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Comparative Analysis of Plasma and Dried Plasma Spots (DPS) of Tofacitinib-A Pan JAK inhibitor using LC-MS/MS; Application to Pharmacokinetic Study in Mice

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Abstract— *Dried plasma spots were employed as an alternative sample collection technique for the quantitative determination of tofacitinib in mice plasma, using liquid chromatography tandem mass spectrometry method.* ***Method****: A simple, highly sensitive and rapid assay method has been developed for the estimation of tofacitinib on mice dried plasma spots (DPS) using liquid chromatography attached to tandem mass spectrometry with electro spray ionization (ESI) in the positive-ion mode. The method employs liquid extraction of tofacitinib from DPS disk of mice dried plasma spots followed by chromatographic separation using 5 mM ammonium formate with 0.1% Formic acid in water: acetonitrile in gradient elution method at a flow rate of 0.350 mL/min on an Acquity UPLC BEH C18 (50 x 2.1 mm, 1.7 µm) column with a total run time 2.5 min. The MS/MS ion transitions monitored were m/z 313→149 for tofacitinib and m/z 477→210 for the internal standard (Loperamide).* ***Result****: The assay was linear in the range of 1.16–1511 ng/mL. In current study this new method has been applied to analyze the DPS (Dried Plasma Spot) and neat plasma samples of Tofacitinib obtained from a pharmacokinetic study in mice.* ***Conclusion****: The quantitative analysis of tofacitinib with dried plasma spot specimens seems to be a prominent and advantageous technique, especially when applied to pharmacokinetic studies, where plasma sampling procedure becomes rapid and required plasma volumes are negligible and storage of samples is an issue.*

Keywords*— Dried plasma spot: JAK inhibitors; LC-MS/MS; mice pharmacokinetics; Tofacitinib.*

I. INTRODUCTION

The novel JAK inhibitors (JAKinibs) tap down cytokine mediated pathway signal via the JAK-STAT pathway, these have crucial role in immune regulation and growth. These 'small molecules' drugs are highly specific for blocking targets identified within cells that is responsible for chronic inflammation [1].

Rheumatoid arthritis is categorized as autoimmune disease and occurred in females than males and more frequently in elder population [2]. The occurrence rate mentioned in year 2002 ranged from 0.5% to 1% [3] The concepts for developing an assay for dry matrices are similar to those for any other matrices; the only difference is that we need the methods to transfer the analytes out of the dry paper and into a form that can be analyzed [4].

Dried plasma spot (DPS) sampling is an attractive but underutilized approach that combines the benefits of easy collection, storage, and transport with the ability to overcome recognised hematocrit (HCT) difficulties in dried blood spot (DPS) analysis [5.] Studied quantitative analysis of gabapentin using dry plasma samples and proved to be an excellent and advantageous technique, especially when applied to pharmacokinetic studies where the plasma sampling is rapid and the amount of plasma available for sample processing is negligible or very small [6].

Instead of analyzing whole blood spots (DBS) directly, a bilayer polymer membrane is used to separate dry plasma spots (DPS) from whole blood. This dry plasma spot analysis (DPS) method eliminates potential problems with different hematocrit values in whole blood samples and provides pharmacokinetic data from plasma rather than whole blood [7.] Dry plasma samples (DPS) were compared relatively well with neat plasma, with sensitivity and specificity values >90% [8.] There is increasing interest in the use of tofacitinib in the management of psoriatic arthritis, following its success and wide use as an approved treatment option for patients with rheumatoid arthritis (RA) [9].

The various literatures showed that DBS and micrsampling are effective ways to be applied in early drug discovery stage where mice are used in sparse sampling methods [10-14].

The stipulation of selective and sensitive bio-analytical strategies should be essential to create of reliable information on pharmacological medicine [16].

Many challenges in pharmaceutical industry that complete by the utilization of analytical technologies and high-throughput automated platforms has been in use; in order to perform high throughput experiments in a shorter time frame with increased data quality [17].

The diverse methods for quantification of tofacitinib were developed by various authors using Spectrometric-Assay, RP-HPLC; The analytical techniques were available in research and development stages [18,19].

The progression of analytical techniques is bringing a new era of development which will serve as a rapid and unequivocal tool in the drug development process [20-23].

