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Determination of residual manganese in Mn porphyrin-based superoxide dismutase (SOD) and peroxynitrite reductase mimics

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Abstract

The awareness of the beneficial effects of Mn porphyrin-based superoxide dismutase (SOD) mimics and peroxynitrite scavengers on decreasing oxidative stress injuries has increased the use of these compounds as mechanistic probes and potential therapeutics. Simple Mn^{2+} salts, however, have SOD-like activity in their own right both *in vitro* and *in vivo*. Thus, quantification/removal of residual Mn^{2+} species in Mn-based therapeutics is critical to an unambiguous interpretation of biological data. Herein we report a simple, sensitive, and specific method to determine residual Mn^{2+} in Mn-porphyrin preparations that combines a hydrometallurgical approach for separation/speciation of metal compounds with a spectrophotometric strategy for Mn determination. The method requires only common chemicals and a spectrophotometer and is based on the extraction of residual Mn^{2+} by bis(2-ethylhexyl)hydrogenphosphate (D2EHPA) into kerosene, re-extraction into acid, and neutralization followed by UV-vis determination of the Mn^{2+} levels via a Cd^{2+}-catalyzed metallation of the H_2TCPP^{4-} porphyrin indicator. The overall procedure is simple, sensitive, specific, and amenable to adaptation. This quantification method has been routinely used by us for a large variety of water-soluble porphyrins.

Introduction

The beneficial effects of Mn porphyrins as superoxide dismutase (SOD) and peroxynitrite reductase mimics in oxidative stress [1] models, particularly cationic Mn(III) 5,10,15,20-tetrakis-*N*-alkylpyridylporphyrins [2–5], have motivated their use as mechanistic probes and potential therapeutics and stimulated the commercialization of some of the compounds of the series. However, along with the bigger demand have emerged a variety of issues related to the quality of the samples that have jeopardized some of the biological data reported so far (for details, see [6–8]). Inappropriate synthetic procedures and unsuitable work-up and purification strategies may leave behind non-innocent impurities that may have biological activity in their own right, may mask the *in vivo* effects of the compounds in study, and could in turn result in questionable conclusions. CalBiochem preparations of Mn(III) 5,10,15,20-tetrakis-*N*-alkylpyridylporphyrins, for example, were recently found to be actually a complex mixture of compounds of different degree of *N*-alkylation that affected dramatically the intrinsic antioxidant capacity and biological activity of such compounds [7,8]. In another case, we have also clearly shown that all commercially available preparations of anionic Mn(III) tetrakis(4-

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carboxylatophenyl)porphyrin, $MnTCPP^{3-}$ (or $MnTBAP^{3-}$) (from Alexis, Porphyrin Products, MidCentury Chemicals and CalBiochem) carried traces of Mn in the form of non-porphyrin species that possessed high SOD-like activity in its own right, while pure $MnTBAP^{3-}$ itself was not an SOD mimic at all [6,7]; such impurities had little effect on the peroxynitrite scavenging ability of the samples, which made pure $MnTBAP^{3-}$ a suitable probe for peroxynitrite over superoxide in mechanistic studies [9].

The two obvious impurities that may arise in all Mn porphyrin-based preparations are the unmetallated porphyrins (metal-free ligands) and residual Mn^{2+} salts left from the metallation procedure. While the unwanted presence of metal-free ligands, which are photosensitizers, in Mn porphyrin samples can be conveniently detected and quantified by a variety of techniques [6,7], the quantification of residual Mn^{2+} species in Mn porphyrin samples (or any other Mn-based antioxidant, for that matter) has never been fully addressed. The catalytic rate constant for the dismutation of $O_2^{\cdot -}$ by simple Mn^{2+} salts ranges from 1.3×10^6 M^1 s^{-1} to ~10^7 M^{-1} s^{-1} [10–12]. Thus, residual Mn^{2+} salts left in the most common Mn-based antioxidants such as Mn porphyrins (MnP), Mn salens, or Mn cyclic polyamines may eventually have SOD-like biological activity in their own right. The critical role of simple Mn^{2+} salts as SOD mimics in cell models of oxidative stress have been documented [13–15].

