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Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry☆

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ABSTRACT

Cyclosporin A (CsA) and tacrolimus are immunosuppressant drugs principally used in solid organ transplant recipients. Therapeutic drug monitoring (TDM) of both drugs is essential to avoid toxicity related to overdosage, and transplant rejection from underdosage. This necessitates frequent hospital visits to phlebotomy services. Capillary blood sampling onto dried blood spots (DBS) provides numerous advantages to venous whole blood sampling, including the ability for patients to send DBS to the laboratory by post, significantly reducing the number of unnecessary hospital visits. We have developed a novel, simple and rapid method for the extraction and simultaneous UPLC–MS/MS measurement of both CsA and tacrolimus from DBS. The extraction method involved a simple 30 min hot solvent extraction with ultrasonication. Extract (10 μL) was injected onto a Waters Acquity UPLC column filter unit security frit, coupled to a Waters Acquity BEH C18 UPLC column, with methanolic mobile phase gradient elution. Eluant was connected to a Waters Quattro Premier XE tandem mass spectrometer operating in ES+ mode. We detected multiple reaction monitoring (MRM) transitions of m/z 1220 > 1203 and 1231.9 > 1215.1 for CsA and d12 CsA respectively which co-eluted at 1.30 min, and 821.6 > 768.5 and 809.6 > 756.5 for tacrolimus and ascomycin respectively which co-eluted at 1.17 min. Ion suppression was negligible. Mean recovery was 95.5% for CsA and 92.8% for tacrolimus. Limit of detection and limit of quantitation were both 8.5 μg/L for CsA, and 0.5 and 2.3 μg/L respectively for tacrolimus. The assay was linear up to 1500 μg/L for CsA (r^2 = 0.9999), and up to 50 μg/L for tacrolimus (r^2 = 0.9994). Mean intra assay imprecision, inter assay imprecision and bias were all <10% for both CsA and tacrolimus. DBS were stable for at least 14 days at room temperature. Comparison of the DBS UPLC–MS/MS method and the routine venous whole blood LC–MS/MS assay demonstrated good agreement between the two methods for both drugs. We have developed a simple and robust method for the extraction and simultaneous measurement of CsA and tacrolimus from DBS. The method will allow TDM of transplant recipients to proceed at home using capillary blood sampling.

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1. Introduction

Cyclosporin A (CsA) and tacrolimus are powerful immunosuppressant drugs which act by inhibiting proliferation of T-lymphocytes. Their direct effect is via inhibition of calcineurin and subsequent impairment of T-cell receptor transcription of the interleukin-2 (IL-2) gene. Although principally used in solid organ transplant recipients to prevent organ rejection, due to their immunomodulatory effects, both drugs are being increasingly used for the management of other autoimmune conditions such as psoriasis and rheumatoid arthritis [1].

Due to their variable pharmacokinetics and narrow therapeutic ranges, therapeutic drug monitoring (TDM) of both drugs is essential [2,3]. Nephrotoxicity is a significant consequence of CsA and tacrolimus overdosage, and transplant rejection can result from underdosage. Frequent monitoring of whole blood drug concentrations necessitates regular patient visits to hospital phlebotomy services, particularly in the first few months following transplantation. This is not an ideal scenario for immunocompromised patients.

CsA and tacrolimus are routinely measured in venous whole blood samples collected by venepuncture. Clinically, capillary blood sampling using dried blood spots (DBS) has become commonplace for the analysis of a number of markers of inherited metabolic disorders in newborn screening. Capillary blood sampling using DBS

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offers several advantages over venous blood sampling including; (1) DBS are amenable to small sample sizes, (2) capillary blood sample collection at home allows patients to post DBS cards to the laboratory for analysis thus reducing frequency of hospital visits, (3) DBS can be sent to the laboratory prior to a hospital clinic consultation allowing more efficient patient–physician contact time, (4) DBS are advantageous for CsA C_2 serum monitoring [4,5], and (5) many analytes exhibit enhanced stability when dried on the blood spot card compared to stability in venous blood [6].

