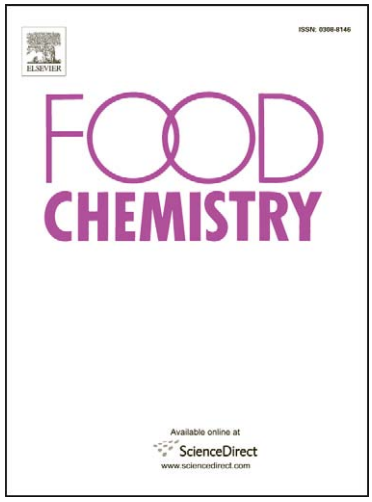
Food Chemistry, Volume 123, Issue 2, 15 November 2010, Pages 281-285

Accepted Manuscript



Ascorbate enhances iron uptake into intestinal cells through formation of a $FeCl_3$-ascorbate complex

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PII: S0308-8146(10)00487-5
DOI: 10.1016/j.foodchem.2010.04.031
Reference: FOCH 9555

To appear in: *Food Chemistry*

Received Date: 29 October 2009
Revised Date: 9 March 2010
Accepted Date: 20 April 2010

Please cite this article as: Thumser, A.E., Rashed, A.A., Sharp, P.A., Lodge, J.K., Ascorbate enhances iron uptake into intestinal cells through formation of a $FeCl_3$-ascorbate complex, *Food Chemistry* (2010), doi: 10.1016/j.foodchem.2010.04.031

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Ascorbate enhances iron uptake into intestinal cells through formation of a $FeCl_3$-ascorbate complex.

Running title: Ascorbate enhances iron uptake by forming a $FeCl_3$-ascorbate complex.

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ABSTRACT

It has been well documented that ascorbate enhances iron uptake, with a proposed mechanism based on reduction to the more absorbable ferrous form. We have performed a study on the effects of ascorbate on ferric iron uptake in the human epithelial Caco-2 cell-line. Ascorbate increased uptake in a concentration-dependent manner with a significant difference between iron uptake and reduction. Uptake kinetics are characteristic of a non-essential activator and the formation of an Fe^{3+}-ascorbate complex. This investigation provides evidence that ascorbate enhances the apical uptake of ferric iron into Caco-2 cells through the formation of a Fe^{3+}-ascorbate complex.

Keywords:

Enterocyte iron uptake; Ascorbate; Ferric iron

Abbreviations:

DMT1: Divalent Metal Transporter-1; IMP: Integrin-Mobilferrin pathway

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1. Introduction

An adequate dietary supply of iron is essential both to maintain red blood cell production and for the normal function of a number of key metabolic enzymes. Body iron is maintained primarily by regulation of the absorption of dietary iron in the proximal small intestine (Sharp, Tandy, Yamaji, Tennant, Williams & Singh Srai, 2002; Sharp & Srai, 2007). Dietary iron is found in two basic forms, as haem or non-haem, in the form of iron oxides, salts or chelates. Non-haem iron is the predominant form in most diets, but the bioavailability of this form is relatively low (1-10%) (Sharp & Srai, 2007). The uptake of dietary non-haem iron into the gastrointestinal (GI) tract requires protein-mediated transport, and two separate enterocyte pathways have been identified, namely the Divalent Metal Transporter-1 (DMT1) and the Integrin-Mobilferrin pathway (IMP) (Conrad & Umbreit, 2000; Conrad et al., 2000). DMT1 requires the iron to be in the ferrous form (Fe^{2+}), and as a major proportion of dietary iron is in the ferric form (Fe^{3+}) it needs to be reduced prior to uptake by DMT1 in the enterocytes. Several mechanisms are potentially available to reduce non-haem iron into the ferrous form. The brush border of duodenal enterocytes and cultured intestinal cells possess ferric reductase enzyme activity (Dcytb) (Latunde-Dada, Van der Westhuizen, Vulpe, Anderson, Simpson & Mckie, 2002), and overexpression of Dcytb in intestinal cells has been shown to stimulate iron uptake (Latunde-Dada, Simpson & Mckie, 2008). Non-haem iron can also be reduced by dietary factors, the most important being ascorbate that can reduce ferric iron to the more absorbable ferrous form (Teucher, Olivares & Cori, 2004). The presence of ascorbate has been previously shown to increase iron uptake in a dose-dependent fashion *in vitro* (Han, Failla, Hill, Morris & Smith, 1995; Kalgaonkar & Lonnerdal, 2008) and to increase the absorption of iron in human studies (Olivares, Pizarro, Pineda, Name, Hertrampf & Walter, 1997; Lopez & Martos, 2004; Moretti, Zimmermann, Wegmuller, Walczyk, Zeder & Hurrell, 2006). Following reduction, the Fe^{2+} becomes a substrate for DMT1 (Sharp & Srai, 2007). In contrast to DMT1, the IMP pathway

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transports solely ferric iron, but not other metals of nutritional importance, and requires the chelation of ferric iron by mucins before interaction with β3-integrin and mobilferrin (Conrad & Umbreit, 2002; Sharp & Srai, 2007).

The purpose of this study was to gain a better understanding of the mechanism by which ascorbate enhances the uptake of ferric iron into the small intestine, using the Caco-2 cell model of human small intestinal enterocytes (Yamaji, Tennant, Tandy, Williams, Singh Srai & Sharp, 2001). We have used a kinetic approach and interpreted the results to show that ferric iron forms a complex with ascorbate, which is subsequently transported into the cells by a DMT1-independent mechanism.

2. Materials and methods

2.1. Cell culture

Caco-2 TC7 cells were maintained in 25-cm^2 plastic flasks. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) from GIBCO (Grand Island, NY), supplemented with 20% v/v foetal calf serum (GIBCO), 1% v/v non-essential amino acids (GIBCO), 1% antibiotic antimycotic (penicillin-steptomycin) solution (GIBCO) and 1% v/v L-glutamine (Biowhittaker Europe; Verviers, Belgium). The cells were maintained at 37 ºC in an incubator with 5% carbon dioxide (CO_2), 95% air atmosphere at constant 95% relative humidity, and the medium replaced every 2 days. For iron uptake experiments, the cells were seeded at a density of 40,000 cells/cm^2 in collagen-treated six-well plates (Costar Corp.; Cambridge, MA) and used at 21 days after seeding (Johnson, Yamaji, Tennant, Srai & Sharp, 2005).

