

ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC) METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF L-ALANYL-L-GLUTAMINE IN PHARMACEUTICAL DOSAGE FORM (INFUSIONS).

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ABSTRACT

A simple, precise, accurate and validated reverse phase UPLC method has been developed for the estimation of L-Alanyl L-Glutamine (20% w/v) in infusion. The quantification was carried out using amino-bonded silica gel column, packed with octadecylsilane (2.1 mm × 100 mm, 1.7 μ m) and the mobile phase used was a mixture of acetonitrile and 0.05 M phosphate buffer, pH 4, (70:30) at a flow rate of 0.25 ml / min. The detection wavelength was 215 nm and column temperature was 40°C. The retention time was found to be 0.77 min. The results obtained showed a good agreement with the declared content. Recovery values for L-Alanyl -L-Glutamine were 99.19 - 100.82 %. The proposed method is reliable, rapid, precise, selective and may be used for the quantitative analysis of L-Alanyl -L-Glutamine in infusions.

Key words: L-Alanyl L-Glutamine, UPLC, method development, validation.

1. INTRODUCTION

Glutamine is the most abundant free amino acid in the extracellular and intracellular compartments, contributing to more than 50% of the body's free amino acid pool(Young VR et al.2001). Glutamine is involved in a wide variety of metabolic and synthetic biochemical processes and supports rapidly proliferating cells, such as lymphocytes and enterocytes (Souba WW.1997). and acts as nitrogen and ammonium carrier to the liver and kidney. In conditions of excessive organ/tissue demand of Glutamine during episodes of sepsis following trauma, major surgery, and other catabolic stress situations, endogenous Glutamine

production may not be sufficient to meet the increased requirements(Fust P and Stehle P, 2004), and up to 50% of Glutamine depletion might be observed (Planas M et al.1993; Houdijk AP et al.1998). In addition, Glutamine depletion has been associated with poor prognosis in hypercatabolic situations such as burn injury (Parry-Billings M et al. 1990). Glutamine limits intestinal permeability in experimental models in animals (Gianotti L et al. 1995) and reduces gut atrophy (Souba WW et al. 1990; O'Dwyer ST et al. 1989). It also preserves intestinal and extraintestinal immunoglobulin A levels (Kudsk KA et al. 2000) and decreases

intestinal proinflammatory cytokine production(Ameho CK et al. 1997). In humans, Glutamine supplementation reduced clinical infections after bone-marrow transplantation(Ziegler TR et al. 1992) and maintained gut integrity during total parenteral nutrition (TPN)(van der Hulst RR et al. 1993).

UPLC refers to Ultra Performance Liquid Chromatography. It improve in three areas: chromatographic resolution, speed and sensitivity analysis. It uses fine particles and saves time and reduces solvent consumption.(Jerkovich AD et al. 2003; Wu N et al. 2001; Unger KK et al. 2000; Swartz ME 2004).

UPLC comes from HPLC. HPLC has been the evolution of the packing materials used to effect the separation. An underlying principle of HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than $2.5\mu\text{m}$, there is a significant gain in efficiency and it's doesn't diminish at increased linear velocities or flow rates according to the common Van Deemter equation(Van Deemter et al. 1956) . By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which is known as Ultra Performance.

The classic separation method is of HPLC (High Performance Liquid Chromatography) with many advantages like robustness, ease of use, good selectivity and adjustable sensitivity. It's main limitation is the lack of efficiency compared to gas chromatography or the capillary electrophoresis(Zhang YH et al. 2000; Zhou C et al. 2005) due to low diffusion coefficients in liquid phase, involving slow diffusion of analytes in the stationary phase. The Van Deemter equation shows that efficiency increases with the use of smaller size particles but this leads to a rapid increase in back pressure, while most of the HPLC system can operate only up to 400 bar. That is why short columns filled with particles of about $2\mu\text{m}$ are used with these systems, to accelerate the analysis

without loss of efficiency, while maintaining an acceptable loss of load.

To improve the efficiency of HPLC separations, the following can be done :-

- a. work at higher temperatures
- b. use of monolithic columns

1.1 Use of the UPLC system

Elevated-temperature chromatography also allows for high flow rates by lowering the viscosity of the mobile phase, which significantly reduces the column backpressure(Zhu J et al. 2005; Greibrokk T et al. 2003). Monolithic columns contain a polymerized porous support structure that provide lower flow resistances than conventional particle-packed columns(Gerber F et al. 2004; Tanaka N et al. 2001; Wu N et al. 2004).

No methods have been reported in the literature for the determination of drug on UPLC.

The goal of this study was to develop a rapid, more accurate, precisely reliable , less expensive and least time UPLC method for the analysis of L-Alanyl L-Glutamine in infusions.

