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Chromatographic analysis of photodynamically significant porphyrin dimers and trimers

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Abstract

Two porphyrin dimers, dihematoporphyrin dimer (DHD) and divinyl dimer (DVD), and two porphyrin trimers, dihematoporphyrin trimer (DHT) and divinyl trimer (DVT), have been analyzed utilizing isocratic ion-pair reversed-phase high-performance liquid chromatography. Results indicate that the vinyl porphyrins can be distinguished by three peaks appearing near 15, 38, and 42 min. The hematoporphyrin complexes are identified by the appearance of a peak located at 35 min. The DVT and DVD complexes present unique chromatographic markers at 28 and 15 min, respectively. Based on the location of these chromatographic markers, it was found that the Photofrin® drug must contain the DVD and the DHT complexes, but does not contain the DVT complex. The purity of the DVT complex is compromised by the presence of DHD and DHT impurities.

Keywords: Porphyrins; Dihematoporphyrin; Divinyl

1. Introduction

Hematoporphyrin derivative, HpD, is an anticancer drug used in conjunction with photodynamic therapy (PDT) [1]. This process involves the use of light, usually from a laser, to induce fluorescence in the HpD molecule. During the de-excitation process, the HpD molecule may transfer energy to an oxygen molecule, causing it to assume a singlet state. Singlet oxygen is toxic to cells. PDT destroys cancer cells in any area of the body which is accessible to a light source and can significantly accumulate the HpD drug. Photofrin®, a purified form of HpD, has undergone stage three clinical trials for the treatment of esophageal, lung, and bladder cancer. Photofrin is reportedly enriched in the so called "good" components of HpD because it contains a higher percentage of the dimeric and oligomeric components.

The composition of Photofrin has been documented [2]. It contains at least 50–70% ether-, ester- and carbon–carbon-linked oligomeric porphyrins. The synthesis, NMR, and FAB mass spectral analysis of several oligomeric components believed to be present in Photofrin have been investigated extensively by Ward [3–5] and by Dougherty and Pandey [6–9]. Kessel [10] has also reported HPLC and photophysical measurements for some of the dimers and trimers believed to be present in Photofrin.

1.1. Ether-linked dimers

This report presents the chromatographic profiles of the following porphyrins: dihematoporphyrin ether (DHD), divinyl ether (DVD), trihematoporphyrin

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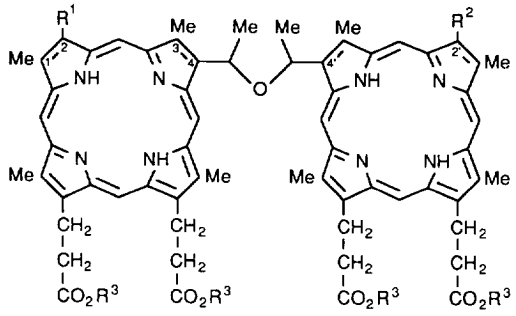

Fig. 1. Structures for the ether-linked divinyl (DVD) and dihematoporphyrin (DHT) dimers. The divinyl (DVT) and dihematoporphyrin trimers (DHT, not shown) contain three porphyrin rings joined by an ether linkage.

ether (DHT) and trivinyl ether (DVT) (Fig. 1). The complex termed DHD in this report is commonly called DHE. In those instances where other works are referenced, the DHE terminology has been retained because the original authors used the term. In 1988, in an effort to identify some of the components present in Photofrin, Ward and Morris synthesized several porphyrin dimers, including dihematoporphyrin ether, DHE [3]. 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. [6] also produced strong evidence which showed that the ether-linked dihydroxyethyl dimer was an ineffective in vivo photosensitizer. In 1991, Kessel et al. [10] produced solid evidence that the ether linked divinyl dimer (DVD) and trimer (DVT) porphyrins were efficient in vivo photosensitizers. Pandey et al. [8] have also reported that the divinyl and dihematoporphyrin trimers are both efficient in vivo photosensitizers, with the divinyl trimer more efficient. If these dimers and trimers are ranked in terms of their efficacy toward cancer, it would decrease in the following order: DVD>DVT>DHT>DHD.

1.2. Chromatography of porphyrins

Kessel et al. [10] have reported reversed-phase HPLC gradient profiles for DHE and DVD utilizing a Waters RP-8 column and a methanol–tetrahydrofuran mobile phase. The isocratic ion-pair reversed-phase technique employed in this report utilizes a C_{18} μBondapak column and a methanol–water mobile phase. Because an isocratic method is employed in this report, the chromatograms reported by Kessel et al. [10] differ markedly from the ones presented here.

