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# Quantification of Tofacitinib in Human Plasma Samples Using Radio-Labeled Internal Standard

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

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## ABSTRACT

A simple, sensitive and accurate liquid chromatography tandem mass spectrometric method has been developed and validated for determination of Tofacitinib in human plasma. The method was developed on Agilent Zorbax SB-$C_8$ 150×4.6 mm, 5 µm column using 10mM ammonium formate: acetonitrile (40:60 v/v) mobile phase for Chromatographic separation of Tofacitinib. The Tofacitinib and Tofacitinib- C3 were monitored by electrospray ionization in positive ion multiple reactions monitoring mode to detect the Tofacitinib at mass/charge 313.200/149.200 and Tofacitinib-$^{13}$C3 (Internal Standard) at 316.500/149.200. Liquid-liquid extraction was employed in the extraction of analytes from human plasma. Both drug and internal standards were stable in plasma samples. The proposed method was validated as per international council of harmonization guidelines over a linear concentration range of 0.2ng/mL to 100.0ng/mL with a correlation coefficient (r) of ≥ 0.9983. Bioavailability and Bioequivalence studies of tofacitinib in biological samples can be achieved by analyzing them using the validated developed method. This study plays a key role in determining routine therapeutic drug monitoring of tofacitinib drug.

*Keywords: Tofacitinib; human plasma; method development; validation.*

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## 1. INTRODUCTION

Tofacitinib is a type of drug known as a Janus kinase (JAK) inhibitor with the chemical name 3-((3R,4R)-4-methyl-3-(methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino)piperidin-1-yl) 3oxopropane nitrile. Molecular formula of Tofacitinib is $C_{16}H_{20}N_6O$ and its molecular weight is 312.369 g/mol. It was used in the treatment of Rheumatoid arthritis, Psoriatic arthritis, and Ulcerative colitis.

Literature review reveals that very few analytical methods have been reported for the determination of Tofacitinib by using various analytical techniques [1-9].

It was found that no suitable validated method was available from the literature for determination of bioavailability and bioequivalence of tofacitinib in biological samples. Therefore the aim of the present study is to develop a validated hyphenated method for determination of routine therapeutic drug monitoring of tofacitinib drug.

## 2. METHODS

### 2.1 Chemicals and Reagents

Tofacitinib reference standard (purity, 99.5%) was purchased from Vivan Life Sciences, Mumbai. Tofacitinib-$^{13}$C3 was internal standard was obtained from Clearsynth Labs. Ltd, Mumbai. Tertiary butyl methyl ether, Ammonium formate (reagent grade) and HPLC grade Water were purchased from Rankem, Mumbai. HPLC grade Methanol was purchased from Merck, Mumbai.

### 2.2 Instrumentation

HPLC system (Shimadzu, UFLC XR). Mass spectrometric detection was performed on an API 4000, Q-TRAP triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM on Analyst 1.6.2 software package (SCIEX).

### 2.3 Calibration Standards and Quality Control Samples

Standard Stock solutions of Tofacitinib (200.0 µg/mL) were prepared in methanol. From each stock solution 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 1250 ng/mL, 2500.0 ng/mL, 4000 ng/mL, 5000.0 ng/mL intermediate dilutions were prepared in human plasma. From the above concentrations 0.2ml were used to spike blank human plasma in order to obtain calibration curve standards of 0.2, 0.4, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0, 80.0 and 100.0ng/mL. Five levels of QC concentrations at 0.584, 3.648, 7.296, 45.600 and 76.000 ng/mL (LQC, MQC3, MQC2, MQC1 and HQC) were prepared by using the different plasma. Spiked calibration curve standards and Quality control standards were stored at -70 ±15°C. Standard stock solutions of Tofacitinib-$^{13}$C3 (200.0 µg/mL) were prepared in methanol. Tofacitinib-$^{13}$C3 was further diluted to 75.0 ng/mL (Spiked concentration of internal standard) using diluent & stored in the refrigerator 2-8 °C until analysis.

