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Research Article

Method Validation Report for the Estimation of Nelarabine in K_3edta-Human Plasma by Using Lc-Esi-Ms/Ms

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ABSTRACT

Author want to define the procedure for robust and sensitive method utilized in bio-analytical research to establish a simple technique for determining the level of nelarabine in K_3EDTA plasma from human beings, is a combination of liquid chromatography, electro spray ionization and mass spectro-photometry (LC-MS/MS) parameters were systematically optimized, by using methanol: 5mM ammonium acetate in water (80:20%v/v) as mobile phase, flow rate of 1.2mL/minute, Zorbax SB-C18; 2.1*50mm, 5µm Agilent Technologies column. Solid-phase extraction (SPE) was further optimized to improve recoveries and minimize matrix effects. The final method achieved instrument detection limits as low as 0.01**fg (femto-grams)** on-column, retention time for nelarabine observed at 2.65 ± 0.03minutes with run time 4.0 minutes. Calibration linearity concentration range of 2.00 to 1000ng/mL with a correlation coefficient (r^2) of ≥ 0.9997, %Mean ISTD recovery with correction factor for Nelarabine = 83.79; %CV of ISTD recovery (Extracted) for Nelarabine = 6.15, recoveries within 70–130%, and intra / inter-day precision (RSD ≤20%) confirmed the robustness and reproducibility of the protocol. The *LC-MS/MS* technique that was created to quantify the amount of Nelarabin in the biological matrix worked well for routine blood sample analysis from patients for pharmacokinetics research and medication monitoring.

Keywords: Nelarabine (STD), Nelarabine-D4 (ISTD), LLOQQC, LQC, MQC, HQC, ULOQ.

INTRODUCTION

Nelarabine is a small molecule drug, The chemical formula $C_{11}H_{15}N_5O_5$ and molecular weight as 297.27 g/mol, chemical structure was given in figure-1a which was water-soluble [2]. O-methyl guanine forms a beta-N (9)-glycosidic bond with arabino furanose in purine nucleoside. As a prodrug of 9-beta-D-arabinofuranosylguanine (ara-G), it inhibits DNA synthesis and induces cell death [1]. It may be used as an anti-cancer agent. (IUPAC) Bibcode: 2R, 3S, 4S, 5RThe compound is a ring structure with two amino groups and six methoxy substituents. 5-(hydroxymethyl). In adults and children whose disease has not responded to or relapsed after two chemotherapy regimens, Nelarabine is prescribed as an anti-neoplastic agent to treat acute T-cell lymphoblastic leukemia, specifically T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL). Abnormal buildup of de-oxy-guanosine tri-phosphate (dGTP) in lymphocytes[3] was shown to be the cause of the severe T-cell immunological deficit seen in people with purine nucleoside phosphorylase (PNP) deficiency. This prompted research into dGTP and its derivatives as possible antileukemic medications [3,4]. On the other hand, PNP in RBCs quickly breaks down dGTP [5]. Adenosine de-aminase in the blood converts the pro-drug nelarabine (compound 506U78; brand name Arranon) to the active de-oxy guanosine analogue, 9-β-arabinofuranosylguanine (ara-G) [6]. Unlike mature T cells, Ara-G can withstand breakdown by PNP and is hazardous to T lymphoblasts.[7] Converted to 5′-triphosphate

(ara-GTP) by de-oxy-cytidinekinase and mitochondrial de-oxy-guanosinekinase after accumulation in T-lymphoblast's, ara-G inhibits DNA synthesis and causes cell death [8,9]. Thanks to Nelarabine, a pro-drug that is eight times more water soluble than ara-G, the difficult synthesis of ara-G and its low solubility in water have been rendered moot.[10]. Rapid conversion of nelarabine to ara-G after intravenous injection is shown by its 2-hour half-life and 10-fold greater area under the concentration time curve compared to nelarabine itself. This indicates that nelarabine is an effective pro-drug for ara-G[11]. T cells are believed to have increased exposure to ara-GTP due to larger amounts of ara-G accumulation, which is connected with their exquisite sensitivity compared to B-cells.[12] A review of the literature shows that there aren't reports of techniques for determining Nelarabine using a stability-indicating high performance liquid chromatographic technique.[13] The presented methodologies had several issues with stability and repeatability when it came to long run analysis. The method's stated goals include improving the analyte's sensitivity in comparison to previously reported methods, both when used alone or in combination with other biological matrices, and achieving a short chromatographic run time of 5 minutes per sample, making the method applicable to high-throughput bio-analysis.[14,15]. Establishing a reliable bio-analytical approach for estimating Nelarabine in K_3EDTA human plasma samples using LC-ESI-MS/MS is the primary objective of this investigation. In accordance with US-FDA regulations, compare with the corresponding deuterated internal standard as Nelarabine-D4 [16] chemical structure was given in figure-1b. In addition, a straightforward extraction process using a tiny volume of plasma that is both extremely sensitive and well linear is required.[17,18].

MATERIALS & METHODS

Study Design

This study aimed to develop and validate a sensitive and selective method for the quantitative determination of nelarabine in K3EDTA human plasma by using HPLC-ESI-MS/MS ultimately met the established performance criteria.

Method optimization was performed in two stages:

(1) LC-MS/MS instrumental parameters that included (i) compound dependent settings, (ii) source conditions, (iii) mobile phase composition, (iv) injection volume, and (v) chromatographic flow rate, to achieve optimal sensitivity and selectivity.

(2) SPE conditions were optimized through stepwise assessment of (i) reconstitution solvent composition, (ii) solvent evaporation, (iii) SPE elution strategies (single, mixed, sequential), (iv) washing conditions, and (v) sorbent selection.

Additional sample pretreatment strategies were tested to address analyte-specific challenges, including acidification. During the optimization process, isotopic-ally labeled internal standards (ISTDs) were introduced directly via the instrument, allowing signal evaluation without applying ISTD correction during extraction. Matrix effects (ME) were assessed using the post-extraction addition method, and method validation included recovery, reproducibility, detection limits, and application to real environmental samples [19].

