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The influence of citrate, maltolate and fluoride on the gastrointestinal absorption of aluminum at a drinking water-relevant concentration: A ^{26}Al and ^{14}C study

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This work was carried out in the College of Pharmacy building, University of Kentucky, Lexington, KY, 40536-0082. AMS analysis was conducted at the PRIME Lab, Purdue University.

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Abstract

The objectives were to test the null hypotheses that (1) citrate, maltolate, and fluoride do not significantly influence oral Al bioavailability, C_{max} or T_{max} at an Al dose relevant to drinking water exposure; and (2) Al citrate and maltolate are absorbed intact from the gastrointestinal tract. Male Fisher rats were given 1 ml of solution intra-gastrically containing 1 nCi ^{26}Al (65 nmol total Al) as the Al^{3+} ion, or as complexes with ^{14}C-citrate, ^{14}C–maltolate or fluoride, during concurrent ^{27}Al iv infusion. Blood was repeatedly collected for serum ^{26}Al, total Al and ^{14}C quantification. Absorption parameters were estimated using WinNonlin. Al bioavailability, C_{max} and T_{max} from the ion, citrate, maltolate, and fluoride were 0.29 ± 0.11, 0.61 ± 0.31, 0.50 ± 0.25, and 0.35 ± 0.10%; 659 ± 195, 1073 ± 250, 881 ± 356, and 880 ± 295 fg/ml; and 1.2 ± 0.9, 1.0 ± 1.1, 1.3 ± 1.0, and 1.0 ± 0.9 h (X ± SD) respectively. Serum ^{14}C was ~100 times higher than ^{26}Al. The results suggest a non-significant enhancement of oral Al bioavailability by citrate and maltolate, some Al complex dissociation in the GI tract, and less absorption of Al than citrate or maltolate. The presence of citrate, maltolate and fluoride, at a similar molar concentration to Al, would not be expected to greatly influence Al absorption from drinking water.

Keywords: Accelerator mass spectrometry, aluminum bioavailability, ^{26}Al, ^{14}C, chemical species

Abbreviations:

Al	aluminum
AMS	accelerator mass spectrometry
C_{max}	maximum blood concentration

cta	citrate
ETAAS	electrothermal atomic absorption spectrometry
malt	maltolate
T_{max}	time after dosing of C_{max}

Introduction

Aluminum (Al) has no demonstrated essential function in mammals. It is a neurotoxicant. It has been suggested that Al is associated with Alzheimer's disease, although this is controversial [1]. In patients receiving renal dialysis, Al can cause dialysis encephalopathy, renal osteodystrophy and a hypochromic microcytic anemia [2]. Experiments with rats and mice demonstrated embryo/fetal toxicity after oral administration of a variety of Al salts [3, 4].

Various Al compounds have been used to study Al toxicity, including Al chloride and nitrate salts, and Al complexes with citrate, fluoride, lactate, and maltolate [5]**.** Little attention was paid to Al speciation in most studies. There is evidence that some Al species are more toxic than others. Citric acid has been used in drinking water treatment as a membrane cleanser and as a pile/well cleaning aid, and is present in many dietary sources such as fruit juices and soft drinks. Al forms relatively strong complexes with citrate by binding through its carboxyate and □-hydroxyl groups [6, 7] . It has been suggested that citrate is a major factor in the toxicity of orally administrated Al [8, 9]. Maltol (3-hydroxy-2-methyl-4H-pyran-4-one), a natural product and an approved food additive in the US and Australia, is used as a flavor enhancer in beverages like coffee and chocolate milk and as a favoring agent in breads and cakes. At neutral pH and mM Al concentrations, $Al(maltolate)_3$ is soluble and stable to hydrolysis [10]. Al maltolate was more toxic to animals and neuronal and glial cells than Al lactate or Al chloride [10, 11]. Oral administration of Al maltolate resulted in increased brain Al [12, 13]. Fluoride is commonly present in drinking water. Al and fluoride can form stable complexes [14]. By mimicking phosphate AlF_3 and AlF_4^- can inhibit GTP_{ase} activity and affect the activity of a variety of phosphoryl transfer enzymes which are important in cell signal transduction or energy metabolism [15]. The addition of

fluoride to i.p. injections of Al increased Al-induced behavioral toxicity [16]. Consumption of Al fluoride in drinking water by rats for 1 year resulted in increased neuronal abnormalities [17]. These studies raised concern about the concurrent presence of fluoride and Al in drinking water.

Major sources of Al intake include drinking water, food and medications [18]. Oral Al bioavailability from drinking water was estimated in several studies in rats to be 0.05 to 0.36%, based on urinary Al output or Al in urine plus Al retained in bone, liver and brain [19-21]. Oral Al bioavailability was estimated to be 0.25 to 0.4% from a comparison of areas under the curve (AUC) of serum Al concentration versus time when Al was given po and iv [22]. Several studies showed that a high dose of citrate enhanced Al absorption from both pharmacological and physiological Al exposures, and at various pHs [19, 20, 23, 24]. However, none of the previous studies determined the Al species at the exposure conditions.

Plasma citrate and Al concentrations were measured in 3 humans after oral administration of 280 mg Al and 3.2 gm citrate [25]. The authors concluded it was unlikely that Al was absorbed as Al citrate because plasma citrate concentrations had returned to baseline values before the Al absorption peak occurred. However a similar study at an Al dose relevant to drinking water has not been reported.

The only reported study that estimated oral Al bioavailability from Al maltolate found it to be ~ 0.1% [21]. However the authors did not report the Al species, or Al:maltolate molar ratio under the conditions studied, preventing calculation of the Al species.

The interaction between fluoride and Al relevant to their oral absorption has been investigated in rats and mice [26]. Co-administration of fluoride or citrate with Al increased plasma Al levels, whereas Al decreased fluoride absorption. However Glynn et al. concluded that fluoride (50 mg/L) did not change Al absorption, based

on right femur Al concentration after 6 week oral exposure to 100 mg/L Al [23]. The bioavailability of Al in the presence of fluoride at a drinking water-relevant level has not been reported.

Accelerator mass spectrometry (AMS) is an ultra-sensitive analytical technique that has been applied to the measurement of rare nuclides such as ^{26}Al and ^{14}C, as in this study, enabling their use as tracers. AMS does not measure radionuclide decay. It counts individual isotope atoms, making it a very efficient technique to measure radioisotopes with long half-lives [27, 28]. It is the only method currently available to study Al absorption and kinetics at physiological concentrations. However, the high cost of ^{26}Al and ^{14}C analysis by AMS limits the use of this experimental method.

