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PRE-FORMULATION CHARACTERIZATION OF CHELATED AMINO ACID COMPLEXES

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ABSTRACT: Amino acid chelated complexes were synthesized and characterized to determine their purity. The purity of the complexes was found to be ranging from 42-75%. The HPLC method for determination of free amino acid content in the complexes was developed and validated and found to be linear, precise and accurate within concentration range of 0.157 mg/mL to 20.14 mg/mL. The method was used to estimate the purity of the amino acid complexes. The changes in the carboxylate and amine group stretches in FT-IR indicated the association of metal ion with the amino acid. The initial studies described above demonstrated that the chelated complexes can produce sufficient osmotic pressure to be used as ergogenic aids.

INTRODUCTION: During intense exercise, the body's demand for metabolic precursors and electrolyte mediated cellular hydration increases tremendously. Normal physiological changes typically occur rapidly to accommodate this increased demand but are unable to meet prolonged demands. First, direct sources of cellular energy are utilized, such as ATP and phosphocreatine [1, 2]. Glycogenolysis and glycolysis are also upregulated with the peak rate of glycolysis reached quickly and maintained based on the availability of oxygen for cellular respiration in the Kreb Cycle [3]. Peak energy production in aerobic cellular respiration produces 38 ATP per glucose molecule. Prolonged or intense exercise can outpace the oxygen supply and necessitate an upregulation of anerobic respiration producing lactic acid [1].

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This is not an efficient process and only yields 3 ATP per glucose molecule. Energy production under prolonged or intense exercise is limited by the source of metabolic activity. For example, lipids are a concentrated source of energy but have a slow metabolic conversion to ATP. Proteins, either ingested or obtained from muscle stores, are catabolized to amino acids that are readily incorporated into the Kreb Cycle [4-7]. Amino acids are metabolized primarily in the liver with additional metabolism in muscles [6]. For example, the production of alanine and glutamate is increased linearly with exercise.

However, amino acid metabolism also causes an exponential increase in nitrogen byproducts including ammonia [8]. After prolonged or intense exercise, there is rebound in the rate of muscle anabolism. The extent of catabolism and the recovery afterwards is partially dependent on the circulating amino acid levels [4, 9, 10]. Therefore, it would be physiologically beneficial to provide amino acid supplementation in cases of prolonged or intense exercise.

Along with alterations to cellular metabolism, exercise causes the body's temperature to increases and compensatory increase in sweating resulting in water and electrolyte loss [11]. Prolonged sweating can deplete electrolyte stores and lead to dehydration which has been correlated with poor athletic performance [12]. Dehydration and electrolyte replenishment has been addressed extensively in the scientific literature with several proposed ergogenic aids. We hypothesized that the complexation of electrolytes with amino acids is a viable strategy to promote electrolyte absorption and provide metabolic precursors. An ergogenic aid helps in more than one way in improving athletic performance [13-15]. The ergogenic aids proposed for hydration and electrolyte replenishment include solutions for fluid replenishment that contain 14-22 grams of sugar per serving, 3-5% of the daily value of sodium per serving (81mg-110mg), and 1% the daily value of potassium per serving (30-48mg) [13-15 16, 17]. These solutions are often consumed before, during, and after prolonged intense exercise to assist in maintaining proper hydration, and electrolyte balance to counter the loss through sweat[18, 19].

Despite extensive reports and use, these sugar-based solutions have several limitations for athletes involved in intense exercise, tonicity and limited electrolyte rates of absorption. First, the addition of sugar increases the solution's residence time in the gut and promotes absorption of water. This extended residence time in the gut can cause upper abdominal pain, nausea, loss of appetite, and feeling full after consuming little amounts of food[20-22].

Second, the tonicity of the dissolved sugar and electrolyte salts affects hydration. Hypotonic solutions, including plain water, causes quenching of thirst before the body achieves proper hydration[23]. Conversely, hypertonic solutions further dehydrate the body and worsen water loss [24, 25]. Most sugar-electrolyte solutions are isotonic to avoid these limitations. Third, the ions involved in electrolyte supplementation, often Na^+, K^+, Ca^{2+}, and Mg^{2+}, are absorbed through rate-limited transport mechanisms in the gastrointestinal system[26-31]. Passive-mediated diffusion or facilitated diffusion is the transport of molecules or ions across a biological membrane via transmembrane proteins in the direction of concentration gradient [9, 32-34]. Facilitated diffusion is an important mechanism for the absorption of various ions, amino acids, and small peptides [35].

There are numerous carrier molecules that facilitate in the transport of these substances across biological membranes. Many times water is involved in the transfer of molecules across membranes, referred to as solvent flow [36]. Water solubilizes ions and molecules to facilitate their absorption either by occupying the unsatisfied valencies of metal ions or through electrostatic interactions. The brush borders or villi present in the small intestine are responsible for absorption of large amounts of water, ions, and amino acids. Despite numerous these various facilitated transport pathways, the involvement of transport proteins can induce rate-limited absorption of ions and metabolic precursors and delay their ergogenic potential in intense exercise.

