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Chromatographic separation and sensitive determination of teriflunomide, an active metabolite of leflunomide in human plasma by liquid chromatography-tandem mass spectrometry

Jignesh M. Parekh [a], Rajendrasinh N. Vaghela [a], Dipen K. Sutariya [a], Mallika Sanyal [b], Manish Yadav [c], Pranav S. Shrivastav [a,*]

[a] Chemistry Department, School of Sciences, Gujarat University, Navrangpura, Ahmedabad 380009, India
[b] Chemistry Department, St. Xavier's College, Navrangpura, Ahmedabad 380009, India
[c] Bioanalytical Research Department, Veeda Clinical Research, Ahmedabad 380015, India

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ABSTRACT

A sensitive, selective and high throughput liquid chromatography tandem mass spectrometry (LC–ESI-MS/MS) method has been developed for the determination of teriflunomide, an active metabolite of leflunomide in human plasma. Plasma samples were prepared by liquid–liquid extraction of teriflunomide and valsartan as internal standard (IS) in ethyl acetate from 200 μL human plasma. The chromatographic separation was achieved on an Inertsil ODS-3 C18 (50 mm × 4.6 mm, 3 μm) analytical column using isocratic mobile phase, consisting of 20 mM ammonium acetate–methanol (25:75, v/v), at a flow-rate of 0.8 mL/min. The precursor → product ion transition for teriflunomide (*m/z* 269.0 → 82.0) and IS (*m/z* 434.1 → 350.3) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and negative ion mode. The method was validated over a wide dynamic concentration range of 10.1–4001 ng/mL. Matrix effect was assessed by post-column infusion experiment and the mean process efficiency were 91.7% and 88.2% for teriflunomide and IS respectively. The method was rugged and rapid with a total run time of 2.0 min and is applied to a bioequivalence study of 20 mg leflunomide (test and reference) tablet formulation in 12 healthy Indian male subjects under fasting condition.

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1. Introduction

Leflunomide (LEF) is a disease-modifying antirheumatic drug of the isoxazol class, with potent anti-inflammatory and immunosuppressive properties. It is used in the treatment of rheumatoid arthritis (RA) and acts by inhibiting dihydroorotate dehydrogenase, the rate limiting enzyme in the pathway for pyrimidine production [1]. LEF is a prodrug, which rapidly and non-enzymatically gets converted to its active metabolite, teriflunomide [3-cyna-3-hydroxy-N-(4-trifluoromethylphenyl)-crotonamide] by first-pass metabolism in the liver and gut after oral administration. It is reported that teriflunomide possesses immunomodulator effects of the drug by reversible inhibition of the enzyme dihydroorotate dehydrogenase and inhibits cell proliferation of lymphocytes. Since the conversion of LEF to its metabolite *in vivo* is essentially complete (>95%), most pharmacokinetic studies have been focused in measuring the plasma concentration of teriflunomide and not LEF [2].

Phase II clinical trials have revealed linear pharmacokinetics of teriflunomide over a dose range of 5–25 mg LEF per day, with a mean plasma half-life of 15–18 days and a bioavailability of nearly 100%. The pharmacologically active metabolite is extensively bound to plasma proteins (>99.3), primarily to albumin, with almost constant portion (0.5%) of free teriflunomide [3–5].

Several high-performance liquid chromatography methods are reported to determine teriflunomide in biological matrices [6–10]. Dias et al. [6] have measured teriflunomide by reversed-phase HPLC in whole blood or plasma from humans or rabbit with a sensitivity of 400 ng/mL. The recoveries obtained for the analyte were in the range of 78–108% in human blood for concentrations ranging from 400 to 100,000 ng/mL. Li et al. [7] studied the pharmacokinetics of LEF in Chinese volunteers and determined teriflunomide levels in human serum over the concentration range of 195–25,000 ng/mL. Serum samples were prepared by liquid–liquid extraction (LLE) in ethyl acetate and 50 μL was used for injection in the chromatographic system. Similarly van Roon et al. [8] presented a simple and rapid method for teriflunomide by HPLC–UV in human serum and discussed its application for optimization of LEF therapy. The assay was linear over the concentration range of 500–100,000 ng/mL,

* Corresponding author. Tel.: +91 079 26300969; fax: +91 079 26308545.
E-mail address: pranav_shrivastav@yahoo.com (P.S. Shrivastav).

