CHAPTER TWO

2.0   BIOCHEMISTRY AND PHOTOCHEMISTRY OF HEMATOPORPHYRIN AND HEMATOPORPHYRIN DERIVATIVE

2.1   Introduction

Due to the claim of its selective retention properties in tumours and its proven efficacy as a photosensitizer, HpD has been developed intensively in cancer therapy research. The clinical usefulness of HpD for the detection and therapy of malignant disease is well documented. On the other hand, its parent compound, Hp was reported to be less effective as a photosensitizer due to its rapid excretion and polar nature. It should be noted that this concept of tumour specificity and retention are no longer a relevant requirement with the possibility of Immediate PDT. Hence, the main aim of this study is to investigate the pharmacokinetics and therapeutic efficacy of Hp using Immediate PDT. This will be discussed at length in chapter 5 and 6 of this thesis. In this chapter, efforts will be aimed at reviewing the preparation of the clinical HpD and Hp according to published procedure and to perform biochemical characterization of the prepared materials.

2.2   The Discovery Of Hematoporphyrin

It was the discovery of Hematoporphyrin and related compounds that heralded the modern use of photodynamic therapy. The early history of porphyrin chemistry was quoted from the reviews of Zalar *et. al.* (1977) and Daniell and Hill (1991). It began in the early 1840s with the chemical treatment of hemoglobin with concentrated sulphuric acid (Scherer, 1841). Concentrated sulphuric acid was added to dried blood and the precipitate

washed free from iron. When the iron free residue was treated with alcohol, a blood red colour solution was obtained. Later it was named as iron-free hematin. In 1867, Thudichum purified and crystallised this compound and named it cruentine, being the product of cruorin, better known now as hemoglobin. Then, Hoppe-Seyler, 1871 introduced the term "Hematoporphyrin" which means blood purple (from the Greek word porphyros meaning reddish purple). The first description of the photodynamic property of hematoporphyrin was by Hausmann, 1908. He showed destruction of paramecia and blood cells on exposure to light following the administration of hematoporphyrin. He later described results of *in vivo* photosensitization on white mice and guinea-pigs. The first *in vivo* effect of hematoporphyrin on humans was reported by Meyer-Betz, 1913 from his famous self-experiment. He exposed himself to the sun and immediately developed erythema, oedema and pain. The photosensitivity lasted for more than 2 months. In the period between the two world wars, there were two significant advances in photodynamic therapy. Firstly, the apparent selective localization of porphyrins to tumours was observed by Policard, 1924.

The second major finding was by Auler and Banzer (1924), who were the first to observe photodynamic action involving hematoporphyrin on tumours. They investigated the uptake of hematoporphyrin into tumour, concluding its specific uptake and retention, with higher levels in the tumour than in the normal surrounding tissues. In 1955, Rasmussen-Taxdal *et. al.* confirmed hematoporphyrin's tumour-localizing ability in a variety of tumours.

In 1955, Schwartz *et. al*, investigated the nature of hematoporphyrin and found it to be crude with a variable mixture of different porphyrins. With partial purification, the pure hematoporphyrin was the poorest tumour localiser and it was the usually the minor fractions that showed the greatest affinity for tumours. They tried to make a new

preparation from this residue by purifying this more active component. Among these new porphyrin preparations was the acetic-sulphuric acid treated porphyrin, which has come to be known as hematoporphyrin derivative, HpD.

Lipson *et al* (1961) further developed Schwartz's procedure to produce a hematoporphyrin derivative, HpD from the parent compound Hp. This HpD was produced in two steps : crude Hp was first treated with a mixture of acetic and sulphuric acids and the product was then treated with dilute sodium hydroxide. He reported that the HpD produced was superior to Hp in terms of tumour visualization and delineation.

The compositions of HpD has been analysed on a number of HPLC studies (Moan *et al*, 1982; Kessel and Cheng, 1985a). It is shown to be a very complex mixture with two main types of components: porphyrin monomers (Hp, HvDs and PP) and a more hydrophobic, poorly resolved material, which later was termed as DHE (dihematoporphyrin ether) (Dougherty *et al*, 1984 and Dougherty, 1984).

2.3   Earlier Works With Hematoporphyrin

In Dougherty 's early work, it was shown that Hp was inferior to HpD in controlling an experimental tumour in rodent (Dougherty *et al* , 1983). Following this, several groups have reported that Hp was ineffective as a photosensitizer whether tested *in vitro* (Kessel and Chou, 1983a) or *in vivo* (Dougherty *et al*, 1983). Nonetheless, reports continue which indicate Hp to be an effective photosensitizer of experimental tumours (Tomio *et al*, 1983 and Zhou *et al*, 1988).

There have been questions raised on the ability of Hp to act as a photosensitizer for cells in *in-vitro* and *in-vivo* studies. The confusion arises from the fact that certain

11

preparations of Hp may contain materials similar to DHE which themselves act as efficient photosensitizers while pure Hp is without effect. The preparations of Hp which contained a small amount of the oligomeric material is responsible, at least in part, for the photodynamic activity of Hp *in vivo or in vitro* (Dougherty, 1983; Tomio *et. al.*, 1983, Zhou *et. al.*, 1988 and Dougherty *et. al.*, 1992). The confusion of pure Hp being without effect stems from the fact that a delayed, conventional PDT (24 hrs post injection) was employed for therapeutic response and Hp being a polar substance would have excreted out of the body system before the treatment is employed. The concentration of Hp would have fallen considerably before the delayed treatment starts.

