RP-HPLC METHOD DEVELOPMENT FOR THE ESTIMATION OF L-ASPARAGINASE FOR INJECTION 10000 IU

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Abstract:

L-asparaginase is an enzyme used primarily in the treatment of certain types of cancer, particularly acute lymphoblastic leukemia (ALL). The primary aim of this study is to develop a new method for the quantification of L-Asparaginase for injection 10000IU using Reverse phase-High Performance Liquid Chromatography (RP-HPLC) after an extensive literature survey. Method was achieved with Welchrom XB-C18, 250 x 4.6mm 5□m column with run time 15 min at a flow rate 0.6ml/min. Retention time was obtained at 2.782 min. Detector wavelength was set at 225nm. The estimated percent of L- Asparaginase in its marketed formulation was found to be 101.67%. Consequently, the author aimed to develop a robust assay method capable of quantifying the drugs in their commercial pharmaceutical dosage form. This method is designed to be simple, specific, precise, and economical, featuring a shorter run time that makes it ideal for routine quality control applications.

Key words: L-Asparaginase, Oncoginase for Injection 10000 IU, RP-HPLC, & Acute lymphoblastic leukemia.

1. Introduction

L-asparaginase is an enzyme used primarily in the treatment of certain types of cancer, particularly acute lymphoblastic leukemia (ALL). It works by breaking down asparagine, an amino acid that some cancer cells rely on for growth. By depleting asparagine levels, L-asparaginase can inhibit the growth of these cancer cells. L-asparaginase is usually administered as an injection, either intramuscularly or intravenously. It is freely soluble in water with several hours to 3-4 days half life. IUPAC name is E-coli L-Asparaginase amidohydrolase and chemical formula is $C_{1377}H_{2208}N_{382}O_{442}S_{17}$. It is mainly used in

combination with other chemotherapy agents for treating ALL and sometimes for other malignancies. Common side effects may include allergic reactions, pancreatitis, liver function abnormalities, and effects on blood clotting. Patients may also experience nausea, vomiting, or changes in blood sugar levels [1-23].

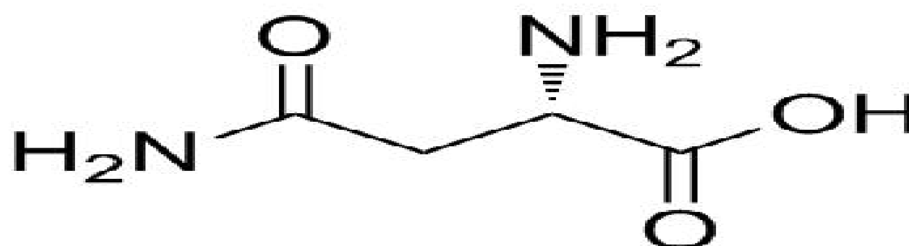

Figure 1. Chemical structure of L-Asparaginase

This Exhaustive literature review [24-37] examines the physicochemical properties of L- Asparaginase and discusses various analytical methodologies used for its quantification. This foundational work supports the validation [38] of a novel reverse-phase high-performance liquid chromatography (RP-HPLC) method for quantifying L-Asparaginase

2. Experimental part:

2.1 Chemicals, Reagents and Solvents:

Table 1. Chemicals, Reagents & Standards

Chemicals & Solvents Standards	**Make**	**Grade**	**Batch No.**	**Assay**
Triethyl amine	Sigma Aldrich	HPLC	614381027LU	99.5%
Acetonitrile	Merck	HPLC	A24B025	≥99.90%
Orthophosphoric acid	Merck	AR	622940712GW	85% w/w
0.9% NaCl	Merck	AR	8489801717	≥99.98%
L-Asparaginase Working Standards	Sigma Aldrich	HPLC	WS-O63	98%
L-Asparaginase injection 10000IU	Chandra Bhagat Pharma	HIS	LAI2401B	-

2.2 Analytical Instrumentation & Equipments:

Table 2. Analytical Instrumentation & Equipments

S.No	Instrument	Make, Model & Details	Identification No.
1.	HPLC	Waters e2695/Alliance Series	ARD/LC/2021009
2.	HPLC-Column	Welchrom XB-C18, 250 x 4.6mm 5□m	CLL/CCC/64/23
3.	Analytical Balance	Sartorius	ARD/BAL/201806
4.	pH Meter	Thermo Scientific:ORIONSTAR A215	ARD/PHM/202103
5.	Sonicator	PCi Ultrasonic bath chiller Model	ARD/SC/2021005
6.	Shakers and Mixers	Thermo Scientific - MaxQ™ 4450	ARD/MIX/TFS/09

