Research Article

Bioanalytical Method for Simultaneous Determination of Rubitecan and it's Metabolite in Human Plasma by LC-MS-MS

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ABSTRACT

A simple, sensitive and fast throughput liquid chromatography tandem mass spectrometry (HPLC-ESI-MS/MS) method has been developed for the combined estimation of cabotegravir and rilpivirine in human plasma, using respective deuterated drug as internal standards. The method involved Liquid-Liquid Extraction (LLE) of the analytes and internal standards from human plasma. The chromatographic separation was achieved on a Column: Kromasil C18 column (150 mm x 4.6 mm i.d., 5 µm) analytical column using isocratic mobile phase, consisting of 10mM Ammonium Format and Acetonitrile (70:30 v/v), at a flow-rate of 0.8 mL/min with 75% flow splitting. The parent→product ion transitions 394.40./29106, 364.4/291.06, 398.10/295.06 and 368.4/295.06 (m/z) for Rubitecan, 9-amino rubitecan, Rubitecan-D4 and 9-amino rubitecan-D4, respectively were monitored on a triple quadrupole mass spectrometer, operating in the multiple reactions monitoring (MRM) positive ion mode. The method was validated over the concentration range of 2.5-500ng for Rubitecan and 9-amino rubitecan. The mean recovery values for both the drugs from spiked plasma samples were reproducible. The method was rugged and rapid with a total run time of 4.0 minutes. The retention times were found to be 2.396 for rubitecan and 1.976 for 9-amino rubitecan.

Keywords: Rubitecan, 9-amino rubitecan LC–MS/MS; Liquid/liquid extraction.

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INTRODUCTION

Rubitecan is a semisynthetic antitumor and antiviral drug that is linked to camptothecin. Rubitecan binds to and inhibits the enzyme topoisomerase I, causing protein-linked DNA single-strand breaks, which hinders DNA and RNA production in dividing cells. It also prevents reversible single-strand DNA break repair. Rubitecan is a pyrano-indolizino-quinoline that is camptothecin with a nitro group in place of the hydrogen at position 9. It's a 9-aminocamptothecin prodrug. It functions as an anticancer agent, a DNA topoisomerase inhibitor, and a prodrug (EC 5.99.1.2). It's a pyrano-indolizino-quinoline, a C-nitro molecule, a semisynthetic derivative, a tertiary alcohol, and a delta-lacton all rolled into one. Rubitecan inhibits tumour growth by preventing DNA from unwinding during replication via DNA topoisomerase 1. Rubitecan is broken down into 9-amino-Rubitecan (the active metabolite), which is then glucuronidated. 5- and 6-hydroxylations predominated in animals, whereas 9-aminolation followed by conjugation to glucuronic acid was the predominant pathway in humans. The objective of this study was to develop and validate a more sensitive and selective high throughput LC-MS/MS method that can be efficiently used in pharmacokinetic studies. The goal of this study was to create and validate a simple, precise, and accurate LC-MS method for determining Rubitecan and 9-aminoRubitecan in human plasma. This method was created specifically to research pharmacokinetic parameters using a non-compartmental design, and it has more selectivity and specificity than the previous methods [1-6].

MATERIALS AND METHODS

Reference-Samples & working standards

The following listed analytes and internal standards were used in the proposed method of liquid chromatography.

Table 1: Reference standards and internal standards

Compound	Description	Source/Supplier
Rubitecan (Drug-1)	Analyte of interest	Samex Overseas, India
9-amino Rubitecan (Drug-2)	Analyte of interest	Toronto Research Chemicals Inc., Canada
Rubitecan-D4	Internal standard for Drug-1	
9-amino Rubitecan-D4	Internal standard for Drug-2	

Reagents & Chemicals

All the chemicals and reagents used were of standard grade and the details of which are as given under in table 2.

Table 2: Reagents and chemicals used in LC-MS method development

Chemical / Reagent	Grade	Manufacturer
Methyl-Alchol	HPLC Grade	Thermo-Fisher Scientific India Pvt. Ltd
ACN(CH3CN)	HPLC Grade	
Water (Milli-Q)	HPLC Grade	
Ammonium format	GR Grade	Merck (Worli, Mumbai, India).
Formic acid	AR Grade	
Dichloromethane	HPLC Grade	
Sodium Hydroxide	GR Grade	
Diethyl Ether	AR Grade	
Blank Plasma	Harvested K_2 EDTA blank plasma obtained from Prime_ Hospital, Blood Bank-Hyderabad	

Equipment and LC-MS/MS Assay Conditions

The evaluation was carried using an HPLC system (Shimadzu, Kyoto, Japan) with LC-20AD pumps for solvent delivery, DGU-20 A3 degasser, CTO-AS VP Column oven, and a high throughput SIL-HTC auto sampler. Multiple reaction monitoring (MRM) mode was used to accomplish mass spectrometric detection using an API-4000 triple quadruple instrument (MDS-SCIEX, Toronto, Canada). The data was processed using Analyst software, version 1.4.1, and a turbo ion spray interface in positive ionisation mode was used (SCIEX).