In vitro studies have shown that although initially Hp was taken up by tumour cells in addition to the non-polar, high molecular weight, porphyrin species but eventually the less hydrophobic monomer species begin to leak out from the cell relatively quickly eventually leaving only the nonpolar hydrophobic species within the cell (Moan and Sommer,1983). Since most clinical practices have reported a one to three day interval between drug injection and treatment to allow for serum clearance, it would be expected that all monomeric species originally taken up by tumour cells would subsequently have leaked out. Various animal studies using Photofrin or Polyhematoporphyrin (PHP) which have the similar components as HpD (Kessel, 1986a) have confirmed that the monomeric components of the drug are rapidly eliminated from the body following intravenous administration (Stribbling, 1991; Bellnier *et al*, 1989). In studies using ^{14}C-labelled PHP in the mouse, Bellnier *et al* (1989) showed that the great majority of radiolabelled drug appeared in feces and the monomeric component was eliminated rapidly.

As a result, Hp was overlooked and has not been widely used in PDT because it does not appear to be selectively localized in tumour relative to the surrounding healthy

tissue, a property which is undoubtedly regarded as important for the successful application of PDT. This has led to confusions about the nature of the drug. Hp apparently has a fast clearance rate from the body and this would reduce skin photosensitivity. Besides this, Hp is a simpler compound. These are the few characteristics of a photosensitizer pursued by workers in PDT. As the clearance rate of Hp from the body is fast, the alternative treatment procedure, which was introduced at the University of Malaya as Immediate PDT is employed. In Immediate PDT, photoradiation was administered immediately (15 mins) after the administration of drug as opposed to the conventional delayed (24-72 hrs) treatment procedure (Olivo *et. al.*, 1989 and Olivo, 1990).

It is thought that PDT caused tumour cell death by affecting their vasculature and not by affecting the cells directly. Several groups (Milanesi *et al,* 1987; Nelson *et al* 1987) have shown that following treatment of an experimental animal tumour by PDT, the tumour cells do not die immediately, but die at the same rate as tumours present in anoxic conditions. In other words, cells do not receive oxygen, due to vascular damage and hence die. Besides that they also would not receive other essential nutrients, which would contribute further to cell death. In addition to the phenomenon of the vascular damage, the porphyrins most frequently used in clinical PDT are injected in the form of isotonic aqueous solutions and have been reported to localize mainly at the level of the vascular stroma (Dougherty, 1984). With a much higher serum concentration of Hp immediately after the intravenous injection of Hp, it could be more efficacious to photo-irradiate the tumour immediately for more effective destruction of the microvasculature of the tumour. Despite fast clearance rate, Immediate PDT would still give enough time for photodynamic action to the place and have the targeted tumour destroyed.

2.4 Hematoporphyrin Derivative: A First-Generation Sensitizer

HpD is the forerunner of the modern photosensitizers. HpD is produced from hematoporphyrin and is a complex mixture of monomeric and polymeric porphyrins. Despite its complexity, a partially purified version, Photofrin, the commercial name of HpD, has been widely used to treat cancer with measurable success. Several thousand patients have now received this treatment and high complete response rates have been achieved from early and superficial cancer with HpD-mediated PDT. Photofrin is the photosensitizer licensed for many therapeutic use, receiving marketing approval in Canada 1993 for the treatment of recurrent superficial papillary bladder cancer. In 1994 it was licensed in the Netherlands for palliation of certain oesophageal and lung cancers. Marketing of Photofrin began in late 1996 in the U.S. (Dougherty, 1997).

HpD, together with its commercial variants Photofrin, Photosan, Photogem and Photocarcinorin hold an important place in the development of tumour phototherapy. These are the first generation photosensitizers. Although they have been used extensively in experimental clinical work, these photosensitizers have three important disadvantages. Firstly they accumulate in the skin and this can lead to the photosensitization of any skin area exposed to light. The symptom can persist for many weeks following the administration of the drug. Secondly, the absorption band in the red (630nm) is weak ie. the material is not a good absorber of the light available, the energy then delivered is only poorly utilised by the photodynamic process. Thirdly, they are variable mixtures from which it has not been proven possible to isolate a single highly active constituent. The complexity of the mixture arises from both positional isomerism and stereoisomerism for the case of DHE. The complex mixtures also complicate other metabolic studies of the drug

and make difficult the identification of the true active components. For these reasons much efforts have been directed towards the development of the second generation photosensitizers.

Part of this study describes the pharmacokinetics and therapeutic response using Hp and HpD in Immediate PDT which overcomes these problems of drug compositions and skin photosensitivity.

2.5 The Development Of Second Generation Photosensitizer

An ideal photosensitizer should have the following characteristics:

(a) The clearance of the drug from the body should be rapid so as to reduce skin photosensitivity.

(b) Toxicity of the drug should be negligible

(c) It should be a single homogenous compound.

(d) It should be water soluble.

(e) The drug should have a high absorption in the far red end of the visible spectrum (750-800nm) and a high quantum yield for activated oxygen species.

(f) The drug should be chemically stable; with a long shelf life.

(g) Its chemical synthesis should be straight forward and relatively cheap.

