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Note

Purification of haematoporphyrin IX by extraction and reversed-phase chromatography

KOICHI SAITOH*, YOSHIHIKO SUGIYAMA and NOBUO SUZUKI
Department of Chemistry, Faculty of Science, Tohoku University, Sendai 980 (Japan)
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Haematoporphyrin IX (Hp, see Fig. 1) was the first porphyrin isolated from natural materials in the last century: by sulphuric acid treatment of blood. Hp and its derivative (HpD) are of increasing interest today as substances for diagnosis and therapy of cancer. These compounds are selectively concentrated by neoplastic tissues, and accordingly the tumours can be distinguished by selective fluorescence under UV light[1,2]. The cells exposed to light in the presence of Hp or HpD can be damaged and killed[3–6].

Hp is by far the most unstable of the natural porphyrins commonly used in the laboratory[7,8]. This is due to the presence of the labile hydroxyethyl side chains. The purification of Hp is very difficult.

Crude Hp can be analyzed by paper chromatography[9], thin-layer chromatography (TLC)[9] or high-performance liquid chromatography (HPLC)[10–12]. However, preparative separation is very difficult. The methods applied have been chromatography on Sephadex gel[13] or extraction[14,15]. Recently, Vever-Bizet *et al.*[16] used the dihydrochloride form of Hp as the starting material, and purified Hp by the combination of multi-step liquid–liquid extraction and column chromatography on silica gel. The Hp was eluted after the impurities. When a large amount of sample is treated on a preparative column, it is possible that the tailing portion of the impurities eluted from the column overlaps with the Hp zone subsequently eluted. In order to avoid this and also to reduce the time needed for complete elution of the Hp zone, the

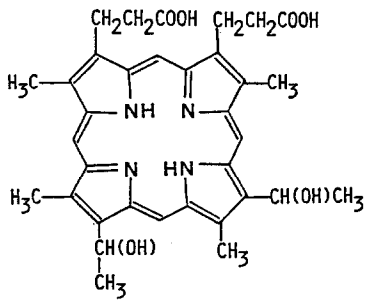

Fig. 1. Structure of haematoporphyrin IX (Hp).

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chromatography should be carried out on a column from which Hp can be eluted ahead of the impurities.

In this paper we describe a method for the purification of Hp by multi-step extraction and reversed-phase preparative column chromatography. The free porphyrin form of Hp is taken as the starting material in the extraction procedure. The purity of the resulting material was checked by reversed-phase high-performance TLC (HPLC), HPLC and mass spectrometry. The visible absorption spectrum was recorded for the purified Hp.

EXPERIMENTAL

Materials

The free porphyrin form of Hp was obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of reagent grade. Water was doubly distilled from glass apparatus.

Chromatography

The column used for the preparative separation was a LiChroprep RP-8 (40–63 μm, 310 mm × 25 mm I.D.; Merck, Darmstadt, F.R.G.). A Kyowa Seimitsu Model KHU-26H pump was used for solvent delivery.

The HPTLC plate used was a RP-8 F_{254S} (10 cm × 10 cm, Merck product No. 13725) which had been coated with octyl-bonded silica gel. Methanol–phosphate buffer (pH 3, $2.2 \cdot 10^{-2}$ mol/l) (85:15, v/v) was used as the developing solvent. The development was carried out in a Camag 28510 horizontal chamber (Muttenz, Switzerland). The compounds on the chromatogram were examined with a Shimadzu CS 920 TLC densitometer (Shimadzu, Kyoto, Japan).

The home-made HPLC system comprised a Model Twinkle pump and a Model VL-611 sample-injection valve (JASCO, Tokyo, Japan) and a rapid-scanning UV–VIS multiwavelength detector of our original design[17]. The column used was a LiChrocart RP-18 (250 mm × 4 mm I.D., Merck), and the mobile phase was methanol–phosphate buffer pH 3) (85:15, v/v).

Purification

Commercial Hp was purified by extraction followed by reversed-phase liquid chromatography.

The extraction procedure of Vever-Bizet *et al.*[16] was improved so that the free porphyrin form of Hp could be applied as the starting material. The present extraction procedure is outlined below.