Chemicals and Reagents

The reagents used during analysis include methanol, acetonitrile, Water (MilliQ), ammonium acetate buffer, Human Plasma, K_3EDTA (Anticoagulant), Nelarabine (Standard) and Nelarabine-D4 (Internal Standard). Other chemicals consumed for this study were AR grade & solvents were HPLC grade.

Instrumentation

The HPLC system used was Shimadzu LC VP Series. Column type as Chromolith RP18 end capped, 100*4.6mm. A model API-4000 triple quadruple instrument (make: MDS Sciex) was used for ESI (Electron Spray Ionization technique) mass spectrometric detection employing multiple reaction mode (MRM) scan type. The Analyst Version 1.4.2 and Watson LIMS Version 7.3 were used for data processing.

Bio-Analytical Chromatographic Conditions: Column, mobile phase, rinsing solution, flow rate, split ratio, sample cooler temperature, injection volume, needle rinsing volume, column oven temperature, rinsing mode, retention time, and run duration were all specified in table-1, which contains bio-analytical conditions.

Table-1: The summary of the bio-analytical chromatographic conditions:

Parameter	Selection
Column	Zorbax SB-C18; 2.1*50mm,5µm(Make: Agilent Technologies)
Mobile phase	HPLC grade methanol: 5mM ammonium acetate in water (80:20v/v)
Rinsing solution	HPLC grade methanol : Milli-Q water or HPLC grade water (50:50,v/v)
Flow rate	0.800 mL/minute (with splitter)
Split ratio	50:50
Sample cooler temperature	15°C
Injection volume	15 µL
Needle rinsing volume	500 µL
Column oven temperature	N/AP
Rinsing mode	Before and after aspiration
Retention time	Nelarabine at 2.65 ± 0.3minutes and Nelarabine-D4 at 2.62 ± 0.3 minutes
Run Time	4.0 minutes

Mass Spectrometry: A triple quadru-pole mass spectrometer from Applied Bio-systems MDS Sciex (Concord, Ontario, Canada) with an ESI (Turbo Ion Spray) interface was used for mass spectrometric detection. We used the negative ion mode to ionize the ESI. Using Analyst Version 1.4.2 and Watson LIMS Version 7.3, the tandem mass spectrometer was run in the single ion monitoring mode (SRM) at unit resolution. At m/z 298.11 (parent) and 166.02 (product), the transitions for multiple reaction monitoring were established for Nelarabine and Nelarabine-D4, respectively. The Harvard infusion pump was used to continuously inject the standard solution at a rate of 10µL/min, as shown in table-2, in order to optimize the mass spectrometric conditions for Nelarabine.

Table-2: Information of MASS spectrometry.

Parameter	Nelarabine	NelarabineD4
Ionization mode	Positive	Positive
Detection (m/z)	m/z 298.11(parent) and 166.02 (product)	m/z 298.11(parent) and 166.02 (product)
Ion spray voltage (ISV)	5000V	5000V
Temperature (Temp. °C)	500°C	500°C
Curtain gas (CUR)	20 psi	20 psi
Collision gas (CAD)	6 psi	6 psi
GS1	21 psi	21 psi
GS2	31 psi	31 psi
De-clustering potential (DP)	125 V	125 V
Collision Energy (CE)	35 V	35 V
Collision cell exit potential (CXP)	15 V	15 V
Entrance Potential (EP)	10 V	10 V
Dwell time	200 ms	200 ms

Preparation of Solution

Nelarabine Stock Solution (Standard): A solution with a concentration of 1mg/mL was prepared by dissolving about 5,000 milligrams of the nelarabine working standard in 5 milliliters of clean, volumetric glass vials, dissolved in HPLC grade methanol and makeup the volume with the same to produce a solution of 1mg/mL Updated the concentration of the Nelarabine solution above to reflect its real weight and potency. You may use the stock solution for up to 7 days after storing it in the fridge at 2-8°C. For spiking into plasma to create CC (Calibration Curve) standards, QC (Quality Control) samples, and DIQC (Dilution Integrity Quality Control) samples, the stock solutions were diluted to appropriate concentrations using a combination of methanol and HPLC grade water (Diluents) in a ratio of (50:50v/v). Quality control samples and standards for calibration curves were prepared using different stock solutions.

Nelarabine-D4 Stock Solution (Internal Standard): A solution with a concentration of 1mg/mL was prepared by dissolving about 5,000 milligrams of nelarabine-D4 in 5 milliliters of HPLC-grade methanol and then filling the remaining volume with the same. Chang the concentration of Nelarabine-D4 above to reflect its potency, molecular weight, and the quantity actually weighed. The stock solution was kept in the fridge between 2 and 8 degrees Celsius and used up within a week. Using diluents for internal standard dilution, the stock solution was diluted to a sufficient concentration.

Reference Points for the Calibration Curve and Quality Control Samples: A series of 10 non-zero concentrations of Nelarabine, ranging from 2.00ng/mL to 250ng/mL, were created to serve as calibration curve standards. The controlled samples for quality control were prepared with different concentrations of Nelarabine, including 2.00ng/mL for LLOQQC (Lower Limit of Quantification Quality Control), 6.00ng/mL for LQC (Low Quality Control), 50.0ng/mL for MQC1 (Middle Quality Control), 400ng/mL for MQC2, and 800ng/mL for HQC (High Quality Control). They were kept at a temperature of -70°C until they were ready to be used. For the purpose of testing their stability at -20°C, twelve sets of LQC and HQC were placed in a deep freezer.

Ammonium acetate buffer (5 mM, w/v): An amount of 1000 milliliters of Milli-Q / HPLC grade water was added to a 1000 milliliter reagent container containing about 385.4 milligrams of ammonium acetate. Blended well and subjected to 5 minutes of ultra-sonication. Within four days of its production, the buffer solution was utilized after being kept at room temperature (20±5 °C).

Mobile phase preparation (20:80 v/v): Added 1600 mL of HPLC-grade methanol to 400mL of 5mM ammonium acetate buffer in a 2000mL reagent container-I sonicated it in an ultrasonicator for five minutes after thoroughly mixing it. You need to utilize the mobile phase within 7 days after preparing it and keep it at room temperature (20±5 °C).