A whole range of compounds have been investigated for the above properties. Most of these compounds are based around the porphyrin/chlorin nucleus. N-aspartyl chlorin e6(NPe6) has been used *in vivo* and *in vitro*. It is a good photosensitizer with somewhat enhanced absorbance of red light, but it showed poor localization (Bonnet, 1989). M-tetrahydroxyphenyl chlorin (mTHPC) showed that skin sensitization had virtually

15

disappeared after two weeks (Bonnet, 1995a). Bacteriochlorin a, a derivative of bacteriochlorophyll a, purpurins, benzoporphyrin derivative (BPD) and it's methyl ester are showing much promise.

The phthalocyanines and napthalocyanines are new synthetic porphyrin analogues. They have great advantage over the porphyrins due to their strong absorbance in the red (Spikes,1986). Tetraphenylporphines has been found that their localization ability depends on their degree of sulphation, with the tetrasulphonated derivative being the best (Kessel *et al,* 1987a).

Details of the synthesis of PpIX from ALA have been studied. ALA methylester was then developed and was found to contain higher lipophilicity than ALA itself and are able to penetrate more easily into cells and produce PpIX at much lower concentrations (Moan and Peng, 1997). Boronated porphyrin compound (BOPP) was initiated in clinical trial and it gave no significant toxicities noted. Laboratory studies indicated that the discrete localization of BOPP occurs predominantly in the mitochondria of glioma cells (Hill, 1999).

2.6 Nature and Biochemistry Of Hematoporphyrin And Hematoporphyrin Derivative

2.6.1 Hematoporphyrin

Hematoporphyrin, a labile porphyrin, has been extensively used in the early studies of porphyrins. Hematoporphyrin is available commercially in the recrystalized dihydrochloride form. Commercial preparations of hematoporphyrin (Hp) or its hydrochloride (Hp-diHCl) are not pure and constitute 70% hematoporphyrin (Hp), 5-10% hydroxyethyl monovinyl-deuteroporphyrin (HVD) and the remaining 15-20% comprises

protoporphyrin (PP) and unknown compounds (Dougherty, 1983). It has been shown that different fractions of Hp show marked differences in their potential for localization in tumours, with minor fractions showing the greatest affinity for neoplastics tissues.

The structure of Hematoporphyrin, upon which the great majority of sensitizers used in clinical work are based, is shown in Fig.2.1. It does not occur naturally. Hematoporphyrin is very closely related to protoporphyrin which occurs naturally as the free porphyrin, but more commonly as the iron complex, heme. The treatment procedure removes the iron from the hemin and hydrated the vinyl side chains to form hydroxyethyl groups. Hematoporphyrin has the basic porphyrin structure of four pyrole groups joined by four methane bridges. This basic structure is called porphin. Different porphyrins are classified by the number, location and types of side chains attached to the pyrole rings. Hematoporphyrin has two hydroxyethyl groups in position 2 and 4 (by notation of Fisher), two propionic acid groups in position 6 and 7 and four methyl groups occupying the remaining positions around the exterior of the tetrapyrole structure. Its nontrivial name is 1,3,5,8 tetramethyl, 2,4 dihydroxyethyl 6,7 dipropionporphyrin, or more commonly 2,4 dihydroxyethyl, 2, 4 devinyl protoporphyrin.
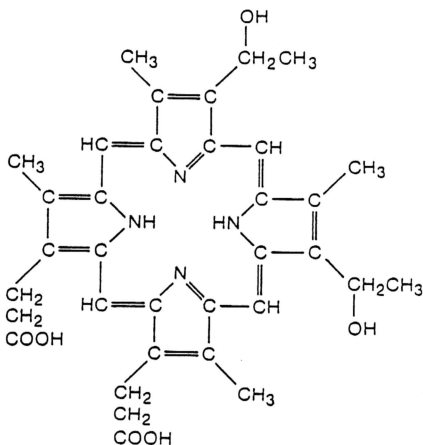
Fig. 2.1 : Chemical structure of hematoporphyrin ($C_{34}H_{38}O_6N_4$, MW: 598.6)

2.6.2 Hematroprophyrin Derivative

HpD is a mixture of porphyrins derived from Hp. The method of preparation was originally devised by Schwartz *et al* (1960) and later improved by Lipson (1960). Hematoporphyrin derivative is prepared by a two-step process. Hematoporphyrin dihydrochloride is first acetylated with a 19:1 acetic-sulfuric acid mixture (Dougherty *et al*, 1979). The initial step gives a purple solid which contains a mixture of acetylation products (Clezy *et al*, 1980): Hp monoacetates (20-30%), Hp diacetates (50-60%) and

18

unchanged Hp (15-20%); the Hp could dehydrate to give two other monomeric species : hydroxyethylvinyl deuteroporphyrins (HVDs) and protoporphyrin (PP). Structure of these compounds are shown in Fig. 2.2. Positional and diastereoisomers add to the complexity of this mixture. In order to produce a solution for injection, this solid is treated with 0.1M NaOH for 60 mins (Dougherty *et. al.* ,1979) and then brought back to neutrality. This causes hydrolysis and elimination of the 2-acetoxyethyl functions, to give back hematoporphyrin and to generate 3(8)-hydroxylethyl-8(3)-vinyldeuteroporphyrin and protoporphyrin. Typically, this mixture consists of approximately 20% Hp, 20-30% HVD and 3-5% PP. The remaining 50% appears to be an oligomeric mixture not yet fully defined chemically. This later fraction is responsible for tumour localization, therefore it made sense to try to concentrate this material from HpD. This was achieved by using techniques that separate molecules due to their size, eg: ultrafiltration (Dougherty *et. al.*, 1984; Kessel and Cheng, 1985b; Ho *et. al.*, 1988). The enriched oligomeric mixture was called Photofrin II, while the commercial name for HpD was Photofrin I.

