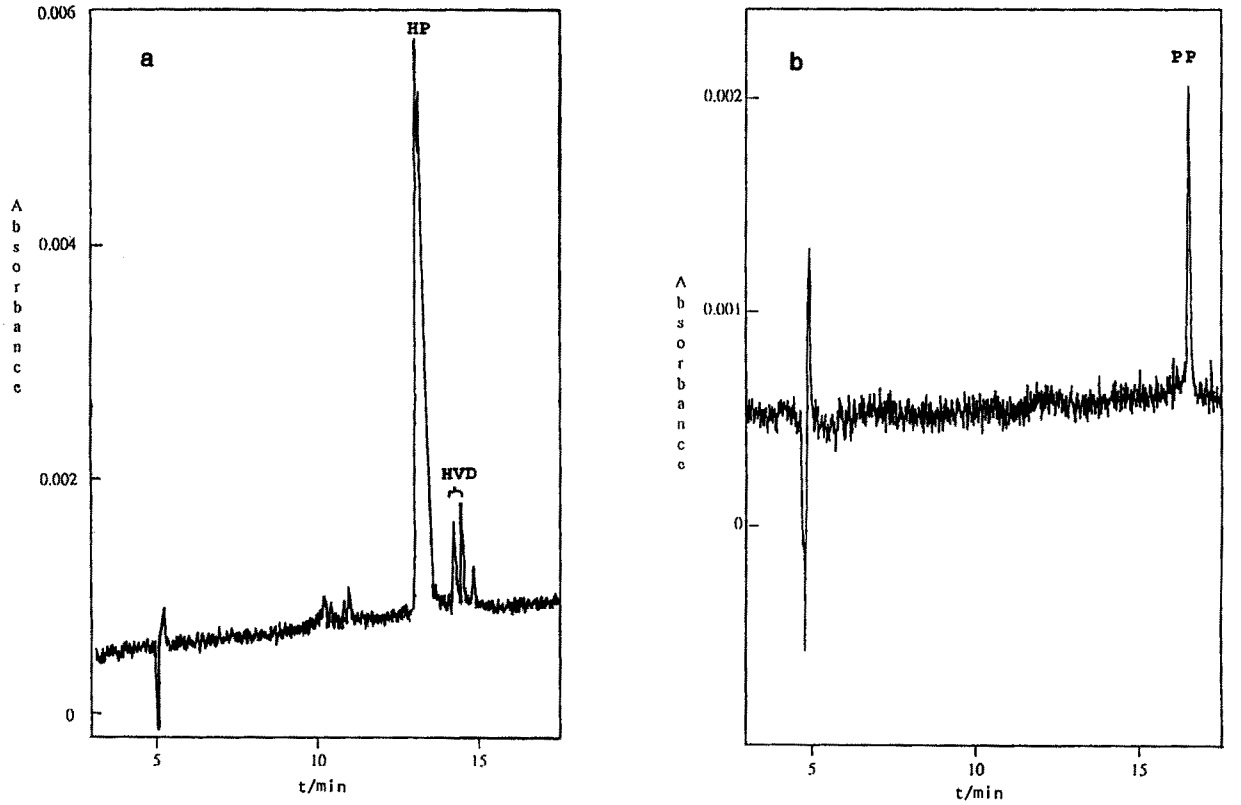

Fig. 2. Electropherograms of (a) haematoporphyrin (HP) in running buffer of 20 m*M* pH 9.2 borate–25 m*M* SDS; (b) protoporphyrin (PP) in running buffer of 20 m*M* pH 9.2 borate–50 m*M* SDS. Capillary, 57 cm × 75 μm I.D., 50 cm to detector. Applied voltage, +15 kV. Injection, 2 s (12 nl). 0.24 mg cm^{-3} sample in DMSO–water (5:95, v/v). Detection, 340 nm. Peaks assigned to HP, PP and hydroxyethylvinyldeuteroporphyrin (HVD) labelled.

tion time for the neutral marker was taken as the onset of the solvent dip [24].

Variation of mobilities of PP and HP with SDS concentration is given in Fig. 3. The curves were drawn to fit data from samples diluted before injection either with water or SDS. In the borate buffer at pH 9.2 the carboxylic acid side chains on the haematoporphyrins are fully ionised, so both HP and PP are negatively charged when binding to the micelles. PP is seen to associate more strongly than HP with the micelles. Binding is known to correlate with hydrophobicity, and the vinyl side chains in PP aer more hydrophobic than the hydroxyethyl side chains in HP. The migration order is the same as the order of elution in reversed-phase HPLC [7,23] using C_8 or C_{18} stationary phases, which also bind components in the order of their hydrophobicity.