The reversed-phase ion-pair technique employed in this report was first described by Bonnett et al. in 1978 [11] for the characterization of the HpD mixture, and later adapted to the isocratic technique by Ward et al. [12] for analysis of the HpD intermediate (termed "HpD solid"). Ward et al. [4] did report a DHE chromatogram utilizing an ion-pair reversed-phase technique. That report described an aqueous methanol mobile phase. The DHD chromatogram reported here is similar to the DHE chromatogram reported by Ward et al. [4]. There are no reports of an isocratic chromatographic comparison of the four porphyrin dimers and trimers: DVD, DVT, DHT, and DHD. This report presents an isocratic chromatographic comparison of Photofrin, hematoporphyrin-IX, protoporphyrin-IX, hydroxyethylvinyldeuteroporphyrin (HVD), the porphyrin dimers (DVD and DHD) and the porphyrin trimers (DVT and DHT). However, it is the dimer and trimer complexes which are featured.

Fluorescence emission spectra and quantum yields are also reported for all of the porphyrin dimers and trimers featured in this report. The fluorescence quantum yields we report are slightly higher than those reported by Kessel et al. [10].

2. Experimental

Hematoporphyrin-IX dihydrochloride, protoporphyrin-IX and HVD were purchased from Porphyrin Products (Logan, UT, USA). Photofrin was graciously supplied by (Quadralogic, Vancouver, Canada). DHD, DVD, DHT and DVT were graciously supplied by Dr. David Kessel. These dimers and trimers

were synthesized according to published procedure [5].

2.1. Chromatographic conditions

HPLC measurements were performed under isocratic conditions on a Beckman System Gold chromatograph housing a Beckman 112 pump, a Beckman 166 detector set to 360 nm, and an Altex 210 injector. A Waters C_{18} reversed-phase μBondapak column (30 cm×3.9 mm I.D., 5 μm particle size) was used for separation. The HPLC system included the use of a Whatman 0.2 μm in-line filter and a silica-based C_{18} μBondapak 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 begin at 2.0 min and was complete after about 48 min. A 1.0 ml/min flow-rate was utilized.

2.2. Spectroscopic procedures

The electronic spectrum of each compound was measured using a Beckman DU spectrophotometer. Spectra were recorded from 750 to 300 nm in 1 cm quartz cuvettes and absorbance values (λ_{max}=400 nm) for each compound was obtained. Absorbance values ranged between 0.03 and 0.05 units for fluorescence quantum yield measurements.

The fluorescence emission spectrum for each compound was obtained using a Hitachi 4010 spectrofluorometer and Rhodamine B (Curtin-Matheson Scientific) for spectral correction. Each porphyrin was excited at 400 nm and its fluorescence emission spectrum scanned from 550 nm to 750 nm.

Quantum yield measurements (within 10% error) were performed utilizing the ratio method and tetraphenylporphine as a standard (Φ_1=0.12) [13]. This technique follows that outlined by Parker and Rees [14], utilizing the following equation:

$$\frac{F_2}{F_1}=\frac{\text{area}_2}{\text{area}_1}=\frac{\phi_2}{\phi_1}\cdot\frac{\epsilon_2 c_2 d_2}{\epsilon_1 c_1 d_1}=\frac{\phi_2}{\phi_1}\cdot\frac{A_2}{A_1}$$

where F=fluorescence area, ϕ=fluorescence quantum yield, ϵ=extinction coefficient and A=absorbance. The area under the curve of each HPLC fraction was calculated utilizing computer software developed by Hitachi.

3. Results and discussions

3.1. Vinyl complexes

Fig. 2A–D contain the chromatograms of the vinyl and hematoporphyrin dimers and trimers. The divinyl dimer (DVD) and trimer (DVT) share two common peaks, appearing near 38 and 42 min (Fig. 3). The DVD complex contains other peaks which elute early (Fig. 2A); one doublet appearing between 12 and 14 min and the second set of peaks appearing between 14 and 16 min. The origin of the peaks appearing between 12 and 14 min is not known, but a similar peak is present in the protoporphyrin chromatogram (Fig. 4A). The peaks located at 14.7 and 15.4 min are unique to the DVD complex. As a result, the chromatographic contribution from the DVD complex reduces to two minor peaks appearing at 14.7 and 15.4 min and two major peaks appearing near 38 and 42 min.

