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Dried Blood Spot Methodology in Combination With Liquid Chromatography/Tandem Mass Spectrometry Facilitates the Monitoring of Teriflunomide

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Background: Teriflunomide, a once-daily oral immunomodulator approved for treatment of relapsing-remitting multiple sclerosis, is eliminated slowly from plasma. If necessary to rapidly lower plasma concentrations of teriflunomide, an accelerated elimination procedure using cholestyramine or activated charcoal may be used. The current bioanalytical assay for determination of plasma teriflunomide concentration requires laboratory facilities for blood centrifugation and plasma storage. An alternative method, with potential for greater convenience, is dried blood spot (DBS) methodology. Analytical and clinical validations are required to switch from plasma to DBS (finger-prick sampling) methodology.

Methods: Using blood samples from healthy subjects, an LC-MS/MS assay method for quantification of teriflunomide in DBS over a range of 0.01–10 mcg/mL was developed and validated for specificity, selectivity, accuracy, precision, reproducibility, and stability. Results were compared with those from the current plasma assay for determination of plasma teriflunomide concentration.

Results: Method was specific and selective relative to endogenous compounds, with process efficiency ~88%, and no matrix effect. Inaccuracy and imprecision for intraday and interday analyses were <15% at all concentrations tested. Quantification of teriflunomide in DBS assay was not affected by blood deposit volume and punch position within spot, and hematocrit level had a limited but acceptable effect on measurement accuracy. Teriflunomide was stable for at least 4 months at room temperature, and for at least 24 hours at 37°C with and without 95% relative humidity, to cover sampling, drying, and shipment conditions in the field. The correlation between DBS and plasma concentrations ($R^2 = 0.97$), with an average blood to plasma ratio of 0.59, was concentration independent and constant over time.

Conclusions: DBS sampling is a simple and practical method for monitoring teriflunomide concentrations.

Key Words: teriflunomide, dried blood spot, LC-MS/MS

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INTRODUCTION

Teriflunomide is a once-daily oral immunomodulator approved for the treatment of relapsing-remitting multiple sclerosis.[1]

Teriflunomide has a long half-life in vivo.[1] If needed, an accelerated elimination procedure using cholestyramine or activated charcoal can be used in cases of overdose, pregnancy, or for any reason that accelerated elimination is determined to be clinically desirable. If used in relation to pregnancy, a teriflunomide plasma concentration of ≤0.02 mcg/mL, which is believed to be nonteratogenic based on animal data, should be achieved. Measurement of teriflunomide plasma concentrations is not practical at sites lacking the facilities to prepare and process blood samples. A dried blood spot (DBS) sampling method, in which a few drops of blood, drawn by lancet from the finger, are applied onto specially manufactured absorbent filter paper, can be used as an alternative to plasma monitoring, and would allow for simplified sample storage and transport.

Blood samples from finger or heel pricks deposited on filter paper date back to the early 1960s when it was used in the screening of newborns for phenylketonuria.[2] Compared with conventional methods of blood sample preparation, the DBS method offers several advantages: more convenient sample collection,[3] smaller sample volumes, no sample centrifugation, reduced risk of infection,[4] and, in many cases, improved sample stability, allowing storage and shipment of samples at room temperature.[5] After the widespread introduction in the 1990s of highly sensitive analytical systems such as tandem mass spectrometry (MS/MS), the

utility of DBS sampling has been demonstrated for therapeutic drug monitoring[6–8] and for epidemiological studies.[4,9] DBS methodology has also been used for quantitative biomarker assessment.[10–14] The objective of the present work was to develop and validate a simple assay using DBS methodology and liquid chromatography coupled with MS/MS to determine teriflunomide concentrations in human blood. The assay was validated using internationally accepted criteria. Investigations on additional parameters [sample collection volume, DBS punching location, hematocrit (HCT) effect, and temperature/long-term stability of test compounds] were conducted as part of the evaluation. As all previous studies had been performed using plasma, we also verified that the in vivo blood to plasma ratio did not depend on the storage temperature of the DBS sample, time, or plasma concentration.

MATERIALS AND METHODS

Chemical Reagents and Equipment

Teriflunomide and its internal standard (IS) [$^2H_4$]-teriflunomide were obtained from Toronto Research Chemicals Inc, (North York, ON, Canada).

Acetonitrile [high-performance liquid chromatography (HPLC) grade] was purchased from ACI Labscan and methanol (HPLC grade) was purchased from Camlab (the Netherlands). Acetic acid and formic acid (RS-For LC/MS) were purchased from Acros Organics (the Netherlands), and Ultrapure Water with a resistance >18.0 Ω/cm was used.

For DBS application, PerkinElmer Bioanalysis Card (Research Use Only) #226–1004, was purchased from PerkinElmer Health Sciences Inc, (Greenville, SC), and was used for the development and validation of the bioanalytical method. PerkinElmer 226 Spot Saver Card—GR2261003 (CE certified), also purchased from PerkinElmer Health Sciences Inc., was used for clinical sampling.

A Harris Uni-core puncher of 6-mm diameter was purchased from Whatman—GE Healthcare (the Netherlands).

Drug-free fresh human blood, with ethylenediaminetetraacetic acid added as anticoagulant, was obtained from healthy volunteers and stored at refrigerator temperature (approximately 4°C) for no longer than 2 days until use. For every batch of fresh blood obtained, the HCT value was determined and recorded in the study file (HCT value in the range of 38%–42% was recommended).

LC-MS/MS Conditions

The HPLC system consisted of Shimadzu LC-20AD pump, SIL-20AC autosampler, and CTO-20AC oven (Kyoto, Japan). Chromatography was performed on an Agilent Zorbax Eclipse XDB-C8 analytical column with 3.5 μm particle size (4.6 mm internal diameter, 50 mm length) (the Netherlands) at a flow rate of 0.7 mL/min with mobile phases consisting of a mixture of water/acetonitrile (vol/vol) containing 0.1% acetic acid. Optimal chromatographic separation was achieved by running for 4.5 minutes isocratically. The column was maintained at 40°C with the column effluent delivered to the mass spectrometer interface without splitting.