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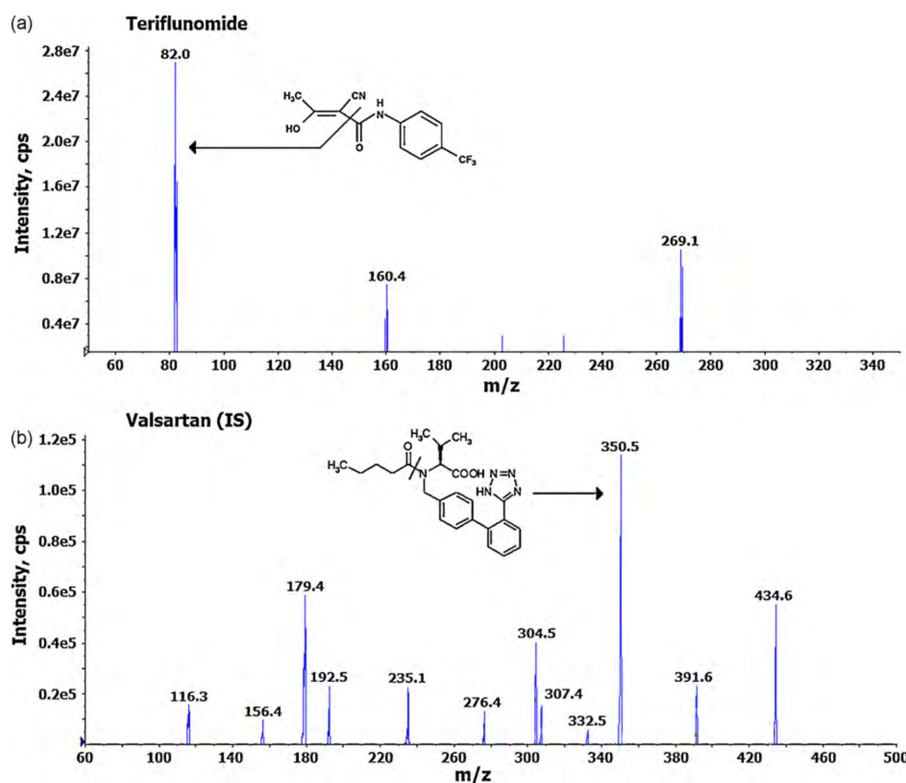

Fig. 1. Product ion mass spectra of (a) teriflunomide (*m/z* 269.1 → 82.0, scan range 50–350 amu) and (b) internal standard, valsartan (*m/z* 434.6 → 350.5, scan range 60–500 amu) in negative ionization mode.

with very long chromatographic run time of 13 min. In another method, Chan et al. [9] determined plasma concentration of LEF via protein precipitation with acetonitrile. The chromatographic separation was performed on a Nova-Pak C18 column in 10 min with the limit of quantification of 800 ng/mL. A method for the simultaneous determination of LEF and its active metabolite, teriflunomide in human plasma is described by Schmidt et al. [10]. Plasma samples were prepared by extraction in ethyl acetate using warfarin as internal standard. The merits of the method included low plasma volume requirement (250 μL), however, the chromatographic analysis time was very high (22 min) and thus may not be suitable for high throughput analysis. An LC–MS/MS assay has been proposed for the determination of LEF and identification of its metabolites under gradient conditions [11]. The study was intended to establish the role of human P450 isozymes in the conversion of LEF to teriflunomide and the effect of pH and temperature on LEF decomposition. In addition, the stability of LEF was investigated in human and rat plasma, whole blood, liver microsomes and cytosol. A rapid method to quantify teriflunomide derivate, FK778 in plasma by LC–MS and HPLC–UV has been demonstrated by Molinaro et al. [12] and applied to pharmacokinetic analysis in animal models. The calibration curves were linear from 1000 to 50,000 ng/mL and the chromatographic run time was 10 min. Therapeutic drug monitoring of teriflunomide in serum has been studied by van Roon et al. [13] to derive a relation between the steady state serum concentration and disease activity using the 28 joint (DAS28) response. Measurement of teriflunomide concentration was based on their reported methodology [8]. Very recently, a rapid and simple HPLC method to determine teriflunomide in renal transplant patients has been reported [14]. The method was linear up to 200,000 ng/mL and each HPLC separation took about 7 min.

In the present study, a sensitive and rapid LC–ESI-MS/MS method has been developed for reliable measurement of teriflunomide in subject samples. The method is highly selective to quantify teriflunomide in the presence of 6 other antirheumatic medications. The validated method requires only 200 μL human plasma for LLE and demonstrated excellent performance in terms of ruggedness and efficiency (2.0 min per sample). Interference due to matrix was ascertained by post column infusion technique. It was successfully applied to a bioequivalence study in 12 healthy Indian males for 20 mg leflunomide tablet formulation under fasting condition.

2. Experimental

2.1. Chemicals and materials

Reference standards of teriflunomide (99.1%) and valsartan (internal standard (IS), 99.4%) were obtained from Alfa-Omega Pharma Pvt. Ltd. (Bangalore, India) and Varda Biotech (P) Ltd. (Mumbai, India) respectively. HPLC grade methanol was procured from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). HPLC grade ethyl acetate, ammonium acetate and formic acid were purchased from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma (K_2EDTA as anticoagulant) was obtained from Prathama Blood Centre (Ahmedabad, India) and was stored at −20 °C until use.