Commercial Hp (178 mg) dissolved in acetone (100 ml) was added to a shaking flask containing chloroform (375 ml) and phosphate buffer (pH 3, $3.5 \cdot 10^{-3}$ mol/l phosphoric acid and $1.89 \cdot 10^{-2}$ mol/l sodium dihydrogenphosphate, 375 ml), and the mixture was shaken in order to extract Hp into the chloroform phase. After separation from the aqueous phase, the organic (chloroform) phase was shaken twice with the phosphate buffer (200 ml each time). Next, Hp was back-extracted from the chloroform solution into an aqueous solution buffered at pH 7.3 ($3.34 \cdot 10^{-3}$ mol/l sodium dihydrogenphosphate and $6.66 \cdot 10^{-3}$ mol/l disodium hydrogenphosphate, 200 ml). The aqueous solution containing Hp was washed three times with chloro-

form (250 ml each time), and then acidified to pH 3 by addition of 0.1 mol/l hydrochloric acid. The Hp was then extracted by ethyl acetate–ethyl formate (8:2, v/v; 250 ml). The organic phase was washed three times with water (150 ml each time), and dried over anhydrous sodium sulphate overnight. It was then concentrated to about 20 ml. Finally, Hp was precipitated by addition of isooctane (about 10 ml). The recovery of Hp, after drying over phosphorus pentoxide under reduced pressure, was 61.2 mg.

The chromatographic purification of Hp was carried out on a column packed with octyl-bonded silica gel (LiChroprep RP-8). Methanol–phosphate buffer (pH 3) (85:15, v/v) was used as the mobile phase at a flow-rate of 1.3 ml/min. A 61-mg amount of the Hp sample which had been processed by extraction was dissolved in 75 ml of the mobile phase. A 5-ml portion of this solution was injected into the column in every chromatographic experiment. Thus, the whole Hp sample to be purified was processed by fifteen experiments.

On the column the Hp sample was resolved into several reddish zones. The fraction corresponding to the zone of highest mobility was collected. After removal of methanol by evaporation under reduced pressure, the Hp contained in the residual aqueous solution was extracted into ethyl acetate (50 ml). The ethyl acetate extract was washed with water, and then dried over anhydrous sodium sulphate. Hp was precipitated by addition of isooctane, and it was finally dried over phosphorus pentoxide under reduced pressure. Thus 20 mg of highly purified Hp were obtained.

RESULTS AND DISCUSSION

HPTLC

Fig. 2a, b and c show the HPTLC chromatograms for the separation of commercial Hp, the material obtained by the extraction procedure and the final material obtained by the whole purification procedure, respectively. It is obvious that the commercial material contains many impurities, some of which are removed by the extraction procedure (see Fig. 2b). The final material obtained after the purification on the octyl-bonded silica gel column gives a single spot, as shown in Fig. 2c. The impurities removed by the extraction or chromatographic procedures have lower R_F values than that of the final material, that is, Hp. Taking into account the general

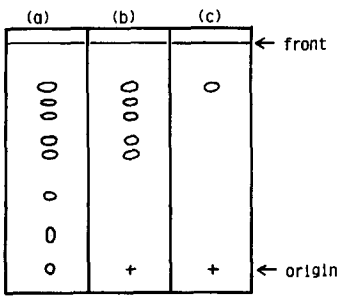

Fig. 2. Reversed-phase TLC chromatograms of commercial Hp (a), and the materials resulting from the extraction procedure (b) and the extraction–chromatography (c). HPTLC plate: RP-8 F_{254S} (Merck). Developing solvent: methanol–phosphate buffer at pH 3 (85:15, v/v). Solvent front: 75 mm.

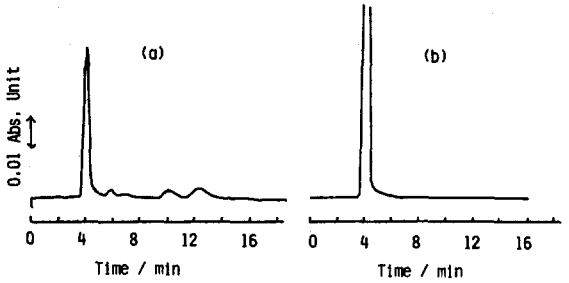

Fig. 3. HPLC separation of commercial Hp (a) and the final material from the purification procedure involving both extraction and chromatography (b). Column: LiChrocart RP-18 (250 mm × 4 mm I.D.). Mobile phase: methanol–phosphate buffer at pH 3 (85:15, v/v), flow-rate 1.0 ml/min. Detection at 396 nm.

