Research Article

Essential Amino Acids Content Determination of Hard Gelatin Capsules from Vitamin Formulation by HPLC

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Received: 10-01-2017; **Revised:** 28-01-2017; **Accepted:** 14-02-2017.

ABSTRACT

The stability indicating analytical method by derivatization and fluorescence detection was developed for the content estimation of essential amino acids present in multivitamin hard gelatin capsules formulation by HPLC. The amino acids L-Histidine HCl, L-Arginine HCl, L-Threonine, L-Alanine, L-Valine, L-Methionine, L-Lysine HCl, L-Isoleucine, L-Leucine and L-Phenylalanine were estimated by using 6-Aminoquinolyl-N-HydroxySuccinimidyl Carbamate (Acc.Q fluor) reagent dervatisation with borate buffer (0.5M) in a max recovery vial and kept at 55°C for 10 minutes. The solutions were injected in to the chromatograph equipped with quaternary gradient pump, column oven, auto sampler with cooler and a fluorescence detector. Similarly marketed capsules amino acid formulation was analysed and compared with in-house developed sample. The system suitability parameters were evaluated and the method was validated as per ICH guidelines. The linearity of the method was established from 25% level to 150% level of working concentration. The regression coefficient (r^2) was reported not less than 0.9991 for each amino acid. The analyte recovery for each amino acid was performed by spiking known quantity of standard solutions in 5 levels and triplicate preparations. The overall recoveries of analytes were 96.72% to 102.78%.

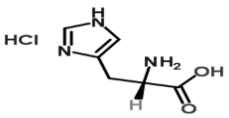
Keywords: Essential Amino Acids, Amino Acids content Determination, Vitamin Formulation, Hard Gelatin Capsules, and Stability Indicating.

INTRODUCTION

The amino acids are organic compounds containing amine group and carboxylic groups with a side chain specific to each amino acid. It comprises of Nitrogen, Carbon, Oxygen, Hydrogen and other elements.[1-3] They are classified based on functional groups location, pH, polarity and side chain groups such as aliphatic, aromatic, hydroxyl or sulphur. The amine and carboxylic groups attached with first alpha carbon atom have specific importance and are known as alpha amino acids. The generic formula for many of the amino acids is $H_2NCHRCOOH$. Our human body is made up of 20%protein which comprises of amino acids in cells, muscles and tissues. Amino acid plays a major role in transportation and storage of nutrition to various parts of the body. Most of the amino acids are supplied from food intake and humans can only able to produce 10 to 20 amino acids called essential aminoacids.[1-4]

They are histidine, isoleucine, methionine, lysine, arginine, phenylalanine, valine, threonine and tryptopan. The deficiency of amino acids causes stomach imbalance such as acidity and alkalinity balance, weak immune system, fatigue, nausea and body water retention balance.[4,5] The molecular structure of amino acids are represented in Fig.1.

Many of the available literature for the estimation of aminoacids in dosage forms deals precolumn derivatization with O-phthaldehyde,1,5-difluro- 2,4-dinitrobenzene,9-fluorenyl methyl-chloroformate and other reagents with florescence detection by HPLC.[4-7] Our present study reveals that stability indicating analytical method development of capsules formulation containing 10 amino acids was developed by precolumn derivatisation with 6-Amino quinolyl-N-Hydroxy Succinimidyl Carbamate (Acc.Q fluor) reagent. The developed method was compared by the estimation of marketed product available in the local market. In order to prove the method is stability indicating it was validated by set of ICH guidelines.[8-13]



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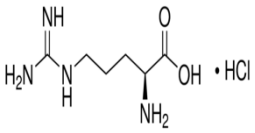

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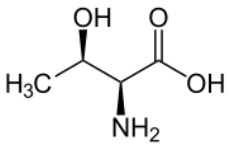

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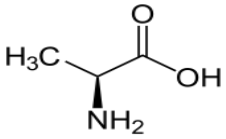

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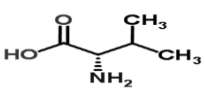