To date, three reports detailing liquid chromatography tandem mass spectrometry (LC–MS/MS) methodologies for the analysis of CsA or tacrolimus from DBS have been published in the literature [5,7,8]. No previous reports have detailed simultaneous recovery of both CsA and tacrolimus from DBS using the same extraction conditions, allowing measurement of both drugs on the same LC–MS/MS run. Simultaneous measurement offers significant advantages for our centre which houses a major heart and lung transplant clinical department. Here, patient samples being analysed for CsA or tacrolimus can be analysed on the same analytical run, thus standardising sample preparation procedures and reducing analysis time.

Herein we report a novel, simple and rapid technique for the simultaneous extraction of CsA and tacrolimus from DBS, and their measurement by UPLC–MS/MS. We believe this method would be suitable for routine clinical assay of finger prick capillary blood samples self obtained by patients in a community setting.

2. Experimental

2.1. Materials

HPLC grade water, HPLC grade methanol and HPLC grade acetonitrile, ammonium acetate, and ascomycin were purchased from Sigma (Poole, Dorset, UK). Formic acid (AnalaR grade) was purchased from VWR International (Leicestershire, UK). CsA was obtained from Novartis (Camberley, UK) and tacrolimus from Astellas (Staines, UK). d12 CsA was purchased from the International Proficiency Testing Scheme (Tooting, UK). In vitro diagnostic 903 grade blood spot cards were purchased from Whatman (GE Healthcare). Polypropylene 2 mL 96-deep well plates, and thermo seal silver foil plate sealers were purchased from Thermo Scientific (Surrey, UK).

2.2. Calibration standards and quality control material

Commercially available calibrators could not be used in the DBS assay as the matrix in these materials is significantly different to whole blood to alter flow dynamics on the DBS card. This causes greater spreading on the card and hence dilution of sample. For this reason, calibration standards and quality controls (QCs) were prepared by spiking whole blood with pure CsA and tacrolimus. Anonymised whole blood samples from ten patients not receiving either drug were pooled and used to prepare CsA and tacrolimus standards and QCs. Briefly, stock solutions of CsA and tacrolimus were prepared by dissolving pure drug in methanol to yield concentrations of 10 g/L and 25 g/L respectively. These stock solutions were serially diluted in 50:50 (v/v) methanol:water to yield final concentrations of 300 mg/L and 10 mg/L of CsA and tacrolimus respectively. A high concentration calibrant containing 1500 μg/L and 50 μg/L of CsA and tacrolimus, respectively, was prepared by addition of 25 μL of each diluted stock solution into 4.95 mL of pooled whole blood. A range of calibrators and QCs were prepared by dilution of this highest concentration calibrator with further pooled blood sample containing neither drug. Final CsA calibrant concentrations were 0, 75, 150, 450, 750, 1200 and 1500 μg/L, and quality control (QC) concentrations 120, 300 and 900 μg/L. Final tacrolimus calibrant concentrations were 0, 2.5, 5, 15, 25, 40 and 50 μg/L, and QC concentrations 4, 10 and 30 μg/L.

2.3. Sample preparation

Standards, QCs and patient samples (25 μL) were spotted onto Whatman 903 DBS cards and left to dry at room temperature for a minimum of 3 h. Discs of 6 mm diameter were punched from the DBS using a stationery paper hole punch. Discs were placed into individual wells of a 96 well 2 mL deep-well polypropylene block. Methanol (250 μL) was added to each well, followed by 25 μL of internal standard solution comprising 50 μg/L of ascomycin and 200 μg/L of d12 CsA in acetonitrile. The plate was thermo sealed to prevent evaporation from the plate, and incubated in an ultrasonic water bath heated to 80 °C for 30 min.

2.4. Liquid chromatography

Chromatography was performed on a Waters® Acquity™ UPLC system. Extracted sample (10 μL) was injected directly from the 96-deep well plate onto a Waters Acquity UPLC column filter unit security frit, 0.2 μm, 2.1 mm, coupled to a Waters Acquity UPLC BEH C18, 2.1 mm × 50 mm, 1.7 μm column (Waters, Hertfordshire, UK).

Mobile phase A contained 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid in water. Mobile phase B contained 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid in methanol. Initial conditions were 50:50 (v/v) mobile phase A:B. Following sample injection, elution was performed by means of a gradient from 50 to 100% mobile phase B over 1 min, followed by 100% mobile phase B, held for 30 s. Following this, the columns were re-equilibrated back to initial conditions and held for 1.5 min prior to the next sample injection. Mobile phase flow rate was maintained at 0.6 mL/min, and chromatography performed at 55 °C.

