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**SYNTHESIS AND CHARACTERIZATION OF AMINO ACID COMPLEXES OF
PHYSIOLOGICAL METAL IONS TO BE USED AS ERGOGENIC AIDS**

By

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A THESIS

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Abstract

Electrolyte replenishments are largely used by athletes and have become an integral part of sports science. Long duration physical exercise causes loss of water and essential electrolytes resulting in dehydration that hampers athletic performance. Hence hydration is necessary during extended physical exercise. Ergogenic aids are very broad range of physical, pharmacological or other aids including electrolyte and hydration supplementation. The current approaches to supplemental hydration therapy uses isosmotic, electrolyte and sugar solutions which help in hydration better than plain water. However this approach to hydration therapy is limited by high sugar content which can cause gastric distress. Additionally, certain metal ions used for electrolyte replenishment are very poorly absorbed in the GI tract when given in inorganic forms and often fail to correct hyponatremia. These limitations suggest alternative approaches to hydration therapy with focus on electrolyte replenishment, would improve the treatment of exercise-induced dehydration.

Amino acid complexes have been proven to be effective in improving the absorption of various metal ions. Amino acids also play a crucial role in regulating muscle physiology during and after increased muscle exercise. Amino acid-based complexes with metal ions could overcome the current limitations in hydration therapy and also function as ergogenic aids during extended physical exercise. The current research focus is to develop amino acid complexes with physiologically relevant metal ions to promote hydration and be used as ergogenic aids. Amino acid complexes with physiologically relevant metal ions were prepared with glycine, alanine, valine, glutamine, and glutamate with sodium, potassium, calcium and magnesium. The synthesis parameter were

optimized to produce complex yields of up to 90%. Amino acid-metal ion complex formation was demonstrated through a variety of characterization methods, including MS and FTIR. Reaction mixtures were also freeze dried for additional characterization. This report describes the synthesis and characterization of physiologically relevant metal ions complexed with amino acids for the eventual use in hydration and electrolyte replenishment and as ergogenic aids.

Preface

Abstracts

1. N. J. Kathe, E.J. Haas, H.V. Chauhan, J. Fujita, B. S. Henriksen “Synthesis and Holistic Characterization of Glucogenic Amino Acid Complexes as Ergogenic Aids” AAPS annual meeting, San Antonio, Nov 2013 (**Poster**)
2. N.Kathe, E. Haas, PhD. B.Henriksen, PhD. “Synthesis and holistic characterization of essential amino acid complexes” ACS regional Meeting, Springfield, Oct 2013 (**Poster**)

Dedicated to my family and friends

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List of common abbreviations

ACN	Acetonitrile
MeOH	Methanol
PDFOA	Pentadecafluorooctanoic acid
EDTA	Ethlyenediaminetetraacetic acid
AAFCO	Association of American Feed Control Officials
BCAA	Branched chain amino acids
ESI-MS	Electron spray ionization mass spectrometry
FT-IR	Fourier Transformed IR
AES	Atomic Emission spectroscopy
M	Metal
Ca	Calcium
Mg	Magnesium
Na	Sodium
K	Potassium
OH	Hydroxide
Cl	Chlorine
N ₂	Nitrogen gas
O ₂	Oxygen
Zn	Zinc

CHAPTER 1:
**Introduction to amino acid
complexes and ergogenic aids**

1.1 Introduction to amino acid complexes

1.1.1 Introduction to complexation

Metal complexes consists of a central atom or ion called the coordination center and a surrounding array of bound molecules or ions, that are in turn known as ligands (1). Complexation phenomenon was discovered by Alfred Werner in 1893. He proposed a new area of chemistry based around the creation of multi-component compounds which consisted of a metal ion bound to different molecules, referred to as ligands, in order to form more stable structures with varying geometries (2,3).

There are certain requirements crucial for a molecule to be a complex (4). First, there must be more than one binding interaction between the metal ion and the ligand, one of which is an electrostatic or ionic interaction other type of interaction possible is covalent or coordinate covalent interaction. Second, the participating metal must have a fixed valency. Alfred Werner discovered that the metal atom have two types of valencies, namely primary and secondary valencies. Third, the coordinate covalent bond may be formed with neutral molecules or charged ions. Fourth, the bonds that the metal and the ligand form must have defined spatial arrangements. These requirements then affect the strength, geometry, and physico-chemical characteristics of complexes.

Metal ions form the central core of the complexes and have great influence on the resulting complexes formed. During complexation the metal ions acts as a Lewis acid and reacts with the ligand. The bond formation or sharing of electrons between the two results in neutralization of the charge. The presence of d-orbital shells in the metal ion imparts the ability to have more than one oxidation state which in turn gives the metal ion a

higher coordination number. Having higher co-ordination number allow metal ions to form multiple bonds with the same or different ligands to form bidentate, tridentate, or polydentate complexes. Transition metal ions (e.g. lanthanide metals) can form stable complexes based on a large potential number of coordinated covalent bonds (5). On the other hand, alkaline earth metals form relatively weaker complexes due to their smaller relative size and lack of available d-orbital shells (6). The weakest complexes are typically associated with monovalent metal ions (e.g. Na⁺ and K⁺) due to the presence of only one valance that allow for only electrostatic (ionic) bonds with various functional groups on potential ligands. Monovalent metal ion interactions with amino, amide, or carbonyl functional groups is rather weak and results in weak complexes that dissociate to a greater extent.

Ligands are molecules that associate with metal ions according to specific geometries to form larger associated complexes. Although there are often wide variety in the physicochemical properties of potential ligands, the minimum requirement is that ligands have at least 2 functional groups which are able to form chemical bonds through either electron donation or by sharing a lone pair of electrons forming a co-ordination covalent bond (7). Some functional groups are common present on a ligand (5) (Figure 1.1, 1.2)

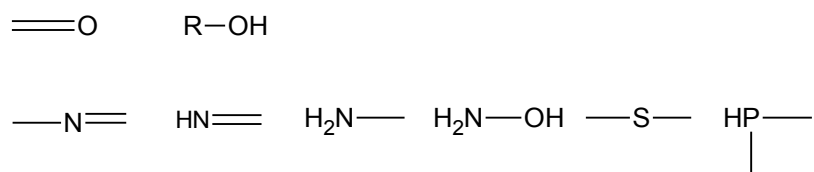


Fig 1.1 The functional group forming co-ordinate covalent bond

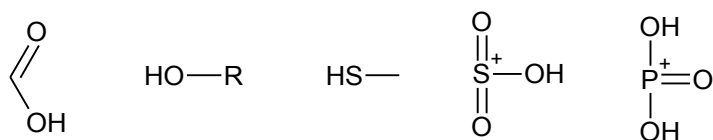


Fig 1.2 The functional group forming co-ordinate covalent bond

Generally the greater the number of coordinating groups the stronger the ligand is able to bind to a metal ion (8). The functional groups on the ligand must act as Lewis base while the metal ion acts as a Lewis acid. The strength of their bond is dependent on the strength of the Lewis base (5). Also steric hindrance and bulk of the ligand plays an important role in the relative stability of the complex. The number of co-ordinations possible for the ligand with the metal ion are limited by steric bulk of the ligand (5,7,9). The relative size of the ligand is also important, chain length up to 4-6 atoms are considered to be optimum for complexation with most metal ions. If the length is too small then the bond angles formed during complexation are too strained. However, if the length is too long (in case of peptides and other molecules) the chain can fold in various conformation and create a steric hindrance for stable complexation (9).

1.1.2 Amino acid complexes

Amino acid complexes are defined according to the Association of American Feed Control Officials (AAFCO) as, “the products resulting from the reaction of metal ion (in salt form) with amino acid in molar ratio of one mole of metal to one to three moles of amino acids to form a coordinate covalent bond. The average weight of hydrolyzed

amino acid must not be more than 150 Da and the molecular weight of the complex must not be exceed 800 Da” (10).

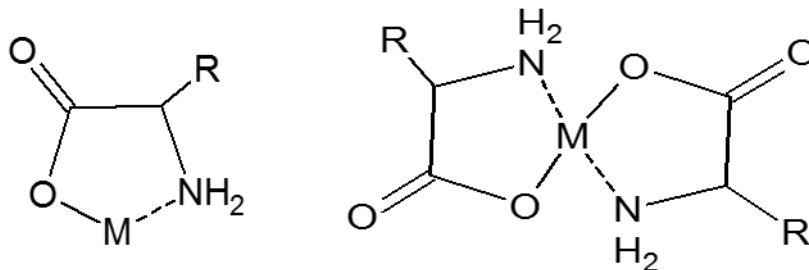


Figure 1.3 Structures of amino acid complexes

Amino acids in a complex provide the metal ion with an organic structure to shield its charge and facilitate its absorption (Figure 1.3). One of initial works on amino acid complexes by Morgan et al. elucidated the structure of these complexes. Through conductometric experiments, the nature of the bond between amino acid and the metal ion was determined to be non-ionic in nature, later termed as coordinate covalent bond (11).

Amino acids function as a good substrate due to the presence of both amino and carboxylate functional groups. They can form complexes with metal ions through electrostatic as well as co-ordinate covalent interaction. Physiologically amino acids are classified as glucogenic, ketogenic, or both. Glucogenic amino acids are those which on metabolism produce can produce glucose or intermediates of glycolysis while Ketogenic are amino acids that produce ketone bodies on metabolism. The amino acids used in the current study were all glucogenic as they have greater utility and less adverse effects. Chemically they belong to different chemical classes. Glycine, valine, and alanine are amino acids with aliphatic side chains while glutamic acid has a polar and charged side

chain and glutamine has a neutral side chain. Amino acids can serve as chemically diverse ligand pools which are physiologically useful. Different amino acids and metal ions have different extents of complexation. The same amino acid can be complexed with different metal ions having wide differences in stability constants (Table 2) (6,12,13). The stability constants of amino acid-metal ion complexes are lower than some synthetic ligands. For example, metal ions such as Ca^{2+} and Mg^{2+} form relatively weaker complexes than metals such as Fe^{3+} and Cu^{2+} (6).