The determination of residual Mn^{2+} in Mn porphyrin samples is critical for assessing if further purification is required and for granting that the effects observed *in vivo* are related to the Mn porphyrin itself rather than to residual Mn^{2+} salts contaminants or their derivatives. We describe herein the first method for determination of residual Mn^{2+} species in Mn porphyrin samples, by combining a hydrometallurgical approach for separation/speciation of metal compounds with a sensitive spectrophotometric strategy for Mn determination.

Materials and Methods

Materials

The following reagents were used as received: 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (H_2TCPP^{4-}, Aldrich, ~85% dye content), $HClO_4$ (Aldrich, 69.0–72.0%), $CdCl_2$ (Aldrich, 99 +%), $Mn(OAc)_2{\cdot}4H_2O$(Aldrich, 99.99%), bis(2-ethylhexyl)hydrogenphosphate (D2EHPA, Aldrich, 97%), kerosene (Aldrich), NaCl (Mallinckrodt, 100.2%), NaOH (Mallinckrodt, 98.4%), hydroxymethylaminomethane (Tris, EM, >99.8%). UV-vis measurements were performed on a Shimadzu UV-2501PC spectrophotometer at 0.5 nm resolution.

Preparation of working solutions—$H_2TCPP^{4-}{}_{(aq)}$ solution (2.03×10^{-4} M) was prepared by diluting 8 ml of H_2TCPP^{4-} stock solution (10.01 mg of porphyrin in 7 ml of 0.01 M NaOH and 3 ml H_2O) with 27.8 ml of water. The concentration of the H_2TCPP^{4-} solution was determined spectrophotometrically (ε_{415nm} = 386,000 M^{-1} cm^{-1}) [6]. Due to light sensitivity, all flasks containing H_2TCPP^{4-} solutions were fully wrapped with aluminum foil. Solutions were found indefinitely stable if stored in the dark. D2EHPA solution in kerosene (0.227 M), aqueous solutions of $CdCl_2$ (1.20 mM), $HClO_4$ (1 M); $NaOH_{(aq)}$ (5 M), NaCl (2.00 M) and Tris buffer (pH 7.8; 1.00 M) were prepared. Freshly prepared aqueous solutions of $Mn(OAc)_2{\cdot}4H_2O$ of various concentration were used for calibration. The concentration of MnPs in their aqueous solutions was determined spectrophotometrically using reported ε values. The ε_{468nm} = 104,700 M^{-1} cm^{-1} of a pure sample of $MnTCPP^{3-}$ was used [6]. Deionized water was used throughout the study.

Methods (Figure 1)

Step 1: Extraction—To a 1.7 ml-snap-cap conical plastic microcentrifuge tube were added 100 µl of 2.00 M NaCl solution, (900 − X) µl of water and X µl of the aqueous MnP sample.

This solution is referred to as "saline solution". Then 500 μl of 0.227 M D2EHPA solution in kerosene was added. The biphasic mixture was heavily shaken (manually) for 5.0 min, after which the phases were separated in a Brinkmann Eppendorf 4515 centrifuge (2.5 min at 3,000 rpm). The volume X of the aqueous MnP sample is chosen in order to give a concentration of residual Mn^{2+} in the saline solution in the range between 1 and 30 μM. For example, if the MnP sample is thought to contain residual Mn^{2+} in the order of (a) ~1 mol% or (b) ~0.1 mol %, then the volume X of a 5 mM MnP stock should be (a) ~ 80 μl of or (b) ~ 800 μl, respectively, to yield a "saline solution" of ~ 4 μM residual Mn^{2+}.

Step 2: Reextraction—An 450 μl aliquot of the organic phase (from the step 1) was transferred to a microcentrifuge tube, to which were added 900 μl of $HClO_{4(aq)}$ (1.00 M). The biphasic mixture was heavily shaken manually for 5.0 min, after which the phases were separated in a centrifuge (2.5 min at 3,000 rpm). The organic layer was carefully and fully discarded using 100 μl Eppendorf pipettes. The use of 1000 μl Eppendorf tips were found unsuitable to carry this phase separation out.

