A PRELIMINARY INVESTIGATION INTO A SIMPLE AND ROBUST PROTEIN PRECIPITATION METHOD FOR THE SIMULTANEOUS DETERMINATION OF IMMUNOSUPPRESSANTS IN TRANSPLANT PATIENT BLOOD USING LC-MS/MS

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Gareth W Hammond, Lisa J Calton and Donald P Cooper.
Clinical Business Operations Group, Waters Corporation, Atlas Park, Simonsway, Manchester, M22 5PP, UK

INTRODUCTION

Simple LC/MS/MS methods have been widely adopted in clinical chemistry laboratories for the therapeutic drug monitoring (TDM) of single immunosuppressant drugs including Tacrolimus, Cyclosporin A and Sirolimus[1,2]. For newer immunosuppressants, such as Everolimus, this may be the only method currently available to the clinical chemist[3]. A demand for multi-analyte methods has arisen from the increasing use of combination therapy and the need to streamline laboratory workflows. Typically, these methods use on-line SPE[4] and are unattractive to some laboratories because they tend to reduce sample throughput. We have therefore investigated the use of more advanced MS/MS technology in combination with simple chromatography to deliver a rapid multi-analyte immunosuppressant TDM method.

Figure 1. System configuration of a Waters Acquity UPLC™ / TQD.

METHODS

Mass Spectrometry

A Waters Acquity Tandem Quadrupole Detector (TQD) coupled to an Acquity UPLC™ (Waters Corporation, Manchester, UK) was used for all analyses. The full system configuration is shown in Figure 1. The instrument was operated in positive electrospray ionization mode. All data acquisition was performed using MassLynx™ 4.1 software with auto data processing using the QuanLynx™ Application Manager.

The compound-dependent cone voltage was optimized to maximize the abundance of the precursor ion entering the source and selected to pass through the first quadrupole to the collision cell. Collision-induced dissociation was facilitated by argon and collision energy to produce characteristic product ions. Using this information a specific Multiple Reaction Monitoring (MRM) experiment was created and is shown in Table 1.

Compound	MRM Function	Dwell (ms)	Cone (V)	Collision Energy (eV)
Ascomycin	809.2 > 756.1	10	36	22
Tacrolimus	821.2 > 768.1	50	36	22
32-desmethoxy-rapamycin	901.3 > 834.1	10	32	18
Sirolimus	931.3 > 864.1	50	32	18
Everolimus	975.3 > 908.1	50	32	18
Cyclosporin A	1219.6 > 1202.3	5	30	20
Cyclosporin D	1233.6 > 1216.3	5	30	20

Table 1. The tuning parameters used when monitoring the four immunosuppressants and their internal standards.

MS Conditions

Polarity	ES+
Capillary (kV)	0.80
Source Temperature (°C)	120
Desolvation Temperature (°C)	350
Cone Gas Flow (L/Hr)	OFF
Desolvation Gas Flow (L/Hr)	800
Collision Gas Flow (mL/Min)	0.12
Collision Cell Pressure (mBar)	$4.75e^{-3}$

UPLC Conditions

Column:	Waters MassTrak™ Column
Mobile Phase A:	2mM ammonium acetate in aqueous + 0.1% formic acid
Mobile phase B:	2mM ammonium acetate in methanol + 0.1% formic acid
Column Temperature:	55°C
Injection Volume:	20µL

Gradient Table

Time (mins)	Flow Rate (mL/min)	%A	%B	Curve
Initial	0.4	50	50	1
0.4	0.4	0	100	11
1.4	0.4	50	50	11

Calibrators and QCs

Mixed analyte calibrators (Chromsystems, Munich, Germany) and controls (Chromsystems, Munich, Germany and Recipe, Munich, Germany) were prepared using the standard MassTrak Immunosuppressant Kit sample preparation procedure.