However, the relative strength of the ligand-ion bond doesn't guarantee increased bioavailability. For example, the synthetic ligand EDTA was initially thought to improve the bioavailability metal ions such as Zn^{2+} , Fe^{2+} , Co^{2+} , and Cu^{2+} etc. (14,15). Bioavailability studies demonstrated that most of the metal-EDTA complexes were excreted unchanged in feces and urine (16,17). This was likely due to strong stability constants of complexes that lead to inhibition of complex metabolism and hydrolysis (18). When natural ligands like amino acids are involved in complexation, they mimic the stability of endogenous substances and can be metabolized or hydrolyzed. The stability constants of the amino acid based metal ion complexes are sufficient to allow dissociation of the metal ion following absorption of complexes (19). Ashmead et al. reported the ability of different natural organic ligands to increase the bioavailability of metal ions over synthetic ligands (20–22). Additionally, some weak dibasic acids (e.g. lactic acid, succinic acid) were proven to not be as effective ligands to increase the bioavailability of metal ions or act as an energy source. Amino acid complexes metal ions can be used as a better alternative for sugar-electrolyte solutions.

Ligand	Mg²⁺	Ca²⁺	Mn²⁺	Fe²⁺	Co²⁺	Ni²⁺	Cu²⁺	Zn²⁺
Glycine	3.44	1.38	3.44	4.30	5.23	5.77	8.62	5.52
Cysteine	~4		4.10		9.30		19.20	9.86
Serine		~0.5						
Arginine				3.87				
Aspartic acid	2.43	1.60	3.74		5.90	7.12	8.57	5.84
Histidine			7.74	9.30	13.86	15.90	18.33	12.88
Lysine			2.0	4.5	6.8	8.8	13.70	7.80
Glutamic acid	1.9	2.05	3.3	4.6	5.06	5.90	7.85	5.45
Glycylglycine	1.06	1.24	2.15		3.49		6.04	3.80
Glycylserine								3.7
Alanylglycine		0.66						3.0
Imidazole		0.08				2.94	4.20	2.58
Proline		~4			9.3		16.8	10.2
Phenanthroline							6.3	
EDTA*	8.69	10.70			16.30	18.55	15.70	16.47
2-Mercapto ethylamine					9.38	10.96		8.07

Table 1.1 Summary of stability constants of different ligands and metals (6) *(23)

1.1.3 Physiological fate of amino acid complexes

Amino acid complexes to be used as electrolyte supplements requires understanding of the mechanism involved in the absorption of the complexes and also the fate of these complexes after absorption.

1.1.3.1 Absorption of amino acid complexes

Mechanisms involved in the absorption of amino acid complexes are different from those for metal ions (22). 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 (Figure 1.4). 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 (22,24–26)

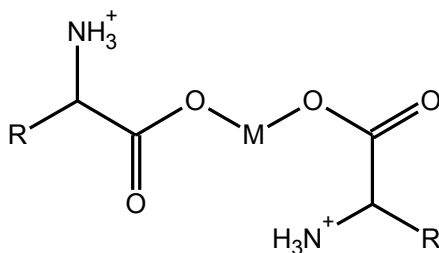


Figure 1.4 Amino acid complexes under acidic conditions.

There are mainly two main proposed mechanism involved in the absorption of amino acid complexes active and passive transport. Amino acid complexes upon exposure to acidic pH of the stomach are converted into protonated complex form (Figure 1.4). This protonated form of complex still holds the metal ion intact. 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 (27–29). Amino acid complexes act as a carrier molecule for metal ion during active transport. 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.

Passive-mediated diffusion or facilitated diffusion is a process of spontaneous passive transport (as opposed to active transport) of molecules or ions across a biological membrane via transmembrane proteins in the direction of concentration gradient (30–33). Facilitated diffusion is also important mechanism for the absorption of various amino acids and small peptides (34). There are various carrier molecules that help in the transport of amino acid complexes. Many times water is involved in the transfer of molecules across membranes, the process is called solvent flow (35). Water solubilizes or forms complexes with molecules to facilitate their absorption. In case of complexes the water molecules assemble with the complexes as shown in (Figure 1.5) either by occupying the unsatisfied valencies of metal ions or through electrostatic interactions. The amino acid complexes can also cross through the intercellular spaces which are well hydrates. The brush borders or villi present in the small intestine are responsible for absorption of large amount of water and solutes along with them and are likely involved in absorbing amino acid complexes. The combined result of these various routes of absorption results in increased net absorption of metal ions (e.g. Ca^{+2} , Mg^{+2} etc.) which is one of the key therapeutic goals for this thesis.

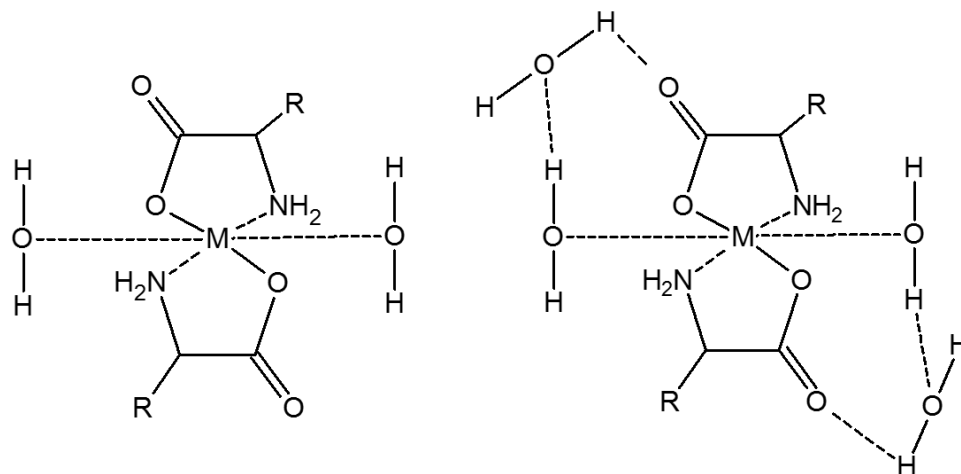


Figure 1.5 Structure of association of water molecules with amino acid complexes

1.1.3.2 Metabolism of amino acid complexes and amino acids

Following absorption, amino acid complexes are subjected to hydrolysis or the metal ion is exchanged with various intracellular ligands that are present in the cells. Radiolabelling studies with the metal ion have shown that the exchange liberates the metal ion from the amino acid and is then found to be assimilated by the body (36). The metal ions and amino acids can be released into the blood or can be incorporated into synthesis of biomolecules, etc.

After the amino acid complexes liberate the metal ion, the utility of these amino acids as ergogenic aids is of particular interest. These amino acids suffer the same fate as ingested amino acids and are digested. Therefore, it is important to understand the metabolism of

amino acids leading to energy production. Some amino acid are glucogenic and can be used to produce glucose or metabolic intermediates of the Krebs Cycle (Figure 1.6).