One potential improvement for ergogenic aids in intense exercise is through the complexation of metal ions for electrolyte replenishment with amino acids for use as metabolic precursors. The potential advantage for amino acid-metal ion complexes is that the mechanisms involved in the absorption of amino acid complexes are different from those for free metal ions [37-40]. Amino acid complexes have been proven to be absorbed by more than one mechanism. For example, after ingestion, complexes encounter stomach acidity and cause the amino acids to acquire charge. Comparative absorption studies between amino acid complexes and soluble metal salts concluded that acidity of the stomach doesn't lead to complete hydrolysis and the complex is absorbed intact [37, 41-43].

Also, amino acid complexes upon exposure to acidic pH of the stomach are converted into protonated complex form [33]. This protonated complex is still associated with the metal ion. As the amino acid complexes enter the gut, the luminal wall has a negatively charged glycoprotein called as glycocalyx. The protonated complex is electrostatically attracted to the glycocalyx layer and taken up into the mucosal cells by transporters like γ-glutamyl transport system[44-46]. Amino acid

complexes act as a carrier molecule for metal ion during active transport [41, 47]. The intracellular fate of the complexes varies with most hydrolyzed or competitive disassociation of the metal ion with endogenous complexing agents with stronger affinities than amino acids [48].

Despite the improved absorption potential for amino acid complexes, the complexation of metal ions with any chelating agent doesn't assure increased absorption or ergogenic utility of complex components as seen in case of ion-complex with EDTA. There are specific considerations for a complex (or a ligand to be used for forming a complex) to be used as an ergogenic aid [31, 49]. Briefly, the stability constants of the complexes should be similar to some biological ligands to ensure the metal ion can be associated in a complex[50]. The complexes should also have low molecular weight (<1000 Da) to promote diffusion across biological membranes and absorption[51]. After absorption the complex must hydrolyze to release the metal ion[50, 52].

Amino acid complexes have stability constants that are optimum. Amino acids also tend to form complexes with metal ions in the ratio of one, two or three molecules of amino acids to one molecule of metal ion (depending on the valence of the metal ion). This complexation ratio of a metal ion with amino acid(s) then leads to molecular weights of approximately 1000 Da or less. Most biological transporters involved in absorption of metal ions in the gastrointestinal tract utilize amino acid residues in the active absorption processes and further suggests their potential use as ligands [53-55].

The focus of the current research describes the creation and preliminary characterization of molecular complexes of amino acids and metal ions as a novel approach to ergogenic aids that avoids the problems with current sugar-electrolyte solutions. The use of amino acids as complexing agents should provide metabolic precursors for use in athletes involved in intense exercise [1, 56]. Amino acid complexes are currently being used as metal supplements and have showed improvement in the bioavailability of Mg^{2+} ,Ca^{2+}, Cu^{2+}, and Mn^{2+} [33]. Their use in electrolyte supplementation and hydration is justified. Amino acid complexes can be a better alternative to the current therapy used in hydration and electrolyte replenishment therapy in intense exercise.

MATERIALS & METHODS:

Sodium hydroxide, potassium hydroxide, ammonium hydroxide, metallic zinc were purchased from Fischer Scientific (Pittsburgh, PA, USA). Magnesium chloride, calcium chloride, L-alanine, L- glutamine, were purchased from Sigma Aldrich (St. Louis, MO, USA). L-Valine, L-glycine, L-glutamic acid were purchased from Acros Organics (NJ, USA) Eriochrome black T was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Disodium EDTA was purchased from Spectrum Chemical Mfg., Company (Gardena, CA, USA). Acetonitrile, was purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (Optima grade) was purchased from Fischer Scientific (Pittsburgh, PA, USA). L-Glycine was purchased from Acros Organics (NJ, USA). Perfluorooctanoic acid was obtained from Matrix Scientific (Columbia, SC, USA). Deionized water was used throughout the experimental procedure.

Succinic acid was purchased from Eastman Organic Chemicals Ltd (Rochester, NY, USA). Disodium EDTA was purchased from Spectrum organic limited (St. Louis, MO, USA). Glycine, glutamic acid was purchased from Acros Organics (NJ, USA). Calcium Chloride was purchased from Sigma Aldrich (St. Louis, MO, USA). Nickel alloy fuse wire was purchased from Parr Bomb Calorimeter (Moline, IL, USA). Benzoic acid was purchased from Fischer Scientific (Pittsburgh, PA, USA). Potassium chloride, sodium chloride were purchased from Fischer Scientific (Pittsburgh, PA, USA), the complexes were obtained from synthesis, Nanopure water was used throughout the experiment.