The DVD complex is unexpectedly "pure". A small amount of protoporphyrin monomer is present but it does not appear to contain any trace of the DHD, DHT, or the DVT complex. The presence of these peaks in all of these dimers and trimers arise from the presence of stereo- and regioisomeric heterogeneity, such that even a 'pure' dimer such as the DVD complex may contain additional isomers [4,5,8].

The chromatogram of the DVT complex is shown in Fig. 2B and Fig. 3. It contains several monomeric impurities. The peaks appearing before 8 min are due to the presence of hematoporphyrin (4–6 min, Fig. 5B) and HVD (6–8 min, Fig. 5C). As seen in Fig. 5A, the peaks occurring between 12 and 14 min, as well as the peak appearing near 20 min appear to be associated with the presence of protoporphyrin. The origin of the peak appearing near 10 min is not known, but its position suggests that it is a monomer (a similar minor peak does appear in the hematoporphyrin chromatogram, Fig. 5B). Fig. 5E and 5F contain evidence that this complex is not pure and contains a significant amount of DHD and DHT. The peaks arising from the DVT complex reduces to four

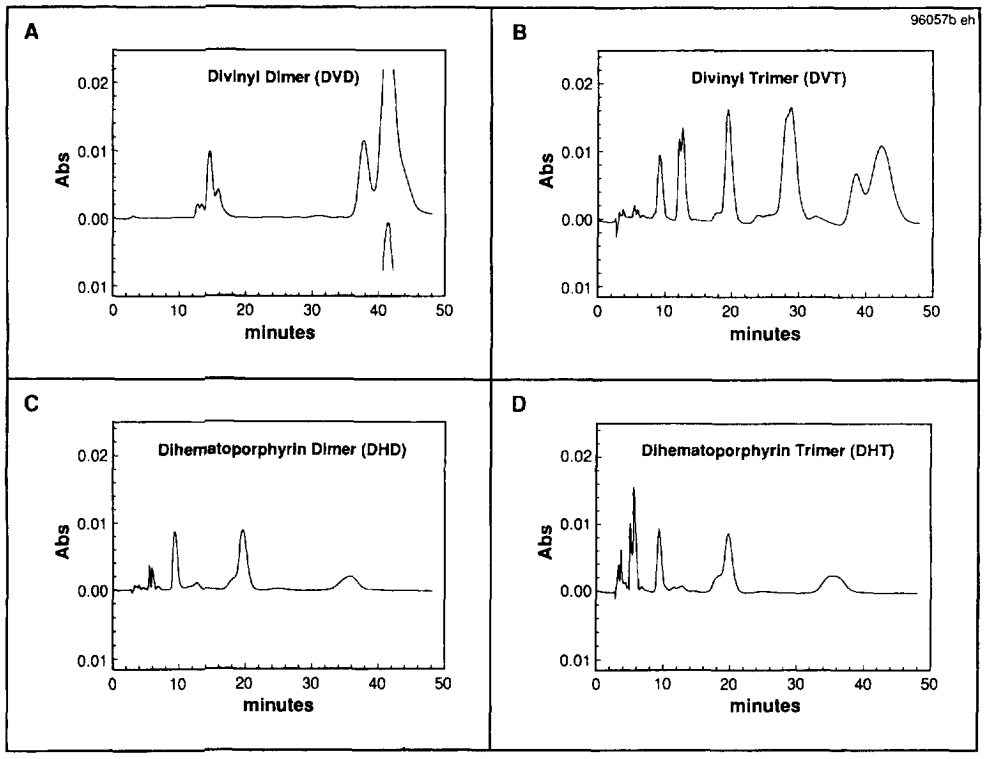

Fig. 2. Chromatograms of (A) DVD, (B) DVT, (C) DHD and (d) DHT. The chromatograms were obtained on a C_{18} μBondapak column. The mobile phase is MeOH–H_2O (80:20).

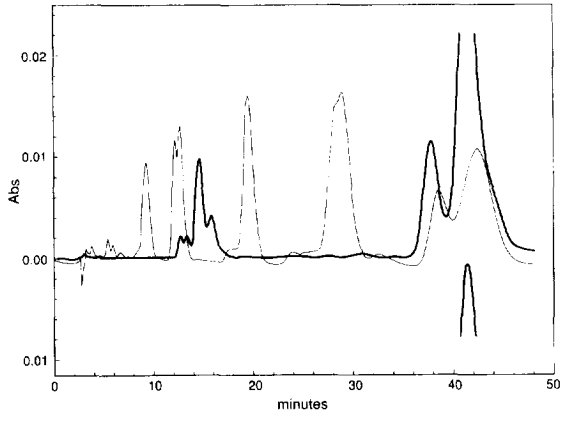

Fig. 3. Chromatographic overlay of DVD (solid line) and DVT. Peaks common to both complexes are located between 36 and 42 min.

major peaks which appear near 10, 28, 38 and 42 min. The single marker peak for DVT appears near 28 min; this peak is not found in the chromatogram of the DVD, DHD, or the DHT complex.

