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High-performance liquid chromatography with spectrophotometric and electrochemical detection of a series of manganese(III) cationic porphyrins

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Abstract

Recent studies have revealed potent pharmacological activities of manganese-containing cationic porphyrins. An analytical method employing high-performance liquid chromatography with spectrophotometric and electrochemical detection (HPLC–UV/EC) suitable for in vivo applications is described for a series of manganese(III) cationic porphyrins with good separation and resolution. In particular, this method resolved the four atropisomers of manganese(III) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP^{5+} or AEOL-10113), verified by mass spectrometry. Electrochemical and spectrophotometric methods of detection were compared using manganese(III) *meso*-tetrakis(1,3-diethylimidazolium-2-yl)porphyrin (MnTDE-2-ImP^{5+} or AEOL-10150), the lead catalytic antioxidant of this series. Both methods of detection were quantitative, but electrochemical detection, although less specific for in vivo applications, appears to be considerably more sensitive than spectrophotometric detection.

Keywords: Manganese; Porphyrins

1. Introduction

Synthetic water-soluble cationic porphyrins and metalloporphyrins are currently under investigation as therapeutic agents, including photosensitizers [1], DNA cleavers [2] and superoxide dismutase (SOD) mimetics [3]. The SOD activity of manganese(III) porphyrins is governed by their metal-centered redox potential [3–6], and a number of structural variations, including *meso* and *beta* substitution (Fig. 1), have been explored in order to control their redox potential [4,5]. Other chemical modifications such as longer *N*-alkyl chains on the *meso*-pyridinium (*ortho*) substituents decreased the interaction of the porphyrins with DNA and therefore their in vivo toxicity [4,5]. Several in vitro and in vivo studies using manganese(III) porphyrins as catalytic antioxidants were reported [7–9], yet a simple and sensitive method of analyses was lacking.

HPLC has been extensively applied to natural

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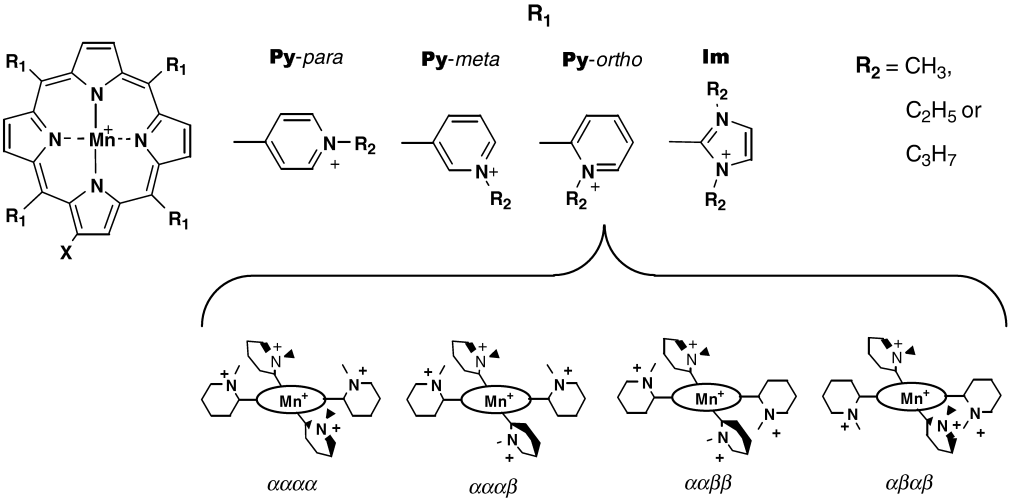

Fig. 1. Structures of the manganese(III) cationic porphyrins analyzed (prepared with chlorines as counter-ions).

anionic porphyrins as well as synthetic anionic and uncharged porphyrins [10,11]. Free ligand porphyrins have also been considered as tools for the detection of metals [12]. However, relatively few HPLC methods have been reported for cationic porphyrins [10,13–15]. HPLC with electrochemical detection (HPLC–EC) is a sensitive method for the analyses of certain redox active compounds, including antioxidants [16] and metal-complexes [17–19]. Electrochemical detection of porphyrin related compounds was reported only for a series of free ligand uroporphyrinogens [20]. Since the metal center of manganese(III) porphyrins is redox active, electrochemical detection seemed a viable alternative method to spectrophotometric (Soret band) detection. A HPLC method for a series of manganese(III) cationic porphyrins is described and electrochemical detection compared to spectrophotometric detection.

