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Quantitation of total and free teriflunomide (A77 1726) in human plasma by LC–MS/MS

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ABSTRACT

The clinical activity of leflunomide, a drug used in the treatment of rheumatoid arthritis, is due to its active metabolite, teriflunomide. *In vitro* studies indicate that at least 99% of teriflunomide is expected to be protein bound in human plasma *in vivo*, leaving <1% in the unbound or 'free' state for clinical activity. To examine details of the relationships between leflunomide dosing and patient response, it is necessary to have an assay that is sufficiently sensitive to measure the minor fraction of free teriflunomide in patient samples. Therefore, we aimed to develop and validate an LC–MS/MS method for the measurement of teriflunomide, and use it to determine the total and free teriflunomide concentration in patients with rheumatoid arthritis. Teriflunomide and its deuterated internal standard were extracted from human plasma and separated using a reversed phase method with a C_{18} column. Detection was conducted with an API 3000 LC–MS/MS System by monitoring selected ions in negative ion MRM. Optimal detection occurred at *m*/*z* 269.1/160.0 (teriflunomide) and *m*/*z* 273.1/164.0 (teriflunomide-D4). Over a linear range of 5–500 μg/L, the inter-batch precision ranged from 1.9 to 8.8% and accuracy from −8.4 to 8.0%. The intra- and inter-batch assay precision for quality control samples ranged from 2.1–5.4% and 5.7–7.1% respectively. The procedure was applied to assess total and free plasma concentrations of teriflunomide in patients with rheumatoid arthritis. Free teriflunomide was approximately 0.11% of total teriflunomide, and there was a significant correlation (r^2 = 0.724) between free and total teriflunomide concentrations. A validated, accurate and sensitive method was developed and successfully applied for the measurement of total and free teriflunomide concentration in human plasma samples. This method has been shown to be reproducible and sensitive and can be applied to clinical samples.

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1. Introduction

Leflunomide is an isoxazol derivative which is converted *in vivo* to teriflunomide (A77 1726) which has both anti-inflammatory and immunosuppressive properties [1,2]. The conversion of leflunomide to teriflunomide occurs in the liver via two cytochrome P450 (CYP450) enzymes, CYP1A2 and CYP2C19. The mode of action of teriflunomide is thought to involve reversible inhibition of the enzyme dihydroorotate dehydrogenase (DHODH), resulting in reduced pyrimidine ribonucleotide levels and a decreased proliferation of activated T lymphocytes [3–5]. *In vitro* studies with human plasma show that 99.4% of the added teriflunomide is bound to plasma proteins, which is consistent with a low volume of distribution (approximately 12.7 L – range 6–30.8 L) [6]. It has a plasma half-life of approximately 15 days and steady state plasma concentrations are highly variable between individuals, ranging from 3 to 150 mg/L and 5–93 mg/L in two independent studies [7,8].

Leflunomide has a role in treating rheumatoid arthritis (RA), an inflammatory arthritis that affects over 1% of the population. It can be used as monotherapy in resistant or recalcitrant disease, or may be added to other disease-modifying anti-rheumatic drugs (DMARDs) if disease is not adequately controlled despite maximal doses [9]. While being an effective agent, leflunomide is associated with a number of adverse effects including hepatotoxicity, pneumonitis and gastrointestinal effects. Up to 60% of RA patients discontinued treatment within 1 year due to intolerance/toxicity [9]. If patients do not achieve adequate response or suffer unacceptable toxicity with leflunomide, they are often treated with other toxic and/or expensive agents such as intramuscular gold,

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cyclosporin or biological DMARDs such as tumour necrosis factor-α inhibitors.

Two independent groups have related efficacy to plasma levels of teriflunomide. In these studies, 90% of patients were taking leflunomide as monotherapy for relapsed or resistant RA, and better treatment responses were reported in patients who achieved a steady-state concentration of >16,000 μg/L [7] and >50,000 μg/L [10]. While the reasons for this discrepancy are unclear, if there are relationships between plasma teriflunomide and patient responses, the free or unbound fraction is likely to be a more accurate predictor. The variability in free teriflunomide concentrations between patients has not been reported due to the difficulty in assaying this minor fraction, but given the high proportion of drug bound to plasma proteins, variability in this free concentration may be significantly higher than the variability in total drug concentration.