Synthesis of chelated complexes:

Monovalent metal ions (Na^{+}, K^{+}) are present as hydroxide salts that react with the amino acids to form complexes. Divalent metal ions (Ca^{2+}, Mg^{2+}) are present as chloride salts that are reacted with amino acids in the presence of potassium hydroxide to form complexes. Amino acids used for creation of complexes include alanine, glutamic acid,

glutamine, glycine, and valine. Briefly, 1mmol of amino acid and 1mmol of monovalent metal hydroxide in 15 mL of deionized water and refluxed at 55^{o}C for 1 hr. Alternatively, 1mmol of divalent metal ion chloride salts along with 2 mmol of potassium hydroxide and 2 mmol of amino acids in 15 mL water and refluxed for 3-4 hours at 65 ^{o}C. Each resulting solution was cooled and sample fractions were dried using lyophilization, described later.

Calculation of Yield and Complex Sample Analysis:

Sample aliquots (200 µL each) of the cooled reaction mixture were diluted to 5 mL with nanopure water and reacted with 1 mL of 1M HCl for 2 minutes and neutralized with 3 mL of 1M ammonium hydroxide. Indicator, eriochrome black T, was then added and titrated against 0.0499 M Na_2EDTA. The titer reading was recorded (A). The analysis was performed in triplicate. This titer reading a gives an estimate of the total metal content incorporated in the mixture.

Sample aliquots (200 µL each) of the cooled reaction mixture were also diluted to 5 mL with deionized water. The resulting suspension was then centrifuged and the supernatant was collected. This supernatant was then subjected to similar treatment as above. The titer reading in this case was recorded (B). The analysis was performed in triplicates. The titer reading B gives an estimate of solubilized metal ion content which corresponds to the amount complexed.

The reaction yield (Y) was calculated using fractions A and B (Equation 2.2). Fraction B represents the amount of metal that has been solubilized and is forming the complex while fraction A represents the amount of total metal ion.

$$Y = \frac{B}{A} \times 100 \quad \text{.....(2.2)}$$

Lyophilization:

All samples were filtered to separate unreacted metal. The filtrates were lyophilized using a Millrock Technology lyophilizer (Kingston, NY, USA). Briefly, samples were initially cooled from room temperature to -50 °C at atmospheric pressure. Primary drying consisted of heating from -20°C to 20°C at 50 µBar. Secondary drying occurred at a constant 20°C for 240 minutes to ensure complete drying. The resulting free-flowing powder samples were used for all the further complex characterization.

Mass Spectrometric analysis:

Powder complex samples were dissolved in a water: acetonitrile mixture (10:90 v/v) at 10mg/mL. Each complex was characterized by using electrospray ionization mass spectrometry (ESI-MS) using Qtrap3200 Mass Spectrometer (Life technologies Corp., CA, USA) with the Analyst software v1.5.1. ESI MS was performed in Q1 positive ion mode. A syringe pump was used for the continuous injection of the sample solutions at a flow rate of 10µL/min. The voltages optimized for each complex individually to optimize the mass/charge ratio for each amino acid complex.

HPLC:

A validated liquid chromatography method was developed for quantification of amino acids. This method was linear, precise, and accurate over the concentrations evaluated in this study. Briefly, A HPLC system consisted of a solvent delivery module (LC-10AT), an auto injector (SIL-10AD) programmed by a system controller (Model SCL-10A), an UV–Visible spectrophotometric detector (model SPD-6AV), purchased from Shimadzu (Tokyo, Japan) was used. The mobile phase consisted of 0.75 mM perfluorooctanoic acid in ACN: Water mixture (17:83) and the flow rate was maintained at 2 ml/min and monitored at 210 nm. Chromatographic separation was achieved at room temperature on a Phenomenex C-18 column (300 × 3.9 mm, 10 µm, CA, USA).

The data analysis was performed using VP-Class software version 7.2.1. The HPLC method was developed and validated for the quantitative analyses of glycine. The chromatographic separation was achieved on a Phenomenex C-18 column (300 × 3.9 mm, 10 µm, CA, USA) with a flow rate of 2.0 mL/min with UV detection at 210nm. Mobile phase was filtered and degassed prion to HPLC use. Standard solutions of glycine were prepared in deionized water. Glycine was obtained as a powdered reagent. It was reconstituted with deionized water to give a stock

of 40 mg/mL. Various standard solutions were prepared from this stock solution after appropriate dilution. The unknown free glycine concentration in the samples was determined by correlating the regression equation relating to the peak area, obtained from the set of standard solutions.

