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PURIFICATION OF PORPHYRIN ESTERS BY GEL FILTRATION*

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SUMMARY

A relatively simple yet reproducible method has been developed for the separation of porphyrin esters on the micro or macro scale. This method is based on the principle of gel filtration. Though separation appears to result primarily as a consequence of size of the porphyrin esters, evidence is presented suggesting that adsorption of the porphyrin ester to the Sephadex LH-20 does play some role.

INTRODUCTION

The number of methods for the chromatographic separation of porphyrin mixtures seems to grow at a rate approximately parallel to the number of chemists and biochemists working with these compounds. In part, this would seem to indicate that existing methods do not satisfy all the requirements imposed by the various investigators.

Two problems that we have encountered are low capacity, and erratic or artifactual results. That is, we have found it difficult to scale up methods that work well for micro quantities of porphyrins so they could be used on the preparative scale. Also, we have found some systems, *e.g.* $CaCO_3$ eluted with chloroform–benzene, that work satisfactorily on some occasions, fail entirely on other occasions. This problem apparently is a function of the crystal structure and degree of activation of the adsorbant. Theoretically, careful standardization techniques can minimize some of these problems. Regretfully, however, in our hands success or failure with any column chromatographic system seems to depend most consistently on luck.

We, therefore, set out to develop a system for separating porphyrin mixtures that would be simple enough so that individuals not having years of experience with porphyrins could achieve success the first time around. In addition to emphasizing reliability, we concentrated on the problem of capacity.

The system that has evolved from our efforts is based on gel filtration. It can be used on either the micro or macro scale, and it functions satisfactorily in the hands of untrained personnel.

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MATERIALS AND METHODS

Uroporphyrin I and coproporphyrin I were isolated from the urine of cattle with erythropoetic porphyria. Deuteroporphyrin IX was prepared according to CHU AND CHU[1]. Mesoporphyrin IX was prepared by the method of BAKER *et al.*[2]. Sephadex LH-20 was purchased from Pharmacia (Piscataway, N.J.). Solvents were analytical grade reagents purchased from commercial suppliers and were distilled in an all glass apparatus before use.

TABLE I

MELTING POINTS (°C) OF METHYL ESTERS AND *n*-BUTYLESTERS OF PORPHYRINS

Porphyrin	*Methyl ester*		*n-Butyl ester*
	Observed	*Literature*[7]	
Mesoporphyrin IX	208	216	138–140
Deuteroporphyrin IX	221–222	218–220	169
Coproporphyrin I	250–265	251–252	117–120
Uroporphyrin I	292–293	292	153–154

TABLE II

ABSORPTION BANDS OF THE METHYL AND *n*-BUTYL ESTERS OF PORPHYRINS

Absorption maxima of porphyrin esters measured in chloroform. Numbers in parentheses are values quoted from FALK[3].

Porphyrin	*Wavelength (mμ)*									
	Methyl ester					*n-Butyl ester*				
	I	*II*	*III*	*IV*	*S*	*I*	*II*	*III*	*IV*	*S*
Mesoporphyrin IX	620 (621)	566 (567)	533 (533)	499 (499)	400 (400)	619	565	534	498	401
Deuteroporphyrin IX	619 (621)	565 (566)	530 (530)	497 (497)	400 (399.5)	619	565	530	497	400
Coproporphyrin I	621 (621)	567 (566)	534 (534)	499 (498)	400 (400)	621	567	533	499	401
Uroporphyrin I	626 (627)	572 (572)	536 (536)	501 (502)	405 (406)	626	571	536	501	406

Prior to esterification the porphyrins were partially purified by solvent extraction. Both the methyl and *n*-butyl esters of the porphyrins were prepared with the corresponding anhydrous alcohol to which either dry HCl gas, or 5 % w/v H_2SO_4 had been added. The porphyrins employed in preparing the *n*-butyl esters had been previously purified and characterized as the corresponding methyl esters. The methyl esters were hydrolyzed in methanolic KOH and then reesterified.

The porphyrin esters were purified by solvent extraction, by chromatography on celite[3], and by gel filtration on Sephadex LH-20. The purity of all samples of porphyrin esters was checked by thin layer[4,5] or paper chromatography[6]. None were used in experiments unless they ran as a single spot in two solvents. The methyl esters of

the porphyrins were crystallized from the same mixture while the butyl esters of coproporphyrin and uroporphyrin were crystallized from chloroform–butanol. The melting points of the esters are given in Table I.