Chromatographic Conditions:

Table 3. Chromatographic Conditions

Column	Welchrom XB-C18, 250x4.6 mm, 5 □m
Flow rate	0.6ml/min
Wavelength (λmax)	225 nm
Run time	15 minutes
Column Temp. & Sample Temp.	25°C & 5°C
Injection Volume	20□L
Elution	Isocratic
Diluent	Buffer:Acetonitrile 70:30

2.3 Sample, Standards & Solutions Preparations 10% (v/v) diluted Orthophosphoric acid:

5.9 mL of Orthophosphoric acid was taken in a volumetric flask of 50mL and made till the mark with water.

Mobile phase A (Buffer): 2 mL of Triethylamine was taken in 2000mL of water and mixed well. The pH was adjusted to 7.02 with 10% orthophosphoric acid. The mobile phase was filtered with 0.45μ filter & sonicated for 5 minutes.

Mobile Phase B: Acetonitrile and water was made in 95:5 ratio.

Diluent: Mobile phase A & Acetonitrile was mixed in 70:30 ratio.

Solutions:

i) Standard Stock Solution-1: Weighed accurately 60.47 mg of L-Asparaginase standard and transferred into volumetric flask of 100mL. 50ml of buffer was added further and shaken vigorously, then 5 mL of Acetonitrile was added and swirled to dissolve. 20 mL of Buffer was added along with 5 mL of acetonitrile again and made upto the mark with buffer.

ii) Standard-1: From the above standard stock solution-1, pipetted out 3 mL and transferred into 50 mL of volumetric flask & made till the volume with the diluent.

iii) Standard stock-2: 60.52mg of L-Asparaginase was weighed accurately & transferred to volumetric flask of 100ml. Then 50 ml of buffer and added 5 mL of Acetonitrile was added & shaken vigorously. The solution was swirled to dissolve and added 20 ml of Buffer and 5 mL of Acetonitrile again and made till the volume with buffer.

iv) Standard-2: From the above standard stock solution-2, pipetted out 3 ml and transferred into volumetric flask of 50ml. The final volume was made up with diluent.

v) Sample stock-1: Two vials were flipped off the seal and reconstituted each vial with 5 mL of readymade 0.9% Sodium chloride saline solution. The vial was shaken vigorously and transferred the solution carefully into 100 ml volumetric flask. The procedure was repeated for three times with buffer, it was ensured that the vials are clear and added 50 mL of buffer further and shaken vigorously. To this, 10 mL of Acetonitrile was added and volume was made to final with the buffer.

vi) Sample solution-1: From the above Sample Stock Solution-1, 3mL was pipetted and transferred into volumetric flask of 100 mL. The final volume was made with the diluents.

vii) Sample stock-2: 2 vials were taken & flipped off the seal, and reconstituted with 5mL of readymade 0.9% Sodium chloride saline solution. Then they were shaken vigorously and transferred the solution carefully into 100 ml volumetric flask. The procedure was repeated for three times with buffer, it was ensured that the vials were clear and then, 50 mL of buffer was added and shaken vigorously.10 mL of Acetonitrile was added to it and the final volume was made up with buffer.

viii) Sample solution-2: From the above Sample Stock Solution-2, 3mL was pipette into 100ml volumetric flask and the volume was made upto mark with the diluent.

3. Results and Discussion

3.1 Data summary of specificity & system suitability

Table 4. Specificity & System Suitability Summary Data

<table>
<tr><th colspan="2">Specificity & System suitability</th><th>Results</th><th>Acceptance criteria</th></tr>
<tr><td colspan="4">Specificity</td></tr>
<tr><td>Parameter</td><td colspan="2">Standard Solution</td><td rowspan="4">RT obtained with the sample solution should be comparable with the standard solution.</td></tr>
<tr><td rowspan="3">Identification & Retention Time (RT) Conformation.</td><td>L-Asparaginase1 & 2</td><td>2.752 & 2.735</td></tr>
<tr><td colspan="2">Sample Solution</td></tr>
<tr><td>L-Asparaginase for inj 10000IU</td><td>2.731</td></tr>
<tr><td colspan="2">Peak purity index</td><td>Single point threshold</td><td rowspan="3">Peak purity should pass. Peak purity index should be greater than single point threshold.</td></tr>
<tr><td>Standard Solution Peak Purity</td><td>1.0000</td><td>0.9998</td></tr>
<tr><td>Sample Solution</td><td></td><td></td></tr>
</table>

