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Determination of amino acid without derivatization by using HPLC - HILIC column

Pravin Bhandare[1], P. Madhavan[1], B.M. Rao*, N Someswar rao[2]

[1]Analytical Research, Custom Pharmaceutical Services, Dr. Reddy's Laboratories Ltd., Visakhapatnam, India
[2]Department of Analytical Chemistry, School of Chemistry, Andhra University, Visakhapatnam, India

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Abstract

The present paper deals with HPLC method development for determination of amino acid. A high performance liquid chromatography (HPLC) method with UV detector is developed for determination of individual 17 Amino acids content in bulk drug substance without derivatisation and without any pretreatment of sample. The method is developed with market available Silica column by using isocratic mobile phase 2.5 mM Potassium dihydrogen phosphate with pH 2.85 and Acetonitrile with ration 25:75. The proposed method contains the high percentage of Acetonitrile in mobile phase, so that silica column works as HILIC column for amino acid analysis. The limit of quantitation for non aromatic Amino acids is in the range 1.2 – 2.1 µg/mL and for aromatic Amino acid is in the range of 8 – 12 ng/mL. The limit of detection for non aromatic Amino acids is in the range 0.4 – 0.65 µg/mL and for aromatic Amino acid is in the range of 2.5 – 3.6 ng/mL.. The method is accurate, linear and precise from LOQ level to 150% level with respect to analyte concentration for the analysis of Amino acid in bulk drug substance. The method is specific to resolve the degradation impurity formed in Acidic, Basic and Oxidative condition.

Key words: Amino acid without derivatisation, HPLC validation, HILIC column.

Introduction

Amino acids are very important for human body for their regular biological activities. Amino acids are the building blocks of the body. Besides building cells and repairing tissue, they forms antibody to combat bacteria and virus; they are part of enzyme and hormonal system. Amino acids are very important for building nucleoproteins (RNA & DNA) [1]. Eight amino acids are regards essential for human body: phenylalanine, valine, Threonine, Tryptophan, Isoleucine, Methionine, Leucine, and lysine.[3] Additionally, Cysteine (or sulphur-containing amino acids), tyrosine (or aromatic amino acids), Histidine and Arginine are required by infants and growing children [1,2]. Individual living with Phenylketonuria (PKU) must have Tyrosine intake in their food because the person living with PKU can't convert the Phenylalanine into Tyrosine [3]. The table-1 lists the WHO recommended daily amounts currently in use for essential amino acids in adult humans.[2] This table indicates that the daily dose for essential amino acid is in the range 0.28 gm to 2.7 gm which is quite high, so HPLC method developed is important to control the total impurity by quantifying amino acid in bulk drug.

Table-1: WHO limit for Amino acid per day

Amino acid(s)	mg per kg body weight
I Isoleucine	20
L Leucine	39
M Methionine + C Cysteine	10 + 4 (15 total)
F Phenylalanine + Y Tyrosine	25 (total)
T Threonine	15
W Tryptophan	4
V Valine	26

The lack chromophores in most amino acids and highly hydrophobic side chain present in most of amino acid makes the HPLC method development more challenging. Various high performance liquid chromatography methods for the analysis of derivatized amino acid have also been studied since the 1970s. Some of the methods are enabled both qualitative and quantitative analysis of most amino acid. The ion exchange chromatography method with postcolumn derivatisation [4] and reverse phase HPLC method with prederivatisation [5] are available in literature. Other analytical methodologies that have been applied to the analysis of amino acid include GC –MS [6], TLC [7], Capillary electrophoresis -MS [8], GC [9], LC-MS, tandem mass spectrometry (MS-MS) [10] and Capillary electrophoresis [11]. Most of the present methods are expensive or required derivatisation and laborious sample preparation procedures. The novelty of present method is analysis of amino acid by using HILIC column technique which gives the liberty for amino acid analysis without derivatisation. The advantages of this method as compared to present published method is that, i) In this method sample preparation and sample pretreatment not required which makes method very simple and fast, ii) This method is with commercial available column and with UV detector which makes method cost effective.
This paper deals with the method development and validation of the developed method.

