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Bioanalytical Method Development and Validation of Ripretinib in Human Plasma by LC–MS/MS in Accordance with Regulatory Standards

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Abstract

The current methodology was established to quantify ripretinib in spiking human plasma via liquid chromatography-mass spectrometry. The liquid-liquid extraction technique was employed, and chromatographic separation was conducted on a Waters Symmetry Shield, C18 (4.6 mm x 50 mm) analytical column with a mobile phase of 5 mM ammonium acetate (pH 4.0) and acetonitrile in a 50:50 v/v ratio. The positive ion mode was employed to acquire m/z 510.1→m/z 417 for ripretinib and m/z 515.19→m/z 256.12 for telmisartan as the internal standard. The calibration curve exhibited linearity within the 5-200 ng/ml range. The intra- and interday accuracy ranged from 94.73% to 99.84%, with precision (%CV) not exceeding 2% across all quality control levels. The extraction recovery percentage exceeded 95%, indicating exceptional matrix and analyte selectivity (% interference = 0), along with satisfactory stability study outcomes across all types (% nominal 94.38% to 98.96%). The experimental data indicate that the newly proposed method is a validated bioanalytical technique suitable for application in blood samples for pharmacokinetic research.

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Introduction

Tyrosine kinase inhibitor rimatetinib blocks PDGFRA and KIT kinase signals. By attaching to the activation loop and switch pockets, ripretinib deactivates the kinase and blocks subsequent signaling and cell proliferation in both wild-type and mutant cells. The formal name for the compound is 1-(4-bromo-5-\[1-ethyl-7-(methylamino)-2-oxo-1,2-dihydro-1,6-naphthyridin-3-yl]-2-fluorophenyl)-3-phenylurea. At 510.36 g/mol, it possesses the molecular weight and the chemical formula $C_{24}H_{21}BrFN_5O_2$.

Ripretinib is a white or off-white solid with a crystalline structure. Lipophilic and mildly basic, ribetinib is practically insoluble in water. For oral administration, ripretinib is available in the form of 50 mg tablets that are oval in shape and range in color from white to off-white. The inert ingredients in each pill are microcrystalline cellulose, magnesium stearate, hypromellose acetate succinate, lactose monohydrate, crospovidone, and silicon dioxide [1-9].

Riprotinib has been successfully measured in the plasma of rats and beagle dogs using a variety of methods. The impact of voriconazole and itraconazole on the pharmacokinetics of ripretinib was investigated, and a technique was devised to quantify ripretinib concentrations in beagle dogs using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [10]. The concentration of ripretinib in rat plasma was determined using an LC-MS/MS technique[11]. The methods for measuring ripretnib and DP-5439 concentrations in human plasma are still underdeveloped, nevertheless. The pharmacokinetics of ripretinib in the absence of complete quantitative methodology[12] due to the impact of CYP3A initiative, CYP3A inspiration, and stomach acid suppression. A method for measuring the concentration of ripretinib in human plasma using LC-MS/MS[13]. This study set out to create and test an LC-MS/MS method for ripretinib measurement in human plasma.

MATERIALS AND METHODS

Chemicals and Reagents

Ripretinib (RTB)and Telmisartan (TES)were obtained from YARROW CHMICALS,India. All other chemicals and solvents were purchased from MERCK fine Chemicals, Mumbai. Thecontrol humanplasma samplewas procuredfromDoctors PathologicalLabs (Hyderabad,India).Chemicalstructuresare presentedinFigure1.

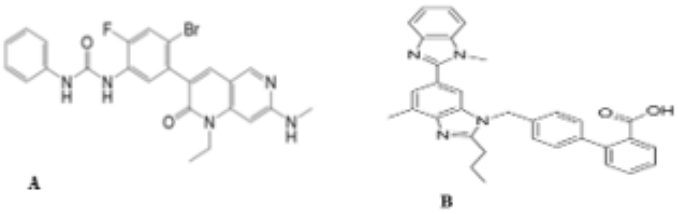


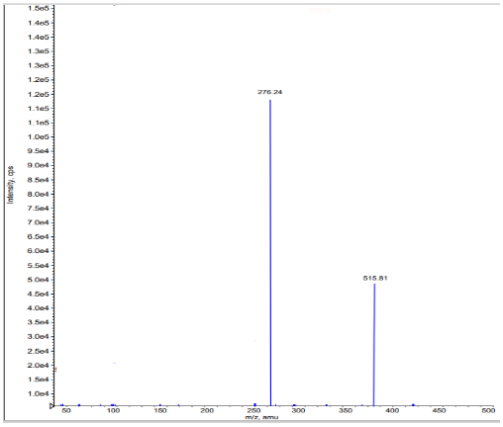
Figure 1. Chemical Structures of A)RipretinibB) Telmisartan.

