

RESEARCH ARTICLE

Ultra-Performance Liquid Chromatography Method for Bioanalysis of Teriflunomide using Dried Blood Spot Methodology

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ABSTRACT:

Plasma slowly loses teriflunomide, an oral immunomodulator used once in a day and majorly it is approved for treating of relapsing-remitting multiple sclerosis. It is not possible to measure the plasma concentrations of teriflunomide at locations without the tools necessary to prepare the processed blood samples. Plasma monitoring can be replaced with DBS technique, dried blood spot sampling, which allows for easier sample storage and travel. A few drops of blood are extracted from the rat tail using a lancet and applied to specially made absorbent filter paper. A UPLC test technique for the measurement of teriflunomide in DBS is created and validated for specificity, accuracy, selectivity, stability and repeatability using blood samples from pharmacokinetic studies. Process efficiency was necessary, method was selective and specific regarding endogenous chemicals, and there was no matrix effect. All concentrations were evaluated for accuracy as well as precision for intra-day and also inter-day analysis. The amount of blood deposited and the punch position within the spot had no bearing on the detection of teriflunomide in the DBS assay, however the hematocrit level had a negligible but tolerable influence on measurement precision. Teriflunomide has a minimum stability of three months at room temperature. With an average ratio of blood to plasma, an association between DBS concentration and plasma concentrations is seen. A straightforward and useful technique for keeping track of teriflunomide concentrations is DBS sampling. The technique has been expanded to the in-vivo determination of teriflunomide in male albino rats and is fully verified in accordance with ICH criteria.

KEYWORDS: Teriflunomide, Dried Blood Spot, UPLC, Immunomodulator, Multiple Sclerosis, *in-vivo*.

INTRODUCTION:

In vivo, teriflunomide has a protracted half-life¹. In cases of overdose, pregnancy, or whenever rapid elimination is deemed to be therapeutically beneficial, a technique using cholestyramine or activated charcoal may be utilised if necessary. Leflunomide's active metabolite, teriflunomide, is sold under the brand name Aubagio® and is used to treat relapsing types of multiple sclerosis. Important language on the FDA label warns users of teriflunomide of the possibility of hepatotoxicity and teratogenicity.

Laboratory equipment for blood centrifugation and plasma storage are needed for the current bioanalytical assay to determine teriflunomide concentration in plasma. Dried blood spot methodology² is an alternate technique that may be more practical. Although it's crucial to keep an eye on children's and babies' medication levels, there are only so many blood samples that can be taken. Using DBS micro-sampling technique, small aliquots of whole blood samples are spotted onto filter paper to analyse drug levels. DBS provides numerous benefits over a traditional plasma assay and in use for analysis of a wide broad range of medications. Especially to carryout paediatric investigations, reduced blood collection using DBS is beneficial in reducing the blood volume used in bioanalysis. To move from plasma to DBS methods, analytical and clinical validations are necessary^{3,4}. A review of the literature reveals that many bioanalytical techniques have been published for

Teriflunomide estimation. The existing methods like HPLC⁵ and LC-MS/MS⁶ are inappropriate for the estimation of Teriflunomide in biological fluids by simple and rapid technique. A single UPLC⁷ method by Qbdand a UHPLC⁸ method reported for Teriflunomide dosage forms. For Teriflunomide a DBS technique applied LC-MS/MS⁹ method also found in literature. Therefore, it is suggested to enhance the current methods and to create new, straightforward techniques for the quick and easy estimate of teriflunomide in biological fluids by UPLC¹⁰. The main objective of this research work focussed on development and further validating a UPLC bioanalytical method for teriflunomide detection and quantification using DBS.

MATERIALS AND METHODS:

The internal standard and Teriflunomide are bought from Sigma Aldrich trading co.ltd. Shanghai, China. All additional chemicals, including HPLC grade CAN (acetonitrile), methanol, ammonium acetate (GR grade) were procured from Merck (Mumbai, India). Millipore (Milford, MA, USA)'s Milli-Q water purification equipment was utilised to prepare the water for the entire analysis. Punching equipment named Harris Micro punch 6.0mm and the Harris cutting mat were purchased from GE Healthcare Ltd., together with FTATM DMPK-C (Drug Metabolism and Pharmacokinetic) blood spot cards. DMPK-C type were chosen since they don't need chemical impregnation like DMPK-A or DMPK-B cards do. The best DBS cards were selected after also looking into Agilent made DBS cards. Ultimately, chosen cards of DMPK-C because they had superior blood spot shapes than other cards. Ethylenediaminetetraacetic acid was added as an anticoagulant to drug-free whole-human-blood that was purchased from Vimta Laboratory (Hyderabad, India) and kept at -20°C until usage (not more than two days). The HCT value was calculated for each batch of freshly drawn blood and recorded. HCT values between 38% and 42% were advised.