The U.S. Environmental Protection Agency currently recommends Al in drinking water be < 0.2 mg/L for aesthetic purposes (http://www.epa.gov/safewater/mcl.html). Additional research on the pharmacokinetics and toxicity of Al species in drinking water (e.g. Al fluoride) was needed as part of the consideration for development of drinking water regulations and guidance [29, 30]. The primary objective of the current study was to test the hypothesis that the absolute oral bioavailability of Al in rats is the same when dosed as the Al^{3+} ion in the absence of added ligands or in the presence of citrate, maltolate, or fluoride at a dose relevant to daily consumption of Al in drinking water by humans. ^{26}Al and AMS were used to address this objective. Al bioavailability, C_{max} and T_{max} in the absence or presence of ligands were also compared to test the null hypothesis that these ligands do not have an effect on Al absorption. By quantifying serum ^{14}C by AMS following oral administration of ^{26}Al ^{14}C-citrate and ^{26}Al ^{14}C-maltolate, the hypothesis that Al does not dissociate from citrate and maltolate in the GI tract and is absorbed as Al complexes was also tested. The Al

species of the administered solutions were predicted by computer modeling based on known Al-ligand binding constants, Al hydrolysis constants, Al and ligand concentrations, and pH. Citrate, maltolate and fluoride were selected for study as Al ligands in the current work because of their presence in the diet and/or drinking water and the concern that they increase Al toxicity.

Materials and Methods

Materials

^{26}Al (0.5 Ci/mole, ^{26}Al:^{27}Al ratio = 1:34) in 0.1 N HCl was obtained from the Purdue Rare Isotope Measurement Lab (PRIME Lab). ^{14}C-citric acid (109 mCi/mmol) was purchased from Amersham Biosciences. ^{14}C-maltol (50.9 mCi/mmol) was custom synthesized for this project by PerkinElmer. Sodium fluoride, sodium hydroxide and all other chemicals were obtained from Sigma. The solutions for oral dosing were prepared the day of their administration by combining the Al and ligand, from stock solutions, and incubating the resulting dosing solution for 1 h at room temperature. The pH was adjusted by addition of dilute NaOH with stirring. The Al solution for iv infusion was prepared by dissolving $AlK(SO_4)_2$ in saline. It was sterilized by filtration through a 0.22 μm filter.

Animals

The subjects were 23 male Fisher 344 rats, weighing 270 ± 18 gm (X ± SD). Animal work was approved by the University of Kentucky Institutional Animal Care and Use Committee. The research was conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

Experimental procedures

All rats were implanted with two femoral venous cannulae 1 day prior to oral dosing. This enabled iv administration through one cannula and blood withdrawal from another. The withdrawal cannula terminated upstream of the Al administration cannula to enable more accurate determination of the serum Al concentration. Oral Al absorption was determined in the un-anesthetized rat.

Systemic Al clearance was estimated in a pilot study following an iv bolus Al injection [22]. Oral Al bioavailability was calculated by comparison of the areas under the plasma Al concentration curves following concurrent oral and iv Al doses, where the tracer ^{26}Al was used in the oral dose and ^{27}Al as the iv infusion dose. We did not give the iv ^{27}Al as a large bolus dose because it would produce plasma Al concentrations in excess of the capacity of transferrin to bind Al, resulting in Al citrate species that would probably be eliminated at a different rate than Al transferrin. This would not model the normal species of Al in plasma, which is > 90% Al transferrin [7]. Rather, we infused ^{27}Al at a rate selected to maintain a plasma Al concentration of ~ 500 ng/ml, as described [22].

Twenty-two rats received an iv infusion of ^{27}Al at 100 µg Al/kg/h as $AlK(SO_4)_2$ in saline from 14 h prior to 24 h after oral dosing. One rat was randomly assigned to receive an iv infusion of saline to measure the endogenous serum Al concentration. The 22 subjects were randomly assigned to receive 1 ml of MilliQ-purified water or 1 ml of a solution containing ^{26}Al (52 ng [1 nCi] ^{26}Al and ~1700 ng ^{27}Al, total 65 nmol Al, therefore 65 µM) by gastric administration. This was given in the absence of ligands or in the presence of citrate, maltolate or fluoride. The MilliQ water-dosed group had 2 rats to monitor ^{26}Al and ^{14}C contamination of samples. Each Al treatment group had 5 rats. The ratio of total Al to ligand was 1:1 for citrate (containing 30 nmol [5764 ng; 3270 nCi] ^{14}C-citric acid), 1:3 for maltolate (containing 30 nmol [3784 ng; 1530 nCi] of ^{14}C-maltol) and 1:4 for fluoride. The pH of the administered solution was adjusted to ~ 5 for the free Al^{3+} ion, ~ 7 for Al in the presence of citrate or maltolate, and ~ 4 for Al in the presence of fluoride. To assess if there was significant loss of Al due to adsorption to the syringe or gastric feeding needle used to deliver the oral Al solution, the oral delivery procedure was simulated by delivery of identical Al

solutions into a plastic tube. The delivered Al concentration was compared to the solution for delivery. The delivered solutions of the Al ion, citrate, maltolate and fluoride, contained 104, 92, 97 and 107% of the Al concentration of the original solution, showing no significant adsorption loss of Al to the syringe or feeding needle.

Al speciation in the solutions prepared for gastric administration was calculated from pH 2 to 8 using the computer modeling program SPECIES (Academic Software, Trimble, Otley, UK). Values for the aluminum hydrolysis constants were taken from [31], the solubility constant for freshly prepared $Al(OH)_3$ from [32], Al citrate binding constants from [7], Al maltolate binding constants from [33], and the Al fluoride constants from [34]. The presence of insoluble Al, presumably amorphous $Al(OH)_3$, was determined by Al analysis in dosing solutions (without ^{26}Al addition) before and after passage though a 0.22 μm filter. The unfiltered and filtered solutions, and the concentrated Al stock solution from which they were prepared, were analyzed by electrothermal atomic absorption spectrometry (ETAAS) to determine their Al concentration.

Blood was withdrawn 1 h prior to, and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 4, 8 and 24 h after oral dosing. The blood withdrawn, 0.4 ml in the first 9 samples, then 0.6 and 2.2 ml in the 8 and 24 h samples, respectively, was replaced by an equal volume of injected saline. Serum was obtained for quantification of total Al, ^{26}Al, and ^{14}C. Blood urea nitrogen (BUN) and creatinine were determined in the 24 h sample to assess renal function. When the BUN or creatinine was above the normal limit (30 mg/dl or 1 mg/dl, respectively), the rat was replaced with another rat.

The absence of food in the stomach was produced by limiting food access to a 10% protein diet that was designed to minimize gastric food retention

(Harlan Teklad 95215). This diet was available from 08:00 to 18:00 h daily for 7 days prior to gastric ^{26}Al dosing. Food and water were removed 14 h before to 4 h after dosing and a fecal collection cup, modified from [35], was installed to prevent fecal recycling. In a pilot study, six rats had access to this diet for 10 h daily for 7 days. Fourteen h after diet removal, no food or feces were found in their stomachs [22].

Analysis of total Al by electrothermal atomic absorption spectrometry (ETAAS)

Al was quantified by ETAAS, using a Perkin–Elmer 4100 ZL spectrometer. Serum samples were diluted ten-fold with 0.2% HNO_3 containing 2.5 mM Mg as a matrix modifier, and compared to Al aqueous standards in the same matrix. All serum samples were repeatedly analyzed until their determined total Al concentration RSD was <10%.

