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## REVERSED-PHASE HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY AND COLUMN LIQUID CHROMATOGRAPHY OF METAL COMPLEXES OF PHEOPHORBIDE *a*

KYOKO ADACHI\*, KOICHI SAITOH and NOBUO SUZUKI\*

*Department of Chemistry, Faculty of Science, Tohoku University, Sendai, Miyagi 980 (Japan)*

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### SUMMARY

The thin-layer chromatographic mobilities of pheophorbide *a* (Phor-*a*) and six metal-Phor-*a* complexes were measured on an octadecyl-bonded silica gel (C<sub>18</sub> silica) thin layer with various polar developing solvents, such as methanol, ethanol, acetonitrile and their mixtures with an aqueous solution containing an electrolyte such as sodium chloride. The mobility of the metal complex decreases in the following order of complexed metal ions: Co<sup>III</sup> > Fe<sup>III</sup> > Zn<sup>II</sup> > Phor-*a* > Ni<sup>II</sup> ≈ Pd<sup>II</sup> > Cu<sup>II</sup>, although the Fe<sup>III</sup> complex shows an exceptionally smaller mobility than the Zn<sup>II</sup> complex when pure acetonitrile or the water-acetonitrile containing sodium chloride is used as the developing solvent. The mobility of a metal-Phor-*a* complex is generally smaller than that of the pheophorbide *b* complex of the same metal. A successful reversed-phase high-performance liquid chromatographic separation was achieved for a mixture of Phor-*a* and its Fe<sup>III</sup>, Co<sup>III</sup>, Cu<sup>II</sup> and Zn<sup>II</sup> complexes by using ethanol-phosphate buffer (pH 3) (85:15, v/v) as the mobile phase.

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### INTRODUCTION

Pheophorbide is one of the significant degradation products of chlorophyll and is found in natural waters<sup>1</sup>, marine sediments<sup>2</sup> and brined green vegetables<sup>3</sup>. Pheophorbide contains in its molecular structure a chlorin macrocycle which has complexing ability with metal ions. The formation of pheophorbide complexes with Cu<sup>II</sup> or Zn<sup>II</sup> is utilized to enhance the green colour of stored and processed vegetables<sup>4,5</sup>.

The usefulness of chromatography for the separation and detection of the Cu<sup>II</sup> and Zn<sup>II</sup> complexes of pheophorbide has been reported<sup>6</sup>. However, the chromatographic characteristics of complexes with other metals have hardly been studied. In previous work, the retention behaviour of pheophorbides, including both *a* and *b* forms (Phor-*a* and Phor-*b*), was investigated in comparison with that of chlorophylls

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\* Present address: Department of Chemistry, Hamamatsu University School of Medicine, Hamamatsu, 431-31 Japan.

and pheophytins in reversed-phase high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC)<sup>7</sup>.

In this paper we describe the reversed-phase HPTLC and HPLC behaviour of the metal complexes of Phor-a, which is one of the predominant derivatives from chlorophyll-a, the most common type of chlorophyll. The complexes with Fe<sup>III</sup>, Co<sup>II</sup>, Co<sup>III</sup>, Ni<sup>II</sup>, Cu<sup>II</sup>, Zn<sup>II</sup> and Pd<sup>II</sup> were studied. In addition, the chromatographic mobility of each metal-Phor-a complex was compared with that of the corresponding metal complex of Phor-b.

This work was carried out as part of a series of studies on the chromatography of porphyrins, metalloporphyrins and related compounds. Porphine<sup>8</sup>, tetraphenylporphine<sup>9,10</sup>, tetratolylporphine<sup>11</sup>, etioporphyrin<sup>12</sup>, haematoporphyrin<sup>13</sup> and their metal complexes have been investigated so far.

## EXPERIMENTAL

### Materials

All procedures for the preparation of pheophorbides and their metal complexes (see Fig. 1) were carried out in the dark.

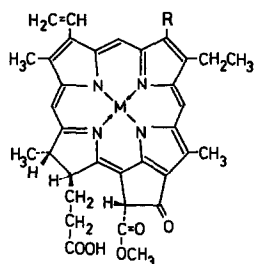


Fig. 1. Formula of a typical complex of Phor-a or Phor-b with a divalent metal M. R = CH<sub>3</sub> for Phor-a and CHO for Phor-b.