UPLC Conditions:

Waters Acquity UPLC system and Empower software made up the system. Agilent column of Zorbax SB-C18 (100 x 2.1mm, particle size 1.8µm) used for UPLC chromatography, which was carried out at 0.5mL/min flow rate with mobile phases made up of an isocratic combination of acetonitrile and 1mM ammonium acetate at a 40:60 ratio. By running for 5 minutes, the chromatographic separation of the analyte and internal standard was optimised. The column was held at 20°C, whereas the autosampler was maintained at 4°C.

Preparation of Calibrators and QC Samples:

By dissolving standard 50mg of teriflunomide in a 50 mL volumetric flask, added 30mL methanol, after which the mixture was subjected to a 10-minute sonication

process at a temperature of no more than 20°C to create a standard stock solution of teriflunomide (TFM). Allowed the solution to reach room temperature before diluting it with methanol to the desired level to create a 1000µg/mL solution. Dilutions/working solutions from the aforementioned stock solution were created by spiking human blood with the following serial concentrations: 50, 100, 200, 300, 400, 500, 800, and 1000ng/mL.

A micro pipette used to mark blood aliquots from calibrators and quality control samples onto filter paper, which was then allowed to air dry for minimum two hours before going to analysis. All QC samples kept at room temperature until needed. QC concentrations are selected as LLOQ (50ng/mL), LQC (200ng/mL), MQC (400ng/mL) and HQC (800ng/mL)

DBS Sample Extraction:

Sample extraction procedures:

DBS discs measuring 6mm in diameter were extracted from the sample region using the Harris Micro punch and transferred to 2.0mL eppendorf tube. 1200µL of methanol and 40µL of IS (working IS in methanol at 0.5 µg/mL) were added. The material was shaken for one hour at the room temperature and then vortexed for about 15 seconds before being centrifuged for 10 minutes at 10,000rpm. A 10µL aliquot of this extract from 150µL was then transferred to a glass injection vial of 200µL capacity and injected into the UPLC machine.

Analytical Validation:

According to Guidance for Industry:

Bioanalytical method validation¹¹ and ICH guidelines¹², all validation studies were carried out.

Assay Specificity and Selectivity:

By confirming that there was no substantial interference with the used biological control medium related to retention duration by the drug (s) to be analysed, and thereby specificity was evaluated. By contrasting the chromatogram (s) of the blank biomatrix and the spiking matrix with the analyte at LOQ level, the specificity of the approach was validated. The RT, retention time was not accompanied by any interfering endogenous peaks.

Linearity:

In each run, a well-defined calibration curve was created for the teriflunomide concentration range of 50-1000ng/mL. The calibration samples were split in half and examined at the starting and at the ending of the run. We used the weighting method and calibration model that were the simplest. The ratio of peaks, teriflunomide/IS regions to the teriflunomide concentration served as the basis for calculating the curve's parameters. The samples' teriflunomide concentrations were calculated

using the derived linear regression equation for the curve.

With the exception of the lowest concentration level, where it should be within 20%, the accuracy of calibration samples obtained using back-calculation should be within 15% of the corresponding nominal concentration.

Except for LLOQ and ULOQ (upper limit of quantification 1000ng/mL), a maximum of 33% of samples per calibration curve may deviate from these requirements. Each curve reflected at least six different concentration levels.

Matrix Effect, Extraction Recovery, and Process Efficiency:

To ascertain the effect of the biological matrix on the quantification of teriflunomide, the instrument response for the injection of low, medium, and high QCs (n = 4 per level) directly into the mobile phase (neat solutions), the addition of the same amount of analyte to extracted blank samples (post extraction spiked samples), and the biological matrix before extraction (pre-extraction spiked samples), were compared. The ratio of mean teriflunomide peak regions in DBS processed validation samples compared to neat unextracted samples was used to determine the overall procedure efficiency. This phrase takes into consideration any signal loss resulting from the extraction procedure or the matrix effect. The amount of teriflunomide that was recovered during extraction was calculated by comparing the mean peak areas of extracted DBS validation samples with that of blank DBS samples which are spiked after the extraction.

The absolute matrix effect can be determined by comparing the average teriflunomide peak areas in neat, unextracted samples to those in DBS blank samples injected post extraction. It was assumed that there would be an external matrix influence if the ratio was 85% or 115%.

Matrix Variability:

To ensure that the biological matrix wouldn't interfere with the assay, the selectivity of the developed method was tested using six different individual lots of blank blood samples spiked with only IS at LLOQ level (n = 3 per lot) and blank blood samples without IS (n = 3 per lot). The results were compared to a calibration curve.

