Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 4323 – 4330
Received 25 April 2021; Accepted 08 May 2021.

Analytical method validation for the determination of Ninhydrin Positive Substances in amino acids by High Performance Liquid Chromatography

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Abstract

The aim of the work is focused on the Validation of an Analytical method for the determination of Ninhydrin Positive substances (NPS) in Amino acids using HPLC Technique in a single method. Since, most of the amino acids are widely used in the determination of Renal and Nutrition drug products.The Chromatographic separation was performed using Sodium Amino Acid Analysis Cation Exchange column using Sodium Eluent Na 315, Sodium eluent Na 425 and Sodium Eluent Na 640 as Mobile phase, performed by Gradient program with detection of wavelength 570 nm using flow rate as 0.4 mL/min. The method has been developed and validated using post column derivatization technique (HPLC/Pinnacle PCX).The Method Validation Parameters/Performance Characteristics like Specificity, Method Precision, Linearity and Range, LOD, LOQ, Ruggedness, Solution stability, Robustness was carried out and all the amino acids were successfully met the acceptance criteria and the method has been validated as per ICH Q2R1 Guideline.The method has been successfully validated for its intended purpose.

Keywords:HPLC Technique, Specificity, Method Precision, Linearity.

Introduction:

Renal and Nutrition products mainly contain amino acids. As part of EP Testing for these substances, the presence of ninhydrin positive substances is determined. This determination is performed by using either TLC or HPLC with post column derivatization technique. The monographs are being revised to remove TLC method and replace with HPLC method. Since most of the amino acids are being revised to include this HPLC method, the amino acids Aspartic acid and Glutamic acid whose method has not been published. However, for Taurine and Ornithine HCL whose monograph not available. This approach assures the method has been developed and validated for all the amino acid substances which used to formulate Nutrition and Renal products. The EP reporting threshold limits for the ninhydrin positive substances HPLC method is0.05% and for ammonium is 0.02%.

The aim of the work is focused on the Validation ofOptimized HPLC method for the Ninhydrin Positive substances in Amino acids using HPLC Technique [1].

The following amino acids has been validatedas per ICHQ2R1in a single method:

Glycine, L-alanaine, L-Arginine, L-Aspartic acid, Glutamic acid, L-Histidine, L-Isoleucine , L-Leucine,L-Lysine acetate, L-Lysine HCl, L-Phenyl alanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine, Taurine and Ornithine HCl [2-21].

Methodology: Refer "Asian Journal of Pharmaceutical and Clinical Research: Vol 11, Special issue 4,2018- "Development Of Analytical Method for the Determination of Ninhydrin-Positive Substances In Amino Acids by High-Performance Liquid Chromatography"[23]

Method Validation Parameters/ Performance Characteristics:

Specificity parameter was performed and published in the Journal [23]. Other parameters like Method Precision, Linearity Accuracy, Range, LOD, LOQ, Stability of Solutions, Robustness were carried out as per the same procedure mentioned in the article [23] and tabulated from Table-1 to9.

Table-1: Method Precision results for each amino acids

Parameters	Experiment	Acceptance criteria	Name of the amino acid	Results/ Figures	
				%RSD for CSTB (Test solution	%RSD for amino acid blend & drug

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				concentration From 0.2% to 1.5%)	**substance** (Test solution concentration From 0.05% to 1.5%)
Method Precision	%RSD for the individual amino acid peak area of amino acids CSTB blend and amino acid drug substances (in water and dil. HCl) linearity at each linearity levels	Not more than 10%(n=6)	Aspartic acid	0.4% to 1%	0.4% to 2%
			Threonine	0.2% to 1%	0.4% to 2%, 1% to 6%
			Serine	0.5% to 1%	0.3% to 1%
			Glutamic Acid	0.2% to 0.5%	1% to 2 %
			Proline	0.1% to 2%	0.5% to 5%, 1% to 4%
			Glycine	0.3% to 0.4%	1% to 2%, 0.3% to 1%
			Alanine	0.2% to 0.5%	0.4% to 1%
			Valine	0.4% to 0.5%	1% to 2%
			Methionine	0.4% to 1%	1% to 3%
			Isoleucine	0.3% to 1%	1% to 6%
			Leucine	0.3% to 1%	1% to 5%, 1% to 8%
			Tyrosine	1% to 3%	0.4% to 3%
			Phenylalanine	0.1% to 1%	1% to 4%
			Tryptophan	0.02% to 1%	1% to 4%, 1% to 6%
			Lysine	0.2% to 1%	0.2% to 1%
			Histidine	0.2% to 1%	0.2% to 2%
			Arginine	0.2% to 1%	1% to 2%, 0.1% to 4%
			Ornithine	NA	0.2% to 1%
			Taurine	NA	1% to 2%, 0.4% to 1%

