

Amino Acids Analysis in Whole Saliva by Reverse Phase High Performance Liquid Chromatography



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Abstract : The objective of the study was to determine optimal conditions for sampling, sample dispensation for amino acid analysis in women saliva with the aid of identify nutritional deficits. We quantify the amino acid concentration by high performance liquid chromatography during salivary amino acids in 10 young women bear with anorexia nervosa during a period of significant loss of body weight, compared with 10 healthy age-matched controls. Free amino acid levels in saliva were similar in both groups, however significantly higher levels of Taurine, Glutamine and phenylalanine were found in anorexia nervosa patients, as well as significantly lower levels of arginine, tyrosine and tryptophan compared with controls. The salivary amino acids difference in anorexia nervosa patients can be explicate with severe protein malnutrition deficit.

Key words : Anorexia nervosa, Amino acids, Saliva, OPA, HPLC

Introduction

Anorexia nervosa is an eating disorder that is characterized by a distorted body image leading to excessive food restrictions that result in a marked loss of weight (Moyano *et al.*, 1998). The biochemical evaluation of the nutritional status is very useful at diagnosis and for the follow-up, allowing for a more specific identification and treatment of deficient factors which results in a shorter and more efficient recovery (Nussbaum, 1992; Madruga, 1993). However, in spite of being a severe nutritional disorder, it is associated with rather mild abnormalities of the classical parameters of malnutrition (Schebendach and Nussbaum, 1992; Halmi *et al.*, 1987). Although amino acid and protein metabolism have commonly been considered to recline at the center of the metabolic complexities of protein malnutrition (Jackson and Grimble, 1990), few reports of amino acid patterns in anorexia nervosa have been published (Halmi *et al.*, 1987; Schweiger *et al.*, 1986; Schreiber *et al.*, 1991).

Amino acids are known to be precursors for a variety of biologically important substances including many neuroactive compounds. Plasma free amino acid concentrations express the balance between uptake (exogenous from the diet and endogenous by proteolysis and synthesis from other metabolites) and utilization (protein synthesis and amino acid catabolism, unusual

losses in stool and urine), which are influenced by hormonal factors and by the availability of vitamins and cofactors involved in intermediary metabolism. The interpretation of plasma amino acid patterns depends on the knowledge of their metabolism during various physiologic and pathologic states (Oberholzer and Briddon, 1990). To date, analysis of amino acid in plasma is considered as a valuable diagnostic tool in cases of suspected inborn errors of amino acid metabolism. The presence of a characteristic pattern of elevated amino acids is very useful in diagnosis of these rare disorders. To overcome this, the preliminary reports prove the changes in the salivary amino acids during anorexia nervosa provide an evidence for protein malnutrition deficits. These applications have led to an increase in the number of salivary amino acid determination and the need for a cost-effective, rapid, reliable and automated method for severe nutritional disorder. The present study reveals that amino acid concentration in anorexia nervosa cases to convey quantitative amino acid results in the most informative way for the understanding of the metabolic abnormalities developed in these patients.

Materials and Methods

Chemicals

Amino acid standards, Sulpho salicylic acid, Krebs ringer solution (NaCl, KCl, CaCl₂, MgSO₄, and

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phosphate buffer, pH 7.4.) and orthophthaldialdehyde (OPA) were from Sigma, Saint Louis, USA. Acetonitrile, Methanol was HPLC grade. Deionised water was filtered from millipore system.

Preparation of standard amino acids and sample solution

To an aliquot (2 ml) of freshly prepared aqueous solution of 19 amino acids, aspartic acid, serine, glutamine, threonine, glutamic acid, asparagine, methionine, GABA, alanine, taurine, tyrosine, phenylalanine, tyrosine, tryptophan histidine, valine, arginine, isoleucine and leucine (each **400 µg/ml**), was added in an aliquot (2 ml) of an aqueous solution of other standard amino acids (400 µg/ml). And then this combined solution was further diluted with 0.2 M hydrochloric acid (4 ml). These amino acids solutions (2 ml) were finally diluted to 20 ml with 0.2 M hydrochloric acid in a volumetric flask prior to use.

Sample preparation

Whole saliva was collected from 10 young women with anorexia nervosa and 10 healthy adults in the morning, at least 2 h after the last intake of food. The mouth was rinsed with water immediately before the collection. Whole saliva was collected and **placed on ice. Protease cocktail inhibitor (1 µL/mL of whole saliva)** was added to saliva immediately after collection to minimize protein degradation (Dodds *et al.*, 2005; Lawrence, 2002). Whole saliva was then centrifuge at 12,000 rpm at 4°C for 10 min. An aliquot of separated **200 µl** of supernatant saliva was pretreated with 200 µl of acetonitrile to remove the proteins by precipitation, then; the moisture was refrigerated at 4°C for 10 mins followed by centrifugation at 14, 000 rpm for 8 mins and filtrated through a 0.2 µm millipore filter. The filtrated 10 µl of saliva samples were diluted with 190 µl of Krebs ringer solution contains pH 3.0. Finally, isolated 10 µl of saliva sample was transferred into micro vials and placed in amber vials with screw caps and stored in the refrigerated sampler of the HPLC Agilent system at 5°C for amino acid analysis (Fekkes *et al.*, 1995).