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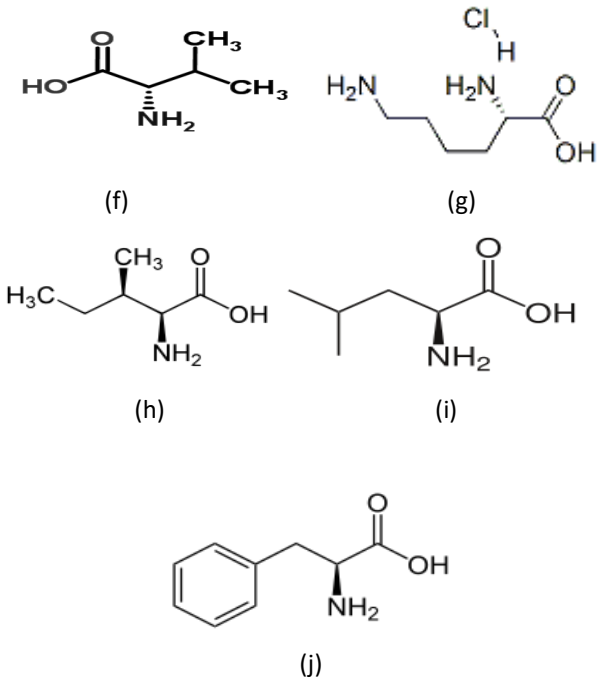

Figure 1: Molecular structure of Amino Acids (a) L-Histidine HCl (b) L-Arginine HCl (c) L-Threonine (d) L-Alanine (e) L-Valine (f) L-Methionine (g) L-Lysine HCl (h) L-Isoleucine (i) L-Leucine (j) L-Phenylalanine

MATERIAL AND METHODS

Reagent and Chemicals used for this Study

The amino acids reference standards for L-Histidine HCl, L-Arginine HCl, L-Threonine, L-Alanine, L-Valine, L-Methionine, L-Lysine HCl, L-Isoleucine, L-Leucine and L-Phenylalanine were procured from Sigma Aldrich and their certified purity were greater than 98%.The nuromin EA capsules marketed by ordain Global health care. Ltd, Chennai was purchased from local market. Acetonitrile, Hydrochloric acid and sodium borate and purified water used for this study were procured from Ranchem, Mumbai, India. The AccQ Fluor reagent and AccQ eluent A was purchased from M/s waters corporation, USA.

Instruments and Equipments used for this Study

The Waters Alliance model e2695 equipped with PDA detector, quaternary gradient pump, auto sampler with cooler, column oven and a fluorescence detector (FLR 2475) was used for this study. The software used for data integration of this analysis was Waters empower 2. The sonicator used to dissolve standard and samples (WENSAR LMUC-4), the balance used for weighing (Shimadzu, AUW220D) and pH meter (Oakton, pH-Con 700) were used for this study.

Reference Standard and Test Solution Preparation

The stock solution of each amino acids were prepared in the following concentrations; 152µg/mL for L-Histidine HCl, 541µg/mL for L-Arginine HCl, 173µg/mL for L-Threonine, 475 µg/mL for L-Alanine,267 µg/mL for L-Valine, 368 µg/mL for L-Methionine,1010 µg/mL for L-Lysine HCl, 233 µg/mL for L-Iso leucine, 722 µg/mL for L-Leucine and 206 µg/mL for L-Phenylalanine in diluent (0.1M HCl). Filter the solution through 0.45µm membrane filter. Dilute the stock solutions appropriately to get the final concentration of 8 µg/mL,27 µg/mL,9 µg/mL,24 µg/mL,13 µg/mL,18 µg/mL, 51 µg/mL, 12 µg/mL, 36 µg/mL, 10 µg/mL respectively. These preparations was carried out below 25°C.