2.2. Liquid chromatographic conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-10ADVP pump, SIL-HTc autosampler, CTO 10 ASvp column oven and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions. The separation of teriflunomide and IS was achieved on an Inertsil ODS-3 C18 (50 mm length × 4.6 mm inner diameter and 3.0 μm particle size) analytical

column from Gilead Sciences' (Foster City, CA, USA) and maintained at 35 °C in a column oven. For isocratic separation, the mobile phase consisted of 20 mM ammonium acetate–methanol (25:75, v/v). The flow rate of the mobile phase was kept at 0.8 mL/min and the total chromatographic run time was 2.0 min. The auto sampler temperature was maintained at 5 °C. The total eluent from the column was split in 45:55 ratio; flow directed to the ISP interface was equivalent to 450 μL/min.

2.3. Mass spectrometric conditions

Ionization and detection of teriflunomide and IS was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API-3000 (Toronto, Canada), equipped with turbo ion spray interface and operating in negative ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions for teriflunomide (m/z 269.1 → 82.0) and IS (m/z 434.6 → 350.5) (Fig. 1). The source dependent parameters maintained for both teriflunomide and IS were Nebuliser gas: 6.0 psig; ion spray voltage (ISV): −4500 V; turbo heater temperature (TEM): 500 °C; collisional activation dissociation (CAD): 6 psig and curtain gas (CUR), nitrogen: 11 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 −13 V for teriflunomide and −46, −27, −10, −250 and −14 V 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 1.4.1 was used to control all parameters of LC and MS.

2.4. 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 10.1, 20.2, 722, 1031, 2061, 2945, 3681 and 4001 ng/mL concentrations respectively, while quality control samples were prepared at five levels, viz. 4001 ng/mL (ULOQ QC, upper limit of quantitation quality control), 3001 ng/mL (HQC, high quality control), 2201 ng/mL (MQC, medium quality control), 28.6 ng/mL (LQC, low quality control) and 10.1 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 valsartan in 25.0 mL of methanol. Its working solution (100 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.

2.5. Sample extraction protocol

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 200 μL of spiked plasma sample, 20 μL of internal standard (100 μg/mL) was added and vortexed for 10 s. Further, 50 μL of 5% formic acid solution was added and vortexed for another 10 s. LLE was carried out with 2.0 mL of ethyl acetate on rotary mixer (rotospin) for 5 min at 32 × g. Samples were then centrifuged at 3204 × g for 5 min at 10 °C. After centrifugation, 1.5 mL of the supernatant organic layer was transferred and evaporated to dryness in a thermostatically controlled water-bath maintained at 40 °C under a gentle stream of nitrogen. The dried samples were reconstituted in 400 μL of mobile phase and 5 μL was used for injection in the chromatographic system.

2.6. Validation Methodology

The bioanalytical method was thoroughly validated following the USFDA guidelines [15]. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of teriflunomide (4001 ng/mL) and valsartan (100 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 re-injecting any sample during method validation. Carry over experiment was performed to verify any carry over of analyte, which may reflect in subsequent runs. The design of the study comprised of the following sequence of injections i.e. mobile phase solution [20 mM ammonium acetate–methanol (25:75, v/v)] → LLOQ sample → extracted blank plasma → ULOQ sample → extracted blank plasma → ULOQ sample → extracted blank plasma.

The selectivity of the method towards endogenous plasma matrix components was assessed in twelve different batches (8 normal of K_2EDTA, 2 haemolysed and 2 lipemic) of blank plasma. Check for interference due to concomitantly used antirheumatic medication (ibuprofen, diclofenac, celecoxib, naproxen, and acetaminophen) 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 (100 μg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (100 ng/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), diclofenac (294/250), celecoxib (380/316), naproxen (229/185) and acetaminophen (150/107) 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 ($1/x^2$) linear regression which was finalized during pre-method validation. A correlation coefficient (r^2) value >0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was atleast ten times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (%CV) not greater than 20% and accuracy within 80–120%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15%.

For determining the intra-batch accuracy and precision, replicate analysis of plasma samples of teriflunomide was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC, HQC and ULOQ QC samples. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ±15% except LLOQ, for which it should be within ±20%. Similarly, the mean accuracy should not deviate by ±15% except for the LLOQ where it can be ±20% of the nominal concentration.

Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by the post column analyte infusion experiment. A standard solution containing teriflunomide (at ULOQ QC level) and IS was infused post column via a 'T' connector into

the mobile phase at 10 μL/min employing external infusion pump. Aliquots of 5 μL of extracted control plasma were then injected into the column by the autosampler and MRM LC–MS/MS chromatograms were acquired for teriflunomide and IS. Any dip in the baseline upon injection of extracted blank plasma (without IS and analyte) would indicate ion suppression, while a peak at the retention time of teriflunomide or IS indicates ion enhancement.

The relative recovery, matrix effect and process efficiency were assessed as recommended by Matuszewski et al. [16]. 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 unextracted samples (spiked after extraction) with mean area of neat standard solutions (in mobile phase). The overall 'process efficiency' (%PE) was calculated as (ME × RE)/100. Further, the effect of plasma matrix (relative matrix effect) on analyte quantification was also checked in six different batches/lots of plasma. 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 aforementioned 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 long-term stability at 2–8 °C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Autosampler stability (wet extract), dry 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. Long-term 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 plasma. 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.

