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DIAGNOSIS OF PORPHYRIAS BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Ion-pair reversed-phase high-performance liquid chromatography together with fluorescence detection is useful in the analysis of urinary porphyrin carboxylic acids. The sensitive and quantitative detection facilitates the clinical diagnosis of porphyrias. The method described permits the detection of porphyrins down to 0.1 ng directly from urine without laborious sample pre-treatment. A linear response curve was obtained from 0.2 up to 200 ng for coproporphyrin I. The within-assay correlation coefficients ranged from 2.5 to 10.1%. Recovery experiments gave an accuracy of 89–109%. The rapidity and simplicity of the method allows its application to the routine analysis of urinary porphyrins in the clinical laboratory.

INTRODUCTION

Porphyrias are caused by inborn or acquired defects of enzymes in the haem biosynthesis. For the diagnosis of the different diseases of porphyrin metabolism it is necessary to identify and quantify the porphyrins from urine, faeces and/or blood. In most work published so far, the porphyrins were extracted, methylated and separated on silica gel by thin-layer chromatography[1,2] or high-performance liquid chromatography (HPLC)[3,4]. Miller and Malina[5] described the conversion of porphyrin methyl esters to the corresponding copper(II) chelates, which were determined down to nanogram levels using a bonded CN column and UV detection. The extraction and esterification, however, are very time-consuming procedures. Evans *et al.*[6] published an example of an ion-exchange HPLC separation of some free porphyrin carboxylic acids. An intricate method for the analysis of free porphyrin carboxylic acids by reversed-phase HPLC was described recently[7]. Bonnett *et al.*[8] reported the direct determination of free urinary porphyrin carboxylic acids by ion-pair reversed-phase HPLC. Under the conditions described, the separation of the porphyrins could not be reproduced in our laboratory. For routine analysis the method applied must be rapid, simple and reproducible. With these objectives in mind we tried to develop a method for the quantification of the individual free porphyrin carboxylic acids. Ion-pair reversed-phase HPLC with multilinear gradient elution was applied to the determination of the porphyrins directly in the urine of patients with porphyric diseases[9].

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EXPERIMENTAL

Apparatus

A Model 8500 high-performance liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) with a solvent programmer was used for the gradient elution. Samples were brought on to the column with a Rheodyne 7105 continuous-flow septumless injector (Rheodyne, Berkeley, CA, U.S.A.). The pre-packed column (300 × 4 mm) contained 10-μm particles of μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.). The column eluate was detected alternatively by two different fluorescence spectrophotometers, a Model 3000 (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Schoeffel FS 970 (Spectra-Physics, Santa Clara, CA, U.S.A.) with a deuterium lamp. With the Perkin-Elmer 3000 the excitation wavelength was set at 399 nm (slit width 15 nm) and the emitted fluorescence light was measured at 615 nm (slit width 20 nm). With the FS 970 detector the fluorescence was excited at a wavelength of 399 nm and for detection a 580 nm cut-off filter was used.

Reagents

Spectro-grade methanol was purchased from E. Merck (Darmstadt, G.F.R.) and J. T. Baker (Phillipsburg, NJ, U.S..A). Water was deionized and then processed with a Milli-Q filtration system (Millipore, Bedford, MA, U.S.A.) to a conductivity of 8 MΩ. The ion-pair reagent tetrabutylammonium phosphate (pH 7.5) was supplied by Waters Assoc. The eluents were prepared by mixing an appropriate amount of tetrabutylammonium phosphate with the solvent to obtain 5 m*M* solutions. The eluents were filtered through a Gelman 0.45-μm Tuffryn membrane filter (Gelman, Ann Arbor, MI, U.S.A.) and degassed under vacuum.

Coproporphyrin I standards (5 μg) were obtained from Sigma (St. Louis, MO, U.S.A.). Coproporphyrin III and the synthetic mixture of 8- to 2-carboxylic porphyrins were purchased from Porphyrin Products (Logan, UT, U.S.A.).

For filtration of urine samples Gelman Acrodisc membrane filters with a pore size of 0.2 μm were used.