Analysis of ^{26}Al and ^{14}C by accelerator mass spectrometry (AMS)

Quality control serum samples containing ^{26}Al were prepared by po administration of Al (52 ng ^{26}Al and 1700 ng ^{27}Al, total 65 nmol Al) to 2 rats. Blood was collected at 4 h. Quality control serum samples containing ^{14}C were prepared by po administration of citric acid containing 30 nmol ^{14}C-citrate given with equimolar ^{27}Al. Blood was collected at 2 h.

Samples were prepared for AMS of ^{26}Al as described [22]. Four mg ^{27}Al (ICP/DCP standard, Aldrich) was added to a 100 µl aliquot of each serum sample (except 200 µl for 8 h and 1000 µl for 24 h sample) to enable determination of the ^{26}Al:^{27}Al ratio by AMS and quantification of serum ^{26}Al by its comparison to the known (4 mg added) ^{27}Al concentration. The sample was dried overnight at 80˚C, digested in 2 ml of a 70:30 mixture of HNO_3 and H_2O_2, heated at 80 ˚C to

evaporate the liquid, which was trapped, the residue dissolved in 0.5 ml 35% nitric acid and transferred to a porcelain crucible, dried overnight and ashed at 1000 ˚C for 2 hr. The radionuclide (^{26}Al) to stable nuclide (^{27}Al) ratio was determined by the PRIME Lab [36].

For ^{14}C sample processing and analysis, 50 µl serum samples frozen in micro-centrifuge tubes were sent to the PRIME Lab. The production of graphite for ^{14}C analysis included lyophilization, combustion and graphitization [37].

One quality control sample containing ^{26}Al or ^{14}C was processed with each batch of serum samples to assess the accuracy and precision of the analysis. Five ^{26}Al replicate quality control serum samples had a RSD of 3.8%. Five ^{14}C replicate quality control serum samples had a RSD of 4.9%. Samples analyzed for ^{26}Al or ^{14}C with a normalized radionuclide/stable nuclide percent error > 10% or 20%, respectively, were not included in the data analysis.

Data analysis

Pharmacokinetic analysis of the ^{26}Al serum results was conducted using WinNonlin. One and two compartment models were used to best fit the ^{26}Al data to estimate AUC, C_{max} and T_{max}. The mean total Al serum concentration was calculated from the AUC of total Al divided by the time period -1 h to 24 h. Oral ^{26}Al bioavailability was calculated from the following:

$$\frac{\text{AUC for } ^{26}\text{Al} \times ^{27}\text{Al infusion rate}}{\text{Mean total Al serum concentration} \times ^{26}\text{Al dose}}$$

Statistical analysis

The results were tested for normality of distribution using the Kolmogorov-Smirnov test and for equal variances using Bartlett's test for the ^{26}Al results (four treatments) and F test for ^{14}C results (two treatments). A one-way ANOVA was conducted to test for significant treatment differences of absolute Al bioavailability, C_{max}, and T_{max} among the 4 Al species. The square root transforms of the ^{26}Al bioavailability results were similarly compared. Results are expressed as X ± SD. Significance was accepted at $P < 0.05$.

Results

The Al species in the freshly prepared solutions for intragastric administration, predicted by calculations, are shown in Figure 1. The dotted lines in Figure 1 denote solutions that would be supersaturated with respect to the precipitation of amorphous $Al(OH)_3$, based on the solubility product of freshly prepared aluminum hydroxide [32].

In the absence of added ligands, the speciation of the Al^{3+} ion is dominated by hydrolysis reactions. A 65 μM solution of Al at pH 5 consists of comparable amounts of Al^{3+} and $Al(OH)_2^+$, with a smaller concentration of $Al(OH)^{2+}$. A neutral solution of a 1:1 mixture of Al and citrate consists primarily of the trimer $Al_3(H_{-1}cta)_3(OH)^{4-}$ and ~ 20% of the $Al(H_{-1}cta)^-$ monomer. In these formulas, cta^{3-} refers to the citrate anion in which all three carboxylate groups are deprotonated, and $H_{-1}cta^{4-}$ refers to a coordinated ligand in which the α-hydroxyl group has also been deprotonated as a result of metal binding. The Al fluoride solution at pH 4 consists of ~ 55% AlF_2^+, 40% AlF_3, 4% AlF^{2+}, and 1.7% AlF_4^-. The speciation calculations indicate that the Al would be fully soluble in the Al ion, Al citrate and Al fluoride administered solutions.

The speciation results for the administered Al maltol solution show a mixture at pH 7 of 64% $Al(mal)_3$, 25% $Al(OH)_3$, ~ 5% $Al(OH)_4^-$ and ~ 5% $Al(mal)_2^+$. Although the calculated $Al(OH)_3$ concentration exceeds the solubility of freshly prepared $Al(OH)_3$, no visible precipitate was observed. It appears that in these dilute solutions, either the formation of insoluble $Al(OH)_3$ is too slow to be observed during the one hour incubation time between their preparation and delivery, or the total mass of the precipitate is too small to be detected visually. Neutralization of a 10 mM Al^{3+} solution to pH 7 led to the formation of colloidal particles of $Al(OH)_3$ with a diameter of ~ 400 nm, which resulted in only a faint

opalescence, rather than an obvious precipitate [32]. Such colloids remain labile and reactive for at least 30 min following neutralization [38]. True equilibration of Al solutions with the less soluble, crystalline form of $Al(OH)_3$ (gibbsite) takes months [39]. Thus any Al present in the dosage solutions as the nominally insoluble $Al(OH)_3$ remained dispersed in solution as a labile, colloidal suspension. The Al in such colloids, if not absorbed directly, would remain bioavailable to some degree due to the ability of the colloid to equilibrate with chelating agents as the solution conditions change.

The Al concentration determined by ETAAS in the unfiltered and filtered Al citrate (pH 7), Al maltolate (pH 7) and Al fluoride (pH 4) solutions at 20, 65, and 200 μM and 2 mM Al was not greatly different from that expected, based on the Al concentration in the solution from which these simulated dosing solutions were prepared. At 65 μM Al, the Al concentration in the unfiltered Al citrate, maltolate and fluoride solutions was 105, 86 and 96%; and in the filtered solutions 91, 86 and 92% of expected, respectively. For citrate and fluoride, the similarity between the results for filtered and unfiltered solutions is consistent with the speciation calculations that indicate that the free Al concentration is below the solubility limit for amorphous $Al(OH)_3$ formation. In the case of maltol, the similarity between the filtered and unfiltered samples shows that any colloidal $Al(OH)_3$ has a small particle size, which supports the hypothesis that the Al within these colloids is capable of equilibrating relatively quickly with the solution as conditions change.

At pH 7, in the absence of ligand, the Al concentration in the 20, 65, and 200 μM and 2 mM Al conditions was 11, 13, 51 and 52% in the unfiltered and 10, 4, 1.5 and 1.5% in the filtered solutions, as expected if significant Al hydroxide is present, as predicted by the results shown in Figure 1, Panel A. For 65 μM Al at

pH 5, only 50% of Al passed through the 0.22 μM filter, even though the speciation results do not predict a significant amount of precipitation at this pH. This discrepancy could reflect some error in the experimental K_{SP} for freshly precipitated $Al(OH)_3$.

