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ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PORPHYRIN ISOMERS

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SUMMARY

A novel ion-pair reversed-phase high-performance liquid chromatographic system for the simultaneous separation of type I, II, III and IV isomers of coproporphyrin has been developed. Complete separation of all isomers was obtained on a 7 μm LiChrosorb RP-18 column. The mobile phase consisted of an aqueous phosphate buffer and a mixture of methanol and acetonitrile containing the ion-pair reagent tetrabutylammonium phosphate. The method was applied to the analysis of the isomeric purity of numerous synthetic coproporphyrins.

Separation of type I and III isomers of uroporphyrin was achieved by a modified ion-pair system using both isocratic and gradient elution. The latter elution technique served for the simultaneous separation of the naturally occurring type I and type III isomers of octacarboxylic through tetracarboxylic porphyrins. Urine samples originating from porphyric patients were analysed with this highly selective profiling technique.

INTRODUCTION

The determination of porphyrin isomers is of great importance in synthetic organic chemistry, biochemistry and clinical chemistry. The correct structure of the porphyrin ring system was determined by Fischer and coworkers merely by the isolation and identification of all the theoretically predictable porphyrin isomers depending on the respective type of substitution[1]. Complete separation of individual porphyrin isomers enables efficient control of the uniformity of a porphyrin synthesis.

Porphyrin carboxylic acids such as uro- and coproporphyrin originating from biological sources can be present as different positional isomers, owing to the simultaneous action of enzymes of the metabolic pathway of the heme biosynthesis. The activity and specificity of these enzymes can be measured by quantitation of the respective porphyrin isomers[2,3]. Abnormal ratios of porphyrin isomers in human materials, such as urine, faeces or blood, offer valuable information for the differential diagnosis of porphyrias and some cases of liver diseases[4].

Numerous procedures have been described for the determination of porphyrins

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from various materials. Chromatographic methods, however, are necessary for the analysis of their positional isomers. Paper chromatographic separation of porphyrin carboxylic isomers was achieved by Falk *et al.*[5] and by Eriksen and Eriksen[6]. Thin-layer chromatography of porphyrin methyl esters[7,8] and free carboxylic acids[9] was applied to separate individual isomers.

High-performance liquid chromatography (HPLC) served for the separation of the methyl ester isomers of coproporphyrin[10–12] and uroporphyrin[13,14]. Reversed-phase HPLC of free porphyrin carboxylic acid isomers is especially useful for the analysis of biological samples, since these materials contain only free acids, and thus an esterification procedure can be omitted. Coproporphyrin I and III isomers and uroporphyrin I–IV isomers were separated by Englert *et al.*[15] and Wayne *et al.*[16] using aqueous phosphate buffers and acetonitrile as mobile phase. Other reversed-phase HPLC separations of free porphyrin carboxylic acid isomers were described by Lim *et al.*[17–20] using aqueous ammonium acetate and acetonitrile as eluents. This highly efficient system, which is based on hydrophobic interactions, was applied to the first complete separation of free coproporphyrin I–IV isomers[17], and the simultaneous determination of nearly all the possible isomers in the series from octa- to tetracarboxylic porphyrins[18–20].

Ion-pair reversed-phase HPLC analysis of free porphyrin carboxylic acids was first reported by Bonnett *et al.*[21]. These authors employed tetrabutylammonium phosphate for ion-pairing, but porphyrin isomers could not be resolved under the conditions applied. This ion-pair system was modified decisively by our group for the analysis of urine[22,23], faeces[24] and blood[25]. Separation of coproporphyrin I and III was possible with our system. Chiba and Sassa[26] were able to separate the type I and III isomers of hexa- through tetracarboxylic porphyrins by applying chromatographic conditions similar to those described by us. Uroporphyrin isomers, however, could not yet be resolved by ion-pair chromatography.

The aim of the present study is to demonstrate the capability of ion-pair reversed-phase HPLC for the simultaneous separation of coproporphyrin I to IV isomers, and for the reliable separation of naturally occurring isomers in the series from uroporphyrin to coproporphyrin.