Method

Patients' urines were collected for 24 h in brown bottles containing 5 g of sodium carbonate. Prior to analysis 2.5 ml of urine were filtered through a 0.2-μm membrane filter to protect the column from solid matter. Urines with very high concentrations of porphyrins were diluted with an appropriate amount of the eluent.

The coproporphyrin I standard and the different porphyrin carboxylic acid solutions were prepared by dissolving the commercially available standards in 2.5 ml of normal urine.

Chromatographic conditions

Ion-pair reversed-phase HPLC separation was performed by multilinear gradient elution. The mobile phase consisted of methanol and water, both containing 5 mmol/l of tetrabutylammonium phosphate as the counter-ion. The starting conditions of 30% methanol *vs.* water were held for 1 min after injection. Then the methanol content was raised to 40% within 1 min and held for 2 min. In the following 2 min the methanol content was increased to 42% and then within 12 min to 64%

at a rate of 2%/min and held there for 2 min. For elution of less polar compounds the solvent composition was brought to 100% for 4 min. Between two analyses the column was reconditioned for 15 min with the starting solvent mixture. The pump was set at a flow-rate of 1 ml/min. The injected sample volumes varied from 10 to 100 μl.

RESULTS AND DISCUSSION

For the separation of the free porphyrin carboxylic acids we chose ion-pair reversed-phase HPLC with tetrabutylammonium phosphate as the counter-ion. In contrast to a previous report[8], we found it necessary to employ gradient elution. A satisfactory chromatographic separation of the porphyrins was achieved with the described multilinear gradient with a solvent system consisting of water and methanol, both containing 5 mmol/l of the counter-ion. The porphyrin carboxylic acids were eluted from the reversed-phase column in order of decreasing polarity. Fig. 1 shows the separation of urine spiked with uro-, heptacarboxylic-, hexacarboxylic-, pentacarboxylic- and coproporphyrin I and III. Amounts of 40 pmol of each porphyrin, except coproporphyrin I (16 pmol), were injected. The porphyrins with 8–5 carboxylic groups are type I isomers. The system also allows the separation and quantification of coproporphyrins I and III. To identify and quantify the individual porphyrins the method of standard additions[10] was applied by adding an appropriate amount of the standard mixture to the urine samples. The chromatogram of a urine sample from a patient suffering from acute intermittent porphyria is shown in Fig. 2a. The separation of the same urine sample spiked with an aliquot of standard

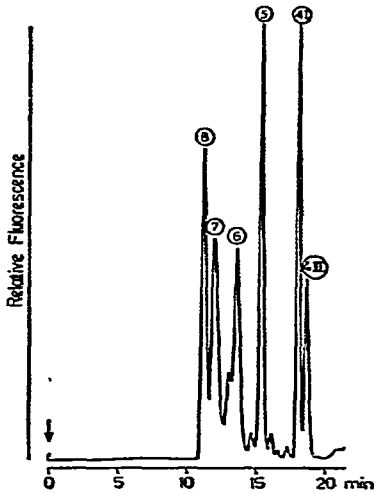


Fig. 1. Separation of a synthetic mixture of free porphyrin carboxylic acids in urine. Peaks: 8 = uroporphyrin I; 7 = heptacarboxylic porphyrin I; 6 = hexacarboxylic porphyrin I; 5 = pentacarboxylic porphyrin I; 4I = coproporphyrin I; 4III = coproporphyrin III.

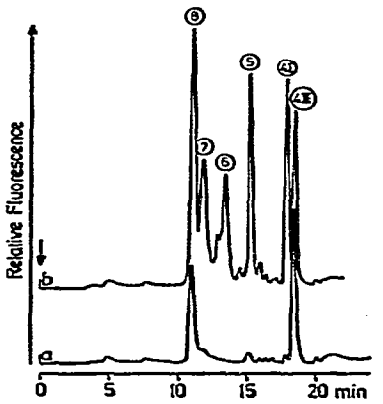
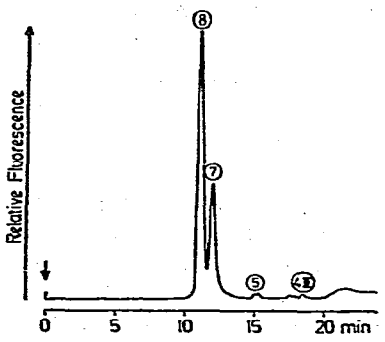


Fig. 2. Porphyrin carboxylic acid profile of a patient suffering from acute intermittent porphyria. (a) 50 μl of patient urine; (b) 50 μl of patient urine spiked with porphyrin standard mixture. Peaks as in Fig. 1.

mixture is demonstrated in Fig. 2b. The coproporphyrin in urine was found to be 95% of the normal type III isomer.

