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ESTIMATION OF TERIFLUNOMIDE ALONG WITH CONCOMITANT DRUGS IN DIFFERENT BIOLOGICAL MATRICES USING LC-MS/MS

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ABSTRACT: A simple, rapid and sensitive liquid chromatography-tandem mass spectrometric (LC–MS/ MS) assay method has been developed and fully validated for the quantification of Teriflunomide (TFM), in different biological matrices like human plasma, rat plasma, rabbit plasma and dog plasma and as well as their serums. The analytes were extracted from 200 µL aliquots of each plasma/serum via protein precipitation using acetonitrile. The extracted samples were chromatographed on a XBridge C18 column by using acetonitrile and 5 mM ammonium formate (pH 9.0) as the mobile phase in gradient mode at a flow rate of 1.0 mL/min. The calibration curves obtained were linear ($r^2 \geq 0.98$) over the concentration range of 1.0–4000 ng/mL. Detection was carried out on a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) with an electro spray ionization (ESI) mode using the respective m/z 268.8→82 for TFM and 281→86 for IS. The method was validated interms of specificity, accuracy, precision and limit of quantification. The results of the intra-and inter-day precision and accuracy studies were well within the acceptable limits. The analyte was found to be stable up to 72 hrs in the matrices. Along with concomitant drug analysis the method is precise and sensitive enough for its intended purpose and can be applied to clinical samples

INTRODUCTION: Teriflunomide ((2Z)-2-cyano-3-hydroxy-N-[4-(trifluoromethyl) phenyl] but-2-enamide) is an immunomodulatory agent with anti-inflammatory properties that selectively and reversibly inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHO-DH), required for de novo pyrimidine synthesis. As a consequence teriflunomide reduces the proliferation of dividing cells including activated T cells which are thought to drive the disease process in Multiple Sclerosis.

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Teriflunomide (**Fig.1**) may decrease the risk of infections compared to chemotherapy like drugs because of its more-limited effects on the immune system [1]. It has been found that teriflunomide blocks the transcription factor NF-kB and also inhibits tyrosine kinase enzymes, but only in high doses not clinically used [2]. Teriflunomide is actively being investigated for use in renal transplant recipients, not only for its immunosuppressive effects, but more important, because it has antiviral effects that assist in clearing infections common in transplant recipients, such as BK polyomavirus (BKV) [3-6] and cytomegalovirus [7]. Several analytical methods including HPLC and LC-MS are reported to determine Teriflunomide in whole human blood, human plasma, rabbit plasma with a sensitivity of 0.4 mcg/mL[8-10]. All the

reported methods have applied different extraction procedures and different sensitivity ranges.

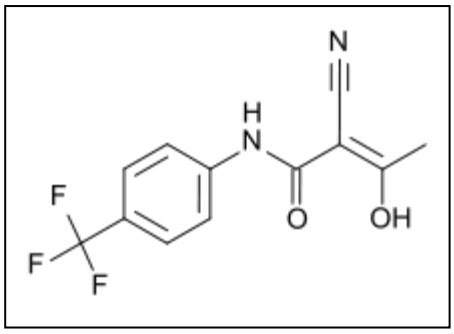


FIG. 1: STRUCTURE OF TERIFLUNOMIDE

Over the last decade, liquid chromatography (LC) combined with mass spectrometry (MS) has become a powerful analytical tool. A breakthrough was the introduction of two atmospheric pressure ionization (API) interfaces, electro spray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Today, LC-MS has evolved into a technique characterized by sensitivity, selectivity, and specificity, allowing for the analysis of trace amounts of target analytes in complex mixtures. Based on these characteristics, one would expect that sample preparation prior to analysis could be minimized or even eliminated. Simplification of sample preparation would reduce time-consuming method development and sample analysis time during routine application of the method.

MATERIALS AND METHODS:

Chemicals and reagents:

TFM was supplied by Selleckhem.com Imipramine (IMP) internal standard, was supplied by Sigma-aldrich. Acetonitrile of MS grade were obtained from Merck, India. Other chemicals were all of analytical grade and purchased from Merck, India. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore. Biological matrices were obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until use.