Quantification of amino acid purity:

Standard curves for remaining amino acids were prepared similarly to glycine. The standard plot equation of each amino acid was used to calculate the amount of free amino acid in their corresponding complexes. Peak area of amino acid was used to calculate the concentration of free amino acid in the respective complexes (Equation 2.6).

$$\%\,Purity = \frac{(Conc\,of\,sample - Calculated\,conc\,of\,amino\,acid)}{Conc\,of\,sample} \times 100 \quad \text{.....Eq.2.6}$$

Metal content analysis method:

The atomic absorption spectrophotometer, Varian Spectra AA200 consisted of flame attachment and acetylene-oxygen burner, purchased from Varian Inc. (CA, USA). All the data was obtained using software spectra AA. The instrument was first calibrated using the standard solution of the respective cations. All standards were run individually. Standard potassium solutions were prepared with 0.1907 g of dried potassium chloride added to 100 mL of Nanopure water to give 1000 µg/mL stock solution diluted to 0.2, 0.4, 0.6, 0.8 and 1 ppm. Standard sodium solutions were prepared with 0.2542 g of dried sodium chloride added to 100mL of Nanopure water to give 1000 µg/mL stock solution diluted to 0.2, 0.6, 1.2, 2 ppm. The amino acid complex solutions were prepared with 0.010 g of dried complexes added to 30 mL of Nanopure water serially diluted to obtain appropriate emission signal strength.

The unknown metal ion concentration in the amino acid complex samples was determined by interpolating from the regression equation relating to the mean intensity, obtained from the set of standard solution. The mean intensity was used to calculate the concentration of the metal in the complexes. These values were then converted to the ratio amino acid to metal ions (Equation 2.7). The amount of amino acid is assumed to be the difference between sample weight and the amount of metal ion.

$$\text{Ratio of amino acid to metal ion} = \frac{\text{Amt of amino acid}}{\text{Amt of metal ion (calculated)}} \times \frac{\text{Mol wt of Metal ion}}{\text{Mol Wt of amino acid}} \quad \text{..} \quad (2.7)$$

Determination of moisture content by Karl-Fischer titration:

Karl Fischer titrimetry (Mettler DL18 Karl Fischer titrator, NJ, USA) was used to determine the moisture content in the lyophilized samples of amino acid complexes. In Karl Fisher titration, powdered sample was added to the autotitrator which determines the total moisture content by titrating the water with the Karl-Fischer reagent. The percentage moisture content in the powders was reported. The experiment was performed in triplicate.

Fourier transform infrared (FT-IR) analysis:

The IR spectra for the complexes were obtained using a Nicolet Avatar 370 DTGS Series FTIR equipped with horizontal attenuated total reflectance (ATR) crystal (ZnSe) all from Thermo Scientific (Madison, WI, USA). Spectra were collected percent transmittance mode using sample powder placed directly onto the ATR crystal. Each spectrum is the result of the average of 32 scans at 4 cm^{-1} resolution. Measurements were recorded between 4000 and 550 cm^{-1}. All spectra were analyzed using IR Solutions® software.

Determination of Stability Constants:

The pH measurement for the experiment were performed using instrument equipped with hydrogen electrode and pH range of 0 to 14 all of which was obtained from Beckman Instruments Inc. (Fullerton, CA, USA). The instrument was calibrated with buffer solution of known pH before starting the pH titration.

Briefly, 1 M NaOH and 1 M HNO_3 solutions were prepared. Additionally, separate 0.1 M solutions of monovalent and divalent metal ion salts and amino acids were prepared. Each solution sets were mixed and purged with nitrogen gas. The mixture was allowed to equilibrate in a temperature bath for 15 min with the pH meter electrode immersed in the solution. Each set was titrated against 1.0 M NaOH. The titration recording was stopped when the pH reached 12. The titration curves were

plotted for all the sets for each amino acid. The titration curve was then used for calculation of the various formation constants $\bar{n}_A$, $\bar{n}$ & pL using the following formula

$$\bar{n}_A = Y - \frac{(V_2 - V_1)(N^0 - E^0)}{(V^0 + V_1)T_{Cl}} \quad (2.11)$$

$$\bar{n} = Y - \frac{(V_1 - V_2)(N^0 - E^0)}{(V^0 + V_1)(\bar{n}_A)(T_{CM})} \quad (2.12)$$

$$pL = log_{10} \frac{\sum_{n=0}^{n} \beta_n^H \frac{1}{(antilog\ \beta)^n}}{(T_{Cl} - \bar{n}T_{CM})} \times \frac{V^0 - V_3}{V^0} \quad (2.13)$$

Where,

$\bar{n}_A$ = Formation function for proton ligand constant

$\bar{n}$ = Formation function for metal ligand constant

N^0 = Molarity of NaOH

E^0 = Molarity of acid in final solution

V^0 = Total volume of the titration solution set (40 mL)

Y = Number of dissociable protons

V1, V2 and V3 = Volume of alkali employed to bring the sets A, B, C to same pH value

