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Quantitative HPLC Analysis of Amino Acids in *Chyavanprash*: A well known Ayurvedic Formulation

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Chyavanprash **is a well known Ayurvedic formulation claimed to be an immunomodulator and is useful in the treatment of chronic conditions such as pulmonary tuberculosis, asthma and cough. It is also widely used as a food supplement for strength and energy. It maintains youthfulness by renewing tissues and counterattacking degeneration. It has been reported to contain proteins which play an important role in fundamental processes of tissue formation, regeneration and function. Since no studies have so far been undertaken to analyze the amino acids which are the building blocks of proteins in *Chyavanprash*, a quantitative HPLC analysis of the different amino acids present was carried out. Six different samples of *Chyavanprash* were analyzed by HPLC using a pre column derivatization technique. Analysis results showed that although the concentration varied, only 3 amino acids, alanine, valine and leucine were present in all 6 samples where as proline was present only in 3, lysine was present only in 2 while one was found to be lacking in both proline and lysine. Also, the results were found to be nearly consistent for the two batches prepared in the lab where as there was marked variation in those procured from the market.**

In the recent years there has been an increasing interest in herbal drugs for health care all over the world. The need of the day, therefore, is to provide scientifically proven and evidence based herbal formulations. *Chyavanprash* is the most well known and popular polyherbal Ayurvedic formulation. It is claimed to be an immunomodulator and is recommended for chronic conditions such as pulmonary tuberculosis, asthma and cough[1]. It is also widely used as a food supplement for strength and energy and maintains youthfulness by renewing tissues and counter attacking degeneration. It has also been successfully used as a preventive and curative tonic and is also useful during chronic infection[1,2]. It has been the subject of study by several workers[3-7]. In the recent past attempts have also been made to develop some parameters for its quality control[8-13].

It has been reported that *Chyavanprash* contains about 5% of protein[3]. Proteins play an intimate role in the fundamental processes of tissue formation, regeneration and function. Amino acids are the fundamental structural units of proteins. Thus considering the medicinal properties attributed to *Chyavanprash*, it was thought worthwhile to look into the amino acid content of *Chyavanprash*. Proper identification and quantitative estimation of the amino acids, either as they occur in the free amino acid pool or as they are obtained in different hydrolysates, is very essential. We thus carried out HPLC analysis in which a pre column derivatisation technique for peptide and protein amino acid identification and estimation was used[14,15].

MATERIALS AND METHODS

Acetonitrile of HPLC grade was purchased from Spectrochem. Water for HPLC was procured from Qualigens. Acetate phosphate buffer concentrate, Waters Acc Q. Fluor Borate buffer, Acc Q. Fluor Reagent powder, Acc Q. Fluor Reagent diluent, amino acid hydrolysate standard were all procured from Waters, USA. All other

reagents used were of analytical grade. Six samples of *Chyavanprash*, of which two (CP-I and CP-II) were prepared according to the standard method[16] and four (CP-A, CP-B, CP-C and CP-D) were procured from the local market.

The HPLC system used was Waters model equipped with two 515 HPLC pumps, a Rheodyne injector, Nova-Pak™ C_{18} column (4 μm, 3.9×150 mm, Waters, USA), 474 fluorescence detector and Millenium[32] chromatography manager (Waters, USA). The column used was specifically certified for the use with Waters Acc Q. Tag method for amino acid analysis.

Sample Preparation:

In all six samples of *Chyavanprash* were studied for a comparative evaluation of their amino acid content. Of these two were prepared in our own laboratory[16] and four were commercial samples of reputed firms procured from the local market. The various ingredients for preparing *Chyavanprash* in the laboratory were either collected by us or procured from the local market and authenticated by matching them with the genuine samples available in the herbal drug museum of our institute. Each sample was hydrolysed[17] to give the free amino acids. For this 0.5 g of *Chyavanprash* sample was heated at 110° for 24 h with 6 N HCl. The reaction mixture was then filtered and the hydrolysate concentrated under reduced pressure and finally dried completely by lyophalisation. Ten milligrams each of the dried hydrolysate was reconstituted in 1 ml of 20 mM HCl solution. Forty microlitres of the amino acid hydrolysate standard was mixed with 960 μl of water.

