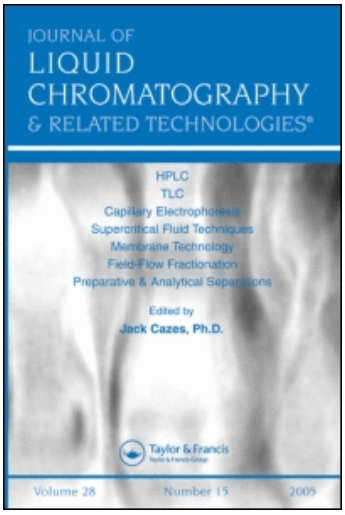


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# Separation of Type I and III Isomers of Copro- and Uroporphyrins Using Affinity Capillary Electrophoresis

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# SEPARATION OF TYPE I AND III ISOMERS OF COPRO- AND UROPORPHYRINS USING AFFINITY CAPILLARY ELECTROPHORESIS

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## ABSTRACT

Naturally occurring 8- and 4-carboxylic porphyrin I and III isomers are separated using a run buffer consisting of bovine serum albumin (BSA) and phosphate as the electrolyte. The method requires the use of deactivated capillaries to minimize protein-wall interactions. The uroporphyrin isomers are resolved in 15 minutes while the separation of the coproporphyrin isomers requires 28 minutes. Retention times are largely characteristic of the number of carboxylic acid side chains as well as the relative affinity for BSA. The binding of porphyrins to BSA is supported by a wavelength maxima red shift in the soret region when using BSA in the run buffer.

## INTRODUCTION

Analysis of porphyrins is important for the differential diagnosis of porphyrias (diseases caused by enzyme deficiencies in heme biosynthesis) (1), and other diseases associated with abnormal porphyrin metabolism. Naturally occurring coproporphyrins type I

and III are decarboxylation products of their corresponding type uroporphyrins. The separation and determination of the coproporphyrin (CP) type I and III isomers is clinically important for the diagnosis of porphyrias and for distinguishing the Dubin-Johnson Syndrome from the Rotor Syndrome (2). CP III is also an intermediate in the synthesis of heme. Of the four possible isomers of uroporphyrin (URO) the type I and III isomers are of biological interest. The fully reduced form of URO III, uroporphyrinogen III is the precursor of all biologically functional tetrapyrroles including the hemes, chlorophylls, corrinoids, and vitamin $B_{12}$. Under normal conditions URO I is encountered in trace amounts in nature, however in certain disease states considerable quantities of this isomer are produced and hence the separation and determination of the URO type isomers is important.



The structures of the two type isomers of CP and URO are shown in Figure 1. The CP type isomers were first separated by chromatography as their free acids using paper chromatography (3). Later the methyl esters were separated using thin-layer chromatography (TLC) (4). The URO I and III type isomers can also be resolved as free acids using paper chromatography (5, 6) and as methyl esters using TLC (7). But the methods listed above are limited in precision and are time consuming. High performance liquid chromatography (HPLC) is now the technique of choice for the separation and determination of porphyrins. The CP type I and III isomers were first separated by recycling HPLC as their methyl esters using adsorption chromatography (8). The method is too complicated and time consuming since it requires ten recycles on two 30-cm μPorasil columns. The methyl esters can be separated by adsorption chromatography on silica with a 1,2-dichloromethane-acetone mobile phase, although resolution is not complete (9). The CP type isomers can be resolved using reversed-phase HPLC (10-16). The relative hydrophobicity of the isomers and hence elution order is determined by the arrangements of the four methyl groups, and the closer these groups are to each other the stronger the compound is retained (12). And as a result the type I isomer with methyl groups symmetrically positioned around it elutes first in reversed-phase

