Journal of Chromatography, 123 (1976) 414–418

CHROM. 9083

Note

Rapid method for the quantitative determination of porphyrin methyl esters by high-pressure liquid chromatography

P. HÖRCHNER and T. RIETVELD

Department of Internal Medicine II, Erasmus University of Rotterdam, Faculty of Medicine, P.O. Box 1738, Rotterdam (The Netherlands)

(Received February 3rd, 1976)

The separation and determination of the various porphyrins is of clinical importance for the diagnosis of the type of porphyria, but it can also be applied for the investigation of porphyrin metabolism. Thin-layer chromatography (TLC) on silica gel, after extraction and methylation of the porphyrins, is the method usually employed. The individual porphyrins can be determined spectrophotometrically after elution from the silica gel by use of their characteristic absorption peaks at *ca.* 400 nm[1].

Because of the rapid development of high-pressure liquid chromatography (HPLC) during the last few years, new separation methods have become available. In comparison to TLC, HPLC has the advantage of giving a better separation in a considerably shorter time. Therefore we decided to investigate whether HPLC could be used for the separation and determination of porphyrin methyl esters and whether it could provide a useful alternative for the common TLC–spectrophotometric determination.

EXPERIMENTAL

Apparatus

A gradient mixer (Type 11300; LKB, Bromma, Sweden), which has been described in detail elsewhere[2], was programmed to produce a linear gradient of two liquids. The mixer was designed primarily to generate gradients over a long period of time (for use in conventional column chromatography), but it can also be used for HPLC work, although the latter type of analysis is performed in a much shorter time. The gradient mixer was connected to a liquid pump (Type CMP 2; Chromatronix, Berkeley, Calif., U.S.A.), which could deliver a maximum pressure of 500 p.s.i.g. and a reasonable pulse-free liquid flow. Essentially, the pump is of the double-piston type, whose pistons deliver liquid alternately and which has a third piston to damp pulses which are generated during switching from the first to the second piston. Because of its relatively large inner volume, this pump is not ideal for gradient work, but in view of the results obtained is satisfactory. A pressure gauge was placed in a side-line of the system.

Glass columns (25 cm × 2.0 mm I.D.) were used. Injection was carried out

by means of an injection valve (Chromatronix, Type SV-8031), which contained a sample loop with a volume of 21.5 μl. All of the connections and fittings were made from inert material such as PTFE, Kel-F or Delrin (Chromatronix). The effluent was monitored by means of a spectrophotometer (Type PMQ II, Carl Zeiss, Oberkochen, G.F.R.) which was equipped with a cylindrical cell of 10-mm light path and 8-μl capacity (Zeiss, Type MR 1 D). The instrument was connected by means of a lin–log converter (Zeiss, Type TKR) to a potentiometric recorder. The peak areas were determined by means of a simple but adequate electronic integrator (Type BC 1; Kipp & Zonen, Delft, The Netherlands).

Materials

Columns were packed with Merckosorb SI 100 (30 μm) (E. Merck, Darmstadt, G.F.R.) by gentle tapping. Protoporphyrin-IX dimethyl ester (PPMe), coproporphyrin-I tetramethyl ester (CPMe) and uroporphyrin-I octamethyl ester (UPMe) were purchased from Sigma (St. Louis, Mo., U.S.A.); their purity was always better than 90%, as determined spectrophotometrically by means of the molar extinction coefficients[1]. All of the liquids were of reagent-grade quality and were used without further purification.

Procedure

In order to condition the column, 20% tetrahydrofuran in heptane (liquid A) was pumped through the column for 15 min. The pumping speed was always 2.0 ml/

