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Research Article

A novel UPLC–MS/MS method for the estimation of an immunosuppressant in human whole blood

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ABSTRACT

To facilitate estimation of Ciclosporine (CsA), an immunosuppressant, in whole blood sample, ultra-performance liquid chromatography–mass spectrometric method employing positive electrospray ionization was developed for the determination of CsA concentration in human whole blood. CsA together with the internal standard (Ciclosporine D) was extracted from 250µL of human whole blood by protein precipitation followed by solid phase extraction. The chromatography was performed using C18, sub 2µ column with a mobile phase consisting of methanol and 0.1% ammonia solution. Multiple reaction monitoring mode was used for the quantification of CsA in whole blood. The validation of the method including sensitivity, linearity, reproducibility and stability was examined. The lower limit of quantification (LLOQ) of the developed assay method for CsA was 10ng/mL and the linear calibration curve was acquired with r > 0.99 between 10 and 2000 ng/ml. The intra-day and inter-day variation of the current assay was evaluated and the coefficient of variation were found within 6.4% at LLOQ and 6.8% for other quality control samples, whereas the mean accuracy ranged from 92.3% to 108.6%. The present method provides a robust, rapid and sensitive analytical tool for Ciclosporine in human whole blood.

Keywords: Ciclosporine A; Human whole blood; Immunosuppressant; UPLC-MS/MS

INTRODUCTION

Ciclosporine (CsA) is a cyclic polypeptide immunosuppressant, consisting of 11 amino acids. It is a potent immunosuppressive agent which prevents rejection in transplants. The exact mechanism of action of CsA is not known, suggests that the effectiveness of cyclosporine is due to specific and reversible inhibition of immunocompetent lymphocytes in the G0- or G1-phase of the cell cycle. Ciclosporine is highly lipophilic and in whole blood it is bound to blood cells and plasma proteins (Lindholm, 1991). It has a narrow therapeutic index and very high pharmacokinetic variability between patients; hence the dose needs to be individualized in patients during the treatment (Jorga et al., 2004).

Various methods have been published for the estimation of CsA in biological fluids like saliva, plasma and whole blood. Previous reported method include analysis by radio immunoassay (Kulkarni et al., 1999) and HPLC (Gulati, 1998), protein precipitation followed by liquid chromatography mass spectrometry (LC-MS/MS) detection [Koster et al., 2009; Bogusz et al., 2007], protein precipitation followed by C18 solid phase extraction and liquid chromatography mass spectrometry (LC-MS/MS) detection (Paul, 2005; Mendoza et al., 2004), or estimation in saliva using C18 cartridge solid phase extraction with LCMS/MS detection (Mendoza et al., 2004). The estimation of ciclosporine in biological

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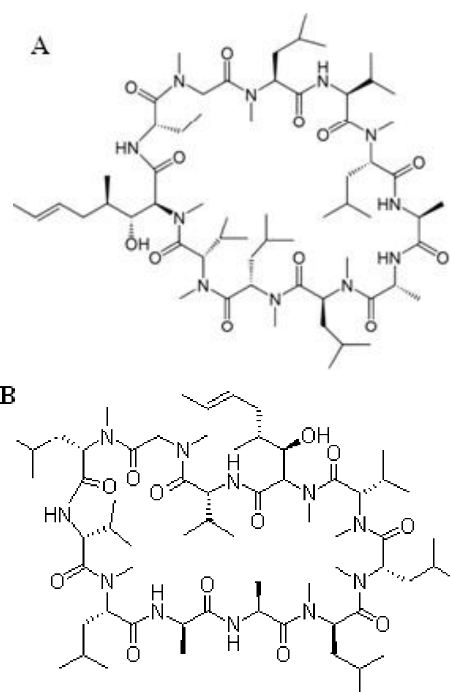

Figure 1: Chemical structures of (A) Ciclosporine and (B) Ciclosporine D

fluids is to be done by LC-MS/MS, which is much sensitive and specific compared to other techniques as described previously. In addition, a sample preparation technique using solid phase extraction gives a cleaner sample with no significant matrix effect compared to simple protein precipitation techniques reported earlier. The advanced development in solid phase extraction field which uses polymer cartridges gives consistent and reproducible results compared to the silica based C18 cartridges used as per the earlier reported methods. The aim of the present study was to develop and validate a sufficiently sensitive, precise, accurate, reproducible and fast LC–MS/MS multiple reaction monitoring (MRM) method for the quantification of CsA in human whole blood which suits the requirement of high throughput and consistent sample analysis for pharmacokinetic or therapeutic monitoring study.

