SECOND DERIVATIVE-HIGH-PERFORMANCE LIQUID CHROMATO-GRAPHIC–FLUOROMETRIC DETECTION OF PORPHYRINS IN CHICK EMBRYO LIVER CELL CULTURE MEDIUM

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SUMMARY

A high-performance liquid chromatographic system is described which is suitable for the separation and quantitative determination of a mixture of porphyrin esters in nanogram quantities from the culture medium used for maintenance of a monolayer culture of chick embryo liver cells. The desired sensitivity was obtained by coupling a high-performance liquid chromatograph with a second derivative-fluorometric detection system. The most effective method for preparation of the porphyrin methyl esters prior to chromatography was found to be lyophilization of the culture medium prior to esterification with 5% sulphuric acid in methanol.

INTRODUCTION

The separation and quantitative determination of porphyrins is often required in biochemical and pharmacological studies. Methods available for this purpose are solvent fractionation[1], counter-current distribution[2], column chromatography of free or esterified porphyrins[3,4] and thin-layer chromatography (TLC)[5]. In recent years the technique of high-performance (pressure) liquid chromatography (HPLC)[6–8] has been found to offer advantages over TLC for the separation and quantitative determination of porphyrins. HPLC analysis of porphyrins is usually preceded by conversion of porphyrins to their methyl esters since HPLC methods for separation of free porphyrins[9] are still in the developmental stage.

The detection system most commonly used for HPLC analysis of porphyrins is the ultraviolet (UV) absorption detector which allows detection of porphyrins in the low microgram range. The UV detector is appropriate for analysis of porphyrins in the urine and feces of a porphyric patient where porphyrins are present in relatively large amounts. However, the UV detector is of insufficient sensitivity for the measurement of nanogram amounts of porphyrins produced by monolayer cultures of chick embryo liver cells[10] following exposure to a variety of chemicals. In the present study a method is described for the quantitation of nanogram amounts of porphyrin methyl esters which should facilitate studies of drug-induced porphyrin biosynthesis in chick embryo liver cells. The technique makes use of HPLC coupled

with a second derivative-fluorometric detection system. In addition several esterification procedures have been compared in order to determine which esterification procedure would result in the highest yield of porphyrin methyl esters from a mixture of porphyrins in chick embryo liver cell culture medium.

MATERIALS AND METHODS

Solvents and chemicals

Hexane (spectro grade), chloroform (spectro grade) and methanol (HPLC grade) were obtained from Caledon Labs. (Georgetown, Canada). Ethyl acetate (HPLC grade) was purchased from Baker (Canlab, Toronto, Canada). The following porphyrin methyl ester standards were purchased from Porphyrin Products (Logan, Utah, U.S.A.): Protoporphyrin IX dimethyl ester, coproporphyrin III tetramethyl ester, pentacarboxyl porphyrin I pentamethyl ester, hexacarboxyl porphyrin I hexamethyl ester, heptacarboxyl porphyrin I heptamethyl ester and uroporphyrin III octamethyl ester. Waymouth MD 705/1 culture medium was obtained from Grand Island Biological Co. (Grand Island, N.Y., U.S.A.). Boron trifluoride in methanol ($BF_3 \cdot 2CH_3OH$) was purchased from Aldrich (Milwaukee, Wisc., U.S.A.).

Instrumental conditions

The liquid chromatographic system was assembled using components from the following manufacturers: Model 110 solvent metering pump (Altex Scientific, Berkeley, Calif., U.S.A.); Model CV-6-UHPa-N60 universal injection valve equipped with a 10-μl injection loop (Valco, Houston, Texas, U.S.A.); spectrofluorometer (excitation 405 nm, detection > 430 nm, slit width 10 nm) fitted with a microflow cell (Farrand, Valhalla, N.Y., U.S.A.); Dual-channel recordall strip chart recorder (Fisher Scientific, Whitby, Canada).

The following procedures were used to reduce background noise and to improve stability; firstly, a pulse dampener was installed between the pump and valve. This consisted of a coil of stainless-steel tubing (1 m × 4.8 mm I.D.) connected to a coil of stainless-steel capillary tubing (2 m). Secondly, two 0.01-μf capacitors, two 1000-μf 50 V capacitors and two 100-Ω resistors were placed across the +12 V and −12 V power supply leads to the photometer electronics (Fig. 1).

