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THE DETERMINATION OF UROPORPHYRINOGEN DECARBOXYLASE IN TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO FLUORESCENCE DETECTION

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SUMMARY

High-performance liquid chromatography coupled to fluorescence detection was utilized for the separation and quantitation of porphyrins as methyl esters. The method (developed for biochemical investigation of porphyrias) permitted quantitation down to 0.2 nanograms of porphyrins per sample. One of the possible applications is the study of the enzyme uroporphyrinogen decarboxylase. No significant difference was found between two methods of methylation and extraction of the samples prior to chromatography.

INTRODUCTION

Porphyrins are tetrapyrrolic compounds synthesized in a variety of biological tissues. They differ in the type and number of carboxylic side-chains.

Disturbances of porphyrin metabolism attributed to drugs, environmental contaminants and genetic errors [1—3] have been described. In many of these situations it is necessary to measure the porphyrin content of biological fluids and tissues.

High-performance liquid chromatography (HPLC) of porphyrin mixtures, both methylated [4—6] and as free acids [7], has been developed with advantages of accuracy and time required when compared with the widely used thin-layer chromatographic (TLC) separation of porphyrin methyl esters. The detection is usually by a UV detector, so sensitivity remains a major problem because in many experimental models using tissue culture [8] or human biopsy material [9], the porphyrins present are in the nanogram range. In these situations radioactive methods were often used [9].

This paper reports an analytical technique utilizing HPLC with fluorometric detection, which permits the quantitation of porphyrins in subnanogram

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amounts. The method, developed for the biochemical investigation of porphyrias, was applied to the determination of the activity of the enzyme uroporphyrinogen decarboxylase which is markedly affected in the liver of patients with porphyria cutanea tarda and of animals intoxicated with porphyrogenic compounds [2, 9].

EXPERIMENTAL

Chemicals

Mesoporphyrin IX dimethyl ester, protoporphyrin IX dimethyl ester, coproporphyrin III tetramethyl ester, pentacarboxylporphyrin I pentamethyl ester, hexacarboxylporphyrin I hexamethyl ester, heptacarboxylporphyrin I heptamethyl ester, and uroporphyrin III octamethyl ester were purchased from Porphyrin Products (Logan, UT, U.S.A.). Ethyl acetate and *n*-heptane (reagent grade) were obtained from Farmitalia Carlo Erba (Milan, Italy). Solvents were filtered under vacuum through a polycarbonate membrane (0.4 μm) (Nucleopore, Pleasanton, Canada) before use. Chloroform (Aristar grade) was purchased from BDH (Poole, Great Britain) and was always washed with distilled water before use.

Apparatus and conditions

The high-pressure liquid chromatograph was a Model Series 3 microprocessor-controlled pump module (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne 7105 injector supplied with 175-μl sample loop injector (Rheodyne, Berkeley, CA, U.S.A.). The column (25 × 0.26 cm I.D.) was packed with Silica A 10 μm (Perkin-Elmer).

A Model 3000 fluorescence spectrometer (Perkin-Elmer) was used with the excitation wavelength set at 404 nm (slit width 10 nm) and the emission wavelength at 623 nm (slit width 10 nm). The attenuation of the fluorescence spectrometer was varied according to the expected porphyrin concentration. The UV detector was a Model LC 56 B (Perkin-Elmer) with the detection wavelength set at 400 nm.

Separation of the porphyrins was obtained by multilinear gradient elution. The initial conditions were 30% ethyl acetate in *n*-heptane for 6 min after injection; then the percentage of ethyl acetate was raised to 45% within 1 min. Over the next 10 min the ethyl acetate percentage was raised linearly up to 80% and this condition was held for 6 min. Elution was completed with a 3-min purge period at 80% of ethyl acetate. Between two analyses the column was reconditioned for 10 min with the starting solvent mixture. The flow-rate was kept constant at 0.8 ml/min.

