SHORT PAPERS

Thin-layer Chromatographic Detection of Metal - EDTA Complexes in Human Faeces

María V. Camacho
Laboratorio de Fisiopatología, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 1827, Caracas 1010-A, Venezuela

Ajoy K. Banerjee
Centro de Química, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 1827, Caracas 1010-A, Venezuela

and Ernesto Candela and Miguel Layrisse
Laboratorio de Fisiopatlogía, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 1827, Caracas 1010-A, Venezuela

The application of a thin-layer chromatographic technique combined with ion-exchange chromatography for the detection of metal - EDTA complexes in human faeces is described. Reproducible separations were obtained on silica gel and cellulose pre-coated plates using appropriate solvent systems. These complexes were easily identified from the specific colours produced after spraying with an appropriate reagent. Using this procedure, it was possible to detect some of these complexes when added to human faeces in amounts corresponding to approximately those which would be formed if a meal (containing the normal requirements for the metals) were fortified with the recommended maximum amount of Fe(III) - EDTA (10 mg of Fe as the complex). However, when the experiment was carried out *in vivo*, that is, the subjects ingested a meal together with the complex, or with Fe(III) - EDTA plus ten times the normal requirements of the metals as inorganic salts, none of the complexes was detected in the faeces in either instance.

Keywords: *Metal - EDTA complexes; human faeces; thin-layer chromatography*

Extensive studies have been carried out over many years to find a suitable iron compound that could be used for food fortification.[1] Iron - ethylenediaminetetraacetic acid (sodium salt) [Fe(III) - EDTA] has several advantages over other proposed iron-fortifying agents, mainly its higher bioavailability. The absorption of its iron is the same when the complex is administered in solution and when it is mixed with foods.[2]

The absorption of iron from vegetable foods fortified with Fe(III) - EDTA is much greater than that from the same foods fortified with iron(II) sulphate,[3–5] suggesting that the absorption of iron from the complex is not affected by the inhibitory ligands present in vegetable foods that considerably reduce the absorption of iron from other iron compounds as well as that of the intrinsic food iron.[6] Moreover, the absorption of iron from Fe(III) - EDTA is increased by the presence in the meal of absorption-enhancing ligands such as meat and ascorbic acid.[5] In addition, at the dose used for iron fortification this compound does not produce adverse effects on the food vehicle such as changes in colour or taste.[4] The main concern regarding its use is the possibility that its ingestion in small amounts over long periods of time might affect the nutritional status of other metallic micronutrients owing to the formation in the gut of EDTA complexes once the iron moiety has been released, and that this might render these metals unavailable for absorption, leading eventually to a nutritional deficiency.

This paper describes an efficient and selective thin-layer chromatographic (TLC) assay for EDTA complexes in human faeces that could be used to determine the possible *in vivo* interactions between Fe(III) - EDTA and metals of nutritional importance.

Experimental

Reagents

All solutions were prepared from analytical-reagent grade chemicals and solvents (E. Merck, Darmstadt, FRG) in de-ionised, distilled water, unless stated otherwise.

Synthesis of Metal - EDTA Complexes

The following inorganic salts were used in preparing the solid chelates: $MgCl_2.6H_2O$, $CaCl_2$, $CuCl_2.2H_2O$, $MnCl_2.4H_2O$, $ZnCl_2$ and $Fe(NO_3)_3.9H_2O$. Equimolar amounts of $Na_2H_2EDTA.4H_2O$ and each inorganic salt in solution were mixed. Sodium hydrogen carbonate was added to neutralise the hydrogen ion released from the ligand. The pH was adjusted to 7.0, except for Fe(III) - EDTA, for which the pH was adjusted to 5.0. The EDTA complexes formed were precipitated with ethanol and recrystallised from water - ethanol. Their infrared spectra were recorded using the KBr technique and compared with those published.[7–9] The complexes were prepared as 0.1% *m/V* aqueous solutions to be used as standards in all the TLC runs.

Procedure A

In vitro studies were carried out by adding the EDTA complexes to control samples of faeces in amounts corresponding to the requirements of the metal moeity for one meal (one third of the recommended daily allowances),[10] that is, to a 100-g sample of faeces were added the complexes Ca(II) - EDTA (1.6 g), Cu(II) - EDTA (6.7 mg), Fe(III) - EDTA (62 mg), Mg(II) - EDTA (1.5 g), Mn(II) - EDTA (15 mg) and Zn(II) - EDTA (50 mg). This was repeated, except that the faeces were sterilised before adding the complexes in order to eliminate any enzymic or bacterial action.