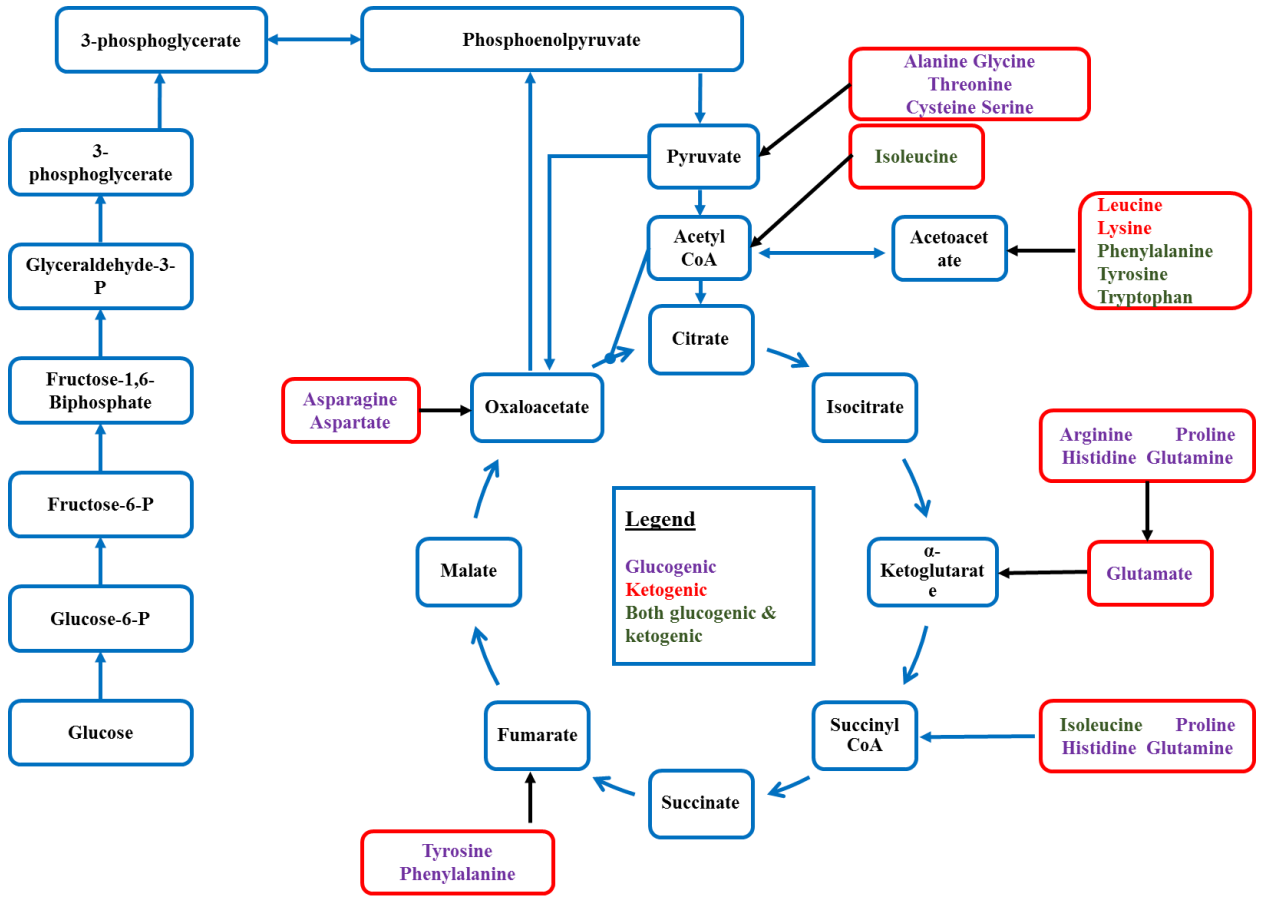


Fig 1.6 Overview of metabolism of amino acids and sugars

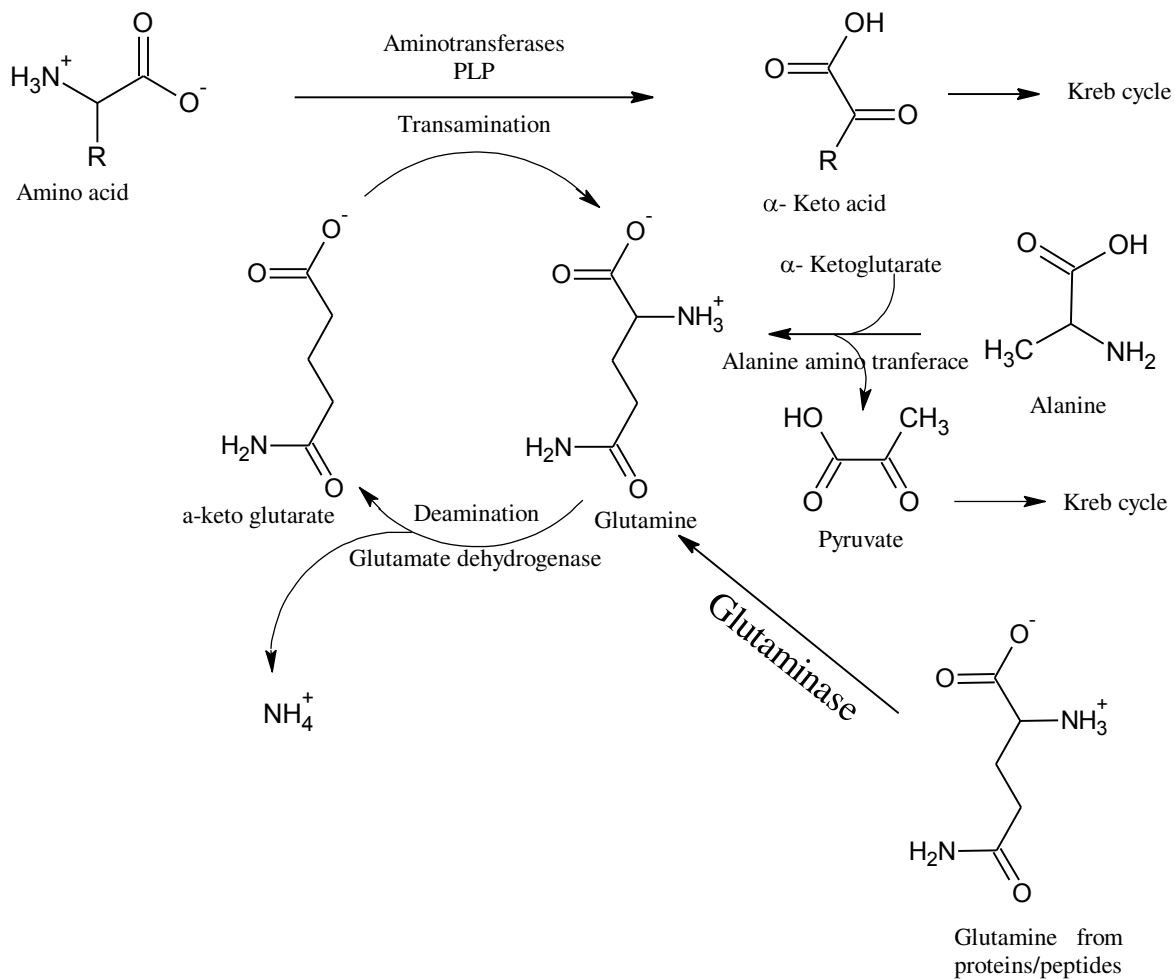


Figure 1.7 Summary of transamination and deamination reactions

Amino acids undergo certain bioprocessing steps before being utilized as an energy source. Firstly, nitrogen on the amino acids must be removed before amino acids become metabolically useful (37,38). Secondly, each of the different amino acids takes different metabolic pathways. To summarize them, there are two principle types of reactions that amino acids undergo, transamination and deamination (figure 1.7) (39). In the first kind of reaction, the enzymes aminotransferases convert amino acids to their respective α -ketoacids by transferring the amino group. This α -ketoacid can then enter

the Krebs cycle for energy production. The second kind of reaction, deamination, removes the amino group of the amino acid in the form of ammonia which is excreted by urea cycle (39,40). In the liver, the oxidative deamination of glutamate results in formation of α -keto-glutarate (a Krebs cycle intermediate) and ammonia, which is converted into urea and excreted (41). Deamination reactions in other organs forms ammonia that is generally incorporated into glutamate to generate glutamine, which is the main transporter of amino groups in blood. Hence, all amino acids through transamination/deamination reactions can be converted into intermediates of Krebs cycle, directly or via conversion to pyruvate and acetyl-CoA. Amino acid complexes can thus be utilized as a source of energy which can be utilized by the body.

1.2 Introduction to electrolyte replenishments and ergogenic aids.

Ergogenic aids are very broad range of physical, pharmacological or other aids that help in improving athletic performance. These ergogenic aids can be classified as nutritional ergogenic aids (e.g. creatine, protein supplements), sugar and electrolyte supplements (e.g. sports drinks), and drug based ergogenic aids (e.g. caffeine and androgenic steroids). These aids works by different mechanisms to improve athletic performances with varying levels of effectiveness and safety (42,43). The use of these agents is very prevalent among athletes and sportsmen (44). Commonly used ergogenic aids have a variety of uses and limitations (Table 1.2).

Product	Current Use	Limitations
Creatinine	Improves muscle stamina and performance (45)	Causes fluid retention. Concerns for possible renal and muscle dysfunction (46)
Caffeine	Ergogenic due to stimulant effect. (47)	Causes tachychardia, diuresis (48)
Anti-oxidants	Helps in reducing the oxidative stress during performance (49)	Limited potential benefits
β -Hydroxy β -methyl butyric acid (HMB)	Increases lean muscle and muscle mass, tissue repair (50)	Limited benefits
Protein	Can improve muscle repair and also play a role in energy production	Excess amino acids and protein can create stress on renal system
Sugar and electrolyte hydration mixtures	Help in hydration and replenish necessary electrolyte and sugar	Gastric distress, Poor absorption of certain ions.

Table 1.2 Summary of different ergogenic aids used in sports nutrition.

1.2.1 Physiological changes during intense physical activity

Ergogenic aids help in more than one way in improving athletic performance. It is therefore essential to understand the physiological changes that are taking place during intense physical activity. It is also helps in understanding how ergogenic aids can help in countering the physiological challenges during intense physical activity.

During exercise, the body's demand for nutrients and metabolic precursors increases tremendously. There are various rapid physiological changes that occur to accommodate this increased demand. First, instant sources of cellular energy are utilized, such as ATP and phosphocreatine (51). 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 Krebs Cycle. 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 anaerobic respiration producing lactic acid. (51). This is not a very efficient process which only yields 3 ATP per glucose molecule.

Energy production under prolonged or intense exercise is based on 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 Krebs Cycle (37,52,53). Amino acids are metabolized primarily in the liver with additional metabolism in muscles (53). 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 (54). After prolonged or intense exercise, there is rebound in the rate of muscle anabolism. The extent of catabolism and the recovery afterwards is dependent on the amino acid levels. 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 increase and compensatory increase in sweating resulting in water and electrolyte loss (55). Sweating can lead to dehydration which impairs athletic performance (56).

1.2.2 Current therapy for hydration and electrolyte supplementation

The focus of the current research is the problem of dehydration and electrolyte replenishment. The ergogenic aids belonging to this class are fluid replenishment containing sugar electrolyte solutions. These solutions are often consumed before, during, and/or after prolonged intense exercise to assist in maintaining proper hydration, and electrolyte balance to counter the loss through sweat (57,58). Sugar addition also increases the solution's residence time in the gut and promotes absorption of water. Extremely hypotonic solutions, including plain water, causes quenching of thirst before the body achieves proper hydration (59). Conversely, hypertonic solutions further dehydrate the body and worsen water loss. Hence to get proper hydration the solutions need to be isotonic. There are various ergogenic aids belonging to this class which counter hydration and electrolyte supplementation (Table 1.3)

Category	Advantages	Disadvantages	Commercial Example
Water	Hydration No calories	Low electrolyte Thirst quenching,	Dasani Fiji
Fruit Juice	Contains vitamins, minerals Electrolytes, antioxidants Sugar etc	High sugar GI distress Tooth enamel decay	Orange Grape Apple Grapefruit
Fruit Punch	Hydration fortified with some vitamins	High sugar, Lacks important nutrients GI distress	Fruit Punch Orange drink
Vitamin Water	Hydration	Lacks important nutrients	Glacéau Vitamin water
Sport Drinks	Hydration Electrolytes (sugar)	High in calories GI distress	Gatorade Powerade

Table 1.3 Summary of different hydration supplements

The sugar electrolyte solutions contain metal nutrients in inorganic salt form. Many physiologically relevant electrolytes (e.g. Ca^{2+} and Mg^{2+}) are poorly absorbed across the gastrointestinal mucosa and play a critical role in nerve and muscle function (60,61). For example, Ca^{2+} and Na^+ are required for firing of action potential and muscle contraction, while K^+ is required for restoration of membrane potential and relaxation of muscle, and Mg^{2+} is required for relaxation of the muscle (62). These electrolytes have rate-limited absorption due to saturable transport mechanism (63,64). Electrolyte replenishment needs in cases of prolonged or intense exercise might then exceed typical physiological capacities for ion absorption. Therefore, approaches to increase the rate of electrolyte absorption or bypass ion transport processes could be used to improve the absorption of supplemented metal ions.

The current use of sugar and electrolyte solutions (e.g. Gatorade[®]) has limitations associated with the correction of electrolyte imbalances (e.g. hyponatremia) in conditions of prolonged or intense exercise (65). These products contain large amount of sugar and have been shown to affect systemic hormonal levels (e.g. insulin) that can impair athletic performance. Additionally, the high sugar content in these electrolyte solutions can cause gastric distress and further affect hydration. The high sugar content can also promote the storage of sugar in the form fat for future energy needs and lead to metabolic syndromes (66,67).