Previous methods used to analyse teriflunomide in plasma have utilised HPLC with UV detection [8,11–13], but methods with this detection technique are unable to reliably detect the minor 'free' fraction in human plasma. A recently developed LC–MS/MS method was more sensitive, but it required valsartan as an internal standard, utilised an inherently lengthy liquid–liquid extraction and did not investigate the 'free' level of teriflunomide in patient plasma [14]. The aim of this study was to develop and validate a LC–MS/MS method and use it to measure the teriflunomide concentration in human plasma according to established criteria [15–17], and apply this method in patients who were taking leflunomide for the treatment of RA.

2. Materials and methods

2.1. Chemicals and reagents

Teriflunomide (A77 1726) and D4-teriflunomide (A77 1726-D4, Isotopic purity: 99%) were obtained from Toronto Research Chemical Inc. (Toronto, Canada). Water was Milli-RQ grade (Millipore Pty. Ltd., Milford, MA, USA). Methanol (analytical grade), was from Mallinckrodt Chemicals Inc. (Phillipsburg, NJ, USA). Acetonitrile (analytical grade), ammonium acetate, zinc sulphate ($ZnSO_4$), di-sodium phosphate (Na_2HPO_4) and sodium chloride (NaCl) were obtained from Merck KGaA (Darmstadt, Germany). Formic acid (98%) was from Fluka Chemie GmbH (Steinheim, Germany). Dimethyl Sulphoxide (DMSO) was purchased from Optigen Scientific Pty. Ltd. (Adelaide, Australia). Drug free ("Blank") human plasma was collected from healthy volunteers and stored at −80 °C until use.

2.2. Preparation of standard and IS solutions

For preparation of the standard stock solution, teriflunomide was dissolved in methanol (1000 μg/L). Internal standard (IS) was prepared by dissolving D4-teriflunomide (1 mg) in 1 mL DMSO, which was further diluted to 100 μg/L with methanol. Stock solution and IS were stored at −80 °C prior to use.

2.3. Instrumentation

Separation of the analytes was accomplished using a reversed phase column (CC125/3 Nucleosil 100-5 C18; Machery-Nagel, Düren, Germany) and a C18 pre-column insert (Newguard RP-18, Applied Biosystems, Toronto, Canada).

Chromatography was performed with Shimadzu HPLC apparatus (Kyoto, Japan) consisting of a gradient pump (model LC-10Ad), an automatic injector (model SIL-HTc) and an on-line degasser (model DGU-14a). Detection was with a triple quadrupole tandem mass spectrometer equipped with a turbo ion spray interface (API 3000 LC–MS/MS, Applied Biosystems, Toronto, Canada) and a Valco 12 port diverter valve. Data acquisition and integration were carried out with LC–MS software (Analyst 1.4) linked directly to the API 3000 LC–MS/MS System.

2.4. Chromatography conditions and MS/MS detection

Separation of teriflunomide and D4-teriflunomide was achieved with a reverse phase gradient method. Mobile phase A was composed of 0.5 mM ammonium acetate in water–acetonitrile–formic acid (95:5:0.02, v/v/v), and mobile phase B was composed of 0.5 mM ammonium acetate in water–acetonitrile–formic acid (5:95:0.02, v/v/v). The flow rate was 0.5 mL/min and column was maintained at room temperature.

The initial mobile phase composition of 70% solvent A and 30% solvent B was maintained for 0.2 min. Between 0.2 and 4.51 min, the percentage of solvent B was increased on a linear gradient to 70%, and between 4.51 and 4.99 min, the percentage of solvent B was maintained at 100%. Between 4.99 and 6.99 min, the percentage of solvent B was decreased to 30%. These conditions were maintained until 7 min, followed by injection of the next sample. Total run time was 7 min. The first 2.5 min were diverted to waste using a diverter valve.

Quantitation of teriflunomide and IS was achieved in negative ion mode with quadrupoles Q1/Q3 set to unit resolution. Teriflunomide was detected using multiple reactions monitoring (MRM) with a dwell time of 200 ms. Optimal detection was achieved with the single charged Q1/Q3 transition for teriflunomide at 269.1/160.0 amu and 273.1/164.0 amu for D4-teriflunomide (Fig. 1). The compound dependant parameters were; nebuliser gas 10 pounds per square inch gauge (psig), Curtain gas 8 psig, Turbo gas temperature 350 °C, Collision cell gas 4 psig, Sprayer voltage −4500 V, Declustering potential −50 V, Focusing potential −200 V, Entrance potential −9 V, collision energy −30 V and collision cell exit potential −12 V.