Instrumentation:

A Shimadzu LC system (Model: SIL-HTC) was coupled with a MS system (AB Sciex), which was equipped with an ESI source and operated with Analyst 1.6.1 software.

Liquid Chromatographic conditions:

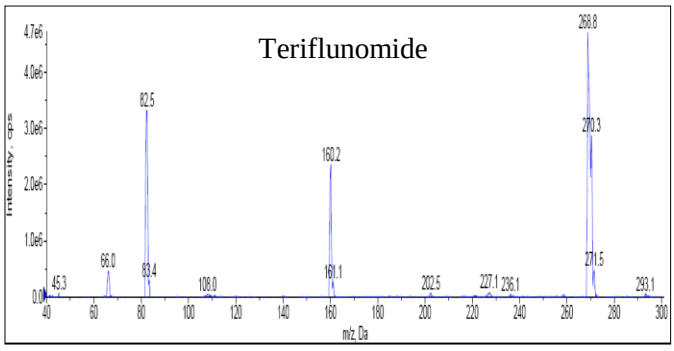
HPLC separation was carried out on a XBridge C18 analytical column (150 mm × 4.6 mm, 5 µm) with mobile phase A-0.005M Ammonium formate pH 9.0 and B-acetonitrile at a flow rate of 1 mL/min in gradient mode and the column temperature was maintained at 30°C. The sample injection volume was 10 µL and the analytical run time was 15 min. The autosampler temperature was maintained at 5°C.

MS conditions:

Ionization and detection of teriflunomide and IS was carried out on a triple quadrupole massspectrometer, MDS SCIEX API-3000 (Toronto, Canada), equipped with electro spray interface and operating in both negative and positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor→product ion transitions for teriflunomide (m/z 268.8→82) and IS (m/z 281→86). The source dependent parameters maintained for both teriflunomide and IS were Nebuliser gas: 12.0 psig; ion spray voltage (ISV): 3500V; heater temperature (TEM): 450°C; collisional activation dissociation (CAD): 5 psig and curtain gas (CUR), nitrogen: 8 psig.

The optimum values for compound dependent parameters like declustering potential (DP), collision energy (CE), entrance potential (EP), focusing potential (FP) and cell exit potential (CXP) set were -46, −30, -10, −283 and −13V for teriflunomide and 40, 25, 12, 230 and 13V for IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms. Analyst software version 1.6.1 was used to control all parameters of LC and MS. The product ion spectra of these analytes are shown in **Fig.2**.

(a)



(b)

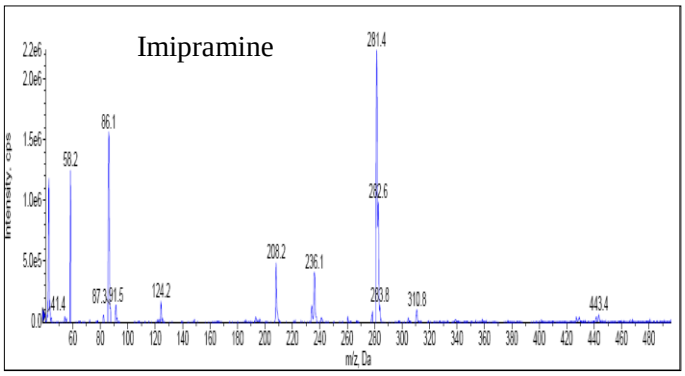


FIG. 2: PRODUCT ION MASS SPECTRA OF (a) TERIFLUNOMIDE (m/z 268.8→82) IN NEGATIVE IONIZATION MODE AND (b) INTERNAL STANDARD, IMIPRAMINE (m/z 281→86) IN POSITIVE IONIZATION MODE

Standard stock, calibration standards and quality control sample preparation:

The standard stock solution of teriflunomide (1000 µg/mL) was prepared by dissolving requisite amount in methanol. Its working solution for spiking was prepared in methanol:water (80:20,v/v). Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with working solution (4% of total plasma volume). Calibration curve standards were made at 1, 5, 10, 50, 100, 500, 1000 and 4000 ng/mL concentrations respectively, while quality control samples were prepared at five levels, viz.4000 ng/mL (ULOQ QC, upper limit of quantitation quality control), 3000 ng/mL (HQC, high quality control), 500 ng/mL (MQC, medium quality control), 5 ng/mL (LQC, low quality control) and 1 ng/mL (LLOQ QC, lower limit of quantification quality control).

Stock solution (1 mg/mL) of the internal standard was prepared by dissolving 25.0 mg of IMP 25.0 mL of methanol. Its working solution (50 µg/mL) was prepared by appropriate dilution of the stock solution in methanol:water (80:20,v/v). Standard stock and working solutions for spiking were stored at 2-8°C, while calibration curve and quality control samples in plasma were kept at −70°C until use.

Sample extraction procedure:

Prior to analysis, all frozen samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 50 µL of spiked plasma/serum sample, 20 µL of internal standard (1 µg/mL) was added and vortexed for 10s. Further, 150 µL of acetonitrile was added and vortexed for 2 min. Samples were then centrifuged at 10000 rpm for 10 min at 10°C. After centrifugation, supernatant was taken and injected into LC conditions.

Validation Methodology:

The bioanalytical method was thoroughly validated following the USFDA guidelines [11]. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of teriflunomide (500 ng/mL) and IMP (1 µg/mL) at the start of each batch during method validation. System performance was studied by injecting one extracted LLOQ sample with IS at the beginning of each analytical batch and before reinjecting any sample during method validation. Carryover experiment was performed to verify any carryover of analyte, which may reflect in subsequent runs. The design of the study comprised of the following sequence of injections i.e. diluent (Methanol:Water 80:20), LLOQ sample, extracted blank plasma/serum, ULOQ sample, extracted blank plasma/serum.

The selectivity of the method towards endogenous plasma matrix components was assessed in twelve different batches (8 normal of K2EDTA, 2 haemolysed and 2 lipemic) of blank plasma. Check for interference due to concomitantly used antirheumatic medication (ibuprofen, fluoxetine, and venlafaxine) was studied for ionization (ion suppression/enhancement), analytical recovery (precision and accuracy) and chromatographic interference (interference with MRM of teriflunomide and IS). Their stock solutions (100µg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (500ng/mL) of each drug were prepared in the mobile phase, spiked in plasma and analyzed under the same conditions at LQC and HQC levels.

The MRM transitions in the negative ionization mode for ibuprofen (205/161), fluoxetine (380/316), and venlafaxine (278/121) were studied. The linearity of the method was determined by analysis of five calibration curves containing eight

non-zero concentrations. The area ratio response for teriflunomide/IS obtained from multiple reaction monitoring was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted (1/x2) linear regression which was finalized during pre-method validation. A correlation coefficient (r2) value > 0.99 was desirable for all the calibration curves.

The relative recovery, matrix effect and process efficiency were assessed. All three parameters were evaluated at HQC, MQC and LQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean area response of pre-spiked samples (spiked before extraction) to that of extracts with post-spiked samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of un extracted samples (spiked after extraction) with mean area of neat standard solutions (in methanol). The overall 'process efficiency'(%PE) was calculated as (ME×RE)/100.