MATERIALS AND METHODS

Chemicals

CsA (Fig 1) was purchased from Concord Biotech Ltd, India and Ciclosporine D (Fig 1) from Splendid Labs, India. Methanol (LCMS grade) was purchased from JT Baker, Ammonia (puriss grade) was purchased from Sigma Aldrich, Zinc sulfate heptahydrate from Fluka, Purified water obtained from Milli Q system was used throughout the experiment. All other reagents were of analytical reagent grade.

Instrument and conditions

LC-MS/MS analysis was performed using Waters Acquity UPLC, coupled with Waters Quattro Premier XE tandem quadruple mass spectrometer (Waters, USA). All data were acquired employing MassLynx quantitative analysis data processing software.

The chromatographic separation was performed on Acquity BEH C18, UPLC column (50 x 2.1 mm, 1.7 µm). The mobile phase was a mixture of methanol and 0.1% Ammonia solution pumped in gradient mode at a flow rate of 0.3 mL/min. The column temperature was maintained at 40°C.

The detection was operated in electrospray positive ionization using multiple reaction monitoring mode and the transitions of m/z 1219.8→ 1202.9 for CsA and 1233.9 →1216.8 for internal standard were monitored.

Standard Solutions

Stock Solution (1mg/mL) of CsA was made in methanol. Separate stock solutions were prepared for calibration standard and quality controls. Further intermediate solutions were prepared by serial dilutions of stock solution in diluent (50% methanol in water). Working solutions for calibration and quality control samples were prepared by diluting these intermediate solutions with diluent. The calibration standard and quality control whole blood samples were prepared by addition of working solutions to drug-free whole blood in volumes not exceeding 5% of the blood volume. The solution of internal standard were obtained by dissolving Ciclosporine D(CsD) in methanol to a concentration of 1mg/mL and further diluting this solution to a concentration of 8 µg/mL with diluent.

Preparation of Samples

The whole blood samples were stored at -20°C. The samples were thawed at room temperature before processing. 250 µL of whole blood sample were aliquoted to 2ml polypropylene tube, 10 µL of internal standard solution was added and vortexed for 10 secs. The sample was then treated with 0.3M zinc sulfate solution followed by methanol, vortexed, centrifuged and supernatant diluted with water. The pretreated samples were then subjected to solid phase extraction (SPE) using Optima UX (from MDI devices, India) 30 mg/1 ml SPE tubes. The sample supernatant was added to the preconditioned cartridge, further washed with water followed by 50% methanol, heptane and finally eluted with 90% heptane in isopropyl alcohol. The eluted samples were dried, reconstituted with methanol and transferred to UPLC vials and a 2 µL aliquot was injected into the chromatographic system.

Calibration curves

The calibration curve was constructed in the range 10 to 2000 ng/mL of CsA. The concentration of calibration samples for CsA are 10, 20, 100, 300, 900, 1300, 1600 and 2000 ng/mL. The calibration curves were obtained by weighted linear regression (weighing factor 1/x): the peak area ratio (analyte/IS) was plotted vs the analyte concentrations. The suitability of calibration model was confirmed by back-calculating the concentration of the calibration standards.

Method Validation

The method validation was performed as per the guidelines for Bioanalytical Method Validation of Food and Drug Administration (CDER, May 2001). Full validation is performed in human whole blood. Whole blood was spiked with standard solutions to obtain a calibration curve with 8 different concentrations. The main QC concentrations quantified were 30 (low), 800(medium), 1680(high) ng/ml and 10ng/ml as LLOQ (Limit of quantification).

