Chromatography Technical Note No AS102

Automating the analysis of selected Immunosuppressants in whole blood using a GERSTEL Multi Purpose Sampler coupled to liquid chromatography tandem mass spectrometry detection.

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Introduction

Cyclosporin A, tacrolimus and sirolimus are routinely measured in whole blood to monitor immunosuppressive therapy in transplant patients.
Cyclosporin A, is a cyclic undecapeptide derived from the fungus *Tolypocladium inflatum*, it is a potent immunosuppressive drug that is effective in combating tissue rejection following organ transplantation. When compared to other commonly used immunosuppressants, e.g. corticosteroids and azathioprine, cyclosporin has greatly improved graft survival in skin, heart, kidney, pancreas, bone marrow, lung, small intestine and liver transplants.
Tacrolimus (FK-506) is an immunosuppressive drug that is mainly used after allogeneic organ transplant to reduce the activity of the patient's immune system and so lower the risk of organ rejection. It is a 23-membered macrolide lactone discovered in 1984 from the fermentation broth of a Japanese soil sample that contained the bacteria *Streptomyces tsukubaensis.*
Sirolimus is a relatively new immunosuppressant drug and is especially useful in kidney transplants. Sirolimus is a macrolide first discovered as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample from an island called Rapa Nui, better known as Easter Island.
Presented is methodology outlining fully automated sample preparation and analysis of whole blood samples including automated protein crash and centrifugation.

Instrumentation

GERSTEL MPS 2, fitted with 1000 µl syringe and LC injection valve
Anatune CF-100 centrifuge
Instrument Top Sample Preparation (ITSP) Hardware Kit
Maestro Version 1.3.7.69
Agilent 6410 Triple Quadrupole Mass Spectrometer with HotBox and electrospray source.
Agilent 1200 Series HPLC
G1312B Binary Pump SL
G1316B Thermostatted Column Compartment SL
G1379B Degasser

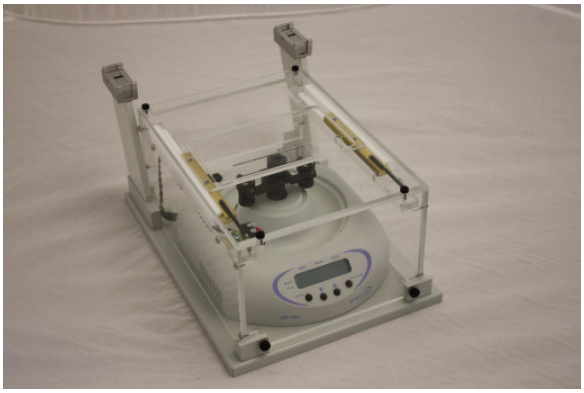

Figure 1 - Anatune CF-100 centrifuge.

Methodology

Two sets of working calibrators were prepared by reconstituting commercially available lyophilized whole blood calibration standards. (Chromsystems, Munchen, Germany) and (Recipe, Munich, Germany) See tables 1 & 2. For sample preparation, 50 µl of the whole blood sample is placed in a standard two ml glass screw top autosampler vial and the vial capped using a magnetically transportable PolyMag™ caps (Gerstel, Germany). The sample is then placed on the vial tray of the multi purpose sampler (MPS). See Figure 2.
The following aspects of sample preparation are fully automated, conducted via the MPS and the CF-100.

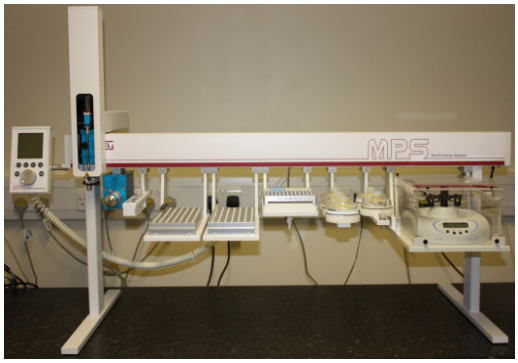

Figure 2 – Gerstel MPS for automated immunosuppressant extraction.