Patient Samples

Whole-blood patient samples containing either Tacrolimus, Sirolimus, Everolimus or Cyclosporin A were a kind donation by Dr Michael Vogeser, Institute of Clinical Chemistry, Hospital of the University of Munich, Germany. The samples were used to compare an existing single-analyte LC/MS/MS method run on a Waters Alliance® 2795 and Quattro micro™ with the multi-analyte LC/MS/MS method.

Sample Preparation

Whole blood (50µL) was pipetted into a microfuge tube, 0.1M zinc sulphate in aqueous was added and vortex mixed (5s) to breakdown the red blood cells. Acetonitrile containing internal standards (5ng/mL 32-desmethoxyrapamycin, 2ng/mL ascomycin, and 25ng/mL cyclosporin D) was added, vortex mixed (20s) and centrifuged (5mins @ 13,000rpm) to precipitate and remove proteins. The supernatant (20µL) was injected using load ahead onto the LC/MS/MS system giving an injection-to-injection time of less than 2minutes.

Ion Suppression Studies

Blank matrix was processed and the extract analyzed with post-column addition of the analytes, each at 100ng/mL and a flow rate of 10µL/min using the integral sample fluidics of the Acquity TQD.

Statistical Analyses

Passing-Bablok linear regression (Microsoft® Office Excel 2003 with Add-In Analyse-It™ version 1.73) was used to compare the patient samples run using the single and multi-analyte LC/MS/MS methods.

RESULTS

- A chromatogram of the lowest extracted whole blood Chromsystems calibrator is shown in Figure 2. Each chromatogram is annotated with the compound name, the peak-to-peak signal-to-noise ratio (SNR) for the analyte and concentration. All responses are well above the limit of detection (SNR 5:1).
- Example ion suppression chromatograms (Figure 3) demonstrate that the analyte peaks elute away from the significant matrix effects. The main ion suppression effect is observed at the start of the chromatogram. The analyte peaks elute after this and before the sharp change in background due to the change in gradient.
- Good linear calibration curves were obtained for all analytes using the Chromsystems calibrators. Example calibration curves are shown in Figure 4. All calibration curves in whole blood were linear (r^2>0.995).

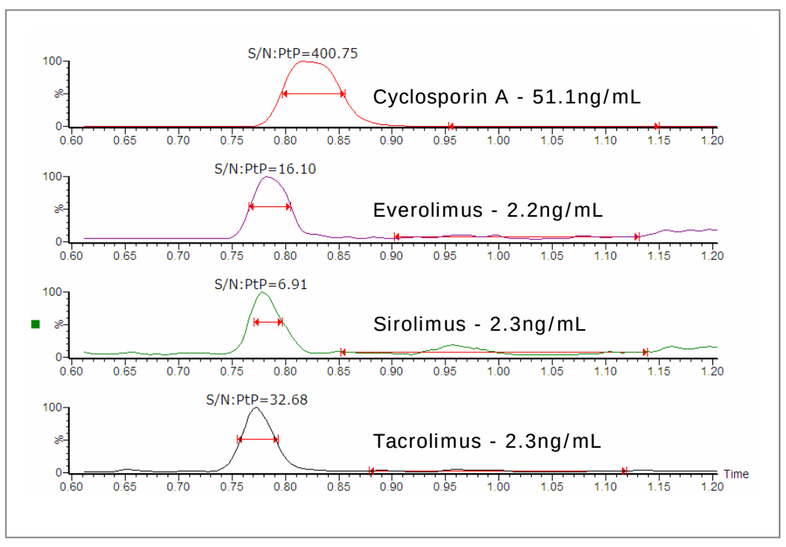

Figure 2. Chromatograms showing the lowest Chromsystems calibrator and the signal-to-noise ratio for each immunosuppressant.

