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Isolation and photodynamic effects of hematoporphyrin derivative components: a chromatographic analysis of the starting materials

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Abstract

Twenty different fractions of hematoporphyrin derivatives (HpD) and eight fractions of an HpD dimer mixture were isolated utilizing isocratic reversed-phase ion-pair high-performance liquid chromatography. These fractions were characterized by UV–visible and fluorescence spectrophotometry. Fluorescence quantum yields and photokill efficiency for each fraction in PTK_2 epithelial cells were obtained. Results indicate that some part of the photoactivity exhibited by HpD may be due to impurities present in the HpD starting material, hematoporphyrin-IX dihydrochloride, depending on its source. It was also found that hematoporphyrin D, a commercial acetylated product formed during synthesis of HpD, contained a higher percentage of monomers than would be expected.

1. Introduction

Photodynamic therapy (PDT) is an experimental treatment for cancer and consists of the administration of a photosensitizing chemical followed by activation of the chemical by light, usually from a laser. The absorption of light at the proper wavelength induces fluorescence in the drug and ultimately leads to the production of excited triplet state molecules. The transfer of energy from the drug to triplet oxygen may result in the production of singlet oxygen, an agent which is toxic to cells. Drugs currently being investigated for use with PDT include porphyrins [1,2], chlorins [3,4], purpurines [5], phthalocyanines [6], and derivatives of all of these [7]. Lederle Pharmaceutical Company has recently completed stage III clinical trials of a specific form of hematoporphyrin derivative (HpD), under the trade name Photofrin® porfimer sodium, for use in the treatment of esophageal, lung, and bladder cancer. HpD is a mixture of several porphyrins, including the monomers: protoporphyrin (PP), hematoporphyrin (Hp), hydroxyethylvinyldeuteroporphyrin (HVD), and other dimeric and/or oligomeric porphyrins [1]. These dimers and oligomers are believed to contain ester linkages, ether linkages, and carbon–carbon linkages [2]. Dougherty [1] originally produced evidence

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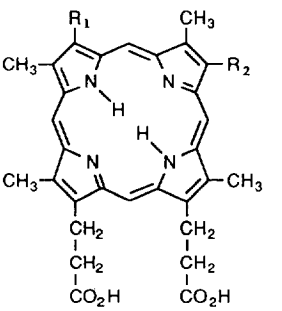
which suggested that dihematoporphyrin ether (DHE) was the only in vivo photoactive component present in the HpD mixture and later produced structural evidence for the existence of this dimeric HpD component [8]. Evidence has since been presented which showed conclusively that the dimeric/oligomeric fractions of Photofrin do not contain DHE [9]; rather, the observed photoactivity is more likely related to the presence of ether-linked [10] and carbon–carbon-linked [11] mono- and/or divinyl dimers or oligomers.

The problem of separating and identifying components present in the HpD mixture has proved to be a formidable task. Much is known about the composition of the HpD mixture but its *exact* composition is still not known. What is known is that this drug is effective in destroying certain types of cancers.



Hematoporphyrin (dihydroxyethyl): $R_1 = R_2 = CH_3CH(OH)$ -
Hydroxyacetate: R_1 = hydroxyethyl, $CH_3CH(OH)$ -
R_1 = acetate, $CH_3CH(OAC)$ -
Hydroxyethylvinyl: R_1 = hydroxyethyl, $CH_3CH(OH)$ -
R_2 = vinyl, $CH_2 = CH$ -
Diacetate: $R_1 = R_2 = CH_3CH(OAC)$ -
Vinylacetate: R_1 = vinyl, $CH_2 = CH$ -
R_2 = acetate, $CH_3CH(OAC)$
Divinyl (protoporphyrin): $R_1 = R_2 = CH_2 = CH$-

Fig. 1. The nine basic structures for the hematoporphyrin derivative precursor, HpDp. Taken from Ref. [10].

1.1. Composition of the starting materials

HpD was synthesized in two ways in this report. It was first prepared in the usual manner by utilizing a two-step synthesis whereby hematoporphyrin-IX dihydrochloride is treated with a mixture of sulfuric and acetic acid [12]. The result of this step produces an HpD intermediate which is composed of eight porphyrin acetates and protoporphyrin. This step-one intermediate is termed hematoporphyrin derivative precursor, HpDp, in this report. Ward et al. [13] was able to isolate all nine of these components of HpDp. The structures are shown in Fig. 1. The HpDp mixture is then treated with a 0.1 *M* sodium hydroxide solution. The pH is then adjusted to 7.4. This produces the product commonly known as hematoporphyrin derivative, HpD.

The step-one intermediate, HpDp, is marketed by Porphyrin Products under the trade name Hematoporphyrin D. Therefore, HpD can also be prepared by a base hydrolysis of Hematoporphyrin D in a 0.1 *M* sodium hydroxide solution. This report provides a comparison of the composition of the synthesized HpDp product with Hematoporphyrin D.

Kessel et al. [14] have also reported a synthetic technique for HpD. It involves the use of hematoporphyrin-IX diacetate as the starting material. Except for some small variations, this HpD synthesis is quite similar to the Lipson method [12]. However, the composition is different. This will be discussed later.

1.2. Composition of hematoporphyrin derivative

The composition of HpD has been studied extensively. It was first synthesized by Lipson in consultation with Dr. S. Schwartz, and published by Lipson in 1961 [12]. This two-step procedure of acid acetylation, followed by alkaline hydrolysis, of hematoporphyrin-IX dihydrochloride has been described above. The first HPLC analysis of the acetylation products was published by Clezy et al. [15] in 1980, but the most elegant was accomplished by Ward et al. [13]. There appears to be universal agreement about the composition of the HpD intermediate, HpDp.

The HpD mixture reportedly contains 20% Hp (hematoporphyrin-IX), 20–30% HVD (hydroxyethylvinyldeuteroporphyrin), 3–5% PP (protoporphyrin), and approximately 50% oligomeric components [13–20]. None of these re-

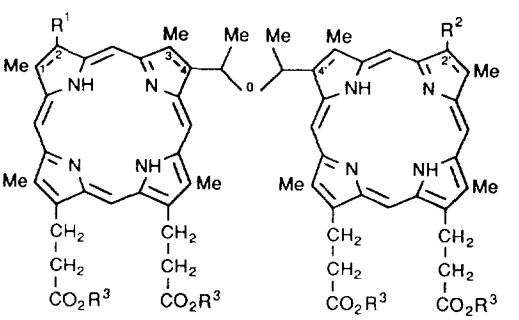
ports utilized Hematoporphyrin D as the starting material for synthesizing the HpD product. As will be shown later, the protoporphyrin content rises to 10% when HpD is prepared from Hematoporphyrin D.