Berenbaum *et. al.* (1982) suggested that the porphyrin oligomers not only have ether bond which is derived from two sec-hydroxyl groups on adjoining hematoporphyrin, and ester bond formed between a carboxyl group on one Hp molecule and a sec-hydroxyl from another Hp molecule, but the oligomers also have direct carbon-carbon bonds. Dougherty (1984) and Dougherty *et. al.* (1984) reported that the structure of the oligomeric mixture was an ether linkage between two hematoporphyrin molecules and was later named DHE for convenience. This was based on hydrolitic reactions towards acids and bases and various NMR spectroscopic methods. However, Kessel *et. al.* (1985), Dougherty (1987a), Kessel *et. al.* (1987b) and Byrne *et. al.* (1987) pointed out that the oligomeric fraction has been determined to be held together by both ether and ester bonds, though the proportions

19

these bonds can change in the first few days following the synthesis of the drug. Byrne *et al* (1990) reported that a typical Photofrin II sample consists of monomers (15%) and ester-linked (10-20%), ether-linked (20-30%) and acid-resistant (35-50%) oligomers. Similar relative ratios can be obtained for the oligomers in HpD in which is less than 50% of the total material. Examples of the types of bond are shown in Fig. 2.3.
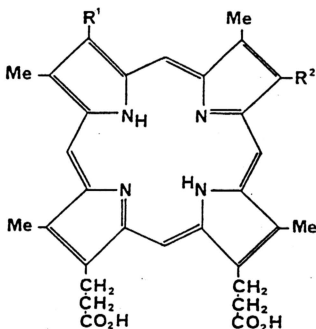
| Formula	Name
(1) $R^1 = R^2 = CH(OH)Me$	Haematoporphyrin
(2) $R^1 = CH(OH)Me$; $R^2 = CH(OAc)Me$	8^1-O-Acetylhaematoporphyrin
(3) $R^1 = CH(OAc)Me$; $R^2 = CH(OH)Me$	3^1-O-Acetylhaematoporphyrin
(4) $R^1 = CH = CH_2$; $R^2 = CH(OH)Me$	8-(1-Hydroxyethyl)-3-vinyldeuteroporphyrin
(5) $R^1 = CH(OH)Me$; $R^2 = CH = CH_2$	3-(1-Hydroxyethyl)-8-vinyldeuteroporphyrin
(6) $R^1 = R^2 = CH(OAc)Me$	O-O-Diacetylhaematoporphyrin(haematoporphyrin diacetate)
(7) $R^1 = CH = CH_2$; $R^2 = CHOAc)Me$	8-(1-Acetoxyethy)-3-vinyldeuteroporphyrin
(8) $R^1 = CH(OAc)Me$; $R^2 = CH = CH_2$	3-1(1-Acetoxyethyl)-8-vinyldeuteroporphyrin
(9) $R^1 = R^2 = CH = CH_2$	Protoporphyrin

Fig. 2.2 : Chemical structure of HpD
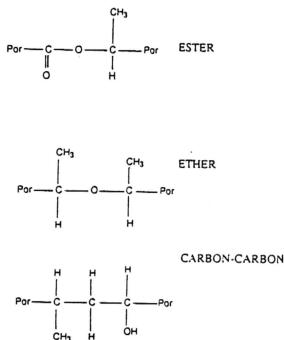
Fig. 2.3: The bonds in the oligomeric component of Photofrin II and HpD

2.7 Photochemistry And Photophysics Of Hp And HpD

2.7.1 Photochemical Interactions

The electronic structure of a photoactive species is described by four quantum numbers which define the energy each electron can possess. Further to the identification of the electronic structure, it is often simpler to refer to the spin multiplicity of the orbitals concerned. The spin quantum number, s, can have values of +1/2 and -1/2 and that each orbital can contain a maximum of 2 electrons. For the pair of electrons, their spin states can either be antiparallel or parallel to give a resulting total spin of 0 (singlet state where S(-1/2+1/2)=0) or 1 (triplet state, S(1/2+1/2)=1). In the common case where the ground state

(i.e., the normal unexcited state) is a singlet, it is called S_0 and the excited states are as follows S_1, S_2 etc in orders of their energies. The triplet states are written as T_1, T_2 etc. Spin multiplicity is generally conserved during electronic transitions (i.e. change in spin multiplicity is a forbidden transition) but this can be violated, on occasion. The violation of this rule is intersystem crossing.

A photosensitizer such as porphyrin has, like most polyatomic molecules, configuration for its ground state, with various excited singlet and triplet states. In fact, the very basis of fluorescence or phosphorescence arises from the radiative transitions from the excited singlet or triplet states to the ground singlet states respectively.

