Journal of Chromatography, 145 (1978) 290—294
Biomedical Applications

CHROMBIO. 111

Note

High-performance liquid chromatographic analysis of biologically important porphyrins

VLADIMÍR MILLER

Research Institute for Pharmacy and Biochemistry, 130 60 Prague 3 (Czechoslovakia)

and

LUBOR MALINA

Dermatological Clinic, Medical Faculty of Hygiene, Charles University, Prague 10 (Czechoslovakia)

(Received July 12th, 1977)

Determination of biologically important porphyrins and their quantitative analysis in tissues and body fluids plays a significant role in the precise classification of the various types of porphyria in clinical practice.

Until the present time, thin-layer chromatography has been considered the best laboratory separation method for the determination of these compounds in biological materials. It has usually been employed after extraction and methylation, with subsequent elution from the silica gel and spectrophotometric detection [1]. Though this procedure does not require any special instrumental equipment, the performance is usually lengthy and elution of the spots can cause experimental errors as well.

Therefore, high-performance liquid chromatography (HPLC) has recently been utilized for the quantitation of porphyrins, giving increased sensitivity and separation in a considerably shorter time. In all communications published so far, the resolution of porphyrins with 2—8 carboxylic groups has been carried out, after conversion to methyl esters, by adsorption chromatography on silica gel columns using gradient elution [2,3] or programmed flow-rate [4,5] with spectrophotometric detection. In the last paper, a considerable increase in sensitivity was achieved by use of a fluorescence detector [6].

A decrease in the precision of porphyrin analyses can occur, especially in

less concentrated samples, owing to spontaneous formation of copper (II) porphyrin chelates, observed in thin-layer chromatography [7] and HPLC [4] chromatograms and identified by mass spectrometry [4].

In this paper, a chemically bonded stationary phase was used with isocratic elution for the separation of porphyrin methyl esters which were converted to their copper (II) chelates with good chromatographic qualities and increased precision and sensitivity.

EXPERIMENTAL

Apparatus and conditions

A Varian (Palo Alto, Calif., U.S.A.) Model 8500 liquid chromatograph, with a pulseless piston pump, septumless injector and spectrophotometric detector (Variscan) operating at 400 and 402 nm, was used.

A stainless-steel column (25 cm × 0.2 cm I.D.) was packed with MicroPak CN, 10 μm (Varian), and the mobile phase, consisting of ethyl acetate — *n*-heptane — isopropanol (40:60:0.5), was pumped through at a flow-rate 50 or 80 ml/hour. Pressure, 70 kg/cm^2 ; chart speed 0.5 cm/min.

Chemicals

All solvents were of reagent-grade quality or for use in spectrophotometry. Standards of protoporphyrin-IX dimethyl ester, coproporphyrin-I tetramethyl ester and uroporphyrin-I octamethyl ester were obtained from Sigma (St. Louis, Mo., U.S.A.).

Sample preparation from urine

The method of Doss [8] was employed, with adsorption on talc, esterification with 5% sulphuric acid in methanol, and extraction with chloroform.

Preparation of cupric porphyrin chelates

The procedure of Doss [8] was modified as follows. A tenfold molar excess of the chelating agent (0.1% solution of copper (II) acetate in chloroform — methanol (19:1)) was added to porphyrins which had been isolated and esterified as described above. The sample was kept for 60 min at laboratory temperature and than evaporated to dryness. The residue was dissolved in a known volume of chloroform and injected into the column.

RESULTS AND DISCUSSION

Under the chromatographic conditions described abcve the mixture of porphyrin methyl esters with 2—8 carboxylic groups was successfully separated on a new column with polar chemically bonded stationary phase. This column did not require lengthy washing with the mobile phase in order to obtain a correct initial equilibrium, as is the case with silica gel columns. The period necessary for the resolution of porphyrins with 2—8 carboxylic groups using isocratic elution did not exceed 16 min (flow-rate 50 ml/h). The separation of porphyrins with 4—8 carboxylic groups required only 10 min (flow-rate 80 ml/h).

Fig. 1 shows the separation of five porphyrins occurring in a urine extract, the retention times of which increase with increasing number of carboxylic groups in the porphyrin molecule. Besides the five main elution peaks of the corresponding porphyrins, the chromatogram shows additional interfering peaks, with shorter elution times, which in some cases significantly reduce the precision of the quantitative evaluation. It is known from literature data [7], and mass spectrometry has proved [4], that these small peaks belong to copper (II) complexes which result from the reaction of porphyrins with copper (II) ions contained in the chemicals used. Chloroform [4] and distilled water [7] used in the preparation of samples of clinical materials were stated as possible sources of these ions.

The interference of copper (II) complexes was low and virtually negligible in clinical samples that had been analysed immediately after extraction and methylation. In the case of a longer time interval (several days), or in samples with a low porphyrin content, the formation of complexes was manifested in a significant way. This was the reason for the additional treatment of the samples before the actual chromatographic analysis. Considering the fact that complexed methyl esters of porphyrins have similar chromatographic properties to free compounds, the methyl esters of porphyrins were purposely converted to their complexed analogues which were then chromatographed. Under the same chromatographic conditions, the mixture of porphyrin chelates with 4—8 carboxylic groups was entirely separated within a time interval that was even shorter than that of non-complexed compounds. Intentional conversion of free methyl esters of porphyrins to copper (II) chelates resulted at the same time in an increase in sensitivity of the analysis, since the millimolar absorption