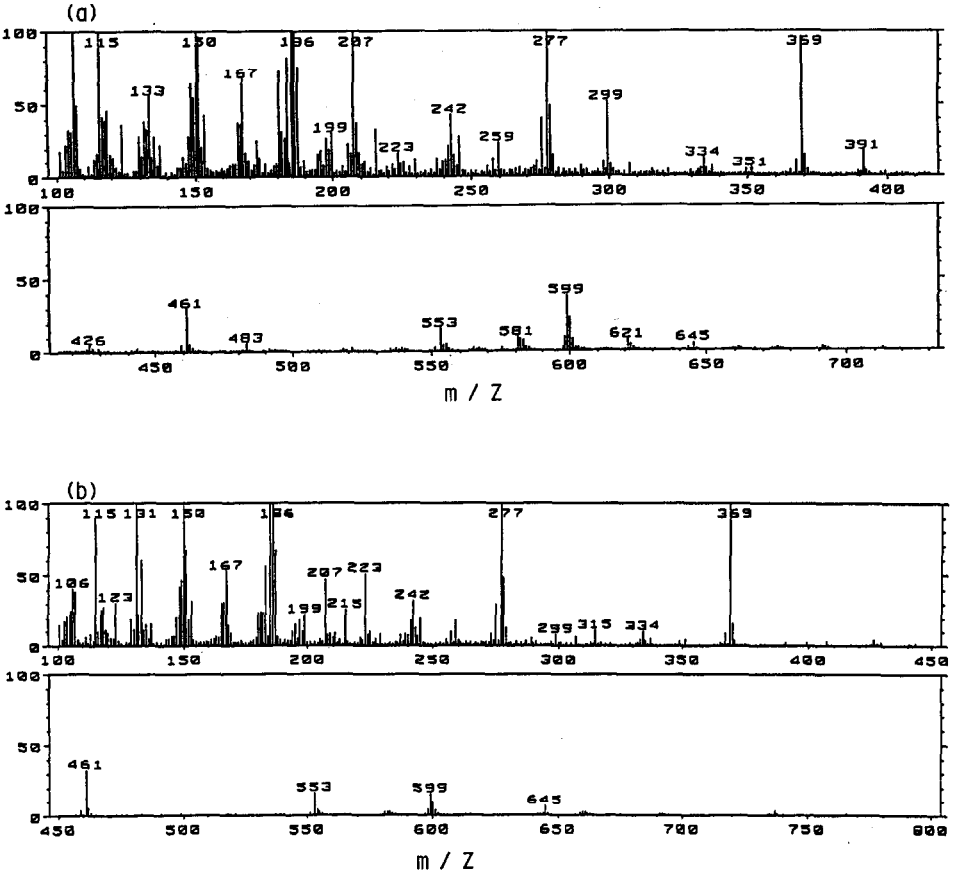

Fig. 4. FAB mass spectra of Hp purified only by extraction (a) and by extraction–chromatography (b). Matrix: glycerol. Ion source: xenon.

trend in mobility in the reversed-phase chromatography, it is estimated that the impurities contained in the commercial material are less polar than Hp.

HPLC

Fig. 3a and b show the HPLC chromatograms of the commercial Hp and the final material obtained by the present purification procedure, respectively. The former chromatogram was recorded for a 5-μl portion of the solution of commercial Hp at a concentration of about $1 \cdot 10^{-4}$ mol/l. At least three minor peaks due to unknown impurities are present. On the other hand, in the latter chromatogram, there is one peak despite the use of scale expansion in order to reveal any small peaks.

The HPTLC and HPLC studies thus confirm the purity of the final material obtained by the purification procedure involving extraction and preparative chromatography.

Identification of the purified material

Fig. 4a and b show the fast atom bombardment (FAB) mass spectra obtained for the purified materials from the extraction procedure and the additional chromatographic procedure, respectively, by using a JEOL JMS-HX100 mass spectrometer equipped with a DA-5000 data-processing system.

Both spectra contain signals around *m/z* 599 which are assigned to the molecular ion, $(Hp + H)^+$ (mol.wt. of Hp 598.70; calc. for $^{12}C_{34}\,^{1}H_{38}\,^{14}N_4\,^{16}O_6$, 598.28), and around *m/z* 581 which are assigned to the fragment ion, $(Hp + H)^+ - H_2O$. The signals found around *m/z* 391, 483 and 621 in the spectrum of the material obtained after extraction (Fig. 4a) have not yet been assigned. It has been confirmed, by comparison with a blank spectrum, that most signals other than those specified above are compatible with those found in the spectrum of glycerol used as the matrix for the Hp samples.