Peak Purity	0.99999	0.99998	
Blank andPlacebo Solution. Interference	L-Asparaginase	NIL	Blank and placebo solution must not elute at the retention time of the analyte peak.
System suitability			
% RSD of standard solution 1 & 2 respectively		0.349 & 0.733	% RSD should be NMT 2.0%
% RSD of the L-ASPase sample solution		0.149	% RSD for RT should be NMT1.0%
The Number of theoretical plates for main peak in L-ASPase standard solutions 1 & 2		1423 & 1194	The theoretical plates for main peak in standard solution should be NLT 1000
The tailing factor for main peak in L-ASP standard solution 1& 2		1.197 & 1.293	The tailing factor for main peak in standard solution should be NMT 0.8 and 2.0

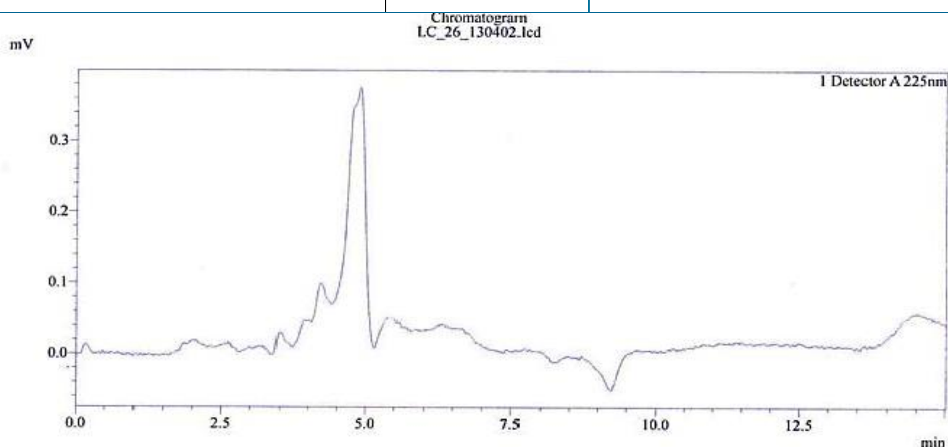

Figure 2. Chromatogram for Blank

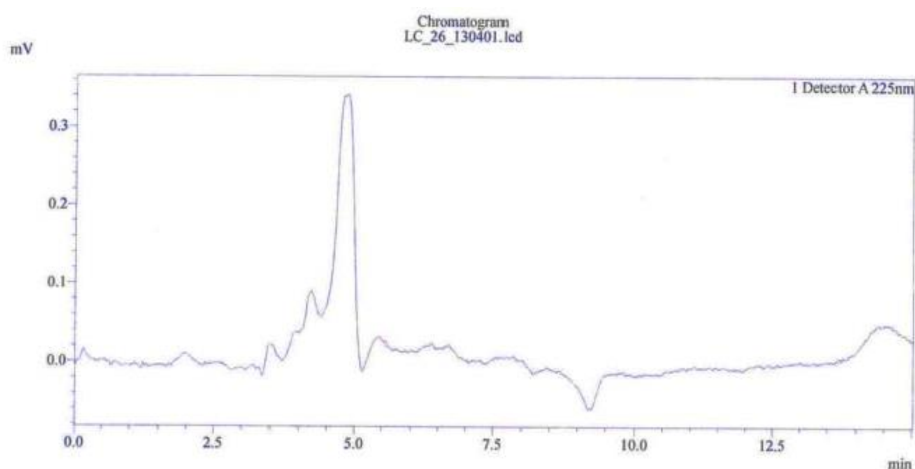

Figure 3. Chromatogram for Placebo

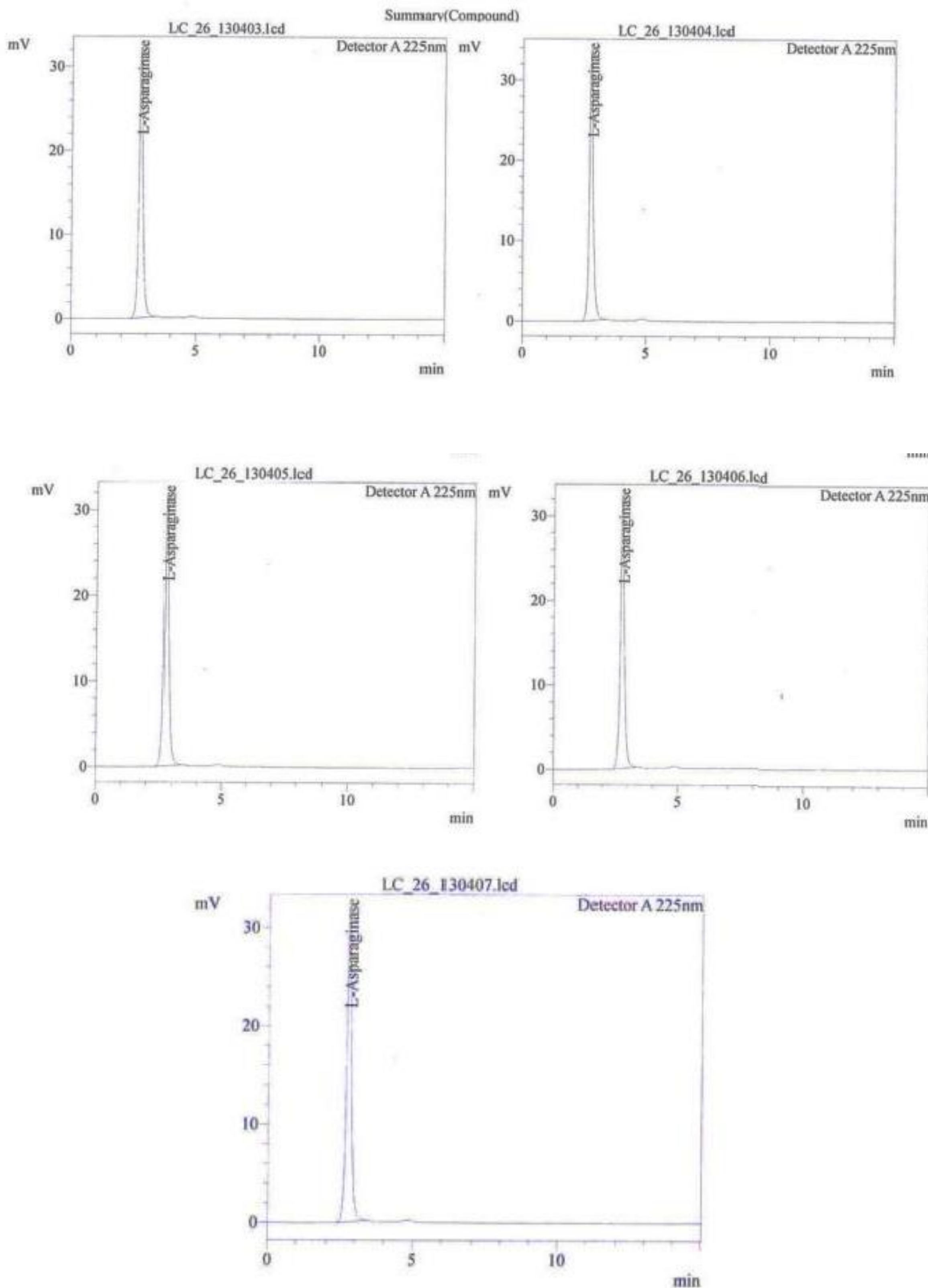

Figure 4. Chromatograms for standard solution-1

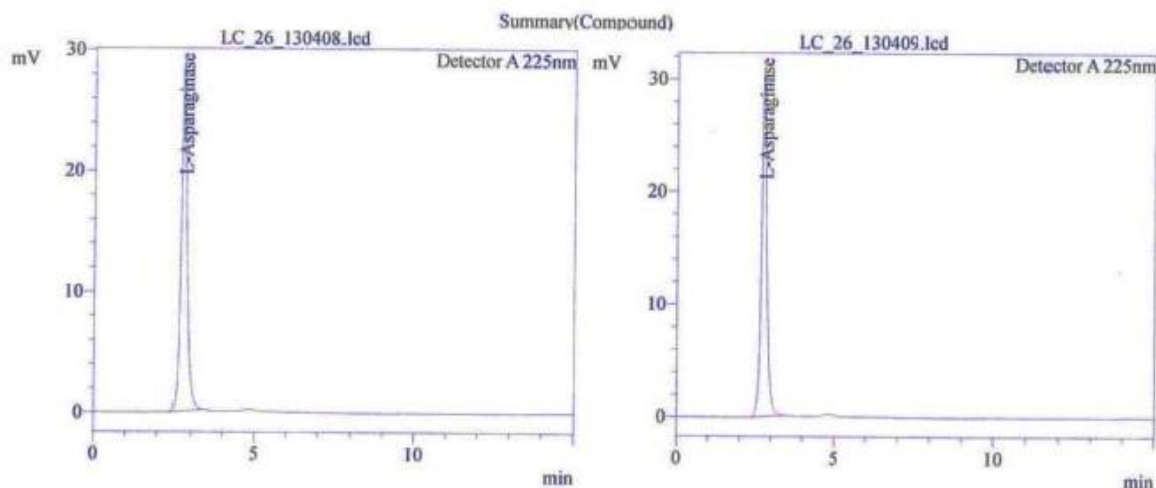

Figure 5. Chromatograms for standard solution-2

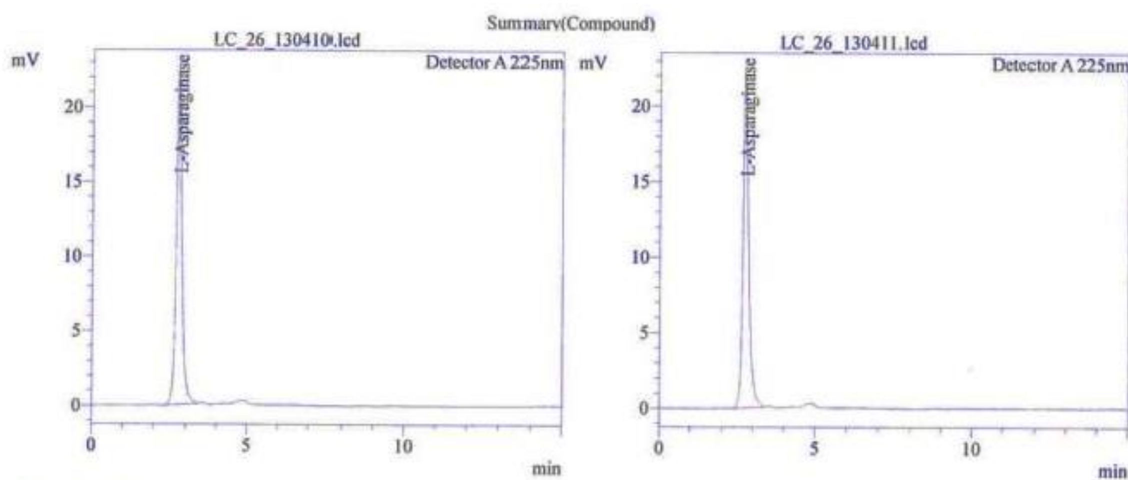

Figure 6. Sample Solutions 1&2 chromatograms

Table 4. System Suitability of Standard Solution-1

Solutions	RT	Area	Tailing factor	Theoretical plates
Standard solution injection-1	2.748	366860	1.208	1387
Standard solution injection-2	2.754	368024	1.213	1549
Standard solution injection-3	2.753	368874	1.189	1362
Standard solution injection-4	2.753	365676	1.186	1417
Standard solution injection-5	2.750	368331	1.187	1402
Mean	2.752	367553	1.197	1423
Standard deviation	0.003	1282	0.013	73
% Relative Standard deviation	0.091	0.349	1.065	5.143
Bracketing Standard solution	2.738	371988	1.297	1171

