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ISOCRATIC ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF UROPORPHYRIN AND COPROPORPHYRIN TYPE II AND IV ISOMERS IN HUMAN URINE

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SUMMARY

Urinary porphyrins of porphyric patients were isolated as their methyl esters by using a simple, modified thin-layer chromatographic system. Existing methods for the isocratic ion-pair high-performance liquid chromatographic separation of uroporphyrin and coproporphyrin isomers were decisively improved by elevating the column temperatures, changing the types of columns used and modifying the eluent compositions. These techniques were applied to the determination of the isomeric distribution of uroporphyrins and coproporphyrins isolated from urines of patients in the acute or latent phase of acute intermittent porphyria. In these urines relatively high contents of the atypical uroporphyrins II (2–5%) and IV (13–19%) were found. The coproporphyrin fractions contained significantly smaller amounts of the atypical isomers II (1–2%) and IV (2–5%), the presence of which was demonstrated for the first time in such urines. Several mechanisms for the formation of the atypical coproporphyrin isomers are discussed. The isocratic ion-pair separation method served also to control the isomeric purity of uroporphyrin specimens of both natural and synthetic origin.

INTRODUCTION

The determination of porphyrins from natural sources has normally been focused on type I and III isomers, as the atypical isomers II and IV were thought to be non-existent in living systems[1]. Substantial improvements in separation and identification techniques for porphyrin isomers now permit detailed analyses for these compounds at trace levels. Isomeric porphyrins can be separated by several chromatographic methods, preferably by high-performance liquid chromatography (HPLC)[2]. Thus, the simultaneous separation of all four isomeric coproporphyrins as free carboxylic acids was performed by using either hydrophobic interaction[3] or ion-pair chromatography[4,5]. Many attempts to separate all four uroporphyrin isomers completely have been reported; however, the separation of uroporphyrins III and IV could not be achieved, even by applying different HPLC techniques[5–10]. Thus, the uroporphyrin isomers must still be converted into the corresponding co-

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proporphyrin isomers by acid-catalysed partial decarboxylation in order to permit a complete analysis of these porphyrins.

Recently, we were able to demonstrate the presence of type II and IV isomers in the total urinary porphyrins of some patients suffering from attacks of acute intermittent porphyria (AIP)[5]. However, the isomeric composition of the individual porphyrin fractions was not elucidated. Formation of the atypical uroporphyrins II and IV, in addition to the normal isomers I and III, was shown to result from non-enzymatic self-condensation of porphobilinogen, which can occur either in the human body or during collection and storage of the urine samples from these patients[5].

In this paper, we report a complete analysis of the isomeric composition of the individual fractions of porphyrins, *e.g.*, uroporphyrins and coproporphyrins, present in the urines of patients with acute or latent forms of AIP. We were especially interested in establishing the isomeric composition of the coproporphyrin fractions, as the formation of these intermediates necessarily involves enzymatic decarboxylation steps under the conditions applied. Application of a modified thin-layer chromatographic (TLC) system allowed a simple separation of the respective urinary uroporphyrin and coproporphyrin fractions. The ratios of the porphyrin isomers were determined by improved isocratic ion-pair HPLC methods, which were based on those described previously[5]. Another aim of this study was to determine the isomeric purity of a series of uroporphyrin specimens originating from both natural and synthetic sources.

EXPERIMENTAL

Reagents

Uroporphyrin I octamethyl ester (original preparation of Fischer and Hofmann[11]), uroporphyrin II octamethyl ester (synthesized by MacDonald and Michl[12]) and a Waldenström uroporphyrin "III" octamethyl ester (original preparation of Waldenström *et al.*[13]) were generous gifts from the Hans-Fischer-Gesellschaft (Munich, F.R.G.). The porphyrin methyl esters were hydrolysed to the free acids by treatment with 6 *M* hydrochloric acid at 25°C for 72 h in the dark. The hydrochloric acid was removed by adsorption of the free porphyrins on Sep-Pak C_{18} cartridges (Waters Assoc., Eschborn, F.R.G.) according to Friedmann and Baldwin[14]. For HPLC analysis the porphyrin free acids were dissolved in a 50 m*M* solution of tetrabutylammonium phosphate in methanol. A statistical mixture of uroporphyrins I (12.5%), II (12.5%), III (50%) and IV (25%) was obtained by the method of Mauzerall[15]. Aqueous phosphate buffer solutions and the ion-pair reagent tetrabutylammonium phosphate were prepared as described elsewhere[5]. Talc was purchased from E. Merck (Darmstadt, F.R.G.).