Cal Level	Analyte ng/ml		
	Tacrolimus	Sirolimus	Cyclosporin A
Std_01	2.1	2.6	23.5
Std_02	5.8	6.6	127
Std_03	11.4	12.8	299
Std_04	17.3	20.0	484
Std_05	23.1	29.0	703
Std_06	40.0	49.2	896

Table 1 – Levels of selected immunosuppressants in the whole blood Chromsystems calibrators.

Cal Level	Analyte ng/ml		
	Tacrolimus	Sirolimus	Cyclosporin A
Std_01	1.67	1.45	20.7
Std_02	3.28	3.10	40.1
Std_03	6.91	6.20	77.2
Std_04	13.9	13.3	151
Std_05	29.2	26.1	383
Std_06	58.1	51.3	1051

Table 2 – Levels of selected immunosuppressants in the whole blood Recipe

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calibrators.

200 µl of a 0.1 M zinc sulphate solution is added to the vial to precipitate proteins, followed by 500 µl of internal standard solution in acetonitrile (ascomycin 5 ng/ml, 3,2-desmethoxyrapamycin 5 ng/ml and cyclosporin D 10 ng/ml). The vial is then moved using magnetic transport to the CF-100 centrifuge whereby the contents are thoroughly vortexed for one minute to assist in the protein precipitation. The vial is then centrifuged at 3000 rpm for one minute to separate the proteins from the supernatant in preparation for injection.

Sample analysis is fully automated by means of an external injection valve and loop fitted onto the MPS, 50 µl of supernatant is injected. Separation is achieved by means of an Agilent Zorbax XDB 2.1 x 30 mm; 3.5 µm particle size. The chromatographic mobile phases consisted of 2 mM ammonium acetate plus 0.1 % formic acid in water (eluent A) and 2 mM ammonium acetate plus 0.1 % formic acid in methanol (eluent B). A gradient elution was performed from 50 % B to 100 % B in 1 minute, with an isocratic hold at 100 % B for 1 minute; the column was then equilibrated to baseline conditions. Column flow rate was 0.5 ml/min throughout the chromatographic run whilst the column temperature was maintained at 60 °C.

An Agilent 6410B tandem mass spectrometer with electrospray source was used in positive ionization mode. Instrument analysis time was 4 minutes per sample using the conditions listed in table 3. Quantification was performed on the ammonium adducts of the various compounds under investigation. See Figure 6 for an example chromatogram showing the internal standards and compounds of interest.

Parameter	Tacrolimus	Sirolimus	Cyclosporin A
Precursor ion	821.7	931.5	1219.8
Product ion (Q)	768.3	864.5	1203.1
Dwell	50	50	50
Fragmentor (V)	180	140	140
Collision Energy (Q)	15	10	15

Parameter	Ascomycin	DMR*	Cyclosporin D
Precursor ion	809.6	901.6	1233.7
Product ion (Q)	756.4	834.7	1217.2
Dwell	50	50	50
Fragmentor (V)	160	180	200
Collision Energy (Q)	15	10	15

* DMR - 3,2-desmethoxyrapamycin
Gas Temp (°C):350 Gas Flow (l/min):5
Nebulizer (psi):40 Capillary (v):4000

Table 3:- Selected MS conditions for analysis

Results

Calibration curves were constructed for all three analytes using both sets of calibrators. Linear calibrations were achieved from the Chromsystems six point calibration standards. Correlation coefficients of 0.999, 0.996 and 0.993 were obtained. See Figure 2. Linear calibrations were also achieved from the Recipe six point calibration standards. Correlation coefficients of 0.996, 0.999 and 0.997 were obtained for tacrolimus, sirolimus and cyclosporin A respectively. See Figure 3.