2.5. Preparation of calibration standards and quality control samples

As patient samples were diluted 1:200 prior to analysis (see Section 2.6), blank plasma was diluted 1:200 with phosphate buffered saline (pH 7.4; NaCl 150 mM and Na_2HPO_4 10 mM), and a standard curve was constructed by spiking increasing amounts of teriflunomide to prepare solutions of 5, 10, 20, 40, 50, 100, 250, 500 μg/L. Quality Control Low (QCL, 8 μg/L), Quality Control Medium (QCM, 80 μg/L) and Quality Control High (QCH, 400 μg/L) solutions were prepared in diluted plasma from a stock solution created from a separate weighing of teriflunomide.

2.6. Sample preparation

To process an analytical run, the standard, quality control and test samples were treated in the same manner, except that methanol was added to the test samples instead of the teriflunomide stock solution. To adjust the concentration of teriflunomide in plasma samples so that it was within the detection range of the assay, these were diluted 1:200 with phosphate buffered saline (pH 7.4; NaCl 150 mM and Na_2HPO_4 10 mM) prior to processing. For each calibration series, a matrix blank sample was prepared from 200 μL of blank 1:200 plasma. Samples were prepared by adding 100 μg/L teriflunomide-D4 (20 μL) to 200 μL of 1:200 plasma, followed by $ZnSO_4$ (200 μL, 0.2 M) and acetonitrile (200 μL) to precipitate plasma proteins. Tubes were capped, vortexed for 10 s and centrifuged at 4000 rpm for 10 min at 4 °C. Reagent blank samples were prepared using water instead of plasma.

For analysis, 60 μL of the supernatant was injected onto the HPLC column. Injection of each blank and standard was made in

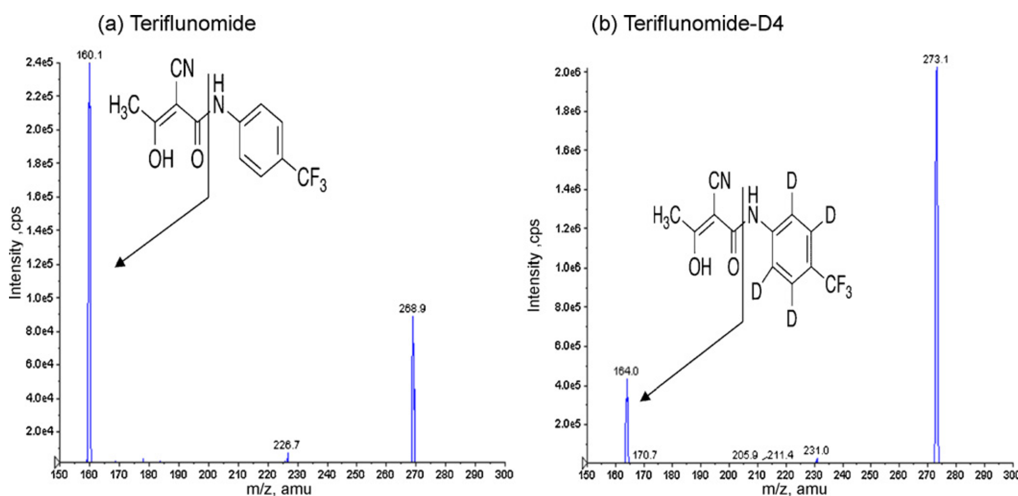

Fig. 1. Product ion mass spectra of (a) teriflunomide (*m*/*z* 269.1/160.0, scan range 150–300 amu) and teriflunomide-D4 (*m*/*z* 273.1/164.0, scan range 150–300 amu).

duplicate, while five replicates of the LOQ and six of the quality control samples were made.

2.7. Determination of unbound teriflunomide concentration

Unbound teriflunomide was determined by Rapid Equilibration Dialysis (RED) [18]. Briefly, plasma samples were adjusted to pH 7.4 using phosphate buffer (pH 7.4; 100 mM) and a 400 μL plasma aliquot was dialysed against 400 μL isotonic phosphate buffered saline solution (pH 7.4; NaCl 150 mM and Na_2HPO_4 10 mM) in a RED plate (ThermoFisher Scientific Inc., Waltham, MA, USA) at room temperature on an orbital shaker at 100 rpm. After dialysis, the resulting plasma and buffer dialysates were recovered, prepared (Section 2.6) and analysed by LC–MS/MS as per Section 2.4, noting that the residual plasma was diluted 1:200 and the dialysate buffer was used undiluted for sample preparation.

