ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Special issue 4, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

# DEVELOPMENT OF ANALYTICAL METHOD FOR THE DETERMINATION OF NINHYDRIN-POSITIVE SUBSTANCES IN AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

**SRINIVASA RAJAGOPALACHARI NK, SHANMUGASUNDARAM P***

**Department of Pharmaceutical Chemistry and Analysis, School of Pharmaceutical Sciences, Vels Institute of Science Technology and Advanced Studies, Chennai, Tamil Nadu, India. Email: srinivasaraja.gopalachari@gmail.com**

***Received: 15 September 2018, Revised and Accepted: 10 October 2018 and 11 December 2018***

**ABSTRACT**

**Aim and Objectives:** The aim of the work is focused on the optimization of the high-performance liquid chromatography (HPLC) method for the determination of ninhydrin-positive substances 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 exist independently in the monograph for each amino acid either by TLC or HPLC.

**Methods:** 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 evaluated using post-column derivatization technique (HPLC/Pinnacle PCX).

**Results:** All the amino acids were eluted correspondingly at the individual retention time and the method shall be validated as per the ICH Q2R1 Guideline.

**Conclusion:** The method has been successfully evaluated and developed for the analytical applications.

**Keywords:** Amino acids, Method development, HPLC, RRT, Specificity

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11s4.31674

## INTRODUCTION

Renal and nutrition products mainly contain amino acids. As part of the European Pharmacopoeia (EP) testing for these substances, the presence of ninhydrin-positive substances (NPS) is determined. This determination is performed using either thin-layer chromatography (TLC) or high-performance liquid chromatography (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, amino acids such as Taurine and Ornithine Hydrochloride (HCl) whose monograph has not been published and not available. This approach assures that the method has been developed for all the amino acid substances which are used to formulate nutrition and renal products. The EP reporting threshold limits for the NPS HPLC method are 0.05% and for ammonium are 0.02%.

The aim of the work is focused on the optimization of the HPLC method for the NPS in amino acids using HPLC technique [1].

The following amino acids are developed with a single HPLC method equipped with post-column derivatizer as follows:

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].

### Ammonium concentration

Molecular weight of ammonium chloride: 53.491 g/mol
Molecular weight of ammonium: 18.038 g/mol

$$\text{Final concentration of ammonium}: \frac{18.038 \times 740.30 \times 10 \times 6 \times 1}{53.491 \times 1000 \times 25 \times 50 \times 100}$$

=0.000119828 mg/mL

## MATERIALS AND METHODS

### Materials

All the amino acids used for this study were purchased from Sigma-Aldrich, and the catalog numbers are mentioned with each amino acid: Glycine-50046, L-alanine-05129, L-arginine-11009, L-aspartic acid-11189, L-glutamic acid-49449, L-histidine-53319, L-isoleucine-4160, L-leucine-4330, L-lysine HCL -L7286, L-phenyl alanine-78019, L-proline-81709, L-serine-4959, L-threonine-89179, L-tryptophan-93659, L-tyrosine-93829, L-valine-V0500, ornithine HCL -O8305, and taurine-T0625 [2-21].

### Reagents and chemicals

Ammonium chloride with catalog number: 09724 and HCL acid with catalog number: 84436, both the chemical have been purchased from Sigma Aldrich with EP grade.

### Instrument and analytical conditions

#### *Mobile phase*

Channel A: Sodium eluent Na 315, catalog no. Na 315; Channel B: Sodium eluent Na 425, catalog no. Na 425; Channel C: Sodium eluent Na 640, catalog no. Na 640; Channel D: Sodium regenerant RG011, catalog No. RG011; and the entire mobile phase (ready to use solution) have been purchased from Pickering Laboratories.

*Channel D: Eluent was used for column flushing, regeneration, and storage only.

#### *Derivatizing solution*

The Post Column Derivatizing solution used for this method is "TRIONE" Ninhydrin reagent, Make: Pickering Laboratories, catalog No. T200[22].