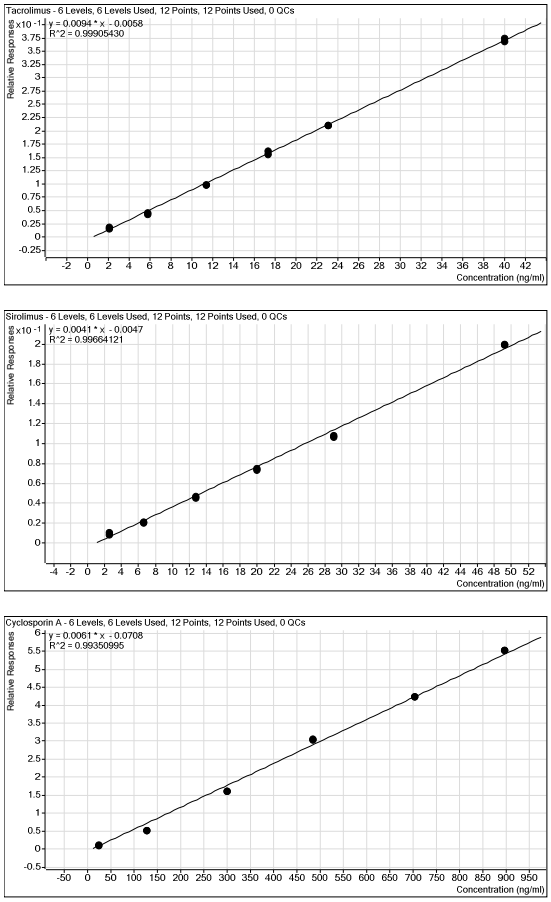
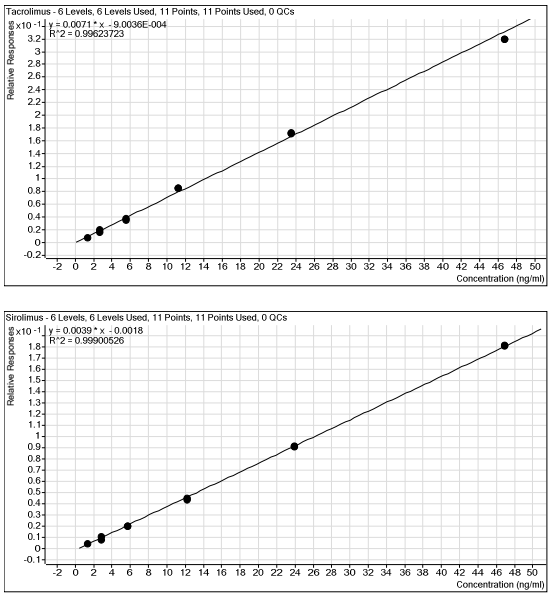


Figure 2 - Calibration curves for tacrolimus, sirolimus and cyclosporin A using the Chromsystems whole blood calibrators.



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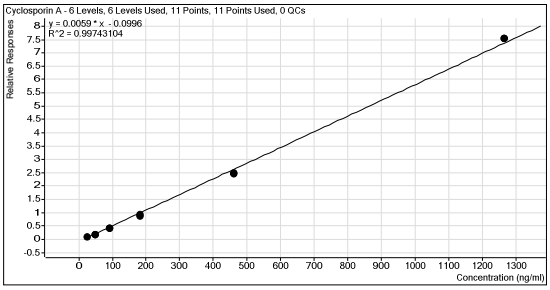

Figure 3 - Calibration curves for tacrolimus, sirolimus and cyclosporin A using the Recipe whole blood calibrators.

In order to fully validate the method, quality control materials obtained from Chromsystems and Recipe were reconstituted as per the manufacturer's instructions, extracted and analysed using the automated procedure, data is presented in tables 4 and 5.

The Anatune CF-100 centrifuge was evaluated for the centrifugation of protein precipitated whole blood samples, results of which proved favourable, even at 3000 rpm the small robotic centrifuge was capable of spinning down whole blood samples in preparation for LC-MS/MS analysis. See Figure 4.