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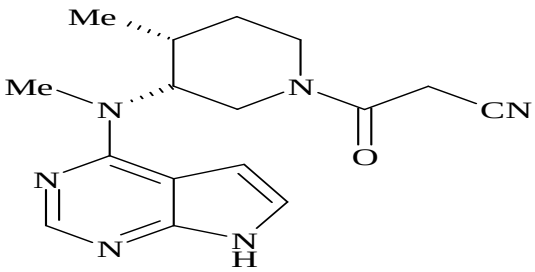
To the best of our knowledge, method for the determination of tofacitinib in mice dried blood spots (DPS) has not been reported in the literature. The current study aims to create a simple and fast LC-MS/MS method for quantifying tofacitinib in mice. Dried plasma spots and the traditional plasma technique can both be used without sacrificing sensitivity. The validated method was compared by both sampling approaches and found that it can be successfully used in a mice pharmacokinetic study following intravenous and oral administration of tofacitinib at 2.0 and 10 mg/Kg dose.

II. METHODS

The intravenous and oral pharmacokinetics study was conducted in male BALB/c Mice. Tofacitinib concentrations from plasma and DPS were measured by liquid chromatographic tandem mass, The AUC exposures of both the matrices were compared by statistical approaches.

Dried plasma spots (DPS) is a relatively new alternative sample collection technique that involves collecting and preserving plasma samples on filter paper, primarily from pharmacokinetic research. It is a rational sampling strategy for pharmacokinetic studies in clinical and preclinical phases of studies where sample size is a prime concern. The utility of DPS to determine the pharmacokinetics of tofacitinib were not studied though it is currently used in potential treatments. So the accuracy of DPS versus plasma samples was evaluated in the current exercise.



Structure of tofacitinib

Chemicals and reagents

Tofacitinib (purity: 99%), loperamide and dipotassium ethylenediaminetetraacetic acid (K_2.EDTA) was purchased from Sigma-Aldrich, Bangalore, India. Acetonitrile, TBME and methanol were purchased from Rankem, Ranbaxy Fine Chemicals Ltd, New Delhi, India. Dimethylsulfoxide (DMSO) from Honeywell Research Chemicals, absolute ethyl alcohol was purchased from Changshu Chemicals, China and Normal saline was purchased from Claris Otsuka, Ahmedabad, India.

Instrumentation and chromatographic conditions

A waters UHPLC Acquity I class system equipped with binary solvent manager (M2OVPA201M), column manager (SNA21UPH017G), auto-sampler organizer (M20U 0021H) along pump (C21UPB130A) was used to inject 2.0 µL aliquots of the processed samples on an Acquity UPLC BEH C18 (50 x 2.1 mm, 1.7 µm) column; Waters, Milford, MA, USA, which was kept at ambient temperature (24 ± 1°C). using 5 mM ammonium formate with 0.1% Formic acid in water: acetonitrile in gradient elution method (as showed in below table) and was filtered through a 0.45 µm membrane filter (Millipore, USA) and then degassed ultrasonically for 5 min was delivered at a flow rate of 0.35 mL/min (with 50 % splitter) into the mass spectrometer electro spray ionization chamber.

Gradient Table:

Time	Flow Rate	%A	%B	Curve
Initial	0.350	20.0	80.0	Initial
0.50	0.350	20.0	80.0	6
1.00	0.350	70.0	30.0	6
1.75	0.350	70.0	30.0	6
1.90	0.350	20.0	80.0	6
2.25	0.350	20.0	80.0	6

Formulations

The intravenous solution formulation of Tofacitinb was prepared using DMSO: Solutol: Ethanol (1:1): Normal Saline (5:5;90 % v/v). It was administered at a dose volume of 10 mL/ kg in male BALB/c mice by tail vein; while for oral dose the formulation was prepared in 1% Tween 80 in 0.5% methyl cellulose at dose volume of 10 mL/Kg.

Animal experiments

The experimental protocol for animal studies was approved by Institutional Animal Ethical Committee (IAEC) HSK college of pharmacy Bagalkot (IAEC/HKCOP/Aug2021/Ph.D3) nominated by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Male BALB/c mice (~6-8 weeks old; *n* = 12/arm) were procured from Vivo Biotech, Hyderabad, India. The subjected animals were quarantined in Animal House for a period of 7 days with a 12:12 h light:dark cycles and had free access to rodent feed (Altromin Spezialfutter GmbH & Co., Germany) and water *ad libitum*.