3.2. Hematoporphyrin complexes

The major peaks common to the hematoporphyrin dimer (DHD, Fig. 2C) and trimer (DHT, Fig. 2D) appear near 10, 20 and 35 min (Fig. 6). There are several peaks occurring between 2 and 10 min which arise from the presence of hematoporphyrin and HVD impurities (Fig. 7B and C). The major peak near 10 min also appears in the hematoporphyrin chromatogram. The low intensity peaks located near 12 min and the major peak appearing near 20 min can be attributed to the presence of protoporphyrin (Fig. 7A). As a result, the only dimeric peak unique to the DHD and DHT complexes appears near 35

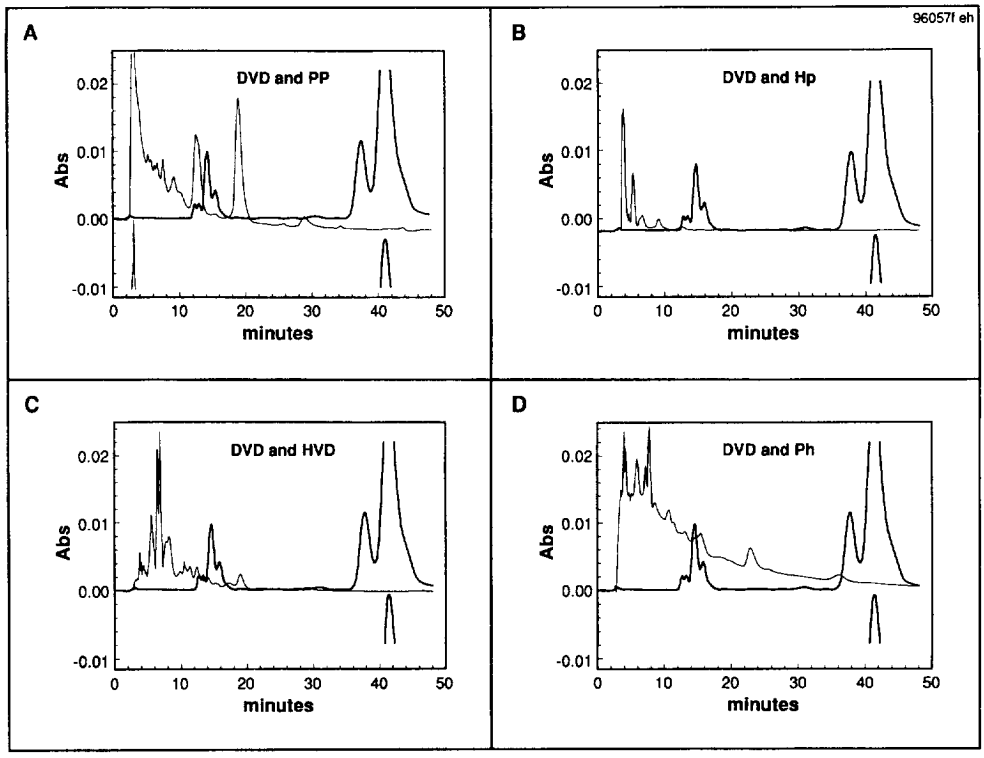

Fig. 4. Chromatographic overlay of DVD (solid line) with (A) protoporphyrin, (B) hematoporphyrin, (C) hydroxyethylvinyldeuteroporphyrin, HVD and (D) Photofrin. No significant protoporphyrin, hematoporphyrin or HVD impurity is present in the DVD complex.

min. The percent composition of hematoporphyrin monomer is much higher in the DHT complex.

3.3. Origin of the monomers

The presence of these monomers in the dimer and trimer complexes could have resulted from incomplete reactions during synthesis or from an incomplete purification procedure. For example, Fig. 5E and Fig. 5F provide evidence for the presence of DHD and DHT in the DVT complex. It is not likely that the low pH (pH 2.5) of the water–ethanol mobile phase destroys the ether bonds, a reaction which could produce monomers. The HPLC technique reported by Ward et al. [4] for analysis of the DHE complex also utilized an aqueous methanol mobile phase. No damage to the ether bond was reported.