1.2.3 Amino acids as ligands for ergogenic complexes

Complexation of metal ions doesn't assure increased absorption or nutritional utility of complex components as seen in case of EDTA (68). There are specific considerations for

a complex (or a ligand to be used for forming a complex) to be used as nutritional aid (68,69). Briefly, it is necessary that the stability constants of the complexes should be similar to some biological ligands (5). The complexes should have low molecular weight (<1000 Da) to ensure easier diffusion and uptake (5,70). After absorption the complex must hydrolyze to release the metal ion (5,71)

Amino acids tend to form complexes with metal ions in the ratio of either one, two or three molecules of amino acids to one molecule of metal ion (depending on the type and valency of the metal ion). This complexation ratio then leads to molecular weights of approximately 1000 Da or less. Amino acid complexes have stability constants that are optimum. 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.

1.3 Summary

Amino acid complexes are currently being used as metal supplements and have showed improvement in the bioavailability(36). Thus 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

Hypothesis and specific aim

The hypothesis of the current thesis is that *amino acids can form complexes with physiological metal ions to be used as ergogenic aids.*

This hypothesis will be accomplished through three aims. First, amino acid complexes will be synthesized using physiologically relevant metal ions and amino acids. Amino acids will be selected which are glucogenic in nature in order to serve as a source of energy. The second aim is to characterize the complexes, including parameters like identity, spectral characteristics, composition, stability constants and purity. All these parameters are critical to ensure quality product. The third aim it to evaluate the potential of selected complexes to act as ergogenic aids, including the evaluation of caloric content and osmotic pressure.

CHAPTER 2:
Synthesis of amino acid complexes
of physiological metal ions

2.1 Introduction

Numerous studies have described various approaches to the synthesis of amino acid-metal ion complexes (20,32,36,69,72–76). Most synthetic schemes react the amino acid functioning as acid, with the metal 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 is 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 the necessary chemical diversity for effective study of their complexes. 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(77). Ca^{2+} , Na^+ is required for firing of action potential and contraction of muscle while K^+ , Mg^{2+} is required for relaxation of muscles. Na^+ , Ca^{2+} is a major extracellular cation while K^+ , Mg^{2+} are major intracellular cations. These ions also form the major component of sweat and were chosen as candidates for synthesis of complexes.

The complexation reaction occurs at elevated temperature and under basic conditions. Specifically, amino acid complexes with sodium and potassium are prepared from the metal hydroxide (Figure 2.1) while complexes with calcium and magnesium are prepared from the chloride salt form in the presence of potassium hydroxide as a base (Figure 2.2).

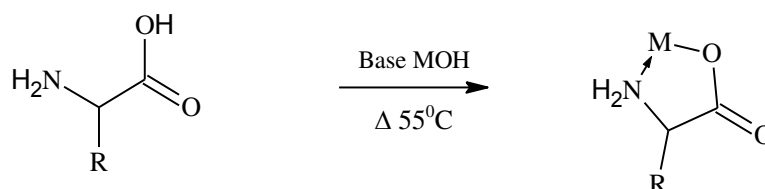


Figure 2.1 Complexation of monovalent metal ions with amino acid

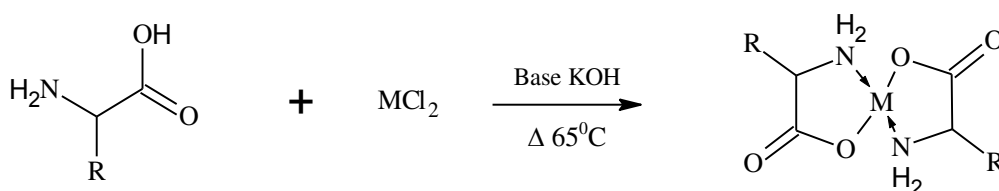


Figure 2.2 Complexation of divalent metal ions with amino acid

The synthesis process is affected by all the various synthesis parameters such as the molar ratio of amino acid to metal ion, temperature, pH, duration of reaction (78). 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 have the appropriate charge for complexation to occur but must be moderated to prevent excessive precipitation of the metal and break down of complex.

2.2 Materials and 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).

2.2 Synthesis

The monovalent metal ions are presented as hydroxides that react with the amino acids the reaction is more favorable and reaches completion faster and lower temperatures are required. On the other hand calcium and magnesium complexes require longer reaction time and higher temperature.

2.2.1 Synthesis of sodium/potassium complexes.

For synthesis of sodium and potassium complexes involves the addition of 1mmol of amino acid and 1mmol of metal hydroxide in 15 mL of deionized water and refluxed at 55°C for 1 hr. The reaction mixture was then cooled and then subjected to lyophilization discussed further.

2.2.2 Synthesis of Calcium/Magnesium complexes.

The optimized synthesis method for calcium/magnesium complexes involves addition of 1mmol of calcium/magnesium chloride along with 2mmol of potassium hydroxide and 2

mmol of amino acids in 15 mL water and refluxed for 3-4 hours at 65°C. The resulting solution was cooled and subjected to further processing as subsequently described.

2.3 Determination of synthesis yield for calcium and magnesium complexes

2.3.1 Introduction

In case of calcium and magnesium complexes, yield determination was carried out using the complexometric titration reaction where insoluble calcium and magnesium was consumed and converted into soluble Ca^{2+} and Mg^{2+} complexes. The extent of calcium and magnesium solubilized then provides an estimate of the extent of reaction.

The complexometric reaction was conducted using a competitive complexing agent, Na_2EDTA , to form complexes with Ca^{2+} or Mg^{2+} ion in the amino acid complex sample. The color of the solution is burgundy, on addition of the indicator, when EDTA displaces the indicator and forms complex with metal ion, the free indicator has a teal blue color which marks the end point of the reaction (Figure 2.3).

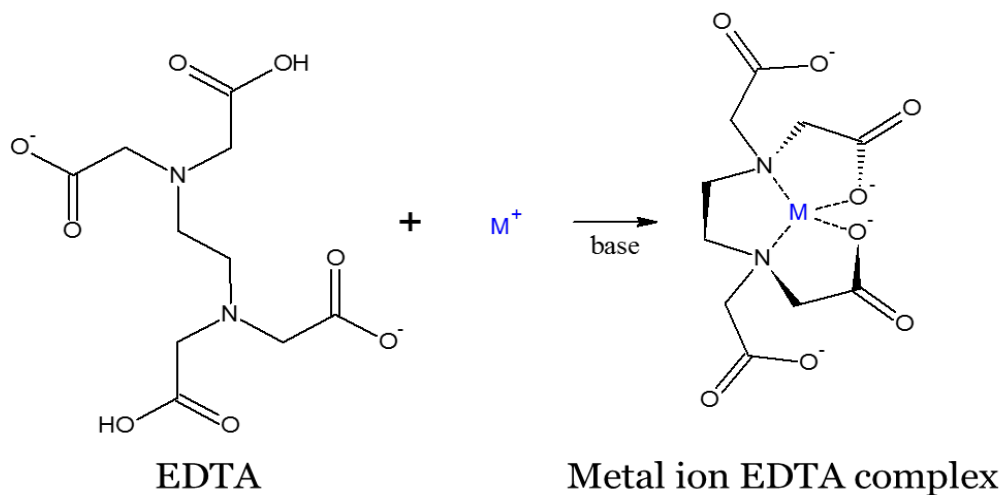


Figure 2.3 Reaction between EDTA and the metal ion.

The calculation of yield using complexometric titration involves 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.

2.3.2 Sample analysis

Aliquot of 400 μL of sample was drawn from the reaction and divided into two fractions (200 μL each). The first fraction was diluted to 5 mL with nanopure water and reacted with 1 mL of 1M HCl for 2 minutes. Then 3 mL of 1 M ammonium hydroxide was added. Indicator, eriochrome black T was then added. The resulting solution was then 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 that went into the reaction.

The second fraction was 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 that is complexed.

2.3.4 Calculation of Yield

The reaction yield (Y) was calculated using fractions A and B (Equation 2.1). 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.

$$Y = \frac{B}{A} \times 100 \quad (2.1)$$

2.3.5 Results and discussion

The yield of the reactions for calcium complexes and magnesium complexes (Figure 2.4) 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 % (Figure 2.4). 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.

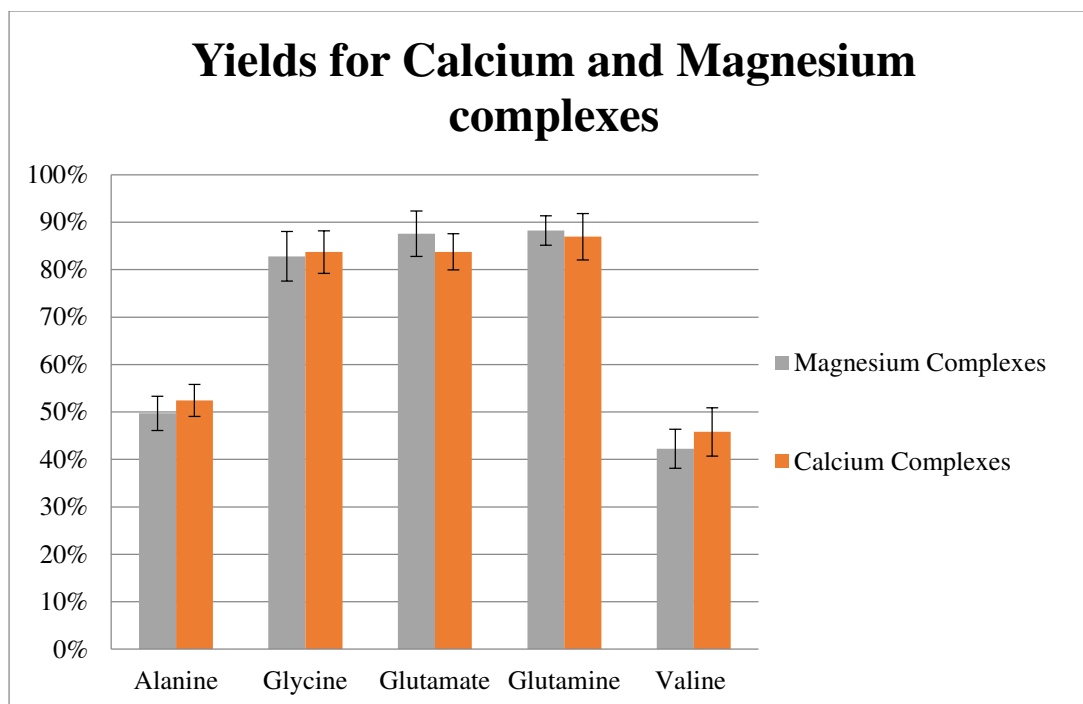


Figure 2.4 Synthesis yield for calcium and magnesium complexes of amino acids

2.4 Post synthesis processing

After the synthesis and establishment of synthesis yield, samples were further processed for subsequent analysis. All samples were filtered to separate out any unreacted metal fraction. The resulting solutions were lyophilized using Millrock Technology (Kingston, NY, USA) lyophilizer. Samples were cooled from room temperature to $-50\text{ }^{\circ}\text{C}$ at atmospheric pressure, followed by primary drying. In this step, the solution were heated from $-20\text{ }^{\circ}\text{C}$ to $20\text{ }^{\circ}\text{C}$ and the pressure was dropped to $50\text{ }\mu\text{Bar}$ and the third step was secondary drying where the sample was held at $20\text{ }^{\circ}\text{C}$ for 240 minutes to ensure complete drying. The resulting samples were used for all the further characterization studies.