#### *Column*

The Analytical Column used for this method is Sodium Amino Acid

**Table 1: Gradient table**

| Time (min) | Flow (mL/min) | % A | % B | % C |
|---|---|---|---|---|
| 0.0 | 0.4 | 100.0 | 0.0 | 0.0 |
| 30.0 | 0.4 | 100.0 | 0.0 | 0.0 |
| 60.0 | 0.4 | 85.0 | 15.0 | 0.0 |
| 85.0 | 0.4 | 10.0 | 90.0 | 0.0 |
| 85.5 | 0.4 | 10.0 | 90.0 | 0.0 |
| 120.0 | 0.4 | 0.0 | 0.0 | 100.0 |
| 130.0 | 0.4 | 0.0 | 0.0 | 100.0 |
| 132.0 | 0.4 | 100.0 | 0.0 | 0.0 |
| 135.0 | 0.4 | 100.0 | 0.0 | 0.0 |

**Table 2: Pinnacle PCX post-column derivatization method**

| | |
|---|---|
| Run time (min) | 134 |
| Equilibration time (min) | 1 |
| Column temperature (°C) | 145 |
| **Column type** | **Sodium** |
| Reactor temperature (°C) | 130 |
| Reactor volume (mL) | 0.3 |
| Pump 1 rate (mL/min) | 0.3 |
| Reagent 1 | T200 |

**Table 3: Pinnacle PCX column temperature gradient**

| Time (min) | Temperature °C |
|---|---|
| 0.0 | 45 |
| 35.0 | 45 |
| 55.0 | 75 |
| 85.0 | 75 |
| 125.0 | 45 |
| 134.0 | 45 |

Analysis Cation Exchange Column, Make: Pickering laboratories, Dimension: 110 x 4.3m, 5 µ, Catalog no. 1154110T.

Gradient Table for HPLC, Pinnacle PCX post column derivatization method and column temperature gradient program are shown in Tables 1-3.

### *HPLC method*

The Instrument parameters used for HPLC, HPLC make / model and software used for this method development were mentioned below:

Flow rate: 0.4 mL/min, injection volume: 50 µL, Run time (min): 135 min, next injection delay: 1 min, detector wave length: 570 nm [22].

HPLC: Make – Waters HPLC, module: e2695 series with PDA detector using pinnacle PCX post column Derivatizer [22].

Software used: Empower-3.

**Diluents: Milli Q water, Make: Millipore or Dilute HCL-R1**

### *Preparation of Dilute HCL-R1*

Diluted 20 g of HCL into a 100 L with water and further diluted 10.0 mL of this solution to 2000mL with water.

**Preparation of ammonium 100 ppm standard**

Diluted 0.741050 g of ammonium chloride to 1000 mL with water. Further, diluted 10 mL of this stock solution to 25 mL with water.

**Preparation of reference (c) stock solution (solution A: Water)**

Diluted 6 mL of 100 ppm ammonium solution to 50 mL with water.

**Preparation of reference (c) stock solution (Solution A: Diluted HCL R1)**

Diluted 6 mL of 100 ppm ammonium solution to 50 mL with diluted HCL -R1.

**Preparation of amino acid mixture stock**

Weighed 30.560 mg of aspartic acid, 30.437 mg of threonine, 30.178 mg of serine, 30.269 mg of glutamic acid, 30.024 mg of proline, 30.462 mg of tryptophan, 30.173 mg of glycine, 30.698 mg of alanine, 30.111 mg of valine, 30.171 mg of isoleucine, 30.121 mg of leucine, 30.498 mg of tyrosine, 30.253 mg of ornithine HCL, 30.138 mg of histidine, 30.614 mg of arginine, 30.612 mg of taurine, 30.103 mg of phenylalanine, and 30.519 mg of lysine HCL, transferred into 50 mL volumetric flask, and diluted to the mark with diluted HCL R1.

