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Note

Normal-phase high-performance liquid chromatography of porphyrin free acids on silica modified with tetraethylenepentamine

C.K. LIM* and JENNY Y.Y. CHAN

Division of Clinical Chemistry, MRC Clinical Research Centre, Harrow, Middlesex, HA1 3UJ (Great Britain)

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The biochemical investigation of the porphyrias has mainly depended on the determination of porphyrins in blood, urine and faeces. Much of the recent work on porphyrin analysis has been made possible by thin-layer chromatography (TLC) but high-performance liquid chromatography (HPLC) has also been used and has superior resolving power and with suitable detectors, greater sensitivity. The porphyrins are usually analysed as methyl esters [1—3] but HPLC separations of porphyrin free acids [4—12] by reversed-phase or reversed-phase ion-pair chromatography have also been reported. Most of the published methods for the porphyrin free acids, however, were insufficient for the resolution of some of the porphyrins important for the differentiation of the porphyrias.

This paper describes a novel HPLC system for the separation of porphyrin free acids using normal-phase chromatography with in situ modification of the silica with tetraethylenepentamine (TEPA). Effective separation of all the clinically important porphyrins including isocoproporphyrin and coproporphyrin I and III isomers is achieved by gradient elution with acetonitrile containing 0.05% TEPA and water containing 0.05% TEPA as the gradient mixture. The method, successfully applied to the analysis of porphyrins extracted from urine and faeces of porphyric patients, is suitable for the differential diagnosis of the porphyrias.

EXPERIMENTAL

Materials and reagents

Acetonitrile was HPLC grade from Rathburn Chemicals (Walkerburn, Great Britain). TEPA was technical grade; chloroform, methanol, ethyl acetate, dimethyl sulphoxide, glacial acetic acid, sodium acetate trihydrate, concen-

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trated hydrochloric and sulphuric acids, all AnalaR grade, and talc (acid washed) were from BDH (Poole, Great Britain). Porphyrin standards were isolated from the faeces of a patient with porphyria cutanea tarda symptomatica (PCT) as methyl esters as previously described [13]. The methyl esters were hydrolysed individually by dissolving in a mixture of concentrated hydrochloric acid (1 drop) and glacial acetic acid (2 drops) and left in the dark overnight. The solution was dried in a vacuum desiccator. The residue was dissolved in dimethyl sylphoxide for HPLC separation.

Sample preparation

Urine. The pH of urine was adjusted to 3.5 with 2 *M* hydrochloric acid. Talc (about 500 mg) was added in batches to 10 ml of urine and thoroughly mixed. The talc was removed by filtration in a small Büchner funnel. After washing with water the adsorbed porphyrins were eluted from the talc with a solution of water—acetonitrile—TEPA (80 : 20 : 0.1, v/v) for HPLC analysis.

Faeces. Concentrated hydrocloric acid (2 ml) was added to about 0.5 g of wet faeces in a centrifuge tube. The particles were broken by mixing in a vortex mixer. Diethyl ether (6 ml) was added and the mixture was again vortex mixed. After thorough shaking with 6 ml of water the mixture was centrifuged. The aqueous acid layer was removed and the pH was adjusted to 3.5 with solid sodium acetate trihydrate. Talc was then added in batches until all porphyrins were adsorbed. The porphyrins were recovered as described for urine.

High-performance liquid chromatography

A Varian 5000 liquid chromatograph with a UV-50 variable-wavelength detector set at 400 nm was used. Injection was via a Rheodyne 7125 injector fitted with a 20-μl loop. The separation was performed on a 10 cm × 5 mm I.D. Hypersil column (5 μm spherical silica) (Shandom Southern Products, Runcorn, Great Britain). The solvents for the gradient elution were acetonitrile containing 0.05% v/v TEPA (solvent A) and water containing 0.05% v/v TEPA (solvent B). The column was equilibrated with 80% solvent A (20% solvent B) before the sample (20 μl) was injected. Porphyrins were eluted with a 20-min linear gradient from 80% A (20% B) to 30% A (70% B). The flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

Silica columns with in situ coating with TEPA have been described for the separation of sugars [14, 15] and bile pigments [16]. For sugars the normal-phase (adsorption) mechanism [15] operated and for bile pigments probably a mixed mechanism was involved, with adsorption, partition and ion-pairing [16]. The porphyrins, especially heptacarboxylic porphyrin and uroporphyrin (with eight carboxylic acid groups) are easily ionizable compounds. Their retention and elution from a silica column would therefore suggest ion-pair or complex formation with TEPA. The ion-pairs or complexes are eluted in the order expected for normal-phase chromatography. It is likely, however, that a mixed mechanism operates here.