2.8. Validation procedure

The limit of quantitation was determined by injecting decreasing concentrations of teriflunomide into the analytical system to determine the minimal concentration providing a signal-to-noise ratio of at least 3:1 with adequate precision and accuracy (80–120%) [15]. Calibration standards and blanks were prepared (as per Section 2.5) and analysed in duplicate to establish the calibration range with acceptable accuracy and precision (85–115%). An eight point calibration curve over a concentration range of teriflunomide (5–500 μg/L in 1:200 blank plasma) was generated on five separate occasions.

The analyte-to-IS ratio (response) was calculated for each sample by dividing the area of the teriflunomide peak by the area of the IS peak. Standard curves of teriflunomide were constructed using linear regression analysis by plotting this ratio against the known teriflunomide concentration in each sample.

The accuracy and precision of the assay was determined by analysing teriflunomide samples at the LOQ, QCL, QCM, and QCH in six replicates (except for the LOQ, where five replicates were analysed), each in five analytical runs, together with the independently prepared calibration curves as described above.

Back-calculated concentrations of calibration and quality controls samples were estimated using the equation generated via linear regression analysis.

Accuracy was calculated as the relative difference (%Diff) between the calculated concentration and the spiked concentration for each standard solution as per the equation:

$$\%\,\text{Diff} = \frac{\text{Calculated Conc.} - \text{Spiked Conc.}}{\text{Spiked Conc.}} \times 100$$

Precision was calculated as the relative standard deviation (%CV) of the calculated concentrations of each standard solution as per the equation:

$$\%\,\text{CV} = \frac{\text{Standard Deviation of Calculated Conc.}}{\text{Mean of Calculated Conc.}} \times 100$$

Intra- and inter-assay precision was calculated for each of the calibration curve and quality control samples.

2.8.1. Recovery

The absolute recovery, or extraction efficiency the assay was determined by comparing, on three separate occasions, the peak areas of each QC concentration (QCL, QCM and QCH, prepared as per Section 2.5) with the peak area from matrix blank samples that were prepared (Section 2.5) and spiked (post-extraction) to the same final concentration with teriflunomide and IS. As the extraction efficiency of teriflunomide and IS was determined simultaneously, the recovery was calculated as the teriflunomide: IS extraction ratio.

2.8.2. Determination of matrix effect

To investigate whether endogenous matrix constituents interfered with the assay for free and total teriflunomide, samples of QCL, QCM, and QCH in reagent blank were prepared as per Section 2.5 above. Plasma from six healthy human volunteers were diluted 1:200, prepared as a matrix blank (Section 2.5), and spiked with teriflunomide and IS post extraction to a final concentration equivalent to QCL, QCM and QCH. Furthermore, blank plasma from the same six individuals was dialysed via RED, recovered, prepared as per Section 2.5 and spiked with teriflunomide and IS to a final concentration equivalent to QCL, QCM and QCH. Matrix effect was calculated by dividing the calculated concentration of the post-extraction spiked 1:200 plasma and dialysed samples by the calculated concentration of teriflunomide in reagent blank.

2.8.3. Carry over effect

To investigate carry over contamination of the assay, reagent blank injections were included at various points within the run, including after patient samples and after injection of the QCH. Carry over was determined by calculating the observed concentration of teriflunomide in each of the reagent blank injections.