EXPERIMENTAL

Reagents and materials

Coproporphyrin I (free acid and tetramethyl ester), coproporphyrin II tetramethyl ester, coproporphyrin IV (free acid and tetramethyl ester), uroporphyrin I octamethyl ester, and a mixture of synthetic coproporphyrin I and III tetramethyl esters were generous gifts of the Hans-Fischer-Gesellschaft (München, F.R.G.). All these porphyrins were the original preparations of Hans Fischer and coworkers.

Coproporphyrin III, uroporphyrin III octamethyl ester and a synthetic mixture containing type I isomers of octa- to dicarboxylic porphyrins were purchased from Porphyrin Products (Logan, UT, U.S.A.).

Porphyrin methyl esters were hydrolysed with 25% hydrochloric acid for 24 h (light protection). After removal of the volatiles with a rotary evaporator, the residue was dissolved in a 40 mmol/l solution of tetrabutylammonium phosphate in methanol.

Methanol and acetonitrile were of LiChrosolv grade and purchased from E. Merck (Darmstadt, F.R.G.). Water was deionized and purified to 16 $M\Omega^{-1}$ by a Milli-Q-filtration system (Millipore, Bedford, MA, U.S.A.). The ion-pair reagent tetrabutylammonium phosphate was prepared from a 10% methanolic solution of tetrabutylammonium hydroxide (Fluka, Buchs, Switzerland) and 85% phosphoric acid (analytical grade, Merck) by adjustment to a pH of 7.4. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate (analytical grade, Merck) were used for the preparation of the aqueous buffer solutions.

Preparation of urine samples

A 10-ml volume of urine (pH 3.5 to 4.0) was adsorbed on 500 mg of talc. The talc was washed twice with 10 ml of water, and the porphyrins were desorbed with 2.5 ml of a 40 mmol/l solution of tetrabutylammonium phosphate in methanol. Prior to injection, the sample was filtered through a 0.5-μm membrane filter.

Apparatus

A Model 8500 HPLC system (Varian, Palo Alto, CA, U.S.A.), equipped with a solvent programmer, was used together with a Rheodyne 7105 injector (Rheodyne, Berkley, CA, U.S.A.). For detection with a filter fluorimeter (Fluorichrom, Varian), the excitation wavelength was set at 400 nm (interference filter) and the emission wavelength at 570 nm (cut-off filter).

Chromatographic conditions

Three different types of column were used for the chromatographic separations: a LiChrosorb RP-18 (7 μm, 25 cm × 4 mm I.D., Merck), a LiChrospher 100 CH-18/2 (5 μm, 25 cm × 4 mm I.D., Merck) and a Bio-Sil ODS-5S (5 μm, 25 cm × 4 mm I.D., Bio-Rad, München, F.R.G.).

Isocratic separation of coproporphyrin I–IV isomers was performed with the eluent systems 1 and 2, and isocratic separation of uroporphyrin I and III isomers with eluent system 3. Gradient elution was applied to the simultaneous separation of uro- to coproporphyrin I and III isomers.

Eluent system 1. This consisted of 52% aqueous phase with a 40 mmol/l phosphate buffer (pH 6.6) and 48% methanolic phase containing 12.5 mmol/l tetrabutylammonium phosphate (pH 7.4).

Eluent system 2. This consisted of 55% aqueous phase with a 40 mmol/l phosphate buffer (pH 6.8) and 45% organic phase containing 10 mmol/l tetrabutylammonium phosphate (pH 7.4) in methanol–acetonitrile (77:23, v/v).

Eluent system 3. This consisted of 60% aqueous phase with a 40 mmol/l phosphate buffer (pH 5.4) and 40% methanolic phase containing 12.5 mmol/l tetrabutylammonium phosphate (pH 7.4).

Gradient elution. The aqueous phase and the methanolic phase had the same composition as in eluent system 3. The programme was started at a concentration of 42% methanolic phase in the buffer phase for 5 min, then increased within 15 min to 57%, then within 5 min to 77%, then within 1.6 min to a final concentration of 90% methanolic phase, which was maintained for 4 min. The column was reconditioned for 10 min with the starting mixture.

