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Micellar electrokinetic capillary chromatography of haematoporphyrin, protoporphyrin and their copper and zinc complexes

Chikara Kiyohara, Koichi Saitoh* and Nobuo Suzuki

Department of Chemistry, Faculty of Science, Tohoku University, Sendai, 980 Miyagi (Japan)

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ABSTRACT

The micellar electrokinetic chromatographic separation of haematoporphyrin IX (HP) and protoporphyrin IX (PP) in the forms of free acids and of metal complexes with zinc or copper is successful in a mixture of a micellar solution of sodium dodecyl sulphate (SDS) at pH 11 and dimethylformamide (10:2, v/v). All the porphyrins migrate in the direction of the electric field, *i.e.*, from the positive end towards the grounded end of the capillary. The migration time of each porphyrin increases with increasing concentration of DMF in the carrier solution. The capacity factor calculated for the distribution of each porphyrin compound between the SDS micelles and the bulk solution varies nearly linearly with the concentration of the SDS micelles. The migration sequence, that is, the increasing order of migration time, for the porphyrins is $Zn(HP) < H_2HP < Cu(HP) < Zn(PP) < H_2PP < Cu(PP)$.

INTRODUCTION

There are two types of separation for porphyrins and metalloporphyrins: (1) their separation in either the free acid form or the complexed form with a certain metal in accordance with the difference in the molecular structure of the porphyrin and (2) their separation in accordance with the complexing metal ion. The latter type of separation is generally the more difficult because each metal ion is surrounded by a bulky macrocyclic structure of porphyrin. High-performance thin-layer chromatography (HPTLC) [1,2] and column high-performance liquid chromatography (HPLC) [3,4] have been promising methods for both types of separation of metalloporphyrins and porphyrins.

Recently, there has been increased interest in the high separation ability of capillary zone electrophoresis (CZE) and also its expanded mode, micellar electrokinetic capillary chromatography (MEKC) [5]. The applicability of MEKC to the separation of metal complexes was previously confirmed for non-charged bis- and trisacetylacetonato complexes of di- and trivalent metals using a micellar solution of sodium dodecyl sulphate (SDS) as the carrier solution [6,7].

This work was undertaken in order to examine the feasibility of MEKC for the separation of metalloporphyrins in accordance with both their central metal ions and their porphyrin structures. With regard to the metal-free forms of porphyrins, MEKC separation of urinary porphyrins has already been reported by Weinberger *et al.* [8]. Two typical bioporphyrins, *viz.*, haematoporphyrin IX (HP) and protoporphyrin IX (PP), were considered in this work. The migration behaviour of the respective porphyrins in the free acid forms and the complexed forms with copper(II) and zinc(II) was investigated using

* Corresponding author.

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different conditions of the electrophoretic carrier solution.

EXPERIMENTAL

Materials

Fig. 1 shows the structures of free acid forms of HP and PP (H_2HP and H_2PP, respectively) and the corresponding metal complexes [M(HP) and M(PP), in general].

H_2HP (Wako, Osaka, Japan) was purified as detailed elsewhere [9]. Copper haematoporphyrin [Cu(HP)] and zinc haematoporphyrin [Zn(HP)] were prepared by reaction of H_2HP with copper chloride and zinc acetate in mixtures of dimethyl sulphoxide (DMSO) and water, respectively [10].

H_2PP was purified from its sodium salt (Green Cross, Osaka, Japan) as follows. The sodium salt of the porphyrin (120 mg) was taken in 40 ml of acetone–water (7:3, v/v), and the non-dissolved fraction was removed by centrifugation. A 20-ml portion of this solution was diluted with 240 ml of buffer solution (3.5 m*M* H_3PO_4–19.9 m*M* NaH_2PO_4, pH 3), followed by shaking with 320 ml of ethyl acetate. The organic phase was washed with 200 ml of water, then H_2PP was precipitated by adding 2,2,4-trimethylpentane. The purified H_2PP was dried under vacuum.