Diluting Agent (50:50 v/v): A 50:50 combinations of MilliQ/HPLC grade water and HPLC grade methanol was made. After that, it was subjected to 5 minutes of ultrasonication. The solution was to be used within 7 days after preparation and kept at room temperature (20±5°C).

Rinsing Solution (Volume/Volume): A diluents was used for this purpose.

Formic acid buffer (0.1% v/v): 0.5mL of formic acid was transferred to a 500mL reagent container was filled with 499.5 mL of MilliQ / HPLC grade water and 0.5 mL of formic acid to create a 0.1% formic acid buffer (v/v). Took everything and sonicated it for five minutes in an ultrasonicator. The buffer was made up as needed and used up within four days after being prepared. The buffer solution was kept at a temperature of 20±5 °C, or room temperature.

Finding the Right System Solution: System suitability testing required the preparation of a combination of analyte and internal standard. The analyte concentration is equal to the working concentration used for spiking (25ng/mL for Nelarabine-D4), whereas the internal standard concentration is equal to the middle concentration of the calibration range (25ng/mL for Nelarabine). An injectable combination of the system suitability test solution was used. On the basis of recovery, aqueous samples are produced. The system suitability sample was prepared by mixing 25µL of analyte, 50µL of working concentration of internal standard, and 925 µL of mobile phase.

Getting the Sample Ready: After thawing at room temperature, the materials were vortex to mix them well. Except for blank plasma samples, which only required 25µL of diluents, 250µL of the plasma sample was pipette into 15mL glass-stopped tubes, and then 25µL of a 7095.241 ng/mL dilution of Nelarabine-D4 was added and mixed well. After that, 100µL of 0.1% formic acid buffer was mixed in and mixed vigorously. 5mL of methyl tert-butyl ether (MTBE) was then added and agitated for 20 minutes on a reciprocating shaker set at 200 rpm. For 10 minutes at 4°C, the samples were spun in a centrifuge. The organic layer supernatant (4.0mL) was thereafter transferred to sterilized glass test tubes that had already been labeled and allowed to evaporate to dryness under a mild nitrogen stream maintained at 40°C. The 500µL of mobile phase was used to reconstitute the samples before injection.

Validation of Analytical Method: Validation of the devised technique was performed in line with the European Medicines Agency, 2011, and Food and Drug Administration, 2001 standards [20,21].

RESULT AND DISCUSSIONS

Method Validation

System Suitability: System suitability was accomplished by acquiring six replicate injections from a single sample of highest standard (HQC), with analytical column Zorbax SB-C18; 2.1*50mm, 5µm. The system was found to be suitable, specific and reproducible for the current analytical run which was summarized in the Table-3. The acceptance requirements should be met by 80% or more of the plasma lots (not including lipidemic, heparinised, or haemolyzed plasma lots).

Table-3: System suitability Day 1 & 2 for Nelarabine (STD) and Nelarabine-D4 (ISTD)

Injection No.	%Coefficient Variation (Day – 1)			% Coefficient Variation (Day – 2)		
	STD Rt	ISTD Rt	%Area Ratio	STD Rt	ISTD Rt	%Area Ratio
1	0.19	0.19	1.27	0.15	0.16	0.63
2	0.21	0.21	0.51	0.18	0.20	0.67
3	0.15	0.16	1.03	0.17	0.14	0.65
4	0.16	0.12	0.97	0.17	0.17	0.77
5	0.22	0.18	1.62	0.16	0.16	1.23
6	0.07	0.06	0.75	0.10	0.08	0.51

Selectivity (LLOQ): The plasma lots that are eligible for acceptance should not include those that have been haemolyzed, lipidemic or heparinised. Plasma was tested for selectivity for Lower limit of quantification (LLOQ) and aqueous lower limit of quantification (AQLLOQ); any reaction shown in haemolyzed, heparinised or lipidemic matrix samples should fall within the acceptance criteria. These lots of standard blank (STDBL) did not show significant interference at the retention time of nelarabine and nelarabine-D4 given in table-4.

Table-4: Selectivity (LLOQ) responses for STD & ISTD

Sr. No.	Drug (STD) Response			ISTD Response		
	LLOQ		% Interference	LLOQ		% Interference
	Area	RT		Area	RT	
01	2572	2.343	0.43	443009	2.322	0.03
02	15786	2.336	0.11	534378	2.321	0.01
03	1931	2.340	1.24	532684	2.321	0.00
04	1867	2.343	0.37	546457	2.323	0.01
05	2123	2.341	0.75	548372	2.317	0.05
06	4149	2.345	0.10	530300	2.320	0.01
Sr. No.	Drug (STD) Response			ISTD Response		
	AQLLOQ		% Interference	AQLLOQ		% Interference
	Area	RT		Area	RT	
01	2935	2.340	0.37	827966	2.318	0.01
02	2935	2.340	0.58	827966	2.318	0.01
03	2935	2.340	0.82	827966	2.318	0.00
04	2935	2.340	0.24	827966	2.318	0.00
05	2935	2.340	0.55	827966	2.318	0.03
06	2935	2.340	0.14	827966	2.318	0.00

Auto-Sampler / Re-Injection Reproducibility: In LC-MS studies, both auto-sampler stability and re-injection reproducibility are fundamental for ensuring the dependability of analytical results. Auto sampler studies assess the stability of samples stored in the auto-sampler carousel over time, while reinjection reproducibility studies confirm the ability to accurately re-inject samples after a period of storage at 53 hours at 5±3°C in Ammonium formate results were given in

table-5. At least 67% of the QCs should be within ±15.00 % (±20.00% for LLOQQC) of their nominal concentration.

Table-5: Auto-Sampler Re-Injection Reproducibility For STD: 53 Hours At 5±3°C In Methanol: Ammonium Acetate (80:20%V/V) Solution.