2.5. Tandem mass spectrometry

Eluate from the analytical column was injected directly into a Waters® Quattro Premier™ XE tandem mass spectrometer operating in the positive electrospray ionisation mode (Waters, Hertfordshire, UK). The instrument conditions were as follows: electrospray capillary voltage 1.0 kV, collision energy 20 eV and sample cone voltage 20 V for CsA/d12CsA and 28 V for tacrolimus/ascomycin. Desolvation gas flow and temperature were maintained at 620 L/h and 350 °C respectively, and the source temperature was 140 °C. CsA, d12 CsA, tacrolimus and ascomycin were all detected in multiple reaction monitoring (MRM) mode with a dwell time of 0.1 s per channel. MRM transitions were m/z 1220 > 1203 for CsA, 1231.9 > 1215.1 for d12 CsA, 821.6 > 768.5 for tacrolimus and 809.6 > 756.5 for ascomycin, these transitions have been utilised previously for whole blood LC–MS/MS methods [9,10]. The extractor voltage was 3 V and RF lens voltage 0.2 V. Resolution was 14.3 for MS1 and 13.5 for MS2, the photomultiplier energy was 645 V.

2.6. LC–MS/MS method validation

2.6.1. Ion suppression

Ion suppression experiments were performed by continuous post-column infusion of CsA or tacrolimus (1 mg/L in 50:50 mobile phase A:B) directly into the mass spectrometer via a T-piece at a flow rate of 10 μL/min. DBS were prepared from randomised whole blood samples from patients not being treated with either drug (n = 9), extracted as described, and injected, along with a methanol blank. Ion suppression or enhancement was interpreted as a fall or

Table 1
Method validation data.

CsA			Tacrolimus		
Linear range (μg/L)	Mean r^2 value		Linear range (μg/L)	Mean r^2 value	
Linearity ($n = 6$)					
0–1500	0.9999		0–50	0.9994	
CsA			**Tacrolimus**		
Target value (μg/L)	CV (%)	Bias (%)	Target value (μg/L)	CV (%)	Bias (%)
Intra assay precision ($n = 15$)					
120	5.6	2.2	4	8.8	−3.2
300	4.3	5.2	10	7.5	4.2
900	5.2	9.8	30	5.9	1.9
Inter assay precision ($n = 15$)					
120	5.2	0.1	4	11.0	3.4
300	4.1	1.7	10	4.2	2.9
900	6.8	7.5	30	7.3	3.1
CsA			**Tacrolimus**		
Target value (μg/L)	Recovery (%)		Target value (μg/L)	Recovery (%)	
Recovery ($n = 5$)					
1000	95.4		50	91.2	
500	94.1		25	97.0	
100	97.1		10	90.1	
Limit of detection					
8.5 μg/L			0.5 μg/L		
Limit of quantitation					
8.5 μg/L			2.3 μg/L		