Derivatization and Separation procedure:

For the derivatization of the amino acids, Acc Q Fluor reagent was prepared by reconstituting Acc Q. Fluor reagent powder (6-aminoquinolyl N-hydroxy succinimidyl carbamate) with Acc Q. Fluor reagent diluent (acetonitrile) to get a 10 mM solution of the reagent followed by heating at 55° for not more than 10 min till the powder dissolved. Ten microlitres of the standard was added to 70 ml of borate buffer and 2 ml of reagent solution. The reaction mixture was allowed to stand for one minute at room temperature and heated at 55° for 10 min to obtain the derivatized standard. Five millilitres of aliquot was injected directly to the HPLC. The samples were also derivatized in a similar manner by taking 20 μl of the reconstituted hydrolysate, 60 μl of borate buffer and 20 μl of the reconstituted reagent for the derivatization of each sample. Five microlitres of each was injected. The derivatives were eluted with acetate phosphate buffer (solvent A) and mixture of acetonitrile and water (60:40, solvent B). Table 1 shows the gradient programme for the separation and time programme for the run. Chromatographic separations of standard and sample were performed at 30° in a column oven (Waters). The detection excitation and emission were set at 250 and 395 nm, respectively.

RESULTS AND DISCUSSION

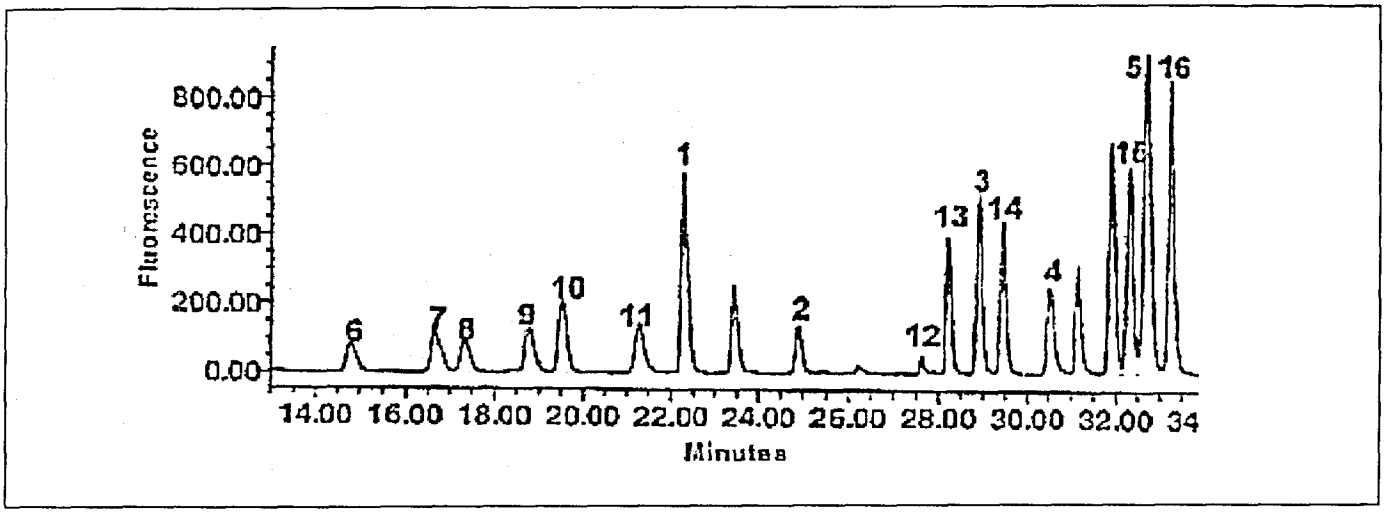
Chyavanprash is claimed to be the best immunomodulator and recommended for all catabolic diseases[1]. *Chyavanprash*, though extensively investigated by several groups, none has actually made an attempt to identify the amino acids that may be present in it. Quantitative analysis of the various amino acids present in different samples of *Chyavanprash* was thus carried out by HPLC using pre column derivatization technique. In this technique, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reacts rapidly with primary and secondary amino acids to yield highly stable ureas which fluoresce strongly at 395 nm. The N-dehydroxy succinimide formed as a side product have been found not to interfere with the analysis of amino acids.