T_{CL} = Total concentration of ligand in the final solution

T_{CM} = Total concentration of metal ion in the final solution

The step-wise stability constants were then determined. The method utilized for the calculation of step-wise stability constant known as half integral method / interpolation at half $\bar{n}$ value. Plot of $\bar{n}$ v/s pL then the corresponding graph can be used to calculate the step wise stability constants log K_1 and log K_2

$$\text{Log}\, K_1 = \log \frac{\bar{n}}{1 - \bar{n}} + \text{pL} \quad (2.14)$$

$$\text{Log}\, K_2 = \log \frac{\bar{n} + (\bar{n} - 1)}{(2 - \bar{n})} + \text{pL} \quad (2.15)$$

Graph of $\bar{n}$ vs pL is plotted

By putting the value of $\bar{n}$ = 0.5 in equation we obtain

log K_1 = pL

By putting value of $\bar{n}$ = 1.5 in equation we obtain
log K_2 = pL

The main focus was on the determination of thermodynamic stability constant as determined by the pH metric method as a more reliable and generally utilized method [57, 58]. The pH change allowed use of the Bejrrum, Calvin and Wilson method in which the amino acid metal ion complex is competitively formed and then disassociates under changing pH conditions of the solution [59].

Determination of Calorific Value:

Powder complexes were pressed into a firm tablet masses by filling 0.6 gm of sample powder into a single punch and die mold at 1100 metric ton pressure using a tablet press (Carer Lab Press Inc., Menomonee Falls, WI, USA). A nickel alloy wire was fused with the compacted mass and used for electrical ignition of the sample inside the bomb calorimeter to cause combustion. The bomb calorimeter consisted of the oxygen bomb, ignition unit and the calorimeter (2L capacity) all purchased from the Parr-Oxygen Bomb Calorimeter (Moline, IL, USA). The temperature change in the calorimeter was measured using a standardized digital thermometer purchased from Cole-Parmer (Court Vernon Hills, IL). The pressurized oxygen used was purchased from Matheson, Trigas Inc. (Basking Ridge, NJ, USA).

The jacketed vessel was filled with 2 L of water. The sample mass fuse wire was connected to the two ends of the electrode inside the bomb, which was then closed and pressurized with oxygen gas until the pressure inside bomb reached 23 atm. The bomb was then submerged in the jacketed calorimeter vessel and the temperature was allowed to equilibrate. The sample was ignited and the temperature constantly recorded for 8 min.

The heat of combustion is directly proportional to the temperature increase (Equation 2.17).

$$\Delta H = -kS\Delta T \quad (2.17)$$

Where

S = Specific heat of water

k = Constant for the instrument

ΔH = Heat of combustion

ΔT = Change in temperature

First the constant for the instrument was calculated using benzoic acid as a standard reagent with known ΔH values. The value of kS was calculated using a series of combustions with benzoic acid. The value was then utilized to calculate the value of heat of combustion which is the calorific value of the substance

Measurement of Osmotic Pressure:

Tonicity was evaluated using a µOsmette osmometer (Precision Systems Inc., Natick, MA, USA) was equilibrated for 20 minutes and calibrated with 500 and 100 mOsm/L standards. 50 µL samples of 2% complex solutions in water and Gatorade® energy drink were analyzed in triplicates.

RESULTS AND DISCUSSION:

Synthesis of amino acid complexes with physiological metal ions:

Published studies have described various approaches to the synthesis of amino acid-metal ion complexes [33, 47, 49, 53, 58, 60-64]. Most synthetic schemes react the amino acid functioning as an acid, with the metal ion functioning as a base. The metal ion can be used in the metallic, hydroxide, or salt form but must be converted into metal hydroxide during the reaction. The selection of both the metal and the amino acid are important for the synthesis of complexes. For example, the chosen amino acids are physiologically relevant and represent different types like amino acids with polar side chains (charged/uncharged) or amino acids with non-polar side chains (aliphatic). Glycine is the simplest amino acid without a side chain and was selected because of its prototypical chemical properties. Alanine and valine were selected due to differing aliphatic side chain lengths with valine having a branched side chain and alanine having a straight side chain. Glutamic acid was also selected due to the presence of acidic functional groups on side chains while glutamine has polar side chain without charge.

These amino acids provided chemical diversity for evaluation of complex formation and characterization. Among the metal ions sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) were chosen due to their physiological relevance in electrolyte loss due to sweating during prolonged or intense physical exercise [63]. Additionally, Ca^{2+} and Na^+ are required for neuromuscular action potentials and muscle contraction K^+ and Mg^{2+} is required for relaxation of muscle. Na^+ and Ca^{+2} are major extracellular cations while K^+ and Mg^{2+} are major intracellular cations.