Table-2: LOD and LOQ results for each amino acids

Parameters	**Experiment**	**Acceptance criteria**	**Name of the amino acid**	**Results/ Figures**		
				Average S/N ratio	LOQ concentration (mg/mL)	LOD concentration (mg/mL)
LOD & LOQ	Signal to noise ratio of individual amino acids peak at LOQ level. Report the concentration for LOD level.	Not less than 10 for LOQ	Aspartic acid	92	0.000301940	0.00000982620
			Threonine	101	0.000301650	0.00000891929
			Serine	124	0.000302260	0.00000733661
			Glutamic Acid	55	0.000302300	0.0000164600
			Proline	23	0.000302980	0.0000397577
			Glycine	120	0.000300380	0.00000749244
			Alanine	84	0.000300930	0.0000108104
			Valine	34	0.000301520	0.0000264263
			Methionine	15	0.000300790	0.0000583322
			Isoleucine	18	0.000300450	0.0000505586
			Leucine	21	0.000301800	0.0000436078

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			Tyrosine	14	0.000303340	0.0000630404
			Phenylalanine	19	0.000303350	0.0000487170
			Tryptophan	17	0.000302480	0.0000532963
			Lysine	78	0.000423600	0.0000162026
			Histidine	47	0.000302410	0.0000193159
			Arginine	30	0.000302470	0.0000305157
			Ornithine	93	0.000383630	0.0000123971
			Taurine	101	0.000302850	0.00000896681

Table-3: Linearity and Range for each amino acids

Parameters	**Experiment**	**Acceptance criteria**	**Name of the amino acid**	**Results/ Figures**			
				Correlation coefficient	**Slope**	**Y intercept**	**Residual sum of squares**
Linearity and Range	Linearity from TS 0.2% to 1.5% CSTB, TS 0.05% to 1.5% for drug substance	Correlation coefficient r ≥ 0.99 , report the slope, y intercept and residual sum of squares, Range must be reported.	Aspartic acid	0.9999	5576503	-207099	76227180782
			Threonine	0.9999	11453450	-43105	13714253221
			Serine	0.9999	10649309	-194683	56708402131
			Glutamic Acid	1.0000	9489908	-93990	27576123918
			Proline	1.0000	4713947	-18494	5679715227
			Glycine	1.0000	25455843	-341312	192697629104
			Alanine	1.0000	45860832	-820503	575786732889
			Valine	0.9999	14716361	-141706	83639310699
			Methionine	0.9999	9137800	-88653	37289089958
			Isoleucine	1.0000	10227505	-105861	32144809690
			Leucine	0.9999	14535358	-118263	81663932364
			Tyrosine	0.9998	358813	-5541	136541341
			Phenylalanine	0.9999	11279527	-89369	51641713919
			Tryptophan	1.0000	1745251	-14495	776326507
			Lysine	0.9999	1796551	-297586	113456859717
			Histidine	1.0000	11355064	-103293	43160540230
			Arginine	0.9999	15847352	-179864	85897269547
			Ornithine	0.9999	457334543	6909	8934020577
			Taurine	0.9999	412032740	-5829	6269433961

Robustness: A gradient separation have the following modifications made to this method as part of robustness. i) Flow of Ninhydrin reagent. ii) Reactor temperature. iii) Change in Column temperature. iv) HPLC Gradient flow. v) Change in column lot and Ninhydrin reagent lot.

Robustness conditions:

i) Ninhydrin reagent flow: Changed ± 10% flow of Ninhydrin reagent flow with respect to nominal flow. Nominal condition: 0.30 mL/min Low : 0.27 mL/min High : 0.33 mL/min

ii) Reactor temperature: Changed -5°C of reactor temperature. Nominal condition : 130°C Low : 125°C Note: Recommended temperature range for Pinnacle PCX reactor is 30°C to 130°C.

iii) Column temperature: Changed ± 2°C column temperature with respect to nominal temperature.