2. Experimental

2.1. Chemicals

Manganese(III) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP^{5+}) and its mono-β-chlorinated analog $MnCl_1$TE-2-PyP^{5+} were prepared as described previously [4]. MnTM-4-PyP^{5+}, MnTM-3-PyP^{5+} and MnTM-2-PyP^{5+} were purchased from Mid Century Chemicals (Posen, IL, USA). Manganese(III) *meso*-tetrakis(1,3-dimethylimidazolium-2-yl)porphyrin (MnTDM-2-ImP^{5+}) and its *N*-ethyl and *N*-*n*-propyl analogs (MnTDE-2-ImP^{5+} and MnTDP-2-ImP^{5+}) were a kind gift from Incara Pharmaceuticals (Research Triangle Park, NC, USA). All porphyrins were provided as chloride salts. Triethylamine (TEA), trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA) and acetonitrile (HPLC grade) were from Sigma–Aldrich. Heparin and pentobarbital were from Elkins-Sinn, Inc. (Cherry Hill, NJ, USA) and Abbott Laboratories (North Chicago, IL, USA), respectively.

2.2. HPLC conditions

The major problem for HPLC separation of cationic porphyrins is the strong interaction of the quaternary amine residues with silica. This interaction remains in reversed-phase silica-based columns, probably due to the presence of incompletely end-capped silanol groups. In order to decrease this interaction, strong acid conditions are required [15]. The column, i.e. YMC-Pack ODS-A™ (4.6×120 mm, 3 μm, 120 Å) (Waters, Milford, MA, USA), was recommended by the manufacturer on the basis of the quality of the full end-capping process and unpublished data supporting the potential stability of the column against hydrolysis.

A mixture of water containing 0.1% of HFBA and variable amounts of acetonitrile (depending on the

compound analyzed) as mobile phase allowed the recovery of the cationic porphyrins, but did not allow the separation of MnTE-2-PyP^{5+} atropisomers. This separation needed an increased ionic strength of the mobile phase that was obtained using an aqueous solution containing 20 m*M* of both TEA and TFA with a pH adjusted to 2.7 (A), and acetonitrile (B= 6%). The flow rate was 1 ml/min, the injection volume 5 μl and the time of run 15 min. The HPLC instrument (Esa, Inc., Chelmsford, MA, USA) was equipped with an autosampler (model 542), and a Coul array detector (model 5600A) in tandem with a spectrophotometric detector (model 520). As observed for other porphyrins and metalloporphyrins, as the temperature was increased from 25 to 45°C, the retention time of cationic metalloporphyrins analyzed became lower and the shape of the peaks higher and narrower [10].

2.3. Mass spectrometry study

Mass spectrometry experiments were performed in order to verify that the four HPLC peaks for MnTE-2-PyP^{5+} were due to isomers. Several mass spectrometry studies have now been reported for cationic porphyrins and metalloporphyrins, and electrospray (positive mode) appears to be the optimal ionization method for this family of metal-complexes. For positive mode MS analyses, elimination of TEA is necessary, so each MnTE-2-PyP^{5+} fraction was collected, lyophilized and re-purified using the same column equilibrated with 0.1% HFBA in a mixture of water/acetonitrile (7:3). The electrospray ionization MS and MS/MS experiments were carried out in a PE/Sciex API 365 mass spectrometer (MDS Sciex, Concord, ON, Canada) equipped with a Cole Parmer 74900 Series diffusion pump (Cole Parmer, Vernon Hills, IL, USA). Samples were injected by loop injection at 5 μl/min. MS/MS experiments were performed at two collision energies, 17 and 37 eV.

2.4. Extraction from rat plasma

Four rats were injected subcutaneously with MnTDE-2-ImP^{5+} (24 mg/kg body wt.) and 3 h later anesthetized with an intraperitoneal injection of pentobarbital (45 mg/kg). The blood was obtained from a cardiac puncture and placed in heparinized tubes to avoid coagulation. The blood was then centrifuged (1600 *g* for 15 min) and the plasma recovered. Proteins were precipitated by adding 100 μl of 0.1% TFA in water to 100 μl of plasma. The samples were vortexed and left to stand for 15 min at 20°C and then centrifuged at 15 000 *g* for 4 min at 4°C. The supernatant was then filtered through Ultrafree-MC 0.22-μm filter units (Millipore, Allen, TX, USA) and transferred to a microvial for HPLC analysis using an aqueous solution containing 20 m*M* of both TEA and TFA (pH 2.7) (A) and acetonitrile (B=12%) as mobile phase. Plasma of non-treated rats was used as a matrix to prepare standards for MnTDE-2-ImP^{5+}, and these samples were handled identically to the treated-rat samples. In order to check the recovery from rat plasma, different amounts of the compound were added to normal plasma. The plasma was left to stand for 15 min at 20°C, and the compound extracted and analyzed as described above. Recovery was greater than 90%.