3.4. Origin of the oligomeric impurities

The dimer and trimer complexes are not expected to present only one chromatographic peak because of the presence of diastereomers and regioisomers [4]. Fig. 2, Fig. 3 and Fig. 6 provide evidence for the presence of DVD, DHD and DHT in the DVT complex. The protocol for separation of these dimers and trimers does include several chromatographic purification steps [5]. The dimer and trimer complexes as synthesized are known to contain various isomers [5]. However, the presence of isomers should produce a chromatographic profile containing fractions which elute within a few minutes of each

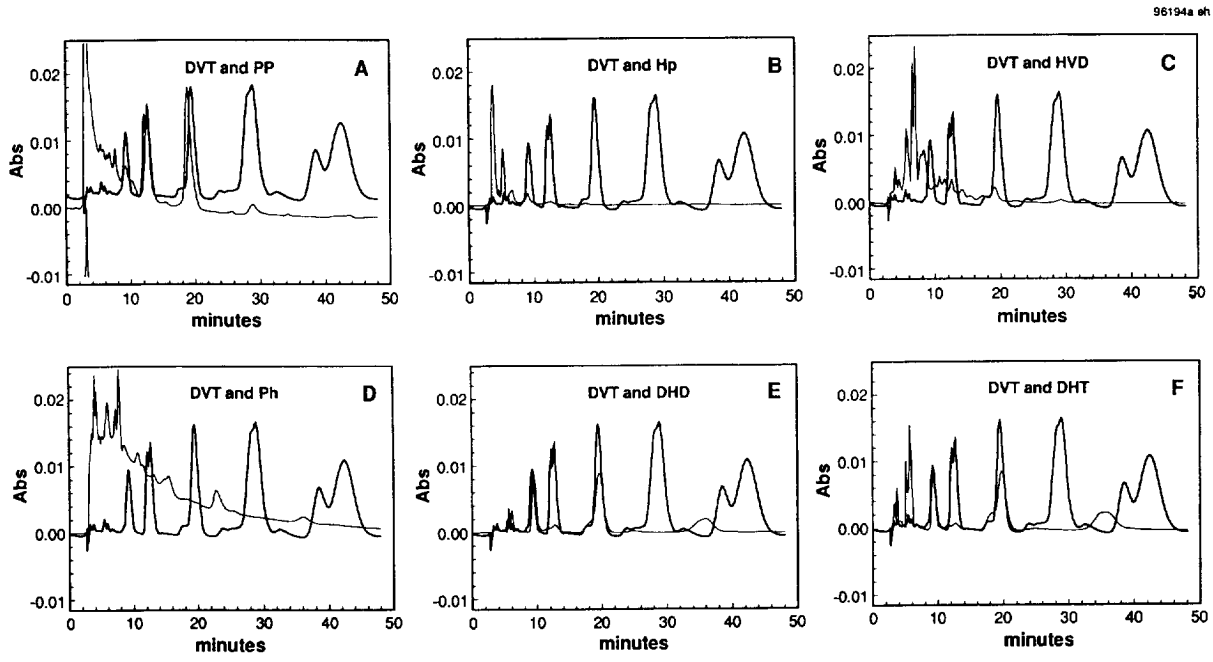

Fig. 5. Chromatographic overlay of DVT (solid line) with (A) protoporphyrin, (B) hematoporphyrin, (C) HVD, (D) Photofrin, (E) DHD and (F) DHT. A significant concentration of protoporphyrin, hematoporphyrin, and HVD is present in the DVT complex. The DVT complex is also contaminated by the presence of both the DHD and the DHT complexes.

other. Fig. 2A–D contain peaks which are well separated. Such patterns are more consistent with a mixture of several compounds.

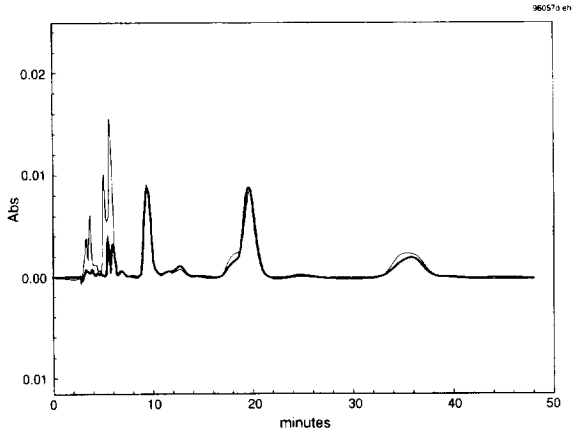

Fig. 6. Chromatographic overlay of DHD (solid line) and DHT. All peaks are common to both complexes.