Copper protoporphyrin [Cu(PP)] was prepared by the reaction of H_2PP (8.6 μmol) and copper acetate (38 μmol) in 4 ml of N,N-dimethylformamide (DMF) at 80°C for 1 h. After

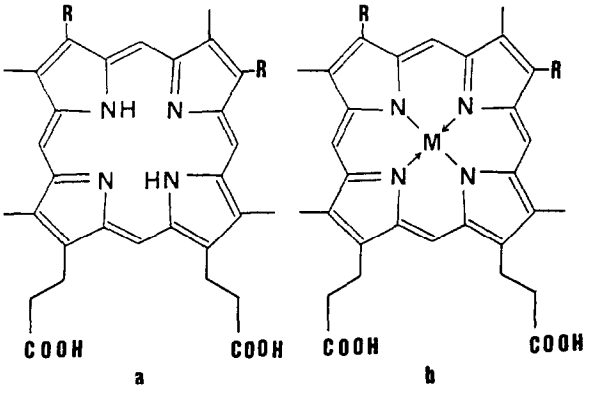

Fig. 1. Structural formulae of (a) porphyrin and (b) its metal (M) complex. R = $-CH(OH)CH_3$ for H_2HP and M(HP) and $-CH=CH_2$ for H_2PP and M(PP).

been cooled near to the room temperature, the reaction mixture was shaken with a mixture of 50 ml of ethyl acetate and 40 ml of 2.4 *M* hydrochloric acid for several minutes. The organic phase was washed four times with 30 ml each of water and then concentrated by removal of the solvent under vacuum. Finally, Cu(PP) was precipitated by addition of 2,2,4-trimethylpentane. Zinc protoporphyrin [Zn(PP)] was synthesized by the reaction of H_2PP (6.8 μmol) with zinc acetate (20.1 μmol) in 2 ml of DMSO at 80°C for 15 h. The reaction mixture was shaken with a mixture of 50 ml of ethyl acetate and 40 ml of water. Zn(PP) was precipitated from the organic phase by procedures similar to those for the preparation of Cu(PP). Identification of the final products of Cu(PP) and Zn(PP) was performed by UV–visible and mass spectrometry.

CAPS buffer (pH 11) was prepared so as to contain 20 m*M* 3-cyclohexylaminopropanesulphonic acid (CAPS) (Dojin Labs., Kumamoto, Japan) and 17 m*M* sodium hydroxide.

The hydrophobic pigment Oil Yellow OB [α-(*o*-tolylazo)-β-naphthylamine] (Tokyo Kasei, Tokyo, Japan) was used as a reference substance for monitoring the migration of the SDS micelle.

Apparatus and conditions

A Model CE890 capillary electrophoretic separation system (JASCO, Tokyo, Japan) was used with a fused-silica capillary (70 cm × 50 μm I.D.). Spectrophotometric detection was carried out 50 cm from the positive end of the capillary. The carrier solution was prepared by mixing, immediately before use, the desired amounts of DMF and the CAPS buffer (pH 11) containing SDS at a certain concentration, unless indicated otherwise. A typical composition of the carrier solution was 40 m*M* SDS in CAPS buffer–DMF (10:2, v/v). The sample solution of a porphyrin or a porphyrin mixture was prepared at a concentration of 10^{-4} *M* for each porphyrin in an identical solution to the carrier solution. Introduction of sample solution (about 4 nl) into the capillary was performed with the aid of an electroinjection technique. Electrophoretic experiments were carried out with an applied voltage of 30 kV (electric field strength along the

capillary 429 V/cm) and at a detection wavelength of 405 nm.

RESULTS AND DISCUSSION

Carrier solution

The electroosmosis of an aqueous solution occurring in a fused-silica capillary generally takes place in the direction of the electric field, *i.e.*, from the positive end towards the grounded end of the capillary, and the velocity of the electroosmotic flow increases with increasing pH of the solution. In this study, the carrier solution filled in the capillary was CAPS buffer of pH 11 (20 m*M* CAPS–17 m*M* NaOH), so that a strong electroosmotic flow of the solution would occur.