CHAPTER 3:
**Characterization of amino acid
complexes**

Characterization of amino acid complexes is critical to ensure the quality of amino acid–metal ion complexes, as it was discovered that many commercial products that claimed to be complexes were physical admixtures of amino acids and metal salts (79). Also, there have been reports of inadequacies in the analytical methods utilized for the quality control analysis of complexes (78–82). It is therefore necessary to develop an array of characterization techniques that would ensure identification and quantification of complexes.

3.1 Materials and Methods

3.1.1 Mass Spectrometric analysis

Mass spectrometric analysis is one of the confirmatory tools used often in analysis of synthesis work. The technique provides data corresponding to the molecular weights of the compound of interest or the molecular fragment of it. These values can confirm the formation of the compound of interest.

Acetonitrile, was purchased from Sigma Aldrich (St. Louis, MO, USA). 10mg/mL sample of amino acid complexes were prepared in a water:acetonitrile mixture (10:90 v/v). 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 10 μ L/min. The voltages optimized for each complex individually to optimize the mass/charge ratio for each amino acid complex.

3.1.2 Determination of moisture content by Karl-Fischer titration.

The freeze drying process converted solutions into powders which are more suitable for analysis and characterization. In (Fourier Transformed Infra-red) FT-IR analysis, moisture content above 5% can cause strong absorption in the 3000 cm^{-1} range and interfere with signals for detection of $-\text{NH}_3^+$ (83).

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 carried in triplicates.

3.1.3 FT-IR analysis.

The process of complexation involves the association of the functional groups on amino acid with the metal ion. The chemical changes that occur during complexation are reflected in the spectra of samples. The infrared spectra has been described as the chemical fingerprint of the molecule. Any changes in the chemical structure causes characteristic changes in the spectra. These characteristic changes can be attributed to the changes in the structure like complexation.

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.

3.1.4 Metal content analysis

The determination of metal content of amino acid complexes is an important aspect of characterization of amino acid complexes and gives an estimate of its purity. If the ratio is too high than the theoretical value then it implies that there is free amino acid and if the ratio is too low then it implies that metal is present in the form of inorganic salts. Product having inorganic salt form of metal ions is of little utility.

Materials and method

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.

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.

For preparation of standard potassium solution, 0.1907 g of dried potassium chloride was added to 100 mL of Nanopure water to give $1000\text{ }\mu\text{g/mL}$ stock solution. Following standard solutions were prepared using stock solutions 0.2 , 0.4 , 0.6 , 0.8 and 1 ppm .

While for the preparation of standard sodium solutions 0.2542 g of dried sodium chloride was added to 100mL of Nanopure water to give 1000 µg/mL stock solution. Following standard solutions were prepared using stock solutions 0.2, 0.6, 1.2, 2 ppm.

Preparation of complexes solutions

0.010 g of dried complexes were added to 30 mL of Nanopure water. Each stock solution was further diluted 30 times to obtain appropriate emission signal strength.

Calculations

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 3.1). The amount of amino acid is assumed to be the difference between sample weight and the amount of metal ion.

$$\text{Ratio of amino acid to metal ion} = \frac{\text{Amt of amino acid}}{\text{Amt of metal ion (calculated)}} \times \frac{\text{Mol wt of Metal ion}}{\text{Mol Wt of amino acid}} \quad (3.1)$$

3.1.5 HPLC method development and validation for glycine

The detection and quantification of amino acid is a challenging task due to the absence of distinct chromophores and often requires derivatization (84). 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 (85,86). 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 (85). Glycine was chosen as a model amino acid for method development and validation.

3.1.5.1 Materials

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.

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). 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.

3.1.5.2 Mobile Phase

1.242 g of perfluorooctanoic acid was dissolved in 680 mL of acetonitrile (Optima HPLC grade). The solution was agitated with a magnetic stirrer for 10 minutes to ensure

complete dissolution. The resulting solution's volume was made up to 4 L using a volumetric flask to make a 0.75 mM solution. The solution was degassed for 30 minutes and then filtered through a 0.45 μm polycarbonate filter (Osmonics, Inc.).

3.1.5.3 Standard Solutions

Standard solutions for 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.

3.1.5.4 Calculation

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.

3.1.5.5 HPLC analysis of glycine

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 \times 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 prior to HPLC use.

3.1.6 Quantification of purity of amino acid complexes

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.

Standard curves for the 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 3.2).

$$\% \text{ Purity} = \frac{(\text{Conc of sample} - \text{Calculated conc of amino acid})}{\text{Conc of sample}} \times 100 \quad (\text{Equation 3.2})$$

3.1.7 Determination of stability constants of complexes

3.1.7.1 Introduction

Stability constants are a measure of the interaction strength of ligand and the metal ion. A stronger interaction results in a more stable complex and can affect hydration and electrolyte supplementation in potential ergogenic aids. Specifically, the bioavailability of the metal ion is influenced by the thermodynamic stability constant.

There are two types of stabilities constants commonly referred to, kinetic stability constant and thermodynamic stability constant (87). The kinetic stability constant deals with the activation energy of the reaction, the rate of reaction, formation of intermediate, etc. It is used to describe the rate under which a reaction can take place. In contrast, the

thermodynamic stability constant is a measure of the extent to which the product will be formed or the likelihood that the complex will remain associated together or be converted to another species when the conditions reach an equilibrium stage.

For the current study the main focus is on the determination of thermodynamic stability constant. pH metric method is the more reliable and generally utilized method for the estimation of stability constant (87,88). The complexation is accompanied by displacement of proton from the ligand. Monitoring the extent of change in pH (hydrogen ion concentration) can be used to calculate the stepwise stability constant of the metal complexes. The method used is Bejrrum's, Calvin and Wilson's method in which the amino acid metal ion complex is competitively formed and then disassociates under changing pH conditions of the solution (89).

3.1.7.2 Materials

NaOH, HNO₃, NaNO₃ were purchased from Fischer Scientific (Pittsburgh, PA, USA). 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)

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.

3.1.7.3 Preparation of reagent solution

4 gm of sodium hydroxide was dissolved in deionized water, the final volume was made up to 100 mL to give 1 M NaOH. 1M HNO₃ was prepared by diluting 7.69 mL of 15.8 M HNO₃, the final volume of the solution was made up to 100 mL to make a 1 M solution. 8.45 g of NaNO₃ was dissolved in deionized water and the volume was made up to 100 mL to make 1 M solution 0.9521 g of MgCl₂ was dissolved in deionized water and the volume was made up to 100 mL. 0.1M CaCl₂ was prepared by dissolving 1.1098 g in deionized water the volume made up to 100 mL. 0.1M of valine solution was prepared by dissolving 1.1715 g of valine in deionized water and making up the volume to 100 mL. 1.471 g of glutamic acid was dissolved in deionized water and the volume was made up to 100 mL to form 0.1 M solution of glutamic acid.

The difference in the pH measurement during the titration of amino acid solution in the presence and absence of metal ion can be used to calculate the formation functions the stability constant. Irving and Rossotti demonstrated a method involving titration of three sets of solutions against standardized sodium hydroxide (89,90). The analysis was performed by using the data from these 3 curves for the particular amino acid

	SET A	SET B	SET C
Acid (HNO₃)	0.8 mL	0.8mL	0.8 mL
Glutamic acid	---	2 mL	2 mL
NaNO₃	4 mL	4 mL	4 mL
Metal ion	---	---	1 mL
Water	35.2 mL	33.2 mL	32.2
Total	40 mL	40 mL	40 mL

Table 3.1 Titration solution sets for glutamic acid

	SET A	SET B	SET C
Acid (HNO ₃)	0.8 mL	0.8mL	0.8 mL
Valine	---	2 mL	2 mL
NaNO ₃	4 mL	4 mL	4 mL
Calcium Chloride			1 mL
Water	35.2 mL	33.2 mL	32.2
Total	40 mL	40 mL	40 mL

Table 3.2 Titration solution sets for valine

Each set of solution sets were mixed and purged with nitrogen gas to displace CO₂. 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 base is added in successions of 0.05 μL and the pH is measured after every subsequent addition of base. The titration recording is stopped when the pH reaches 12.