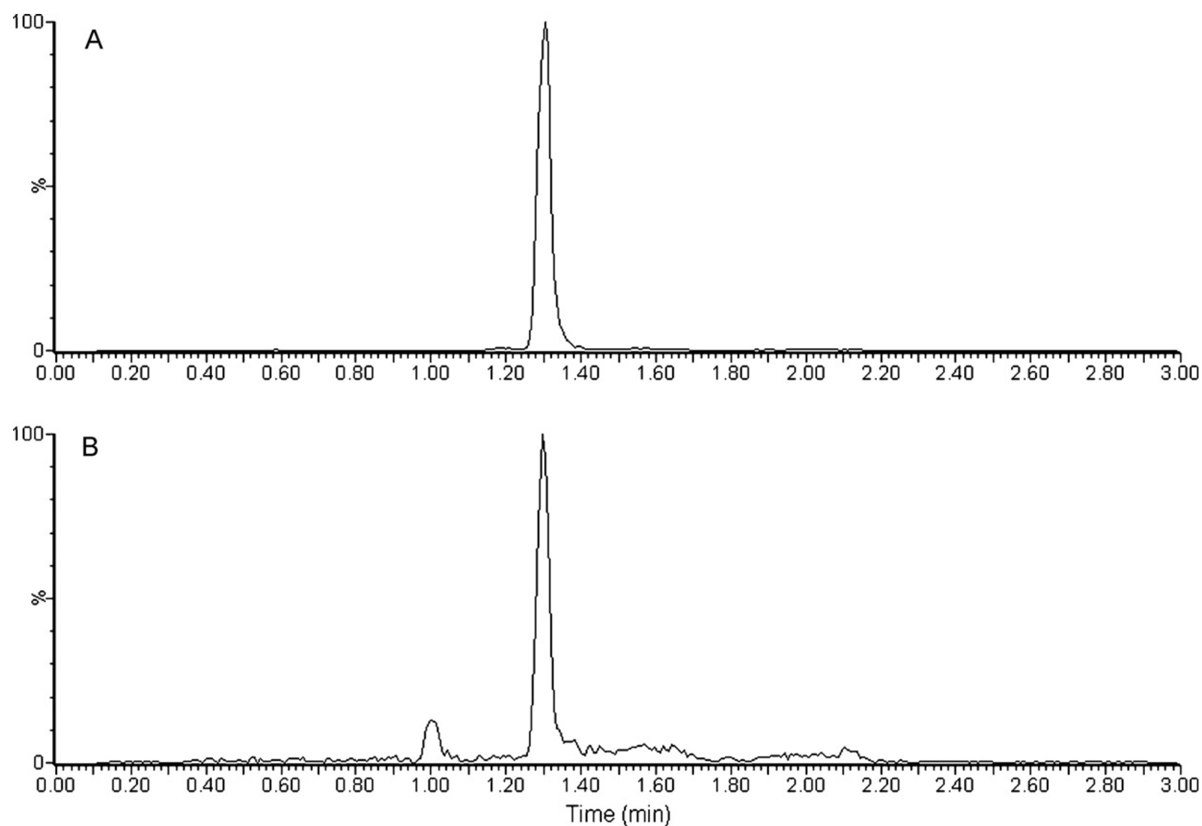

Fig. 1. Typical chromatograms for CsA and d12 CsA internal standard produced by the new LC–MS/MS method. (A) Chromatogram of a patient sample with a detector response of 3.95×10^5 cps, yielding a CsA concentration of 247.7 μg/L and (B) chromatogram of the d12 CsA internal standard with a detector response of 3.55×10^4 cps (200 μg/L). Both chromatograms illustrate negligible interference in the immediate region of elution and both CsA and d12 CsA have a similar retention time of 1.30 min.