All the porphyrin compounds studied were regarded as anionic in a basic solution owing to the dissociation of carboxylic protons from respective molecules. It was reasonable to consider that the migration of the anionic form of each porphyrin compound would be governed by its electrophoretic motion toward the positive end of the capillary in addition to the electroosmotic flow of the carrier solution in the opposite direction. When the feasibility of the CZE separation of H_2HP, Cu(HP) and Zn(HP) was preliminarily examined simply using the CAPS buffer alone, these three HP compounds moved towards the negative end of the capillary and were detected at migration times of 6.5, 6.8 and 5.6 min, respectively, at an electric field strength of 269 V/cm. This implied that the velocity components of the electrophoresis for these negatively charged HP species were smaller than that of the electroosmosis of the solution.

When SDS was added to the buffer solution noted above, the solubility of each porphyrin was improved. The addition of SDS, however, resulted in a decrease in the reproducibility of the migration velocities (and times) of porphyrins. The migration times (t_s) of H_2HP and Cu(HP) in particular increased successively with repetition of the CZE runs. For example, the t_s values for H_2HP were 9.83, 10.39, 11.26 and 14.50 min in the first, third, fifth and eighth runs, respectively, with CAPS buffer containing 25 m*M* SDS and at an applied voltage of 30 kV. Such undesirable effects were not observed with Zn(HP) and Oil Yellow OB. However, the migration velocity of these two compounds also decreased after the sample solution containing H_2HP and/or Cu(HP) had been injected into the capillary. These undesirable phenomena were presumably caused by the adsorption of H_2HP and/or Cu(HP) on the inner surface of the capillary.

When DMF was added at levels up to 10% (v/v), instead of SDS, to the buffer solution, the reproducibility was not improved, although the solubility of each porphyrin was improved. It was found that the problems of both the reproducibility of the migration velocity and low solubility of these HP compounds could be solved by using buffer solution containing both SDS and DMF. For example, the t_s values for H_2HP were 7.14 and 7.16 min in the first and third runs, respectively, when using the DMF–CAPS buffer (pH 11) (20:80, v/v) containing 25 m*M* SDS and an applied voltage of 30 kV.

Effect of DMF concentration

In this study DMF was used as an organic additive to the carrier solution, considering its high dissolving capability for porphyrins, high dielectric constant (36.7 D) and low viscosity (0.80 cP).

When a buffer solution (pH 11) containing 50 m*M* SDS was used as the carrier solution, the migration times of each porphyrin (t_s), carrier solution (t_0) and SDS micelle (t_{mc}) increased with increasing concentration of DMF in the carrier solution, as shown in Fig. 2, where t_0 and t_m were estimated from the migration times of a non-retained system peak and Oil Yellow OB, respectively. It is obvious that the difference in t_s values among the porphyrin compounds increases with increasing DMF content.

Effect of SDS concentration

The formation of micelles of SDS in a DMF-containing solution was examined by conductimetry. Fig. 3 shows the variation of the specific conductivity change ($\Delta\kappa$) and the molar conductivity (Λ) of SDS with the total concentration of SDS (C_{SDS}) in CAPS buffer (pH 11)–DMF (10:2, v/v). The break of the proportionality between the specific conductivity change ($\Delta\kappa$)

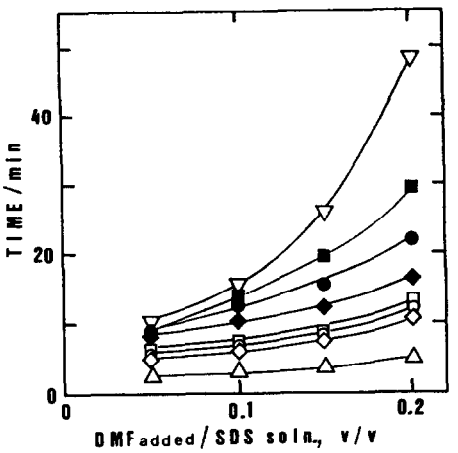

Fig. 2. Effect of addition of DMF to a carrier solution (volume ratio) on t_s of porphyrin compounds, t_0 and t_{mc}. Carrier solution, 50 m*M* SDS–20 m*M* CAPS–17 m*M* NaOH; capillary, 70 cm × 50 μm I.D.; effective migration length, 50 cm; applied voltage, 30 kV. ○ = t_{s,H_2HP}; □ = $t_{s,Cu(HP)}$; ◇ = $t_{s,Zn(HP)}$; ● = t_{s,H_2PP}; ■ = $t_{s,Cu(PP)}$; ◆ = $t_{s,Zn(PP)}$; △ = t_0; ▽ = t_{mc}.