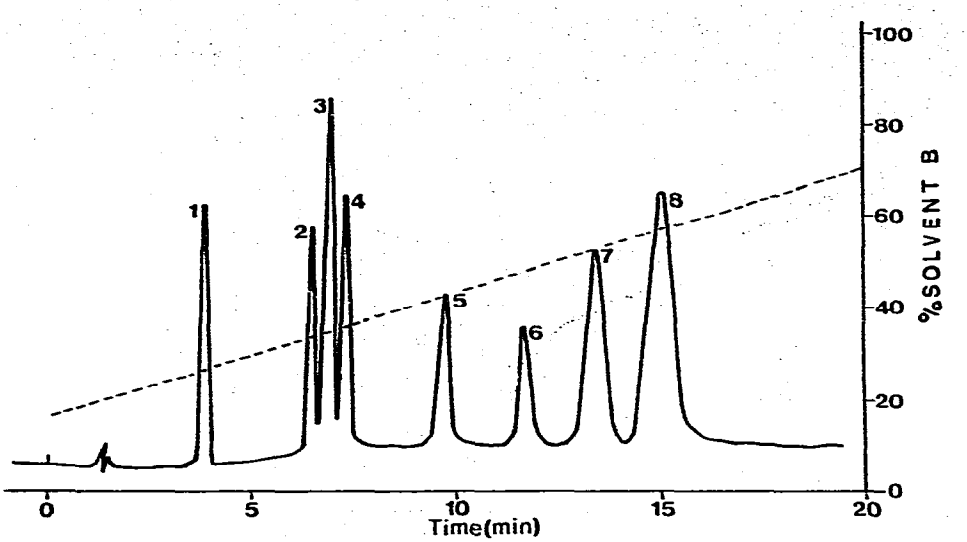

Fig. 1. HPLC separation of porphyrin standards. Peaks: 1, protoporphyrin; 2, coproporphyrin III; 3, coproporphyrin I; 4, isocoproporphyrin; 5, pentacarboxylic porphyrin; 6, hexacarboxylic porphyrin; 7, heptacarboxylic porphyrin; 8, uroporphyrin. Column, Hypersil (10 cm × 5 mm); solvent A, acetonitrile containing 0.05% v/v TEPA; solvent B, water containing 0.05% v/v TEPA; elution, 20-min linear gradient from 20% B to 70% B; flow-rate, 1 ml/min; detection, 400 nm.

Fig. 1 shows the separation of a standard porphyrin mixture using the present HPLC system. All the clinically important porphyrine, including protoporphyrin, coproporphyrin I and III, isocoproporphyrin, heptacarboxylic porphyrin and uroporphyrin were separated in less than 20 min. This is comparable to or better than other systems [4—12] described for the separation of porphyrin free acids. The improvement in the resolution of coproporphyrin I and III from isocoproporphyrin and heptacarboxylic porphyrin from uroporphyrin, however, is important for the differentiation of PCT from other forms of porphyrias since the disease is characterised [2] by the presence of isocoproporphyrin in the faeces and excess heptacarboxylic porphyrin and uroporphyrin in both urine and faeces (Fig. 2a and b).

The suitability of the present method for porphyrin profiles and differential diagnosis of the porphyrias is furhter demonstrated by the analysis of faecal and urinary porphyrins from a patient with congenital erythropoietic porphyria (EP) as shown in Fig. 3a and b. The porphyrin profiles were typical [2] of the disease, in which there was considerable amount of coproporphyrin with small quantities of other porphyrins in the faeces (Fig. 3a) and excessive uroporphyrin and coproporphyrin together with smaller amounts of penta-, hexa- and heptacarboxylic porphyrins in the urine (Fig. 3b).

The separation of porphyrin free acids permits rapid analysis of urinary and faecal porphyrins, an improvement on methods requiring esterification before HPLC separation. However, esterification followed by solvent extraction of porphyrin methyl esters into chloroform is a convenient way of isolating porphyrins from biological materials. Thus a simple method for the isolation