2.7. Bioequivalence study design

The design of the study comprised of "An open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover bioequivalence study of a test formulation of leflunomide (20 mg tablets of an Indian Company) and a reference formulation (ARAVA® tablets containing 20 mg leflunomide from Aventis Pharmaceuticals Inc., USA) in 12 healthy adult Indian male subjects in the age group of 20 to 50 years under fasting conditions". Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. An independent ethics committee constituted as per Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [17]. The subjects were orally administered a single dose of test and reference formulations after recommended wash out period of 2 weeks with 200 mL of water. Blood samples were collected at 0.0 (pre-dose), 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5, 13.0, 13.5, 14.0, 16.0, 18.0, 20.0, 24.0, 48.0 and 72.0 h after oral administration of the dose for test and reference formulation in labeled K_2EDTA-vacuettes. The maximum volume of blood withdrawn during the entire study was approximately 310 mL, which included (other than for measurement) up to 10 mL for screening, about 10 mL for post study safety assessment (hematology and biochemical tests) while 0.5 mL of heparinised blood was discarded prior to each sampling through venous cannula. Plasma was separated by centrifugation and kept frozen at −70 °C until analysis. During study, subjects had a standard diet while water intake was free. An incurred sample re-analysis (ISR) was also conducted by computerized random selection of 20 subject samples. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ±20% [18].

3. Results and discussion

The present study was undertaken to develop a sensitive, selective and a high throughput method to determine teriflunomide, the pharmacologically active metabolite of LEF, especially for subject sample analysis. As LEF get rapidly metabolized *in vivo*, its circulating concentration is mostly below the limit of detection [19], thus most pharmacokinetic studies have been conducted for measuring teriflunomide and not LEF. Teriflunomide is formed by N—O bond cleavage in the isoxazole ring which has the same oxidation state as the parent drug [20]. This metabolite is mainly responsible for the anti-inflammatory and disease-modifying properties of LEF.

3.1. Method development

The electrospray ionization (ESI) of teriflunomide and valsartan (IS) was conducted in negative ionization mode as the metabolite has high electron affinity due to the presence of trifluoromethyl group. Similarly, valsartan too gave higher response in the negative mode on account of carboxylic acid group. Q1 MS full scan spectra for teriflunomide and IS predominantly contained deprotonated precursor $[M-H]^-$ ions at m/z 269.1 and 434.6 respectively. The most abundant and consistent product ions in Q3 MS spectra for teriflunomide and IS were observed at m/z 82.0 (corresponding to cyanomethyl methyl ketone) and m/z 350.5 at −30 and −27 V 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 250 μL 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 LLE to be used as the preferred extraction tech-