Instrumentation

HPLC system (1200 Series Agilent Technologies, Germany) connected withtriple quadrupole Mass spectrometer instrument (API 4000, Toronto, Canada). Data processing was performed withthe Analyst 1.4.1 software package (SCIEX)Ionization was performed by electro-spray positive mode with Unit Resolution.

Detection

At a source temperature of 550 °C and an ion spray voltage of 5000 volts, the mass parameters were fine-tuned to obtain the product ions of m/z: 417.10 and 276.24, respectively, from their respective precursor ions of Ripretinib[M+H]+ (m/z: 510.1) and Telmisartan [M+H]+ (m/z: 515.81). All gas channels with nitrogen: 35 psi for heater gas, 22 psi for nebulizer gas, 10 psi for CAD gas, and 22 psi for curtain gas. Without a split, the source flow rate is 500 µL/min. The potentials for



the analyte and internal standard are 65 and 85 V, respectively, during clustering; 45 V for collision energy; and 34 V and 17 V, respectively, for the analyte and internal standard, at the exit of the collision cell. Figures 2.0–3.0 show the mass spectra of the parent and product ions.

Figure 2. Mass spectra (Q1 and Q3) of Ripretinib.

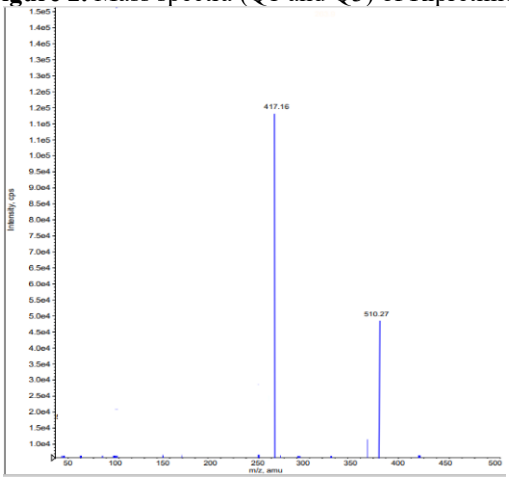


Figure 3. Mass spectra (Q1 and Q3) of Telmisartan.

Chromatographic conditions

The analytical column used for chromatography was a Hypurity advance C18 column (50 mm×4.6 mm, 5 µm; Thermo Electron). The procedure was carried out at 40°C with a mobile phase consisting of 5 mM Ammonium acetate (pH 4.0): Acetonitrile (50:50 v/v) with a flow rate of 0.5 mL/min. For the purposes of chromatography and extractability, TES served as an internal standard. With a total run time of 4 minutes, the drug and internal standard were eluted at 1.51±0.2 and 1.65±0.2 minutes, respectively.

Preparation of Standards and Quality Control (QC) Samples

Methanol was used to generate standard stock solutions of RTB (1000.0µg/mL) and TES (1000.0µg/mL). The TES standard stock solution was diluted with 50% methanol to create the internal standard spiking solution, which had a concentration of 250.0ng/mL. All solutions, including internal standard spiking solutions and standard stock solutions, were kept in the fridge between 2 and 8 degrees Celsius until the analysis. Before analysis, the screened drug-free plasma was mixed with the RTB standardstock solution to achieve concentration levels of 5, 10, 20, 40, 60, 80, 100, and 200 ng/mL for analytical standards and 5 (Lower limit QC), 15 (Low QC), 50 (Mid QC), and 190 ng/mL for quality control (QC) standards. The mixture was then frozen at -80°C. When ready for analysis, the corresponding aqueous standards were reconstitution-dissolved in the mobile phase and kept in the fridge between 2 and 8 degrees Celsius.

