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Determination of arginine, lysine and histidine in drug substance and drug product without derivatisation by using HILIC column LC technique

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ABSTRACT

The present paper deals with HPLC method development and validation for determination of Arginine, Lysine and Histidine in drug substance and drug product without derivatisation by High Performance Liquid Chromatography (HPLC) using HILIC column technique. The above amino acids are eluted by using isocratic mobile phase which contains solution A as 50 mM Potassium dihydrogen phosphate and solution B as Acetonitrile in the ratio of 70:30. The Limit of Quantitation (LOQ) of Arginine, Lysine and Histidine is 0.22 µg/mL, 6.7 µg/mL and 0.14 µg/mL respectively. The Limit of Detection (LOD) of Arginine, Lysine and Histidine is 0.066 µg/mL, 2.01 µg/mL and 0.042 µg/mL respectively. The method is accurate, linear and precise from LOQ level to 150% level with respect to analyte concentration for the analysis of Arginine, Lysine and Histidine in bulk drug substance. The proposed method is accurate to analyse the Arginine, Lysine and Histidine in drug product at 50% level to 150% level with respect to analyte concentration. The method is specific to resolve the degradation impurity formed under Acidic, Basic and Oxidative condition.

Key words: Arginine, Lysine, Histidine, Amino acid without derivatization, HILIC column, Silica column.

INTRODUCTION

Lysine is an essential amino acid which means that human body cannot synthesize it. Lysine is indispensible amino acid that is required preformed in the diet of humans. Lysine is also first limiting amino acid for protein synthesis in persons consuming a predominantly cereal based diet such as wheat & rice [1].Young adults need about 23 mg of this amino acid per day per kilogram (10 mg per lb) of body weight. Deficiency of L-Lysine may lead to anemia, blood shoot eyes, enzyme problems, hair loss, inability to concentrate, irritability, lack of energy, poor appetite, reproductive problems, retarded growth and weight loss [3].

Histidine is one of the 22 proteinogenic amino acids. In terms of nutrition, Histidine is considered an essential amino acid in human infants. After reaching several years of age, humans begin to synthesize it, at which point it becomes a non-essential amino acid [4]. Histidine was accepted in 1985 by the FAO/WHO/UNU as an indispensible amino acid in human adult, despite controversy regarding its essentiality [5]. The lack of Histidine in the diet for a prolonged period resulted in an accommodation of protein turnover and phenylalanine oxidation, measured by the ^{13}C- phenylalanine indicator amino acid. Significant decrease in albumin, transferrin and hemoglobin concentration occurred slowly over the Histidine depletion period [6].

Table-1: Daily Requirement table [7]

Amino Acid	mg/kg body weight/day
Lysine	30
Histidine	10

Arginine is a conditionally indispensable amino acid [7]. Arginine is a nitric oxide precursor. Nitric oxide is formed from Arginine via the enzyme nitric oxide syntheses (NOS) [9]. A significant nutritional problem in preterm infants is a severe deficiency of Arginine (hypoargininemia), which results in hyperammonemia, as well as cardiovascular, pulmonary, neurological, and intestinal dysfunction. Arginine deficiency may contribute to the high rate of infant morbidity and mortality associated with premature births. [8]

The lack chromophores and highly hydrophobic side chain present in Lysine, Arginine & Histidine 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 [10] and reverse phase HPLC method with prederivatisation [11] are available in literature. Other analytical methodologies that have been applied to the analysis of amino acid include GC –MS [12], TLC [13], Capillary electrophoresis -MS [14], GC [15], LC-MS, tandem mass spectrometry (MS-MS) [16] and Capillary electrophoresis [17]. Most of the present methods are expensive or required ion pair reagent, derivatisation or laborious sample preparation procedures. The novelty of present method is analysis of amino acid by using HILIC technique which gives liberty for amino acid analysis without derivatisation and without ion pair reagent. 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

Materials

Lysine and Arginine was purchased from Sigma and Histidine was purchased from Fluka, GR grade Potassium dihydrogen phosphate was purchased from Merch Ltd., Mumbai, HPLC grade Acetonitrile was purchased from Ranbaxy fine chemicals, New Delhi, India.