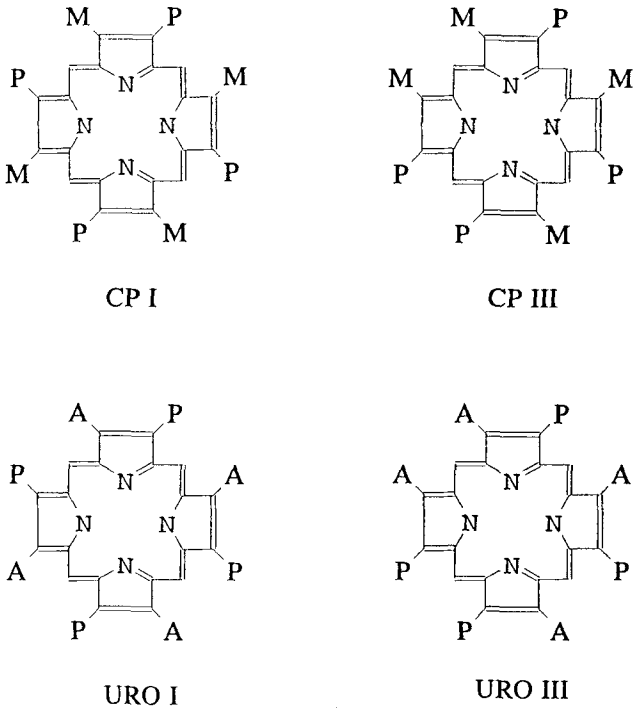


**Figure 1.** Molecular structures of the type I and III isomers of copro- and uroporphyrin. Key for the groups: A = $CH_2$-COOH (acetyl); M = $CH_3$ (methyl); and P = $CH_2$-$CH_2$-COOH (propionyl).

HPLC. The CP type isomers can also be resolved using reversed-phase ion-pair chromatography (17). The first method published for the separation of the URO type I and III isomers utilizes a mobile phase consisting of 0.3 % water in acetone that is adjusted to pH 7.6 with tributylamine and a silica column (18). The methyl esters can be resolved by recycling HPLC (19) or on two μPorasil columns in less than 4 hours (20). The separation of the methyl esters can be accomplished using quaternary mobile phases with silica or aminopropyl-bonded silica (21, 22). The type I and III isomers can be resolved as their free acids using reversed-phase HPLC (23, 24) and reversed-phase ion-pair chromatography (17).

In the URO molecule the propionyl (Pr) group is the most hydrophobic and the type III isomer which has two adjacent Pr groups is more hydrophobic than the symmetric type I isomer and is more strongly retained.

An alternative to HPLC is capillary electrophoresis (CE) (25-28). This method is capable of separating ionic species based on charge to mass ratios, approaching efficiencies of a million theoretical plates. Electrokinetic chromatography (EKC) is a technique that can be considered to be a branch of CE and is capable of separating both neutral and charged analytes (29). Here analytes are separated by their differential distribution into an electrically migrating phase and the surrounding aqueous phase, where the two phases have different migration characteristics. Affinity CE (ACE) is an new EKC technique where components are resolved based on their relative affinity for the electrically migrating phase (30). In the present study resolution of the type I and III isomers of CP and URO free acids using CE and bovine serum albumin (BSA) as the electrically migrating phase is investigated. The use of BSA as an affinity run buffer additive has already been demonstrated for the enantiomeric separation of leucovorin (31).



## EXPERIMENTAL

### Materials

All solutions were prepared in doubly deionized water using a NANOpure Ultrapure Water System (Model D4741, Barnstead, Dubuque, Iowa). Type I and III isomers of copro- and uroporphyrin were obtained from Porphyrin Products (Logan, Utah). BSA was purchased from Sigma Chemical Company (St Louis, MO). Sodium hydroxide, methylene chloride, and reagent grade methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Reagent grade potassium phosphate was purchased from J.T.Baker Chemical Company

(Phillipsburg, NJ). The polyethylene glycol (PEG) CEP-3 was obtained by Innophase Corporation (Portland, CT).

## Apparatus

The EKC experiments were performed in fused silica capillaries of 75$\mu$m i.d. x 360$\mu$m o.d. (Polymicro Technologies, Phoenix, AZ). Typical lengths were 70 cm. An automated CE instrument (Model Phoresis 1000, Spectra Physics Analytical, Fremont, CA) connected to a Spectra Physics 4400 integrator was used to obtain the data. The wavelength spectra was measured using the data software supplied with the instrument on an IBM model 70 computer. Vacuum injection was employed and detection measured at 396 nm. A Varian model 3700 gas chromatograph (Varian Instrument Group, Sunnyvale, CA) was used in the coating of the capillaries.