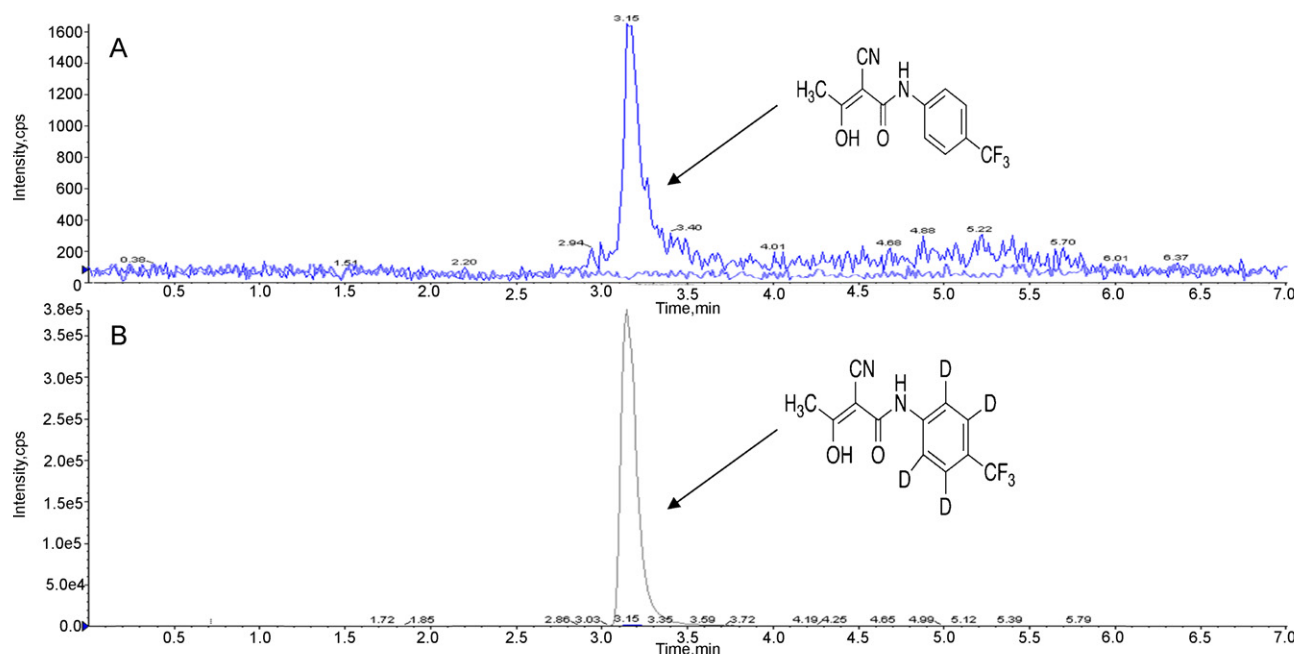

Fig. 2. Representative chromatogram of: (a) Teriflunomide (*m/z* 269/160, 3.1 min) extracted from blank plasma at the QCL concentration of 8 μg/L (top trace) and control human plasma (bottom trace); (b) Teriflunomide-D4 (IS, *m/z* 273.1/164.0, 3.1 min) added to blank plasma, 20 μL of 100 μg/L.

2.8.4. Stability

The stability of teriflunomide was determined in whole and 1:200 diluted plasma after three freeze–thaw cycles (−80 °C – room temperature (RT)), after 0, 2, 6 and 24 h on the benchtop (approximately 20 °C), after storage at −80 °C for up to 1 month and over 12 h in the LC–MS/MS autosampler. All stability studies were conducted at three concentration levels (QCL, QCM and QCH) with two determinations for each. As the optimal operating range of the MS assay was within from 5 to 500 μg/L, we created quality control patient samples by adding appropriate quality control (QCL = 1600 μg/L; QCM = 16,000 μg/L; QCH = 80,000 μg/L) to whole plasma. After storage, the whole plasma samples were diluted 200 times and the resultant teriflunomide concentration was equivalent to the QCL, QCM and QCH. Stability was expressed as the percentage recovery of the assayed solution relative to a freshly prepared solution (day 0, *t* = 0).

2.9. HPLC-UV assay

HPLC-UV determination of total teriflunomide concentration in plasma samples was conducted using a modified method of Schmidt et al. [8]. Briefly, a Shimadzu HPLC-UV apparatus (Kyoto, Japan) consisting of dual liquid chromatography pumps (LC-20AD), UV/VIS detector (SPD-20A), communication bus module (CBM-20A), auto-sampler (SIL-20A HT) and in-line degasser (DGU-20A3) was utilised. The stationary phase was as per the LC–MS/MS method above (Section 2.3).

Mobile phases consisted of acetonitrile-water-formic acid, with mobile phases A (5:94.8:0.2, v/v/v) and B (95:4.8:0.2, v/v/v). The total run time was 15 min, with the gradient starting at 35% B and increasing linearly to 100% of B over 10 min. This was followed by 5 min at 35% B to re-equilibrate the column to the starting acetonitrile concentration. Elution of the drugs was achieved at a flow rate of 0.6 mL/min and UV detection was set at 280 nm.

Sample preparation was as per Section 2.6 above, except that neat plasma (i.e., undiluted) was used. Teriflunomide eluted at approximately 5.3 min.