Nominal Condition		Low		High	
Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)
0	45	0	43	0	47
35	45	35	43	35	47

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55	75	55	73	55	75*
85	75	85	73	85	75*
125	45	125	43	125	47
135	45	135	43	135	47

* Since, recommended temperature for pinnacle PCX column heater is 30°C to 75°C, hence above 75°C is not preferred

iv) Change in HPLC Gradient flow: Nominal condition: 0.40 mL/min, Low: 0.36 mL/min, High: 0.44 mL/min

v) Change in column lot and Ninhydrin reagent lot:The study was performed by the different column lot number (Serial No.) and different lot number of Ninhydrin reagent to prove the method is robust with different column and reagent lots also.

Table-4: Robustness at Ninhydrin Flow variation

Parameters	**Experiment**	**Acceptance criteria**	**Name of the amino acid**	**Results for % agreement with respect to nominal condition**	
				Low variation	**High variation**
Robustness	Injection of TS solutions at 0.5% level.	% Agreement between mean peak area in modified conditions and in nominalcondition 100% ± 25%	Threonine	97%	101%
			Proline	105%	96%
			Glycine	96%	102%
			Leucine	95%	102%
			Tryptophan	100%	101%
			Arginine	99%	101%
			Taurine	94%	102%

Table-5: Robustness at Reactor Temperature variation

Parameters	**Experiment**	**Acceptance criteria**	**Name of the amino acid**	**Results for % agreement with respect to nominal condition**
				Low variation
Robustness	Injection of TS solutions at 0.5% level.	% Agreement between mean peak area in modified conditions and in nominal condition 100% ± 25%	Threonine	88%
			Proline	90%
			Glycine	91%
			Leucine	90%
			Tryptophan	93%
			Arginine	90%
			Taurine	80%

Table-6: Robustness at Column Temperature variation

Parameters	**Experiment**	**Acceptance criteria**	**Name of the amino acid**	**Results for % agreement with respect to nominal condition**	
				Low variation	**High variation**
Robustness	Injection of TS solutions at 0.5% level.	% Agreement between mean peak area in modified conditions and in nominal condition 100% ±	Threonine	99%`	100%
			Proline	99%	100%
			Glycine	104%	101%
			Leucine	101%	100%
			Tryptophan	101%	100%

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		25%	Arginine	100%	100%
			Taurine	97%	98%

Table-7: Robustness at Gradient Flow variation

Parameters	**Experiment**	**Acceptance criteria**	**Name of the amino acid**	**Results for % agreement with respect to nominal condition**	
				Low variation	**High variation**
Robustness	Injection of TS solutions at 0.5% level.	% Agreement between mean peak area in modified conditions and in nominal condition 100% ± 25%	Threonine	117%	86%
			Proline	110%	92%
			Glycine	117%	88%
			Leucine	116%	82%
			Tryptophan	117%	88%
			Arginine	115%	90%
			Taurine	121%	82%

Table-8: Robustness at varied column and reagent lot

Parameters	**Experiment**	**Acceptance criteria**	**Name of the amino acid**	**Results for % agreement with respect to nominal condition**	
				Varied column	**Varied regent lot**
Robustness	Injection of TS solutions at 0.5% level.	% Agreement between mean peak area in modified conditions and in nominal condition 100% ± 25%	Threonine	100%	100%
			Proline	99%	100%
			Glycine	100%	101%
			Leucine	99%	101%
			Tryptophan	100%	101%
			Arginine	99%	101%
			Taurine	100%	100%

ACCURACY: Accuracy for specified impurities Valine in isoleucine andLeucine in Isoleucine were carried out from TSI-0.05% to TSI-1.5% Test solutions and the results were tabulated under Table-9.

Table-9: Accuracy for Specified Impurities

Experiment	Acceptance criteria	Test identification solution	Mean % Recovery for Valine in Isoleucine	Mean % Recovery for Leucine in Isoleucine
Analysis of test solution	% Recovery 100% ± 30% for TSI-0.05 (LOQ) for other levels 100%±15%.	TSI-0.05	101	97
		TSI-0.2	99	97
		TSI-0.5	99	99
		TSI-1.0	99	99
		TSI-1.5	100	99

Stability of solutions: Test solutions and System suitability were stored at room temperature for 4 days and there was no anomaly observed, i.e. No obvious precipitates or discoloration in solutions, no interference from

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close eluting peaks, Meet system suitability requirements and % Agreement experimental concentration at stability time point and the first injection of the solution i.e within the acceptance criteria of 100% ±20%.