Derivatization and analysis

OPA reagent was made 24 h before first use by dissolving OPA at 54 mg mL⁻¹ in methanol and adding 200 µL to 1.8 mL 0.5 M sodium borate (pH 9.5) and 40 µL 2-mercaptoethanol. The reagent was filtered into an auto sampler vial and used for up to 3 days. Precolumn derivatization was performed in the injection loop by automated mixing of 10 µL sample and 15 µL OPA

reagent, followed by a delay of 2 min prior to injection. The chromatographic separation was performed by gradient elution at 40°C.

Chromatography

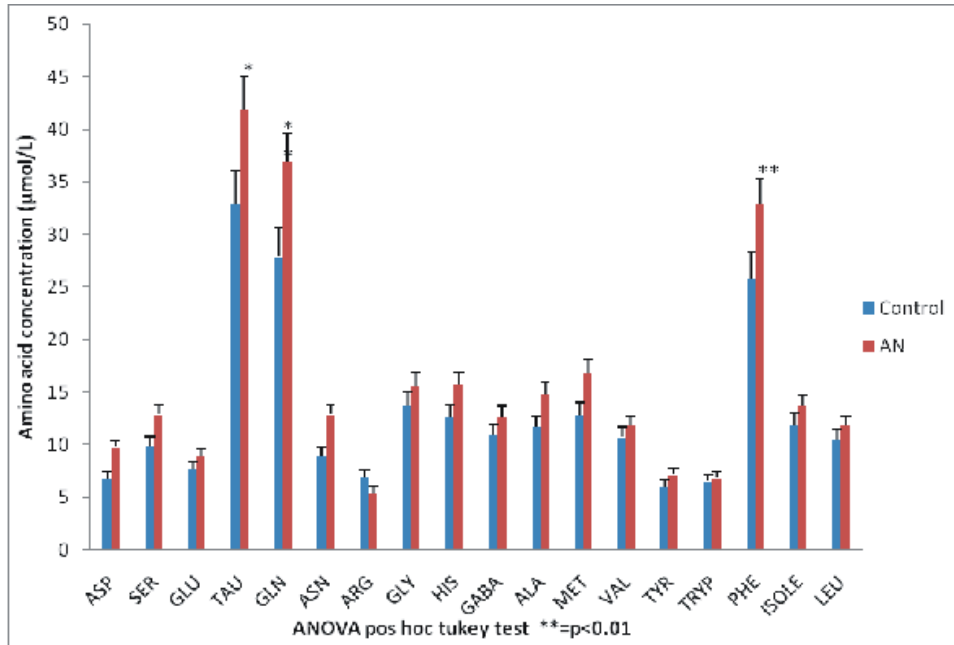
Flow rate	: 1.0 ml/min
Detection	: 340 nm
Injection quantity	: 20 µL
Column used	: Agilent Zorbax Eclipse-AAA 3.0 x 150 mm, 3.5 µm
Column temperature	: 40° C
Mobile phase	: Na ₂ HPO ₄ 40 mM (5.68 g Sigma Ultra Anhydrous Na ₂ HPO ₄ in 1 liter of deionized water (Millipore company, USA), adjusted to pH 7.8 with H ₃ PO ₄ solution 10 N), the mobile phase B composed of acetonitrile, methanol and water (40:45:10). Total run time per injection was 35 min. Peak identity was confirmed by co-elution with authentic standards.

Statistical Analysis

The values of amino acids concentration in saliva were averaged; standard error of the mean was calculated in anorexia nervosa verses healthy young women were compared and confirmed through one way ANOVA posthoc Tukey test (p= 0.01).

Results

The results of the analysis of saliva samples from anorexia nervosa and healthy young women (Age: 16-30 years) are given in Fig. 1. These results were very close to those obtained by other HPLC systems with the same (Moller, 1993; Einarsson *et al.*, 1983) or other methods (Feste, 1992; Ziegler *et al.*, 1992 and Dorresteyn *et al.*, 1996). However, the results for present study revealed that predominantly high in amino acid concentration such as taurine, glutamine and phenylalanine which compared to control samples (p= 0.01). Furthermore, in our study, the mean concentration for tyrosine and tryptophan was specifically low shown in Fig.1. The age ranges were selected to represent physiological periods such as the newborn period, infancy, early and late childhood, puberty, and adulthood. The results of the within-run precision



Results are expressed in µmol/L of mean ± S.E.

This study was performed with saliva sample collected from two set from Anorexia nervosa and control samples after an overnight fast.