2.7.2 Mechanisms Of Photodynamic Action: Photoprocesses Of Porphyrins

The photochemical process of PDT starts with the absorption of light energy by a photosensitizer and it then becomes excited. Intersystem crossing may occur to a lower energy triplet manifold instead of being emitted as a photon (fluorescence) or by internal conversion of the excited state to a high vibrational level of the singlet ground state. Energy loss from the triplet state can be by intersystem crossing and internal conversion back to the singlet ground state by phosphorescence or by the transference of energy to another system. This triplet states decays only very slowly and so, it has time to react with its environment forming active species of oxygen. These reactions can proceed by two possible mechanisms which depend on substrate and reactant formation.

In a type I (or radical) mechanism the excited sensitizer state reacts with a substrate resulting in hydrogen atom or electron transfer to produce free radicals. These then react with molecular oxygen, to give an oxidised product.

The type II (or singlet oxygen) mechanism, in contrast, involves exclusively with

22

molecular oxygen to form an electronically excited state of oxygen known as singlet oxygen ($^1O_2$). It is now becoming increasingly recognized as an important chemical and biological agent of sensitizers in PDT. Although singlet oxygen is thought to be the cytotoxic species generated during PDT, other active oxygen species are generated. Das *et al* (1985) reported that a small amount of hydroxyl radical is presented. It has been shown that reactions of reactive secondary products *in vivo* such as superoxide, hydrogen peroxides and cyclic peroxides can be formed at the right pH (Buettner and Hall, 1987: Bonnet, 1995b)

In general, the competition between Type I and Type II reaction pathways (Table 2.1) is controlled by the relative concentrations of the oxygen and substrate as well as the rate of reaction of the triplet porphyrin sensitizer with substrate and oxygen. In biological system, the substrate could be lipids in membranes, amino acid residues of proteins and bases in nuclei acids. The overall effects of Type I and II reactions are disturbances in metabolism, membrane damage leading to changes in permeability and even mutations. Low drug doses favour the singlet oxygen pathway; on the other hand, high drug doses enhance the probability of radical mechanisms (Grossweiner *et al*, 1982) . The porphyrin/ lipid ratio is an important time dependent factor. When this ratio is small, monomeric porphyrin predominates and the photosensitzation mechanism is of Type II. At higher porphyrin concentrations, aggregate predominates and a Type I mechanism becomes more important even in well oxygenated system (Emilliani and Delmelle, 1983).

Table 2.1: Photosensitization kinetics in photodynamic mechanisms (Boulnois, 1986)

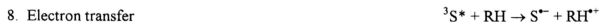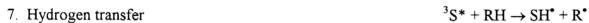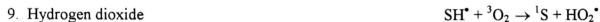*Photosensitizer excitation*

Resonant excitation

1. Singlet state absorption $\qquad ^1S + h\nu \rightarrow {}^1S^*$

Decays

2. Radiative decay (fluorescence) $\qquad ^1S^* \rightarrow {}^1S + h\nu'$

3. Non-radiative singlet decay $\qquad ^1S^* \rightarrow {}^1S$

4. Intersystem crossing $\qquad ^1S^* \rightarrow {}^3S^*$

5. Triplet state decay $\qquad ^3S^* \rightarrow {}^1S$

6. Triplet phosphorescence decay $\qquad ^3S^* \rightarrow {}^1S + h\nu''$

Type I mechanisms:

Free radical derivations

7. Hydrogen transfer $\qquad ^3S^* + RH \rightarrow SH^\bullet + R^\bullet$

8. Electron transfer $\qquad ^3S^* + RH \rightarrow S^{\bullet-} + RH^{\bullet+}$

Reactant formations

9. Hydrogen dioxide $\qquad SH^\bullet + {}^3O_2 \rightarrow {}^1S + HO_2^\bullet$

10. Superoxide anion $\qquad S^{\bullet-} + {}^3O_2 \rightarrow {}^1S + O_2^{\bullet-}$

Oxidation

Type II mechanism:

<u>Reactant formation</u>

7. Intermolecular exchange $\qquad$ $^3S* + {}^3O_2 \rightarrow {}^1S + {}^1O_2*$
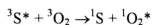
<u>Oxidation</u>

8. Cellular oxidation $\qquad$ $^1O_2* + X \rightarrow X(O)$
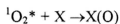
^1S $\qquad$ ground singlet state of sensitizer;

$^3S^*$ $\qquad$ excited singlet state of sensitizer;

T_1 $\qquad$ excited triplet state of sensitizer;

$^3O_2$ $\qquad$ ground triplet state of oxygen;

$^1O_2*$ $\qquad$ excited singlet state of oxygen;

RH $\qquad$ substrate;

X $\qquad$ cellular target;

X(O) $\qquad$ oxidized cellular target.

2.8 Method Of Preparation Of Clinical HpD

Clinical HpD was prepared following the procedure of Lipson (1961) as described by Kessel and Cheng (1985a). All reagents used were of analytical grade.

Hematoporphyrin dihydrochloride > 60% (Fluka Biochemika) (5 gm) was dissolved in 100 ml of glacial acetic acid-concentrated sulfuric acid mixture (19;1, v/v). This mixture was stirred for 1 hr and was then filtrated through the Whatman No. 1 filter paper. The filtrate was let to drop into 667 ml of 5% sodium acetate solution. The precipitate form was referred as HpD solid. A centrifuge was used to wash the HpD solid. Washing was extensively done 6-8 times at 3000g (5010rpm) and the final wash was at 6000g

(10000rpm) to ensure good packing. The above washing was carried out using water for injection. The HpD solid was then dried overnight in vacuum at room temperature.