Mobilities in Fig. 3 together with the pattern and relative absorbance of peaks in the electropherograms (*e.g.*, Fig. 2) aid the assignment of HP and PP as components in mixtures at any SDS concentration. The impurity peak with the longest migration time in the electropherogram of HP (Fig. 2) is found to be PP. Since the two positional isomers of HVD are expected, by analogy with the separation order in reversed-phase HPLC, to give a doublet of peaks between HP and PP, the doublet migrating between HP and PP in Fig. 2 may be identified as HVD. This assignment was checked by heating HP at 60°C in DMSO for 5 h and for 24 h. Such treatment causes decomposition of HP to HVD [11] and PP by successive elimination of water, with the relative

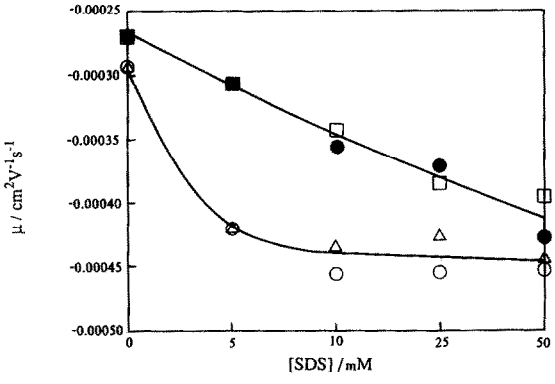

Fig. 3. Variation of mobilities of haematoporphyrin (HP) and protoporphyrin (PP) with SDS concentration. HP diluted in (□) water or (●) SDS; PP diluted in (○) water or (△) SDS.

concentration of HVD going through a maximum after approximately 5 h [25]. In the electropherograms in phosphate–SDS buffers of the reaction mixture sampled at $t = 0$, 5 and 24 h, four peaks were observed. The area of the fastest migrating peak decreased over this time period, that of the slowest migrating peak increased, whilst the central doublet went through a maximum at 5 h, confirming that the assignment has been correctly made.

Free solution electrophoresis and micellar electrokinetic chromatography of polyhaematoporphyrin

The effect of pH on FSCE separations of PHP was studied using running buffer solutions of phosphate and borate at pH 6.9 and 9.2, respectively, both with ionic strength 0.040 mol kg^{-1}. The best electropherograms were obtained in borate buffers.

As complete separation of the components in PHP was not obtained in FSCE, MECC was employed to improve the resolution. Two micellar species were studied, anionic SDS and cationic CTAB, in a range of buffers. It was found that SDS gave much better separations than CTAB.

Fig. 4 shows electropherograms obtained over a range of SDS concentrations in the pH 9.2 20 m*M* borate buffer, while Fig. 5 gives a series of electropherograms in the pH 6.9 20 m*M* phosphate buffer. A common trend is evident in both Figs. 4 and 5. With increasing SDS concentration, the broad underlying distribution, evident in the FSCE electropherograms of PHP, shifts to later migration times and become more skewed in shape. At low SDS concentrations, early migrating sharp peaks are separated from the broad distribution. At high SDS concentrations, most peaks merge into the front of the distribution.

SDS is known to break up aggregates of porphyrins, and to test for the effect of any aggregation in PHP a comparison was made of samples injected from water and from SDS solution. No significant differences were observed in the MECC electropherograms, showing aggregation is not a problem in the present work.

Using the assignment of monomer peaks as discussed in the previous section, peaks from HP and HVD were located in the electropherograms of PHP. Assignments were also confirmed by spiking PHP samples with HP and PP.