The synthesis of amino acid-metal ion complexes was affected by the reaction parameters such as the molar ratio of amino acid to metal ion, temperature, pH, duration of reaction [65]. The molar ratio of reagents is important to ensure sufficient interaction of the amino acid with the metal ion to form the complexes. The reaction temperature helps provide the necessary energy to drive the reaction forward and also keep the reactants in solution. The pH is required to be kept alkaline to ensure the carboxylate group and the amine group has the appropriate charge for complexation to occur but must be moderated to prevent excessive precipitation of the metal and break down of complex.

Formation of amino acid complexes:

Successful association of the amino acid and metal ion resulted in clear solutions of solubilized complex with unreacted metal forming an insoluble precipitate in the reaction mixture. Following lyophilization, all complexes formed white free-flowing powders. In addition to a physical description of reaction mixture appearance, the characterization of amino acid complexes was critical to ensure the production and quality of amino acid–metal ion complexes. For example, many commercially available products described as comparable complexes were reported to be physical mixtures of amino acids and metal salts[54]. Also, there have been reports of inadequacies in the analytical methods utilized for the quality control analysis of complexes [54, 65-68]. It is therefore necessary to develop an array of characterization techniques that would ensure identification and quantification of complexes.

The evaluation of reaction yield for divalent metal ion complexes was determined using different a complexometric titration reaction. Monovalent complexes are not able to participate in the competitive complexometric titration. Complexes

with Ca^{2+} and Mg^{2+} ions were evaluated using a competitive complexing agent, Na_2EDTA, to form competitive complexes with metal ions in the sample. The color of the unreacted solution is burgundy that turned teal blue in color when EDTA displaces the indicator and forms complex with metal ion. This indicator-based color change was used for determination of solubilized metal ion content and determination of total metal content in the reaction mixture.

The metal present is converted into hydroxide during the reaction which is insoluble. As complexation progresses the insolubilized metal hydroxide is converted into solubilized metal complexes. The solubilized metal content corresponds to the amount of metal converted into complex. Hence the yield would be the ratio of solubilized metal content to the total metal content of the reaction mixture.

The yield of the reactions for calcium complexes; alanine 52%, glycine 83%, glutamate 82%, glutamine 88%, and valine 46% and magnesium complexes; alanine 50%, glycine 82%, glutamate 87%, glutamine 89%, valine 43%, indicate that the amino acid with polar side chains (glycine, glutamine and glutamic acid) have higher yields of 85-90% than the amino acids with aliphatic side chains (valine and alanine) with yield values of 40-55 %. Metal ions being charged and polar would tend to associate more with polar amino acids leading to higher yields.

The literature reports yields ranging from 60-90%. Use of elemental form of metal or oxide form was reported to give better yields [61]. In addition to yield values, the mass spectrometric analysis provides the m/z peaks corresponding to the molecular weight of the amino acid complexes. Mass spectrometric analysis of all complexes identified either the intended complex molecular mass peak or the protonated complex molecular mass with the exception for the magnesium glutamine complex. The moderate to high yield values for divalent amino acid complexes as well as the presence of complex peaks by mass spectrometric analysis confirm the formation of the amino acid complexes.

Characterization of amino acid complexes:

The determination of amino acid to metal ion ratio in formed complexes gives an estimate of purity (**Table 1).** A higher-than-expected ratio suggests the presence of free amino acids with a lower-than-expected ratio suggesting the presence of uncomplexed metal salts. The ratio values in case of monovalent metal ions was between 1.1-1.7: 1 with divalent metal ions between 2.4-4.2: 1. These values are higher than the theoretically calculated value for the complexes and suggest the presence of excess free amino acid (**Fig.1**).

TABLE 1 MOLAR RATIO OF AMINO ACID TO METAL ION

Amino acid per mole of metal ion	Na^+	K^+	Ca^{2+}	Mg^{2+}
Valine	1.6	1.4	4.2	3.7
Alanine	1.5	1.5	3.9	3.5
Glycine	1.6	1.4	2.8	2.7
Glutamate	1.6	1.1	2.7	2.5
Glutamine	1.7	1.2	2.6	2.4

Fig. 1 Purity of amino acid complexes

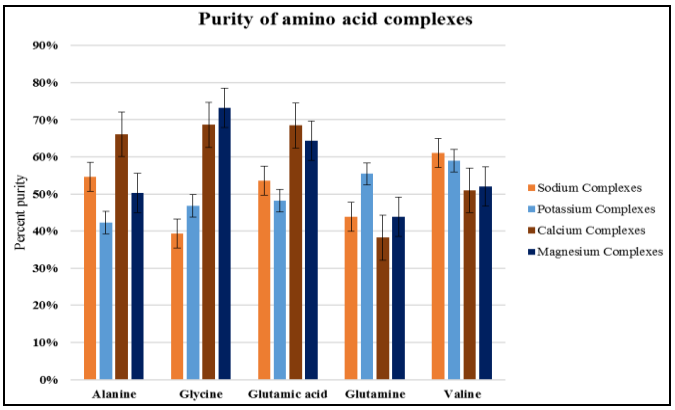

FIG.1: FIVE AMINO ACIDS (ALANINE, GLYCINE, GLUTAMIC ACID, GLUTAMINE AND VALIVE) WERE CHELATED WITH FOUR ELECTROLYTE SALTS (SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM) TO GENERATE CHELATED COMPLEXES FOR PRE-FORMULATION STUDIES.