The BUN and serum creatinine values of the rats ranged from 3.9 to 17.8 mg/dl and 0.2 to 0.5 mg/dl, respectively, and were within normal limits. Therefore data from all subjects were used in the analysis.

^{26}Al in all serum samples was determined by AMS with an analytical error of ≤ 10%. There were 7 ^{14}C serum samples with an analytical error > 20%; they were not included in the data analysis. Since these samples were from 7 different rats, this did not greatly influence the data analysis. All results had a normal distribution with the exception of the T_{max} values for ^{26}Al. The variances did not differ among/between treatment groups with the exception of the C_{max} values for ^{14}C ($p = 0.028$).

The average ^{26}Al concentration in the serum samples obtained from all rats prior to ^{26}Al dosing was 0.71 ± 0.76 fg/ml (X ± SD, range = 0 to 3.27 fg/ml). The ^{26}Al concentration in serum samples from non-^{26}Al treated rats was 1.07 ± 1.16 fg/ml (range = 0 to 4.64 fg/ml). The peak serum ^{26}Al concentration after oral ^{26}Al dosing was ≥ 70 times the ^{26}Al concentration in serum from non-^{26}Al-dosed rats. For both ^{14}C-citrate and ^{14}C-maltol, the peak ^{14}C concentration after the oral ^{14}C dose was ≥ 30 times that seen in non-^{14}C-dosed rats. The total serum Al concentration in the rat that did not receive the ^{27}Al infusion was ~ 50 ng/ml. The mean total serum Al concentration in the rats that did receive the ^{27}Al infusion was 639 ± 168 ng/ml. The time courses of serum ^{26}Al concentration following oral ^{26}Al dosing are shown in Figure 2. Absolute bioavailability, C_{max} and T_{max} values for ^{26}Al are shown in Table 1. Although the mean oral bioavailability and C_{max} of

Al as the citrate, maltolate and fluoride was 2.1, 1.7 and 1.2 and 1.6, 1.3 and 1.3 times higher than in the absence of ligands, respectively, no statistically significant differences were observed among these 4 Al species. The time courses of serum ^{26}Al and ^{14}C after Al citrate and Al maltolate administration, as a percentage of the administered dose per ml serum, are shown in Figure 3. The shapes of the serum ^{14}C and ^{26}Al concentration versus time curves for individual rats were similar, although serum ^{14}C was ~100-fold higher than serum ^{26}Al. ^{26}Al and ^{14}C concentrations returned close to the baseline by 24 h.

Discussion

The current study tested the following null hypotheses: 1) citrate, maltolate, and fluoride do not influence Al bioavailability, C_{max} or T_{max} at an Al dose relevant to human consumption of Al in drinking water; 2) Al citrate and maltolate complexes do not dissociate in the GI tract and are absorbed intact.

The Al dose given in this study was similar to the daily oral Al intake from water by humans. Humans consume an average of 1.4 L per day of drinking water [40] containing 50 to 100 µg (1.85 to 3.7 □mol) Al/L [41]. This yields a typical daily oral Al intake of ~ 0.14 mg, (2 µg/kg for a 70 kg human). The rats (~ 300 g) were dosed with 1 ml of 65 µM Al (5.85 µg/kg b.w.). Given the rat surface area of ~ 425 cm^2 and human surface area of ~18,000 cm^2, the dosage of Al to the rats was ~ 0.0041 µg/cm^2, about 1/2 of the human daily Al exposure from drinking water (~ 0.0078 µg/cm^2).

The molar ratio of Al to ligands was as employed in Al transport and uptake studies in Caco-2 cells [42]. Species calculations show that at pH 7 nearly all of the citrate and ~ 70% of the maltolate should be bound to Al in the prepared dose solutions. In the rat stomach pH (~ 3.2) [43] there would be significant dissociation of Al maltol and Al citrate to produce mixtures containing higher concentrations of the free ligand and unchelated Al^{3+} ion. As the pH increases from the stomach to the rat jejunum (~ 7) [44] Al complexes with citrate and maltol would form again. The Al-F complexes remain largely intact at the pH of the stomach, but would be expected to convert to Al-hydroxo complexes at the neutral pH of the jejunum. The absence of food and feces in the stomach, and probably upper intestine, at the time of dosing enabled us to test the hypothesis that Al maltol and Al citrate were absorbed intact. If the Al citrate complex was absorbed intact, the time course and extent of absorption of ^{26}Al and ^{14}C-citrate

should be comparable. Our results suggested considerable dissociation of the Al citrate complex in the GI tract.

The average fluoride concentration in drinking water, obtained from surface water, was 0.1-0.3 mg/L (5.5-15.8 μM) (http://sfwater.org/detail.cfm/MC_ID/13/MSC_ID/166/MTO_ID/299/C_ID/1456/ListID/1). Fluoride is often added to the "optimal' level of ~ 0.7 mg/L (36.8 μM) to prevent tooth decay (http://www.fluoridation.com/enviro.htm). We studied an Al:F ratio = 4 because 1) almost all Al binds to F^- under this condition and 2) the ratio is close to the Al:F ratio in non-fluoridated drinking water.

The administration of two isotopes of Al (^{26}Al and ^{27}Al) and the much greater iv administration of ^{27}Al made it possible to concurrently determine the AUCs of oral and iv Al administration in the same subject. This approach reduced inter-subject variability when calculating absolute bioavailability. It is possible that the elevated concentration of Al in the blood from the iv infusion could influence GI tract Al absorption, but there are no reports suggesting this. Recent literature relevant to the mechanism(s) of Al absorption from the GI tract suggests roles for passive paracellular diffusion between cells and active transport. As the Al concentration in the delivered oral solution (~ 1755 ng/ml) exceeded the plasma Al concentration, the elevated blood Al would not be expected to significantly inhibit paracellular diffusion of Al. The transferrin metal binding capacity for Al was not saturated under this condition, suggesting elevated blood Al would not affect speciation of the absorbed ^{26}Al.

A disadvantage of the use of ^{26}Al as a tracer is the high cost of its analysis by AMS, which limits the experimental design to a small number of samples. For substances that have very low oral bioavailability, such as Al, it is difficult to detect small differences in the percentage absorbed without a fairly

large number of subjects, especially in the presence of considerable variability. In the current study, although the mean Al bioavailability in the presence of citrate was 2-fold of that in the absence of ligands, these differences were not statistically significant. Based on the results of the mean and SD of Al bioavailability, the power of these results is 0.42, 0.30 and 0.12 for the Al ion group compared to the Al citrate, Al maltolate and Al fluoride groups, respectively, at a significance level of 0.05, using (http://calculators.stat.ucla.edu/powercalc/). To increase the power to 0.9 to see a significant difference between these 2 groups, at least 6 and 16 rats, 11 and 23 rats, and 69 and 63 rats would be required for the Al ion and Al citrate groups, Al ion and Al maltolate groups, and Al ion and Al fluoride groups, respectively. Even though the bioavailabilities for these Al complexes were not statistically significantly different, their differences might be relevant. Bioequivalence is a term in pharmacokinetics generally used to assess the expected *in vivo* biological equivalence of two proprietary preparations of a drug (http://en.wikipedia.org/wiki/Bioequivalence). If two products are said to be bioequivalent it means that they have the same bioavailability and potency, assuming equal doses. In the United States, FDA considers two products bioequivalent if the 90% confidence interval of relative bioavailability (rate and extent of availability, e.g. C_{max} and AUC) of the test to reference lie within an acceptable range (80%-125%). In the current study, the mean Al oral bioavailability and C_{max} in the presence of citrate, maltolate and fluoride (as test product) versus the absence of ligands (as reference) were 210, 170 and 120% and 160, 130 and 130%, respectively. The relative bioavailability and C_{max} were above the upper limit 125%. Therefore, Al in the presence of ligands failed to demonstrate bioequivalence to Al in the absence of ligands.

