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MS/MS Method Validation for the Quantitative Determination of Vadadustat in *In-Vitro* Spiked Saliva

[1]Kishore Konam,[2]Dr Koduru Swathi, [2]E.Sushma, [*] Dr Mitta Chaitanya

[1]School of Pharmacy, Dhamma Dipa International Buddist University, Bishnupur, Manu Bankul, Sabroom, South Tripura 799145

[2]Assistant Professor, [*]Associate Professor, Department of Pharmaceutical Analysis,Bojjam Narsimhulu Pharmacy College for Women, Hyderabad 500059

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KEYWORDS

lorem ipsum, Vadadustat, LOD, LOQ, ICH Q2 (R1), LC-MS/MS

ABSTRACT:

Introduction: Vadadustat is an orally administered hypoxia-inducible factor prolyl hydroxylase inhibitor (HIF-PHI) developed for the treatment of anemia associated with chronic kidney disease (CKD). It mimics the body's natural response to hypoxia (low oxygen levels) by stimulating endogenous erythropoietin (EPO) production, thereby increasing red blood cell (RBC) levels.

Objectives: To prevent frequent blood withdrawal during bio analysis,there is a necessity to develop convenient method for patients.So far there are no existing methods for determination of the same in in-vitro saliva by LC-MS/M which rapid in estimation.

Methods: The max of Vadadustat was observed at 245 nm by UV spectrometry establishing a very good linearity along with sensitivity. The detection limit (LOD) =0.2845µg mL-1 and quantitation limit (LOQ)= 0.9013 µg mL-¹ were obtained from the linear concentrations taken in the range of 1.5-15 µg mL-1. The correlation coefficient (r2) of 0.999 was found. The method validation parameters according to ICH Q2 (R1) were performed.

Results: The developed method described here UPLC-MS/MS method was found to be novel, sensitive and rapid with improved results when successfully tested for human saliva samples without significant differences in the steady state Vadadustat concentrations.

Conclusions: Current method could answer the safety issues during therapeutic drug monitoring and pharmacokinetic behavior of the drug when tested clinically.

1. Introduction

Tyrosine kinase inhibitor and anti-cancer drug Vadadustat is used to treat chronic myelogenous leukemia and other advanced cancers. Its formal chemical name is 2-(2-(2,3-dichlorophenyl)acetyl)-1,3,4-oxadiazole-5-carboxylic acid[1]. Vadadustat is a white-to-off white, crystalline powder that is slightly soluble in water; soluble in organic solvents like DMSO, methanol, and ethanol.Vadadustat is an orally administered hypoxia-inducible factor prolyl hydroxylase inhibitor (HIF-PHI) used to treat anemia associated with chronic kidney disease in adults undergoing dialysis. Upon oral administration, vadadustat is absorbed and undergoes extensive metabolism, primarily through glucuronidation by uridine diphosphate-glucuronosyltransferase (UGT) enzymes. Following a single radiolabeled oral dose in healthy adults, approximately 85.9% of the administered dose is recovered: 58.9% in urine (with less than 1% as unchanged drug) and 26.9% in feces. This indicates that Vadadustat is extensively metabolized, with renal excretion playing a significant role in the elimination of its metabolites. The drug's pharmacokinetic profile supports its once-daily dosing regimen, providing a convenient oral alternative to injectable erythropoiesis-stimulating agents for patients with anemia due to chronic kidney disease. Few UV spectroscopy methods were reported [1-6] Much literature is available on the

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liquid chromatographic determination as well as detection with hyphenated mass spectrometric method for simultaneous estimation of tyrosine kinase inhibitors have been reported [7-15]. The findings could suggest that the gradient elution and the simple, economical bio-analytical methods with lower costs and high sensitivity which are validated are necessary to analyze huge samples clinically in therapeutic drug monitoring. Current study involves developing a novel bio-analytical method in order to quantify the Vadadustat in human saliva by LC-MS/MS and UV spectrometry. Studies have shown a positive correlation between salivary and plasma IM concentrations, suggesting saliva as a viable surrogate matrix for assessing systemic drug exposure. This simplifies monitoring and reduces costs compared to frequent blood draws.Stability indicating study following ICH guidelines were executed [16-20].

2. Objectives

Instruments used:

ELICO SL 210 UV-VIS spectrophotometer with two sample holders was employed. For noting down the absorbance, sample filled with quartz cuvettes were taken. Samples weighed on balance having readability of 0.0001 g. Acquity SDS Ultra Performance LC system (Waters, India) for liquid-liquid extraction method coupled with MASS spectrometer Mass Lynx version 4.1 SCN805 was used.

Standards and Reagents: Vadadustat reference standard was obtained as a gift sample from Hetero Labs, Sanathnagar , Hyderabad. Standard drug in its dosage of 400 mg procured from store, Hyderabad, Telangana. The drug has showed complete solubility in distilled water which was used as a solvent throughout the experiment. Acetonitrile, formic acid and ammonium acetate were of HPLC grade. Chemicals used in the study were of highest analytical grade.**UV-Vis Method**

Standard stock solution of Vadadustat:

VDS was precisely weighed at 10 mg, then taken into a flask with 10 mL capacity. A little amount of distilled water was added to dissolve, and the volume (1000 µg mL^{-1}) was made up with the same. Pipetted 1.0 mL from the above into a flask having 10 mL capacity, filled with water until graduation, to get 100 µg mL^{-1} of strength. In order to establish the lambda max and determine the linearity, the dilutions from the stock solutions were further processed to create a 10 µg mL^{-1} solution, which was then scanned by a UV-Vis spectrophotometer in the range of 180-380 nm. The results are presented in Fig. 1.

3. Methods

METHOD VALIDATION

Precision:

Both the intra-day and inter-day accuracy of the approach were investigated. It involved evaluating the medication Vadadustat (10 µg mL^{-1}) six times during the day. The outcomes are shown in Table III. By measuring the same concentration of solution under several conditions such as a different day, analyst, and instrument inter day precision was ascertained. The data gathered were those shown in Table V.

