DOSIMMUNE®: Fully automated analysis of immunosuppressant drugs in whole blood using stable isotope labeled internal standards

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1. Introduction

Immunosuppressant drugs are used to reduce activity of the immune system to prevent rejection of transplants. The major drugs used are calcineurin inhibitors, Cyclosporin A and Tacrolimus, and the mTOR inhibitors, Sirolimus and Everolimus. Circulating concentrations of these compounds should remain within a narrow therapeutic range, as overdosing can cause serious toxicity and long-term morbidity, and underdosing can cause rejection. As immunosuppressant drugs result in a high pharmacokinetic variability between individual patients TDM is now an established approach to mitigate the risks associated organ transplantation.

Several commercial immunoassays are available for the TDM of immunosuppressant's, however, all immunoassays show a significant positive bias compared to LC-MS/MS methods. Despite the availability of automated immunoassays each test is restricted to one analyte for each test when in many clinical settings multiple immunosuppressants are used in one individual patient. In this example, an automated LC-MS/MS method is described for the routine TDM analysis of immunosuppressants.

2. Method

The analysis of immunosuppressant drugs was performed using a fully automatic sample preparation platform integrated with LC-MS/MS detection (CLAM-2000 & LCMS-8050). To create a method for routine clinical pathology the reagent kit DOSIMMUNE® (Alsachim, France) was used.

Hemolyzed whole blood sample was loaded directly into the automated sample preparation system. The treated samples were trapped and then separated at 65 °C with an isocratic mode at a flow rate of 0.8 mL/min in 1.4 min.

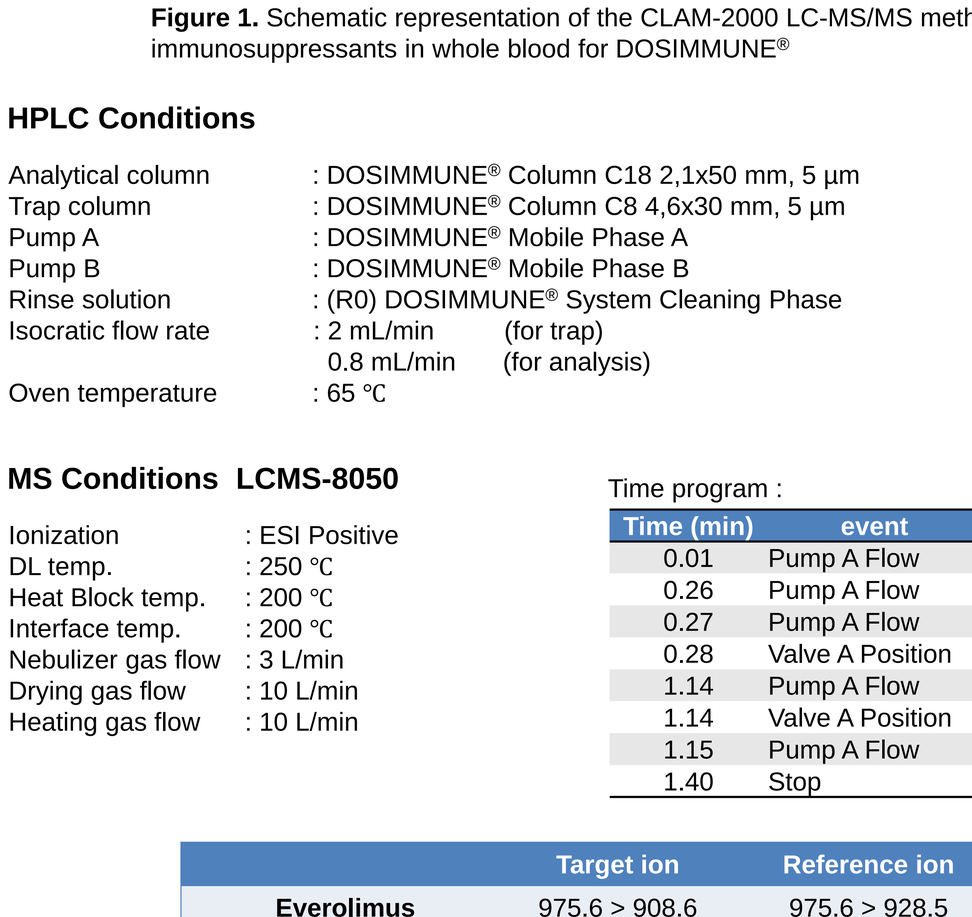

Figure 1. Schematic representation of the CLAM-2000 LC-MS/MS method for immunosuppressants in whole blood for DOSIMMUNE®