If accuracy ranged from 80% to 120% and from each matrix lot precision was less than 20%, then results for the LLOQ samples were deemed satisfactory. The examination of the blank samples from the six distinct lots was conducted using the raw peak areas identified at

the retention times of teriflunomide and IS as the foundation for the acceptance criterion. The average peak area of teriflunomide in the LLOQ QCs could not exceed 20% in more than 10% of the blank samples.

Stability:

When drying, storing, and transporting spiked DBS samples under various conditions, the stability of teriflunomide was examined. Each evaluation was carried out both at low QC and high QC concentrations in triplicate. In order to emulate expected sample and transport conditions, an optimum temperature of 25°C and 95% of relative humidity was used for the current investigation. These circumstances should be evaluated on basis of cases and were thought of as the worst case scenario. Internal data shows that drying time, which takes around 3 hours, was covered by assessing stability at working room temperature for 24hours. Freshly made QC DBS samples (from pooled blood) were kept at 25°C with 95% RH (relative humidity) for 24hours before processing and analysis to account for the effects of temperature and humidity during sampling and drying. QCs were kept at room temperature for 90 days before to process and analyse in order to evaluate the short-term as well as long-term storage of DBS extracted samples. Each and every QC sample utilised for these stability evaluations was tested against recently made spiked blood spots. Teriflunomide's stability in processed samples was also examined for autosampler stability up to 36hours.

Effect of HCT:

Blood viscosity is significantly influenced by the HCT value,¹³⁻¹⁶ and this could affect the blood's flow and diffusion properties into the filter paper. This result can lead to uneven blood distribution throughout the paper at high values of HCT. HCT reference ranges for men and women are 41%-51% and 37%-47%, respectively. By diluting blood cells with plasma taken from a blood collection, reconstituted blood samples with specific HCT levels (30%, 35%, 40%, 45%, 50%, and 60%) were made following 10-minute spinning at around 2000 rpm of fresh human blood in order to evaluate the significance of HCT on teriflunomide quantification. QC samples were created for each HCT level at low as well as high concentrations, then spotted onto the given filter paper. Then, QC samples made from freshly drawn human blood which has 41% HCT value were generated and analysed (n = 9 for each level per HCT value) against a drawn calibration curve.

Concentrations found at the low QC and high QC levels prepared with 40% value of HCT in blood were utilised as the optimised value for statistical data analysis in order to account for the bias seen in blood after reconstitution. The 95% confidence interval for the

mean and statistical study consisted of a bias determination as proper function of HCT level. If the estimated ratio's 95% confidence interval was wholly inside the 15% specified limit, the HCT value was regarded as unaffected.

Blood Spot Size Effect:

Since the sampling method enables the deposition of a varied volume of blood onto the DBS filter paper, the impact of blood spot size needs evaluation on determination's accuracy was carried out. By spotting increasing volumes (n = 4 per volume; 10, 20, 40, 60, and 80 μ L) onto the sample card, DBS samples at low QC and high QC concentrations were evaluated. Four replicate 6.0mm discs were removed from the centre of each QC sample after drying, and they were examined in addition to calibrators. TFM concentrations and nominal values were compared. DBS samples made with varied blood volumes would not appear to differ if accuracy fell between 85% and 115% of nominal values.

Punch Position Effect:

Due to a potential interaction between analyte and/or blood and the components of DBS filter paper, the distribution of testing chemicals in the area may be influenced by blood distribution¹⁷. By piercing the DBS discs (n = 3) from the centre and periphery of DBS samples at low QC and high QC concentrations, the homogeneity within the spot was evaluated, followed by analysis and calibrators. Results from both peripheral and central discs would indicate that there is no discernible distribution effect if the computed teriflunomide concentrations were accurate to within 85% to 115% of nominal concentrations.

Dilution Integrity Test:

This test was evaluated using DBS validated samples spiked with given compound at 2-, 4-, and 10-fold of high QC concentration to prove the procedure is appropriate for a DBS sample with test compound higher than ULOQ concentration. By raising the concentration of IS by the proper dilution factor, this test employing DBS samples (n = 9 per dilution) was carried out. Following extraction, a volume of injection solvent that was likewise multiplied by same factor was added to the dried extract. A DBS teriflunomide sample containing at a concentration higher than ULOQ level can be diluted using the previously tried dilution procedure, according to accuracy of the predicted concentrations within the range of 85%-115% of the nominal values.

Puncher Cross-Contamination Test:

Contrary to liquid samples, cross contamination is prevented by using disposable pipette tips, DBS method using a puncher that physically contacts the sample

while cutting each DBS disc. As a result, methanol was used to clean the cutting mechanism in between usage. Every validation run with a proper calibration curve includes 3 samples of blank punched immediately following at ULOQ calibration level, the sample punch to utilise a manual puncher for analysing spot-to-spot carry-over. These samples were taken and put through analysis. In the blank sample, any response noted for interfering peak(s) shouldn't be more than 20% of TFM response at the concentration of LLOQ.