Further, the effect of plasma/serum matrix (relative matrix effect) on analyte quantification was also checked in six different batches/lots of plasma/serum. From each batch, six samples at LLOQ level were prepared (spiked after extraction) and checked for the %accuracy and precision (%CV). The deviation of the standards should not be more than ±15% and at least 90% of the lots at each QC level should be within the fore mentioned criteria. All stability results were evaluated by measuring the area response (teriflunomide/IS) of stability samples against freshly prepared comparison standards at LQC and HQC levels. Stock solutions of teriflunomide and IS were checked for short-term stability at room temperature and longterm stability at 2–8°C.

solutions were considered stable if the deviation from nominal value was within ±10.0%. Autosampler stability (wet extract), bench top (at room temperature) and freeze–thaw stability were performed at LQC and HQC using six replicates at each level. Freeze–thaw stability was evaluated by successive cycles of freezing (at −20 and −70°C) and thawing (without warming) at room temperature. Longterm stability of spiked plasma samples stored at −20 and −70°C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%. To authenticate the ruggedness of the proposed method, it was performed on two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was studied on two different columns.

Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at 8000 ng/mL (2×ULOQ) teriflunomide concentration in the screened matrix. The precision and accuracy for dilution integrity standards at 1/5th (1600 ng/mL) and 1/10th (800 ng/mL) dilution were determined by analyzing the samples against freshly prepared calibration curve standards.

RESULTS AND DISCUSSION: The present study was undertaken to develop a sensitive and selective method to determine teriflunomide, the pharmacologically active metabolite of LEF in different biological matrices. Typically, suppression or enhancement of analyte response is accompanied by diminished precision and accuracy of subsequent measurements[12-13]. One limitation associated with LC-MS analysis is its susceptibility to matrix effect[14]. Matrix effect is defined as the effect of co-eluting residual matrix components on the ionization of the target analyte. Matrix effect thus limits the utility of LC-MS for quantitative analysis. Teriflunomide is mainly responsible for the anti-inflammatory and disease-modifying properties of LEF[15-16]. The current method is proved for its ruggedness to different biological matrices like human plasma, rat plasma, rabbit plasma, dog plasma and their serums. This method can be applied in studying the preclinical as well as clinical sample analysis of teriflunomide.

Method development:

The electro spray ionization (ESI) of teriflunomide was conducted in negative ionization mode as it has high electron affinity due to the presence of trifluoro methyl group. Internal standard, IMP has given higher response in positive mode. Q1MS full

scan spectra for teriflunomide predominantly contained deprotonated precursor [M−H]− ions at m/z 268.8 and IS at m/z 281 respectively. The most abundant and consistent product ions in Q3MS spectra for teriflunomide and IS were observed at m/z 82.0 and m/z 86 at −30 and 25V collision energy respectively.

The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and adequate response for the analyte. Reported procedures have employed either protein precipitation or liquid–liquid extraction (LLE) for sample preparation from human plasma. Chan et al.[9] have reported extraction of teriflunomide by protein precipitation with acetonitrile using 100 µL human plasma. The mean extraction recovery at different QC levels was 101.3%. Quantitative extraction of LEF and its metabolite, teriflunomide by LLE has been demonstrated by Schmidt et al.[10]. Both the analytes were extracted in ethyl acetate employing 250µL plasma in presence of sodium acetate buffer (pH 5.0).

In the present study, based on sensitivity, matrix effect and reproducibility requirements both these extraction techniques were tried during method development. Reproducibility and recovery data for teriflunomide and IS supported protein precipitation to be used as the preferred extraction technique for all the matrices. The recovery in other solvent systems was between 50% and 80%, but was in consistent with some ion suppression (greater than 15% CV).

The chromatographic separation of teriflunomide and IS was initiated to achieve a short runtime, symmetric peak shapes, minimum matrix interference and solvent consumption. Previous studies have reported different columns with 5µm particle size, 3–4 mm inner diameter and columns lengths (125–150 mm) with runtimes ≥15 min[8–10]. Thus, in the present work chromatographic separation was tried on XBridge C18 (150 mm×4.6 mm, 5 µm), Waters X-Terra MS C18 (100 mm×3.9 mm, 5 µm) and Inertsil ODS-3 C18 (150 mm×4.6 mm, 5 µm) columns. To find the best eluting solvent system, various combinations of methanol/acetonitrile with additives like ammonium acetate and ammonium formate in different concentration and volume ratios were tested. Best results were obtained in terms of higher sensitivity, superior retention and better peak shapes on XBridge C18 and also on XTerra MS C18 column. The total run time of 15 min ensured separation of teriflunomide and IS at 6.7 and 9.6 min respectively. Representative chromatograms in **Fig. 3 & 4** of extracted blank plasma, fortified with teriflunomide at Mid QC (m/z 268.8→160) and IS (m/z 281→86).