## Procedures

The capillaries were coated by the following procedures: (1) the capillary was pretreated with 0.1M NaOH at 80 degrees C for 4 hours, (2) the capillary was rinsed with $H_2O$ then methanol, and dried at 130 degrees C for 6 hours, (3) a solution of 20 % (w/v) PEG was physically pushed through the capillary, and (4) the capillary was heated at 225 degrees C for 24 hours. The coating on the outside of the capillary was dissolved using hot $H_2SO_4$ to prepare a window in the desired position on the capillary for detection. New capillaries were conditioned with the running buffer for at least 6 hours before use. The capillary was cleaned frequently during runs by washing with methanol, 15mM SDS, and methanol, in that order.

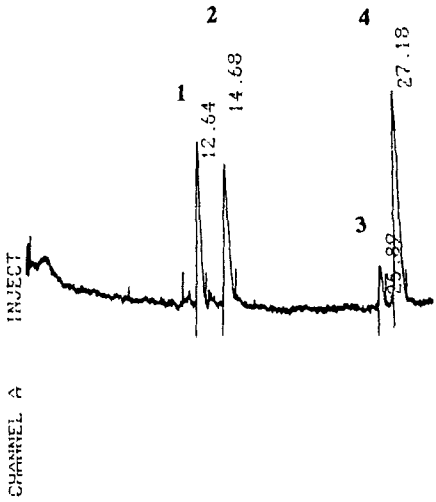


**Figure 2.** Electropherogram shown for the porphyrin type isomers using affinity CE on a PEG-coated capillary. Key (for this and subsequent figures): (1) URO I; (2) URO III; (3) CP III; and (4) CP I. Conditions: capillary dimensions; 75 $\mu$m i.d. x 360 $\mu$m o.d., electric field strength 270 V/cm, 20 mM phosphate, pH 7.6, BSA concentration 0.030 mM, and detection wavelength 396 nm.



## RESULTS AND DISCUSSION

Figure 2 shows an electropherogram representing the separation of the type I and III isomers of CP and URO at pH 7.6 and a BSA concentration of 0.030 mM in the run buffer. The URO type isomers can be separated at pH 7.6 and 0.015 mM BSA concentration in less than 10 minutes. The same experiment in the absence of BSA (all other conditions being identical) shows only one peak for the type I and III isomers of CP and one peak for URO I and III. At the conditions in Fig. 2 the porphyrin isomers are anionic and therefore have an electrophoretic mobility ($\mu_{ep}$) in the direction of the anode in the presence of an electric field

(E). The BSA molecule consists of a single polypeptide chain made up of 582 amino acid residues and has an isoelectric point of 4.7 (32). At a pH value of 7 BSA is also anionic and has an $\mu_{ep}$ in the direction of the anode in the presence of an E. The magnitude of $\mu_{ep}$ for the porphyrins is larger than that for BSA as determined experimentally by measuring the migration time of each molecule, and therefore the porphyrins move with a greater $\mu_{ep}$ than the porphyrin-BSA-bound complex. The $\mu_{eo}$ is much smaller than the corresponding $\mu_{ep}$ values as a result of the deactivated capillary, resulting in injection at the cathode.

The $\mu_{ep}$ and hence retention time of the sample is largely characteristic of the number of carboxylic acid side chains as demonstrated by the earlier elution time of the URO type isomers in comparison with the CP type isomers, as well as a function of the amount of BSA in the run buffer. The separation of the type I and III isomers is achieved through differential affinities for the BSA molecule. The URO type isomers can be resolved with 0.015 mM concentration BSA in the run buffer while a concentration of 0.030 mM BSA is needed to resolve the CP type isomers.



It is interesting that the retention time of the porphyrins increased by more than a factor of two when increasing the BSA concentration from 0 to 0.030 mM in the run buffer, indicating a relatively large affinity of the porphyrins molecules for the BSA. This concept is supported by earlier work that shows significant binding constants for the interaction of these porphyrin type isomers with human serum albumin (HSA), a protein similar to BSA (33). The binding constants were determined by measuring the shift in the wavelength maxima ($\lambda_{max}$) of the porphyrin molecule in the visible region as a function of protein concentration in solution. Figure 3 shows the influence of BSA added to the run buffer on the wavelength spectral scans for the URO type I and III isomers. There is a shift in the $\lambda_{max}$ to longer wavelengths for both isomers when BSA is added to the run buffer, providing more evidence for binding. The CP type isomers also undergo a red shift in the presence of BSA and this is illustrated by a comparison of the $\lambda$ scan for the porphyrin type I isomers in

