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SEPARATION OF METALLOPORPHYRINS FROM METALLATION REACTIONS BY LIQUID CHROMATOGRAPHY AND ELECTROPHORESIS

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SUMMARY

The analytical separation of the indium and manganese complexes of three synthetic, *meso*-substituted, water-soluble porphyrins from their respective free bases in metallation reaction mixtures is described. The ligands tetra-3N-methylpyridyl porphyrin, tetra-4N-methylpyridyl porphyrin and tetra-N,N,N-trimethylanilinium porphyrin are complexed with In(III) and Mn(III) and are separated from residual free base by high-performance liquid chromatography (HPLC) in acidic conditions with gradient elution on ODS bonded stationary phase. Electrophoretic separation is achieved on both cellulose polyacetate strips and polyacrylamide tube gels under basic conditions. Although analytical separations can be achieved by both HPLC and electrophoresis, only HPLC is suitable for the development of preparative scale separations. Column chromatography, ion-pairing and ion-

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suppression HPLC techniques fail to separate such highly charged and closely related aromatic compounds.

INTRODUCTION

Porphyrins and metalloporphyrins are biologically significant molecules that may be viewed as derivatives of porphine, a tetrapyrrolic macrocycle. Heme, chlorophyll, vitamin B_{12} and various cytochromes are examples of naturally occurring porphyrins. In 1924, Policard [1] noted the selective biodistribution of porphyrins administered to test animals. Subsequent research has shown the selective affinity of porphyrins for neoplastic, embryonic, traumatized and lymphatic tissue [2–10]. Work in our laboratory has focused on the evaluation of indium complexes of cationic, water-soluble porphyrins for use as radiopharmaceuticals to delineate lymph nodes [9] and melanomas [10] in laboratory animals. The manganese complexes of the same ligands are being evaluated as contrast enhancement agents for magnetic resonance imaging. Pure metalloporphyrin is required for in vivo animal studies, and its purification requires the separation of free base from the product of the metallation reactions.

Most naturally occurring porphyrins that result from the breakdown of heme occur as free carboxylic acids. Separations of the methyl esters of a series of porphyrins with two to eight carboxylic side-groups have been described [11–16], and ion-pairing high-performance liquid chromatography (HPLC) of the free acids has also been documented [17–22]. Other separation schemes for a series of metal chelates of free [23] or esterified [24] porphyrins have also been reported. Most of these separation methods are applied to the diagnosis of various porphyrias through the analysis of blood, urine or feces. Separations of the particular synthetic cationic porphyrins used for delineating malignancies, specifically the *meso*-substituted N-methylpyridinium and the N,N,N-trimethylanilinium derivatives (Fig. 1), have not been described in the literature.

We wish to report our evaluation of several liquid chromatographic and electrophoretic techniques and their effectiveness in achieving both analytical and preparative separations of metallated cationic porphyrins from their respective free bases. Ideal purification requires a minimum of handling steps to prevent product loss, especially with mixtures from radiolabeling reactions. Two analytical electrophoretic techniques and the high-performance liquid chromatographic (HPLC) conditions required for separation are presented herein. All compounds evaluated have chromophores which absorb in the visible range. The relatively small structural differences between free base and metalloporphyrin are offset by distinct charge differences that are pH-dependent.

EXPERIMENTAL

Materials

Free base porphyrins were purchased from Midcentury (Posen, IL, U.S.A.), Porphyrin Products (Logan, UT, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.)