3.5. Correlation with photofrin

Chromatograms of Photofrin and these dimers and trimers appear in Fig. 4D, Fig. 5D, Fig. 7D and Fig. 8D. The presence of monomers in the Photofrin mixture is easily seen. The hematoporphyrin isomer appears at 3.94 and 4.21 min and its impurity is seen at 5.89 min. The presence of the isomeric HVD complex is seen at 7.2 and 7.8 min. The protoporphyrin complex is observed near 23 min. Although there is no Photofrin peak in the 38–42 min range, the region of the chromatogram assigned to the DVD and DVT complexes, the two peaks appearing at 14.72 and 15.38 min are markers for the presence of DVD (see Fig. 2A and Fig. 4D). The Photofrin peak appearing near 36 min has been assigned to a DHT, rather than a DHD, complex. Ward, a respected pioneer in this field, reported no significant concentration of DHE (the compound we call DHD) in the Photofrin mixture [3]. Ward's conclusion was based on HPLC, NMR, and FAB analysis. Accepting

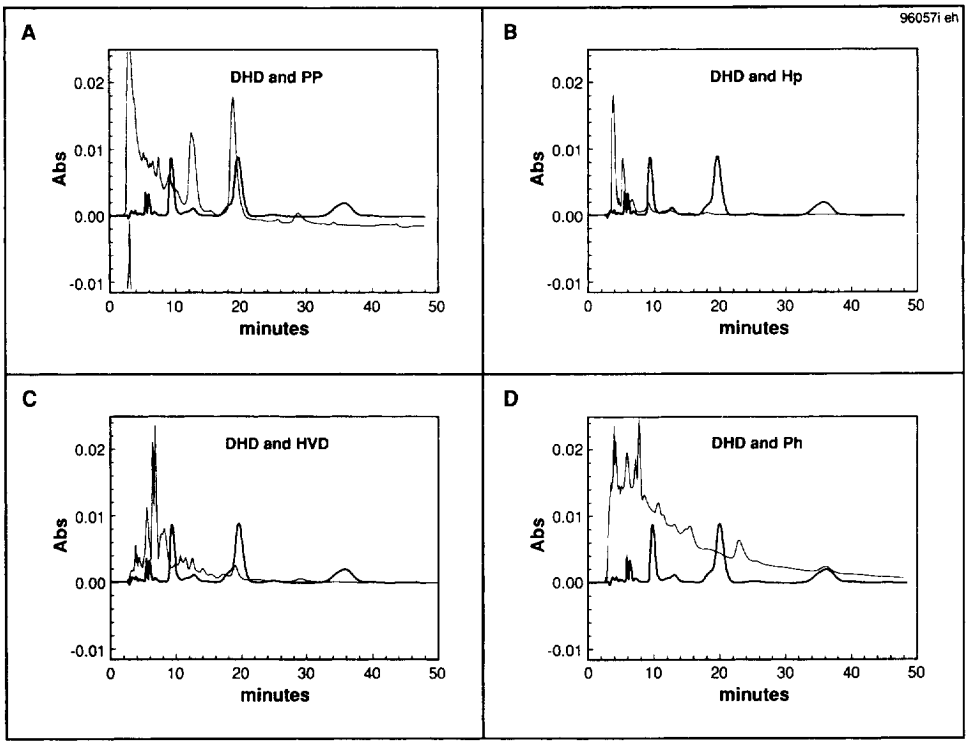

Fig. 7. Chromatographic overlay of DHD (solid line) with (A) protoporphyrin, (B) hematoporphyrin, (C) HVD and (D) Photofrin. A significant amount of protoporphyrin, hematoporphyrin and HVD is present in the DHD complex.

those findings, the chromatographic match observed in Fig. 7D, occurring near 35 min must be attributed to the presence of the DHT complex. Pandey et al. [8] have previously reported the presence of a hematoporphyrin trimer in the Photofrin mixture. There is not enough chromatographic evidence to support the existence of a relevant concentration of the DVT complex in the Photofrin mixture. Fig. 4D clearly shows that Photofrin does not present a peak near 28 min, as would be expected if the DVT oligomer were present.

Owens et al. have reported the existence of an early-running oligomeric component (believed to be related to the DVD complex) in the HpD mixture. Their findings were based on isocratic ion-pair reversed-phase chromatography, fluorescence quantum yields, and in vitro photokill measurements [15]. The chromatographic profile of the DVD complex presented here supports that conclusion.