2. MATERIALS AND METHODS

All the chemicals and reagents used were of AR/HPLC grade were from Merck . Pure standard and samples of L-Alanyl L-Glutamine were obtained from Kyuwa Hakko Bio Ltd, Singapore. The purities of this standard was 99.92 %.

A reverse phase UPLC system (WATERS, ACQUITY UPLC), consisting of Binary Solvent Manager along with TUV detector and Sample Manager, having Empower2 software was used for analysis. UPLC Amino Acid Column, AccQ-Tag Ultra consisted of Amino-Bonded silica gel, packed with octadecylsilane (2.1 mm X 100 mm), $1.7\mu\text{m}$ was used for analysis.

2.2 Preparation of Mobile Phase

A degassed mixture of 0.05 M Potassium Dihydrogen *o*-Phosphate and Acetonitrile in the ratio of 30:70 (v/v) was prepared and the pH was

adjusted to 4.0 with *o*-phosphoric acid/tri ethyl amine. The mixture was filtered through 0.22 μ membrane filters and was degassed.

2.3 Standard Preparation

A 40.0 mg of L-Alanyl L-Glutamine working Standard was weighed and transferred accurately into a 100 ml clean and dry volumetric flasks. It was further diluted to volume with HPLC grade water. Resulting into final concentration of 400 ppm

2.4 Sample Preparation

A 1 ml of sample solution from L-Alanyl L-Glutamine infusion (20% w/v) was taken and transferred accurately to 50 ml clean and dry volumetric flask. It was diluted to volume with HPLC grade water. From this further dilution was prepared to get the final concentration of 400 ppm. The solution was filtered through 0.22 μ membrane filters and it was degassed.

2.5 Chromatographic Conditions

Freshly prepared Buffer and Acetonitrile 30:70 (v/v) mobile phase and adjust pH to 4, were filtered through 0.22 μ membrane filter and sonicated before use.

Flow rate of Mobile phase was maintained at 0.25 ml/min. The column temperature was maintained at $40^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. The detection was carried out at 215 nm, Injection volume was 0.4 μ l and total run time was 3 min. Column used was AccQ-Tag Ultra consisted of Amino-Bonded silica gel, packed with octadecylsilane (2.1 mm X 100 mm), 1.7 μ m.

2.6 Assay Procedure

A 0.4 μ l of placebo, standard preparation (6 times) and sample preparation (3 times) were separately injected into the chromatographic systems. Then the chromatograms and the peak responses were measured. The placebo chromatogram was examined for any extraneous peaks that were observed in the chromatograms of sample and standard preparations. Chromatogram of the standard preparation was recorded and the peak responses were measured. The tailing factor for the principal peak should not be more than 4.0 and the

number of the theoretical plates should not be less than 5000. The % RSD (Relative Standard Deviation) should not be more than 2.0.

A 0.4 μ l of standard preparation and assay preparation was separately injected in the chromatogram, the chromatograms were recorded and the responses for the major peaks were measured.

3. RESULT AND DISCUSSION

Chemical structure and chemical properties are the most important facts that predict chromatographic behavior. In the present investigation the best resolution

was achieved using a AccQ-Tag Ultra consisted of Amino-Bonded silica gel, packed with octadecylsilane (2.1 mm X 100 mm), 1.7 μ m and mobile phase Buffer and Acetonitrile 30:70 (v/v). The lower percentage of acetonitrile in mobile phase resulted in peak broadening of the component and long analysis duration, while higher percentage of acetonitrile in mobile phase resulted peak splitting of L-Alanyl L-Glutamine peak. Optimal retention time, 0.77 min minutes L-Alanyl L-Glutamine was achieved when the pH of mobile phase was adjusted to 4 with 85 % phosphoric acid. Small changes in pH of the mobile phase had a great influence to the chromatographic behavior of these substances. Higher pH of the mobile phase resulted in peak tailing, and lower pH resulted in broadening of peak.

3.1 Accuracy and precision

The accuracy of the method was evaluated by analyzing prepared solutions of L-Alanyl L-Glutamine. The precision of the method was investigated with respect to repeatability. Calibration graphs of the injected concentration of L-Alanyl L-Glutamine, against recovered concentration of L-Alanyl L-Glutamine showed a straight line, with a recovery of $99.19\text{--}100.82 \pm 0.61$ % of drug. The recovery data is expressed in tables 1-3. These tables show that the method is accurate for determination of L-Alanyl L-Glutamine. All calibration curves have a correlation coefficient of at least 0.9999.