3. Results and discussion

3.1. Structure and interaction with the stationary phase

The structures of the manganese(III) cationic porphyrins analyzed are shown in Fig. 1. The mobile phase of choice for the analyses of this series of porphyrins in a reversed-phase (C_{18}) silica HPLC column was an isocratic mixture containing 20 m*M* of both TEA and TFA (pH 2.7) with variable amounts of acetonitrile, at 45°C. For each structure, the amount of acetonitrile (% B) and the retention time (R_t) are reported in Table 1.

Varying the position of the nitrogen of the pyridinium ring from *para* to *ortho*, which implies the loss of planarity of the porphyrin, slightly increases the interaction with the stationary phase. As expected, a longer *N*-alkyl substituent further increases the hydrophobic interactions with the stationary phase, and the same rule applies to the presence of a halogen in *beta* position. The *meso*-imidazolium substituted porphyrin with *N*-methyls (MnTDM-2-ImP^{5+}) interact more with the stationary

Table 1
Manganese-porphyrins analyzed, optimal detection parameters (Sb, Soret band and OP, optimal potential vs. H/Pd electrode), amounts of acetonitrile (% B) and retention times (R_t), using a reversed-phase HPLC column (C_{18}) and an aqueous solution containing 20 m*M* of both TEA and TFA (pH 2.7) as mobile phase (A) at 45°C

Compound	Structure	Detection	B (%)	R_t (min)
MnTM-4-PyP^{5+}	R_1=Py-*para* R_2=CH_3	Sb=462 nm OP=−0.6 V	0	4.0
MnTM-3-PyP^{5+}	R_1=Py-*meta* R_2=CH_3	Sb=459 nm OP=−0.6 V	0	4.7
MnTM-2-PyP^{5+}	R_1=Py-*ortho* R_2=CH_3	Sb=454 nm OP=−0.4 V	0	8.0 8.4
MnTE-2-PyP^{5+}	R_1=Py-*ortho* R_2=C_2H_5 X$^-$=H	Sb=454 nm OP=−0.4 V	5	7.7 8.5 9.0 9.6
			6	6.0 6.4 6.7 7.2
$MnCl_1$TE-2-PyP^{5+}	R_1=Py-*ortho* R_2=C_2H_5 X$^-$=Cl	Sb=456 nm OP=−0.4 V	6	11.1 11.9 12.4 13.1 13.5 13.9
MnTDM-2-ImP^{5+}	R_1=Im R_2=CH_3	Sb=446 nm OP=−0.4 V	1 3	9.6 5.5
MnTDE-2-ImP^{5+}	R_1=Im R_2=C_2H_5	Sb=446 nm OP=−0.4 V	10 12	14.2 7.8
MnTDP-2-ImP^{5+}	R_1=Im R_2=C_3H_7	Sb=446 nm OP=−0.4 V	22 24	11.6 7.3

phase than the *N*-methyl *meso*-pyridinium substituted porphyrins (MnTM-4-PyP^{5+}, MnTM-3-PyP^{5+} and MnTM-2-PyP^{5+}). Eight longer *N*-alkyl groups (MnTDE-2-ImP^{5+} and MnTDP-2-ImP^{5+}) drastically increase this interaction.