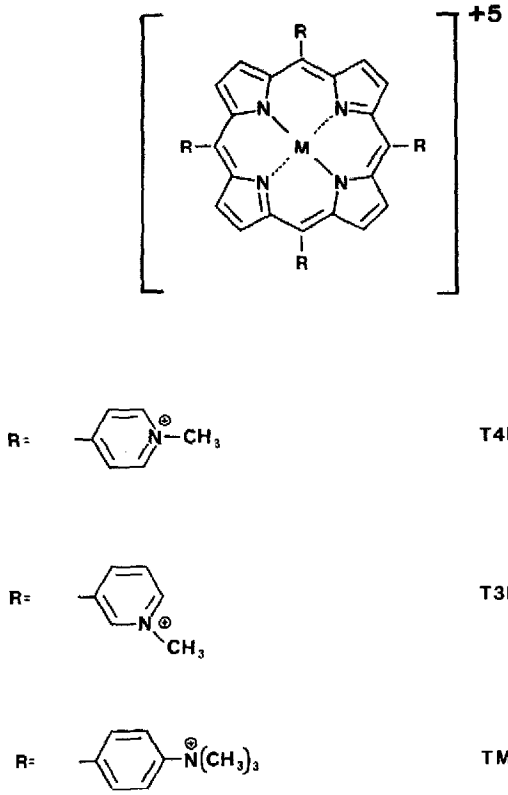

Fig. 1. Structure of *meso*-substituted synthetic porphyrins examined in this study. The metals complexed where either In(III) or Mn(III). The counterions for the free base porphyrins were either tosylate, iodide or chloride. The counterion for the metalloporphyrins was perchlorate. T4NMPyP = tetra-4N-methylpyridyl porphyrin; T3NMPyP = tetra-3N-methylpyridyl porphyrin; TMAnP = tetra-N,N,N-trimethylanilium porphyrin.

and were metallated by refluxing with indium chloride in acidified aqueous solution for several hours as has been previously described for the In(III) complexes [25, 26] and the radiolabeling reactions [9]. The Mn(III) complexes are obtained by refluxing with manganese chloride in aqueous solution for 1 h and precipitating the metalloporphyrin with lithium perchlorate. Repeated recrystallization in water with lithium perchlorate and subsequent elemental analysis gave the following results. Calculated for $MnTMAnP \cdot 5ClO_4$: C, 48.14; H, 4.33; N, 8.02. Found: C, 48.20; H, 4.28; N, 7.78; calculated for $MnT4NMPyP \cdot 5ClO_4 \cdot 3H_2O$: C, 41.19; H, 2.99; N, 8.73. Found: C, 41.10; H, 2.86; N, 8.58; calculated for $MnT3NMPyP \cdot 5ClO_4 \cdot 4H_2O$: C, 40.61; H, 3.41; N, 8.61. Found: C, 40.48; H, 2.90; N, 8.66.

Paper electrophoresis

Gelman (Ann Arbor, MI, U.S.A.) Sepraphore III cellulose polyacetate strips were saturated with 0.5 *M* sodium bicarbonate buffer, pH 8.1, and spotted with aliquots of mixtures from radiolabeling reactions using In(III) and T4NMPyP,

T3NMPyP or TMAnP. Electrophoresis was carried out in a Gelman Semi-Micro II paper electrophoresis chamber using a Heathkit (Benton Harbor, MI, U.S.A.) Model IP-32 regulated power supply at 20 mA, 100–150 V for 20 min. Bands were cut from the strips and eluted by soaking in hot water for several hours.

Column chromatography

Pierce (Rockford, IL, U.S.A.) chromatography columns (152 mm × 8 mm I.D.) were packed with Sephadex LH20 from Pharmacia (Piscataway, NJ, U.S.A.). The resin was prepared by boiling for two 2 h and deaerating in a vacuum dessicator with stirring. Bed height in columns was 10 cm and 0.05% sodium azide was added to control bacterial growth.

High-performance liquid chromatography

The chromatographic system consisted of an IBM Instruments (Danbury, CT, U.S.A.) LC/9533 ternary gradient liquid chromatograph and a Rheodyne Model 7125 syringe-loading sample injector. Chromatographic detection was achieved using a Varian (Palo Alto,CA, U.S.A.) Varichrom multiwavelength UV–VIS detector or a Groton Technology (Waltham, MA, U.S.A.) LC/S FT-UV HPLC detector.

J.T. Baker (Phillipsburg, NJ, U.S.A.) solid-phase extraction minicolumns were used to screen available bonded-phase resins for potential use in HPLC separations.

The cyano, methyl and phenyl HPLC columns were IBM reversed-phase (particle size 5 μm, 250 mm × 4.5 mm I.D.); the cyanoamine column, of 10 μm particle size, was from Perkin-Elmer (Norwalk, CT, U.S.A.) and the C_{18} column was a Vydac (Hesperia, CA, U.S.A.) 218TP54.

Methanol, acetonitrile and water were HPLC grade and tetrahydrofuran, isopropyl alcohol and glacial acetic acid were reagent grade, all obtained from Fisher. Hexanesulfonic acid was reagent grade obtained from Aldrich. All solutions were filtered through 0.45-μm Millipore membrane filters, and sample solutions were filtered through 0.45-μm Millipore filter tips attached to 5-ml B-D insulin syringes.

Gel electrophoresis

Polyacrylamide, bisacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulfate were all electrophoresis grade and purchased from FMC (Rockland, ME, U.S.A.). A Hoefer Scientific (San Francisco, CA, U.S.A.) GT 3 tube gel apparatus with 130 mm × 14 mm I.D. tubes and a PS 1200 DC power supply were run at 100 V, 200 mA in 0.05 *M* Tris buffer, pH 8.0, and 0.05% Triton X-100 non-ionic detergent.