Specificity of the method was assessed by comparing the chromatograms of six different lots of blank human whole blood with those of corresponding spiked whole blood sample at LLOQ level. Linearity was assessed by the correlation coefficient(r) and relative error of calibration standard samples. Intra- and inter-day precision and accuracy were determined by analyzing six replicates of each QC level on 3 separate days, and on each day samples were analyzed together with an independently prepared calibration curve. The precision and accuracy were expressed as coefficient of variation (%CV) and %assay, respectively. The LLOQ was defined as the lowest concentration at which both precision and accuracy were less than or equal to 20% and signal

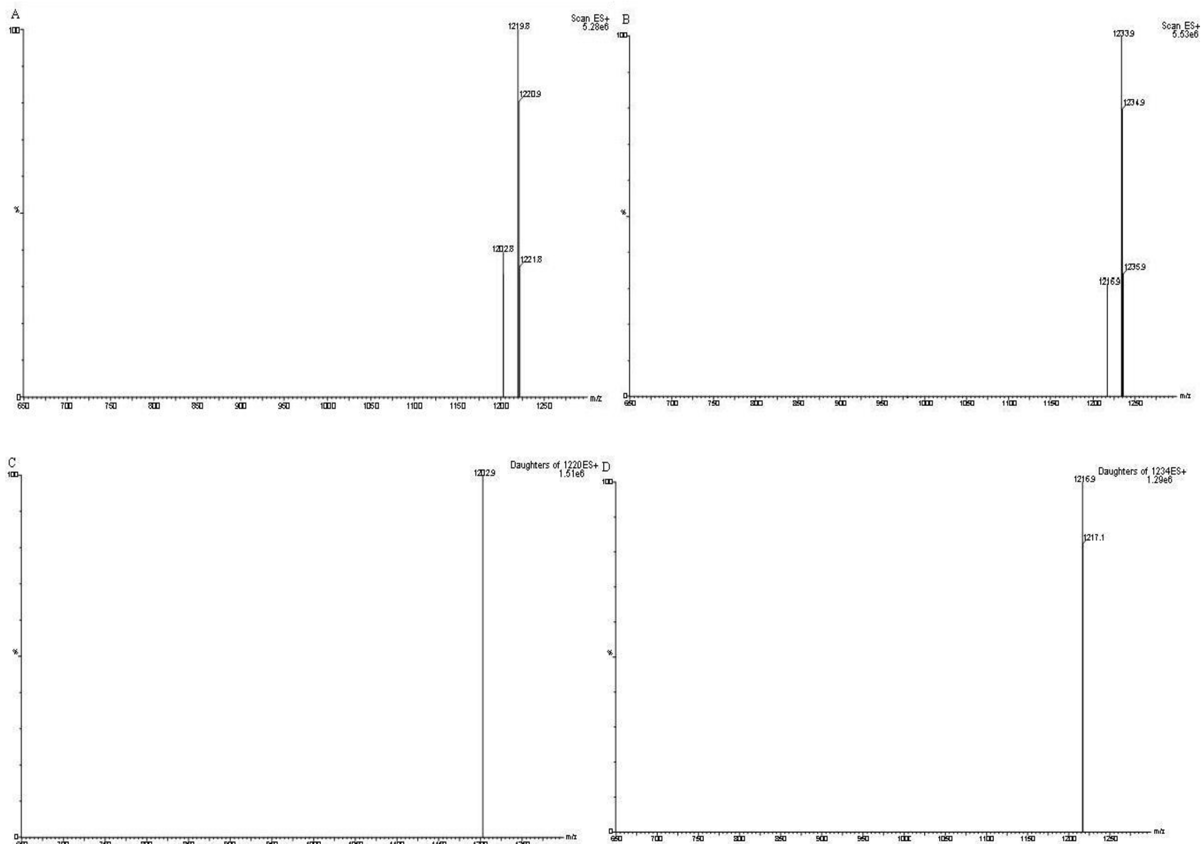

Figure 2: Mass spectrum of Ciclosporine and IS: (A) full scan spectrum of Ciclosporine; (B) full scan spectrum of IS (C) product ion spectrum of Ciclosporine; (D) product ion spectrum of IS

to noise was >5. The extraction recovery of CsA was determined at three QC levels (n = 6) by comparing the peak areas of CsA in the extracted QC samples with corresponding neat solutions of CsA. Matrix factor was evaluated by determining the precision of peak response ratio of CsA to CsD at two concentration levels (low QC and high QC) in six different lots of matrix. The matrix factor was determined by comparing the response ratio of the post spiked samples to the neat solution with similar concentration and IS normalized matrix effect reported as (matrix factor-1)*100. Stability of CsA was evaluated in QC samples at room temperature after storing for 5 h, after three freeze–thaw cycles and after 30 days at -20°C. Stability of CsA in processed samples stored in auto sampler at 5°C for > 72 h was also assessed. The sample stability in dried extract form was also analysed after storing at -20°C for >72h.