The mass spectrometric conditions consisted of a Sciex API4000 triple quadrupole mass spectrometer (MDS SCIEX; Applied Biosystems, Concord, ON, Canada) controlled by Analyst 1.4.2 software. This software was also used for data acquisition and processing. The mass spectrometer was operated in the negative multiple reaction monitoring mode by using a Turbo Ion Spray source. The source conditions were: temperature, 400°C; curtain gas, 25; on-source gas, 1 and 2 at 40. The multiple reaction monitoring transitions monitored were m/z 269 → 81.9 for teriflunomide and m/z 273.1 → 81.9 for [$^2H_4$]-teriflunomide with unit resolution. The optimized declustering potential was −57 eV; the collision energy was −28 eV; and the dwell time was set at 150 millisecond.

Preparation of Calibrators and QC Samples

Calibration samples were prepared by mixing human blood (0.98 mL) with an aliquot (20 μL) of teriflunomide stock solutions in methanol (0.5, 1.25, 2.5, 5, 12.5, 50, 250, and 500 mcg/mL) to give nominal concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 1.0, 5.0, and 10 mcg/mL.

Quality control (QC) samples were prepared by mixing human blood (0.98 mL) with an aliquot (20 μL) of teriflunomide stock solutions in methanol (0.5, 1.5, 25, and 450 mcg/mL) to give lower limit of quantification (LLOQ), low QC, medium QC, and high QC samples with nominal concentrations of 0.01, 0.03, 0.5, and 9 mcg/mL, respectively.

For blood spotting, aliquots (25 μL) of calibrators and QC samples were spotted onto filter paper with a repeat pipette and allowed to dry at room temperature for at least 2 hours before analysis. When required, QC samples were stored at room temperature.

DBS Sample Extraction

In a typical extraction process, 6-mm diameter blood spot disks were punched from the sample area and placed into a 2-mL Eppendorf tube, and 40 μL of IS (working IS in methanol at 0.5 mcg/mL) and 1200 μL of methanol were added. After vortex mixing for 15 seconds followed by mixing using a shaker for 60 minutes at room temperature, the sample was centrifuged at ×16,000*g* for 10 minutes. Then, 150 μL of the extract was transferred to a 200-μL glass injection vial and an aliquot of 2 μL was injected into the HPLC system.

Analytical Validation

All work was performed in compliance with the Good Laboratory Practice principles of the Organization for Economic Cooperation and Development.[15] All validation experiments were performed according to the Bioanalytical Method Validation Guidance for Industry[16] and the European Medicines Agency guidelines on validation of bioanalytical methods.[17]

Assay Specificity and Selectivity

Specificity was assessed by verifying the absence of significant interference in the biological control medium with regard to the retention time of the compound (s) to be assayed and of the IS.[18] The following chromatograms were obtained:

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(1) blank DBS, (2) blank DBS spiked with teriflunomide at the LLOQ concentration and with IS, and (3) blank DBS spiked with the IS at the concentration used in the study.

Linearity

A calibration curve was prepared within the range of 0.01 to 10 mcg/mL teriflunomide in each run. Half of the calibration samples were analyzed at the beginning of the run and half at the end. The simplest calibration model and weighting procedure were used. The calculations of the curve's parameters were based on the ratio of the peak areas of teriflunomide/IS versus the concentration of teriflunomide. Teriflunomide concentrations for samples were calculated from the curve's equation obtained by means of linear regression.

Accuracy of back-calculated calibration samples should be within 100 ± 15% of the corresponding nominal concentration, except at the lowest concentration level, where the accuracy should be within 100 ± 20%.

Per calibration curve, a maximum of 33% of the calibration samples, except the LLOQ and upper limit of quantification (ULOQ, 10 mcg/mL), may differ from these specifications. At least 6 concentration levels were represented in each curve.

Matrix Effect, Extraction Recovery, and Process Efficiency

The influence of the matrix on electrospray ionization and on the quantification of teriflunomide was monitored using quantitative methods[19] and involved a comparison of: (1) the instrument response for the low, medium, and high QCs (n = 4 per level) injected directly in mobile phase (neat solutions), (2) the same amount of analyte added to extracted blank samples (postextraction spiked samples), and (3) the same amount of analyte added to the biological matrix before extraction (preextraction spiked samples).

Total process efficiency was calculated from the ratio of mean peak areas of teriflunomide in extracted DBS validation samples versus neat unextracted samples. This term accounts for any loss in signal attributable to the extraction process or matrix effect.

Extraction recovery was calculated from the ratio of mean peak areas of teriflunomide in extracted DBS validation samples versus blank DBS samples spiked after extraction.

The ionization efficiency, defined as absolute matrix effect,[20] was calculated from the ratio of mean peak areas of teriflunomide in blank DBS samples spiked after extraction versus neat unextracted samples. If the ratio was <85% or >115%, an exogenous matrix effect was inferred.[21,22]

Matrix Variability

To confirm that the biological matrix would not interfere with the assay, the selectivity of the developed method was tested by analyzing 6 different lots of blank blood samples spiked with IS at the LLOQ level (n = 4 per lot), and blank blood samples with no IS (n = 4 per lot) from the same 6 individuals, against a calibration curve.

The results for the LLOQ samples were considered acceptable if the imprecision from each matrix lot was ≤20% and the accuracy was within the range of 80%–120%. The acceptance criterion for the analysis of the blank samples from the 6 individual lots was based on the raw peak areas found at the retention times of teriflunomide and IS. No more than 10% of the blank samples could have raw peak areas greater than 20% of the average peak area of teriflunomide in the LLOQ QCs.

Assay Variability

The assay variability of the method was determined from 3 independent runs, each run containing a full calibration curve, with 6 replicate samples at each of the 4 validation levels (ie, LLOQ QC, low QC, medium QC, and high QC).

For each nominal concentration, the observed concentrations were individually analyzed through a 1-way random effects analysis of variance (ANOVA). Mean and 2-sided 95% CIs were calculated within the ANOVA[23] framework. The observed mean and CIs were converted to the percentage difference from nominal, using nominal concentration as the denominator.

Within-run, between-run, and total variances were estimated by equating observed and expected mean squares within the 1-way random effects ANOVA. Two-sided 95% CIs were calculated using the χ^2 method for within-run variance, the Modified Large Sample method for between-run variance, and the Graybill-Wang method for the total variance. Variance estimates and CIs were converted to percent coefficient of variation (CV) by applying the square root transformation and dividing by the observed concentration mean.