Instrumentation

The LC system used for method development and validation was Agilent 1100/1200 series equipped with photodiode array detector. Liquid Chromatography (LC) operated with Chemstation software, Agilent technology.

Method

Lysine, Histidine and Arginine analysis method was developed by using HILIC technique with silica column. 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 30:70, where as mobile phase A was 50 mM Potassium dihydrogen phosphate and mobile phase B was Acetonitrile, for the quantitation of amino acids in bulk drug substance as well as drug product with run time 30 minutes. The detector wavelength was selected as 200 nm and column oven temperature was maintained 30°C in all experiments.

Preparation of Standard solution

Histidine & Arginine was prepared with the concentration 0.5 mg/mL and Lysine was prepared with the concentration 1 mg/mL.

Preparation of Test solution

Drug substance

Histidine & Arginine was prepared with the concentration 0.5 mg/mL and Lysine was prepared with the concentration 1 mg/mL.

Drug product

The capsule content was transferred into 25 mL volumetric flask and then added about 20 mL of water, sonicated about 20 minutes and made up to mark with water. Then this solution filtered through 0.22 µm filter paper.

RESULTS AND DISCUSSION

The objective of this work was to develop a stability indicating chromatographic method to determine the Lysine, Arginine and Histidine content in bulk drug substance and drug product without derivatisation by using single method.

Mobile phase selection

The method was screened by using Silica column with various buffers which includes Ammonium acetate, Ammonium format and phosphate. Since, selected amino acids are non chromophoric, so phosphate buffer chosen for method development. The various concentration of buffer was used, where observed that the increasing in buffer concentration tailing of Lysine and Arginine was decreasing, so that 50 mM phosphate buffer selected for further analysis. The various composition of mobile phase A: 50 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 70% Acetonitrile where tailing factor ranging from 0.8 to 1.2. The tailing factor and plate number has been tabulated in Table-3.

Method validation

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. Lysine, Arginine and Histidine were found stable in 0.1 N Hydrochloric acid and 0.1N Sodium hydroxide for 24 h but degraded in 3% v/v 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. The purity factor and stress study results are summarized in table no. 2.

Table-2: Stress study results

Amino acid Name (Abbreviation)	* Degradation of Amino acid under stress condition (% w/w)			Peak purity factor		
	Acidic	Basic	Oxidative	Acidic	Basic	Oxidative
Lysine (LYS)	ND	ND	13.4	999.244	998.784	998.002
Histidine (HIS)	ND	ND	54.8	999.751	999.791	999.822
Arginine (ARG)	ND	ND	72.6	999.148	999.121	997.142

ND: No degradation observed after 24 hours.

** Degradation of amino acid under stress condition (% w/w) is calculated as follows,*

= 100 – Amino acid content (% w/w) after stress study

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 [18-19].

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 1.0% in Method precision and Intermediate precision.

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 [18-19].

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 150% with respect to analyte concentration. The results are tabulated in table-3.

Table-3: Linearity results

Amino acid	Linearity Equation	Y-Axis Intercept (% wrt 100% analyte concentration)	R^2
Lysine	y = 10.11x - 4.209	0.43	0.999
Arginine	y = 114.8x + 206.9	1.73	0.998
Histidine	y = 175.9x + 134.0	0.76	0.999

Accuracy

The accuracy of method was demonstrated in drug product in presence of ingredient which is most commonly used in formulation of Lysine, Arginine and Histidine. The most common ingredients are tabulated in table-5. The accuracy was performed by spiking Lysine, Histidine and Arginine in drug product at 50%, 100% and 150% level with respect to analyte concentration. Each level solution was prepared in triplicate and injected each solution once in chromatographic system. The results are tabulated in Table-4.