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2.2. Iron uptake studies

Caco-2 cells were transferred to serum-free medium for 24 hours before the start of experiments. The cells were washed by incubation with buffer (140 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 10 mM MES, 0.5 mM of $MgCl_2$, 1 mM $CaCl_2$ pH 6.0) for 2 minutes at 37 ºC. In order to measure iron uptake, 60 kBq of ^{55}Fe(II) were added to each culture well and incubated for 15 minutes at 37 ºC. Uptake was stopped by washing the cells three times with ice-cold buffer and the cells were solubilised in 1 ml of 200 mM NaOH overnight (4 ºC). Intracellular radioactivity was determined by scintillation counting.

The data was fitted to a hyperbolic equation by non-linear regression (SigmaPlot, Systat Software Inc. San Jose):

$$\text{Uptake} = V_{max} \times [FeCl_3]/(K_m + [FeCl_3])$$

where $[FeCl_3]$ is the concentration of substrate, V_{max} is the calculated maximum velocity of the reaction and K_m is the calculated Michaelis constant, an indicator of substrate affinity (Segel, 1975).

2.3. Cell viability assay

To ensure that our iron and ascorbate treatments did not induce cytotoxicity which might in turn influence iron reduction and uptake, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine cell viability. This assay measures the cleavage of MTT to a formazan derivative by mitochondrial enzymes and there is a linear relationship between the amount of formazan produced and the number of viable cells (Mosmann, 1983; Ferrari, Fornasiero & Isetta, 1990). To each well of a 96-well culture plate was added 10 µl MTT reagent and the plate was incubated for 3-4 hours (37 ºC), before the addition of 100 µl DMSO per well to solubilise the cells. The absorbance of formazan product was determined at 570 nm.

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2.4. Ferric reduction assay

The conversion of Fe^{3+} to Fe^{2+} in the absence or presence of ascorbate was measured spectrophotometrically by using a chromogenic ferrous iron chelator (bathophenanthrolinedisulfonic acid, BPDS) (Timmerman & Woods, 1999). Uptake buffer (700 µl) containing various concentrations of Fe^{3+} (0-30 µM) was added to a cell culture well containing 21-day post-confluence Caco-2 cultures and incubated for 15 minutes (37 ºC). The buffer was collected (4 ºC) and 500 µl of 2 mM BPDS added before incubation for 1 hour at 37 ºC. The concentration of Fe(II)-BPDS complex was measured at 534 nm, using a $FeCl_2$ standard curve.

2.5. Statistics and data analysis

Kinetic parameters were determined by fitting the data to a hyperbolic curve by non-linear regression (Prism 5 for Windows, GraphPad Software Inc.). Two-way ANOVA with Bonferroni post-test was used to determine statistical significance (Prism 5 for Windows, GraphPad Software Inc.).

3. Results and Discussion

The aim of this study was to kinetically investigate the uptake of non-haem iron by Caco-2 cells, and its promotion by ascorbate. The uptake of $FeCl_3$ across the apical membrane of Caco-2 cells has been well-documented to be a protein-mediated process, with several proteins capable of playing the role of iron transporter (Garrick et al., 2006). This observation was confirmed by the saturable, hyperbolic nature of $^{55}Fe(II)$ uptake (Fig. 1), with a calculated V_{max} of 304 pmol/h and K_m of 13.2 µM. A previous study showed that ferrous iron uptake by Caco-2

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cells was also saturable, with a K_m of 7 µM (Linder, Moriya, Whon, Kassa & Gilley, 2006), a value that is essentially identical to that obtained in our study (Fig. 1). It has previously been shown that the reduction of ferric iron is required for uptake into Caco-2 cells (Han et al., 1995), and a potent enhancer of iron uptake is ascorbate (vitamin C) (Teucher, Olivares & Cori, 2004), which is capable of reducing ferric to ferrous iron (Johnson, Yamaji, Tennant, Srai & Sharp, 2005). In our studies the presence of ascorbate in the cell culture media significantly increased the rate of iron uptake in a concentration-dependent manner (Fig. 2; Table 1), and had no effect on cell viability at concentrations up to 30 µM ascorbate (data not shown).

The process by which ascorbate increases iron uptake has previously been ascribed to the reductive properties of ascorbate that increase the conversion of ferric to ferrous iron (Teucher et al., 2004), the latter then being a substrate for the membrane transporter DMT1 that is specific for ferrous iron (Sharp et al., 2002; Sharp & Srai, 2007). Indeed, it is believed that ascorbate secreted in gastric juice is present to reduce dietary iron, preventing the formation of non-bioavailable ferric chelates (Atanasov, Li, Bjarnason, Tzatchev & Simpson, 2005). If the reduction of ferric iron by ascorbate, or any other dietary reductant, was obligatory for the intestinal uptake of dietary ferric iron, then we should not observe a protein-mediated ferric iron uptake process. As shown here (Figs. 1 and 2) and in other studies (Han et al., 1995), this is clearly not the case, as the Caco-2 cells take up a low, basal level of iron in the absence of ascorbate. One proposed mechanism to explain this observation involves the presence of a membrane-bound ferric reductase, Dcytb, that can catalyse the reduction of ferric iron in the absence of ascorbate, or other dietary reducing agents, to stimulate iron reduction and uptake into Caco-2 cells (Yamaji et al., 2001; Latunde-Dada et al., 2002; Sharp & Srai, 2007; Latunde-Dada et al., 2008). However, the endogenous expression of Dcytb in Caco-2 cells is very low (Latunde-Dada et al., 2008).

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An alternative proposal is that ferric iron reduction is not essential and that there are separate pathways for the uptake of ferric and ferrous iron (Han et al., 1995). In order to separate the processes of reduction and increased uptake, both of which could potentially be catalysed by ascorbate, we determined ferric iron uptake rates and reduction under identical conditions (Fig. 3). Both iron uptake (Fig. 3A) and ferric iron reduction (Fig. 3B) were significantly increased in the presence of ascorbate. We surmised that if the promoting effect of ascorbate on iron uptake was due only to its role as a reductant, then a comparison of ^{55}Fe uptake *vs.* ferrous reduction should show a direct, linear correlation with a slope equal to one. This was observed in the absence of ascorbate, but there was a significant difference in the presence of ascorbate (Fig. 3C). This observation implies that a process distinct from iron reduction may explain the promoting effect of ascorbate and therefore a kinetic approach was used to determine the mechanism of rate enhancement observed with ascorbate.