Step 3: Neutralization—An 800 μl aliquot of the acidic solution (aqueous phase from step 2) was transferred to a microcentrifuge tube. Then were added 40 μl of Tris buffer (pH 7.8; 1.00 M) and ~160 μl of NaOH (~5 M). The exact volume of NaOH solution was determined in advance as that necessary to bring a control solution of $HClO_4$ (800 μl, 1 M) and Tris (40 μl) to pH 7.0–7.1. The resulting neutralized Mn^{2+}– containing solution was used in last step.

Step 4: Mn^{2+} determination (spectrophotometric measurement)—Into a 1.5 ml quartz cuvette (1 cm optical path) were added 40 μl of Tris buffer (1 M), 120 μl of H_2TCPP^{4-} (2.03×10^{-4} M) solution, and 800 μl of the neutral solution from step 3. The UV-vis measurements were carried out in the following manner: (1) the initial absorbance at 468 nm (λ_{max} of $MnTCPP^{3-}$ product) is recorded as A_0; (2) addition of 40 μl of $CdCl_2$ (1.20 mM) to the cuvette marks time zero (the absorbance maximum shifts from 413 nm for H_2TCPP^{4-} to 433 nm for $CdTCPP^{4-}$); (3) the formation of MnTCPP3- is marked by a band at 468 nm, whose change in absorbance is monitored for 30 min; (4) the absorbance at 30 min is recorded as A_{30}. The concentration of residual Mn^{2+} may be expressed in mol % of the MnP by the quotient between the concentrations of the residual Mn^{2+} and MnP in the "saline solution" times 100%.

Calibration curves—Calibration curve was obtained using the procedure described above with different concentrations of $Mn(OAc)_2\ 4H_2O$ (0.6, 2.0, 4.0, 8.0, 16.1, 24.1 and 32.1 μM) (Figure 2A and 1B). The content of Mn^{2+} in these sample was calculated from the recorded absorbance measurements using the following relationships: (a) $A_0' = 0.96 \times A_0$ (where 0.96 corresponds to the dilution factor upon addition of $CdCl_2$ to the cuvette); (b) $\Delta A = A_{30} - A_0'$; (c) a calibration curve constructed from ΔA values versus known concentration of Mn^{2+} in the "saline" solution.

Results and Discussion

A common hydrometallurgical practice for the separation of metal ions involves the controlled liquid-liquid extraction of the cations into an organic phase containing an appropriate extractant. Among the systems that have been extensively studied for hydrometallurgical Mn^{2+} extraction are those using di-(2-ethylhexyl)phosphoric acid (D2EHPA) as extractant [16–20]. It follows, therefore, that the separation of the residual Mn^{2+} salts from the aqueous Mn porphyrin samples was carried out by liquid-liquid extraction using D2EHPA in kerosene. No *in situ* demetallation of Mn(III) porphyrins by D2EHPA was observed. Of note, common water-soluble Mn(III) porphyrins are indefinitely stable toward metal loss even in concentrated sulfuric acid. The use of a large excess of D2EHPA in kerosene (0.227 M) relates to the

extraction equilibrium constant of ~10^{-3} [16] for the Mn^{2+}/D2EHPA system. Of note, the Mn^{2+} loading capacity of 0.1 M D2EHPA is 0.026 M [18]. NaCl was added to the aqueous phase to yield a "saline solution" and to avoid, thus, the partition of some of the Mn porphyrins (*e.g.*, PEG-ylated Mn(III) *N*-pyridylporphyrin [2]) into the kerosene phase. The residual Mn^{2+} species extracted with D2EHPA/kerosene is re-extracted into an aqueous medium with a strong acid. This re-extracted solution is, then collected and neutralized with NaOH. Perchloric acid was the acid of choice to minimize interference with the spectrophotometric assay (see below).

The use of this extraction method to metal complexes of low stability to acids is precluded. Attemtps to measure the free Mn^{2+} content of Mn(III) salen samples (EUK-8, Mn(III) N,N′-ethylenebis(salicylideneiminato) resulted in the *in situ* demetallation of Mn(III) salen. The acidity of D2EHPA prevented also the quantification of residual Mn^{2+} species in commercial $MnTBAP^{3-}$, as this porphyrin precipitates in the acidic form and co-precipitation of residual Mn^{2+} could not be ruled out, as observed with other acids [6].

