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INTRODUCTION

- Routine monitoring for the immunosuppressive drug cyclosporin A (CsA) is required to maintain an effective, sub-toxic drug concentration.
- Current practice is to measure trough concentrations of CsA but "C2" measurements (2 hours \pm 10minutes post-dose) are advocated as a more informative indicator of drug exposure¹.
- In a busy clinic, accurate timing for C2 sampling could present considerable difficulties.
- C2 monitoring and pharmacokinetic profiling could be facilitated by using capillary (finger-prick) blood sampling but current immunoassays do not have sufficient sensitivity and additional tests for kidney function would still be required.
- We have therefore developed and validated a liquid chromatography - tandem mass spectrometry (LC-MS/MS) method for the simultaneous, routine analysis of CsA and creatinine in low-volume (finger-prick) whole blood samples.

METHODS

Patient Samples

- Ethical Committee approval & informed consent were obtained.
- Venous & capillary (finger prick) blood samples were collected into Vacutainer & Microtainer tubes (Beckton Dickinson), respectively.
- 130 paired samples were collected from 65 heart and lung transplant recipients.

Sample analysis by EMIT

- For comparison, CsA concentrations were also measured using EMIT (Dade Behring Ltd, Milton Keynes, UK) and an Olympus AU600 analyser.
- Over-range samples were diluted with CsA-free whole blood according to the manufacturers protocol.

Internal Standards and Calibrators

- CsA (Sigma Aldrich) was dissolved in methanol and diluted into CsA-free blood to produce a 5000 μ g/L calibrator.
- Additional CsA calibrators were purchased from Abbott Laboratories (M Maidenhead UK).
- Acetonitrile containing ascomycin (45 μ g/L) was used as precipitant.
- Pre-treatment solution was 0.4M zinc sulphate in water containing deuterated creatinine (CDN Isotopes, Canada) at a concentration of 22 μ mol/L.
- For comparison, serum creatinine was also measured using a kinetic Jaffe method and an Olympus AU600 analyser.

Mass Spectrometry

- A Quattro micro tandem mass spectrometer (Micromass, Manchester, UK) with **Z** SPRAY ion source was used for all analyses.
- Electrospray positive ionisation mode, directly coupled to the HPLC system.
- System control and data acquisition was performed using MassLynx v3.5 software with automated data processing by the QuanLynx and Neolynx Application Managers.

- In the presence of ammonium acetate, cyclosporin A forms a strong ammoniated species (m/z 1220) that produces a characteristic fragment (m/z 1203) upon CID (Figure 1).
- Sample analysis was performed in multiple reaction monitoring (MRM) mode:
Function 1 (0-0.5 min) m/z 114>44 (creatinine), m/z 117>47 (d3-creatinine).
Function 2 (0.5-1.4 min) m/z 1220>1203 (CsA), m/z 809>756 (ascomycin).

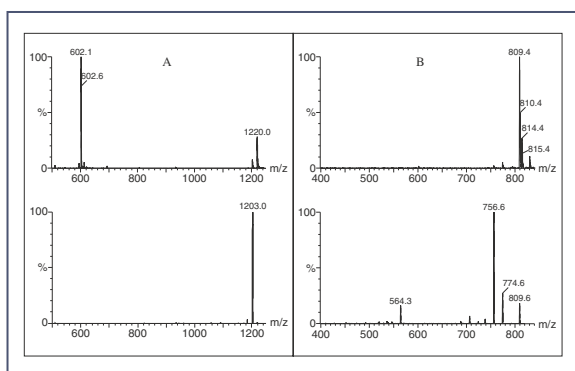
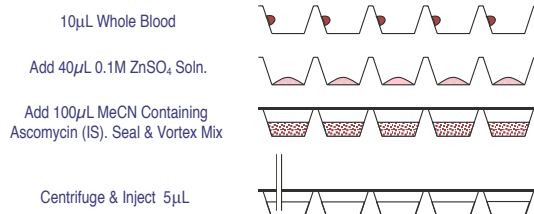


Figure 1. Positive ionisation electrospray mass (upper) and CID (lower) spectra for cyclosporin A (A) and ascomycin (B) in the presence of ammonium acetate. In both cases, the mass spectra show $[M+NH_4]^+$ and for cyclosporins A, there is an additional peak at lower mass representing $[M+2H]^{2+}$. The CID spectra show the product ions from $[M+NH_4]^+$ and the conditions were optimised to maximise the intensity of the most abundant ion

Sample Preparation for LC-MSMS



High Performance Liquid Chromatography

- Waters 2795 Alliance HT LC system (Waters Ltd., Watford, UK)
- Inject 5 μ L supernatant directly from the 96 well microtitre plate.
- Analyse using SecurityGuard C18 cartridge column (4.0mm x 3.0mm; Phenomenex, Macclesfield, UK) maintained at 55 $^{\circ}$ C and eluted with a step gradient (Table 1).
- Cycle time approximately 2.5 minutes injection to injection.