To prepare clinical HpD, 0.5 gm of HpD solid was dissolved in 25 ml of 0.1 N sodium hydroxide and allowed to stir continuously for 1 hr. The pH of the solution was then adjusted to 7.1 with 0.1N hydrochloric acid. The solution was finally made isotonic with sodium chloride and made up to volume with water for injection to 50 ml at a concentration of 10 mg/ml. Subsequently, this solution was filtered through 0.2μm Millipore filter.

Clinical HpD was then stored immediately in the dark at -20°C and used within 6 months of preparation. The entire preparation of HpD solid and clinical HpD were carried out in dim lights to prevent photodegradation.

2.9 Method Of Preparation Of Hp

Hematoporphyrin in the form of dihydrochloride (Hp 95% purity) was obtained from Porphyrin Product, Utah. Reagents used were of analytical grade. 0.5 gm of hematoporphyrin 95% purity was dissolved in 50 ml of 0.1 N sodium hydroxide solution. The solution was stirred for 5 min and then neutralized to pH 7.1 with 0.1 N hydrochloric acid. The solution was finally made isotonic with sodium chloride and brought to a concentration of 5 mg/ml. Following this, it was filtered with a 0.2μm Millipore filter. It was then stored in the dark and frozen and used within 6 months of preparation. The entire preparation was also carried out in dim lights as the preparation of HpD to prevent photodegradation.

2.10   Analyses And Characterization Of Clinical Hp And HpD

The photochemical behaviour and the characteristics of HpD and Hp were determined by spectroscopic analyses. Hence, absorption and fluorescence spectroscopy were carried out. Chromatography analysis such as HPLC was carried out to ascertain the chemical composition of the prepared samples. Clinical drug test such as pyrogenicity, abnormal toxicity and sterility were carried out to determine the biological purity and safety of the clinical HpD.

2.10.1 Absorption Spectra Of Clinical HpD And Hp

HpD and Hp are characterized by intense electronic absorption spectra, a property which is widely used in their analysis. These porphyrins displayed a major absorption band in the region of 400nm, known as the Soret band with four minor peaks at longer wavelengths between 450-650nm. This structure is due to the strong resonance of the ring structure of the basic porphin molecule. The different side chains cause relatively minor spectral shifts in these bands.

The absorption spectra of HpD and Hp in saline are shown in Fig. 2.4. The absorption were obtained using a UV-VIS-NIR scanning spectrophotometer (UV-3101PC Shimadzu). The intensity and the exact peak positions are concentration and solvent dependent. It is well known that aggregated porphyrins have their absorption maxima at 360-370nm (eg. HpD). Aggregation is the only phenomenon which has been consistently related to deviation from Beer's Law (in aqueous solution) indicating the presence of more than one absorbing species in solution. On the other hand, the corresponding maxima absorption for monomers (eg. Hp) are located at or above 390nm (Brown et al, 1976;

Moan, 1984). The absorption spectrum carried out in University of Malaya could be compared with the spectrum obtained by other workers (Moan and Sommer, 1981; Brown and Vernon, 1991). The last absorption peak between 620-630 nm is very useful in therapy because of its greater tissue penetration capability.

2.10.2 Fluorescence Spectra Of Clinical HpD And Hp

Fluorescence spectra of various species of porphyrins show variations in fluorescence band peaks. This is because shifts in fluorescence band peaks are pH and solvent dependent.