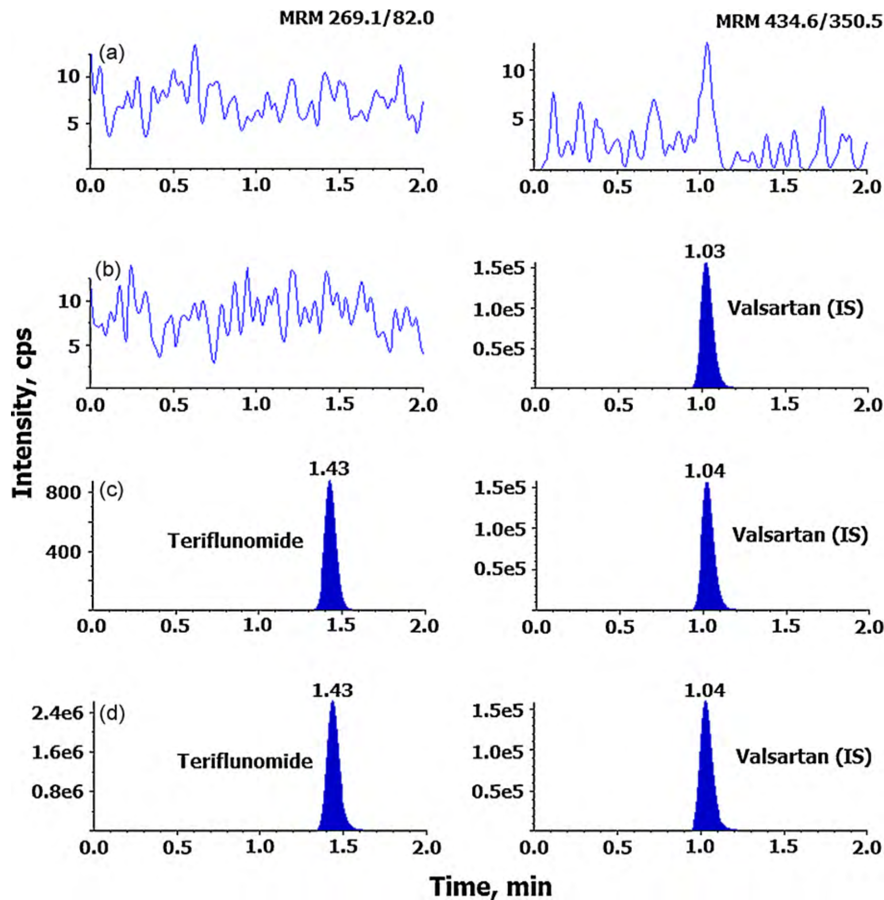

Fig. 2. MRM ion-chromatograms of (a) extracted blank plasma (without IS and analyte), (b) blank plasma with valsartan (IS, m/z 434.6 → 350.5), (c) teriflunomide at LLOQ (m/z 269.1 → 82.0) and IS (d) real subject sample at C_{max} after administration of 20 mg dose of leflunomide.

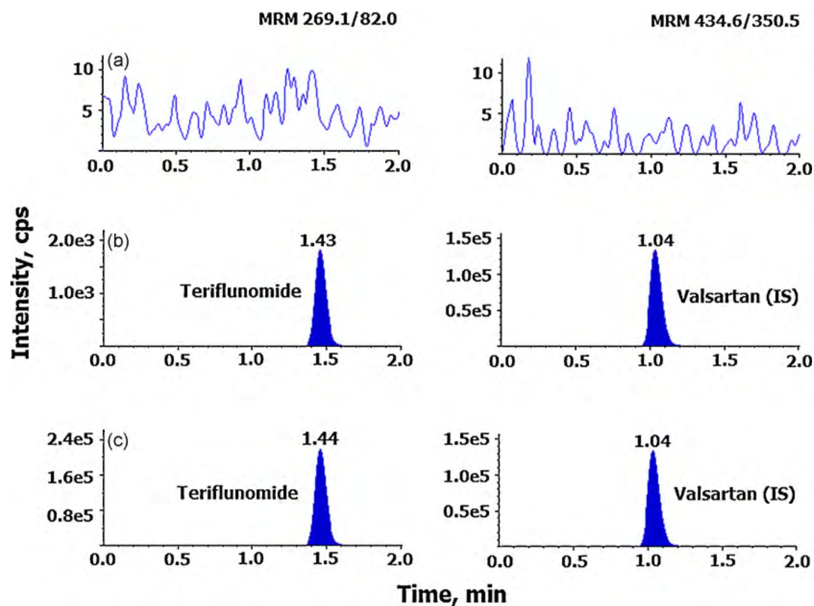

Fig. 3. MRM ion-chromatograms of blank plasma with antirheumatic medications (ibuprofen, diclofenac, celecoxib, naproxen, and acetaminophen) in (a) absence of teriflunomide and IS, (b) teriflunomide at LQC and IS (c) teriflunomide at HQC and IS.

nique. LLE was tested to isolate teriflunomide from plasma using diethyl ether, dichloromethane, methyl *tert*-butyl ether, *n*-hexane and ethyl acetate as extracting solvents and also by varying the pH of plasma from 2.0 to 7.5. As reported earlier [11] there was no major effect of pH on the extraction of teriflunomide, however, quantitative and consistent recoveries for teriflunomide and IS were obtained at all QC levels with ethyl acetate using 50 μL, 5% formic acid (pH 2.15). The recovery in other solvent systems was between 70% and 80%, but was inconsistent with some ion suppression (greater than 15% CV).

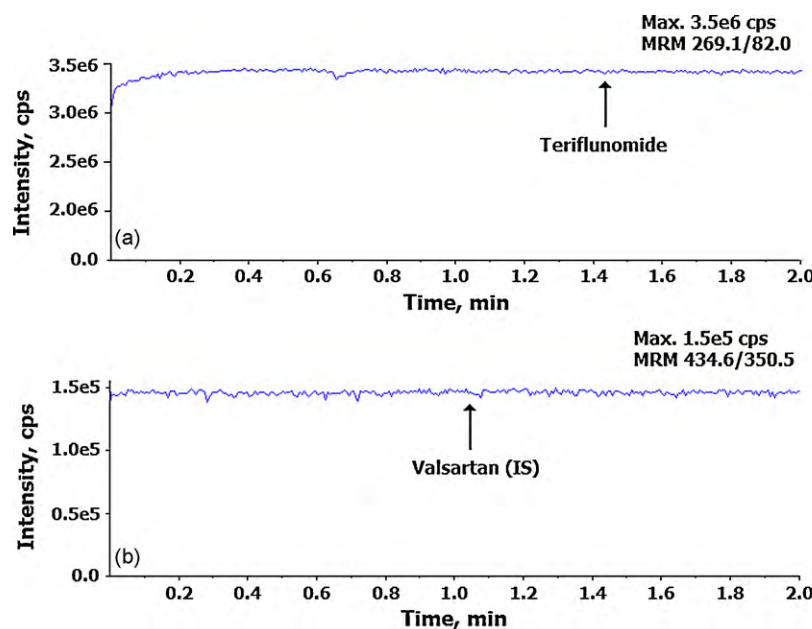

Fig. 4. Post column analyte infusion experiment for (a) teriflunomide and (b) valsartan.