A flow-rate of 1 ml/min was used for all separations. Sample volumes between 5 and 25 μl were injected.

RESULTS AND DISCUSSION

Simultaneous separation of coproporphyrin I, II, III and IV isomers

The retention behaviour of coproporphyrin I–IV isomers was investigated under isocratic conditions using the synthetic coproporphyrins originally prepared by Fischer and coworkers. We studied in detail the separation characteristics of three different columns, the proportions of the organic solvent, the concentration of the ion-pair reagent tetrabutylammonium phosphate, and the ionic strength and pH value of the aqueous phosphate buffer.

Among the columns tested, we obtained the highest selectivity with the 7 μm LiChrosorb RP-18 column, whereas the 5 μm LiChrospher 100 CH-18/2 and the 5 μm Bio-Sil ODS-5S columns offered lower selectivities under comparable conditions. Therefore, further experiments were performed exclusively with the LiChrosorb column.

The proportions of the methanolic phase in the eluent system were checked in the range from 20 to 80%. An optimum concentration was found between 45 and 50%, depending on the respective batches of columns used. Thus, the exact content of methanolic phase has to be adjusted from column to column.

The concentration of the ion-pair reagent, which was present only in the methanolic phase, was examined in the range from 4 to 20 mmol/l. The effects on selectivity are relatively moderate: only a slight increase of retention times with increasing tetrabutylammonium phosphate concentration was observed, as with other porphyrins[23].

The ionic strength of the aqueous phosphate buffer was varied from 2 to 100 mmol/l. No significant influence on retention times or on selectivity was observed, confirming the results of our earlier studies with coproporphyrin I[23].

The most significant effects on separation selectivity and retention time of the individual isomers were achieved by changing the pH value of the aqueous phosphate buffer (Fig. 1). In these experiments a 48% concentration of the methanolic phase, which contained 12.5 mmol/l ion-pair reagent, and a buffer concentration of 40 mmol/l in the aqueous phase were used (eluent system 1). A large increase in capacity ratios (k') for all isomers occurred when the pH value of the buffer phase was decreased from 7.2 to 5.1. However, different retention times for all four isomers could be observed only in the pH range between 6.1 and 6.9. Below pH 6.1 isomers III and IV are totally unresolved, whereas above pH 6.9 isomers II and IV cannot be separated. The order of elution was I, III, IV and II, which corresponds to the same sequence as with the hydrophobic interaction technique[17]. The chromatogram obtained at pH 6.6 reveals that the isomers II to IV are not completely separated owing to their relatively broad peaks (Fig. 2).

We attempted to improve the resolution by addition of a modifying agent such as acetonitrile. Indeed, with an optimum concentration of 23% acetonitrile in the methanolic phase we obtained better selectivities for isomers III and IV. The influence of pH in the buffer phase on the capacity ratios of isomers I–IV with this modified system is shown in Fig. 3. At pH 6.8 eluent system 2 enabled complete separation of isomers II, III and IV (Fig. 4). Thus the simultaneous separation of all four coproporphyrin isomers by ion-pair reversed-phase HPLC was achieved for the first time.

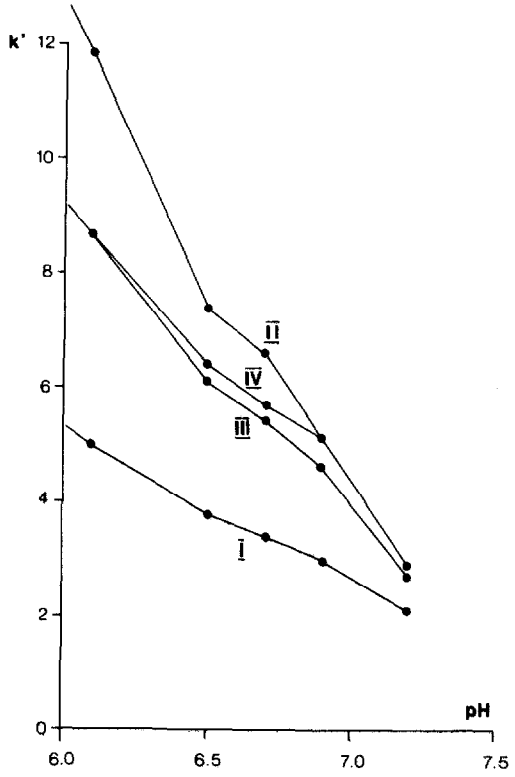


Fig. 1. Influence of pH value of the aqueous phosphate buffer on capacity ratios (k') of coproporphyrin I, II, III and IV isomers (eluent system 1).