Sample preparation from tissues

Porphyrinogens formed as reaction products of the in vitro uroporphyrinogen decarboxylase activity were oxidized to porphyrins, adsorbed on Zerolit FF (ip) resin (BDH), methylated for 48 h with methanol—sulphuric acid and extracted into chloroform as described by Smith et al. [10]. Alternatively, the methylation was performed with boron trifluoride—methanol (14%, Merck-Schuchardt) as described by Smith and Francis [11] with slight modifi-

ıtions: to 2 ml of boron trifluoride—methanol, 4 ml of chloroform were ıdded; the tubes were mixed on a Vortex for 40 sec and centrifuged at 5090 *g* ›r 10 min. This procedure was repeated twice with a mixture of chloroform—ıethanol (6 ml in the ratio 2:1 and 2 ml in the ratio 1:1). The pooled extracts ere combined and washed twice with distilled water. After the second wash-ıg the chloroform layer was taken, mixed with ethanol (10 ml) and evaporated nder a stream of nitrogen at 37°C. Precautions were taken during the whole rocedure to avoid contact with direct light, and samples were kept in the dark ; −20°C until injected. Immediately before the HPLC analysis, samples were issolved with a chloroform solution of mesoporphyrin dimethyl ester (0.5 mol/ml). This compound, not present in biological tissues, was used as ıternal standard.

alibration curves

Porphyrin methyl esters of known concentrations for the calibration curve ere prepared from individual porphyrin methyl esters dissolved in chloroform. he concentrations of these original solutions were determined spectrophoto-ıetrically by measuring the absorption at the Soret band and using the ıtinction coefficients reported by Falk [12] for meso-, proto-, copro- and roporphyrin and by Doss [13] for pentacarboxylic, hexacarboxylic and eptacarboxylic porphyrin. The standard mixtures were prepared ready to dis-›lve shortly before use with 2.5 ml of a cloroform solution of mesoporphyrin imethyl ester (0.5 nmol/ml). When dissolved, they were stable for at least one eek if stored in the dark at −20°C.

ESULTS

The multilinear gradient elution for the separation of mesoporphyrin IX, rotoporphyrin IX, copro-, pentacarboxylic, hexacarboxylic, heptacarboxylic nd uroporphyrin methyl esters obtained using a mixture of pure compounds is ıown in Fig. 1. No interfering peak(s) were found in extracts from liver, pleen, kidneys and red blood cells; occasionally, additional peaks were bserved, quite likely due to the formation of trace amounts of the zinc omplex of the porphyrins.

Absolute quantitation of porphyrins present in samples was performed by omparison with a calibration curve obtained by plotting the peak area ratio of ach porphyrin to mesoporphyrin versus the concentration of the porphyrin tandard solutions. The calibration was linear for all the porphyrins considered ithin the concentration range 20—2000 ng/ml. Fig. 2 shows the part of the urves up to 1000 ng. The relative correlation coefficients (which also take into ccount the 2000 ng/ml concentration point) were all greater than 0.999. The alibration curves sloped differently depending on the specific fluorescence of ach porphyrin. The best sensitivity was obtained for coproporphyrin etramethyl ester. On some occasions, the HPLC system was coupled to the UV letector and the two methods of detection were compared. Fig. 3 presents the ›rofiles obtained using a UV or a fluorometric detector after injecting the same ımple containing the reaction products of a preparation of human red blood ell uroporphyrinogen decarboxylase. In this example, the chromatogram was