Mass-Spectral Conditions

MS works on the principle of producing ions, which are subsequently separated and identified based on their mass-to-charge ratio (m/z). Scanning the analytes for the parent and its fragment ions in a mass spectrometer is part of technique development. 100 ng/mL solutions of analytes and internal standards were generated in a mobile phase solvent system.

Solution of each analyte / ISTD was infused separately at a flow rate of 10µL using the Hamilton syringe pump and scanned parent and product masses in full scan mode. Mass spectra of each analyte and internal standard were recorded in the range of 50 to 400 amu. Once the parent ion was obtained it was further scanned for product ions using MS/MS mode. Nitrogen gas was used as collision gas, zero air as sheath gas and the resolution was set to unit mass. The fragment ion having higher intensity was selected for multiple reactions monitoring (MRM). After selecting the parent and product ion, compound parameters were optimized in infusion mode and the gas parameters were optimized in flow injection analysis with mobile phase. A "T" connector was used to connect the LC pump and syringe pump to the detector and optimized the gas parameters at a flow rate of 0.3 mL/min. to get appropriate gas parameter values. A Turbo ion spray interface (TIS) operated in positive ionization mode was used for the detection. The MRM transitions monitored were 394.40/291.06 (m/z), 364.4/291.06 (m/z), 398.10/295.06 (m/z) and 368.4/295.06 (m/z) in the Q1 MS full scan spectra for Rubitecan, 9-amino Rubitecan, Rubitecan-D4 and 9-amino Rubitecan-D4 respectively.

Table 3: The optimized chromatographic conditions

Parameter	Condition
Mobile Phase Solvents	Mobile Phase Buffer: Organic Mixture, 30:70 (v/v)
Columns/Stationary phase	Kromasil C18 column (150 mm x 4.6 mm i.d., 5 µm)
Flow _Rate	1.0mL/minute, 75% flow splitting
Injection_ Volume	10 µL
Column_Oven_ Temp(°C)	40(°C) ± 3°C
Auto-Sampler_Temp(°C)	5(°C) ± 3°C
Retention_Times	Rubitecan (Analyte-1): 2.396 minutes
	9-amino Rubitecan (Analyte-2): 1.972 minutes
	Rubitecan D4 (ISD-1): 2.382 minutes
	9-amino Rubitecan D4 (ISD-2): 1.976 minutes
Chromatographic_ Run_ Time	5 minutes
Mode-of-Detection	Mass detector
Rinsing_ volume	500µLD
Needle_ Stroke	52mm
Rinsing_ Speed	35µL/second
Sampling_ speed	10.0µL/second
Purge_ time(minutes)	1.0 minute
Rinse_dip_time (minutes)	1 second
Rinse_mode	Before and after aspiration

The quadruples 1 and 3 were set at unit resolution and had a dwell period of 200ms each transition. The following are some of the most common mass-spectral properties used in this method:

Mode-of-Synchronization : LC Sync
Ion_Source : Turbo_Ion_ Spray (TIS)
Type-of-Scan : Multiple_ Reaction_ Mode (MRM)
Polarity
Rubitecan : Positive
9-amino Rubitecan : Positive
Rubitecan-D4 : Positive(+Ve)
9-amino Rubitecan D4 : Positive(+Ve)

MRM-Transition	Rubitecan	:	394.40./29106 (m/z)
	9-amino Rubitecan	:	364.4/291.06 (m/z)
	Rubitecan-D4	:	398.10/295.06 (m/z)
	9-amino Rubitecan D4	:	368.4/295.06 (m/z)

Preparation of Primary-Standard-Stock-Solutions

A 99.9% pure Rubitecan D4 (ISD-1) and 9-amino rubitecan and 9-amino Rubitecan–D4 (ISD-2) are acquired from Sigma-Aldrich, Hyderabad. Rubitecan was obtained from M/S. Samex Overseas, Surat-I, India, used as standard reference materials to prepare the stock solutions for both calibration and quality control standards.

Preparation of Rubitecan (Analyte-1) primary Stock Solution 100 µg/mL

Accurately weighed Rubitecan standard equivalent to 2.5mg of Rubitecan and add appropriate volume of Methyl-Alchol to make final concentration of Rubitecan equivalent to 100µg/mL.

