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L-Arginine monohydrochloride analysis by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

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ABSTRACT

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) has demonstrated to be a decent method for examination of biochemical samples, such as, amino acids. L-Arginine monohydrochloride is an amino acid which seems to be white in colour and is available in crystal in addition to lyophilized powder form. The current examination intends to the analysis of standard L-Arginine monohydrochloride for two different concentrations i.e. 100 and 200 parts per million (ppm). A simple sample preparation strategy was embraced for the analysis. The lyophilized powder of standard amino acid was solubilised in HPLC grade water. The gradient HPLC system was used as an analytical instrument with reverse phase (C18) column and isocratic pump system. The detection of target analyte was done by diode array detector. As a result of the study, the data of standard amino acid (L-Arginine monohydrochloride) in two different concentrations of 100 and 200 ppm were archived in chromatographic reports and in the chromatograms; the Peak Concentration in percentage, Retention time in minutes and Area in milli absorbance units at 100 ppm are 36.659, 3.040, 42915 and at 200 ppm are 93.626, 2.487, 37186 respectively.

Keywords: Arginine, Reverse Phase High Performance Liquid Chromatography, RP-HPLC, C18 column, Peak Concentration, Retention time, Primaide organizer, Isocratic Pump System.

INTRODUCTION

Amino acids are low molecular weight nitrogenous substances which builds proteins and peptides (Wu, 2013). L-Arginine monohydrochloride appears white in colour; it may be crystal or powder form. It has the pH (10% water a 25ºC) 4.70 - 6.20. Molecular formula of L-Arginine monohydrochloride is $C_6H_{15}N_4O_2Cl$. Molecular weight is 210.66 kg mol (http://www.himedia-labs.com/intl/en/products/Chemicals/Biochemicals-Amino-Acids/L-Arginine-mono-hydrochloride-GRM039). To estimate the presence and quantitation

of L-Arginine monohydrochloride into the suspected samples, a very specialized and reliable analytical instrumentation is necessary. And the most important factor for confirmation of analysis is the standard data to which the unknown concentrations of samples can be compared. L-Arginine monohydrochloride may be found in body fluids such as urine, and blood (to be precise, plasma) (Markowski *et al.* 2007 and Alkaitis *et al.* 2016). Quantitative analysis of L-Arginine monohydrochloride was carried out in pharmaceutical products (Belikova, 2016). L-Arginine can be found in microbiological samples and in fermentation processes (Ginésy *et al.* 2019). It was also found in wines (Wang *et al.* 2014). Chromatographic analysis is used to affirm the identity of sample, extent of its purity and its composition quantitatively. In case of high-performance liquid chromatography, the analysis of samples can be carried out conveniently at faster pace. This analytical method gives reliable quantitative results due to its very sensitive and highly specific system (Sato, 1977). Amino acid analysis has its root long back in history. From classical methods to highly specialized methods. Although quantitative analysis of arginine can be carried out by colorimetric and enzymatic method, but the present study is focused over depending on a technique which provides fast and reliable results. (Mira de Orduña, 2001; Micklus and Stein, 1973 and Faby, 1986).

The experiment of Ng *et al*. (2015) is based on analysis of underivatized amino acids by HPLC with mass detection. Such study has advantageous as it provides convenience, simplicity and repeatability. Unlike the current study, gradient solvent system was used to analyse amino acids. Analysis time for each amino acid was 25 minutes, flow rate of 5 µL amino acids was 0.400 ml/min. During the analysis oven temperature was 35°C and pressure of 2100 psi was maintained consistently. Detection of amino acids was carried out by Altus SQ MS. Though derivatization of amino acids were not involved but system responded with great sensitivity and specificity. In watermelons, the separation and quantification of L-citrulline and L-arginine was studied in Malaysia. Individually, L-citrulline and L-arginine stock solution were prepared in distilled water and filtered through a 0.45 μm syringe filter (Bioflow). A mixed standard solution was prepared by mixing an equal volume of each standard stock solution. A series of working standard solutions was prepared by diluting the stock solution with dH_2O. A similar method to this has adopted for this study to prepare the stock solution of standard L-Arginine monohydrochloride, it was solubilized into the chromatographic grade water and filtered through the RanDisc™ 0.22 μm syringe filter. And a working solution was made by serially diluting the stock solution. (Ridwan *et al.* 2018).

Reverse Phase High Performance Liquid Chromatography is used in this study to analyse the standard amino acid L-Arginine monohydrochoride since it has hydrophobicity and it was based on the principle that separation of compounds on polarity of stationary and mobile phase. In RP-HPLC, organic solvent as a mobile phase is of much importance as it has capability to modify the surface tension in chromatographic eluent (Hughes, 2013). Along with the features like sensitivity and specificity, this system is also versatile in nature that it can analyse the samples of toxicology, biology with same ease (Patil *et al.* 2012).