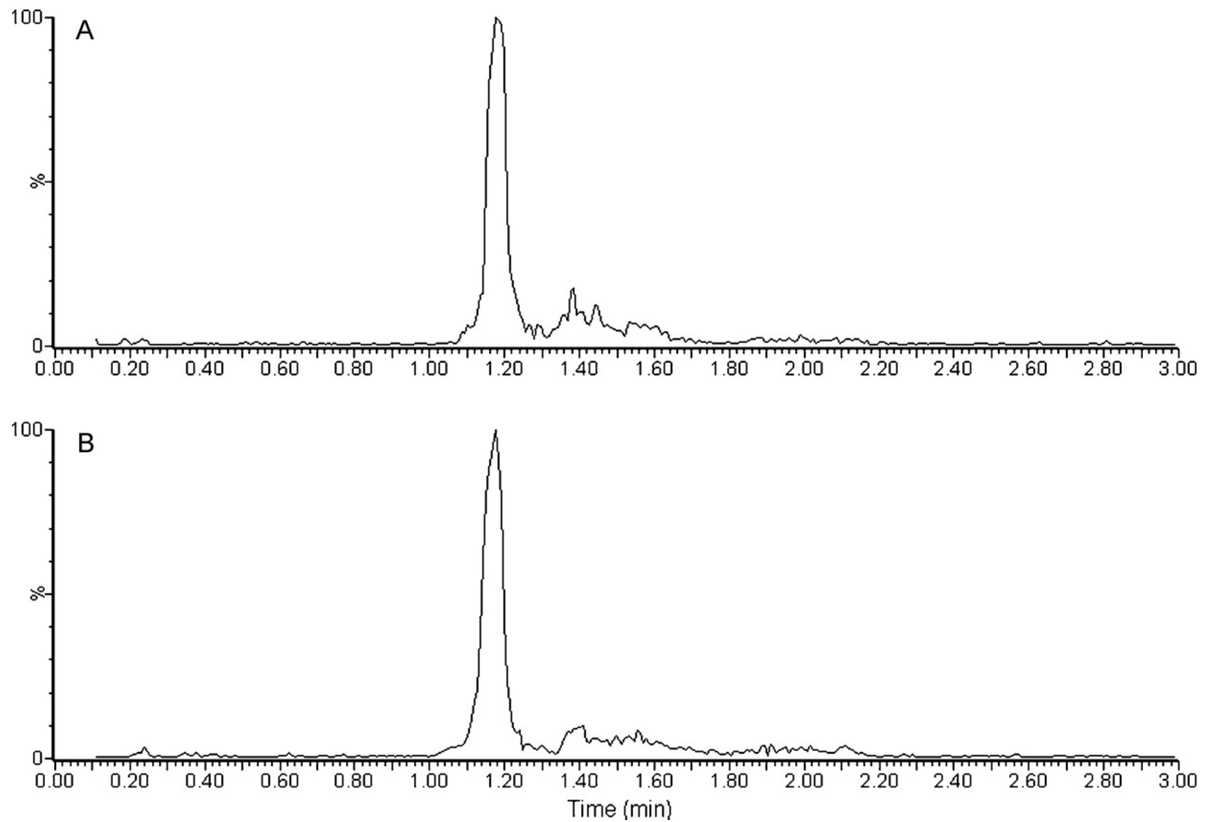

Fig. 2. Typical chromatograms for tacrolimus and ascomycin internal standard produced by the new LC–MS/MS method. (A) Chromatogram of a patient sample with a detector response of 2.25×10^4 cps, yielding a tacrolimus concentration of 12.9 μg/L and (B) chromatogram of the ascomycin internal standard with a detector response of 2.22×10^4 cps (50 μg/L). Both chromatograms illustrate negligible interference in the immediate region of elution and both tacrolimus and ascomycin have a similar retention time of 1.17 min.

increase in baseline count greater than 10% at the retention time of the analyte.

2.6.2. Linearity

Linearity of the assay was assessed by repeat ($n = 6$) analysis of calibrators, with concentrations ranging from 0 to 1500 μg/L for CsA, and 0 to 50 μg/L for tacrolimus. LC–MS/MS response was plotted against nominal concentration values using the QuanLynx™ software (Waters, Hertfordshire, UK). Linearity of the assay was confirmed by weighted linear regression with a correlation coefficient r^2 value > 0.99.

2.6.3. Recovery

Recoveries of CsA and tacrolimus were determined by comparing the amount of each drug measured before and after pooled whole blood samples were spiked with a known amount of pure CsA (100, 500 and 1000 μg/L, $n = 5$) and tacrolimus (10, 25 and 50 μg/L, $n = 5$). Spiked blood samples were used to prepare DBS and recoveries calculated as a percentage, following DBS analysis.

