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# THE PURIFICATION OF HAEMATOPORPHYRIN IX AND ITS ACETYLATED DERIVATIVES

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## SUMMARY

The purification of haematoporphyrin IX by extraction followed by chromatography on silica gel is described. Thin-layer chromatography and NMR spectroscopy confirmed the high purity of the haematoporphyrin thus obtained. The different components of acetylated haematoporphyrin and those of haematoporphyrin derivative (HpD), a material used in cancer phototherapy, were also separated on silica gel.

## INTRODUCTION

It is well known[1,2] that tumours take up porphyrins to a greater extent than do surrounding tissues. Diagnostic methods for tumors[3–7] have been developed by taking advantage of the red porphyrin fluorescence under UV light. Further, biological materials containing porphyrins are severely damaged on irradiation with light[8–10]. This "photodynamic effect" combined with the localizing properties of porphyrins forms the basis of a new cancer therapy developed by Dougherty and coworkers[8–10] and others[11–13]. The therapy consists of administering the porphyrin followed by irradiation with red light. The method has been used successfully with external tumours and, through the use of optical fibres[14] and lasers[15,16], it can now be applied to deeply located tumours.

Haematoporphyrin IX (Hp) (Fig. 1a) and haematoporphyrin derivative (HpD) have been the most widely used porphyrins in cancer diagnosis and therapy. Also, haematoporphyrin is used in many laboratories as a common photosensitiser[8,12,17–19]. However, their use gives rise to concern because of the general uncertainty about the purity of commercial haematoporphyrin and the complex nature of the haematoporphyrin derivative (HpD).

Commercial haematoporphyrin is usually contamined with 8(3)-(1-hydroxyethyl)-3(8)-vinyldeuteroporphyrin (HVD, two isomers; Fig. 1b), protoporphyrin (Pp; Fig. 1c) and a small amount of more polar porphyrins and unidentified brown impurities. The preparation of HpD by treatment of commercial Hp with acetic acid–sulphuric acid (19:1, v/v) for times ranging from 15 min to 12 h[3,9] followed by neu-

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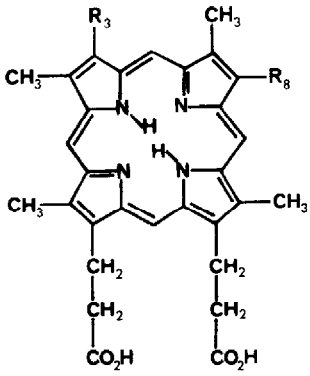


| | $R_3$ | $R_8$ |
|---|---|---|
| a : | —CH(OH)Me | —CH(OH)Me |
| b : | —CH(OH)Me | —CH=$CH_2$ |
| | or —CH=$CH_2$ | or —CH(OH)Me |
| c : | —CH=$CH_2$ | —CH=$CH_2$ |
| d : | —CH(OH)Me | —CH(OAc)Me |
| | or —CH(OAc)Me | or —CH(OH)Me |
| e : | —CH(OAc)Me | —CH(OAc)Me |
| f : | —CH(OAc)Me | —CH=$CH_2$ |
| | or —CH=$CH_2$ | or —CH(OAc)Me |

Fig. 1. Structures of compounds in commercial haematoporphyrin (Hp) and haematoporphyrin derivative (HpD). (a) Haematoporphyrin (Hp); (b) 8(3)-(1-hydroxyethyl)-3(8)vinyldeuteroporphyrin (HVD); (c) protoporphyrin (Pp); (d) O-acetylhaematoporphyrin (MAHp); (e) O,O'-diacetylhaematoporphyrin (DAHp); (f) 8(3)-(1-acetoxyethyl)-3(8)vinyldeuteroporphyrin (AVD).

tralization and precipitation was first thought to yield purified Hp. Actually, as shown by chemical and high-performance liquid chromatographic (HPLC) studies[20,21], the resulting material is a mixture of monoacetylated haematoporphyrin (MAHp, two isomers; Fig. 1d), diacetylated haematoporphyrin (DAHp; Fig. 1e), unreacted haematoporphyrin and other minor components. Moreover, the preparation[9] of the HpD solutions used in clinical studies involves the dissolution of the acetylated haematoporphyrin in 0.1 *N* sodium hydroxide solution followed by neutralization. As shown by HPLC studies[20,21], the acetylated derivatives do not survive this treatment. In fact, in addition to Hp, clinical HpD solutions consist primarily of HVD and Pp, which are the usual impurities in commercial Hp but now in increased amounts. HpD solutions have been reported to have better therapeutic activity than crude Hp[22]. However, it is possible that components other than the HVD and Pp could be responsible for this effect[23]. Also, clinical results obtained with HpD solutions prepared in various laboratories have been reported to differ greatly[24].