It can be concluded that the materials resulting from both the extraction and the extraction–chromatography are Hp of high purity. Some impurities were found in the extracted material, but these become negligible after the additional purification by reversed-phase chromatography.

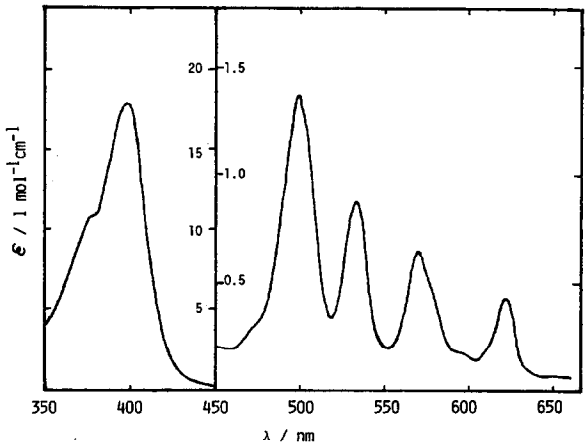

Fig. 5. UV–VIS absorption spectrum of pure Hp in methanol at 25°C.

UV–VIS absorption spectrum

Because of the difficulty in purification of Hp, few UV–VIS spectral data have so far been reported for this compound at high purity.

Fig. 5 shows the UV–VIS absorption spectrum of Hp in methanol at 25°C; the sample was obtained by the extraction–chromatography procedure. The absorption maxima are found at the following wavelengths (in nm) with the molar absorption coefficients (in l/mol · cm) given in parentheses: 396 (Soret, $1.79 \cdot 10^5$), 497 ($1.36 \cdot 10^4$), 531 ($8.47 \cdot 10^3$), 568 ($6.07 \cdot 10^3$), 620 ($3.76 \cdot 10^3$). Beer's law was valid, at each absorption maximum, up to concentrations of at least $5.18 \cdot 10^{-4}$ mol/l.

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REFERENCES

1 R. L. Lipson, E. J. Baldes and A. M. Olsen, *J. Natl. Cancer Inst.*, 26 (1961) 1.
2 D. Kessel, *Cancer Res.*, 41 (1981) 1318.
3 T. J. Dougherty, G. B. Grindey, R. Fiel, K. R. Weishaupt and D. G. Boyle, *J. Natl. Cancer Inst.*, 55 (1975) 115.
4 T. J. Dougherty, J. E. Kaufman, A. Goldfarb, K. R. Weishaupt, D. Boyl and A. Mittleman, *Cancer Res.*, 38 (1978) 2628.
5 T. W. Sery, *Cancer Res.*, 39 (1979) 96.
6 C. J. Gomer, D. R. Doiron, J. V. Jester, B. C. Szirth and A. L. Murphree, *Cancer Res.*, 43 (1983) 721.
7 R. K. Dinello and C. K. Chang, in D. Dolphin (Editor), *The Porphyrins*, Vol. 1, Academic Press, NY, 1978, p. 297.
8 E. Becker, R. Bradley and C. Watson, *J. Am. Chem. Soc.*, 83 (1961) 3743.
9 R. V. Belcher, S. G. Smith, R. Mahler and J. Campbell, *J. Chromatogr.*, 53 (1970) 279.
10 R. Bonnett, R. J. Ridge, P. A. Scourides and M. C. Berenbaum, *J. Chem. Soc., Perkin Trans. 1*, (1981) 3135.
11 R. Bonnett, A. A. Charalambides, K. Jones, I. A. Magnus and R. J. Ridge, *Biochem. J.*, 173 (1978) 693.
12 P. A. Cadby, E. Dimitriadis, H. G. Grant, A. D. Ward and I. J. Forbes, *J. Chromatogr.*, 231 (1982) 273.
13 M. Momenteau, C. Ropars and M. Rougee, *J. Chim. Phys. Phys. Chim. Biol.*, 65 (1968) 110.
14 R. J. Porra and T. G. Jones, *Biochem. J.*, 87 (1963) 186.
15 P. H. Hynninen and N. Ellfolk, *Acta Chem. Scand.*, 27 (1973) 1795.
16 C. Vever-Bizet, O. Delgado and D. Brault, *J. Chromatogr.*, 283 (1984) 157.
17 K. Siatoh and N. Suzuki, *Anal. Chem.*, 51 (1979) 1683.