### 2.4 Sample Preparation

75.0 ng/mL drug and internal standard samples were prepared by Liquid-liquid extraction method. This was followed by addition of 200.000µL of extraction buffer and 2.0 mL of methyl tertiary butyl ether and vortexed for approximately 15minutes on rotospin at 50rpm. Then the samples were centrifuged at 4500 rpm for 5 minutes at 4°C and then evaporated with nitrogen gas at 40°C (reconstituted with Acetonitrile: 10mM ammonium formate (60:40%V/V)) and vortexed for 1 minutes prior to injection.

## 3. RESULTS

### 3.1 Method Development

Several method trials were performed to optimize chromatographic conditions, especially, composition of mobile phase, selection of suitable column etc. Different extraction methods like solid phase extraction, Liquid-liquid extraction, precipitation methods were optimized for extraction of Tofacitinib and Tofacitinib-$^{13}$C3 from plasma sample. Liquid-liquid extraction was chosen to optimize the drug and internal standard.

### 3.2 Optimized Chromatographic Conditions

Chromatographic separation was achieved with acetonitrile:10 mM ammonium formate: (60:40v/v), gave the best peak shape and low baseline noise was observed using the Agilent Zorbax SB-C8150×4.6mm,5µm, column.

Tofacitinib-$^{13}$C3 was selected as internal standard due to its compatibility with analyte chromatographic conditions. Both Tofacitinib and Tofacitinib-$^{13}$C3 were eluted at 1.99 minutes with total runtime of 3.5 minutes for each injection.

### 3.3 Detection

The mass transitions were selected as *m*/*z* 313.200/149.200 and *m*/z 316.500/149.200 for quantification of Tofacitinib and Tofacitinib-$^{13}$C3 respectively.