and C_{SDS} indicates the formation of micelles of SDS in the solution. From the break point, the critical micelle concentration (CMC) of SDS is estimated to be about 8 m*M* in this instance.

When the solution contained SDS micelles, the partitioning phenomenon between the micelles and the bulk aqueous solution was taken into account for a non-charged solute having hydrophobic characteristics [5]. The capacity factor, k', defined as the ratio of the amount of a solute in the micelle phase to that in the bulk aqueous phase, is given as a combined function of t_s, t_0 and t_{mc} [11]:

$$k' = (t_s - t_0)/[t_0(1 - t_s/t_{mc})] \quad (1)$$

and in that case k' varies with the total concentration of SDS (C_{SDS}) in the solution, according to the following approximate equation:

$$k' = KV_{SDS}(C_{SDS} - CMC) \quad (2)$$

where K is the distribution coefficient, defined as the concentration ratio of the solute in the micelle phase to that in the bulk aqueous phase, V_{SDS} is the partial molar volume of SDS in the micelle and CMC is the critical micelle concentration of SDS. It is noted that eqn. 2 is valid in principle under the condition $C_{SDS} > CMC$.

The effect of the SDS concentration on the migration behaviour of porphyrins was investigated using CAPS buffer (pH 11)–DMF (10:2, v/v). It was found that the k' values calculated for the porphyrins according to eqn. 1 did not always increase with increasing C_{SDS}, as shown in Fig. 4. This result is reasonable when it is considered that a considerable proportion of each porphyrin compound is not electrically

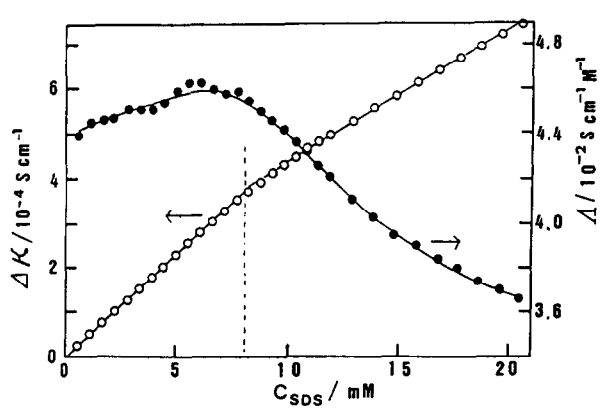

Fig. 3. Variation of conductivity with total concentration of SDS (C_{SDS}) in CAPS buffer (pH 11)–DMF (10:2, v/v) at 25°C. Λ = Molar conductivity of SDS; Δκ = specific conductivity change.

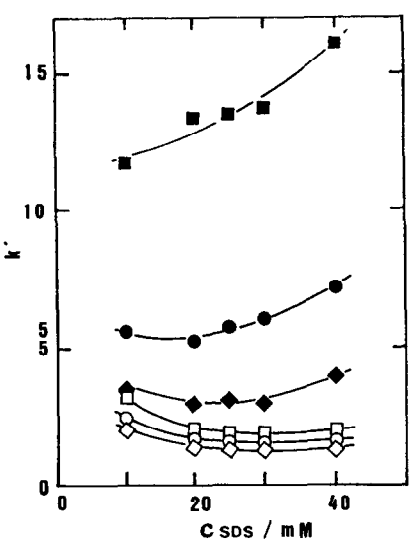

Fig. 4. Plot of capacity factor (k') calculated from eqn. 1 *versus* total concentration of SDS in the carrier solution CAPS buffer [(pH 11)–DMF (10:2, v/v)]. ○ = t_{s,H_2HP}; □ = $t_{s,Cu(HP)}$; ◇ = $t_{s,Zn(HP)}$; ● = t_{s,H_2PP}; ■ = $t_{s,Cu(PP)}$; ◆ = $t_{s,Zn(PP)}$.