HPLC Conditions

Analytical column	: DOSIMMUNE® Column C18 2,1x50 mm, 5 µm
Trap column	: DOSIMMUNE® Column C8 4,6x30 mm, 5 µm
Pump A	: DOSIMMUNE® Mobile Phase A
Pump B	: DOSIMMUNE® Mobile Phase B
Rinse solution	: (R0) DOSIMMUNE® System Cleaning Phase
Isocratic flow rate	: 2 mL/min (for trap) 0.8 mL/min (for analysis)
Oven temperature	: 65 °C

MS Conditions LCMS-8050

Ionization	: ESI Positive
DL temp.	: 250 °C
Heat Block temp.	: 200 °C
Interface temp.	: 200 °C
Nebulizer gas flow	: 3 L/min
Drying gas flow	: 10 L/min
Heating gas flow	: 10 L/min

Time program :

Time (min)	event	
0.01	Pump A Flow	2 mL/min
0.26	Pump A Flow	2 mL/min
0.27	Pump A Flow	0.02 mL/min
0.28	Valve A Position	1
1.14	Pump A Flow	0.02 mL/min
1.14	Valve A Position	0
1.15	Pump A Flow	2 mL/min
1.40	Stop	

	Target ion	Reference ion
Everolimus	975.6 > 908.6	975.6 > 928.5
[$^{13}C_2$,$^{2}H_4$]-Everolimus	981.6 > 914.5	981.6 > 932.6
Sirolimus	931.5 > 864.4	931.5 > 882.3
[^{13}C,$^{2}H_3$]-Sirolimus	935.5 > 864.5	935.5 > 882.5
Tacrolimus	821.5 > 768.3	821.5 > 576.2
[^{13}C,$^{2}H_4$]-Tacrolimus	826.5 > 768.3	826.5 > 581.4
Cyclosporin A	1219.8 > 1202.9	1202.7 > 425.0
[$^{2}H_{12}$]-Cyclosporin A	1231.7 > 1214.9	1231.7 > 1196.8

Table 1. MRM transition **[M+NH_4]$^+$** of immunosuppressants and stable isotope labeled internal standards

3. Result and discussion

3-1. Validation method for Immunosuppressant drugs

Therapeutic drug monitoring (TDM) has become a key clinical tool to help individualize therapy, check compliance and maximize response while lowering side effects. Liquid chromatography-tandem mass spectrometry has become a major technology in TDM given its inherent specificity, sensitivity and quantitative capability for small molecule drug analysis. Within the context of a routine clinical pathology environment there are considerable advantages in integrating mass spectrometry into small molecule drug monitoring when compared to immunoassays. For high throughput assays, the design of the sample preparation method also takes into account automated systems. One such example is the Clinical Laboratory Automated sample preparation Module (CLAM-2000) integrated with LC-MS/MS (LCMS-8050).

Samples preparation for manual handling

1. 50 µL of samples/calibrators in 1.5 mL microtube
2. Add 25 µL of Internal Standard
3. Add 350 µL of Extraction buffer
4. Shake for 1 min
5. Centrifuge at 15,000 g during 7 min
6. Transfer 200 µL of supernatant to vial

Samples preparation for CLAM-2000

1. Take 20 µL of IPA/H_2O (75/25) to sample cup
2. Add 20 µL of samples/calibrators
3. Add 150 µL of Extraction buffer
4. Add 12.5 µl of Internal Standard
5. Shake for 1 min at 1,900 rpm
6. Filtrate for 1 min

The linearity and accuracy of the method was evaluated using 6 calibrators levels. Calibration curves were linear over the concentration range studied for all compounds (2 to 35 µg/L for Tacrolimus, Sirolimus and Everolimus; 25 to 1800 µg/L for Cyclosporin A). The linear regression analysis was typically R^2>0.998. For 4 drugs linearity and accuracy were within the analytical acceptable range and showed accuracies between 90 and 110%.

