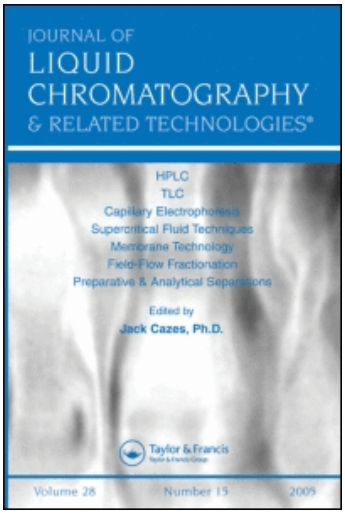

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ANALYTICAL AND PREPARATIVE REVERSE PHASE HPLC OF PORPHYRIN C AND N,N'-DIACETYL PORPHYRIN C

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ABSTRACT

Efficient high-performance liquid chromatographic (HPLC) conditions have been developed for the routine analysis of the therapeutically useful form of porphyrin c as the free acid. Porphyrin c and its diacetyl derivative have been resolved into their diastereoisomers. Preparative HPLC conditions for the purification of porphyrin c and its diacetyl derivative have also been developed.

INTRODUCTION

There has been recent interest in porphyrin c(I) and its N,N'-diacetyl derivative(II) because Porphyrin c has been found to selectively localize in tumors(5,6,7). This property has been used to selectively sensitize tumors to kill both in vivo and in vitro(5,6,7). Porphyrin c is a well characterized molecule that is rapidly excreted from the body(6,7) and so has a significant

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advantage over hematoporphyrin derivative (HpD). Hematoporphyrin derivative is a complex mixture of porphyrins which following in vivo administration (8-11) selectively localize in tumors of many different histologic types and sensitize them to kill by light of appropriate wavelength. HpD is not yet fully characterized and, although it localizes in tumors, it persists in the skin for over one month causing generalized photosensitivity on exposure to bright daylight. The rapid excretion of porphyrin c results in less photosensitization of the skin and makes it potentially a useful photosensitizer for both the detection and phototherapy of tumors.

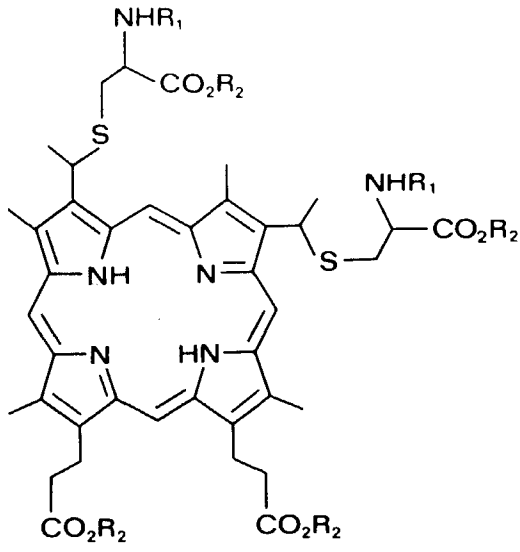
Porphyrin c (PC)(I) shown in Figure 1, occurs naturally(1) as the iron-porphyrin, hemin c, which is the chromophore in cytochrome c. This cytochrome is an ubiquitous protein found in all aerobic organisms. Porphyrin c is linked to the apoprotein via sulphide bonds through two cysteinyl residues attached at the 2(a) and 4(a) positions(2). In 1950 it was shown(3) that hydrolysis of the thioether bonds produced optically active hematoporphyrin. More recent work(4) showed the presence of possibly four diastereoisomers RR, RS, SR and SS in synthetic samples of bis-(N-benzyloxy carbonyl) porphyrin c tetramethyl ester, while porphyrin c isolated from horse heart was found to be one diastereoisomer.

A number of synthetic methods for the preparation of porphyrin c have appeared in literature(12-17) but all produce mixtures of porphyrins requiring purification. Because of this we developed a preparative reverse phase HPLC method for the purification of porphyrin c and its N,N'-diacetyl derivative.