Every validation run using a calibration plot curve contained a blank sample that was analysed immediately following the sample at ULOQ concentration level to look into carry-over effect from one sample to the next sample in the autosampler. In the blank sample, the interfering peak (s) response shouldn't be more than 20% of the component peak response at the LLOQ sample concentration.

Blood-to-plasma ratio:

Teriflunomide's blood-to-plasma ratio (B/P) was assessed at low QC and high QC. Whole blood was spiked with teriflunomide and IS, followed by an equilibrium incubation period of five minutes at 37°C in a preheated water bath. To obtain plasma samples, the remaining whole blood samples underwent centrifugation before being divided into aliquots (10 μ L) and spotted onto DBS cards (in duplicates). Methanol was used as a precipitating agent during the extraction of plasma samples. To determine the B/P ratio at low QC and high QC concentrations, the blood concentration in the DBS was divided by the matching plasma concentration.

Using the DBS technique for a pharmacokinetic investigation:

The albino rats (220 \pm 20g) were housed in a sterile setting with a temperature of 22°C \pm 2°C, 12hour light/dark cycles, and RH level of 50 \pm 5%. All rats were kept in allotted cages with access to free water and standard laboratory food. In every study, the animals (n=6) were given free access to water but were denied food 12hours before to treatment. A pharmacokinetic study successfully used the newly designed UPLC technology by administering To test the sensitivity and selectivity of the devised approach in a real-time analysis, teriflunomide was administered orally to six male albino rats using a single solution utilising a BD syringe equipped with an oral gavage needle (size 18). At 0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48hours after the dose, the sample was obtained. Each rat had a total blood volume taken from it of about 0.5mL. The spots were then perforated, dried, and centrifuged at 4,000rpm for 10 minutes in diluent. The acquired samples of supernatant were put into micro vials that had already

been labelled. The resulting blood samples were kept at -20°C until analysis. WinNonlin® software version 5.2 and SAS® software version 9.2 were used to calculate the pharmacokinetic parameters.

Sample Qualification and Sample Analysis:

Prior to analysis and after the DBS samples arrived in laboratory of bioanalytical, a visual examination of the spot's quality was carried out. The following conditions have to be satisfied for a place to be valid: A single drop of blood was used to create the spot, which was dark red in colour and had a diameter more than or equal to 7.0 mm. On the sampling paper, the spot was equally distributed on both sides. Following validation, the UPLC technique was used to analyse the DBS samples.

RESULTS:

Method development:

One of the essential elements in the DBS process to ensure correct diagnosis is blood spotting. Therefore, it is important to look into any potential blood spotting handling errors when developing the approach. Blood samples containing medications of interest are typically spotted with a pipette, one drop per spot. However, clinics may duplicate drop blood samples onto DBS cards. We evaluated the potential effects of this sample handling error on the assay accuracy. When spotting, pipettes were supposed to be held just above the DBS card without contacting it, according to the laboratory instructions. However, volumetric pipettes can have their tips touch the cards' surfaces. As a result, we assessed the effects by contact of the pipette tip while detecting cards. With a fixed spot volume of 10 μlitres of naive blood, the possible effects of punching various disc sites were evaluated using four punching positions as lower right, upper right, upper left, and lower left. The areas for the analyte peak were calculated for both low QC (200ng/mL) and high QC (800ng/mL) values. In addition to the disc with the centre-punched calibration samples, discs from the four periphery sites were also analysed. The peak area ratios of discs punched out from the disc's centre and its periphery were identical, showing that the impact of the disc punching locations was minimal. The 6 examined animals' reported analyte concentrations were similar, indicating reproducibility of blood spotting on the card across the board. Since there were no problems with selectivity or matrix effects during the method development, the UPLC settings employed in this investigation were the same as those for the test in human plasma. First, we concentrated on the extraction process, namely choosing the proper extraction solvents from acetonitrile, acetonitrile: water (8:2, v/v), acetonitrile: water (1:1, v/v), and methanol, methanol: water (8:2, v/v), and methanol: water (1:1, v/v). Even though acetonitrile's extraction efficiency was limited, other solvents had comparable extraction

performance. We chose 50% v/v acetonitrile rather than 100% v/v organic solvents or blends that contained 80% v/v organic solvent since there were less endogenous peaks in the chromatograms. Lower sensitivity of this DBS approach compared to conventional plasma-based assays is a potential concern that needs to be addressed during method development. We tested in a way such that increase in the punching spot area resulted in higher sensitivity given the desired LLOQ. In addition to the 3.0mm diameter of disc punching, the sensitivity of a 6.0mm diameter punch was evaluated. The peak intensity of 6.0mm disc punch increased three to four times, matching the expected increase of four times. According to this study, expanding the punching disc area might boost the assay's sensitivity for teriflunomide in human whole blood. If a more sensitive blood-based assay is needed in the future, a bigger punch might be helpful in supporting clinical investigations.