Pharmacokinetic studies

Tofacitinib was administered to male BALB/c mice by intravenous bolus dose and oral dose. The administered dose was 2 mg/ kg for IV and 10 mg/Kg for PO dose, Post dosing serial blood samples [~100 µL /mice (sparse sampling; *n* = 3 at each time point) were collected at 0.083 (only for IV), 0.25, 0.5, 1, 2, 4, 8 and 24 hr from retro-orbital plexus post dose. Blood samples were collected in tubes containing K_2.EDTA as the anticoagulant and centrifuged for 10 min at 10,000 rpm in a refrigerated centrifuge (Biofuge, Heraeus, Germany) maintained at 4 °C for plasma separation and stored frozen at -20 ± 10 °C until analysis.

Plasma samples processing and bioanalysis

The plasma samples were analyzed using a validated method previously reported [10]. Briefly, to an aliquot of 25 µL plasma sample, 10 µL of internal standard solution (Loperamide; 100 ng/mL) was spiked and to this mixture 0.4 mL of acetonitrile was added and vortexed for 3 min, followed by centrifugation at 10,000 rpm for = 5 min. Post centrifugation, clear supernatant was transferred into vials and 2 µL was injected onto LC-MS/MS system for analysis. The

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linearity range was 1.16 - 1511 ng/mL. In-study quality control (QC) samples, supplemented with concentrations of 3.49, 929.9 and 1453 ng/ mL of Tofacitinib, were analyzed with the unknown study samples

For plasma samples analysis the criteria for acceptance of the analytical batch includes the following:

(i) 67 % of the QC samples must be within 85-115 % of the nominal concentration.

(ii) Not less than 50 % at each QC (Low /Mid /High QC's) concentration level must meet the acceptance criteria.

Following completion of the bioanalysis both the linearity and quality control (QC) samples values were found within the accepted limits as per above criteria.

Mice pharmacokinetics study

The plasma concentration versus time profiles for tofacitinib was generated in mice. The key pharmacokinetic parameters such as area under curve (AUC), half-life ($T_{1/2}$), clearance (CL), and volume of distribution at steady state (Vss) were determined by non-compartmental analysis performed using Phoenix WinNonlin software (ver 8.1)

Plasma spotting

The DPS cards were prepared from freshly harvested whole plasma; on the same time by spotting 25 µL of the respective spiked CC/QC or whole plasma from the tofacitinib dosed mouse on sampling paper employing a calibrated pipette. The plasma samples were placed for drying in the dark for at least 3-4 h before storing at controlled temperature (24 ± 1 °C) until analysis; while DPS cards were placed for drying at ambient temperature for 2-3 h and post drying the samples were transferred in plastic pouch as per labeled time points.

DPS homogeneity

The spot homogeneity was evaluated by punching out the disc from the periphery of the DPS. Plasma spots at QC low and QC high level were prepared in triplicate. The obtained DPS were processed and analyzed as described in the sample preparation section.

Recovery

By comparing the responses of the analyte (Tofacitinib) extracted from repeated QC samples (n = 6) with the response of the analyte (Tofacitinib) extracted from standards at equal concentrations by liquid-liquid extraction procedure, the extraction efficiency of tofacitinib and the IS from DPS samples was assessed by liquid extraction process. Recovery of tofacitinib was determined at three QC concentration ie. LQC, MQC and HQC concentrations, whereas the recovery of the IS (Loperamide) was determined at a single concentration of 10 ng/mL. Samples were prepared by punching a 3.0 mm diameter disk from the center of the DPS dried area (Dried plasma spot) using a hole punch and mat (Harri-Micro-Punch®, 3 mm circular) into a 2.0 mL microcentrifuge tube using the 250 µL of extraction solvents ie. TBME (Ter butyl methyl ether), ethyl acetate, methanol, acetonitrile, methanol/acetonitrile: water (in different proportions).

Sample preparation

To each DPS card (3 mm) in a microcentrifuge tube, 200 µL of methanol:water (20:80) was added vortex mixed for 10 min (Thermomixer®, Eppendorf) and sonicated for 10 min at room temperature in an ultrasonicator (Elmasonic S 300H). Post sonication 200 µL of acetonitrile with the IS (100 ng/mL) was added vortex mixed for 10 min followed by centrifugation 10 min. Clear supernatant (200 µL) was separated and transferred into a HPLC vial for injection and 2.0 µL was injected onto LC-MS/MS system.