Although atomic absorption is a classical technique to measure metal levels in water, we chosed to determine the residual Mn^{2+} by spectrophotometry given the wide availability of spectrophotometers in biochemical and biomedical laboratories. A method utilizing H_2TCPP^{4-} (λ_{max} = 415 nm) as indicator [21–23] gives results comparable to those obtained by atomic absorption, is simple and, given the specific nature of the $MnTCPP^{3-}$ complex formed (λ_{max} = 468 nm), is subjected to little interference by other metal ions [21–23]. The insertion of Mn^{2+} in the H_2TCPP^{4-} porphyrin does not occur at room temperature in absence of a catalyst, such as Cd^{2+}. The coordination of Cd^{2+} to H_2TCPP^{4-} occurs readily to yield a deformed $CdTCPP^{4-}$ porphyrin (λ_{max} = 433 nm) that enhances the Mn^{2+} insertion into the porphyrin by several orders of magnitude *via* a Cd-to-Mn metathesis reaction [24]. The very low reduction potential of the $Mn(II)TCPP^{4-}$ [6] favors its immediate aerobic oxidation to Mn (III)$TCPP^{3-}$, which has a sharp, characteristic band at 468 nm of high molar absortivity (log ε = 5.04 [6]). The reaction is, thus, monitored at this wavelength (Fig. 2A-insert) as it is specific to the Mn product and falls in a spectral region that remains unmasked by the high absorptions due to the excess H_2TCPP^{4-} and $CdTCPP^{4-}$ species (Fig. 2A). The reaction was carried out in Tris buffer instead of borate and/or imidazole buffers [21–23] because of its common use in biochemical and biomedical laboratories.

The large excess of $NaClO_4$ (derived from the NaOH-neutralization of $HClO_4$) in the cuvette slows the $MnTCPP^{3-}$ formation in a classical kinetic salt effect manner, as the $CdTCPP^{4-}$ complex and the residual Mn^{2+} species are of opposing charges. The use of HCl instead of $HClO_4$ was prohibited by the high coordination ability of Cl^- compared to ClO_4^-. In excess Cl^-, formation of the catalytic intermediate $CdTCPP^{4-}$ was precluded, as indicated by UV-vis spectra (the major species in solution was still H_2TCPP^{4-}), and formation of $MnTCPP^{3-}$ did not occur. Excess Cl^- competes effectively with H_2TCPP^{4-} for the metal ions and inhibits, thus, metalloporphyrin formation [25].

The method gives a linear response of ΔA *vs* [Mn^{2+}] (Fig. 2B) up to ~35 μM Mn^{2+} in the "saline solution" (*i.e.*, ~22.4 μM of Mn^{2+} in the cuvette), after which H_2TCPP^{4-}/$CdTCPP^{4-}$ (24.36 μM in the cuvette) is mostly depleted and the system is no longer responsive to excess Mn^{2+}, unless more H_2TCPP^{4-} is added. The limits of detection (LOD, 3s) and of quantification (LOQ, 10s) were 0.4 and 1.3μM of Mn^{2+} in the "saline solution", respectively. An artificial "saline solution" prepared by deliberate mixing of a PEG-ylated Mn(III) *N*-pyridylporphyrin (290 μM) and $Mn(OAc)_2$ (24.1 μM) was subjected to the whole extraction/quantification procedure and compared to a control sample of $Mn(OAc)_2$ alone (24.1 μM); the Mn^{2+} recovery was 99.2 %, which confirmed also that excess MnP does not interfere in neither the extraction of Mn^{2+} by D2EHPA nor the subsequent quantification steps.

This method has been routinely used by us for a large variety of water-soluble porphyrins. The cationic Mn(III) *N*-alkylpyridylporphyrins are regularly isolated through double precipitation and extensive washings; both procedures are critical for effective removal of nearly all residual Mn^{2+} from the preparations. Mn porphyrins that we have prepared via this route [7,26–28] usually have low levels of residual Mn^{2+}, often in the range of 0.05 – 1 mol % on a MnP basis. The extensive ultrafiltration procedure used previously for Mn(III) porphyrins that do not precipitate readily [2,27] gave samples of 0.1 – 1 mol % on a MnP basis.

