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Note

Gel permeation chromatography of porphyrins and hemins

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The synthesis and modification or porphyrins and their derivatives continues to be an intensively investigated area of research[1,2]. Traditionally, methods based on adsorptive interactions have been used almost exclusively for purification[3]. The sensitivity to solvent strength and adsorbent activity, however, may sometimes unnecessarily complicate development of routine purification procedures due to time-consuming standardization. On the other hand, a number of purification problems continually occur which would be most effectively solved by utilization of a more general system. For example, during porphyrin modification studies, it may be necessary to isolate porphyrins from multicomponent mixtures containing substances of higher or lower molecular weight. Another example involves the separation of dimeric species such as hemin-oxo-dimers from monomeric porphyrin derivatives. Our objective in undertaking this study was to develop a simple and efficient system of resonable capacity which would be directly applicable, with little or no modification, to a large number of porphyrin derivatives for the types of separation problems discussed above.

There have been very few reports of utilization of gel permeation chromatography (GPC) for the purification of porphyrin esters and various synthetic porphyrins which are soluble in organic solvents. Bachmann and Burnham[4] developed a useful system utilizing a lipophilic Sephadex derivative (Sephadex LH-20). However, these workers were careful to point out that adsorptive interactions played a significant role in controlling separation and provided convincing evidence of such control. Other workers, using Sephadex LH-20 have reported separations based on the number of carboxyl groups present in individual components[5].

Several research groups have utilized Sephadex LH-20 for the purification of chlorophylls and chloroplast pigments[6–8]. In these works also, a significant degree of adsorption was clearly observed and separation could not be ascribed to a steric exclusion mechanism. For our studies, we have chosen to utilize polystyrene–divinylbenzene copolymers[9]. In order to test the versatility of the system, we have investigated the retention characteristics of a variety of porphyrins and hemin-oxo-dimers. These include porphine, tetraphenylporphine (TPP), octaethylporphine (OEP), mesoporphyrin IX dimethyl ester (MPDME) and a mixture of isomers of tetraacetyltetraethylporphine (TATEP).

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MATERIALS AND METHODS

All of the porphyrins and hemins used in this study were prepared according to published procedures[1,10]. The mixture of TATEP isomers was synthesized from 3-ethyl,4-acetylpyrrole[11] according to a published method[12], although at a much lower yield than that reported.

The polystyrene–divinylbenzene copolymer, Bio-Beads, was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) in several different pore sizes which provide a wide range of effective exclusion limits.

The beads were allowed to swell overnight in an appropriate solvent which had been shaken over calcium oxide, filtered and degassed. The slurry (*ca.* 1.5 times the "wet settled volume") was poured, all at once, into a 100 × 1.5 cm glass column (Altex No. 252-14) which was fitted with a packing reservoir and solvent was allowed to flow until the bed reached a stable height. Excess solvent and the packing reservoir were removed and an adjustable plunger (Altex) was attached and solvent allowed to flow.

Solvent was supplied by a reservoir suspended above the column. The injection device consisted of a common narrow-bore 3-way PTFE stopcock. Polyethylene or PTFE tubing (0.5 mm I.D.) was used for both inlet and outlet lines. Flow-rates were controlled with a Glenco metering valve placed on the outlet side of an Isco Model UA-5 absorbance monitor equipped with a 405 nm filter.

The tendency for the beads to float in high-density solvents (*e.g.*, chloroform or methylene chloride) induced us to swell and pour the beads in a methylene chloride–toluene (2:3) mixed solvent system in which they were more easily manipulated. After packing, the column may be re-equilibrated with other (*i.e.*, high-density) solvents if desired. Columns fitted with adjustable plungers are available from several manufacturers or can be relatively inexpensively constructed as described by Mulder[9]. Conventional, open-bed, gravity-fed columns may be used provided a mobile phase of sufficiently low density is employed.