EXPERIMENTAL

Equipment

The HPLC system used consisted of a model 510 pump and Lambda-Max Model 418 LC Spectrophotometer from Waters Associates, Melbourne Australia. A Rheodyne model 7105 injector was used



I $R_1 = H$ $R_2 = H$

II $R_1 = COCH_3$ $R_2 = H$

III $R_1 = CO_2CH_2Ph$ $R_2 = CH_3$

IV $R_1 = COCH_3$ $R_2 = CH_3$

Figure 1. The structures of porphyrin c and some of its derivatives.

equipped with a 2ml loop. Analytical HPLC was carried out on a Nova Pak C18 column 3.9mmx15cm packed with spherical particles of 4μm size with 60-100Å pore size, end capped. Preparative HPLC was carried out on a custom packed column of dimensions 15cmx19mm internal diameter, packed with the same silica as the analytical column.

Materials

The solvents used for HPLC were Chromar HPLC grade obtained from Mallinckrodt, Melbourne Australia. Ammonium acetate, ammonium

bicarbonate and acetic acid as well as other solvents and reagents used in the work up procedures and synthesis, were of analytical reagent grade. The water used for HPLC mobile phase was purified through a Millipore Milli-Q water purification system, Melbourne Australia. ^{14}C labeled acetic anhydride was obtained from Amersham Australia.

Methods

Porphyrin c was prepared from hemin by the method of Neilands(12) and N,N'-diacetyl porphyrin c was prepared from porphyrin c with aqueous acetic acid and acetic anhydride using standard literature procedures. ^{14}C labeled diacetyl porphyrin c was similarly prepared using labeled acetic anhydride. HPLC eluents were prepared by dissolving the required amount of buffer or ionic modifier in the water component of the eluent adjusting the pH where applicable and then adding the methanol component of the eluent and degassing prior to use. For preparative HPLC samples of diacetyl porphyrin c were dissolved in the minimum amount of dimethyl sulphoxide, diluted with methanol and filtered prior to injection. Where volume reduction of the solution was required it was carried out using a stream of nitrogen. Porphyrin c samples were prepared for injection by dissolving the porphyrin in a solution of ammonium bicarbonate (6%, w/v) in water followed by filtration of the solution prior to injection. The volume injected was in the region 0.5-1ml.

Isolation of the diacetyl porphyrin c from HPLC eluent was carried out as follows. The bulk of the methanol was removed from the combined fractions under vacuum and the residual solution was saturated with sodium chloride. An equal volume of ethyl acetate was added and the solution was acidified with 5M hydrochloric acid, with stirring and shaking until the bulk of the porphyrin was in the ethyl acetate layer (approximately pH4-5). The water layer was extracted with a second volume of ethyl acetate and the combined extracts washed with saturated aqueous sodium chloride, the organic layer was separated and the ethyl acetate removed under vacuum.

When salt-free porphyrin samples were required, residual traces of sodium chloride were removed by precipitation from suitable solvents.

Porphyrin c was isolated from HPLC eluents by evaporation of the eluent and of the ammonium bicarbonate under vacuum to leave the pure solid.

The tetramethyl ester of diacetyl porphyrin c was prepared by esterification of a solution of pure diacetyl porphyrin c (II) in moist tetrahydrofuran at 0°C with an ethereal solution of diazomethane(8). Evaporation of the solvents under a stream of nitrogen gas gave the required N,N'-diacetyl porphyrin c tetramethyl ester (IV).

All handling of the porphyrin solutions was carried out as far as practicable in the absence of air, under reduced light and using degassed solvents and solutions.

Porphyrins were routinely detected on HPLC at 401nm and for preparative runs detection was carried out at longer wavelengths to keep the trace on scale.

RESULTS AND DISCUSSION

Porphyrin c (I)

Samples of porphyrin c were prepared from hemin and cysteine hydrochloride(12). The purity of the prepared crude porphyrin c was found by HPLC to be in the range 50-85%. The reasons for this variation in the purity of different batches of porphyrin c involve both the instability of the highly reactive intermediates formed in the reaction, as well as the reaction conditions used. Since the free acid form of the porphyrins was needed for *in vitro* and *in vivo* work, a method was needed for both the analysis and purification of samples in that form. We chose a reverse phase C-18 silica support for HPLC, with eluents and ionic modifiers that were volatile and readily adaptable to preparative work. Useful applications of volatile buffers with good selectivity and sample recovery have been previously reported(18,19).