The column (28 × 0.2 cm I.D.) was packed with silica gel (Partisil 5; Whatman, Clifton, N.J., U.S.A.) by a balanced density slurry method[11]. The eluting solvent was hexane–ethyl acetate–methanol (60:37:3, v/v/v). Before use the eluting solvent was degassed under vacuum. The column was stabilized at room temperature by means
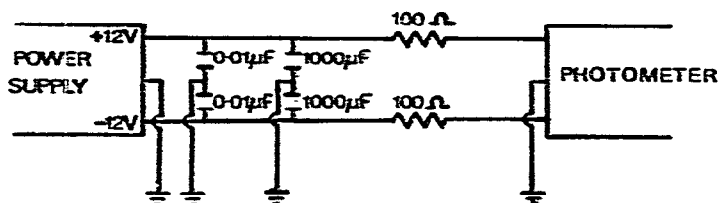

Fig. 1. Circuit diagram for stabilization of the photometer power supply.

of a noncirculating water jacket. The electronically generated second derivative was obtained from a unit constructed in our laboratory according to the circuit diagram shown in Fig. 2. Electronic components were purchased from Electrosonic (Willowdale, Canada).
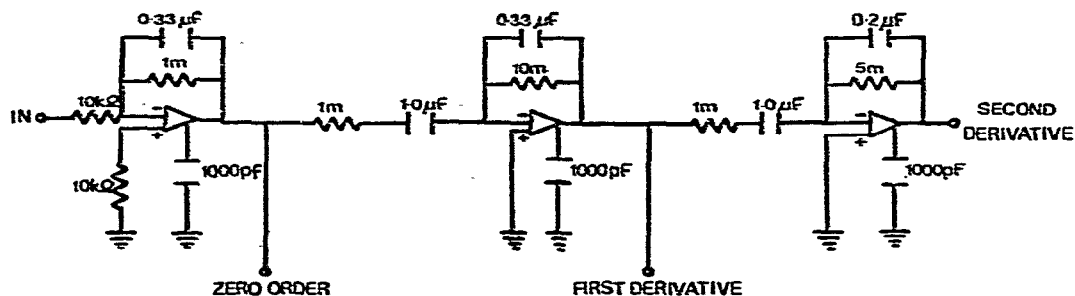

Fig. 2. Circuit diagram for generation of the second derivative. All IC operational amplifiers are low-noise type (LM 312 H). m = MΩ.

Preparation of porphyrin methyl ester standards

The following porphyrins were weighed out in 80–120 μg quantitites and dissolved in 1 ml of chloroform: proto-, copro- and uro-porphyrin, penta-, hexa- and hepta-carboxyl porphyrin methyl esters. Quantitative determination of the porphyrin methyl esters in chloroform was carried out spectrophotometrically by measuring the absorption of the Soret band and using the known extinction coefficients for proto-, copro- and uro-porphyrin methyl esters[12]. In order to calculate the extinction coefficients for the penta-, hexa- and hepta-carboxyl porphyrins[13], a curve was constructed by plotting the reciprocal of the extinction coefficient (1/E mM) against the number of carboxyl groups for proto-, copro- and uro-porphyrin (coefficient of determination r^2 = 0.99). Reciprocals of the extinction coefficients for the penta-, hexa- and hepta-porphyrins were then read from this curve. Dilution of standards was carried out using the mobile phase solvent. The standards which were prepared shortly before use, were kept in the dark until injected onto the column.

Esterification of porphyrins from Waymouth MD 705/1 medium

Free carboxyl porphyrins were prepared from proto- and uro-porphyrin methyl esters by hydrolysis in the dark in 25% (w/v) HCl for 5 and 24 h respectively[12]. Coproporphyrin dihydrochloride was obtained from Porphyrin Products. The concentration of free porphyrins in HCl was measured spectrophotometrically using the known extinction coefficients[12]. Known quantities of proto-, copro- and uro-porphyrin were then added to 5 cm petri dishes containing 5 ml of Waymouth MD 705/1 medium. Porphyrins in the medium were subsequently esterified and prepared for HPLC by one of the following methods.

