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## Overview

A rapid LC-MS/MS method for the analysis of the immunosuppressant cyclosporin A (CsA) in whole blood is presented. The method has good precision (intra-assay CV<5%, inter-assay CV<10%) and the accuracy is demonstrated by the analysis of UK NEQAS (n=27) and patient (n=180) samples. Calibration is linear to at least 5000µg/L and the superior characteristics of the assay will allow for the analysis of CsA at peak or trough concentrations in a routine environment.

## Introduction

Cyclosporin A is a powerful immunosuppressive drug that has revolutionised solid organ and bone marrow transplantation. Therapeutic drug monitoring of CsA is advocated because inter-individual variations in ADME are marked (1) and nephrotoxicity or graft rejection can occur if the correct CsA concentration is not maintained (2).

Most laboratories use immunoassays to allow the prompt reporting of results although there is increasing concern that none of the currently available immunoassays fully satisfies the required performance criteria (3). Furthermore, the limited dynamic range of most immunoassays will make them unsuitable for the analysis of CsA concentrations at early times after treatment when it is thought to correlate more closely with clinical outcome (4).

LC-MS/MS methods for the analysis of CsA in whole blood have previously been investigated as reference methods (5, 6). We have now validated in a clinical setting an improved and simplified LC-MS/MS method that will provide an extended dynamic range whilst maintaining a high sample throughput.

## Materials and Methods

The use of patient samples for this study was approved by the Wythenshawe Hospital Local Ethical Committee. Blood samples were collected into Vacutainer tubes containing EDTA from 180 heart and lung transplant recipients attending the follow-up clinic. Ascomycin (internal standard) and CsA for preparing calibrators were from Sigma-Aldrich (Poole, UK).

For comparative purposes, CsA concentrations were also measured using EMIT (Dade Behring Ltd. Milton Keynes, UK) immunoassay kits as directed by the manufacturer.

## Mass Spectrometry

A Quattro LC triple quadrupole mass spectrometer (Micromass UK Limited, UK) fitted with a Z-Spray ion source was used for all analyses. In positive electrospray ionisation mode in the presence of ammonium acetate, cyclosporin A forms a strong ammonium adduct ( $m/z$  1220, **Figure 1**) that can be deaminated by CID to reform the protonated molecule ( $m/z$  1203, **Figure 1**).

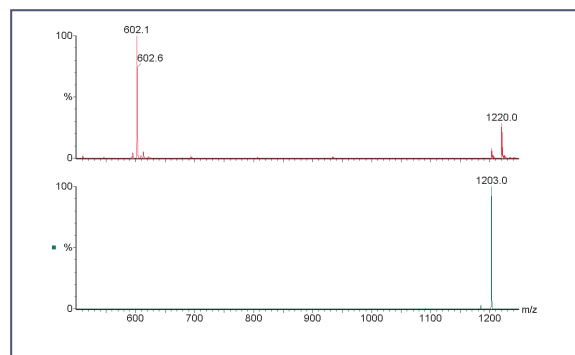


Figure 1. Positive electrospray mass (upper) and product ion (lower) spectra for Cyclosporin A

### Sample Preparation for LC-MS/MS

- Add 100 $\mu$ L whole blood or calibrator to 200 $\mu$ L MeCN containing ascomycin (internal standard, 45 $\mu$ g/L)
- Vortex mix vigorously to disperse precipitate
- Centrifuge 10000xg for 3 minutes
- Analyse 5 $\mu$ L of the supernatant

### Chromatography

Samples were analysed using a Supelco LC CN column (3 $\mu$ m, 3.0mm x 33mm) eluted with 65% aqueous acetonitrile containing 2mM ammonium acetate and 0.1% formic acid at a flow rate of 0.5mL/min. CsA and ascomycin were monitored in MRM mode using the transitions  $m/z$  1220>1203 and  $m/z$  809>756, respectively (**Figure 2**). The analysis time was less than 2 minutes, injection-to-injection, and HPLC columns were suitable for >1000 analyses.