Analytical method validation (AMV):

Specificity and Selectivity:

The retention durations for tenofovir and teriflunomide were around 2.6 and 3.4 minutes according to the represented chromatograms for blank spot and blank spot spiked with teriflunomide at LLOQ and internal standard, IS shown in Figure 1. In blank chromatogram that was eluted with teriflunomide or tenofovir, there were no discernible interference peaks. As a result, the technique is focused and specific with regard to endogenous substances.

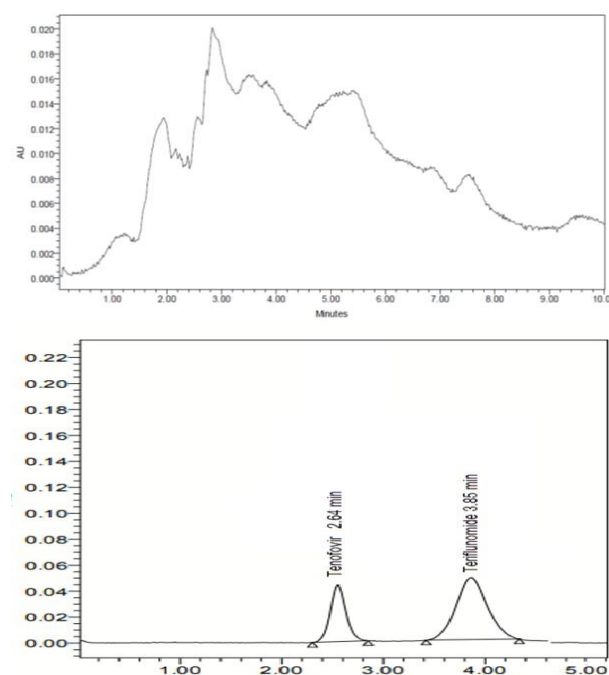


Fig. 1: Chromatograms for blank DBS, and blank DBS spiked with teriflunomide at LLOQ and IS

Linearity and Accuracy:

Teriflunomide calibration curves were found linear and ranged from 50 to 1000ng/mL. The calibration curve's parameters in given Table 1 demonstrate that the value of correlation coefficient (R) is above 0.99. The variability test of assay, ISR, and regular analysis showed that there was no effect on the validity or the robustness of the method because the slopes variance and intercepts of the calibration curves were acceptable. The mean for back-calculated concentrations listed in Table 2 and they were in between 98.1 and 106.9% of nominal, with CVs for teriflunomide ranging from 1.6% to 2.3%. All individual concentrations which are back-calculated fell within the specified range.

Matrix Effect:

Investigating the effect of the sample matrix occurs throughout the development and validation of the assay. Table 3 displays the information regarding the teriflunomide matrix impact. No matrix effect was noticed regardless of testing substance concentration, and process efficiency was about 89%. Teriflunomide is not significantly lost during the extraction process, according to the recovery data at different concentrations, and there is also no considerable binding to the employed polypropylene and glass components.

Matrix Variability:

The variable ranges of accuracy and precision utilising 6 distinct lots of human blood were within predetermined acceptable requirements at the LLOQ for teriflunomide. The same six different blood lots' blank samples (without IS) had no interfering effects on the raw peak areas that were more over 20% of LLOQ's peak area. Quantification of teriflunomide was unaffected by the influence of endogenous components, which was confirmed using 6 distinct lots of human blood samples (matrix variability).

Assay Variability:

Tables 4 and 5 give data for intra-day (within the same day) and inter-day (on different days) accuracy and precision of current method for teriflunomide determination. QC samples were examined over the course of two days, and assessed the method's intra- and inter-day accuracy parameters. The results showed that the method had intra-day (same day) precisions that ranged from 93.9 to 107.0% and inter-day (two different days) precisions that ranged from 92.5 to 109.2%. Over 4 QC concentrations, the overall precision (% CV) ranged from 1.3% to 4.5%. As a result, the approach has a respectable level of accuracy as well as precision.

Stability Study:

The test substance remained stable in dried condition of blood spot for at least 90 days with and without relative humidity for 24 hours when it was packed along with

desiccant and kept at working room temperature. This outcome avoids the potential problem of extremely constrained medical infrastructures for drying, storing, and transportation of taken samples. Additionally, when kept at the autosampler temperature of 4°C, processed DBS samples of teriflunomide remained stable for minimum 36 hours. Table 6 displays the findings of the evaluation of Teriflunomide's long-term stability in DBS. Tables 7 and 8 exhibit the results of short-term stability study for low and high QC, respectively.