Sample preparation

Isolation of the medicine and IS from plasma samples was accomplished by means of liquid-liquid extraction. Specifically, 100µL of IS (50ng/mL) and 100µL of plasma sample (corresponding concentration) were introduced into labelled polypropylene tubes and briefly vortexed for this purpose. Then, 2.0 mL of di-ethyl ether, an extraction solvent, was added and mixed by vortexing for 10 minutes. The samples were subsequently spun in a centrifuge at 5,000 rpm for 10 minutes at a temperature of 20°C. The next step was to transfer the sample supernatant to their own polypropylene tubes. Subsequently, at 40°C, all of the samples were placed in a nitrogen incubator to allow them to evaporate. After the residue had dried, 500 µL of reconstitution solution was added and the mixture was quickly vortexed. The last step was to inject the extracted material into the LC-MS/MS system after transferring it into auto-sampler vials.

Selectivity and specificity

B y pretreating and analyzing six separate blank plasma samples, we were able to gauge the method's selectivity and identify any endogenous chemicals that could interfere with the analyte and IS during elution. The retention durations and MRM responses were used to identify the chromatographic peaks of the analyte and IS. There should be no more than 20% overlap between the mean peak areas of the Lower limit QC of RTB and Baricitib peak areas in blank samples at the relevant retention times. In a similar vein, the blank sample TES peak area at the corresponding retention time shouldn't exceed 5% of the mean LOQ TES peak area. Plasma blank and lower limit QC typical chromatograms are shown in Fig. 4.0 to 5.0.

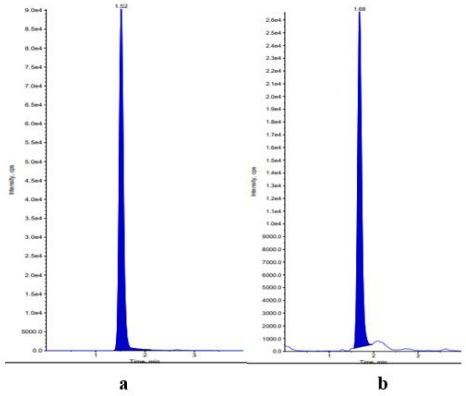


Figure 4. Blank plasma chromatogram of interference-freea) RTB and b) TES (Internal standard).

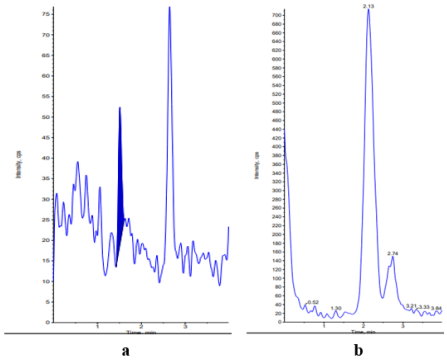


Figure 5. Chromatogram of LLOQ sample a) RTB and b) TES.

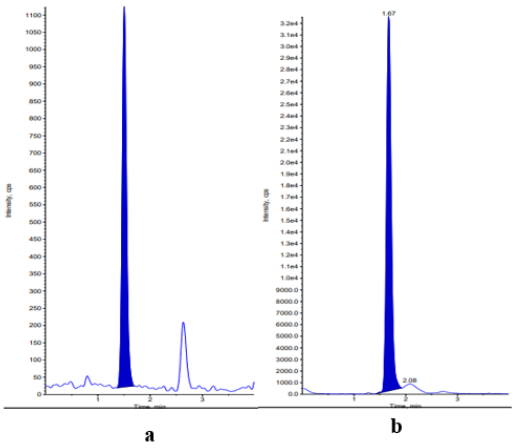


Figure 6. Chromatogram of LQC sample a) RTB and b) TES.

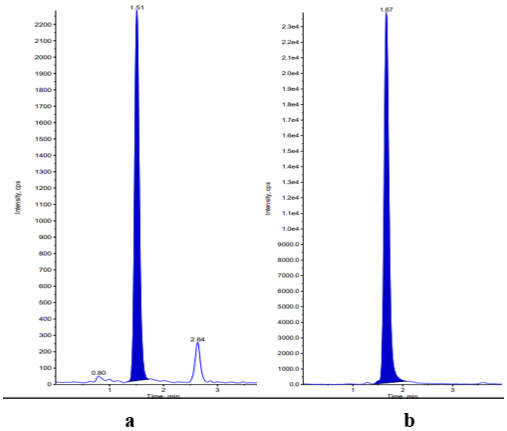


Figure 7. Chromatogram of MQC sample a) RTB and b) TES.