Study of Linearity & Range:

Using the appropriate aliquot of samples from the stock solution, a series of concentrations in the range of 1-12 µg mL^{-1} as displayed in Fig. 2 were created. These solutions absorbances were measured at 258 nm after the volume was adjusted to the mark using diluent (Table I), and a calibration curve plotting absorbance vs. concentration was created. The approach was discovered to be linear between 1 and 12 µg mL^{-1}.

Limit of Detection and Limit of Quantification: According to ICH recommendations, the LOD and LOQ of Vadadustat were calculated using the slope method and a standard error of response, discovered as 0.8977 and 0.2925 µg mL^{-1} respectively.

Accuracy: Three distinct levels of triplicate absorbance, namely 50 %, 100 %, and 150 %, were measured. By adding a specified quantity of a standard solution to the previously examined sample solution, recovery tests were conducted. The percentage recovery was then determined using the method.

Robustness: Three aliquot of 10 ppm test solution were prepared and was scanned at wavelength of +1 nm of λ_{max}. The absorbance values were tabulated in table IV.

Ruggedness: The table V provides information on the level of test findings repeatability. The reproducibility

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was tested by two independent analyzers on different days, which shows that the procedure was built to withstand harsh conditions.

DRUG STABILITY STUDIES

Acid And Base Degradation:
To a 25 mL volumetric flask, 2.5 mL of VDS and 5 mL of 0.1 N HCl/0.1 N NaOH were transferred from the aforementioned stock solution (100 µg mL^{-1}). After 24 h, 0.1 N NaOH/0.1 N HCl in a volume of 5 mL was used in order to neutralize the solution.The solution was left to stand at room temperature. With the goal of achieving a concentration of 10 µg mL^{-1}, the final volume was adjusted to the proper level. Each 25 mL volumetric flask contained 5 mL of 0.1 N HCl and 5 mL of 0.1 N NaOH, which were brought up to the specified level with distilled water (blank solutions) and then tested for absorbance.

Oxidation (Hydrogen peroxide):
In a 25 mL volumetric flask, 2.5 mL of VDS and 5 mL of 0.3 % V/V H_2O_2 were added after 2.5 mL of the aforementioned stock solution (100 µg mL^{-1}) was transferred there from. The mixture was then left unattended for 24 h at room temperature. The solution is ultimately diluted with distilled water to a volume of 25 mL after 24 h. 25 mL capacity flask with 5 mL of 0.3 % H_2O_2, 20mL of water poured up to the lower meniscus (Blank). The absorbance was measured against this blank.

Photolytic conditions:
Vadadustat, weighing about 10 mg, was put in a petri dish and subjected to UV light with a greater wavelength for five h. The drug component was then dissolved in diluent produced up to the volume to produce a 10 µg mL^{-1} solution. For the purpose of generating the major degradants found in pharmacological material, photo stability experiments are carried out.

Solution stability studies:

Bench top and refrigerator: To determine the bench top solution stability up to 8 h, the test solution (10 µg mL^{-1}) is stored on the bench top under standard laboratory conditions and tested at sufficient intervals. By keeping the solution in the freezer and refrigerator for three days each, the solution stability was assessed.

LC-MS/MS ANALYSIS

Sample preparation:
Using the liquid-liquid extraction technique, a sample of human saliva was analyzed. A 100 mL sample of human saliva was mixed with 50 mL of the 0.1 M ammonium acetate solution and 60 mL of the IS working solution (50 µg mL^{-1}) A vortex mixer was used to combine the solutions. After that, 1.2 mL of formic acid was added, and it was gently blended for 5 minutes. For the purpose of evenly mixing the solution, the centrifuge was run at 4 ℃ and 15,000 rpm for around 10 minutes. The extract-containing organic phase was poured into a clean, dry beaker, and the beaker was then dried at a temperature of 40 °C. The resulting trash was collected by centrifugation at 4 ℃ for three minutes after being dissolved in 100 mL of 60% acetonitrile. 20 µl of clear supernatant liquid was poured onto the column for examination.

Application of developed method:
VDS concentrations at a steady state in a sample of human saliva were determined using the developed UPLC-MS/MS method, which was also used to precisely record the sampling time points. Then the human saliva samples were centrifuged (4000 rpm for 5 min at 4 ℃). The saliva supernatant until analysis was stored at -70 ℃. The samples were processed after being freeze-thawed at room temperature.

Mass Spectrophotometric and Chromatographic conditions:
An Acquity Ultra Performance LC system from Waters was used for the analysis. The separation was accomplished by chromatography using an X-Terra column (2.1 mm,100 mm, 3.5 µm, Sigma Aldrich, India). A and B, (40:60 %V/V) were utilized in a binary gradient separation with A serving as the organic phase and 0.1 % formic acid and 0.05 % ammonium acetate serving as the aqueous phase. The mobile phase flow (0.5 mL min^{-1}) was kept constant at during the three-minute test. In contrast to the column temperature, which was limited at no more than 30 ℃ the auto-sampler temperature was set to 5 ℃. The Mass Lynx version 4.1 SCN805 mass

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spectrometer was connected to the UPLC system. The electrospray ionization (ESI-positive ion ES+) source was used to run the mass spectrometer in MRM mode. The standard mass spectra was produced by injecting 1 µg mL^{-1} concentration during gradient elution in the appropriate mobile phase ratio.

In order to comprehend the precursor and product ions, the Vadadustat sample that was run in human saliva that included VDS was analyzed using the Mass Scan function and Daughter Scan function. VDS peaks were most clearly seen at respective frequencies of 494.1 m/z and 394.1 m/z. As a result, the transition m/z 494.5,394.5 in MRM mode was created. The collision energy (CE), a factor that might influence the transition, was steadily optimized throughout a number of injections. To maintain the optimization of the collision energy for 494.5,394.5 m/z, the mode was operated between 10 and 30 V with a 5 V interval between each injection. The parameter optimization process was handled carefully in order to approach the maximum point. The strongest signal for the channel 494.5,394 m/z was consequently acquired at 30 V. The optimal cone power supply was 30 V for Vadadustat and the internal standard. While maintaining a cone gas flow (102 L h^{-1}), the desolvation gas flow (850 L h^{-1}). Mass Lynx Software was used for data processing and collecting.