The objective of the study is to find standard retention time values for the L-Arginine monohydrochloride of two different concentrations (in parts per million). A single solvent system of acetonitrile was used with isocratic pump system and diode array detector detected the target analyte.

MATERIALS & METHODS

Chemicals:

Acetonitrile for HPLC and Spectroscopy (S D Fine Chem-Limited) was used as a solvent phase for standard amino acid, L-Arginine monohydrochloride. *Methanol of HPLC grade* was used for pre wash and post wash of the column of system. *Water of chromatographic grade* was used to dissolve the standard amino acids. L-Arginine monohydrochloride is used from *Himedia standard amino acids kit*.

Instruments and apparatus*:*

Hitachi High Performance Liquid Chromatography system by Hitachi Instrument (Dalian) Co. Ltd. with Primaide organizer, reverse phase (C18) column, isocratic pump system was used for the analysis of sample and analyzed data was recorded on HPLC connected hardware and Primaide System Manager software, *Ultrasound bath*

sonicator was used to degas the solvent system and the column washing solvent. Both the solvents were sonicated for 25 minutes and they were carried undisturbed to the HPLC system, *LG Refrigerator* with temperature 2-4°C was used to store the amino acids, *Borosil glass bottles* (500 ml) were used to store the solvent system (acetonitrile), washing solvent (methanol) dipped pump channels of RP-HPLC, *Hi*-pette micropipettes (Vol. 1-10 µL) for dissolution of standard amino acids in eppendorf tubes; as well as it was used for preparation of stock and working solutions of different concentrations, 2 ml Eppendorf tubes for the storage of stock and working solution of arginine, Polylab eppendorf tube storage box with cover lid was used to store the samples as it was handy and easy to carry. It has the capacity to store 64 eppendorf tubes of 1.5 - 2 ml, Shimadzu electronic balance was used to measure the standard amino acids to make them aqueous. Dispo van 2 ml syringes were used to filter the standard amino acid solutions through the syringe filter before loading them into the HPLC system's loop. HAMILTON Microliter™ syringe of 25 µL capacity is used to inject standard amino acid solution into the loop of HPLCsystem. RanDisc™ 0.22 µm syringe filters were used to filter the out the impurities from standard amino acid solution to a great extent, thus allowing it smooth run of solvent for analysis. Print-a-stick sticky labels and Luxor Marker pen were used to label the eppendorf tubes.

Sample preparation:

Stock solution of standard amino acids such as L-Arginine monohydrochloride was made by weighing 2 mg of each amino acids on Shimadzu weighing balance and dissolved in 2 ml of HPLC grade water in 2 ml of eppendorf tube to make up the stock solution of 1000 parts per million (ppm) concentration. The stock solutions were filtered and further used to make concentrations of standard amino acids of working solutions in 100 and 200 ppm Table 1.

Table 1: Sample preparation of standard amino acid concentrations.

Stock solution (in µL)	HPLC grade water (in µL)	Working solution (in ppm)
100	900	100
200	800	200

Each standard amino acid samples were prepared by separate centrifugation process for 5-10 minutes and filtered through the RanDisc™ 0.22 µm in other eppendorf tubes. These samples were ready to load into the system.

HPLC Hardware/Software:

Experimental set up included the complete HPLC system which was used to analyse the standard L-Arginine monohydrochloride. This system was incorporated with a manual injector coupled with Reverse Phase/Eclipse Plus C18 column (5 µm diameter and 4.6 × 250 mm dimensions), to separate the target analytes from the desired sample. 1110 isocratic pump, which pumps the solution of single type. 1430 Diode Array Detector detected the target analytes of interest and displayed on the chromatograms by using ultra-violet radiations. HAMILTON 25 µL Microliter™ syringe was used to inject the sample into the column. Primaide System Manager software was installed in the computer system to operate the HPLC system. It was used to perform the functionalities like initiating the HPLC system, purging the column, set ultra-violet (UV) wavelength range of detection of analyte, run time of sample. The data of analysis was stored on the system's memory. Analyzed data was stored and can be accessed easily through the present software.