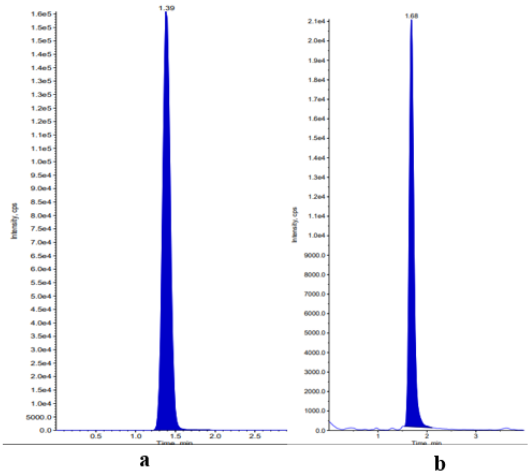


Figure 8. Chromatogram of HQC sample a) RTB and b) TES.

Recovery

Analyzing quality control samples allowed us to determine the extraction recovery of RTB and TES from plasma. By comparing the peak regions of the plasma sample and the standard solution spiked with the blank plasma residue, we were able to determine recovery at three concentrations: 15, 50, and 190 ng/mL. To achieve the necessary recovery, a recovery rate of above 85% was deemed sufficient.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

If you want to know how low a concentration in a sample can go before it becomes undetectable due to noise, you need to know its limit of detection (LOD). The limit of detection (LOD) was established by comparing the test findings of samples with known analyte concentrations to those of blank samples, with a signal-to-noise ratio (s/n) of 3:1. One definition of analyte concentration that can be established with adequate precision and accuracy is the limit of quantitation (LOQ). Testing a series of mobile phase and plasma standards with established RTB concentrations allowed us to determine the LOQ.

Matrix effect

To predict the variability of matrix effects in samples from individual plasma lots, the matrix effect was quantified by determining the matrix factor, which was calculated as follows:

Matrix Factor = Peak response ratio in presence of extracted matrix (post-extracted)
Peak response ratio in aqueous standards

For this study, we used aqueous standards of the same concentration to post-spike six lots of blank biological matrices at the mid-QClevel. Each lot was extracted in triplicate. The coefficient of variation (CV %) represents the overall accuracy of the matrix factor, and it should be less than 15%.

Calibration curve, Precision and Accuracy

A range of 5 to 200 ng/mL of RTBin in human plasma was used to generate the calibration curve. A quadratic model with weighted 1/x2 regression analysis was used to get the calibration curve. The RTB concentration in ng/mL was plotted against the RTB/TES peak area ratio. To ensure accurate results, we created the calibration curve, standard samples, and quality control samples in duplicates (n=5). Regression analysis, which was computed using least-squares regression analysis, was used to assess the method's linearity and the validity of the linear regression. The back-calculated concentrations of the calibration points should be within ≤15 and ±15% of their nominal values, respectively, in terms of precision and accuracy. Nevertheless, the Precision and Accuracy ought to fall between < 20 and ± 20%, respectively, for LLOQ. Tables 1.0 to 2.0 display the results.

Stability (Freeze-thaw, Autosampler, Benchtop, Long-term) ofRTB in Plasma

Samples of low-quality and high-quality controls (n=6) were taken from a deep freezer following three cycles of freezing and thawing in accordance with the clinical protocol. After each of three 24-, 36-, and 48-hour cycles, the samples were frozen at -80 °C. Furthermore, analysis was performed after 60 days of storage at -80oC to assess the long-term stability of RTB in quality control samples. After storing the autosampler tray with control concentrations for 48 hours, researchers examined the stability of the device.

Control concentrations were used to study benchtop stability for 24 hours. In addition to the recently spiked calibration curve standards, stability samples were also processed and extracted. The stability samples should have an accuracy of no more than 15% and a precision of no more than 15% of their nominal concentrations, respectively. To see the outcomes, look at Table 3.0.