III. RESULTS

a) Calibration curve

The plasma calibration curve was constructed using eight calibration standards (viz., 1.16, 2.32, 18.6, 81.3, 232.5, 581, 1162 and 1511 ng/mL). The calibration standard curve was observed reliable, reproducible over the standard concentrations across the tested calibration range. Calibration curve of tofacitinib was prepared by determining the best fit of peak area ie. Analyte (Tofacitinib) /peak area IS ratios versus concentration, and fitted to the y = mx + c using weighing factor ($1/X^2$). The average slope and intercept values were found to be 0.0112 and 0.0050, respectively. The regression was found to be $\geq$0.99. The lowest concentration with the RSD <20% was taken as LLOQ and was found to be 1.16 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for calibration curves for tofacitinib was within 85.4–110.

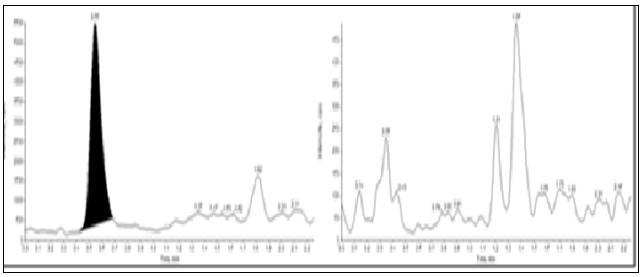

Figure 1: Plasma spiked with tofacitinib at LLOQ (1.16 ng/mL) and Typical MRM chromatograms of DPS blank sample.

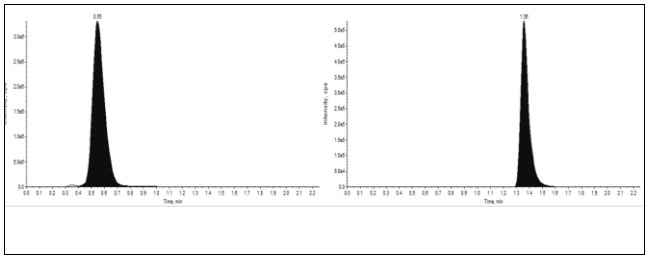

Figure 2: Plasma spiked with tofacitinib at ULOQ (1511 ng/mL) and Typical MRM chromatograms of loperamide at 1.4 min RT.

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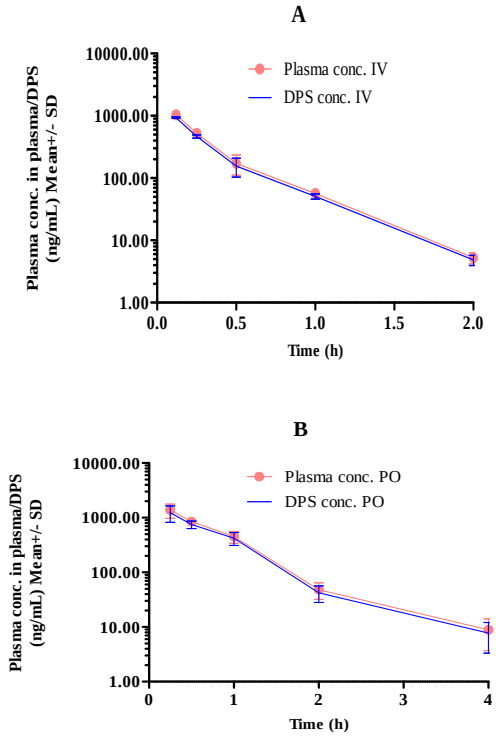

Figure 3 (A): Time vs. plasma concentration profile of tofacitinib in neat plasma and DPS following intravenous route at 2 mg/Kg dose;
Figure 3 (B): Time vs. plasma concentration profile of tofacitinib neat plasma and DPS following oral route at 10 mg/Kg dose;

TABLE 1 (A): Comparative intravenous pharmacokinetic parameters tofacitinib neat plasma vs. dried plasma spot (DPS) in male BALB/c mice at 2 mg/Kg dose.

IV (2 mg/Kg)	Unit	Plasma	DPS
$T_{1/2}$	(h)	0.30	0.30
Cmax	(ng/mL)	1036	933
C_0	(ng/mL)	1944	1774
AUC_{0-t}	(ng·h/mL)	455	410
$AUC_{0-\infty}$	(ng·h/mL)	458	413
CL	(mL/min/kg)	72.9	80.8
Vd	(L/kg)	1.87	2.09
Vss	(L/kg)	1.20	1.33
T_{last}	(h)	2.00	2.00
Time points considered for $T_{1/2}$ calculation:		0.5-2.0 h	0.5-2.0 h

TABLE 1 (B): Comparative oral pharmacokinetic parameters of tofacitinib neat plasma vs. dried plasma spot (DPS) in male BALB/c mice at 10 mg/Kg dose.