Plots of ^{55}Fe uptake rates ($1/v$ *vs.* $1/[^{55}Fe]$) in the presence of ascorbate display mixed-type activation kinetics (Fig. 4) (Segel, 1975), with activation of ^{55}Fe uptake by ascorbate being saturable, as indicated by the finite V_{max} obtained (Fig. 4 inset). These observations are characteristic of a non-essential activator process (Segel, 1975) and indicate that ascorbate forms a complex with ferric iron that is then taken up into the Caco-2 cells. This finding implies that ascorbate functions as a chelating agent of ferric iron and suggests that an iron-ascorbate complex enters the cells directly, although we cannot determine whether the iron in this complex is in the Fe^{3+} or Fe^{2+} form.

To further explore the kinetics of ascorbate-induced iron uptake we undertook similar experiments in the presence of a known inhibitor. Previous studies have shown that Zn^{2+} inhibits the intestinal uptake of iron (Yamaji et al., 2001; Han et al., 1995; Solomons, Pineda, Viteri & Sandstead, 1983; Arredondo, Martinez, Nunez, Ruz & Olivares, 2006; Conrad, Umbreit, Moore, Uzel & Berry, 1994), but its effects are not mediated through DMT1 (Tandy et al., 2000; Garrick

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et al., 2003; Mackenzie, Takanaga, Hubert, Rolfs & Hediger, 2007). We thus investigated the inhibitory effect of Zn^{2+} on ferric iron uptake into Caco-2 cells, both in the absence and presence of ascorbate. The results showed mixed-type (non-competitive) inhibition of iron uptake (Fig. 5), with calculated K_i values of 141 and 236 μM Zn^{2+}, in the absence and presence of ascorbate, respectively. The similarity of the inhibition constants (K_i) is interpreted to indicate that the same protein complex is involved in ferric iron uptake across the apical membrane of Caco-2 cells. As there is an unidentified transporter pathway for iron and zinc in Caco-2 cells that is not DMT1 (Yamaji et al., 2001) and DMT1 is specific for ferrous iron (Alonso-Vale et al., 2008), our findings would imply that DMT1 is not involved in the uptake of the iron-ascorbate complex. Thus, a different transporter system must be involved in the uptake of the iron-ascorbate complex and one possibility is that this complex interacts with IMP-β3-intergrin-mobilferrin pathway, in a manner similar to that of Fe^{3+}-mucin transport complex (Sharp & Srai, 2007), while other options include Zip14, a transporter of both iron and zinc in the duodenum, and the SVCT1 ascorbate transporter (Liuzzi, Aydemir, Nam, Knutson & Cousins, 2006; Wilson, 2005).

In conclusion, this investigation provides evidence that ascorbate, in addition to its well-characterised reduction of ferric iron to provide a bioavailable supply of ferrous iron to DMT1, also enhances directly the uptake of ferric iron into Caco-2 cells through the formation of an iron-ascorbate complex. DMT1 is not involved in the uptake of the iron-ascorbate complex and further work is required to identify the transporter system involved in the uptake of the iron-ascorbate complex into intestinal enterocytes.

Acknowledgements: Funding was provided for Aswir Abd Rashid by the Public Service Department of Malaysia, Kuala Lumpur, Malaysia.

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Figure legends:

Figure 1: Uptake of ^{55}Fe into Caco-2 cells in the absence of ascorbate is saturable and displays hyperbolic kinetics. The data were fitted (solid line) to a hyperbolic equation: rate of Fe^{3+} uptake (v) = [S] × V_{max}/(K_m + [S]), where [S] is the concentration of $FeCl_3$ substrate, V_{max} is the calculated maximum rate of ^{55}Fe uptake and K_m is the calculated Michaelis constant at every ascorbate concentration. The calculated V_{max} and K_m values were 304 ± 18 pmol/h and 13.2 ± 1.6 μM, respectively

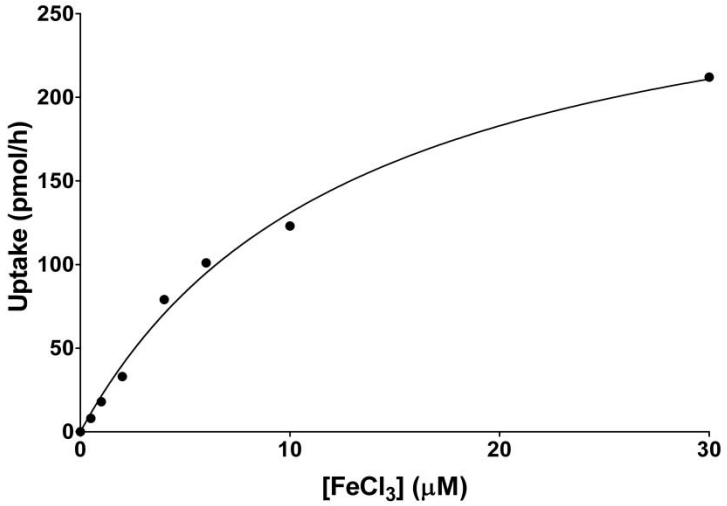

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Figure 2: Uptake of ^{55}Fe into Caco-2 cells is increased by the presence of ascorbate at concentrations of 0 (■), 0.5 (▲), 1.0 (□), 5.0 (▼) and 10.0 (○) μM ascorbate. The data were fitted separately for every ascorbate concentration to a hyperbolic equation: rate of Fe^{3+} uptake (v) = [S] × V_{max}/(K_m + [S]), where [S] is the concentration of $FeCl_3$ substrate, V_{max} (pmol/h) is the calculated maximum rate of ^{55}Fe uptake and K_m (μM) is the calculated Michaelis constant at every ascorbate concentration. $r^2 > 0.985$ for all fitted data.

