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DEVELOPMENT AND VALIDATION OF A RAPID, ROUTINE LC-MS/MS ASSAY FOR THE ANALYSIS OF TACROLIMUS (FK506) USING MICROLITRE VOLUMES OF WHOLE BLOOD AND SINGLE POINT CALIBRATION

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Overview

A rapid LC-MS/MS method for routine therapeutic drug monitoring of the immunosuppressant Tacrolimus (FK506) is presented. Microlitre volumes of whole blood are used making the assay useful for analysis of paediatric and capillary blood samples. The assay is precise (intra-assay CV<7%, inter-assay CV<6%) and accurate. Comparison with an existing immunoassay (n=99 heart and lung transplant recipients) demonstrates the superior specificity of the LC-MS/MS method. Reanalysis of the data using single-point calibration gives results that are not significantly different (p=0.32; paired t-test) allowing rapid analysis of critical samples.

Introduction

Tacrolimus (FK506) is an immunosoppressive drug of considerable value for the protection of transplanted organs[1]. Nephrotoxicity or graft rejection can result from inappropriate dose regimens and therapeutic drug monitoring is mandatory so that an effective, sub-toxic drug concentration can be maintained. However, the immuno-assays commonly used for this purpose are compromised by cross-reactivity with FK506 metabolites. In contrast, tandem mass spectrometry (LC-MS/MS) can provide the required sensitivity and specificity for accurate analysis of FK506 but reported methods require relatively large sample volumes with multiple clean-up steps[2]. We describe here the development and validation of a rapid, micro-scale LC-MS/MS assay for FK506 with minimal sample processing.

Methods

Patient Samples

The use of patient samples for this study was approved by the Wythenshawe Hospital local ethical committee. Venous blood samples were collected into Vacutainer tubes (Beckton Dickinson, Oxford, UK) containing EDTA from 99 heart and lung transplant recipients attending the follow up clinic.

Sample analysis by MEIA

For comparative purposes, Tacrolimus concentrations were measured using a microparticle-enzyme immunoassay (MEIA, Abbott Diagnostics, Maidenhead, UK) and an IMx analyser, according to the manufacturers instructions.

Standards and Calibrators

The Tacrolimus analogue Ascomycin (Sigma, Ltd., Poole, UK) was used as internal standard. Tacrolimus was a gift from Fujisawa (Munich, Germany) and a series of Tacrolimus calibrators were purchased from Abbott Diagnostics. External QC samples were from the International Tacrolimus Proficiency Testing Scheme (Analytical Unit, St George's Hospital, London, UK).

Mass Spectrometry

A Quattro micro tandem mass spectrometer fitted with a **Z** *SPRAY* ion source was used for all analyses (Waters Corporation, Manchester, UK). The instrument was operated in electrospray positive ionisation mode and was directly coupled to the HPLC system. System control and data acquisition was performed using MassLynx v4.0 software with automated data processing by the QuanLynx Application Manager.

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In the presence of ammonium acetate, Tacrolimus forms a strong ammonium adduct (*m/z*821) that produces a characteristic fragment (*m/z*768) upon CID (**Figure 1**).

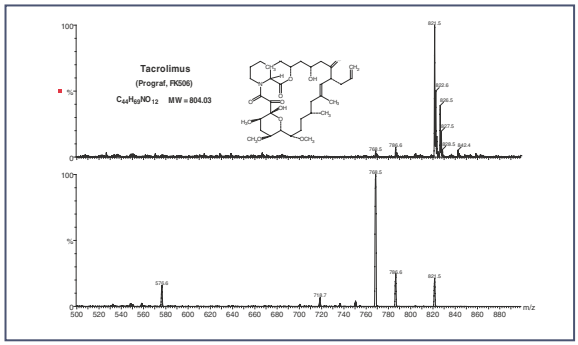
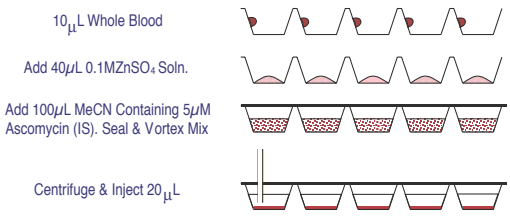


Figure 1. Electrospray mass (upper) and CID (lower) spectra of Tacrolimus dissolved in 50% aqueous methanol containing 2mM ammonium acetate and 0.1% formic acid

- Sample Preparation for LC-MSMS



High Performance Liquid Chromatography

- Waters 2795 Alliance HT LC system (Waters Ltd., Watford, UK)
- Inject 20µL 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°C and eluted with a step gradient (**Table 1**).
- Monitor Tacrolimus and Ascomycin in MRM mode using the transitions *m/z*821>768 and *m/z*809>756 respectively (**Figure 2**).
- 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 Tacrolimus
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.*