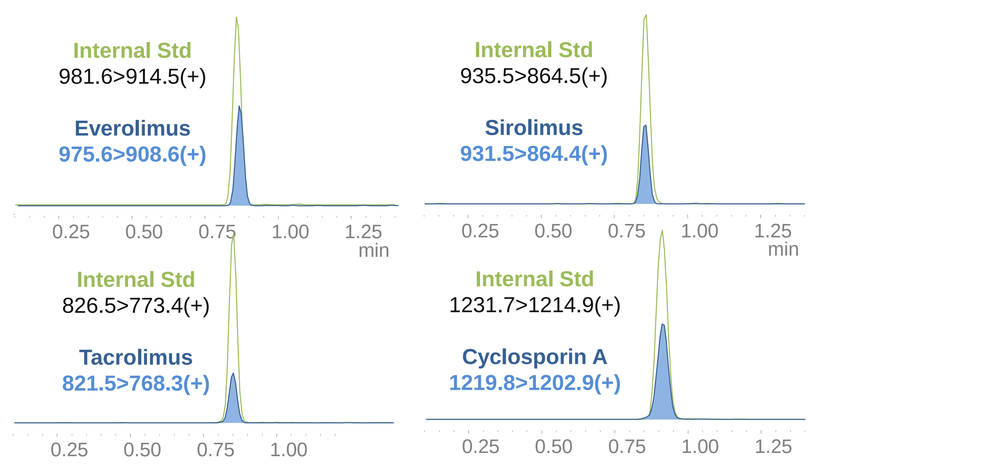

Figure 2. MRM chromatograms of each immunosuppressant and internal standard

Compounds	Everolimus				Sirolimus				Tacrolimus				Cyclosporin A			
Levels	**QC 1**	**QC 2**	**QC 3**	**QC 4**	**QC 1**	**QC 2**	**QC 3**	**QC 4**	**QC 1**	**QC 2**	**QC 3**	**QC 4**	**QC 1**	**QC 2**	**QC 3**	**QC 4**
Target conc. (µg/L)	4.4	8.7	13.4	26.5	4.9	8.8	13.4	26.7	4.4	8.7	12.9	26.7	49.0	174.0	706.3	1300.2
Average conc. (µg/L)	4.3	7.5	13.0	27.5	4.9	8.9	14.0	28.6	4.5	8.7	14.3	25.4	49.6	194.3	782.2	1370.2
CV (%)	**13.1**	**12.4**	**10.6**	**13.7**	**16.5**	**14.7**	**6.7**	**11.2**	**13.2**	**7.7**	**8.0**	**10.9**	**4.4**	**4.9**	**1.7**	**4.1**
Accuracy (%)	**96.7**	**86.8**	**96.7**	**103.9**	**99.5**	**100.6**	**104.7**	**107.3**	**102.4**	**110.7**	**10.7**	**95.2**	**101.2**	**111.7**	**110.7**	**105.4**

Table 2. Repeatability (n=5) of 4 immunosuppressants using LCMS-8050 with CLAM-2000

3-2. Comparative studies between LC-MS/MS and Immunoassay

Patient blood samples undergoing treatment with Tacrolimus were determined by immunoassay and CLAM-2000 & LCMS-8050. The agreement between both methods is shown in figure 3.

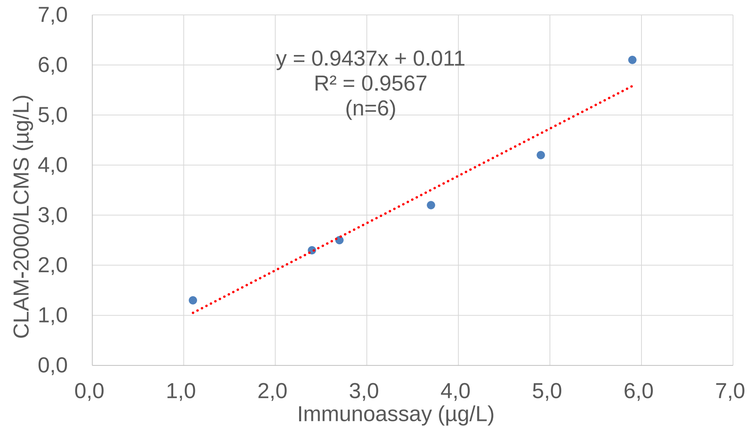

Figure 3. Patient sample comparison between LC-MS/MS and Immunoassay

4. Conclusion

- Simple sample preparation protocol and using stable isotope labeled IS are increased data quality and throughput.
- Excellent correlation exists comparison between LC-MS/MS and immunoassay.
- The automation of the method increases the analytical performance, reduces the risk for human operators, and due to the reduced reagent consumption, reduces also the cost of the analysis.