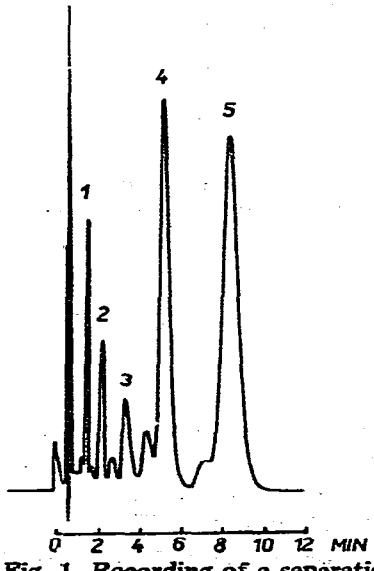

Fig. 1. Recording of a separation of porphyrin methyl esters from a urine sample of a porphyric patient. 1, coproporphyrin methyl ester; 2, pentacarboxylicporphyrin methyl ester; 3, hexacarboxylicporhyrin methyl ester; 4, heptacarboxylicporphyrin methyl ester; 5, uroporphyrin methyl ester. Detection at 402 nm.

coefficients of chelated porphyrins are on average more that 50% higher than non-complexed porphyrins [8].

Fig. 2 shows two chromatograms of a week-old extract of porphyric urine before (A) and after (B) conversion to copper (II) complexes. It is evident that chelation of the sample has yielded a better chromatogram with increased sensitivity of the analysis.

The above findings have been utilized in the detection and determination of porphyrins in clinical samples of skin of porphyric patients in which the porphyrin levels lie in the nanogram region [9].

Possible removal of contaminating trace amounts of metal ions and utilization of complexed porphyrins in the HPLC analysis clinical materials are the subject of our next studies.

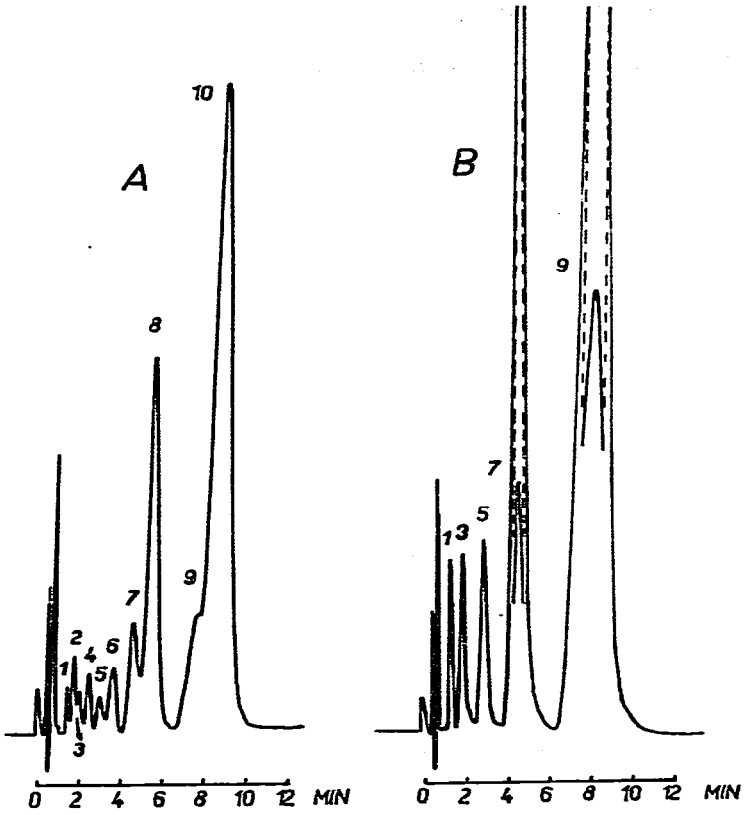

Fig. 2. Chromatogram of porphyrin methyl esters from a week-old sample of porphyric urine before (A) and after (B) conversion to cupric complexes. 1, cupric chelate of coproporphyrin methyl ester; 2, coproporphyrin methyl ester; 3, cupric chelate of pentacarboxylicporphyrin methyl ester; 4, pentacarboxylicporphyrin methyl ester; 5, cupric chelate of hexacarboxylicporphyrin methyl ester; 6, hexacarboxylicporphyrin methyl ester; 7, cupric chelate of heptacarboxylicporphyrin methyl ester; 8, heptacarboxylicporphyrin; 9, cupric chelate of uroporphyrin methyl ester; 10, uroporphyrin methyl ester. Detection at 400 nm.

ACKNOWLEDGEMENT

The authors thank Mrs. Hejlova for technical assistance.

REFERENCES

1 M. Doss and W. Mannheim, Z. Klin. Chem. Klin. Biochem., 5 (1967) 260.
2 N. Evans, D.E. Games, A.H. Jackson and S.A. Matlin, J. Chromatogr., 115 (1975) 325.
3 P. Horchner and T. Rietveld, J. Chromatogr., 123 (1976) 414.
4 N. Evans, A.H. Jackson, S.A. Matlin and R. Towill, J. Chromatogr., 125 (1976) 345.
5 C.H. Gray, C.K. Lim and D.C. Nicholson, in P.F. Dixon, C.H. Gray, C.K. Lim and M. S. Stoll (Editors), High Pressure Liquid Chromatography in Clinical Chemistry, Porphyrin Profiles in Porphyrias, Proceedings of a Symposium held at King's College Hospital Medical School, London, December 15—16, 1975, p. 79—85.
6 R.F. Adams, W. Slavin and A.R. Williams, Chromatogr. Newsl. 4, No. 2 (1976) 24.
7 T.K. With, Ann. Clin. Res., 8, Suppl. 17 (1976) 9.
8 M. Doss, Anal. Biochem., 39 (1971) 7.
9 L. Malina, V. Miller and I.A. Magnus, Clin. Chim. Acta, in press.