SHIMADZU
Excellence in Science

PO-CON1787E

Fully automated sensitive determination of immunosuppressant drugs in whole blood, using high quality internal standardization

IATDMCT 2017

Eishi IMOTO[1], Aurore JAFFUEL[2], Fanny DAYOT[3], Jean-François HOEFFLER[3], Alban HUTEAU[2]
[1]SHIMADZU Corporation, Kyoto, Japan,
[2]SHIMADZU France, Noisiel, France,
[3]ALSACHIM,Illkirch, France

ALSACHIM

Fully automated sensitive determination of immunosuppressant drugs in whole blood, using high quality internal standardization

Introduction

Measurement of immunosuppressant drugs (Figure 1.) is essential during organ transplantation. Under-dosing can lead to organ rejection, while over-dosing can cause serious toxicity. Traditional methods to measure immunosuppressant drugs in whole blood are based on either immunoassays or chromatography. Immunoassays, though, are affected by matrix interferences and lack of specificity. LC-MS/MS has then become the gold standard due to its specificity, precision and sensitivity. However, it has still one major drawback: current LC-MS/MS platforms demand personnel with expertise and, for whole blood samples, tedious sample preparation. As a consequence, sample throughput is generally much lower than for immunoassays. We here report a fully automated procedure for the quantitation of four major immunosuppressant in whole blood samples, using of 13C labelled internal standards.

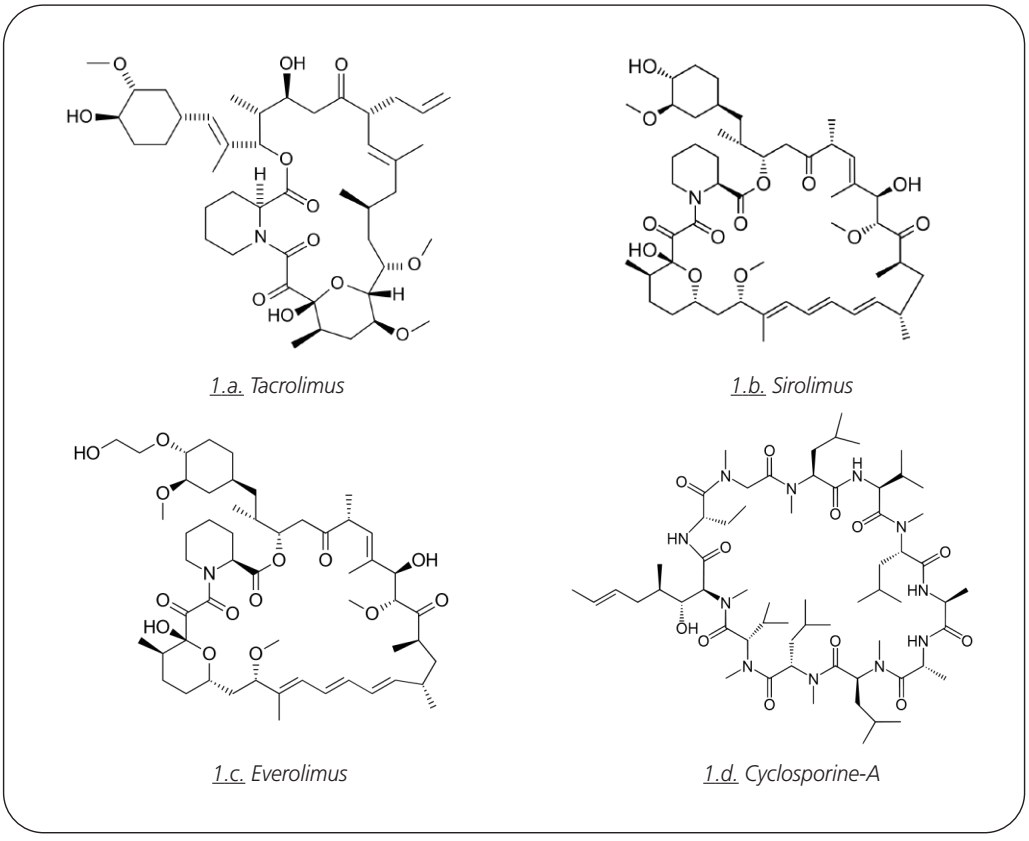

Figure 1: Structures of tacrolimus (a), sirolimus (b), everolimus (c), and cyclosporine-A (d)