RESULT

In the initialization of experiment, the HPLC column was given pre-wash with HPLC grade methanol for 25 minutes. Soon after, the solvent system (100% acetonitrile) for HPLC and spectroscopy was employed with each concentration of standard amino acid L-Arginine monohydrochloride. This solvent was run for next 25 minutes; within that time interval and the amino acid was eluted. Further, post wash of column was carried out with HPLC grade methanol for 25 minutes. Total run time taken was of 75 minutes. Flat runs of methanol prior and later (pre-wash and post-wash respectively) to the sample were monitored to ensure that the column was clean and impurities-free. Flow cell was properly maintained, flow rate of the solvent for every wash and sample run was set as 1.0 ml/min. Chromatographic profile of two different concentrations i.e. 100 and 200 ppm of standard amino acid arginine was obtained (Table 2).

Table 2: HPLC parameters for standard L-Arginine monohydrochloride.

HPLC Parameters	Arginine (100 ppm)	Arginine (200 ppm)
Injection Volume (µL)	20.0	20.0
Mobile phase Solvent-A	Acetonitrile	Acetonitrile
Flow rate (ml/min)	1.0	1.0
Monitoring ultra-violet wavelength (nm)	240-260	240-350
Run time (in minutes)	25	25
Peak Concentration (%)	36.659	93.626
Retention time (in minutes)	3.040	2.487
Area (in milli Absorbance Units)	42915	37186

The present study was focused on standard amino acid, L-Arginine monohydrochloride of its two concentrations i.e., 100 ppm and 200 ppm. For both the concentrations of L-Arginine monohydrochloride, acetonitrile was used as a solvent system and the pump conditions i.e. the rate of solvent run flow was kept constant (1.0 ml/min). The analysis time for each standard concentration of volume 20 µL as the elution of standard amino acid can be achieved within lesser period of time. The HPLC system used for this study has in-built detection feature with the varying wavelength of ultra-violet radiations. This wavelength can be set according to need of monitoring elution of various standard amino acids according to their absorption limits.

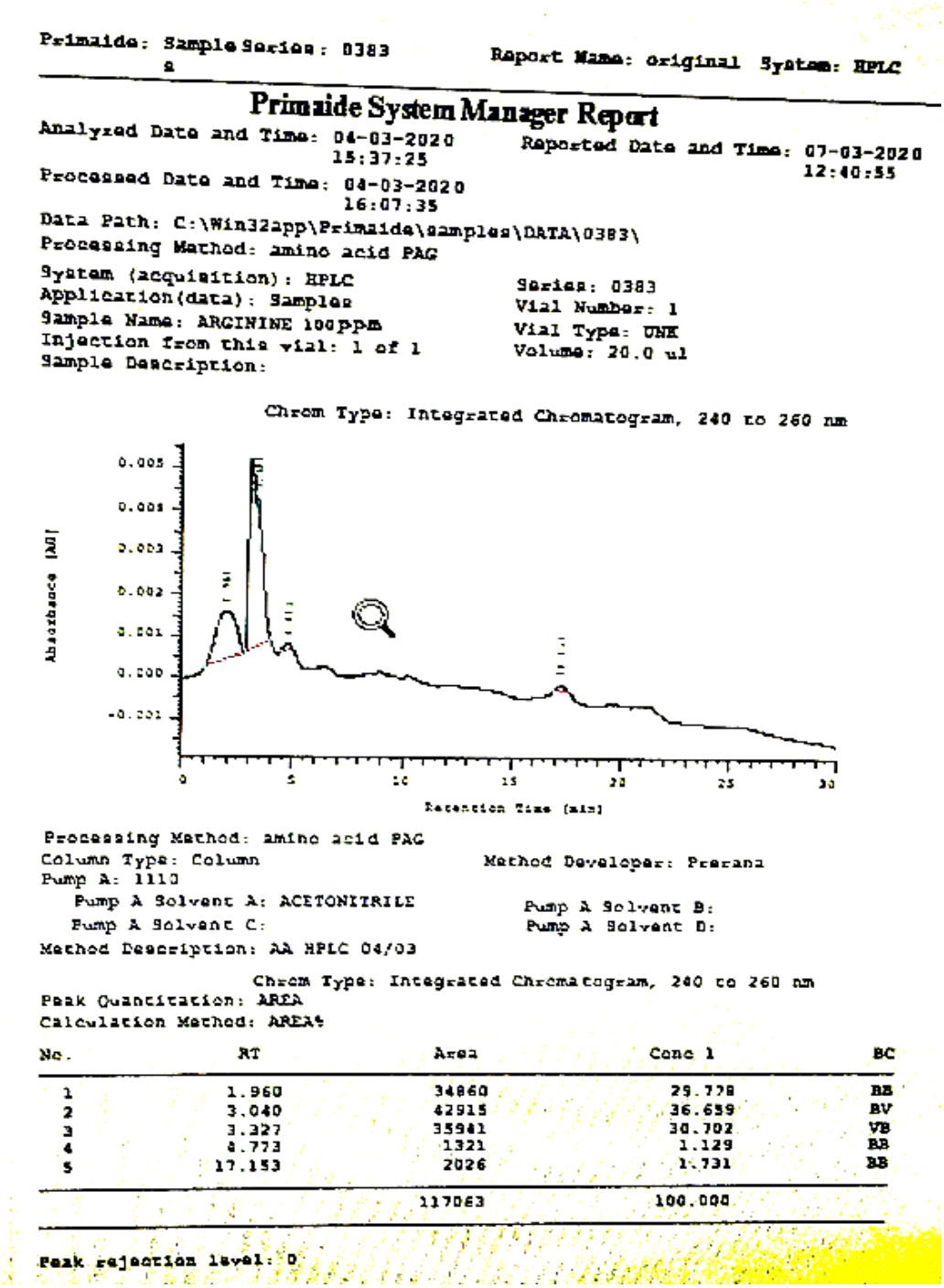
In the present result, for 100 ppm arginine, 20 µL of injection volume loaded into the system's loop, the retention time was 3.040 seconds and a highest peak was observed within the area of 42915 mAU (milli Absorbance Units). This peak was resultant to the 36.659% at 240-260 nm monitoring ultra-violet wavelength (Figure 1).