Apparatus

The HPLC system consisted of a Model 1090A liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.), equipped with a column oven, a Model F-1000 spectrofluorimeter (excitation at 394 nm, emission at 624 nm) (Merck-Hitachi, Darmstadt, F.R.G.) and a Model 1040 diode-array detector (wavelength range 200–590 nm) (Hewlett-Packard).

Urine samples

Urine specimens (24-h) of patients with acute or latent phases of AIP were collected in dark-brown bottles at nearly neutral pH, refrigerated as soon as possible and stored at −20°C[16].

Sample preparation

A 50-ml sample of urine (pH 5–6) was oxidized with a solution of 3 mg of iodine and 6 mg of potassium iodide in 3 ml of water for 10 min at room temperature. After treatment with 5 mg of sodium thiosulphate, the urine was adjusted to pH 3.5 with acetic acid. The porphyrins were adsorbed on 600 mg of talc and washed twice with 20 ml of water. The talc was dried and the porphyrins were dissolved in 10 ml of methanol–concentrated sulphuric acid (9:1, v/v) and kept at 38°C for 40 min. The methanol was removed under vacuum and the residue was dissolved in 10 ml of water. The porphyrin methyl esters were adsorbed on Sep-Pak C_{18} cartridges according to Kennedy *et al.*[17], washed with water and eluted with 10 ml of acetone.

Separation of the porphyrin esters was performed by preparative TLC on silica gel (Kieselgel 60 F, PSC-Fertigplatten, Merck) using toluene–methanol (85:15, v/v) as the solvent. The R_F values of uroporphyrin and coproporphyrin methyl esters were 0.4 and 0.7, respectively. The porphyrin zones were eluted with acetone–methanol (1:1, v/v) and the solvents were removed under vacuum. Hydrolysis of the porphyrin methyl esters with 6 *M* hydrochloric acid and adsorption of the free acids on Sep-Pak C_{18} cartridges were carried out as with the porphyrin methyl ester standards (see above). Uroporphyrins were partially decarboxylated at 180°C to the corresponding coproporphyrins and purified on Sep-Pak C_{18} cartridges, as described elsewhere[5].

Chromatographic conditions

Separation of uroporphyrin isomers. The separations were carried out on LiChrosorb RP-18 (7 μm) columns (250 mm × 4 mm I.D.; Merck), which were protected by a LiChroCART guard cartridge (LiChrosorb RP-18, 5 μm; 4 mm × 4 mm I.D.; Merck). The mobile phase was aqueous phosphate buffer (28 m*M*, pH 5.75)–methanol containing 5 mmol/l of tetrabutylammonium phosphate (pH 7.13) (66:34, v/v). The flow-rate was 1.0 ml/min at a column temperature of 38°C.

Separation of coproporphyrin isomers. The separations were performed on LiChrospher RP-18 (5 μm) columns (125 mm × 4 mm I.D.; Merck) protected by a guard cartridge (see above). The mobile phase was aqueous phosphate buffer (44 m*M*, pH 6.63)–organic phase [methanol–acetonitrile–water (72:21:7, v/v)] containing 8.9 mmol/l of tetrabutylammonium phosphate (pH 7.25) (59:41, v/v). The flow-rate was 1.5 ml/min at a column temperature of 40°C.

RESULTS AND DISCUSSION

Preparation of urine samples

Urine from patients suffering from AIP normally contain large amounts of porphobilinogen, which artificially forms all four uroporphyrin isomers in various ratios under physiological conditions[5]. The non-enzymatic self-condensation of this porphyrin precursor within the human body cannot be avoided, but refrigeration and deep-freezing of the freshly excreted urine largely suppress this reaction during

