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Characterisation of the photochemotherapeutic agent disulphonated aluminium phthalocyanine and its high-performance liquid chromatographic separated components

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ABSTRACT

Disulphonated aluminium phthalocyanine ($AlPcS_2$), a potential clinical photosensitiser, has been synthesised in a reproducible form and shown by reversed-phase HPLC to consist of at least eight components which are believed to be individual $AlPcS_2$ regioisomers. These components have been isolated either as single bands or mixtures of two using preparative reversed-phase HPLC methods. The number and position of sulphonate groups per phthalocyanine macromolecule for each component has been determined using a chemical degradation and HPLC assay. Results suggest that the bulk $AlPcS_2$ material consists mostly ($>60\%$) of an amphiphilic α-,α-disubstituted regioisomer, with both sulphonate groups substituted to the same side of the molecule (adjacent form). Possible structures for some of the other separated components of $AlPcS_2$ are also presented.

INTRODUCTION

Photodynamic therapy (PDT) is a treatment for neoplastic disease that involves the selective destruction of tumours using light-activated sensitiser compounds (photosensitisers) that preferentially accumulate in target tissue areas [1–3]. Phthalocyanines (Pc) are one of the second generation photosensitisers that show significant therapeutic advantages over the presently used hematoporphyrin-based compounds owing to superior light absorption at longer wavelengths and lower skin photosensitivity [4–6]. Much interest has centred around the use of water-soluble sulphonated metallophthalocyanines (Fig. 1), $MPcS_n$ (where M = Al, Zn, Ga and $n = 1$, 2, 3 or 4). The disulphonated compound $AlPcS_2$ has shown particularly promising results in biological studies [7–9] and is under investigation as a potential clinical candidate.

Chromatographic analysis of symmetric sulphonated metallophthalocyanines, such as $GaPcS_2$ and $AlPcS_2$, shows them to consist of many components [8–11]. These have proven difficult to isolate and identify by HPLC and other analytical techniques. Recently a method for the analysis of asymmetric sulphophthalocyanines using a diode array HPLC detector has been reported that identifies components of synthetic fractions based on their spectral properties [12].

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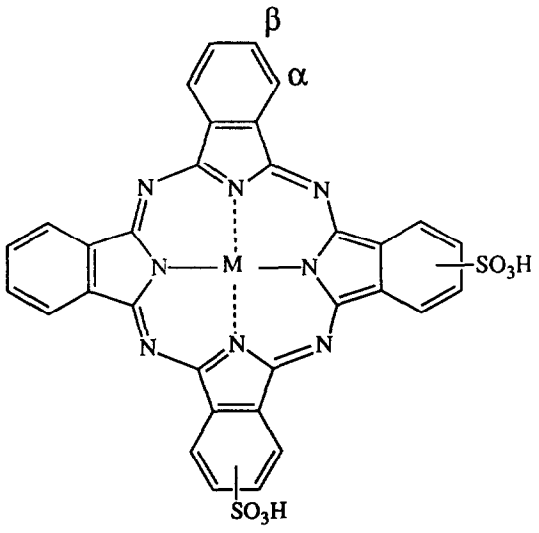

Fig. 1. The structure of disulphonated metallophthalocyanine ($MPcS_2$) showing the two possible positions (α and β) of substitution per Pc ring.

A mixture of differently sulphonated aluminium phthalocyanines ($AlPcS_{1-4}$) can be synthesised either by direct sulphonation of aluminium phthalocyanine chloride (AlPcCl) with oleum or by the condensation of phthalic and sulphophthalic acids with aluminium chloride [10]. $AlPcS_2$ is then obtained from $AlPcS_{1-4}$ using preparative reversed-phase medium-pressure liquid chromatography (MPLC). Material prepared in this way is composed of several components as demonstrated by reversed-phase HPLC. It is believed that these components are all disulphonated but differ in the location of sulphonate substitution around the phthalocyanine ring. The $AlPcS_2$ fraction obtained from the oleum reaction and MPLC process has been shown to be highly reproducible [10] and for this reason this material has been synthesised in bulk for evaluation as a clinical photosensitiser. A knowledge of the properties of the individual components of this $AlPcS_2$ fraction is essential in order to fully characterise it for future studies. Due to the low volatility and tendency to aggregate in solution of these compounds usual methods of structural analysis (NMR and mass spectroscopy) are inconclusive. This paper describes the separation and isolation of these components from the $AlPcS_2$ fraction using preparative reversed-phase HPLC methods, their subsequent reversed-phase HPLC analysis and characterisation of each using a chemical degradation and HPLC assay technique.