The moisture content for the amino acid complexes was found to be between 2-3.5% for the complexes. Alanine and glutamic acid complexes with Na^+ and K^+ had slightly higher moisture content than the remaining complexes (**Table 2**). The moisture content of the complexes was found to be within acceptable limits so as to not interfere with other analytical procedures.

TABLE 2: AVERAGE PERCENT MOISTURE CONTENT OF AMINO ACID COMPLEXES.

Amino acid complexes of	Na^+	K^+	Ca^{+2}	Mg^{+2}
Alanine	3.25 ± 0.22	3.3 ± 0.19	1.94 ± 0.17	1.83 ± 0.12
Glutamic acid	2.92 ± 0.17	2.67 ± 0.12	1.85 ± 0.20	1.88 ± 0.09
Glutamine	1.89 ± 0.14	1.96 ± 0.11	1.68 ± 0.08	1.76 ± 0.09
Glycine	2.12 ± 0.13	2.22 ± 0.17	1.84 ± 0.15	1.76 ± 0.11
Valine	2.08 ± 0.16	1.99 ± 0.21	1.89 ± 0.08	1.98 ± 0.22

The detection and quantification of amino acid is a challenging task due to the absence of distinct chromophores and often requires derivatization [69,70]. However derivatization is not a valid approach due to the breakdown or disruption of complex giving false estimation of the free amino acid content. To circumvent this issue the HPLC/UV analysis was performed at a lower and nonspecific wavelength of 210 nm as reported previously [64,71]. At this wavelength the carboxylate group of amino acid, complex shows ultraviolet absorption. The column and mobile phase conditions facilitate separation of the free amino acid when used with an ion pairing reagent [64]. Glycine was chosen as a model amino acid for method development and validation

The estimation of amino acid complex purity is a critical aspect of characterization of complexes. Amino acid complexes which were synthesized still had amino acid present in them, which is difficult to separate due to similar physicochemical characteristics. The amount of free amino acid was quantified using the validated HPLC method. The amino acids peak show a retention time between 2.5 to 3.5 min while the complexes show a peak between 14-16min. The components are well separated and the free amino acid can be easily quantified.

The specificity of this reverse phase HPLC method was determined by comparing the chromatograms obtained by injecting the mobile phase alone (blank), glycine in mobile phase, mixture of glycine and metal salts in mobile phase and the metal-glycine complex in mobile phase. Mobile phase was spiked with stock solution of glycine, glycine and metal salt mixture and metal glycine complex respectively. Each of these sample was injected into the system. The representative chromatograms of mobile phase alone without analyte mobile phase contacting glycine, mobile phase containing glycine and calcium ion salt, and mobile phase contacting calcium glycine calcium complex, clearly indicate separation and detection of amino acids and amino acid complexes. It is therefore proved that glycine and the complex have distinguished peaks from one another. The retention time for glycine was found to be 2.89 min, that of the complex was found to be 8 to 10 minutes and that of metal ion was found to be 14-16minutes. Since glycine peak can be distinguished from other peaks without and interference from the mobile phase signal it can be said that the method is specific for analysis of free glycine present in the complex.

The amino acid-based purity of complexes ranged from approximately 40% to 72%. Calcium and magnesium complexes showed higher purity values yields as compared to sodium and potassium complexes. This can be attributed to the fact that sodium and potassium form weaker complexes which might lead to lower purity. Glutamine slowly degrades over time in presence of moisture explaining the general lower purity values[72]. Additionally, calcium and magnesium along with aliphatic amino acid tend to form complexes with higher purity.

Determination of complex stability:

These results indicate amino acids and metal ions were physically co-located in the reaction mixture and lyophilized powders. Additionally, the low moisture content, moderate yield, and moderate purity of amino acid-metal ion associations suggest complexes likely formed. To prove amino acids and metal ions were not only co-located, their associations were measured using changes in the FT-IR spectra for complexes compared to free amino acids. Specifically, amino acids associate with the metal ions through the amino and carboxylate groups. The FT-IR spectra showed -NH_3^+ peaks for the acids that are replaced by a broad $–NH_2$ stretch for the complexes. The $–COO^-$ stretch is also shifted slightly to a shorter wave

number for complexes. These changes were consistent with all the amino acid complexes and indicate complexes formed (**Fig. 1**).