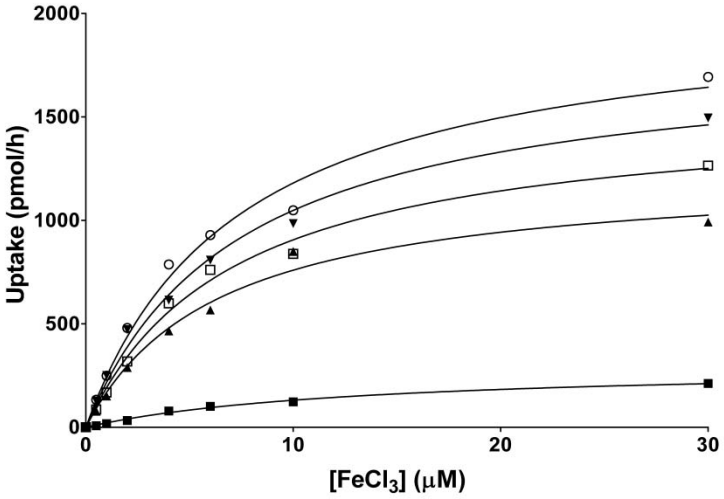

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Figure 3: Effect of ascorbate on the uptake of ^{55}Fe or the reduction of Fe^{2+} to Fe^{3+} into Caco-2 cells. (A) Uptake of ^{55}Fe (□, ■) and (B) reduction of Fe^{2+} to Fe^{3+} (○, ●) was measured in the presence (□, ○) or absence of 10 μM ascorbate (■, ●). Data were analysed by two-way ANOVA: ***, $p < 0.001$; ** $p < 0.01$ for comparison of relevant data sets at each time point. (C) Comparison $FeCl_3$ uptake *vs.* ferrous formation in the presence (●) and absence (○) of 10 μM ascorbate. Linear regression analysis indicates a significant difference between the slopes (P = 0.01), with values of 0.75 ± 0.03 (r^2 0.99) and 1.03 ± 0.09 (r^2 0.97) in the presence and absence of ascorbate.

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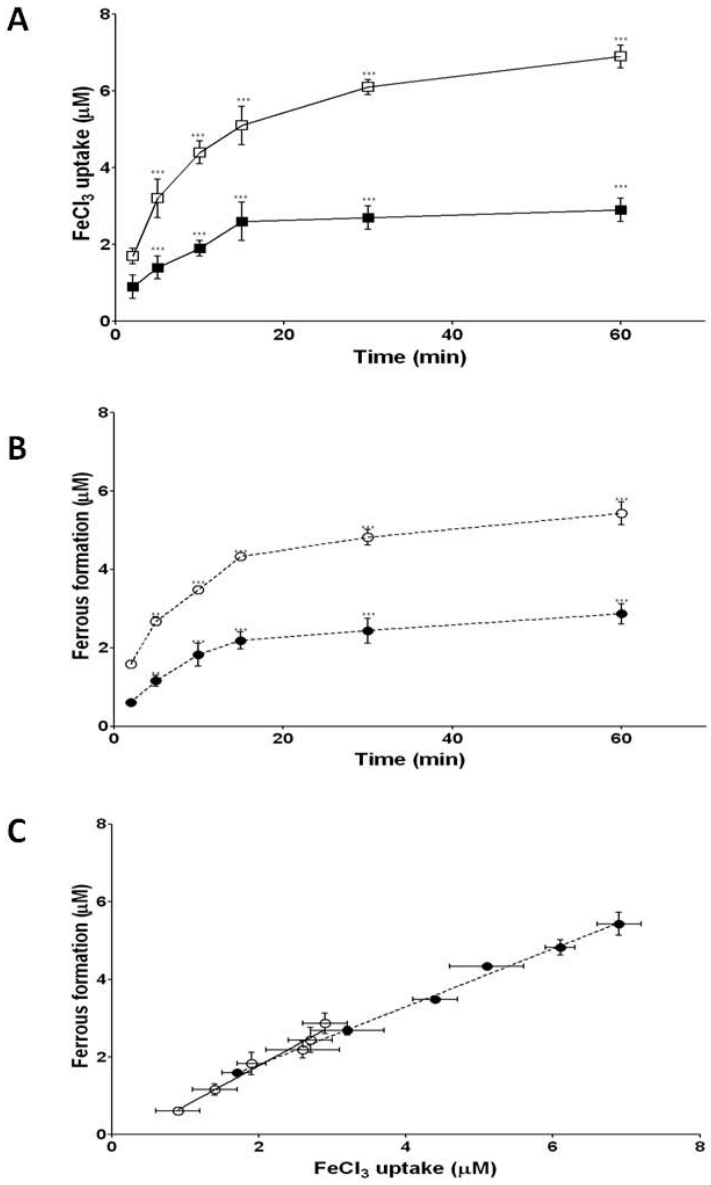

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Figure 4: Effect of ascorbate on the uptake of ^{55}Fe into Caco-2 cells. Data from the same experiment as in Figure 1 are plotted as 1/*v vs.* [ascorbate]), at 0 (■), 1 (Δ), 2 (□), and 30 (◆) μM $FeCl_3$. The data were fitted to a hyperbolic equation ($v = [S] \times V_{max}/(K_m + [S])$, as indicated by the solid lines. The insert shows a plot of $1/V_{max}$ *vs.* [ascorbate], with V_{max} determined by fitting of the experimental data to the hyperbolic equation.

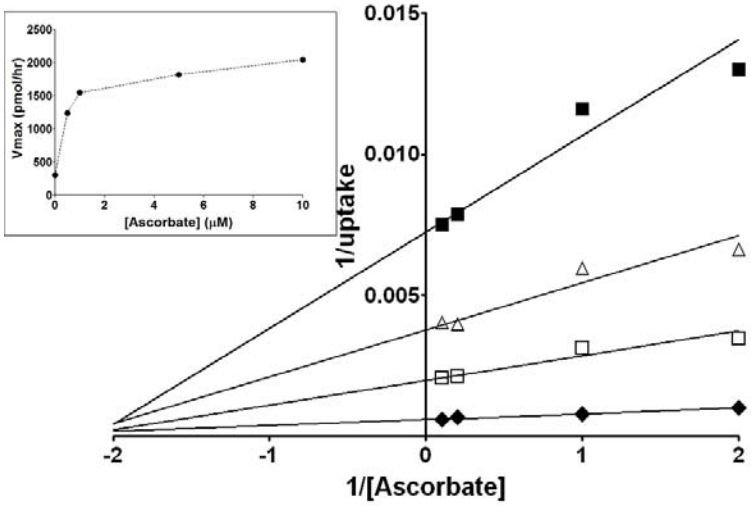

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Figure 5: Effect of zinc on ^{55}Fe uptake into Caco-2 cells in the (A) presence or (B) absence of ascorbate. Zinc concentrations were 0 (○), 100 (□) and 200 (Δ) μM in the presence of ascorbate, and 0 (●), 100 (■) and 200 (▲) μM in the absence of ascorbate. The data was fitted to a hyperbolic equation, as indicated by the solid lines. (C) A replot of the calculated K_m/V_{max} ratio *vs.* zinc concentration in the presence (▽) or absence (◆) of 10 μM ascorbate. The data were fitted by linear regression, as indicated by the solid lines.