Table 1
Intra-batch and inter-batch precision and accuracy for teriflunomide.

QC ID	Nominal concentration (ng/mL)	Intra-batch				Inter-batch			
		n	Mean concentration observed (ng/mL)[a]	%CV	%Accuracy	*n*	Mean concentration observed (ng/mL)[b]	%CV	%Accuracy
ULOQ QC	4001	6	3944	5.8	98.6	30	4044	4.5	101.1
HQC	3001	6	3041	4.3	101.3	30	2979	6.4	99.3
MQC	2201	6	2121	5.3	96.4	30	2186	3.6	99.3
LQC	28.6	6	27.4	3.5	95.8	30	27.6	2.4	96.5
LLOQ QC	10.1	6	10.2	2.8	101.0	30	10.3	4.2	102.0

CV: coefficient of variance; *n*: total number of observations.
[a] Mean of 6 replicates at each concentration.
[b] Mean of 6 replicates for five precision and accuracy batches.

The chromatographic separation of teriflunomide and IS was initiated to achieve a short run time, symmetric peak shapes, minimum matrix interference and solvent consumption. Previous studies have reported different columns with 5 μm particle size, 3–4 mm inner diameter and columns lengths (125–150 mm) with run times ≥10 min [8–10]. Thus, in the present work chromatographic separation was tried on Phenomenex, Gemini C18 (50 mm × 4.6 mm, 5 μm; surface area 404 m^2/g, pore size 101 Å), Waters X-Terra RP C18 (50 mm × 4.6 mm, 5 μm; surface area 170 m^2/g, pore size 141 Å), ACE C18 (50 mm × 4.6 mm, 5 μm; surface area 300 m^2/g, pore size 100 Å) and Inertsil ODS-3 C18 (50 mm × 4.6 mm, 3.0 μm; surface area 450 m^2/g, pore size 100 Å) 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 Inertsil ODS-3 C18 column using 20 mM ammonium

Table 2
Absolute matrix effect, relative recovery and process efficiency for teriflunomide.

A[a] (%CV)[b]	*B*[c] (%CV)[b]	*C*[d] (%CV)[b]	Absolute matrix effect (%ME)[e]	Relative recovery (%RE)[f]	Process efficiency (%PE)[g]
LQC					
0.0094 (3.7)	0.0090 (5.2)	0.0085 (4.2)	95.7 (99.1)[h]	94.4 (89.4)[h]	90.3 (88.6)[h]
MQC					
0.75 (3.6)	0.73 (3.5)	0.70 (2.6)	97.3 (98.1)[h]	95.9 (89.6)[h]	93.3 (87.9)[h]
HQC					
0.95 (1.6)	0.93 (4.2)	0.87 (2.4)	97.9 (97.8)[h]	93.5 (90.1)[h]	91.6 (88.1)[h]

[a] Mean area ratio (analyte/internal standard) response of six replicate samples prepared in mobile phase (neat samples).
[b] Coefficient of variation.
[c] Mean area ratio (analyte/internal standard) response of six replicate samples prepared by spiking in extracted blank plasma.
[d] Mean area ratio (analyte/internal standard) response of six replicate samples prepared by spiking before extraction.
[e] $(B/A) \times 100$.
[f] $(C/B) \times 100$.
[g] $(C/A) \times 100 = (\mathrm{ME} \times \mathrm{RE})/100$.
[h] Values for internal standard, valsartan.

Table 3
Stability of teriflunomide under different conditions ($n = 6$).

Storage condition	Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	
		Mean, stability samples ± SD	%Change[a]
Bench top stability; 9 h			
HQC	3001	3143 ± 84.6	4.72
LQC	28.6	27.6 ± 0.3	−3.50
Wet extract stability; 36 h			
HQC	3001	3094 ± 149.0	3.10
LQC	28.6	28.2 ± 0.6	−1.40
Dry extract stability; 49 h			
HQC	3001	3111 ± 74.6	3.54
LQC	28.6	28.5 ± 0.9	−0.35
Freeze and thaw stability; 4 cycles, −20 °C			
HQC	3001	3069 ± 106.3	2.26
LQC	28.6	28.8 ± 1.3	0.70
Freeze and thaw stability; 4 cycles, −70 °C			
HQC	3001	2999 ± 120.5	−0.06
LQC	28.6	27.3 ± 0.8	−4.55
Long term matrix stability; 104 days, −20 °C			
HQC	3001	3170 ± 78.0	5.63
LQC	28.6	29.6 ± 2.3	3.50
Long term matrix stability; 104 days, −70 °C			
HQC	3001	3160 ± 108.7	5.30
LQC	28.6	30.1 ± 2.1	5.24