3.2. The atropisomers of MnTE-2-PyP^{5+} and MnCl$_1$TE-2-PyP^{5+}

The loss of the free rotation of the pyridinium ring in the case of the *ortho meso*-substituent results in a mixture of four atropisomers (or rotational isomers), $\alpha\alpha\alpha\alpha$, $\alpha\alpha\alpha\beta$, $\alpha\alpha\beta\beta$ and $\alpha\beta\alpha\beta$ (Fig. 1). Atropoisomers of cationic porphyrins have been previously separated on silica gel thin-layer chromatography plates [21] and by capillary electrophoresis [22]. In our HPLC system, MnTM-2-PyP^{5+} exhibited two peaks, whereas MnTE-2-PyP^{5+} exhibited four peaks, thus showing that longer *N*-ethyl chains improve the separation of the four atropisomers (Fig. 2a). This separation was confirmed by electrospray mass spectrometry on each HPLC fraction of MnTE-2-PyP^{5+}. The four fractions MnTE-2-PyP^{5+} showed the same

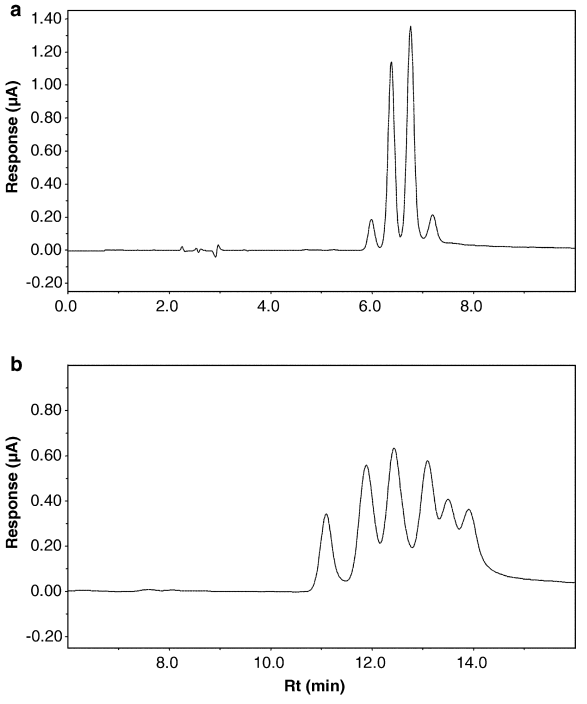

Fig. 2. HPLC chromatograms (EC detection, inverted signal) of MnTE-2-PyP^{5+} (a) and $MnCl_1$TE-2-PyP^{5+} (b) using a reversed-phase HPLC column (C_{18}) and an aqueous solution containing 20 m*M* of both TEA and TFA (pH 2.7) (A) and acetonitrile (B=6%) as mobile phase.

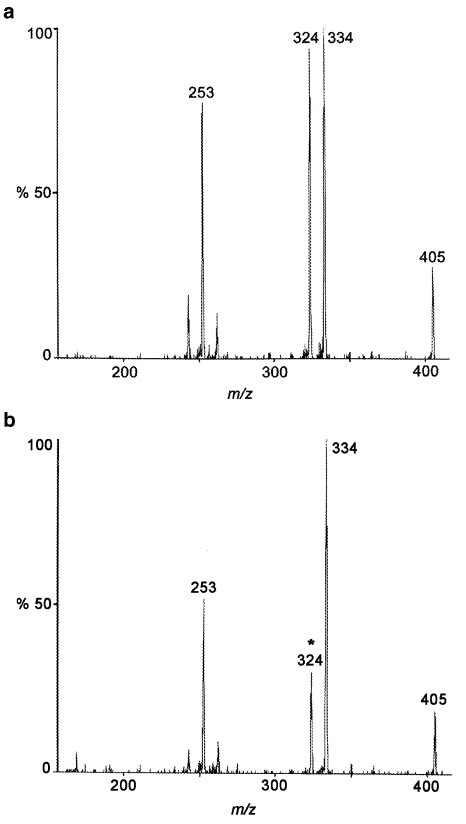

Fig. 3. MS/MS spectra (17 eV) of the HPLC peak 1 (presumably the $\alpha\alpha\alpha\alpha$ isomer) (a) and the HPLC peak 4 (presumably the $\alpha\beta\alpha\beta$ isomer) (b) of MnTE-2-PyP^{5+}. Peaks m/z=405, 334, 324 and 253 correspond to $[LMn^{III}+2HFBA^-]^{3+}/3$, $[LMn^{II}+HFBA^-]^{3+}/3$, $[LMn^{III}+HFBA^-\text{-Et}]^{3+}/3$ and $[LMn^{II}\text{-Et}]^{3+}/3$, respectively (L, free ligand porphyrin). The relative intensity of the m/z=324 peak is lower for HPLC peaks 3 and 4.