Materials and Methods

2.1 Materials

Glutamine, Phenyl Alanine, Proline, Alanine, Methionine, Aspartic acid, Valine and Tryptophan was purchased from Loba Chemie, Asparagine hydrochloride and Isoleucine was purchased from Fluka, Cystine from Aldrich, Leucine, Glutamic acid was purchased from Qualigens, Glycine was purchased from S.D. fine chemicals, Serine, Tyrosine was purchased from Spectrochem.

GR grade Potassium dihydrogen phosphate was purchased from Merch Ltd., Mumbai, HPLC grade Acetonitrile was purchased from Ranbaxy fine chemicals, New Delhi, India. Analytical Reagent grade Acetic acid was purchased from Qualigens fine chemicals, Mumbai, India.

2.2 Instrumentation

The LC system used for method development and validation was Agilent 1100 series equipped with photodiode array detector. Liquid Chromatography (LC) operated with Chemstation software, Agilent technology.

2.3 Method

Silica columns are well known for Normal phase chromatographic application but few applications are available as HILIC. This paper demonstrates the application of Silica column as like HILIC column for amino acid quantization at detector wavelength 200 nm. The Kromasil SIL column used for amino acid analysis has a 250 mm length, 4.6 mm diameter and 5 µm particle sizes. The isocratic mobile phase used with the ratio of mobile phase A and B as 25:75, where as mobile phase A is 2.5 mM Potassium dihydrogen phosphate with pH=2.85 and mobile phase is Acetonitrile, for the quantification of amino acid in bulk drug with run time 20 min. The column oven temperature is maintained 30°C in all experiments.

2.4 Sample preparation

Alanine, Asparagines, Aspartic acid, Cysteine, Glutamic acid, Glutamine, Glycine, Isoleucine, Leucine, methionine, Proline, Serine, Threonine and valine was prepared with the concentration 1 mg/mL and Phenylalanine, Tryptophan and Tyrosine was prepared with concentration 0.1 mg/mL.

Results and Discussion

The objective of this work was to develop the stability-indicating chromatographic method to determine the amino acid content in bulk drug substance without derivatisation.

3.1 Column selection

Since, amino acids are well known for their hydrophobic property so method development trial was started with special column chemistry instead of regular C8, C18 etc. column. Various column chemistry was screened which includes the Merck HILIC (zwitterionic bonded phases) column, Acclaim mixed mode (Reverse phase and weak anion exchange property in single column) column and finally freeze the Kromasil SIL column with 250 mm length, 4.6 mm diameter and 5 µm particle sizes.

3.2 Mobile phase selection

The method was screened by using Silica column with various buffers which includes Ammonium acetate, Ammonium format and phosphate. Since, amino acids are UV inactive, so phosphate buffer chosen for method development. The various composition of mobile phase A: 2.5 mM Potassium dihydrogen phosphate and mobile phase B: Acetonitrile were screened where found that the increasing percentage of Acetonitrile was increased the retention time as well as tailing factor while decreasing the percentage of Acetonitrile was decreased the retention time as well as tailing factor. The method has been finalized with 75% Acetonitrile where tailing factor ranging from 0.8 to 1.2 and retention time ranging from 6 minute to 15 minutes. The tailing factor and plate number has been tabulated for all amino acid in Table-3.

The method has been screened for various pH range (pH from 2.0 to 7.5) where found that the lower pH is giving more tailing while at higher pH Aspartic acid and Glutamic acid was eluted in void volume, other 15 amino acid was not observed major impact on retention time and tailing factor due to high pH. Finally mobile phase A with pH 2.85 was chosen for regular analysis where tailing factor is within range and Glutamic acid & Aspartic acid were retaining on column.

1.4 Method validation

3.4.1 Specificity

The method specificity has been demonstrated in presence of degradant by stress study. The stress study was performed in Acidic, Basic and Peroxide condition. Most of the amino acid found stable in 0.1 N Hydrochloric acid and 0.1N Sodium hydroxide for 24 hours but most of the amino acids was degraded in 3% Hydrogen peroxide solution. The proposed method is capable to resolve all degradant from main peak which indicates that the method is stability indicating. The peak purity results confirm that the Amino acids peak is homogenous in all stress conditions. Stress study results are summarized in table no. 2 for all amino acids.