A comparison of the HPLC fingerprint profiles of amino acid hydrolysate standard (fig. 1) and those of amino acid hydrolysates obtained from the six samples of *Chyavanprash* (fig. 2) showed that only valine, alanine, leucine and proline or lysine were present, although the amount of the individual amino acids varied. The amino

TABLE 1: GRADIENT TABLE USED FOR HPLC SEPARATION OF AMINO ACIDS.

Time (min.)	%A	%B
Initial	100	0
0.5	98	2
15.0	93	7
19.0	90	10
32.0	67	33
33.0	67	33
34.0	0	100
37.0	0	100
38.0	100	0
64.0	100	0

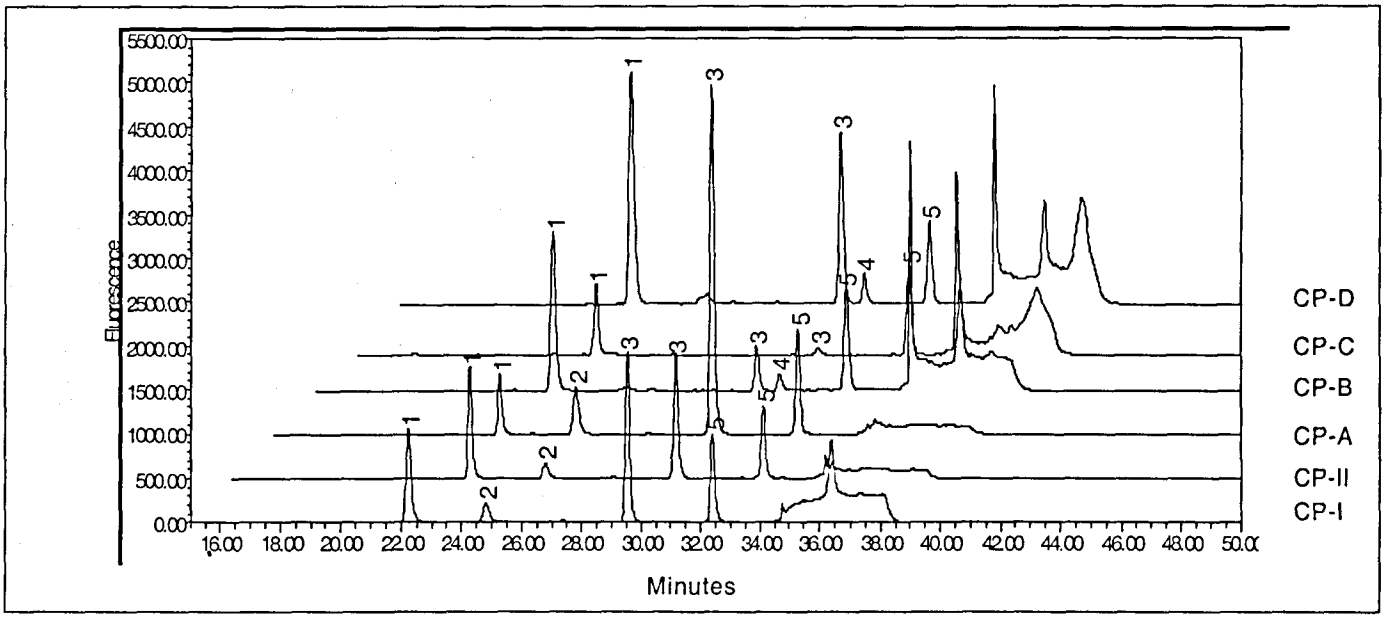
Fig. 1: HPLC chromatogram of amino acid hydrolysate standard detected on fluorescence detector.



