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ANALYSIS OF FREE STOOL PORPHYRINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The determination of stool porphyrins is necessary for the diagnosis of some porphyrias in clinical laboratories. Quantitative methods for the analysis of faeces for porphyrins are unpleasant and difficult to perform. An extraction and ion-pair reversed-phase high-performance liquid chromatographic procedure is described for the separation and determination of individual free stool porphyrins. The within-assay coefficients of variation range from 2 to 6%. A linear response curve is observed between 38 and 380 nmol/g for coproporphyrin I in dry stool. The method can be applied to the routine analysis of free stool porphyrins in the clinical laboratory.

INTRODUCTION

The determination of naturally occurring porphyrins in human excreta and blood is important for the diagnosis of porphyric diseases. For most types of porphyrias observed in central Europe it is generally sufficient to analyse porphyrins and their precursors in urine, which thus constitutes the first step in the diagnostic procedure for porphyria in the clinical laboratory. For the diagnosis of the rarely found porphyria variegata and erythrohepatic protoporphyria it is necessary to examine the stool porphyrins.

However, the methods used for the analysis of faeces for porphyrins are time-consuming and tedious and it is difficult to make them quantitative. Earlier procedures for the determination of porphyrins included extractions and solvent fractionations followed by spectrophotometry[1,2] or fluorimetry[2], but only groups of porphyrins designated as "coproporphyrin" and "protoporphyrin" could be measured. The separations of the individual porphyrins have been greatly enhanced by the introduction of thin-layer chromatography[3,4] and high-performance liquid chromatography (HPLC)[5,6]. In all of these procedures the stool porphyrins were converted into the methyl esters prior to the chromatographic step.

Recently, the reversed-phase HPLC of free porphyrin carboxylic acids from urine and blood has been shown[7–10] to be a rapid, simple and accurate separation technique. Up to now, no methods for the determination of free stool porphyrins by HPLC have been described. The results obtained with our method for the determina-

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tion of free porphyrins from urine and blood encouraged us to adapt this ion-pair technique to the analysis of stool porphyrins.

EXPERIMENTAL

Apparatus

Gradient elution was performed with a Model 8500 high-performance liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) consisting of two syringe pumps attached to a solvent programmer. The injection system used was a Rheodyne 7105 sample valve (Rheodyne, Berkley, CA, U.S.A.). The pre-packed columns were a LiChrosorb RP-18 (10 μm) (25 cm × 4.6 mm I.D.) (E. Merck, Darmstadt, G.F.R.) or a μBondapak C_{18} (10 μm) (30 cm × 4 mm I.D.) (Waters, Milford, MA, U.S.A.). An RP-18 (10 μm) pre-column (3 cm × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.) was used together with the LiChrosorb column. The fluorescence of the column eluate was measured with a Model 3000 spectrofluorimeter (Perkin-Elmer, Norwalk, CT, U.S.A.) and recorded on an SP-4100 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). The excitation wavelength was set at 399 nm (slit width 15 nm) and the emitted fluorescence was detected at 615 nm with a slit width of 20 nm.

Reagents

Spectro-grade methanol was purchased from E. Merck and J. T. Baker (Phillipsburg, NJ, U.S.A.). Water was de-ionized 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 0.45-μm Tuffryn membrane filter (Gelman, Ann Arbor, MI, U.S.A.) and degassed under vacuum.

Coproporphyrin I standards were obtained from Sigma (St. Louis, MO, U.S.A.). The other porphyrins applied as proto-, meso-, deutero-, uroporphyrin and the synthetic mixture containing 8- to 2-carboxylic porphyrins were purchased from Porphyrin Products (Logan, UT, U.S.A.).

Method

The method consists of four principal steps: freeze-drying, extraction, centrifugation and HPLC analysis.

About 500 mg of faeces were freeze-dried and pulverized, and 50 mg of dry stool were extracted three times with 1 ml of a 60 m*M* solution of tetrabutylammonium phosphate in methanol–water (80:20). Samples with high porphyrin contents must be extracted until the liquid phase shows no further red fluorescence under UV light. The extracts were combined and centrifuged for 5 min at 2500 *g*. An aliquot of the supernatant was injected into the sample valve. Solutions with very high concentrations of porphyrins were diluted with an appropriate amount of the eluent.

The porphyrin standards were prepared by dissolving the commercially available porphyrins in the eluent. Dried samples (50 mg) of the same faeces were mixed with increasing volumes of a coproporphyrin I solution (1.1 μM), then processed as described above and used for checking the linearity and accuracy of the method.

