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SEPARATION AND ANALYSIS OF HAEMATOPORPHYRIN DERIVATIVE COMPONENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography has been used to separate and analyse the components of haematoporphyrin derivative, a material used in cancer phototherapy. Both haematoporphyrin derivative in the solid form and the solution derived from it have been quantitatively analysed on reversed-phase columns. The factors (low pH, presence of ion-pairing reagent and solvent) that are of importance in optimising these separations are discussed.

INTRODUCTION

The ability of porphyrins to accumulate in human and animal tumours has been known for some time [1—3] and has been used as a means of identifying tumour tissue [3]. Initially haematoporphyrin was used as the tumour localizing agent but in 1960 Lipson et al. [4] introduced haematoporphyrin derivative (HPD) which they claimed was a superior localizing agent. This material was subsequently used for the identification of a variety of tumour tissues by observing the fluorescence of the porphyrin material within the tumour cells [5—12].

In the early 1970's the use of these porphyrins for cancer therapy was suggested by the work of Dougherty and co-workers [13, 14] and others [15—18]. Dougherty et al. [14] reported in 1975 that HPD, in conjunction

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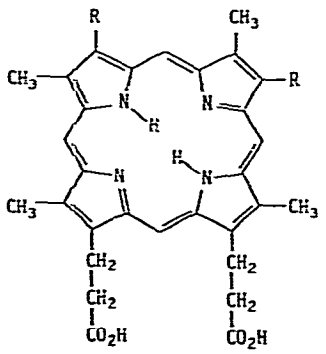
with locally applied red light delivered 1 day after administration of HPD, prevented recurrences for at least 90 days in about half of the mice bearing a subcutaneous mammary tumour. Kelly et al. [18] showed, at the same time, that HPD activated by white light caused necrosis of human bladder tumour transplanted into mice. Since that time, work on the identification of tumour tissue and the treatment of experimental animals and human patients using HPD has been considerably extended [19, 20].

HPD is prepared [4, 19] in two steps from haematoporphyrin. The first step, involving treatment of haematoporphyrin with acetic acid—concentrated sulphuric acid (19:1), produces a solid which is referred to in the sequel as HPD solid. This solid material is then dissolved in dilute sodium hydroxide solution for up to 60 min, the pH is adjusted to 7.4 using dilute hydrochloric acid then salt and saline solution are added to make the material suitable for injection. This solution, which is that used for treatment by irradiation or for detection by fluorescence of tumour material is referred to in the sequel as HPD clinical.



(1) R = $CH_3CH(OH)$– (haematoporphyrin)
(2), (3) R = $CH_3CH(OH)$–, CH_2=CH–
(4), (5) R = $CH_3CH(OAc)$–, $CH_3CH(OH)$–
(6) R = $CH_3CH(OAc)$– (haematoporphyrin diacetate)
(7), (8) R = $CH_3CH(OAc)$–, CH_2=CH–
(9) R = CH_2=CH– (protoporphyrin)

Both HPD solid and HPD clinical are mixtures of porphyrins as shown by thin-layer chromatography (TLC) [19]. Separation of the methyl esters of the porphyrin components of HPD solid was achieved by silica chromatography and nine components (1—9) were identified in this manner [21]. These compounds differ only in the nature of the side chains (R_1 and R_2) and it is convenient to refer to them by indicating the nature of these side chains. Thus compounds 2 and 3 containing a 2-hydroxyethyl and a vinyl side chain are designated hydroxyvinyl derivatives; 4 and 5 are the hydroxyacetates and 7 and 8 are the vinylacetates. On this designation haematoporphyrin (1) is the dihydroxy compound, protoporphyrin (9) is the divinyl compound and the diacetate derivative (6) of haematoporphyrin becomes the diacetate. The advantage of this designation is that it is possible to discuss the isomeric pairs (e.g. 2 and 3) without necessarily having to indicate the exact structure of both.