4. Results

Specificity and recovery:

For the purpose of ensuring that no interfering substances were present, six samples of blank human saliva were administered. Mass spectrum of VDS in its pure form was depicted in Fig.4A whereas the m/z values for daughter ion peaks and spiked human saliva were shown in Fig.4B and Fig.4C respectively. Chromatograms made from blank as pictured in Fig.5A and spiked peaks were compared in order to determine the technique selectivity. The chromatogram of empty human saliva is shown in Fig.5B and 5C. With VDS, shows the normal spiking MRM chromatograms of human saliva. VDS had a retention period of around 0.82 minutes. There were no indications of interference in the retention periods of the analyte or the samples of blank saliva. At each stage of recovery, the test samples (n=5) at various concentration levels were analyzed. Illustrations exhibited with the help of chromatograms in Fig.7 at HQC, in Fig.8 for MQC along with LQC level in Fig.9.

Precision, Linearity, LLOQ and Accuracy:

There were created several concentrations ranging from 0.5 to 500 ng mL^{-1}. While the least squares linear regression (r^2) was determined to be 0.9939, the calibration curve was discovered to be linear as depicted in Fig.6. The LLOQ is 1 ng mL^{-1}, while the LQC is 3 ng mL^{-1}.

UV-Vis chromatographic analysis:

The estimated amount of Vadadustat in bulk and dose form may be determined effectively using the established Spectrophotometric approach. It is discovered that the relative standard deviation is well within the parameters. The accuracy was found to be statistically significant with RSD in the range of 0.9-1.03 % for 50 to 150 % (Table II). The precision was found to be validated (Table III) in the concentration range of 10-50 µg mL^{-1} with RSD 0.89 %. The robustness (Table IV) was found to be validated at 257 nm and 259 nm with RSD 0.12-0.16 %. The ruggedness (Table V) was calculated for two days with two analytes and the RSD was found to be in the range of 0.13-0.17 %.

5. Discussion

UPLC coupled Mass Spectrometric analysis

Based on the molecule's chromatographic behavior (VDS), an Ultra performance liquid chromatographic elution with MS/MS detection was used. This produced the ideal peak shape, and other Suitability parameters were found to be well within the acceptable limits. The range of the mean recovery, which ranged from 90.3 % to 114.5% (RSD: 8.0 %), is shown in tables VI and VII. With ideal MS/MS conditions, the parent and product ion peaks were at 494.1 and 394.1 m/z, respectively. Table VII lists the recovery of quality control samples. Extraction recovery was found to range from 99.3 % to 114.7 % for linear concentrations between 0.5 ng mL^{-1} and 500 ng mL^{-1}. Table VI lists the retention times corresponding to each concentration level with their respective chromatographic data supported in Fig. 10.For the purpose of determining VDS in human saliva and bulk formulations, a brand-new, quick, selective, and highly sensitive UPLC MS/MS assay was created and

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validated. This method allows for a more convenient and patient-friendly approach to monitoring the drug in cancer patients which eliminates the need for time-consuming and potentially discomforting blood collection procedures.Research has established a positive correlation between VDS levels in saliva and plasma, indicating that saliva holds potential as a reliable alternative matrix for assessing the drug's systemic exposure.

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Table I Linearity of Vadadustat by UV-visible spectrophotometer

Concentration [a]	Measured Absorbance
1.5	0.1689
3.0	0.3578
6.0	0.5215
9.0	0.6908
10.5	0.8791
15.0	1.0612

[a] *measured in µg mL^{-1}*

Table II Table showing Accuracy data obtained through UV Visible spectrophotometer

Accuracylevel	Sample (bulk +formulation)	Absorbance	%Recovery	%RSD
50 %	2.5+5	0.5578	99.16	1.03 %
	2.5+5	0.5499	97.76	
	2.5+5	0.5611	99.75	
100 %	5+5	0.7834	97.05	0.903 %
	5+5	0.7953	98.52	
	5+5	0.7962	98.63	
150 %	7.5+5	1.0298	98.56	0.914 %
	7.5+5	1.0268	98.27	
	7.5+5	1.0123	96.88	

Table III Table showing Precision data

S. no.	Concentration [a]	Measured Absorbance
1.0	10	0.8832
2.0	10	0.8795
3.0	10	0.8815
4.0	10	0.8627
5.0	10	0.8842
6.0	10	0.8785
%RSD		**0.89 %**

[a] *measured in µg mL^{-1}*

Table IV Table illustrating Robustness data

S. no	Concentration (µg mL^{-1})	257 nm	259 nm
1	10	0.8512	0.8825
2	10	0.8493	0.8798
3	10	0.8515	0.8826
%RSD		**0.12 %**	**0.16 %**

Table V Table illustrating Ruggedness data

Day 1			Day2	
Analyst 1[a]		Analyst 2[b]	Analyst 1[a]	Analyst 2[b]
Concentration[c]	Absorbance	Absorbance	Absorbance	Absorbance
10	0.8832	0.8568	0.8793	0.8642

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10	0.8795	0.8493	0.8548	0.8756
10	0.8815	0.8536	0.8675	0.8845
%RSD	**0.17 %**	**0.13 %**	**0.14 %**	**0.15 %**

[a] absorbance measured by first analyst [b] absorbance measured by second analyst [c] measured in µg mL^{-1}

Table VI Extraction recovery of Vadadustat in bulk formulation

Drug	Sample name	Conc (ng mL^{-1})	Retention time	Extraction Recovery
vadadustat	BLK	0.4	0.79	-
	BLK	1.2	0.80	-
	CC-1	-	-	-
	CC-2	1	0.83	99.3
	CC-3	4.1	0.81	101.8
	CC-4	22.8	0.80	91.3
	CC-5	114.7	0.81	114.7
	CC-6	249.2	0.80	99.3
	CC-7	391.4	0.82	97.9
	CC-8	474.5	0.82	94.9