Table-2: Stress study results

Amino acid Name	Amino acid Name (Abbreviation)	** Degradation of Amino acid under stress condition (% w/w)		
		Acidic	Basic	Oxidative
Proline	PRO	ND	ND	9.5
Alanine	ALA	ND	ND	13.1
Isoleucine	ILE	ND	ND	66.4
Cysteine	CYS	ND	67.8	* 42.9
Phenyl alanine	PHE	ND	ND	4.4
Valine	VAL	ND	ND	53.3
Serine	SER	ND	ND	36.8
Glycine	GLY	ND	ND	13.1

Tyrosine	TYR	ND	ND	ND
Tryptophan	TRP	ND	ND	ND
Glutamine	GLN	ND	10.0	5.4
Asparagine	ASN	ND	ND	10.0
Leucine	LEU	ND	ND	93.3
Threonine	THR	ND	ND	10.0
Methionine	MET	ND	ND	100
Glutamic acid	GLU	ND	ND	ND
Aspartic acid	ASP	ND	ND	ND

ND: No degradation observed after 24 hours.
** After 24 hours precipitation observed so sample filtered through filter paper and filtrate used for analysis.*
*** Degradation of amino acid under stress condition (% w/w) is calculated as follows,*
= 100 – Amino acid content (% w/w) after stress study

3.4.3 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogenous sample under prescribed conditions [12-13].

The precision of method has been performed by injecting six preparation of each amino acid and determined the content against injecting the respective standard amino acid. Intermediate precision also performed by changing column, system, analyst on different day.
The % RSD of amino acid content in drug substance for all amino acid was found below 2.0% in Method precision and Intermediate precision.

3.4.4 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample [12-13].

Linearity for every amino acid was demonstrated by injecting test solution at five different concentration including lower concentration at LOQ level and higher concentration at 200% with respect to analyte concentration.

The method was found linear for every amino acid from LOQ level to 200% level with respect to analyte concentration. The correlation coefficient was found more than 0.99 and y intercept was found less than ± 5 % against 100% response.

3.4.5 Accuracy

The accuracy of method was demonstrated by injecting the standard at 50%, 100% and 150% level with respect to analyte concentration. Accuracy solution was prepared in triplicate at each level and injected each preparation once into chromatographic system.

The percentage recovery for each amino acid was found within the range of 98.0 to 102.0%.

3.4.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection represents the concentration of analyte that would yield a signal to noise ratio at about 3 (12-15) and the limit of quantification represents the concentration of analyte that would yield a signal to ratio at about 10 [12-13].

The LOD and LOQ were established for individual amino acid and the results are tabulated in table-3. The system precision was performed at LOQ level for each amino acid and the % RSD was found below 7.3%.

Table-3: Stress study results

Name	LOQ	LOD	* Plate number	* Tailing factor
Proline	2.1 µg/mL	0.65 µg/mL	13736	1.06
Alanine	1.4 µg/mL	0.50 µg/mL	11233	0.83
Isoleucine	1.4 µg/mL	0.45 µg/mL	12866	0.9
Cysteine	0.1 µg/mL	0.03 µg/mL	8174	1.03
Phenyl alanine	12 ng/mL	4 ng/mL	13039	1.1
Valine	1.25 µg/mL	0.40 µg/mL	11574	0.87
Serine	1.6 µg/mL	0.50 µg/mL	8298	0.93
Glycine	1.6 µg/mL	0.60 µg/mL	4558	0.68
Tyrosine	8 ng/mL	2.5 ng/mL	8717	1.13
Tryptophan	10 ng/mL	3.0 ng/mL	13261	1.04
Glutamine	0.3 µg/mL	0.09 µg/mL	8508	0.95
Asparagine	0.2 µg/mL	0.06 µg/mL	7790	0.78
Leucine	1.4 µg/mL	0.40 µg/mL	11053	0.97
Methionine	50 ng/mL	15 ng/mL	14832	1.03
Glutamic acid	1.4 µg/mL	0.45 µg/mL	11173	1.03
Aspartic acid	1.2 µg/mL	0.40 µg/mL	7480	1.73
Threonine	1.6 µg/mL	0.55 µg/mL	14288	0.91