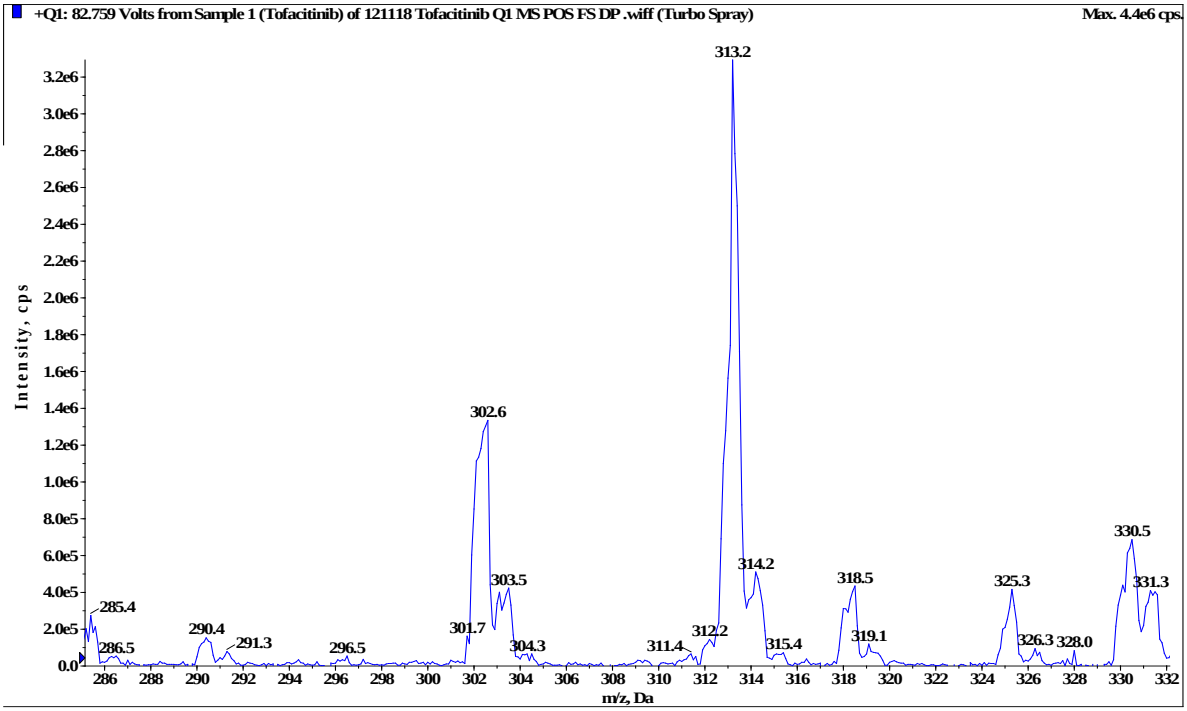


**Fig. 1. MRM scan of tofacitinib at Q1**

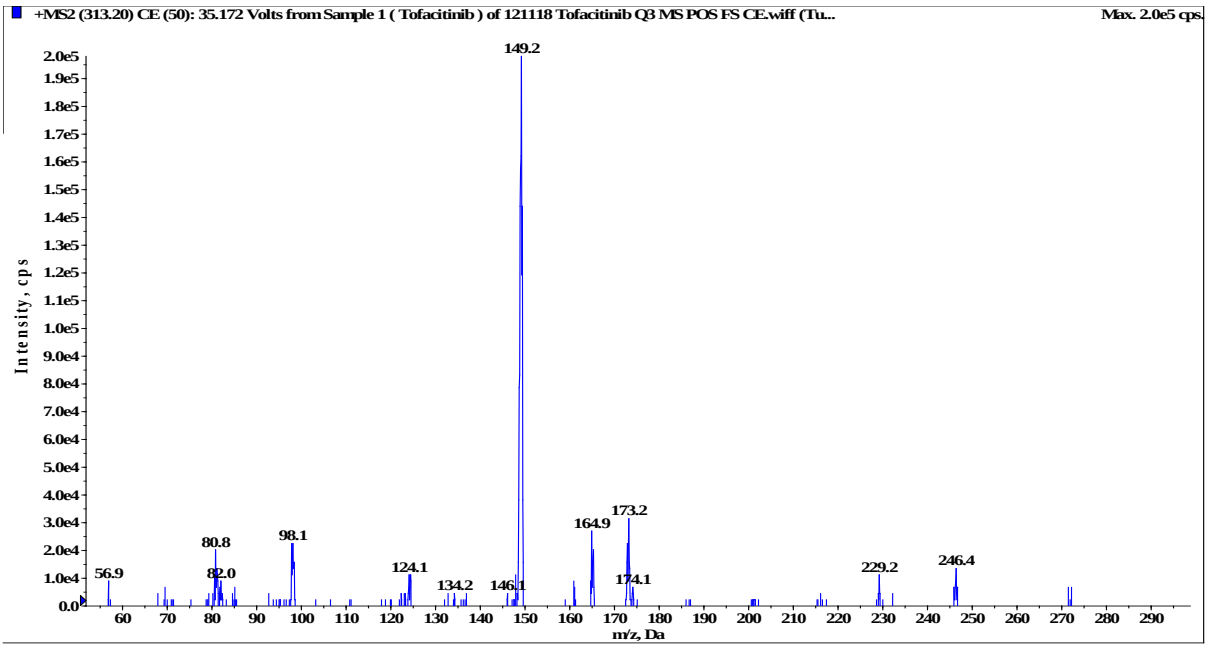


**Fig. 2. MRM scan of tofacitinib at Q3**

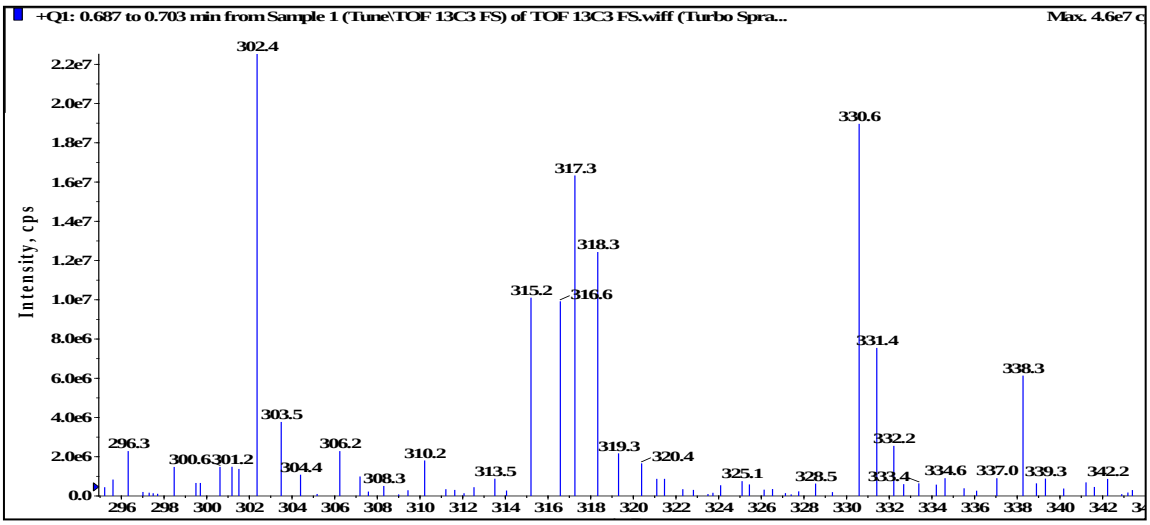


**Fig. 3. MRM scan of tofacitinib-$^{13}$C3 at Q1**

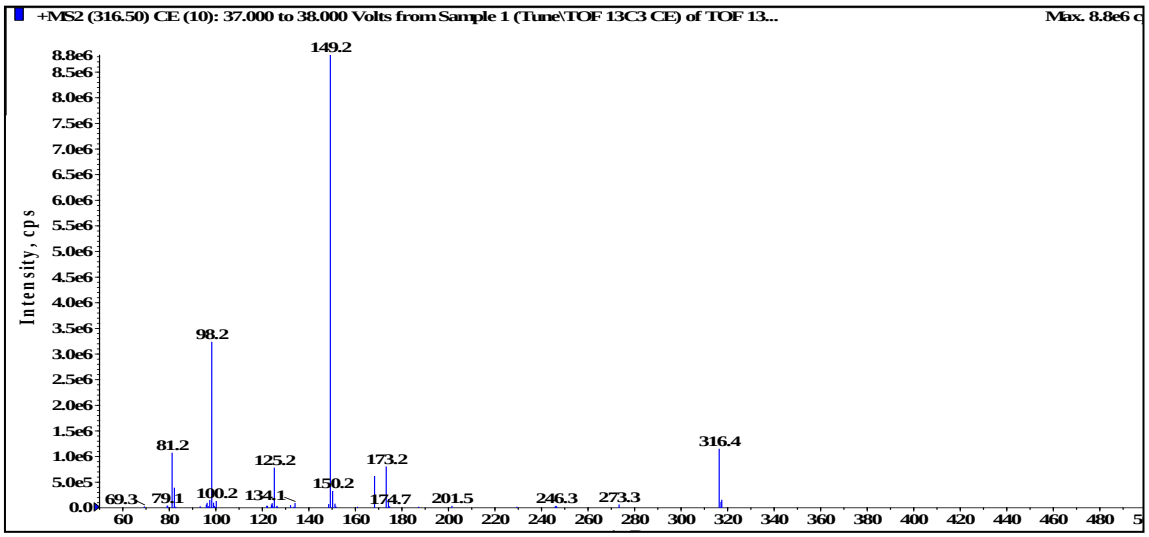


**Fig. 4. MRM scan of tofacitinib-$^{13}$C3 at Q3**

## 3.2 Method Validation

**Table 1. Calibration curve details of tofacitinib**

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Nominal Concentration Spiked plasma (ng/mL)** | 0.201 | 0.403 | 1.007 | 2.014 | 5.034 | 10.068 | 25.169 | 50.338 | 80.541 | 100.677 |
| **01** | 0.194 | 0.421 | 1.039 | 2.163 | 5.152 | 10.656 | 25.201 | 45.858 | 79.836 | 90.853 |
| **02** | 0.202 | 0.385 | 1.079 | 2.090 | 5.195 | 10.390 | 24.999 | 48.151 | 76.926 | 97.079 |
| **03** | 0.201 | 0.388 | 1.075 | 2.121 | 5.155 | 10.253 | 25.316 | 46.201 | 78.381 | 98.448 |
| **Concentration measured (mean) (ng/mL) (n=3)** | 0.1990 | 0.3980 | 1.0643 | 2.1247 | 5.1673 | 10.4330 | 25.1720 | 46.7367 | 78.3810 | 95.4600 |
| **% CV** | 2.19 | 5.02 | 2.07 | 1.72 | 0.46 | 1.96 | 0.64 | 2.65 | 1.86 | 4.24 |
| **% Mean Accuracy** | 99.00 | 98.76 | 105.69 | 105.50 | 102.65 | 103.63 | 100.01 | 92.85 | 97.32 | 94.82 |

### 3.3 Selectivity

The selectivity of the method assessed by comparing chromatograms of blank plasma and Tofacitinib and Tofacitinib-$^{13}$C3.