3.6. Photophysical properties

The fluorescence emission spectra are shown in Fig. 9 and their emission optima appear in Table 1. Each compound was excited at 400 nm in dimethylformamide (DMF). Both the DVD and DVT complexes emit at slightly shorter wavelength (625 nm) than the DHD and DHT complexes (630 nm). These spectra are in good agreement with those reported by Kessel et al. [10] for these same complexes. The fluorescence quantum yields for each complex in this report were calculated using tetraphenylporphyrin (TPP) in benzene as a standard ($\Phi_f = 0.12$).

Only the DVD complex exhibits a relatively low fluorescence quantum yield, 0.027. The DVT, DHD and DHT complexes exhibit typical fluorescence quantum yields for porphyrin complexes, 0.0678, 0.058 and 0.055, respectively. Kessel et al. [10] reported fluorescence quantum yields for DHE (the

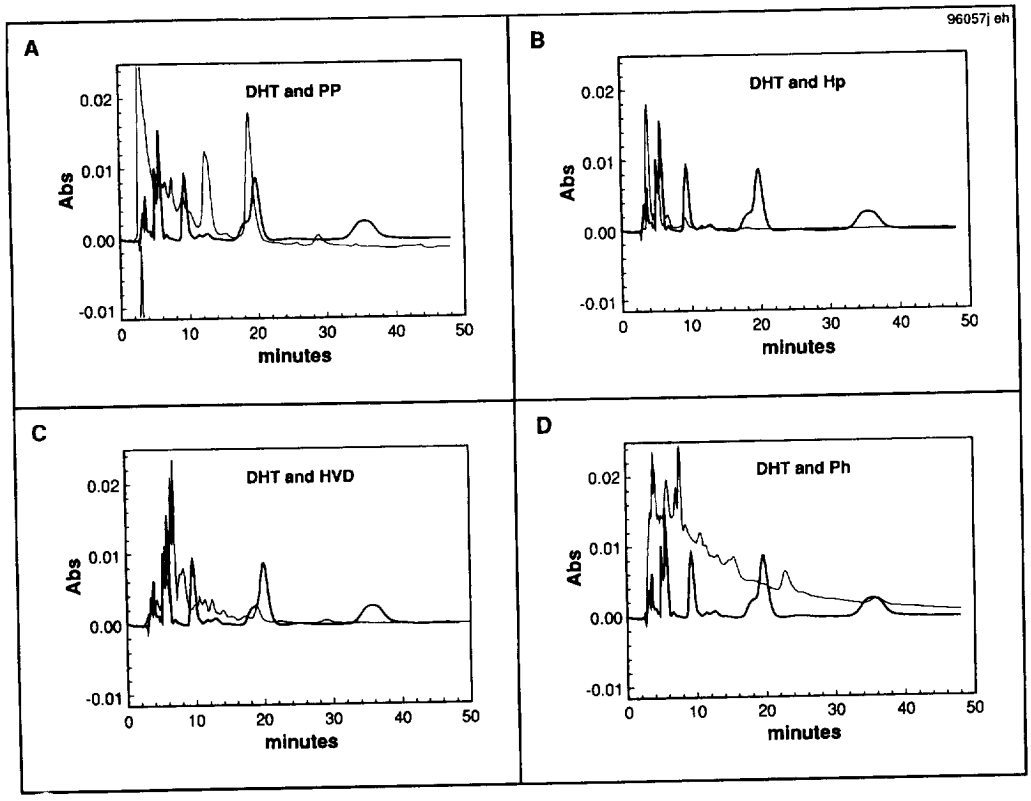

Fig. 8. Chromatographic overlay of DHT (solid line) with (A) protoporphyrin, (B) hematoporphyrin, (C) HVD and (D) Photofrin. A significant concentration of protoporphyrin, hematoporphyrin and HVD is present in the DHT complex.

compound we call DHD) and DVE (the compound we call DVD) complexes using quinine in 0.1 *M* H_2SO_4 as the standard and tetrahydrofyran, ethanol, and formamide as solvents. The values they report for the DVE and DHE complexes in formamide are somewhat smaller than those shown in Table 1. The fluorescence quantum yields reported here are higher because each complex, except DVD, contains monomers. This hypothesis is based on the fact that only the chromatographically pure DVD complex presents a more traditional fluorescence quantum yield.