Fig. 1: Salivary amino acids in Anorexia nervosa (AN) and Healthy subjects (Control) (N=10)

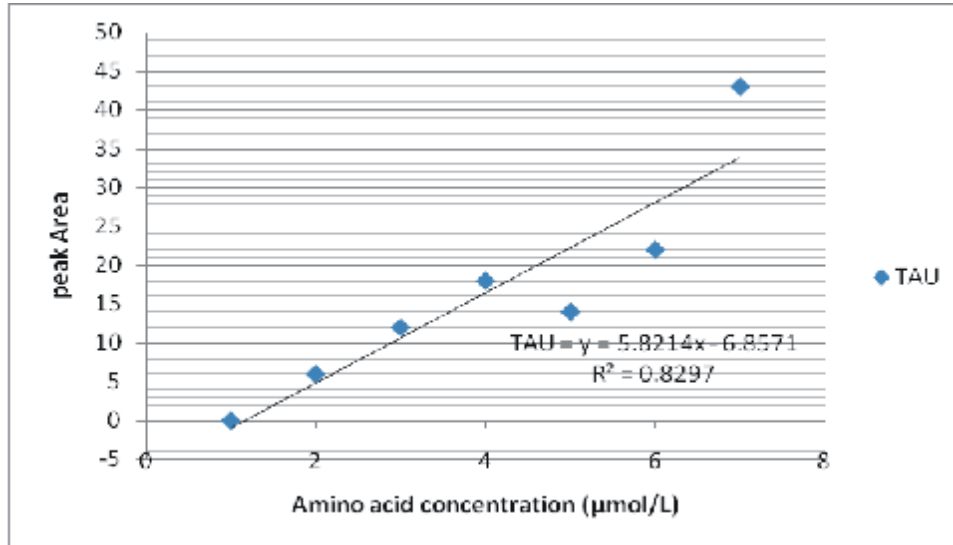


Fig. 2: Linear plot of amino acids under optimized conditions shows maximum levels of taurine in saliva during anorexia nervosa

assay for the retention time of amino acids from the saliva sample were similar to those obtained with serum. The calibration curve of linearity plot in salivary amino acids showed the upper limit of regression value as ($y = 5.8214x - 6.8571$; $R^2 = 0.8297$) in taurine compare to other amino acids (Fig.2). To support this result, taurine plays an

important role in cell membrane stabilization, modulation of intracellular calcium levels, osmoregulation and detoxification (Schaffer *et al.*, 2000; Shimzu and Satsu, 2000), it is likely to modulate various physiological functions, which are disturbed in a broad range of clinical situations.

Discussion

Anorexia nervosa is characterized by aversion to food and a progressive weight loss to the point of emaciation. During early fasting, fat mobilization is promoted by falling levels of insulin and increased sympathetic nerve activity to the adipose tissue. Early rapid proteolysis occurs, with amino acid mobilization from muscle gluconeogenesis and production of urinary urea nitrogen. As part of the life-saving adaptation to total starvation, the body gradually converts from glucose and amino acid economy to a fat-derived fuel economy (White *et al.*, 1978).

Taurine takes part in several biochemical reactions; cell membrane protection seems to be the major physiological role either by reducing toxic substances or by acting as an osmoregulator (Schaffer *et al.*, 2000)

Further, alanine and glutamine are involved in shuttling the nitrogen from branched-chain amino acids to the liver, intestinal mucosa and kidney. Other protein amino acids, such as the aromatic ones, are also released in muscle breakdown caused by a catabolic state (Castillo *et al.*, 1994). Tyrosine and phenylalanine are extensively catabolized in the liver, because of the hepatic localization of phenylalanine hydroxylase and tyrosine amino transferase, but tyrosine is more rapidly cleared from the plasma than phenylalanine (Wannemacher *et al.*, 1976). The high glutamine concentration, apart from its gluconeogenic function, might have a beneficial action at the gastrointestinal mucosa and on the immune system (Calder, 1994), that might contribute to the low rates of infection in these patients (Madruga *et al.*, 1993), in contrast with other malnutrition states (Jackson and Grimble, 1990).

Moreover, different causes of malnutrition such as renal (Ceballos *et al.*, 1990) or liver failure (Byrd *et al.*, 1993), cancer cachexia (Pisters and Brennon, 1990), HIV infection (Hortin *et al.*, 1994) might also contribute to differences in amino acid patterns. The plasma amino acid profile in anorexia nervosa demonstrates a rather different pattern to those of other severe malnutrition states, showing a marasmic pattern of balanced protein energy malnutrition. However, a decline in total amino acids, essential and non-essential (except for glycine) seems to be the most generalized abnormality in severe undernutrition (Bremer *et al.*, 1981). However, these results vary widely among laboratories (Parvy *et al.*, 1993), owing to pre-measurement and methodologic factors (De Jonge *et al.*, 1996), such as fasting state, time of sampling, deproteinization, and storage conditions.

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