Chromatographic conditions

The separation of free porphyrin carboxylic acids was performed by ion-pair reversed-phase HPLC with multilinear gradient elution. The mobile phase consisted of methanol and water, both containing 5 m*M* of tetrabutylammonium phosphate as counter-ion.

The gradient programme for the LiChrosorb column began at 65% methanol–water for 1 min, then the methanol content was increased at 10%/min for 1 min to 75% methanol–water (maintained for 2 min), followed by 5%/min for 3 min to a final methanol concentration of 90%, which was maintained for 5 min. Between two analyses the column was reconditioned for 10 min with the starting mixture.

Other gradients have to be used with the μBondapak column. The starting composition of 50% methanol–water was maintained for 2 min, then the methanol content was increased to 58% in 2 min and to 68% in a further 2 min, and finally to 78% in a further 1 min maintained there for 10 min. The time for reconditioning with starting solvent mixture was 8 min. The separation of uro- and heptacarboxyporphyrin was achieved with the μBondapak column by employing gradients described in previous papers[7,11].

For both columns the flow-rate was set at 1 ml/min. The sample volumes injected varied from 5 to 25 μl.

RESULTS AND DISCUSSION

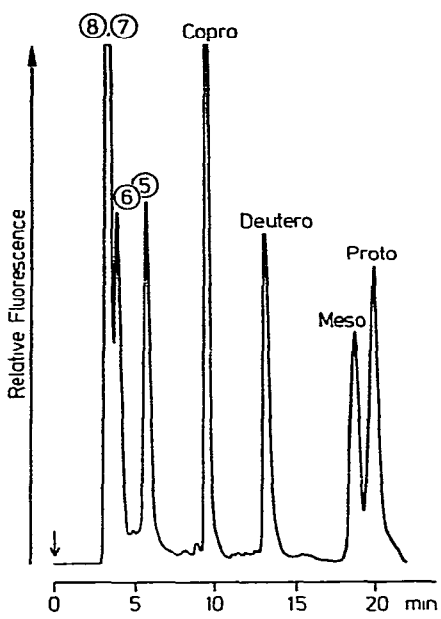
Extraction

The procedures including the esterification step commonly used for the extraction of stool porphyrins[3–6] are not applicable to free porphyrin carboxylic acids. Therefore, we first modified the extraction methods described for stool analysis by solvent partitioning[6,12] and determination of free porphyrins in blood[10]. However, with these solvent mixtures we could not overcome difficulties with the extraction of uroporphyrin. A 60 m*M* solution of tetrabutylammonium phosphate in methanol–water, however, extracted all porphyrins from stool specimens that had been added to the faeces. Freeze-drying led to nearly complete deodoration of the stool sample.

Chromatographic separation

The separation of extracted free porphyrin carboxylic acids was performed by ion-pair reversed-phase HPLC with tetrabutylammonium phosphate as the counter-ion. To obtain a good resolution and a short separation time for both columns, gradient elution systems were chosen. These gradients allowed the satisfactory separation from coproporphyrin to protoporphyrin; even mesoporphyrin was well resolved from protoporphyrin.

A synthetic mixture containing uro-, heptacarboxy-, hexacarboxy-, pentacarboxy-, copro-, deutero-, meso- and protoporphyrin was separated with the μBondapak column (Fig. 1). The porphyrins were eluted in order of decreasing polarity. Dicarboxylic porphyrins with saturated side-chains show shorter retention times than those with unsaturated functional groups. Uro- and heptacarboxylic porphyrin, however, are not fully separated under these conditions. For the analysis of stool specimens with significant amounts of these porphyrins, which were found, for example, in



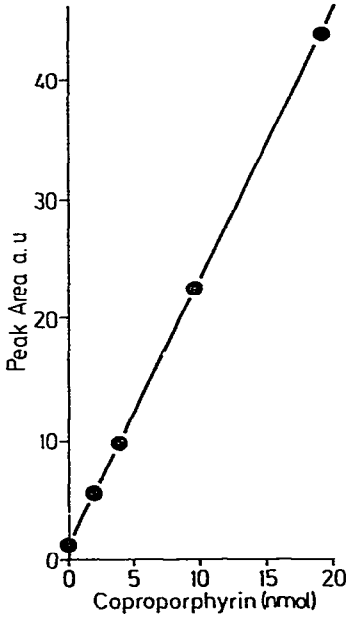


Fig. 1. Separation of a synthetic mixture of porphyrins (μBondapak column). Peaks: 8 = uroporphyrin I; 7 = heptacarboxylic porphyrin I; 6 = hexacarboxylic porphyrin I; 5 = pentacarboxylic porphyrin I; Copro = coproporphyrin I; Deutero = deuteroporphyrin IX; Meso = mesoporphyrin IX; Proto = protoporphyrin IX.