### 3.4 Precision and Accuracy

Precision and accuracy for this method were controlled by calculating the within-run and between-run variations at five concentrations (0.584, 3.648, 7.296, 45.600 and 76.000 ng/mL) of QC samples in six replicates. The within-run precision and accuracy were between 1.40 to 2.92 and 90.21 to 93.71% for tofacitinib. Similarly, the between-run precision and accuracy were between 2.24 to 5.05 and 92.63 to 95.51 % for tofacitinib.

### 3.5 Recovery

The % recoveries of Tofacitinib were determined at three different concentrations 0.584, 45.600 and 76.000 ng/mL, were found to be 76.97, 80.09 and 81.26%. The overall average recoveries of Tofacitinib and Tofacitinib-$^{13}$C3 were found to be 79.44 and 78.74%.

**Table 2. Precision and accuracy (analysis with spiked plasma samples at seven different concentrations)**

| S.No | HQC | MQC1 | MQC2 | MQC3 | LQC | DQC | LLOQ |
|---|---|---|---|---|---|---|---|
| **Nominal Concentration (ng/mL)** | 76.675 | 46.005 | 7.361 | 3.680 | 0.582 | 251.563 | 0.201 |
| **01** | 71.943 | 43.540 | 6.877 | 3.440 | 0.534 | 246.339 | 0.221 |
| | 74.562 | 43.941 | 6.879 | 3.501 | 0.540 | 239.628 | 0.198 |
| | 70.579 | 42.562 | 6.894 | 3.438 | 0.539 | 240.000 | 0.202 |
| | 71.797 | 43.623 | 6.840 | 3.482 | 0.519 | 242.029 | 0.195 |
| | 70.485 | 42.439 | 6.650 | 3.378 | 0.501 | 239.018 | 0.190 |
| | 70.184 | 42.549 | 6.726 | 3.393 | 0.517 | 237.291 | 0.209 |
| **Mean (n=6)** | 71.5917 | 43.1090 | 6.8110 | 3.4387 | 0.5250 | 240.7175 | 0.2025 |
| **SD** | 1.62605 | 0.66391 | 0.09986 | 0.04802 | 0.01535 | 3.15126 | 0.01111 |
| **% CV** | 2.27 | 1.54 | 1.47 | 1.40 | 2.92 | 1.31 | 5.49 |
| **% Mean Accuracy** | 93.37 | 93.71 | 92.53 | 93.44 | 90.21 | 95.69 | 100.75 |
| **02** | 72.124 | 42.375 | 7.143 | 3.537 | 0.534 | 214.096 | 0.210 |
| | 71.371 | 48.071 | 6.460 | 3.521 | 0.532 | 266.147 | 0.192 |
| | 70.391 | 43.941 | 7.095 | 3.672 | 0.537 | 245.532 | 0.203 |
| | 70.841 | 40.149 | 6.991 | 3.343 | 0.550 | 249.411 | 0.194 |
| | 68.706 | 43.407 | 6.862 | 3.451 | 0.503 | 246.473 | 0.186 |
| | 72.204 | 43.380 | 6.373 | 3.464 | 0.536 | 247.348 | 0.208 |
| **Mean (n=6)** | 70.9395 | 43.5538 | 6.8207 | 3.4980 | 0.5320 | 244.8345 | 0.1988 |
| **SD** | 1.30286 | 2.59187 | 0.32874 | 0.10928 | 0.01556 | 16.90864 | 0.00960 |
| **% CV** | 1.84 | 5.95 | 4.82 | 3.12 | 2.92 | 6.91 | 4.83 |
| **% Mean Accuracy** | 92.52 | 94.67 | 92.66 | 95.05 | 91.41 | 97.33 | 98.91 |
| **03** | 73.557 | 45.470 | 7.213 | 3.570 | 0.559 | 251.783 | 0.213 |
| | 72.758 | 49.340 | 6.610 | 3.587 | 0.564 | 266.736 | 0.200 |
| | 74.511 | 44.615 | 6.663 | 3.585 | 0.582 | 252.180 | 0.194 |
| | 73.684 | 41.156 | 7.218 | 3.515 | 0.576 | 261.870 | 0.202 |
| | 70.764 | 45.268 | 7.135 | 3.585 | 0.515 | 256.664 | 0.201 |
| | 73.156 | 45.090 | 7.240 | 3.739 | 0.566 | 255.006 | 0.207 |
| **Mean (n=6)** | 73.0717 | 45.1565 | 7.0132 | 3.5968 | 0.5603 | 257.3732 | 0.2028 |
| **SD** | 1.27381 | 2.60438 | 0.29440 | 0.07484 | 0.02374 | 5.86872 | 0.00649 |
| **% CV** | 1.74 | 5.77 | 4.20 | 2.08 | 4.24 | 2.28 | 3.20 |
| **% Mean Accuracy** | 95.30 | 98.16 | 95.28 | 97.74 | 96.27 | 102.31 | 100.90 |
| **Between Batch Precision and Accuracy** | | | | | | | |
| **Mean (n=18)** | 71.8676 | 43.9398 | 6.8816 | 3.5112 | 0.5391 | 247.6417 | 0.2014 |
| **SD** | 1.61143 | 2.21790 | 0.26342 | 0.10171 | 0.02352 | 12.25833 | 0.00891 |
| **% CV** | 2.24 | 5.05 | 3.83 | 2.90 | 4.36 | 4.95 | 4.42 |
| **% Mean Accuracy** | 93.73 | 95.51 | 93.49 | 95.41 | 92.63 | 98.44 | 100.20 |