2.10. Application of the assay

This component of the study was approved by Royal Adelaide Hospital (RAH), Research and Ethics Committee and the University of South Australia, Division of Health Sciences Ethics Committee. Patients who were receiving leflunomide for treatment of RA at the RAH Rheumatology Unit were asked to participate. After obtaining written informed consent, venous blood samples were taken for determination of total and unbound teriflunomide concentration. After sampling, blood was directly centrifuged and plasma frozen at −80 °C. Samples were prepared as above (Section 2.6) and analysed via LC–MS/MS (Section 2.4) to determine total and free teriflunomide concentration, and HPLC-UV (Section 2.9) was also used to determine the total teriflunomide concentration.

3. Results and discussion

3.1. Chromatography

Teriflunomide and the deuterated IS both eluted at a retention time of approximately 3.1 min. A representative chromatogram of teriflunomide and IS extracted from 1:200 plasma is displayed in Fig. 2.

3.2. Validation of the LC–MS/MS assay

3.2.1. Calibration curve and limit of quantification (LOQ)

Five validation runs were conducted on separate days and the standard curves obtained for teriflunomide were linear with a mean coefficient correlation (±SD) of 0.999 (±0.001).

3.2.2. Accuracy and precision

The intra- and inter-assay accuracy and precision for the calibration curve samples is reported in Table 1, and demonstrates that the inter- and intra-assay accuracy and precision was <8.8% for each sample.

For the quality control samples, the intra-assay coefficient of variation was <5.4%, and accuracy ranged from −6.5 to 5.2% (Table 2). The accuracies and intra- and inter-assay precisions for

Table 1
Assay performance of the teriflunomide calibration curve over the concentration range of 5–500 μg/L in 1:200 blank human plasma, prepared and analysed by LC–MS/MS as per Sections 2.4–2.6.

Concentration (μg/L)	Mean observed concentration (μg/L) ±SD	Accuracy (%)	Precision intra-assay (%) ($n=2$)[a]	Precision inter-assay (%) ($n=5$)
5	5.3 ± 0.5	5.2	4.5	8.8
10	10.8 ± 0.8	8.0	3.3	7.4
20	19.6 ± 1.4	−1.9	2.4	7.3
40	38.3 ± 1.3	−4.2	1.9	3.3
50	53.6 ± 2.1	7.3	4.8	3.9
100	91.6 ± 1.8	−8.4	2.5	1.9
250	254.4 ± 10.5	1.8	1.8	4.1
500	491.3 ± 13.6	−1.7	1.5	2.8

[a] Except for the LOQ: $n=5$.

Table 2
Assay performance of LOQ, QCL, QCM and QCH teriflunomide, prepared in 1:200 blank human plasma, prepared and analysed by LC–MS/MS as per Sections 2.4–2.6.

Concentration (μg/L)	Mean observed concentration (μg/L) ± SD	Accuracy (%)	Precision intra-assay (%) ($n=6$)[a]	Precision inter-assay (%) ($n=5$)
5 (LOQ)	5.3 ± 0.5	5.2	4.5	8.8
8 (QCL)	7.6 ± 0.5	−5.1	5.4	7.1
80 (QCM)	74.6 ± 5.7	−6.5	3.3	5.7
400 (QCH)	411 ± 27.6	2.5	2.1	6.7

[a] Except for the LOQ: $n=5$.

the all of the concentration tested were within the defined acceptance criteria as described in Section 2.8.

3.2.3. Recovery

The recovery (extraction efficiency) of the teriflunomide:teriflunomide-D4 was >88% for the QCL, QCM and QCH, with the coefficient of variation ranging from 1.3 to 6.6%.

3.2.4. Time taken to reach equilibrium for the RED device

There was an increase in free concentration of teriflunomide between 2 and 6 h with no further increase up to 24 h (Fig. 3). Therefore, equilibration dialysis was deemed to be complete by 6 h, and this time was used in all subsequent experiments.

3.2.5. Assay specificity, carry over and matrix effect

A comparison of matrix blank chromatograms (from six healthy volunteers) with those obtained after spiking the blank plasma samples and dialysate (following RED) with teriflunomide and IS ascertained that endogenous substances did not interfere with the assay. The mean calculated amount of teriflunomide in the six individual plasma samples ranged from 97.8% to 110.9%, indicating the matrix effect was less than 11% (Table 3). Finally, reagent blank samples that were included within analytical runs did not show noticeable peak areas, indicating that there was no significant carry-over effect.