Fig. 2. Relationship between area of the detected fluorescence peak and amount of coproporphyrin I (nmol/g) added to dry stool.

cases of porphyria cutanea tarda[13], the chromatographic conditions used for urine samples were applied.

The individual porphyrins were identified and quantified by the method of standard additions[14] by adding appropriate amounts of porphyrins to the extracts. One column allowed more than 400 analyses of extracts without loss of column performance.

Criteria of the method

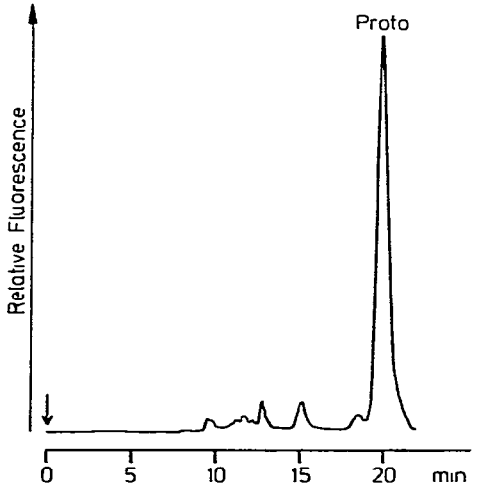
The relationship between the area of the chromatographic peak and the amount of coproporphyrin I added to the stool specimen was linear in the range from 38 to 380 nmol/g (dry stool) with a correlation coefficient of 0.999 (Fig. 2). The accuracy was checked by recovery experiments, comparing the same samples with standard solutions of identical concentrations. The recovery observed was between 105 and 110%. The determination of the within-assay precision was performed by analysing five samples from the same faeces. The coefficients of variation were 2.3% for copro-, 4.8% for deutero-, 6.1% for meso- and 4.0% for protoporphyrin.

Quantitative determinations of faecal components are possible only with limitations because of wide changes in bowel habits, intestinal transit time and bulk of stool. In contrast to urinary sampling, the bowel cannot be emptied completely at will. Moreover, variations in daily faecal porphyrin excretion and the difficulties of

ensuring complete collection mentioned above make absolute quantitation almost impossible to achieve. Fortunately, for the diagnosis of porphyrias the porphyrin profile is the most important feature. Therefore, it is not necessary to determine the absolute amounts of the 24-h excreted individual porphyrins.

Porphyrin profiles

Separation of a stool extract from a patient suffering from erythrohepatic protoporphyria was performed with the method described here (Fig. 3). In the faeces we found an excessive concentration of protoporphyrin of 2250 nmol/g (dry stool), whereas other porphyrins were excreted in normal amounts. The protoporphyrin concentration for healthy persons lay between 21 and 150 nmol/g. These values agree well with those reported by Christensen and Romslo[6], which were obtained by HPLC using methyl esters.



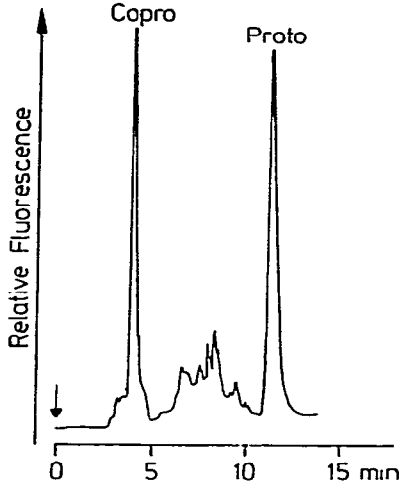


Fig. 3. Porphyrin carboxylic acid profile of a stool extract from a patient suffering from erythrohepatic porphyria (μBondapak column). Peaks as in Fig. 1.

Fig. 4. HPLC separation (LiChrosorb column) of an extract from faeces from a patient with porphyria variegata. Peaks as in Fig. 1.

Elevated excretion of coproporphyrin and protoporphyrin in the faeces is usually observed in cases of porphyria variegata[15]. Chromatographic separation of a stool extract from a patient with tentative diagnosis of this disease yielded high concentrations of protoporphyrin and coproporphyrin, with an excretion ratio of 2:1 (Fig. 4). This result enabled us to confirm the diagnosis of a porphyria variegata.

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