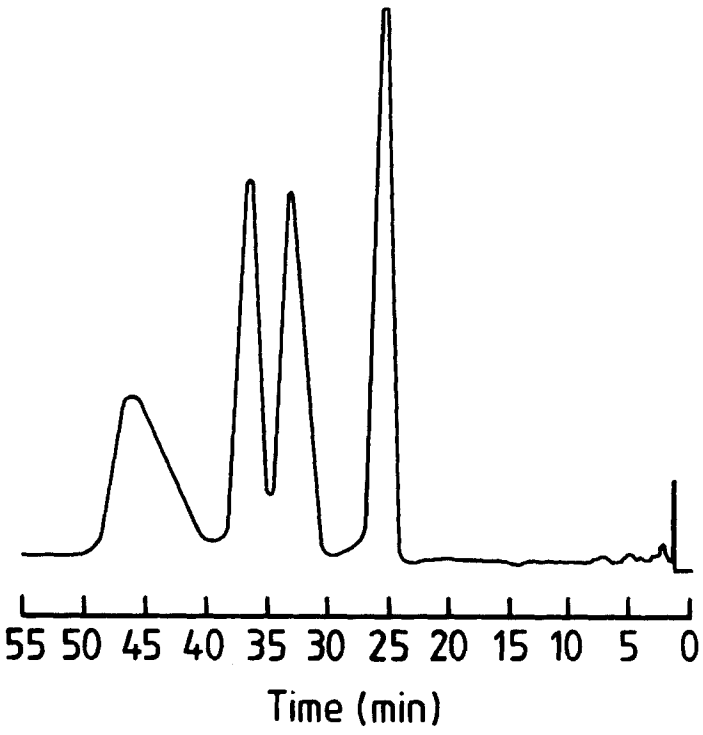


Figure 2. Analytical HPLC trace or porhyrin c (I) eluted at 1ml/min with methanol-water (58:42) containing 6% w/v ammonium formate and brought to pH5.4 with formic acid.

Kojo et al.(4) developed an analytical HPLC procedure by which bis-(N-benzyloxycarbonyl) porphyrin c tetramethyl ester (III) was chromatographed successfully. However, they were only able to resolve the derivatized material into 3 peaks, whose area ratios 1:2:1 represented the four theoretical diastereoisomers. When a synthetic sample prepared from DL-cysteine and consisting of all 8 theoretical diastereoisomers was chromatographed the same trace was obtained by them.

In our HPLC work with porphyrin c a number of ionic modifiers were tried in search of good eluents that would offer high resolution on the analytical scale. These included reagents such

as ammonium formate, triethyl amine-phosphoric acid mixtures, sodium acetate-acetic acid and others. We found that the best resolution of the free acid form of porphyrin c (I) could be achieved with ammonium formate-formic acid at pH5.4 as shown in Figure 2. This result is in good agreement with the earlier reported optimum value of pH5.16 for the separation of porphyrin isomers(21). As shown, synthetic porphyrin c (I) can be resolved into the four diastereoisomers. When ammonium acetate-acetic acid were used in place of ammonium formate-formic acid porphyrin c could only be resolved into three peaks with two of the diastereoisomers remaining unresolved.

Shorter retention times for porphyrin c were desirable in the routine examination of fractions obtained during preparative chromatography of this porphyrin. On such occasions retention time could be decreased at the expense of resolution. Thus for faster elutions the eluent used was normally a methanol-water mixture containing 53-60% methanol and ammonium bicarbonate (6%, w/v) as the ionic modifier.

The type of trace obtained for porphyrin c under such conditions is shown in Figure 3, where three of the isomers are seen to co-elute and the fourth is partially resolved.

N,N'-diacetyl porphyrin c (II)

N,N'diacetyl porphyrin c was successfully chromatographed on the same reverse phase column with methanol-water based eluents. The trace in Figure 4 shows the base line resolution of this porphyrin into 3 peaks with area ratios of 1:2:1. Under these conditions two of the diasteroisomers coincide and are not resolved. The ionic modifier in this case consisted of ammonium bicarbonate (2%, w/v) without pH adjustment. A similar result could be obtained using ammonium acetate (6%, w/v) as the ionic modifier without adjustment of the pH, Figure 5. The latter eluent modifier results in a pH value close to 7.0 which makes it a preferable system in this case.