The bioavailability of Al when introduced as the ion in the current study was 0.28%, consistent with results using the same experimental methods [22]. A 2-fold increase of the mean absorption of Al in the presence of citrate was seen compared to Al alone. Enhanced Al absorption in the presence of citrate has been repeatedly reported from studies that used ^{27}Al [45]. There are several reported studies, conducted under conditions that model drinking water Al concentration, which used ^{26}Al to investigate the effect of citrate on Al absorption. It was reported that the oral administration of 200 µL citrate (62 g/L) enhanced ^{26}Al absorption 5- to 10-fold [19]. The ^{26}Al was given as 3.8 ng ^{26}Al and 63 ng ^{27}Al (2.5 nmol total Al) at pH 1.6 to 2 and an Al to citrate molar ratio of 1:25,000. Concomitant intake of 1 mmol of citrate (molar Al to citrate ratio = 1:40,000; 3.8 ng of ^{26}Al and 63 ng of ^{27}Al, 2.5 nmol total Al) increased median Al absorption by about 2- to 5-fold [20]. However in another study conducted under the same conditions, no significant enhancement by citrate was seen [46]. In these 3 studies, where a high citrate to Al ratio (Al:citrate = 1:25,000 or 1:40,000) was used, Al absorption increased 2- to 10-fold. In the current study, a much lower citrate dose (65 nmol) and Al:citrate ratio (1:1) were used. The effect of citrate on Al absorption was not statistically significant. Increasing the citrate to Al ratio would favor formation of a smaller 1:2 Al:citrate complex ($Al(H_{-1}cta)(cta)^{4-}$), which might more easily diffuse through the paracellular pathway than the $Al_3(H_{-1}cta)_3(OH)^{4-}$ trimer formed at lower citrate:Al ratios. The absorption of Al, when administered as the citrate (5 ng of ^{26}Al and 80 ng of ^{27}Al, 3.1 nmol total Al, pH 6.2, citrate dose not reported), was greater than when Al hydroxide was given (2.7 ng of ^{26}Al and 43.2 ng ^{27}Al, 1.7 nmol total Al, pH 7), 0.7 versus 0.1% [21]. When 1 mmol/kg sodium citrate was added to 12.1 ng of ^{26}Al as Al citrate at pH = 8.3 (Al:citrate 1:40,000), Al absorption increased to 5%. Based on the speciation

model used to prepare Figure 1, we would predict that such a large excess of citrate would completely suppress the formation of the Al-citrate trimer and that essentially 100% of the Al would be the 1:2 $Al(H_{-1}cta)(cta)^{4-}$ complex.

The overall results of the previous studies and current study suggest the effect of citrate on Al absorption might be citrate dose dependent. This is consistent with results using Caco-2 cells where citrate had a different effect on the flux of 2 μM versus 8 mM Al-citrate. 2 μM citrate did not have a significant effect on Al flux whereas at 8 mM Al-citrate, the citrate affected tight junction integrity to influence Al flux [42]. In previous rat studies, the high dose of citrate (65 to 100 μmol in most ^{26}Al rat studies) may have interacted with the GI tract to facilitate Al absorption by the paracellular pathway while the much lower dose (65 nmol in the current study) had a less obvious effect. Exposure to Al citrate resulted in markedly enhanced transmural Al transport *in vitro* in duodenal and jejunal everted gut preparations compared to Al chloride [47]. This was associated with increased deposits in intercellular spaces of ruthenium red (a marker used to evaluate tight junction structural integrity) and a prolonged significant reduction in transmural resistance. Similarly, permeability of Al in the Caco-2 cell study was low, suggesting poor oral absorbance, independent of the absence or presence of ligands, as long as the integrity of the cell monolayer was maintained [42]. However, when tight junction permeability increased, Al flux similarly increased. This is in agreement with the results of the current study where Al absorption was < 1% at 65 nmol, and citrate, maltolate, and fluoride had no significant effect on Al bioavailability, C_{max} and T_{max}. Administration of much larger doses of Al and citrate has a much greater potential to change the GI milieu and produce non-physiological absorption results. This adds to the difficulty of comparing the results among these studies.

The absorption of Al when administered as the maltolate approximated that of Al hydroxide (~ 0.1%) [21]. The Al was given as 5 ng ^{26}Al and 80 ng ^{27}Al, 3.1 nmol total Al, pH = 6, in 2 ml water (1.55 μm). The Al to maltolate molar ratio was not reported. Al is present primarily as a $Al(maltolate)_3$ complex at 65 μM Al and 195 μM maltolate at pH = 7, as in the present study, whereas at 1.55 μM Al and 4.65 μM maltolate at pH 6 virtually all the Al would be a mixture of $Al(OH)_2^+$ and $Al(OH)_3$. Therefore, the predominant Al species in this study might have been Al hydroxide. In the present study, the bioavailability of Al, introduced as the maltolate, was 0.51%. The presence of maltolate did not significantly change the bioavailability, C_{max} or T_{max} of Al compared to the absence of ligands.

This is the first study to investigate the effect of fluoride on Al absorption at a physiologically-relevant Al concentration using ^{26}Al. Since fluoride was also studied at a physiologically-relevant concentration, the expected increase of fluoride in rat serum was not predicted to be discernable from endogenous fluoride. As there is no appropriate fluoride tracer that could have been used in this study only serum Al was measured in rats that received Al fluoride. The presence of fluoride did not significantly change the bioavailability, C_{max} or T_{max} of Al.

The present study was the first to use ^{26}Al and ^{14}C to address the null hypothesis that Al citrate and Al maltolate do not dissociate in the GI tract, resulting in their absorption intact. The serum ^{14}C concentration from oral administration of ^{26}Al ^{14}C-citrate or ^{26}Al ^{14}C-maltolate was ~100 times higher than expected if the ^{14}C was absorbed as an Al-ligand complex. One interpretation is that there was considerable dissociation of Al citrate and Al maltolate in the GI tract. As ~ 80% of the citrate in the Al citrate dosing solution was associated with Al (Figure 1, Panel B) the free citrate does not account for the ~ 60% absorption of ^{14}C from ^{14}C-citrate, as Al bioavailability was ~ 0.6% and citrate ~ 100-fold greater. For Al maltolate,

speciation calculations predict that ~ 35% of the Al would not be associated with maltolate, leaving sufficient non-Al associated maltolate to account for the absorption of ^{14}C from ^{14}C-maltolate. At the lower pH of the rat stomach even though a higher percentage of Al would be associated with maltol, there would still be sufficient free maltol to account for the observed uptake of ^{14}C. Serum ^{14}C approached zero by 24 h, suggesting no free citrate or maltol remained in the intestine available for absorption.