It was reported that the in vivo anti-tumor activity widely reported for HpD was contained in the oligomeric fraction [6]. Quadrologic then introduced the oligomerically rich drug, Photofrin. In 1984, Dougherty et al. [8] reported that this oligomeric portion of HpD contained a dihematoporphyrin ether (DHE) linked through the 2 and/or 4 hydroxyethyl groups. Two years later, Kessel et al. [21], utilizing lithium aluminum hydride, reported the existence of ester linkages through the hydroxyethyl groups and the propionic side chains. In 1987, Dougherty used this same technique to show the existence of both ether and ester linkages in Photofrin [20]. Dougherty's group reported that Photofrin contained approximately 40–50% of porphyrins linked via an ester bond, and that approximately half of these ester linkages contained vinyl groups. This group also concluded that a significant portion of the remaining Photofrin drug was composed of ether linkages. It now appears that the ether/ester linkages are dependent upon the synthetic technique employed for HpD; e.g. the use of hematoporphyrin-IX dihydrochloride as the starting material (the Lipson method) primarily yields ether linkages, while the use of hematoporphyrin diacetate as the starting material (Kessel's method) primarily yields ester linkages. In addition, the ester linkages formed during HpD synthesis utilizing Kessel's method have been shown to convert to ether linkages after a few days when dissolved in water [13].

The Photofrin drug contains a smaller percentage of monomers than does HpD, but also contains a higher percentage of late running oligomeric components. Based on mass spectral analysis, Pandey et al. [10] was able to isolate some fractions of Photofrin. They reported the existence of ether-linked dimers, trimers, and various monomeric dehydration products. This work by Pandey et al. suggested that the oligomers present in Photofrin contained up to six porphyrin rings, confirming an earlier report by Kessel et al. [22].

1.3. Ether-linked dimers

In an effort to identify some of the dimeric components present in Photofrin, Morris and Ward, in 1988, synthesized several porphyrin dimers, including dihematoporphyrin ether, DHE [9]. The structures for these dimers are shown in Fig. 2. Based on some preliminary measurements, these researchers reported that the divinyl dimer (DVD) produced a significant in vivo photoactivity while the ether-linked dihydroxyethyl dimer (DHE) was photodynamically inactive. In 1990, Pandey et al. [23] also produced strong evidence which showed that the ether-linked dihydroxyethyl dimer was an ineffective in vivo photosensitizer. In 1991, Kessel et al. [24] produced solid evidence that the ether-linked divinyl dimer (DVD) and trimer (DVT) porphyrin derivatives were efficient in vivo photosensitizers. There is presently little doubt that ether-linked porphyrin dimers must contain



Tetramethyl ester of monohydroxy-monovinyl hematoporphyrin ether dimer:
$R^1 = CH_3CH(OH)$, $R^2 = CH_2{=}CH_2$, $R^3 = CH_3$
Tetramethyl ester of dihematoporphyrin ether dimer:
$R^1 = R^2 = CH_3CH(OH)$, $R^3 = CH_3$
Tetramethyl ester of divinyl ether dimer:
$R^1 = R^2 = CH_2{=}CH_2$, $R^3 = CH_3$

Fig. 2. The basic structures for the ether-linked dihematoporphyrin, the ether-linked divinyl porphyrin, and the ether-linked monovinyl porphyrin. Taken from Ref. [7].

at least one vinyl group to be an effective in vivo photosensitizer.

1.4. Ester-linked dimers

As part of the vigorous research conducted by Pandey and Dougherty [25], these researchers not only provided evidence that the ester-linked dimers of deuteroporphyrin and protoporphyrin were not present in Photofrin, but also provided evidence that the ester-linked dimers were photodynamically weak in vivo photosensitizers. In 1990, Ward et al. [26] reported that di-hematoporphyrin ester was not a major component of Photofrin. Despite the fact that ester-linked porphyrins are believed to be present in Photofrin, there is presently little interest in any ester-linked porphyrin dimer for use as a photosensitizer.

1.5. Carbon–carbon-linked dimers

The existence of carbon–carbon-linked dimers in the HpD and Photofrin mixtures was first reported by Byrne and Ward [27]. This report assumed that these dimers were inactive in vivo. However, Pandey et al. synthesized various porphyrin dimers with carbon–carbon linkages [11], and have shown that all of them possess a significant in vivo photoactivity.

1.6. Chromatographic analysis of HpD and Photofrin

Previous chromatographic analyses of the HpD mixture have been reported. Most reversed-phase systems employ gradient elution which allows separation of porphyrin-free acids with two to eight carboxylic groups. The first ion-pair reversed-phase HPLC analysis of the base hydrolyzed HpD mixture was presented in 1978 by Bonnett et al. [28]. Tetrabutylammoniumphosphate (TBAP) was used as the ion-pairing agent. The ion-pair technique has since been adapted to an isocratic method by Ward et al. [13] and is the chromatographic technique employed in this report. Kessel et al. [14], Hilf et al. [16], Berns et al. [17], Moan et al. [29], Kessel and Chou [30], and Swincer et al. [31] have also reported reversed-phase HPLC analyses of HpD. These results also outline the composition of HpD as 20% Hp, 20–30% HVD, 3–5% PP, and approximately 50% oligomeric components. Dellinger and Brault [19] have reported an effective normal-phase HPLC technique for the separation of HpD mixtures.

This report describes a chromatographic separation of twenty different HpD fractions. Each fraction was characterized by chromatographic retention time and peak shape, UV–visible and fluorescence emission spectrophotometry, fluorescence quantum yield measurements, and by photokill efficiency on kangaroo rat PTK_2 epithelial cells. These HpD parameters are compared with those for hematoporphyrin-IX dihydrochloride (the starting material for HpD, from three different vendors), protoporphyrin, hydroxyethylvinyldeuteroporphyrin, Photofrin, HpDp (the HpD precursor formed after the acetylation of hematoporphyrin-IX with sulfuric–acetic acid), and a synthesized mixture of ether-linked dihydroxyethyl (DHE), divinyl (DVD), and monohydroxyethyl monovinyl dimers (MVD).