Table-4: Accuracy results

S. No.	Analyte spiked (% wrt analyte concentration)	Accuracy (In %)		
		Histidine	Arginine	Lysine
1	50	100.1	99.8	96.9
2	100	95.5	97.3	96.1
3	150	96.1	96.3	97.5

Table-5: Drug product ingredient

S. No.	Ingredient	Content (mg)
1	L-Leucine	18.3
2	L-Isoleucine	5.9
3	L-Lysine HCl	25.0
4	L-Phenylalanine	5.0
5	L-Threonine	4.2
6	L-Valine	6.7
7	L-Tryptophan	5.0
8	DL-Methinonie	18.4
9	5-Hydroxy Anthranalic acid	0.2
10	Folic acid	0.75
11	Nicotinamide	25.0

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 [18-19] and the limit of quantification represents the concentration of analyte that would yield a signal to ratio at about 10 [18-19].

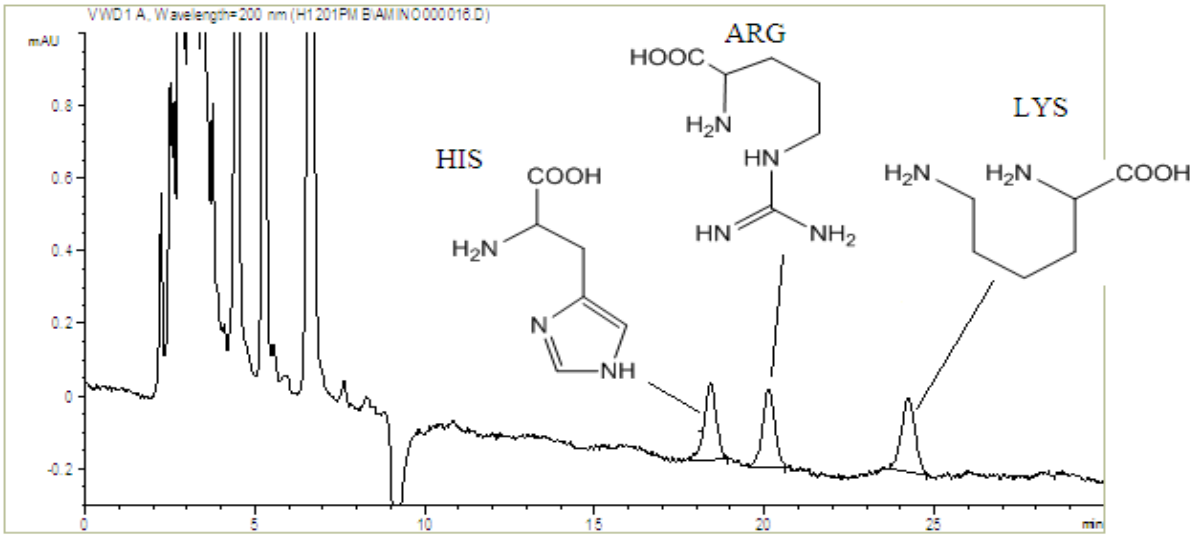
The LOD and LOQ were established for individual amino acid and the results are tabulated in table-6. The system precision was performed at LOQ level for Lysine, Histidine and Arginine and the % RSD was found 6.5%, 3.9% and 5.3% respectively.

Table-6: LOD, LOQ and System suitability results

Name	LOQ	LOD	* Plate number	* Tailing factor
Histidine	0.14 µg/mL	0.042 µg/mL	12772	0.90
Arginine	0.22 µg/mL	0.066 µg/mL	14449	0.98
Lysine	6.7 µg/mL	2.01 µg/mL	12491	0.93

** 1. Plate number and Tailing factor calculated for the analyte peak, obtained by injecting individual amino acid at their 100% analyte concentration.*
2. Theoretical plate number calculated by Tangent method and Tailing factor calculated by USP method.

Fig. 1: LOQ solution chromatogram of Histidine, Arginine and Lysine



Solution Stability

Solution stability of these amino acids 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.

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 Lysine, Arginine and Histidine content in respective drug substance and drug product by HPLC is precise, linear, robust, accurate and stability indicating. This method can be used to release the amino acid drug substance, drug product and during stability study of amino acid in Quality Control department.

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