Both sets of samples were subjected to aqueous extraction by 1 : 2 dilution in de-ionised water followed by homogenisation, centrifugation at 1800 *g* for 30 min (MSE-Fisons, Coolspin) and at 28000 *g* for 15 min (Beckman, 15L65). The resulting supernatant was lyophilised and redissolved in the smallest possible volume of de-ionised water. Approximately half of this extract was run through an anion-exchange column containing carboxymethylcellulose (Whatman CM 52) activated with 0.1 M NaCl solution and eluted with de-ionised water. Both the extract and the eluate were subjected to TLC.

Procedure B

In vivo experiments were carried out with twelve healthy volunteers who consented to collaborate in this study. Before the experiment started, a sample of faeces was collected from each subject to serve as a control. Two consecutive samples of faeces were collected following a single oral dose of Fe(III) - EDTA (70 mg) corresponding to 10 mg of iron (the maximum daily amount recommended for food fortification) with a meal. Aliquots of these samples of faeces were subjected to aqueous extraction as described above. This was repeated, except that the subjects ingested an excess of cations (ten times the recommended allowance for a meal) in the form of inorganic salts, together with Fe(III) - EDTA (70 mg), also during a meal.

Thin-layer Chromatography

TLC was conducted using 20 × 20 cm aluminium sheets, pre-coated with silica gel 60 and cellulose, without fluorescent indicator, the layer thickness being 0.2 and 0.1 mm, respectively. Standard and test solutions were applied to the TLC plates (Merck, Darmstadt, FRG) in 10-μl aliquots at a distance of 2 cm from the bottom edge of the adsorbent layer at 1-cm intervals. They were placed in equilibrated tanks (9 × 23 × 23 cm), lined with Whatman No. 3MM chromatographic paper and containing 100 ml of mobile phase.

The chromatograms were developed to a height of 15 cm by the one-dimensional ascending technique with a development time varying from 2 h (cellulose) to 6 h (silica gel) in the pH range 6.5–7.5 at ambient temperature. Following development, the plates were dried under a constant flow of warm air (30–40 °C). The spots were revealed by spraying with an appropriate reagent and the colours observed in daylight. Each complex was run on two different adsorbents and each of these was developed with two different solvents. The solvent systems chosen and the compositions of the spray reagents are given in Table 1.

Results and Discussion

In either solvent system, a 0.05% *m/V* solution of the complexes could be reproducibly detected with a sensitivity 20-fold greater than that previously reported.[11,12]

In the *in vitro* experiments in which the complexes had been added to the faeces, the TLC separation showed a tailing effect when the metal - EDTA complexes were added to a control sample of faeces or to the sterilised faeces and the aqueous extract was subjected to the TLC procedure. Similar observations have been reported in the literature with aqueous solutions of EDTA complexes when the solvent system was outside the pH range 6.5–7.5.[11,13] Although the stability of these complexes is highly dependent on pH, in this instance the result could not be explained either by an effect of the pH of the faeces (as this was determined before the addition of the complexes and it was found to fall within the above range) or by a change in the pH of the solvent system, as this was carefully controlled throughout the experiment. However, when the faeces extract was run through an anion-exchange column containing carboxymethylcellulose, the complexes were detected in the eluate without the tailing effect, suggesting the presence of an interfering anion in the faeces, which was retained by the column.

The R_F values shown in Table 1 correspond to the average of the values observed with standard solutions and in the faeces eluates from the *in vitro* experiment, in all the TLC determinations. These values agree well with those reported previously,[11–13] despite a minor modification to the solvent system, which contained ethylene glycol monoethyl ether instead of ethylene glycol monomethyl ether. The selection of the solvent system depends on the availability of the components and the use of cellulose as the adsorbent is

Table 1. Chromogenic behaviour and R_F values of metal - EDTA complexes in solution and in the faeces eluates from the *in vitro* experiment

Complex	Adsorbent	Solvent system*	Spray reagent†	Colour	R_F
Ca - $Na_2EDTA.3.5H_2O$	Cellulose	A	Murexide	Yellow - orange‡	0.58
	Cellulose	B			0.52
	Silica gel	A			0.54
	Silica gel	B			0.47
Mg - $Na_2EDTA.2H_2O$	Cellulose	A	Murexide	Bright yellow‡	0.60
	Cellulose	B			0.53
	Silica gel	A			0.57
	Silica gel	B			0.49
Cu - $Na_2EDTA.3H_2O$	Cellulose	A	PAN	Mauve	0.74
	Cellulose	B			0.62
	Silica gel	A			0.68
	Silica gel	B			0.64
Zn - $Na_2EDTA.3.5H_2O$	Cellulose	A	PAN	Pink	0.68
	Cellulose	B			0.63
	Silica gel	A			0.70
	Silica gel	B			0.67
Fe - Na_2EDTA	Cellulose	A	DPC	Red	0.56
	Cellulose	B			0.54
	Silica gel	A			0.57
	Silica gel	B			0.53
Mn - $Na_2EDTA.2H_2O$	Cellulose	A	Benzidine	Blue	0.73
	Cellulose	B			0.66
	Silica gel	A			0.73
	Silica gel	B			0.68

* A = water - ethyl methyl ketone - butanol - acetone (40 + 10 + 10 + 40); B = water - ethylene glycol monoethyl ether - butanol - acetone (45 + 20 + 25 + 10).