Preparation of 9-amino Rubitecan (Analyte-2) primary Stock Solution, 100µg/mL

Weigh accurately 9-amino Rubitecan reference compound which is equivalent to 2.5mg of 9-amino Rubitecan is dissolved in 10 of Methyl-Alchol to make monophasic-clear and transparent solution. Further it is diluted up to the volume with Methyl-Alchol to make final concentration of 9-amino Rubitecan equivalent to 100µg/mL.

Preparation of mixed intermediate working solutions of analytes

The combined working solutions of Rubitecan and 9-amino Rubitecan were prepared by diluting the stock solutions in Methyl-Alchol. The mixed drug intermediate solution (Rubitecan-2.5µg/mL and 9-amino Rubitecan-2.5µg/mL) is made by collecting 1.25 mL of Rubitecan stock solution (100µg mL^{-1}) and 1.25 mL 9-amino Rubitecan stock solutions (100µg mL^{-1}) into a 50-mL calibrated-volumetric-flask. The final volume was made up to the 50 mL with Methyl-Alchol. All the volumetric measurements were made using calibrated micropipettes.

Preparation of Rubitecan-D4 (ISTD-1) Stock Solution, 100 µg/mL

Accurately weighed deuterated D4-derivative of Rubitecan standard equivalent to 2.5 mg of Rubitecan-D4 and add appropriate volume of Methyl-Alchol to make final concentration of Rubitecan-D4 equivalent to 100µg/mL.

Preparation of 9-amino Rubitecan-D4(Internal-STD-2) Stock Solution, 100µg/mL

Accurately weighed deuterated 9-amino Rubitecan-D4 standard equivalent to 2.5mg of 9-amino Rubitecan-D4 and add appropriate volume of Methyl-Alchol to make final concentration of 9-amino Rubitecan-D4 equivalent to 100µg/mL. Correct the final concentration for 9-aminoRubitecan-D3 accounting for its potency and the amount weighed.

Preparation of mixed ISTD dilution (25ng/mL of Rubitecan-D4 & 25ng/mL of 9-amino Rubitecan D4)

Take 25µL of Rubitecan-D4 (ISTD-1, 100µg/mL) stock solution and 25 mL of 9-amino Rubitecan D4 (Internal-STD-2, 100µg/mL) stock-solution into a 100mL volumetric flask with methanol. The solution further diluted up to 100mL with the diluent solvent to get each concentration of 25 ng/mL of Rubitecan-D4 & 25 ng/mL of 9-amino Rubitecan D4.

Preparation of spiking stock solutions

Preparation of calibration standards spiking stock

Prepare the CC spiking solutions in diluent using mixed drug and metabolite intermediate solution (Rubitecan-2.5µg/mL & 9-amino Rubitecan 2.5 µg/mL) as described in the table below. Volume of intermediate stock taken for the preparation of calibration standards was as per the tale given below.

Table 4: Preparation of calibration standards spiking stock

Parent Solution Concentration		Volume Taken (mL)	Vol.of diluent in (mL)	Final vol in (mL)	SS Concentration		SS ID
Analyte-1 (pg/mL)	Analyte-2 (pg/mL)				Analyte-1 (picogm/mL)	Analyte-2 (picogm/mL)	
2500000	2500000	1.000	4.000	5.000	500000	500000	SS STD1
500000	500000	2.500	2.500	5.000	250000	250000	SS STD2
250000	250000	2.500	2.500	5.000	125000	125000	SS STD3
125000	125000	2.500	2.500	5.000	62500	62500	SS STD4
62500	62500	2.000	3.000	5.000	25000	25000	SS STD5
25000	25000	2.000	3.000	5.000	10000	10000	SS STD6
10000	10000	2.500	2.500	5.000	5000	5000	SS STD7
5000	5000	2.500	2.500	5.000	2500	2500	SS STD8
Analyte-1: Rubitecan (Drug)_Analyte-2: 9-amino Rubitecan (metabolite) Diluent-Formic acid in Methyl-Alcohol (0.1%v/v): Water 50:50v/v)							

Sample Extraction Procedure

Take the needed number of plasma/spiked samples from the deep freezer, defrost them at room temperature or in a water bath kept at room temperature, and vortex the tubes to mix them. Transfer 0.2 mL of material into a pre-labelled tube and follow the sample preparation instructions below. To all the samples except the STD blank, add 50L of mixed ISTD dilution (25ng/mL Rubitecan-D4 & 9-amino Rubitecan–D4) and vortex for around 20 seconds. 50µL of extraction buffer (0.1N sodium hydroxide solution) was added to all samples and vortex for about 20 seconds. 2.5 mL of binary mixture containing Diethyl ether: Dichloromethane, 70:30 (v/v) was added as solvent extraction system. All samples are centrifuged at 4000-rpm for 120-seconds by using refrigerated-centrifuge operated at 10°C ±2°C. The supernatant liquid was transferred in to prelabelled tubes for evaporation to dryness under nitrogen environment at 40 ± 5°C. Reconstitute the dried residual sample with 100µL of mobile phase [Organic Mixture: 10mM ammonium formate, (70:30v/v)] and vortexes thoroughly. Using HPLC-ESI-MS/MS, transfer the required volume of samples into pre-labelled auto sampler vials and inject.