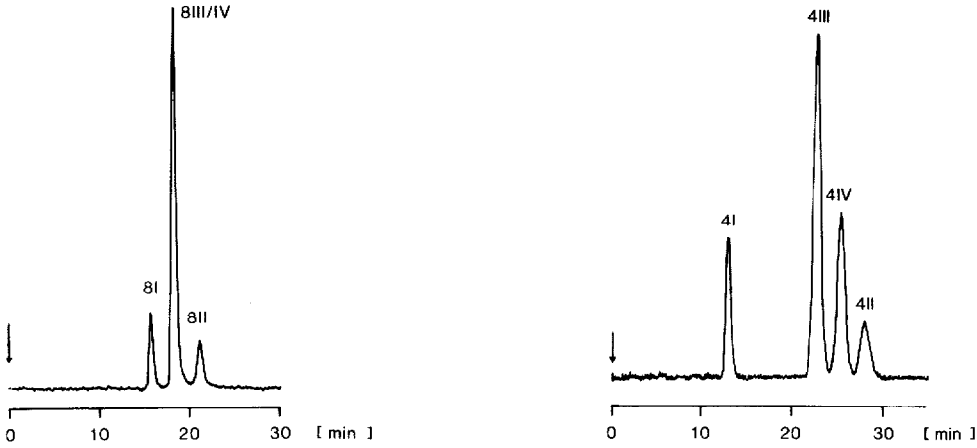

Fig. 1. HPLC separation of a statistical mixture of uroporphyrins I, II and III/IV on a 7-μm LiChrosorb RP-18 column at 38°C. Fluorescence detection.

Fig. 2. HPLC separation of coproporphyrins I, II, III and IV obtained by partial decarboxylation of a statistical mixture of uroporphyrins. Column: 5-μm LiChrospher RP-18 at 40°C. Fluorescence detection.

specimen collection and storage. Furthermore, porphobilinogen can be removed from such urines by treatment with talc, which retains only porphyrins[18].

Porphyric patients excrete considerable amounts of porphyrinogens[19], which are easily isomerized by strong acids[20]. We therefore oxidized the urine samples with iodine at nearly neutral pH according to Mauzerall[20] in order to prevent isomerization of the respective porphyrinogens. Indeed, some isomerization might occur when the oxidation step is carried out under strongly acidic conditions, as applied by Martasek *et al.*[21] and Westerlund *et al.*[22].

Preparative isolation of the individual porphyrin fractions as their methyl esters is normally performed by TLC. We developed a simple solvent system (toluene–methanol) for the TLC separation of uroporphyrin and coproporphyrin methyl esters, thus avoiding the highly toxic benzene present in other solvent systems[23].

Isocratic HPLC separation of uroporphyrin and coproporphyrin isomers

We have previously performed the ion-pair HPLC separation of uroporphyrin isomers under isocratic conditions on a 7-μm LiChrosorb RP-18 column at room temperature[5]. When we now increased the column temperature to 38°C and slightly modified the composition of the mobile phase, *e.g.*, the ionic strength and pH of the aqueous phosphate buffer and the concentration of the ion-pair reagent tetrabutylammonium phosphate in the organic modifier methanol, a sharp reduction in the retention times of all uroporphyrin isomers resulted without any loss of chromatographic resolution (Fig. 1). Using these conditions, the separation of uroporphyrins I, II and III/IV was possible within *ca.* 20 min. However, the isomers III and IV could not be resolved by the eluent system applied, even when the retention time was prolonged to more than 1 h.

The simultaneous ion-pair separation of coproporphyrins I–IV was formerly carried out on a 7-μm LiChrosorb RP-18 column at 38°C[5]. Application of a 5-μm

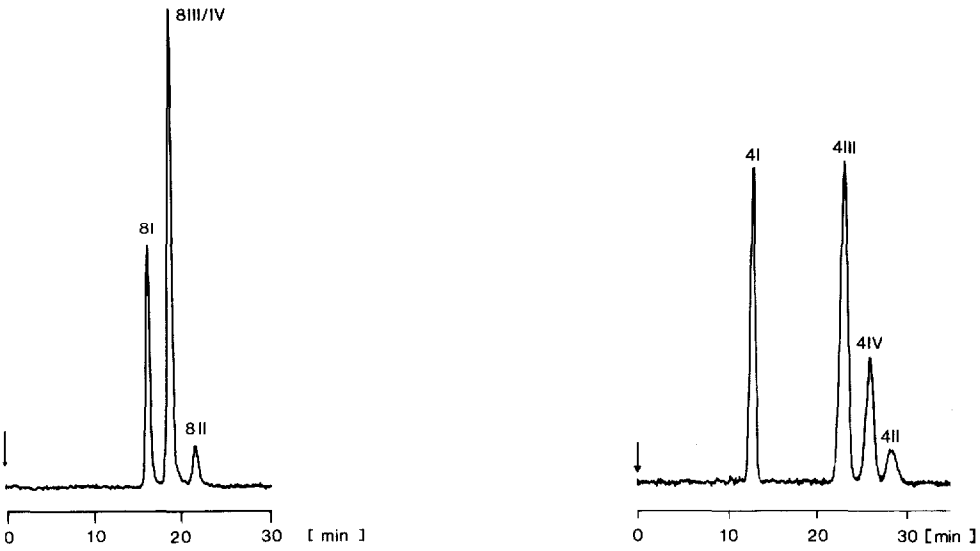

Fig. 3. HPLC separation of urinary uroporphyrins I, II and III/IV isolated from a patient with acute intermittent porphyria (case 1). Conditions as in Fig. 1.