The above problems emphasize our interest in using purified Hp to prepare HpD, as this should provide the answer to the important question of whether the "activation" of crude haematoporphyrin via the acetylation–alkaline hydrolysis procedure involves Hp itself, other contaminant porphyrins or other impurities present in various amounts in commercial haematoporphyrin.

Although the analysis of crude haematoporphyrin can be performed relatively easily by paper chromatography[25,27], thin-layer chromatography (TLC)[27] or HPLC[20,21,28,29], preparative separation is much more difficult. Using low-pressure column chromatography, only Sephadex gels[30] were found to separate the dicarboxylic porphyrins. This method has been applied to haematoporphyrin purification by Momenteau *et al.*[31]; other methods involve tedious multiple extractions[32,33] or liquid–liquid partitions[34]. Alternatively, haematoporphyrin esterification allows easier purification[35], but the esterified product must be hydrolysed before biological assays can be made. Owing to the sensitivity of secondary alcohols to dehydratation reactions (even under mild conditions), uncertainty about the purity often remains and storage must be avoided.

In this paper, we describe a convenient method for the purification of haematoporphyrin involving extraction and column chromatography on silica gel. The purity of the resulting material can be checked by TLC with the same eluent system. This method has also been applied to the separation of the major components of HpD before alkaline treatment (*i.e.*, MAHp and DAHp) and after hydrolysis. HpD was prepared from purified Hp.

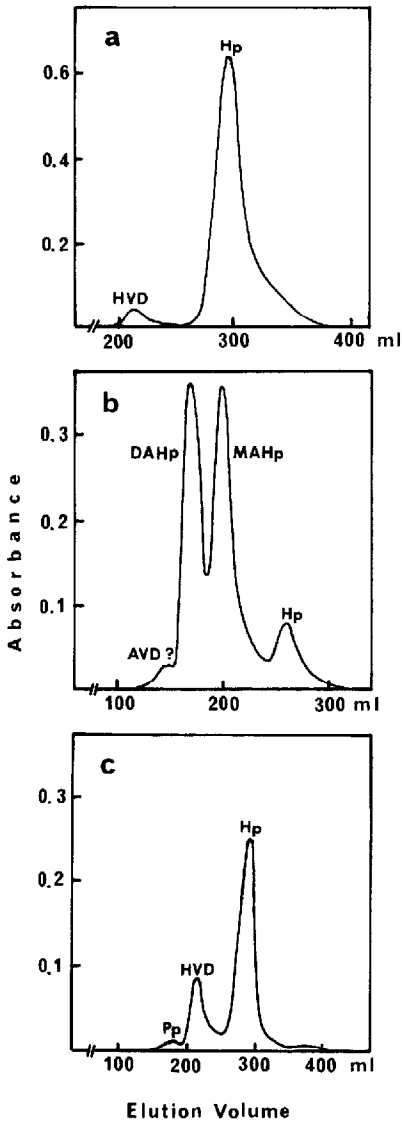


Fig. 2. Chromatography of pre-purified Hp (a), acetylated Hp (b) and acetylated Hp after hydrolysis (c). Silica gel column, 45 × 2.5 cm I.D.; solvent, acetone–ethyl acetate–water (16:13:7); flow-rate, 1–2 ml/min). Elution profile ($\lambda$ = 498 nm, 1-mm cell).

## EXPERIMENTAL AND RESULTS

### *Materials*

Haematoporphyrin IX was obtained as its dihydrochloride from Sigma. Solvent of analytical-reagent grade were purchased from Merck (acetic acid was *ca.* 100% pure and sulphuric acid 96%). Silica gel 60 (230–400 mesh) (Merck) was used for column chromatography. Gravity elution was generally performed with an eluent flow-rate of 1–2 ml/min. Photometric detection at a wavelength corresponding to the most intense porphyrin peak in the visible region (*ca.* 500 nm) was used. TLC was performed by using silica gel 60 on plastic sheets (Merck). NMR spectra were recorded in [$^2H_6$]dimethyl sulphoxide using a Bruker WM 500 instrument. Resonances are quoted relative to tetramethylsilane.