Table VII Results of in vitro saliva spiked with Vadadustat QC samples

Drug	Sample name	Concentration (ng mL^{-1})	Retention time	%Recovery
vadadustat	Blank			
	Saliva	136.1	0.72	-
	Saliva	120.6	0.73	-
	HQC-1	435.8	0.82	102.5
	HQC-2	450.6	0.81	450.6
	MQC-1	321.5	0.82	133.9
	MQC-2	242.0	0.81	100.8
	LQC-1	2.7	0.82	90.5
	LQC-2	3.0	0.80	100.9
	LLOQ-1	1.9	0.82	-
	LLOQ-2	1.6	0.82	-

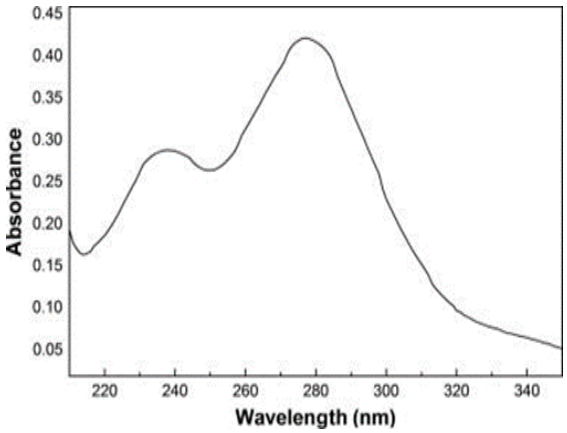

Fig. 1 Spectrum showing Vadadustat in distilled water

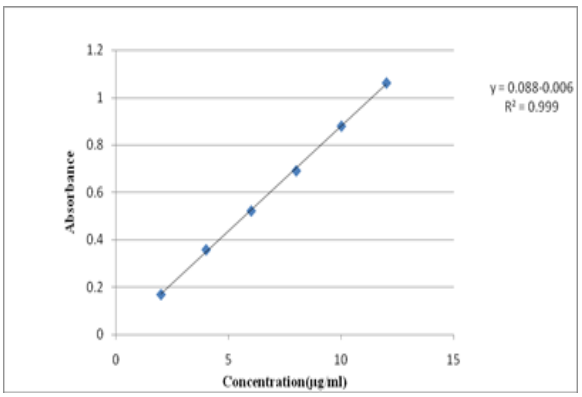

Fig. 2 UV Calibration Curve of Vadadustat at 245 nm

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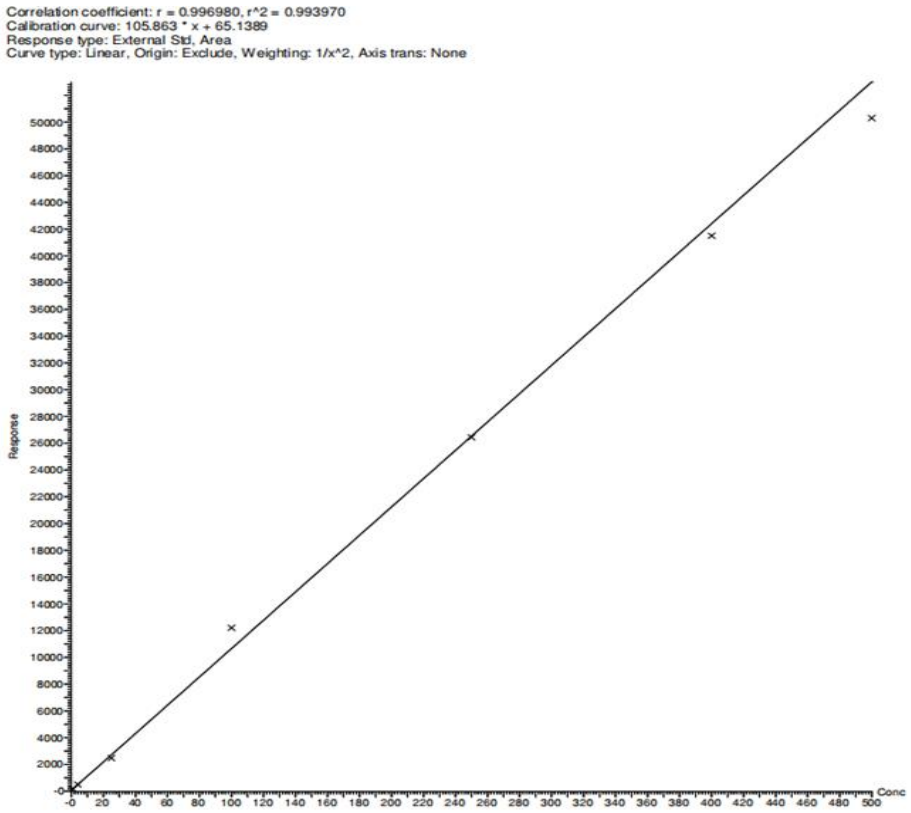
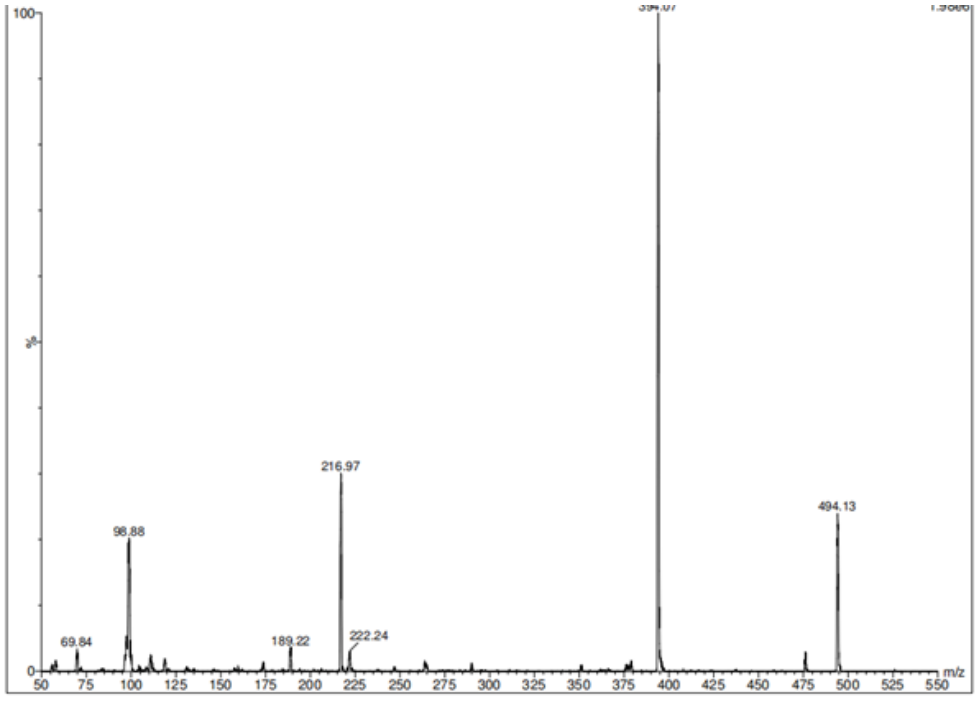