Phor-a was prepared from spinach leaves as described previously<sup>7</sup>. The metal complexes of Phor-a were prepared by the procedure detailed below, which were similar to those used for the Phor-a complexes of Cu<sup>II</sup> and Zn<sup>II</sup> by Jones *et al.*<sup>14</sup>.

In the first step of the preparation, 10 mg of a metal compound (see below) and a few small crystals of ascorbic acid were added to an acetone solution (2 ml) containing about 1 mg of Phor-a. The mixture was warmed at 40°C in an atmosphere of nitrogen in order to prevent undesirable oxidation of the Phor-a structure in the reaction procedure. The chlorides of Fe<sup>II</sup> and Pd<sup>II</sup> and acetates of Co<sup>II</sup>, Ni<sup>II</sup>, Cu<sup>II</sup> and Zn<sup>II</sup> were used as the metal compounds. In preparing both Ni and Co complexes, acetic acid was used as the reaction solvent instead of acetone. The reaction time was 2 h for each reaction, except for the Ni complex, which required 10 h. In each reaction process, a small portion of the reaction mixture was regularly sampled in order to check the Phor-a consumption and the formation of the desired complex by monitoring the changes in the visible absorption spectra and the disappearance of the fluorescence of Phor-a under UV radiation, and also by HPTLC.

In the second step of the preparation, the crude metal-Phor-a complex was extracted with 6 ml of diethyl ether from the reaction mixture that had preliminarily been cooled and then diluted with 10 ml of water. The ether phase, after separation from aqueous phase, was washed with 3 ml of 6 *M* hydrochloric acid to remove unreacted Phor-a (this procedure was omitted with of Zn<sup>II</sup> complex, because undesirable demetallation occurred). The ether phase was then washed repeatedly with 2-ml portions of water until the pH of the aqueous phase became higher than about 5. After removal of the ether by evaporation, the solid product was dried *in vacuo* and then stored in a phosphorus pentoxide desiccator in an atmosphere of nitrogen at -20°C.

In the washing step with 6 *M* hydrochloric acid, the metal complexes other than the Co complex did not transfer from their ether solutions to the acid, whereas part of Co complex was back-extracted into the acid. The fraction of Co complex that had been transferred from the ether solution to the 6 *M* hydrochloric acid was so stable that it was no longer re-extracted with a new portion of diethyl ether. This fraction of the Co complex is hereafter denoted Co(Phor-a)<sub>acid</sub> in order to distinguish it from the fraction that is non-extractable into 6 *M* hydrochloric acid; the latter fraction is denoted Co(Phor-a)<sub>ether</sub>. The Co(Phor-a)<sub>acid</sub> complex was almost completely transferred into diethyl ether after 10-fold dilution of the acidic solution with water. It was solidified, dried and stored as for Co(Phor-a)<sub>ether</sub>.

The final product of each metal-Phor-a complex was checked by fast atom bombardment mass spectrometry. A significant signal appeared at the *m/z* value corresponding to the molecular ion (M + 1)<sup>+</sup> of the desired metal complex. The mass spectrometric results for Co(Phor-a)<sub>ether</sub> are considered under Results and Discussion.

Phor-b and its metal complexes were prepared by using similar procedures to those used for Phor-a and its metal complexes. The purity of each product was checked by HPTLC and HPLC. Contamination with a small amount of unreacted Phor-b was unavoidable in the procedures used, because both Phor-b and its metal complexes were so soluble in 6 *M* hydrochloric acid that the contaminating Phor-b could not be removed selectively from the metal complex product by washing with the acidic solution.

### HPTLC

RP-18 F<sub>254s</sub> HPTLC plates (10 × 10 cm) coated with C<sub>18</sub>-silica were obtained from Merck (Darmstadt, F.R.G.). A frontal portion of the thin-layer coating was scraped off the plate so that the development would automatically stop when the solvent front had run 75 mm from the sample origin.

Sample solutions of Phor-a and its metal complexes in diethyl ether were prepared in the concentration range 0.1–1 *mM*. A 0.5- $\mu$ l portion of the sample solution was spotted at the origin and development was carried out horizontally in a Model 28510 chamber (Camag, Muttenz, Switzerland) in a thermostated room at 25°C until the migration of the solvent front had automatically stopped after 75 mm.

The compounds in the chromatogram were detected spectrophotometrically with a Shimadzu (Kyoto, Japan) CS-920 densitometer at a detection wavelength 410 nm for Phor-a and its metal complexes, except for the Fe<sup>III</sup> complex (390 nm), and at 430 nm for Phor-b and its metal complexes.