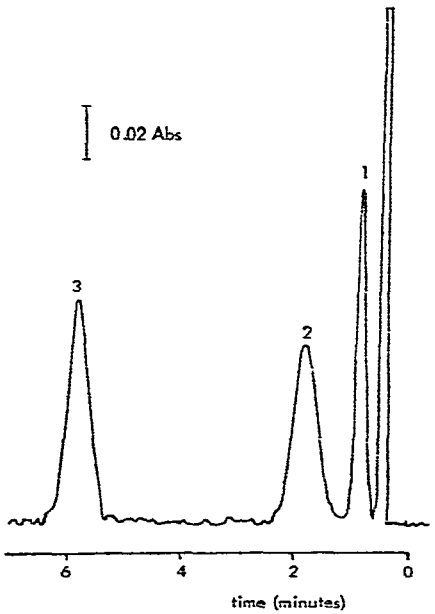

Fig. 1. Recording of a separation of porphyrin methyl ester standards. Sample size, 21.5 μl; column (25 cm × 2.0 mm) packing, Merckosorb SI 100 (30 μm); mobile phase, linear gradient from 20 to 50% tetrahydrofuran in heptane; flow-rate, 2.0 ml/min; column pressure, 375 p.s.i.g.; detector (photometer) sensitivity, 0.2 absorbance units full scale; wavelength, 400 nm. Peaks: 1 = protoporphyrin methyl ester (PPMe); 2 = coproporphyrin methyl ester (CPMe); 3 = uroporphyrin methyl ester (UPMe).

min, which generated a pressure drop of *ca.* 375 p.s.i.g. over the column and a flow velocity of 1.1 cm/sec. A linear gradient, from 20% tetrahydrofuran in heptane (A) to 50% tetrahydrofuran in heptane (B) in 7 min, was started directly after injection of the sample. The spectrophotometer was set at 400 nm. When a run had been completed, the column was regenerated by pumping liquid A through the column for 5 min. The total time needed for one analysis was only 11–12 min.

RESULTS AND DISCUSSION

In the preliminary investigations we tried various mixtures of chloroform and heptane as the eluent. A liquid gradient appeared to be necessary in order to elute all of the porphyrin methyl esters in a reasonable time since their polarities differ greatly. With the apparatus used, gradients of chloroform in heptane produced a strongly pulsating baseline, presumably as a result of inadequate mixing of the primary solutions. Therefore we replaced chloroform by tetrahydrofuran. However, this caused an injection problem. In HPLC, the samples to be injected are often dissolved in a liquid of similar composition to that of the starting eluent, in this case 20% tetrahydrofuran in heptane. Generally, porphyrin methyl esters have only a low solubility in tetrahydrofuran–heptane mixtures, therefore it was necessary to use the porphyrin esters in a chloroform solution, which restricted the volume

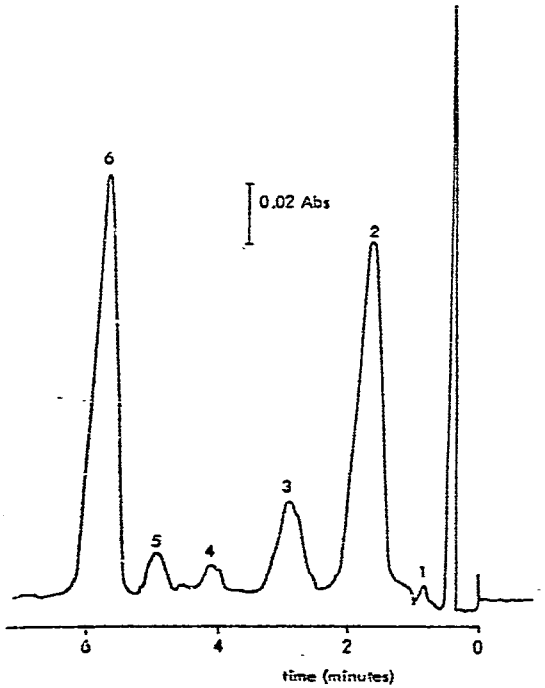

Fig. 2. Chromatogram of porphyrin methyl esters extracted from an urine sample of a patient with acute intermittent porphyria. Chromatographic conditions were the same as in Fig. 1, the detector sensitivity of peak 6 being 0.5 absorbance units full scale. Peaks: 1 = PPMe; 2 = CPMe; 3 = pentacarboxylicporphyrin methyl ester; 4 = hexacarboxylicporphyrin methyl ester; 5 = heptacarboxylicporphyrin methyl ester; 6 = UPMe.