Run Number	LLOQQC 2.00ng/mL	LQC 6.00ng/mL	MQC 400 ng/mL	HQC 800 ng/mL
1	~2.90	5.70	391	808
2	2.28	5.86	401	802
3	2.15	6.08	397	796
4	2.18	5.76	394	800
5	2.25	6.45	397	800
6	2.27	6.04	401	787
Mean	2.34	5.98	397	799
S.D.	0.280	0.274	3.92	7.00
% CV	11.97	4.58	0.99	0.88
% Accuracy	117.00	99.67	99.25	99.88
%Bias	17.00	-0.33	-0.75	-0.13
N	6	6	6	6

Auto-Sampler Carryover Effect: Where a small amount of an analyte from a previous sample remains in the system and is detected in subsequent blank or sample injections, is a common issue in LC-MS/MS batch runs. It can affect the accuracy and reproducibility of analysis, particularly when quantifying low concentrations values were given in table-6. The STDBL inject-able after ULOQ should have a carryover of 20.0% for the analyte and 5.0% for the ISTD response, in comparison to the analyte and ISTD response in the LLOQ sample, respectively.

Table 6: %Carry over during batch RUN

Sr.No.	Acquisition Batch ID	STD Area of Carry-over Blank	ISTD Area of Carry over Blank	Area of LLOQ1	Area of LLOQ2	Average ISTD area	% Carry Over for STD	%Carry Over for ISTD
1	P&A:01	0	8	1777	1660	514203.1	0.00	0.00
2	P&A:02	69	22	2155	1609	417904.4	4.29	0.01
3	P&A:03 DC:01	47	40	2594	1512	530287.3	3.11	0.01
4	P&A:03 DA:01	58	61	1566	1820	387610.0	3.70	0.02
5	P&A:04	0	14	1974	1958	460890.0	0.00	0.00
6	HLE:01,DI:01	5	20	2018	2136	480829.5	0.25	0.00
7	BSE:01	125	55	1715	1569	453321.7	7.97	0.01
7	FT01	117	85	3875	2316	803394.3	5.05	0.01
8	BT:01,DE:01,SE:01	21	13	2538	2116	730138.9	0.99	0.00
11	P&A:05	16	32	1043	976	306107.0	1.64	0.01
12	CME:01	22	128	1757	1500	463550.9	1.47	0.03
13	ASRR:02	22	38	3087	1609	611599.7	1.37	0.01

Note: P&A=Precision & Accuracy; DC=Different Column; DA=Different Analyst; DI=Dilution Integrity; BSE=Batch Size Experiment; FT=Freez Thaw; BT=Bench Top, DE=Dry Extract, SE=Stability of Extract; CME=Concomitant Medication Experiment; ASRR=Auto-sampler Re-injection Reproducibility.

Biological Matrix Screening and Specificity: An LC-MS-MS method showed specificity in standard plasma samples. It refers to the method's ability to precisely measure the analyte in the presence of other sample constituents, ensuring it's not influenced by interfering substances. This is crucial for

accurate quantification and is typically assessed during method validation. Specificity studies were done for Plasma Blank, drug with Biological Matrix table-7a and Selectivity experiment in presence of Concomitant medication drug Table-7b. There was no detectable interference for Nelarabine or the internal standard in any of the plasma lots tested, including the haemolytic and lipemic plasma. The planned anticoagulant matrix must include not less than 80% of the whole matrix in order to be considered acceptable. For screening evaluate the lot by using accepting standard blank and LLOQ and AQLLOQ sample

Table-7a: Specificity of Biological Matrix.

Sr.No.	Matrix Batch / Lot No.	STD Response			ISTD Response		
		LLOQ		% Interference	LLOQ		% Interference
		Area	RT		Area	RT	
01	1441	2169	2.652	1.89	470648	2.624	0.01
02	1442	2314	2.651	0.17	492693	2.615	0.00
03	1443	1691	2.652	0.47	413426	2.635	0.00
04	1444	2024	2.650	0.40	478343	2.623	0.00
05	1445	2430	2.653	0.41	519770	2.633	0.00
06	1446	1751	2.652	0.34	482317	2.627	0.01
07	1447	1923	2.646	0.62	429775	2.627	0.00
08	Lipidemic	1927	2.673	0.62	522461	2.628	0.00
09	Haemolyzed	1817	2.653	2.20	463570	2.631	0.08
10	Na-Heparin	1985	2.640	3.07	444782	2.614	0.01
Sr.No.	Matrix Batch / Lot No.	STD Response			ISTD Response		
		AQLLOQ		% Interference	AQLLOQ		% Interference
		Area	RT		Area	RT	
01	1441	3407	2.646	1.20	766295	2.618	0.01
02	1442	3407	2.646	0.12	766295	2.618	0.00
03	1443	3407	2.646	0.23	766295	2.618	0.00
04	1444	3407	2.646	0.23	766295	2.618	0.00
05	1445	3407	2.646	0.29	766295	2.618	0.00
06	1446	3407	2.646	0.18	766295	2.618	0.01
07	1447	3407	2.646	0.35	766295	2.618	0.00
08	Lipidemic	3407	2.646	0.35	766295	2.618	0.00
09	Haemolyzed	3407	2.646	1.17	766295	2.618	0.05
10	Na-Heparin	3407	2.646	1.79	766295	2.618	0.00

Table-7b: CME: Concomitant Medication Experiment Comparison with LQC & HQC.

Run Number	CME: LQC 6.00ng/mL	LQC 6.00 ng/mL	CME: HQC 800ng/mL	HQC 800 ng/mL
Mean	5.98	6.15	799	806
S.D.	0.172	0.481	9.05	2.12
% CV	2.88	7.82	1.13	0.26
%Accuracy	99.67	102.50	99.88	100.75
%Bias	-0.33	2.50	-0.13	0.75
N	6	2	6	2

Ruggedness: Ruggedness studies in LC-MS assess how well a method performs despite variations in experimental conditions. These studies help determine the method's ability to yield consistent results when faced with unexpected variations in parameters like mobile phase composition, column performance, or sample preparation,

ensuring reliability during routine use, here a study was performed with human Plasma Nelarabine concentrations in quality controls for two separate batch runs with different analyst with different column results were given in table-8 the precision % CV for LLOQC for different column and analyst less than the value as 6.15% and 9.53%, for LQC, MQC, HQC to be less than or equal to 6.52% and 6.05% respectively.