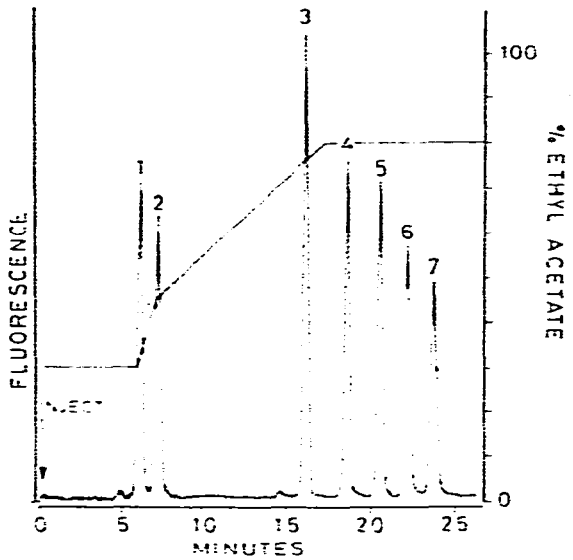


Fig. 1. HPLC separation of standard mixtures of porphyrin methyl esters. Peaks: 1 = mesoporphyrin dimethyl ester; 2 = protoporphyrin dimethyl ester; 3 = coproporphyrin tetramethyl ester; 4 = pentacarboxylporphyrin pentamethyl ester; 5 = hexacarboxylporphyrin hexamethyl ester; 6 = heptacarboxylporphyrin heptamethyl ester; 7 = uroporphyrin octamethyl ester. Conditions of elution are as described in the text.

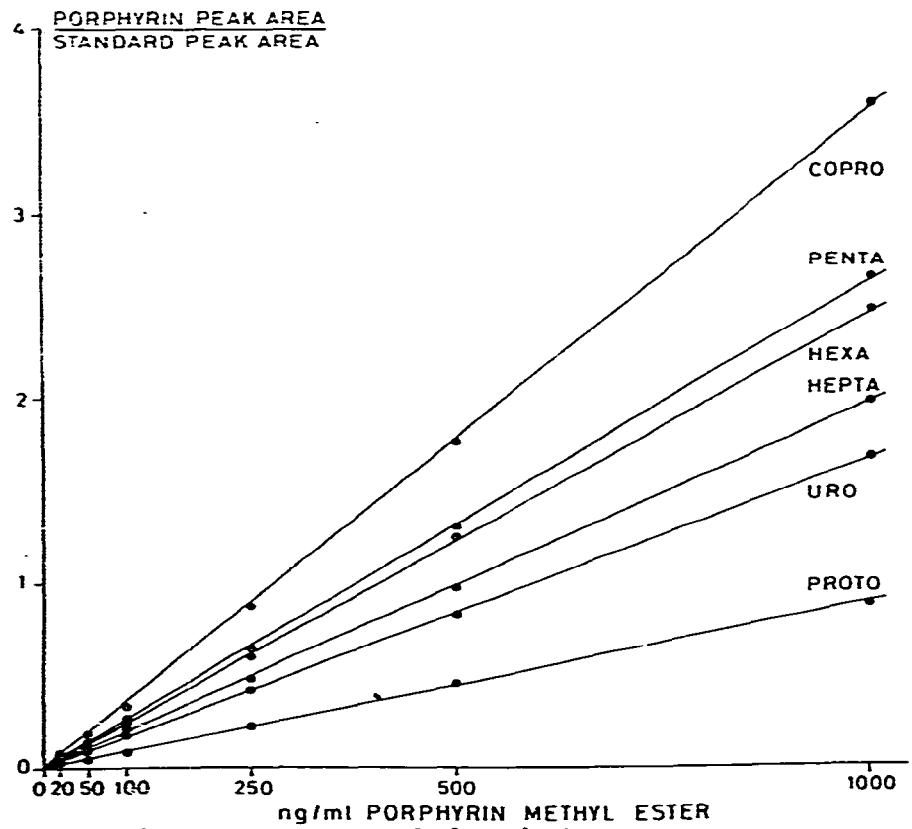
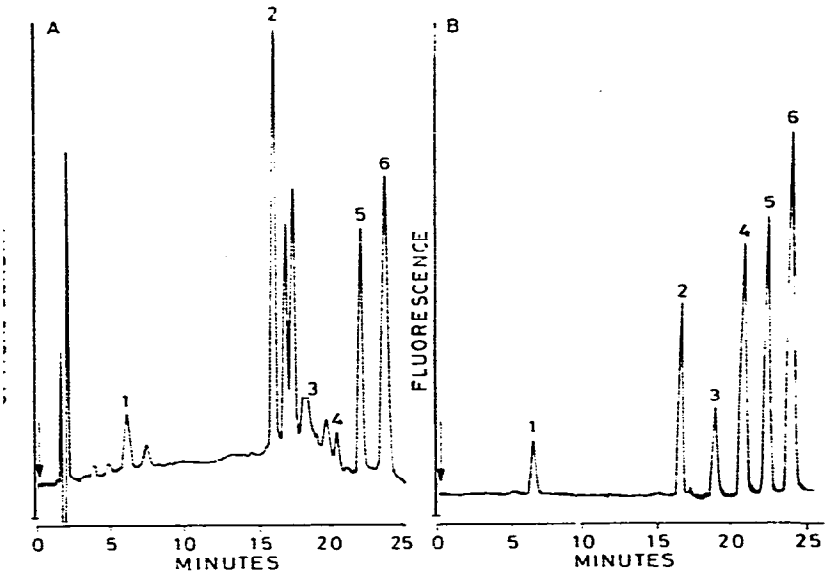