EXPERIMENTAL

Aluminium disulphonated phthalocyanine

Disulphonated aluminium phthalocyanine was prepared in our laboratory via the sulphonation of AlPcCl with oleum and preparative-scale MPLC (elution with water–methanol, 30:70), as described elsewhere [10]. An analytical HPLC trace of this material is shown in Fig. 2 with the major component peaks labelled a–h.

Preparative and analytical HPLC

The chromatograph used for both analytical and preparative operations was a Gilson Autoprep System consisting of a Model 305 and two Model 306 piston pumps, an 806 manomeric module, an 811B dynamic mixer with 1.5-ml mixing chamber, a Rheodyne 7125 injection valve fitted with a 20-μl loop, an Applied Biosystems 759A absorbance detector and a Gilson Model 201 fraction collector. The apparatus incorporated a Rheodyne 7030 switching valve allowing both analytical and preparative columns to be connected to the system. Automatic control of the system and collection and analysis of data was facilitated through a Gilson 506B system interface using a Gilson 712 software package on an Elonex 286M computer.

Analytical separations were achieved on a

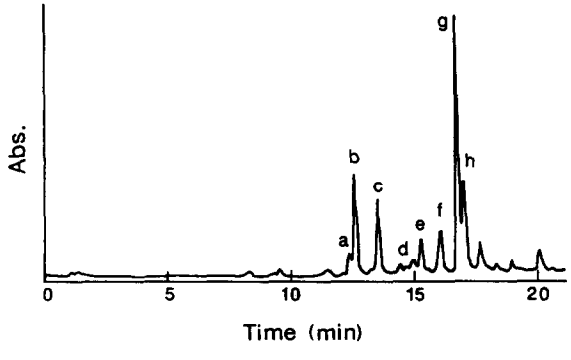

Fig. 2. Chromatogram of bulk $AlPcS_2$ material composed of components a–h which are probably different regioisomers.

Nova Pak Radial-Pak C_{18} 6 μm 100 × 8 mm Waters radial compression column contained within an RCM 8 × 10 cartridge holder with a guard column of the same packing material. In a typical analysis a 20-μl sample of analyte (of concentration *ca.* 30–1000 μg/ml) in water was injected onto the column and eluted with a linear gradient of 100% 20 m*M* phosphate buffer (pH 7.0) changing to 80% methanol over 20 min, running at a flow-rate of 2 ml/min. The eluted phthalocyanine components were detected by their absorption at 360 nm.

Preparative separations of the $AlPcS_2$ components were carried out on a Prep Nova Pak HR Prep Pak 6 μm 100 × 25 mm Waters radial compression column with a Guard Pak insert, both contained within a RCM 25 × 10 cartridge holder and using a mobile phase of 20 m*M* ammonium acetate (pH 6.4) and methanol at a flow-rate of 10 ml/min. Gradient elution methods developed to separate the components are described in the Results and discussion section.

In a typical preparative run 100–300 μl of a 5 mg/ml solution of $AlPcS_2$ was injected onto the column using one of the Model 306 pumps. Milligram quantities of each individual eluted component were collected by running the apparatus over many cycles. Samples were evaporated to dryness at 120°C and re-dissolved in water at a concentration of *ca.* 50 μg/ml prior to assay using the analytical method described above.