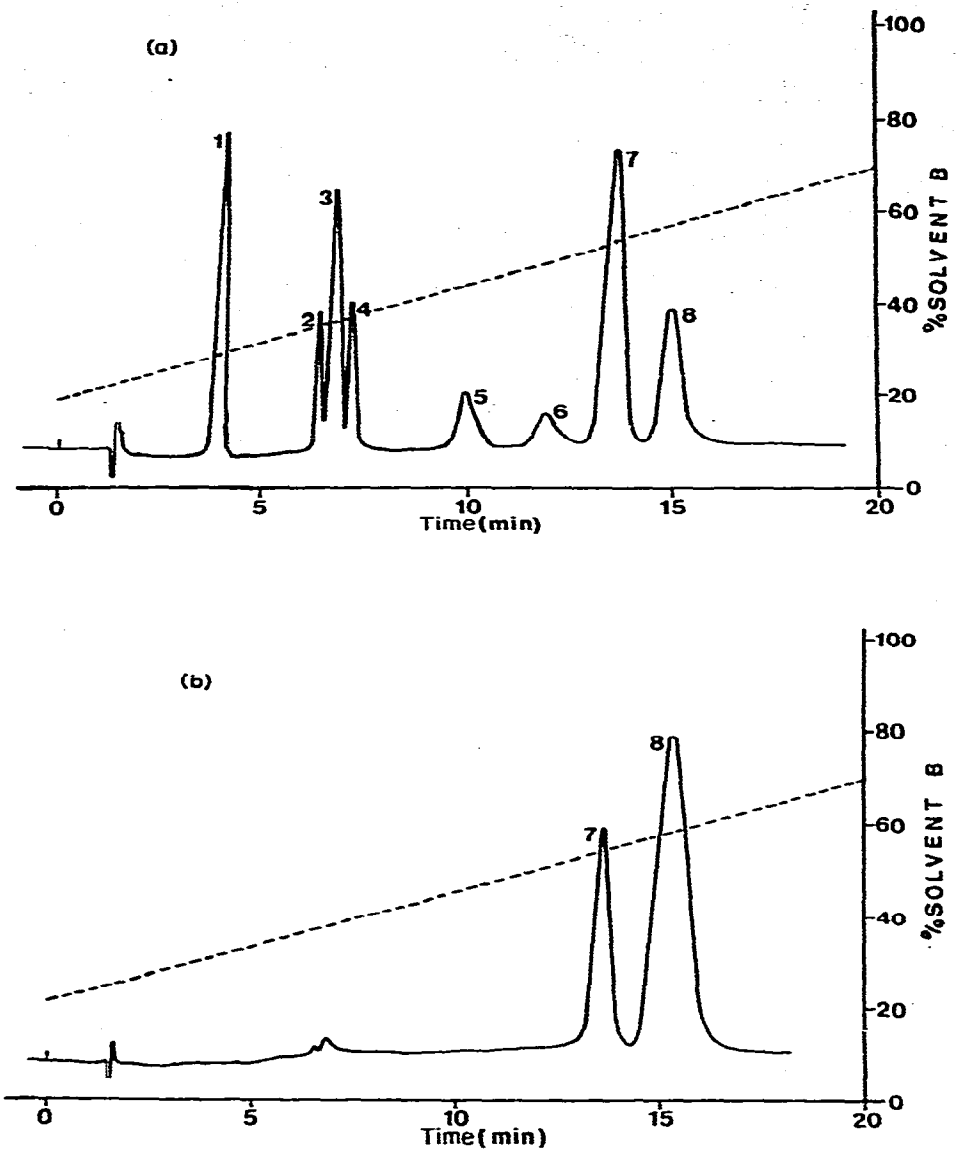

Fig. 2. HPLC separation of porphyrins from (a) faeces and (b) urine of a patient with porphyria cutanea tarda symptomatica. Peaks and HPLC conditions as in Fig. 1.

of porphyrin free acids in a suitable form for HPLC is needed. For this we used the talc method which is both simple and effective. The recoveries of proto-, copro- and uroporphyrin were greater than 95%. The present HPLC method coupled with the fast and simple extraction procedure therefore appears suitable for the routine analysis of both urinary and faecal porphyrins.