Fig. 3 Calibration curve obtained by LC detection of Vadadustat

(A)



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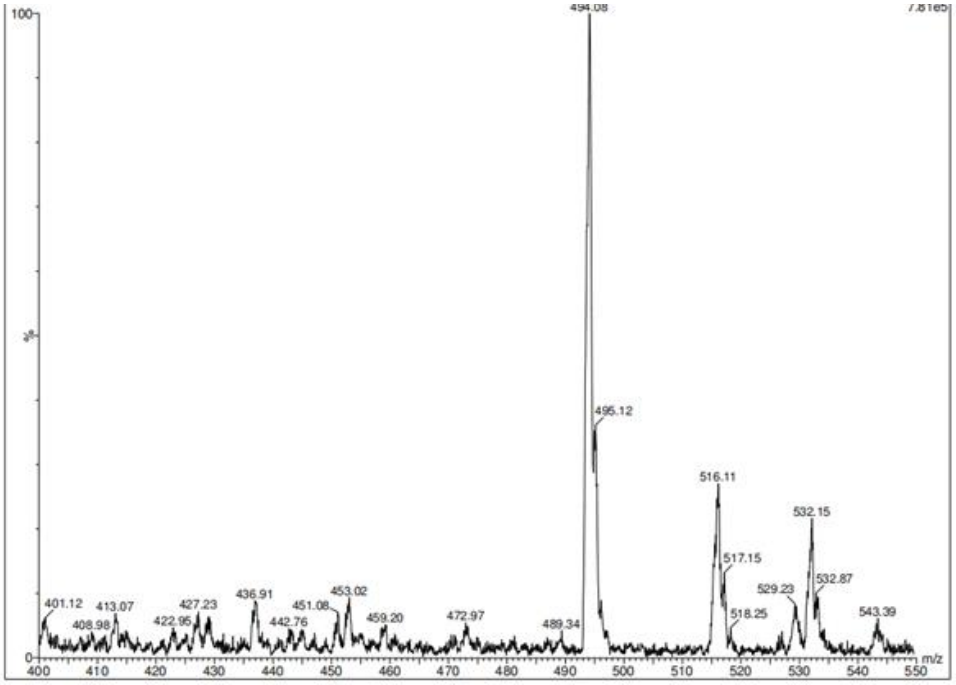
(B)



(C)

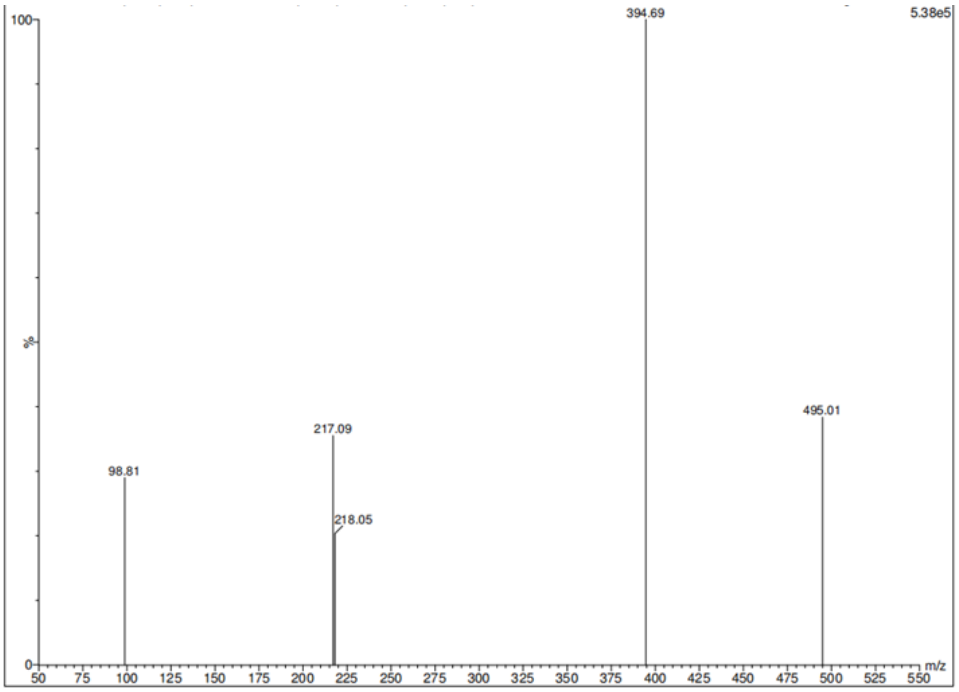


Fig.4 A-Mass Spectra of VDS m/z values in its pure form, B-Mass Spectra of daughter ion peaks in its dosage form, C-MRM spectra showing *In vitro* saliva spiked with VDS