All calculations were performed using SAS software release 8.2.[23]

No more than 33.3% of individual validation samples within a given concentration level should be greater than ±15.0% of nominal, except at the LLOQ where the acceptance criterion was ±20.0% of nominal. The mean estimates for accuracy (bias) and variance (imprecision) for each validation level should not exceed 15.0%, except at the LLOQ where the acceptance criterion was ±20.0%.

Stability

The stability of teriflunomide in spiked DBS samples was investigated under different conditions for drying, storage, and transport. Each assessment was performed, in triplicate, at low and high QC concentrations. For the purposes of the current work, a temperature of 37°C ± 95% relative humidity was chosen to simulate anticipated sampling and transport conditions. These conditions were considered as the worst case scenario and should be assessed on a case by case basis.

Stability during drying time (approximately 3 hours) was covered by measurement of stability at room temperature over 24 hours, using human fresh liquid blood (internal data). To take into account the effect of humidity and temperature during sampling and drying,[24] freshly prepared DBS QC samples were stored at 37°C with 95% relative humidity for 24 hours before processing and analysis.

To mimic expected transportation conditions, further DBS QC samples were stored 24 hours at 37°C without humidity, before processing and analysis.

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To assess short- and long-term storage of DBS samples, QCs were stored for 1, 3, and 7 days, and for 1, 3, and 4 months at room temperature before processing and analysis.

All QC samples used for these stability assessments were assayed against freshly prepared spiked blood spots.

In addition, the stability of teriflunomide was tested for up to 3 days at room temperature in processed samples.

Effect of HCT

The HCT value has a profound effect on blood viscosity,[14,25–28] and may thereby influence flux and diffusion properties of blood into filter paper. Owing to this effect, blood distribution through the paper can be nonuniform at high HCT values (ie, >55%).

Reference values for HCT are 41%–51% for men and 37%–47% for women.[14]

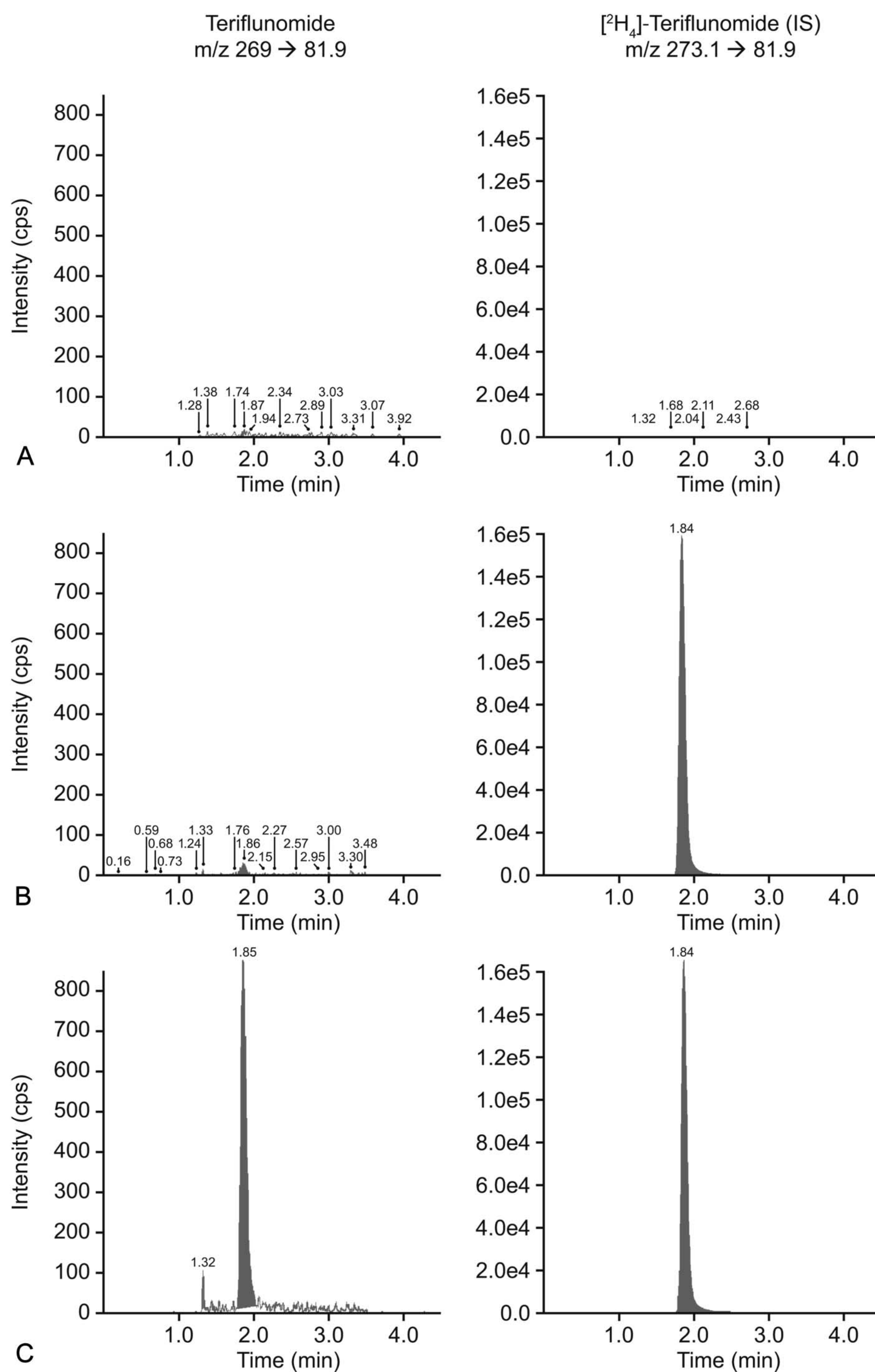

FIGURE 1. Chromatogram of (A) blank DBS, (B) blank DBS spiked with IS, and (C) blank DBS spiked with teriflunomide at the LLOQ (0.01 mcg/mL), and IS.