Serum citrate peaked after 32 min and returned to baseline by 90 min, whereas blood Al peaked after 87 ± 19 min, then decreased slowly over 24 h in 3 humans who drank a solution containing 280 mg (10.37 mmol) ^{27}Al, as the hydroxide, and 3.2 g citrate (15.45 mmol) at pH = 4.5 [25]. There was 100-fold greater citrate than Al absorption. Based on these results the authors concluded that it was unlikely that Al was absorbed as Al citrate. The administered solutions in this study were very concentrated; 0.1 M Al^{3+} and 0.17 M citrate. The authors attempted to assess the speciation of the aluminum in the discussion of their results. However, the model that they used included only mononuclear complexes. Speciation calculations for these conditions using the model from the present study indicate that about 60% of the Al would have been the trimer and about 40% $Al(cta)_2^{3-}$. At the more alkaline pH of the intestine, the percentage of trimer would have increased to almost 90%. Owing to the use of ^{26}Al and the exquisite sensitivity of AMS, the Al dose in the present study was 0.0006% of this previous study. In the present study, the serum ^{14}C and ^{26}Al peaks occurred at a similar time. Considering that the magnitude of citrate absorption was 100 times that of Al absorption, it is very possible that a small fraction of the absorbed citrate was as Al citrate, e.g., the Al was absorbed as the Al citrate complex.

The pH increase from the stomach to duodenum to jejunum is due to carbonate and bicarbonate secretion. Although the interactions between Al and carbonate or bicarbonate ions are so weak that they can be neglected [48], the results of the present study suggest considerable Al dissociates from citrate. The T_{max} of Al in the present study was approximately 1 h and that of citrate ~ 2 h, consistent with their absorption from the proximal small intestine. The non-absorbed Al in the intestine that was freed from the ligand may have formed Al hydroxide or associated with mucus on the wall of the GI tract to be taken up into epithelial cells and sequestered in the cell nuclei, or excreted into feces.

In summary, the results of the current work advanced the understanding of the importance of the chemical species of Al on its absorption from the GI tract and the risk assessment of Al toxicity, when administered as the Al^{3+} ion, or as Al citrate, maltolate or fluoride in drinking water. Generally, these results did not reject the null hypothesis that citrate, maltolate and fluoride have no significant effect on Al absorption (bioavailability, C_{max}, and T_{max}) under the studied conditions. At an Al dose relevant to that consumed by the human in drinking water the absolute bioavailability of Al was < 1%. Citrate and maltolate absorption were much greater than Al. Although Al bioavailability didn't significantly increase after a single oral dose in the presence of citrate, maltolate or fluoride compared to the Al ion under the conditions studied, the absorption of Al when given as the ion, citrate, maltolate and fluoride failed to demonstrate bioequivalence. Furthermore, this study only addressed oral bioavailability. The distribution of Al to target organs, such as the brain, and the resulting effects, as well as the rate of clearance by the kidney and/or the liver may not be equivalent for these Al species. Further study of the effect of ligands on Al absorption, distribution and elimination under conditions that

model Al consumption in drinking water during long term exposure is needed for a more informed risk assessment of Al.

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Conflict of Interest Statement

The authors have no conflict of interest for this work.

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Figure legends

Figure 1. Results of Al speciation calculations at a total of 65 μM Al in the absence of ligands (Panel A); and in the presence of citrate (cit) (65 μM) (Panel B), maltolate (mal) (195 μM) (Panel C), and fluoride (F) (260 μM) (Panel D) in the pH range 2 to 8. The dotted lines indicate solutions which exceed the solubility product of freshly prepared $Al(OH)_3$, i.e. the pH range in which the calculated concentration of $Al(OH)_3$ exceeds its solubility.

Figure 2. Time courses of serum ^{26}Al concentrations following oral administration of 52 ng ^{26}Al in the absence of ligands or in the presence of citrate, maltolate or fluoride. Values are mean ± SD from the 5 rats of each Al treatment group. The X axis is shown in three segments to expand the results from the early time points.

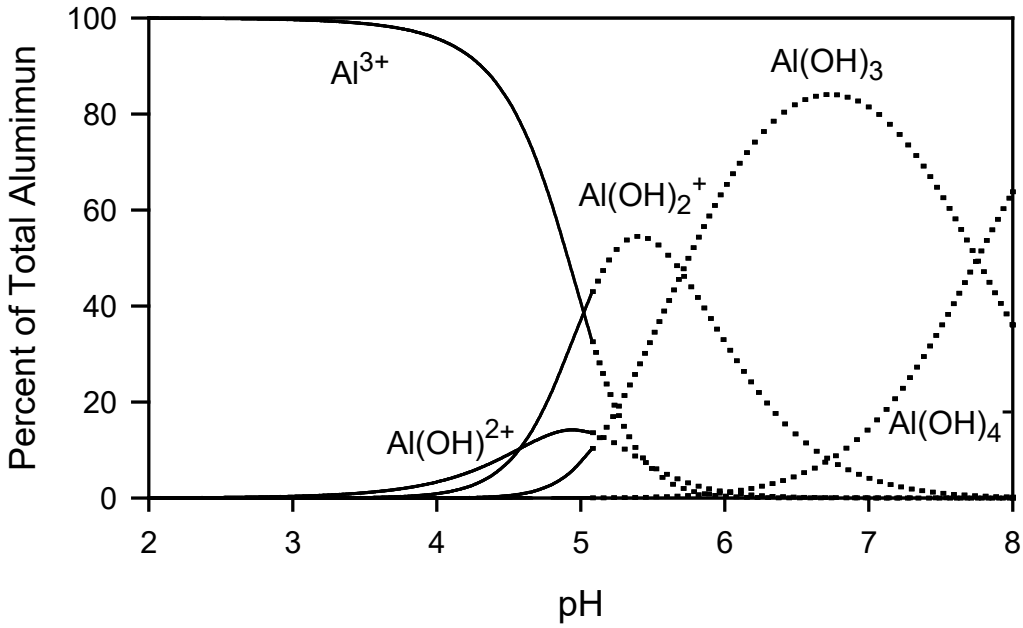
Figure 3. Serum ^{26}Al and ^{14}C in each of the five rats after oral administration of ^{26}Al-^{14}C-citrate (upper five panels) and ^{26}Al-^{14}C-maltolate (lower five panels), shown as a percentage of the administered dose/ml serum. Serum ^{26}Al shown as squares and dashed line with the scale on the left axis. Serum ^{14}C shown as triangles and solid line with the scale on the right axis. Each panel shows results from one rat with a connecting line among the points. Note the 100-fold difference in the Y scales on all graphs.