MS base peak (m/z=405, $[LMn^{III}+2HFBA^-]^{3+}/3$, with L the free ligand porphyrin) [2,23,24]. A MS/MS experiment at low collision energy (17 eV) on the base peak revealed a variation on relative intensity of *N*-ethyl fragmentation (m/z=324, $[LMn^{III}+HFBA^-\text{-Et}]^{3+}/3$) as 97, 100, 57 and 31% for HPLC peaks 1 to 4, respectively (Fig. 3). At higher collision energy (37 eV), no difference was observed.

The statistical ratio of the $\alpha\alpha\alpha\alpha$, $\alpha\alpha\alpha\beta$, $\alpha\alpha\beta\beta$ and $\alpha\beta\alpha\beta$ isomers is 12.5, 50, 25 and 12.5%, respectively, whereas, experimentally using both spectrophotometric and electrochemical detection, the ratios of the four HPLC peaks are approximately 6, 44, 44 and 6% (slightly varying from batch to batch) (Fig. 2a). The non-statistical ratio is likely due to kinetic factors during the *N*-alkylation, i.e. the distance between the charges [4,22]. Kaufmann et al. have characterized the $\alpha\alpha\alpha\alpha$ isomer as being the most polar [21], thus the most likely isomer corresponding to the first peak in our HPLC system.

The substitution with halogens in *beta* position further increases the possibilities of structural isomers. Mono-substitution, for example, theoretically increases the number of total stereo-isomers from four to 16 (two $\alpha\alpha\alpha\alpha$, eight $\alpha\alpha\alpha\beta$, four $\alpha\alpha\beta\beta$ and two $\alpha\beta\alpha\beta$), yet careful analysis of the structures suggests that certain isomers may have the same retention time. Six HPLC peaks were observed for $MnCl_1$TE-2-PyP^{5+} (Fig. 2b), probably one peak corresponding to the $\alpha\alpha\alpha\alpha$ isomers, two peaks to the

$\alpha\alpha\alpha\beta$ isomers, two peaks to the $\alpha\alpha\beta\beta$ isomers and one peak to the $\alpha\beta\alpha\beta$ isomers.

3.3. HDV plots of MnTM-3-PyP^{5+} and MnTDM-2-ImP^{5+}

The manganese-centers of all the metalloporphyrins analyzed are in the 3+ redox state, as demonstrated by the 20-nm hypsochromic shift of the Soret band upon the reduction of the metal center by ascorbic acid [5]. Although a current response is observed when applying a positive potential (up to 1 V vs. H/Pd electrode), the optimal signal is obtained by applying a negative potential, thus leading to the reduction of the metal.

Hydrodynamic voltammetry (HDV) plots show the intensity of the current response (peak height) as a function of the potential applied [16]. The HDV plots of MnTM-3-PyP^{5+} and MnTDM-2-ImP^{5+} are shown in Fig. 4. The optimal potential was chosen as the highest response current consistent with a ''good'' HPLC signal (minimum tailing), which is defined here as the potential corresponding to one-third of the maximum intensity of current response obtained, i.e. −0.6 and −0.4 V (vs. H/Pd electrode) for MnTM-3-PyP^{5+} and MnTDM-2-ImP^{5+}, respectively (Fig. 4). The HDV plots of MnTM-2-PyP^{5+} and MnTDM-2-ImP^{5+} were similar, and varying *N*-substituents had a negligible effect on either their redox potentials [4] and HDV plots (not shown).

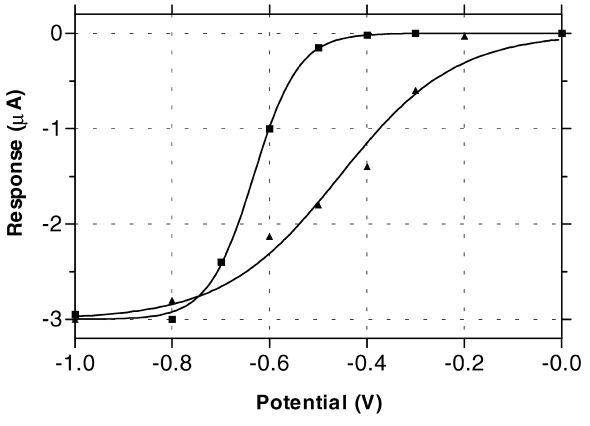

Fig. 4. Hydrodynamic voltammetry (HDV) plots (peak height vs. potential applied — vs. H/Pd electrode) of MnTM-3-PyP^{5+} (■) and MnTDM-2-ImP^{5+} (▲) (amount ~75 pmol). The mobile phase was an aqueous solution containing 20 m*M* of both TEA and TFA (pH 2.7) (A), and 0 and 2% of acetonitrile (B), respectively.