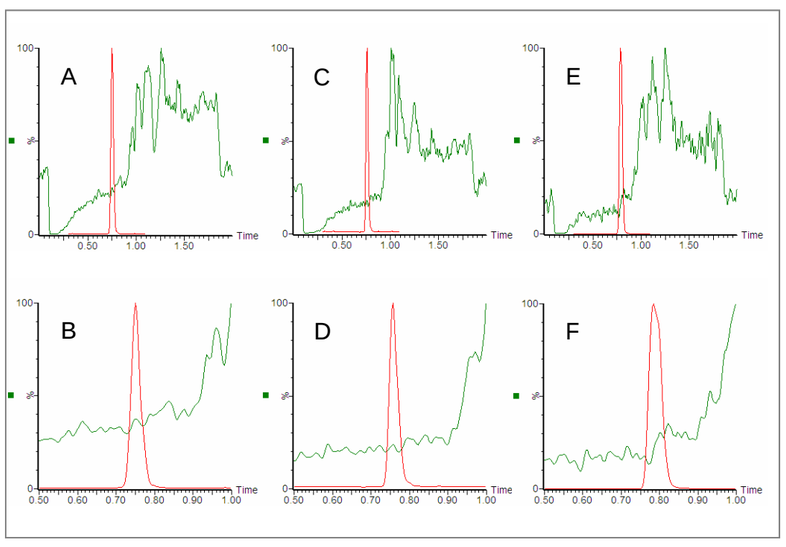

Figure 3. Ion suppression profiles for Tacrolimus (A and B), Sirolimus (C and D) and Cyclosporin A (E and F). For each analyte a chromatogram of a matrix standard is shown and a blank blood with post-column addition of the analyte. The top chromatogram displays the full acquisition whilst the expanded chromatogram beneath more clearly shows any matrix effects near the eluting peak.

- Chromsystem controls used for the intra-day precision study (n=5) is summarised in Table 2. Acceptable precision was also obtained for inter-day (n=5) precision using Recipe controls. All %CVs for intra- and inter-day precision studies were < 10%. The average linearity correlation coefficient (r^2) for each analyte over the 5 day study is also in Table 3.

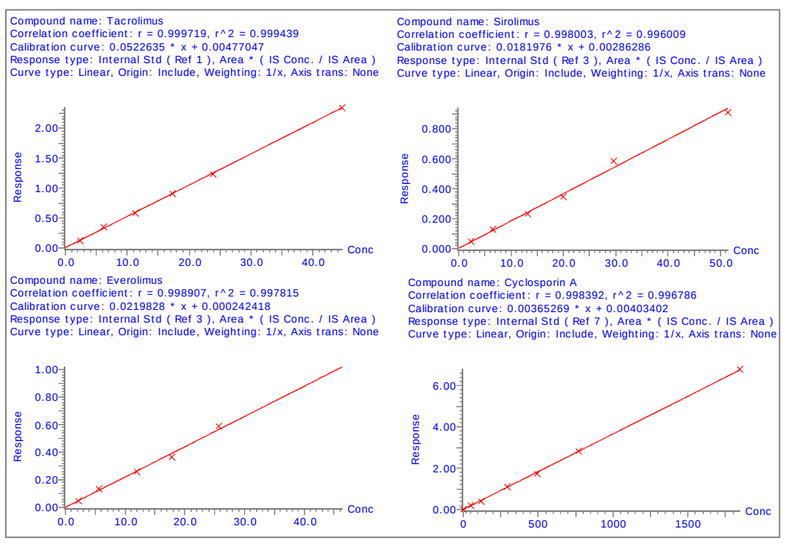

Figure 4. Chromsystems calibration curves for Tacrolimus, Sirolimus, Everolimus and Cyclosporin A.