RESULTS AND DISCUSSION

Selection of Internal Standard

CsD was selected as internal standard for the assay as it is well separated from the analyte, it showed similar ionization efficiency and adequate, reproducible and similar extraction recovery from whole blood employing the sample pretreatment protocol described above. In addition, it is not an endogenously occurring substance.

Method development and optimization of MS/MS conditions

In order to develop the ESI MS/MS method for the determination of CsA, the full scan precursor and product ion spectra of the analyte (CsA) and the IS were acquired in positive ion mode by infusing each compound separately at a concentration of 1 µg/mL in Methanol: water (50:50) at a flow rate of 10µL/ min. The scan gave rise to predominant ammonium adduct peak at m/z 1219.8 and 1233.9 respectively for CsA and IS (Fig 2). Following detailed optimization procedure for fragmentation of the parent molecule, product ion spectra was obtained for CsA and IS (Fig. 2). The results showed that the most sensitive mass transition was m/z 1219.8→ 1202.9 for CsA and m/z 1233.9 →1216.8 for the internal standard. These deaminated product ions were used for quantification.

Liquid chromatographic conditions

The chromatographic conditions were optimized for the rapid and efficient separation of CsA and IS from whole blood components. For this reason, a C18 stationary phase and methanol as the organic modifier of the mobile phase were chosen, over C8 and acetonitrile respectively, as this combination gave the best results in terms of peak shape (width, symmetry, sharpness). Further optimization was performed by using high pH 0.1% ammonia solution in the mobile phase and increasing the column temperature, which

