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The determination of iron as its EDTA complex in *Helix aspera* by hydrophilic interaction liquid chromatography coupled to Fourier transform electrospray ionisation mass spectrometry

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ABSTRACT

Metal complexes of Fe(III) such as Fe(III) ethylene diamine tetraacetic acid (FeETDA) have been observed to be effective molluscicides. The mechanism of toxicity of FeEDTA complex on molluscs is not clear and it is also not known if Fe(III) in the form FeEDTA is absorbed more effectively by snails than simple iron salts. Snails were fed with molluscicide pellets containing the FeEDTA complex and also with pellets containing $FePO_4$ after 3–4 days the hearts, kidneys and dart sacs removed and analysed for Fe(III) content. Hydrophilic interaction liquid chromatography (HILIC) coupled with Fourier transform electrospray ionisation mass spectrometry (FT-ESIMS) was used to analyse the sample extracts. The method had a very wide linear range from 2 to 10,000 ng mL^{-1}, intra- and inter-day precisions of *ca.* ±0.5% were observed for the analysis of extracts from snail tissues spiked with Fe(III). The limit of detection was of 0.5 ng mL^{-1} for a 20 μL injection. The levels of Fe(III) in tissues from snails fed Fe EDTA pellets were 10–100 times higher than the levels in snails fed $FePO_4$ pellets. The analysis of Cu, Zn, Ca and Mn could also be carried out using the same analytical procedure.

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1. Introduction

Iron(III) phosphate containing pellets are used as molluscicides but relatively little is known about the mechanism of action of these pellets although iron has been observed to be deposited in digestive gland and body wall of the mollusc [1]. It has been reported that the ability of metals to act as molluscicides was improved when they were formulated as organic complexes [2] and in 1995 a patent was taken out on the use of ferric EDTA and related complexes as molluscicides [3]. Recently, it has been observed that earthworms are also very sensitive to molluscicides based on $FePO_4$ [4]. The aim of the current study was to develop a method for the determination of iron in the organs of *Helix aspera* as a model for what may occur in other molluscs. A number of studies have been conducted to determine whether or not the uptake of iron via the gut is more effective when the iron is administered as a complex such as FeEDTA compared with administration of a simple salt such as $FeSO_4$. In one study [5], it was found that iron was accumulated to a lesser extent in liver and spleen of rat when FeEDTA was administered than when $FeSO_4$ was administered but other organs were not examined. Zhu et al. [6] found that the penetration of iron through Caco-2 cells, used as a model for the cells of the gut, was less effective in the case of Fe(III)EDTA compared with $FeSO_4$ and $FeCl_3$. However, an *in vivo* study by the same group found that NaFeEDTA produced different tissue distributions of iron compared to $FeSO_4$ in the rat [7]. Levels of iron in the kidney were 83% higher when rats were fed NaFeEDTA and levels in the liver were 53% lower when compared with feeding $FeSO_4$.

Among a large number of chelating reagents for formation of complexes with metals, particularly iron, EDTA is considered to be very effective. EDTA can normally be represented as H_4Y, in the fully protonated form because of four acidic groups. The pK_a values of four corresponding hydrogens are $pK_{a1} = 1.99$, $pK_{a2} = 2.67$, $pK_{a3} = 6.16$, and $pK_{a4} = 10.26$ [8]. The hexadentate ligand has an unusual chelating power with metals forming 1:1 complexes with many metals. Of all the important metals, Fe^{3+} has the highest complexation stability constant ($\log K$ of 25.1), followed by Cu ($\log K$ 18.8), Zn ($\log K$ 16.3), Co^{2+} ($\log K$ 16.2) and so on [9]. In aqueous solution EDTA and Fe(III) forms an octahedral complex $[FeY]^-$ exhibiting a negative charge.

The determination of Fe(III) as its FeEDTA complex has been carried out by a number of researchers. Owens et al. [10] studied EDTA complexes by capillary electrophoresis (CE) with UV detection. Complexes of various metal ions were formed prior to analysis including Fe^{3+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}. The authors reached a detection limit of $[FeY]^-$ at 4 μg mL^{-1}. Blatny et al. [11]

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studied metal ions in water with CE; EDTA was added to water to form a complex which was then detected at 254 nm. The limit of detection for Fe was 13 ng mL^{-1}. Sillanpää et al. [12] performed ion pair HPLC to measure EDTA complexes with ferric ions. They used a buffer solution containing the ion-pairing agent cetrimide as the mobile phase. The complex was detected at 260 nm and the limit detection was 0.5 μg mL^{-1}. A similar ion pair method was used by Lucena et al. with tetrabutylammonium chloride as ion pair reagent and UV detection at 280 nm [13]. Chen et al. [14] developed an analysis of $[FeY]^-$ by CE coupled with electrospray ionization-mass spectrometry. The authors reached a detection of limit at 4 ng mL^{-1} for $[FeY]^-$ in the negative ion mode. Quintana and Reemtsma [15] used liquid chromatography coupled with ESI-MS to detect the EDTA–Fe(III) complex. They added the volatile ion-pairing reagent tributylamine to the mobile phase. A phenyl–hexyl column was used and the detection of limit was 1 ng mL^{-1} with a 50 μL injection volume. Álavarez-Fernández et al. used an ESI-time of flight method to determine a number of Fe(III) chelates used in fertilisers in various agricultural matrices. The used reverse phase chromatography in the negative ion mode, high sensitivity was achieved through the use of ^{57}Fe-labelled internal standards and the LOQ for Fe(III) EDTA achieved was 328 pmol/mL (114.1 ng/mL) [16]. Inductively coupled plasma-mass spectrometry (ICP-MS) has been also used to determine Fe. Yeh and Jiang [17] use ICP-MS to speciate V, Cr and Fe with CE separation, the limit of detection was 10 ng mL^{-1} for $[FeY]^-$. Xuan et al. [18] introduced a ZIC–hydrophilic interaction liquid chromatography (HILIC) column to separate and identify the different phytosiderophores of the mugineic acid family as their metal complexes. Various metals (Zn, Cu, Ni, and Fe) were added to 2′-deoxymugincic acid (DMA) and nicotinamine to form complexes. They used a mobile phase consisting of NH_4AC and CH_3CN at pH 7.3. When authors checked the stability of the complexes during separations using EDTA, they found EDTA–Fe(III) peaks $[FeY]^-$ at 21.7 min with *m*/*z* 344.0. However, they had not shown chromatograms or validate the method.