Effect of HCT:

Regardless of the teriflunomide concentrations (Low QC and High QC), the bias rose linearly according to statistical study analysis as a function of HCT level with 95% confidence interval (Figure 2). As a result, at low nominal concentrations and using an acceptance limit of 15% for the bias, the quantitation of teriflunomide in DBS sample satisfies the requirements for HCT accepted interval (35%–57%). Teriflunomide concentrations in DBS are high enough to meet the HCT interval's acceptable parameters (30%-55%). The validated values of intervals easily fall within the range of reference values for both men (41%-51%) and women (37%-47%) according to the HCT reference values. Calibration and QC samples should be made from freshly taken blood with HCT as near to 40% as much as possible to reduce the impact of HCT during regular sample analysis.

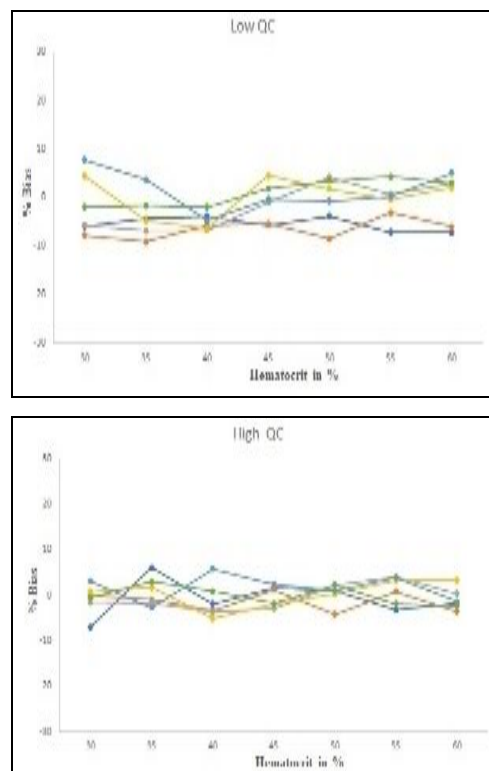


Fig. 2: Influence of HCT on quantification of teriflunomide at low and high QC concentrations

Blood Spot Size Effect:

Accuracy of the measured concentration relative to the nominal value was constant with the volume of blood deposited and remained within acceptable limits, as evidenced by increased deposition of QC samples at low and high concentrations.

Punch Position Effect:

In Table 9, the accuracy measurement of teriflunomide using various discs punched from the DBS's centre and edges is contrasted. There was no observable dispersion influence, as evidenced by the analyte concentrations that were measured with bias values from the centre and peripheral discs of DBS samples, which varied from 85% to 115%.

Dilution Integrity Test:

DBS samples can be precisely diluted into the calibration curve's range by a factor of 2, 4, or 10, according to the findings of trials to assess the accuracy values of teriflunomide quantification after diluting the sample (data not shown).

Tests on the puncher's cross-contamination and autosampler carry-over:

When each punch was followed by a methanol clean of the cutting system, the puncher cross contamination test demonstrated that there was negligible cross contamination. Furthermore, there was no evidence that teriflunomide had been carried over from the autosampler when a blank DBS sample was administered right after injecting ULOQ sample.

Pharmacokinetic research Sample eligibility:

By visual examination, all samples revealed that about 99% of the spots on all DBS cards were genuine.

Sample Evaluation:

T_{max} (time to observe maximum drug concentration), K_{el} (apparent terminal velocity constant calculated from as semi-logarithmic plot of blood concentration versus time curve using squared regression minimum) and $t_{1/2}$ are the values of C_{max} (maximum drug concentration observed during the study), AUC_{0-48} (area under the blood concentration-time curve measured in 48 hours according to the trapezoid rule) and AUC_{0-48} . The devised method was used to analyse all of the samples, and Figure 3 displays the mean teriflunomide concentrations over time. Table 10 displays the calculated pharmacokinetic parameters.

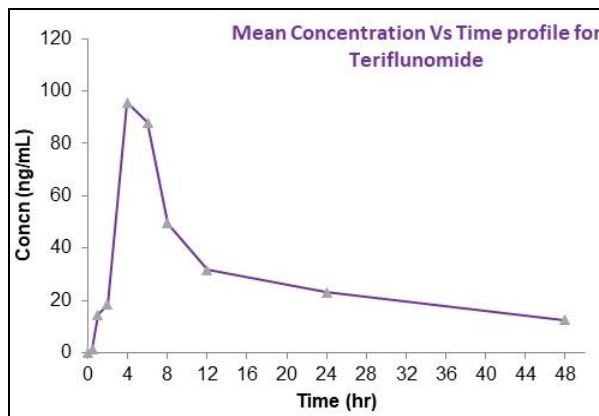


Fig. 3: Concentration-Time Profile of Teriflunomide in Pharmacokinetic study

DISCUSSION:

Analytical validation and a PK investigation in accordance with accepted standards were carried out to encourage the technical use of DBS in the measurement of teriflunomide bio concentrations. Spotting 10 μ L of blood sample onto the specified filter paper for the analytical validation made it simple to punch a partial disc for quantification with a diameter of 6 mm. By minimising the variability of HCT level, means the amount of deposited blood, and the placement of Harris punch within the spot, a 6.0 mm punch maximised the precision value of the procedure in comparison to a smaller punch diameter. The quantity of blood to the filter paper applied and the placement of the punch position had no impact on the measurement of testing chemical. Additionally, the accuracy of the data was only slightly but acceptably impacted by the HCT value within the range (30%-55%). The median HCT values observed in MS patients are covered by this range¹⁸. Blood samples with teriflunomide were stored for at least three months at working room temperature after being drawn up on filter paper, for at least 36 hours in processed samples stored at room temperature, and for 24 hours at 37°C with and without relative humidity. Additionally, teriflunomide remained stable in blood samples for at least 24 hours. Together, these stability experiments took into account the sample's anticipated drying, transport, and sampling environments.

CONCLUSION:

By doing away with the necessity for a phlebotomist, refrigerated storage for sample transportation, and centrifugation during the processing of plasma, DBS sampling is an easy and practical way to monitor teriflunomide concentrations. This greatly simplifies drug monitoring regardless of the location of the patient or the availability of lab space. These preliminary results imply that DBS sampling instead of the conventional plasma assay could be utilised for teriflunomide determination in all pharmacokinetic evaluations, while

additional developed research in patients and application in a bigger clinical trial are still required. Since all of the pharmacokinetic parameters fell within a reasonable range, it can be said that the current study offers solid support for clinical pharmacokinetic investigations for future research on the chosen medicine. The results of all the validation parameters lead us to the conclusion that the developed approach may be used with the requisite precision and accuracy for bioavailability and bioequivalence (BA/BE) investigations and regular therapeutic medication monitoring.

COMPETING INTERESTS:

The authors have no material financial or non-financial interests to disclose.

AUTHOR CONTRIBUTIONS:

The study's inception and design involved input from all authors. The final manuscript was read and approved by all writers.

Table 1: Linearity data set of Teriflunomide

Concentration in (ng/mL)	Teriflunomide Peak Area
50	2514
100	5425
200	10328
400	19942
500	26977
600	31285
800	40069
1000	51005

Table 2: Recovery Results of Teriflunomide

Recovery	LLOQ QC-50 ng/mL		LOW QC-200 ng/mL		MID QC-400 ng/mL		HIGH QC-800 ng/mL	
	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery
	51.960	103.920	206.337	103.146	409.960	102.490	796.038	99.147
	49.070	98.140	212.187	106.070	416.029	104.007	831.002	103.502
	49.370	98.740	211.563	105.758	406.003	101.501	815.204	101.534
	50.920	101.840	213.854	106.903	409.565	102.391	815.934	101.625
	51.170	102.340	213.618	106.786	415.116	103.779	831.872	103.610
	51.230	102.460	202.146	101.051	395.052	98.763	825.952	102.873
Mean	50.620	101.240	209.951	104.952	408.621	102.155	819.334	102.049
SD	1.142		4.696		7.625		13.479	
CV(%)	2.257		2.237		1.866		1.645	

Table 3: Matrix effect Results

Unit No.	Teriflunomide 500 ng/mL	
	Neat standard Concentration	Extracted blank along with spiked sample peak concentration
Unit No.: 1		
Unit No.: 2	506.020	512.309
Unit No.:3	477.504	522.804
Unit No.: 4	495.620	525.138
Unit No.: 5	487.158	512.167
Unit No.: 6	495.506	505.899
Mean	490.658	514.694
SD	10.411	7.586
CV(%)	2.122	1.474
Matrix effect (%)	1.049	

Table 4: Intra-day Accuracy and Precision Results

Teriflunomide								
	LLOQ QC-50 ng/mL		LOW QC-200 ng/mL		MID QC-400 ng/mL		HIGH QC-800 ng/mL	
	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery
Intra-day	48.150	96.300	212.266	105.900	392.798	98.199	856.038	107.005
	52.260	104.520	209.268	104.404	408.998	102.250	751.002	93.875
	50.100	100.200	208.107	103.825	404.206	101.052	815.204	101.901
	51.452	102.904	211.809	105.672	420.145	105.036	787.934	98.492
	50.450	100.900	208.073	103.808	376.069	94.017	791.872	98.984
	50.690	101.380	214.878	107.203	396.971	99.243	825.952	103.244
Mean	50.517	101.034	210.733	105.135	399.864	99.966	804.667	100.583
SD	1.395		2.713		15.087		36.129	
CV(%)	2.760		1.288		3.773		4.490	