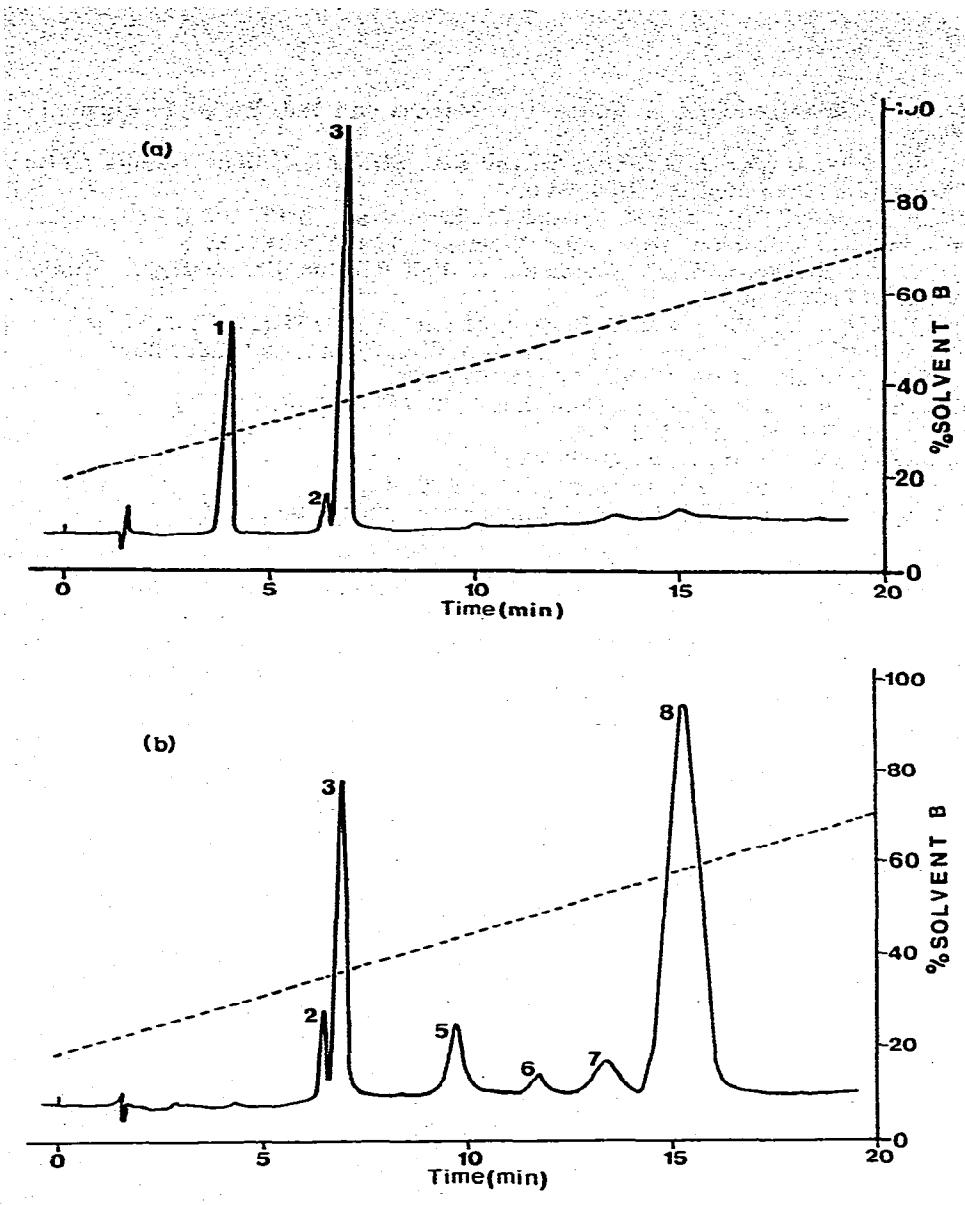

Fig. 3. HPLC separation of porphyrins from (a) faeces and (b) urine of a patient with congenital erythroppoietic porphyria. Peaks and HPLC conditions as in Fig. 1.

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REFERENCES

1 N. Evans, A.H. Jackson, S.A. Matlin and R. Towill, J. Chromatogr., 125 (1976) 345.
2 C.H. Gray, C.K. Lim and D.C. Nicholson, Clin. Chim. Acta, 77 (1977) 167.
3 V. Miller and L. Malina, J. Chromatogr., 145 (1978) 290.

4 R. Bonnet, A.A. Charalambides, K. Jones, I.A. Magnus and R.J. Ridge, Biochem. J., 173 (1978) 693.
5 E. Englert, Jr., A.W. Wayne, E.E. Wales, Jr. and R.C. Straight, J. High Resolut. Chromatogr. Chromatogr. Commun., 2 (1979) 570.
6 H.D. Meyer, K. Jacob, W. Vogt and M. Knedel, J. Chromatogr., 199 (1980) 339.
7 M.O. Longas and M.B. Poh-Fitzpatrick, Anal. Biochem., 104 (1980) 268.
8 R.H. Hill, Jr., S.L. Sirmans and L.L. Needham, J. High Resolut. Chromatogr. Chromatogr. Commun., 3 (1980) 588.
9 R.M. Smith, D. Doran, M. Mazur and B. Bush, J. Chromatogr., 181 (1980) 319.
10 G.R. Gotelli, J.H. Wall, P.M. Kabra and L.J. Marton, Clin. Chem., 26 (1980) 205.
11 M. Salmi and R. Tenhunen, Clin. Chem., 26 (1980) 1832.
12 R.E. Ford, C.N. Ou and R.D. Ellefson, Clin. Chem., 27 (1981) 397.
13 G.H. Elder, J. Clin. Pathol., 28 (1975) 601.
14 K. Aitzetmüller, J. Chromatogr., 156 (1978) 354.
15 B.B. Wheals and P.C. White, J. Chromatogr., 176 (1979) 421.
16 C.K. Lim, R.V.A. Bull and J.M. Rideout, J. Chromatogr., 204 (1981) 219.