Fully automated platform for determination of immunosuppressant drugs in whole blood

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Introduction

Therapeutic drug monitoring of four major immunosuppressant drugs, Cyclosporine A, Tacrolimus, Sirolimus and Everolimus, is well established. Overdosing with these critical dose drugs can cause serious toxicity and long term morbidity, while organ rejection can occur if a patient is under dosed. Nowadays clinical laboratory has two main choices in technologies: immunoassay or chromatography based methods. LC–MS/MS's superior specificity makes it the presumptive gold standard in immunosuppressant quantitation. It relieves the method

from common interferences that plague immunoassays such as metabolites that have structural resemblance and interfering antibodies. However, current LC–MS/MS platforms demand personnel expertise and tedious sample preparation and sample throughput is generally much lower compared to immunoassays. We report a fully automated procedure for the quantitation of four major immunosuppressant in whole blood samples, increasing data quality/precision, throughput and safety (The work described herein is for research use only).

Methods

The quantitative analysis of Immunosuppressant was performed using reagents provided in Chromsystems "MassTox®" ONEMinute Kit (ref, 93900). The Immunosuppressant and Internal standard were monitored using UHPLC-MS/MS system (Nexera X2 and LCMS-8050, Shimadzu, Kyoto, Figure 1)).

Sample preparation was performed using Precipitation reagent, Extraction buffer and Internal standard set. 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 1.

Figure 1:   CLAM-2000 online with Nexera X2 system and LCMS-8050 triple quadrupole mass spectrometer.

Result and discussion

LC-MS/MS analysis

The Immunosuppressant standards and the Internal were firstly analysed by flow injection to optimize mass spectrometer parameters. All compounds were detected as positive ion choosing the MRM transitions listed in Table 2. Then Immunosuppressant standard mix was used to set-up chromatographic separation (Table 1).

Table 1:   Analytical Condition

[LC] NexeraX2 System	
Column Temp.	: 65 °C
Time Program	: 0.3 min (trap load); 1.5 min (elution); 2.3 min (stop)
Injection Volume	: 5 µL
[MS] LCMS-8050	
Ionization	: ESI Positive
Nebulizer Gas	: 3 L/min
Interface temperature	: 300 °C
Desolvation Line	: 250 °C
Heat Block temperature	: 400 °C
Drying Gas	: 10 L/min
Scan Type	: MRM

Table 2:   MRM Transitions

Compound	MRM transition
Cyclosporin A	1219.90 > 1202.80
Tacrolimus	821.60 > 768.30
Sirolimus	931.70 > 864.50
Everolimus	975.70 > 908.50
Cyclosporin A-d$_{12}$	1231.90 > 1214.80
Everolimus-d$_4$	979.60 > 912.40
Sirolimus-d$_3$	934.60 > 864.40
Tacolimus-^{13}Cd$_2$	824.60 > 771.40

Fully Automated sample preparation

The CLAM-2000 was programmed to perform sample extraction and protein precipitation followed by filtration and sample collection. The filtrated sample was then automatically transported using an arm from the CLAM-2000 to the HPLC for LC-MS/MS analysis and no human intervention was required (Fig 2).

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Parallel/Sequential sample preparation and analysis

Reagent Dispensing	Reagent Dispensing	Sample Dispensing	Reagent Dispensing	Stirring	Incubation	Reagent Dispensing	Stirring	Filtration	Direct UHPLC connection for LCMS analysis (2.3 min)
MetOH 20 ul	Extr. Buffer 50 ul	Whole blood 25 ul	Internal STD 12.5 ul	2200 rpm	Room temperature	Prec. Reagent 125 ul	2200 rpm	Aspiration	