neutral but negatively charged owing to the dissociation of carboxylic protons under such basic conditions as pH 11. Accordingly, the contribution of the electrophoretic motion occurring in the bulk aqueous phase should be taken into account in the calculation of k'. In such a case, eqn. 2 should be altered by using the distribution ratio (D) in the place of the partition coefficient (K) because the solute distribution phenomenon is not related to the concentration of a particular species (C_i) but to those of all (n) species of the solute:

$$k' = DV_{SDS}(C_{SDS} - CMC) \quad (3)$$

where

$$k' = \frac{\sum_{i=1}^{n} C_{i,\text{ micelle phase}}}{\sum_{i=1}^{n} C_{i,\text{ bulk aqueous phase}}}$$

Capacity factor

Under the practical experimental conditions of CZE, the bulk aqueous solution and SDS micelles moved towards the negative end of the capillary at apparent velocities v_{eo} and v_{mc}, respectively. When the migration of a solute is a combined function of the electrophoresis in the bulk aqueous phase and the distribution between the SDS micelle phase and the bulk aqueous phase, the velocity of the solute, v_s, is given by the following equation:

$$\begin{aligned} v_s &= [n_{aq}/(n_{aq} + n_{mc})](v_{eo} + v_{s,ep}) \\ &\quad + [n_{mc}/(n_{aq} + n_{mc})]v_{mc} \\ &= [1/(1 + k')](v_{eo} + v_{s,ep}) + [1/(1 + k')]v_{mc} \end{aligned} \quad (4)$$

where n_{aq} and n_{mc} are the numbers of solute molecules in the bulk aqueous phase and the SDS micelle phase, respectively, and $v_{s,ep}$ is the electrophoretic velocity vector of the solute in the bulk aqueous phase. By rearranging this equation, the capacity factor, k', is represented by

$$k' = (v_{eo} - v_s + v_{s,ep})/(v_s - v_{mc}) \quad (5)$$

The k' values of the porphyrins and their metal complexes were calculated from eqn. 5. The values of v_{eo}, v_s and v_{mc} were calculated from the values of t_0, t_s and t_{mc}, respectively, measured in the experiments using a micellar solution of SDS, and $v_{s,ep}$ was approximately estimated from the values of v_s and v_{eo} that were measured in an SDS-free carrier solution. It was found that the capacity factor increased with increasing C_{SDS} in the solution, as shown in Fig. 5. When the result of the plot of k' *versus* C_{SDS} was fitted to a linear relationship, the intercepts on the C_{SDS} axis were found to be in the range 5–10 mM.

When the linear relationship given by eqn. 3 is fitted to the experimental plots (k' *versus* C_{SDS}) shown in Fig. 5, the slope and the intercept on the C_{SDS} axis correspond to the product DV_{SDS} and the CMC of SDS in the carrier solution, respectively. The values of the intercept on the C_{SDS} axis for the plots for the porphyrin compounds of interest are in the range 5–10 mM, which agrees with the value of the CMC of SDS estimated by conductimetry (*ca.* 8 mM; see Fig. 3). It has been clarified at this stage that the capacity factor of each porphyrin compound depends little on the concentration of the free (*i.e.*, not aggregated) form of SDS but considerably on the concentration of the micellar