Table 1. The absolute oral bioavailability (non-transformed and square root transformed), C_{max}, and T_{max} of ^{26}Al administered in the absence of ligands (ion) or in the presence of citrate, maltolate or fluoride, and C_{max}, and T_{max} of ^{14}C from citrate and maltolate.

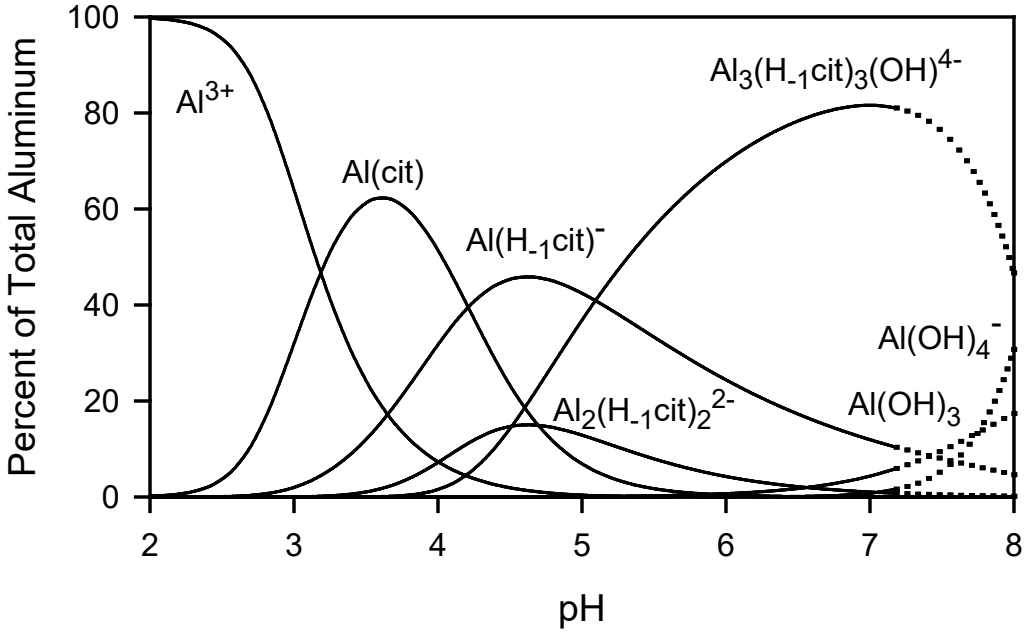
	Ion	Citrate	Maltolate	Fluoride	ANOVA or t-test result
^{26}Al oral bioavailability (%)	0.29 ± 0.11	0.61 ± 0.31	0.50 ± 0.25	0.35 ± 0.10	p = 0.11
^{26}Al oral bioavailability (%) (square root transform)					p = 0.14
^{26}Al C_{max} (fg/ml)	659 ± 195	1073 ± 250	881 ± 356	880 ± 295	p = 0.18
^{26}Al T_{max} (h)	1.2 ± 0.9	1.0 ± 1.1	1.3 ± 1.0	1.0 ± 0.9	p = 0.90
^{14}C C_{max} (ng/ml)		1.8 ± 0.7	0.6 ± 0.2		p = 0.0059
^{14}C T_{max} (h)		2.9 ± 2.2	1.2 ± 1.1		p = 0.15

Figure 1.

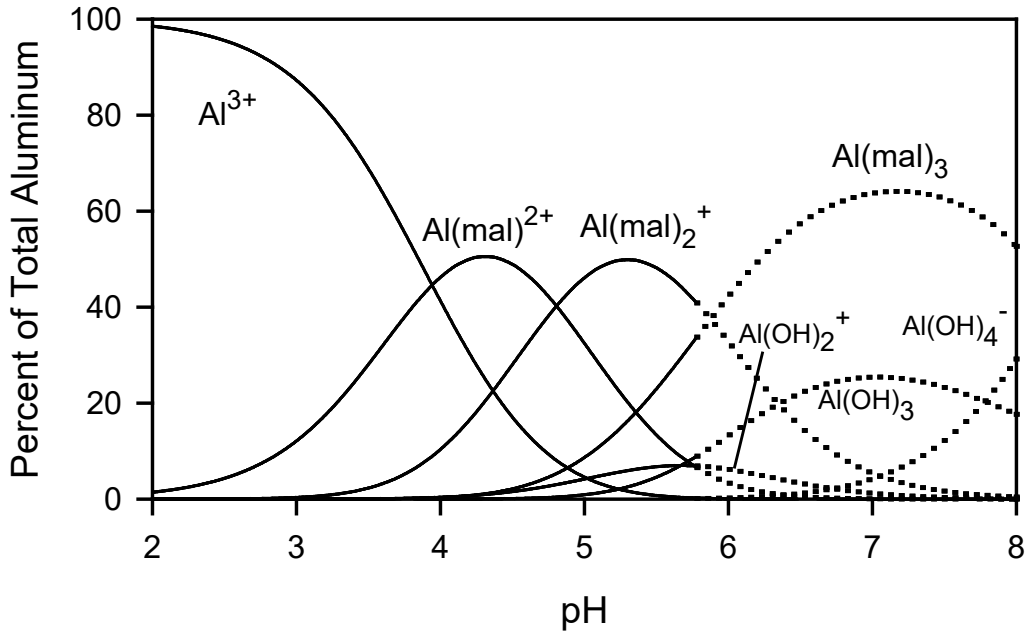
Panel A



Panel B



Panel C



Panel D

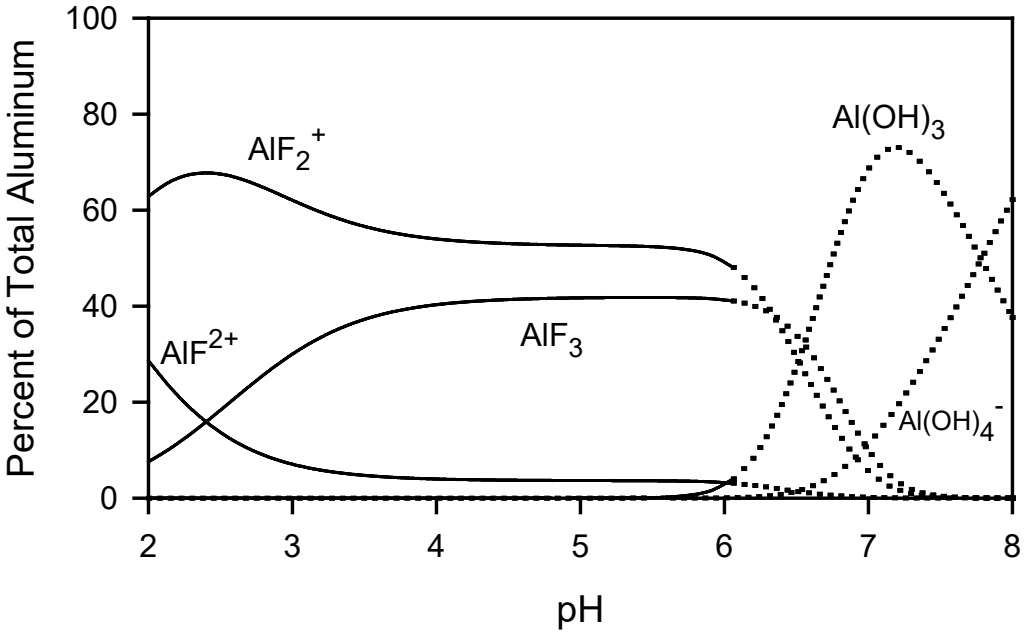


Figure 2.

