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Short Communication

Application of ion-pair high-performance liquid chromatography to detection of the atypical coproporphyrin isomers II and IV in human faeces

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ABSTRACT

A highly selective and sensitive method has been developed for the detection of small amounts of the atypical isomers II and IV of coproporphyrin in human faeces. This method combines liquid–liquid extraction and solid-phase sampling techniques using talc and C_{18}-modified silica gel as the sorbents. Simultaneous separation of the four coproporphyrin isomers I–IV was achieved by isocratic ion-pair high-performance liquid chromatography. Stool samples of healthy subjects ($n = 12$) contained $1.1 \pm 0.4\%$ (mean ± S.D.) isomer II and $2.2 \pm 0.9\%$ isomer IV of total coproporphyrins. A somewhat higher content of isomer II (2.7%) and isomer IV (5.4%) was found in faeces of a patient suffering from porphyria variegata.

INTRODUCTION

The occurrence of coproporphyrins of the series I and III as the principal isomers in human urine and faeces has been recognized for many years, whereas the isomers II and IV were previously thought to be non-existent in biological materials. Recently, we were able to detect for the first time small amounts of the atypical coproporphyrin isomers II and IV in urine of both porphyric patients [1] and healthy persons [2] with the help of ion-pair high-performance liquid chromatography (HPLC). The formation of the atypical isomers was shown to result mostly from non-enzymic isomerization of the naturally occurring coproporphyrinogens, especially in relatively acidic urines [3]. These unexpected findings prompted us to investigate human faeces for the putative presence of coproporphyrins II and IV.

This paper describes a highly efficient preparation method for the purification of stool samples, suitable for a reliable HPLC analysis of trace amounts of coproporphyrin isomers in such a complex matrix.

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EXPERIMENTAL

Reagents

Methanol and acetonitrile were of LiChrosolv grade (E. Merck, Darmstadt, Germany). The ion-pair reagent tetrabutylammonium phosphate (pH 7.2) and the aqueous phosphate buffer (pH 6.6) were prepared as described elsewhere [1].

Apparatus

A Hewlett-Packard (Waldbronn, Germany) Model 1090A liquid chromatograph, equipped with a column oven, a Model F-1000 spectrofluorimeter (Merck-Hitachi, Darmstadt, Germany) (excitation at 395 nm, emission at 625 nm) and a Model 1040 diode-array detector (Hewlett-Packard), was used.

Stool samples

Stool samples of apparently healthy subjects of both sexes (25–40 years old) were studied. In addition, faeces from a patient (female, 36 years old) suffering from porphyria variegata were analysed.

Sample preparation

A 500-mg sample of freshly excreted stool was suspended in 10 ml of water and disintegrated. After oxidation with an aqueous solution of 10 mg of iodine and 20 mg of potassium iodide for 10 min at room temperature, the excess iodine was eliminated with sodium thiosulphate. The suspension was freeze-dried for 10–20 h, and the residue was then vortex-mixed with 6 ml of concentrated hydrochloric acid. Vortex-mixing was repeated twice after the successive addition of 18 ml of diethyl ether and 18 ml of water. The mixture was centrifuged for 10 min at 1000 *g*; the ethereal layer and the insoluble middle phase were discarded. The aqueous acid layer was diluted with 100 ml of water and brought to pH 3.5 with sodium acetate. The porphyrins were adsorbed on 250 mg of talc, washed with water, eluted with 10 ml of methanol–concentrated sulphuric acid (9:1, v/v), and esterified for 40 min at 40°C. The porphyrin methyl esters were separated by semipreparative thin-layer chromatography (TLC) on silica gel (Kieselgel 60 F, Merck) with *n*-hexane–dichloromethane–acetone (41:41:18, v/v) as the solvent. The coproporphyrin esters were hydrolysed, and the free acids were isolated as described previously [1].

Chromatographic conditions

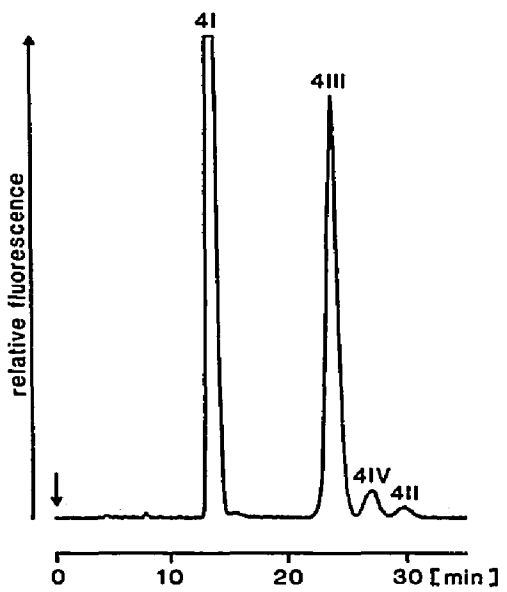
Separations of the coproporphyrin isomers I–IV were performed on LiChrospher RP-18 (5 μm) columns (125 mm × 4 mm I.D.; Merck) protected by a LiChroCART guard cartridge (LiChrospher RP-18, 5 μm; 4 mm × 4 mm I.D.; Merck). The mobile phase consisted of 56–58% aqueous phosphate buffer (44 m*M*, pH 6.6) and 42–44% organic phase, containing 8.9 m*M* tetrabutylammonium phosphate (pH 7.2) in methanol–acetonitrile–water (72:21:7). The flow-rate was 2.0 ml/min at a column temperature of 40°C.