Chemical degradation and HPLC assay

This method is a modification of a procedure described elsewhere [11] and is used to determine both the average number of sulphonate groups per phthalocyanine molecule and ratio of α:β sulphonate substitution, from the HPLC peak ratios of the three degradation products 3- and 4-sulphophthalimide and phthalimide. A similar method used for the trace analysis of insoluble phthalocyanine pigments has recently also been described [13]. Briefly, a small quantity of phthalocyanine material was dissolved in a minimal quantity of concentrated nitric acid (Fisons, analytical-reagent grade) and heated to 50°C until the characteristic phthalocyanine green colour disappeared (1–3 min). The resulting solution was then neutralised with 1 *M* sodium hydroxide (BDH, AristaR grade) and analysed by HPLC. The HPLC method was adapted to allow for more sensitive detection using typically <100 μg analyte. Separation of the degradation products was achieved using a Spherisorb S5ODS2 25 cm × 4.6 mm column and a binary mobile phase of 0.1% trifluoroacetic acid in water and methanol running an elution programme of 0% methanol for 10 min followed by a linear gradient to 70% methanol by 35 min at a flow-rate of 1 ml/min. The eluent was monitored at 220 nm. Elution times of degraded components were: 3-sulphophthalimide, 7 min; 4-sulphophthalimide, 11 min; phthalimide, 29 min, as determined from the standard compounds 3- and 4-sulphophthalimide (prepared as described in ref. 14) and phthalimide (Aldrich). Ratios of sulphonated to unsulphonated and 3-sulpho to 4-sulpho substitution were calculated from the peak areas of 3-sulphophthalimide + 4-sulphophthalimide:phthalimide and 3-sulphophthalimide:4-sulphophthalimide respectively.

UV–visible spectroscopy

UV–visible spectra of the $AlPcS_2$ fraction and its separated components were obtained over the range 300–800 nm using a Perkin-Elmer Lambda 2 spectrophotometer. Concentrations of the methanolic and aqueous solutions was *ca.* 1 μ*M*.

RESULTS AND DISCUSSION

The chromatogram of the bulk $AlPcS_2$ fraction is shown in Fig. 2 and is seen to consist of at least eight components (labelled a–h) with one of the more lipophilic components, g, comprising over 60% of the material (by peak area). The profile of this chromatogram was seen to be unchanged over a range of injected $AlPcS_2$ concentrations indicating it unlikely that any of the peaks are due to dimers or higher aggregates. Two reversed-phase preparative methods were developed to isolate these components: (a) Components a, b, c and d ; 100% 20 m*M* ammonium acetate (pH 6.4), then linear gradient to 40% methanol over 8 min which was then held isocratically for a further 20 min.

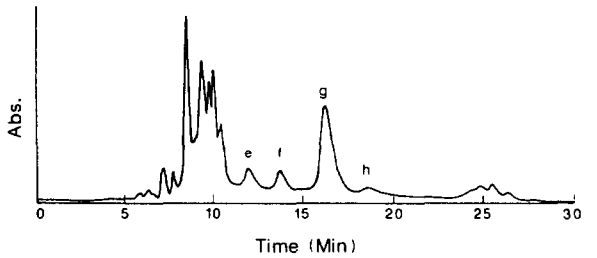

Fig. 3. Example chromatogram from the preparative reversed-phase HPLC method (conditions described in the text) used to isolate components e, f, g and g/h from the bulk $AlPcS_2$ material.

Elution times: a = 13.8 min, b = 14.3 min, c = 16.2 min and d = 19.8 min. (b) Components e, f, g and h; 100% 20 m*M* ammonium acetate (pH 6.4) then linear gradient to 52% methanol over 6 min which was then held isocratically for a further 22 min. A typical chromatogram from this method is shown in Fig. 3. Elution times: e = 12.0 min, f = 13.8 min, g = 16.2 min and h = 18.6 min.

Single-peak components were obtained with >90% purity (by peak area) but components a and b were inseparable (Fig. 4A) on a preparative scale and isolation of h from g was not possible. These were therefore isolated as mixed fractions a/b and g/h, respectively. A typical analytical HPLC chromatogram for a single-peak component is shown in Fig. 4B. Retention times for each component are a = 12.5 min, b = 12.9 min, c = 13.6 min, d = 14.4 min, e = 15.4 min, f = 16.1 min, g = 16.9 min, h = 17.1 min. A 3-mg loading of $AlPcS_2$ per run for the preparative chromatography was found to give the optimum recovery of each component. Increased loading caused peak broadening in the chromatography resulting in poor separation. Solubility of these component fractions was found to be lower in water compared to that of the bulk $AlPcS_2$ starting material. The UV–visible spectra of these species showed the formation of typical phthalocyanine aggregates and dimers (λ_{max} = 640 nm [15]). This increased aggregation following HPLC is probably due to reductions in electrostatic repulsions between phthalocyanine molecules caused by either a change of the axial ligands of aluminium phthalocyanine from OH/H_2O to acetate, or a change in the salt form of the sulphonated substituents from SO_3Na to SO_3H induced by the acidic nature of the eluent. The UV–visible spectra of all components in methanol, except component fractions a/b and f which still showed the some aggregate, were typical of monomeric metallated phthalocyanine species (λ_{max} = 670 nm [15]).