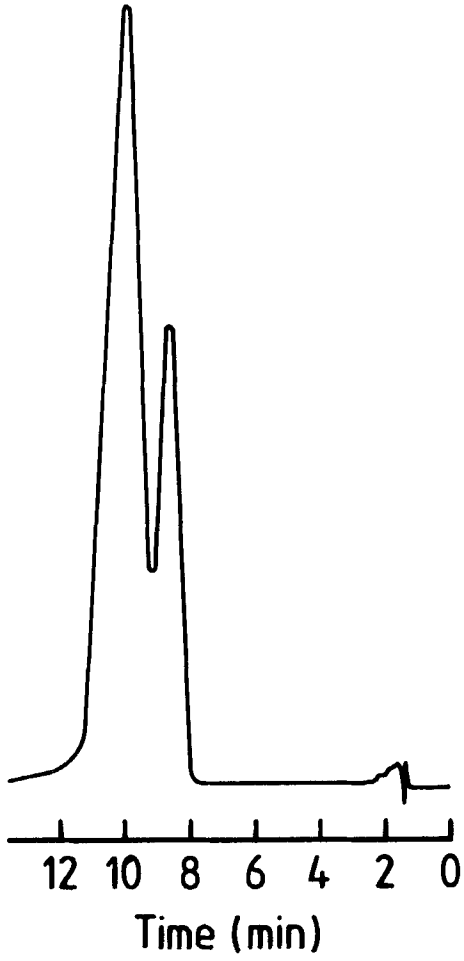


Figure 3. Analytical HPLC trace of porphyrin c (I) eluted at 1ml/min with methanol-water (58:42) containing 6% w/v ammonium bicarbonate.

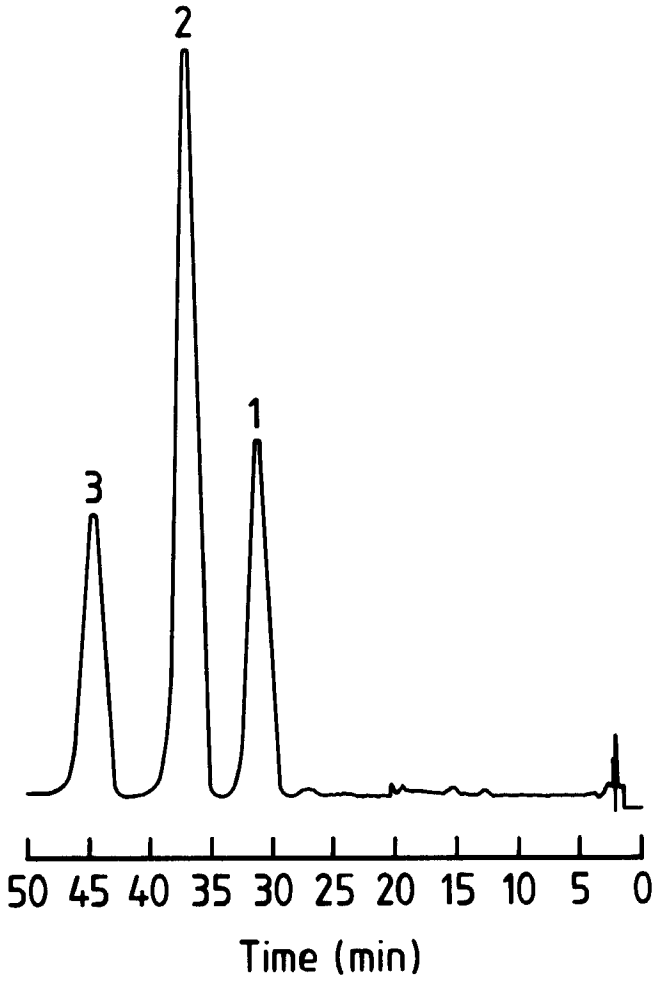


Figure 4. Analytical HPLC trace of N,N'-diacetyl prophyrin c (II) eluted at 1ml/min with methanol-water (1:1) containing 2% w/v of ammonium bicarbonate.