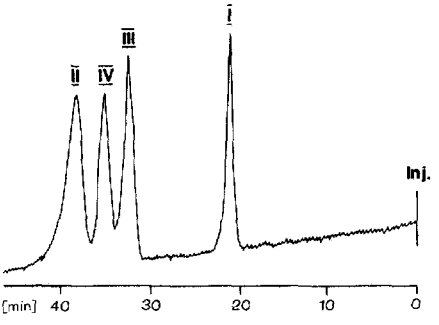
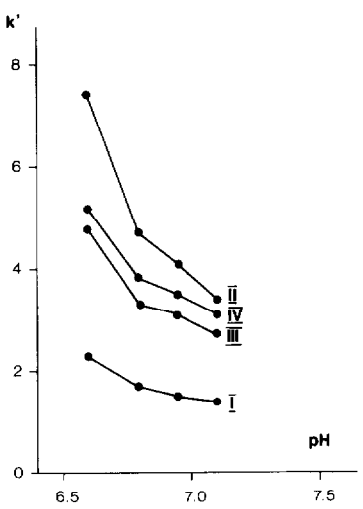


Fig. 2. Isocratic separation of coproporphyrin I, II, III and IV isomers on a LiChrosorb RP-18 column at pH 6.6 of the aqueous phosphate buffer (eluent system 1). Fluorescence detection.

Control of the isomeric purity of synthetic coproporphyrins

We applied eluent system 2 to the purity control of the coproporphyrin isomers synthesized by Fischer and coworkers. All the compounds studied had been stored for more than 50 years without detectable decomposition. They had been purified



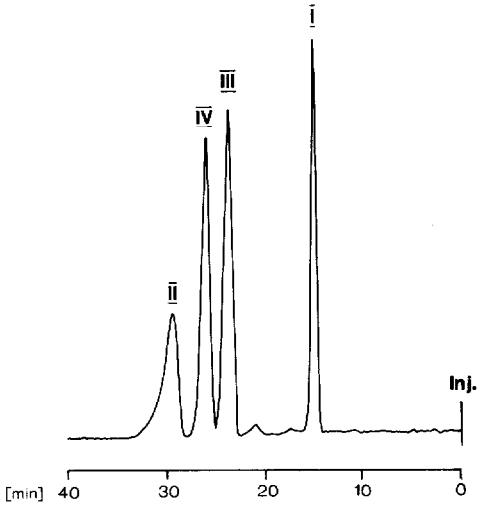


Fig. 3. Influence of pH value of the aqueous phosphate buffer on capacity ratios (k') of coproporphyrin I, II, III and IV isomers (eluent system 2).

Fig. 4. Isocratic separation of coproporphyrin I, II, III and IV isomers on a LiChrosorb RP-18 column at pH 6.8 of the aqueous phosphate buffer (eluent system 2). Fluorescence detection.

only by fractional crystallization of the respective methyl esters, since the HCl numbers of coproporphyrin isomers are identical[27]. No other separation techniques were available at that time.