The spectra presented in Fig.2.6 were obtained using a Perkin Elmer Luminescence Spectrometer (LS 50B). Clinical HpD and Hp have a characteristic red fluorescence with emission peaks at 615nm and 675nm in saline. These peaks shift to 630nm and 690nm in serum. The above spectra is comparable to the commercial clinical HpD (Photofrin I) and Hp with a peak at 615nm and 675nm (Doiron, 1982; Brown and Vernon, 1991). Aggregation or dimerize porphyrins have generally a much lower fluorescence quantum yield than the corresponding monomers (Moan and Sommer, 1981; Moan, 1984). These characteristics are in agreement with Fig. 2.5.
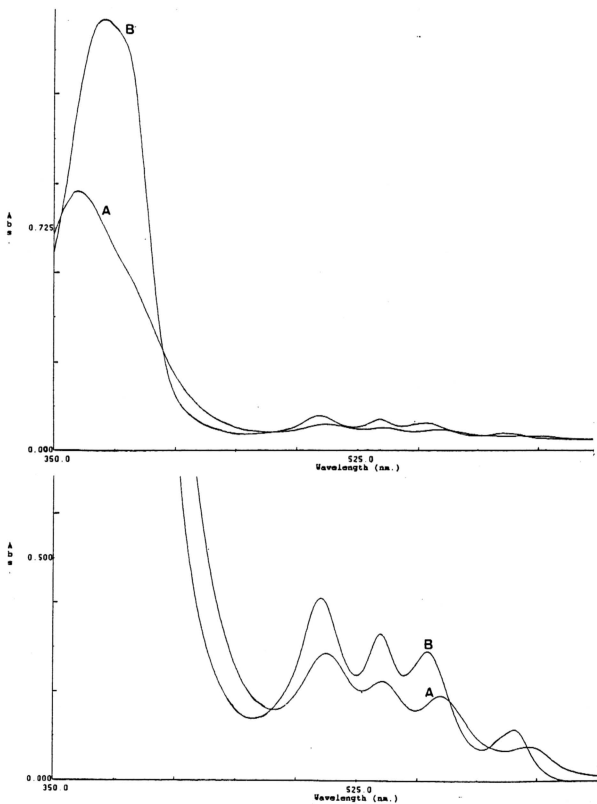
Fig. 2.4 Absorption spectra of HpD (A) and Hp (B) in saline. Both the porphyrins have a concentration of 20 mg/ml respectively
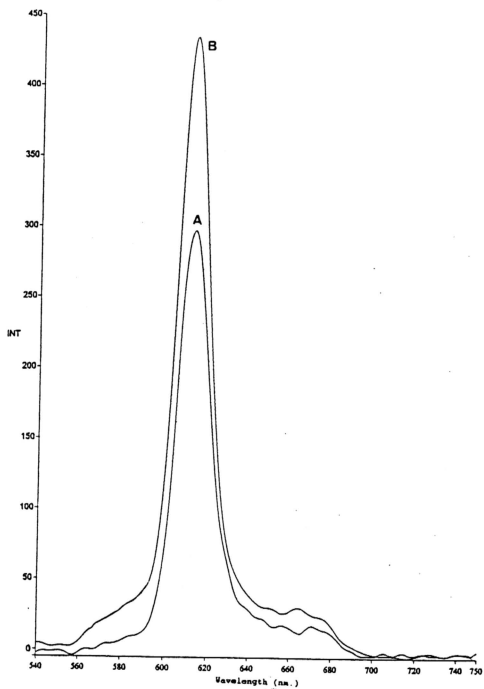
Fig. 2.5 Fluorescence spectra of HpD (A) and Hp (B) in saline. Both the porphyrins have a concentration of 1.0 μg/ml respectively

2.10.3. High Performance Liquid Chromatography For Clinical HpD And Hp

HPLC analysis was performed with a liquid chromatograph (Shimadzu LC-6A) equipped with a UV-VIS detector (SPD-6AV) set at a wavelength of 397nm. A shim-pack, reverse phase LC 18 column (6 mm X 150 mm) was used for the analysis. The mobile phase was a gradient of methanol and water in a volume ratio of 70:30 , containing 2.5 mM of tetra-n-butylammonium phosphate and phosphoric acid (0.2%) at pH 2.3. The flow rate was 1 ml/min. An aqueous concentration of 1 mg/ml of clinical HpD and Hp with a volume of 20µl each was detected

The chromatogram of both the porphyrins were compared with retention times of eluting fractions of the clinical HpD sample reported by other investigators (Moan and Sommer, 1983 and Cadby *et al*, 1983). Results of the HPLC analysis (Fig. 2.6) showed that the clinical HpD prepared at this centre was comparable with commercial clinical HpD (66% of oligomers and 34% of monomers) (Moan *et al*, 1982). The chromatogram of Hp that is shown in Fig. 2.7. has a high percentage of monomers (95%) and a low percentage of oligomers (5%). The smaller peaks were due to the 5% contaminants in the Hp which were impossible to isolate.

2.10.4 Pyrogen, Sterility And Toxicity Test For Clinical HpD

Pyrogen, sterility and toxicity test were only carried out on clinical HpD. This is to ensure that the clincial HpD prepared at this laboratory was safe to use on patients. The following tests were carried out at the Drug Control Laboratory, Ministry of Health, Malaysia and the Department of Microbiology, Faculty of Medicine, University of Malaya.

A random sampling of 20% of the total number of dispensed vials, each containing 10 ml of clinical HpD at a concentration of 10 mg/ml were used.
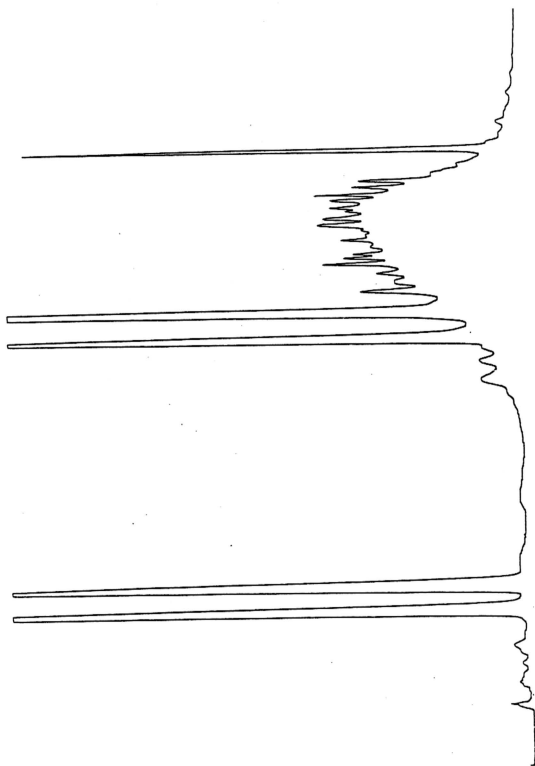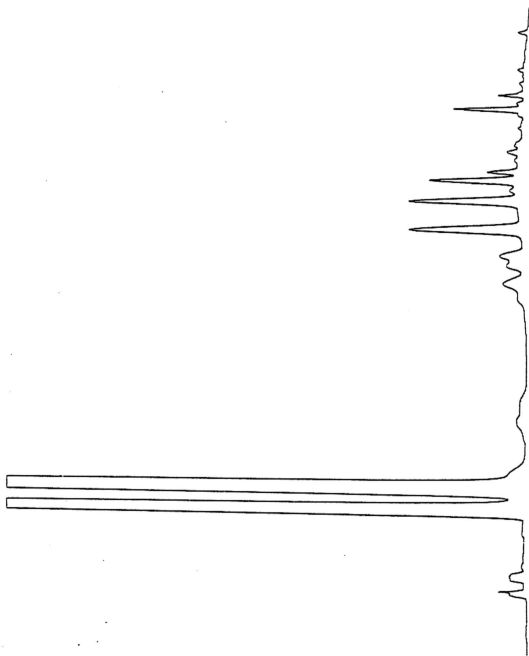
Fig.2.6   HPLC profile of HpD