In the current work a HILIC chromatography method interfaced with Fourier transform electrospray ionisation mass spectrometry (FT-ESIMS) was developed for the determination of uptake of iron(III) into tissues of the snail following feeding of a commercially available molluscicide based on iron(III).

2. Experimental

2.1. Chemicals and materials

All chemicals used were analytical grade. EDTA disodium salt dihydrate, EDDS trisodium salt solution, iron chloride hexahydrate were purchased from Sigma–Aldrich. HPLC grade acetonitrile and formic acid were from VWR International, UK. For all eluents and standards preparations, sample preparations, deionised water was provided by a water purification system (Milli-Q system, Millipore). Snails (*H. aspera*) were purchased from Blades Biological Systems (Kent, UK). Ferramol slug pellets (W. Neudorff, Germany) were purchased locally. Iron(III) phosphate pellets (1%, w/w) were provided by Lonza Ltd. and contained iron(III) phosphate without addition of EDTA.

2.2. Microscopy

All snails were dissected under a stereo-binocular microscope from Brunel, UK with magnification power objectives 4× and an eyepiece 10×. A micro-dissection tool kit was used for dissecting the snails. The dissected tissue was homogenised using a hand held homogenizer (LabGen 7B, USA).

2.3. Standards and sample preparation

FeEDTA: $FeCl_3{\cdot}6H_2O$ (*ca.* 77.7 mg; MW = 270.3 g mol^{-1}) and $C_{10}H_{14}O_8N_2Na_2{\cdot}2H_2O$ (*ca.* 106.9 mg; MW = 372.2 g mol^{-1}) were weighed out and both dissolved in 100 mL of water in the same volumetric flask, thus preparing solution A with FeEDTA (referred to hereafter as the FeEDTA complex standard) at a concentration of 1 mg mL^{-1}. Solution A was diluted 1000× with acetonitrile/0.1% (v/v) formic acid (70:30) producing a stock solution of FeEDTA complex standard at a concentration of 1 μg mL^{-1}. From this solution calibration series was prepared with 2, 10, 50, 200, 500, 1000, 5000 and 10,000 ng mL^{-1} of FeEDTA.

2.4. Feeding and dissection of snails

The snails were fed manually with pellets which contained either $FePO_4$ or commercial pellets containing FeEDTA and kept in an aquarium. Once they had died (after 3 days), dissections were carried under the microscope. Firstly, the outer shells were removed by breaking bits of shell away with forceps taking care not to damage the soft tissue. Pieces of the shell and any other surface contamination was removed by washing the body in water (3× 0.5 L). The mantle skirt was located and cut away; another cut was made transversely from the pneumostome to the left side of the mantle along the posterior edge of skirt. The body was cut posteriorly along the left side of the mantle to the posterior end of the lung. Thus, the mantle was laid aside and the atrium, aorta, ventricle and kidney were removed from the lung roof. The dart sac in the body cavity was also removed. The kidney, heart and dart sac were placed into 5 mL vials and extracted with either 1 mL of 0.1% (v/v) formic acid in water in order to determine FeEDTA in the tissues after feeding with FeEDTA pellets or 1 mL of 0.1% (v/v) formic acid in water containing 10 μg mL^{-1} of EDTA where total iron in the tissues was compared between snails fef on FeEDTA pellets, $FePO_4$ pellets and a control group not fed on pellets. The tissue was then homogenised with a hand held homogeniser. A drop of 1% (v/v) Triton solution was added to the vials and the samples were left sonicate for 1 h at 40 °C. Acetonitrile (1.5 mL) was then added to each vial and all vials were vortexed for 5 min. The solutions were filtered using a syringe filter and transferred to HPLC autosampler vials.

2.5. Caco-2 assay system

Caco-2 cells (HTB-37) were obtained from the ATCC. Permeability studies were carried out using the BIOCOAT HTS Caco-2 assay system purchased from Becton Dickinson Labware Europe. This system provides an *in vitro* intestinal model for the transport of drugs and natural compounds consisting of a fibrillar collagen coated 24-well insert plate and a Multiwell feeder tray for culturing cell monolayers; combined with basal seeding medium, Entero-STIM a serum free medium containing butyric acid to induce cell differentiation and MITO + serum extender containing the hormones, growth factors and metabolites required for serum free cell culture.

A suspension of Caco-2 cells at a density of 4×10^5 cells/mL was prepared in basal medium supplemented with MITO + serum extender, 500 μL of which was pipetted into each of the 24 collagen inserts to give a seeding density of 2×10^5 cells per insert. A volume of the same medium (35 mL) was pipetted into the Multiwell feeder tray and the system was incubated at 37 °C, 5% CO_2 and 100% humidity for 24 h The medium was then removed from the feeder tray and from each insert taking care not to damage the cell monolayer. 500 μL of Entero-STIM medium supplemented with MITO + serum extender was added to the interior of each insert and 35 mL of the same medium was added to the feeder tray. The system was then incubated under the conditions previously described

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