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TABLE 1. Summary of Calibration Curve Parameters

Run No	Slope (a)	Intercept (b)	Correlation Coefficient (R)
1	0.535	−1.43E-04	0.9979
2	0.508	−4.19E-05	0.9993
3	0.535	−3.36E-04	0.9986
4	0.491	−3.83E-05	0.9981
5	0.492	−2.54E-04	0.9996
6	0.493	−1.09E-04	0.9991
7	0.475	5.19E-04	0.9992
8	0.487	−6.22E-05	0.9984
9	0.478	1.80E-04	0.9972
10	0.364	1.41E-04	0.9952
11	0.356	9.70E-04	0.9957
12	0.431	3.66E-05	0.9988
Mean	0.470		
SD	0.058		
CV, %	12.4		

Accepted results n = 10 for each run.

To evaluate the influence of HCT on the quantification of teriflunomide, reconstituted human blood samples with defined HCT levels (30%, 35%, 40%, 45%, 50%, 55%, and 60%) were produced by diluting blood cells with plasma obtained after 10-minute centrifugation at ×2000*g* (at 4°C) of fresh human blood taken from a healthy adult.

At each HCT level, QC samples were prepared at both low and high concentrations, and spotted onto filter paper. DBS samples were then prepared and analyzed (n = 9 per level per HCT value) against a calibration curve, and QC samples prepared from fresh human blood with an HCT value at 41%.

To take into account the bias obtained in reconstituted blood, concentrations determined at the low and high QCs prepared in blood with an HCT value of 40% were used as the nominal value for statistical analysis.

Statistical evaluation consisted of a determination of bias as a function of the HCT level with 95% CI for the mean. It was compared with the usual threshold of ±15%. The software used was SAS v9.2 on the Windows 7 operating system.

The HCT value was considered without influence if the 95% CI for the estimated ratio fell entirely within the 15% specification limit.

Effect of Blood Spot Size

The use of the finger-prick sampling method allows deposition of a variable blood volume onto the filter paper, and thus an evaluation of the influence of blood spot size on the accuracy of the determination was performed. DBS QC samples at low and high concentrations were examined by spotting increasing volumes (20, 40, 60, and 80 μL; n = 4 per volume) onto the card. After drying, 4 replicate 6-mm discs were taken from the center of each DBS QC sample and analyzed along with calibrators. The teriflunomide concentrations were compared with the nominal values. Accuracy in the range of 85%–115% of the nominal values would suggest no apparent difference for the DBS samples made with different blood volumes.

Effect of Punch Position

The distribution of test compounds in the spot can be affected by blood distribution because of possible interaction of blood and/or the analyte with the materials of the filter paper.[25,29] The homogeneity within the spot was assessed by punching the DBS discs (n = 3) from the center and peripheral area of DBS QC samples at low and high concentrations (using a spotting volume of 80 μL), followed by analysis along with calibrators. Results from both the central and peripheral discs would suggest no apparent distribution effect if the accuracies of the calculated teriflunomide concentrations were within the range of 85%–115% with reference to nominal concentrations.

Dilution Integrity Test

To demonstrate that the method is suitable for a DBS sample with test compound concentration higher than the ULOQ, the dilution integrity was assessed using DBS validation samples spiked with the test compound at 2-, 4-, and 10-fold the concentration of the high QC.[30]

The dilution test using DBS samples (n = 9 per dilution) was performed by increasing the concentration of IS by the appropriate dilution factor. After extraction, the dry extract was taken up with a volume of injection solvent also multiplied by the same factor.

Accuracy of the calculated concentrations within the range of 85%–115% of the nominal values would suggest that a DBS sample containing teriflunomide at a higher concentration than the ULOQ can be diluted using the above tested dilution method.

TABLE 2. Mean, CV, and Accuracy of Back-Calculated Concentrations of Calibrators for Teriflunomide

Nominal, mcg/mL	Cal 1 0.01	Cal 2 0.025	Cal 3 0.05	Cal 4 0.1	Cal 5 0.2	Cal 6 1	Cal 7 5	Cal 8 10
Number, n	24	12	12	12	12	12	12	24
Mean, mcg/mL	0.010	0.025	0.049	0.099	0.244	1.041	5.116	9.888
SD	0.0005	0.0016	0.0022	0.0040	0.0062	0.0337	0.2322	0.5435
CV, %	5.3	6.3	4.4	4.0	2.5	3.2	4.5	5.5
Accuracy, %	100.5	99.4	97.5	99.1	97.7	104.1	102.3	98.9

Cal, calibration.

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Puncher Cross-Contamination Test

Unlike liquid samples, where the use of disposable pipette tips avoids cross contamination, the DBS method uses a puncher that cuts each DBS disc and comes into direct contact with the sample.[31] Therefore, the cutting system was cleaned with methanol between uses.

To investigate spot-to-spot carry-over using a manual puncher, each validation run containing a calibration curve included 3 blank samples punched directly after the sample punch at the ULOQ calibration level. These samples were extracted and analyzed.

The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration.

Autosampler Carry-Over Test

To investigate carry-over from one sample to the other in the autosampler, each validation run containing a calibration curve included a blank sample analyzed directly after the sample at the ULOQ calibration level.

The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration.

Clinical Validation

Sample Collection

The samples for clinical validation were taken from healthy male and female volunteers (N = 18) who received repeated oral dosing of teriflunomide 70 mg once daily for 5 days followed by an accelerated elimination procedure using colesevelam hydrochloride for 11 days. The study protocol was approved by the local institutional ethics committee. Written informed consent was obtained from the subjects. The study was conducted at Biotrial, Rennes, France.

On days 1, 3, 6, 7, 9, 13, and 17, venous blood samples, approximately 4 mL in volume, were obtained by nurses. Immediately after homogenization with anticoagulant, blood was divided into 2 aliquots. The first aliquot was centrifuged at ×1500*g* for 10 minutes at 4°C, and the plasma fraction was transferred into a 2-mL polypropylene tube and stored at −20°C before analysis. From the second aliquot, 3 samples of 30 μL and 2 samples of 10 μL of the venous blood were spotted onto the filter paper. After drying at room temperature for at least 3 hours, venous DBS samples were packaged in a zip-lock bag containing desiccant and stored at room temperature. The remaining liquid blood was also stored at −20°C before shipment. For each subject, the HCT of the venous sample was measured and recorded.