### HPLC

A Twinkle solvent-delivery pump and a VL-611 sample injection valve (Jasco, Tokyo, Japan), a TSK ODS-80TM column (particle diameter 5  $\mu\text{m}$ , packed in a 150 mm  $\times$  4.6 mm I.D. stainless-steel tube) (Toyo Soda, Tokyo, Japan) and a rapid-scanning UV-visible multi-wavelength spectrophotometric detector of our original design<sup>15</sup> were assembled into a liquid chromatograph system. A mixture of alcohol (methanol or ethanol) and an aqueous solution (pH buffered solution or unbuffered aqueous sodium chloride solution) was used as the mobile phase at a flow-rate of 0.78 ml/min.

Sample solutions of Phor-a and its metal complexes were prepared at a 0.1 mM concentration in acetone and a 2- $\mu\text{l}$  aliquot of each solution was injected into the column. Spectrophotometric detection was carried out at 410 nm, except for Fe (Phor-a), which was detected at 390 nm. In-flow recording of UV-visible absorption spectra of the column effluent was continued through every HPLC experiment by using the rapid-scanning UV-visible spectrophotometric detector in order to identify the substance eluted from the column.

### Spectroscopy

Visible absorption spectra were recorded with a Hitachi (Tokyo, Japan) U-3200 spectrophotometer. The bandpass was set at 1 nm.

Mass spectra were obtained by using a JEOL JMS-HX100 mass spectrometer equipped with a fast atom bombardment ionization system (JEOL, Tokyo, Japan). The sample, which had been preliminarily dissolved in dimethyl sulphoxide, was added to a glycerol-triglycerol (1:1) mixture used as a matrix. They were coated on the ionization target, followed by bombardment with a xenon atom beam (at 6 kV and 20 mA) under  $5.0 \cdot 10^{-6}$  Torr pressure.

Electron spin resonance (ESR) spectra were recorded at 77 K with a JEOL JES-FE2XG ESR spectrometer.

## RESULTS AND DISCUSSION

### Spectral characteristics

Phor-a has two major absorption bands in the visible region, which shifted on metal complex formation. The wavelengths of maximum absorption and relative absorptivities measured for Phor-a and its metal complexes are summarized in Table I. The metal complexes other than those of Ni<sup>II</sup> and Pd<sup>II</sup> have two absorption maxima in common with Phor-a, one in the blue region and the other in the red. The complexes of Ni<sup>II</sup> and Pd<sup>II</sup> have two maxima with comparable absorptivities in the blue region. The absorption bands of the Fe<sup>III</sup> and Co<sup>III</sup> complexes were broad in comparison with the others. According to the relative absorptivities shown in Table I, the absorption bands in the blue, rather than those in the red region, are suitable for the sensitive spectrophotometric detection of Phor-a and its metal complexes by HPTLC and HPLC, although exceptions are found for the complexes of Ni<sup>II</sup> and Pd<sup>II</sup>.

Table I indicates that the absorption spectra observed for Co(Phor-a)<sub>ether</sub> and Co(Phor-a)<sub>acid</sub> are different from each other, implying that these types of Co complex have different chemical forms. In order to clarify the oxidation state of the cobalt in these complexed forms with Phor-a, their ESR characteristics were examined. It is

TABLE I

VISIBLE ABSORPTION SPECTRAL CHARACTERISTICS FOR PHEOPHORBIDE *a* AND ITS METAL COMPLEXES IN DIETHYL ETHER AT 25 °C

Substance	Wavelength of maximum absorption, blue/red (nm)	Relative absorptivity (blue/red)
Phor-a	409/667	2.00
Fe(Phor-a)Cl	387/623	2.27
Co(Phor-a) <sub>ether</sub> *	411/652	1.63
Co(Phor-a) <sub>acid</sub> *	430/662	1.41
Ni(Phor-a)	393, 417/648	0.95**
Cu(Phor-a)	422/650	1.37
Zn(Phor-a)	426/655	1.37
Pd(Phor-a)	390, 418/638	0.67**

\* A fraction of the product.

