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RESEARCH ARTICLE

Development of a Validated Reverse Phase Liquid Chromatographic Assay-Method for determination of Tofacitinib in pure form and in Physical Admixtures

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ABSTRACT:
Aim: The aim is to develop simple validated analytical method for analysis of Tofacitinib by RP HPLC in pure and laboratory prepared physical admixtures. **Method:** Tofacitinib was estimated by RP HPLC using methanol , water mixture as mobile phase. Linearity range was found to be 10-50 mcg/ml. The correlation coefficient was 0.9998. The limit of detection and quantification were found to be 0.053 and 1.63 µg /ml respectively. The drug solutions were scanned in UV spectrophotometry and the maximum absorption was found to be 285.9 nm and the same was fixed as detecting lambda by RP HPLC method. The Column used was C_{18}.with the dimensions of 150 mm X 4.6 mm and 5 micron particles. **Results and Conclusions:** The proposed method was successfully applied for the determination of tofacitinib in pure and laboratory prepared physical mixtures. The % RSD value of Tofacitinib in bulk and physical admixture was calculated at different time intervals for recovery , precision (Iintraday and Interday experiments) and quantification studies were found to be less than 2 %.

KEYWORDS: Tofacitinib, RP HPLC, Validation, ICH guidelines, Isocratic.

INTRODUCTION:
Tofacitinib(3-((3R,4R)-4-methyl-3-(methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino) piperidin-1-yl)-3-oxopropanenitrile) is a new class of drug called Janus kinase inhibitor. Tofacitinib, a first oral non-biologic disease-modifying anti-rheumatic drug (DMARD) can be used as monotherapy or in combination with methotrexate or other non-biologic DMARD's, for treating adults with moderate or severe rheumatoid arthritis.[1,2] It is contraindicated for use with biologic DMARDs or with immunosuppressive agents, such as azathioprine and cyclosporine.

Rheumatoid arthritis is an autoimmune disease characterized by a dysregulation of pro-inflammatory cytokines including IL7, IL15, IL21, IL6, IFN-alpha, and IFN-beta. Cytokines signaling results in tissue inflammation and joint damage by stimulating the recruitment and activation of immune cells via the Janus kinase signaling pathway.[3,4,] Tofacitinib is a partial and reversible Janus kinase (JAK) inhibitor that will prevent the body from responding to cytokine signals. By inhibiting JAKs, tofacitinib prevents the phosphorylation and activation of STATs. The JAK-STAT signaling pathway is involved in the transcription of cells involved in hematopoiesis, and immune cell function. Tofacitinib works therapeutically by inhibiting the JAK-STAT pathway to decrease the inflammatory response. It is approved by USDFDA in November 2012 [5,6]

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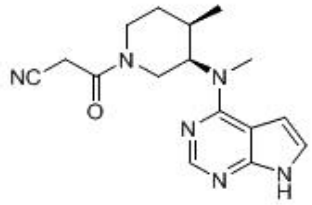

Figure 1: Structure of Tofacitinib

laboratory prepared physical mixtures. Confirmation of the applicability of the developed method was done by validating according to the International Conference on Harmonization (ICH).

MATERIALS AND METHOD:

All reagents used in the experimental work were HPLC grade. HPLC grade methanol, acetonitrile and water were purchased from Qualigens, Mumbai, India. A Shimadzu HPLC system consists of a LC-20AD solvent delivery system (pump), SPD-M20A photodiode array detector, Rheodyne injector with 20 μL loop volume, and LC-Solution assisted for data collections and processing. The Chromatographic separation was performed using phenomenex C_{18} 150 x 4.6 mm, 5µ (I.D) column, detection wavelength is 285 nm and run time is 6.5 min.

Selection of Mobile phase:

In first trial mobile phase used was acteonitrile and water (50:50) which gave a broad peak In second trial mobile phase used was actenonitrile and water (70:30) it did not give sharp and narrow peak. At last the mobile phase was then changed to water and methanol in the ratio of equal proportion with this we got sharp and narrow peak that found to have satisfying system suitability parameters were within the limits.

Preparation of the mobile phase and diluents:

The Mobile phase was prepared by mixing methanol and water in the ratio of 50:50 (v/v) .The resultant solution was thoroughly mixed and filtered through a poly-tetra-fluoro ethanol (PTFE) filter of 0.45 µm pore size using vacuum pump and degassed by sonication to expel the dissolved gases in solvent system.