In addition, in parallel to venous sampling, finger-prick DBS samples were prepared. Finger-prick sampling is a major advantage for DBS methodology,[16,32,33] because it is less stressful for the patients, no anticoagulant is required, no binding to materials (syringe and tubes) is observed, and homogeneity of the samples is generally good. Furthermore, less blood volume is required and shipping can be performed at ambient temperature. To maximize sample quality, clinical staff received practical training on blood-sampling techniques and assessment of spot quality before the study. Finger-pricks were performed according to the technique described elsewhere,[34,35] and blood was dropped directly onto filter paper. The finger-prick DBS samples were left to dry for at least 3 hours, and then stored in a sealed plastic bag at room temperature with desiccant before shipment.

Liquid samples were shipped from the clinical center to the bioanalysis laboratory in a frozen condition, and the DBS samples were shipped at room temperature in a waterproof container containing desiccants.

Venous DBS samples and liquid blood were collected to be analyzed only if the correlation between plasma and finger-prick DBS samples presented unexpected variations, or if the number of valid spots from finger-prick was insufficient to perform statistical analysis.

Sample Qualification

On arrival of the DBS samples in the bioanalytical laboratory and before analysis, a visual inspection of the quality of the spot was performed. A spot was considered valid if the following criteria were met: (1) spot diameter was superior or equal to 7 mm, (2) spot was spread symmetrically on both sides of the sampling paper, and (3) spot was made from a single drop of blood and dark red in color.

Sample Analysis

All analyses of the plasma and DBS samples were conducted by Eurofins Medinet, Breda, the Netherlands.

The DBS samples were analyzed using the method described above, and plasma samples were analyzed by a validated, LC-MS/MS analytical method with an LLOQ of 0.01 mcg/mL (data on file).

Incurred sample reanalysis (ISR) is applicable to DBS samples.[14,36,37] ISR tests were conducted on plasma samples and on the second spot of the same DBS sample for 10% of study samples. Samples with a concentration of at least 3 × LLOQ up to near the higher concentration of the analyte were selected. The samples were assayed in a separate analytical run shortly after their initial analysis and within established stability. The difference from the initially obtained concentration was calculated for each sample and matrix.

The ISR test was considered acceptable if no more than 33.3% of reassayed samples exceeded ±20% of the original value.

Statistical Analysis

The relationship between plasma and DBS concentrations was explored graphically with linear regression including the Passing–Bablok method.[38] In addition, a linear model was applied to the difference D = log (DBS Concentration) − log

TABLE 3. Matrix Effect, Extraction Recovery, and Process Efficiency of Teriflunomide

	Low QC (0.03 mcg/mL)	Medium QC (0.5 mcg/mL)	High QC (9 mcg/mL)
Matrix effect	95.2	99.3	99.2
Extraction recovery	92.6	88.9	88.1
Process efficiency	88.2	88.3	87.4

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TABLE 4. Inaccuracy and Imprecision of DBS Assay

Nominal Conc., mcg/mL	Mean Calculated Conc., mcg/mL	Mean Difference (95% CI), %	Within-run Imprecision, %	Total Imprecision, %
0.01	0.00985	−1.46 (−5.53 to 2.60)	8.48	8.48
0.03	0.0297	−0.963 (−3.42 to 1.49)	4.59	4.59
0.5	0.499	−0.189 (−12.5 to 12.1)	3.85	6.07
9	8.92	−0.846 (−2.43 to 0.743)	4.69	4.69

n = 18 for each concentration (conc.).

(Plasma Concentration) and the average M = [log (DBS Concentration) − log (Plasma Concentration)]/2 (Bland–Altman method[39,40]). Blood to plasma ratio and 90% CI were derived from the model using the antilog transformation.

RESULTS

Analytical Method Validation

Assay Specificity and Selectivity

The chromatograms for blank DBS, blank DBS spiked with teriflunomide at LLOQ and IS, and blank DBS spiked with IS, presented in Figure 1, show that the retention times for teriflunomide and [$^2H_4$]-teriflunomide were approximately 1.8 minutes. No significant interfering peaks were evident in blank DBS that eluted with teriflunomide or the IS. The method is therefore selective and specific relative to endogenous compounds.

Linearity

Calibration curves for teriflunomide in DBS were linear using a weighting factor 1/X × X in the range of 0.01 to 10.0 mcg/mL. The calibration curve parameters given in Table 1 show that the correlation coefficients (R) of the calibration curves were above 0.99. The variation of the slopes and intercepts of the calibration curves were acceptable, meaning that no effect was observed on the validity of the runs and on the robustness of the method, as shown by the assay variability test, the ISR test and routine analysis.

All individual back-calculated concentrations were within acceptable limits, and the mean back-calculated concentrations given in Table 2 were between 97.5% and 104.1% of nominal, with CVs in the range of 2.5%–6.3% for teriflunomide.

Matrix Effect, Extraction Recovery, and Process Efficiency

The ionization efficiency (or matrix effect), extraction recovery, and overall process efficiency for teriflunomide is shown in Table 3. Regardless of the test compound and the concentration tested, no matrix effect was observed and process efficiency was in the range of 88%. The recovery data at various concentrations indicate that there is no major loss of teriflunomide during the extraction process, and no significant binding to the glass and polypropylene materials used.

Matrix Variability

At the LLOQ for teriflunomide, the ranges of accuracy and precision using 6 different lots of human blood were within established acceptance criteria (data not shown). Blank samples (without IS) from the same 6 individual lots of blood did not produce any interference with raw peak areas that were greater than 20% of the peak area of the LLOQ.