Results and discussion

Method development and validation

Clinical pharmacokinetics has made extensive use of LC-MS/MS because to the method's exceptional selectivity, sensitivity, and repeatability. Developing and validating an assay method for the quantitative detection of RTB from human plasma samples that is simple, sensitive, quick, rugged, and reproducible is the main objective of this work. Experiments were conducted to optimize the chromatographic conditions, including the mobile phase's composition and nature, the columns used, and the extraction methods (solid phase, precipitation, and liquid-liquid extraction) in order to achieve the best resolution and increase the signal of RTB and TES. The MS optimization was carried out by directly injecting RTB and TES solutions into the mass spectrometer's ESI source. In order to achieve a better spray shape and achieve better ionization of the protonated ionic RTB and TES molecules, the ESI source's critical parameters, including the needle (ESI) voltage, capillary voltage, source temperature, and other variables like nebulizer gas, heater gas, and desolvation gases, were optimized. In the case of ripretiniband m/z 515.19 → 256.12 telmisartan, the combined ion spectra of RTB and TES showed a large abundance of fragment ions at 510.1→m/z 417. To achieve a rapid and selective LC approach, optimization of chromatographic conditions, including mobile phase, column, and extraction procedure, was carried out after mass spectrometer parameters were fine-tuned. Using a mobile phase consisting of 5 mM Ammonium acetate (pH 4.0): Acetonitrile (50:50 v/v), a flow rate of 0.5 mL/min, and an injection volume of 10 µL, effective separation and elution were accomplished. The best chromatography was achieved by optimizing the following: the Liquid-liquid extraction method; the C18 (4.6 mm x 50 mm) column; and the Waters Symmetry Shield.

Selectivity and Specificity

The use of MRM (Multiple Reaction Monitoring) functions allowed for a highly selective examination of RTB and TES, free of any substances that could interfere. Plasma spiked with 5 ng/mL of RTB and 50.0 ng/mL of TES were used to generate the chromatograms.

Limit of Detection (LOD) and Quantification (LOQ)

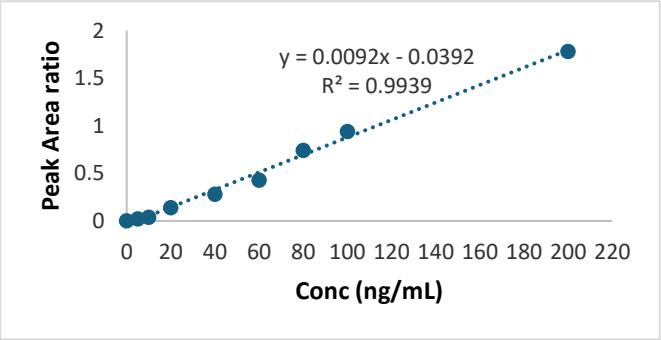
The instrument's detection values for RTB, even at low concentrations, were determined using the limit of detection. S/N values > 3-5 were used to calculate an estimated LOD of 290 pg/mL. The method's limit of quantification was determined to be 755 pg/mL, the lowest quantity shown on the calibration curve.

Matrix effect

The aqueous standard was added to six sets of blank biological matrices in triplicate for both the Low and High QC (Quality control) levels. The samples were then compared with neat standards of the same concentration that were injected alternately. Both the LQC and HQC matrix factors have a precision (%CV) of 0.94 and 0.86, respectively. At the various retention times, neither the IS nor the analyte had any effect on the suppression nor enhancement of ions.

Calibration curve standards,Precision and Accuracy

The peak area ratio (RTB/TES) vs RTB concentration was used to plot the calibration curves. The calibration remained



constant across the concentration range of 5–200 ng/mL. Accuracy levels varied between 97.16 and 98.52%, while the CV% was below 5%. Table 1 shows that all five analytical curves had R2 values close to 1, indicating very good linearity. Every single curve had a determination coefficient (r2) higher than 0.9939. Fig. 9 shows that the approach satisfied the validation criteria for being linear over the concentration range that was examined. During the two days between the repeatability and intermediate precision tests, six individuals were injected with three different concentrations of curcumin working solutions (5, 15, 50, and 195 ng/mL) in triplicate. This allowed for the analysis of intra- and inter-day precision. The accuracy varied between 96.71% and 98.54%, and the intra-batch CV% was below 15%, as demonstrated in Table 2. The accuracy varied between 94.73% and 99.84%, while the inter-batch CV% was under 15%. The results show that this procedure is reliable and reproducible within the analytical range.