Spectral characterization

Spectral identification of eluted bands was done on a Perkin-Elmer Lambda 5 UV–VIS spectrophotometer. Discrimination between metalloporphyrin and free base is provided by their visible spectra as shown in Fig. 2. The Soret band has the highest molar extinction coefficient and is found between 412 and 421 nm for the free base compounds. Metal complexation shifts the maximum absorption to

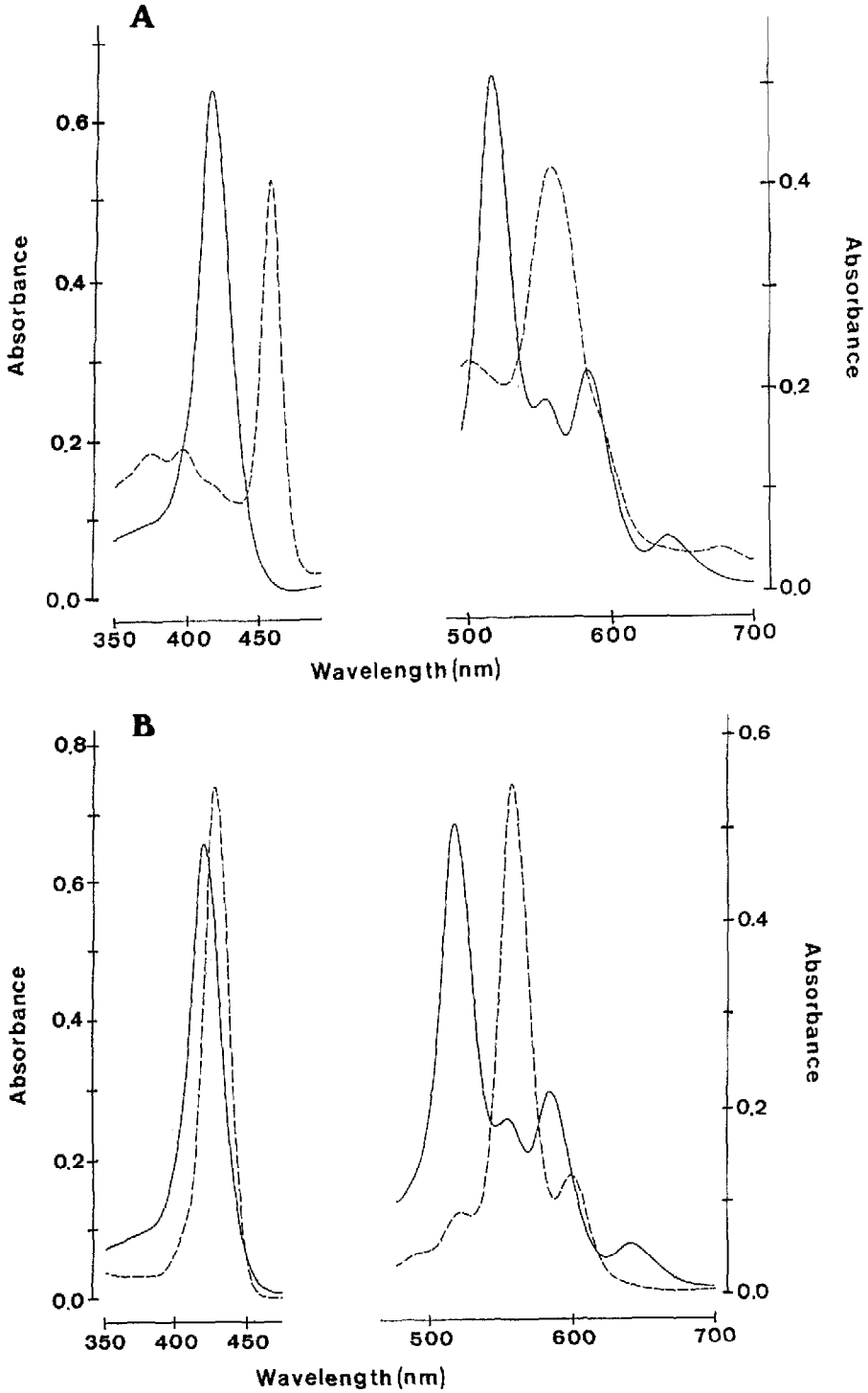

Fig. 2. Overlay of the visible spectra of T4NMPyP with MnT4NMPyP (A) or InT4NMPyP (B) which shows characteristic shifts of absorption maxima. The solid line is the free base and the dashed line is the metalloporphyrin. Spectra of the Q bands were obtained with 1 mg porphyrin or metalloporphyrin dissolved in 20 ml water, which was diluted ten-fold to obtain the Soret region of absorption.

