Thermodynamic Insights into the Interplay between Calcium and Iron(II) Hydroxycarboxylates: Impacts on Solubility, Speciation, and Bioavailability

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1. Methods

1.1 Solubility Determination

Saturated aqueous solutions were prepared to determine the solubility of calcium L-lactate, calcium D-gluconate monohydrate, iron(II) lactate hydrate, and iron(II) D-gluconate dehydrate at various temperatures. For calcium compounds, 10.0 g, 15.0 g, and 25.0 g of calcium L-lactate or 5.0 g, 10.0 g, and 15.0 g of calcium D-gluconate monohydrate were dissolved in 100 mL of water. For iron compounds, 3.0 g, 4.0 g, 5.0 g of iron(II) lactate hydrate or 15.0 g, 20.0 g, 30.0 g of iron(II) D-gluconate dehydrate were used. All solutions were stirred for 2 hours at 25 ℃, 37 ℃, and 49 ℃, respectively. After filtration, the concentrations were determined. Total calcium concentration was measured by EDTA titration, while iron(II) concentration was determined by $KMnO_4$ titration. The conductivity and pH of the saturated solutions were also measured at the three temperatures using an electrolytic conductivity meter (DDS-11A, Shanghai Leici) and a pH meter (FE28-Standard, METTLER TOLEDO), respectively.

1.2 Electric Conductivities Measurements

The electrical conductivity of dilute solutions of calcium L-lactate pentahydrate, calcium D-gluconate monohydrate, iron(II) lactate hydrate, and iron(II) D-gluconate dehydrate was measured to study their dissociation behavior. Solutions with concentrations of 2×10^{-4}, 4×10^{-4}, 6×10^{-4}, 8×10^{-4}, and 1×10^{-3} mol/L were prepared and measured at 25 ℃, 37 ℃, and 49 ℃ using a conductivity electrode calibrated with a 0.01 M potassium chloride standard solution (1408 μS/cm). The molar conductivity ($S\cdot dm^2/mol$) was plotted against the square root of the solution concentration, and the infinitely diluted molar conductivity, Λ_∞, was derived by fitting.[1]

1.3 Density Functional Theory Calculation

Quantum chemical calculations were performed using density functional theory (DFT) through the Gaussian 16 program. Geometry optimization and frequency calculations were carried out using Becke's three-parameter hybrid

functional with Lee-Yang-Parr correlation function theory (B3LYP) and the 6-311G(d,p) basis set. The solvent (water) effect was accounted for using the integral equation formalism of the polarizable continuum (IEFPCM) model. It operates by defining a cavity around the solute molecule, which is then embedded within a continuous, polarizable dielectric medium representing the bulk solvent (water). The obtained frequencies were checked to confirm that they corresponded to actual minima on the potential energy surface, as indicated by the absence of imaginary frequencies.[2,3]

1.4 Mixed Calcium Hydroxycarboxylates and Iron(II) Chloride Solutions

Two separate calcium hydroxycarboxylate solutions were prepared: 10.0 g of calcium L-lactate and 5.0 g of calcium D-gluconate were each dissolved in 100 mL of distilled water. These solutions were then combined with varying concentrations of iron(II) chloride dihydrate ranging from 1×10^{-5} to 0.1 mol/L (1×10^{-5}, 2×10^{-5}, 4×10^{-5}, 6×10^{-5}, 8×10^{-5}, 0.01, 0.02, 0.03, 0.04, 0.05 and 0.1 mol/L). The mixtures were stirred continuously at 25 ℃ for two hours to ensure equilibrium. The resulting saturated solutions were filtered to remove any undissolved solids. The total calcium and iron concentrations in the filtered samples were determined using EDTA titration and $KMnO_4$ titration, respectively. The calcium ion activity ($\alpha_{Ca^{2+}}$) was measured using a calcium ion selective electrode. The electrode was calibrated with standard calcium chloride solutions of known concentrations (1.0×10^{-4}, 1.0×10^{-3}, and 1.0×10^{-2} mol/L) at 25 ℃. The calcium ion activity was calculated from the linear relationship based on the Nernst equation.[4]

1.5 Mixed Calcium and Iron(II) Hydroxycarboxylates Solutions

1.5.1 Mixed Calcium L-lactate and Iron(II) Lactate Solutions

To prepare saturated solutions of mixed calcium and iron(II) L-lactate, 10.0 g of calcium L-lactate and 5.0 g of iron(II) L-lactate were combined with 100 mL of deionized water. The mixture was stirred continuously for two hours to ensure complete dissolution and equilibration, with this process repeated at three different temperatures:

25 ℃, 37 ℃, and 49 ℃. After the equilibration period, each solution was filtered to remove any undissolved particles. The filtered samples were then analyzed to determine the total concentrations of calcium and iron.

1.5.2 Mixed Calcium D-gluconate and Iron(II) D-gluconate Solutions

To prepare saturated solutions of mixed calcium and iron(II) D-gluconate, 5.0 g of calcium D-gluconate and 15.0 g of iron(II) D-gluconate were combined with 100 mL of deionized water. The mixture was stirred continuously for two hours to ensure complete dissolution and equilibration, with this process repeated at three different temperatures: 25 ℃, 37 ℃, and 49 ℃. After the equilibration period, each solution was filtered to remove any undissolved particles. The filtered samples were then analyzed to determine the total concentrations of calcium and iron.