The nuromin EA capsules contents were transferred in to mortar and triturate in to a fine powder. The average net content of the capsules was found to be 332.8mg.The powdered capsule content equivalent to average filled weight of capsules was transferred in to a 100 ml volumetric flask, add 30 ml of 0.1M hydrochloric acid and sonicate for 10 minutes at below 25°C to dissolve the contents and make up the volume with diluent. Filter the solution through 0.45µm membrane filter. Further dilute 5 ml of the resulting solution to 50 ml with diluent. The percentage label claim of each amino acid present in market formulation i) L-Histidine HCl-3.71mg/capsule ii) L-Arginine HCl-13.28mg/capsule iii) L-Threonine-4.20mg/capsule iv) L-Alanine-11.9mg/capsule v)L-Valine-6.7mg/capsule vi) L-Methionine-9.2mg/capsule vii) L-Lysine HCl-25.0mg/capsule viii) L-Isoleucine-5.9mg/capsule ix) L-Leucine-18.3 mg/capsule x)L-Phenylalanine-5.0mg/capsule.

Dervatisation Procedure and Chromatographic Parameters:

The gradient elution time programme was set with 1 ml pump flow from 3 channels which A) 100% buffer B) 100% Acetonitrile c) 100% water. The gradient time programme is listed in Table.1.The buffer solution was prepared by dissolving 100ml of AccQ. TaQ (Eluent A) with 1000ml water. The diluent was prepared by dissolving 8.5 ml HCl with 1000ml water.

Preparation of AccQ Fluor Reagent

Transfer 1ml of AccQ Fluor reagent diluent in to Acc.Q Fluor reagent powder vial, shake well, sonicate for 10 minutes to dissolve the contents and kept the solution at 55°C for 10 minutes.

Dervatisation Procedure

Pipetted out and transfer 420µL of 0.1M sodium borate buffer in to 3 max recovery vials and add 60µl blank solution to the vial marked as blank, 60µL of standard solution to the vial marked as standard, 60µL of sample solution to the vial marked as sample and 120µL of Acc.Q Fluor reagent, close the vial and shake well. Kept the vial at 55°C for 10 minutes.

The HPLC system was set as 1 mL of pump flow per minute with 45 minutes gradient elution programme, column oven temperature at 37°C,sample cooler temperature at 10°C, Acc. Q. TaQ (3.9 X 150mm) analytical column, the fluorescence detection of excitation wavelength at 250nm and emission wavelength at 395 nm. The precolumn derivatized amino acids

eluents were subjected to fluorescence detection and the peaks were eluted in the following retention times (RT); 17.8, 20.9, 21.2, 21.8, 27.7, 28.3, 30.9, 31.7, 32.3 and 33.8 minutes respectively.

Table 1: Gradient Elution Time Programme

Gradient Elution					
Time	**Flow rate (mL/min)**	**Solvent – A**	**Solvent – B**	**Solvent - C**	**Curve**
0.0	1.0	100	0	0	-
0.5		99	1.0	0	11
18.0		95	5.0	0	6
19.0		91	9.0	0	6
29.5		83	17	0	6
33.0		0	60	40	11
36.0		100	0	0	11
45.0		100	0	0	6

Comparison between In-house formulation and Marketed Formulation

The hard gelatin amino acids capsules were formulated by sifting of amino acids, lactose monohydrate and magnesium stearate in 40# and filled in capsules shells. This in-house formulated amino acids capsules was analysed by the developed method and the results were reported. The available marketed formulation (nuromin EA) was analyzed as per the developed method and the results were compared to prove the method is stability indicating. The results were listed in Table.2.

Validation of Analytical Method as ICH Guidelines:

The developed method was validated by set of parameters i) Precision ii) Linearity iii) Analyte recovery iv) LOD and LOQ v) Robustness. The precision of the method was established by performing six determination of assay of amino acids as per proposed concentration. The percentage limit for six time assay RSD should not be more than 2.

The linearity range was studied from 25% level to 150% level of working concentration and the linear regression coefficient of variation should be not less than 0.999.The linearity curve was plotted against concentration of each level against peak area.