2. Experimental

2.1. Synthetic procedures

HpD was prepared by treating one part hematoporphyrin-IX dihydrochloride (Sigma) in a 19:1 v/v acetic acid–sulfuric acid mixture, following a procedure outlined by Dougherty and Gomer [18]. This solution was then stirred in the dark for 1 h at room temperature, filtered, and precipitated with 3% sodium acetate. This synthesis leads to what we call the HpD intermediate, HpDp. HpDp was then washed and dried. An aliquot of 1.0 g of this HpDp powder was then base-hydrolyzed in 50 ml of 0.1 *M* NaOH and stirred for 1 h, leading to the synthesis of what is commonly known as HpD. The HpD mixture was adjusted to pH = 7.0, then diluted to a final concentration of 5 mg/ml with 0.9% NaCl. The base hydrolysis of HpDp

produces the photoactive oligomeric HpD components while preserving the monomers protoporphyrin-IX, hematoporphyrin-IX, and hydroxyethylvinyldeuteroporphyrin (HVD). Hematoporphyrin-IX dihydrochloride, protoporphyrin-IX, and HVD were purchased from Porphyrin Products (Logan, UT, USA). Photofrin was graciously supplied by Quadrologic (Vancouver, Canada).

HpD was also prepared by hydrolyzing Hematoporphyrin D (Porphyrin Products) in 0.1 *M* NaOH and proceeding as above.

The synthesis of the HpD dimer mixture, believed to be the ether-linked tetramethyl esters of divinyl (DVD), dihydroxyethyl (DHE), and monohydroxyethyl monovinyl dimers (MVD), was carried out following a technique outlined by Morris and Ward [9]: 25 ml of a saturated solution of hematoporphyrin dimethyl ester in dichloromethane was mixed with 25 ml of a saturated solution of hydrogen bromide in dichloromethane, and the mixture stirred for 1 h. The course of the reaction was followed chromatographically by monitoring the disappearance of the hematoporphyrin dimethyl ester peak. Chromatographic separation of the dimeric fractions was achieved as described for HpD fractions.

The ether-linked hematoporphyrin dimer (DHE), the ether-linked divinyl dimer (DVD), and the ether-linked monovinyl trimer (MVT) were graciously supplied by Dr. D. Kessel.

2.2. Chromatographic conditions

HPLC measurements were performed under isocratic conditions on a Beckman 342 chromatograph housing a Beckman 110 pump, a Beckman 340 controller, a Beckman 165 detector set to 280 nm, and an Altex 210 Injector. A Waters C_{18} reversed-phase μBondapak cartridge was used for separation. The HPLC system included the use of a Whatman 0.2-μm in-line filter and a silica guard column. The mobile phase was an 80:20 v/v methanol–water mixture containing a 2.5 m*M* tetrabutyl ammonium phosphate (TBAP) buffer (pH = 2.5). Elution of the HpD components began at 2.0 min and was complete after about 60 min. A 2.0 ml/min flow-rate and a chart speed of 10 mm/min was utilized. Each fraction was collected manually into amber vials, based on their known retention times. Reinjection of these HpD fractions showed that each fraction contained between 50 and 90% of the fraction of interest, with the early and late running fractions being most resolved. The column was cleaned with mobile phase for 1 h after each injection. The identification of some of the HpD fractions was assessed via co-elution chromatography of known components, where possible. After spectroscopic analysis, each HpD fraction was dried overnight under vacuum to remove the methanol–water mixture.

2.3. Spectroscopic procedures

The electronic spectrum of each fraction was measured using a Perkin-Elmer Lambda 4B spectrophotometer. Spectra were recorded from 750 nm to 300 nm in 1-cm quartz cuvettes and absorbance values ($\lambda_{max} = 400$ nm) for each fraction were obtained. The absorbance of each fraction was adjusted to read between 0.05 and 0.1 units for the fluorescence quantum yield measurements.

The fluorescence emission spectrum for each fraction was obtained using a Hitachi 4010 spectrofluorometer and Rhodamine B (Curtin-Matheson Scientific) for spectral correction. Each HpD fraction was excited at 400 nm and its fluorescence emission spectrum scanned from 550 nm to 750 nm. Peak resolution was checked by taking the first derivative of each observable absorption band. Quantum yield measurements (within 10% error) were performed utilizing the ratio method and tetraphenylporphine as a standard ($\Phi_f = 0.12$) [32]. This technique follows that outlined by Parker and Rees [33], utilizing the following equation:

$$\frac{F_2}{F_1} = \frac{area2}{area1} = \frac{\phi_2}{\phi_1} \cdot \frac{\epsilon_2 c_2 d_2}{\epsilon_1 c_1 d_1} = \frac{\phi_2}{\phi_1} \cdot \frac{A_2}{A_1} \quad (1)$$

where F = fluorescence area, ϕ = fluorescence quantum yield, and A = absorbance.

The area under the curve of each HPLC fraction was calculated utilizing computer software developed by the Hitachi Company.

2.4. Photokill measurements

Preparation of the photosensitizer

Each of the eighteen HpD fractions was collected into amber vials. The mobile phase was removed in a Savant speed vacuum concentrator. Phosphate buffered saline (PBS) (Sigma) was then added to the resultant powder to a final concentration of 25 mg/ml. The fractions were then warmed at 37°C for several hours, mixed well, and filtered with a Corning 0.2-μm collection system. These solutions were then refrigerated until ready for use.

Preparation of the kangaroo rat PTK$_2$ cells

Kangaroo rat kidney epithelial cells (ATCC registration No. CCL56) were harvested in log phase (about 6 days) in 150 mm^2 culture dishes (Costar) containing Eagle's Minimum Essential Medium (MEM) (10% fetal bovine serum, FBS; 2.5% $NaHCO_3$; and 5% penicillin–streptomycin solution). Once confluent, the cells were lifted from the culture dish bottom with 2.0 ml of a Trypsin/EDTA solution. An aliquot of 8.0 ml of MEM was then added to the culture dish. The 10.0-ml aliquot of cells was then transferred to a 15-ml centrifuge tube (Corning) which received a gentle but constant vortex mixing to ensure an even distribution of cells throughout the media.