Preparation of standard solution:

25 mg of Tofacitinib standard powder was weighed and transferred into 25 ml volumetric flask and diluted with methanol: water mixture (50:50) ratio, to make the concentration 1000 mcg/ml. 2.5 ml of this standard stock solution was pipetted out and transferred into 25 ml volumetric flask and diluted with methanol: water mixture (50:50) ratio, to give the concentration 100 mcg/ml.

Preparation of sample solutions:

Laboratory prepared physical admixture of Tofacitinib was accurately weighed and the average weight was calculated. Then, the amount of powder equivalent to 1 dose (5mg) was transferred to a volumetric flask, diluted with diluents and shaken for about 10 minutes then filtered through filter paper. The filtered solution was further diluted in the mobile phase to make the final concentration of working sample equivalent to 10 mcg/ml.

Only few

Chromatographic condition:

The samples were introduced by injector with a 20-µl loop. The analysis was carried out under isocratic conditions using a flow rate 1ml/min at 30˚C temperature. Chromatograms were recorded at wavelength 285nm using a detector Shimadzu UV-VIS.

Methodology:

The HPLC system was stabilized for thirty minutes by passing mobile phase, detector was set at 285 nm, flow rate of 1.0 mL/min to get a stable base line. One blank followed by six replicates of a single standard solution was injected to check the system suitability. Six replicates of each standard solutions 10, 20,30,40,50 µg/mL. were injected. Calibration graph was plotted using concentration of Tofacitinib on X-axis and peak area on Y-axis and linearity curve was shown in Figure 2. The amount of drug present in sample was computed by calibration graph. Chromatographic conditions for estimation of Tofacitinib were described in Table 1

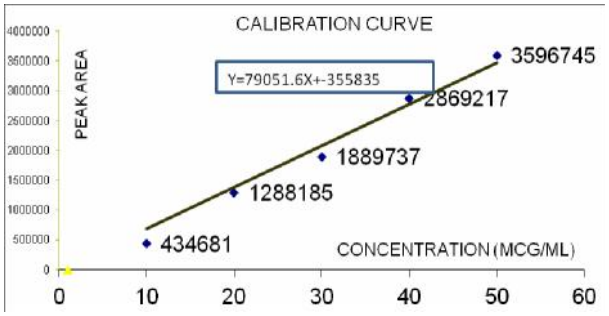

Fig 2: Linearity curve of Tofacitinib

Table no. 1: Optimized chromatographic conditions of Tofacitinib parameters

Parameters	Values
Mobile phase	Methanol: water (50:50)
Column	C_{18}
Diluents	Methanol
Column temperature	30 C
Wavelength	285 nm
Injection volume	20 L
Flow rate	1.0 ml / min
Run time	10 min
Retention time	6.62
Theoretical plates	7072.58
Asymmetry	1.22
Capacity factor	2.79

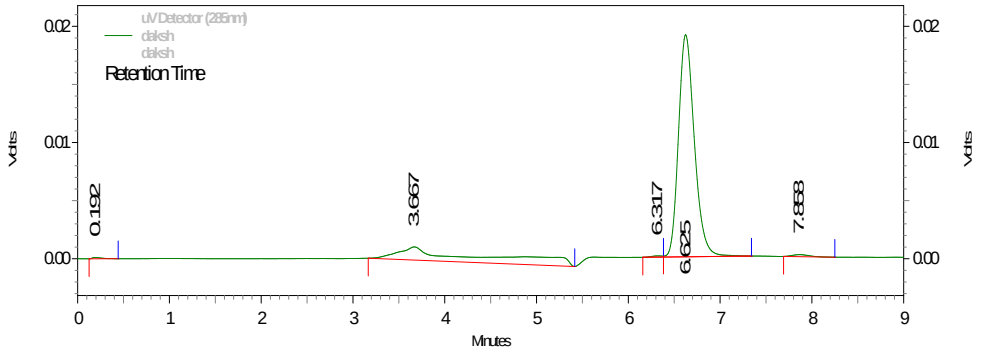

Fig 3: Typical chromatogram of Tofacitinib

RESULTS AND DISCUSSION:

The objective of the present work is to develop simple, precise and reliable HPLC method for the analysis of Tofacitinib in bulk and Laboratory prepared physical admixture of Tofacitinib. This is achieved by using the most commonly employed phenomenex RP 18 (150 x 4.6 mm, 5 µm particle size) column detection at 285 nm. The representative chromatogram indicating Tofacitinib is shown in Figure 3.