Results and Discussion

As per European Pharmacopoeia (EP) testing of these amino acids, the presence of ninhydrin -positive substances was determined by TLC and also some of the amino acids such as Taurine and Ornithine HCl whose monograph was not published. This developed HPLC method using Post column derivatization technique was evaluated for all the amino acids and validated successfully and also all the amino acids were eluted in a single method.

The Results for thedetermination of ninhydrin positive substances (NPS) in amino acids by High performance liquid chromatography equipped with post column derivatizer (HPLC/Pinnacle PCX) for the individual amino acids and amino acid blend drug substances were tabulated from Table-1 to 8 and all the validated parameters were meeting the acceptance criteria. The Chromatograms for system suitability, TSA-1.0 % sample chromatogram at 570 nm and 440 nm, overlay chromatograms were provided from Figure-1 to 4.

Figure-1: Typical chromatogram for system suitability

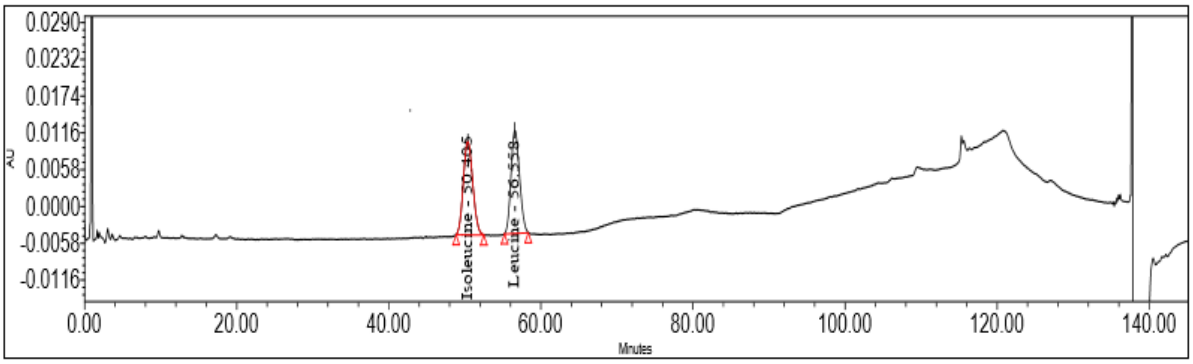


Figure-2: Typical chromatogram for TSA-1.0 % sample chromatogram at 440 nm

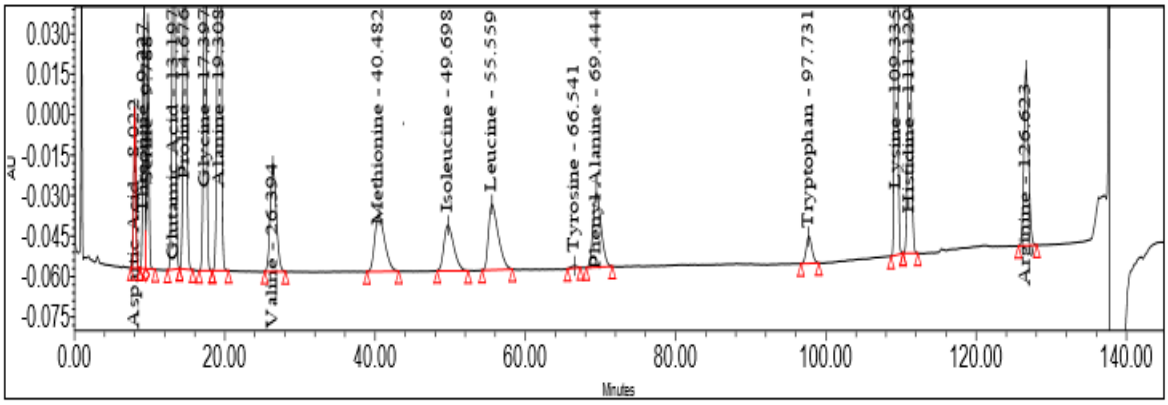
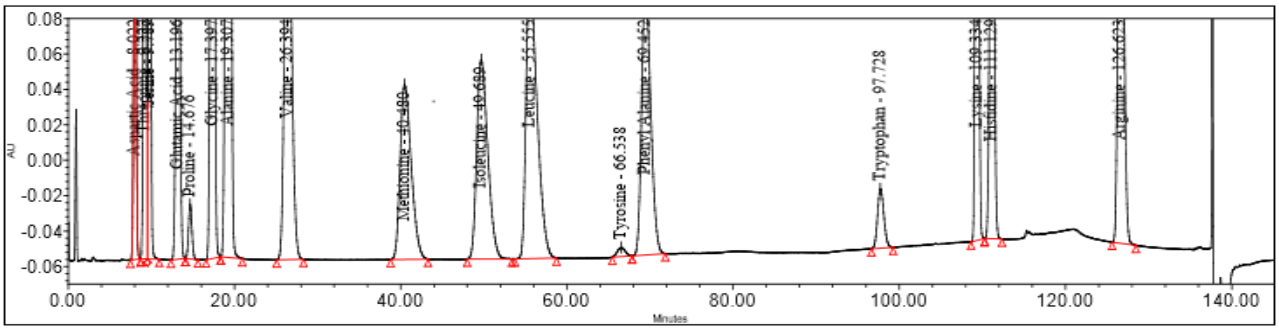