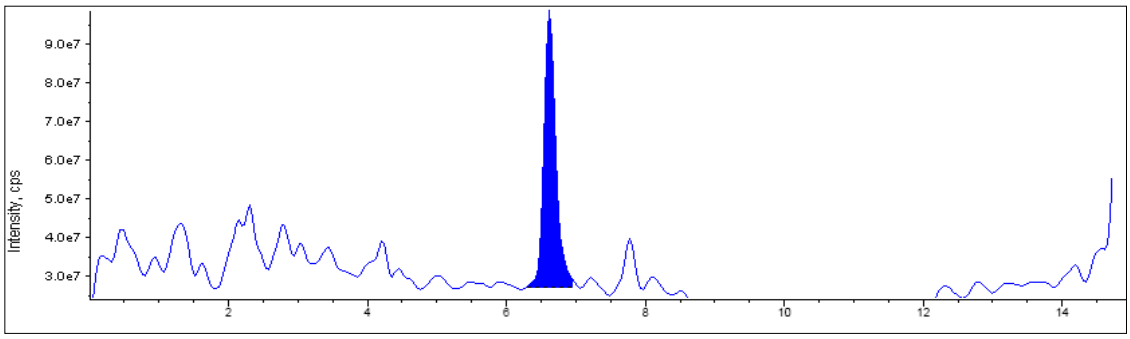


FIG. 3: MRM-ION CHROMATOGRAM OF TERIFLUNOMIDE

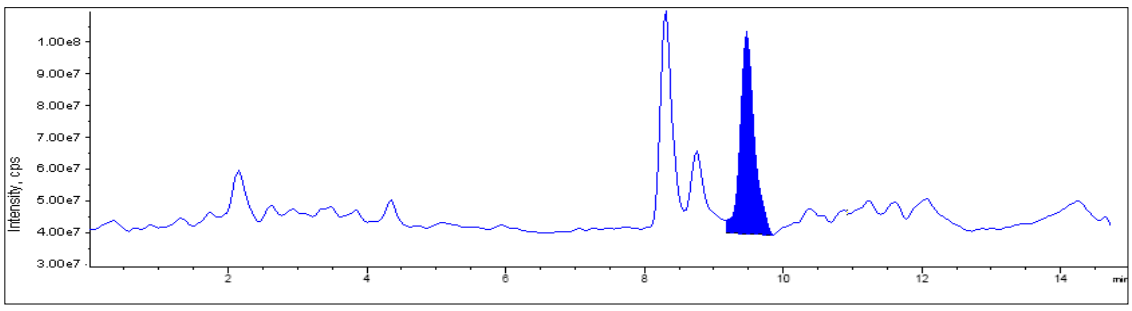


FIG. 4: MRM-ION CHROMATOGRAM OF IMIPRAMINE

The average matrix factor value calculated as the response of post spiked sample/response of neat solutions in methanol at LLOQ levels was 0.98, which indicates a minor suppression of 2%. A general internal standard was used to minimize any possible analytical variation due to solvent evaporation, extraction efficiency, and ionization efficiency of fluoxetine, IMP, Duloxetine, and Ibuprofen were tested as internal standards. IMP used as an internal standard in the present study had similar chromatographic behaviour, similar protein binding and was easily extracted with acetonitrile. Moreover, there was no effect of IS on analyte recovery, sensitivity or ion suppression. **Fig.5** has shown for the proper separation of all the concomitant drugs along with IS for TFM.