** Plate number and Tailing factor calculated for the analyte peak, obtained by injecting individual amino acid at their 100% analyte concentration.*

Fig. 1: LOQ solution chromatogram of Tryptophan, Leucine, Glutamic acid and Glycine

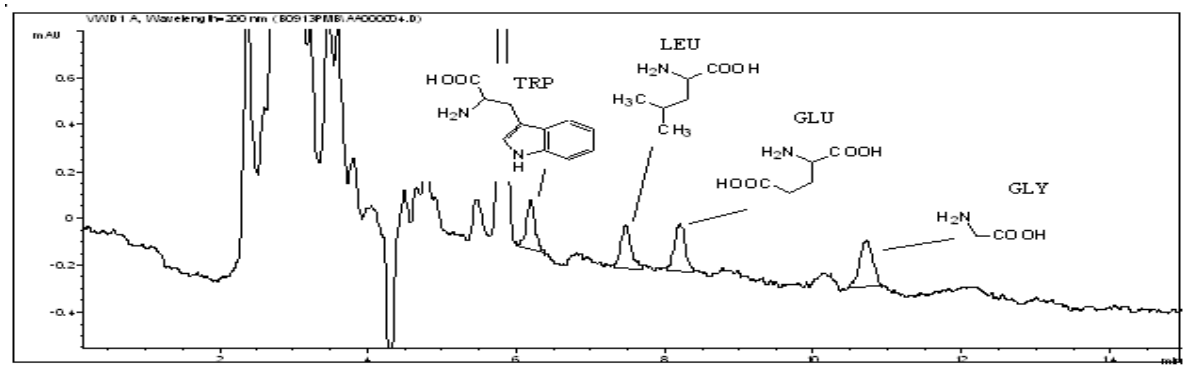


Fig.2: LOQ solution chromatogram of Methionine, Aspartic acid, Threonine and Asparagines

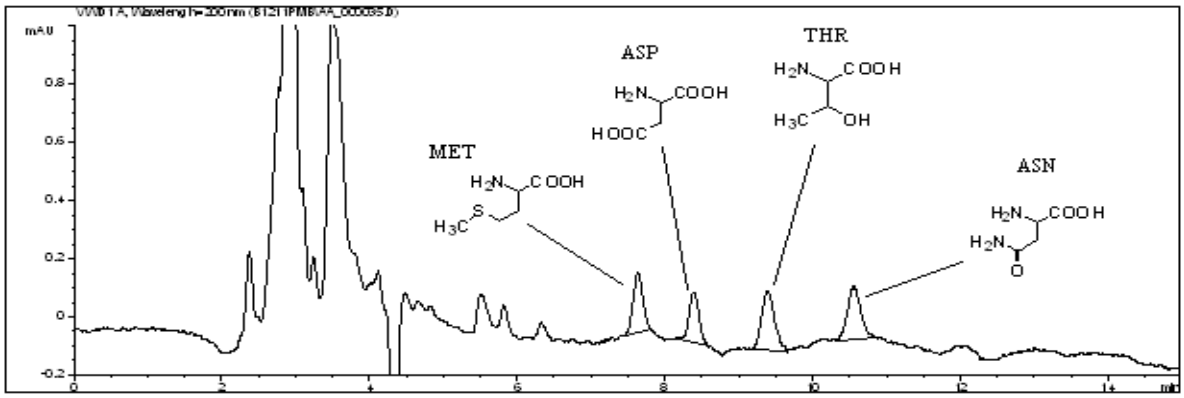


Fig.3: LOQ solution chromatogram of Phenyl Alanine, Isoleucine, Alanine and Proline