Table-8: Ruggedness Study: STD Concentrations In Quality Controls For 2 Separate Batch Runs

Parameter	**LLOQQC 2.00ng/mL**		**LQC 6.00ng/mL**		**MQC 400ng/mL**		**HQC 800ng/mL**	
Analyst & Column	1	2	1	2	1	2	1	2
Intra run Mean	1.92	2.13	6.07	6.03	402	400	798	810
Intra run SD	0.118	0.203	0.396	0.365	7.82	3.50	22.2	6.51
Intra run %CV	6.15	9.53	6.52	6.05	1.95	0.88	2.78	0.80
Intra run %Bias	-4.00	6.50	1.117	0.50	0.50	0.00	-0.25	1.25
N	6	6	6	6	6	6	6	6
Mean Concen. Found (ng/mL)	2.03		6.05		401		804	
Inter-run SD	0.191		0.364		5.81		16.9	
Inter-run %CV	9.41		6.02		1.45		2.10	
Inter-run %Bias	1.50		0.83		0.25		0.50	
N	12		12		12		12	

Recovery: Recovery studies in LC-MS/MS are essential for assessing the effectiveness of a bio-analytical method. They determine how well an analyte can be extracted from a matrix and analyzed without significant losses. A good recovery rate (typically 85-90% or higher) indicates a reliable method. Recovery studies help identify and quantify analyte losses at different stages of the process, from sample collection to analysis, allowing for optimization of the bio-analytical conditions results were given in table-9. All QC levels should have a %CV of at least 15.00%.At all stages, the CV of the recovery should be within 15%.

Table-9: Recovery Studies for Nelarabine

Replicate No.	**HQC**		**MQC**		**LQC**	
	Extracted Peak Area	Un-extracted Peak Area	Extracted Peak Area	Un-extracted Peak Area	Extracted Peak Area	Un-extracted Peak Area
1	779111	1117607	54310	68298	5935	8185
2	736269	1127551	52789	68719	5220	8918
3	721212	1245455	51784	70905	5060	8560
4	764700	1223297	52137	68785	5420	8409
5	770962	1232020	50933	69041	6541	9082
6	743630	1239998	51334	73870	6683	9686
Mean	752647.3	1197654.7	52214.5	69936.3	5809.8	8806.7
SD	22444.43	58717.10	1210.79	2130.98	689.18	541.64
% CV	2.98	4.90	2.32	3.05	11.86	6.15
% Mean Recovery	62.8		74.7		71.3	
Correction Factor	1.250					
% Mean Recovery With Correction	78.55		93.33		82.46	
Overall %Recovery	68.70					
Overall %Recovery With Correction	85.87					
Overall % CV	7.71					

Calibration Curve: During validation, calibration curve was linear for standard concentrations ranging as 2.00, 4.00, 10.00 & 25.0, 50.0, 125, 250, 500 & 1000ng/mL. Linearity studies are essential for technique validation to ensure that the method can accurately quantify analytes across a relevant nine concentration range results were given in table-10 shown with calibration curve in figure-2. Linearity studies evaluate the capacity of LC-MS technologies to provide findings that are directly proportional to the analyte concentration within a specified range. This is crucial for accurate quantification of analytes across a range of concentrations.

Table-12: Calibration Curve Parameters for Nelarabine

Concentration (ng/ml)	**Analyte response (N = 24)**
2	2.02 ± 0.149
4	4.01 ± 0.278
10	9.73 ± 0.362
25	24.1 ± 0.678
50	48.6 ± 1.47
125	126 ± 2.80
250	254 ± 5.20
500	511 ± 11.0
1000	1040 ± 27.3
Slope	**0.0021 ± 0.0001**
Intercept	**-0.0003 ± 0.0003**
R-Squared	**0.9969 ± 0.0021**

Accuracy & Precision: Accuracy and precision are crucial for reliable quantitative analysis. The degree to which a measured value approaches the real or accepted value is known as accuracy, and the degree to which a measurement can be reliably reproduced is known as precision. Both are essential for validating LC-MS methods and ensuring the trustworthiness of results, intra and inter day run assay precision and accuracy in human plasma Nelarabine concentrations in quality controls for four separate batch runs results were given in table-11.

Table-11: Intra- Inter Run - Assay Precision & Accuracy Human Plasma Nelarabine Concentrations in Quality Controls for 3 separate batch runs

Run Number	LLOQQC 2.00ng/mL	LQC 6.00ng/mL	MQC2 50.0ng/mL	MQC1 400ng/mL	HQC 800ng/mL
Day – 1 Batch – 1 (N = 6)					
Intra run Mean	2.00	6.35	48.5	386	820
Intra run SD	0.0703	0.201	0.850	4.20	13.2
Intra run %CV	3.52	3.17	1.75	1.09	1.61
Intra run %Bias	0.00	5.83	-3.00	-3.50	2.50
Day – 2 Batch – 2 (N = 6)					
Intra run Mean	2.07	5.95	50.0	404	767
Intra run SD	0.156	0.109	0.946	5.18	7.59
Intra run %CV	7.54	1.83	1.89	1.28	0.99
Intra run %Bias	3.50	-0.83	0.00	1.00	-4.13
Day – 3 Batch – 3 (N = 6)					
Intra run Mean	1.97	6.11	50.1	399	803
Intra run SD	0.144	0.486	0.695	3.67	7.38
Intra run %CV	7.31	7.95	1.39	0.92	0.92
Intra run %Bias	-1.50	1.83	0.20	-0.25	0.38
Mean Concentration Found (ng/mL)	1.99	6.15	49.2	399	800

Inter-run SD	0.150	0.318	1.07	9.88	24.2
Inter-run %CV	7.54	5.17	2.17	2.48	3.03
Inter-run %Bias	-0.50	2.50	-1.60	-0.25	0.00
n	18	18	18	18	18

Matrix Effect: Matrix effect refers to changes in the analytical signal due to the presence of non-target compounds in the sample matrix. These compounds can interfere with the ionization or ionization efficiency of the target analyte, leading to changes in peak area or retention time. It can arise from various sources, including endogenous compounds (proteins, lipids, salts) or exogenously introduced components (anticoagulants, dosing vehicles). It can lead to inaccurate quantification, reduced precision, and even the mis-identification of analytes. Matrix effect was done for both standard (Nelarabine) and internal standard (Nelarabine-D4) along with aqueous samples estimated for HQC, MQC, and LQC results were given in table-12.