HPLC analysis of synthetic coproporphyrin I, prepared by Fischer *et al.*[28], demonstrated an isomeric purity of 93%. This preparation contained 5% of isomer III and 2% of isomer IV (Fig. 5). A mixture of coproporphyrin I and III, synthesized

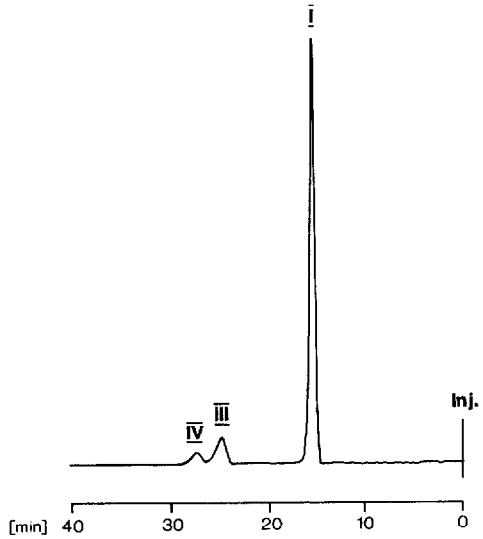


Fig. 5. HPLC analysis of synthetic coproporphyrin I prepared by Fischer *et al.*[28]. Conditions as in Fig 4.

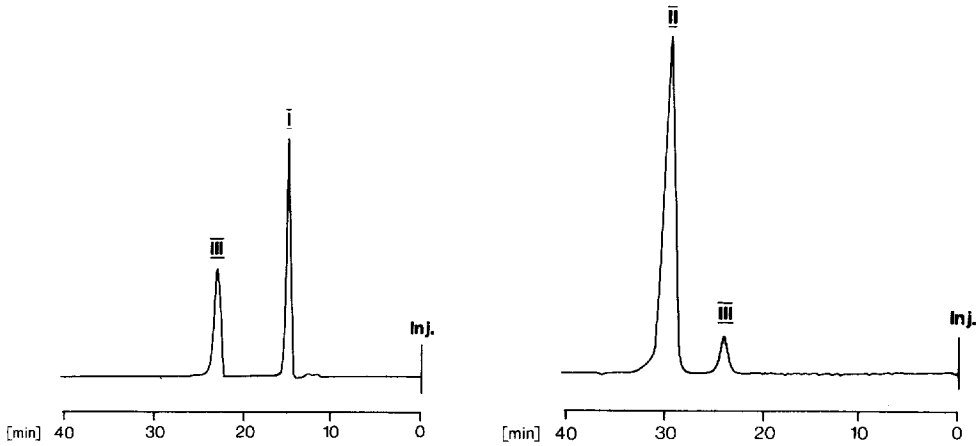


Fig. 6. HPLC analysis of a mixture of synthetic coproporphyrin I and III, prepared by Fischer and Hierneis[29]. Conditions as in Fig. 4.

Fig. 7. HPLC analysis of synthetic coproporphyrin II, prepared by Fischer and Lamatsch[30]. Conditions as in Fig. 4.

by Fischer and Hierneis[29], contained 59% of isomer I and 41% of isomer III (Fig. 6). No other isomers were present in this preparation. The synthetic coproporphyrin II, prepared by Fischer and Lamatsch[30], exhibited an isomeric purity of 92%, being contaminated with 8% of isomer III (Fig. 7). An isomeric purity of 98% was found

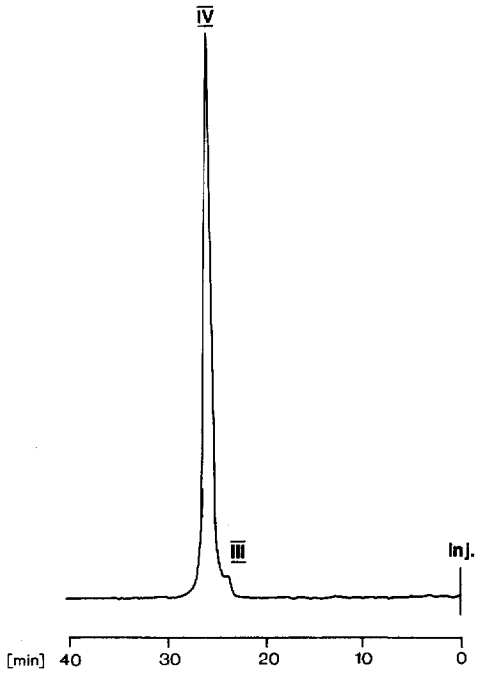


Fig. 8. HPLC analysis of synthetic coproporphyrin IV prepared by Fischer *et al.*[31]. Conditions as in Fig. 4.

for coproporphyrin IV synthesized by Fischer *et al.*[31]. Only 2% of isomer III was present in this preparation (Fig. 8).