Figure 9. Calibration plot of spiked plasma samples.

Table 1. Calibration curve details.

Spiked Plasma Conc (ng/mL)	Concentration Measured(n=5)	*CV (%) (n = 5)	Accuracy %
5.00	5.04	0.32	95.26
10.00	10.24	0.85	97.83
20.00	20.11	0.71	99.36
40.00	40.06	0.26	101.27
60.00	60.03	0.17	97.49
50.00	50.13	0.34	96.32
80.00	80.21	0.65	98.46
100.00	100.42	0.82	98.59
200.00	200.34	0.59	97.47

*Coefficient of variation

Table 2. Precision and accuracy results.

Spiked Plasma Conc (ng/mL)	Within-run			Between-run		
	[a]Concentration Measured (n=6) (ng/mL)	[b]%CV	% [a]Accuracy	[a]Concentration Measured (n=30) (ng/mL)	[b]%CV	% [a]Accuracy
5 (Lower limit QC)	5.34	1.43	98.34	5.28	1.52	94.73
15 (Lower QC)	15.12	2.05	97.47	15.87	2.95	98.21
50 (Mid QC)	50.04	1.96	96.81	50.42	3.73	96.42
195 (High QC)	195.31	3.72	97.45	195.36	2.82	99.84

[a]Mean of samples, [b]Replicate analysis on different days.

Stability(Freeze-thaw,Autosampler, Benchtop, Long term)

Measurement of the RTB in plasma after three cycles of freezing and thawing (-30oC to room temperature) reveals the analyte's stability. Volumes were between 97.54% and 99.996% of their theoretical maximums. Despite being stored in the autosampler tray for 48 hours, the RTB showed no signs of degradation, and the final concentration accuracy ranged from 94.38% to 97.41% of the theoretical values. A final %Accuracy of concentrations of RTB ranged from 95.69% to 97.52% of the theoretical values, and no discernible deterioration of the RTB was noted even after a 24-hour room temperature storage period. Furthermore, it was also examined whether RTB in QC samples could withstand 60 days of storage at -80 oC. Concentration %Accuracy readings were between 95.66% and 97.06% of the theoretical values. The stability of RTBin human plasma at -80oC for at least 45 days was validated by these results (Table 3).

Table 3. Stability studies details.

Spiked Plasma Conc (ng/mL)	Room temperature stability		Autosampler Stability		Long term stability		Freeze and Thaw stability	
	24Hr		48hr		60 days		Cycle 3 (48 hr)	
	Concn measured (n-=6) (ng/mL) (mean)	CV (n=6) (%)	Concn measured (n-=6) (ng/mL) (mean)	CV (n=6) (%)	Concn measured (n-=6) (ng/mL) (mean))	CV (n=6) (%)	Concn measured (n-=6) (ng/mL) (mean)	CV (n=6) (%)
15 (Lower QC)	15.43	2.63	15.16	1.48	15.16	1.52	15.08	2.85
190.00 (High QC)	190.31	1.97	190.34	2.94	190.08	3.91	190.65	3.67

Recovery

By comparing the peak areas of RTBin plasma samples with solvent samples, the recovery following sample preparation utilizing Liquid-liquid extraction using Methyl tertiary butyl ether could be determined. At the RTB control levels, it was estimated. At doses of 15, 50, and 190 ng/mL, the RTB recovery was reported to be 96.55%, 97.59%, and 96.57%, respectively. Overall, 96.85% recovery was achieved with RTB and 92.59% with TES.

Conclusions

Innovative and efficient, the presented approach offers great sensitivity, selectivity, and robustness in assessing RTB in biological fluids using LC-MS/MS. It is the best option for trustworthy analysis since it can reach high recovery rates, which sets it apart from other methods that have been described. The method's consistent performance is further assured by its ruggedness, which suggests that it could be used extensively in bioanalytical studies.

Acknowledgments

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