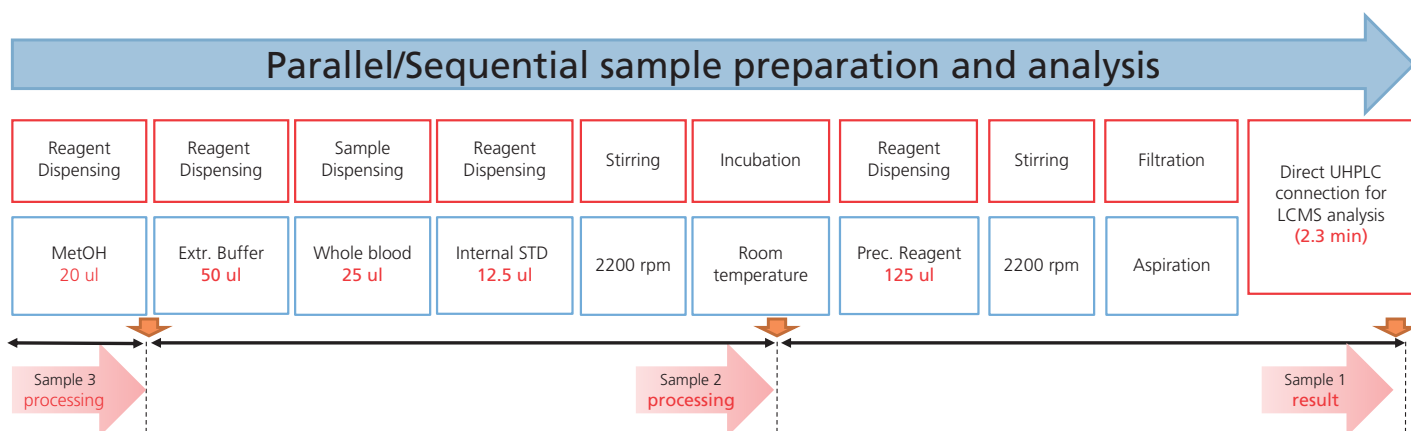Sample 3 processing

Sample 2 processing

Sample 1 result

Figure 2: CLAM2000 fully automated sample preparation and analysis.
Due to the overlapped sample preparation the throughput of the instrument was
1 result each 3.7 minutes for Immunosuppressant quantification.

Linearity, Accuracy and Precision

Linearity and Accuracy were evaluated using reference whole blood calibrators (7 levels) spanning from a wide range of concentrations (Cyclosporin 16.5 – 1100 µg/mL, Everolimus 0.86 – 32.7 µg/mL , Sirolimus 0.9 – 32.7 µg/mL, Tacrolimus 0.96 – 34.8 µg/mL). For all analytes

Linearity was >0.99 (see Table 3) with S/N >25 for LLOQ levels (Figure 3). Precision of the assay was evaluated using Chromsystems reference materials (whole blood controls levels) spanning from low concentration to highest concentration for each analyte (Table 4).

Table 3: Linearity & Accuracy.

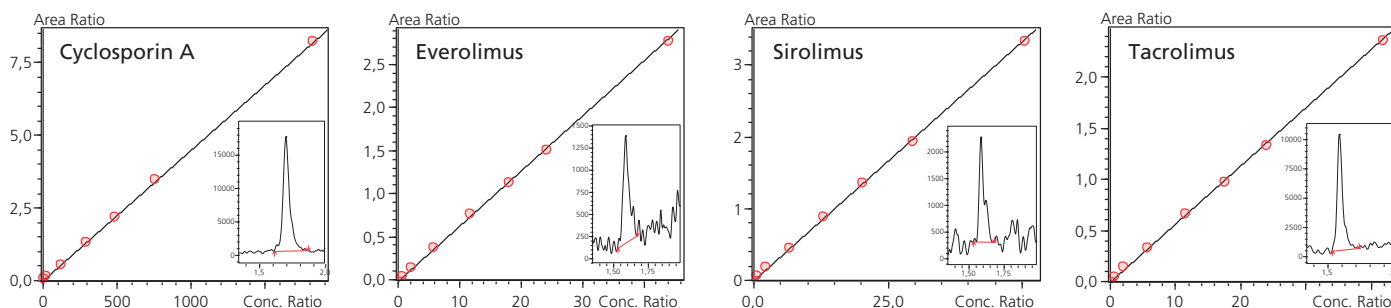| Compound	Accuracy	r^2
Cyclosporin A	90.4%-103.6%	0.998
Tacrolimus	99.7%-101-3%	0.997
Sirolimus	95.1%-105.5%	0.998
Everolimus	96.9%-107%	0.998

Figure 3: Linearity and LLOQ.

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Table 4: Repeatability, Reproducibility , Accuracy, evaluated using Chromsystems reference controls.
* n=7 . ** n=3 non consecutive days with instrument shutdown.