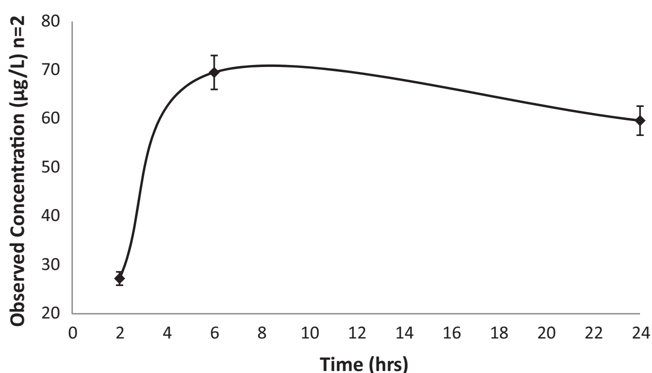

Fig. 3. Determination of the optimal time of dialysis using RED Plates. Pooled plasma was dialysed by RED for 2, 6 and 24 h, (Section 2.7) and the dialysate and retentate analysed by LC–MS/MS (Sections 2.4–2.6) to determine the unbound concentration at each time point.

3.2.6. Stability studies

Plasma stored at −80 °C with teriflunomide present and extracted plasma samples stored in the LC–MS/MS autosampler were stable for 1 month and 12 h respectively. Furthermore, the freeze/thaw study indicated that teriflunomide was stable in whole and 1:200 plasma for three freeze/thaw cycles, with the amount of teriflunomide present ranging from 83.5 to 113.1% with a % CV between 0.5 and 9.2%. The results of bench top stability indicate that plasma samples spiked with teriflunomide were stable for 24 h, with 100.0 to 108.5% of teriflunomide present and the % CV between 0.32 and 10.4%.

3.3. Application of the LC–MS/MS assay

Seventeen patients provided plasma samples; 14 patients provided two samples and 3 patients a single sample for analysis. In patients who had both samples taken at steady state (defined as patients who had been taking the same leflunomide dose for at least 8 weeks), there was less than 6% difference between the two samples. The total drug concentration in all patients was found to range from 7600 to 148,000 μg/L for total concentration and the unbound level from 7 to 125 μg/L for sample 1. For sample 2, the range was 11,000–169,000 μg/L for total concentration and the unbound

Table 3
Matrix effect of LC–MS/MS assay following analysis of teriflunomide from 6 different human plasma ($n=3$ from each of six volunteers) following dilution (1:200 with phosphate buffered saline) or RED as per Section 2.8.2.

	QCL	QCM	QCH
Dialysate (%)	110.9	97.8	98.6
1:200 Drug free plasma (%)	110.9	98.7	100.0
Mean	7.74	80.04	397.96
SD	0.46	0.91	3.25
% CV	5.90	1.14	0.82

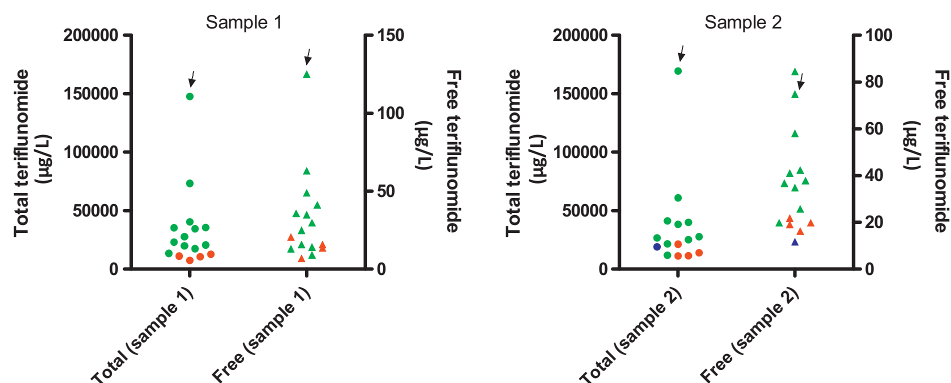

Fig. 4. Plasma concentration of free and total teriflunomide in patients with rheumatoid arthritis treated with leflunomide at 6.5 (blue dot), 10 (red dots) and 20 mg (green dots) as determined by LC–MS/MS as per Sections 2.4 and 2.6. Note that sample 1 was taken approximately 6 weeks before sample 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