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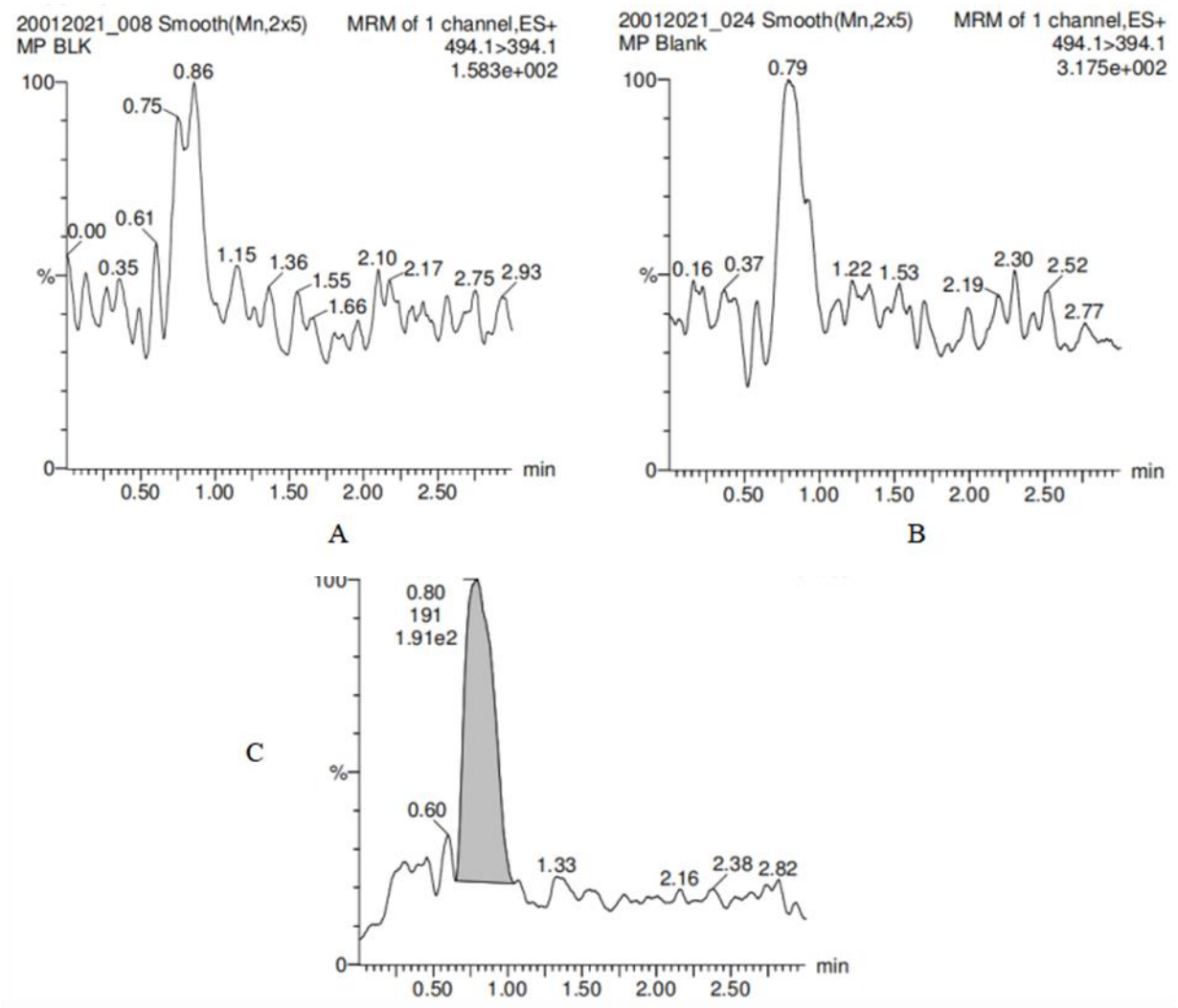
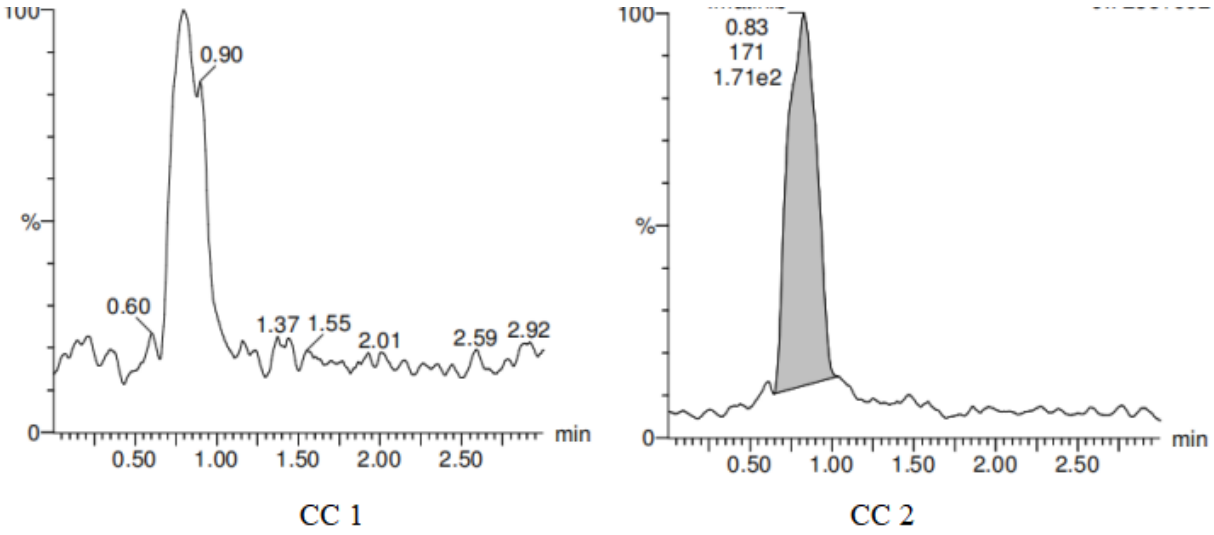