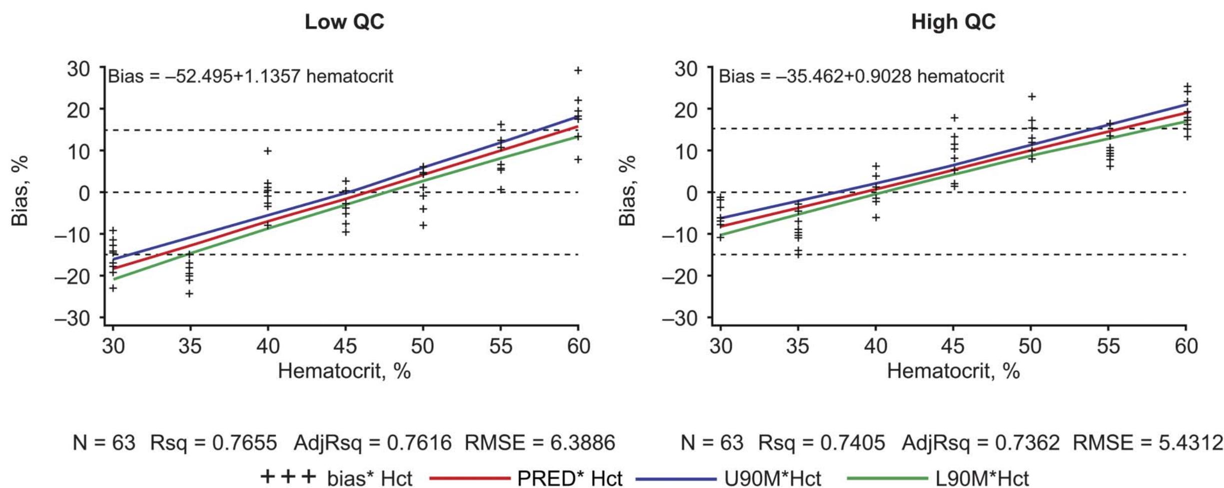

FIGURE 2. Influence of HCT on quantification of teriflunomide at low and high QC concentrations: linear regression with 95% mean CIs of bias depending on HCT level. AdjRsq, adjusted R square; L90M, lower 90% confidence limit for the mean; PRED, predicted value for % bias; Rsq, R square; RMSE, root mean square error; U90M, upper 90% confidence limit for the mean.

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TABLE 5. Influence of Spot Volume on Quantification of Teriflunomide

	Low QC				High QC			
Spot Volume, μL	**20**	**40**	**60**	**80**	**20**	**40**	**60**	**80**
Mean conc., mcg/mL	0.0311	0.0312	0.0307	0.0316	8.91	8.70	9.45	8.55
SD	0.0009	0.0013	0.0009	0.0001	0.49	0.29	0.57	0.34
CV, %	2.9	4.2	3.0	0.3	5.5	3.3	6.1	4.0
Accuracy, %	104	104	102	105	99	97	105	95

n = 4 for observations for each spot volume.

The influence of endogenous components, demonstrated using 6 different lots of human blood (matrix variability), had no effect on the quantification of teriflunomide.

Assay Variability

Data for within-day and between-day inaccuracy and imprecision of the method for determination of teriflunomide are presented in Table 4. The accuracy (% mean difference) over 18 replicates of 4 QC concentrations ranged from −1.46% to −0.189%. The total imprecision (% CV) over 4 QC concentrations ranged from 4.59%–8.48%. Overall, inaccuracy and imprecision values for within-day and between-day were <20% at LLOQ and <15% at all other concentrations tested. Thus, the method possesses acceptable accuracy and precision.

Stability

The test compound was stable in DBS in a sealed bag with desiccant at room temperature for at least 4 months, and at 37°C with and without relative humidity for 24 hours. This result circumvents the possible issue of very limited medical infrastructures concerning the drying, storage, and transport of samples. In addition, processed teriflunomide DBS samples were stable for at least 3 days when stored at room temperature.

Effect of HCT

The statistical evaluation of bias as a function of the HCT level with 95% CI showed that, regardless of the teriflunomide concentration (Fig. 2), the bias increased linearly as a function of HCT level. Thus, based on an acceptance criterion of ±15% for the bias, at low nominal concentration, the measure of teriflunomide in DBS fits acceptable criteria for HCT interval (35%–57%). For high nominal concentration, the value of teriflunomide concentrations in DBS fits acceptable criteria for HCT interval (30%–55%).

TABLE 6. Influence of Position of Punch on Quantification of Teriflunomide

Nominal Concentration	Low QC		High QC	
Position of Punch	**Central**	**Peripheral**	**Central**	**Peripheral**
Mean conc., mcg/mL	0.034	0.033	9.8	10.2
SD	0.002	0.002	0.27	0.69
CV, %	7.8	5.4	3.0	7.7
Accuracy, %	112	111	109	114

According to the HCT reference values in women (37%–47%) and in men (41%–51%),[14] the validated intervals fall comfortably within the reference values.

To minimize the effect of HCT during routine analysis of samples, calibration and QC samples should be prepared from fresh blood with HCT as close as possible to 40%.

Effect of Blood Spot Size

Deposition of an increasing volume of QC samples at low and high concentrations showed that the accuracy of measured concentrations against the nominal values (Table 5) is constant as a function of the volume of blood deposited and remains within acceptance criteria. These results validate the variability of blood volume (from 20 to 80 μL) spotted when sampling is performed from a finger-prick.

Effect of Punch Position

A comparison of the accuracy of teriflunomide quantification using discs punched from the center and peripheral areas of the DBS is shown in Table 6. The bias values for the measured analyte concentrations from the discs at both the center and peripheral areas of the DBS samples were comparable and were all within 85%–115%, confirming no apparent distribution effect.

Dilution Integrity Test

The results of experiments to evaluate the accuracy of quantification of teriflunomide after sample dilution indicate

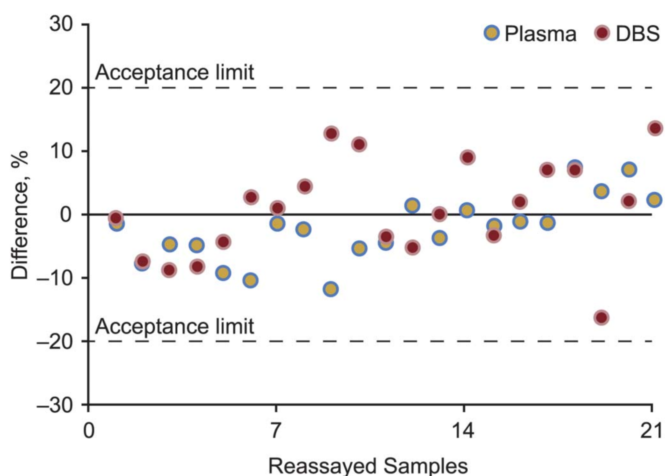

FIGURE 3. Incurred sample reproducibility in plasma (yellow dots) and in DBS from finger-prick (red dots).

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that DBS samples can be accurately diluted by a factor of 2, 4, or 10 into the range of the calibration curve (data not shown).

Puncher Cross-Contamination and Autosampler Carry-Over Tests

The puncher cross-contamination test demonstrated that, when the cutting system was cleaned with methanol after each punch, there was no cross contamination. In addition, there was no evidence of autosampler carry-over of teriflunomide when a blank sample was injected just after a ULOQ sample.