level from 12 to 85 µg/L. The data is displayed in Fig. 4. According to these results, unbound teriflunomide represents 0.11% (range 0.04–0.22%) of the total level. Regression analysis demonstrated a significant correlation between the total and free teriflunomide concentration in the patient samples ($r^2 = 0.724$, $P < 0.0001$, Fig. 5). The steady state total plasma teriflunomide concentration in our patient group is consistent with the previously reported steady state concentrations for patients with RA who were administered leflunomide at doses of 10 or 20 mg per day [7,8]. *In vitro* studies have reported that the fraction unbound was approximately 0.6% of the total concentration [6], and while the method used to determine this was not reported, similar discrepancies between the fraction unbound reported in the literature and those obtained with equilibration dialysis and rapid equilibration dialysis have been observed [18]. Regardless, our data support the finding that teriflunomide is highly protein bound, with >99% bound to plasma proteins.

When the samples were analysed by HPLC-UV, a peak that co-eluted with teriflunomide was identified in one patient, but its identity was not able to be determined. Regardless, when the total teriflunomide concentration determined by LC–MS/MS was compared with the results obtained by HPLC-UV, the ratio of the concentration calculated by HPLC-UV:LC–MS/MS was 0.99, and a high degree of similarity was observed ($r^2 = 0.973$, Fig. 6).

In comparison with the published HPLC-UV methods [8,11–13], the LOQ of our method is 20–100 fold lower, which has allowed us to quantify the unbound fraction of teriflunomide in human plasma. A further advantage is the lack of interfering peaks, as was observed in one of the 17 patient samples analysed by HPLC-UV, although it made little difference to the correlation between the two methods.

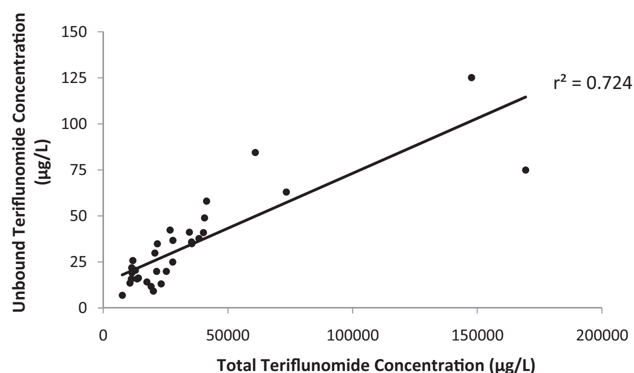

Fig. 5. Relationship between total and free teriflunomide concentration as determined by LC–MS/MS. N.B. of the 17 patients included in the study, 14 had 2 samples included in this analysis, and 3 had only one sample, $n = 31$.

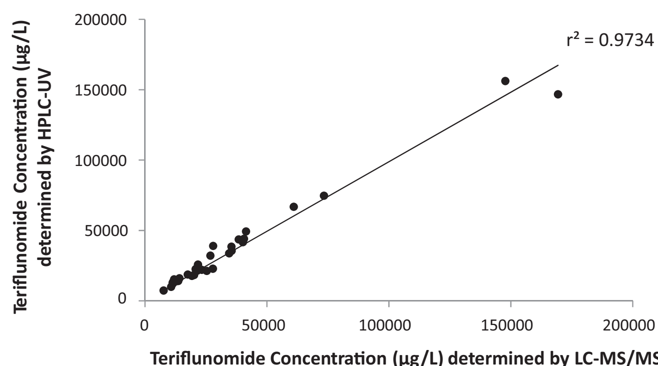

Fig. 6. Relationship between total teriflunomide concentration determined by LC–MS/MS and HPLC-UV assays. N.B. of the 17 patients included in the study, 14 had 2 samples included in this analysis, and 3 had only one sample, $n = 31$.

A recently published LC–MS/MS method had a 2 fold higher LOQ [14], involved liquid–liquid extraction for sample preparation, and it was not used to examine unbound teriflunomide. In comparison, protein precipitation with zinc sulphate and acetonitrile used in the present study is relatively straightforward and was made possible by the use of a deuterated internal standard, which overcame any issues regarding matrix effect.

4. Conclusion

We have developed and validated a sensitive analytical method with tandem mass spectrometric detection for the quantitation of teriflunomide, the active metabolite of leflunomide in human plasma. We subsequently applied the method to determine the total and free teriflunomide concentration in 17 patients who were taking leflunomide for RA. Our results indicate that the fraction unbound to plasma proteins is 0.11%. Further studies are needed to ascertain whether this highly sensitive assay for determining total and free teriflunomide concentrations in human plasma can be used to optimise patient outcomes.

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