Introduction of the photosensitizer to PTK$_2$ cells

An aliquot of 50 μl of PTK$_2$ cells from the 15 ml centrifuge tube was added in triplicate to 54 wells of a sterile 96-well cluster plate (PGC Scientific) using wide bore pipette tips (PGC Scientific). Aliquots of 50 μl each of the eighteen HpD fractions (25 mg/ml) were then added to the 54 wells (each fraction was evaluated three times). Control measurements were carried out in two ways. One set of controls consisted of four wells containing 50 μl of PTK$_2$ cells and 50 μl of PBS (these cells would receive light but no drug). The other set of controls consisted of eighteen wells containing 50 μl of PTK$_2$ cells and 50 μl of each HpD fraction (these cells received the drug but no light). Five standards were used to establish a survival curve—cell number verses absorbance at 570 nm. These five wells contained 50, 40, 30, 20, and 10 μl of cells, respectively, 50 μl of PBS each, and 0, 10, 20, 30, and 40 μl of MEM, respectively. The standards were not irradiated. The cluster was then placed in a Forma CHP Incubator (37°C, 5% CO_2) overnight.

Photodynamic measurements

At 24 h after mixing, the cluster plate was removed from the incubator. The sensitized cells were then irradiated for 3 min (four wells per treatment) with a 630-nm light from a Coherent Nd–YAG pumped dye laser (Quartronic, 50 mW).

Cell viability measurements

One hour after irradiation, 15 μl of Cell Titer 96 (Promega) was added to each test sample. The formazan dye is taken up only by living cells. Comparisons of MTT, thymidine incorporation, and LDH release have been reported. The Promega titer compares favorably [34–37]. After 6 h, 100 μl of solubilizer was added to each well, and the cluster plate was left in the incubator (37°C, 5% CO_2) overnight. The following day, the absorbance of each well at 570 nm was determined using a 96-well Elisa Reader (Bio-tek). Cell viability (%) was assessed by plotting the absorbance for each well against the standard plot.

3. Results and discussion

There are no reports describing the exact preparation and/or storage conditions employed for Hematoporphyrin D. As a result, it is not widely used in the synthesis of HpD. It is known that Hematoporphyrin D is a mixture of porphyrin acetates. Such acetates are variable in composition because they lose acetic acid easily with time and tend to polymerize. The primary difference between commercial HpDp, Hematoporphyrin D, and that synthesized in this report

is that the former contains a lower diacetate content and a higher HVD and protoporphyrin content (see Fig. 3). This suggests either that Hematoporphyrin D is synthesized from a hematoporphyrin-IX product whose composition resembles the Aldrich product in Fig. 4c, or, that the long-term storage methods employed by Porphyrin Products for its Hematoporphyrin D allows for loss of acetic acid and/or dehydration. One or both of these occurrences would lead to a decrease in the presence of the acetate groups and to an increased presence of both HVD and protoporphyrin.

Of particular importance is the presence in Hematoporphyrin D of an undesirably high 18% protoporphyrin content. Because a large protoporphyrin concentration would ultimately lead to increased skin sensitivity on exposure to light, it would also limit the effectiveness of any PDT treatment.

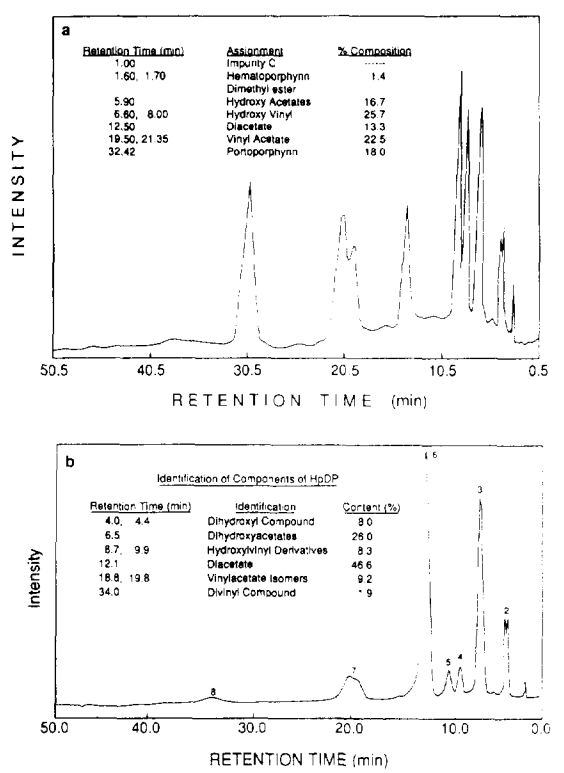


Fig. 3. (a) Hematoporphyrin D. Chromatographic conditions are similar to those outlined for Fig. 7. (b) Chromatogram of hematoporphyrin derivative precursor (synthesized in our lab).

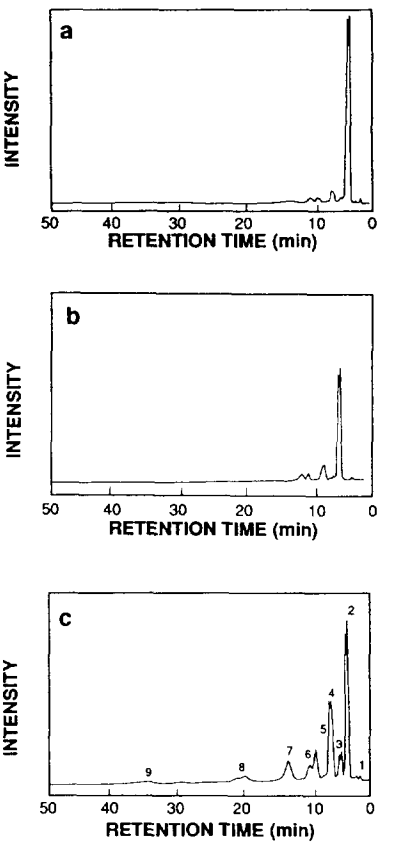


Fig. 4. HPLC chromatograms of hematoporphyrin-IX dihydrochloride from Porphyrin Products (a), Sigma (b), and Aldrich (c), obtained under the same conditions as those outlined for Fig. 7.