Electropherograms of the base-hydrolysed PHP

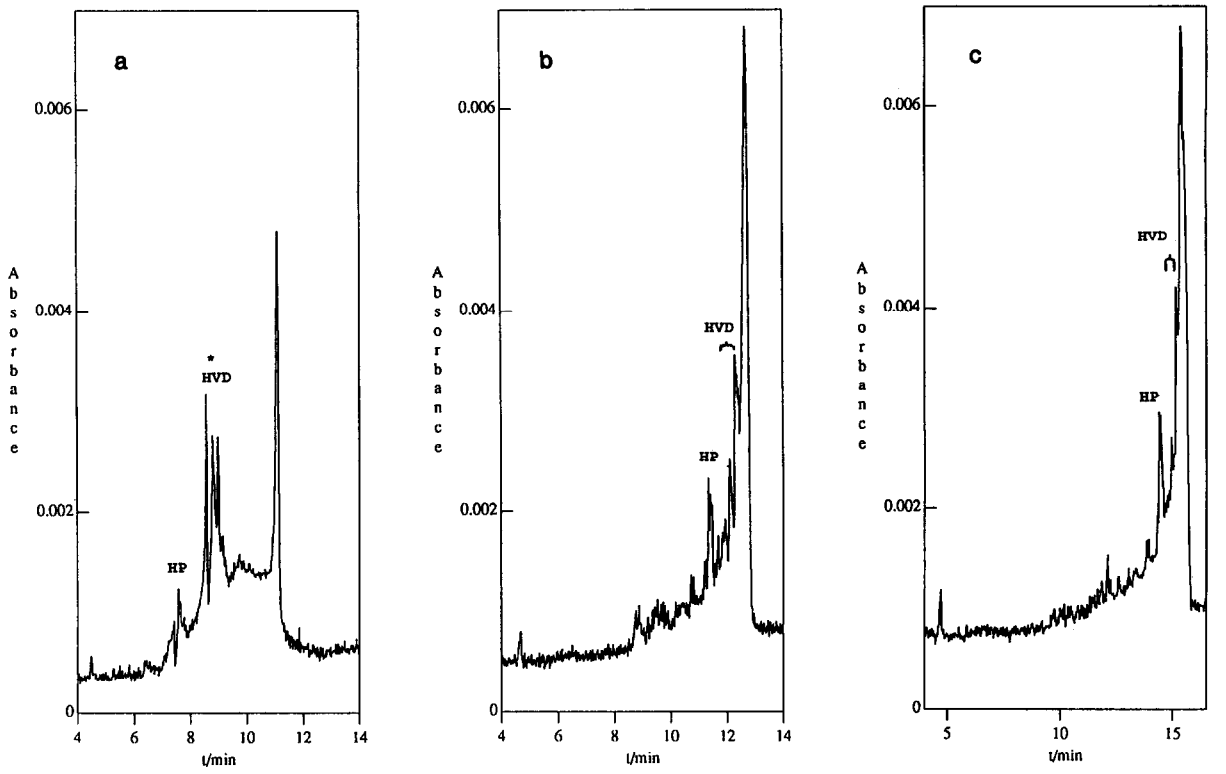

Fig. 4. Electropherograms of polyhaematoporphyrin (PHP) in 20 m*M* pH 9.2 borate at a range of SDS concentrations: [SDS] = (a) 0, (b) 25, (c) 50 m*M*. Sample: 0.24 mg cm^{-3} in 1:20 diluted phosphate-buffered saline. Other CE separation conditions and labelling of monomer peaks as in Fig. 2. Group of peaks starred contains oligomers in addition to HVD.

are given in Fig. 6I and acid-hydrolysed PHP in Fig. 6II. Base hydrolysis is known to break down ester-linked oligomers, whilst acid hydrolysis cleaves both ester- and ether-linked oligomers [6,7,9]. Carbon–carbon linked oligomers are unaffected by both processes [10]. Analysis of electropherograms in Figs. 4 and 6 for solutions with 50 m*M* SDS reveals changes in proportions of components in PHP on hydrolysis. Subtraction of peak areas of HVD and HP from the total area (absorbance integrated over time) for PHP gave an area ascribed to oligomers. Comparison of the area of the oligomers in phosphate–SDS or borate–SDS before and after acid hydrolysis (Figs. 4c, 5c, 6IIb, 6IIc) showed that the percentage of oligomers remaining following acid hydrolysis was 45% of its starting value. This is within the range 35–50% previously measured using HPLC for samples of Photofrin II [10], which are similar to PHP. HP and HVD peak areas increased on hydrolysis, with HP dominant after acid hydrolysis. The HVD–HP peak area ratio from CE is 0.6:1, similar to the ratio 0.7:1 from HPLC analysis of an acid-hydrolysed sample [25].

Whilst the analysis of CE data thusfar has provided similar results to those obtainable by HPLC, additional information on the pattern of depletion of the oligomer distribution on hydrolysis is obtained from the present CE study. The alteration in PHP peak profile on hydrolysis is most marked in the electropherograms in borate buffer without any added SDS, *i.e.*, under FSCE conditions. Base hydrolysis depletes the slowest migrating peak in Fig. 4a, which can therefore be assigned to ester-linked oligomers. The FSCE mobility of such species