Fig. 2. Calibration curves for standard porphyrin esters.



ig. 3. Comparison of the profiles obtained with (A) UV and (B) fluorometric detectors of a mple containing the reaction products of a preparation of human red blood cell uroorphyrinogen decarboxylase. Peaks: 1 = mesoporphyrin dimethyl ester; 2 = coproporphyrin tramethyl ester; 3 = pentacarboxylporphyrin pentamethyl ester; 4 = hexacarboxylporhyrin hexamethyl ester; 5 = heptacarboxylporphyrin heptamethyl ester; 6 = uroporphyrin ctamethyl ester. Conditions of elution are as in Fig. 1.

btained at the highest sensitivity of the UV detector (0.02 a.u.f.s.) (Fig. 3A). /ith the fluorometric detector (Fig. 3B), sensitivity could be further enhanced —10 times, depending upon the porphyrin, without changes in the signal-toise ratio.

Tables I and II report the results of recovery experiments on the effectiveess of two methylation and relative extraction procedures, one with ıethanol—sulphuric acid and one with boron trifluoride—methanol. Known mounts of porphyrins (copro-, pentacarboxylic, hexacarboxylic, heptaarboxylic and uroporphyrin) were adsorbed as free acids on Zerolit FF resin nd then methylated and extracted as previously described.

ABLE I

ECOVERY OF PORPHYRINS AFTER METHYLATION WITH METHANOL—SULHURIC ACID

alues represent the mean ± S.E. of two determinations.

mount of each porphyrin dded to the incubation ixture (nmol)	Recovery (%)				
	Copro-	Penta-	Hexa-	Hepta-	Uro-
.083	90 ± 5	83 ± 5	74 ± 5	65 ± 1	106 ± 1
.160	90 ± 2	81 ± 1	73 ± 3	61 ± 3	102 ± 8
.800	93 ± 1	84 ± 1	77 ± 1	67 ± 0	103 ± 1
.600	98 ± 2	90 ± 1	84 ± 5	68 ± 1	107 ± 2

TABLE II

RECOVERY OF PORPHYRINS AFTER METHYLATION WITH BORON TRIFLUORIDE

Values represent the mean ± S.E. of two determinations.

Amount of each porphyrin added to the incubation mixture (nmol)	Recovery (%)				
	Copro-	Penta-	Hexa-	Hepta-	Uro-
0.083	83 ± 3	73 ± 1	67 ± 3	58 ± 6	89 ± 6
0.160	88 ± 3	77 ± 3	67 ± 6	57 ± 6	90 ± 6
0.800	88 ± 3	81 ± 3	73 ± 3	61 ± 5	98 ± 3
1.600	86 ± 6	75 ± 10	69 ± 6	58 ± 5	95 ± 8

In both procedures, no interfering peaks were visible in the chromatograms and recovery was not influenced by changes in the amount of porphyrin adsorbed on the resin. The efficiency of methylation and extraction, relative to each porphyrin, increased in the order: uro- > copro- > penta- > hexa- > hepta-. The recovery was always higher than 57%.