At the 200 ppm concentrated arginine, 240-350 nm of ultra violet radiation wavelength was set prior to analysis. Out of the total volume of 200 ppm arginine, 20 µL of injection volume loaded into the system's loop, the retention time was 2.487 seconds and a highest peak was observed within the area of 37186 mAU (milli Absorbance Units). This sharp peak was resultant to the 93.626% (Figure 2).

DISCUSSION

The complete analysis in RP-HPLC was carried out with the reverse phase (C18) column. Two different chromatographic modes were used to check their effects on profile of sweat metabolites i.e., C18 column and HILIC (Hydrophilic Interaction Liquid Chromatography). Results using C18 column were more accurate and gives good resolution of chromatographs than HILIC column were also analysed standard amino acids by using C18 column in HPLC (Calderón-Santiago et al. 2014; Delgado-Povedano *et al*. 2016). In HPLC (Hitachi Instrument (Dalian) Co. Ltd.) 20 µL of standard amino acid and sweat sample were injected. Isocratic solvent system of acetonitrile was used as a mobile phase. Analysis time for each sample and standard was 25 minutes. Flow rate for standard amino acid concentrations and samples was 1.0 ml/min. Prior to analysis samples were filtered through syringe filter of 0.22 µm. Ng *et al*. (2015) were analysed

underivatized amino acids in Perkin Elmer Altus™ HPLC system. For the study 0.45 µm filters were used and injected 5 µL of samples using solvent system A (acetonitrile, tetrahydrofuran, ammonium formate, formic acid), solvent system B (acetonitrile and ammonium formate) for the run time of 25 minutes, at the flow rate of 0.400 ml/min and detected using mass spectrometry. Amino acids in sweat by tandem mass spectroscopy were analyzed by Calderón-Santiago *et al*. (2014). But in present study target analytes were detected on in built ultra-violet radiations and no other specific mass detection was employed.



Primaide: Sample Series: 0383 Report Name: original System: HPLC
a

Primaide System Manager Report

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Processed Date and Time: 04-03-2020 16:07:35
Data Path: C:\Win32app\Primaide\samples\DATA\0383\
Processing Method: amino acid PAG
System (acquisition): HPLC Series: 0383
Application(data): Samples Vial Number: 1
Sample Name: ARGININE 100ppm Vial Type: UNK
Injection from this vial: 1 of 1 Volume: 20.0 ul
Sample Description:

Chrom Type: Integrated Chromatogram, 240 to 260 nm

Processing Method: amino acid PAG
Column Type: Column Method Developer: Prerana
Pump A: 1110
Pump A Solvent A: ACETONITRILE Pump A Solvent B:
Pump A Solvent C: Pump A Solvent D:
Method Description: AA HPLC 04/03

Chrom Type: Integrated Chromatogram, 240 to 260 nm
Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Conc 1	BC
1	1.960	34860	29.778	BB
2	3.040	42915	36.659	BV
3	3.327	35941	30.702	VB
4	8.773	1321	1.129	BB
5	17.153	2026	1.731	BB
		117063	100.000	