The peaks are (Rt) 1. Alanine (22.27), 2. Proline (24.91), 3. Valine (28.90), 4. Lysine (30.51), 5. Leucine (32.67), 6. Aspartine (14.81), 7. Serine (16.66), 8. Glutamine (17.32), 9. glycine (18.78), 10. histidine (19.53), 11. NH_3 (21.29) 12. cystine (27.64), 13. Tyrosine (28.22), 14. methionine (29.45), 15. isoleucine (32.30), 16. Phenyl alanine (32.22).

acids, alanine, valine and leucine were present in all the six samples, whereas proline was present only in the two batches, CP-I and CP-II, prepared in the lab and in only one market sample, CP-A. Out of the four market samples, CP-A, CP-B, CP-C and CP-D analyzed, two others, CP-B and CP-D, contained lysine instead of proline whereas the fourth, CP-C, was found not to contain both proline and lysine.

Fig. 2: HPLC chromatograms of different samples of *Chyavanprash*



The peaks are, 1. alanine, 2. proline, 3. valine, 4. lysine, 5. leucine

TABLE 2: AMOUNT OF AMINO ACIDS IN VARIOUS SAMPLES OF *CHYAVANPRASH*

Sample Code	Weight of amino acid (ng/ 5 µl)				
	Alanine	Valine	Leucine	Proline	Lysine
CP-I	12.5	23.3	6.62	9.75	-
CP-II	9.35	15.1	6.26	6.66	-
CP-A	5.05	43.5	9.24	21.1	-
CP-B	13.8	5.72	8.16	-	5.60
CP-C	5.39	1.26	19.2	-	-
CP-D	20.2	22.5	6.62	-	10.0

Besides these five amino acids, none of the samples were found to contain any other amino acids. It can thus be inferred that essentially only these amino acids are present in *Chyavanprash*. The results showed that for each 5 µl of the sample injected, the amount of alanine varied from 5.1 to 20.2 ng, while valine varied from 1.26 to 43.5 ng and leucine varied from 6.26 to 19.2 ng (Table 2). The peak heights were used to calculate the amount of each amino acid in samples as compared to the standards. The identification was made on the basis of their retention time.

The results have shown that there was lesser variation in the amino acid contents of the two batches prepared in the laboratory than those in the marketed samples of different companies indicating a possible variation in the mode of preparation and/or plant materials used. It may also be due to improper storage of raw materials. Also since a large number of ingredients are present in it, there is a possibility of adulteration/substitution on commercial scale.

Amino acids constitute a very important group of compounds. They are essential for the human body as they exhibit specific functions (http//www.realtime.net/anr/aminoacd). Lysine insures adequate absorption of calcium, helps form collagen and aids in the production of antibodies, hormones and enzymes. Its deficiency may result in tiredness, inability to concentrate and irritability. Leucine is used for the production of other essential biochemical components in the body, some of which are utilized for the production of energy, stimulants to the upper brain and keep one more alert. Valine promotes mental rigor, muscle coordination and calms emotions. Proline is extremely important for the proper functioning of joints and tendons and also helps in maintaining and strengthening heart muscles. Alanine is an important source of energy for muscle tissue, the brain and central nervous system. It strengthens the immune system by producing antibodies.

The present study shows that *Chyavanprash* is a good source of these amino acids. These results help give a rational explanation for and lend support to the therapeutic use of *Chyavanprash* in Ayurveda. These findings can be taken as one of the parameters, along with the other reported parameters, for quality control of *Chyavanprash.*

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