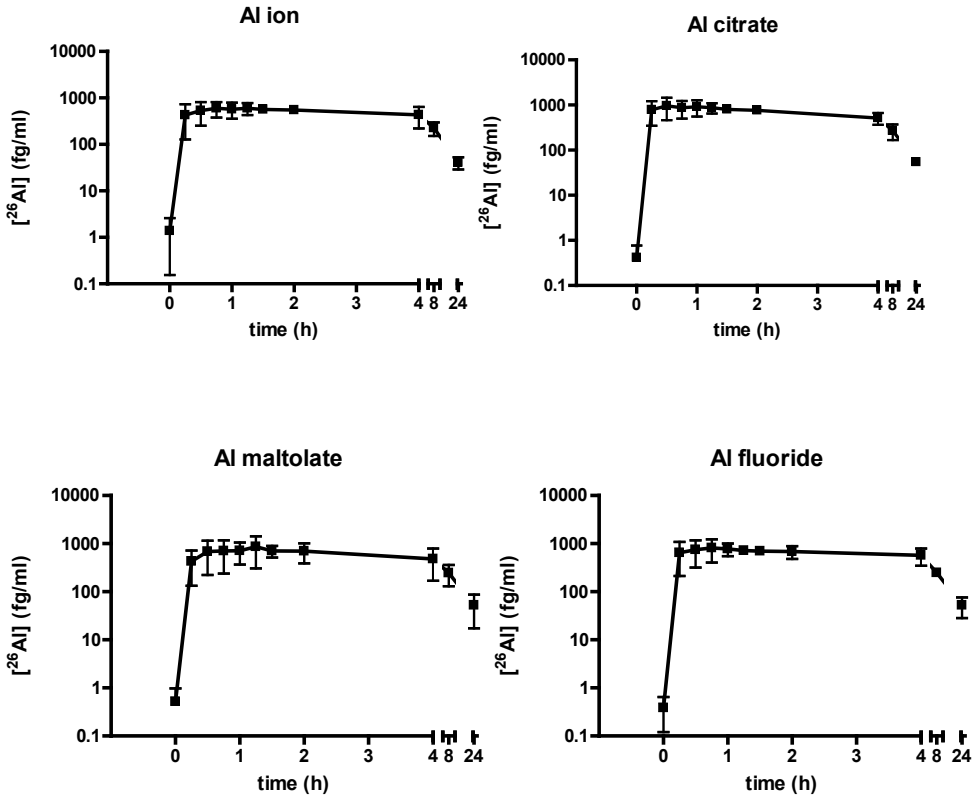
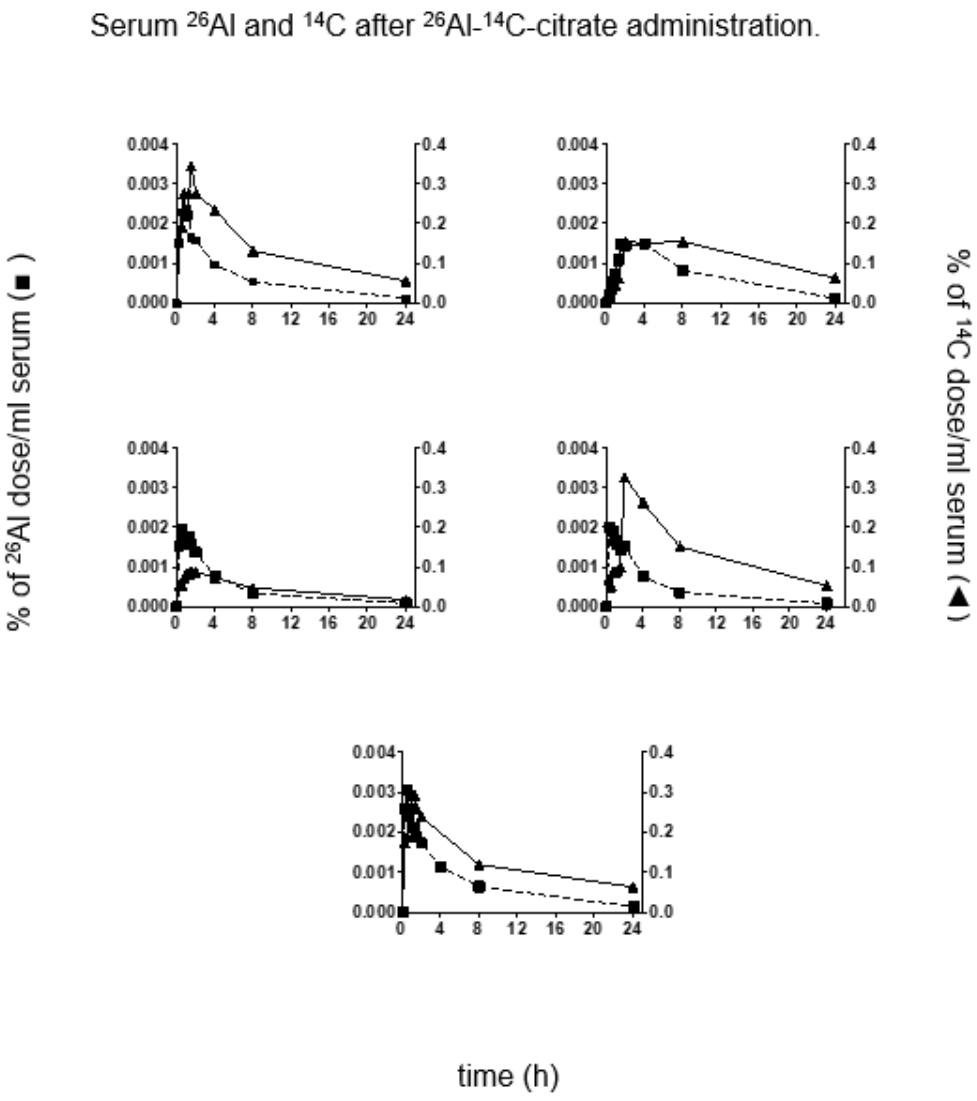

Al ion
Al citrate
Al maltolate
Al fluoride
[26Al] (fg/ml)
10000
1000
100
10
1
0.1
0
1
2
3
4 8 24
time (h)

Figure 3.


Serum 26Al and 14C after 26Al-14C-citrate administration.
% of 26Al dose/ml serum (■)
% of 14C dose/ml serum (▲)
0.004
0.003
0.002
0.001
0.000
0.4
0.3
0.2
0.1
0.0
0
4
8
12
16
20
24
time (h)

Serum ^{26}Al and ^{14}C after ^{26}Al-^{14}C-maltolate administration.