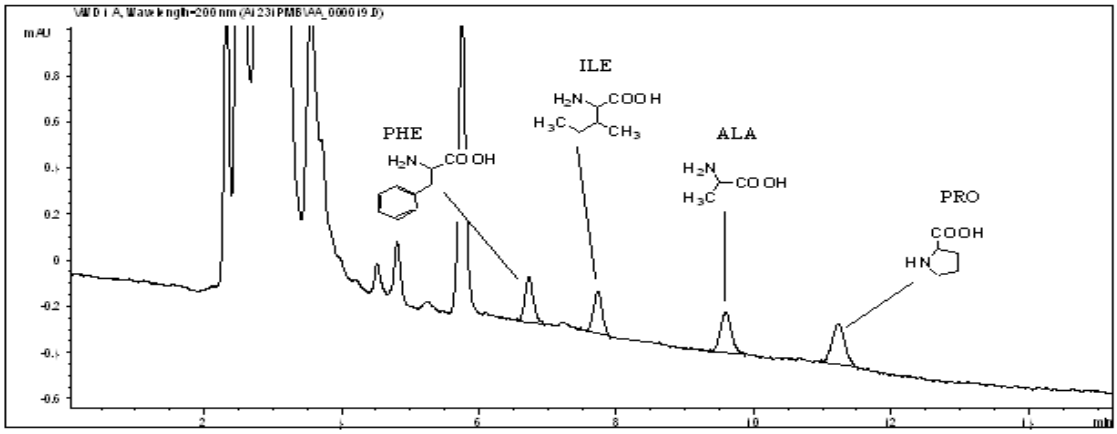
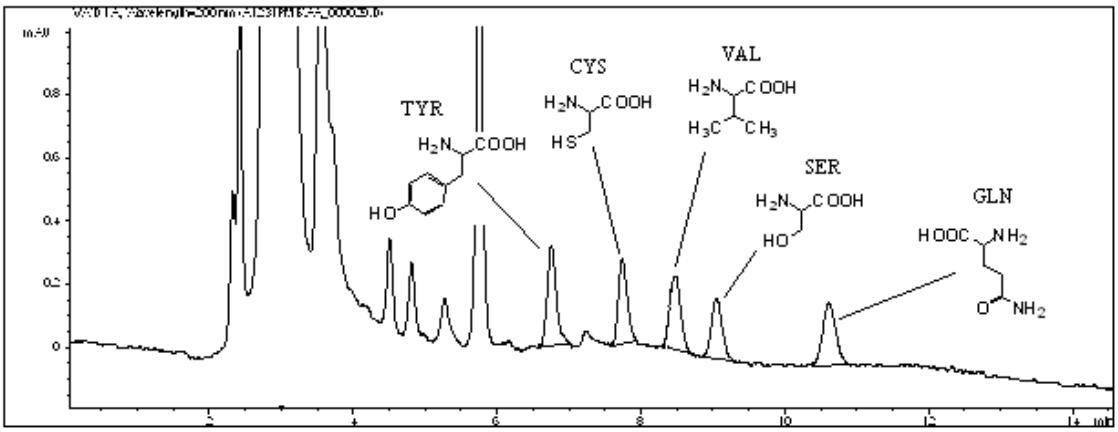


Fig 4: LOQ solution chromatogram of Tyrosine, Cysteine, Valine, Serine and Glutamine



3.4.7 Solution Stability

Solution stability of all amino acid was established by leaving the test solution in tightly closed volumetric flask at room temperature on a laboratory bench for 24 hours. The content of amino acid was checked after 24 hours against freshly prepared test solution.

The variation in amino acid content after 24 hours was found within 0.5%. This indicates that the test solution is stable for 24 hours.

3.4.8 Robustness

Robustness of the method was studied by making small deliberate changes in the flow rate, column temperature and mobile phase composition separately by keeping other parameter intact. The flow rate was changed by ± 0.1 mL/min, column temperature was changed by ± 2°C and mobile phase composition changed by ± 2% , the content of amino acid was determined with changed parameter were found that the amino acid content was not varied more than 1%. This indicates that the method is robust.

Conclusion

The new method developed for determination of amino acid content in respective drug substance by HPLC is precise, linear, robust, accurate and stability-indicating. This method can be used to release the amino acid drug substance and during stability study of amino acid in Quality Control department.

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