Fully automated sensitive determination of immunosuppressant drugs in whole blood, using high quality internal standardization

Method and Materials

The quantitative analysis of Immunosuppressant (Figure 2.) was performed using reagents provided in Alsachim Dosimmune® kit. The Immunosuppressant and Internal standard were monitored using UHPLC-MS/MS system (Nexera X2 and LCMS-8050, Shimadzu, Kyoto). Sample preparation was performed using extraction buffer and internal standard set provided in Alsachim Dosimmune® kit. Analytical performance of the method was monitored using whole blood calibrators and whole blood QC. Automatic sample preparation was performed using CLAM-2000 module (Shimadzu, Kyoto).

Figure 2: Sample workflow overview.

Sample preparation : CLAM-2000 and Dosimmune® kit

Whole blood sample tube is placed into the CLAM-2000 system : (1) 25µL of whole blood sample is mixed with 12.5µL of Internal standard and 175µL of extraction buffer solution, (2) followed by a 30s stirring and (3) a 1min filtration. The extracted sample is transferred to the autosampler of the Nexera X2 system and injected immediately.

UHPLC conditions : Nexera X2 and Dosimmune® kit

Analytical column	: Ascentis® C18 2,1x50 mm, 5 µm
Trap column	: Ascentis® C8 4,6x30 mm, 5 µm
Injection volume	: 20 µL
Mobile Phase A	: 90% 3mM Ammonium formate (pH=3.6) 10% MeOH
Mobile Phase B	: 10% 3mM Ammonium formate (pH=3.6) 90% MeOH
Isocratic flow rate	: Mobile Phase A: 2 mL/min (trap column), Mobile Phase B: 0.8 mL/min (analytical column)
Oven temperature	: 65°C

MS conditions : LCMS-8050

Nebulizing Gas	: 3 L/min (N2)	HESI	: 200°C	Pause time	: 1 msec
Heating Gas	: 10 L/min (Air)	DL	: 250°C	Polarity switching	: 5 msec
Drying Gas	: 10 L/min (N2)	HB	: 200°C	Points per peak	: > 30

Fully automated sensitive determination of immunosuppressant drugs in whole blood, using high quality internal standardization

Table 1: Formula, exact mass and MRM transition for each compound

Compound	Formula	Monoisotopic Mass	MRM
Everolimus	$C_{53}H_{83}NO_{14}$	957,6	975,6 → 908,5
Everolimus $^{13}C_2d_4$	$C_{51}{}^{13}C_2H_{79}D_4NO_{14}$	963,6	981,5 → 914,5
Sirolimus	$C_{51}H_{79}NO_{13}$	913,5	931,6 → 864,5
Sirolimus $^{13}Cd_3$	$C_{50}{}^{13}CH_{76}D_3NO_{13}$	917,5	935,4 → 864,5
Tacrolimus	$C_{44}H_{69}NO_{12}$	803,5	821,5 → 768,6
Tacrolimus $^{13}Cd_4$	$C_{43}{}^{13}CH_{67}D_4NO_{12}$	808,5	826,4 → 773,6
Ciclosporine	$C_{62}H_{111}N_{11}O_{12}$	1201,8	1219,9 → 1202,8
Ciclosporine d_{12}	$C_{62}H_{99}D_{12}N_{11}O_{12}$	1213,8	1231,8 → 1214,9