RESULTS AND DISCUSSION

Using TPP as a test substance we have been able to obtain efficiencies of up to 9000 plates/m (height equivalent to a theoretical plate, HETP $\approx$ 0.1 mm), as is shown in Fig. 1. In the earlier reports of the use of gels for porphyrin esters and chloroplast pigments it was clearly demonstrated that adsorption played a significant role in controlling separation and that the retention volumes were dependent upon the number of carbonyl groups present. In order to demonstrate the absence of such effects in the present system we have investigated several porphyrins which are similar in size but which differ in the number and nature of carbonyl bearing substituents. Thus, OEP has no carbonyl substituents, MPDME has two ester groups and the TATEP isomers have four acetyl groups. Nearly identical retention volumes are observed for these three samples.

In order to evaluate the importance of association of aromatic systems with the polystyrene gel, we have investigated mixtures of porphine, TPP and OEP. Porphine elutes much later than either TPP or OEP. The TPP, possessing four aromatic groups, elutes with a retention volume which is similar to that of OEP which has no aromatic

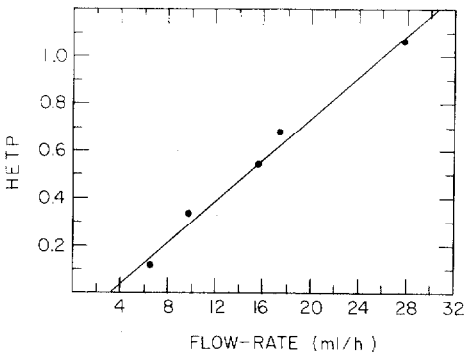

Fig. 1. HETP *versus* flow-rate.

substituents but is of comparable size. There also appears to be no effect on chromatographic behavior upon incorporation of metal ions. Thus, OEP elutes with a retention volume which is identical with that of its nickel complex. The choice of solvent system also has little or no effect on retention behavior for systems studied here.

The results discussed above are all entirely consistent with the proposal that the retention behavior of porphyrin derivatives in this system is based on a steric exclusion mechanism. This is in contrast to systems based on lipophilic polydextrans where separation is often controlled by adsorptive interactions with carbonyl or aromatic groups.

This form of chromatography is clearly suitable for separation of dimeric species from the corresponding monomers. One such problem which is frequently encountered is the separation of hemin-μ-oxo-dimers from monomeric hemins or free porphyrins. Such separations have been attained by conventional adsorption chromatography[13]. However, yield may be low or optimization of the chromatographic system for a particular separation may be time consuming[14].

As is shown in Fig. 2, this GPC method very effectively separates $(FeTPP)_2O$ from FeTPPCl. Furthermore, hemin-μ-oxo-dimers can be isolated from mixtures

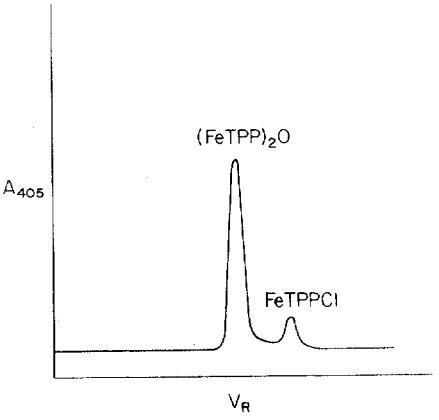

Fig. 2. Monomer–dimer separation. V_R = Retention volume; A_{405} = obsorbance at 405 nm.

containing both monomeric hemins and free base porphyrins. Thus, this method is extremely useful for removal of trace amounts of unreacted free porphyrins from hemin-oxo-dimers following iron incorporation reactions.

Although we have been able to accomplish the separation of $(FeTPP)_2O$ from FeTPPCl with several types of Bio-Beads, the SX-4 type appears to be most effective for this particular separation problem. Using these same mobile and stationary phases we have been able to separate hemin-oxo-dimers of several different substitution types from their corresponding monomers. Thus, we have separated the hemin-μ-oxo-dimers of deuteroporphyrin IX dimethyl ester (DPDME), OEP and TPP from their corresponding monomeric chloride derivatives using pure toluene or methylene chloride–toluene (3:2) as eluent on SX-3 or SX-4. It is of interest to note that the hemin-oxo-dimers of OEP and DPDME appear to be more susceptible than that of TPP to conversion to monomeric derivatives by impurities, presumably trace HCl, in halogenated solvents.

We have routinely used this system for the separation of $\approx$10 mg samples. Depending upon the application, *ca.* 20 mg of sample tends to approach the maximum capacity of the column used here.

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