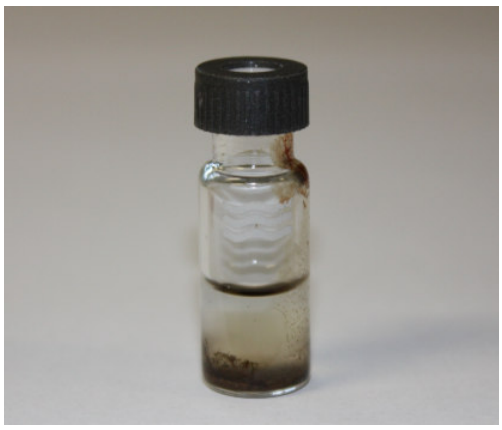

Figure 4 – Showing the whole blood sample following protein precipitation and centrifugation.

Optional Solid Phase Extraction Clean-up stage

In addition to the zinc sulphate and acetonitrile precipitation, a second method incorporating the ITSP C8 solid phase extraction (SPE) (Microliter, USA) was also investigated. Sample processing was identical to that mentioned previously except, prior to centrifugation 1000 µl of HPLC grade water was added to the extract. This was performed to ensure the extract was suitable for SPE. Following centrifugation, 1500 µl of sample extract was loaded onto a C8 SPE cartridge that had previously been solvated with 100 µl of 50/50 methanol/acetonitrile and equilibrated with 100 µl of water.
The cartridge was then rinsed with 100 µl of 30/70 methanol/water, before being purged with 500 µl of air to dry the cartridge. Analytes were finally eluted from the cartridge using 100 µl of 50/50 methanol/acetonitrile.

Calibration results from this second method using the Chromsystems calibrators are presented in Figure 5.

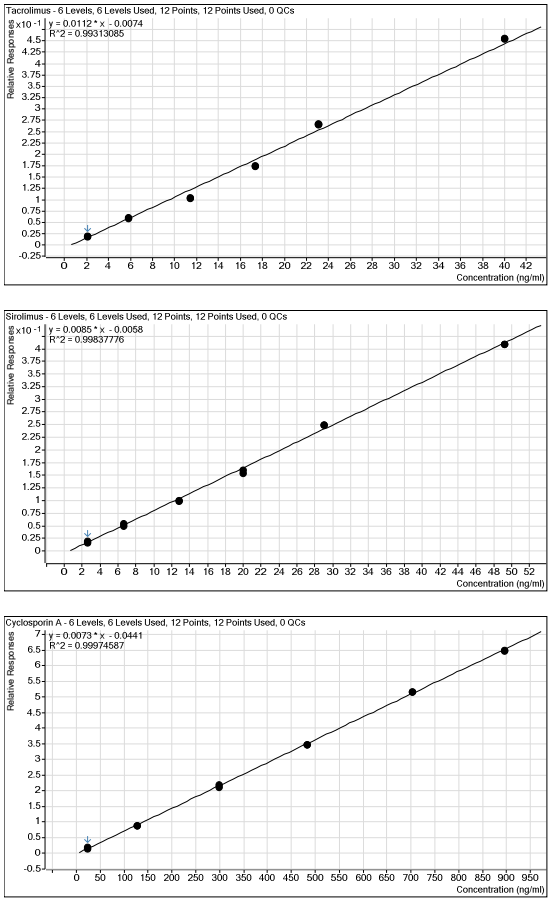

Figure 5 - Calibration curves for tacrolimus, sirolimus and cyclosporin A using the Chromsystems whole blood calibrators following SPE.

Linear calibrations were achieved for all three analytes. Correlation coefficients of 0.993, 0.998 and 0.999 were obtained for tacrolimus, sirolimus and cyclosporin A respectively.

In order to validate this additional SPE protocol, quality control materials were reconstituted, extracted and analysed in order to assess the suitability of the method, data is presented in table 6.