**Table 3. Recovery of analyte (Tofacitinib)**

| Replicate No. | HQC | | MQC1 | | LQC | |
|---|---|---|---|---|---|---|
| | Post extracted Response | Extracted Response | Post extracted Response | Extracted Response | Post extracted Response | Extracted Response |
| **1** | 3332093 | 2484851 | 2126492 | 1602347 | 29956 | 21535 |
| **2** | 3342270 | 2783479 | 2111431 | 1741349 | 28795 | 23509 |
| **3** | 3401387 | 2842140 | 2152001 | 1738898 | 29987 | 23295 |
| **4** | 3391415 | 2792742 | 2083372 | 1716164 | 28954 | 22452 |
| **5** | 3292164 | 2765224 | 2091824 | 1688719 | 28724 | 22605 |
| **6** | 3333957 | 2659957 | 2135353 | 1684011 | 29711 | 22173 |
| **Mean (n=6)** | 3348881.0 | 2721398.8 | 2116745.5 | 1695248.0 | 29354.5 | 22594.8 |
| **SD** | 40819.38 | 130516.55 | 26257.57 | 51512.32 | 593.28 | 727.69 |
| **% CV** | 1.22 | 4.80 | 1.24 | 3.04 | 2.02 | 3.22 |
| **% Mean Recovery** | 81.26 | | 80.09 | | 76.97 | |
| **Overall % Mean Recovery** | 79.44 | | | | | |
| **Overall SD** | 2.218 | | | | | |
| **Overall % CV** | 2.79 | | | | | |

**Table 4. Stability of tofacitinib in human plasma samples**

| Stability experiments | Storage condition | Spiked plasma concentration (pg/ml) | Concentration measured (*n*=6) | RSD (*n*=6) (%) | Accuracy (%) |
|---|---|---|---|---|---|
| **Bench top in plasma** | Room Temperature 6hr 47Min | 0.582 | 0.5358 | 6.91 | **97.72** |
| | | 76.675 | 74.0697 | 5.74 | **92.49** |
| **Processed (extracted sample)** | Autosampler 51Hrs 31Min | 0.582 | 0.5538 | 4.30 | **95.6** |
| | | 76.675 | 74.9542 | 2.35 | **98.89** |
| **Freeze/Thaw stability** | -28±5°C Cycle-3 | 0.582 | 0.5450 | 3.77 | **98.6** |
| | | 76.675 | 74.1577 | 3.99 | **98.98** |
| **Long-term stability in human plasma** | 2-8ºC 9 days 17Hr 40Min | 0.582 | 0.5635 | 5.45 | **97.27** |
| | | **76.675** | **75.7820** | **4.74** | **99.98** |