3.1.7.4 Calculation

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

$$\bar{n}_A = Y - \frac{(V_2 - V_1)(N^o - E^o)}{(V^o + V_1)T_{Cl}} \quad (3.3)$$

$$\bar{n} = Y - \frac{(V_1 - V_2)(N^o - E^o)}{(V^o + V_1)(\bar{n}_A)(T_{CM})} \quad (3.4)$$

$$pL = \log_{10} \frac{\sum_{n=0}^n \beta_n^H \frac{1}{(\text{antilog } \beta)^n}}{(T_{Cl} - \bar{n}T_{CM})} \times \frac{V^o - V_3}{V^o} \quad (3.5)$$

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

V_1, V_2 and V_3 = 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

There are various method for determination of step-wise stability constant. 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 $\log K_2$

$$\log K_1 = \log \frac{\bar{n}}{1-\bar{n}} + pL \quad (3.6)$$

$$\log K_2 = \log \frac{\bar{n}+(\bar{n}-1)}{(2-\bar{n})} + pL \quad (3.7)$$

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

$$\log K_2 = pL$$

3.2 Results and Discussion

3.2.1 Mass Spectrometric analysis

The mass spectrometric analysis provides the m/z peaks corresponding to the molecular weight of the amino acid complexes (Table 3.3). The m/z values obtained were M+H, where M is the molecular weight and H is a proton. The values confirm the formation of the amino acid complexes.

m/z values	Na ⁺	K ⁺	Ca ⁺²	Mg ⁺²
Alanine	111.9	127.89	217	201
Glutamic Acid	170.2	186.2	333.3	317.3
Glutamine	169.2	185.2	331.3	-
Glycine	97.1	113.9	189.3	173.3
Valine	140.3	156.3	273	257.3

Table 3.3 Summary of m/z values for complexes.

3.2.2 Determination of moisture content by Karl-Fischer titration

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 higher moisture content than the remaining complexes (Table 3.4).

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

Table 3.4 Average percent moisture content of amino acid complexes.

3.2.3 FTIR analysis

Amino acid forms bond with the metal ion through the amino and carboxylate groups. Spectral changes corresponding to the stretches of these groups provides evidence of complexation. The FTIR spectra showed characteristic changes confirming the formation of complexes. -NH₃⁺ peak appearing in the amino acid spectra is not seen in the complexes. A broad -NH₂ stretch appears instead of it. The -COO⁻ stretch is shifted slightly to a shorter wavenumber, this is give the summary of the spectral changes that were seen in the complexes v/s amino acids. These changes were consistent with all the amino acid complexes (Table 3.5).

	Amino acid	Amino acid complexes
NH ₃ ⁺ stretch	Present	Absent
-NH ₂ stretch	Absent	Present and broadened
-COO ⁻ stretch	Present	Present and shifted to a shorter wave number

Table 3.5 Summary of FT-IR spectral changes

3.2.4 Metal content analysis

The ratio values in case of monovalent metal ions is between 1.2:1 to 1.7:1 and in case of divalent metal ions is between 2.4:1 to 4.2:1. These values are higher than the theoretically calculated value for the complexes. Which indicates the presence of excess free amino acid (Table 3.6).

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

Table 3.6 Amino acid per mole of metal ion

3.2.5 HPLC method development and validation for glycine

3.2.5.1 Specificity

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 metalsalt mixtur and metal glycine complex respectively. Each of these sample was injected into the system. The representative chromatograms of mobile phase alone without analyte (Figure 3.1), mobile phase contacting glycine (Figure 3.2), mobile phase containing glycine and calcium ion salt (Figure 3.3), and mobile phase contacting calcium glycine calcium complex (Figure 3.4) 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 14-16 minutes. 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.

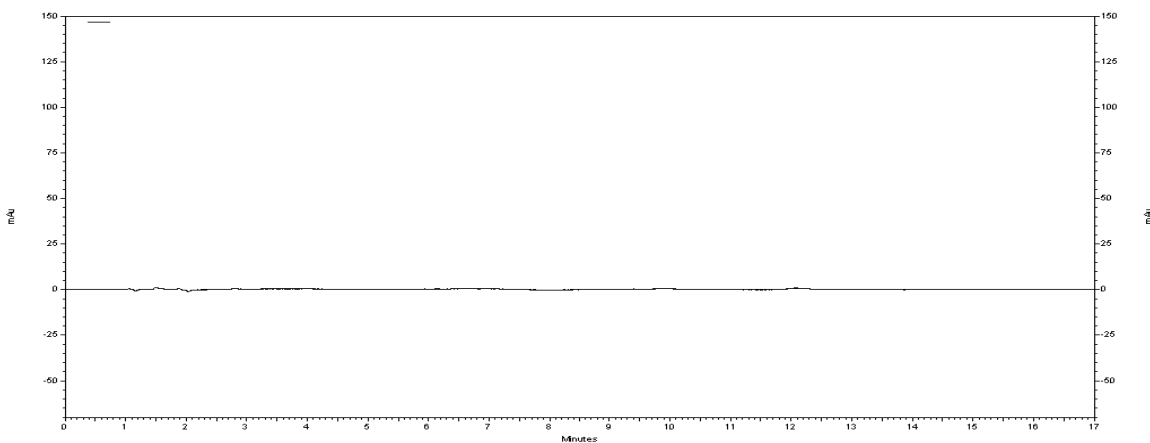


Figure 3.1 Chromatogram of mobile phase

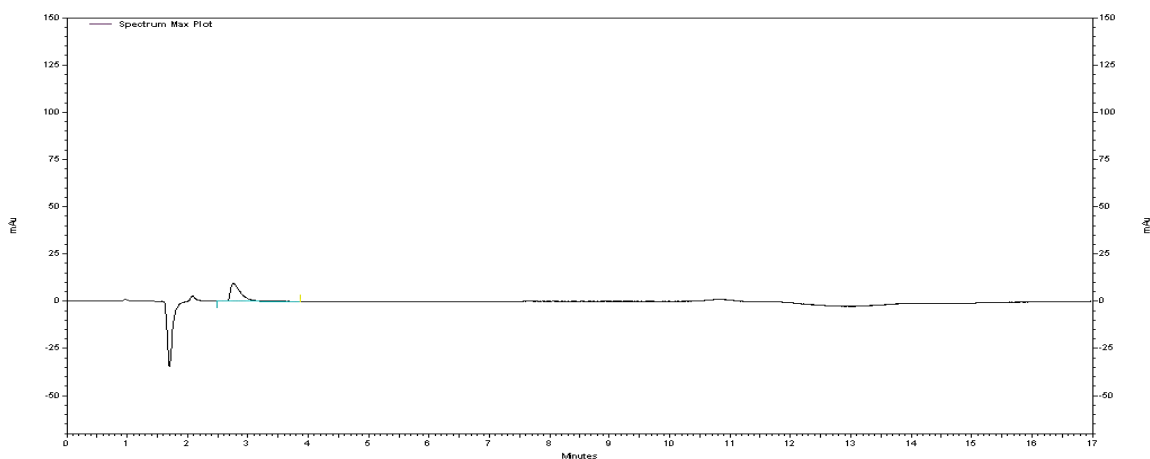


Figure 3.2 Chromatogram of glycine (0.157 mg/mL)

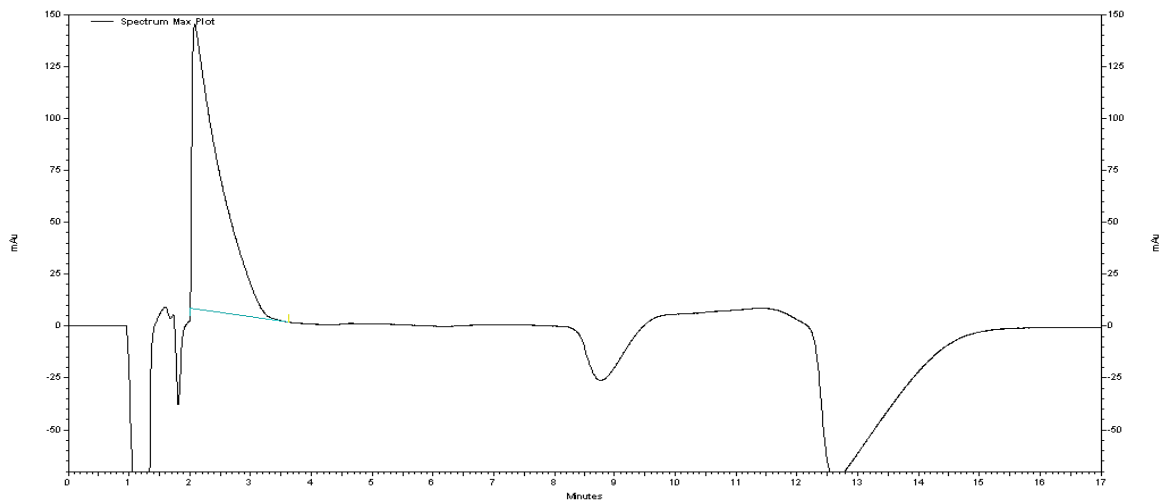


Figure 3.3 Chromatogram of glycine + CaCl₂ mixture

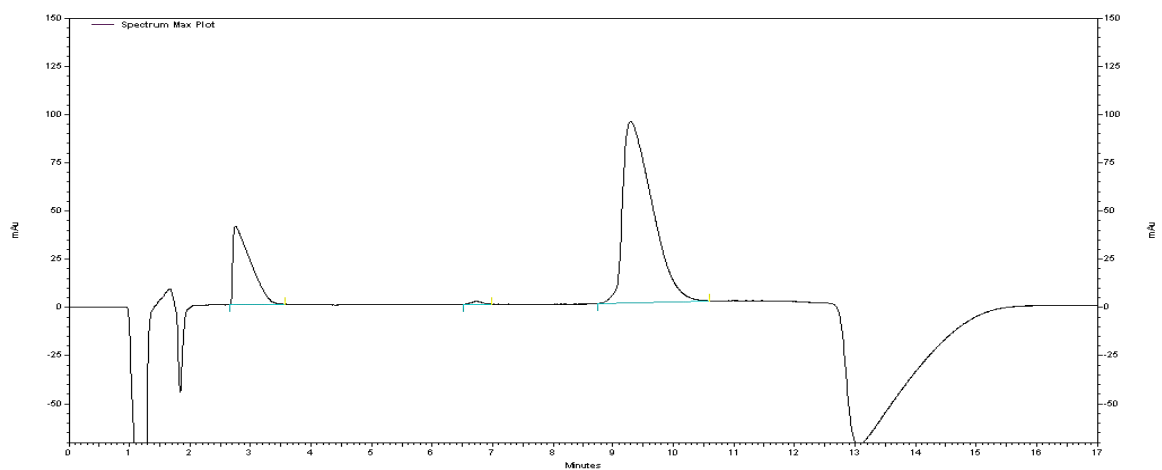


Figure 3.4 Chromatogram of synthesized calcium glycine complex

3.2.5.2 Linearity

Linearity study is a study which correlates the concentration range with UV absorption as measured by peak area. The standard curve was found to be linear over the concentration range of 0.157 mg/mL to 20.14 mg/mL (Figure 3.5).