TABLE I

ABSORPTION MAXIMA OF PORPHYRINS AND METALLOPORPHYRINS

Compound	Soret band (nm)	Q bands (nm)
TMAnP	412	518, 554, 585, 640
InTMAnP	418	520, 559, 598
MnTMAnP	465	516, 560, 678
T4NMPyP	421	514, 550, 579, 635
InT4NMPyP	431	515, 555, 593
MnT4NMPyP	462	513, 561, 592
T3NMPyP	416	513, 581, 653
InT3NMPyP	420	517, 555, 594
MnT3NMPyP	462	505, 559, 679

between 418 and 431 nm for the indium complexes and between 462 and 465 nm for the manganese complexes. The Q band absorption patterns given in Table I also distinguish free base from metalloporphyrin and are used as a qualitative measure of the composition of porphyrin solutions.

RESULTS AND DISCUSSION

Paper electrophoresis

Application to the center of a cellulose polyacetate strip of product mixture from the radiolabeling reaction of ^{111}In(III) and T4NMPyP resulted in two distinct bands. The radioactive band migrated the farthest towards the cathode and a colored non-radioactive band remained closer to the origin. The migration bands from non-radioactive reactions using these same compounds were characterized by UV–VIS after electrophoresis and their positions compared in order to identify the free base, at 7 mm, and pure labeled metalloporphyrin at 10 mm from the origin. Although 75% of the applied activity was recovered in the [^{111}In]T4NMPyP band and the eluted product has been used for animal studies, the gel strips were easily overloaded, extensive handling was required of the radiolabeled material, and many strips were needed to obtain sufficient injectable material for clinical use.

Further complications arose with product mixtures of the metallation reactions of ^{111}In(III) and T3NMPyP. Electrophoresis resulted in a non-radioactive colored band which remained at the origin, a migration band at 2 mm that contained 65% of the activity, a band due to the free base at 3 mm, and a fourth band identified as the pure labeled metalloporphyrin at 7 mm which only contained 35% of the activity applied to the strip. The two additional bands have remained unidentified despite spectral characterization, although the high percentage of activity of one band confirmed that it contained radioactive indium and the color of both bands suggested that they contain porphyrin. Electrophoresis of TMAnP and its ^{111}In(III) complex invariably yielded a radioactive band at 2 mm that

would not migrate further and contained 75% of the applied activity. The absence of other radioactive bands that migrated towards the anode in all of the separations supported the lack of unreacted indium.

Attempts were made to purify only the ^{111}In(III) complexes of the compounds of interest by paper electrophoresis since the amount of radiolabeled porphyrin used in tumor delination is relatively small. The quantities of manganese porphyrins needed for imaging are much greater and purification by paper electrophoresis is impractical.

Column chromatography

A mixture of T4NMPyP and InT4NMPyP applied to a column of Sephadex LH-20 resin swollen with a 75:25 (v/v) methanol–water mixture separated into two bands and caused a yellow discoloration of the resin. The band that stopped 3 cm from the top of the resin adsorbed so strongly to the resin that 9 *M* urea was required to remove it. The eluted band was spectrally characterized as enriched in the metalloporphyrin compared to the applied mixture but nevertheless impure. The other compounds would not elute from the resin without urea, and mixtures of free base and metalloporphyrin would coelute when urea was added to the mobile phase. Elution mixtures of methanol–water from 50:50 to 90:10 failed to improve separation.

High-performance liquid chromatography

A variety of mobile phases and gradients was evaluated in an effort to separate metalloporphyrins from free base porphyrins by HPLC on reversed-phase columns. Bonded-phase resins of weakly polar nature such as cyano and cyanoamine, low-interactive phenyl and methyl resins and ODS were tested with water mixtures from 10 to 90% (v/v) methanol, tetrahydrofuran, acetonitrile and isopropyl alcohol under isocratic and gradient conditions. Despite wide variations in solvent polarity, strong adsorption to the column could not be avoided and only resulted in gradual porphyrin elution over a long period of time. After screening available bonded-phase resins under more extreme conditions with Baker minicolumns, low pH seemed to minimize adsorption on several resins. Acetic acid–water mixtures from 75 to 90% water gave reproducible coelutions at 7.0 min at a flow-rate of 1.0 ml/min on 25-cm columns for the indium-complexed metalloporphyrins and their free bases. Even the addition of ion-pairing agents such as hexanesulfonic acid and sodium dodecyl sulfate (SDS) could not resolve the problem of coelution, despite the use of long-chain pairing agents to maximize the differences between the two compounds.