Table 5. System Suitability of Standard Solution-2

Solutions	RT	Area	Tailing factor	Theoretical plates
Standard solution injection-1	2.727	372349	1.362	1090
Standard solution injection-2	2.744	368507	1.224	1298
Mean	2.735	370428	1.293	1194
Standard deviation	0.012	2716	0.098	147
% Relative Standard deviation	0.438	0.733	7.587	12.312

Table 6. L-Asparaginase Sample Solution Specificity

Solutions	RT	Area	Tailing factor	Theoretical plates
Sample solution injection-1	2.734	285870	1.334	1160
Sample solution injection-2	2.729	286475	1.460	1060
Mean	2.731	286173	1.397	1110
Standard deviation	0.003	427	0.089	70
% Relative Standard deviation	0.127	0.149	6.392	6.326

3.2 Precision

Precision describes the closeness between serial measurements that were obtained from multiple sampling conditions from the homogeneous sample.

3.2.1 System precision:

System precision describes the instrument performance under the prescribed chromatographic parameters. System precision studies were carried out by single preparation of standard solution and injecting the sample under the same conditions with six determinations.

3.2.2 Method Precision:

Method Precision studies were carried out with test solution of six preparations andinjecting the sample under the same chromatographic conditions.

Table 7. Precision summary of L-Asparaginase

System precision	Results	Acceptance limits
% RSD of standard solution (6 replicates)	0.582	% RSD of the standard for six replicate injections should be NMT 2.0%
No. of theoretical plates for main peak in standard solution	1381	Theoritical plates should be NLT 1000
Tailing factor	1.213	The tailing factor for main peak in standard solution should be NMT 0.8 and 2.0
Method precision		
Calculated %RSD for % Assay content L-ASPase from six sample preparations	1.468	%RSD should be NMT 2.0 with all the individual values within limit.

4. Proposed procedures for marketed Pharmaceutical Formulation:

The Marketed Formulation (**Oncoginase 10000IU**) was analysed separately by injecting the standard & sample solutions of 10µ L volume into the HPLC. The quantity of the drug found in the formulation was calculated by comparison of the peak area of the standard and sample.

Table 8. Results of Assay in dosage form

Formulation	Batch No	Label claim(mg)	% Assay*	Estimated amount
ginase injection10000IU	LSI2406CB	Oncoginase 10000IU	101.67	10167.37 IU

*= Average assay % of 3 replicate injections of individual batch.

Conclusion

The precision results reflected minimal variability, underscoring the method's reliability for routine analysis. Key parameters such as specificity and precision were within acceptable limits, confirming the method's efficacy in quantifying L-Asparaginase accurately. Overall, the HPLC method provided a comprehensive analytical tool for the quality control of Oncoginase for injection 10000 IU ensuring that pharmaceutical products meet regulatory standards.

Conflict of Interest

Authors declared that there was no conflict of interest.

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References

1. Gaynon P, Schrappe M. Childhood ALL. In: Cairo M, Perkins S, editors. Hematological Malignancies in Children, Adolescents and Young Adults. Hackensack, NJ: World Scientific Publishing Company; 2012. pp. 197–235.
2. Howlader N, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF, et al., editors. SEER Cancer Statistics Review, 1975-2011. Bethesda, MD: National Cancer Institute; 2014. National Cancer Institute. Section 28: Childhood cancer by site, incidence, survival, and mortality.
3. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. N Engl J Med. 2006;354:166–78. doi: 10.1056/NEJMra052603.
4. Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. Lancet. 2008;371:1030–43. doi: 10.1016/S0140-6736(08)60457-2.