\*\* The absorbance at the longer wavelength of the two peaks in blue region was taken for the calculation.

predicted, in principle, that an ESR signal should be observed for the complex of Co<sup>II</sup> (d<sup>7</sup>), but not for Co<sup>III</sup> (d<sup>6</sup>) species. The ESR parameters determined for Co(Phor-a)<sub>ether</sub> and Co(Phor-a)<sub>acid</sub> are compared with those for the Co complexes of protoporphyrin IX dimethyl ester (P<sub>p</sub>DME)<sup>16</sup> in Table II. Although the samples to be applied in ESR were prepared at nearly the same concentrations, a strong ESR signal was recorded for the Co(Phor-a)<sub>ether</sub> sample, whereas only a faint response was obtained for the Co(Phor-a)<sub>acid</sub> sample. The intensity of the ESR signal (peak-to-peak height) for the latter sample was smaller than 1/15th of that for the former. It was found that the ESR parameters determined for the Co(Phor-a)<sub>ether</sub> sample were close to those for the oxygen adduct of the Co<sup>II</sup>-P<sub>p</sub>DME complex rather than the simple Co<sup>II</sup>-P<sub>p</sub>DME complex, as shown in Table II. Accordingly, it is considered that Co(Phor-a)<sub>ether</sub> contains Co(Phor-a)O<sub>2</sub>. In the mass spectrum recorded for the Co(Phor-a)<sub>ether</sub> sample, significant peaks appeared at *m/z* ca. 682 and 650, which

TABLE II

ESR PARAMETERS FOR THE COBALT COMPLEXES OF PHEOPHORBIDE *a* AND PROTOPORPHYRIN IX DIMETHYL ESTER (P<sub>p</sub>DME) AT 77 K

No.	Sample or known complex	Solvent	$g_{\perp}$	$g_{\parallel}$	$ A_{\perp}  \cdot 10^4 \text{ cm}^{-1}$	$ A_{\parallel}  \cdot 10^4 \text{ cm}^{-1}$	Ref.
1	Co(Phor-a) <sub>ether</sub>	DEE*	1.992	2.076	14.9	20.9	This work
2	Co(Phor-a) <sub>acid</sub> **	DEE	1.992	2.084	14.2	19.3	This work
3	Co(P <sub>p</sub> DME)-MTE*	Toluene	2.360	2.016	37.7	90.1	16
4	Co(P <sub>p</sub> DME)-MTE-O <sub>2</sub>	Toluene	2.002	2.072	21	12	16
5	Co(P <sub>p</sub> DME)-ME*	Toluene	2.384	2.018	54.4	93.2	16
6	Co(P <sub>p</sub> DME)-ME-O <sub>2</sub>	Toluene	2.003	2.059	19.2	14	16

\* DEE = diethyl ether; MTE = 2-(methylthio)ethanol; ME = mercaptoethanol.

\*\* Very weak response.

TABLE III  
 $R_F$  VALUES OF PHEOPHORBIDE  $a$  AND ITS METAL COMPLEXES ON A  $C_{18}$ -BONDED SILICA GEL THIN LAYER AT 25°C  
 RP-18 F<sub>2548</sub> HPTLC plate (Merck No. 13724).

Developing solvent	$R_F \cdot 100$						
	Phor- $a$	$Fe^{III}$	$Co^{III}$	$Ni^{II}$	$Cu^{II}$	$Zn^{II}$	$Pd^{II}$
Methanol	42	47-62*	81-92*	31	30	73	32
Methanol-water (95:5)**	19	28-40*	87	14	13	47	13
Methanol-water (95:5) containing 0.01 M NaCl	19	75	91	14	13	50	14
Ethanol	80	100	100	73	72	92	73
Ethanol-water (87.5:12.5)	42	46-54*	62-71*	36	31	68	35
Ethanol-water (87.5:12.5) containing 0.01 M NaCl	42	85	92	35	30	67	34
Acetonitrile	32	65	1-9*	25	23	72	24
Acetonitrile-water (95:5)	31	61	86-97*	24	24	73	25
Acetonitrile-water (95:5) containing 0.01 M NaCl	31	61	100	27	24	76	25

\* Broadened spot.