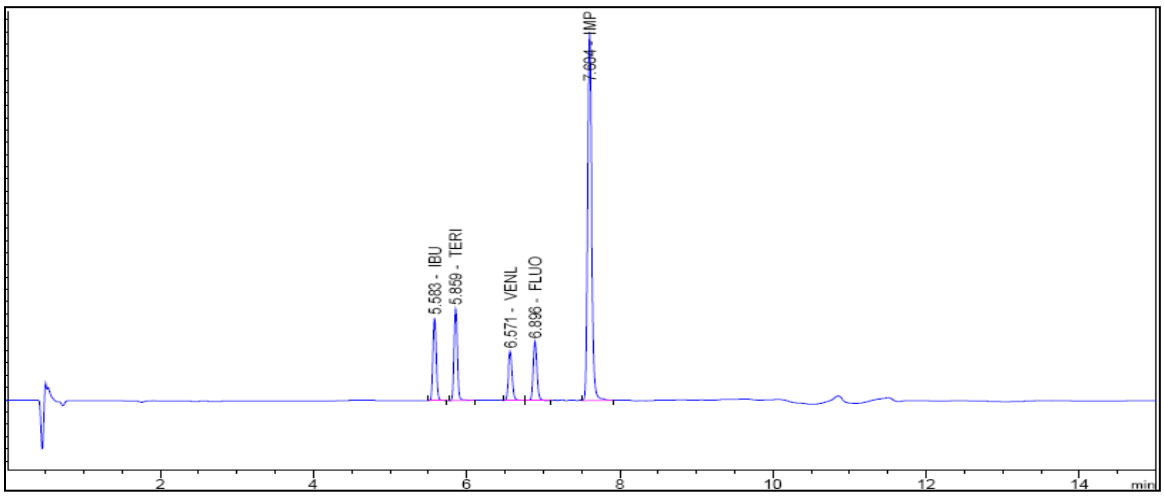


FIG. 5: LC CHROMATOGRAM SHOWING THE PROPER SEPARATION OF DRUGS

Assay performance and validation:

Throughout the method validation, the precision (%CV) of system suitability test was observed in the range of 0.15–0.65% for the retention time and 2.2–3.8% for the area response of both the drugs (teriflunomide and IS), which is not more than the acceptance criteria of 4%. The signal to noise ratio for system performance was ≥ 10 for teriflunomide and IS. Carryover evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method.

There was no carry-over observed during auto sampler carryover experiment. No enhancement in the response was observed in extracted blank plasma/serum (without IS and analyte) after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of teriflunomide and IS respectively. All five calibration curves were linear over the concentration range of 1–4000 ng/mL. A straight line fit was made through the data points by least square regression analysis to give the mean linear equation y = 0.0030x−0.00300 where y is the peak area ratio of the analyte/IS and x the concentration of the analyte. The mean standard deviation value for slope, intercept and correlation coefficient (r2) observed were 0.000045, 0.00058 and 0.00095 respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 91.4% to 110.6% and 1.4% to 9.0% respectively.

The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 1 ng/mL in plasma at a signal-to-noise ratio (S/N) of ≥ 10. The intra-batch and inter-batch precision and accuracy were established from validation runs performed at ULOQ QC, HQC, MQC, LQC and LLOQ QC levels (**Table 1**).

The intra-batch precision (%CV) ranged from 1.0 to 6.3 and the accuracy was within 86–111%. For the inter-batch experiments, the precision varied from 0.8 to 4.4 and the accuracy was within 90.5–110.9%. The relative recovery, absolute matrix effect and process efficiency data for teriflunomide and IS at LQC, MQC and HQC levels is presented in **Table 2**.