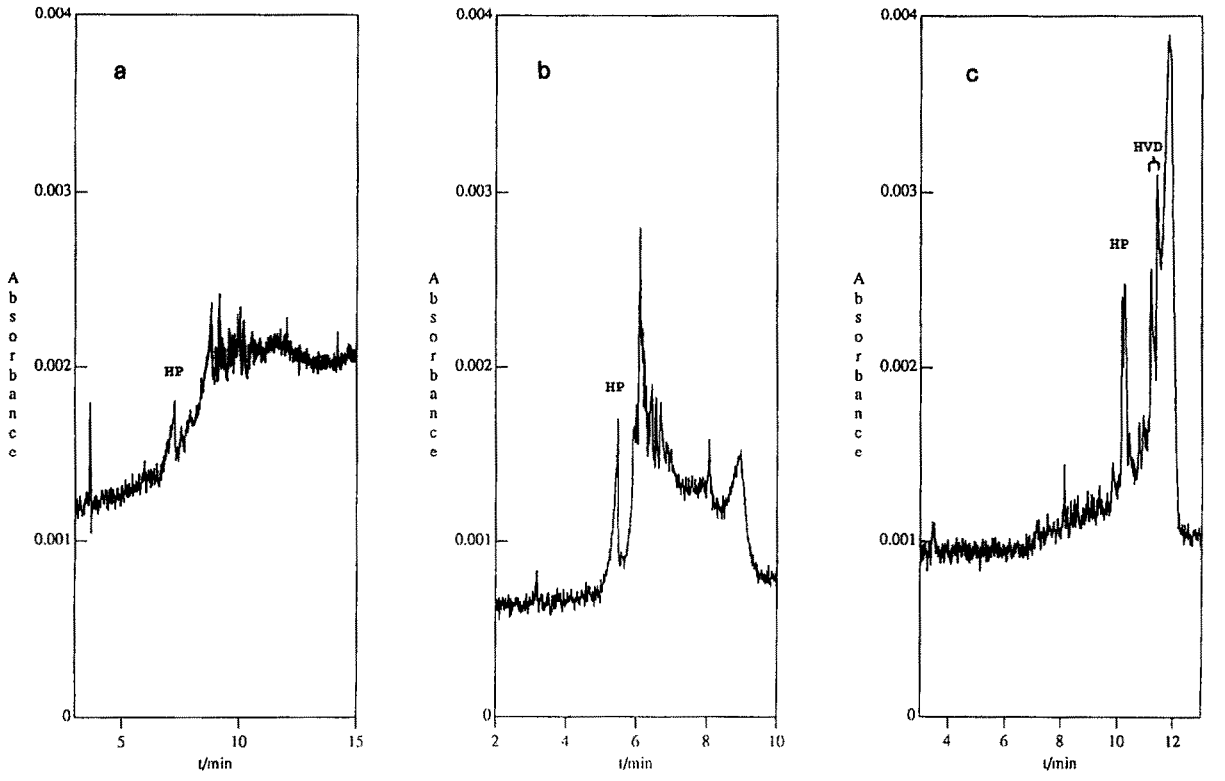

Fig. 5. Electropherograms of polyhaematoporphyrin (PHP) in running buffer of pH 6.9 20 m*M* phosphate at a range of SDS concentrations: [SDS] = (a) 0, (b) 25, (c) 50 m*M*. Sample and separation conditions as in Fig. 4, except applied voltage = +20 kV.

is 1.5 times greater in magnitude than HP, indicating a higher charge-to-size ratio. Other depleted peaks in the broad envelope of PHP in Fig. 6a cannot be individually resolved. Acid hydrolysis causes further depletion in the centre of the distribution. Since the difference between acid and base hydrolysis is cleavage of ether-linked oligomers, this suggests that the ether-linked oligomers have a relatively narrow distribution of mobilities centred on the mean value for all oligomers. Fine structure is evident in all electropherograms in Figs. 4 and 6, indicating the potential for resolution of individual oligomers in further studies.

CONCLUSIONS

Analysis of polyhaematoporphyrins has been carried out using capillary electrophoresis. A 20 m*M* borate buffer, pH 9.2, with 0–50 m*M* SDS is suitable for separation. At the upper end of this range of surfactant, the order of resolution of the monomers HP, HVD and PP is the same as that in reversed-phase HPLC [6,7,11], consistent with MECC separation according to hydrophobicity. Without added SDS the FSCE mobility order, which depends on charge-to-size ratio, gives new information on the distribution of types of oligomers in PHP. In future work, we aim to focus on optimising CE conditions for resolution and collection of fractions of individual oligomers.