In order to identify the structure and/or the origin of any HpD or HpDp impurity, an investigation of the starting material, hematoporphyrin-IX dihydrochloride, is necessary. Our chromatogram of hematoporphyrin dihydrochloride from three different vendors appears in Fig. 4. Based on the correct assumption that the major chromatographic peak (e.g. peak 2 in Fig. 4c) belongs to the hematoporphyrin-IX product, it is clear that the products from Porphyrin Products and Sigma are least contaminated, while that from Aldrich is the most contaminated. Although all commercial hematoporphyrin samples are variable and highly im-

pure materials, the Aldrich sample appears to contains a higher number of impurities. Referencing Dinello and Chang [38], commercial hematoporphyrin-IX dihydrochloride is known to contain substantial amounts of HVD and protoporphyrin. The presence of the isomeric HVD impurity is seen as peaks 5 and 6 in Fig. 4c, for all three commercially available hematoporphyrin-IX dihydrochloride products. The protoporphyrin impurity is best evidenced by peak 8 in Fig. 4c. The identity of these impurities was easily determined by co-elution chromatography (see Figs. 5 and 6).

Chromatograms of hydroxyethylvinyldeuteroporphyrin (HVD) and protoporphyrin (PP) appear in Figs. 5a and 6, respectively. These two

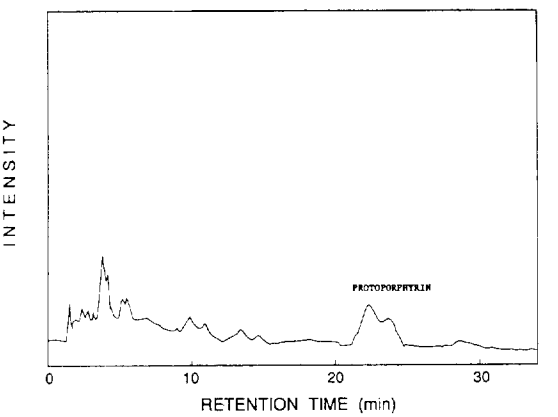


Fig. 6. HPLC chromatogram of protoporphyrin. Spectrum was obtained on a Beckman 340 chromatograph using a mobile phase of 80:20 (v/v) MeOH–H_2O containing a 2.5-m*M* TBAP buffer (pH = 2.5) and a Waters C_{18} μBondapak reversed-phase column. Flow-rate = 2.0 ml/min.

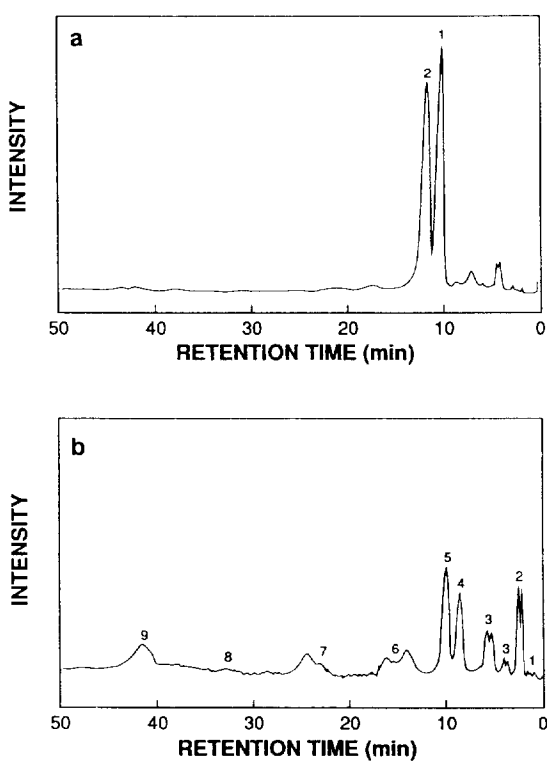


Fig. 5. HPLC chromatogram of hydroxyethylvinyl deuteroporphyrin, HVD (a) and Photofrin (b). Spectrum was obtained on a Beckman 340 chromatograph using a mobile phase of 80:20 (v/v) MeOH–H_2O containing a 2.5-m*M* TBAP buffer (pH = 2.5) and a Waters C_{18} μBondapak reversed-phase column. Flow-rate = 2.0 ml/min.

porphyrins are also contaminated. HVD clearly contains hematoporphyrin and protoporphyrin. Protoporphyrin clearly contains hematoporphyrin and HVD. Apparently, manufacturers of these three commercial porphyrins make no attempt to remove these impurities, presumably because of the hydration/dehydration equilibrium of the very labile hydroxyethyl side chains.

Fig. 4 contains evidence of other hematoporphyrin-IX dihydrochloride impurities other than HVD and PP. All three chromatograms of hematoporphyrin dihydrochloride in Fig. 4 contain two major impurities bearing retention times of 7.3 and 6.0 min. These impurities, although present in all three hematoporphyrin samples, are best observed as peaks 4 and 3 in Fig. 4c, respectively. Ward and Swincer [39] have also reported the presence of both monomeric and oligomeric impurities in hematoporphyrin-IX. For simplicity, the impurity appearing near 7.0 min will be referred to as impurity A and that appearing near 6.0 min as impurity B. Another minor impurity is also found in hematoporphyrin-IX dihydrochloride, impurity C, appearing near 2.0 min in Fig. 4c. Based on the magnitude of the fluorescence quantum yields (Table 2) and spectrophotometric behavior, impurities A and B appear to be monomeric and

impurity C is oligomeric. The degree of polymerization for hematoporphyrin impurities A and B apparently changes during base hydrolysis in sodium hydroxide, as does hematoporphyrin itself. Under the conditions of this experiment, any porphyrin fraction presenting an absorbance value near 370 nm and a fluorescence quantum yield below 0.05 will be considered dimeric or oligomeric. Porphyrin fractions presenting absorbancies above 386 nm *and* fluorescence quantum yields above 0.05 will be considered monomeric. Previous works have determined that the position and shape of the Soret band (the major band occurring near 400 nm for almost all porphyrins) are effective markers for the determination of the degree of polymerization: Soret bands appearing near 393 nm are indicative of monomers and bands near 370 are indicative of oligomers [40]. Later, it will be shown that impurities A, B and C behave as oligomeric components in culture.