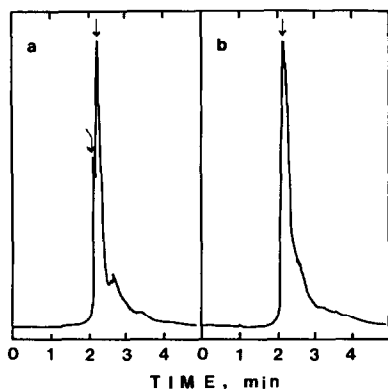


Fig. 2. HPLC traces of (a)  $\text{Co(Phor-a)}_{\text{ether}}$  and (b)  $\text{Co(Phor-a)}_{\text{acid}}$ . Mobile phase: methanol-(0.02 M  $\text{NaH}_2\text{PO}_4$ -0.02 M  $\text{H}_3\text{PO}_4$ , pH 3) (95:5, v/v); flow-rate, 0.78 ml/min. Detection at 415 nm.

could be assigned to the molecular ions of  $\text{Co(Phor-a)O}_2 [(M + 1)^+]$  and its deoxygenated form,  $\text{Co(Phor-a)} [(M - 32) + 1]^+$ , respectively. It was confirmed by HPLC (see Fig. 2) that  $\text{Co(Phor-a)}_{\text{acid}}$  apparently contained one particular form of the complex (eluted at retention time,  $t_R$ , 2.18 min) and that  $\text{Co(Phor-a)}_{\text{ether}}$  contained at least two components ( $t_R$  2.17 and 2.28 min, respectively).

From the above results, it was concluded that the material obtained in the  $\text{Co(Phor-a)}_{\text{acid}}$  fraction was a complex of  $\text{Co}^{\text{III}}$ , such as  $\text{Co(Phor-a)Cl}$ , with considerable purity, and that obtained in the  $\text{Co(Phor-a)}_{\text{ether}}$  fraction was a mixture of the  $\text{Co}^{\text{III}}$  complex,  $\text{Co}^{\text{II}}$  complex- $\text{O}_2$  adduct and others. It was considered that the complex first synthesized from  $\text{Co}^{\text{II}}$  under reductive conditions had been converted into its oxygen adduct or a  $\text{Co}^{\text{III}}$  in the subsequent washing, extraction and other steps.

To the following chromatographic studies were applied the  $\text{Co}^{\text{III}}$  complex obtained as  $\text{Co(Phor-a)}_{\text{acid}}$ .

#### HPTLC of Phor-a and its metal complexes

The HPTLC mobility was measured for Phor-a and its metal complexes with different developing solvents, including pure methanol, ethanol and acetonitrile and their mixtures with aqueous solutions. Three examples of chromatograms are illustrated in Fig. 3 and the  $R_F$  values determined are summarized in Table III.

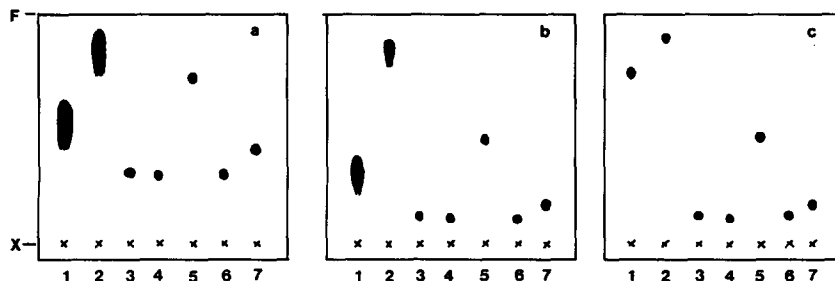


Fig. 3. HPTLC traces of pheophorbide and its metal complexes on RP-18  $\text{F}_{254s}$  plates. Developing solvents: (a) methanol; (b) methanol-water (95:5, v/v); (c) 0.01 M sodium chloride in methanol-water (95:5, v/v). Compounds: Pheo-a complexes of (1)  $\text{Fe}^{\text{III}}$ , (2)  $\text{Co}^{\text{III}}$ , (3)  $\text{Ni}^{\text{II}}$ , (4)  $\text{Cu}^{\text{II}}$ , (5)  $\text{Zn}^{\text{II}}$ , (6)  $\text{Pd}^{\text{II}}$ ; (7) Phor-a.  $\times$ , Sample origin; F = solvent front.

When a pure organic solvent or its mixture with water was used as the developing solvent, the complexes of trivalent metals, such as  $\text{Fe}^{\text{III}}$  and  $\text{Co}^{\text{III}}$ , gave diffuse spots on the chromatogram (see in Fig. 3a and b). When an electrolyte such as sodium chloride was added to the developing solvent, the chromatograms of both the  $\text{Fe}^{\text{III}}$  and  $\text{Co}^{\text{III}}$  complexes were improved to sharp spots with enhanced mobilities ( $R_F$  values), as shown in Fig. 3c. In contrast, the complexes of divalent metals, such as  $\text{Ni}^{\text{II}}$ ,  $\text{Cu}^{\text{II}}$ ,  $\text{Zn}^{\text{II}}$  and  $\text{Pd}^{\text{II}}$ , always gave sharp spots on the chromatograms obtained with different solvents, and the mobilities of the complexes were almost independent of the presence of an electrolyte in the developing solvent. These results imply that dissociation or exchange of the anion ( $\text{Cl}^-$ ) bonded with the central metal ion ( $\text{Fe}^{\text{III}}$  or  $\text{Co}^{\text{III}}$ ) of the complex occurred during the migration process on the chromatogram developed with the pure water-containing organic solvent, and that the metal complex was stabilized when sodium chloride was added to the developing solvent.