### *Purification of haematoporphyrin IX*

The purification of haematoporphyrin involved two steps: solvent extraction followed by chromatography on silica gel.

Commercial haematoporphyrin IX dihydrochloride (200 mg) was dissolved in a mixture of water (375 ml) and acetone (100 ml). The solution was extracted with chloroform (375 ml) and the organic phase washed with water (2 × 250 ml). It should be pointed out that, as haematoporphyrin was in the dihydrochloride form, the aqueous phase from the first extraction step was acidic (pH $\approx$ 3). If the neutral form of haematoporphyrin is used, the pH must be adjusted accordingly. The aqueous phases used for washing are kept below pH 5.5. The aqueous phases containing primarily polar impurities that appear as broad brown spots on TLC plates (see below) were discarded. Next, the chloroform phase was extracted with phosphate buffer (0.01 *M*, pH 7.5, 200 ml) and the aqueous phase washed three times with fresh chloroform (3 × 250 ml). This step partly eliminated contaminant hydrophobic porphyrins. The aqeous phase was acidified and extracted with ethyl acetate–ethyl formate (8:2, 250 ml. The organic phase was then thoroughly washed with water, dried over anhydrous sodium sulphate and concentrated. Addition of isooctane precipitated an amorphous solid which was dried (reduced pressure, phosphorus pentoxide to give pre-purified haematoporphyrin (yield *ca.* 50%). It was found to contain less than 5% of HVD and no detectable amounts of Pp by chromatography.

Pre-purified Hp (12 mg) dissolved in the eluent solvent was placed on a 45

TABLE I

$R_F$ VALUES OF PORPHYRINS ON THE SILICA GEL TLC SYSTEM

Development in acetone–ethyl acetate–water (16:13:7).

| *Porphyrin* | $R_F$ |
|---|---|
| Impurities | 0.51–0.59 |
| Hp | 0.62 |
| MAHp | 0.66 |
| HVD | 0.67 |
| DAHp | 0.69 |
| Pp | 0.72 |

× 2.5 cm I.D. silica gel column. Elution with acetone–ethyl acetate-water (16:13:7) yielded two fractions (Fig. 2a). Each of them was added to a mixture of water and ethyl formate, which led to phase separation. The organic phases containing the porphyrins were then washed with water, dried over anhydrous sodium sulphate, concentrated and precipitated with isooctane. The first fraction eluted was identified as HVD by comparison on TLC plates (see below) with that of an authentic sample prepared according to Bonnett *et al.*[21]. The major fraction (*ca.* 95%), which moved as a single spot on TLC plates, was identified as haematoporphyrin IX. Its MMR spectrum {$\delta$ [$(C^2H_3)_2SO$]:12.33 (bs, 2 × $CO_2H$); 10.72, 10.71, 10.69, 10.68, 10.30, 10.22 (all s, *meso*-H); 6.54, 6.53, 6.51 (bq, 2 × $CHOHCH_3$); 6.12 (bs, 2 × O*H*); 4.37, 4.36, 4.35 (t, *J* 6.4 Hz, 2 × $CH_2CH_2CO_2H$); 3.71, 3.68, 3.65, 3.62 (all s, $ArCH_3$); 3.21, 3.19, 3.18 (t, *J* 7 Hz, 2 × $CH_2CH_2CO_2H$); 2.15, 2.14 (d, *J* 6.5 Hz, 2 × $CHOHCH_3$, coupled with $CHOHCH_3$); −3.94 ppm (s, 2 × N*H*) agrees with that expected from literature data[21]. It can be pointed out that the resonances at 10.72, 10.71, 10.69 and 10.68 ppm each account for half a proton. This pattern is due to the presence of two diastereoisomers of haematoporphyrin in equal amounts.

Acetone–ethyl acetate–water (16:13:7) was also used in the analysis of the crude haematoporphyrin by TLC on silica gel. In this system, Hp moved as a single spot whereas HVD and Pp gave distinct spots. $R_F$ values are given in Table I. Other brown impurities usually found in commercial haematoporphyrin remained spread over the range $R_F$ = 0.51–0.59.

Purified Hp was stored in the dark at −20°C. Its purity was checked periodically by TLC.