Results

Method conditions

The method enables the quantification of tacrolimus, sirolimus, everolimus and cyclosporine-A in whole blood samples. The established quantification strategy for this compounds is to use internal calibration using deuterium labeled standards. However, they generally suffer from poor isotopic enrichment, leading to overestimation of the unlabeled form. We here use 13C labeled internal standards for tacrolimus, sirolimus and everolimus. This guaranties better isotopic enrichment, better precision of the results, long term stability of the standards and perfect co-elution with the analytes, leading to a better correction of matrix effects. Linearity was confirmed in the range 0.5 to 40 ng/mL for tacrolimus, sirolimus, everolimus, and in the range 5 to 1500 ng/mL for cyclosporine-A (Figure 3.). For all analytes, r^2 of linearity models was above 0.99, with S/N > 25 for LLOQ levels (Figure 4.). Controls showed accuracies comprised in between 85 and 115% for all analytes (Table 2.).

Calibration in whole blood

Linearity was confirmed, in whole blood, in the range 0.5-40 ng/mL for tacrolimus (Figure 3.a.), sirolimus (Figure 3.b.), everolimus (Figure 3.c.), and 5-1500 ng/mL for cyclosporine-A (Figure 3.d.).

Fully automated sensitive determination of immunosuppressant drugs in whole blood, using high quality internal standardization

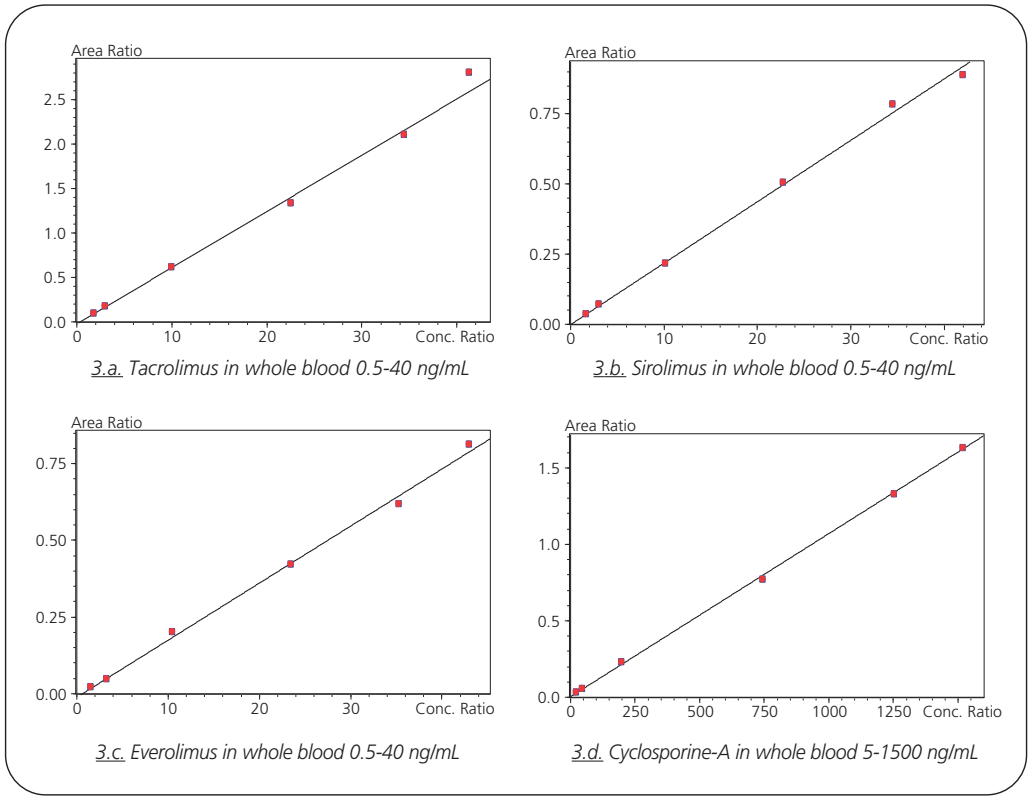

Figure 3: Calibration curves for tacrolimus (a), sirolimus (b), everolimus (c), and cyclosporine-A (d), in whole blood.