A series of other synthetic coproporphyrins prepared by H. Fischer and co-workers was analysed, resulting in similar isomeric purities, which varied between 90 and 99%.

Separation of uroporphyrin I and III isomers

Chiba and Sassa[26] achieved ion-pair HPLC separation of hexacarboxylic through tetracarboxylic porphyrin I and III isomers by adjusting the buffer phase to pH 6.0. In our system, decreasing the pH value of the phosphate buffer to 5.4 led to almost a baseline separation of uroporphyrin I and III isomers (Fig. 9). Isocratic conditions with 40% methanolic phase, which contained 12.5 mmol/l of the ion-pair reagent, were used for this application (eluent system 3). In contrast to the separation of coproporphyrin isomers, acetonitrile must be omitted in this case owing to its adverse effect on the resolution of the uroporphyrin isomers.

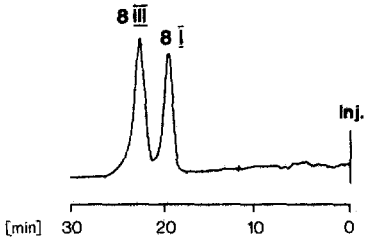


Fig. 9. Isocratic separation of uroporphyrin I and III isomers on a LiChrosorb RP-18 column at pH 5.4 of the aqueous phosphate buffer (eluent system 3). Fluorescence detection.

Simultaneous separation of uro- and coproporphyrin isomers

The simultaneous determination of naturally occurring isomers in the series from uroporphyrin to coproporphyrin is of special interest for the analysis of samples from clinical or biological materials. For this purpose, an ion-pair method was developed consisting of the same components as in eluent system 3 for the isocratic separation of uroporphyrin I and III. However, gradient elution had to be performed in order to obtain acceptable retention times for the coproporphyrin isomers. Application of this elution technique resulted in the simultaneous formation of both a solvent and a pH gradient, since the aqueous buffer phase had a pH value of 5.4 and the methanolic phase with the ion-pair reagent was of pH 7.4. Thus, the actual pH value of the mobile phase changed from 6.2 to 7.0 in the course of the gradient programme when the proportion of the methanolic phase was increased from 42 to 90%. A satisfactory resolution of a synthetic mixture of uro- and coproporphyrin I and III isomers was obtained in less than 30 min (Fig. 10).

Analysis of a commercially available mixture of octa- to dicarboxylic porphyrins of type I with this gradient elution system confirmed the isomeric purity of these porphyrins with the exception of an extra peak, eluted just before the hexacarboxylic porphyrin (Fig. 11). Additional peaks were found in the region from octa- to heptacarboxylic porphyrins when another lot of this mixture (Lot No. 68) was

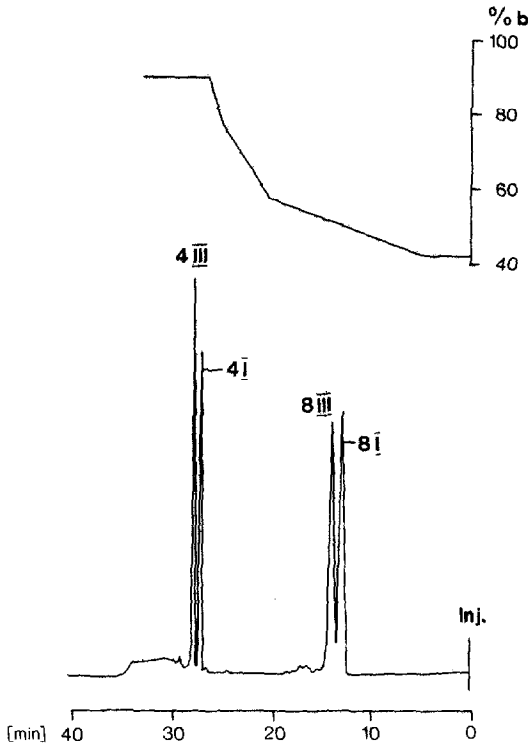


Fig. 10. Separation of uroporphyrin and coproporphyrin I and III isomers on a LiChrosorb RP-18 column (gradient elution). Fluorescence detection. Aqueous phase, 40 mmol/l phosphate buffer (pH 5.4); methanolic phase (b), 12.5 mmol/l tetrabutylammonium phosphate (pH 7.4). Peaks: 8 = uroporphyrin, 4 = coproporphyrin.