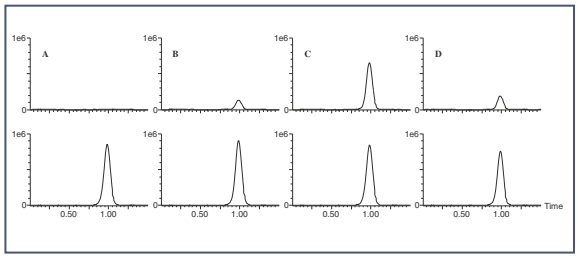


*Figure 2. LC-MS/MS chromatograms for FK506 (upper traces; m/z921>768) and for Ascomycin internal standard (lower traces; m/z809>756). **A**, zero calibrator; **B**, 6µg/L calibrator; **C**, 30µg/L calibrator and **D**, a patient sample found to contain 10.4µg/L FK506*

Results

- The calibration curve was linear over the range examined (up to 30µg/L; **Figure 3**).
- The lower limit of quantitation (CV<10%) was 0.5µg/L (**Figure 4**).
- Intra- and inter- assay variation were both <7% (**Table 2**)
- No matrix effects on the ionisation efficiency of Tacrolimus or Ascomycin were evident (**Table 3**).
- Analysis of external QC samples (n=18) showed good accuracy of the LC-MS/MS method (**Figure 5**).

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- Comparison of the Tacrolimus concentration measured by MEIA and LC-MS/MS for 99 heart and lung transplant recipients showed good agreement between the methods and confirmed the expected negative bias of the LC-MS/MS analysis caused by metabolite interference in the MEIA (**Figure 6A &B**).
- There was no significant difference between the LC-MS/MS results for the patient samples when calculated using either a multi-point or single point (30µg/L calibrator extrapolated through zero) calibration curve (p=0.32, paired t-test) and there was no bias between the two methods of calibration (**Figure 7A & 7B**).
- There was no loss in sensitivity for Tacrolimus or Ascomycin when a single extract was analysed repeatedly over a 27hr period (**Figure 8**; 2.9% CV for peak area ratio; 6.9%CV and 7.6% CV for Tacrolimus and Ascomycin peak areas respectively).

	Tacrolimus Concentration (µg/L)			
	Intraassay (n=15)		Interassay (n=10)	
Pool	Mean ± SD	%CV	Mean ± SD	%CV
Low	2.7 ± 0.25	6.4	2.6 ± 0.14	5.2
Medium	6.6 ± 0.34	5.1	6.5 ± 0.27	4.1
High	14.8 ± 0.52	3.5	15.2 ± 0.45	3.0

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

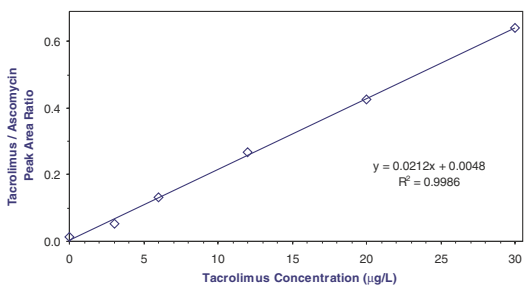

Figure 3. Multipoint Calibration Curve for the LC-MS/MS Assay

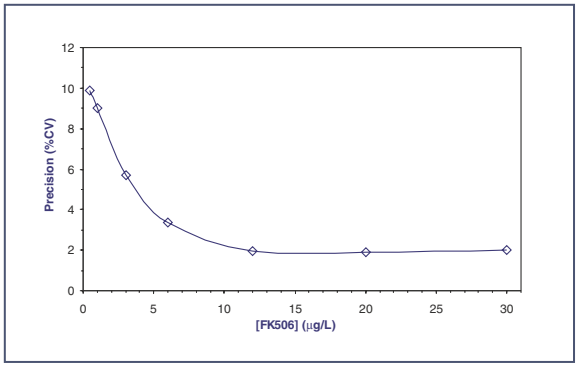

Figure 4. FK506 Intra-assay Precision Profile: LC-MS/MS Assay using 10µL Blood n=10

	Whole blood			Water		
Tacrolimus (µg/L)	Tacrolimus Peak Area	Ascomycin Peak Area	Peak Area Ratio	Tacrolimus Peak Area	Ascomycin Peak Area	Peak Area Ratio
5	487± 28*	2731±143	0.18±0.01	583±14	3319±27	0.18±0.01
10	1082±56	2865±100	0.38±0.01	1102±104	3071±191	0.36±0.01
20	2287±138	2943±100	0.78±0.02	2042±17	2736±71	0.75±0.01