The variation of the mobility of each metal-(Phor-a) complex with the concentration of the organic component in the developing solvent was examined by using ethanol-water and acetonitrile-water mixtures. The results are shown in Figs. 4 and 5, respectively. With both solvent systems, the  $R_M$  values [ $R_M = \log(1/R_F - 1)$ ] of Phor-a and its divalent metal complexes decrease linearly with increase in the volume percentage ( $\phi$ ) of the organic component in the developing solvent, and the  $R_M$  vs.  $\phi$  plots for these complexes are parallel to each other. The  $R_M$  values of  $\text{Fe}^{\text{III}}$  and  $\text{Co}^{\text{III}}$  complexes were so small in the methanol-water that a reliable relationship between  $R_M$  and the ethanol content of the developing solvent could not be confirmed. In the acetonitrile-water binary solvent  $\text{Co}^{\text{III}}$  was not retarded ( $R_M$  values smaller than  $-1.5$ ), but the  $\text{Fe}^{\text{III}}$  complex exhibited moderate mobility, thus being smaller than Phor-a and larger than the  $\text{Zn}^{\text{II}}$  complex. It is considered that acetonitrile does not solvate the  $\text{Fe}^{\text{III}}$ -(Phor-a) complex as strongly as methanol.

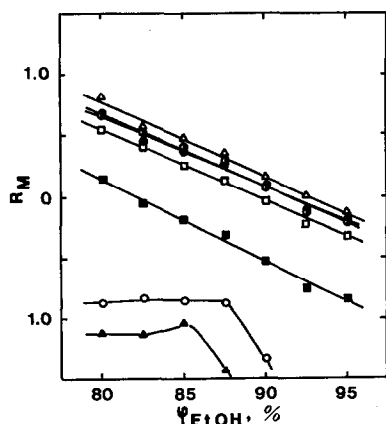


Fig. 4.  $R_M$  values of Phor-a and its metal complexes as a function of the ethanol content ( $\phi_{\text{EtOH}}$ ) of the ethanol-water containing 0.01  $M$  sodium chloride used as the developing solvent. Compounds: ( $\square$ ) Phor-a and its ( $\circ$ )  $\text{Fe}^{\text{III}}$ , ( $\blacktriangle$ )  $\text{Co}^{\text{III}}$ , ( $\bullet$ )  $\text{Ni}^{\text{II}}$ , ( $\triangle$ )  $\text{Cu}^{\text{II}}$ , ( $\blacksquare$ )  $\text{Zn}^{\text{II}}$  and ( $\circ$ )  $\text{Pd}^{\text{II}}$  complexes.

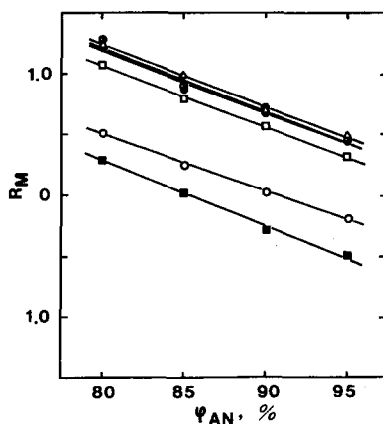


Fig. 5.  $R_M$  values of Phor-a and its metal complexes as a function of the acetonitrile content ( $\phi_{\text{AN}}$ ) of the acetonitrile-water containing 0.01  $M$  sodium chloride used as the developing solvent. Symbols as in Fig. 3. The plots for the  $\text{Co}^{\text{III}}$  complex are omitted because of the extremely small  $R_M$  values.