While providing information on the composition of the porphyrin mixture, analysis of the methyl esters does not necessarily reflect the composition of the mixture of carboxylic acid used clinically. Bonnett et al. [22] have analysed the porphyrin acids by high-performance liquid chromatography (HPLC) and were able to identify haematoporphyrin (1) the diacetate (6) (partially

resolved only) and protoporphyrin (9) in the mixture. A more recent publication from Bonnett and co-workers [23] describes the further separation of HPD solid and the identification of components 1—8 in the mixture.

In this paper we describe the first complete analysis of both HPD solid and HPD clinical and discuss the factors that are of importance in achieving a complete resolution by HPLC of a very similar group of porphyrins as well as some of the factors that affect the composition of HPD clinical.

EXPERIMENTAL

All solvents were distilled, degassed and filtered through a 0.45-μm Millipore filter prior to use.

HPLC was performed using a Waters Model 6000A solvent delivery system and U6K injector. The detector normally used was a Waters Model 440 UV absorbance type operating at 405 nm. Columns used were Waters μBondapak C_{18} (10 μm) and Waters Radial-Pak C_{18} 5 mm and 8 mm I.D. (5 and 10 μm).

Haematoporphyrin was obtained in the form of its dihydrochloride from Roussel. Protoporphyrin was prepared from haematoporphyrin by brief heating in dimethylformamide. Haematoporphyrin diacetate was prepared by treating haematoporphyrin with acetic anhydride and pyridine and was approximately 80% pure. Haematoporphyrin dimethyl ester, prepared using diazomethane, was separated by HPLC into two peaks, resolved to the baseline, using the conditions described in Fig. 1. Each peak was collected, the solvent was removed and the residue was analysed by mass spectrometry (AEI MS-30).

The ion-pairing reagent (IPR) was tetra-*n*-butylammonium phosphate (Unichrom). The reagent is prepared by diluting the Unichrom concentrate in

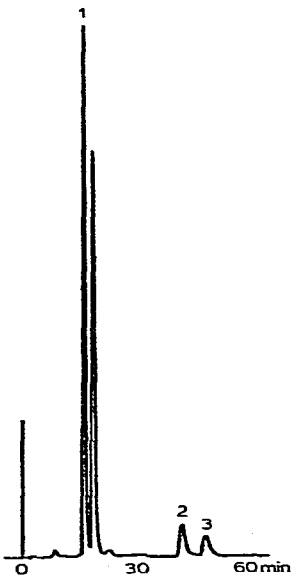


Fig. 1. Chromatogram of haematoporphyrin dihydrochloride in dimethyl sulfoxide solution. Peak numbers correspond to the structure numbers in the text. Column: Waters Radial-Pak C_{18} (5 μm). Solvent: methanol—aqueous IPR, pH 2.0 (80:20); flow-rate: 1.5 ml/min.

distilled water to 1 l, providing a 5 m*M* solution. This solution is then diluted with distilled water (1:1) before use, providing a 2.5 m*M* solution whose pH was adjusted using phosphoric acid and measured with a digital pH meter.

RESULTS AND DISCUSSION

In order to achieve a satisfactory analysis of either form of HPD, any analytical technique must be quantitative, reproducible and preferably relatively rapid. We considered that HPLC would be satisfactory on all these counts. Our initial attempts to reproduce the literature separation [22] using the reported conditions showed that there were considerable differences between the results reported in the literature and the results we could achieve. Accordingly we undertook an investigation into the effect of changing individual parameters in order to ascertain their importance in the separation technique.

Our best separations have been achieved on either a Waters Radial-Pak C_{18} column containing 5-μm adsorbent or a Waters μBondapak C_{18} (10 μm) column. However, the conditions required for best separation and resolution on these columns vary considerably. We attribute this difference to the extent to which the silica in each column has been silanised. With the μBondapak column, any silica remaining unreacted after the treatment with the C_{18} silylating reagent is end-capped with trimethylsilyl groups. The Radial-Pak columns do not receive this treatment. Hence the type of silica, the mesh size and the procedure used for silylation all combine to produce column materials whose performance can vary considerably yet which can all provide good resolution and peak shape of the porphyrin acids.