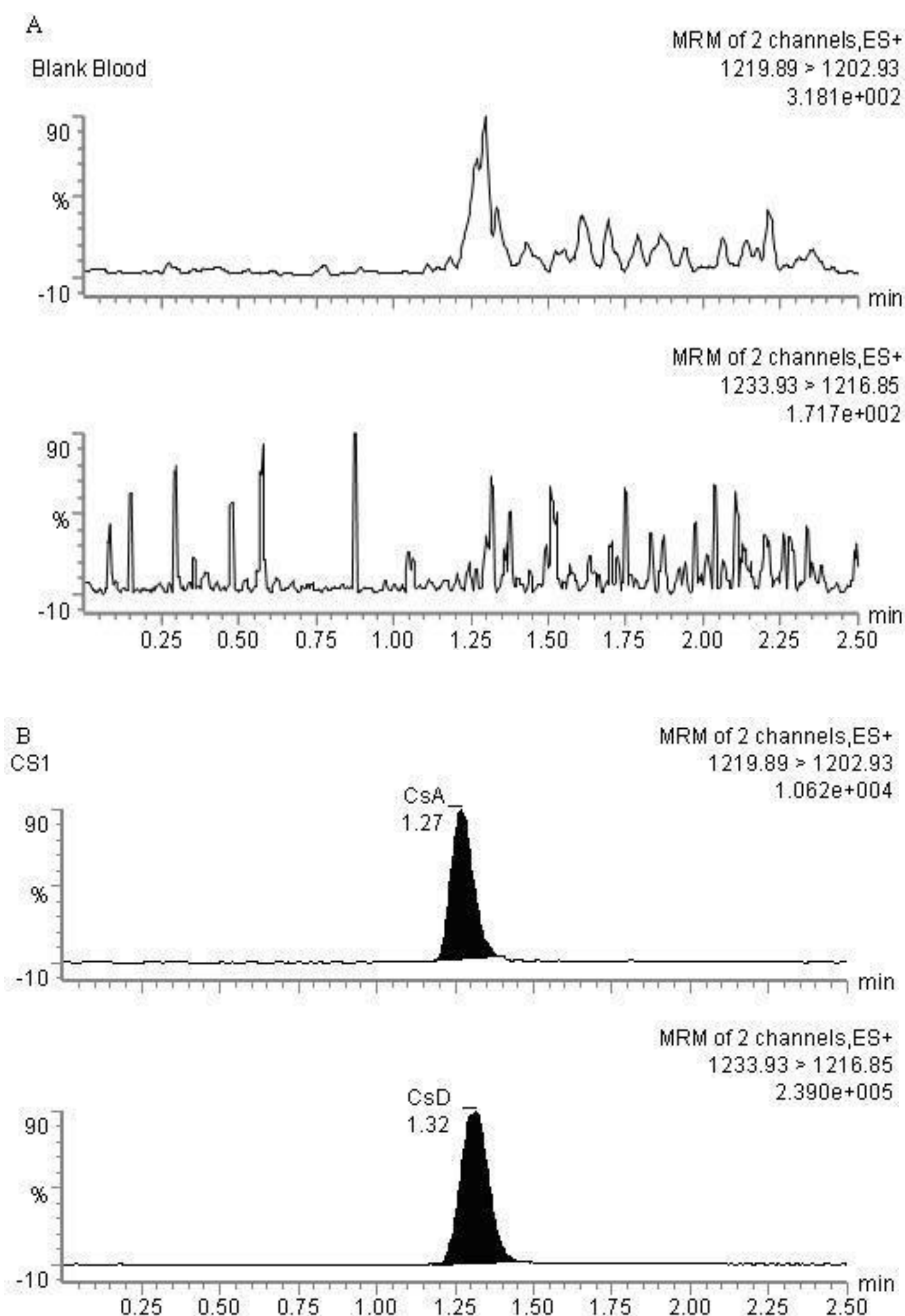

Figure 3: Representative MRM chromatograms of Ciclosporine and IS in human whole blood: (A) blank whole blood; (B) whole blood sample spiked with Ciclosporine (at LLOQ conc) and IS

improved even more the peak shape of the analyte, accelerated analysis time and enhanced ionization efficiency. Finally, under the optimized chromatographic conditions CsA eluted at 1.27 min and CsD (IS) at 1.32 min, respectively, as shown in Fig. 3.

Sample Preparation

The solid phase extraction of whole blood samples with Optima UX 30mg/1ml (from MDI) cartridge was selected over liquid–liquid and simple protein precipitation for efficient sample clean-up. It was found that the protein precipitation by zinc sulphate and methanol was required for the deproteination as well as to free the bound drug and further matrix clean up by SPE provided a consistent recovery of CsA and no interferences from whole blood matrix. Furthermore, due to its simplicity and speed, this sample pretreatment protocol can be considered compatible with high throughput analysis.

Method Validation

Selectivity

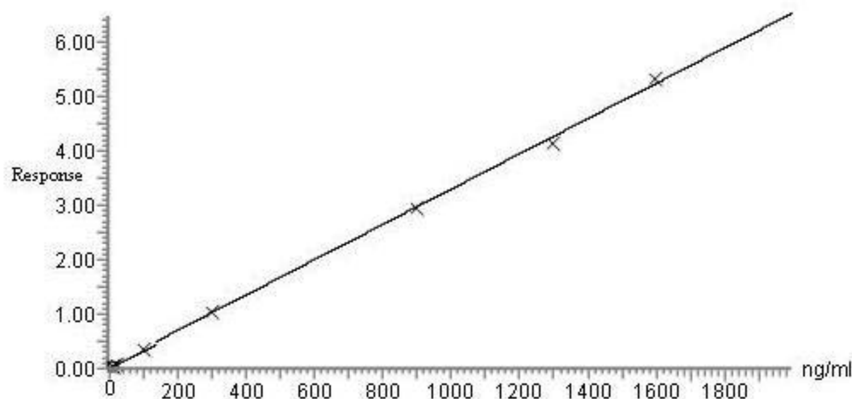

Figure 4: Representative calibration curve in human whole blood (10-2000ng/ml)

Table 1: Intra-day and Inter-day mean, standard deviation, accuracy and precision for Ciclosporine

Nominal Conc. ng/ml	Intra- day (n=6)				Inter-day (n=18)			
	Mean ng/ml	SD* ng/ml	% Accuracy	Precision (%CV)	Mean ng/ml	SD* ng/ml	% Accuracy	Precision (%CV)
10	9.555	0.61	95.8	6.4	9.644	1.24	96.7	12.9
30	28.646	1.94	95.7	6.8	27.625	1.44	92.3	5.2
800	877.897	42.0	110.0	4.8	870.304	36.8	108.6	4.2
1680	1784.545	117.0	106.5	6.6	1702.233	106.7	101.6	6.2

* SD – standard deviation

Table 2: Stability of Ciclosporine under different conditions (n=6)