5. Kidd JG. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. J Exp Med. 1953;98:565–82. doi: 10.1084/jem.98.6.565.
6. Broome JD. Evidence that L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. Nature. 1961;191:1114–5. doi: 10.1084/jem.118.1.99.
7. Broome JD. Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. I. Properties of the L-asparaginase of guinea pig serum in relation to those of the antilymphoma substance. J Exp Med. 1963;118:99–120. doi: 10.1084/jem.118.1.99.
8. Broome JD. Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. II. Lymphoma 6C3HED cells cultured in a medium devoid of L-asparagine lose their susceptibility to the effects of guinea pig serum in vivo. J Exp Med. 1963;118:121–48. doi: 10.1084/jem.118.1.121.
9. Ho DH, Whitecar JP, Jr, Luce JK, Frei E., 3rd L-asparagine requirement and the effect of L-asparaginase on the normal and leukemic human bone marrow. Cancer Res. 1970;30:466–72.
10. Onuma T, Waligunda J, Holland JF. Amino acid requirements in vitro of human leukemic cells. Cancer Res. 1971;31:1640–4.
11. Sobin LH, Kidd JG. A metabolic difference between two lines of lymphoma 6C3hed cells in relation to asparagine. Proc Soc Exp Biol Med. 1965;119:325–7. doi: 10.3181/00379727-119-30169.
12. Wade HE, Elsworth R, Herbert D, Keppie J, Sargeant K. A new L-asparaginase with antitumour activity? Lancet. 1968;2:776–7. doi: 10.1016/s0140-6736(68)90977-x.
13. Schwartz JH, Reeves JY, Broome JD. Two L-asparaginases from E. coli and their action against tumors. Proc Natl Acad Sci U S A. 1966;56:1516–9. doi: 10.1073/pnas.56.5.1516.
14. Boyse EA, Old LJ, Campbell HA, Mashburn LT. Suppression of murine leukemias by L-asparaginase. Incidence of sensitivity among leukemias of various types: Comparative inhibitory activities of guinea pig serum L-asparaginase and Escherichia coli L-asparaginase. J Exp Med. 1967;125:17–31. doi: 10.1084/jem.125.1.17.
15. Keding R. Discontinuation of Elspar®, (asparaginase for injection) 10,000 IU. Deerfield, IL: Lundbeck LLC; 2012. [Last accessed on 2015 Jul 30]. Available from: http://www.fda.gov/downloads/Drugs/DrugSafety/DrugShortages/UCM321556.pdf
16. Prescribing information. Deerfield, IL: Baxalta, Incorporated (formerly Gaithersburg, MD: Sigma Tau Pharmaceuticals, Inc.); 2015. Oncaspar® (pegaspargase).
17. Prescribing Information. Palo Alto, CA: Jazz Pharmaceuticals; 2014. Erwinaze® (asparaginase Erwinia chrysanthemi).
18. Dolowy WC, Henson D, Cornet J, Sellin H. Toxic and antineoplastic effects of L-asparaginase. Study of mice with lymphoma and normal monkeys and report on a child with leukemia. Cancer. 1966;19:1813–9. doi: 10.1002/1097-0142(196612)19:12<1813::aid-cncr2820191208>3.0.co;2-e.
19. Hill JM, Roberts J, Loeb E, Khan A, MacLellan A, Hill RW. L-asparaginase therapy for leukemia and other malignant neoplasms. Remission in human leukemia. JAMA. 1967;202:882–8.