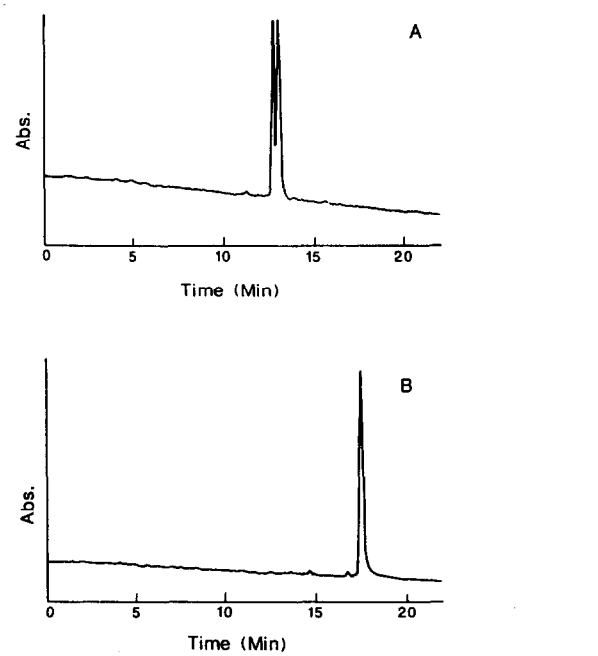

Fig. 4. Chromatograms of the isolated components a/b (A) and g (B). HPLC conditions and retention times of other components are described in the text.

The results from the chemical degradation and HPLC assay of the bulk $AlPcS_2$ fraction and some of the individual components are shown in Table I. Figure 5 shows a typical chromatogram from a degraded sulphonated phthalocyanine sample. Each component was assayed at least twice, the error in the results obtained being ± 15%. Both the bulk $AlPcS_2$ fraction and its major component g show evidence of being disulphonated species with the sulphonate groups predominantly substituted on the α positions of the phthalocyanine ring. Components a/b, d and f are also consistent with disulphonated species, though component c had a sulphonated:unsulphonated ratio indicating some monosulphonate character. In general, as lipophilicity of the component fractions increased (*i.e.* as retention time increased) then the sul-

TABLE I

RATIOS DETERMINED FROM THE PEAK AREAS OF THE CHEMICALLY DEGRADED PRODUCTS OF $AlPcS_2$ AND ISOLATED COMPONENTS SHOWING DEGREE OF SULPHONATION (A RATIO OF 1:1 UNSULPHONATED: SULPHONATED INDICATES A DISULPHONATED PHTHALOCYANINE) AND POSITION OF SULPHONATE SUBSTITUTION, ON ORIGINAL PHTHALOCYANINE RING (FROM RATIO OF 3-SULPHOPHTHALIMIDE:4-SULPHOPHTHALIMIDE)

Component	a/b	c	d	f	g	$AlPcS_2$
Sulphonated:unsulphonated	1:1.1	1:1.7	1:1.1	1:1.2	1:0.9	1:0.8
3-Sulphophthalimide:4-sulphophthalimide	1.75:1	1.6:1	2.1:1	9.0:1	7.4:1	6.4:1

phonate substitution changed from α/β to almost pure α.

There are sixteen possible regioisomers of $AlPcS_2$ (Fig. 6 shows three of the possibilities) of which five are β-,β-disubstituted, five are α-,α-disubstituted and six are α-,β-disubstituted. Further, of all sixteen possibilities there are ten isomers with sulphonate groups on the same side (adjacent form) and six with groups on opposite sides of the ring (opposite form). Previous chromatographic studies with $GaPcS_2$ [16] and $AlPcS_2$ [8,9] by other workers have shown them to also be composed of a number of components. These studies have postulated single isomer structures for these components based on their amphiphilic properties and *in vitro* photobiological efficacy. The more lipophilic components are suggested to be the adjacent form isomers. Using the same reasoning it would also be expected that isomers with α-sulphonate substitution would show increased lipophilicity over those with β-substitution. Based on this previous work and upon the lipophilicity (elution time) and degradation assay presented here, structures for the isolated components of $AlPcS_2$ can be proposed. The major component g is therefore probably an adjacent α-,α-disubstituted regioisomer (*e.g.* Fig. 6A) and similarly, the components corresponding to f, d and a/b, for example, are consistent with opposite α-,α-, adjacent α-,β- and opposite α-,β-disubstituted species, respectively.

