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High Throughput Quantification of Immunosuppressant Drugs in Human Blood by Liquid Chromatography - Tandem Mass Spectrometry for Clinical Research

Magnus Olin, Thermo Fisher Scientific, Hägersten, Sweden; Victoria Barclay, Anna Hansson, Sara Bildsten, Miguel Gambell-Barroso, Louise Gustafsson, Clinical Pharmacology, Karolinska University Hospital, Huddinge, Sweden

ABSTRACT

Purpose: To perform analytical validation of a method for quantification of immunosuppressant drugs (Cyclosporin A, Tacrolimus, Everolimus and Siroliumus) in human whole blood for clinical research.

Methods: The method is based on protein precipitation of whole blood followed by direct injection into a two-channel UHPLC system coupled to a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer equipped with a heated electrospray ion source.

Results: Results were evaluated using Thermo Scientific™ TraceFinder™ software. The method performance was evaluated by testing accuracy and precision, selectivity, carry over, and linearity in the calibrated range.

INTRODUCTION

The analytical validation of a clinical research method for the quantification of four immunosuppressant drugs (Cyclosporin A (CyA), Tacrolimus (TAC), Sirolimus (SIR) and Everolimus (EVE) in whole blood is reported. The present method allows low-level quantitation (CyA 10 ng/mL, TAC, SIR, EVE at 0.5 ng/mL), which makes it especially suitable for clinical research studies. At the same time, the method was adapted for high throughput analysis using multi-channeling, which delivers results in *less than one minute* per injection. Method performance was evaluated in terms of linearity of response within the calibration ranges, selectivity, intra- and inter-assay accuracy, intra- and inter-assay precision and carry-over for each analyte.

MATERIALS AND METHODS

Test Samples

The method was evaluated using the MassTox® calibrators and Quality Control Samples from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). An additional level for both calibrator and Quality Control sample at LLOQ was obtained by dilution of a low-level Quality Control sample with blank whole blood, providing calibration samples at 7 levels and Quality Control samples at 5 levels. The calibrated range was 10 -1950 ng/mL (CyA), 0.5-42 ng/mL (EVE, TAC) and 0.5 – 47 ng/mL (SIR). The nominal concentrations of the Calibrators and Test samples are presented in Table 1 and 2. Five batches of data were collected on two identical LC-MS/MS systems.

Table 1. Nominal concentrations of Calibrators

Compound	Cal0.5*	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6
EVE	0.534	2.18	5.9	11.3	16.9	23.5	41.6
SIR	0.578	2.25	6.44	12.4	18.6	28.2	47
TAC	0.546	2.18	6.64	12	18	24.4	42.4
CYA	9.88	24.1	123	296	492	783	1941

*Cal 0.5 was obtained by dilution of 1 part of QCI with 4 parts of blank blood

Table 2. Nominal concentrations of Test Samples (ng/mL)

Compound	QC0.5*	QCI	QCII	QCIII	QCIV
EVE	0.534	2.46	4.71	9.48	33.7
SIR	0.578	2.58	9.2	19.1	39.3
TAC	0.546	2.56	7.39	15.8	34.9
CYA	9.88	47.5	225	474	1202

*QC0.5 was obtained by dilution of 1 part of QCI with 4 parts of blank blood

Sample Preparation

Whole blood was extracted by off-line protein precipitation and internal standard addition into a 96-well plate by an automated procedure using a Hamilton™ robotic liquid handler[1]. Samples were positioned in Hamilton sample racks and placed on a large size blood rocker and agitated for 2 min. 85 µL aliquots of samples were pipetted into the wells followed by 85 µL of water and 300 µL of internal standard working solution. The internal standard working solution consisted of Methanol / 0.4 M $ZnSO_4$ in water (80/20) with internal standard concentrations of 20 ng/mL (CyA) and 2 ng/mL (TAC, EVE, SIR). The plate was sealed and vigorously shaken on a vortex mixer (Multi TubeVortexer, VWR, Radnor, PA) for 5 min. The plate was allowed to rest for 2 min and was then centrifuged for 5 min at 2050 ×g. The plate was then transferred to the autosampler and kept at 12ºC pending analysis.