Table-9: Matrix Factor Experiment for STD & ISTD

Replicate No.	**AQHQC**		**AQMQC**		**AQLQC**	
	STD	**ISTD**	**STD**	**ISTD**	**STD**	**ISTD**
1	1231479	708692	602440	719127	9416	729128
2	1220157	716338	616063	735329	9045	728445
3	1248244	719982	627391	751017	9594	748534
4	1278097	735230	608021	724291	9021	728305
5	1239104	702919	601267	708942	8695	717611
6	1241883	713644	611901	711712	8991	692449
Mean	**1243160.7**	716134.2	**611180.5**	725069.7	**9127.0**	724078.7
SD	**19647.43**	11097.08	**9713.85**	15830.15	**323.94**	18454.01
% CV	**1.58**	1.55	**1.59**	2.18	**3.55**	2.55

Stability Studies: The nelaraine kept out of the fridge for 8 hours at room temperature to ensure their short-term stability. LQC and HQC's were tested for a 10-day, 16-hour, and 20-minute period at temperatures ranging from 2.0°C to 8.0°C for long-term stability as shown in table-13. The samples were frozen at −20°C±5°C and at −78°C±10°C and thawed at room temperature (25°C) 3times. QC sample solutions were spiked and left to stand for 17 hours and 28 minutes on the bench top. To test their durability, the prepared controls were kept in an auto sampler for 2 days, 20 hours, and 27minutes at 5°C±3°C. Wet extract stability was assessed by keeping spiked QC samples at room temperature for 23hours and 42minutes. At 2°C–8°C, the half-life of a wet extract was 2 days, 20 hours, and 23 minutes. The shelflife of dry extracts of spike controls was evaluated over a period of 2 days, 20 hours, and 2 minutes at −28°C ± 5°C. All readings fell within acceptable parameters, as shown in table-14.

Table-13: Short-Term & Long-Term Working Solution Stability for Nelarabine (STD).

Replicate No.	**Short Term Stability**		**Long Term Stability**	
	LLOQ 100 ng/mL	ULOQ 50000 ng/mL	LLOQ 100 ng/mL	ULOQ 50000 ng/mL
	Area Stability samples	Area Stability samples	Area Stability samples	Area Stability samples
01	2094	1307850	2268	1332605
02	2149	1297118	1880	1349630
03	2153	1289492	2438	1357579
04	1937	1257676	2076	1349354
05	2189	1280589	2067	1325340
06	2057	1267607	2244	1317021

Mean	2096.5	1283388.7	2158.8	1338588.2
SD	91.12	18666.24	193.46	15968.29
%CV	4.35	1.45	8.96	1.19
%Mean stability	95.65	92.28	98.45	96.25

Table-14: Stability studies data for Nelarabine

Stabilities level	Concentration level	Concn. Mean with SD (n=6)	% CV	%Mean stabilities
Freeze & thaw at -20±5°C	HQC	823 ± 7.61	0.92	99.64
	LQC	6.13 ± 0.0598	0.98	101.83
Freeze & thaws at −78°C ± 10°C	HQC	823 ± 12.3	1.49	99.64
	LQC	6.09 ± 0.265	4.35	101.16
Bench top -78±5°C	HQC	894 ± 7.86	0.88	109.83
	LQC	6.77 ± 0.211	3.12	112.09
Dry extract -20±5°C	HQC	884 ± 20.8	2.35	108.60
	LQC	6.83 ± 0.169	2.47	113.08
Stability of extract Ambient temp.	HQC	883 ± 12.0	1.36	108.48
	LQC	6.69 ± 0.204	3.05	110.76
stability of extract 5°C± 3°C	HQC	886 ± 10.8	1.22	
	LQC	6.84 ± 0.283	4.14	113.25

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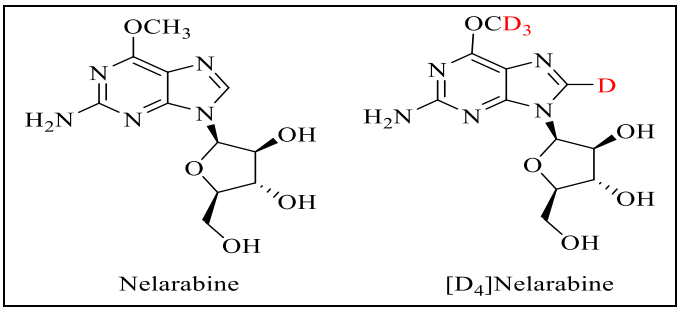

Figure 1: Chemical structure for Nilarabine (STD) and Nilarabine D4 (ISTD)