4. Conclusion

A full chromatographic analysis of the divinyl and hematoporphyrin dimers and trimers has shown that the presence of the divinyl complex can be confirmed by two sets of peaks appearing near 15 min and between 38 and 42 min. The DVT complex is not pure and contains a significant concentration of both the DHD and DHT complexes. The DVT complex presents a unique chromatographic marker near 28 min. The hematoporphyrin dimer and trimer complexes (DHD and DHT) share three common peaks occurring near 10, 20, and 35 min. Although there are no unique peaks for either of these latter complexes, the presence of DHT can be discerned by the appearance of a chromatographic marker appearing near 35 min. In order to unambiguously identify each component present in these porphyrins, a full NMR and mass spectral (fast atom bombardment) analysis is required. These studies are in progress.

A chromatographic comparison of these dimers and trimers with the Photofrin drug suggests that Photofrin definitely contains the DVD complex, probably contains the DHT complex, but does not contain the DVT or the DHD complex.

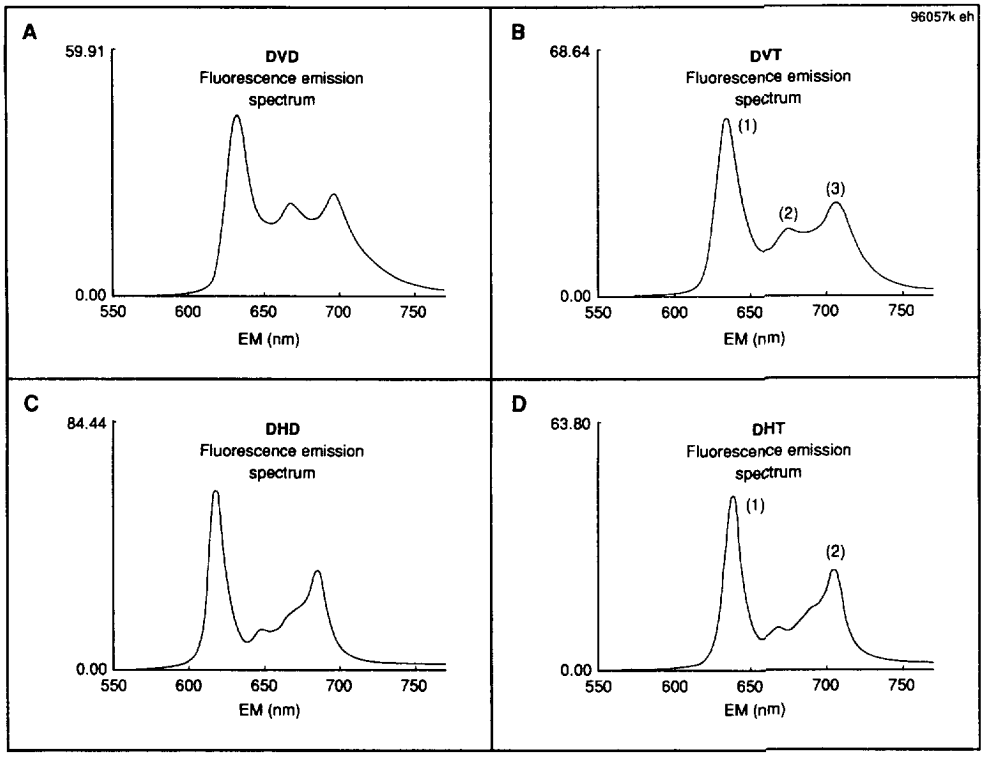

Fig. 9. Fluorescence emission spectra for (A) DVD, (B) DVT, (C) DHD and (D) DHT. The solvent is dimethylformamide. Each solution was excited at 400 nm.

The fluorescence quantum yield for the DVD complex is consistent with that for a porphyrin complex. However, the magnitude of the fluorescence quantum yield for the DVT, DHD and DHT complexes is a little higher than those reported by Kessel's group. This trend can probably be traced to a lack of purity.

Table 1
Photophysical parameters for porphyrin dimers and trimers

Porphyrin	UV-Vis (λ_{max})	Fluorescence emission (nm)	Quantum yield
DVD	400	630, 665, 695	0.027
DVT	400	630, 667, 694	0.068
DHD	400	625, 691, –	0.058
DHT	400	625, 691, –	0.055

The solvent is dimethylformamide. The quantum yields were calculated using tetraphenylporphyrin in benzene as a standard. A refractive index correction was used in the calculation of the quantum yields: $R_f^2(\text{dmf})/R_f^2(\text{bnz})$. The error is ±10%.

Acknowledgments

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Kessel for his support and for supplying us with the porphyrin dimers and trimers.

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