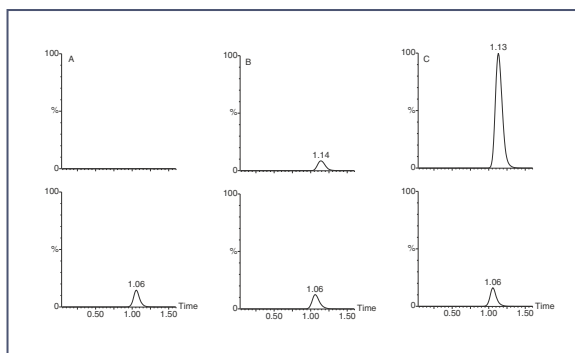


Figure 2. LC-MS/MS analysis of whole blood calibrators containing (A) 0 $\mu$ g/L (B) 100 $\mu$ g/L and (C) 1000 $\mu$ g/L cyclosporin A. Upper traces are for cyclosporin A and lower traces are for ascomycin internal standard. All chromatograms are normalised to the cyclosporin A peak for the highest standard

### Results

Extracts prepared from whole blood calibrators containing up to 5000 $\mu$ g/L CsA produced a linear calibration curve (**Figure 3**) with good precision (**Figure 4**). The LLOD was 0.03 $\mu$ g/L (2.5 x SD of zero calibrator) and the LLOQ was approximately 1 $\mu$ g/L.

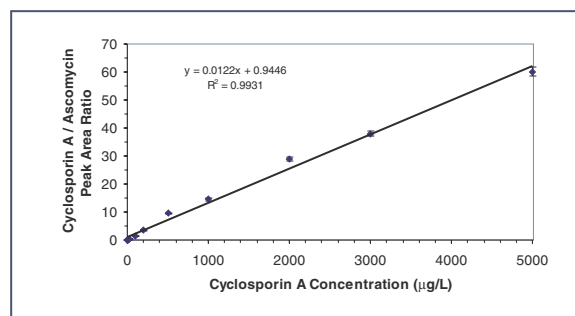


Figure 3. Calibration curve for LC-MS/MS analysis of cyclosporin A. Values are the means  $\pm$ SD of 10 determinations

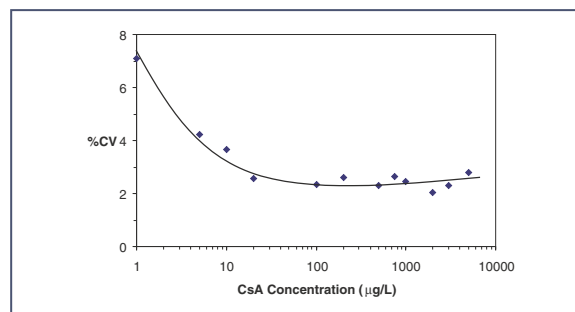


Figure 4. Intra-assay precision profile for the analysis of cyclosporin A by LC-MS/MS. The CV is calculated from the responses (CsA/ascomycin peak ratio) for 10 replicate injections of each standard

The whole procedure, including sample extraction and analysis, was also shown to have good intra- and inter-assay precision (**Table 1**).

	Cyclosporin A Concentration (µg/L)			
	Intra-assay (n=20)		Inter-assay (n=15)	
	Mean ± SD	%CV	Mean ± SD	%CV
Low	85.2 ± 3.5	4.5	93.7 ± 8.5	9.0
Medium	204.7 ± 9.5	4.6	204.4 ± 19.7	9.7
High	817.2 ± 39.5	4.8	777.8 ± 38.1	4.9

Table 1. Analytical precision of the LC-MS/MS assay for cyclosporin A. The coefficient of variation (CV) was calculated as SD/mean for the analysis of *n* replicate extractions of low, medium and high, whole blood QC samples

The repeat analysis of a pooled sample maintained at ambient temperature over a 14 hour period gave a CV of <4% indicating that both the extract and the LC-MS/MS procedure are stable. (Figure 5)