3.4. Electrochemical versus spectrophotometric detection

MnTDE-2-ImP^{5+} was chosen to compare electrochemical (EC) to spectrophotometric (UV) detection because of its optimal properties as a catalytic antioxidant [25]. The standard curves for both detection methods (peak height vs. amount injected) were linear up to 60 μ*M* (5-μl injection volume). Typically, the 95% confidence interval for the slope of the standard curve (five points) employing UV detection was (0.0021–0.0024), with a $r^2=0.9996$, whereas it was (0.0126–0.0136), with a $r^2=0.9991$, for EC detection. Slope intercepts were $Y=3.3\times10^{-5}$ μA and $X=0.003$ μ*M* for UV detection, and $Y=0.013$ μA and $X=0.279$ μ*M* for EC detection.

Using UV detection, the limit of detection (LOD) was 2 μ*M* (concentration with a signal/noise ratio equal to 3), and the lower limit of quantitation (LOQ) 10 μ*M* (lowest concentration with ±5% accuracy). In contrast, using EC detection and applying a potential of −0.4 V, LOD and LOQ were 0.05 and 0.1 μ*M*, respectively. EC detection is therefore at least 40-fold more sensitive than UV detection.

Nevertheless, the specificity of porphyrins absorption band (Soret band) allows ''cleaner'' chromatograms to be obtained from plasma extracts (Fig. 5). EC detection is less specific, showing a response from other compounds present in the plasma extract when the optimal potential is applied, but is necessary when detection of a manganese(III) cationic porphyrin under 1 μ*M* is required.

4. Conclusion

A new HPLC method that allows the separation of a series of manganese(III) cationic porphyrins, including the atropisomers of MnTE-2-PyP^{5+}, is described. The Soret band of porphyrins offers a specific and sensitive detection method. Nevertheless, the 3+ state of the manganese-center and its subsequent reduction when applying a negative potential also offers an effective electrochemical method of detection. As shown using MnTDE-2-

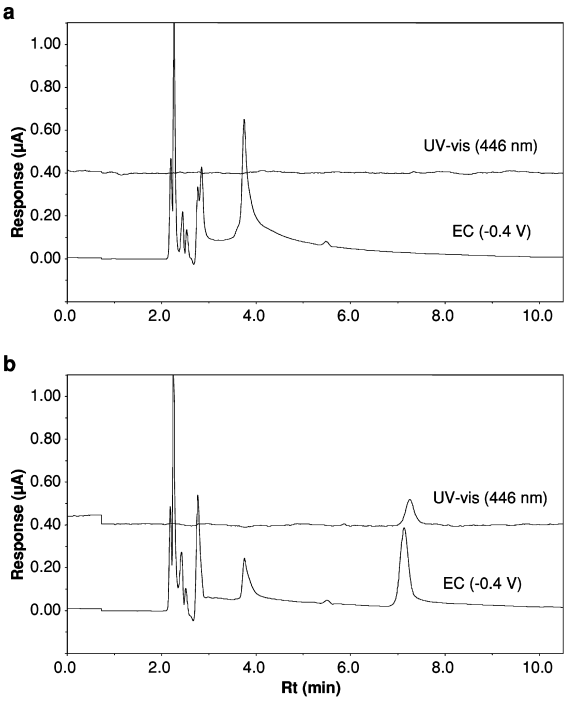

Fig. 5. HPLC–UV/EC chromatograms of a plasma extract from a rat (a) and from another rat 3 h after a subcutaneous injection of MnTDE-2-ImP^{5+} (24 mg/kg body wt.) (b) using an aqueous solution containing 20 m*M* of both TEA and TFA (pH 2.7) (A) and acetonitrile (B=12%) as mobile phase. The concentration in sample from the treated rat was estimated as 6.5 μ*M* (13 μ*M* in the plasma).

ImP^{5+}, electrochemical detection, although less specific, appears to be considerably more sensitive than spectrophotometric detection. The method of analysis described here may be used for in vivo applications, for instance to study the pharmacodynamics of cationic metalloporphyrins.

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