20. Tallal L, Tan C, Oettgen H, Wollner N, McCarthy M, Helson L, et al. E. coli L-asparaginase in the treatment of leukemia and solid tumors in 131 children. Cancer. 1970;25:306–20. doi: 10.1002/1097-0142(197002)25:2<306::aid-cncr2820250206>3.0.co;2-h.
21. Clarkson B, Krakoff I, Burchenal J, Karnofsky D, Golbey R, Dowling M, et al. Clinical results of treatment with E. coli L-asparaginase in adults with leukemia, lymphoma, and solid tumors. Cancer. 1970;25:279–305. doi: 10.1002/1097-0142(197002)25:2<279::aid-cncr2820250205>3.0.co;2-7.
22. Sutow WW, Garcia F, Starling KA, Williams TE, Lane DM, Gehan EA. L-asparaginase therapy in children with advanced leukemia. The Southwest Cancer Chemotherapy Study Group. Cancer. 1971;28:819–24. doi: 10.1002/1097-0142(1971)28:4<819::aid-cncr2820280403>3.0.co;2-9.
23. Ortega JA, Nesbit ME, Jr, Donaldson MH, Hittle RE, Weiner J, Karon M, et al. L-Asparaginase, vincristine, and prednisone for induction of first remission in acute lymphocytic leukemia. Cancer Res. 1977;37:535–40.
24. Jeong HC, Kim T, Yang DH, Shin KH. Development of a UPLC-MS/MS method for the therapeutic monitoring of L-asparaginase. Translational and Clinical Pharmacology. 2018 Sep;26(3):134.
25. Nath CE, Dallapozza L, Eslick AE, Misra A, Carr D, Earl JW. An isocratic fluorescence HPLC assay for the monitoring of l-asparaginase activity and l-asparagine depletion in children receiving E. colil-asparaginase for the treatment of acute lymphoblastic leukaemia. Biomedical Chromatography. 2009 Feb;23(2):152-9.
26. Yao H, Vancoillie J, D'Hondt M, Wynendaele E, Bracke N, De Spiegeleer B. An analytical quality by design (aQbD) approach for a L-asparaginase activity method. Journal of Pharmaceutical and Biomedical Analysis. 2016 Jan 5;117:232-9.
27. Magri A, Soler MF, Lopes AM, Cilli EM, Barber PS, Pessoa A, Pereira JF. A critical analysis of L-asparaginase activity quantification methods—colorimetric methods versus high-performance liquid chromatography. Analytical and bioanalytical chemistry. 2018 Nov;410:6985-90.
28. Sanghvi G, Bhimani K, Vaishnav D, Oza T, Dave G, Kunjadia P, Sheth N. Mitigation of acrylamide by L-asparaginase from Bacillus subtilis KDPS1 and analysis of degradation products by HPLC and HPTLC. SpringerPlus. 2016 Dec;5:1-1.
29. Tan YQ, Loh CK, Saffian SM, Makpol S. Improved HPLC method with automated pre-column sample derivatisation for serum pegylated L-asparaginase activity measurement in paediatric acute lymphoblastic leukaemia patients. Journal of Pharmaceutical and Biomedical Analysis. 2024 Sep 1;247:116243.
30. Anand Gupta, Vaishali Jadhav, Ashish Jain. Analytical Method Development and Validation of Ticagrelor from Bulk and Formulation. Asian J. Pharm. Res. 2019; 9(3):141-146. doi: 10.5958/2231-5691.2019.00022.4
31. Revathi R, Ethiraj T, Chandru S, Dilip I, Faisul Rahman S. Method Development and Validation of Spectroscopic Method for Content Analysis of Cefaclor with Stability Studies. Asian J. Pharm. Res. 2019; 9(2): 75-79. doi: 10.5958/2231-5691.2019.00012.1

32. Awdhut D. Pimpale, Rajendra B. Kakde. Stability-Indicating Method Development and Validation for Estimation of Clopidogrel Bisulfate in Pharmaceutical Dosage Form by Reverse-Phase High-Performance Liquid Chromatography. Asian J. Pharm. Res. 2020; 10(4):253-259. doi: 10.5958/2231-5691.2020.00044.1
33. Zainab A. Bagalkote, Ganesh Gajeli. UV Spectrophotometric Method Development and Validation of Carbimazole in Bulk and Tablet Dosage form. Asian Journal of Pharmaceutical Research. 2021; 11(3):163-6. doi: 10.52711/2231-5691.2021.00030
34. Hamid Khan. Analytical Method Development in Pharmaceutical Research: Steps involved in HPLC Method Development. Asian J. Pharm. Res. 2017; 7(3): 203-207. doi: 10.5958/2231-5691.2017.00031.4
35. Kasad Pinaz A, K.S. Muralikrishna. Base Degradation Study and Method Development of Rivaroxaban by RP-HPLC in bulk. Asian J. Pharm. Res. 3(3): July-Sept. 2013; Page 109-113.
36. Shweta Jogdand, Karuna Mane, Rohit Jadhav, G K Dyade. AQBD Approach in Chemo metric assisted Method Development for the Estimation of Ciprofloxacin and Metronidazole by UV-VIS Spectrophotometry. Asian Journal of Pharmaceutical Research. 2022; 12(3):183-1. doi: 10.52711/2231-5691.2022.00030
37. Sunandana Akkala, Govinda Gopalakrishna Kilaru, Gopi Bandreddy, Suman Baindla Madhusudhan Gutta. Two Steps Non-Enzymatic Synthesis of Molnupiravir, which is Free from Mutagenic Impurity and Analytical Method Development for Estimation of Genotoxic Impurity (Hydroxylamine Hydrochloride Content) by using RP-HPLC Technique. Asian Journal of Pharmaceutical Research. 2024; 14(2):188-6. doi: 10.52711/2231-5691.2024.00031.
38. ICH guidelines Q2 (R1), Validation of Analytical procedures, Text and Methodology 1995.