Separation was achieved with a Vydac 5-μm ODS column under gradient conditions. When solution A was 0.1 *M* potassium acetate adjusted to pH 3.0 with formic acid and solution B contained equal amounts of acetonitrile and water, a 10-min gradient of 5% B to 70% B separated the T4NMPyP free base from its metalloporphyrins, a gradient of 20% B to 80% B separated the TMAnP from its metalloporphyrins and a gradient of 40% B to 80% B separated the T3NMPyP from its metalloporphyrins. Fig. 3 illustrates the separation of InT4NMPyP from

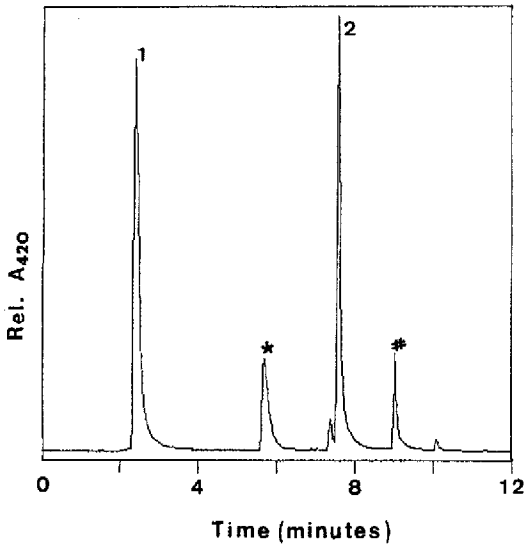

Fig. 3. Chromatogram of the separation of InT4NMPyP (1) from T4NMPyP (2). The peak labeled * is an unknown product of the metallation reaction and the peak labeled # is an unknown compound in the starting material.

T4NMPyP and retention times for the compounds evaluated are given in Table II.

Gel electrophoresis

Analytical separation of metalloporphyrin from free base was also achieved on polyacrylamide gel matrix. The pore size of polyacrylamide is greater than the size of the porphyrin molecules, so acrylamide concentration was adjusted to accommodate ease of band elution and maximum allowable voltage. Ionic strength was also adjusted to allow separation within a reasonable time because only minor changes in migration patterns were noted at different applied voltages. The

TABLE II

HPLC AND POLYACRYLAMIDE GEL ELECTROPHORESIS DATA

Compound	HPLC retention time (min)	Electrophoresis distance (cm)
TMAnP	5.8	4.0
InTMAnP	2.5	8.0
MnTMAnP	2.3	9.0
T4NMPyP	7.6	3.5
InT4NMPyP	2.4	7.5
MnT4NMPyP	2.0	10.0
T3NMPyP	4.7	3.0
InT3NMPyP	2.0	6.0
MnT3NMPyP	2.0	7.5

migration rates were pH-dependent, and ionization of the porphyrins could account for qualitative differences in migration. Separation clearly occurred between free base and metalloporphyrin for all three pairs of compounds, and migration differences were noted between the In(III) and Mn(III) compounds of each ligand as shown in Table II. Results were confirmed by comparison of visible spectra of pure product and starting material with the spectra from eluted bands.

Though different running conditions were investigated for their effect on migration of these compounds, Tris buffer was chosen to maintain constant states of ionization of the compounds throughout the run. The pH of 8 was chosen to maximize charge differences among the compounds being separated without risking demetallation which can occur at pH extremes [27]. Band migration was qualitatively based on charge as expected, with electrophoresis at 100 V, 200 mA for about 3 h giving the cleanest separations.

Polyacrylamide gel electrophoresis provides effective analytical separation of metalloporphyrin from free base for both In(III) and Mn(III) complexes of the ligands chosen. Reversed-phase HPLC on the ODS 5-μm Vydac column gives the most useful separation with a minimum of steps and could most easily be scaled up to the preparative level for biodistribution studies. The ion-pairing and ion-suppression techniques attempted do not enable separation of the metal complexes from their respective free bases. Column chromatography on Sephadex LH-20 is the simplest separation procedure but also fails to separate these compounds. Paper electrophoresis is apparently useful for only two of the radiolabeling reactions studied.

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