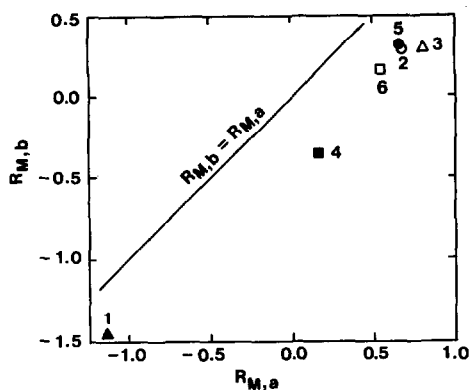


Fig. 6. Correlation between the  $R_M$  values of metal complexes of Phor-a ( $R_{M,a}$ ) and -b ( $R_{M,b}$ ). Developing solvent: 0.01 *M* sodium chloride in ethanol–water (80:20, v/v). Compounds: 1 =  $\text{Co}^{\text{III}}$ ; 2 =  $\text{Ni}^{\text{II}}$ ; 3 =  $\text{Cu}^{\text{II}}$ ; 4 =  $\text{Zn}^{\text{II}}$ ; 5 =  $\text{Pd}^{\text{II}}$ ; 6 = metal-free Phor.

The mobility order for the divalent metal complexes is the same for both the methanol–water and acetonitrile–water binary developing solvents:  $\text{Zn(Phor-a)} > \text{Phor-a} > \text{Ni(Phor-a)} \approx \text{Pd(Phor-a)} > \text{Cu(Phor-a)}$ . This order is consistent with those for metal complexes of porphyrins, such as porphine<sup>8</sup> and its *meso*-tetraphenyl<sup>9,10</sup> and *meso*-tetratolyl<sup>11</sup> derivatives and haematoporphyrin<sup>13</sup>, in reversed-phase HPTLC on an octadecyl-bonded silica gel plate.

#### *Comparison with the HPTLC behaviour of Phor-b and its metal complexes*

In order to examine the difference in the chromatographic behaviour between the metal complexes of Phor-a and Phor-b, the  $R_M$  values of these complexes are compared in Fig. 6. The  $R_M$  value of a metal complex of Phor-b is about 0.4  $R_M$  unit smaller than that of the corresponding Phor-a complex. The replacement of the non-polar  $\text{CH}_3$  group bonded to the chlorin macrocycle (in a Phor-a molecule) with a polar CHO group (in a Phor-b molecule) results in a decrease in the retention, (that is, a decrease in  $R_M$ ) in the reversed-phase liquid chromatographic mode.

#### *HPLC behaviour of metal complexes of Phor-a*

It was pointed out in the HPTLC study that the addition of a salt to the developing solvent was effective in achieving non-diffuse spots on the chromatograms, particularly for the complexes of trivalent metals. In this HPLC study, three mobile phases containing different salts were used for comparison, consisting of 85:15 (v/v) mixtures of ethanol and aqueous solutions of (a) 0.067 *M* sodium chloride, (b) 0.02 *M* sodium acetate adjusted to pH 3 with hydrochloric acid and (c) 0.02 *M* sodium dihydrogenphosphate adjusted to pH 3 with phosphoric acid.

In order to examine the effects of a metal (stainless steel) coming into contact with the liquid at the column wall and tubing, the UV–visible absorption spectrum was recorded for each metal complex in the column effluent by using a rapid-scanning spectrophotometric detector, and it was compared with that recorded for the same metal complexes in comparable solutions prepared in glassware. These spectra were comparable to each other in every instance. It was concluded that the metal parts of

the column and tubing had little effect on the metal complexes chromatographed in the instrument used.

It was found that both the peak shape and the retention time of the  $\text{Fe}^{\text{III}}$ -Phor-a complex depended considerably on the salt added to the mobile phase, whereas such a salt effect was not significant for the Phor-a complexes with divalent metals. The elution curves recorded for Phor-a and its  $\text{Fe}^{\text{III}}$ ,  $\text{Co}^{\text{III}}$  and  $\text{Zn}^{\text{II}}$  complexes with these mobile phases are shown in Fig. 7. The  $\text{Fe}^{\text{III}}$  complex gave two poorly resolved peaks with the mobile phase containing sodium chloride (Fig. 7a), whereas it gave sharp peaks with the other mobile phases (Fig. 7b and c).

Taking account that the  $\text{Fe}^{\text{III}}$  complex injected into the column was prepared in the form  $\text{Fe}(\text{Phor-a})\text{Cl}$ , it is considered that the anion ( $\text{Cl}^-$ ) bonded to the iron was exchanged during the chromatographic process with a different anion present in the mobile phase used. The chemical species assigned to the poorly resolved peaks obtained for the  $\text{Fe}^{\text{III}}$  complex with the mobile phase containing sodium chloride (Fig. 7a) had not yet been clarified.