Parameter fixation:

In developing this method, a systemic study of effects of various parameters was under taken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.

Mobile phase characteristics:

In order to get sharp peak with base line separation from interfering peaks carried out a number of experiments by varying the composition of solvents and mobile phase flow rate. To have an ideal separation of the drug under isocratic conditions, HPLC grade methanol and water were used in the ratio of 50:50 (v/v) was proved to be the most suitable of all the combinations, since the chromatographic peak obtained was better defined and resolved and almost free from tailing.

Linearity:

A linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentration. The linearity of response for the present method was determined by analyzing standard solution in the concentration range of 10 - 50 mcg / ml. The results are showed that the peak areas are linear within the concentration of analysis with a correlation coefficient of 0.9998. (Table 2)

Table:2 :Linearity studies of Tofacitinib

S.NO	Concentration in mcg / ml	Area
1	10	434681
2	20	1288185
3	30	1889737
4	40	2869217
5	50	3596745
	R^2	0.9998
	Slope	79051.6
	Intercept	-355835

Accuracy:

The accuracy of the method was determined by standard addition method at 20,40 and 60 % levels. A known amount of standard drug was added to the fixed amount of pre-analyzed standard solution. The percent recovery and % RSD was calculated and results are presented in Table 3. Satisfactory recoveries ranging from 98 – 102 % were obtained by the proposed method. This indicates that the proposed method was accurate.

Intra-day precision:

To study the intra-day precision, six replicate standard solutions of Tofacitinib were injected. The percent relative standard deviation (% RSD) was calculated and it was found to be 1.698 which are well within the acceptable criteria of not more than 2.0. (Table 3).

Inter-day precision:

To study the inter-day precision, six replicate standard solutions (300 ppm) of Tofacitinib were injected. The percent relative standard deviation (%RSD) was calculated and it was found to be 1.858 which are well within the acceptable criteria of not more than 2.0 (Table 3).

Specificity:

The effect of wide range of excipients and other additives usually present in the physical admixture of Tofacitinib in the determinations under optimum conditions were investigated. Chromatographic

parameters maintained are specific for physical admixture of Tofacitinib.(Table 3).

Limit of detection and limit of quantification:
The detection limit of the method was investigated by injecting standard solutions of Tofacitinib into the HPLC column. By using the signal-to-noise method the peak-to-peak noise around the analyte retention time is measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio is estimated. A signal-to-noise ratio (S/N) of 3 is generally accepted for estimating LOD and signal-to- noise ratio of 10 is used for estimating LOQ. This method is commonly applied to analytical methods that exhibit baseline noise. The limit of detection (LOD) and limit of quantification (LOQ) for Tofacitinib were found to be 0.053 µg/ml and 0. 163 µg/ml respectively.

Robustness:
Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust.

Table :3 Results of accuracy, precision and specificity studies.

Quantification parameter	Average % of drug found	S.D	%RSD
Recovery * 20%	99.8	1.714	1.717
Recovery* 40%	101.7	1.689	1.689
Recovery * 60%	98.2	1.636	1.645
Intraday studies*	102.2	1.698	1.698
Interday studies*	100.1	1.858	1.857
Quantification in Physical admixtures *	99.4	1.510	1.518

* In six replicates

System suitability:
A system suitability test was performed to evaluate the chromatographic parameters (number of theoretical plates, tailing of the peak) before the validation runs. The analytical method validation was carried out as per ICH method validation guidelines.

CONCLUSION:
In this study a simple, fast and reliable HPLC method was developed and validated for the determination of Tofacitinib in laboratory prepared physical admixture. As these proposed methods have the lowest LOQ values and wider linear range is more sensitive method. From the results obtained, we concluded that the suggested methods showed high sensitivity, accuracy, reproducibility and specificity. Moreover, these methods were simple and inexpensive and these can be employed for the routine quality control of Tofacitinib in physical admixtures.

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