TABLE 1: INTRA-BATCH AND INTER-BATCH PRECISION AND ACCURACY FOR TERIFLUNOMIDE IN HUMAN PLASMA

QC ID	Nominal concentration (ng/mL)	Intra-batch (n=6)			Inter-batch (n=6)		
		Mean Conc. found (ng/mL)	% Recovery	% RSD	Mean Conc. found (ng/mL)	% Recovery	% RSD
High QC	3000	2668	88.9	3.3	2717.9	90.5	4.4
Mid QC	500	506	101.2	1.1	508	101.5	0.8
Low QC	5	5.4	107.6	5.7	5.2	103.8	4.6
LLOQ QC	1	1.1	106.9	4.1	1.1	110.9	2.2

TABLE 2: ABSOLUTE MATRIX EFFECT, RELATIVE RECOVERY AND PROCESS EFFICIENCY FOR TERIFLUNOMIDE (N=6)

A[a] (%RSD)	B[b] (%RSD)	C[c] (%RSD)	%Matrix effect (B/A*100)	% Relative recovery (C/B*100)	% Process Efficiency (C/A*100)
Low QC- 0.048 (9.8)	0.05 (4.9)	0.05 (5.0)	107.4	92.3	99.1
Mid QC- 0.59 (2.0)	0.59 (2.7)	0.56 (4.4)	99.9	95.3	95.2
High QC- 5.9 (2.4)	6.2 (3.2)	5.9 (4.3)	103.3	94.9	98.1

[a] Mean area response of 6 replicate samples prepared in methanol (neat samples)
[b] Mean area response of 6 replicate samples prepared by spiking in extracted blank plasma
[c] Mean area response of 6 replicate samples prepared by spiking before extraction

The process efficiency/absolute recovery obtained for teriflunomide and IS was greater than 90% at all QC levels. Further, the more important parameter in the evaluation and validation of a bioanalytical method using biofluids is the demonstration of absence of relative matrix effect, which compares the precision (%CV) values between different lots (sources) of plasma/serum (spiked after extraction) samples. The precision results varied from 2.0% to 9.8% for different plasma/serum lots with accuracy between 92% and 107% at all levels.

The stability of the teriflunomide and IS in biological matrices and stock solutions was examined under different storage conditions. Samples for short-term stability remained unchanged up to 24h, while the stock solutions for long term stability of teriflunomide and the internal standard were stable for minimum of 5 days at refrigerated temperature below 8°C. Teriflunomide in control plasma/serum (bench top) at room temperature was stable at least for 72h at 25°C and for minimum of five freeze and thaw cycles at −20 and −70°C.

Spiked plasma/serum samples stored at −20 and−70°C for long-term stability experiment were found stable for a minimum period of 32 days. Auto sampler stability (wet extract) of the spiked quality control samples maintained at 5°C was determined upto 72h without significant drug loss. The percentage change for different stability experiments in plasma/serum at two QC levels varied from -0.11% to 11.9% as shown in Table 3. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis.

The precision for dilution integrity of 1/5th and 1/10th dilution were 3.9% and 3.4%, while the accuracy results were 90.5% and 91.6% respectively which is within the acceptance limit of 15% for precision (%CV) and 85–115% for accuracy.

Method ruggedness was evaluated using re-injection of analyzed samples on two different columns of the same make and also with different analysts. The precision (%CV) and accuracy values for two different columns with different analysts ranged from 0.31% to 11.1% and 91.3% to 112.4% respectively at all five quality control levels.

TABLE 3: STABILITY OF TERIFLUNOMIDE UNDER DIFFERENT CONDITIONS (N=6)

Storage condition	Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL) ± SD	%Change
Bench top stability, 24 h			
High QC	3002.88	2970 ± 45.6	-0.19
Low QC	5.044	5.31 ± 0.4	-0.65
Auto sampler stability, 72 h			
High QC	3002.88	3040.5 ± 2.1	2.18
Low QC	5.044	4.97 ± 0.4	-6.73
Freeze and thaw stability; 5 cycles, -20°C			
High QC	3002.88	3011.2 ± 28.7	1.19
Low QC	5.044	5.26 ± 0.4	-1.53
Freeze and thaw stability; 5 cycles, -70°C			
High QC	3002.88	3014.5 ± 73.8	1.31
Low QC	5.044	5.34 ± 0.4	-0.11
Long term matrix stability; 32 days, -20°C			
High QC	3002.88	2587.2 ± 44.2	11.89
Low QC	5.044	5.23 ± 0.5	-3.79
Long term matrix stability; 32 days, -70°C			
High QC	3002.88	2894.6 ± 98.1	10.20
Low QC	5.044	5.03 ± 0.42	-1.86