Time (min)	%A	%B	Flow Rate (mL/min)	Curve*
0	50	50	0.6	-
0.4	0	100	0.6	11
0.8	50	50	0.6	11

Table 1. Step gradient used for the LC-MS/MS analysis of Cyclosporin A

A = water containing 2mM ammonium acetate and 0.1% formic acid

B = methanol containing 2mM ammonium acetate and 0.1% formic acid

*Curve 11 steps to the indicated solvent composition at the end of the time segment

RESULTS

Creatinine

- Creatinine eluted at \sim 0.4min along with deuterated creatinine (internal standard) and was quantified using a pseudo-isotope dilution technique (Figure 2a).
- Intra- and inter- assay precision were both less than 6% (Table 2).
- Good correlation with the plasma Jaffe results was obtained but in general the LC-MS/MS results were lower than the Jaffe results (Figure 3; slope = 1.06 y-intercept = -54), as reported for other specific creatinine assays.

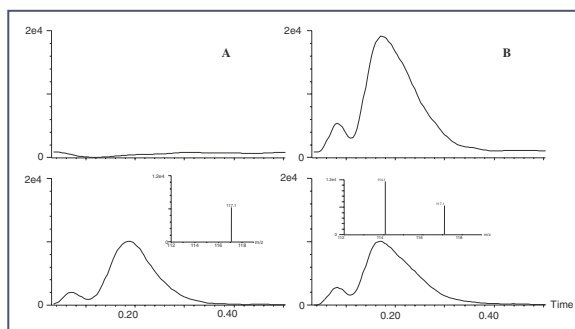


Figure 2a. LC-MS/MS chromatograms from scan function 1 (0 - 0.5 min) for creatinine (upper traces, $m/z114>44$) and d3-creatinine internal standard (lower traces, $m/z117>47$) from (A) cyclosporin A zero calibrator and (B) a patient sample found to contain 163 $\mu\text{moles/L}$ creatinine. Insets show the spectra generated from the chromatographic peak and the relative intensities of the analyte and internal standard from which the creatinine concentration was calculated

Pool	Cyclosporin A Concentration ($\mu\text{g/L}$)			
	Intraassay (n=15)		Interassay (n=10)	
	Mean \pm SD	%CV	Mean \pm SD	%CV
Low	35 \pm 1.3	3.6	80 \pm 5.3	7.0
Medium	411 \pm 10.4	2.5	536 \pm 38.0	7.0
High	1499 \pm 44.6	3.0	1490 \pm 91.0	6.0

Pool	Creatinine Concentration ($\mu\text{moles/L}$)			
	Intraassay (n=15)		Interassay (n=12)	
	Mean \pm SD	%CV	Mean \pm SD	%CV
Low	52 \pm 2.6	4.9	82 \pm 4.6	5.7
Medium	286 \pm 7.0	2.4	174 \pm 3.0	1.8
High	760 \pm 18.8	2.5	592 \pm 19.5	3.3

Table 2. Analytical imprecision of the LC-MS/MS assay

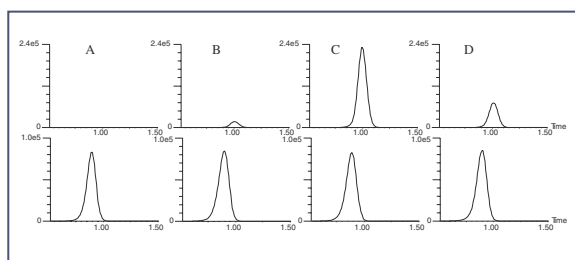


Figure 2b. LC-MS/MS chromatograms from scan function 2 (0.5 to 1.5 min) showing cyclosporin A (upper traces, $m/z1220>1203$) and ascomycin internal standard (lower traces, $m/z809>756$) from (A) zero calibrator (B) 100 $\mu\text{g/L}$ calibrator (C) 1500 $\mu\text{g/L}$ calibrator and (D) a patient sample found to contain 513 $\mu\text{g/L}$ cyclosporin A