### 3.6 Stability (Freeze - Thaw, Auto Sampler, Room Temperature, Long Term)

Stock solution stability was performed to check stability of Tofacitinib and Tofacitinib-$^{13}$C3 in stock solutions prepared in methanol freshly and stored samples at 2-8°C in a refrigerator before 20 days. Room temperature and auto sampler stability for Tofacitinib was investigated at LQC and HQC levels. The results revealed that Tofacitinib was stable in plasma for at least 06 Hrs 47 mins at room temperature, and 51 Hrs 13 mins in an auto sampler. The long-term stability results also indicated that Tofacitinib was stable in a matrix up to 09 days 17 hrs 40 mins at a storage temperature of 2-8°C. Precision (%CV) is less than 5 for Room temperature, long-term, Freeze thaw, auto sampler stability.

## 4. DISCUSSION

The most common goals in the pharmacokinetic studies is to develop a suitable bioanalytical method to analyze a wide range of drugs in biological samples. Initially method development attempt for the HPLC separation was to employ conventional C1, C8 and C18 columns, but tofacitinib was not retainable under gradient elution starting at 100% aqueous mobile phase by the conventional reversed-phase stationary phases routinely employed in our laboratory (data not shown). Sufficient chromatographic retention in the quantitative determination of the drug components in biological samples using mass spectrometric detection is highly recommended to avoid possible interferences from drug-related biotransformation products or ionization suppression due to co-eluted endogenous materials.

When developing any new HPLC–MS/MS assays, it is important to check for possible matrix ionization suppression. Matrix ionization suppression is considered to be more problematic when using the protein precipitation method for sample preparation as compared to the liquid–liquid and the solid phase extraction methods. Generally composition of the eluent affects the chromatographic resolution and the ionization efficiency of the tofacitinib in various atmospheric pressure ionization sources. Greater the organic content in the mobile phase will generate higher ionization efficiencies for small molecules in most atmospheric pressure ionization interfaces.

Influence of the mobile phase composition on various factors like retention factors (k) of ara-C with a commercial reversed-phase/ cation exchange column under isocratic elution was determined in this research work. These facts can be used to optimize the effectiveness of separation of tofacitinib mixtures. Mobile phase variables were tried in different proportions to obtain an insight into the retention and separation mechanism for a commercial mixed-mode column. According to the linear solvent strength theory for the reversed-phase retention alone, the values of log(k) of the analytes decrease linearly as the percent fraction of organic modifier in the mobile phase increases due to the weaker hydrophobic interaction. For the mixed-mode chromatography, the relative contribution of each mechanism includes the hydrophobicity and charge character of analytes as well as mobile phase composition within the same column. By changing the mobile phase conditions, the mode of separation might be thereby changed which allows the chromatographer to achieve the desired selectivity in the separations.

The drug sample was completely dissolved in the optimized mobile phase combination. The linearity studies performed proved the results to be linear over the concentration range of 0.2 – 100.0ng/mL and the correlation coefficient ($r^2$) was greater than 0.9983 for all curves. There were no significant endogenous peaks were observed at respective retention time of Tofacitinib and Tofacitinib-$^{13}$C3 proving the specificity or selectivity of the method. The accuracy and precision results indicate the adequate reliability and reproducibility of the developed method within the analytical range. The ion suppression/enhancement in the signal at HQC level was found to be % CV 5.83 indicating that there is no matrix effect on ion suppression and ion enhancement. The Recoveries of the analyte and internal standard were consistent, precise and reproducible. The stability of the sample solutions were performed by using Freeze – thaw studies.

## 5. CONCLUSION

The proposed research work is highly specific and prior over other described methods reported previously. Quantification of tofacitinib was compared with respective radio-isotope labeled internal standards. Extraction of analyte and internal standard were achieved by using Liquid Liquid Extraction. Chromatographic conditions were improved. Hence the proposed method has significant advantages over previously reported methods in-terms of Selectivity, sensitivity, Linearity, Reproducibility, Accuracy.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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