Additionally, the determination of a stability constants gives a measure of the interaction strength of amino acid and the metal ion. A stronger interaction results in a more stable complex and can affect hydration and electrolyte supplementation in potential ergogenic aids. The bioavailability of the metal ion is influenced by the thermodynamic stability constant. The difference in the pH measurement during the titration of amino acid solution in the presence and absence of metal ion was used to calculate the formation functions the stability constant for calcium-glutamic acid and calcium-valine complexes [59, 73]. There is a difference between titration curves of amino acid v/s amino acid and metal ions. The stability constant for the amino acid complexes was calculated using the titration curves. The amino acid complexes had stability constants of log K glutamic acid 1.51 $mol^{-4}dm^{12}$ and valine log K 1.85 $mol^{-4}dm^{12}$ indicating weak associations that should survive the GI tract but still promote absorption.

Measurement of Osmotic Pressure:

The amino acid-metal ion complexes should be capable of generating sufficient osmotic pressure to be near isotonic to function as potential ergogenic aids for the replenishment of water and electrolytes. Often, iso-tonic or mildly hypertonic solutions are used before or during intense exercise. In contrast, hypertonic solutions (e.g. 10-15% dextrose solution) are often used after exercise for replenishing the liver stores of glucose and glycogen[15]. Arbitrarily chosen complex concentrations of 2% for all complexes showed isotonic or near isotonic values (**Tables 3** and **4**).

In contrast, a 2% solution of calcium gluconate was hypotonic as was a commercially available hydration formulation. Amino acid-metal ion complexes can therefore be prepared so that the colligative property of osmotic pressure ensures isotonic values that can still provide physiologically-relevant electrolyte replenishment and amino acids as a precursor.

TABLE 3: OSMOTIC PRESSURE VALUES OF COMPLEXES (IN mosm/kg)

Amino acid complexes	Na^+	K^+	Ca^{2+}	Mg^{2+}
Alanine	257 ± 0.6	233 ± 0.6	251 ± 0.6	304 ± 0.6
Valine	277 ± 1.2	194 ± 1.15	278 ± 1.2	341 ± 7.5
Glycine	259 ± 0.5	273 ± 12.1	306 ± 3	362 ± 1.1
Glutamine	136 ± 1	167 ± 1.5	189 ± 1	248 ± 1
Glutamic Acid	170 ± 1	143 ± 0.5	134 ± 0.5	235 ± 0.5

TABLE 4: OSMOTIC POTENTIAL VALUES OF FOR COMPARISON

Samples	Osmotic pressure (mosm/kg)
Ca Gluconate	88 ± 1.5
Gatorade® (2%)	134 ± 1
Blood	290*(literature)

Determination of Calorific Value:

The calorific values of all glycine complexes as well as all calcium complexes were determined in the similar fashion (**Table 5**).

TABLE 5: CALORIFIC VALUES OF AMINO ACID COMPLEXES AND CALCIUM GLUCONATE

Sample	Calorific Value (Kcal/ gm)
Calcium Gluconate	2.694
Calcium Glycine	2.018
Magnesium Glycine	1.816
Sodium Glycine	2.058
Potassium Glycine	2.066
Calcium Alanine	1.8927
Calcium Valine	1.989
Calcium Glutamine	2.099
Ca Glutamic acid	2.144

Summary: Amino acid chelated complexes were synthesized and characterized. The initial studies described above demonstrated that the chelated complexes can produce sufficient osmotic pressure to be used as ergogenic aids. If the amino acid complexes are mixed in nutritionally relevant doses of each amino acid and metal ions the resulting solution's osmotic pressure can be suitably adjusted to make it isotonic. Also amino acid complexes have sufficient calorific value to be used for ATP production but further studies are required to determine the electrolyte absorption, potential cellular hydration, or ATP production provide a significant benefit to the athlete. The calorie content in the amino acid complexes described in this manuscript is less than the corresponding sugar based products and may exhibit better potential ergogenic utility since they act as electrolyte carriers.

Analysis of changes in the spectra for complexes compared with free amino acids. The changes in the carboxylate and amine group stretches in FT-IR indicated the association of metal ion with the amino acid. This proved the complexation of metal ion by the amino acid. The metal content analysis indicated that there was presence of excess amino acid over metal ion. The HPLC method for determination of free amino acid content in the complexes was developed and validated and found to be linear, precise and accurate within concentration range of 0.157 mg/mL to 20.14 mg/mL. The method was used to estimate the purity of the amino acid complexes. The purity of the complexes was found to be ranging from 42-75%.

This further supports the presence of free amino acids along with complexes. The stability constant was determined for two amino acids valine which has aliphatic side chain and glutamic acid which has a carboxylate group on the side chain. In spite of the difference in the functional groups the stability constants for both the amino acid complexes were similar indicating that the side chain functional group did not participate in the complexation to provide additional stability. Hence the type of amino acid has little influence on the stability of complexes.

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