Figure-3: Typical chromatogram for TSA-1.0 % sample chromatogram at 570 nm



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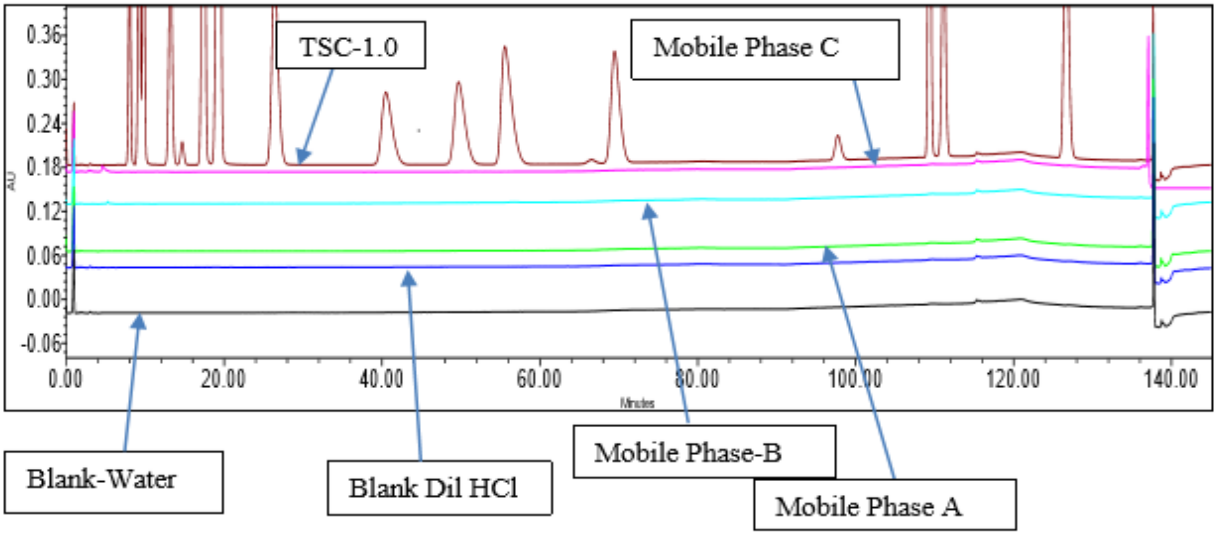
Received 25 April 2021; Accepted 08 May 2021.

Figure-4:Overlaychromatogram of TSA-1.0%, Blank(Water and dil.HCl) , and Mobile Phase A,B,C



Conclusion

The developed HPLC Method for the determination of ninhydrin positive substances (NPS) in amino acids by High performance liquid chromatography equipped with post column derivatizer (HPLC/Pinnacle PCX) for the individual amino acids and amino acid blend drug substances has been validated successfully as per ICHQ2R1 guidelines for its intended purpose.The validated method shall be used for routine analysis for the determination of Ninhydrin positive substances in amino acids.

Competing interests

The authors declare no conflict of interest.

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