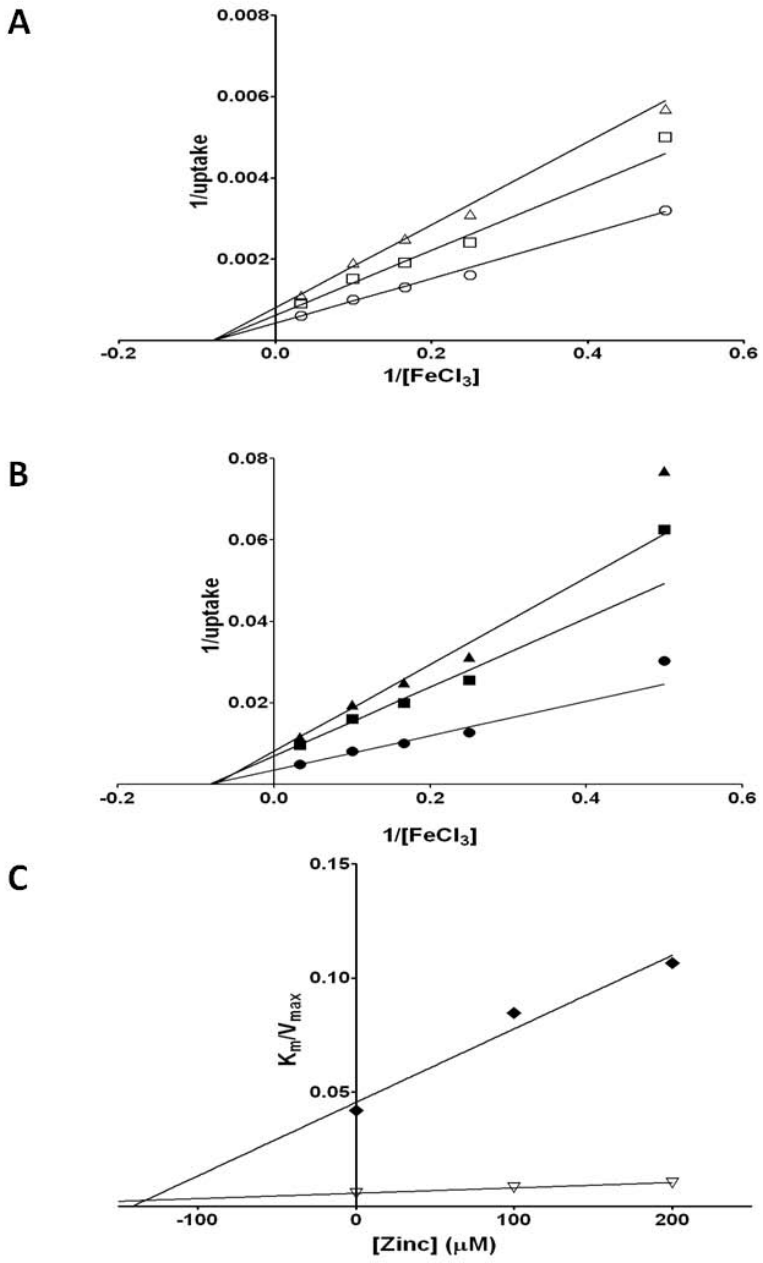

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Table 1: Kinetic parameters for ^{55}Fe uptake into Caco-2 cells in the presence of 0, 0.5, 1.0, 5.0 and 10.0 μM ascorbate.

[**Ascorbate**] (μM)	K_m (μM)	V_{max} (pmol/h)
0	13.2 ± 1.6	304 ± 18
0.5	6.4 ± 1.0	1242 ± 75
1.0	7.1 ± 0.8	1546 ± 77
5.0	7.4 ± 0.9	1820 ± 91
10.0	7.3 ± 1.1	2044 ± 123

The data were fitted separately for every ascorbate concentration to a hyperbolic equation: rate of Fe^{3+} uptake (v) = [S] × V_{max}/(K_m + [S]), where [S] is the concentration of $FeCl_3$ substrate, V_{max} (pmol/h) is the calculated maximum rate of ^{55}Fe uptake and K_m (μM) is the calculated Michaelis constant at every ascorbate concentration. $r^2 > 0.985$ for all fitted data. Two-way ANOVA indicated a significant effect of ascorbate concentration on K_m and V_{max} ($p < 0.001$).