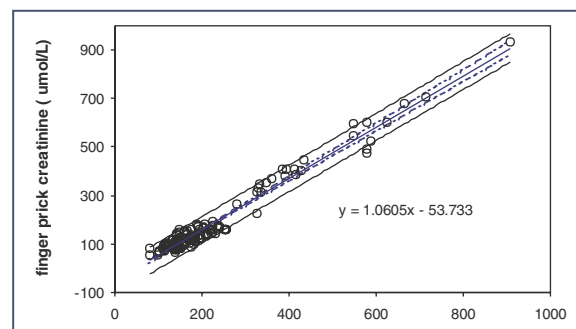


Figure 3. Correlation between creatinine concentrations measured by LC-MS/MS in capillary whole blood (finger prick) and measured by the Jaffe method in conventional serum samples. Linear regression line with 99% and 95% confidence intervals

Cyclosporin A

- Cyclosporin A and ascomycin eluted in the second time window (Figure 2b).
- No matrix effects on the ionisation efficiency of cyclosporin A or ascomycin were evident (Table 3).
- The calibration curve for CsA was linear over the extended working range (0 - 5000 $\mu\text{g/L}$; Figure 4).
- The lower limit of quantitation as derived from the precision profile ($\text{CV}<10\%$) was $<5 \mu\text{g/L}$ (Figure 5).
- Intra- and inter- assay variation were both $<7\%$ (Table 2).
- There was a good correlation between the CsA concentration in venous blood when measured by EMIT and by LC-MS/MS (Figure 6).
- There was a good correlation between the CsA concentration measured by LC-MS/MS in capillary blood vs venous blood from the same patient (Figure 7).
- The assay showed excellent stability when a pooled sample was analysed repeatedly over a 22hr period (Figure 8).

Cyclosporin A g/L	Whole blood			Water		
	Cyclosporin A Peak Area	Ascomycin Peak Area	Peak Area Ratio	Cyclosporin A Peak Area	Ascomycin Peak Area	Peak Area Ratio
50	12955 ± 442*	12810 ± 403	1.0 ± 0.02	12862 ± 720	12820 ± 635	1.0 ± 0.01
100	23094 ± 887	11576 ± 462	2.0 ± 0.07	22605 ± 560	11030 ± 603	2.0 ± 0.02
500	109199 ± 5369	12588 ± 570	8.7 ± 0.02	105358 ± 6704	11169 ± 703	9.4 ± 0.04

Table 3. Summary of the peak area counts and their ratios for Ascomycin and Tacrolimus extracted from whole blood or water after addition of Tacrolimus at the indicated concentrations. *Mean ± standard deviation for 6 replicates

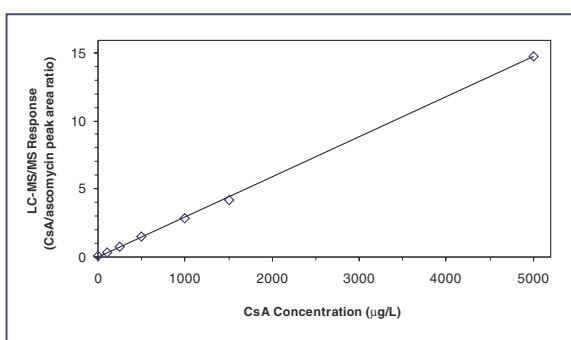


Figure 4. Calibration curve for the LC-MS/MS analysis of cyclosporin A using commercial calibrators (0, 100, 250, 500, 1000 & 1500 µg/L) with an additional in-house calibrator (5000 µg/L) to extend the linear working range to include all potential physiological concentrations of cyclosporin A