LC-MS/MS Method

The supernatant was injected onto a Thermo Scientific™ Transcend II™ system connected to a TSQ Quantis triple quadrupole mass spectrometer equipped with a heated electrospray ion source (HESI), operated in positive ion mode. The LC column used was a Thermo Scientific™ Accucore™ RP-MS 2.1 x 30 mm. Mobile phase A contained methanol/water (50/50) and 2mM ammonium formate + 0.1% formic acid. Mobile phase B contained methanol and 2 mM ammonium formate + 0.1% formic acid. The analytes were eluted using a step gradient starting at 100 % A (50%methanol), elution at 80% B (90%methanol) followed by a wash step using 100% B and an equilibration step using 100% A. The LC method is described in detail in Table 3. The system was used in multi-channel mode, and the cycle time was less than 2 min/channel providing results from one injection in less than one minute.

Table 3. LC Method

Time (min)	Flow mL/min	Gradient	%A	%B
0	1	Step	100	0
0.5	0.6	Step	20	80
1	1	Step	0	100
1.4	1	Step	100	0
1.8	1	Step	100	0

Mobile phase A: Methanol/Water + 2mM ammonium formate + 0.1% formic acid.

Mobile phase B: Methanol + 2 mM ammonium formate + 0.1% formic acid.

Detection was performed by selected reaction monitoring (SRM) using one isotopically labeled internal standard with mass difference of at least four amu for each compound. The ammonium adducts were used as precursors for all analytes and internal standards. One transition was acquired for quantitation, and one transition was monitored as a qualifying transition. The ion source parameters are presented in Table 4, and the mass spectrometer settings are presented in Tables 5 and 6.

Table 4. Ion Source Settings

	Setting
Spray Voltage (V)	3000
Sheath Gas (arb)	70
AUX gas (arb)	10
Sweep gas (arb)	2
Ion Transfer Tube temp (C)	300
Vaporizer temp (C)	350

Table 5. General Mass Spectrometer Settings

	Setting
Cycle Time (s)	0.25
Q1 Resolution (FWHM)	0.7
Q3 Resolution (FWHM)	1.2
CID gas (arb)	2
Source Fragmentation (V)	0
Chromatographic Peak width (s)	3

Table 6. Mass Spectrometer Settings - SRM Transitions

Compound	Precursor (m/z)	Product (m/z)	Collision Energy (V)	Min Dwell Time (ms)	RF Lens (V)
Tacrolimus Quan	821.5	768.4	19	18.923	186
Tacrolimus Qual	821.5	786.4	15	18.923	186
Tacrolimus 13CD4	826.5	773.4	19	18.923	186
Sirolimus Quan	931.5	864.4	16	18.923	202
Sirolimus Qual	931.5	882.4	11	18.923	202
Sirolimus 13CD3	935.5	864.4	16	18.923	202
Everolimus Quan	975.5	908.4	16	18.923	207
Everolimus Qual	975.5	926.4	11	18.923	207
Everolimus13C2D4	981.6	914.5	16	18.923	207
Cyclosporin A Quan	1219.7	1202.6	16	18.923	212
Cyclosporin A Qual	1219.7	1184.6	32	18.923	212
Cyclosporin A D12	1231.8	77.777	16	18.923	212