Interestingly, hematoporphyrin impurities A, B and C also appear in the chromatograms for Photofrin (Fig. 5b) and the HpD mixture (Fig. 7). Therefore, these three early running fractions, as seen in the chromatograms for HpD and Photofrin, do originate from impurities present in hematoporphyrin-IX dihydrochloride, regardless of the vendor, and not from any impurity carried over from HpDp.

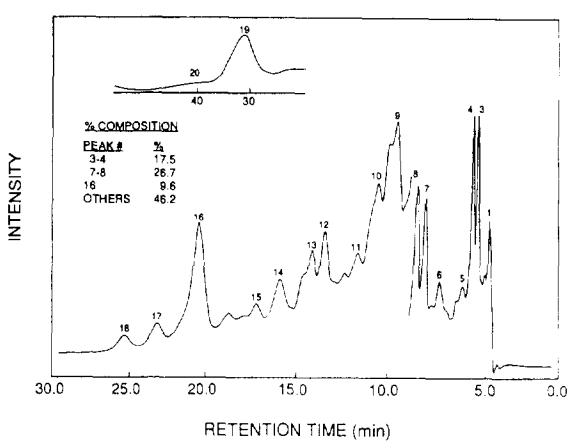


Fig. 7. HPLC chromatogram of HpD prepared from Hematoporphyrin D. Obtained with a Beckman multi-component 420 Chromatograph, using a TBAP solvent of 80:20 (v/v) MeOH–H_2O (pH = 2.5) and a C_{18} Waters reversed-phase μBondapak column. Fluorescence quantum yield measurements and electronic behavior were measured for these fractions.

3.1. Composition of HpD and Photofrin

The HpD product shown in Fig. 7 was synthesized from Porphyrin Products' Hematoporphyrin D. The retention times for these fractions are shown in Table 1. Each porphyrin fraction (labelled numerically in Fig. 7) was collected manually based on its retention time, and major components were identified by literature values and by co-elution chromatography. As many as 33 different peaks were counted in the isocratic HpD chromatogram, but it was not possible to resolve each peak well enough to collect it. Dellinger and Brault [19] have also reported counting in excess of 30 peaks in a normal-phase HPLC chromatogram of HpD (prepared by the Lipson method).

The retention times for some HpD components shown in Fig. 7 are similar to retention times for some components present in the HpDp mixture (Fig. 3). However, except for hematoporphyrin, HVD, protoporphyrin and a few impurities, no other major HpDp component is found in the HpD mixture. Based on a comparison of the chromatographic, electronic, and fluorescence quantum yield data for HpD and HpDp components (Tables 1–3), there is no evidence supporting the presence of an acetate complex present in HpD.

The Photofrin drug (Fig. 5b) contains a higher percentage of late running oligomeric components than the HpD mixture. Photofrin is believed to be prepared by "aging" freshly prepared HpD at pH = 9 and then using membrane filtration to remove the monomers. Whereas HpD fractions 9–15 in Fig. 7 appear as broad, partially resolved peaks, the chromatogram for Photofrin exhibits one well defined component in this range (peak 6). Although the spectrophotometric band positions and fluorescence quantum yields for HpD fractions 9–15 are consistent with the presence of both monomers (peaks 12 and 13) and oligomers (peaks 9–11

Table 1
Electronic, fluorescence, and quantum yield data for hematoporphyrin derivative components shown in Fig. 7

Peak[a]	Retention time (min)	Soret band	Fluorescence emission (nm)	Quantum yield
1	4.4	373.6	608, 622, 656	0.035
2	4.8	372.0	–, 623, 668	0.034
3	5.2	389.0	606,–, 656	0.080
4	5.5	389.0	606,–, 656	0.093
5	6.0	376.8	608, 656, 665	0.065
6	7.6	375.3	611, 622, 668	0.039
7	7.8	393.2	615,–, 672	0.077
8	8.5	399.0	614,–, 672	0.103
9	9.0	379.8	614,–, 671	0.069
10	10.4	377.7	613,–, 668	0.059
11	12.0	376.1	–, 624, 671	0.054
12	13.0	392.6	614, 623, 669	0.074
13	14.0	396.8	615, 624, 672	0.075
14	15.2	376.8	–, 623, 672	0.068
15	16.8	371.6	–, 624, 671	0.060
16	20.0	399.8	–, 629, 678	0.091
17	22.0	378.6	615,–, 668	0.072
18	24.0	380.6	–, 625, 672	0.070
19	32.0	380.4	615, 624, 671	0.052
20	42.0	380.0	–, 625, 671	0.060

[a] See Fig. 7. Solvent is 80:20 (v/v) MeOH–H_2O, 2.5 m*M* TBAP, pH = 2.5. Fractions were obtained on a Beckman 340 HPLC equipped with a Waters C_{18} reversed-phase column.

and 14–15; see Table 1), a collective analysis of peaks 9–15 exhibit a Soret band near 375 nm and a fluorescence quantum yield near 0.052 (see Table 3). This collective analysis suggests that component 6 of Photofrin is related to HpD peaks 9–11. Pandey et al. [10] was able to isolate a fraction of Photofrin similar to HpD peaks 9–11 in Fig. 1. Based on mass spectral analysis, they found this peak derived from ether-linked dimers, trimers, and various dehydration products. Byrne and Ward [27] have also reported a carefully detailed composition of Photofrin based on HPLC analysis. These authors have identified the presence of ether-, ester-, and carbon–carbon-linked dimers, trimers, and oligomers, some of which elute with retention times very close to those observed for some monomers. Utilizing normal-phase HPLC techniques, Dellinger and Brault [41] have also demonstrated that at least one component of Photofrin can be taken up and retained by the human cultivated lymphoblastic cell line, Reh6. The "minor" component described by Dellinger and Brault was isolated and also shown to contain more than one hydrophobic compound. Our observations regarding HpD fractions 9–15 which are based on photophysical behavior, are in agreement that some fractions of HpD and Photofrin which elute as one peak are in fact composed of both monomers and oligomers.