† Murexide = 1% aqueous solution of murexide followed by 15 N NH_3 solution; PAN = 0.25% ethanolic solution of 1-(2-pyridylazo)-2-naphthol followed by 15 N NH_3 solution; DPC = 1% ethanolic solution of diphenylcarbazide followed by 15 N NH_3 solution; benzidine = 5% aqueous solution of sodium peroxide followed by a 1% solution of benzidine in glacial acetic acid.

‡ The colour disappears on drying.

recommended owing to the shorter development time achieved.

In the *in vivo* experiments, none of the twelve volunteers excreted a compound in their faeces with the chromatographic characteristics of these complexes, either after the ingestion of 10 mg of Fe as Fe(III) - EDTA with a meal or after the ingestion of both Fe(III) - EDTA and an excess of cations with the meal.

Many studies have been carried out during the last 40 years to find a suitable salt that could be used for iron fortification. Despite its many advantages, the use of Fe(III) - EDTA has been limited to two field trials, one in Thailand, in which a fish sauce was fortified with this compound,[14] and another in Guatemala, with sugar as the food vehicle.[15] In both studies the parameters of iron nutrition improved in comparison with those of a control population. However, before Fe(III) - EDTA can be used in a large-scale iron fortification programme, it is necessary to determine whether the complex interacts with other trace mineral nutrients present in food.

Previous studies with swine to which ^{55}Fe - [^{14}C]EDTA (sodium salt) was administered orally indicated that more than 97% of the iron from the EDTA complex is split in the lumen of the gastrointestinal tract, and the question arose as to whether such EDTA free from iron could have reacted with other cations contained in the meal. Evidence from other studies seems to indicate that Fe(III) - EDTA does not have an adverse effect on the bioavailability of copper and zinc.[15–17] The results presented here indicate that apparently such a reaction did not take place because either the EDTA suffers a structural modification during the process of digestion that affects its property as a chelating agent, or the conditions present in the lumen of the gut are not suitable for the formation of these complexes.

Partial financial support provided by Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT) and United Nations University (Japan) is gratefully acknowledged.

References

1. Technical Report Series, No. 580, World Health Organization, Geneva, 1975.
2. Gonzalo, G., Faradori, A., Navarrete, C., Burgos, G., and Lira, P., *Rev. Med. Chile*, 1979, **107**, 105.
3. Layrisse, M., and Martinez-Torres, C., *Am. J. Clin. Nutr.*, 1977, **30**, 375.
4. Viteri, F., Garcia-Ibañez, R., and Torun, B., *Am. J. Clin. Nutr.*, 1978, **31**, 961.
5. McPhail, A., Bothwell, T., Torrance, J., Derman, D., Bezwoda, W., and Charlton, R., *Br. J. Nutr.*, 1981, **45**, 215.
6. Layrisse, M., *Excerpta Med. Int. Congr. Ser.*, 1975, **25**, 366.
7. Sawyer, D., and Paulsen, P., *J. Am. Chem. Soc.*, 1958, **80**, 1597.
8. Sawyer, D., and Paulsen, P., *J. Am. Chem. Soc.*, 1959, **81**, 816.
9. Sawyer, D., and McKinnie, J., *J. Am. Chem. Soc.*, 1960, **82**, 4191.
10. "Recommended Dietary Allowances," Ninth Edition, National Academy of Sciences, Washington, 1980.
11. Masoomi, Z., and Haworth, D., *J. Chromatogr.*, 1970, **48**, 581.
12. Vanderdeelen, J., *J. Chromatogr.*, 1969, **39**, 521.
13. Tsunoda, Y., Takeuchi, T., and Yoshino, Y., *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo*, 1964, **14**, 64.
14. Garby, L., *Ann. Trop. Med. Parasitol.*, 1974, **68**, 467.
15. Viteri, F., "Minutes of the International Anemia Consultative Group, New Delhi, April 1983."
16. Candela, E., Camacho, M., Martínez-Torres, C., Perdomo, J., Mazzari, G., Acurero, G., and Layrisse, M., *J. Nutr.*, 1984, **114**, 2204.
17. Solomons, N., Jacob, R., Pineda, O., and Viteri, F., *J. Nutr.*, 1979, **109**, 1519.

Paper A5/200
Received June 5th, 1985
Accepted June 13th, 1985