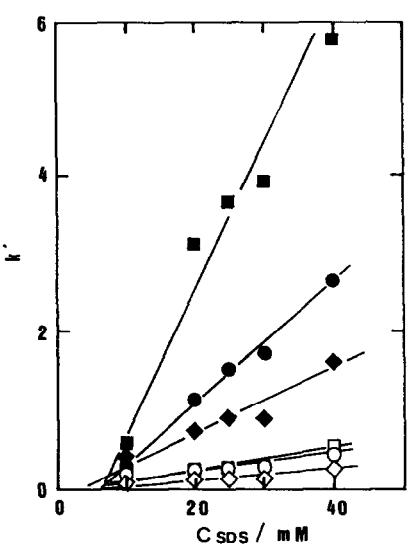

Fig. 5. Plot of capacity factor (k') calculated from eqn. 5 *versus* total concentration of SDS in the carrier solution. Carrier solution and symbols as in Fig. 4.

form of SDS in the solution. According to the slopes of the k' *versus* C_{SDS} plots shown in Fig. 5, the distribution ratio (D) increases in the order $Zn(HP) < H_2HP < Cu(HP) < Zn(PP) < H_2PP < Cu(PP)$.

Separation of haematoporphyrin, protoporphyrin and their copper and zinc complexes

It was found in both series of haematoporphyrin and protoporphyrin that the migration time increased in the order Zn complex < free acid form < Cu complex. This migration sequence agrees with the retention sequences observed for various porphyrin families in reversed-phase HPLC [1–3].

The feasibility of the CZE separation of six porphyrin compounds, *viz.*, H_2HP, Cu(HP), Zn(HP), H_2PP, Cu(PP) and Zn(PP), was examined using a solution containing 20–40 m*M* SDS in CAPS buffer (pH 11)–DMF (10:2, v/v). All the porphyrin compounds could be detected within 30 min. When the SDS concentration was increased, the time span from t_0 to t_{mc} in which all sample components should be detected was extended and accordingly the resolution for each pair of adjacent peaks was improved. The separation of these six porphyrin compounds using the recommended conditions is demonstrated in Fig. 6.

The separation of Zn(HP), H_2HP and Cu(HP) was successful, with elution in that order, using an octadecyl-bonded silica gel (ODS) column (25 cm × 4 mm I.D.) with a methanol–phosphate buffer (pH 3) (85:15, v/v) within 5 min at a flow-rate of 1 ml/min [12]. Using MEKC, complete separation of these three HP compounds was possible in less than 2 min (see Fig. 6). Few data are available at this stage for comparing MEKC with HPLC with respect to sensitivity.

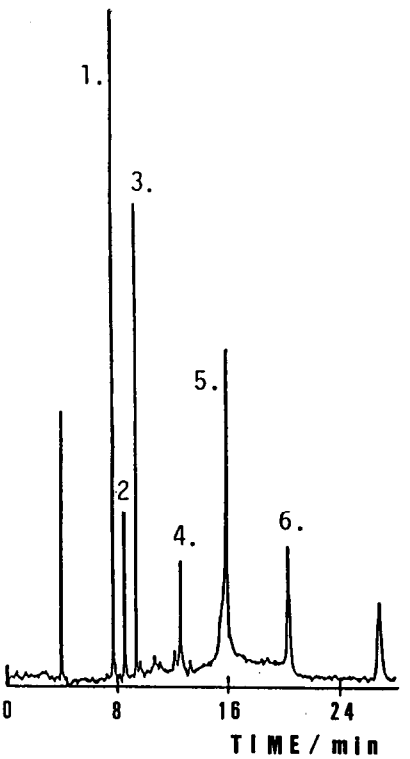

Fig. 6. MEKC separation of porphyrin compounds. Carrier solution, 40 m*M* SDS in CAPS buffer (pH 11)–DMF (10:2, v/v). Applied voltage, 30 kV (429 V/cm); UV detection at 405 nm. Peaks: 1 = Zn(HP); 2 = H_2HP; 3 = Cu(HP); 4 = Zn(PP); 5 = H_2PP; 6 = Cu(PP).

CONCLUSION

It has been confirmed that MEKC is a promising method for the separation of haematoporphyrin and protoporphyrin and their complexes with copper and zinc. A micellar solution of SDS containing DMF is an effective carrier solution. For the successful separation of the six porphyrin compounds, a 40 m*M* SDS solution in CAPS buffer (pH 11)–DMF (10:2, v/v) is recommended.

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