Fig. 4. HPLC separation of coproporphyrins I, II, III and IV obtained after partial decarboxylation of the respective urinary uroporphyrins from a patient with acute intermittent porphyria (case 1). Conditions as in Fig. 2.

LiChrospher RP-18 column at 40°C, together with some modifications of the mobile phase, markedly improved the selectivity, especially for the separation of the isomers II, III and IV (Fig. 2).

HPLC determination of urinary uroporphyrins

The urinary uroporphyrin fractions of four patients (females, 31–40 years old) suffering from acute attacks of AIP were isolated by TLC as their methyl esters. Isocratic ion-pair HPLC analysis of the uroporphyrin free carboxylic acids yielded fairly clean chromatograms, containing only the uroporphyrin isomers I, II and III/IV (Fig. 3). No additional peaks could be detected, in contrast to the chromatograms that we had obtained previously by extraction of urine on talc without the TLC purification step[5]. Partial decarboxylation of the uroporphyrins to the corresponding coproporphyrins and isocratic ion-pair HPLC separation allowed a complete analysis of the isomeric composition originally present in the uroporphyrin fraction (Fig. 4). Table I shows the isomeric distribution of the uroporphyrin fractions from four patients with AIP, obtained by this method. The composition of the resulting coproporphyrin isomers correlated satisfactorily with that of the original uroporphyrin isomers (Table I). In these urines, we found 2–5% of the atypical isomer II and 13–19% of the atypical isomer IV. Thus, we were able to confirm the presence of uroporphyrins II and IV in urines of AIP patients, which we recently described[5]. Owing the mostly non-enzymatic formation of the uroporphyrins from porphobilinogen in AIP patients, the respective isomeric composition depends largely on the retention time and pH of the urine within

TABLE I

ISOMERIC COMPOSITION (%) OF URINARY UROPORPHYRINS (U) FROM FOUR CASES OF ACUTE INTERMITTENT PORPHYRIA

Coproporphyrins (C) were obtained after partial decarboxylation.

Case	*U I*	*U II*	*U III/IV*	*C I*	*C II*	*C III*	*C IV*
1	28.9	5.4	65.7	28.5	4.9	47.8	18.8
2	45.3	2.7	52.0	46.3	3.3	35.9	14.5
3	38.7	3.3	57.9	39.3	3.0	44.0	13.7
4	31.8	3.8	64.4	32.4	3.3	46.1	18.2

the human body. Therefore, the differing ratios of the uroporphyrin isomers (Table I) cannot be used for additional characterization of the individual AIP cases. In these patients, the percentages of the isomers II and IV are comparable to those formed by chemical condensation of porphobilinogen under physiological conditions[5]. In urine samples from control subjects, we observed 80 ± 10% of uroporphyrin I and 20 ± 10% of uroporphyrin III, whereas the atypical isomers II and IV could not be detected until now.

Doss and Schermuly[24] determined the isomeric distribution of all types of porphyrins in the series from uroporphyrin to coproporphyrin in the urine of patients with AIP. In these urine samples only the isomers I and III could be detected. However, the TLC method[25] used by these workers permitted only the separation of the normal isomers I and III, and the questionable presence of the non-typical isomers was not investigated.

HPLC determination of urinary coproporphyrins

The isomeric composition of the urinary coproporphyrin fractions, isolated by TLC, was determined by isocratic ion-pair HPLC in four cases of acute attacks of AIP (cases 2, 4, 5 and 6; females, 31–45 years old; Table II) and two cases in the latent phase of AIP (cases 7 and 8; females, 34 and 42 years old; Table II). Again, clean chromatograms were obtained by this method (Fig. 5).