The analytes recovery study was performed from 50% level to 150% level of working (50, 75, 100, 125 and 150% levels) concentration spiked with known concentration amino acids in to placebo mixture. The concentration for L-histidine hydrochloride; 4, 6, 8, 10 & 12µg/mL, L-arginine; 13.5, 20.3, 27.0, 33.8 & 40.5 µg/mL, L-threonine; 4.5, 6.8, 9.0, 11.3 & 13.5 µg/mL, L-alanine; 12, 18, 24, 30 & 36, L-valine; 6.5, 9.8, 13.0, 16.3 & 19.5 µg/mL, L-methionine; 9, 13.5, 18.0, 22.5 & 27.0, L-lysine HCl; 25.5, 38.3, 51.0, 63.8 & 76.5, L-isoleucine; 6, 9, 12, 15 & 18, L-leucine; 18, 27, 36, 45 & 54 and L-Phenyl alanine; 5, 7.5, 10.0, 12.5 & 15.0. The Average percentage recovery of amino acids should be between 90% to 110% and the percentage RSD should be less than 2.0.

The LOD &LOQ was determined by injecting 4µg/mL of placebo spiked solution serially diluting to find out lowest amount of analyte to be detected for each amino acid and the lowest amount of analyte to be quantified. The limit for LOD is signal to noise ratio should be more than 3.0 and for LOQ is should be more than 10.

The robustness study was conducted by slightly changing the chromatographic parameters such as flow rate, column temperature and mobile phase ratio. The percentage RSD of results obtained should not be more than 2.0.The system suitability parameters were evaluated as the system is not valid unless the following conditions were met; i) the tailing factor of all amino acids peaks in the standard preparation should be not more than 2; ii)the number of theoretical plates for all active peaks should not be less than 2000;iii)the RSD for the area of all active peaks for five replicates of standard preparation should not be more than 6.0%

RESULTS AND DISCUSSION

The stability indicating analytical method was developed with precolumn dervatisation of amino acids with 6-Aminoquinolyl-N-Hydroxy Succinimidyl Carbamate and florescence detection. The developed method was adopted for in house formulated hard gelatin capsules as well as marketed sample available in the market. The results were compared with in- house formulation and found percentage assay greater than 95% of each amino acid. The blank, standard and marketed sample chromatograms were displayed in Fig.2, 3 & 4.