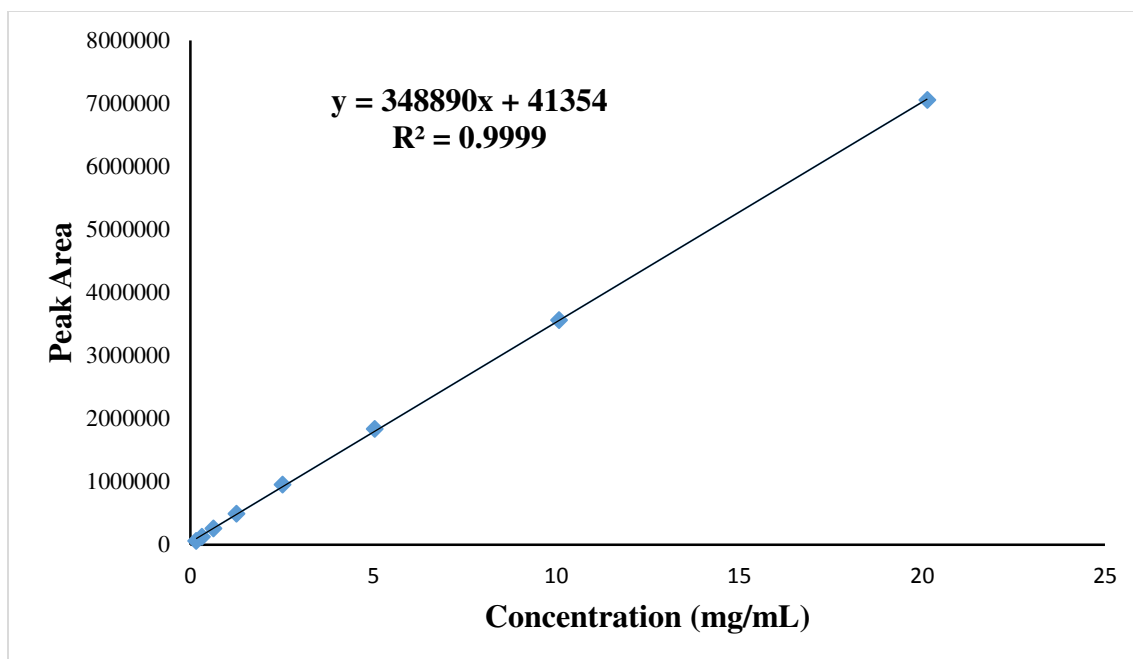


Figure 3.5 Standard curve for glycine

3.2.5.3 Precision

The precision studies for the current method included within day precision and day to day precision. The within day precision study was performed by evaluating repeated injection of standard solutions three times on the same day. The day to day precision was determined by injecting one set of standard solutions on three different days over a period of three weeks. The relative standard deviation (RSD) values were calculated for both within day as well as day to day precision and were all found to be within the acceptable limits as defined by USP (Table 3.7).

Concentration (mg/mL)	Within day		Day to day	
	Mean Peak Area	% RSD	Mean Peak Area	% RSD
0.157344	53259	0.97	3304.44	5.93
0.314688	105394.66	0.95	8453.70	7.47
0.629375	205786.33	1.25	15473.85	6.93
1.25875	411063.66	0.96	29962.29	6.80
2.5175	845619.33	0.82	41236.15	4.63
5.035	1627662	1.59	67629.037	3.92
10.07	3142678.33	1.45	153091.18	4.59
20.14	6118316.33	0.57	314198.59	4.75

Table 3.7 Precision data for within day and day to day variability

3.2.5.4 Accuracy

To determine the accuracy of the given method, three standard sample concentrations were selected to represent a broad concentration range. Each sample was evaluated in triplicate using the HPLC method described previously. The peak areas were used to obtain the calculated value of glycine concentration. The percent accuracy was found to be > 90% with a percentage relative standard deviation (% RSD) of less than 5%, which was within acceptable limits as defined by USP (Table 3.8).

Theoretical concentration (mg/mL)	Experimental concentration (mg/mL)	% Accuracy
0.75	0.724	96.54 ± 1.99
8	8.227	102.84 ± 1.15
16	16.713	104.46 ± 1.27

Table 3.8 Percent accuracy data for glycine

3.2.5.5 Conclusion

An analytical method for quantification of free glycine was developed and validated. The analytical method validated using standard validation protocol in accordance with USP was found to be specific, linear over the concentrations tested, accurate and precise.

3.2.6 Quantification of purity of amino acid complexes

The purity of complexes ranged from 40-72%. Calcium and magnesium complexes showed higher yields as compared to sodium and potassium complexes (Table 3.6). 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 lower purity of the same (91). In all, the purity of divalent metal ions was found to be great which can be correlated to the strength of the metal ion as a Lewis acid. Calcium and magnesium along with aliphatic amino acid tend to form complexes with higher purity.

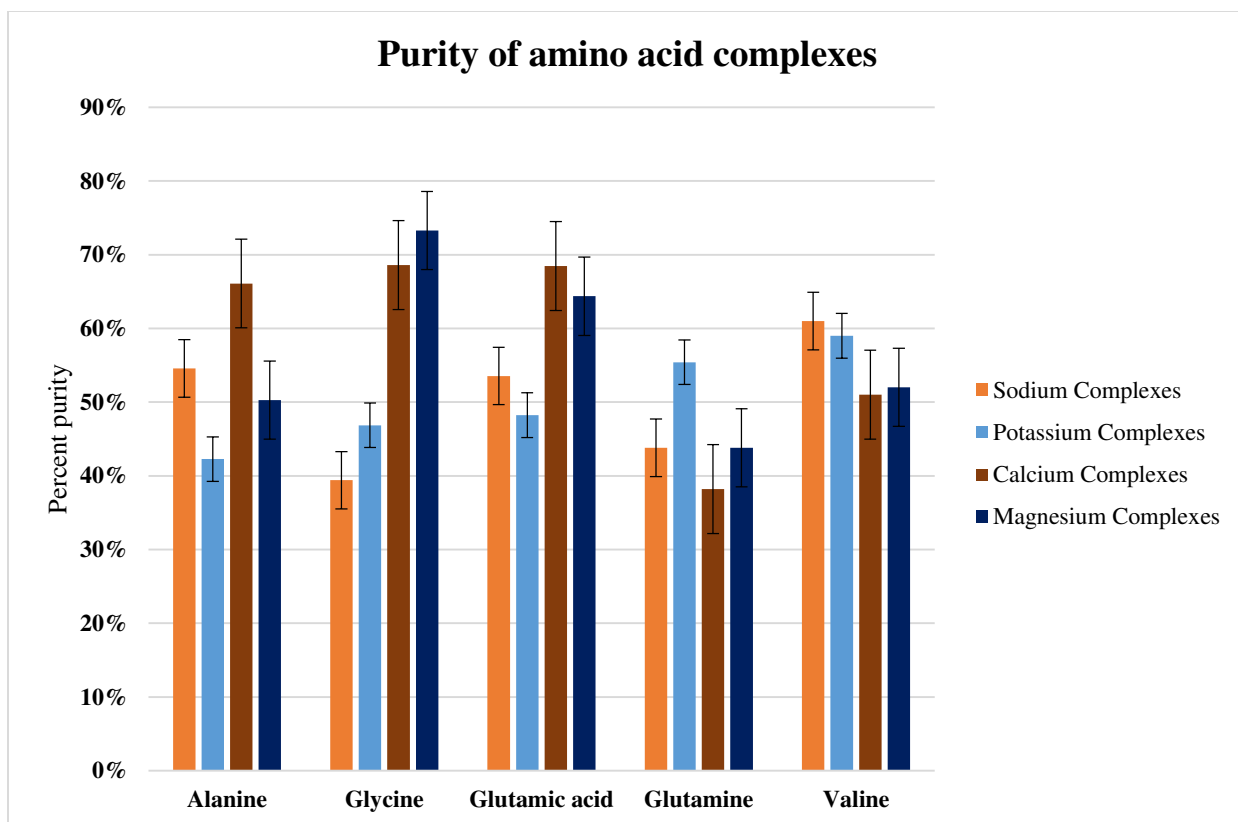


Figure 3.6 Purity of amino acid complexes

3.3.4 Determination of stability constants of complexes

There is a difference between titration curves of amino acid v/s amino acid and metal ions (Figure 3.12, 3.13). The stability constant for the amino acid complexes was calculated (Table 3.9) using the titration curves.

	Glutamic acid	Valine
$\log K_1^H$	4.2	3.33
$\log K_2^H$	10.10	9.80
Log K	1.58 ± 0.2	1.51 ± 0.13