Peak rejection level: 0

Figure 1: Standard L-Arginine monohydrochloride 100 ppm

Primaide: Sample Series: 0384 Report Name: original System: HPLC
a

Primaide System Manager Report

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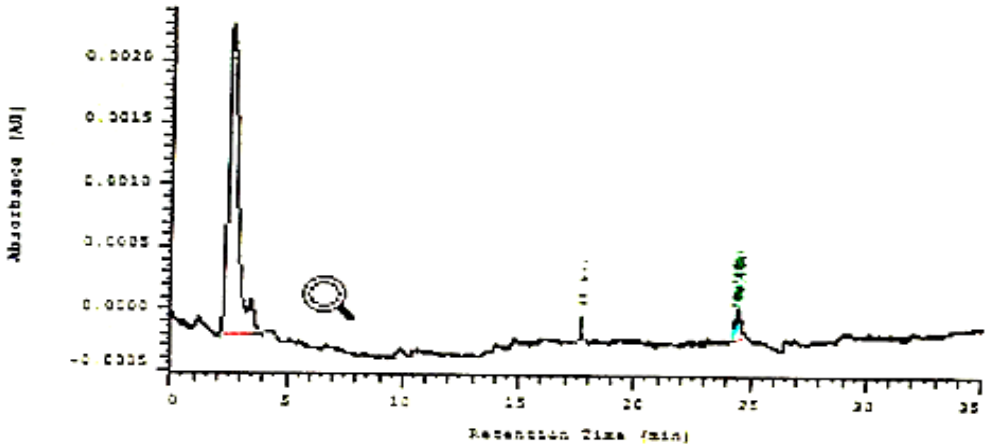
Processed Date and Time: 04-03-2020 17:16:29
Data Path: C:\Win32app\Primaide\samples\DATA\0384\
Processing Method: amino acid PAC

System (acquisition): HPLC Series: 0384
Application(data): Samples Vial Number: 1
Sample Name: ARGININE 200ppm Vial Type: UNK
Injection from this vial: 1 of 1 Volume: 20.0 ul
Sample Description:

Chrom Type: Integrated Chromatogram, 240 to 350 nm



Processing Method: amino acid PAC
Column Type: Column Method Developer: Prerana
Pump A: 1110
Pump A Solvent A: ACETONITRILE Pump A Solvent B:
Pump A Solvent C: Pump A Solvent D:
Method Description: AA HPLC 04/03

Chrom Type: Integrated Chromatogram, 240 to 350 nm
Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Conc 1	BC
1	2.487	37186	93.626	BB
2	17.632	345	0.880	BB
3	24.200	354	0.894	BB
4	24.287	451	1.138	BB
5	24.373	467	1.178	BV
6	24.460	907	2.285	VB
		39714	100.000	

Peak rejection level: 0

Figure 2: Standard L-Arginine monohydrochloride 200 ppm

Immediately after initialising the system, the separation column needs to be purged for 10-15 minutes at the flow rate of 3 ml/min. Himedia standard amino acids kit was used for optimization of all standard amino acids. A working solution of standard amino acids was made in HPLC grade water and was stored at 4°C while Delgado-Povedano *et al.* (2016) used a multi-standard solution of amino acids and made working solution in 0.1 M formic acid and was stored at -20 °C.

The retention time of standard amino acids, arginine at 100 ppm was 3.040 minutes and at 200 ppm was 2.487 minutes which nearly resembles to the retention time (3.1 minutes) observed by Delgado-Povedano *et al.* (2016), in study of standard arginine.

Although no complex methodologies for sample preparation were used in the present study, our findings resembled to the experiments performed by Calderón-Santiago *et al.* (2014) and Delgado-Povedano *et al*. (2016). Calderón-Santiago et al. (2014) used three different sample preparation strategies; sample dilution, hydrolysis and its clean up. Dilution to the sample was done to get representative components from sweat. Followed by this, clean up method reduces unnecessary materials and impurities and increases the sensitivity. Delgado-Povedano et al. (2016) solely carried out for experimenting a sweat sample preparation method for HPLC analysis. It had adopted two sample preparation ways; serial dilution of sweat sample and centrifugal micro solvent phase extraction(c-µSPE) method. The dilution method reduced the matrix effects and the c-µSPE is good for less amount of sample.

CONCLUSION

The Reverse Phase High Performance Liquid Chromatography (RP- HPLC) is an analytical technique used for the study for its specificity and sensitivity. The RP- HPLC instrument has isocratic pump system with the detection unit of ultra-violet radiations. By using very simple sample preparation methodology for standard amino acid L-Arginine monohydrochloride of 100 ppm and 200 ppm concentrations showed sharp peaks at retention time of 3.040 and 2.487 minutes covering the area of 42915 and 37186 mAU on absorbing 36.659% and 93.626% of arginine concentrations respectively. The present study is very useful for detection of arginine in standard amino acids and comparative study in other bodily fluids which helps in forensic investigations.

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