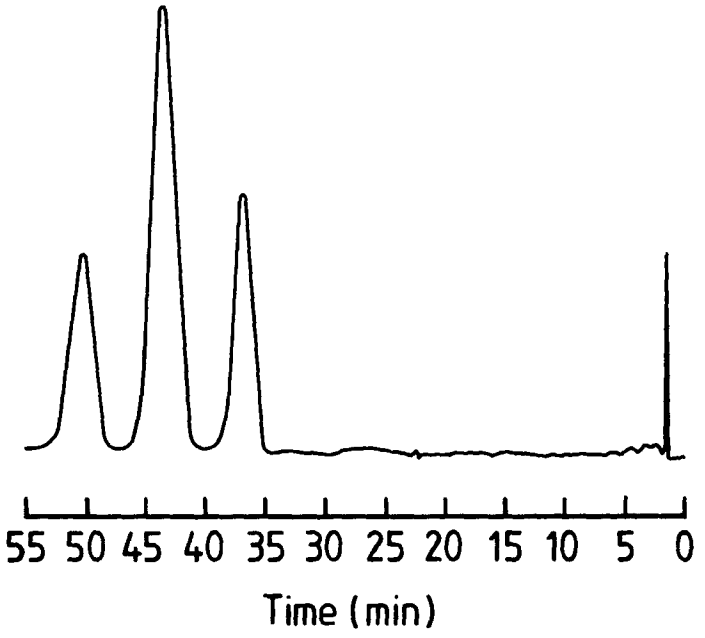


Figure 5. Analytical HPLC trace of N,N'-diacetyl porphyrin c (IX) eluted at 1ml/min with methanol-water (53:46) containing 6% w/v ammonium acetate.

For routine analyses of solutions of diacetyl porphyrin c where resolution of isomers was unnecessary but faster elution was desirable the eluent used for this purpose consisted of methanol-water mixtures with a methanol content in the range 55-60% v/v and 2% w/v of ammonium acetate as ionic modifier. Elution under such conditions gave only partial resolution of the diastereoisomers of diacetyl porphyrin c, but short elution times. Figure 6 shows a typical trace for diacetyl porphyrin c using methanol-water (55-45%) containing 2% w/v of ammonium acetate. ^{14}C labeled diacetyl porphyrin c was separated by analytical HPLC as shown in Figure 4 and each of the three peaks was collected as one fraction. The ^{14}C content of each fraction was determined using a scintillation counter and the result is shown in Table 1. A ratio

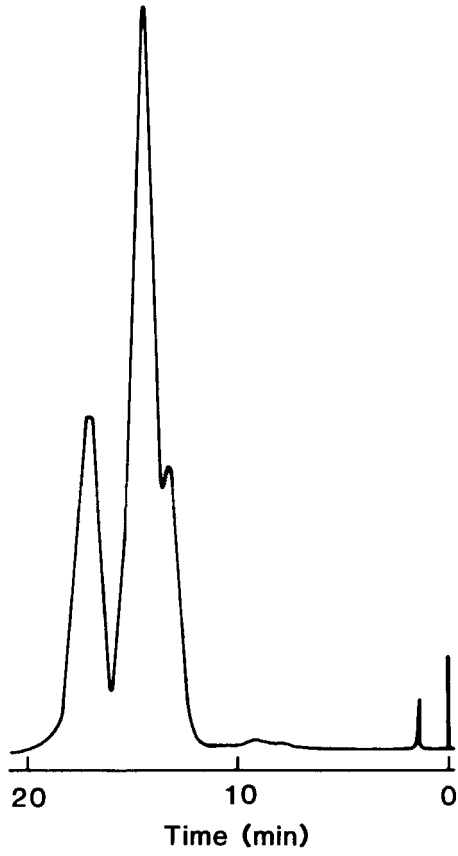


Figure 6. Analytical HPLC trace of N,N'-diacetyl porphyrin c (II) eluted at 1ml/min with methanol-water (55:45) containing 2% w/v ammonium acetate.

TABLE 1

Fractionation of ^{14}C-labeled Diacetyl Porphyrin C (II)

Peak Number	Peak Area	Number of Counts
1	158	418
2	350	927
3	159	455

of 1:2:1 was obtained confirming the presence of porphyrins substituted with two cysteinyl residues per porphyrin molecule in each of the peaks resolved.

The nature of porphyrin c (I) and its diacetyl derivative (II) was further established by I.R. spectroscopy, ^{1}H and ^{13}C nmr and elemental analysis. Fast atom bombardment mass spectra were recorded for both porphyrin c (I) (Figure 7) and of the tetramethyl ester of the diacetyl derivative of porphyrin c (IV)(Figure 8). Both mass spectra show the expected molecular ion and expected characteristic fragmentation patterns.

Preparative HPLC Results

Preparative HPLC was carried out on a column of dimensions 150mmx19mm i.d., packed with the same reverse phase silica as the analytical column. This enabled direct transfer of conditions and methods developed on the analytical columns to the preparative column.