(1) Talc method[5]. The contents of each 5 ml dish was acidified with glacial acetic acid to pH 4–5. About 0.5 g of talc (U.S.P.) was added to each dish and the mixtures stirred for about 2 min. The talc was separated by filtration, and dried in a

desiccator overnight. In order to esterify the porphyrins absorbed on the talc, the talc samples were added to 25 ml erlenmeyer flasks containing 5 ml of 5% sulphuric acid in methanol and shaken gently at room temperature for 24 h. Chloroform (4 ml) was added to each sample and after thorough mixing the suspensions were centrifuged. The talc precipitate from each dish was re-extracted three times with chloroform (2 ml). The pooled chloroform extracts were washed once with 1% ammonia (5 ml) and once with water (5 ml). After drying (anhydrous sodium sulfate) the chloroform solutions were evaporated to dryness at 37° with a stream of air. The residues were dissolved in 200 μl chloroform–mobile phase solvent (1:1, v/v) and injected onto the HPLC system.

(2) Boron trifluoride method[14]. The method used was the same as described above except that treatment of talc samples with 5% sulphuric acid in methanol was replaced by refluxing with 5 ml of boron trifluoride (10% in methanol) for 30 min.

(3) Lyophilization method. The contents of each dish were freeze-dried and then esterified with 5 ml of 5% sulphuric acid in methanol for 11 h with gentle shaking at room temperature. Chloroform (5 ml) was added to each sample followed by 5 ml of water. The aqueous layer was removed and washed twice with 2 ml of chloroform. The pooled chloroform extracts were washed with 1% ammonia (5 ml), water (5 ml) and then dried (anhydrous sodium sulfate). After evaporating to dryness at 37° the residues were made up in 200 μl of chloroform– mobile phase (1:1) and injected onto the column.

RESULTS AND DISCUSSION

A dual-channel strip chart recorder was used to monitor both the zero order and second derivative fluorescence of the porphyrins, and an example of such a chromatogram is shown in Fig. 3. Quantitation was carried out using the second derivative peak heights which were measured as the distance between two large peak deflections (Fig. 3). When the flow-rate was kept constant at 0.5 ml/min all six porphyrin methyl esters were well resolved from each other but efficiency (zone spreading) was low for the higher carboxylated porphyrins (Fig. 4). In order to retain the shape of the peaks for the six to eight carboxyl porphyrins and to decrease their retention times, the flow-rate was increased from 0.5 to 1.5 ml/min after the penta-carboxyl porphyrin was eluted (Fig. 5). By running standard porphyrin samples of different concentration through the HPLC and using the peak height of the second derivative to quantitate the porphyrin esters, a calibration curve was constructed (Fig. 6) with 1–15 ng of porphyrin methyl esters. A coefficient of determination of 1.00 was found for the line relating peak height to the amount of porphyrin methyl ester.

Fluorescence detection of the porphyrins has an important advantage over the commonly used UV detection system. Since porphyrins are often analyzed in biological materials, many extraneous substances are introduced into the chromatographic system which will also absorb light in the Soret region characteristic for porphyrins, *viz.*, 400–410 nm, thus adding many unwanted peaks into the chromatogram. While many substances may absorb in this range of porphyrin fluorescence excitation, few will also fluoresence at the emission wavelength characteristic for
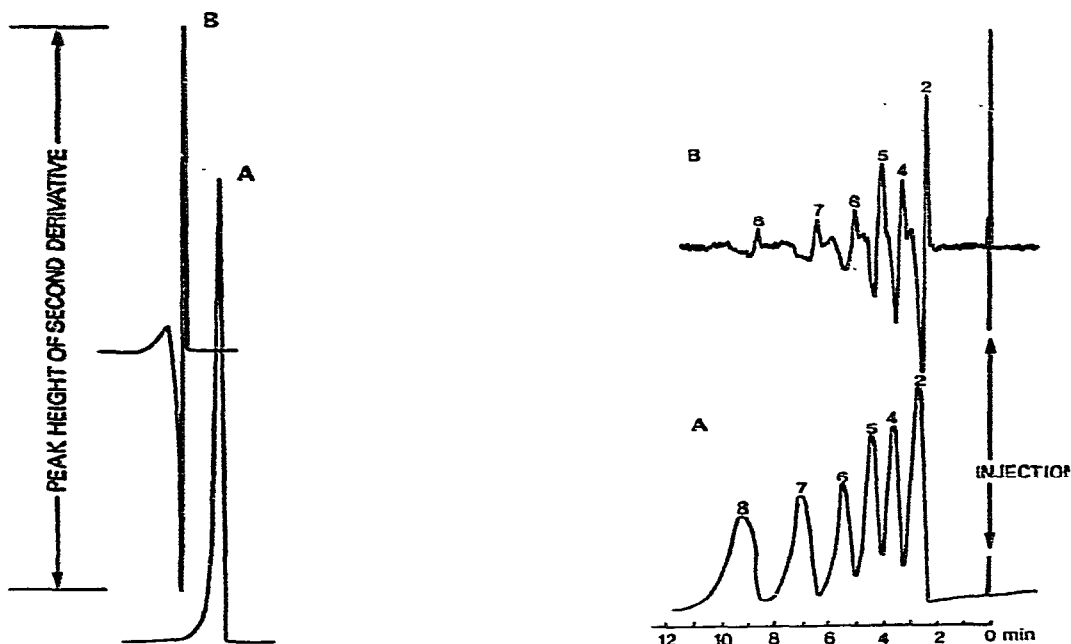
Fig. 3. Zero order (A) and second derivative (B) peaks for a porphyrin methyl ester using HPLC. Column: 28 × 0.2 cm I.D., Partisil 5. Mobile phase: hexane–ethyl acetate–methanol (60:37:3, v/v/v). Detection: fluorescence, excitation 405 nm, detection > 430 nm.