[a] $\%\text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$.

acetate–methanol (25:75, v/v) as the mobile phase. This may be attributed to the large surface area (450 m^2/g) compared to other columns tested. The total run time of 2.0 min ensured separation of teriflunomide and IS at 1.43 and 1.04 min respectively. Representative chromatograms in Fig. 2 of extracted blank plasma (without IS and analyte), blank plasma fortified with IS (m/z 434.6 → 350.5), teriflunomide at LLOQ (m/z 269.1 → 82.0) and an actual subject sample at maximum plasma concentration (C_{max}) demonstrates the selectivity of the method. None of the antirheumatic medications studied showed interfering signals at the retention time of teriflunomide or the IS as shown in Fig. 3. Results of post-column infusion experiment in Fig. 4 indicate no ion suppression or enhancement at the retention time of teriflunomide and IS. The average matrix factor value calculated as the response of post spiked sample/response of neat solutions in mobile phase at the 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 teriflunomide. Efavirenz, fluoxetine, valsartan and oxazepam were tested as internal standards due to non-availability of deuterated standard. Valsartan used as an internal standard in the present study had similar chromatographic behaviour, similar protein binding and was easily extracted with ethyl acetate. Moreover, there was no effect of IS on analyte recovery, sensitivity or ion suppression.

3.2. Assay performance and validation

Throughout the method validation, the precision (%CV) of system suitability test was observed in the range of 0.05–0.15% for the retention time and 1.0–1.5% 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 ≥50 for teriflunomide and IS. Carry-over 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 autosampler carryover experiment. No enhancement in the response was observed in extracted blank plasma (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 10.1–4001 ng/mL. A straight-line fit was made through the data points by least square regression analysis to give the mean linear equation $y = 0.00033x - 0.00052$ 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 (r^2) observed were 0.00003, 0.00093 and 0.0013 respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 98.1% to 101.3% and 1.4% to 5.4% respectively. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 10.1 ng/mL in plasma at a signal-to-noise ratio (S/N) of ≥50.

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 2.8 to 5.8 and the accuracy was within 95.8–101.3%. For the inter-batch experiments, the precision varied from 2.4 to 6.4 and the accuracy was within 96.5–102.0%.

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. The process efficiency/absolute recovery obtained for teriflunomide and IS was greater than 90% and 87% respectively 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 (spiked after extraction) samples. The precision results varied from 2.08% to 5.53% for different plasma lots with accuracy between 97.1% and 102.5% at the LLOQ level.

The stability of the teriflunomide and IS in human plasma and stock solutions was examined under different storage conditions. Samples for short-term stability remained unchanged up to 12 h, while the stock solutions for long-term stability of teriflunomide and the internal standard were stable for minimum of 6 days at refrigerated temperature below 8 °C. Teriflunomide in control human plasma (bench top) at room temperature was stable at least for 9 h at 25 °C and for minimum of four freeze and thaw cycles at −20 and −70 °C. Spiked plasma samples stored at −20 and −70 °C for long-term stability experiment were found stable for a minimum period of 104 days. Dry extract stability of the spiked quality control samples stored at 2–8 °C was determined up to 49 h.

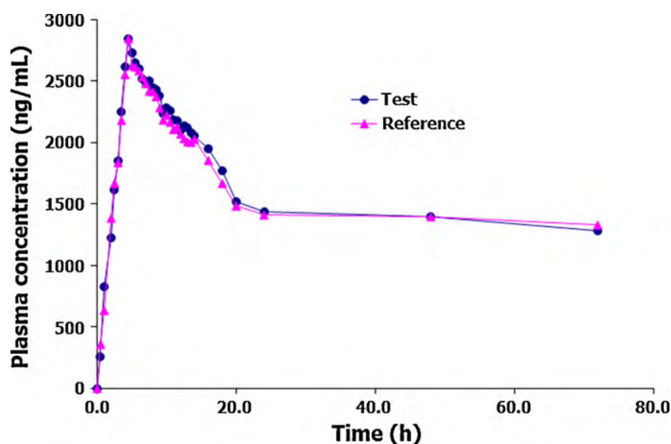

Fig. 5. Mean plasma concentration–time profile of teriflunomide after oral administration of test (20 mg leflunomide tablets of an Indian Company) and a reference (ARAVA® tablets containing 20 mg leflunomide) formulation to 12 healthy volunteers under fasting condition.

Autosampler stability (wet extract) of the spiked quality control samples maintained at 5 °C was determined up to 36 h without significant drug loss. The percentage change for different stability experiments in plasma at two QC levels varied from −4.55% to 5.63% 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. However, none of the subject samples measured showed concentration above the ULOQ. The precision for dilution integrity of 1/5th and 1/10th dilution were 1.82% and 2.27%, while the accuracy results were 99.2% and 102.6% 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 ranged from 1.6% to 4.0% and 97.2% to 103.5% respectively at all five quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 2.4–4.4% and 96.0–101.4% respectively at these levels.