Note: Stock solution for Amino acids was not prepared with water as the majority of the amino acids are sparingly soluble in water.

**Preparation of amino acid mixture spiked with ammonium (Solution A: water)**

Diluted 1 mL of amino acid stock mixture and 2 mL of ammonium ref (c) (diluted in water) into 200 mL with water.

**Preparation of amino acid mixture spiked with ammonium (Solution A: Diluted HCL R1)**

Diluted 1 mL of amino acid stock mixture and 2 mL of ammonium ref (c) (diluted in diluted HCL-R1) into 200 mL with diluted HCL-R1.

Concentration of amino acid mixture: 0.003 mg/mL and concentration of ammonium: 0.00012 mg/mL.

**Preparation of individual amino acid for specificity**

Individually prepared Threonine, Serine, Proline, Tryptophan, Alanine, Valine, Isoleucine, Leucine, Tyrosine and Phenylalanine using Dil HCl R1 as diluent. Individually prepared Aspartic acid, Glutamic acid , Ornithine HCl, Histidine, Arginine, Taurine, Glycine and Lysine HCl using water as diluent. All the amino acids were prepared with approximate concentration as 0.0012 mg/mL and ammonium in water with concentration as 0.000119828 mg/mL.

## RESULTS

Relative retention time (RRT) was calculated with respect to isoleucine for amino acids injected individually as part of specificity study. No blank interference was observed at the retention time of any of the amino acids and ammonium. The chromatograms for blank, ammonium, and amino acid mixture chromatogram with blank overlay are shown in Figs. 1-3.

The specificity results shown the individual RRT as mentioned below:

Taurine eluted at 3.793 min with 0.07 RRT, aspartic acid eluted at 8.342 min with 0.16 RRT, threonine eluted at 9.452 min with 0.18 RRT, serine eluted at 10.037 min with 0.19 RRT, glutamic acid eluted at 13.906 min with 0.27 RRT, proline eluted at 15.461 min with 0.29 RRT, glycine eluted at 17.645 min with 0.34 RRT, alanine eluted at 20.060 min with 0.38 RRT, valine eluted at 28.79 min with 0.55 RRT, isoleucine eluted at 52.442 min with 1.00 RRT, leucine eluted at 58.050 min with 1.11 RRT, tyrosine eluted at 68.507 min with 1.31 RRT, phenylalanine eluted at 71.371 min with 1.36 RRT, ammonium eluted at 82.095 min with 1.57 RRT, tryptophan eluted at 97.663 min with 1.86 RRT, ornithine HCL eluted at 106.397 min with 2.03 RRT, lysine eluted at 109.354 min with 2.09 RRT, histidine eluted at 111.384 min with 2.12 RRT, and arginine eluted at 126.571 min with 2.41 RRT. All the RRTs have been calculated with respect to isoleucine RT.

## CONCLUSION

In this present work, it has been concluded that all the amino acids were eluted separately with no blank interference with the specificity study performed as part of the method development. The method has been successfully developed for the inclusion of amino acids such as taurine and ornithine HCL. The HPLC method shall be validated as per the ICHQ2R1 guidelines for its intended purpose. Once the method being validated, the validated method shall be used for routine analysis for the determination of NPS in amino acids.