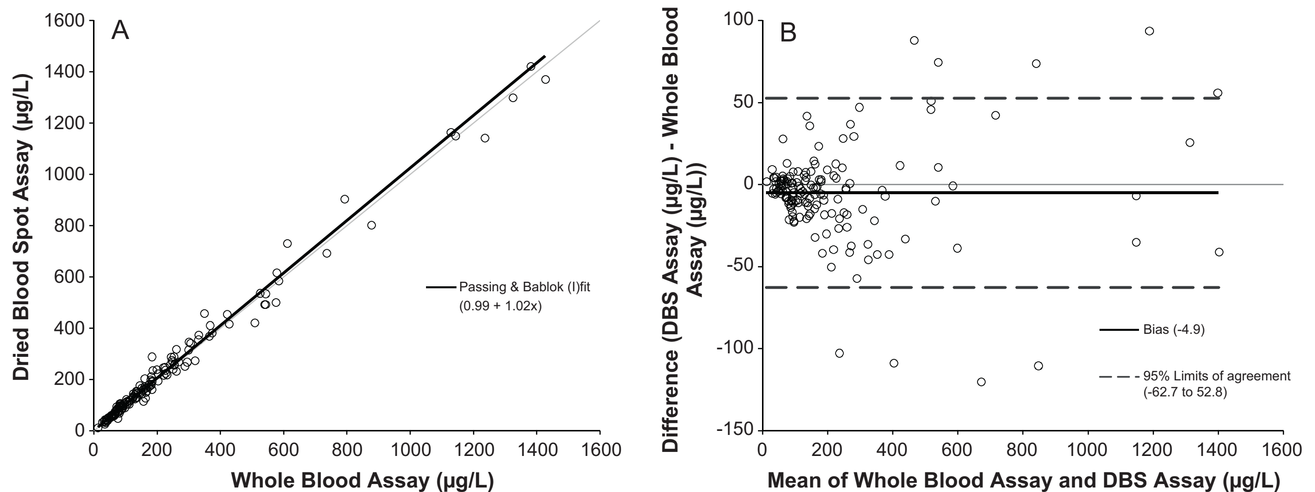

Fig. 3. Method comparison for CsA between the DBS and venous whole blood LC–MS/MS methods; (A) Passing–Bablock analysis demonstrated good correlation between methods, yielding the equation DBS = 0.99 (blood assay) + 1.02, linear regression $r^2 = 0.99$ ($n = 153$). (B) Bland–Altman plot demonstrated a mean bias of −4.9 μg/L (95% limits of agreement −62.7–52.8 μg/L).

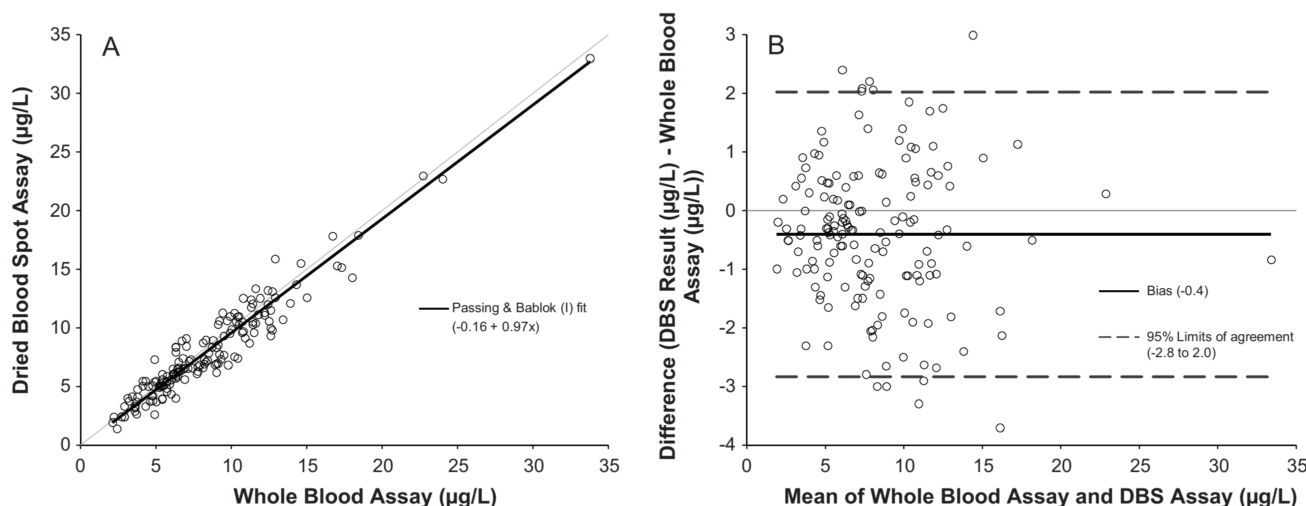

Fig. 4. Method comparison for tacrolimus between the DBS and venous whole blood LC–MS/MS methods; (A) Passing–Bablock analysis demonstrated good correlation between methods, yielding the equation DBS = −0.16 (blood assay) + 0.97, linear regression r^2 = 0.92 (n = 158). (B) Bland–Altman plot demonstrated a mean bias of −0.4 μg/L (95% limits of agreement −2.8–2.0 μg/L).