analysed. Contamination with metal complexes was avoided by addition of EDTA prior to analysis.

Ion-pair gradient elution was also used for the analysis of urine samples of patients suffering from different types of porphyria. The separation of a urinary extract from a patient with acute intermittent porphyria demonstrates the practicability of our method for clinical samples. This patient excreted large amounts of porphyrin precursors, porphobilinogen and δ-aminolaevulinic acid, together with elevated levels of uroporphyrin I and III, and coproporphyrin III (Fig. 12). Two unidentified peaks were still present in this urine sample. Their fluorescence characteristics were similar to those of porphyrins.

CONCLUSIONS

Isocratic ion-pair reversed-phase HPLC can be applied to the simultaneous separation of coproporphyrin I–IV isomers, enabling control of the isomeric purity of coproporphyrins from biological or synthetic sources.

Gradient elution with a similar ion-pair reversed-phase system is a useful tech-

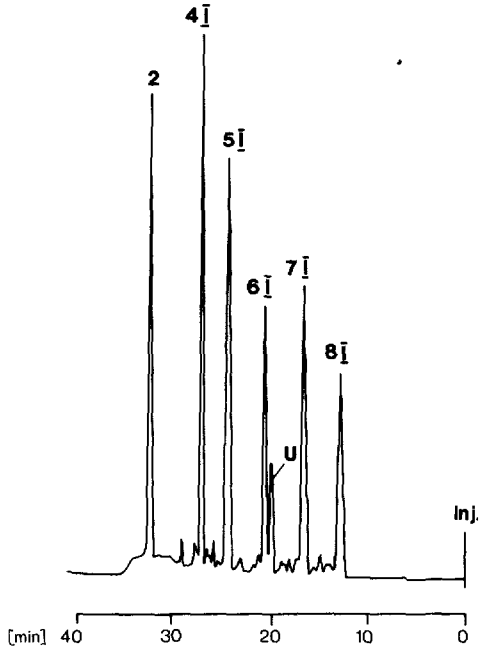


Fig. 11. Separation of a synthetic mixture of porphyrin standards (gradient elution). Conditions as in Fig. 10. Peaks: 8I = uroporphyrin I; 7I = heptacarboxylic porphyrin I; 6I = hexacarboxylic porphyrin I; 5I = pentacarboxylic porphyrin I; 4I = coproporphyrin I; 2 = mesoporphyrin, U = unidentified compound.

nique for the reliable separation of naturally occurring isomers in the series from uroporphyrin to coproporphyrin. The method is suitable for profiling of porphyrins from clinical samples.

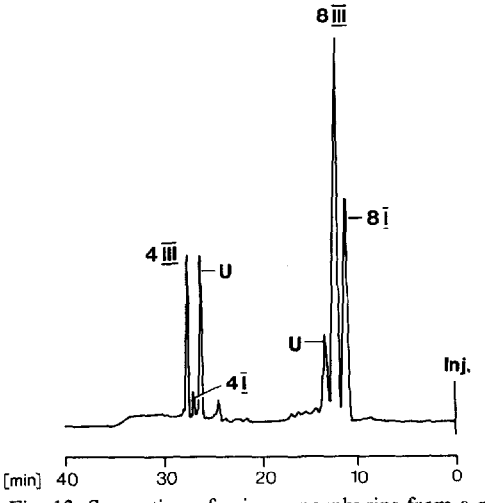


Fig. 12. Separation of urinary porphyrins from a patient with acute intermittent porphyria. Conditions and peaks as in Fig. 10. U = unidentified compounds.

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