Fig.2.7 HPLC profile of Hp

2.10.4.1. Pyrogen Test

The pyrogen test was designed to limit to an acceptable level the risk of febrile reactions in the patient due to the administration of clinical HpD. The test involved measuring the rise in body temperature of rabbits following the intravenous injection of the test solution. The test rabbit was given a maximum drug dose of 10 mg/kg weight intravenously within a period of 10 mins.

All syringes, needles and glassware were rendered pyrogen free by heating to 250°C for not less than 30 mins. Temperature probes with a precision digital display were used. This enable a sensitivity of $\pm 0.1°C$ within a ± 1.0 range.

Female rabbits weighing 1.5-3.5 kg, were used. These rabbits were housed in an area of uniform tempertature between 20°C-23°C and free from disturbance likely to excite them. Food and water were withheld from the rabbits during the test. Not more than 30 mins prior to the injection of the HpD test dose, the "control temperature" of each rabbit was determined. This formed the basis for the determination of any temperature rise resulting from the injection of the HpD solution. In any group of test rabbits, only those rabbits whose control temperatures did not vary by more than 1°C from each other were used. Any rabbit having a temperature exceeding 39.8°C was not used. For each experiment, 5 rabbits were used. A HpD dose of 10 mg/kg body weight was injected into the ear vein of each rabbit. Their rectal temperatures were recorded at 1, 2 and 3 hrs subsequent to the HpD injection.

Any temperature decrease was considered a zero rise. Since no rabbit showed an individual temperature rise of 0.6°C or more above its respective control temperatures and the sum of 3 individual maximum temperature rise did not exceed 1.4 °C. The clinical HpD

34

met the requirements for the absence of pyrogen. According to the standard set by Malaysian Drug Control Authority (D.C.A., Ministry of Health) and based on the U.S., Food and Drug Administration regulations (USPX-1), the HpD prepared was accepted as pyrogen free.

2.10.4.2. Aerobic And Anaerobic Sterility Test

A few random samples of the clinical HpD were streaked onto agar plates that were specially prepared. For aerobic tests, plates containing blood agar or nutrient broth containing Lab-Lemco powder, yeast extract, peptone and sodium chloride were used. The pH of the nutrient broth was maintained at 7.4 ± 0.2. The plates were then streaked with HpD using a wire loop following asceptic techniques. The plates were then incubated at 37°C in aerobic conditions. For the anaerobic tests, either Robinson's cook meat medium (RCMM) or nutrient broth medium consisting of ox heart muscle was used. The plates were then asceptically streaked with HpD and incubated at 37°C in an anaerobic chamber. After 24-72 hrs the plates were analysed for bacterial colony formation. It was found that the clinical HpD samples were free of aerobic and anaerobic bacterial contamination.

2.10.4.3. Abnormal Toxicity Test

In this test, 5 mice were injected intravenously via the tail vein each with a HpD dose of 15 mg/kg body weight which was three times the therapeutic dose (5 mg/kg) at a concentration of 1 mg/ml of HpD. The clinical HpD was diluted to 1mg/ml concentration with 0.9% sodium chloride (sterile and pyrogen free). The mice were observed for 24-48 hrs for any abnormality or death. Results of this test showed no abnormal toxicity.

Therefore, according to the standard set by the Malaysian D.C.A. and based on the U.S. F.D.A. (USPX-1) the sample was accepted as non-toxic.

2.11 Conclusion

A detail description on the origins of Hp and its derivatives (HpD and DHE) were reviewed in this thesis. It was necessary also to include their biochemistry, photochemistry and photophysics of these modern photosensitizers. Preparation of Hp and its biochemical characterization were presented and this would be use as a ground-work for clinical trials in the second phase of PDT work in this centre. Some second generation photosensitizers that show much promise for the future work were reviewed.

Following this, an established method of clinical HpD preparation was presented as well. To ensure biochemical purity of locally prepared HpD for its use in clinical applications, spectroscopic and chromatographic analyses were carried out together with clinical drug tests. The results of the various analyses and tests showed that the locally prepared drug was comparable to the commercially available product. Based on the results of this study, HpD has been certified to be safe and approved for clinical use at this centre by the Malaysian Drug Control Authority (Ministry of Health).