3.3. Application to a pharmacokinetic/bioequivalence study

The validated method has been successfully used to quantify teriflunomide concentration in human plasma samples after the administration of a single 20 mg oral dose of leflunomide. Fig. 5 shows the plasma concentration of teriflunomide vs. time profile in human subjects under fasting condition. The method was sensitive enough to monitor the teriflunomide plasma concentration up to 72.0 h. In all approximately 1200 samples including the calibration, QC and volunteer samples were run and analyzed during a period of 3 days and the precision and accuracy were well within the acceptable limits. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table 4. The 90% confidence interval of C_{max} and AUC_{0-72} are within the bioequivalence acceptance limits of 80–125% for test and reference formulation. The mean values for C_{max}, T_{max}, and AUC_{0-t} obtained for a pharmacokinetic study in 6 healthy Chinese subjects (4 males and 2 females, age 20–22 years) with single dose of 20 mg leflunomide [7] were to some extent different from the results of the present study. This may be due genetic difference, age, gender (body size and muscle mass), type of food, etc. which may result in pharmacokinetic differences. However, the mean $t_{1/2}$ value was comparable with the present work. The %change in the randomly selected subject samples for incurred samples (assay reproducibility) analysis was within ±9%. This authenticates the reproducibility and ruggedness of the proposed method. Further, there was no adverse event during the course of the study.

Table 5
Comparison of selected analytical methods developed for teriflunomide in biological matrices.

Sr. 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 human serum); demoxepam; (–)	LiChrospher 100 RP-18e (125 mm × 4 mm, 5 μm); isocratic; methanol: 45 mM potassium dihydrogen phosphate, pH 3 (50:50, v/v); 1.0 mL/min; 20 μL; 3333 pg	8.9 min (13 min); HPLC–UV (295 nm)	500–10,000 ng/mL	[8]
2	PP[a] with acetonitrile (0.1 mL human plasma); α-phenyl cinnamic acid; (100%)	Waters Nova-Pak C18 (150 mm × 3.9 mm, 4 μm); isocratic; acetonitrile: 0.05 M 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)	500–60,000 ng/mL	[9]
3	LLE[b] with 10 mL ethyl acetate (0.25 mL human plasma); warfarin; (90–96%—leflunomide and 85–90%—teriflunomide)	Nucleosil 100-5 C18 (125 mm × 3 mm, 5 μm); isocratic; acetonitrile: water: formic acid (40:59.8:0.2, v/v/v); 0.5 mL/min; 50 μL; 3125 pg of teriflunomide and 6250 pg of leflunomide	16.2 min for leflunomide and 8.2 min for teriflunomide (22 min); HPLC–UV (261 nm)	50–100,000 ng/mL for leflunomide and 100–100,000 ng/mL for teriflunomide	[10]
4	LLE[b] with ethyl acetate (0.2 mL human plasma); valsartan; (98.2%)	Inertsil ODS-3 C18 (50 mm × 4.6 mm, 3.0 μm); isocratic; 20 mM ammonium acetate, pH 6.5–methanol (25:75, v/v); 0.8 mL/min; 5 μL; 25 pg	1.43 min (2.0 min); LC–ESI-MS/MS	10.1–4001 ng/mL	Present method

[a] Protein precipitation.
[b] Liquid–liquid extraction.

Table 4
Mean pharmacokinetic parameters of teriflunomide following oral administration of 20 mg leflunomide tablet formulation (test and reference) to 12 healthy human subjects.

Parameter	Mean ± SD	
	Test	Reference
C_{max} (ng/mL)	2921 ± 378	2939 ± 322
T_{max} (h)	5.58 ± 2.87	5.38 ± 2.74
$t_{1/2}$ (h)	302 ± 133	313 ± 176
$AUC_{0-72\,h}$ (h ng/mL)	111,722 ± 25,018	111,002 ± 21,939

C_{max}: maximum plasma concentration.
T_{max}: time point of maximum plasma concentration.
$t_{1/2}$: half life of drug elimination during the terminal phase.
AUC_{0-t}: area under the plasma concentration–time curve from zero hour to 72 h.

3.4. Comparison with reported methods

The method presented employs low plasma volume (200 μL) for processing and has the highest sensitivity compared to other procedures [8–10]. Moreover, the total analysis time (extraction and chromatography) is the shortest for teriflunomide compared to existing methods. Also, the on-column loading of teriflunomide at LLOQ was only 25 pg 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 5.

4. Conclusions

The proposed validated method for the estimation of teriflunomide in human plasma is highly sensitive and rapid compared to published reports. The method offers significant advantages over those previously reported, in terms of lower sample requirements, simplicity of extraction procedure and overall analysis time. The efficiency of liquid–liquid extraction and a chromatographic run time of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of teriflunomide. The linear dynamic range established was adequate to measure the plasma concentration of teriflunomide in a clinical study involving Indian subjects. In addition, the carry-over test, post column infusion study and the effect of commonly used medications by subjects are also studied in the present work.

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