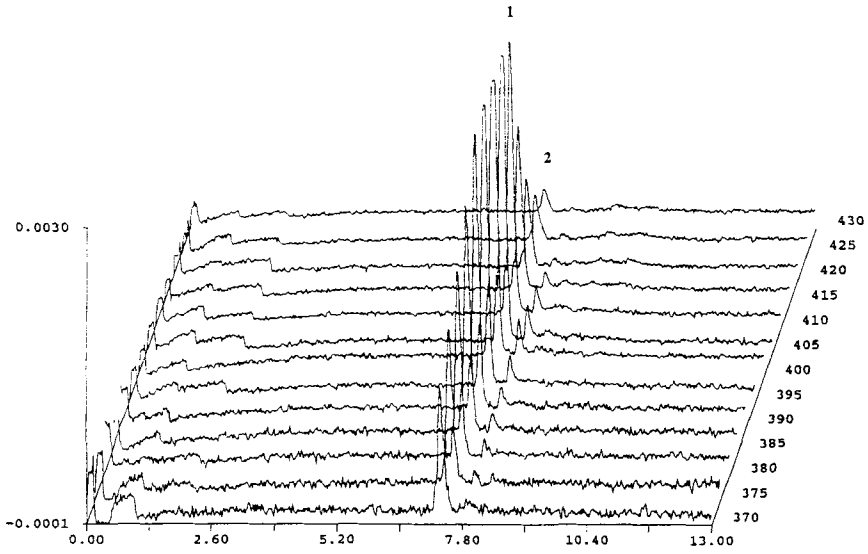


**Figure 3.** Separation of the URO type isomers using affinity CE on a PEG-coated capillary, (URO I concentration 0.04 mg/mL, URO III concentration 0.003 mg/mL). Conditions: capillary dimensions; 75 $\mu$m i.d. x 360 $\mu$m o.d., electric field strength 282 V/cm, 10 mM phosphate, pH 7.0, BSA concentration 0.030 mM, and detection wavelength 396 nm.



the absence of BSA (Fig. 4) and in the presence of BSA (Fig. 5). The CP I molecule undergoes an 8 nm shift in the $\lambda_{max}$ and the URO I isomer experiences a 4 nm shift, and this reinforcing the concept that the CP I isomer has a greater affinity for the protein in this study compared to the URO type I isomer. Previous work measured dissociation constants for the porphyrin isomers binding to HSA (33). It was found that the URO III type isomer has a much higher affinity for HSA than the URO I isomer. The dissociation constants for the CP I and III molecules interacting with HSA were determined to be 7.7 $\mu$M and 6.1 $\mu$M, respectively . The elution order of the URO type isomers in this paper agrees with the data presented in previous work, with the type I isomer eluting first and the URO III isomer being

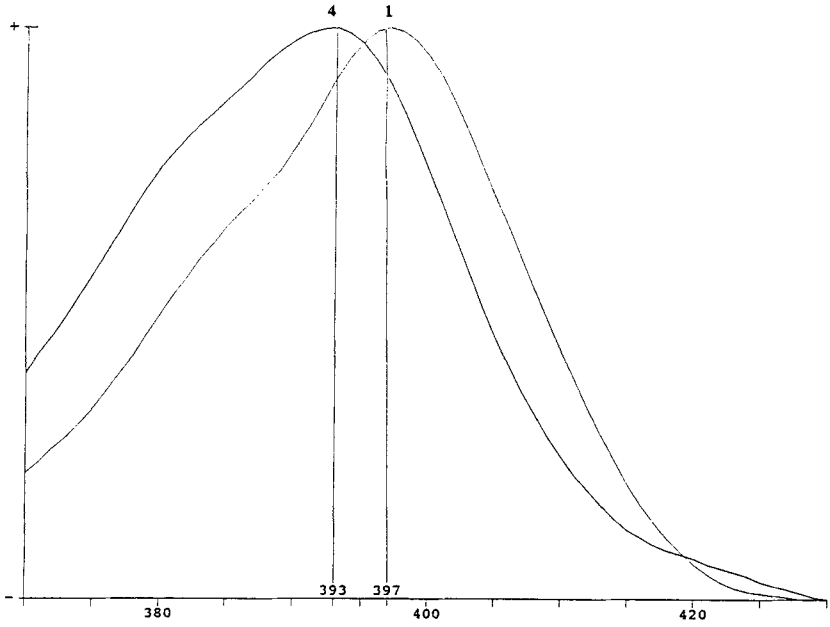


**Figure 4.** Wavelength scan for the type I isomers of Copro- and Uroporphyrin in the absence of BSA, all other conditions the same as in Fig. 2.