Fig. 4. Separation of porphyrin methyl esters (2–8 carboxyl groups) by HPLC using isocratic elution with a constant flow-rate of 0.5 ml/min. A, Zero order; B, second derivative. Numbers above the peaks indicate the number of carboxyl groups (2 = protoporphyrin dimethyl ester; 4 = coproporphyrin tetramethyl ester; 5 = pentacarboxyl porphyrin pentamethyl ester; 6 = hexacarboxyl porphyrin hexamethyl ester; 7 = heptacarboxyl porphyrin heptamethyl ester; 8 = uroporphyrin octamethyl ester). Conditions as in Fig. 3.

porphyrins. Thus specificity is increased by use of a fluoresence detector. By using the second derivative to monitor the fluorescence spectra, sensitivity and resolution are further increased. As can be seen in Fig. 5, peaks in the second derivative mode are larger than in the zero order.

The advantage of using the second derivative rather than zero order spectra is illustrated by examining the hexacarboxyl porphyrin peaks in Fig. 5. Three possible hexacarboxyl porphyrin standards were commercially available, viz., the two individual isomers shown in Fig. 7 and a considerably less expensive standard consisting of a mixture of the two isomers. It was assumed that the difference between the two isomers was so small that no separation would occur on HPLC. For this reason the mixture of the two isomers was purchased and used as a standard. As can be seen in Fig. 5, the zero order peak for hexacarboxyl porphyrin shows a barely detectable inflection on the leading edge, but in the second derivative tracing two different compounds can be seen eluting close together. Clearly, resolution is increased by using the second derivative for quantitation. This increased resolution opens up the possibility for detection of any abnormal porphyrins, such as isocoproporphyrin[15], produced by porphyrin-inducing drugs.
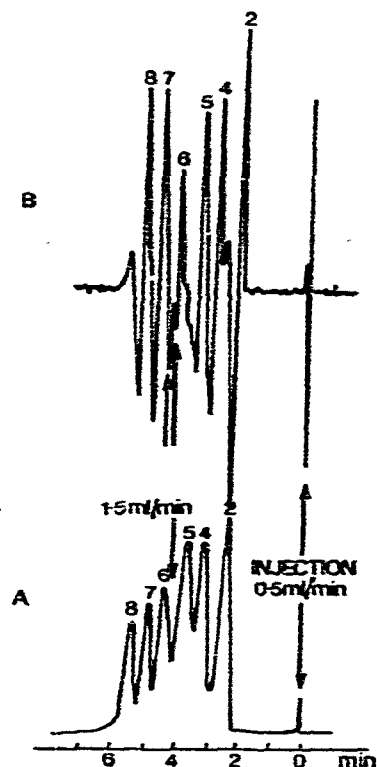
Fig. 5. Separation of porphyrin methyl esters (2–8 carboxyl groups) by HPLC using isocratic elution with flow programming. A, Zero order; B, second derivative. Arrows indicate hexacarboxyl porphyrin isomers (see text). Other details as in Fig. 4.
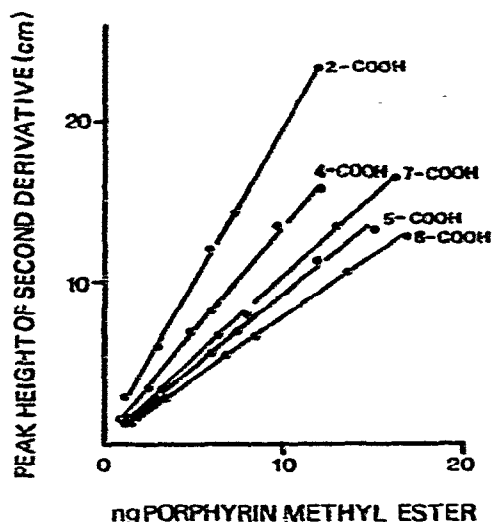
Fig. 6. Calibration curve for standard porphyrin esters. The number of carboxyl groups is indicated by each curve (2-COOH = protoporphyrin dimethyl ester; 4-COOH = coproporphyrin tetramethyl ester; 5-COOH = pentacarboxyl porphyrin pentamethyl ester; 6-COOH = hexacarboxyl porphyrin hexamethyl ester; 7-COOH = heptacarboxyl porphyrin heptamethyl ester; 8-COOH = uroporphyrin octamethyl ester).
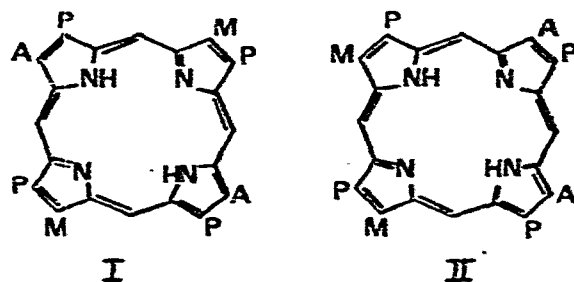