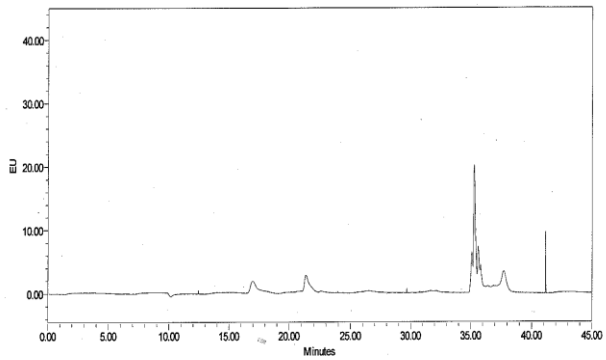

Figure 2: Chromatogram for Blank Preparation

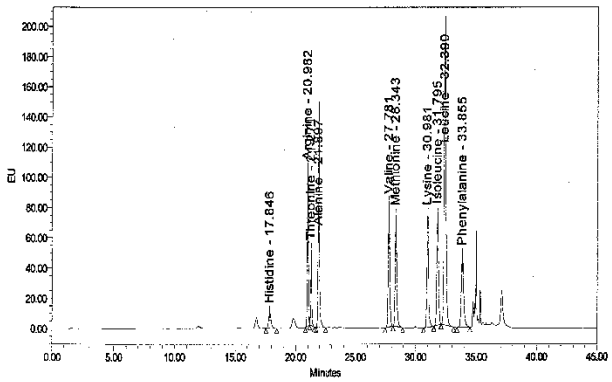

Figure 3: Chromatogram for Standard amino acids preparation

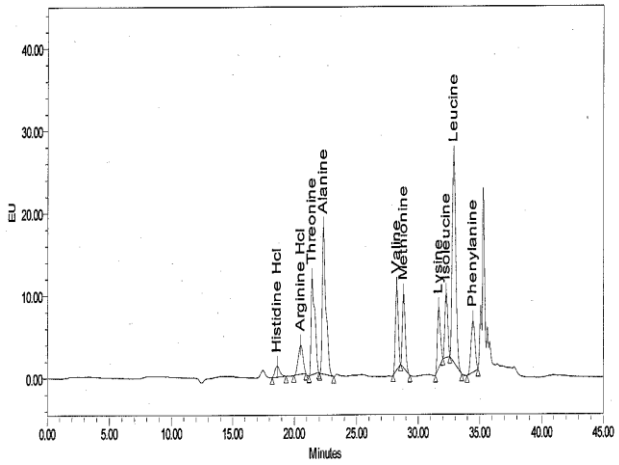

Figure 4: Chromatogram for Marketed sample preparation

The precision of the method was established by six times assay determination of samples for In-house formulation and marketed formulation. The average assay percentage and RSD for In-house formulation was 98.6% to 129.3%; 0.76 to 1.28%. The average assay percentage and RSD for marketed formulation was 108.4% to128.8%; 0.53 to 1.23%.The results were reported in Table.2.

Table 2: Comparison of Test results between In-house and Marketed Formulation

Compound Name	In-house Formulation				Marketed Formulation		
	Assay%	RSD	LC in mg	Overages %	Assay %	LC in mg	RSD
L-Histidine HCl	119.5	0.81	3.71	20.0	115.6	3.71	0.53
L-Arginine HCl	107.1	0.93	13.28	10.0	108.4	13.28	0.86
L-Threonine	115.5	1.02	4.20	20.0	114.8	4.20	0.97
L-Alanine	108.4	1.14	11.90	10.0	109.5	11.90	1.23
L-Valine	120.2	0.76	6.70	20.0	118.7	6.70	1.14
L-Methionine	108.7	1.20	11.20	10.0	106.3	18.40	0.77
L-Lysine HCl	109.6	1.28	25.00	10.0	108.9	25.00	0.90
L-Isoleucine	129.3	0.96	5.90	30.0	128.8	5.90	1.16
L-Leucine	119.8	1.18	18.30	20.0	120.1	18.30	1.21
L-Phenyl alanine	118.9	1.22	5.00	20.0	117.2	5.00	0.99

Table 3: Linearity data results.

Compound Name	Concentration µg/mL	Regression Coefficient	Slope	Intercept	RSD
L-Histidine HCl	6.0-36.0	0.9997	13540	3622	0.78
L-Arginine HCl	3.25-19.5	0.9993	58505	-46312	0.65
L-Threonine	4.5-27.0	0.9994	32954	-13333	0.83
L-Alanine	2.25-13.5	0.9995	105916	-38133	0.91
L-Valine	3.3-20.0	0.9992	73403	-144333	0.85
L-Methionine	2.5-11.25	0.9996	67811	-111467	0.97
L-Lysine HCl	2.0-12.0	0.9997	72323	20000	0.46
L-Isoleucine	12.75-76.5	0.9998	70996	37333	0.37
L-Leucine	6.75-40.5	0.9992	221955	-456608	0.89
L-Phenyl alanine	2.5-15.0	0.9991	63584	-12100	0.82

Table 4: Recovery study data results.

Compound Name	Concentration Added in µg/mL	Concentration Recovered in µg/mL	Mean Recovery	SD	RSD
L-Histidine HCl	4.0,6.0,8.0,10.0 and 12.0	4.1,6.2,7.9,10.04 and 12.21	101.35	1.80	1.78
L-Arginine HCl	13.5,20.3,27.0,33.8 and 40.5	13.4,20.1,27.2,33.6 and 40.3	99.59	0.67	0.67
L-Threonine	4.5,6.8,9.0,11.3 and 13.5	4.3,6.5,8.7,11.0 and 12.8	96.72	1.23	1.27
L-Alanine	12.0,18.0,24.0,30.0 and 36.0	12.2,18.1,24.3,29.8 and 36.3	100.73	0.89	0.88
L-Valine	6.5,9.8,13.0,16.3 and 19.5	6.2,9.3,12.7,16.0 and 19.1	96.82	1.55	1.60
L-Methionine	9.0,13.5,18.0,22.5 and 27.0	9.1,13.7,18.2,22.6 and 27.3	101.05	0.38	0.37
L-Lysine HCl	25.5,38.3,51.0,63.8 and 76.5	25.3,38.1,50.7,63.3 and 76.2	99.39	0.17	0.17
L-Isoleucine	6.0,9.0,12.0,15.0 and 18.0	6.1,9.2,12.1,14.8 and 17.8	100.46	1.61	1.60
L-Leucine	18.0,27.0,36.0,45.0 and 54.0	17.7,26.7,35.8,44.7 and 53.9	99.16	0.57	0.57
L-Phenyl alanine	5.0,7.5,10.0,12.5 and 15.0	5.4,7.8,10.5,12.7 and 15.3	102.78	1.63	1.59