Initially, two solvent systems were found to be satisfactory, either methanol—water (approx. 8:2) or acetonitrile—water (approx. 6:4). Both solvent mixtures give best results when used at low pH (either added phosphoric acid or acetic acid) although lower pH values resulted in longer retention times. For example, protoporphyrin has a retention time of 60 min at pH 2 but a retention time of 15 min at pH 5 when analysed on the same column using identical conditions except for the pH of the solvent. A lowering of retention time generally resulted in less satisfactory resolution. At higher pH values (4—7) the porphyrin peaks often appeared as quite sharp but tailing peaks superimposed on a broad hump. This behaviour, which has been observed on a number of different columns using different solvents, is tentatively attributed to the aggregation which occurs readily with porphyrin acids. It is known [24] that the monomer is substantially favoured at lower pH values and we suggest that the broad unresolved peaks observed at higher pH values are due to dimers (or higher aggregates).

A feature of the use of the methanol—water solvent system was that it consistently gave two peaks of approximately equal response for haematoporphyrin on a variety of columns. This complete resolution of the two very close peaks (see Fig. 1) could not be achieved using the acetonitrile—water solvent system which generally only partially separated the two peaks. These two peaks were observed in samples of HPD, solid or clinical, as well as in commercial samples of haematoporphyrin from two different sources. The doublet of peaks was also observed when haematoporphyrin dimethyl ester was analysed

using the methanol—water solvent system. The two components of haematoporphyrin and of the dimethyl ester have been separated and collected using semi-preparative conditions and gave substantially single peaks after reinjection and analysis. This result is not compatible with the expected behaviour of two isomers that are equilibrating. A fraction containing only two peaks associated with the dimethyl ester of haematoporphyrin was collected from the HPLC column and subjected to hydrolysis (pH 12, 10 h, room temperature), neutralised and the material was analysed by HPLC. Two peaks of identical elution volume to those of haematoporphyrin were obtained. Mass spectral analysis of each separated component from the ester sample gave a molecular ion at *m/e* 626 corresponding to that of haematoporphyrin dimethyl ester and showed the expected fragmentation pattern for a compound with two hydroxyethyl side chains. We therefore suggest that the two peaks correspond to the two diastereoisomers of haematoporphyrin due to the presence of the two chiral carbon atoms in the hydroxyethyl side chains.

Other peaks in the chromatograms of HPD solid and of HPD clinical can be readily identified. The two monohydroxyethyl monovinyl isomers, 2 and 3, are always present to a small extent in commercially available haematoporphyrin which enables their retention volume to be determined. Acetylation of haematoporphyrin using acetyl chloride—pyridine gives the diacetate (6) as the major product. The diacetate can be separated from other products by chromatography on silica. Minor products from the acetylation reaction are the hydroxy acetates, 4 and 5, and the vinyl acetates, 7 and 8. These pairs can be separated from other components by HPLC (see Fig. 2) esterified and

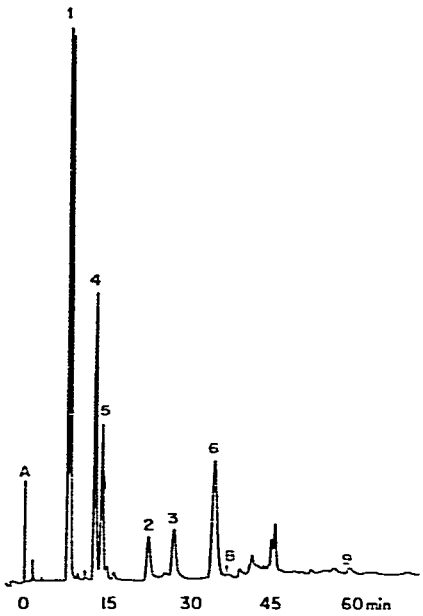


Fig. 2. Chromatogram of HPD solid. Peak numbers correspond to the structure numbers in the text. Column: Waters Radial-Pak C_{18} (5 μm). Solvents: (A) acetonitrile—aqueous IPR, pH 2.5—methanol (3:2:1); (B) methanol—aqueous IPR, pH 2.5 (90:10); flow-rate: 2 ml/min.

identified by spectral methods [21, 25]. We have not, thus far, attempted to ascertain which peak of each particular pair of isomers belongs to a particular structure for that pair.