3.2. Composition and purity of the ether-linked dimers

There is widespread interest in the synthesis of ether-linked and carbon–carbon-linked vinyl-containing porphyrin dimers (DVD) and trimers (DVT) but very little interest in the photodynamically weak ether-linked and ester-linked dihydroxy dimer (DHE). Even protoporphyrin, a divinyl monomer, has been shown to produce a slight in vivo photokill efficiency [23,42]. A similar conclusion regarding the importance of

Table 2
Fluorescence quantum yields for HPLC separated parent components[a]

Fraction	HP	PP	HVD	HPDP	DHE	HPD
1	0.051	0.070	0.096	–	0.094	0.035
2	0.100	–	0.115	0.102	0.158	0.034
3	0.128	–	–	0.103	0.038	0.080
4	0.104	–	–	0.102	0.086	0.093
5	0.100	–	–	0.098	0.088	0.065
6	0.100	–	–	0.103	0.039	0.039
7	0.101	–	–	0.117	0.046	0.077
8	0.089	–	–	0.089	0.042	0.103
9	0.100	–	–	–	0.032	0.069
10	0.102	–	–	–	–	0.059
11	–	–	–	–	–	0.054
12	–	–	–	–	–	0.074
13	–	–	–	–	–	0.075
14	–	–	–	–	–	0.068
15	–	–	–	–	–	0.060
16	–	–	–	–	–	0.091
17	–	–	–	–	–	0.072
18	–	–	–	–	–	0.070
19	–	–	–	–	–	0.052
20	–	–	–	–	–	0.060

[a] Peaks are numbered as shown in Figs. 3–7.

the vinyl group in the HpD mixture was theorized by Forbes and Cowled as early as 1985 [43].

A chromatogram of our synthesized ether-linked porphyrin dimer mixture is shown in Fig. 8. The retention times, electronic, spectrofluorimetric, and fluorescence quantum yields are collected in Table 4. Electronic spectra for each dimer fraction exhibited a broad band centered around 400 nm. Peak derivation for all fractions clearly demonstrated the presence of two bands. The observed Soret band splitting is characteristic of all dimerized porphyrins [44].

Table 3
Spectrophotometric and fluorescence quantum yields for the best resolved HpD fractions

Peak	Soret band	Quantum yield
1–2	370.0	0.027
3–4	393.0	0.120
5–6	373.1	0.051
7–8	393.5	0.111
9–15	395.5	0.052
16	397.0	0.084
17–18	375.4	0.031
19–20	370.0	0.021

Solvent is an 80:20 (v/v) MeOH–H_2O mixture, 2.5 m*M* TBAP, pH = 2.5. Each HpD fraction was collected via HPLC. In this table, HpD peaks 1 and 2 in Fig. 7 were examined as one fraction, etc.

The products believed to be formed during the treatment of Hp.DME (DME = dimethyl ester) with hydrogen bromide (the synthetic procedure which produces the porphyrin dimer mixture), have been reported [9]. The mixture contains a monovinyl monohydroxyethyl ether-linked dimer (abbreviated here as MVD), a divinyl ether-linked dimer (abbreviated here as DVD), and a dihydroxyethyl ether-linked dimer (DHE, to conform with tradition).

A spectrophotometric, chromatographic, and photophysical comparison of the ether-linked dimers in Fig. 8 with the HpD mixture in Fig. 7 would suggest that hematoporphyrin impurities A, B and C are present. Based of previous chromatographic analyses [2,10,24] and co-elution with pure DHE and DVD, we offer a

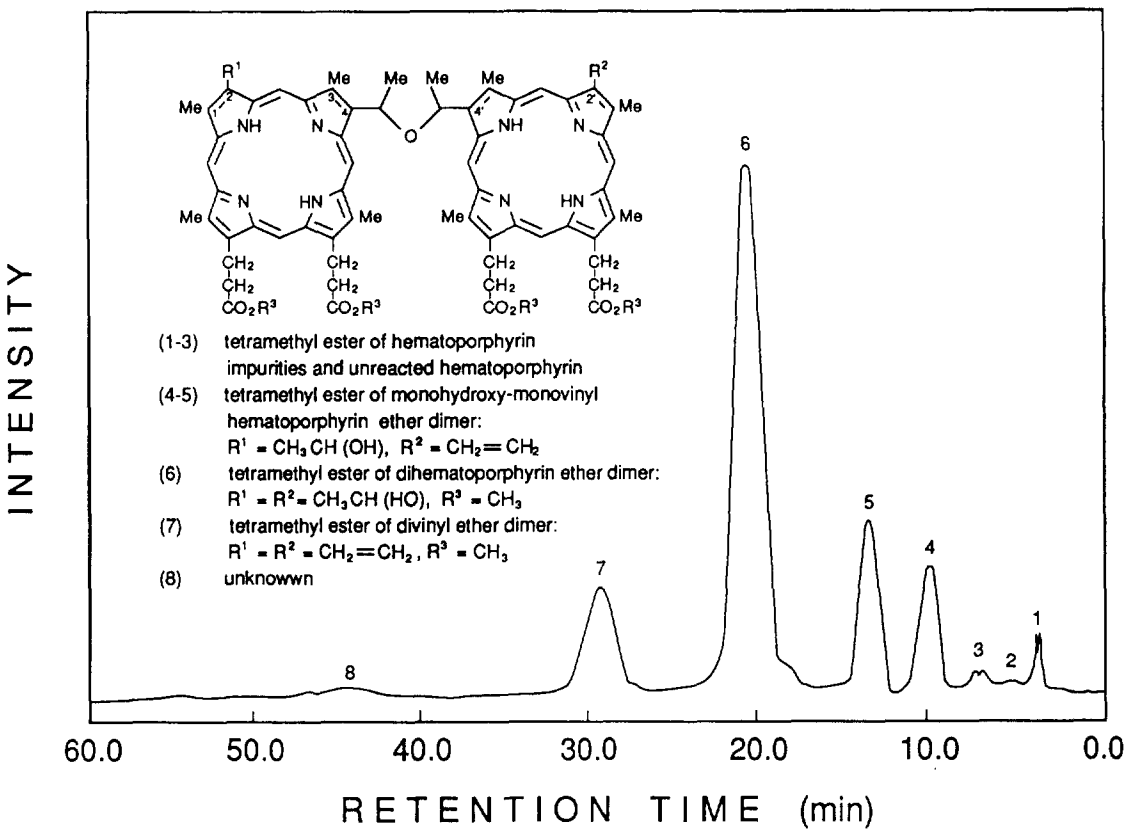


Fig. 8. HPLC chromatogram of HpD dimers. Spectrum was obtained on a Beckman 340 chromatograph using a mobile phase of 80:20 (v/v) MeOH–H_2O containing a 2.5-m*M* TBAP buffer (pH = 2.5) and a Waters C_{18} μBondapak reversed-phase column. Flow-rate = 2.0 ml/min.