PO (10 mg/Kg)	Unit	Plasma	DPS
$T_{1/2}$	(h)	0.56	0.55
T_{max}	(h)	0.25	0.25
C_{max}	(ng/mL)	1376	1232
AUC_{0-t}	(ng·h/mL)	1083	977
$AUC_{0-\infty}$	(ng·h/mL)	1090	983
T_{last}	(h)	4.00	4.00
Time points considered for $T_{1/2}$ calculation:		1-4.0 h	1-4.0 h
Oral bioavailability	%	47.6	47.6

T1/2: terminal Half-life; Tmax: peak plasma time; C_{max} : Maximum plasma concentration; AUC0-∞:area under curve from time zero to infinity; AUC_{0-t}: area under curve from time zero to last observed concentration; Tlast: Time of Last Measurable Concentration; CL: Clearance; Vd: Volume of distribution; Vss: Volume of distribution at steady state.

The % variation of neat plasma vs. DPS (AUC, C_{max} and other key parameter) were within ±10% of variation and will be considered as within acceptable limit of variation.

The DPS method analysis can be used as an alternative tool for quantification of tofacitinib in preclinical and clinical setting where plasma volumes are negligible and storage of samples is an issue.

IV. Discussion

The DPS [Whatman DMPK FTA] cards are used for the analysis of various drugs. It is becoming an increasingly important tool in preclinical drug development, pharmacokinetics / toxicology research, newborn screening, clinical pharmacology, forensic toxicology, doping analysis, and therapeutic drug monitoring. The main advantages of the DPS are reduced matrix volume (plasma / serum) per time point, minimal invasiveness, easy and safe handling, and reduced risk of infection compared to traditional wet sampling. In addition, it offers potential cost savings such as storage, transportation, sample processing time (typically room temperature), and increased throughput. [13].

The objective of the present method was to develop a new simple, rapid and sensitive DPS method for tofacitinib in mice plasma and apply to a pharmacokinetic study in mice. During method development we first focused on the recovery of tofacitinib from DPS with various organic solvents like TBME, ethyl acetate, methanol, acetonitrile and found that recovery was very minimal (ranged between 10−15%). Then we systematically explored combination of methanol/acetonitrile/water in various combinations. The DPS samples extracted with solvents like Acetonitrile: Water (80:20; 50:50 and 20:80, v/v) have shown low recovery. The mixture of methanol:water showed good recovery results. At 20:80 methanol: water v/v ratios the recoveries were 47%. Therefore methanol:water (20:80, v/v) was selected as an extraction solvent as this combination gave consistency and reproducibility in results. The optimized gradient mobile phase [A:Acetonitrile, B:5mM ammonium formate with 0.1% Formic acid in water, Gredient] and chromatographic conditions (Acquity UPLC BEH C18 (50 x 2.1 mm, 1.7 µm) showed better separation with less back ground noise in LC-MS/MS optimized method and good elution power. In order to increase the assay precision, limit the variability between tofacitinib and the IS, to mimic the analyte during ionization, extraction and chromatography we have used loperamide as an IS. The attained LLOQ (1.16 ng/mL) was sufficient to quantify pharmacokinetic parameters for tofacitinib in a pharmacokinetic study in mice and indicating that the developed method is precise and accurate for the quantification of tofacitinib using DPS. Finally we have established an excellent correlation between tofacitinib measured in DPS and determined in plasma in parallel.

V. Conclusion

We have developed an analytical method for quantification of tofacitinib in mouse DPS samples. This method is selective, linear and accurate in the range of 1.16 to 1511 ng / mL. The developed DPS method has several advantages, including low

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plasma volume (25 µl), reduced use in large numbers of animals, continuous bleeding in mice and usefulness in toxicokinetics and pharmacokinetic studies.

In early drug discovery stage the developed method can be used for microsampling where mice are the commonly used species for pharmacokinetics. Traditionally, due to limited blood volume in smaller species; composite PK profiles are obtained from mice. With advancements in bioanalytical instrumentation and in blood sampling techniques, analysis with small blood volume (~50 µL) became feasible from the mouse for PK studies.

Conflict of interest: NO

All the authors have no conflict of interest to declare.

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