*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.*

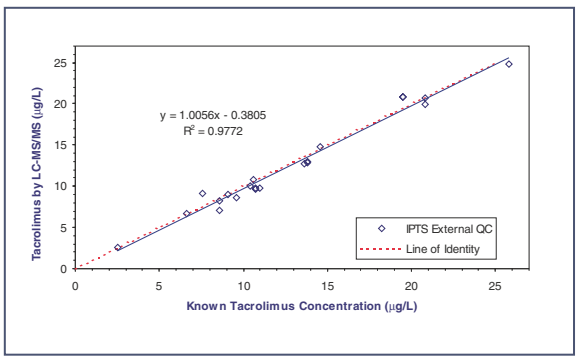

Figure 5. IPTS External QC Samples Analysed by LC-MS/MS

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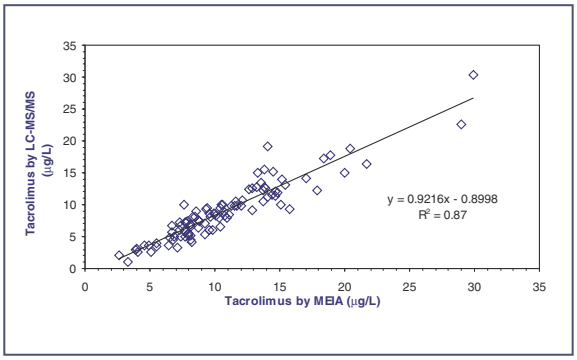

Figure 6A. Comparison of the Tacrolimus concentrations in 99 whole blood samples analysed using LC-MS/MS or MEIA

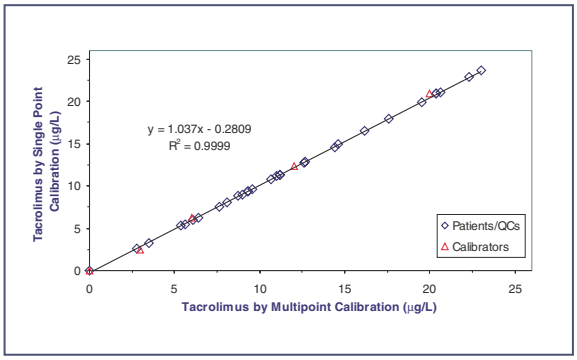

Figure 7A. Comparison of the Tacrolimus concentrations in 99 whole blood samples calculated using single point or multi-point calibration

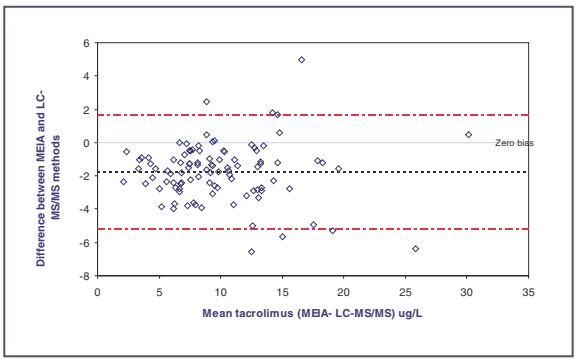

Figure 6B. Bland-Altman difference plot for the analysis of Tacrolimus in 99 whole blood samples using LC-MS/MS or MEIA

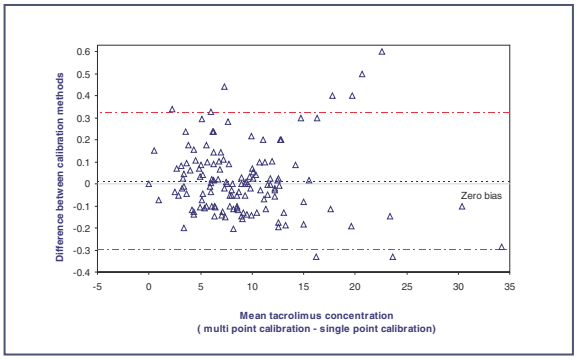

Figure 7B. Bland-Altman difference plot for the analysis of Tacrolimus in 99 whole blood samples using single point or multi-point calibration

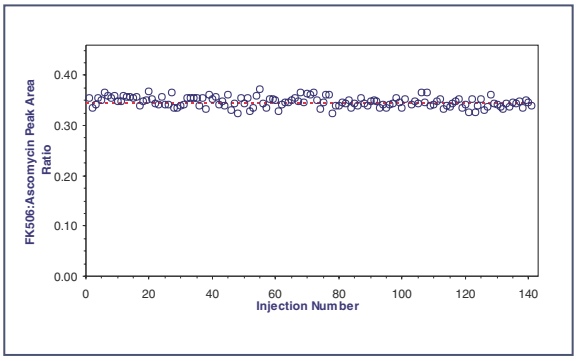

Figure 8. Replicate extractions of a single whole blood sample were analysed by LC-MS/MS for tacrolimus with an injection interval of approximately 10min. Injections were made over a 27hr time period

Conclusion

- A rapid, precise and robust LC-MS/MS method has been developed and validated for the routine analysis of Tacrolimus.
- The method is cost-effective and can be used without modification for the analysis of cyclosporin A and Sirolimus[4,5].

References

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