As an example of the applicability of the method, Table III reports the results of determination of the activity of uroporphyrinogen decarboxylase in animal and human tissues. The decarboxylated products (copro-, penta-, hexa-, and heptacarboxylic porphyrinogen) obtained with 1 h of incubation of the enzymatic preparation at 37°C under nitrogen, were oxidized to porphyrins with light and then methylated with methanol—sulphuric acid. The amount of uroporphyrin present at the end of incubation represents the residual substrate.

With all the tissues considered, it was possible to measure quantitatively all the reaction products simultaneously.

DISCUSSION

The present method measured porphyrins in tissues or formed in in vitro incubations down to a concentration of 0.2 ng/sample. This sensitivity was achieved by the use of fluorescence detection which is more sensitive than the more commonly used UV detection [4—6]. The amount of sample in the optimal range for analysis with fluorescence detection is about one tenth that required with UV detection. Another major advantage of using a fluorometric detector is its specificity, which makes it easy to obtain a clean baseline in the chromatogram, eliminating many interfering substances present in biological samples which absorb light but do not fluoresce in the same range as porphyrins. Furthermore, the gradient elution program described here efficiently separates all the porphyrins without the need for a second derivative system coupled to the detector as described by other authors [14].

HPLC methods of separation of free porphyrins are still in the developmental stage and have been applied mainly to analysis of urines [7, 15]. As a consequence, methylation of the porphyrins is still a necessary step in sample preparation, especially when the amounts present are fairly low. We have

TABLE III

ACTIVITY OF UROPORPHYRINOGEN DECARBOXYLASE IN ANIMAL AND HUMAN TISSUES

Values represent the mean ± S.E. of two samples. Each sample was done in duplicate. The enzymatic preparations had a protein concentration of 2.5—4.6 mg/ml, except blood (11.4 mg/ml).

Tissue	Uroporphyrinogen added (nmol)	Porphyrins recovered (nmol)					Recovery (%)	Uroporphyrinogen decarboxylase activity (pmol per min per mg protein)
		Copro-	Penta-	Hexa-	Hepta-	Uro-		
Rat liver	8	1.223 ± 0.14	0.190 ± 0.01	0.459 ± 0.04	1.820 ± 0.14	3.933 ± 0.16	95	13.209 ± 1.16
Rat kidneys	8	0.455 ± 0.04	0.116 ± 0.00	0.314 ± 0.01	1.336 ± 0.03	4.778 ± 0.20	87	12.724 ± 0.43
Rat spleen	8	0.311 ± 0.02	0.103 ± 0.00	0.296 ± 0.01	1.394 ± 0.04	5.910 ± 0.02	100	9.162 ± 0.40
Human liver	8	0.051 ± 0.00	0.033 ± 0.00	0.100 ± 0.00	0.671 ± 0.07	5.997 ± 0.59	86	10.490 ± 0.47
Human blood	8	0.304 ± 0.02	0.088 ± 0.00	0.270 ± 0.01	1.964 ± 0.17	3.074 ± 0.31	71	3.820 ± 0.04

shown that two commonly used procedures of methylation do not seem to differ significantly in efficiency.

A somewhat higher percentage of recovery and better reproducibility were obtained with the methanol—sulphuric acid method, but the boron trifluoride—methanol method has the advantage of a considerably shorter reaction time.

Finally, we have shown that the use of HPLC coupled to fluorescence detection provides a technique suitable for the biochemical investigation of porphyrias; for example, measurement of the activity of uroporphyrinogen decarboxylase. By our method all the reaction products can be determined simultaneously with advantages in specificity in comparison to radioactive assays, and in sensitivity and accuracy in comparison to TLC separation coupled to spectrophotometric quantitation previously described by other authors [9—11]. This should facilitate the characterization of the various steps of this enzymatic process and of its response to stimuli of different origin.

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