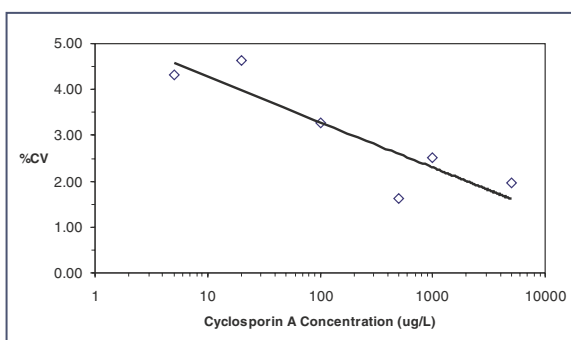


Figure 5. Precision profile for the LC-MS/MS analysis of cyclosporin A. The CV was calculated from 10 replicate injections of single extracts of whole blood calibrators

Figure 6. Correlation between the cyclosporin A concentration measured in venous whole blood by LC-MS/MS and by EMIT

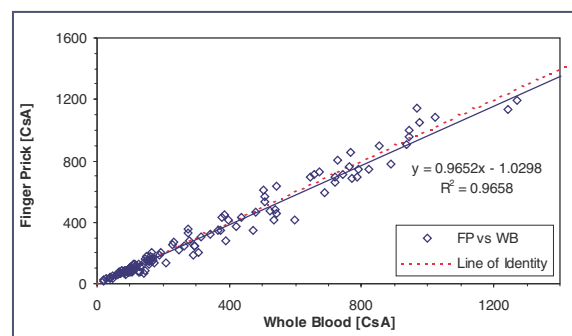


Figure 7. Correlation of cyclosporin A concentration measured in whole blood by LC-MS/MS using venous or capillary (finger prick) sampling

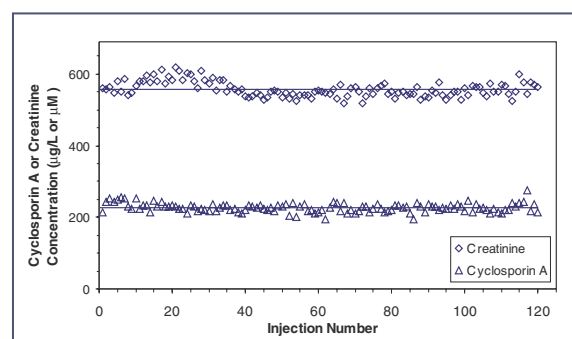


Figure 8. Replicate samples (120) were prepared and analysed by LC-MS/MS with a 10 min injection interval giving a total analysis time of 22hr. The concentrations (mean ± SD) of cyclosporin and creatinine were 227 ± 12.5 µg/L and 558 ± 21 µmoles/L respectively, giving CVs of 5.5% and 3.8%

Acyclovir	Allopurinol	Amlodipine	Amphotericin B	Aspirin	Atenolol
Azathioprine	Bricanyl	Bumetanide	Calcichew	Carvedolol	Clarithin
Codeine	Creon	Cyclazine	Doxazosin	Enalapril	Fluxanase
Flixatide	Fluoxetine	Fluvastatin	Fosamax	Frusemide	Insulin
Itraconazole	Lactulose	Lansoprazole	Lasix	Losec	Methyldopa
Minocycline	Mycophenolic acid	Panadol	Pravastatin	Prednisolone	Prozac
Quinine	Ramipril	Seprin	Seprin	Serevent	Stemetil
Tacrolimus	Temaze pam	Vitamin E	Zopiclone	Zolan	

Table 4. Drugs found not to interfere in the LC-MS/MS analysis of cyclosporin A and creatinine

CONCLUSION

- A rapid, precise and robust LC-MS/MS method has been developed and validated for the routine analysis of cyclosporin A and creatinine in low volumes of capillary blood
- The novel sampling method will enable home monitoring for trough or C2 CsA concentrations in those patients willing to adopt it
- The assay is cost-effective and can also be used for the analysis of tacrolimus and sirolimus^{2,3}

REFERENCES

1. Cantarovich M, Besner JG, Barkun JS, Elstein E, Loertscher R. Two hour cyclosporin level determination is the appropriate tool to monitor Neoral therapy. Clin Transplantation 1998;12;243-249.
2. Keevil BG, McCann SJ, Cooper DP and Morris M. Evaluation of a rapid micro-scale assay for tacrolimus by liquid chromatography-tandem mass spectrometry. 2002: in press.
3. Cooper DP, Keevil BG, Tierney DP and Morris M. Tandem mass spectrometry: A versatile tool for the monitoring of immuno-suppressive drug combinations. Ther. Drug Monit., 2001: 23;463.

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