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Figure1A

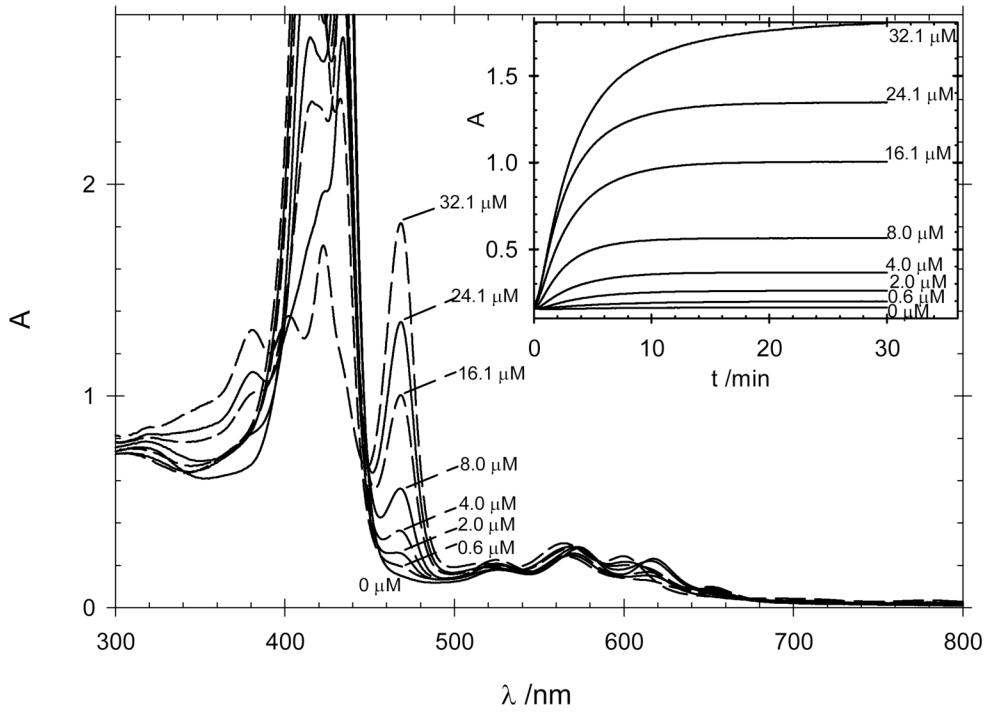

Figure 1B

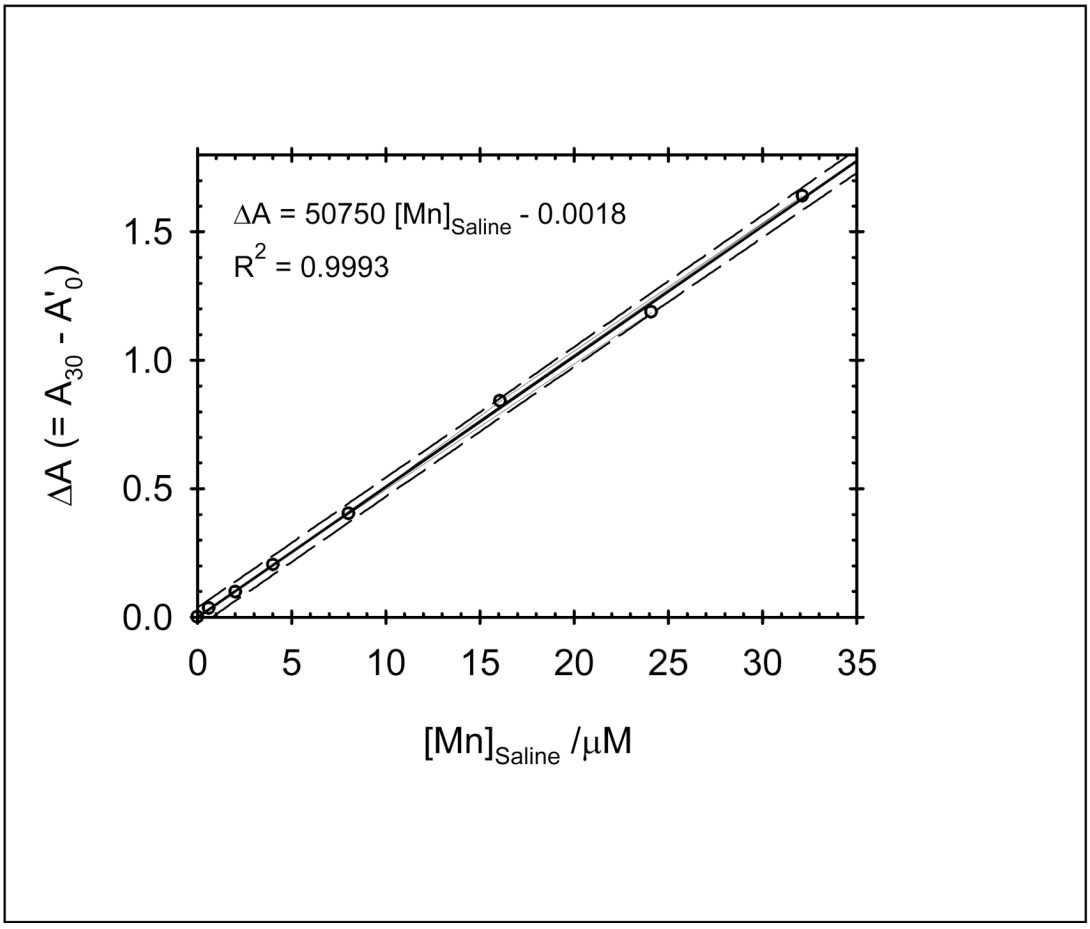

Figure 1.
Schematic representation of the procedure for the determination of residual Mn^{2+} in Mn porphyrin samples.

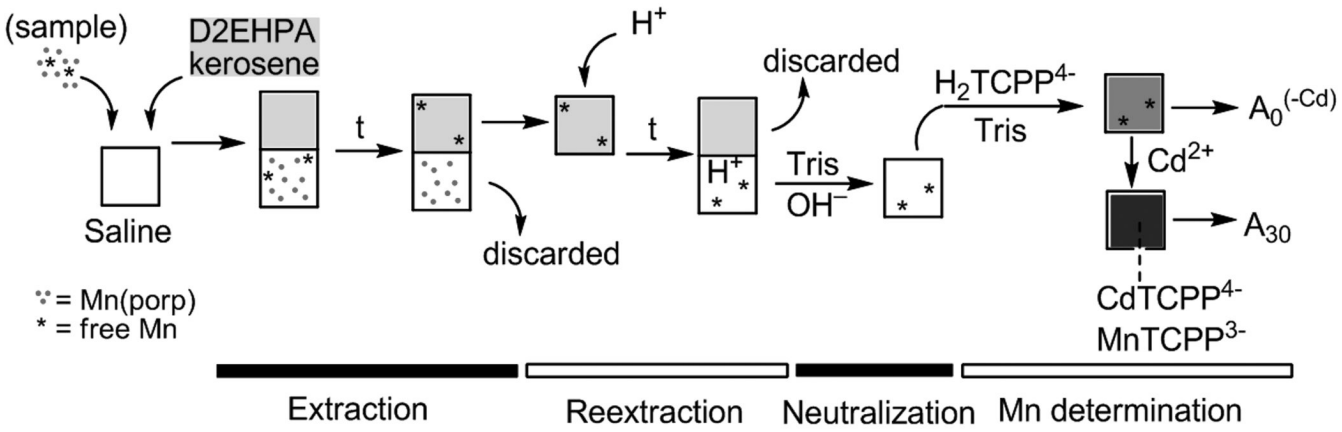

Figure 2.

Calibration curves for the procedure for the determination of residual Mn^{2+} in Mn porphyrin samples: **A)** UV-vis spectra of the cuvette solution at the end of the reaction time (30 min); insert: time-dependent change in the absorbance at 468 nm at various concentrations of Mn^{2+}. **B)** Absorbance change as a function of $Mn(OAc)_2$ concentrations: 0.6, 2.0, 4.0, 8.0, 16.1, 24.1 and 32.1 μM.