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Fig 1

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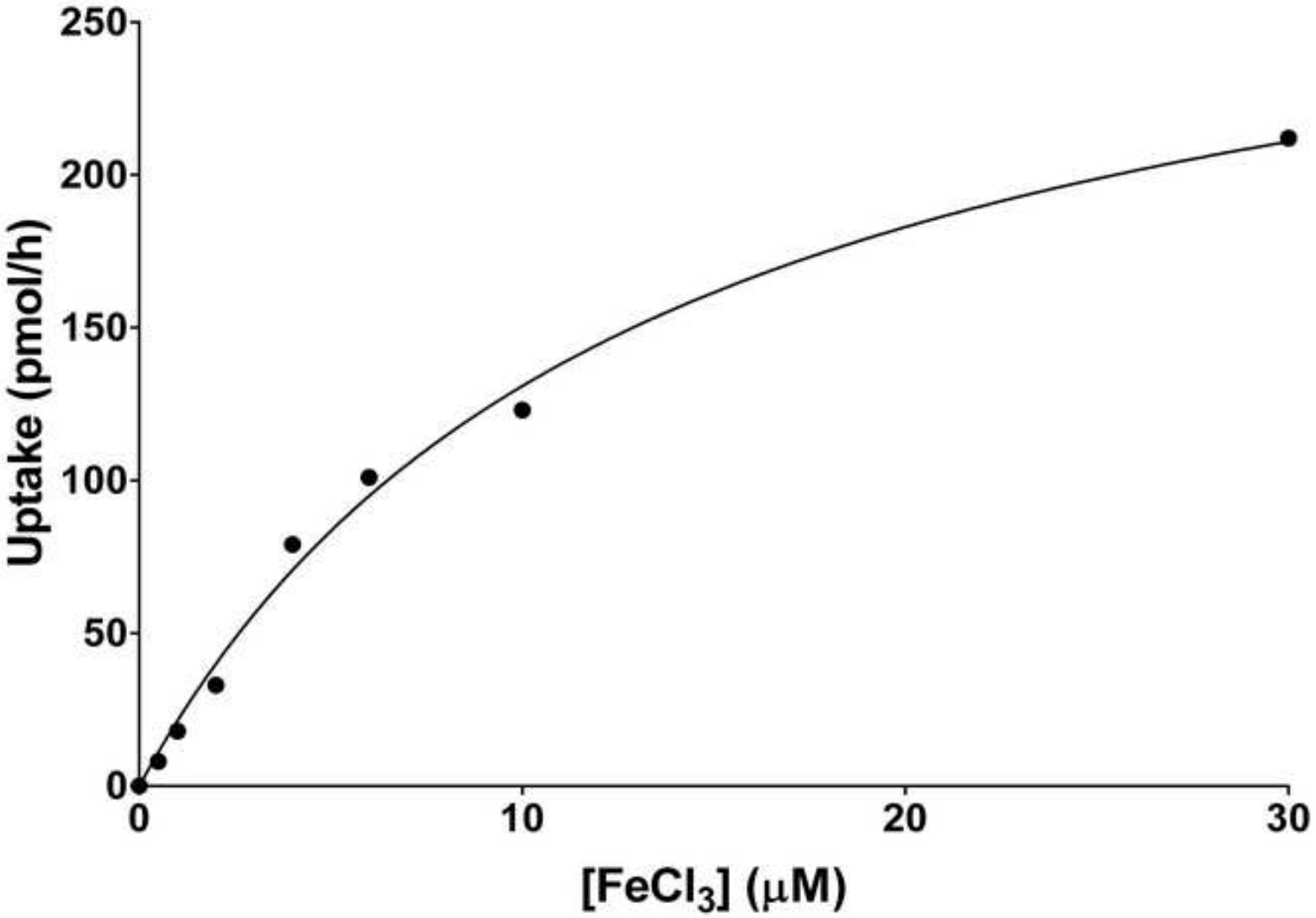

Fig 2

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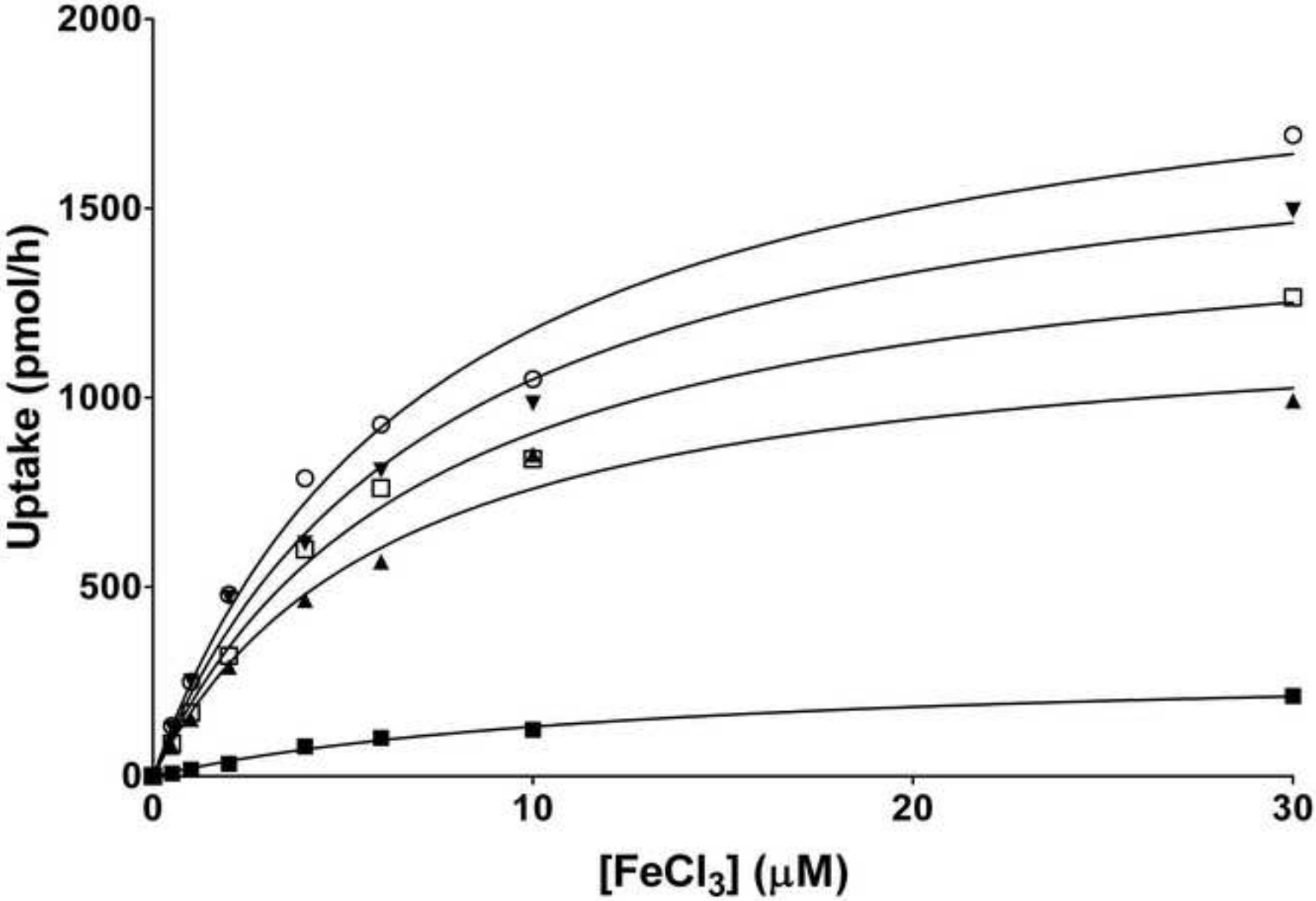