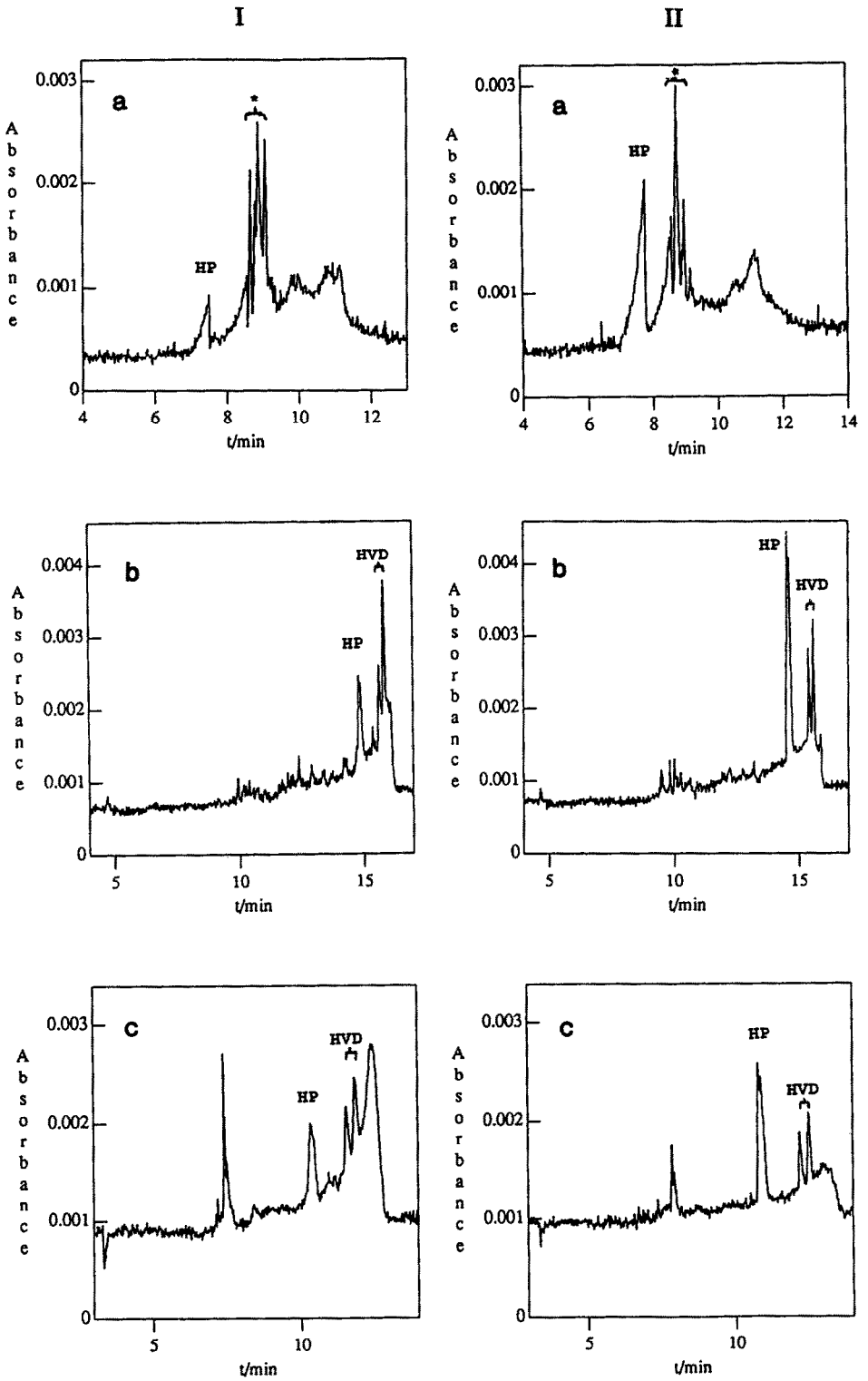

Fig. 6. Electropherograms of (I) base- and (II) acid-hydrolysed polyhaematoporphyrin (PHP) in running buffers of: (a) pH 9.2 borate; (b) pH 9.2 borate–50 m*M* SDS; (c) pH 6.9 phosphate–50 m*M* SDS. Sample: 0.17 mg cm^{-3} hydrolysed PHP in 67 m*M* NaCl. Separation conditions as in Figs. 4 and 5. Peaks due to haematoporphyrin (HP) and hydroxyethylvinyldeuteroporphyrin (HVD) labelled. Groups of peaks starred in (a) contain oligomers in addition to HVD.

ACKNOWLEDGEMENTS

We wish to thank Professor S. B. Brown and Dr. D. I. Vernon of the Department of Biochemistry, University of Leeds, for gifts of polyhaematoporphyrin and encouragement throughout this project.

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