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Note

Separation and quantitation of urinary porphyrins by high-performance liquid chromatography

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Quantitative determination of porphyrins in urine has been tedious, inaccurate and time-consuming [1—4]. A variety of analytical procedures has been proposed for the diagnosis and differentiation of the porphyrias, including thin-layer chromatography [5] and high-performance liquid chromatography (HPLC) [6—8]. Evans et al. [7] reported the HPLC separation of porphyrins as free carboxylic acids on an ion-exchange resin. Bonnett et al. [9] and Meyer et al. [10] used reversed-phase ion-pair HPLC for the separation of the urinary porphyrin carboxylic acids.

We present a method for the identification and quantitation of the urinary porphyrins, uro- (8-carboxyl) and copro- (4-carboxyl) porphyrin, as free carboxylic acids, together with some decarboxylation intermediates, by reversed-phase chromatography using a step gradient system with lithium citrate buffer and increasing methanol concentration. An internal standard, deuteroporphyrin, was used to correct for losses of porphyrins during extraction.

EXPERIMENTAL

Apparatus

The HPLC instrument consisted of a Waters Model 6000 pump (Waters Assoc., Milford, MA, U.S.A.) interfaced to a simple three-way solenoid valve (Lee Company, Westbrook, CT, U.S.A.), a U6K loop injector and a Waters Model 440 absorbance detector. The magnetic valve was used for step gradient elution and was controlled by a homemade two-periodic timer. The absorbance of the porphyrins was monitored at 400 nm. A reversed-phase column (300 mm × 4 mm I.D.), slurry-packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) was used for the analytical separation. Before

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use, all solvents were carefully vacuum filtered (Millipore Type GS 0.22-μm filters, (Millipore, Bedford, MA, U.S.A.). Air bubbles were removed by bubbling nitrogen through the solution.

Chemicals

Water was deionized and Milli-Q-filtered (Millipore) before use. Methanol, hydrochloric acid, calcium chloride, sodium hydroxide (analytical grade) and hemin were purchased from Merck. Trilithium citrate, analytical grade, was obtained from BDH Chemicals (Poole, Great Britain). The porphyrin standard, consisting of mesoporphyrin, coproporphyrin, penta-, hexa- and hepta-carboxylic acid substituted porphyrin, and uroporphyrin was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). Deuteroporphyrin was synthesized according to the procedure of Dinello [11]. The compound was dissolved in 0.1 *M* hydrochloric acid and stored at −70°C (when not in use).

Sample preparation

Urine samples were collected over 24 h in dark plastic bottles containing 5 g of sodium carbonate. A 2.5-ml aliquot of urine was mixed in a 10-ml screw-capped glass tube with 40 μl of deuteroporphyrin solution (48 nmol/l). The porphyrins were adsorbed on calcium hydroxide by adding 2.0 ml of 3% (w/v) calcium chloride and 4.0 ml of 1.0 *M* sodium hydroxide solution. After centrifugation and washing of the precipitate with 1 ml of 0.1 *M* sodium hydroxide, 100 μl of concentrated hydrochloric acid were added to each tube. It is important to check the pH (<2.0) of the solution to ensure that the porphyrins are completely protonated and dissolved. Urine specimens known to contain a high concentration of porphyrins were diluted 1:5 with normal urine, i.e. urine with non-detectable amounts of uro- and coproporphyrin.

The porphyrin standard solution containing porphyrin carboxylic acids (0.4 nmol/l each) was prepared by dissolving the freeze-dried standard substances in 100 μl of 0.1 *M* hydrochloric acid and 2.4 ml of normal urine. The solution was treated as above.