Selectivity

The selectivity of the method towards endogenous plasma matrix was ascertained in six batches of human plasma by analysing blanks and spiked plasma samples at LLOQ concentration. No endogenous peaks were observed at the retention time of the analytes for any of the batches. Method selectivity is proven by evaluating human K2 EDTA plasma blank matrices from eight different individual matrix lots, as well as one lipemic, one heparinized, and one haemolytic plasma lot. During the analyte and ISTD retention durations, no interferences were identified in any of the tested lots when comparing peak responses in blank lots to the mean response of spiked LLOQ samples (n = 6). Figures 3.2, 3.5, and 3.6 show chromatograms of blank plasma, LLOQ sample, and blank plasma with internal standards, respectively, demonstrating the approach's selectivity.

Sensitivity

Six replicates of extracted LLOQ samples were injected against a calibration curve to achieve this. Rubitecan had a lower limit of quantification (LLOQ) of 50 pg/mL and 9-amino Rubitecan had an LLOQ of 50 pg/mL. The accuracy (percent RE) for all three analytes was between 98 and 100 percent at LLOQ, with a percent CV of less than 2%. Rubitecan and 9-amino Rubitecan had mean signal to noise ratios of 98.6 and 99.2, respectively, at LLOQ (n=6). All of the outcomes were deemed to be within acceptable bounds.

Linearity

Matrix-based calibrators must cover the complete calibration range. A minimum of five to eight concentration levels should be used to cover the dynamic linear range, excluding the blank and zero samples. All three calibration curves were reviewed during the course of the inquiry validation were linear for the standards ranging from 50 pg/mL to 10000 pg/mL for both Rubitecan and 9-amino Rubitecan. Least square regression analysis was used to create a straight-line fit through the data points, and a constant proportionality was seen. Back-calculated calibration concentration was obtained to find the appropriate weighting factor. The 1/x2 weighting factor produced the best results. The most exact parameter estimates are obtained by using weighted least squares with weights that are inversely proportional to the variance at each level of the explanatory variables.