Table 5: Inter-day Precision and Accuracy Results

Teriflunomide								
	LLOQ QC-50 ng/mL		LOW QC-200 ng/mL		MID QC-400 ng/mL		HIGH QC-800 ng/mL	
	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery
Inter-day	50.850	101.396	214.587	107.270	392.977	98.244	814.627	101.828
	52.510	104.706	215.581	107.767	370.998	92.750	800.305	100.038
	50.380	100.459	218.360	109.156	394.206	98.552	808.002	101.000
	49.560	98.824	211.452	105.703	370.145	92.536	826.329	103.291
	51.400	102.493	210.851	105.402	396.000	99.000	790.015	98.752
	51.200	102.094	217.062	108.507	392.710	98.178	833.311	104.164
Mean	50.983	101.662	214.649	107.301	386.173	96.543	812.098	101.512
SD	0.996		3.004		12.143		16.140	
CV (%)	1.953		1.399		3.145		1.987	

Table 6: Long-term stability study Results (n=6) after 90 days

Long term stability after 90 days	TFM			
	0 Hr-Low QC	0 Hr-HQC	Day-90-LQC	Day-90-HQC
	209.623	757.042	203.140	756.038
	200.733	792.642	204.920	791.002
	201.580	800.428	201.400	815.204
	199.496	770.430	197.000	755.934
	189.832	797.042	193.800	771.872
203.946	843.042	195.560	825.952	
Mean	200.868	793.438	199.303	786.000
SD	6.486	29.587	4.478	29.918
CV(%)	3.229	3.729	2.247	3.806
% Change	N/A	N/A	-0.779	-0.937

Table 7: Short-term stability study Results (n=6) for LOW QC Concentration

Short term stability	TFM (LOW QC-200 ng/mL)					
	At 0 Hour		At 4 Hour		At 24 Hour	
	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery
	209.623	104.812	203.400	101.700	207.840	103.920
	200.733	100.366	204.020	102.010	199.880	99.940
	201.580	100.790	196.240	98.120	207.748	103.874
	199.496	99.748	193.112	96.556	211.661	105.830
	189.832	94.916	191.256	95.628	186.047	93.023
203.946	101.973	194.125	97.062	207.692	103.846	
Mean	200.868	100.434	197.026	98.513	203.478	101.739
SD	6.486		5.426		9.368	
CV(%)	3.229		2.754		4.604	
% Change	N/A		-1.913		1.299	

Table 8: Short-term stability study Results (n=6) for High QC concentration

Short term stability	TFM (High QC-800 ng/mL)					
	At 0 Hour		At 4 Hour		At 24 Hour	
	Concn. found	% of Recovery	Concn. found	% of Recovery	Concn. found	% of Recovery
	757.042	94.630	776.038	97.005	753.978	94.247
	792.642	99.080	793.046	99.131	780.290	97.536
	800.428	100.054	814.000	101.750	792.138	99.017
	770.430	96.304	782.028	97.754	768.488	96.061
	797.042	99.630	815.220	101.903	810.990	101.374
843.042	105.380	818.724	102.341	804.592	100.574	
Mean	793.438	99.180	799.843	99.980	785.079	98.135
SD	29.587		18.567		21.760	
CV(%)	3.729		2.321		2.772	
% Change	N/A		0.807		-1.053	

Table 9: Effect of Punch position on Teriflunomide recovery

Punch position	TFM			
	LOW QC-200 ng/mL		HIGH QC-800 ng/mL	
	Central	Peripheral	Central	Peripheral
	95.420	95.780	102.006	97.715
	98.260	99.260	99.708	96.022
	100.400	97.028	96.750	99.650
	98.000	95.578	96.558	99.146
	92.260	104.738	96.875	98.223
	96.340	106.420	102.675	105.143
Mean	96.780	99.801	99.095	99.317
SD	2.802	4.694	2.776	3.121
CV(%)	2.895	4.703	2.801	3.142

Table 10: Pharmacokinetic parameters for Teriflunomide in Rat blood (n=6, Mean±SD)

Parameter	TFM
C _{max} (ng/mL)	95.721 ± 9.363
T _{max} (h)	4.00 ± 1.021
t _{1/2} (h)	11.745 ± 4.604
K _{el} (h ⁻¹)	0.059 ± 0.098

C_{max}: maximum concentration. T_{max}: time point of maximum concentration. t_{1/2}: half life of drug elimination during the terminal phase.

K_{el}: elimination rate constant

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