Fig 3

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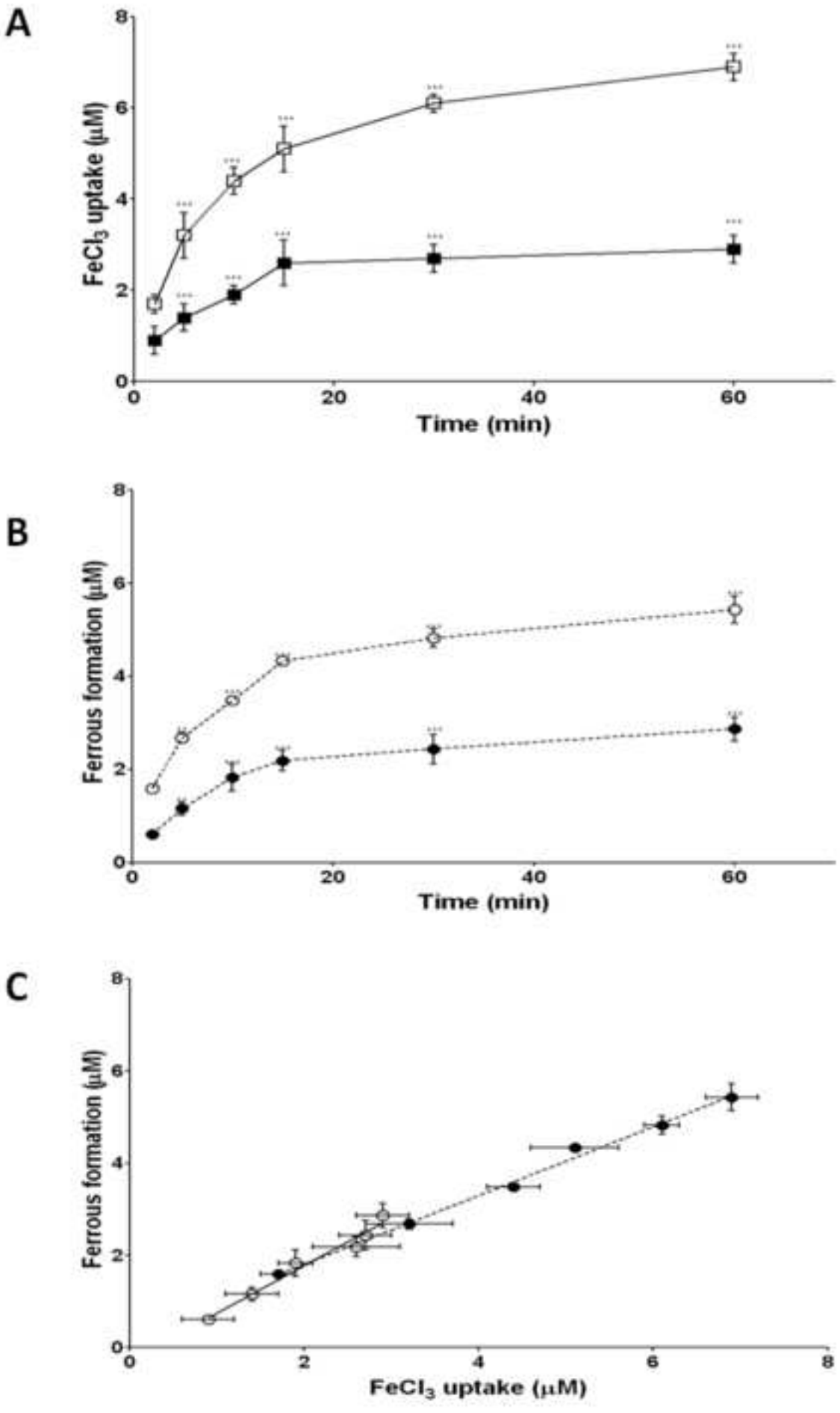

Fig 4

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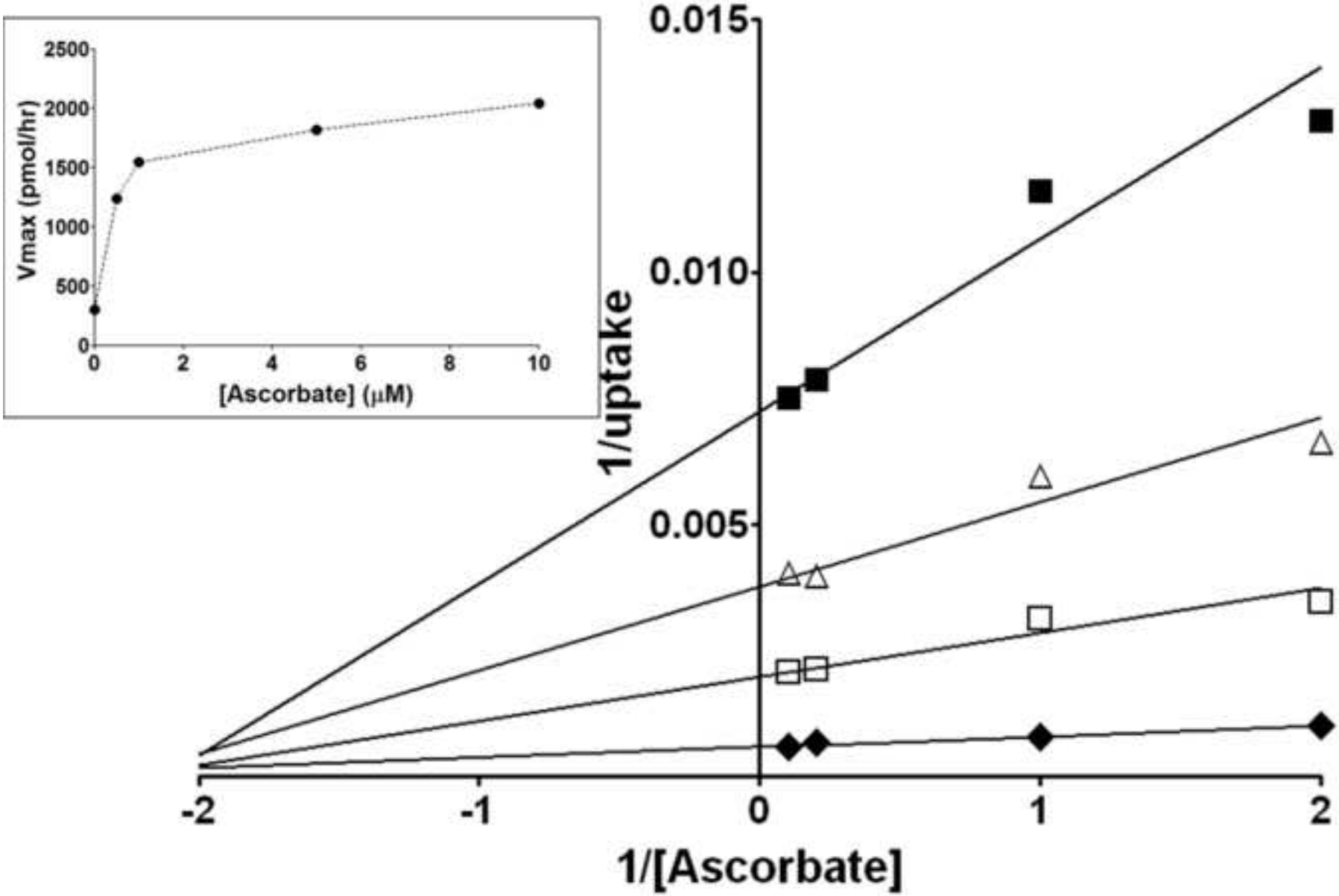