According to the chromatograms shown in Fig. 7, the mobile phase containing sodium dihydrogenphosphate-phosphoric acid at pH 3 is effective in separating the  $\text{Fe}^{\text{III}}$  complex from the  $\text{Co}^{\text{III}}$  complex. By using this mobile phase, the separation of several metal complexes was tested. Phor-a and its  $\text{Fe}^{\text{III}}$ ,  $\text{Co}^{\text{III}}$ ,  $\text{Cu}^{\text{II}}$  and  $\text{Zn}^{\text{II}}$  complexes

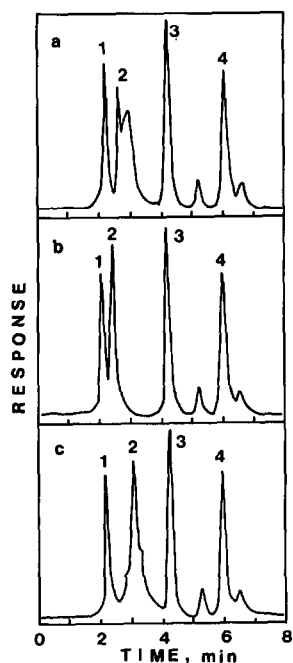


Fig. 7. HPLC elution curves for Phor-a and its  $\text{Fe}^{\text{III}}$ ,  $\text{Co}^{\text{III}}$  and  $\text{Zn}^{\text{II}}$  complexes recorded by using 85:15 (v/v) mixtures of ethanol and aqueous solutions containing different salts. Aqueous solutions: (a) 0.067 *M* sodium chloride; (b) 0.02 *M* sodium acetate adjusted to pH 3 with hydrochloric acid; (c) 0.02 *M* sodium dihydrogenphosphate-0.02 *M* phosphoric acid (pH 3). Flow-rate, 0.78 ml/min. Detection at 390 nm for the  $\text{Fe}^{\text{III}}$  complex and 410 nm for the others. Peaks: 1 =  $\text{Co}^{\text{III}}$ ; 2 =  $\text{Fe}^{\text{III}}$ ; 3 =  $\text{Zn}^{\text{II}}$ ; 4 = Phor-a.

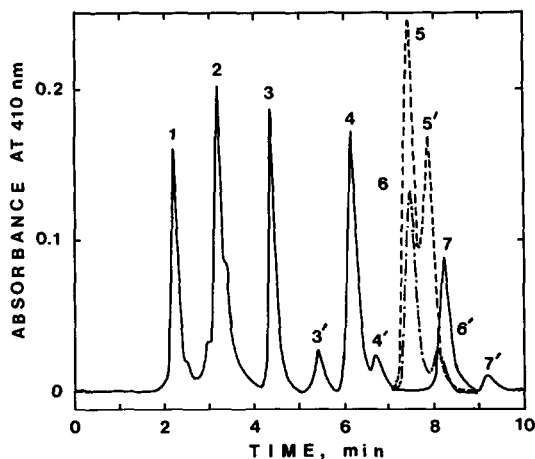


Fig. 8. HPLC separation of Phor-a and its metal complexes. Mobile phase: ethanol-(0.02 M sodium dihydrogen phosphate-0.02 M phosphoric acid, pH 3) (85:15, v/v); flow-rate, 0.78 ml/min. Peaks: 1 =  $\text{Co}^{\text{III}}$ ; 2 =  $\text{Fe}^{\text{III}}$ ; 3 =  $\text{Zn}^{\text{II}}$ ; 4 = Phor-a; 5 =  $\text{Ni}^{\text{II}}$ ; 6 =  $\text{Pd}^{\text{II}}$ ; 7 =  $\text{Cu}^{\text{II}}$ .

were successfully separated from each other, as shown in Fig. 8. The complexes of  $\text{Ni}^{\text{II}}$  and  $\text{Pd}^{\text{II}}$  were hardly resolved, as shown by the superimposed broken curves in Fig. 8. A small peak (or a shoulder in some instances) appeared behind every major peak in the chromatogram. From the real-time monitoring of absorption spectra with the aid of a rapid scanning spectrophotometric detector, the column effluents assigned to every minor peak and its preceding major peak gave the same absorption spectra in the UV-visible region (350–700 nm). The small peak is assigned to the isomer or allomer of the substance that gives the preceding major peak on the chromatogram.

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