Notwithstanding the generally highly acceptable performance of the binary solvent systems (methanol or acetonitrile plus aqueous IPR) for analysing HPD clinical, it was found that applying these to the more complex mixtures encountered such as HPD solid, provided less than ideal results. For example, with the methanol-based system, the peak attributed to the diacetate (6) was found to have identical retention time to one of the hydroxy vinyl isomers and no resolution could be obtained of the hydroxy acetate isomers. The acetonitrile based solvent (60% + 40% aqueous IPR, pH 2.5) on the other hand, provided greatly improved separation of the relatively polar components though at the tolerable expense of complete loss of resolution of the haematoporphyrin diastereoisomers.

The aqueous acetonitrile with the IPR solvent also gave no resolution of the haematoporphyrin peaks on the analytical μBondapak column, However, resolution of this peak can be obtained using the aqueous acetonitrile—acetic acid solvent mixture on this column, thus clearly indicating that strong hydrogen bonding agents are required for the selective HPLC separation of the haematoporphyrin diastereoisomers. Consequently, addition of 10—20% methanol to the aqueous acetonitrile solvent gave not only partial resolution of these two components but also provided improved separation of the other components in this region, notably the hydroxy acetates. In this system, the peak attributed to the diacetate (6) was then clearly separated with a longer retention time from the hydroxy vinyl isomers (Fig. 2).

Despite the considerably improved resolution and peak shape afforded by this ternary solvent system in the polar region of the chromatogram, the protoporphyrin peak showed considerable tailing and was virtually unobservable when present in less than 5—8% concentration. This deficiency was overcome by utilising a dual solvent system for each analysis whereby, in the absence of a solvent programmer, the aqueous acetonitrile—methanol—

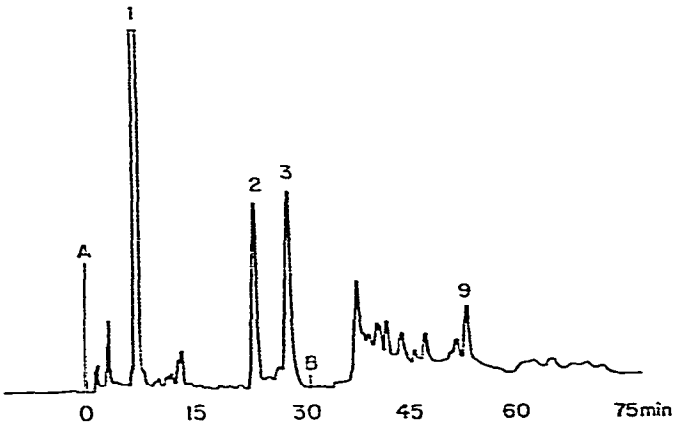


Fig. 3. Chromatogram of HPD clinical. Conditions as for Fig. 2.

IPR mixture was changed to 90% aqueous methanol—IPR (pH 2.5) after elution of the second hydroxy vinyl peak (or diacetate peak if present). This approach provided a chromatogram with greatly improved peak shape and convenient retention volume for protoporphyrin in addition to revealing, by virtue of resultant decreased peak width, a number of previously obscured minor components. The majority of these were observed in the region between the diacetate compound and protoporphyrin but several minor peaks eluted after the latter, usually considered to be the least polar porphyrin of interest in this investigation.

Integration of the chromatogram for HPD clinical (Fig. 3) shows that haematoporphyrin is present to the extent of 45—50%, the hydroxy vinyls comprise 20—25% and protoporphyrin constitutes approximately 3—5% of the total mixture. Minor peaks eluting near haematoporphyrin make up 5—10% of the material and minor peaks eluted by the more polar solvent (B) but excluding protoporphyrin account for 10—15%. However, this last value is less accurate than the others because of the difficulty in allowing for the baseline shift that occurs on changing solvent.