Chromatographic conditions

The column was first equilibrated with a buffer containing 0.05 *M* lithium citrate (pH 2.5)—methanol (30:70, v/v) (buffer 1). Fourteen minutes after sample injection, a buffer containing 0.05 *M* lithium citrate (pH 2.5)—methanol (5:95, v/v) (buffer 2) was introduced into the pump for 10 min. The solvent flow-rate was 1 ml/min and the detector sensitivity 0.05 absorbance units full scale (a.u.f.s.). A 25-μl volume of the sample or standard solution was injected to the column.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a standard mixture of uroporphyrin, hepta-, hexa- and pentacarboxylic acid porphyrin, and copro-, deutero- and meso-porphyrin. The acids are eluted in order of decreasing polarity. The retention time for the least-polar mesoporphyrin is 29 min. Buffer change from 1 to 2 is made after 14 min. A switch back to the equilibration buffer (buffer 1) is

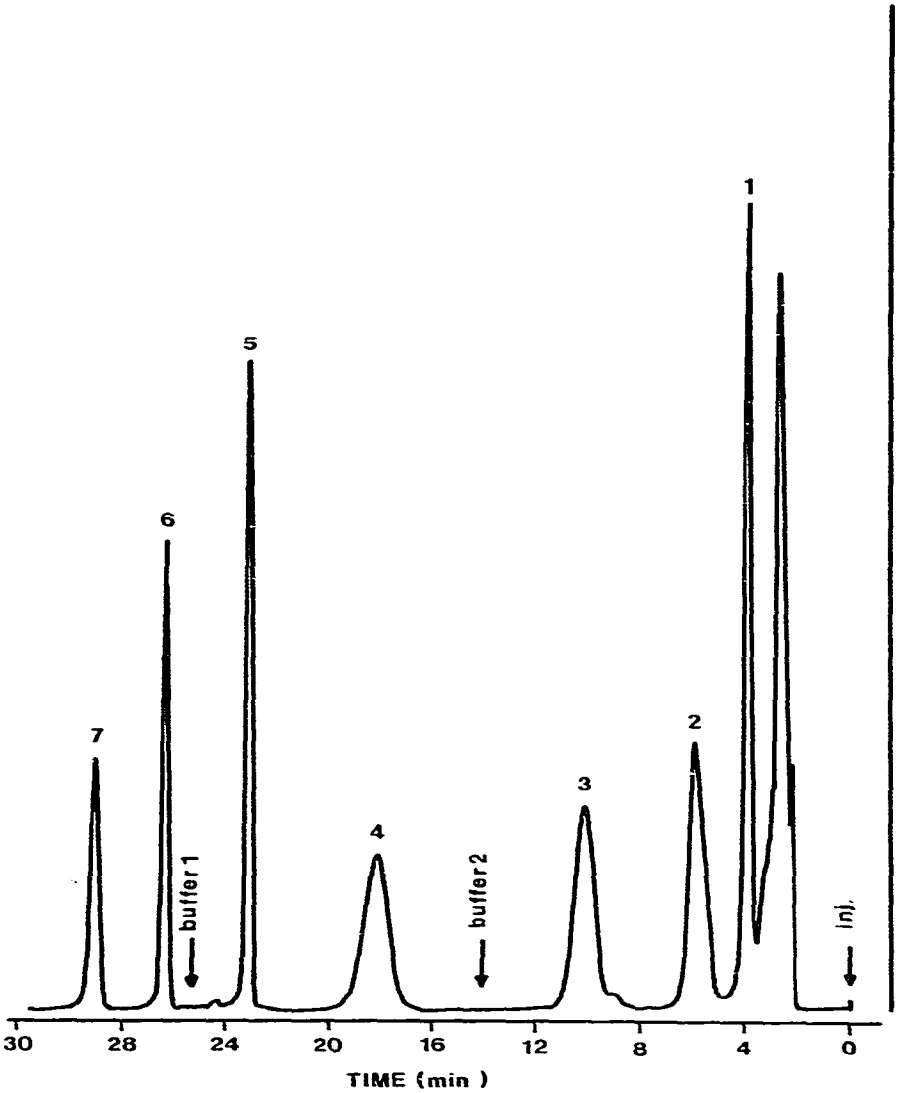

Fig. 1. HPLC separation of a porphyrin carboxylic acid standard mixture, containing uroporphyrin (1), hepta- (2), hexa- (3), and pentacarboxylic acid (4), and copro- (5), deutero- (6) and mesoporphyrin (7). A 10-μm LiChrosorb RP-18 column (300 mm × 4 mm I.D.) was used. Elution system: 0.05 *M* lithium citrate (pH 2.5)—methanol (30 : 70, buffer 1; and 5 : 95, buffer 2). Flow-rate 1 ml/min; absorbance monitored at 400 nm.