Fig. 5 U P L C C hromatogram S h o w i n g VDS (A-Blank, B, C-Inj-1,2)



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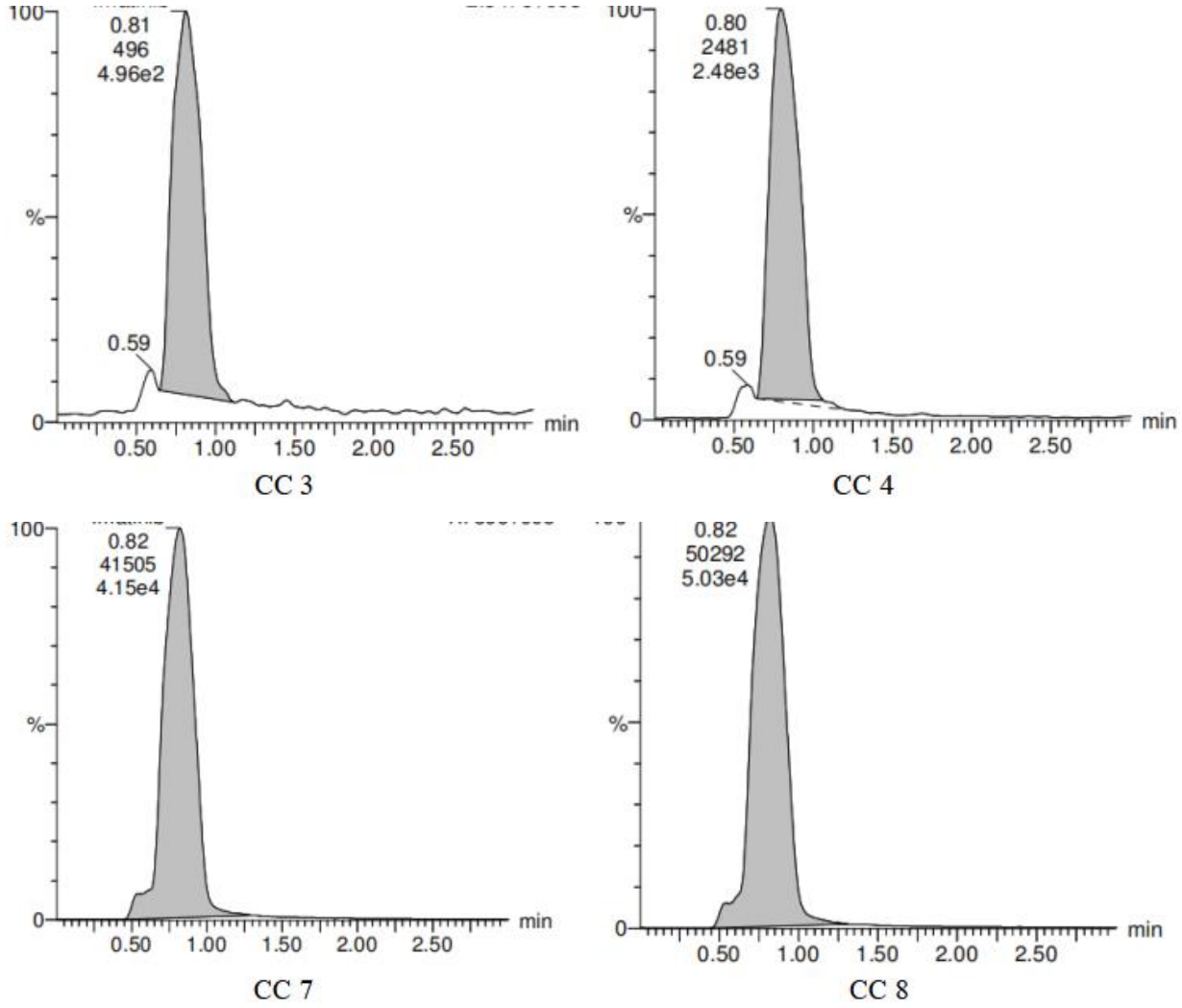

Fig. 6 Linearity chromatograms of V a d a d u s t a t CC 1-0.5ng mL^{-1} to CC 8- 500ng mL^{-1}

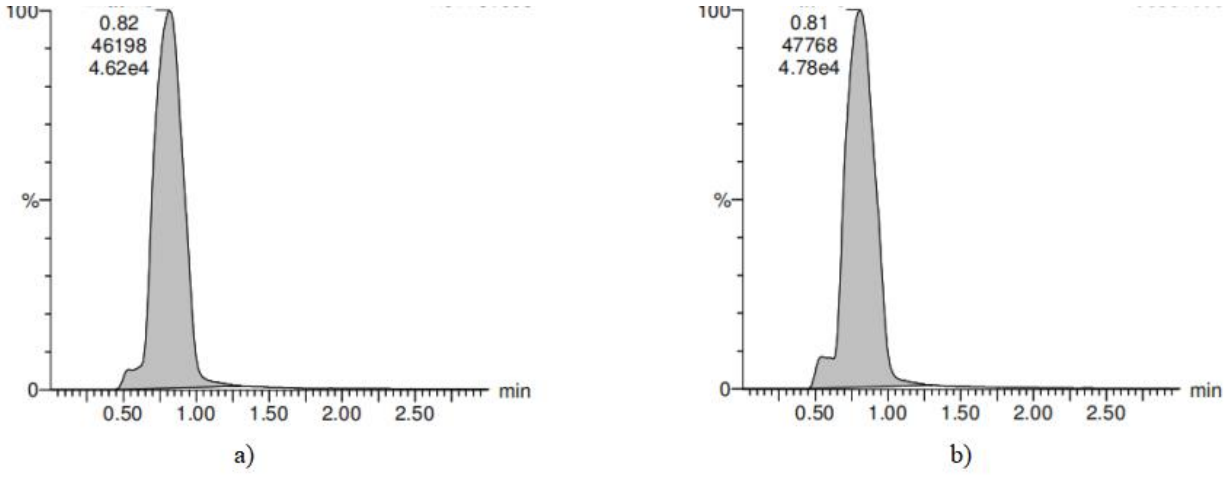

Fig.7 LC chromatograms showing of VDS at HQC Level a) Injection -1 b) Injection-2