Concentration	Room Temp 4hr	Freeze thaw 3 cycles (-20°C)	Auto sampler (5°) 72hr	Dry extract (-20°C) 72hr	Long term 90days (-20°C)
30 ng/ml	97.8%	103.6%	91.1%	94.8%	108.9%
1680 ng/ml	107.6%	107.4%	100.5%	107.2%	102.2%

The calibration standard in whole blood analyzed by the developed analytical methodology showed that the retention time (RT) of CsA and IS was 1.27 min and 1.30 min, respectively. All batches of blank drug-free human whole blood analyzed under the same conditions were found to be free of interference peaks from endogenous whole blood substances at the RT of the analyte and the IS, demonstrating the selectivity of the assay. Representative chromatograms of blank human whole blood and spiked blank whole blood with CsA at the LLOQ are shown in Fig. 3a and b, respectively.

Calibration curve and LLOQ

Calibration curves were constructed by plotting the peak area ratios of CsA to IS of whole blood calibration standards versus nominal concentrations of the analyte. The calibration model was selected based on the analysis of the data by linear and non-linear regression as well as with and without weighting. Calibration curves of five different lots of whole blood calibration standards were constructed over the concentration range 10–2000 ng/mL. The best linear fit and least square residuals for the calibration curves were achieved with 1/x weighting factor, giving a mean linear regression equation for calibration curve : $y = 0.003241x + 0.003663$ where y is the peak ratio of the analyte to IS and x is the concentration of the analyte. The correlation coefficient (r) of the calibration curve was > 0.999, indicating good correlation (Fig. 4). The lower limit of quantification with the proposed method was 10 ng/mL (Table 1) with signal to noise ratio (S/N) > 5. The mean accuracy and %CV (precision) for LLOQ were 95.8% and 6.4% respectively.

Intra and Inter-day Precision and Accuracy

The accuracy and precision data for intra- and inter-day whole blood samples are presented in Table 1. The assay values for both occasions (intra- and inter-day) were found to be within the accepted variable limits. The data indicated that the present method has a satisfactory accuracy, precision and reproducibility.

Recovery

The extraction recovery data (%CV) of Ciclosporine from human whole blood was calculated by analyzing six replicates at 30, 800, and 1680.0 ng/mL together

with the recovery of the IS at the concentration of 8µg/mL. The extraction recoveries of assay were 48.1, 46.2, 54.5% respectively and 48.5% for IS. The data showed that the recovery is almost quantitative for both Ciclosporine and IS.

Matrix effect

To estimate the matrix effect blank blood samples from six different healthy volunteers were processed in accordance with the assay method. The reconstituted extracts were spiked with CsA at low (30ng/mL) and high (1680ng/mL) QC concentrations and with IS at working concentration and injected into the LC-MS/MS system. The precision of peak area ratio(CsA/CsD) across six lots of whole blood was determined.

The %CV values are < 15% for response ratio at low and high QC levels in all the six matrix lots and mean matrix effect of 4% was observed for LQC and HQC which shows that method is efficient enough to clean up the sample.

Stability

Samples at low and high QC concentrations were analysed in six replicates for studying the possible conditions to which the samples might be exposed during storage and handling. The results of the investigated stability parameters (Table 2) showed that stock solutions in methanol were stable for 15days at -20°C and the spiked whole blood sample stored at -20°C for long term duration found to be stable up to 90days. Whole blood samples were stable for 5 h at room temperature and for 32 days at −20°C. However, study samples may require re-analysis, which results in extra freeze/thaw cycles. Stability of CsA in whole blood is adequate during three freeze (−20 ◦C)/thaw cycles. The treated whole blood samples were stable for 72h at auto sampler temperature (5°C). The dry extract of the processed sample also found to be stable for 72h at -20°C. All the stability experiment results were found to be within the assay variability limits.

CONCLUSION

The described UPLC–MS/MS validated analytical methodology enables rapid and selective assay of Ciclosporine in human whole blood. The proposed method presents high sensitivity, accuracy, precision, recovery and stability combined with high accuracy mass measurement, thus being suitable for monitoring of pharmacokinetic, bioavailability and bioequivalence studies. The simplicity of the sample pretreatment and the rapid analysis time could render this method as an ideal high-throughput method for the efficient analysis of a large number of clinical samples.

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