HPD solid is much more variable in composition due to changes that may occur on standing particularly if the material is not adequately washed and dried. However, the diacetate (6) is the major component usually accounting for more than 50% of the freshly prepared material. Depending on how the HPD solid is stored this value may drop considerably on standing. However, in spite of these possible changes the analysis of the HPD clinical made from the HPD solid does not vary to a large extent suggesting that the changes on

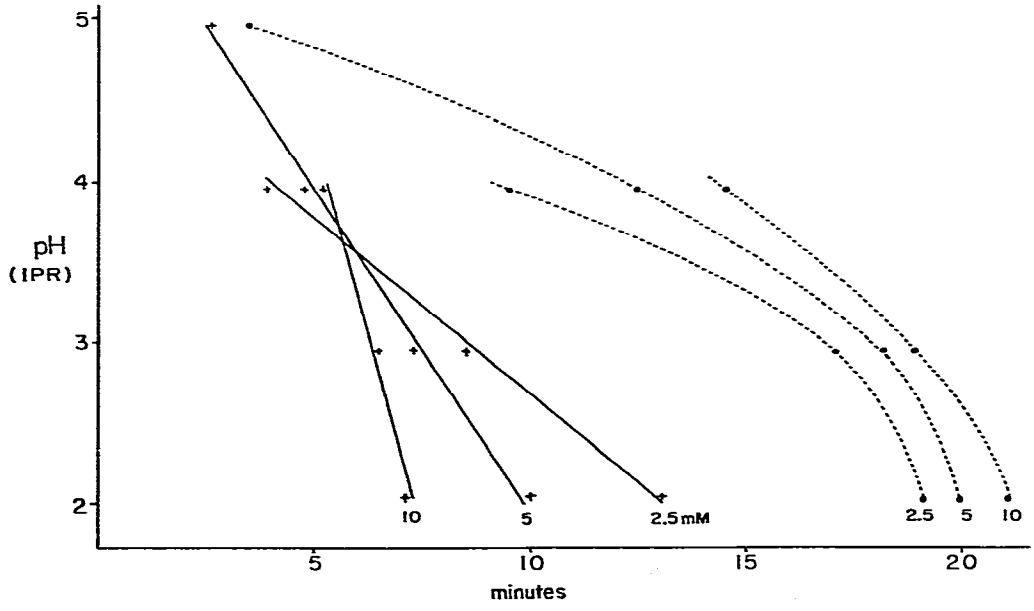


Fig. 4. Plot showing the effect of aqueous IPR solution pH and concentration on the retention time of haematoporphyrin (——) and protoporphyrin (----, multiply time scale × 3). Column: Waters Radial-Pak C_{18} (10 μm); solvent: methanol—aqueous IPR (80:20); flow-rate: 2 ml/min.

storage to HPD solid are similar to those caused by the alkali treatment. The major difference is that the relative amount of the hydroxy vinyls is increased in HPD clinical when it is prepared from fresh HPD solid.

It may be noted that acetonitrile, possessing relatively low hydrogen bonding potential, enhances the resolution of the more polar components as a consequence of minimised solvent—solute interactions. Methanol, on the other hand, appears to provide better resolution and peak shape for the less polar constituents eluting after the diacetate compound. Thus, this dual solvent (or gradient elution) approach would appear to offer the most acceptable compromise for the convenient analysis of HPD solutions.

IPRs have been used quite extensively in the separation of polar, water soluble materials on reversed-phase columns. The role of the IPR is complex and is currently poorly understood [26]. However, at least in part, one reason why IPRs are so useful was shown by the necessity to use them with the Radial-Pak reversed-phase columns which strongly suggests that in this case they function to some extent as an alternative to end-capping by being adsorbed onto the unprotected OH groups on the silica.

Fig. 4 indicates the different effect a change in the ion-pairing concentration has on haematoporphyrin (a polar component) compared to protoporphyrin (a non-polar component). Fig. 4 also shows the substantial change in retention time of these components as the pH of the solution changes.

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