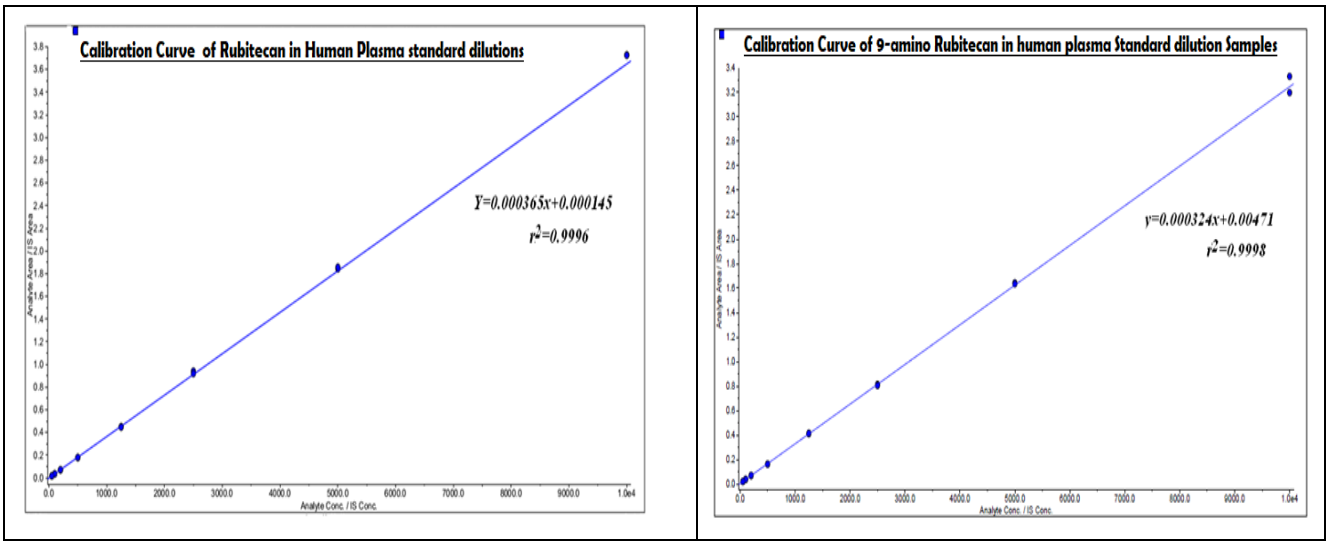

Figure 1: Calibration-Curve- of Rubitecan and 9-aminoRubitecan in human plasma

Precision and Accuracy

Each precision & accuracy run consisting of QC samples (6 replicates each of the LLOQC, LQC MQC and HQC) were analyzed and back calculated against a set of calibration curve standards. Inter and intra batch accuracy and precision evaluation was done using four different 4

batches analysed on different days. Results of inter and intra batch accuracy and precision were presented in *table 2.8.* For Rubitecan, the precision (%CV) for intra batch and inter batch is ≤ 5.71 and ≤ 5.10 respectively for all control samples. For all control samples of 9-amino Rubitecan, the precision (percent CV) for intra batch and inter batch is 5.47 and 6.68, respectively. The percent bias for Rubitecan ranged from -6.67 to 5.00 for intra batch and -4.00 to 3.75 for inter-batch bias. The percent bias for 9-amino Rubitecan ranged from -7.80 to 2.60 for intra batch and -6.00 to -0.25 for inter-batch. Table 3 shows the details of the findings.

Table 5: Summery of Intra- and Inter batch Precision and accuracy study

QC	Rubitecan (picograms/mL)				9-aminoRubitecan (picograms/mL)			
Intra-batch	LLOQ- QC-(50)	LQC-(150)	MQC-(4000)	HQC-(8000)	LLOQ-QC(50)	LQC-(150)	MQC-(4000)	HQC -(8000)
Mean	48.7	140	4070	8200	51.3	141	3910	7910
SD	1.71	4.16	43.9	43.8	2.68	2.59	30.8	86.2
%CV	3.51	2.97	1.08	0.53	5.22	1.84	0.79	1.09
% Bias	-2.60	-6.67	1.75	2.50	2.60	-6.00	-2.25	-1.13
N	5	5	5	5	5	5	5	5
Inter-batch	LLOQ- QC-(50)	LQC-(150)	MQC-(4000)	HQC-(8000)	LLOQ-QC(50)	LQC-(150)	MQC-(4000)	HQC -(8000)
Mean	47.2	144	4180	8400	46.1	140	4010	7980
SD	1.26	3.21	48.5	147	2.52	4.21	31.6	101
%CV	2.67	2.23	1.16	1.75	5.47	3.01	0.79	1.27
% Bias	-5.60	-4.00	4.50	5.00	-7.80	-6.67	0.25	-0.25
N	5	5	5	5	5	5	5	5

Matrix effect

Co-eluting matrix components can hinder or encourage ionization but may not result in a quantifiable response in matrix blanks due to the selectivity of MS detection, impacting the method's precision and accuracy. To investigate the matrix effect, blank plasma was taken from six different sources, including one hemolytic and one lipemic lot. The residue from each blank lot was reconstituted with mobile phase containing a set amount of analyte (LQC level plus ISTD; post extracted samples) and compared to equivalent aqueous samples after extraction. The response ratio obtained from each of the post extracted matrix lots was compared to the mean response ratio of aqueous samples. There was no significant signal suppression/enhancement owing to endogenous plasma matrix during the retention periods of Rubitecan, 9-aminoRubitecan, Rubitecan-D4, and 9-aminoRubitecan-D4 using post column infusion. The percent mean accuracy of back calculated concentration for LLOQ samples from six different matrix lots was 93 percent with a precision of 5.59 percent for Rubitecan and 96 percent with a precision of 5.98 percent for 9-aminoRubitecan. The mean IS normalized matrix factor for all the three analytes was ranges between 0.96 to 1.02 with a % CV of 3.08 as shown in Table-2.9 (A) and (B)

Freeze and Thaw Stability

In practice, it is common to have to subject study materials to repeated freeze-thaw cycles before getting final analytical results. The stability of freeze-thaw (FT) was tested at the LQC and HQC levels. The stability samples were frozen at -205°C and -78°C for the freeze cycles, while the thaw cycles were performed at room temperature without assistance. The initial freeze cycle lasted 24 hours, with successive cycles lasting 12 hours. Results are depicted in Table 6.