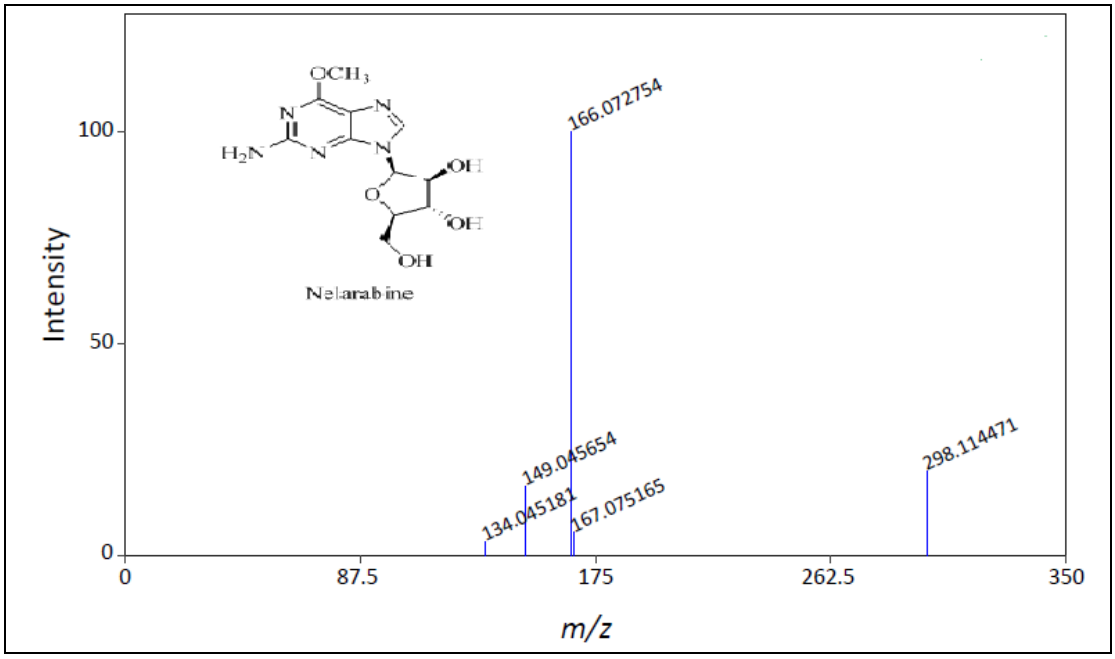

Figure 2: Mass Spectrum of Nilarabine

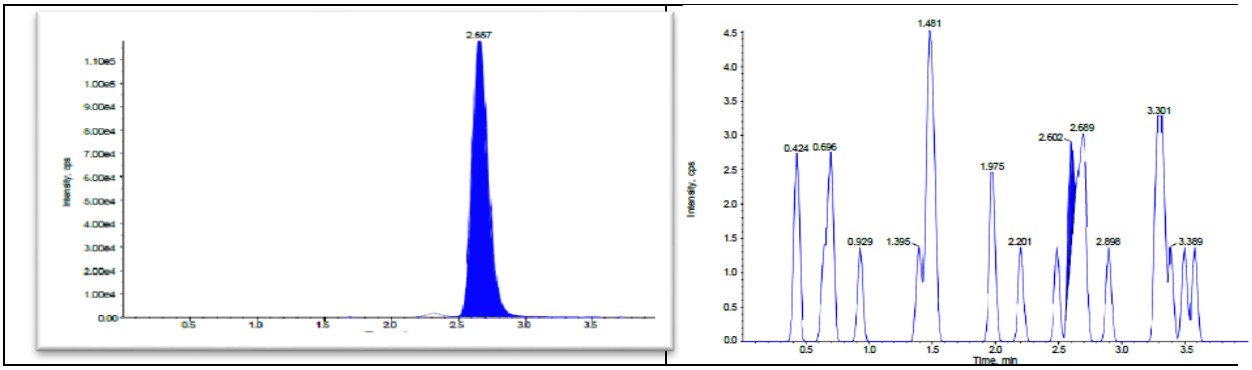

Figure 3: Chromatogram of Standard (STD) Nilarabine

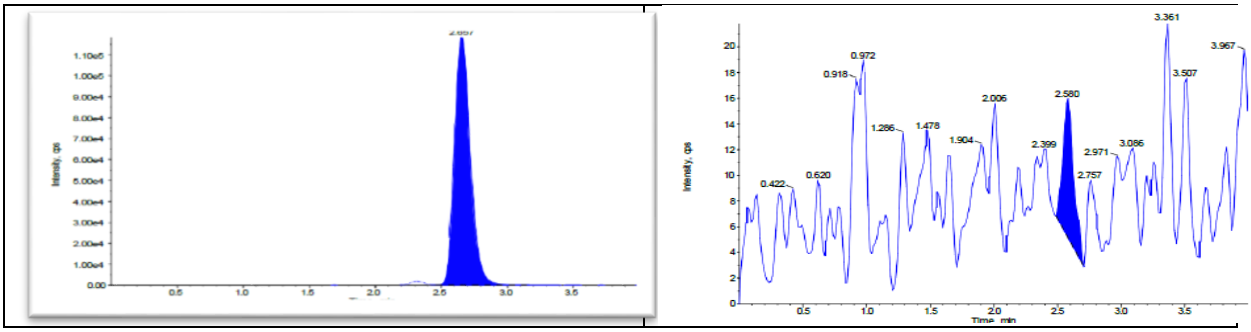

Figure 4: Chromatogram of Internal Standard (ISTD) Nilarabine-D4

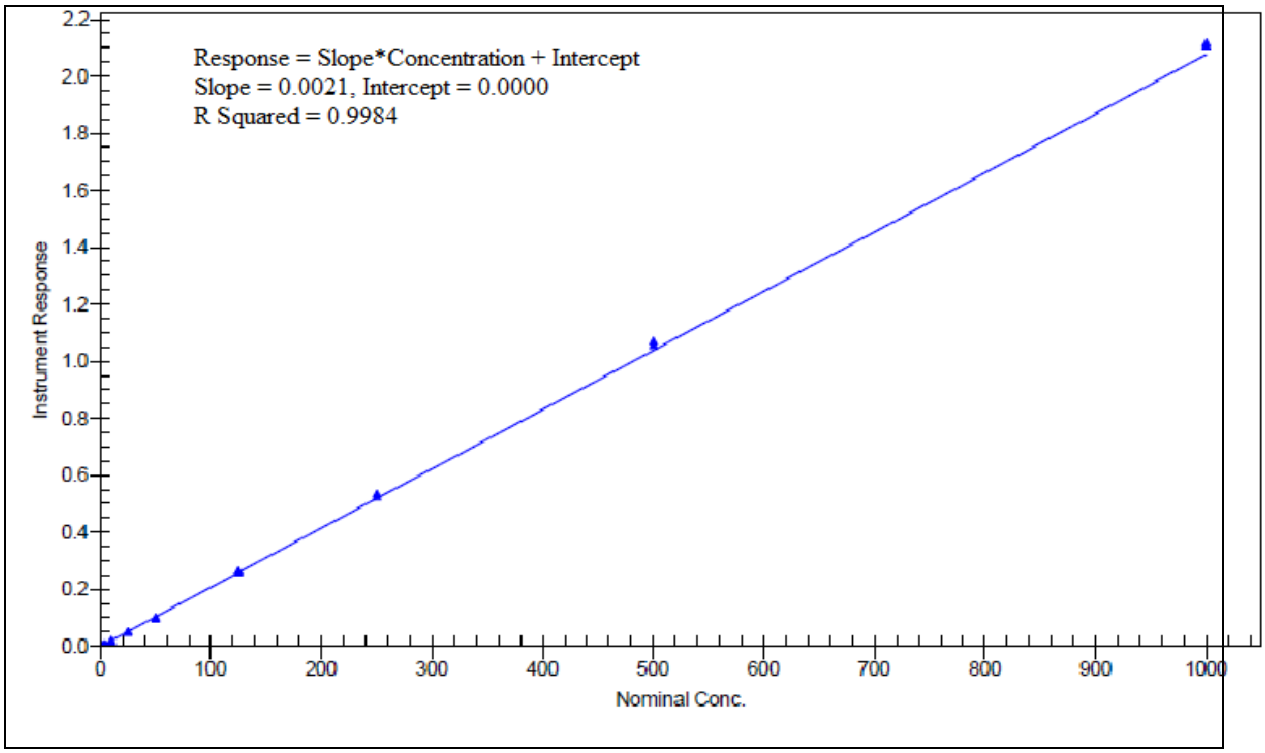

Figure 5: Representative Calibration Curve for Different Concentrations of Standard Nilarabine.

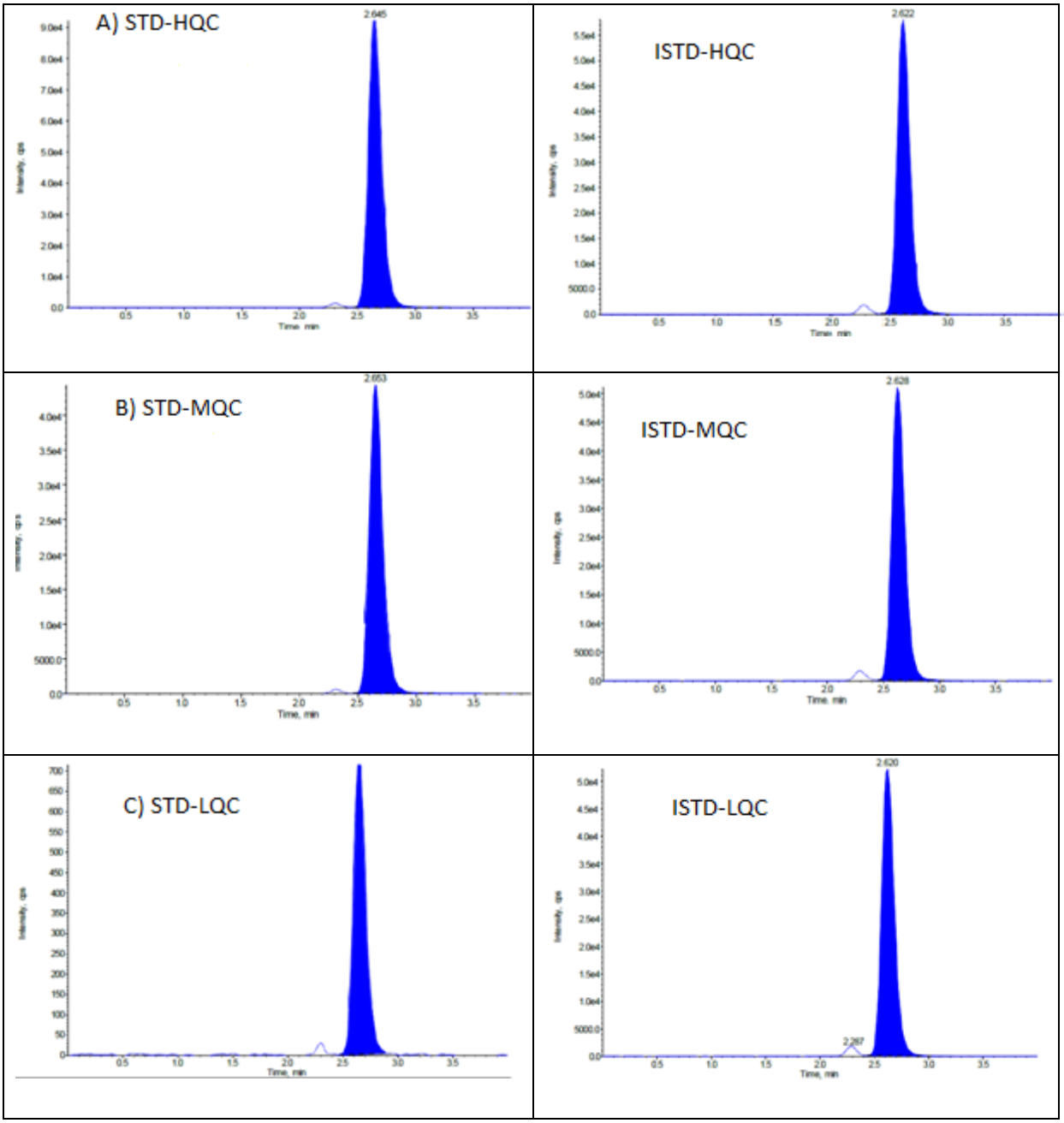

Figure 6: Representative Chromatograms of Aqueous A) HQC, B) MQC, And C) LQC Solutions

CONCLUSION

The proposed research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other described methods in previously. Quantification of Nelarabine was compared with respective isotope labeled internal standard. Extraction of STD and ISTD were achieved by using LLE. Linearity range, column, mobile phase, flow rate, injection volume's plasma usage volume for analysis was improved. The sensitivity of the assay is sufficient to follow accurately the pharmacokinetics of Niraparib. Hence this method has significant advantages over previously reported methods in-terms of Selectivity, sensitivity, Linearity, Reproducibility.

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