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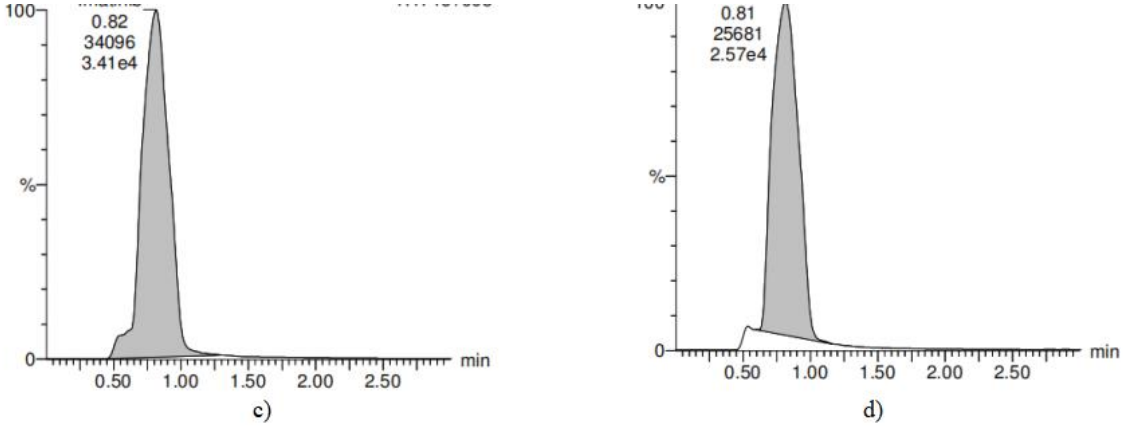

Fig. 8 LC chromatograms showing of VDS at MQC Level c) Injection -1 d) Injection-2

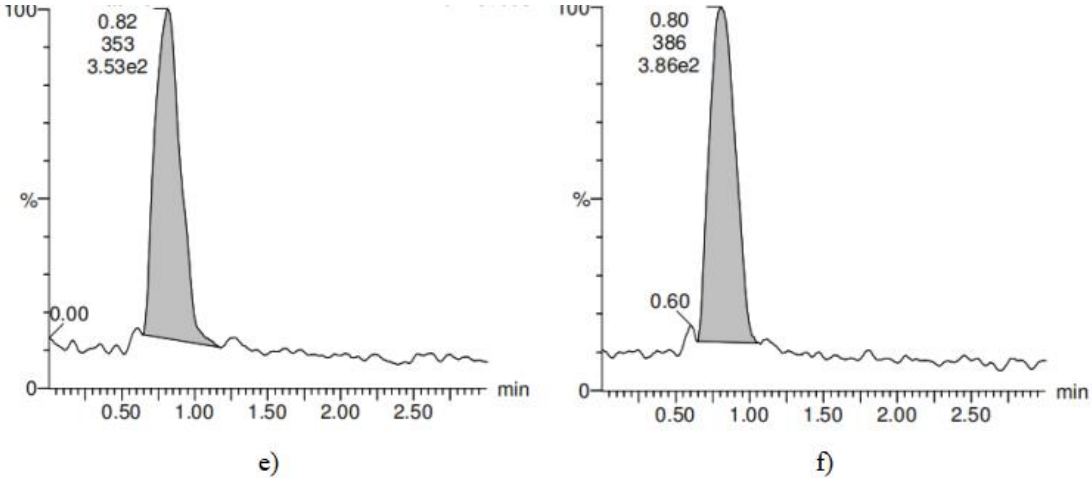

Fig. 9 LC chromatograms showing of VDS at LQC Level e) Injection -1 f) Injection-2

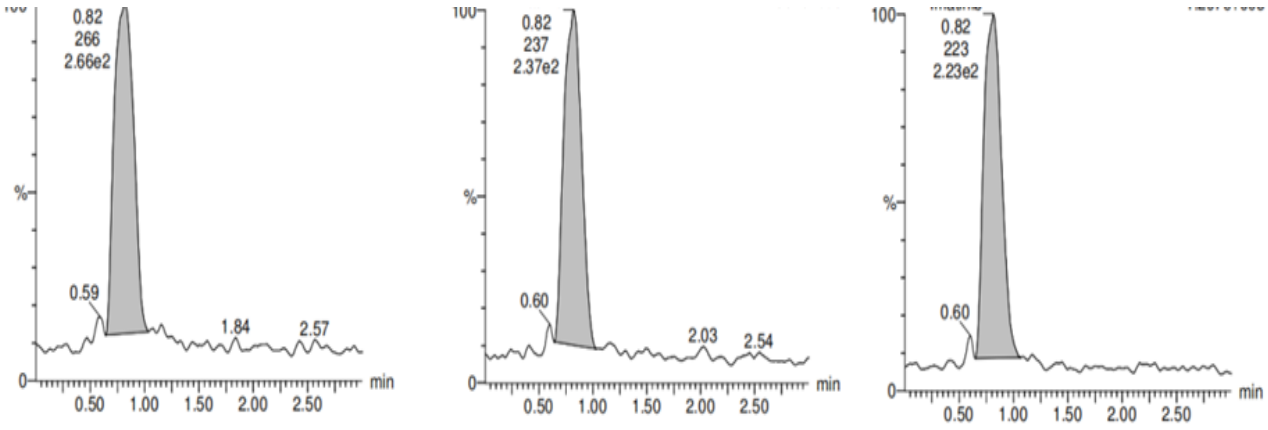

Fig. 10 Chromatograms showing triplicate injections of Vadadustat in human saliva at Lower Limit of Quantification Level