Clinical Validation

Sample Qualification

The visual inspection of samples showed that >99.5% spots from all DBS cards were valid.

Sample Analysis

The original plasma and DBS samples were analyzed and results were reported according to the established procedures. All ISR results were within specifications, as shown in Figure 3.

Statistical Analysis: Correlation Between Plasma and DBS From Finger-Prick

An evaluation of the bridging study (N = 18) showed that a linear relationship between DBS and plasma concentrations was observed over the concentration range assessed. Data were distributed symmetrically along the regression line. The slope from the linear regression was 0.62 and the coefficient of correlation R^2 = 0.97 (data not shown). Similar results were obtained when the Passing–Bablok method was used, with a slope of 0.60, and a coefficient of correlation R^2 = 0.97 (Fig. 4).

The plot of the ratio of the DBS to plasma concentrations versus the plasma concentrations (Fig. 5) shows that the ratio stays constant regardless of the plasma concentration of teriflunomide. The ratio of DBS to plasma concentrations was shown to be constant over time (Fig. 6).

A blood to plasma ratio of 0.59 with 90% CI (0.58, 0.60) was estimated from the statistical model and is close to the blood to plasma ratio of 0.53 determined in a clinical study in healthy subjects using [^{14}C]-teriflunomide (data on file).

The correlation between DBS from finger-prick and plasma concentrations also shows that there is no difference between peripheral and venous site. Moreover, the absence of anticoagulant (ethylenediaminetetraacetic acid) in DBS from finger-prick has no effect on the quantification of teriflunomide.

DISCUSSION

To support the use of DBS methodology in the monitoring of teriflunomide concentrations, analytical and clinical validations according to standard acceptance criteria were performed.

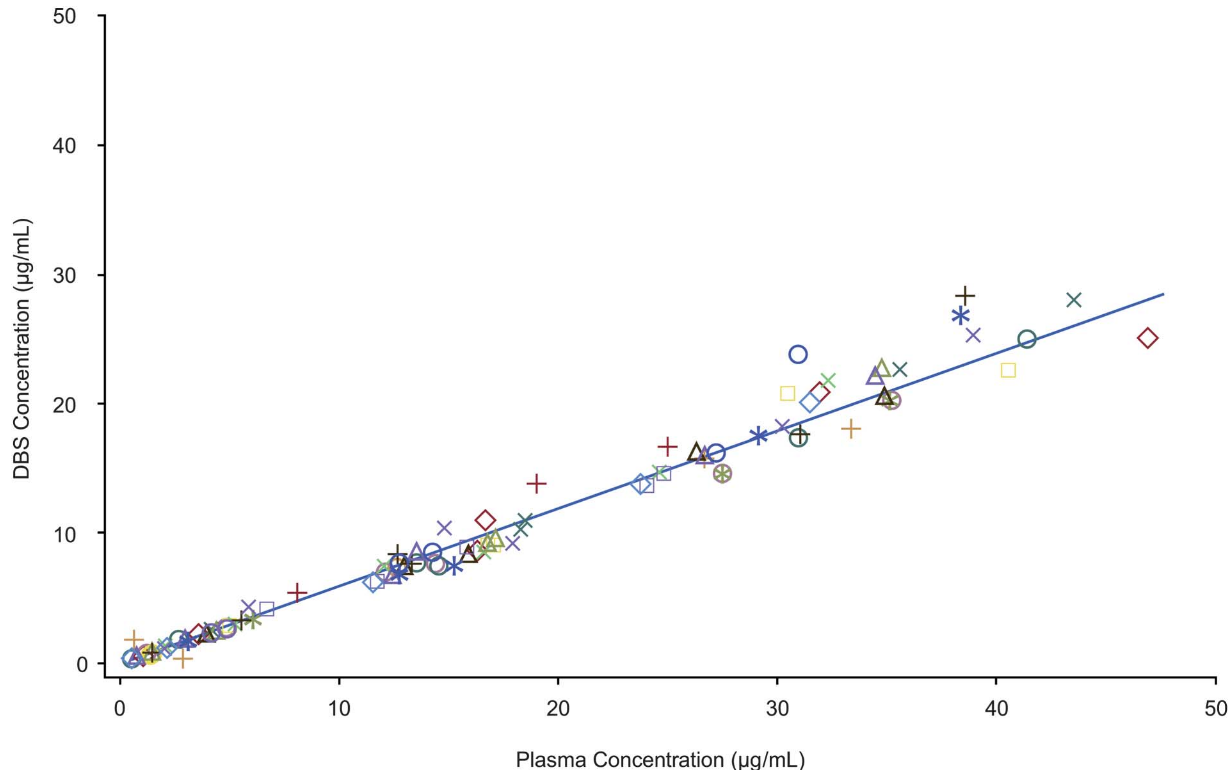

FIGURE 4. Passing–Bablok relationship obtained from plotting plasma and DBS teriflunomide concentrations. Each symbol represents 1 subject. All data of the same subject are represented with the same symbol. Passing–Bablok regression: R^2 = 0.97, slope 0.60 [95% CI 0.58–0.61], intercept −0.04 (95% CI −0.15 to 0.01).