Ruggedness

The toughness test was conducted using two different analysts, instruments, and columns. A different analyst looked at the first batch, a different column looked at the second batch, and a different piece of equipment looked at the third batch. Rubitecan had a precision of 8.04 percent and a bias of 12.92 percent in all tests, while 9-amino had a precision of 8.04 percent and a bias of 12.92 percent in all investigations. Both 3.22 percent precision and 8.00 percent mean bias are allowed limits of 15 percent precision and 8.00 percent mean bias, respectively. The locations differed just little, and the retention times were fairly constant. The method's robustness is demonstrated by the great degree of consistency of detector responses and retention times. Table-2.12 contains the details of the experiment

Table 6: Freeze-Thawing stability data of Rubitecan and 9-amino rubitecan

Freeze-Thawing stability data of Rubitecan (analyte-1)								
Replicate Number	**Freeze-Thawing (-20±5⁰C) (LQC- 150 pg/mL)**		**Freeze-Thawing (-78±8⁰C) (LQC-150 pg/mL)**		**Freeze-Thawing (-20±5⁰C) (HQC-8000pg/mL)**		**Freeze-Thawing (-78±8⁰C) (HQC-8000pg/mL)**	
	Cycle-1	**Cycle-2**	**Cycle-3**	**Cycle-4**	**Cycle-1**	**Cycle-2**	**Cycle-3**	**Cycle-4**
1	145	143	143	138	8320	8400	8180	8200
2	148	140	143	150	8200	8350	7980	8510
3	146	143	137	146	8320	8410	8210	8250
4	143	138	143	141	8290	8150	8370	8450
5	138	149	146	137	8310	8430	8540	8250
Mean	144	143	142	142	8290	8350	8260	8330
S.D.	3.81	4.16	3.29	5.50	50.7	115	211	138
% CV	2.65	2.91	2.32	3.87	0.61	1.38	2.55	1.66
% Accuracy	96.00	95.33	94.67	94.67	103.63	104.38	103.25	104.13
% Bias	-4.00	-4.67	-5.33	-5.33	3.63	4.38	3.25	4.13
N	5	5	5	5	5	5	5	5
Overall % CV		1.98						
Freeze-Thawing stability data of 9-amino Rubitecan (Metabolite)								
Replicate Number	**Freeze-Thawing (-20±5⁰C) (LQC- 150 pg/mL)**		**Freeze-Thawing (-78±8⁰C) (LQC-150 pg/mL)**		**Freeze-Thawing (-20±5⁰C (HQC-8000pg/mL)**		**Freeze-Thawing (-78±8⁰C (HQC-8000pg/mL)**	
	Cycle-1	**Cycle-2**	**Cycle-3**	**Cycle-4**	**Cycle-1**	**Cycle-2**	**Cycle-3**	**Cycle-4**
1	~124	141	144	136	8230	7900	8040	8010
2	137	143	138	135	8150	7910	7880	8020
3	131	136	132	137	7870	7920	7920	8120
4	141	132	137	148	8030	7950	7900	8230
5	140	143	140	143	8000	7990	8190	8080
Mean	135	139	138	140	8060	7930	7990	8090
S.D.	7.09	4.85	4.38	5.54	139	36.5	130	89.3
% CV	5.25	3.49	3.17	3.96	1.72	0.46	1.63	1.10
% Accuracy	90.00	92.67	92.00	93.33	100.75	99.13	99.88	101.13
% Bias	-10.00	-7.33	-8.00	-6.67	0.75	-0.88	-0.13	1.13
N	5	5	5	5	5	5	5	5
Overall % CV		2.54						

Table 7: Stability studies of Rubitecan and 9-amino Rubitecan in Human plasma at two QC levels (n=5)

Stability Condition	**Analyte**	**Nominal Concentration (ng/mL)**	**Calculated concentration**	
			Mean ± SD	**% Bias**
Bench Top stability	Rubitecan	6	5.94±0.240	-1.00
		480	477±9.560	-0.63
	9-aminoRubitecan	1500	1360±27.9	-9.33
		120000	117000±836.66	-2.50
Wet extract Stability	Rubitecan	6	6.07±0.113	1.17
		480	481±7.530	0.21
	9-aminoRubitecan	1500	1410±42.1	-6.00
		120000	122000±1140	1.67
Freeze thaw stability after 5 cycles at -20°C	Rubitecan	6	6.69±0.243	11.50
		480	463±8.44	-3.54
	9-aminoRubitecan	1500	1630±74.8	8.67
		120000	119000±2610	-0.83
Freeze thaw stability after 5 cycles at - 78°C	Rubitecan	6	6.56±0.254	9.33
		480	469±8.26	-2.29

	9-aminoRubitecan	1500	1650±53.9	10.00
		120000	122000±1870	1.67
Auto sampler Stability	Rubitecan	6	6.18±0.166	3.00
		480	498±15.3	3.75
	9-aminoRubitecan	1500	1580±56.3	5.33
		120000	128000±4760	6.67