These urines surprisingly contained the atypical coproporphyrins II and IV, just as found in the uroporphyrin fractions. Positive identification of these unexpected

TABLE II

ISOMERIC COMPOSITION (%) OF URINARY COPROPORPHYRINS (C) FROM SIX CASES OF ACUTE INTERMITTENT PORPHYRIA

Case	*C I*	*C II*	*C III*	*C IV*
2	12.9	1.3	83.2	2.6
4	10.1	1.6	84.6	3.7
5	19.8	1.5	75.3	3.4
6	9.0	0.9	88.3	1.8
7	9.9	2.1	83.0	5.0
8	11.3	2.2	82.1	4.4

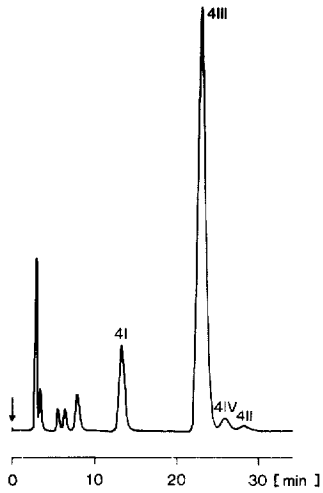

Fig. 5. HPLC separation of urinary coproporphyrins I, II, III and IV isolated from a patient with acute intermittent porphyria (case 2). Conditions as in Fig. 2.

compounds was performed by chromatography after addition of authentic substances and by on-line diode-array detection of their absorption spectra in the wavelength range 300–590 nm. Fig. 6 shows the absorption spectra of the individual coproporphyrin isomers which were overlayed for comparison. The spectrum of isomer IV shows exactly the same curve shape as that of the normal isomers I and III. Isomer II shows a slightly different curve shape at wavelengths above 530 nm. This deviation is caused by the low concentration of isomer II in the urine sample, resulting in an absorption below 0.0005 a.u.f.s. Application of these enhanced separation and

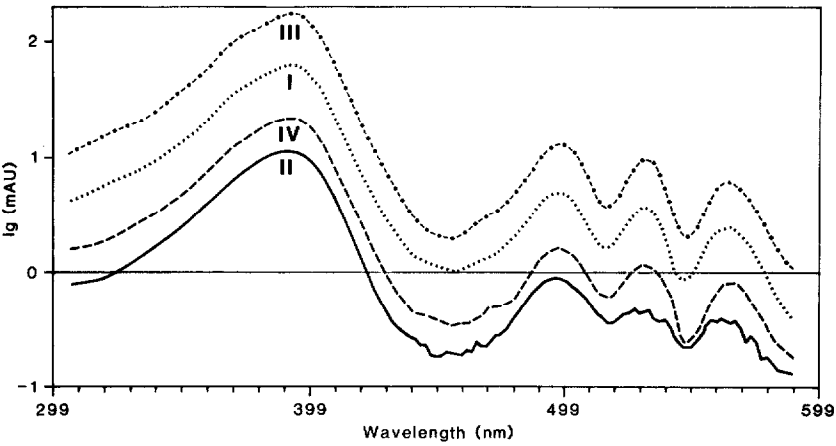

Fig. 6. Absorption spectra of the urinary coproporphyrins I, II, III and IV isolated from a patient with acute intermittent porphyria (case 2). The spectra were recorded by on-line diode-array detection (300–590 nm) and overlayed for comparison.

identification techniques has enabled us to prove the presence of trace amounts of the atypical coproporphyrin isomers II and IV for the first time in such urines. The isomeric proportions of the urinary coproporphyrins of six AIP cases are summarized in Table II. The percentages of the isomers II and IV are significantly lower than those of the atypical uroporphyrins, because we found only 1–2% of isomer II and 2–5% of isomer IV. On the other hand, the ratios of the isomer III are markedly increased and those of the isomer I are decreased in comparison with the ratios of the respective uroporphyrin isomers. Owing to the small number of cases investigated so far, the diagnostic importance of these results still has to be proved by additional data. Urine samples from healthy persons contained the normal coproporphyrin isomers I (20 ± 10%) and III (80 ± 10%) in concentrations similar to those in the AIP patients. The atypical isomers II and IV were found only occasionally in these urines owing to their low concentration. The reference values for normal urines have to be established.

Several mechanisms can be envisioned for the formation of the non-typical coproporphyrin isomers: (1) enzymatic decarboxylation of the respective uroporphyrinogens, formed non-enzymatically from porphobilinogen within the human body; (2) enzymatic decarboxylation of uroporphyrinogens outside the human body by cellular enzymes; this mechanism can be rejected, as only one of the urines investigated contained leukocytes and erythrocytes; (3) chemical isomerization of coproporphyrinogens inside the human body. The last hypothesis is supported by the results of preliminary experiments in which we observed a measurable non-enzymatic rearrangement of coproporphyrinogen III to the other isomers under physiological conditions[26].