Table 3.9 Stability constants of calcium glutamic acid and calcium valine

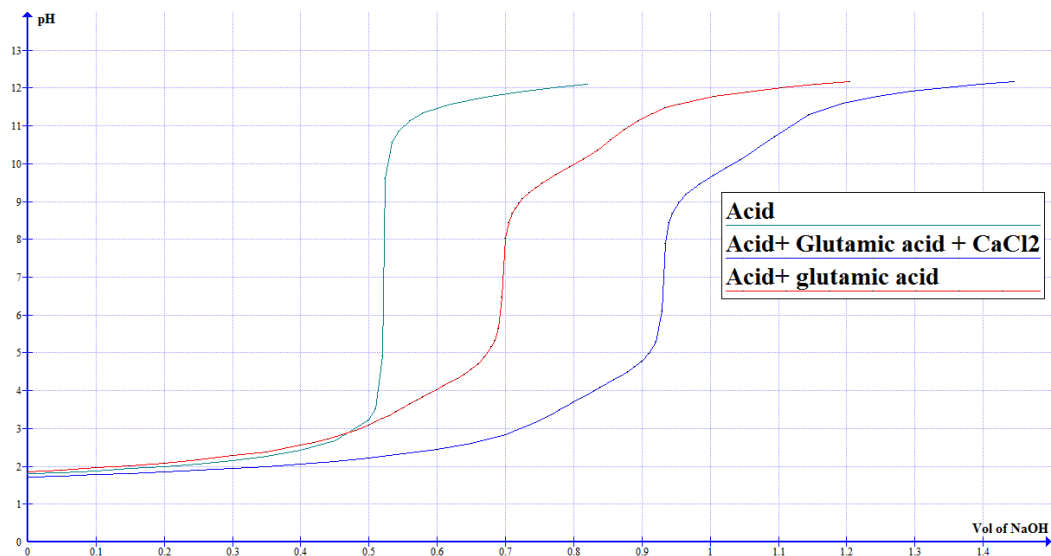


Figure 3.7 Titration curve for glutamic acid

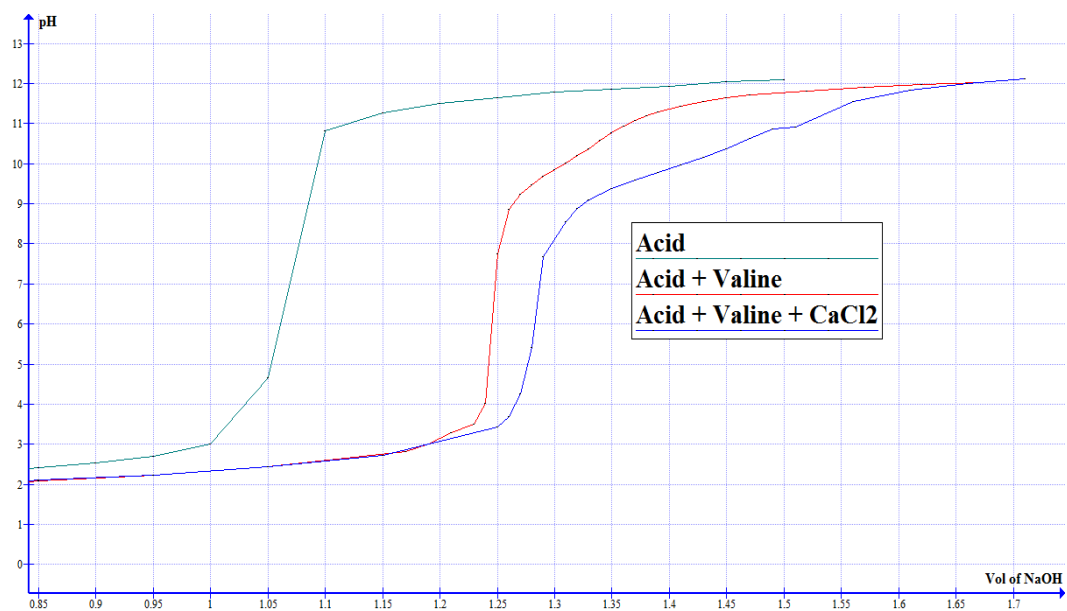


Figure 3.8 Titration curve for valine

3.3 Conclusion

The moisture content of the complexes was found to be within acceptable limits so as to not interfere with other analysis procedures. The structural analysis of the amino acid was performed using mass spectrometry and FT-IR. In mass spectrometric analysis the m/z signals were obtained corresponding to the molecular weight of the complexes. 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.

CHAPTER 4:
**Determination of ergogenic
potential**

4.1 Introduction

4.1.1 Measurement of Osmotic Pressure

The amino acid-metal ion complex should be capable of generating sufficient osmotic pressure to act as an ergogenic aid for the replenishment of water and/or electrolytes. Specifically, iso-tonic or mildly hypertonic solutions (e.g. 5-7% solutions) are often used before or during workout. As discussed previously solution should be isotonic for proper hydration and proper nutrition during workout. In contrast, hypertonic solutions (e.g. 10-15% dextrose solution) are often used after exercise for replenishing the liver stores of glucose and glycogen.

4.1.2 Determination of calorific value

Amino acids were selected as both complexing ligands to metal ions and as potential energy sources during prolonged or intense exercise. However, the ergogenic potential of these complexes must be evaluated to determine potential calorie production from the complexes. The calorific value is generally expressed in Kilocalories (written as calorie on food labels). 1 Kilocalorie is defined as the amount of energy required to warm one gram of air-free water from 14.5 to 15.5 °C at standard atmospheric pressure.

The determination of calorific value of non-fiber containing food or chemicals is evaluated using a bomb calorimeter. The bomb calorimeter consists of an oxygen bomb, which is a small chamber holding sample for combustion. The combustion occurs in presence of 23 atm of pure oxygen. The bomb is submerged in a jacketed calorimeter vessel filled with water to absorb the released heat after combustion. The temperature

change of water following combustion is monitored. The rise in temperature is correlated to the amount of heat liberated in combustion (calorific value)

4.2 Materials and methods

4.2.1 Measurement of osmotic pressure

The analysis was performed on μ Osmett, Model 5004 by automatic precision system (MA, USA). 20 mg of amino acid complexes, calcium gluconate were dissolved in 1 mL of deionized water to make a 2% w/v solutions. The Gatorade[®] energy drink was diluted to make a 2% w/v solutions. Each solution was analyzed in triplicates.

4.2.2 Determination of calorific value

4.2.2.1 Material

20 mg of amino acid complexes were dissolved in 1 mL of deionized water to make 2% solutions. The osmometer was equilibrated for 20 minutes and calibrated with 500 and 100 mOsm/L standards. 50 μ L of sample solutions were transferred to conical vials and analysis was performed in triplicate. Nickel alloy fuse wire was purchased from Parr Bomb Calorimeter (Moline, IL, USA). Benzoic acid was purchased from Fischer Scientific (Pittsburgh, PA, USA). The bomb calorimeter consisted of the oxygen Bomb, ignition unit and the calorimeter (2 L 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). Tablet press used for making the sample fuses consisted

of hydraulic unit (model no 3912) and press (serial no– 35000-650) which was from Carer Lab Press Inc.(Menomonee Falls, WI, USA).

4.2.2.2 Sample preparation

Powdered complex and standard masses were pressed into a firm pellet and a nickel alloy wire was fused with the compacted mass. 0.6 gm of sample powder was filled into a single punch and die mold and compacted at 1100 metric ton pressure along with the wire. The fuse wire was used for electrical ignition of the sample inside the bomb to cause combustion. The wire is positioned in the tablet punch to allow the two ends of the wire be available to connect to the electrodes.

4.2.2.3 Sample analysis

The jacketed vessel was filled with 2 L of water. The sample pellets 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.

Calculation

As discussed earlier the heat of combustion is directly proportional to the temperature increase.

$$\Delta H = -kS\Delta T \quad (4.1)$$

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.

4.3 Results and discussion

4.3.1 Measurement of Osmotic Pressure

Arbitrarily chosen complex concentrations of 2% for all complexes showed osmotic pressure values comparable to those of blood serum (Table 4.1, 4.2). Also analysis of one of the current marketed preparation revealed a consistent results (Gatorade[®] Lime-lemon).

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 4.1 Osmotic pressure values of complexes (in mosm)

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

Table 4.2 Osmotic potential values of for comparison

4.3.2 Determination of Calorific Value

The calorific values of all glycine complexes as well as all calcium complexes were determined in the similar fashion (Table 4.3). The calorific value of the complexes was found to be less than the calorific value of glucose based counterparts.

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

Table 4.3 Calorific Values of amino acid complexes and calcium gluconate

4.4 Conclusion

Amino acid complexes can produce sufficient osmotic pressure to be used as ergogenic aids. If the amino acid complexes are mixed in therapeutic doses of each amino acid and metal ions the resulting solution's osmotic pressure can be suitable adjusted to make it isotonic. Also amino acid complexes have sufficient calorific value to be used for energy

production. The calorie content is lesser than the corresponding sugar based complex but their potential utility could be better.

CHAPTER 5:

Summary and future directions

5.1 Summary

Amino acid complexes of physiologically relevant metal ions were synthesized utilizing glucogenic amino acids. The synthesis yields were estimated for calcium and magnesium complexes and were found to be up to 88% for polar amino acid glycine, glutamine and glutamic acid while amino acid with non-polar side chains showed lower yield. The synthesized complexes were then filtered and lyophilized for further characterization

The structural characterization of amino acid complexes was performed using mass spectrometry and FT-IR. m/z peak obtained in MS analysis corresponded with the calculated molecular weight of the complexes conclusively confirming the formation of complexes. The FT-IR analysis showed consistent spectral changes, compared with spectra of amino acids, which confirmed the association of amino acid with the metal ion. Both the studies indicate that complexes of amino acids with physiological metal ions can be formed. The metal content analysis of the complexes revealed that the amino acid to metal ion ratio was higher than theoretical value indicating presence of excess amino acid in the mixture.

An HPLC method was developed and validated for the accurate and precise quantification of free amino acid using glycine as a prototype, to calculate the percent purity of the complexes. The chromatographic separation was achieved by isocratic elution on Phenomenex C18 column (300 × 3.9 mm, 10 μm). The mobile phase consisted of 0.75mM PDFOA in acetonitrile:water in the ratio 17:83 v/v. The chromatographic method was used to determine the percent purity of complexes by indirect estimation. The percent purity was found to be between 40-75% for the complexes. This correlated with the metal content analysis data for the complexes.

The stability constant for determined for valine as a prototype for aliphatic amino acid and glutamic acid as a polar charged side chained amino acid with calcium metal. The stability constant values were found to be 1.51 for valine calcium complex and 1.56 for calcium glutamate complex. This suggested that the binding modes for both the amino acids were similar and the spare carboxylate group on amino acid did not participate to a significant extent complexation with calcium.

The ergogenic potential of the complexes was tested by testing the osmotic pressure and calorific value of the complexes. The osmotic potential 2% w/v of the complexes generated comparable osmotic pressure as that of Gatorade which is a conventional therapy for hydration while calcium gluconate which is a sugar based complex of calcium showed low osmotic pressure. The calorific value of the complexes was found to be comparable with that of calcium gluconate which is a sugar based complex. The complexes show potential to generate necessary osmotic pressure and have sufficient calorific value to serve as ergogenic aid

5.2 Future directions

The present study illustrated that amino acid complexes of physiological metal ions complexes can be formed and used as ergogenic aids. However further optimization of the reaction parameters needs to be done in order to improve the purity of the complexes. This would allow for further characterization of these complexes by other techniques like XRD to determine the crystal structure, better estimation of calorific value and metal content ratio.

Additionally absorption studies have to be performed on various tissues from different regions of GI tract to determine the rate and extent of absorption at various site, also determine the improvement in absorption over sugar-electrolyte supplements.

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