Very high concentrations of uro- and heptacarboxylic porphyrin were found in the urine of a patient with porphyria cutanea tarda (Fig. 3). Prior to analysis this urine sample had been diluted 25-fold with the eluent.

Another urinary porphyrin profile from a patient with porphyria cutanea tarda in a latent period is illustrated in Fig. 4. In addition to high concentrations of uro- and heptacarboxylic porphyrin, increased levels of pentacarboxylic- and coproporphyrin I and III are observed. An unidentified porphyrin is eluted between pentacarboxylic- and coproporphyrin I. This could be isocoproporphyrin, which is sometimes found in the urine of patients with porphyria cutanea tarda[11,12].



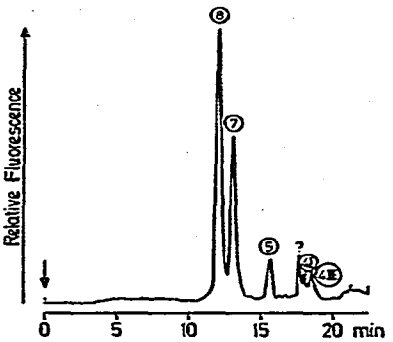


Fig. 3. Chromatogram of a urine sample from a patient with porphyria cutanea tarda in an active period. Prior to analysis the urine sample was diluted 25-fold with eluent. Peaks as in Fig. 1.

Fig. 4. HPLC separation of porphyrins from the urine of a patient with porphyria cutanea tarda in a latent period. Peaks as in Fig. 1.

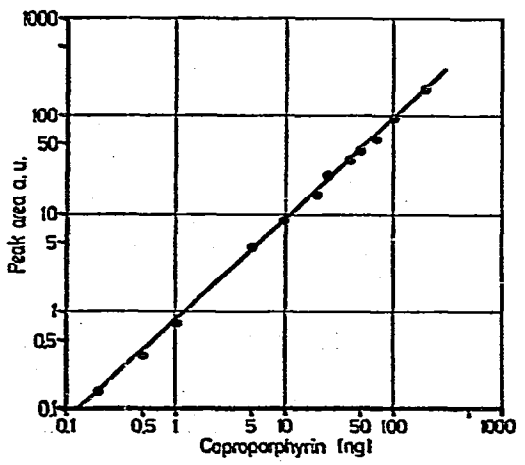


Fig. 5. Relationship between the amount of coproporphyrin I injected and the area of the detected fluorescent peak.

The relationship between the area of the chromatographic peak and the injected amount of coproporphyrin I dissolved in urine was linear in the range 0.2–200 ng with a correlation coefficient of 0.999 (Fig. 5). This range allows the quantification of porphyrins even in urine samples with very low concentrations. The detection limits were 0.2 ng for uroporphyrin I and 0.1 ng for coproporphyrin I.

The within-assay precision was determined by analysing a urine sample spiked with 40 pmol (corresponding to 25–33 ng) of each porphyrin five times without the use of an internal standard. Peak areas were determined manually by the triangular method. The coefficient of variation ranged from 2.5 to 10.1% (uro- 4.0%, heptacarboxylic- 2.5%, pentacarboxylic- 10.1% and coproporphyrin I 6.7%). The accuracy for the same compounds was checked by recovery experiments (89–109%, $\bar{x} = 25$ pmol).

The method described allows the precise determination of urinary porphyrins by two chromatographic runs, which need overall about 80 min. The pre-treatment of the urine samples consists only of a simple and very rapid filtration step.

Despite the simplicity of the method it is intended to screen patient urines first for elevated porphyrin excretion by ion-exchange chromatography or by second derivative UV spectroscopy at the Soret band[13].

Preliminary results indicate that the method can be adapted to other human materials such as faeces and blood.

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