Determination of Tacrolimus in Rat Whole Blood Using Dried Blood Spot (DBS) Analyses by LC-MS/MS

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INTRODUCTION

Tacrolimus (FK-506) is an immunosuppressive agent that is used to lower organ rejection following transplantation and to treat various autoimmune diseases of the skin. However, tacrolimus has significant toxicity, in particular cardiotoxicity.

In the development of a novel dosage form of tacrolimus, a dried blood spot (DBS) method was desirable for toxicokinetic assessment from serial sampling from rats. DBS is an appropriate approach, since tacrolimus is highly sequestered into red blood cells.

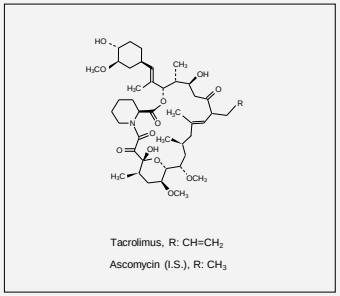

Figure 1. Structures of Tacrolimus and Ascomycin (I.S.)

METHOD

Serial blood samples (40 μL) were removed from cannulated rats following oral gavage administration, placed onto DBS cards (Ahlstrom), and allowed to dry at ambient conditions. DBS punches (7 mm) were then placed into methanolic extraction solvent containing internal standard (ascomycin) and extensively vortexed using a multitube vortexor. The resulting extracts were combined with ammonium formate buffer (10 mM, pH 3.0) 1:1 and briefly vortexed, prior to analysis by LC-MS/MS.

The samples were chromatographed using a Betasil Cyano column and methanol-based mobile phase solutions. A valve switching system with a trap column (Betasil Cyano) was used for eliminating matrix interference with injections of extracted samples. The analytes were detected by Turboion Spray API 3000 LC-MS/MS system under positive MRM mode.

Liquid Chromatography

HPLC : Shimadzu 10ADvp
Autosampler : Perkin Elmer 200
Trap Column : Betasil Cyano (10 x 2.1 mm)
Analytical Column : Betasil Cyano (100 x 2.1 mm)
MP A : MeOH/10mM ammonium acetate pH 5.8 (20/80)
MP B : MeOH
MP C : MeOH/DIH_2O (80/20)

Mass Spectrometry

AB Sciex API 3000
Polarity : Positive
Scan type : Multiple Reaction Monitoring
Tacrolimus : 821.5 → 768.5
Ascomycin (I.S.) : 809.5 → 756.5

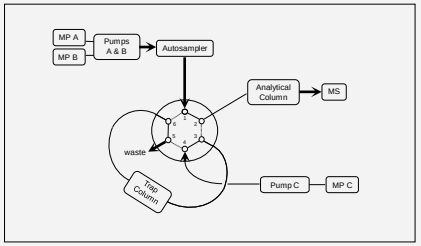

Figure 2. Diagram of LC/MS Instruments.

RESULTS

The method was validated over **a range of 5 to 250 ng/ml** with linear regression ($1/x^2$). The correlation coefficients for first three validation batches were 0.996 or better (Figure 3).

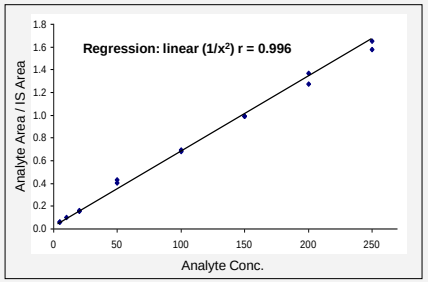

Figure 3. A Typical Calibration Curve for Tacrolimus

Figures 4 through 6 show typical chromatograms for selected samples. The S/N ratio for tacrolimus peak at LLOQ level was greater than 30.

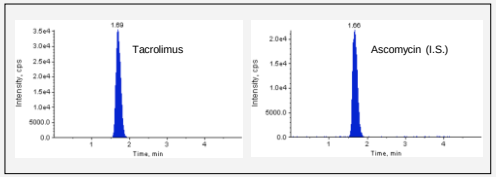

Figure 4. Chromatogram of an Extracted ULOQ Sample (250 ng/mL).

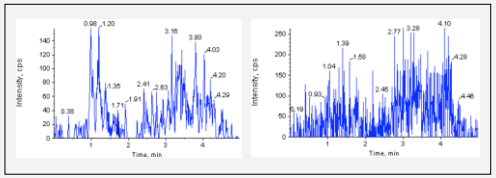

Figure 5. Chromatogram of a Blank Matrix (No I.S.).

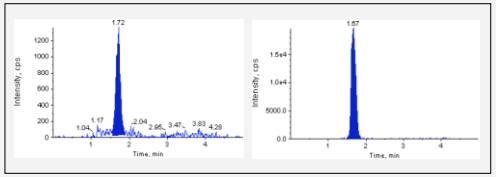

Figure 6. Chromatogram of an Extracted LLOQ sample (5 ng/mL).

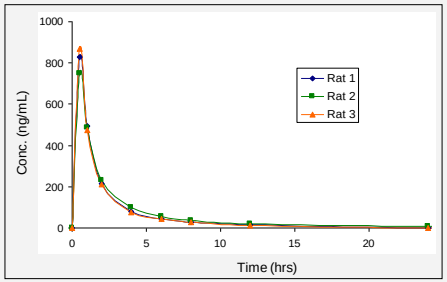

Figure 7. Tacrolimus PK Profile (3 mg/Kg, PO)

The inter- and intra-batch results for QC samples are shown in **Table 1**.

Table 1. Precision and Accuracy Results for Tacrolimus Quality Control Samples (n = 6 replicates per batch).

Batch		5.0 ng/mL (LLOQ)	15 ng/mL (Low)	50 ng/mL (Mid)	200 ng/mL (High)
1	**Mean**	5.15	16.4	55.5	210
	% CV	12.7	7.8	2.4	3.1
	% Bias	2.9	9.1	11.0	5.2
2	**Mean**	5.06	14.4	56.3	204
	% CV	16.4	5.6	5.2	3.6
	% Bias	1.1	-4.2	12.6	2.1
3	**Mean**	5.33	14.9	48.2	214
	% CV	14.9	10.6	3.3	4.1
	% Bias	6.6	-0.7	-3.6	7.0
Overall	**Mean**	5.17	15.2	53.5	210
	% CV	14.0	9.6	7.9	4.0
	% Bias	3.4	1.4	7.0	4.8

Table 2. Long-Term Stability (LTS) and Carry-Over

LTS at RT	DBS Punch Carry-Over
37 days	< 1%

OBSERVATIONS

Methanol was determined as the optimum extraction solvent. Alternate solvents (acetonitrile, methanol containing 0.1% formic acid or mobile phase) resulted in lower extraction recoveries or higher background peaks. A tighter acceptance criteria for the internal standard (± 20%) was employed to improve assay performance, in the absence of a stable-label internal standard.

CONCLUSION

A robust and reliable method has been established for the measurement of Tacrolimus in rat whole blood, with K_2EDTA as anticoagulant, using dried blood spot analysis by LC-MS/MS. The method meets FDA guidelines for bioanalytical procedures and is suitable for pre-clinical analysis.

ACKNOWLEDGEMENTS

The authors would like to thank Julie Showalter and Rodney Boughner for assistance with extraction and data processing and Grant Gaillard and Cory Zuehlsdorf (ID Biological Systems) for providing Ahlstrom DBS cards.