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	Tacrolimus				Sirolimus				Cyclosporin A			
Sample	QC Level 1	QC Level 2	QC Level 3	QC Level 4	QC Level 1	QC Level 2	QC Level 3	QC Level 4	QC Level 1	QC Level 2	QC Level 3	QC Level 4
Replicate_1	2.46	7.09	13.7	31.3	2.83	7.14	12.9	28.2	50.6	220	491	1123
Replicate_2	2.61	7.29	15.3	29.4	2.52	8.11	15.2	28.0	49.2	235	516	1235
Replicate_3	2.66	7.31	13.8	36.8	3.51	7.68	14.4	31.5	53.0	226	457	1199
Replicate_4	3.17	7.07	16.7	34.5	2.53	7.22	14.8	29.3	49.4	232	387	1138
Replicate_5	3.08	7.15	13.6	34.2	2.80	6.94	12.8	30.2	44.4	221	433	1096
Replicate_6	2.17	7.20	15.1	32.3	2.37	8.28	15.1	28.4	45.4	228	465	1041
Replicate_7	2.81	7.26	14.8	36.4	2.75	7.28	13.2	30.3	49.7	253	443	1106
Replicate_8	2.97	7.17	14.1	36.3	2.53	7.19	13.3	29.3	49.4	221	447	1185
Target Value	2.68	7.52	14.8	32.0	2.70	9.01	16.8	34.8	52.8	260	476	1047
Range	1.88-3.48	5.64-9.40	11.1-18.4	24.0-40.0	1.76-3.65	6.76-11.3	12.6-21.0	27.8-41.7	42.3-63.4	208-311	381-571	838-1256
Average	2.74	7.19	14.65	33.90	2.73	7.48	13.97	29.40	48.9	229	455	1140
Pass/Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
S.D	0.33	0.09	1.04	2.70	0.35	0.49	1.03	1.21	2.8	11.0	38.6	62.8
% RSD	12.20	1.24	7.11	7.96	12.92	6.54	7.34	4.13	5.6	4.8	8.5	5.5

Table 4 – Data for the Chromsystems quality control samples.

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	Tacrolimus			Sirolimus			Cyclosporin A		
Sample	QC Level 1	QC Level 2	QC Level 3	QC Level 1	QC Level 2	QC Level 3	QC Level 1	QC Level 2	QC Level 3
Replicate_1	3.91	8.25	12.9	4.84	10.1	15.2	62.6	132	235
Replicate_2	3.04	7.88	14.8	3.98	11.6	21.5	65.1	125	233
Replicate_3	3.56	5.28	12.7	2.83	12.1	15.4	66.8	131	235
Replicate_4	4.10	7.30	12.4	4.04	13.3	17.7	64.8	135	245
Replicate_5	3.26	7.15	13.1	2.72	10.7	19.5	61.2	120	237
Replicate_6	3.30	6.39	15.7	4.41	13.0	16.8	63.5	123	244
Replicate_7	3.17	7.50	12.3	4.74	12.3	19.2	61.9	138	244
Replicate_8	3.57	7.24	12.1	3.61	12.6	14.1	68.0	126	214
Target Value	3.28	6.67	13.3	3.64	11.2	18.9	62.5	132	258
Range	2.30-4.26	5.00-8.34	9.98-16.6	2.55-4.73	8.40-14.0	14.2-23.6	46.9-78.1	106-158	206-310
Average	3.49	7.12	13.3	3.90	12.0	17.4	64.3	129	236
Pass/Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
S.D	0.37	0.92	1.30	0.80	1.10	2.52	2.36	6.14	10.22
% RSD	10.54	12.94	9.79	20.55	9.18	14.44	3.67	4.77	4.33

Table 5 – Data for the Recipe quality control samples.