Fig. 7. Two hexacarboxyl porphyrin hexamethyl ester isomers with opposite rings decarboxylated (I) and with adjacent rings decarboxylated (II). A = CH_2COOCH_3; P = $CH_2CH_2COOCH_3$; M = CH_3.

In order to examine the effects of porphyrin-inducing drugs in chick embryo liver cell culture, accumulated porphyrins are first isolated and then esterified. The

first method investigated for this purpose was the talc method. Recovery from chick embryo liver cell culture medium of uroporphyrin esters ranged from 58 to 68% while recovery of coproporphyrin esters ranged from 41-57% (Table I). The results with protoporphyrin were considerably lower (Table 1) and levels of 100 ng protoporphyrin added to the medium yielded no detectable protoporphyrin ester. The probable reasons for our failure to detect protoporphyrin ester were (1) the presence of a large interfering peak eluting just prior to protoporphyrin ester which prevented detection of small amounts and (2) the poor stability of protoporphyrin. The boron trifluoride method was not suitable as an esterification procedure since an even larger unknown peak appeared which prevented measurement of both proto- and copro-porphyrins. The lyophilization method on the other hand gave very clear samples with no interfering peak. Removal of the interfering peak allowed detection of the esterification of small quantities of protoporphyrin (100 ng). Clearly the lyophiliza-tion method appears to be the most desirable for the isolation and esterification of porphyrin mixtures prior to their separation by HPLC.

TABLE I

RECOVERY OF PORPHYRINS FROM WAYMOUTH MD 705/1 MEDIUM

Porphyrin	Amount of porphyrin added to medium (ng)	Recovery (%)*	
		Talc method	Lyophilization method
Protoporphyrin	100	0 ± 0 (12)	16 ± 3 (3)
	500	14 ± 5 (15)	9 ± 0 (3)
	1000	9 ± 3 (15)	12 ± 1 (3)
Coproporphyrin	100	41 ± 5 (9)	44 ± 12 (3)
	500	52 ± 5 (9)	60 ± 8 (3)
	1000	57 ± 3 (9)	52 ± 2 (3)
Uroporphyrin	100	58 ± 9 (6)	62 ± 12 (3)
	500	62 ± 8 (6)	76 ± 2 (3)
	1000	68 ± 10 (6)	79 ± 1 (3)

* Values shown are the mean ± S.E.M. of the number of determinations shown in parentheses.

Therefore by coupling HPLC–fluorescence detection and monitoring the second derivative, detection of porphyrin methyl esters can now be done in the nanogram range rather than the microgram range, which is commonly the limit of detection with UV-zero order peak monitoring systems. This method should facilitate the elucidation of the nature and amounts of porphyrins formed in response to numerous drugs added to chick embryo liver cell culture[10]. Such information will in turn provide insight into the mechanism of action of porphyrin-inducing drugs.

ACKNOWLEDGEMENTS

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