2.6.4. Precision and accuracy

Assay imprecision was assessed by the analysis of 3 QC samples with concentrations of 120, 300 and 900 μg/L of CsA, and 4, 10 and 30 μg/L of tacrolimus. Samples were analysed 15 times within a single analytical run to determine within batch precision, and analysed in separate batches (n = 15) over a period of two weeks to determine between batch precision. Precision was assessed as a function of the variation (%CV), and accuracy as a function of deviation from theoretical values. According to validation guidelines issued by the US Food and Drug Administration [11] precision and accuracy are deemed acceptable if <15%.

2.6.5. Lower limit of detection and quantitation

The lower limit of detection (LOD) was determined as the smallest detectable peak in DBS prepared from whole blood containing no CsA or tacrolimus, above baseline noise (signal:noise ratio > 5:1, peak to peak). The lower limit of quantitation (LLOQ) was determined by measuring low spiked concentrations of CsA and tacrolimus in DBS. Each concentration was measured 15 times and the %CV and deviation from the theoretical target value determined. The LLOQ was assigned to the lowest concentration with a CV < 20%, and mean value < 20% from the theoretical target value.

2.6.6. Stability

Stability of DBS was determined by preparing blood spots from 10 anonymised patients receiving CsA and tacrolimus therapy. These DBS were prepared, extracted and analysed on the day of preparation and remaining DBS stored at room temperature. The DBS were analysed again after 7 and 14 days. From the results, the mean percentage change in measured CsA and tacrolimus concentrations were calculated.

2.6.7. Dried blood spot and liquid blood assay method comparison

The DBS method was compared to the liquid blood LC–MS/MS assay in clinical use in the laboratory which is calibrated using commercially available calibrators and QCs (Chromsystems, Munich, Germany). Patient venous whole blood samples taken for CsA and tacrolimus TDM were used to prepare DBS using the method described. Concentrations determined by each method were compared using Analyse-It^{TM} statistical analysis software (Analyse-It Software Ltd., Leeds, UK).

3. Results and discussion

3.1. Sample preparation and liquid chromatography

Multiple factors were observed to impact on the efficient extraction and measurement of CsA and tacrolimus from DBS. These included the length of time the DBS were incubated in the ultrasonic water bath, the temperature of extraction and the chromatographic elution gradient conditions. Extraction and chromatographic conditions described in Sections 2.3 and 2.4 were those which yielded optimal extraction of both drugs from DBS, and accurate quantitation by UPLC–MS/MS.

The chromatographic retention time was 1.30 min for both CsA and d12 CsA, and 1.17 min for tacrolimus and ascomycin. The total run time was 3 min which is significantly shorter than the previously published method for tacrolimus measurement from DBS [8], and is an important consideration if the method is to be introduced into routine clinical practice. The resulting chromatograms demonstrated the specificity of the assay with clean elution peaks and no observed interference in the region of elution (Figs. 1 and 2). Ion suppression experiments using continuous post-column infusion of CsA or tacrolimus exhibited minimal signal interference (defined as a fall or increase in baseline count no greater than 10% at the retention time of the analyte), following the injection of extracted DBS and methanol into the mass spectrometer.

3.2. Method validation

Validation of the method was performed according to US FDA guidelines for industry [11], and validation data is summarised in Table 1. Data averaged from 6 individual calibration curves demonstrated that the assay was linear up to at least 1500 μg/L of CsA (r^2 = 0.9999; y = 0.0623x + 0.1965) and 50 μg/L (r^2 = 0.9994; y = 0.0575x − 0.0137) of tacrolimus. These are similar concentration ranges to those used in the current venous whole blood LC–MS/MS assay in routine clinical use. This also compares well to the linear ranges quoted in previously published methods for measurement of CsA (25–1440 μg/L) [7] and tacrolimus (1–30 μg/L) [8] from DBS.

Recovery from pooled whole blood spiked with three different concentrations of CsA and tacrolimus yielded a mean recovery of 95.5% (range 94.1–97.1%) for CsA and 92.8% (range 90.1–97.0%) for tacrolimus, which is within acceptable limits according to US

FDA guidelines. For tacrolimus, our recovery values from DBS are superior to those previously reported of 78% [8].

