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A simple, cheap but reliable method for evaluation of zinc chelating properties

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ABSTRACT

Background: Zinc is an essential trace element. Both its lack and excess are associated with pathological states. The former is more common and can ensue from the excessive treatment with clinically used iron/copper chelators.

Aim and method: The aim of this work was to prepare a reliable, rapid and cheap method for the screening of zinc chelation. Spectrophotometric assessment using a known zinc indicator dithizone was selected.

Results: Initial screening performed by comparison of spectra of dithizone and its complex with zinc suggested 530 and 570 nm as suitable wavelengths for determination of zinc at pH 4.5 while 540 and 590 nm for pH 5.5–7.5. Additional research showed the lower wavelengths to be more suitable. The sensitivity of the method was always bellow 1 μM with good linearity relationship between absorbance and zinc concentration. The method suitability was confirmed by use of two known zinc chelators, ethylenediaminetetraacetic acid (EDTA) and N,N,N′,N′-tetrakis(2-pyridylmethyl)-1,2-ethylenediamine (TPEN).

Conclusion: This method represents a sufficiently precise method for zinc chelation screening usable at pathophysiologically relevant pH conditions. Such method can be employed for both screening of novel zinc chelators and for testing affinity of other metal chelators for zinc.

1. Introduction

Zinc ion (Zn^{2+}) is an essential trace element and one of the most abundant transition metal ions in living organisms. It is involved in many physiological processes, e.g. as essential cofactor maintaining protein structure and regulating its function [1–4]. Zinc proteins include numerous enzymes (e.g. alcohol dehydrogenase, Cu-Zn-superoxide dismutase, and RNA polymerases), zinc dependent transcription factors and hormone insulin. Summarizing its function, Zn^{2+} has an important role in various signalling pathways, including neurotransmission, cell division, development and differentiation [5,6]. Since zinc is an essential component in some of these processes, its removal by chelators (from many enzymes or transcription factors) is rendering them inactive.

Both zinc deficiency and excess can occur in human. Zn deficiency is much more frequent and can be related to inadequate dietary intake, increased requirements and/or excretion. Increased excretion can, for example, take place inadvertently due to use of metal chelators since they have mostly low selectivity. Mice model showed possible zinc depletion caused by two iron chelator agents, desferrioxamine and deferiprone [7]. Similarly, D-penicillamine, a drug used in copper excess can cause severe zinc depletion [8]. Symptoms of Zn deficiency include chronic diarrhea associated with malabsorption, regional enteritis, coeliac sprue, and cystic fibrosis [9,10]. Studies in pregnant experimental animals have demonstrated a high occurrence of foetal malformations [11,12]. Contrarily to zinc deficiency, zinc toxicity is rather a rare and probably mostly reversible state. Zn is considered a nontoxic micronutrient at moderate supplementation levels (≤100 mg/day) however, intoxication can occur at high doses. Intoxications can occur in the workplaces (e.g. from inhalation of $ZnCl_2$ fumes) and in population with excessive oral exposure to Zn dietary supplements or in patients hemodialyzed with water stored in galvanized steel tanks. Intoxication depends on the route of entry for Zn into the organism. There are more mechanisms which can participate on zinc toxicity. It is known e.g. (1) $ZnCl_2$ is a corrosive agent that causes irritation of the GIT after ingestion and irritation of the respiratory tract after inhalation, (2) inhalation of ZnO causes the

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immune reaction in the airways ("metal fume fever") and (3) Zn^{2+} ions stimulate the formation of free oxygen radicals. Oral Zn intoxication may produce within 30 min to 1 h after ingestion burns of the mucosa, haemorrhagic gastroenteritis (with hematemesis, sloughing of mucous membranes, ulcer formation) and later acute renal tubular necrosis and interstitial nephritis. Symptoms may rapidly progress and the patient may suffer from gastrointestinal haemorrhage, shock, and cardiovascular collapse. Current treatment of Zn poisoning is supportive. Chelation is very effective for reducing elevated Zn levels by increasing urinary Zn excretion. Used chelating agents for Zn are calcium disodium ethylenediaminetetraacetic acid (EDTA), BAL (British anti-lewisite, dimercaprol), D-penicillamine and *N*-acetylcysteine. The former two are usually preferred in patients with significant Zn toxicity. The current therapy is far from being ideal: there are still problems regarding the toxicity and the activity appears to be insufficient [13]. Based on the above-mentioned data, screening of zinc chelation activity can lead to both, the development of novel and selective zinc chelators for zinc intoxication treatment and to check if current metal (e.g. iron and copper) chelator cannot influence Zn homeostasis and thus have potential side effects. Moreover, currently clinically used zinc chelators are non-selective and there are side effect drawbacks. For this reason, the main aim of this work is to develop a rapid, cheap but reliable method for screening of zinc chelation. We selected a known indicator [14–16] dithizone and prepared a standardized method for this purpose. This method can be employed for testing of both (1) novel zinc chelators and (2) possible interference of known iron/copper chelators or other drugs with zinc.