n=3; SD-standard deviation; RSD-Relative Standard Deviation

Table 5: Robustness study data results.

Compound Name	Pump Flow rate				Temperature				Mobile Phase composition			
	Lower(0.8ml)		Higher(1.2ml)		Lower(35°C)		Higher(39°C)		Lower (90% organic)		Higher (110% organic)	
		RSD	Assay%	RSD	Assay%	RSD	Assay %	RSD	Assay%	RSD	Assay%	RSD
L-Histidine HCl	118.7	0.78	121.2	0.86	120.2	0.67	119.3	0.75	120.5	0.88	119.0	0.57
L-Arginine HCl	108.6	0.83	109.3	0.97	107.5	0.98	107.7	0.82	107.9	0.96	106.8	0.64
L-Threonine	114.4	1.15	116.4	1.18	115.8	1.08	116.0	1.05	115.7	1.11	115.2	1.07
L-Alanine	107.2	1.14	109.8	1.07	107.9	1.17	108.7	1.10	108.9	1.17	107.6	0.98
L-Valine	119.5	0.77	121.3	0.81	120.6	0.79	120.4	0.82	120.9	0.79	121.0	0.88
L-Methionine	108.6	1.23	110.4	1.03	109.0	1.10	109.3	1.07	109.8	1.05	108.9	1.02
L-Lysine HCl	109.3	1.30	111.2	1.08	109.1	1.15	109.5	1.28	109.2	1.01	109.0	1.18
L-Isoleucine	127.7	0.98	130.6	0.99	130.4	0.84	129.8	0.96	129.6	0.90	129.7	0.68
L-Leucine	119.9	1.22	120.8	1.15	119.3	1.06	119.9	1.21	120.3	1.01	119.5	1.07
L-Phenyl alanine	118.8	1.34	119.7	1.20	119.0	1.02	119.3	1.14	118.4	1.27	119.3	1.33

Table: 6 System Suitability data results

Compound Name	Theoretical Plates	Tailing Factor	Resolution	Standard RSD %
L-Histidine HCl	39269	1.3	-------	0.6
L-Arginine HCl	310471	1.2	4.48	0.4
L-Threonine	245873	1.4	0.83	0.5
L-Alanine	237013	1.4	1.04	0.3
L-Valine	237517	1.2	6.92	0.4
L-Methionine	239798	1.4	0.86	0.4
L-Lysine HCl	252327	1.4	4.06	0.5
L-Isoleucine	251677	1.2	1.36	0.8
L-Leucine	214605	1.3	0.79	0.7
L-Phenyl alanine	154896	1.3	1.63	0.6

The LOD and LOQ values were found from L-histidine HCl 0.004 to L-phenyl alanine 0.007µg/mL; 0.05 to 0.075µg/mL. In Robustness two variables were studies in the lower and higher level and results compared with method precision values. The percentage RSD were found 1.26% to 1.86% from L-histidine HCl to L-phenylalanine. The results were reported in Table.5.The system suitability parameters studied were listed in Table.6.

CONCLUSION

Based on the above study the developed method was specific, precise, linear, accurate and robust for the repeatable application of In-house formulation as well as marketed formulation of essential aminoacids content estimation.

The method was found suitable for routine quality control analysis and formulation development studies for further improvement.

Acknowledgement: We thank to Sathyabama University, Department of chemistry, and Chennai for their continual support to complete the work.

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Source of Support: Nil, **Conflict of Interest:** None.