The spectral purity of the esters was examined using a Bausch and Lomb Spectronic 505 calibrated with a mercury lamp. The positions of the major absorption bands are given in Table II.

RESULTS AND DISCUSSION

Small scale preliminary runs were conducted on columns 1 × 15 cm employing a number of solvent mixtures to see if a mixture of uroporphyrin octamethyl ester and mesoporphyrin dimethyl ester could be resolved. No resolution was observed with the following systems: nitroethane–trichloroethylene (1:1), chloroform–ethyl acetate–benzyl alcohol (30:15:0.1), ethyl acetate–trichloroethylene–cyclohexanone (15:10:0.1), pyridine–*n*-butanol (1:1), pyridine–2-methoxy ethanol (1:1). Some separation was achieved with the following solvents: dioxane, trichloroethylene–methanol (1:1), chloroform–methanol (1:1). Best results were obtained with chloroform–methanol and this mixture was adopted for further studies. In the course of further experiments with chloroform–methanol we occasionally observed erratic resolution of mixtures of porphyrin esters. The addition of a very small amount of aqueous NH_4OH to the solvent mixture improved the performance of the system. Subsequently to avoid the addition of any water to the system, Tris base, which is soluble in methanol, was added to the chloroform–methanol mixture at a concentration of 1 g/l. Using this solvent system we were able to achieve adequate and reproducible resolution. The individual methyl esters of the four porphyrins were passed through a 84 × 2.5 cm column of Sephadex LH-20 and the optical density of the column effluent was measured at 500 mμ in a Bausch and Lomb Spectronic 20. Fig. 1 shows the composit results obtained in four such runs. Fig. 2 shows the elution pattern obtained when a mixture of the four porphyrin esters was run on a similar column. When samples from the peak

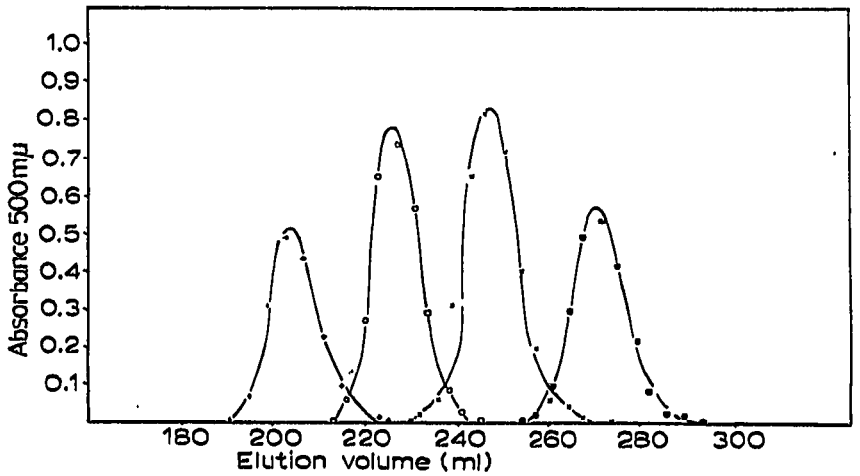

Fig. 1. Elution profile of porphyrin methyl esters run separately on a 2.5 × 87 cm column of Sephadex LH-20. Uroporphyrin octamethyl ester (+); coproporphyrin tetramethyl ester (○); mesoporphyrin dimethyl ester (×); deuteroporphyrin dimethyl ester (●). Solvent, chloroform–methanol (1:1) containing 1 g Tris base per l.

tubes were run on paper chromatograms only a single fluorescent spot was observed for each porphyrin. The entire absorption spectrum of each of the peak tubes were recorded and the position of the absorption bands was identical within limits of the instrument to that of the corresponding starting material. We were particularly concerned in establishing the purity of each of the porphyrin bands. We thought that the high concentration of porphyrin esters employed in these runs might promote aggregation, and we were concerned that mixed aggregates might have formed while still giving apparent resolution. Repeated checks, however, failed to reveal this as a problem in our system.