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Sample	Tacrolimus QC Level 1	Tacrolimus QC Level 2	Tacrolimus QC Level 3	Tacrolimus QC Level 4	Sirolimus QC Level 1	Sirolimus QC Level 2	Sirolimus QC Level 3	Sirolimus QC Level 4	Cyclosporin A QC Level 1	Cyclosporin A QC Level 2	Cyclosporin A QC Level 3	Cyclosporin A QC Level 4
Replicate_1	2.65	8.19	16.7	33.5	2.06	6.77	14.3	28.3	49.3	237	426	912
Replicate_2	2.84	7.32	14.3	32.3	2.31	6.76	13.2	28.2	51.0	222	413	990
Replicate_3	2.92	7.68	16.8	36.7	2.09	7.06	13.9	29.1	49.2	236	426	923
Replicate_4	2.89	8.25	14.2	36.1	2.02	7.02	12.9	28.8	48.6	257	415	939
Replicate_5	2.82	7.94	17.1	35.6	2.11	7.07	13.0	29.9	49.7	244	435	908
Replicate_6	3.20	6.58	15.1	39.6	1.66	6.88	13.6	24.4	47.8	225	432	1088
Replicate_7	2.55	7.75	17.4	39.8	1.98	7.15	13.4	38.8	48.5	242	455	928
Replicate_8	3.28	7.57	14.8	37.3	2.27	6.85	11.5	33.1	51.9	233	450	904
Target Value	2.68	7.52	14.8	32.0	2.70	9.01	16.8	34.8	52.8	260	476	1047
Range	1.88 -3.48	5.64-9.40	11.1-18.4	24.0 -40.0	1.76-3.65	6.76-11.3	12.6-21.0	27.8-41.7	42.3 - 63.4	208 - 311	381 - 571	838 - 1256
Average	2.89	7.66	15.8	36.4	2.06	6.95	13.2	30.1	49.5	237	431	949
Pass/Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
S.D	0.25	0.54	1.33	2.65	0.20	0.15	0.84	4.24	1.37	11.03	15.17	62.57
% RSD	8.53	7.01	8.39	7.29	9.66	2.16	6.38	14.11	2.77	4.65	3.52	6.59

Table 6 – Data for the Chromsystems quality control samples following C8 SPE.

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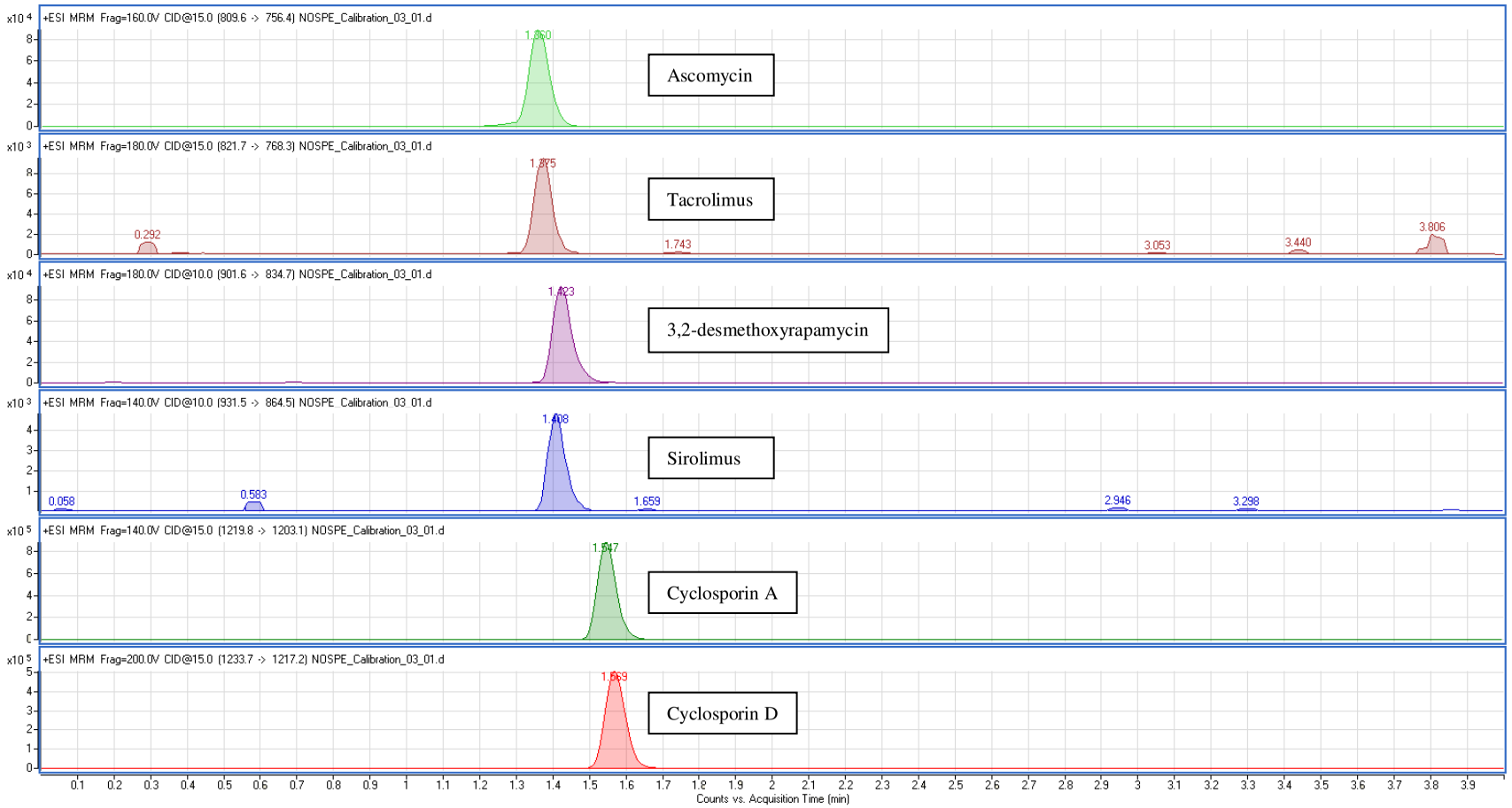