Table 1
Recovery of L-Alanyl L-Glutamine by proposed method

Injected (ppm)	Reference Drug			Infusion		
	Found	% Recov.	Mean	Found	% Recov.	Mean
0.5	0.4953	99.0584	24271	0.4972	99.4344	22377
1	0.9876	98.6738	48354	0.9945	99.4505	44762
1.5	1.4981	99.8702	73411	1.5123	100.823	68070
2	1.9949	99.7456	97759	2.0052	100.2617	90255
2.5	2.4846	99.3841	121756	2.48	99.1989	111623
3	3	100	147013	3	100	135068

Table 2
Interday accuracy and precision of L-Alanyl L-Glutamine by proposed method

Injected (ppm)	Day 1		Day 2		Day 3		Day 4	
	RSD	%Recov.	RSD	%Recov.	RSD	%Recov.	RSD	%Recov.
0.5	0.0045	99.06	0.0032	99.43	0.0014	99.57	0.0049	100.12
1	0.0076	98.67	0.0009	99.45	0.0013	99.23	0.0016	100.26
1.5	0.0006	99.87	0.0007	100.82	0.0013	100.30	0.0011	99.91
2	0.0006	99.75	0.0004	100.26	0.0008	100.52	0.0004	99.96
2.5	0.0073	99.38	0.0008	99.20	0.003	99.42	0.0042	99.67
3	0.0008	100	0.0021	100	0.0036	100	0.0015	100

For intra-day precision, six concentration of each compound were analyzed on the same day. Each concentration of sample was injected 4 times a day. Table 3 summarizes the relative standard deviation (RSD). Generally acceptable repeatability of the results within one day and day-to-day was

observed. Data of the relative retention times obtained in a series of four consecutive injections also showed acceptable repeatability when analyzed not only on the same day but also on four consecutive days.

Table 3
Recovery and regression characteristics of L-Alanyl L-Glutamine by proposed method

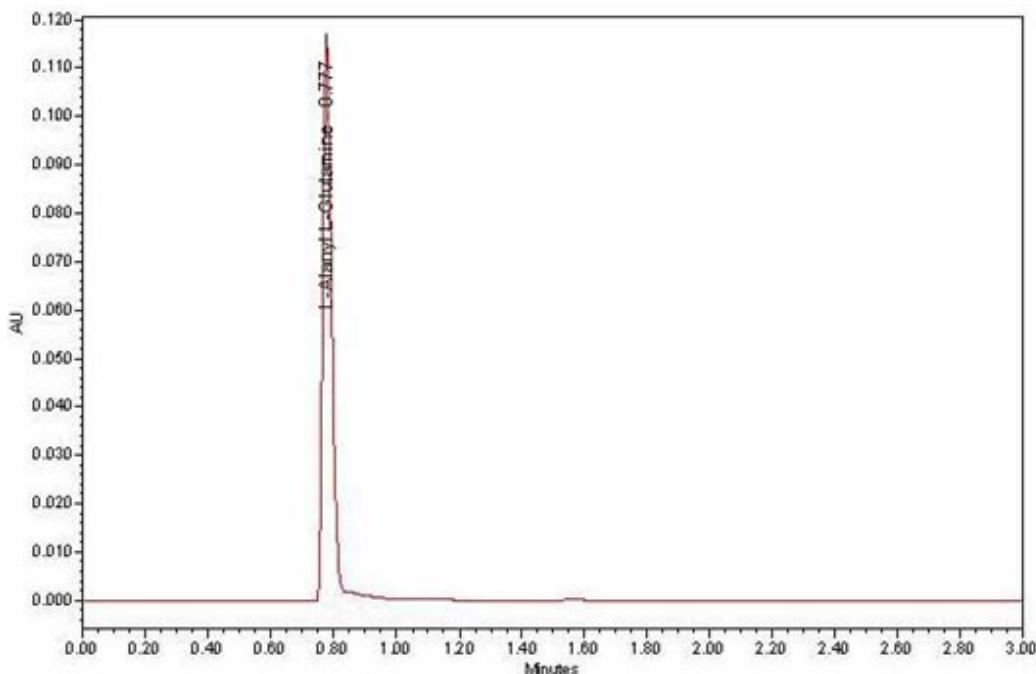
Conc. injected (ppm)	Recovered concentration			
	Day 1	Day 2	Day 3	Day 4
0.5	0.4953	0.4972	0.4979	0.5006
1	0.9867	0.9945	0.9923	1.0026
1.5	1.4981	1.5123	1.5045	1.4986
2	1.9949	2.0052	2.0105	1.9993
2.5	2.4846	2.48	2.4856	2.4918
3	3	3	3	3
Correlation Coefficient (R)	0.9999	0.9999	0.9999	0.9999

3.2 System suitability and specificity

System suitability of the method was evaluated by analyzing the symmetry L-Alanyl L-Glutamine, peaks, theoretical plates of the column.

A typical chromatogram of L-Alanyl L-Glutamine is shown in figure 1.

Figure 1



3.3 Ruggedness.

Ruggedness of this method was evaluated on two different instruments of the same make in two different QC laboratories at Nirlife Healthcare Division, Nirma Ltd.

4. CONCLUSION

The proposed method was found to be simple, specific and highly accurate, required less

time consumption for analysis and this can be employed for the routine analysis.

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