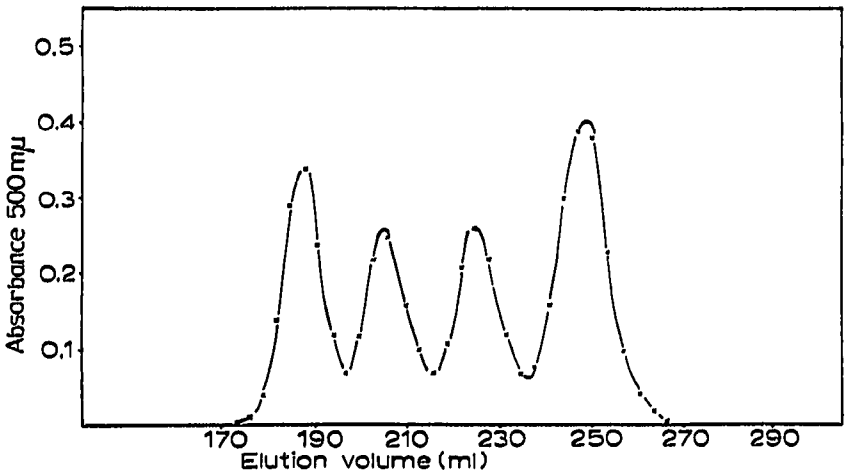

Fig. 2. Elution profile of a mixture of porphyrin methyl esters run on a 2.5 × 78 cm column of Sephadex LH-20. Uroporphyrin octamethyl ester ;coproporphyrin tetramethyl ester; mesoporphyrin dimethyl ester; deuteroporphyrin dimethyl ester. Solvent, chloroform–methanol (1:1) containing 1 g Tris base per l.

In a semi-preparative scale run, 5 mg of each of the methyl esters of uroporphyrin, coproporphyrin, mesoporphyrin and deuteroporphyrin were weighed out and dissolved in chloroform. This mixture was then applied to a pair of 2.5 × 90 cm columns connected in sequence. The first 340 ml of effluent was collected in bulk, then 4 ml samples were collected on an automatic fraction collector. The uroporphyrin peak appeared at 368 ml, coproporphyrin at 404 ml, mesoporphyrin at 442 ml, and deuteroporphyrin at 470 ml. The corresponding troughs between peaks were at 386 ml, 430 ml, and 456 ml. The average ratio between optical density at the peaks to the optical density at the trough was 10. The major part of the material is well enough resolved for preparative work. The tubes in the region of overlap, which contain only a small portion of the total porphyrin esters can be pooled and added to subsequent runs.

Most of the columns were prepared and run at room temperature. Thinking we might see less spreading and thus better resolution of the bands (caused in part by diffusion) at lower temperatures, we also carried out experiments at 3°. At this lower temperature resolution was virtually identical to that obtained at room temperature. The porphyrin esters, however, ran relatively slower, each appearing some 7 ml later than those shown in Fig. 2.

Once the solvent system containing Tris had been developed to the point that satisfied us, we carried out a series of experiments in an effort to find out why Tris had

an apparent beneficial effect. Thinking that Tris might be important due to its basic properties, we tried chloroform–methanol–pyridine (2:2:1) as a solvent. The performance of the column with this solvent was essentially the same as with chloroform–methanol–Tris. Subsequently, using a different batch of Sephadex LH-20 we found we could achieve reproducible resolution of the four porphyrin esters using chloroform–methanol without Tris. We are unable at this time to account for our earlier difficulties in the absence of Tris. We no longer include Tris in our own work, though other investigators may wish to include it as insurance.