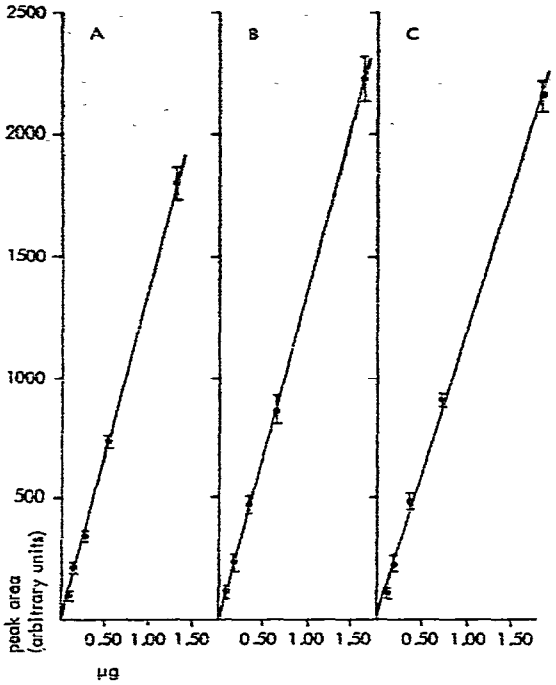

Fig. 3. Graphs of the relationship between the peak area and the injected quantity of PPMe (A), CPMe (B) and UPMe (C).

of the sample that could be injected. The 21.5-μl volumes which we usually applied to the column did not give problems, but injection of 50 μl resulted in a severely disturbed chromatogram.

PPMe and CPMe were separated adequately by 20% tetrahydrofuran in heptane, but UPMe has a capacity factor, k', of 60 in this eluent, which is unfavourable. A linear gradient of 20–50% tetrahydrofuran in heptane provided a good separation of all of the components of the mixture within 6 min (Fig. 1). However, one

TABLE I

REPEATABILITY OF THE ANALYSIS OF PORPHYRIN METHYL ESTERS BY HPLC

Each value represents the mean of five separate measurements of samples taken from the same solution.

Protoporphyrin methyl ester		*Coproporphyrin methyl ester*		*Uroporphyrin methyl ester*	
Amount injected (μg)	*Standard deviation (% of the mean peak area)*	*Amount injected (μg)*	*Standard deviation (% of the mean peak area)*	*Amount injected (μg)*	*Standard deviation (% of the mean peak area)*
1.29	4	1.60	4	1.83	3
0.52	1	0.64	5	0.73	2
0.26	1	0.32	4	0.37	5
0.13	4	0.16	6	0.18	3
0.06	9	0.08	14	0.09	6

has to keep in mind that the choice of this particular gradient was partly determined by the time (*ca.* 1.5 min) needed for the front of the gradient to reach the top of the column. This delay was caused by the relatively large dead volume of the inlet port preceding the column. This means that in a different set-up a slightly different gradient may be necessary. With the use of a steeper gradient the retention time of UPMe could be shortened further, but in this case it was impossible to separate methyl esters of porphyrins with 5, 6 and 7 methyl groups. These compounds occur in relatively small quantities in biological fluids. An example of a separation of porphyrin methyl esters from an urine sample (pathological) is shown in Fig. 2.

In Fig. 3 is shown the linear relation which exists between the peak area and the injected quantity over a range of at least 0.1–1.3 μg, for PPMe, CPMe and UPMe (higher concentrations were not measured). The standard deviation varied between 1 and 6% (Table I). Thus the repeatability of the HPLC separation is comparable to that of TLC separation followed by spectrophotometric determination[1], except for the lowest measured concentration. The total recovery in the eluent of a mixture of porphyrin methyl esters was always between 95 and 100%.

In conclusion, the method described above offers a significant reduction in analysis time compared with the method hitherto used. Moreover, the procedure is simple to perform routinely.

ACKNOWLEDGEMENT

We gratefully acknowledge the assistance of Dr. J. W. O. van den Berg in the preparation of the manuscript.

REFERENCES

1 M. Doss, *Z. Klin. Chem. Klin. Biochem.*, 8 (1970) 197.
2 K. Keck, *Anal. Biocnem.*, 39 (1971) 288.