Purification of diacetyl porphyrin c (II) by preparative HPLC was carried out by elution with methanol-water (55-45%) containing 2% (w/v) of ammonium acetate. Crude samples of the diacetyl porphyrin c contained porphyrin impurities with both longer and shorter retention times than the diacetyl porphyrin c. Because of this the peak containing the required porphyrin was collected in a number of fractions, which were subsequently examined by analytical HPLC prior to deciding which were of the required purity and could be combined. We found that unless precautions were taken to prevent exposure of the collected fractions to light and air, substantial amounts of decomposition products were produced. In order to overcome this, fractions were collected under subdued light in receivers charged with solid carbon dioxide and stored in the dark in an atmosphere of carbon dioxide. The quantity of pure diacetyl porphyrin c obtained from a typical preparative HPLC run involving an injection of around 60mg, was in the range of 30-35mg (based on $\approx 3.12 \times 10^{5}$ at 406nm in 1M HCl)(12). The purity of the sample obtained as determined by analytical HPLC was ca 98%, most

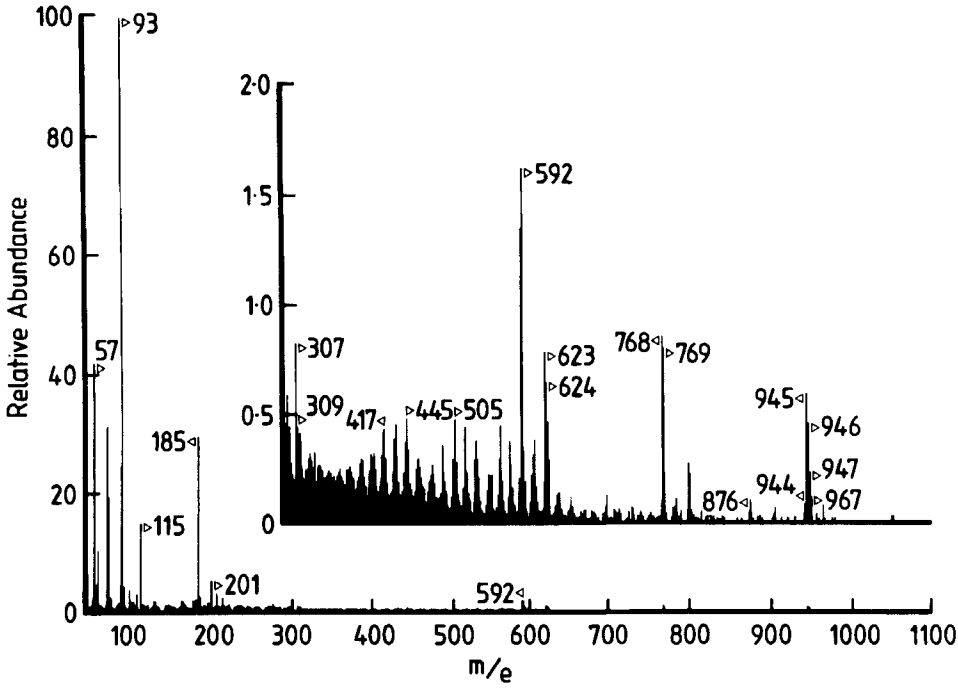


Figure 7. The positive ion fast atom bombardment mass spectrum of the tetramethyl ester of N,N'-diacetyl porphyrin c (IV), recorded from a thioglycerol matrix.

of the impurities arising from handling during the work up procedure. Figure 9 shows a typical preparative HPLC trace for diacetyl porphyrin c, (II). Non-acetylated porphyrin c (I) was also purified by preparative HPLC on the same column used for the diacetyl derivative. The eluent used for preparative runs consisted of methanol-water (53:47) containing 6% (w/v) ammonium bicarbonate as ionic modifier. The quantity of porphyrin c loaded onto the column was typically in the range 40-50mg. Prior to injection, samples were dissolved in distilled water containing ammonium bicarbonate 6% (w/v).