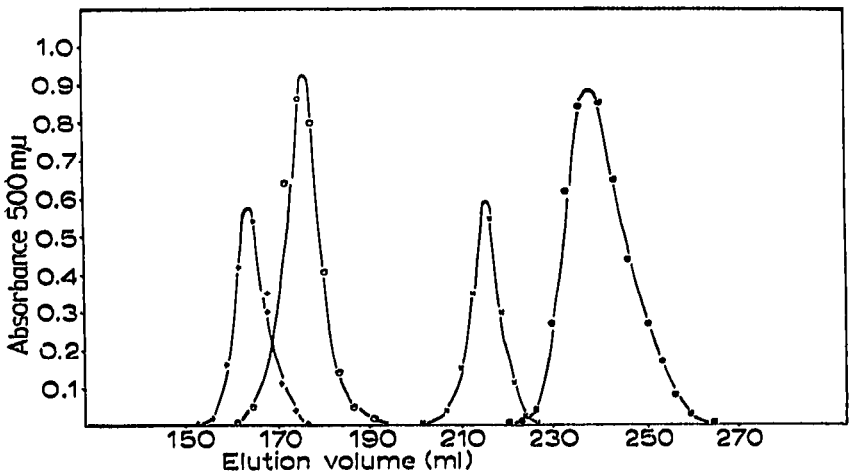

Fig. 3. Elution profile of porphyrin *n*-butyl esters run separately on a 2.5 × 87 cm column of Sephadex LH-20. Uroporphyrin octamethyl ester (+); coproporphyrin tetramethyl ester (○); mesoporphyrin dimethyl ester (×); deuteroporphyrin dimethyl ester (●). Solvent, chloroform-methanol (1:1) containing 1 g Tris base per l.

At the outset of our investigation we thought that it most likely would be necessary to employ a large alcohol to esterify the porphyrins since the large alcohol would exaggerate the difference in the size of porphyrins with different numbers of carboxyl groups. This anticipated advantage would, of course, not hold when separating the esters of mesoporphyrin and deuteroporphyrin, and, in fact, would produce just the opposite effect. To the nearest whole number the molecular weights of the methyl esters of uroporphyrin, coproporphyrin and mesoporphyrin are 943, 710, and 595, respectively. Thus relative to mesoporphyrin dimethyl ester the molecular weight of coproporphyrin tetramethyl ester is 1.21 and uroporphyrin octamethyl ester is 1.58. The molecular weights of the butyl esters of uroporphyrin, coproporphyrin and mesoporphyrin are 1262, 870, and 674 making coproporphyrin tetrabutyl ester 1.29 and uroporphyrin octabutyl ester 1.87 relative to mesoporphyrin dibutyl ester. Presumably, if separation was strictly a function of relative size, the butyl esters should allow better resolution. Fig. 3 shows the elution pattern obtained when the four butyl esters were run on a Sephadex LH-20 column with chloroform–methanol as solvent. As expected, the butyl esters emerged from the column earlier than the corresponding methyl esters. However, it would appear that essentially no gain in resolution has been achieved. In fact, uroporphyrin and coproporphyrin appear to be less well resolved, while mesoporphyrin and deuteroporphyrin remain about the same. These results seem to indicate that the separation of porphyrin ester on Sephadex LH-20 is

not achieved simply on the basis of size. Further evidence that size, *i.e.* molecular weight, is not the sole determining factor in separation is the fact that coproporphyrin tetrabutyl ester, mol. wt. 870, emerges from the column earlier than uroporphyrin octamethyl ester, mol. wt. 943. Tentatively, we interpret these results to indicate that there is interaction between the conjugated ring system and the Sephadex LH-20 matrix and that interaction is decreased by steric interference of the bulky side chains. If this were the case, separation of the porphyrin esters would be due to size differences, though not in the strict sense implied in the definition of gel filtration.

The technical data sheet on Sephadex LH-20 provided by Pharmacia indicates that aromatic compounds tend to bind to Sephadex LH-20. That such interaction is of consequence in our system is indicated by the results obtained from room temperature and cold room runs as well as the comparative performance of Sephadex LH-20 in resolving the butyl and methyl esters of porphyrins.

RIMINGTON AND BELCHER have reported a method for separating porphyrin free acids on Sephadex G-25 (ref. 8). These workers conclude that separation is effected largely as a consequence of hydrogen bonding between the carboxyl groups of the porphyrin and the hydroxyl groups of the dextran matrix. If hydrogen bonding between carboxyl and hydroxyl groups were a major factor we would expect that uroporphyrin with eight carboxyls would emerge from the column more slowly than coproporphyrin with four, or deuteroporphyrin with two. Since the opposite is true, we suspect that adsorption of the aromatic porphyrin nucleus to the gel is important, and just as in the case of the porphyrin esters, the bulky side chains interfere with adsorption.

Separation methods having a high capacity have, in addition to the obvious utility for preparatory work, the advantage of allowing one to detect and isolate minor impurities. For instance, on several occasions we detected minor bands of porphyrins in preparations that showed only a single fluorescent spot when run as the free acid using the time honored lutidine–water paper chromatographic system.

We feel that since gel filtration can be carried out on any scale limited only by the size of available columns, since it is reproducible, and since it requires comparatively little skill and special apparatus, it has considerable utility in porphyrin research.

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