Fig 5

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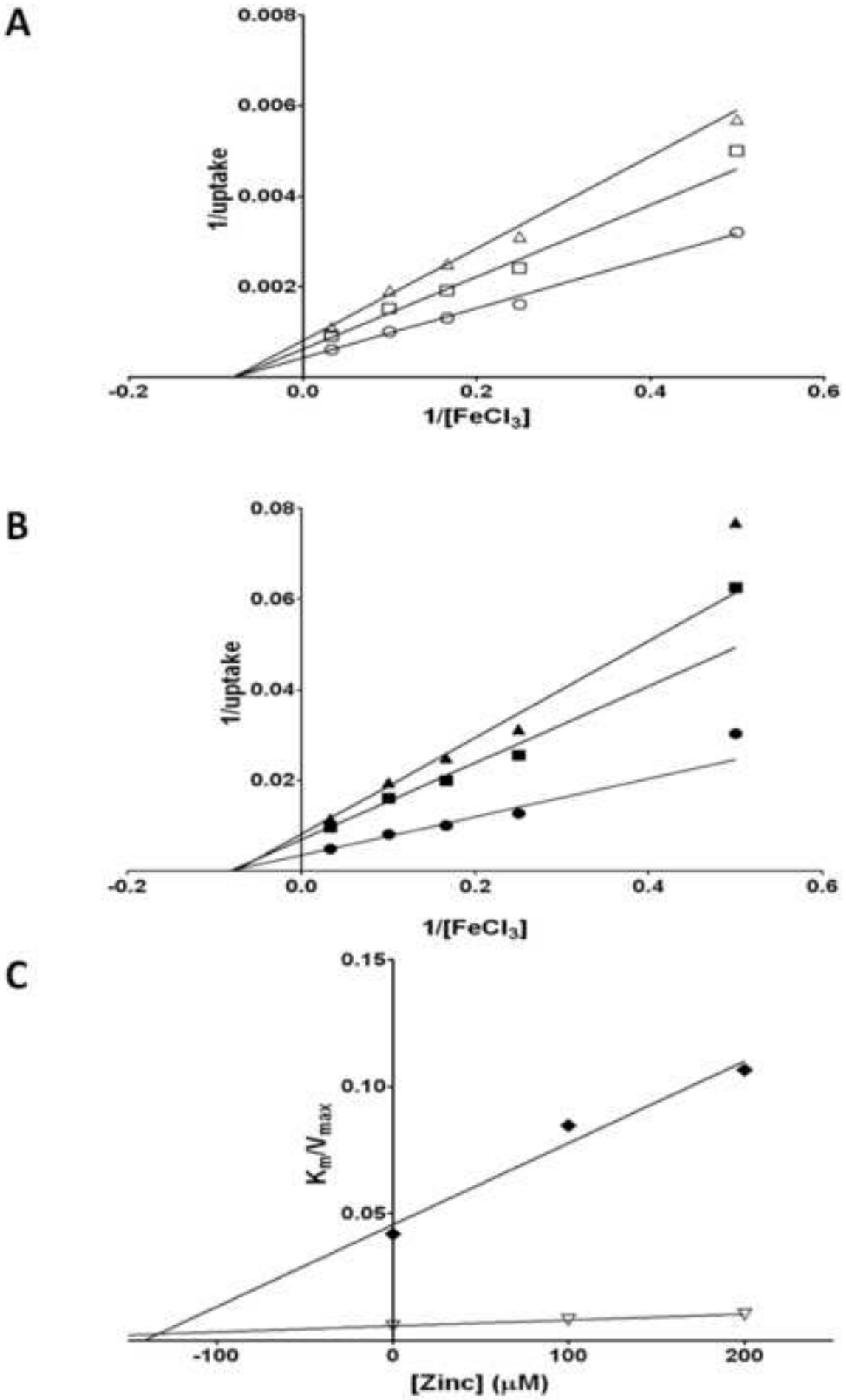

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Ascorbate enhances iron uptake into intestinal cells through formation of a FeCl3-ascorbate complex

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2010-11-15T00:00:00Z
Elsevier Science B.V., Amsterdam.
http://dx.doi.org/10.1016/j.foodchem.2010.04.031
Alfred E. Thumser, Aswir Abd Rashed, Paul A. Sharp, John K. Lodge; Ascorbate enhances iron uptake into intestinal cells through formation of a FeCl3-ascorbate complex, Food Chemistry, Volume 123, Issue 2, 15 November 2010, Pages 281-285

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