made after 25 min. Analysis of new samples can be started every half an hour. The retention times for the biologically most interesting porphyrins, uro- and coproporphyrin, are 4 and 23 min, respectively. It was found necessary to add an internal standard to obtain reproducible results if the analysis was not performed on the same day. A sample analysed one day, three days and one week after sampling gave a coefficient of variation (C.V.) of 1.2% for uroporphyrin and 5.5% for coproporphyrin using deuteroporphyrin as internal standard. Previous reports on HPLC analysis of porphyrins were based on the use of external standards.

Fig. 2 shows a chromatogram from the urine of a patient with porphyria cutanea tarda. Apart from high levels of uro- and coproporphyrin, trace levels of hepta-, hexa- and pentacarboxylic porphyrins were also detected but not quantitated. A linear correlation (r = 0.998, four points) was found between the area (height × width at half height) ratios of pure standard substances and internal standard vs. the uro- and coproporphyrin concentrations in the range 0.05—0.8 μmol/l (1.2 μmol/l deuteroporphyrin added). The standard preparations were treated in the same way as the urine samples. The mean recoveries of uro- and coproporphyrin are 99% and 97%, respectively.

Some of the samples were also analysed by Askevold's method [4]. Plots

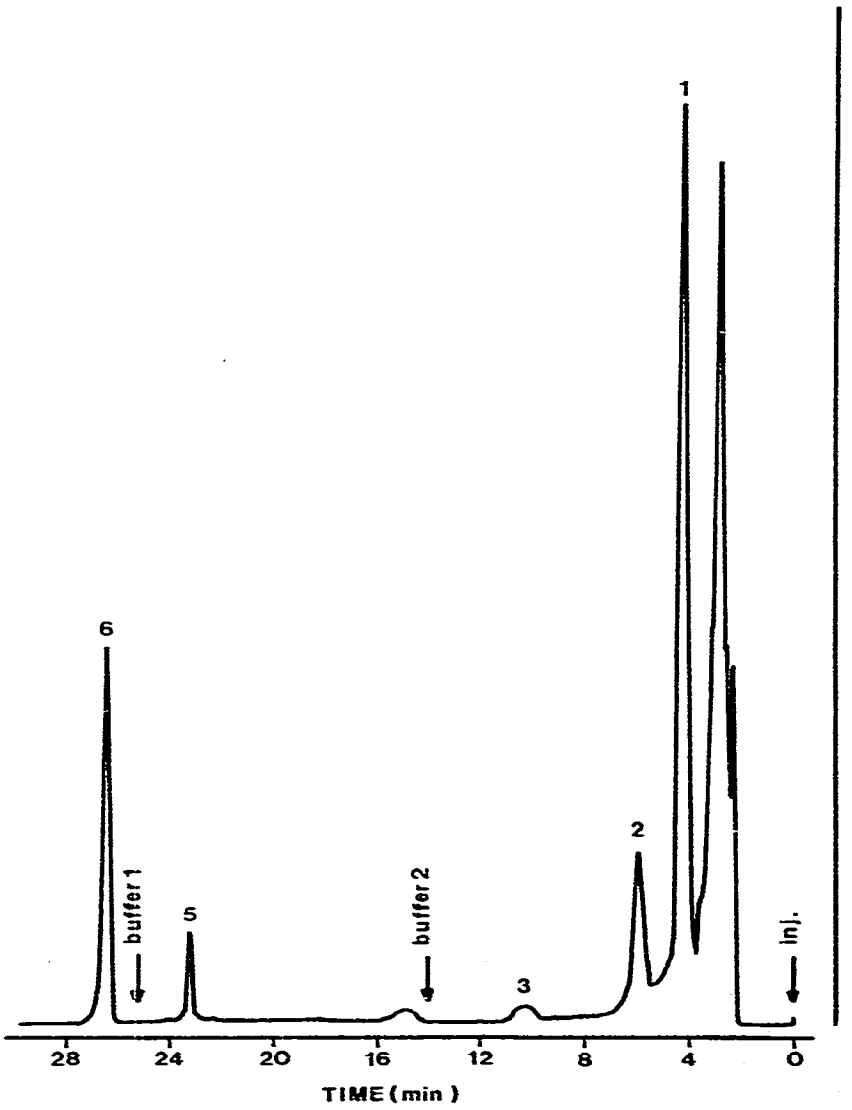

Fig. 2. HPLC separation of porphyrin carboxylic acids obtained from the urine of a patient with porphyria cutanea tarda. Column and elution system as in Fig. 1.

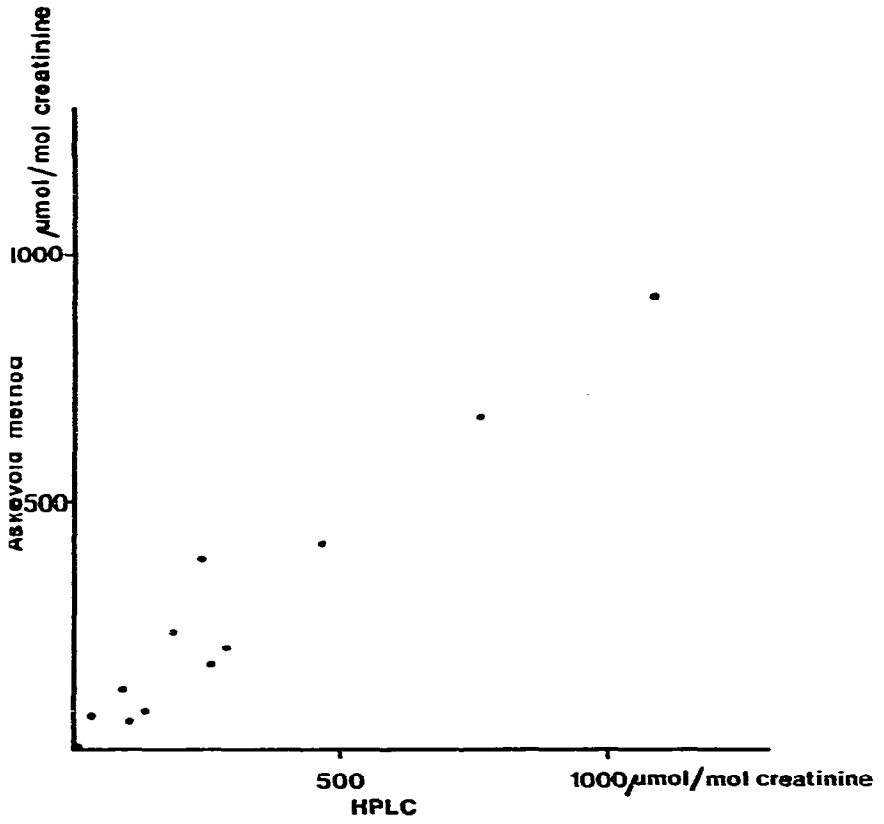

Fig. 3. Correlation of uroporphyrin concentration values, analysed by HPLC and by the method of Askevold.

between data obtained by our HPLC technique and Askevold's method are presented in Figs. 3 (uro-) and 4 (copro-). The molar values are related to creatinine excretion because some of the urines were not collected for 24 h. The correlations are good if the two points (marked with arrows) in the coproporphyrin plot are neglected. For this reason no correlation coefficients are presented. The deviations in µmol values for these latter coproporphyrin points are almost 100%. The HPLC traces of the corresponding urine samples revealed, apart from high uro- and coproporphyrin levels, also detectable levels of hepta- and hexacarboxylated products (see Fig. 2). It is most plausible that these compounds are co-determined in the Askevold method, giving rise to high concentration values and a large deviation in the correlation plot.

CONCLUSION

The described HPLC method offers a good alternative to the classical Askevold method as it is faster and more accurate, especially in cases where decarboxylation products appear.