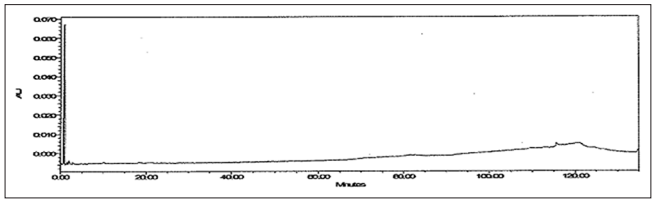

**Fig. 1: Typical chromatogram for water**

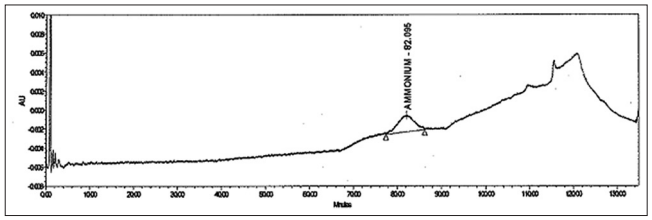


**Fig. 2: Typical chromatogram for ammonium**

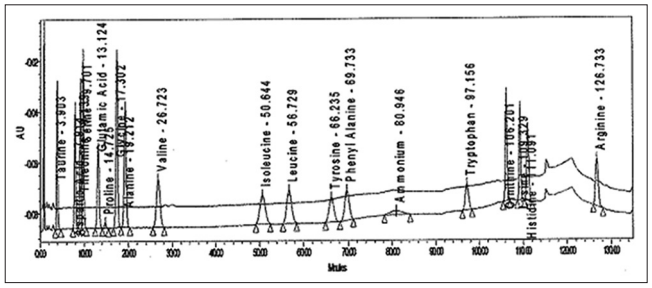


**Fig. 3: Typical chromatogram for amino acid mixture and blank overlay**

## REFERENCES

1. European Pharmacopoeia. Amino acid Analysis. 9th ed., Vol. 1. New York: European Pharmacopoeia; 2017. p. 93.
2. European Pharmacopoeia. Glycine. 9th ed., Vol. 2. New York: European Pharmacopoeia; 2017. p. 2608.
3. European Pharmacopoeia. Alanine. 9th ed., Vol. 2. New York: European Pharmacopoeia; 2017. p. 1656.
4. European Pharmacopoeia. Arginine. 9th ed., Vol. 2. New York: European Pharmacopoeia; 2017. p. 1753.
5. European Pharmacopoeia. Histidine. 9th ed., Vol. 2. New York: European Pharmacopoeia; 2017. p. 2654.
6. European Pharmacopoeia. Isoleucine. 9th ed., Vol. 2. New York: European Pharmacopoeia; 2017. p. 2814.
7. European Pharmacopoeia. Leucine. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 2881.
8. European Pharmacopoeia. Lysine Hydrochloride., 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 2935.
9. European Pharmacopoeia. Phenylalanine. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 3318.
10. European Pharmacopoeia. Proline. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 3424.
11. European Pharmacopoeia. Serine. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 3539.
12. European Pharmacopoeia. Threonine. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 3771.
13. European Pharmacopoeia. Tryptophan. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 3858.
14. European Pharmacopoeia. Tyrosine. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 3870.
15. European Pharmacopoeia. Valine. 9th ed., Vol. 3. New York: European Pharmacopoeia; 2017. p. 3890.
16. European Pharmacopoeia. Glutamic Acid. 9th ed., Vol. 2. New York: European Pharmacopoeia; 2017. p. 2593.
17. European Pharmacopoeia. Aspartic Acid. 9th ed., Vol. 2. New York: European Pharmacopoeia; 2017. p. 1764.
18. Nakano Y, Nohta H, Yoshida H, Todoroki K, Saita T, Fujito H, *et al.* Liquid chromatographic determination of ethambutol in serum samples based on intramolecular excimer-forming fluorescence derivatization. Anal Sci 2004;20:489-93.
19. Al-Ghannam S, Belal F. Spectrophotometric determination of three anti-ulcer drugs through charge-transfer complexation. J AOAC Int 2002;85:1003-8.
20. Marchei E, Pellegrini M, Pacifici R, Palmi I, Pichini S. Development and validation of a high-performance liquid chromatography-mass spectrometry assay for methylxanthines and taurine in dietary supplements. J Pharm Biomed Anal 2005;37:499-507.
21. Available from: https://www.sgs.com/en/news/2016/06/amino-acid-analysis.
22. Available from: https://www.pickeringlabs.com/wp-content/uploads/2015/03/MA391_010415_Web.pdf.