2. Experimental procedures

2.1. Chemicals, solutions and equipment

Dithizone (purity ≥ 99.0%), N,N,N′,N′-tetrakis(2-pyridylmethyl)-1,2-ethylenediamine (TPEN, ≥98%), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, ≥98.5%), zinc chloride (≥98.0%) and DMSO were purchased from Sigma-Aldrich Inc. (Germany) while methanol was from J.T. Baker (Avantor Performance Materials, Inc., USA). Ultrapure water (Milli-Q RG, Merck Millipore, Massachusetts, USA) was used throughout this study.

Stock solutions of TPEN (1 mM) were prepared in DMSO, whereas that of zinc chloride (5 mM) and EDTA (5 mM) in ultrapure water. Dithizone was prepared either in DMSO or in methanol depending on the type of experiment.

Most experiments were performed in 96-well microplates (BRAND GmbH + CO KG, Germany) with use of Synergy HT Multi-Detection Microplate Reader (BioTec Instruments, Inc., USA). The only exception were stoichiometric experiments, which were carried out in semi-micro polystyrene or ultraviolet-transparent cuvettes (BrandTech Scientific Inc., UK) with spectrophotometer Helios Gamma equipped with VisionLite 2.2 software (ThermoFisher Scientific Inc., USA). Acetate buffers (15 mM) were used thorough the study for the pH values of pH 4.5 and 5.5 whereas HEPES buffers (15 mM) for pH 6.8 and 7.5. The pH values were always controlled by the Calibration Check Microprocessor pH Meter HI221 (Hanna Instruments S.L., Spain) and buffers were used only if they were in the acceptable range of ±0.05 pH units.

2.2. Dithizone method

2.2.1. Comparison of spectra of dithizone and its complex with zinc

DMSO solution of dithizone (50 μL, 250 μM) was mixed with 50 μL of different Zn^{2+} solutions or water subsequently in four above mentioned buffers (150 μL). Spectra were then measured in the range of 400 to 700 nm every 10 nm and after different time intervals.

2.2.2. Verifying linearity and detection of the sensitivity

These tests were performed in conditions as planned for the future standardization, e.g. firstly zinc solutions of different concentrations (50 μL) or water were added to the buffers (150 μL). Thereafter 50 μL of DMSO was added as planned for future chelation testing since DMSO is the solvent usable for most compounds. After 2 min of mixing, 50 μL of DMSO or DMSO solution of dithizone (250 μM) was added and the absorbance was measured immediately at wavelengths selected according to results from step 2.2.1. After linearity determination, similar experiments with lower concentration of zinc (final concentration ranging from 0.5 to 1.25 μM) were performed in order to detect method sensitivity.

2.2.3. Verification of the stability of absorbance and the reagents

Verification of the reagents stability was performed by comparison of freshly prepared and older stock solutions of zinc (in water) and dithizone (in DMSO). Measurements were performed as previous ones in buffers (150 μL) with two fixed concentrations of zinc and without zinc. Again, dithizone (50 μL, 250 μM) or DMSO was added in the last step. Measurements were performed in the following time intervals: 0, 2, 5, 24, 48 and 72 h with both new freshly prepared and older stock solutions.

2.2.4. Testing the methodology on known chelators

Testing of compounds was performed in similar conditions like previous measurements. Shortly: the zinc solution (50 μL, 60 μM) was mixed in a buffer (150 μL). After that, 50 μL of a tested compound in different concentrations was added. After 2 min of mixing, 50 μL of DMSO or DMSO solution of dithizone (250 μM) were added and the absorbance was measured immediately at wavelengths selected according to results from step 2.2.1.

2.2.5. Mathematical and statistical analysis

All mathematical and statistical calculations were performed by use of GraphPad Prism 7 for Windows (GraphPad Software, USA). All data are presented as means ± SD. The sensitivity and reagent stability were determined by using one-way ANOVA followed by the Dunnett test comparing measured data with the control (solvent or fresh solution, respectively).

2.3. Assessment of dithizone complex stoichiometry

The assessment of the stoichiometry was carried out according to the previously established protocol for other metals used in our laboratory [17].

2.3.1. Determination of molar absorption coefficients

Firstly, dithizone dissolved in methanol was mixed with a water solution of zinc ions [16] for 1 min at different molar concentration ratios ranging generally from 1:4 to 1:25 (dithizone:zinc) in order to establish the molar absorption coefficients. Afterwards absorption spectra vs. the blank, composed of a buffer and methanol in the ratio 2:1, were immediately measured. Experiments were performed at the same pH conditions as above (pH 4.5, 5.5, 6.8 and 7.5).

2.3.2. Job's method

The standard Job's method was employed [18]. Briefly, the total molar concentration of dithizone and Zn^{2+} solutions was kept constant (50 μM) while their molar concentration ratios were continuously changed from 1:3 to 6:1 throughout the series of samples.