Figure 6 – Example chromatogram from the Chromsystems Standard 3 showing the internal standards and compounds of interest quantified as ammonium adducts.

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Conclusions

Presented is a fully automated method for immunosuppressant analysis featuring automated, internal standard and reagent addition, protein precipitation and centrifugation. Sample preparation is coupled directly to LC-MS/MS and is fully integrated within the Masshunter software. Alternatively it is possible to configure a standalone workstation. The system is capable of handling 98 samples in 8 hours and 22 minutes if using the simple extraction procedure without the SPE. Sample processing, time is under 4 minutes, fitting within the analytical runtime of the LC analysis ensuring highest possible throughput. See Figure 7.

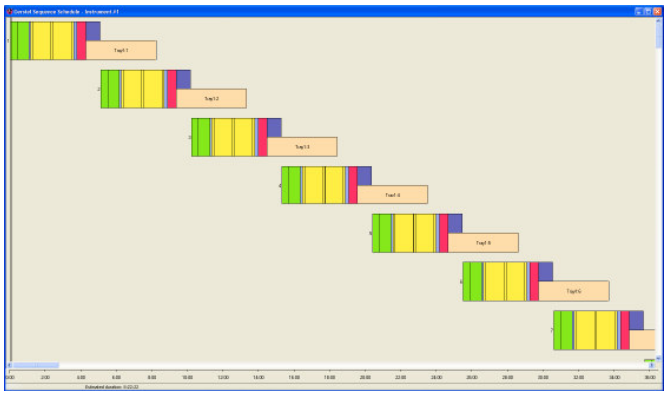

Figure 7 – Illustrating the Prepahead functionality of the automated system.

If cleaner extracts are required, potentially minimising source cleaning, the SPE method maybe employed. Greater levels of sensitivity are achieved using this method due to the concentration factors applied during sample preparation. One downside of the optional SPE step is the time required to prepare samples for analysis, sample processing time is 8 minutes and 45 seconds, which is double the time required to prepare the simple extracts, a batch of 98 samples would take 16 hours and 33 minutes.

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