Because of the presence of impurities eluting both before and after porphyrin c, the eluted porphyrins were collected in a number

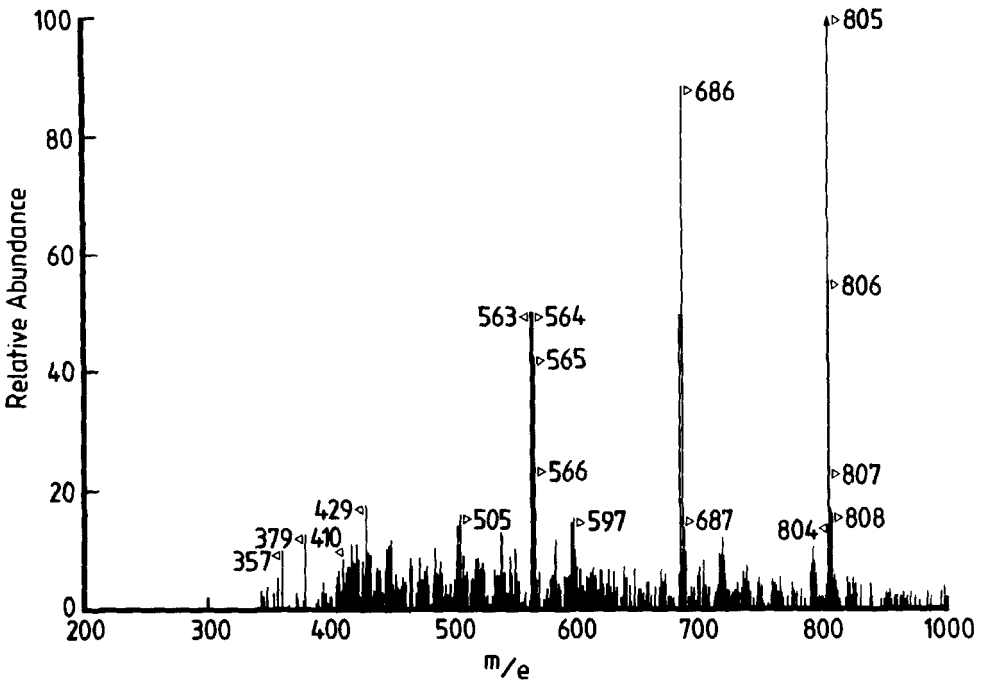


Figure 8. The positive ion fast atom bombardment mass spectrum of porphyrin c (I), recorded from a thioglycerol matrix.

of fractions which were examined by analytical HPLC prior to combining those of the required purity. During the fraction collection and subsequent handling of solutions the precautions described for the diacetyl porphyrin c (II) were also used for porphyrin c (I). The quantity of pure porphyrin c obtained from a preparative HPLC run was in the range 50-70% recovery. The purity of samples of porphyrin c obtained by this method was ca. 97% as determined by analtyical HPLC.

The preparative HPLC column was packed with the same reverse phase packing material as the analytical column and was capable of equally good resolution when used in an analytical mode. The flow rate used for preparative HPLC runs was usually 8ml/min, which is

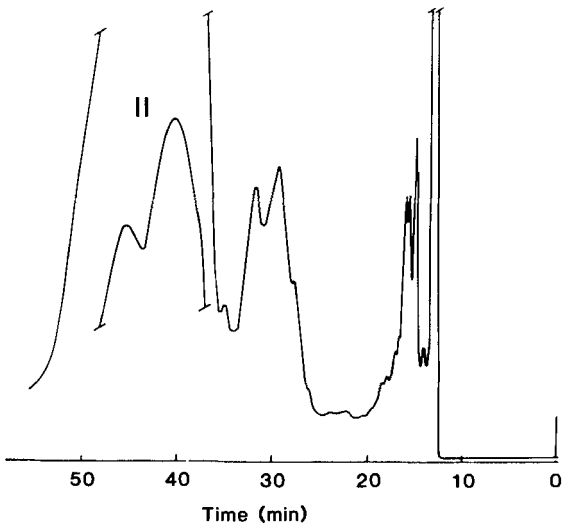


Figure 9. Preparative HPLC trace of N,N'-diacetyl porphyrin c (II) eluted at 8ml/min with methanol-water (55:45) containing 2% w/v ammonium acetate.

approximately one third of the flow needed on the preparative column to reach the equivalent flow of 1ml/min on the analytical column. The lower flow rate would have improved the resolution on the preparative column. However the resolution observed in preparative runs was generally lower than expected on the basis of analytical runs, and became progressively lower as the quantity of sample being chromatographed was increased. This progressive loss of resolution was due to overloading effects(20).

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