Limits of quantification in whole blood

The limits of quantification (LLOQ), in whole blood, are 0.5 ng/mL for tacrolimus, sirolimus, and everolimus, and 5 ng/mL for cyclosporine-A. The signal to noise ratio is above 25 at LLOQ levels.

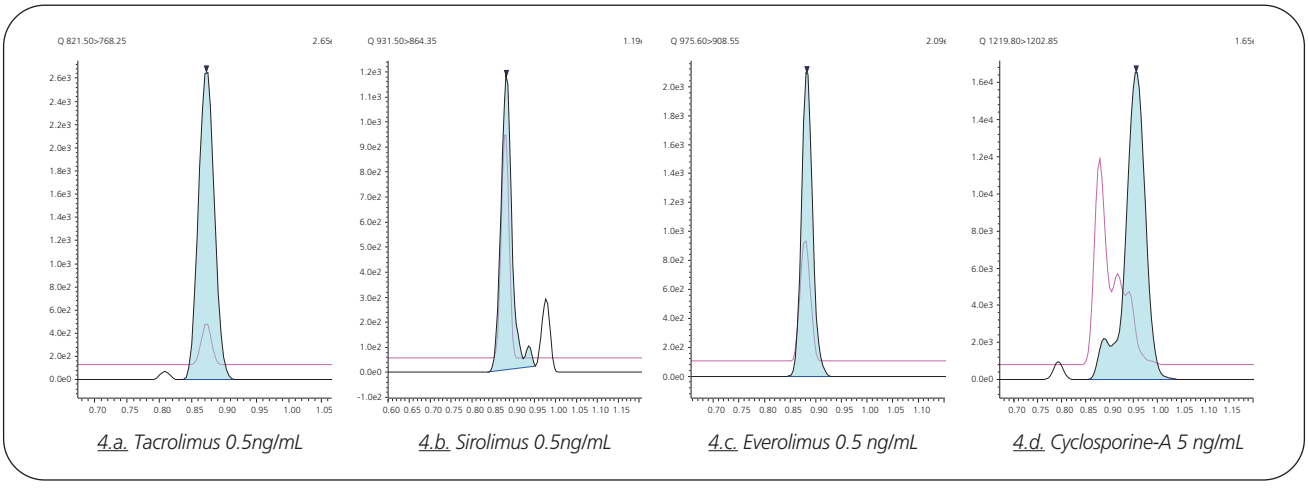

Figure 4: MRM chromatograms, at LLOQ levels, for tacrolimus (a), sirolimus (b), everolimus (c), and cyclosporine-A (d), in whole blood.

SHIMADZU
Excellence in Science

Fully automated sensitive determination of immunosuppressant drugs in whole blood, using high quality internal standardization

Performance Evaluation

Whole blood controls showed accuracies comprised in between 85% and 115% for all analytes.

Table 2: Whole blood control samples accuracies.

Tacrolimus	
Conc. (µg/mL)	Accuracy (%)
2.9	114
5.4	108
13.3	87
40.5	95

Sirolimus	
Conc. (µg/mL)	Accuracy (%)
3.2	115
5.7	105
13.3	93
40.6	103

Everolimus	
Conc. (µg/mL)	Accuracy (%)
3.2	114
5.8	92
13.4	85
42.1	94

Cyclosporine-A	
Conc. (µg/mL)	Accuracy (%)
36.1	98
223.4	106
454.6	85
1693	95

Conclusion

Fully automated sensitive quantification of immunosuppressant drugs in whole blood, using high quality 13C labelled internal standards, increasing data quality, throughput and safety.

For Research Use Only. Not for use in diagnostic procedures. Not available in the USA, Canada, and China.

First Edition: November, 2017

SHIMADZU

Shimadzu Corporation

www.shimadzu.com/an/