The assay exhibited acceptable levels of intra- and inter-assay precision and accuracy. For CsA, mean co-efficients of variation (CV) for intra- and inter-assay precision were 5.0% (mean bias 5.7%) and 5.4% (mean bias 3.1%) respectively. For tacrolimus mean CVs for intra- and inter-assay precision were 7.4% (mean bias 1.0%) and 7.5% (mean bias 3.1%) respectively. At the concentration ranges assessed, these values compare well to accuracy and precision data quoted by prior CsA and tacrolimus DBS LC–MS/MS methodologies [7,8].

For CsA, the LOD and LLOQ was 8.5 μg/L, for tacrolimus the LOD was 0.5 μg/L and the LLOQ was 2.3 μg/L. Our data equates to prior DBS extraction methods, which quoted an LOD of 0.26 μg/L and LLOQ of 1 μg/L for tacrolimus [8], and LLOQ of 25 μg/L for CSA [7].

Patient samples, calibrants and QCs were stable as DBS for up to 14 days at ambient room temperature, exhibiting negligible (≤10%) deterioration in measured concentration after 7 and 14 days following application and drying on the DBS. A previous study in our centre found that the average time taken for DBS to reach the laboratory through the postal system was 1 day from dispatch, with the maximum time being 5 days [4]. Hence, we believe that stability of the DBS through the postal system would not pose any significant problems. Extended stability of CsA and tacrolimus has also been demonstrated previously on DBS stored at ambient temperature [7,8].

Taken together, these data confirm the utility of the UPLC–MS/MS method to accurately, precisely and consistently measure CsA and tacrolimus following extraction from DBS.

3.3. Method comparison

Around 150 anonymised patient venous whole blood samples previously assayed using the in-house whole blood LC–MS/MS method were used to prepare DBS, extracted and assayed using the new LC–MS/MS method. Dosages received by patients were dependent upon the time post transplantation and renal function. For CsA, the average dose was 100–125 mg/bd (range 2.5–225 mg/bd). For tacrolimus, the average dose was 3–4 mg/bd (range 0.5–4.5 mg/bd).

For CsA, Passing and Bablock analysis demonstrated good correlation between the two methods yielding the equation 0.99 + 1.02, linear regression $r^2 = 0.99$, $n = 153$ (Fig. 3a). Bland–Altman analysis demonstrated good agreement between methods, exhibiting a mean bias of −4.9 μg/L (95% limits of agreement −62.7–52.8 μg/L) (Fig. 3b). For tacrolimus, Passing and Bablock analysis demonstrated good correlation between the two methods yielding the equation −0.16 + 0.97, linear regression $r^2 = 0.92$, $n = 158$ (Fig. 4a). Bland–Altman analysis demonstrated good agreement between methods, exhibiting a mean bias of −0.4 μg/L (95% limits of agreement −2.8–2.0 μg/L) (Fig. 4b).

4. Conclusions

We have developed a novel, rapid and simple procedure for the extraction of CsA and tacrolimus from DBS allowing simultaneous measurement by a highly sensitive and specific UPLC–MS/MS method. This method is first reported using a single heated solvent/ultrasonication extraction procedure, which yields efficient extraction of both drugs from DBS. Our intention was to simplify extraction conditions compared to previously published methods, such that the assay could be utilised in routine clinical use. This new method has the following advantages: (1) extraction conditions reliably extract both CsA and tacrolimus from the DBS, (2) the extraction procedure is rapid taking only 30 min, (3) samples are injected into the HPLC system directly from the extraction plate avoiding manual transfer of solvent supernatants and (4) UPLC–MS/MS conditions allow simultaneous measurement of CsA and tacrolimus. We believe heated solvent extraction with ultrasonication may be a useful method for the extraction of a range of analytes that could be applied to DBS.

The new DBS method was compared to the current venous whole blood assay in routine clinical use in our centre. Comparison of around 150 patient samples each for CsA and tacrolimus demonstrated very good agreement between the two methods. Previous studies have demonstrated comparable measured CsA [10,12] and tacrolimus [13,14] concentrations in capillary and venous blood. Additionally, comparable CsA concentrations measured in venous blood, and capillary blood samples taken onto DBS, has been demonstrated [5]. Taken together this data suggests the new LC–MS/MS method will allow simultaneous measurement of CsA and tacrolimus from fingerprick capillary blood samples taken onto DBS by transplant recipients in the community.

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