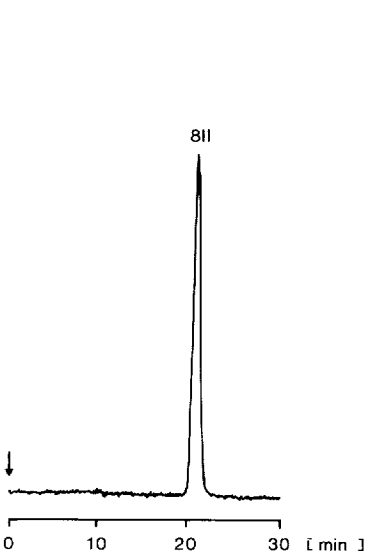

Fig. 7. HPLC analysis of a uroporphyrin II specimen synthesized by MacDonald and Michl[12]. Conditions as in Fig. 1.

Fig. 8. HPLC analysis of a natural uroporphyrin I specimen prepared by Fischer and Hofmann[11]. Conditions as in Fig. 1.

HPLC analysis of uroporphyrin specimens

Isocratic ion-pair separation was applied to check the isomeric purity of a series of uroporphyrin specimens of synthetic or natural origin. The highest isomeric purity was found for a uroporphyrin II specimen synthesized by MacDonald and Michl[12] via the pyrromethene method. The chromatogram in Fig. 7 demonstrates the excellent purity of this specimen, which contained exclusively the isomer II. Lower carboxylated porphyrins were absent, as demonstrated by applying our gradient elution technique[4]. HPLC analysis of a natural uroporphyrin I preparation is shown in Fig. 8. This specimen was isolated and purified by Fischer and Hofmann[11] from the excreta of the famous porphyria case Petry, who suffered from congenital erythropoietic porphyria. The content of isomer I was 99%; only 1% of isomer III was found in this specimen.

We also analysed a so-called Waldenström porphyrin, which had been considered earlier to be uroporphyrin III. The Waldenström porphyrins had been obtained by different isolation procedures from urines of patients in the acute phase of AIP[13]. The nature of these porphyrins has been studied intensively by Nicholas and Rimington[27] with the aid of paper chromatography, Debye–Scherrer X-ray photography and melting-point curves. On the basis of their results, they suggested that the Waldenström uroporphyrins are a mixture of *ca.* 75% of isomer III and 25% of isomer I. HPLC analysis of such a uroporphyrin, originally prepared by Waldenström *et al.*[13], now revealed the presence of isomer I (21%), isomer II (9%) and isomers III/IV (70%) (Fig. 9). Partial decarboxylation to the corresponding coproporphyrins gave 20% of isomer I, 8% of isomer II, 53% of isomer III and 19% of isomer IV. Owing to the presence of substantial amounts of the atypical isomers II and IV in Waldenström's uroporphyrin the mostly non-enzymatic formation of such porphyrins, recognized previously as chemical artifacts by Cookson and Rimington[28], is confirmed by the findings in our study. In addition, we checked the isomeric purity of a series of commercially available uroporphyrin standards. Most of them met the specified

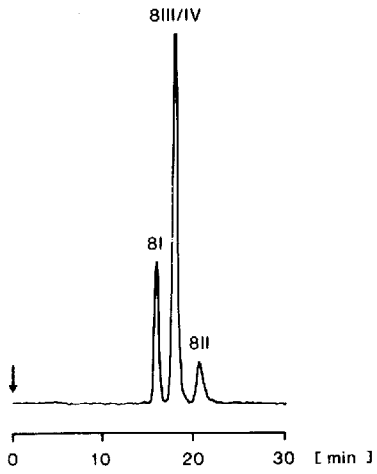

Fig. 9. HPLC analysis of a Waldenström uroporphyrin "III" specimen prepared by Waldenström *et al.*[13]. Conditions as in Fig. 1.

isomeric composition, but in some instances we observed considerable contamination with other isomers, amounting to nearly 50%.

CONCLUSION

Improved isocratic ion-pair HPLC methods are suitable for the determination of the atypical porphyrin isomers II and IV in the urine of patients suffering from AIP. The presence of uroporphyrin isomers II and IV in such urine was confirmed, and the occurrence of the coproporphyrin isomers II and IV was demonstrated for the first time. Formation of the latter is probably due to non-enzymatic isomerization at the porphyrinogen level within the human body.

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