*Acetylated haematoporphyrin*

Purified haematoporphyrin (35 mg) was stirred in 1.5 ml of acetic acid containing 5% sulphuric acid for different intervals of time (15, 30, 60 min) at 18.5 or 25°C. The various solutions were precipitated using 3% aqueous sodium acetate (20 ml). The solids were filtered, thoroughly washed with water and dried (reduced pressure, phosphorus pentoxide). The resulting samples were chromatographed on silica gel columns using acetone–ethyl acetate–water (16:13:7) as the eluent. A typical elution profile is shown in Fig. 2b. Three major fractions were observed and were readily identified in order of elution as diacetylated, monoacetylated and unreacted haematoporphyrin. This order corresponded to increasing polarity, as expected. Moreover, the ratio of the three fractions was found to depend both on the acetylation reaction time and on the temperature. At 18.5°C, the proportions of DAHp, MAHp and Hp were found to be 8:41:50, 15:48:36 and 29:50:20 for reaction times of 15, 30 and 60 min, respectively. The extent of the reaction depended strongly on the temperature. Thus, at 25°C, a 60-min reaction led to 96% pure DAHp. The NMR spectra of DAHp and MAHp were found to agree with those reported by Bonnett *et al.*[21]. A small unresolved fraction that increased with reaction time and temperature was eluted before DAHp. However, it did not exceed 3–4% of the total porphyrin concentration, and is tentatively attributed to acetylated derivative of HVD (Fig. 1f).

TLC analysis of acetylated haematoporphyrin was also performed on silica gel sheets using acetone–ethyl acetate–water (16:13:7). Hp, MAHp and DAHp gave distinct spots; $R_F$ values are given in Table I. A small spot with $R_F$ = 0.74 might be due to acetylated HVD.

*Hydrolysis of acetylated haematoporphyrin*

Acetylated haematoporphyrin (10 mg) was mixed with 0.5 ml of 0.1 *N* sodium hydroxide solution for 1 h in darkness at room temperature. The solution was then brought to pH $\approx$ 5.5 by addition of hydrochhloric acid, extracted with ethyl acetate and taken to dryness using a rotatory evaporator. The resulting solid dissolved in the eluent solvent (acetone–ethyl acetate–water, 16:13:7) was chromatographed on silica gel. Fig. 2c displays the elution profile from the hydrolysis of purified haematoporphyrin that had been treated with acetic acid containing 5% sulphuric acid for 15 min at 20°C. The major fractions were identified as Hp (73%) and HVD (22%). A small fraction (<3%) eluting at first is probably protoporphyrin. A small amount (<2%) of a polar porphyrin eluting last remained unidentified.

## DISCUSSION

The extraction method used in the purification of haematoporphyrin significantly improves its purity. The proportions of chloroform, acetone and water were optimized. The presence of acetone increases the final yield but it makes the elimination of polar impurities more difficult. This was overcome by further washing the organic phase with water. The second part of the extraction procedure made it possible to reduce significantly the amount of hydrophobic porphyrins, thus avoiding overlapping of fractions during the final chromatographic step.

The chromatographic system described is suitable for the separation of closely related dicarboxylic porphyrins, as shown by the results obtained with acetylated haematoporphyrin and hydrolysed HpD. Also, TLC on silica gel allows an easy separation of various dicarboxylic porphyrins. However, obviously, resolution of individual isomers would require more sophisticated methods. Owing to the limited solubility of dicarboxylic porphyrins in the eluent solvents, a good separation is obtained only when operating on a small scale. Otherwise, unwanted adsorption of porphyrin on silica gel may occur at the top of the column. In this respect, silica gel 60 (230–400 mesh) was found to give the best results. It must be emphasized that these eluting solvents do not lead to the solvolysis reactions reported for other systems[36]. In some cases, separation was improved by using acetone–ethyl acetate–water (16:13:7.5).

It appears that the simplicity and cheapness of the chromatographic methods described here should make them valuable for routine checking of the purities of some dicarboxylic porphyrins and the composition of HpD used in clinical studies. If chromatography on silica gel is performed in parallel with reversed-phase chromatography, additional information is obtained; the chromatographic behaviour of the porphyrins is expected to be opposite for the two methods.

This study also confirmed the observation that the treatment of haematoporphyrin by acetic acid followed by hydrolysis drastically decreases the purity of the original material. Biological assays with purified porphyrins and materials derived from purified porphyrins are in progress.

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