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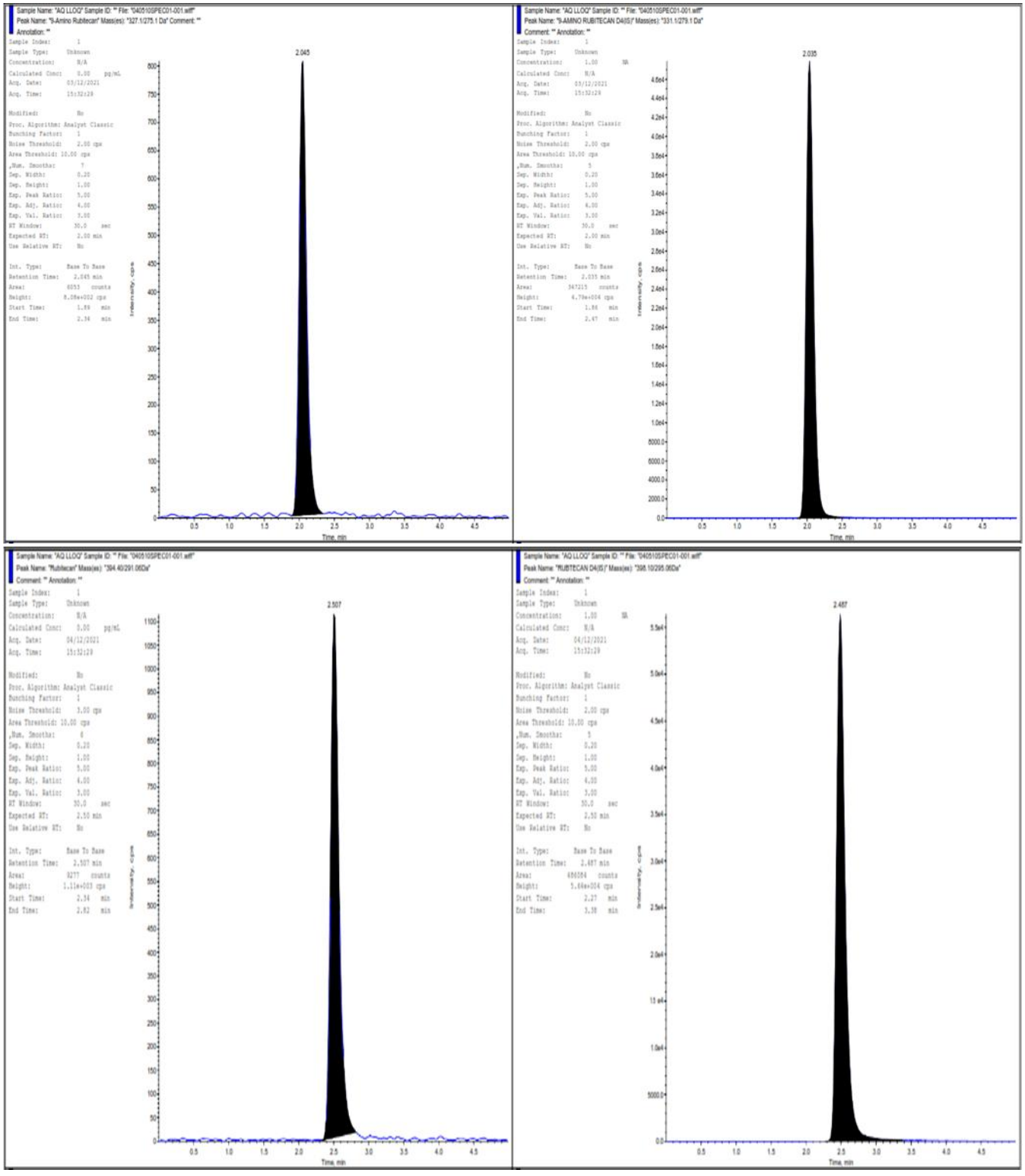

Figure 2: Typical-LLOQ-chromatograms of (A) Rubitecan and (B) 9-amino Rubitecan in matrix with Deuteurated-internal-Standards