RESULTS AND DISCUSSION

The presence of both coproporphyrins and coproporphyrinogens in human faeces has been described by Fischer and Orth [4] in a porphyric patient and by Watson *et al.* [5] in healthy subjects. We therefore converted the acid-labile porphyrinogens into the corresponding stable porphyrins with iodine at nearly neutral pH [5]. This was to prevent the well known non-enzymic isomerization of porphyrinogens under acidic conditions [6].

Isocratic ion-pair HPLC analysis of faecal coproporphyrin extracts, prepared by the method described, produced clean chromatograms showing no detectable interfering substances. The coproporphyrin profile of a normal stool specimen is presented in Fig. 1. The chromatogram in Fig. 2 was obtained from the faecal coproporphyrin fraction of a patient with porphyria variegata. The presence of all four coproporphyrin isomers was confirmed by co-injection of authentic reference compounds as well as by on-line diode-array recording of their absorption spectra in the 310–580 nm wavelength range (Fig. 3).

The isomeric distribution of the four coproporphyrins from twelve stool specimens of healthy subjects was analysed. We observed 69.3 ± 4.9% (mean ± S.D.) of isomer I, 27.4 ± 4.5% of isomer III, 1.1 ± 0.4% of isomer II and 2.2 ± 0.9% of isomer IV. The preponderance of coproporphyrin I in normal human faeces has been previously reported by several authors [7,8]. In the case of a patient with



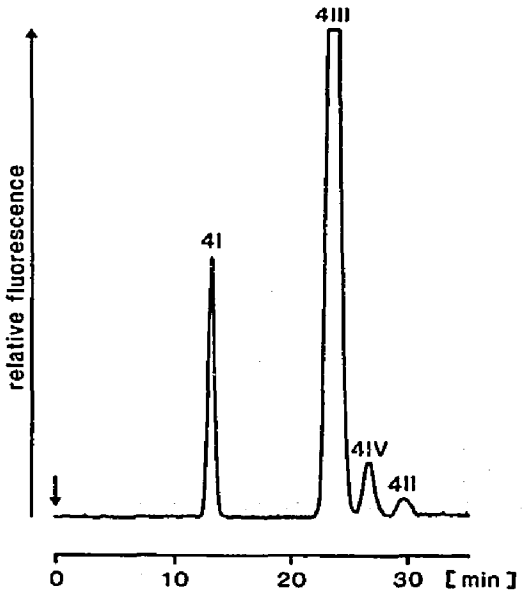


Fig. 1. Isocratic ion-pair HPLC separation of faecal coproporphyrins I–IV on a 5-μm LiChrospher RP-18 column at 40°C. The porphyrins were isolated from faeces of a healthy subject. Peaks: 4I–4IV = coproporphyrins I–IV.

Fig. 2. Isocratic ion-pair HPLC separation of faecal coproporphyrins I–IV from a patient with porphyria variegata. Conditions and peaks as in Fig. 1.

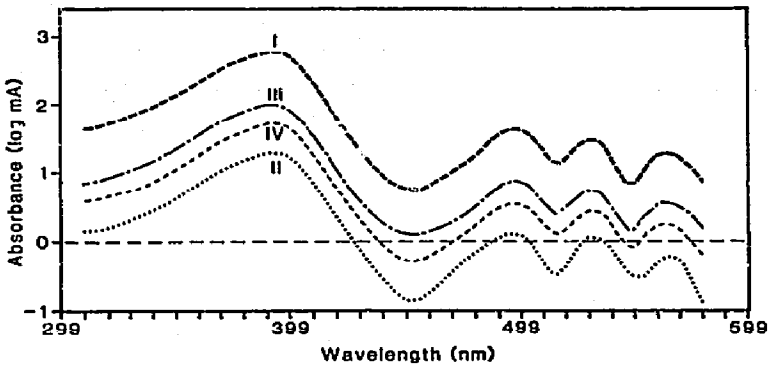


Fig. 3. Absorption spectra of coproporphyrins I–IV isolated from faeces of a healthy subject. The spectra were recorded by on-line diode-array detection (310–580 nm) and overlaid for comparison.

porphyria variegata we found an increased proportion of isomer III, together with a significantly higher content of the atypical isomers (12.8% of isomer I, 79.1% of isomer III, 2.7% of isomer II and 5.4% of isomer IV) (Fig. 2).

The atypical faecal coproporphyrins II and IV may be formed by a similar mechanism as has been shown for the urinary porphyrins, namely by non-enzymic isomerization of natural coproporphyrinogens.

The clinical significance of the newly developed method for the diagnosis of primary or secondary porphyrias has to be tested in future studies.

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