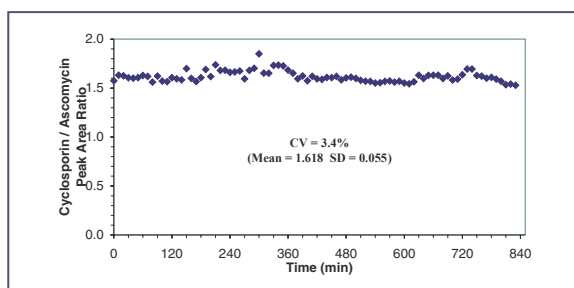


Figure 5. Eighty-four replicate LC-MS/MS analyses of a single extracted whole blood sample containing approximately 50 µg/L cyclosporin A over a 14 hour period

The potential for other drugs to interfere in the assay was assessed by spiking CsA into CsA-free whole blood from an untreated individual and from individuals (n=20) receiving a wide range of therapeutic drugs (Table 2). No significant difference in the CsA or ascomycin peak areas or the CsA / ascomycin peak area ratio was noted (CV<7%; data not shown).

Acyclovir	Allopurinol	Amlodipine	Amphotericin B
Atorvastatin	Atrovent	Azathioprine	Bendroflumazide
Bumetanide	Calcichew	Captopril	Collistin
Creon	Diltiazem	Doxazocin	Fluoxetine
Folic acid	Frusemide	Gancyclovir	Insulin
Losac	Mycophenolate mofetil	Nasoprin	Nystatin
Omeprazole	Paracetamol	Pravastatin	Prednisolone
Quinine	Ranitidine	Salbutamol	Seprin
Serevent	Tacrolimus	Temazepam	Tobramycin
Uro-deoxycholic acid			

Table 2. Drugs found not to cause interference or ion suppression in the LC-MS/MS analysis of cyclosporin A.

The accuracy of the method was evaluated by analysing external quality control samples that are prepared by adding a known amount of CsA to CsA-free whole blood (Figure 6).

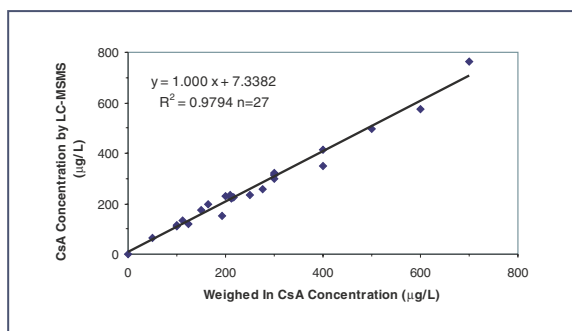


Figure 6. The analysis of cyclosporin A UK National External Quality Assurance Scheme samples by LC-MS/MS

To assess the overall performance of the assay as a routine method in a clinical laboratory, 180 whole blood samples from heart and lung transplant recipients were analysed by the LC-MS/MS method and compared to the existing EMIT immunoassay (Figure 7). An excellent correlation was observed with a slight negative bias towards the LC-MS/MS assay. This bias has been observed for other LC-based CsA monitoring techniques and is caused by cross-reactivity of the antibodies used in the EMIT assay with CsA metabolites (7).

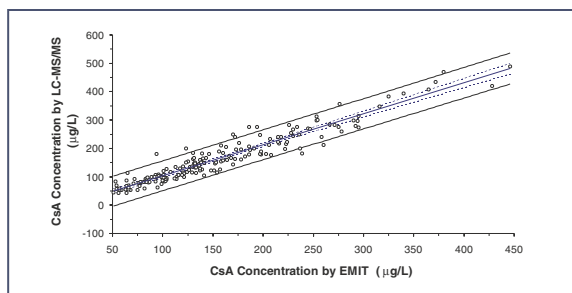


Figure 7. Passing & Bablock regression analysis of the cyclosporin A concentrations determined by LC-MS/MS and EMIT for 180 whole blood samples from heart and lung transplant recipients

## Conclusion

- We have developed and validated a rapid LC-MS/MS procedure for the analysis of cyclosporin A in whole blood.
- The method is precise, accurate and free from interferences.
- The dynamic range is extended in comparison to existing immunoassays.
- The procedure is robust and can be incorporated into a routine clinical laboratory.
- LC-MS/MS should no longer be considered as a "Gold Standard" method but can offer a flexible and cost-effective solution to many problems encountered in the modern clinical laboratory.

## References

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