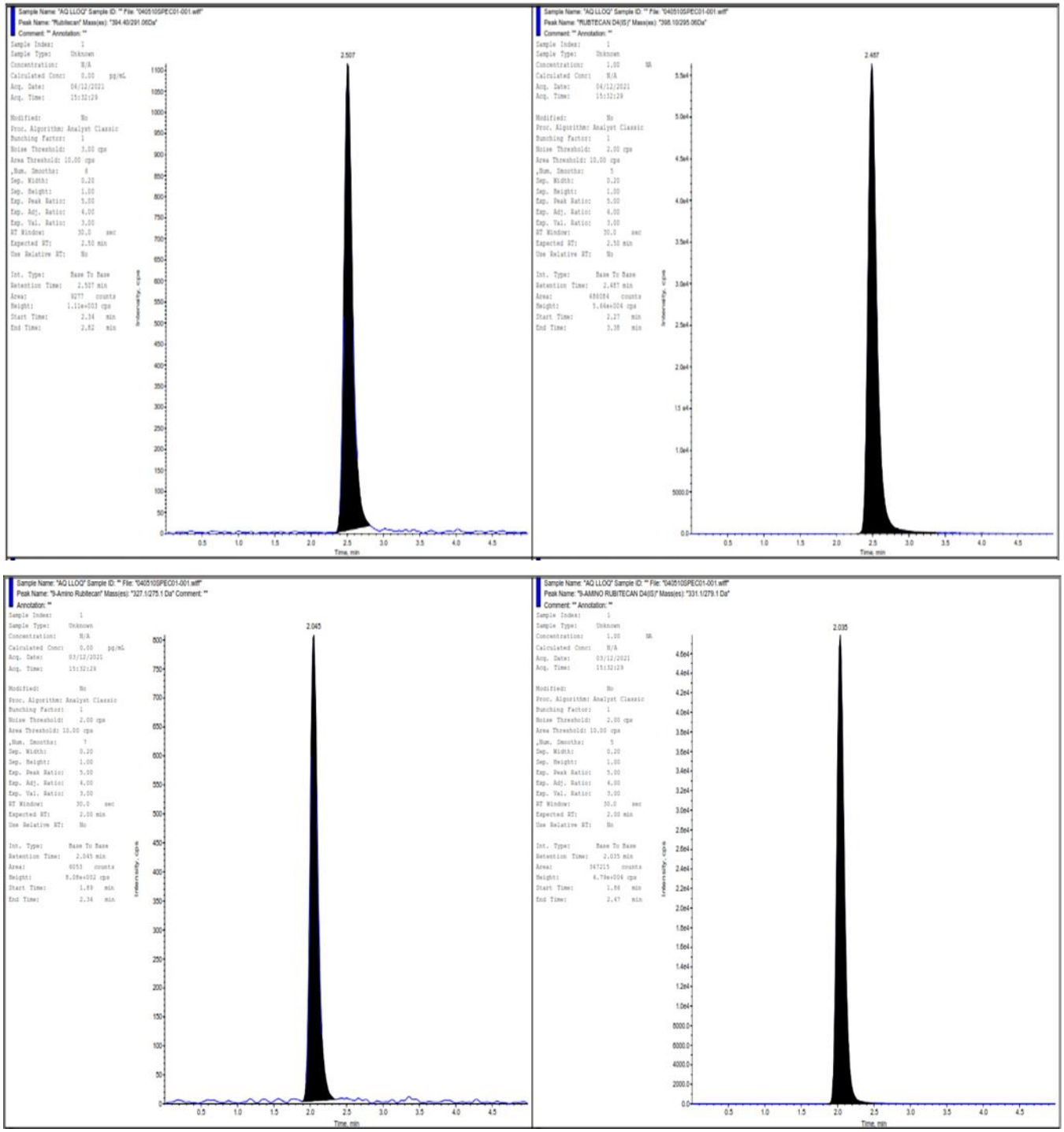

Figure 3: Typical-ULOQ-chromatograms of (A) Rubitecan and (B) 9-amino Rubitecan in matrix with Deuteurated-internal-Standards

CONCLUSION

A simple, rapid, and robust LC–MS/MS technique was established and validated for the simultaneous measurement of Rubitecan and 9-aminoRubitecan in human K2EDTA plasma. The method was validated in accordance with the US Food and Drug Administration's "Guidance for the Industry: Bioanalytical Method Validation, 2001" standards. The mass spectra of each analyte were obtained independently, and the most abundant product ions were chosen for MRM. The MRM transitions observed in the Q1 MS full scan spectra for Rubitecan, 9-aminoRubitecan, Rubitecan-D4, and 9-aminoRubitecan-D3 were m/z 344.10, 229.20, 347.30, and 232.20, respectively, with a dwell duration of 200 ms per transition with quadruples 1 and 3 set at unit resolution. Deuterated derivatives of Rubitecan-D3 and 9-aminoRubitecan-D3 were chosen as internal standards based on their physicochemical features and compatibility with the mobile phase. The specificity/selectivity was tested using several types of plasma, including hemolyzed and lipemic plasma.

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