Compound	Control	Mean Conc (ng/mL)	St Dev	%CV
Tacrolimus	Level 1	2.9	0.1	4.9
	Level 2	7.1	0.3	4.6
	Level 3	15.3	0.7	4.8
	Level 4	26.9	0.9	3.3
Sirolimus	Level 1	2.4	0.3	9.4
	Level 2	9	0.3	3.8
	Level 3	15.5	0.5	3.3
	Level 4	27.9	1.2	4.2
Everolimus	Level 1	2.4	0.1	5.4
	Level 2	4.4	0.3	6.8
	Level 3	8.1	0.7	8.2
	Level 4	23.4	1.5	6.2
Cyclosporin A	Level 1	102.3	5.1	4.9
	Level 2	247.7	10.2	4.1
	Level 3	561.6	16.2	2.9
	Level 4	1584.7	66.5	4.2

Table 2: Summary of the intra-day precision (n=5) determined using Chromsystems controls.

Compound	Linearity (r^2)	Control	Mean Conc (ng/mL)	St Dev	%CV
Tacrolimus	0.9987	Low	3.6	0.1	3.6
		Medium	7.3	0.2	3.2
		High	14.2	0.2	1.6
Sirolimus	0.9975	Low	4.5	0.2	4.4
		Medium	12.9	0.8	6.1
		High	25.2	1.5	5.8
Everolimus	0.9978	Low	3.6	0.3	7.9
		Medium	12.0	0.5	4.6
		High	20.1	1.3	6.4
Cyclosporin A	0.9966	Low	52.2	2.7	5.2
		Medium	105.5	4.4	4.2
		High	217.8	9.2	4.2

Table 3: Summary of the linearity for the immunosuppressants using Chromsystems calibrators and the inter-day precision (n=5) for each Recipe control.

- The Passing-Bablok linear regression analysis between single and multi-analyte methods is shown in Figure 5.

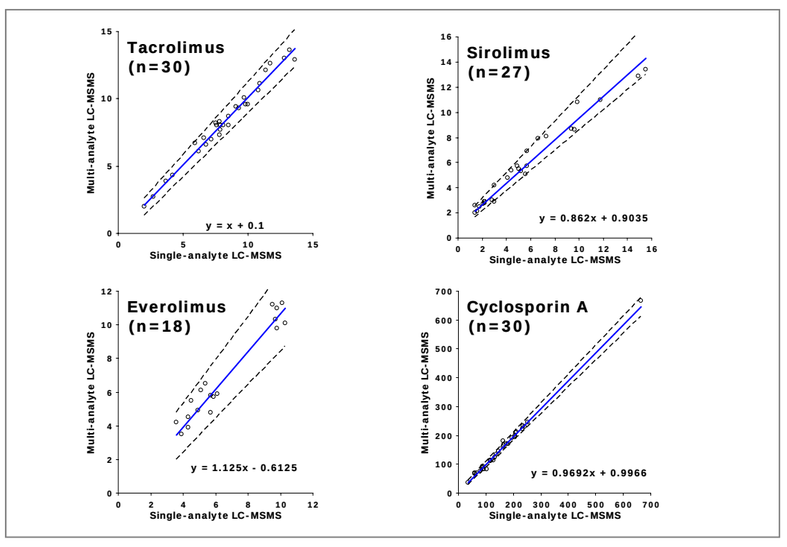

Figure 5. Passing-Bablok linear regression analysis for each analyte comparing the multi-analyte method to results obtained using a single-analyte method on a Waters Alliance 2795 / Quattro micro.

CONCLUSIONS

- **The Acquity-TQD with its fast scanning capabilities has been demonstrated to be able to quantify multiple immunosuppressants in a single analytical run with simple sample preparation.**
- **The analytes are detectable at the required limit of detection, linear, with good intra and inter-day precision.**
- **The analytes elute away from significant ion suppression regions.**
- **Comparison of patient samples by a single analyte LC/MS/MS method give good agreement with statistical processing using Passing-Bablok linear regression analysis.**

References

1. Taylor PJ, Salm P, Lynch SV and Pillans PI. *Ther. Drug Monit.* 2000;22:608-612. Simultaneous quantification of Tacrolimus and Sirolimus, in human blood, by high-performance liquid chromatography-tandem mass spectrometry.
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