	Ref. Level (ug/L)	CV% (intra-assay)*	BIAS% (intra-assay)*	CV% (inter-assay)**	BIAS% (inter-assay)**
Cyclosporin	Low (16.5)	6.0%	1.70%	7.9%	1.68%
	Mid (49.7)	2.2%	1.33%	4.3%	1.22%
	High (1100)	1.83%	2.56%	5.8%	3.08%
Tacrolimus	Low (2.86)	3.29%	2.95%	5.6%	3.58%
	Mid (7.73)	2.69%	8.00%	4.7%	12.33%
	High (34.8)	2.76%	1.85%	4.4%	6.57%
Sirolimus	Low (2.76)	1.02%	2.68%	3.7%	5.59%
	Mid (9.89)	2.44%	12.18%	4.1%	14.77%
	High (32.7)	2.59%	6.01%	4.9%	12.99%
Everolimus	Low (2.6)	2.04%	4.54%	8.9%	6.44%
	Mid (4.79)	5.98%	7.16%	8.2%	11.77%
	High (32.7)	2.55%	4.75%	3.3%	7.64%

Methods correlation

Real samples (EDTA whole blood from patients treated with Tacrolimus) were analyzed using both manual sample preparation and fully automated sample preparation (CLAM-2000). The Tacrolimus concentration obtained by the two sample preparation methods shown a good agreement (Figure 4 B). Moreover the Tacrolimus concentration in 59 whole blood real samples was evaluated with Immunoassay and CLAM-2000 LC-MS/MS approaches. As shown by Passing and Bablok plots (Figure 4 A) there was a good agreement between the two different methods of quantification.
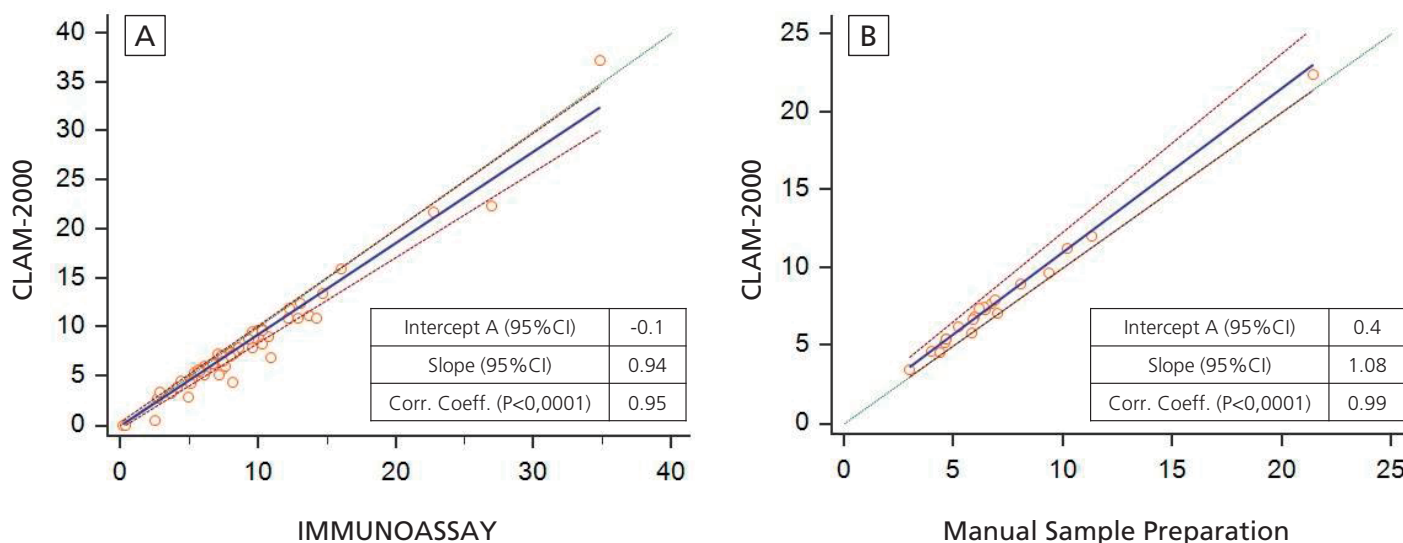

Intercept A (95%CI)	-0.1
Slope (95%CI)	0.94
Corr. Coeff. (P<0,0001)	0.95

Intercept A (95%CI)	0.4
Slope (95%CI)	1.08
Corr. Coeff. (P<0,0001)	0.99

Figure 4: Methods comparison. A) n=59 real samples Tacrolimus. B) n=20 real samples Tacrolimus.

Conclusions

- Fully Automated sample preparation procedure resulted suitable for the quantitation of Immunosuppressant by elimination of all manual preparation steps.
- 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.

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