tentative assignment of the peaks shown in Fig. 8. Of course, the focus here is primarily the hematoporphyrin impurities present in the dimer mixture. Peaks 1–3 in Fig. 8 derive from the presence of Hp.DME (peak 1) and associated hematoporphyrin impurities A and B (peaks 2–3). Clearly, the hematoporphyrin-IX impurities present in HpD and in Photofrin are also present in this dimer mixture. The importance of these impurities lies in the fact that they are also present in the dimer mixture as oligomeric components.

The photophysical lifetime and fluorescence quantum yields for some of the ether-linked dimer fractions were reported by Kessel et al. [24]. They reported fluorescence quantum yields of 0.036 and 0.050 for the ether-linked divinyl dimer and the ether-linked hematoporphyrin dimer (DHE), respectively. The reported fluorescence quantum yields shown in Table 4 are somewhat higher than those reported by Kessel. This could be due to the alcohol–water solvent employed for this study [45]. Kessel's work does support the conclusion that the best photosensitizers are those containing vinyl groups, and, lends credence to the hypothesis that the best photosensitizers are those which present a comparatively low fluorescence quantum yield.

3.3. Photokill efficiency

The emphasis of these photokill measurements, along with chromatographic, spectrophotometric, and photophysical parameters, lies

Table 4
Electronic, fluorescence, and quantum yield data for HpD dimers shown in Fig. 8

Peak	Retention time (min)	Soret band	Fluorescence emission (nm)	Quantum yield
1	4.4	400.2	607, 620, 667	0.029
	4.8	390.0		
2	6.8	401.3	610, 623, 669	0.072
		390.3		
3	8.4	401.3	610, 623, 669	0.072
		390.3		
4	11.0	400.7	608, 622, 668	0.060
		391.0		
5	14.8	400.3	–, 622, 669	0.076
		391.6		
6	21.0	400.1	–, 623, 670	0.065
		391.6		
7	31.0	397.0	–, 626, 676	0.075
		393.0		
8	44.0	400.9	609, 623, 670	0.042

Solvent is 80:20 (v/v) MeOH–H_2O, 2.5 m*M* TBAP, pH = 2.5. Fractions were obtained on a Beckman 340 HPLC equipped with a Waters C_{18} reversed-phase column. The presence of two peaks was based on the appearance of derivative peaks.

in the identification of various components found in HpD and Photofrin. Hematoporphyrin-IX (photokill fractions 2–3 in Table 5), hydroxyethylvinyldeuteroporphyrin (photokill fractions 6–7 in Table 5) produce moderate cell kills but protoporphyrin (photokill fraction 14 in Table 5) produces a 95% cell kill. While all of the HpD components are photoactive in culture, most of the photoactive effects are demonstrated by the late running fractions. Under these in vitro conditions, these are the so called *good* components. Hematoporphyrin impurities A, B and C (photokill fractions 1, 4 and 5 in Table 5) are more photoactive than the monomers. These hematoporphyrin impurities may play some role in the photoactivity observed for HpD and Photofrin.

It is widely recognized that the in vitro and in vivo behavior of HpD components can not be compared. The reason has been linked to the molecular folding interaction of HpD components with low density lipoproteins [46]. It was originally accepted that the primary mechanism for the in vivo uptake of HpD oligomers was mediated exclusively through low-density lipoproteins. Recently, Kongshaug et al. [47], Maziere et al. [48], Korbelik et al. [49], and Wang and Dougherty [50] have addressed the troubling question of the relevance of low-density lipoproteins in photodynamic therapy.

The role of low-density lipoproteins in the uptake and transport of photosensitizers to tumor cells remains under investigation.

4. Conclusion

A detailed electronic, fluorescence, and quantum yield analysis of twenty HpD fractions, eight HpD ether-linked dimeric fractions, and a variety of commercial porphyrins, shows that there are impurities present in HpD, in Photofrin, and in the ether-linked dimers which are carried over from the original starting material, hematoporphyrin-IX (whether containing the dihydrochloride, the diacetate, or the methyl ester group). Hematoporphyrin impurities A, B and C are photoactive in vitro, and may play a role in the photoactivity of HpD or Photofrin.

Hematoporphyrin-IX dihydrochloride, as marketed by Porphyrin Products and by Sigma appears to be much less contaminated than the

Table 5
Photokill efficiency for the HpD components shown in Fig. 7[a] on PTK2 epithelial cells

Fraction	Peak (see Fig. 1)	PTK2 cells killed (%)
1	1–2	87.5
2	3	63.0
3	4	50.0
4	5	80.0
5	6	84.0
6	7	50.0
7	8	31.4
8	9	87.5
9	10	87.5
10	11	81.7
11	12–13	94.2
12	14	91.1
13	15	93.8
14	16	90.5
15	17	93.7
16	18	95.2
17	19	94.6
18	20	93.4

[a] Photokill efficiency was assessed by irradiating PTK2 cells with the HpD fractions at 630 nm for 3 min. Cell viability was determined using a cell titer. and a standard curve of % cell versus absorbance (see Section 2). In this table, photokill fraction 1, e.g., consists of HpD peaks 1 and 2 in Fig. 7, and photokill fraction 18 consists of HpD component 20 in Fig. 7, and so on.

Aldrich version. The Hematoporphyrin D marketed by Porphyrin Products contains less diacetate and more protoporphyrin and HVD than is found in the non-commercial synthesized HpDp mixture. This unusual composition leads to an HpD mixture whose composition includes 10% protoporphyrin. This is clearly undesirable because an increase in the protoporphyrin concentration will surely have a negative effect on skin photosensitivity during photodynamic therapy.

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