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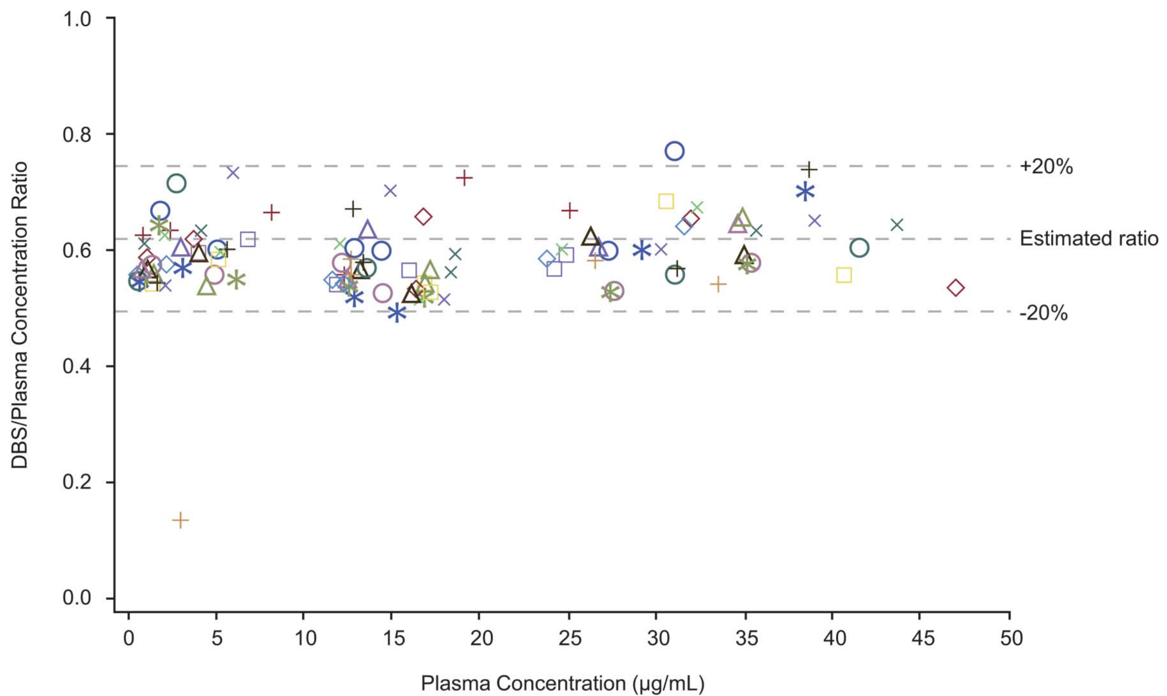

FIGURE 5. Diagnostic plot comparing ratio of DBS to plasma concentration versus plasma concentration. Each symbol represents 1 subject. All data of the same subject are represented with the same symbol. One ratio of 2.77 has not been shown for plasma concentration value of 0.67 mcg/mL.

For the analytical validation, spotting 25 μL of blood onto the filter paper allowed a partial disc of 6 mm in diameter to be easily punched for the quantitation. A 6-mm punch maximized the precision of the method compared with a smaller punch diameter by reducing the variability caused by the HCT level, the volume of blood deposited, and the position of the punch within the spot.[41,42]

Blood volume deposited onto filter paper and position of punch had no effect on the quantification of the test compound. In addition, HCT value within the interval (30%–55%) had

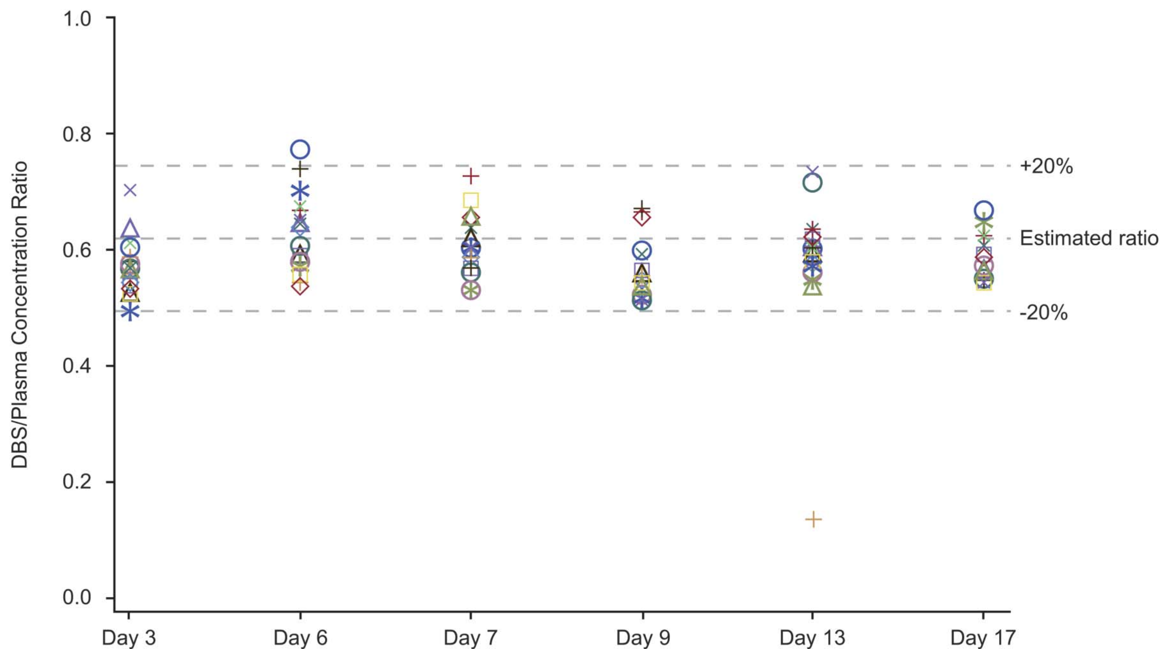

FIGURE 6. DBS to plasma teriflunomide concentration over time. Each symbol represents 1 subject. All data of the same subject are represented with the same symbol. One ratio of 2.77 has not been shown for day 17.

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a limited but acceptable effect on accuracy of the measurements. This interval covers the median HCT levels seen in patients with MS.[43]

Blood samples with teriflunomide collected onto filter paper were stable in long-term storage, for at least 4 months at room temperature, in processed samples at room temperature for at least 3 days, and for 24 hours at 37°C, with and without humidity. Teriflunomide was also stable in liquid blood, after venous sampling, for at least 24 hours. In combination, these stability tests covered the anticipated sampling, drying, and transport conditions of the samples.

Valid spots from finger-prick sampling were ensured with face-to-face practical staff training and sample QC.

Incurred specimen reproducibility was confirmed for DBS and plasma samples, confirming that the DBS method is as repeatable and reproducible as was the reference method validated in plasma.

Clinical validation in 18 healthy subjects showed that the blood to plasma ratio of teriflunomide concentration stayed constant over time, regardless of the plasma concentration of teriflunomide.

CONCLUSION

DBS sampling is a simple and practical method for monitoring teriflunomide concentrations, obviating the need for a phlebotomist, the centrifugation in plasma preparation, and the refrigerated storage requirements for the transport of samples, and therefore greatly facilitating drug monitoring irrespective of the availability of laboratory facilities or location of the patient. Although further studies in patients and in a larger clinical trial are needed, these initial results suggest that DBS sampling could be used for the determination of teriflunomide in all pharmacokinetic evaluations instead of the standard plasma assay.

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