The bulk $AlPcS_2$ fraction used in these studies has previously been shown to be a very effective photosensitiser *in vitro* and *in vivo* [7,17]. This is most probably related to the amphiphilic nature of its major components which would be expected to have excellent cell penetrating prop-

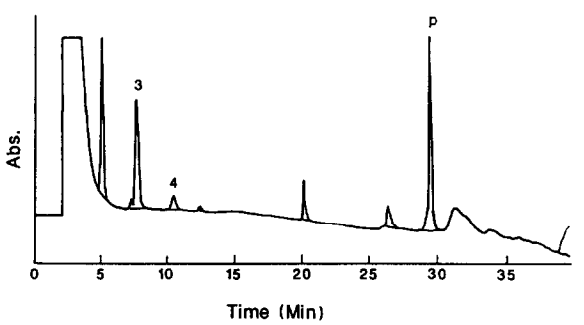

Fig. 5. The separation of the degradation products 3-sulphophthalimide (3), 4-sulphophthalimide (4) and phthalimide (p) from the $AlPcS_2$ component g. Table I shows the peak area ratios determined for unsulphonated:sulphonated and 3:4 sulphonate substitution.

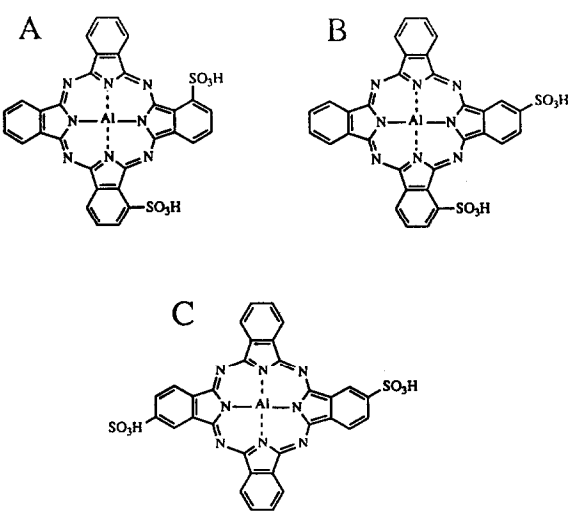

Fig. 6. Examples of possible $AlPcS_2$ isomeric structures for the isolated components: (A) adjacent α-,α-, (B) adjacent α-,β- and (C) opposite β-,β-.

erties. It is possible that a photosensitiser comprising of only this major component would be more efficacious and photobiological studies are underway using isolated component g. However, recent work [18] has suggested that a mixture of sulphonated metallophthalocyanine compounds varying in lipophilicity may have advantages *in vivo* by targeting different areas of the tumour (*i.e.* more lipophilic taken up by cells and more hydrophilic residing in the vascular stroma) and potentially increasing tumour kill. Clearly if this is the case then the role played by the different fractions may now be studied and compared to the mixture. The bulk $AlPcS_2$ material described in this paper may demonstrate such an effect and this is also under investigation.

CONCLUSIONS

An $AlPcS_2$ mixture synthesised via the oleum method for potential clinical use has been separated into seven component fractions by preparative HPLC. The direct sulphonation of aluminium phthalocyanine chloride with oleum results in the production of phthalocyanine species with predominantly α-substituted groups. The analysis of these component fractions using a chemical degradation and HPLC assay has suggested that this clinical $AlPcS_2$ material consists of different regioisomers that vary in lipophilicity, though it most resembles the major lipophilic component. Isomeric structures for these component fractions have been proposed based on their lipophilicity and degree and position of sulphonate substitution. Further work to assess the *in vitro* and *in vivo* phototoxicity and uptake of these individual component fractions compared to the clinical $AlPcS_2$ material is underway.

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