Comparison with reported methods:

The method presented employs low plasma volume (200µL) for processing and has the highest sensitivity compared to other procedures[8–10]. The on-column loading of teriflunomide at LLOQ was only 5pg per sample injection volume, is significantly lower which helps to maintain the column efficiency for more number of injections. A detailed comparison of selected procedures with the present method for teriflunomide determination in biological matrices is given in **Table 4**. One method also reported for estimation of free and total bound teriflunomide in human plasma which has shown the sensitivity in the range of 5-500 µg/mL[17].

TABLE 4: COMPARISON OF SELECTED ANALYTICAL METHODS DEVELOPED FOR TERIFLUNOMIDE IN BIOLOGICAL MATRICES

S.No.	Extraction procedure (sample volume); Internal standard; mean recovery(%)	Column; elution process; mobile phase; flow rate; injection volume; maximum on-column loading at LLOQ per injection	Retention time (analytical run time); detection technique	Linear dynamic range (ng/mL)	Ref.No.
1	Centrifugation (0.1 mL of human serum); demoxepam	Lichrospher 100 RP-18e (125 mmx4 mm, 5µm); isocratic; methanol:45 mM KH2PO4 pH 3(50:50 v/v); 1.0 mL/min; 20 µL, 3333 pg	8.9 min (13 min); HPLC-UV (295 nm)	500-10000 ng/mL	8
2	PPT with acetonitrile (0.1 mL human plasma); α-phenyl cinnamic acid; (100%)	Waters Nova-Pak C18 (150 mmx 3.9 mm, 4µm); isocratic; acetonitrile: 0.05M sodium acetate pH 2.5 (35:65 v/v); 1.5 mL/min; 50 µL; 8333 pg	2.2 min (10 min); HPLC-UV (305 nm)	500-60000 ng/mL	9
3	LLE with 10 mL ethyl acetate (0.25 mL human plasma);Warfarin;85-90%	Nucleosil 100-5 C18 (125 mm x 3 mm, 5µ);); isocratic; acetonitrile: water:HCOOH(40:59.8:0.2 v/v); 0.5 mL/min; 50 µL; 3125 pg	8.2 min (22 min) HPLC-UV (261 nm)	100-100,000 ng/mL	10
4	LLE with ethyl acetate (0.2 mL human plasma); valsartan; 98.2%	Inertsil ODS-3 C18 (50x4.6 mm, 5µ); isocratic, 0.02M CH3COONH4 ph 6.5:methanol (25:75); 0.8 mL/min, 5 µL; 25 pg	1.43 min (2.0 min) LC-ESI-MS/MS	10.1-4000 ng/mL	17
5	PPT with acetonitrile (0.2 mL matrix); acetonitrile; Imipramine (100%)	XTerra MS C18 analytical column (100 mm × 3.9 mm, 5 µm) with mobile phase A-0.005M Ammonium formate pH 9.0 and B-acetonitrile at 1.0 mL/min in gradient mode 10 µL; 5 pg	6.7 min (15 min) LC-ESI-MS/MS	1-4000 ng/mL	Present Method

CONCLUSIONS: The proposed validated method for the estimation of teriflunomide in different biological matrices is highly sensitive and rapid compared to published reports. The method offers significant advantages over those previously reported, interms of lower sample requirements, simplicity of extraction procedure without any matrix effect. The efficiency of protein precipitation extraction and without any interference from the concomitant drugs make it an attractive procedure in bio analysis of teriflunomide.

The linear dynamic range established was adequate to measure the concentration of teriflunomide in any preclinical and clinical study involving different biological species. The concomitant drugs also can be estimated along with the target analyte which is more advantageous than single compound analysis and also useful in drug interaction and toxicology studies.

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