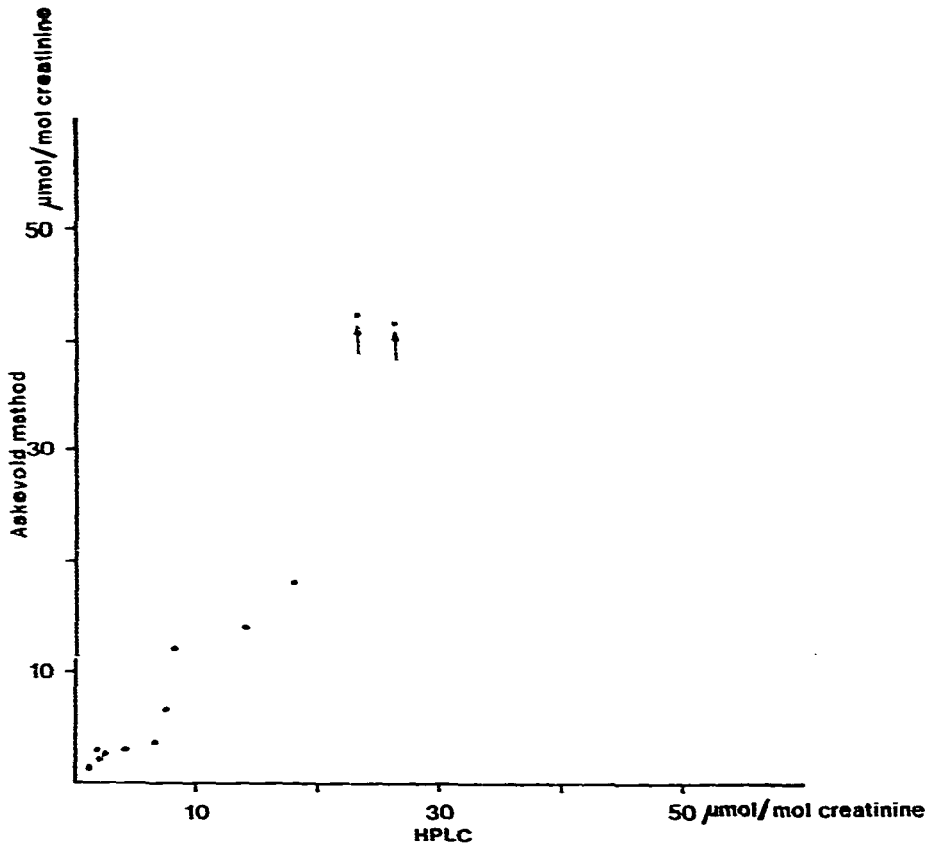

Fig. 4. Correlation of coproporphyrin concentration values, analysed by HPLC and by the method of Askevold.

REFERENCES

1 C.A. Martincz and G.C. Mills, Clin. Chem., 17 (1971) 199.
2 R.A. Cardinal, I. Bossenmaier, Z.J. Petryka, L. Johnson and C.J. Watson, J. Chromatogr., 38 (1968) 100.
3 Y. Grosser and L. Eales, S. Afr. Med. J., 47 (1973) 2162.
4 R. Askevold, Scand. J. Clin. Lab. Invest., 3 (1951) 318.
5 R.C. Scott, R.F. Labbe and J. Nutter, Clin. Chem., 13 (1967) 493.
6 Z.J. Petryka and C.J. Watson, Anal. Biochem., 84 (1978) 173.
7 N. Evans, D.E. Games, A.H. Jackson and S.A. Matlin, J. Chromatogr., 115 (1975) 325.
8 C.H. Gray, C.K. Lim and D.C. Nicholson, Clin. Chim. Acta, 77 (1977) 167.
9 R. Bonnett, A.A. Charalambides, K. Jones, I.A. Magnus and R.J. Ridge, Biochem. J., 173 (1978) 693.
10 H.D. Meyer, K. Jacob, W. Vogt and M. Knedel, J. Chromatogr., 199 (1980) 339.
11 K.R. Dinello and C.K. Chang, in D. Dolphin (Editor), The Porphyrins, Vol. 1, Academic Press, New York, 1978, p. 294.