more strongly retained by the BSA eluting last. The fact that the URO type isomers have a large difference in their relative affinities for HSA explains the ability to separate these isomers in less than 10 minutes using ACE. However, the elution order of the CP type isomers is opposite to that expected from the previous work. The authors are not sure of the reason for the reversal in elution order, but the fact that this work involves the use of BSA instead of HSA may help to explain it. The time and BSA concentration required to separate the CP isomers is larger than that for the URO type isomers and can be explained by the similar affinities the CP type isomers have for HSA.

Migration time reproducibility for the porphyrin isomers is limited by the capillary stability and exhibited a 2.5 % RSD. There are two major factors contributing to the

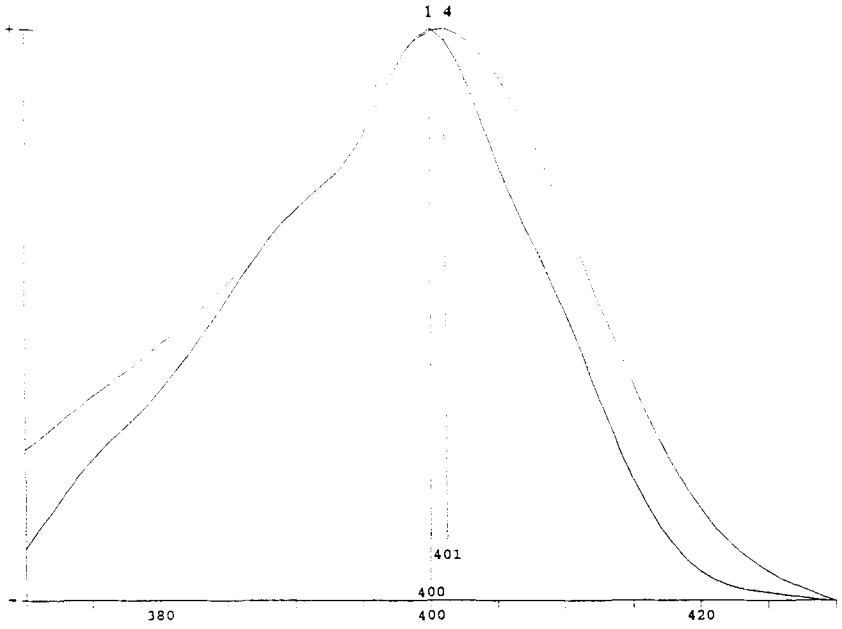


**Figure 5.** Wavelength scan for the type I isomers of Copro- and Uroporphyrin in the presence of BSA, all other conditions the same as in Fig. 2.



instability of the capillaries. One factor involves the adsorption of the analytes to the capillary wall. This phenomena was encountered by Weinberger et al. in their attempt to separate urinary porphyrins using MECC (34). The purpose of employing MECC was not to contribute to the separation mechanism, but to compete with the porpyrins for any electrostatic or hydrophobic sites on the wall. The other factor involves the adsorption of BSA to the capillary wall. There is an inverse relationship between the amount of BSA added to the run buffer and the capillary stability. Although the PEG layer minimizes this effect, it is still a limiting factor. In addition, there is a strong possibility that the protein adsorbs irreversibly to the PEG layer as shown in an earlier study (35).

It is noteworthy that under the conditions described in this paper, ACE resolved a mixture of type I and III isomers of copro and uroporphyrin free acids. This is a rather quick and reliable method applicable to the identification and determination of important types of porphyrins in biological specimens. The major advantages of using an instrument that is capable of doing on-line scanning is the possibility of measuring the degree of binding via a $\lambda_{max}$ shift for species that exhibit this phenomena in addition to the ability to confirm a peak. This technique is limited by irreversible adsorption of the analytes and run buffer additive to the capillary wall. It is important to recognize that the sample and/or run buffer additive carries a net charge not equal to zero to obtain resolution in a reasonable amount of time.



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