1.5.3 Mixed Calcium L-lactate and Iron(II) D-gluconate Solutions

The mixed solutions of calcium L-lactate and iron(II) D-gluconate were prepared by dissolving varying amounts of the two compounds in 100 mL of water and stirring continuously at 25 ℃. Three initial solutions were prepared with calcium L-lactate and iron(II) D-gluconate at (11.10 g, 2.17 g), (13.41 g, 5.30 g), and (17.88 g, 12.10 g), respectively. It was observed that some, but not all, of the resulting solutions maintained complete clarity. The turbid solutions were subjected to ongoing analysis. Samples were filtered and analyzed for calcium and iron concentrations at 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, and 96 hours, while maintaining continuous stirring throughout the experiment. Additionally, three more solutions were prepared with a constant amount of calcium L-lactate (13.41 g) and varying amounts of iron(II) D-gluconate at 11.67 g, 13.80 g, and 15.91 g. The mixtures were stirred constantly at 25°C. Samples from these solutions were filtered and analyzed for calcium and iron concentrations at 0.5, 2, 4, 6, 8, 10, 12, 24, and 48 hours, while maintaining continuous stirring.

1.5.4 Mixed Calcium D-gluconate and Iron(II) lactate Solutions

The mixed solutions of calcium D-gluconate and iron(II) L-lactate were prepared by dissolving varying amounts

of the two compounds in 100 mL of water and stirring continuously at 25 ℃. Four initial solutions were prepared with calcium D-gluconate and iron(II) L-lactate at (4.71 g, 1.40 g), (5.69 g, 1.99 g), (6.64 g, 2.57 g), and (7.53 g, 3.08 g), respectively. Additionally, three more solutions were prepared with a constant amount of calcium D-gluconate (15.69 g) and varying amounts of iron(II) L-lactate at 7.02 g, 8.19 g, and 9.36 g. All solutions were continuously stirred, filtered, and analyzed for calcium and iron concentrations at 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours.

1.6 EDTA Titration

The EDTA titration method was employed for the determination of calcium concentrations. A 0.05 mol/L EDTA solution was prepared and standardized against a 0.02 mol/L aqueous solution of calcium chloride to ensure accuracy. For each analysis, a 1 mL sample was transferred to a titration flask. The sample was then diluted with 40 mL of deionized water to provide sufficient volume for titration. To maintain a basic pH for the reaction, 2 mL of a 2 mol/L NaOH solution was added to each sample. As an indicator, 0.3 mL of a 0.50% murexide solution was added, imparting an initial pink color to the solution. The sample was then titrated with the standardized EDTA solution. The endpoint of the titration was identified by a distinct color change from pink to dark purple, signaling the complete complexation of calcium ions by EDTA.[5]

1.7 $KMnO_4$ Titration

The permanganate titration method was employed for the determination of iron(II) concentrations. A 0.01 mol/L potassium permanganate solution was prepared and standardized against 0.01 g of sodium oxalate to ensure accuracy. For each analysis, a 0.5 mL sample was transferred to a titration flask. The sample was then diluted with 20 mL of deionized water to provide sufficient volume for titration. To create an acidic environment for the reaction, 1.0 mL of a 1:1 mixture of concentrated H_2SO_4 and H_3PO_4 was added to each sample. The sample was then titrated with the standardized potassium permanganate solution. The endpoint of the titration was identified

when the solution turned a light pink color that persisted for at least 30 seconds, indicating the complete oxidation of iron(II) to iron(III) by permanganate.

1.8 X-ray diffraction

Precipitates were collected from the 100% supersaturated calcium L-lactate and iron(II) D-gluconate solution after 96 hours of stirring. The precipitates were filtered, air-dried overnight, and then analyzed using X-ray diffraction. Calcium D-gluconate, calcium L-lactate, iron(II) D-gluconate, and iron(II) lactate standards were obtained from Sigma-Aldrich (Shanghai, China). The X-ray diffraction patterns were collected using a diffractometer equipped with a monochromatic Co-Kα radiation source ($\lambda = 0.179021$ nm). The diffractometer scanned from 5° to 40° (2θ) at a rate of 10° per minute, with operating conditions of 40 kV and 40 mA.[6]

1.9 Fourier Transform Infrared Spectroscopy

FTIR spectra were acquired using potassium bromide (KBr) pellets. The samples were pressed into KBr pellets and analyzed in the spectral range of 4000 - 400 cm^{-1}. The resolution was set to 4 cm^{-1}, and 16 scans were collected for each sample. A KBr salt plate was used as a background reference.[7]

1.10 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were conducted using a Nano-ITC instrument (TA Instruments) equipped with a gold sample cell (500 μL). All samples were dissolved in 100 mM MES buffer (pH 5.0) and degassed for 20 minutes to remove dissolved gases. Calcium L-lactate, calcium D-gluconate, iron lactate, and iron D-gluconate solutions were loaded into the sample cell (5 mM) and injection syringe (50 mM), respectively. The control group contain 100 mM MES without calcium/iron salts in sample cell. The sample cell was stirred at 250 rpm and equilibrated at 25 °C for 90 minutes. Titrations were performed by injecting the appropriate solution into the sample cell at a rate of 180 seconds per injection. The following titrations were carried out: calcium L-lactate into iron(II) D-gluconate, calcium D-gluconate into iron(II) lactate, iron(II) lactate into calcium D-gluconate, and

iron(II) D-gluconate into calcium L-lactate. A control experiment was performed by titrating calcium or iron(II) solutions into 100 mM MES buffer (pH 5.0).[8]

1.11 Statistical analysis

Statistical analyses were performed using R and Python scripts with the different libraries and Origin 2021. Prior to parametric testing, data normality was confirmed by Shapiro-Wilk tests ($p > 0.05$) and variance homogeneity by Levene's test ($p > 0.1$). Results are expressed as mean ± SD from three independent measurements. Significant differences were assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with $p < 0.05$ defined as statistically significant.

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