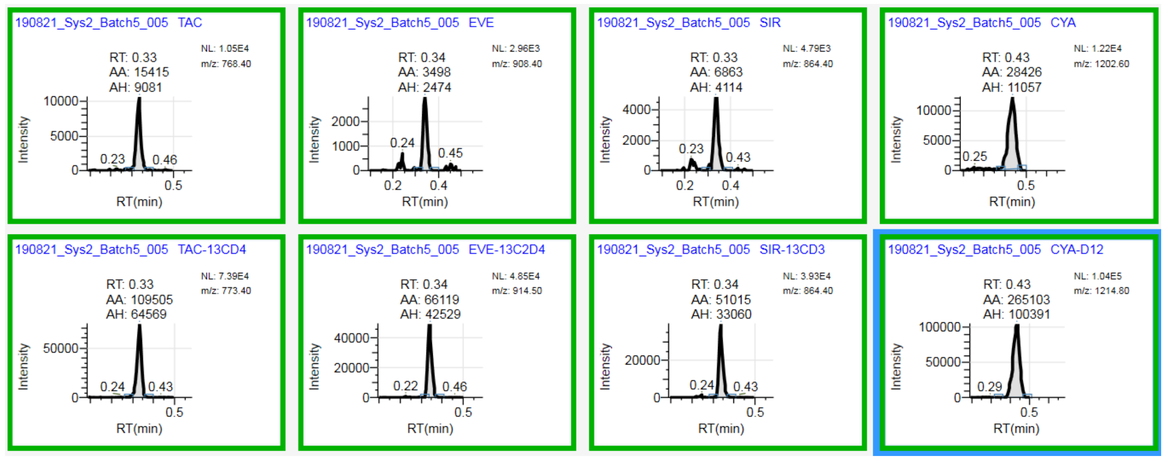
Data Analysis

Data was acquired and processed using TraceFinder 4.1 software. No manual integrations were used in the evaluation. No samples were omitted from evaluation except a few blank samples where sample had run out.

RESULTS

A typical chromatogram is shown in Figure 1. Please note that in this multichannel approach, the retention time is not recorded from the injection but from the start of the data window. The data window starts at 0.5 minutes and thus the actual retention times are 0.5 minutes longer than depicted in the figure. With multi-channeling, there is a 58-second interval between injections and thus the method delivers results in less than one minute per sample.

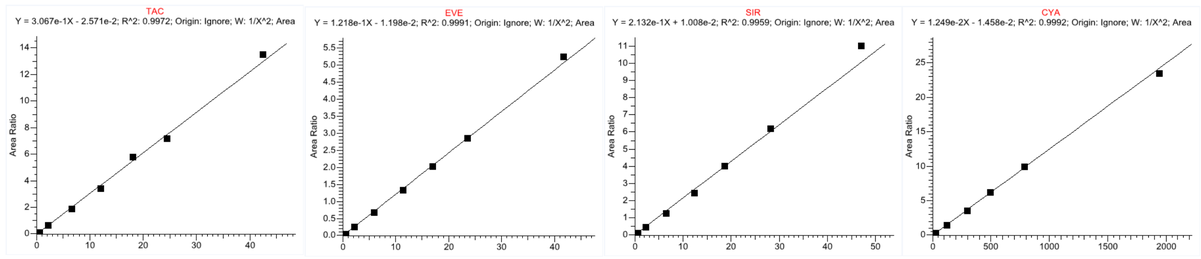
Figure 1. A typical chromatogram, of the lowest standard. N.B. that retention time is from start of the data window, and the actual retention time is 0.5 minutes longer



Linearity

The calibration curves were fitted to a linear regression with weighting $1/X^2$. The back-calculated accuracies for all calibrators in all five batches ranged from 89.9 – 110.4%, and the R^2 values ranged from 0.9957-0.9996. Typical calibration curves are presented in Figure 2.

Figure 2. Typical Calibration Curves



Accuracy and Precision

The accuracy and precision were evaluated using six replicates of each level of test samples in five batches. The samples were run on two identical systems. The intra-assay accuracy and precision are presented in Table 7 and Table 8, and the inter-assay accuracy and precision are presented in Table 9 and Table 10.

Table 7. Intra–Assay Accuracy (%)

	System 1				System 2			
Level	CYA	EVE	SIR	TAC	CYA	EVE	SIR	TAC
QC0.5	93.8-106.1	91.6-107.6	87.2-122.2	87.9-107.0	97.3-111.8	87.0-108.6	83.5-101.7	96.6-103.4
QCI	98.8-103.6	98.1-104.1	94.1-107.4	100.2-106.2	98.2-107.7	98.0-100.2	95.5-103.1	99.3-105.5
QCII	108.3-112.9	99.7-103.7	102.6-109.6	98.5-101.9	104.5-115.9	96.8-99.3	101.3-107.7	97.5-103.4
QCIII	103.4-110.5	100.3-106.1	97.0-106.3	98.2-105.1	99.9-110.7	95.6-103.3	98.9-103.7	99.3-102.1
QCIV	98.2-104.0	104.2-108.1	95.5-101.4	98.9-103.3	94.8-102.4	97.8-104.3	97.3-103.3	99.2-103.7

Table 8. Intra–Assay Precision (CV%)

	System 1				System 2			
Level	CYA	EVE	SIR	TAC	CYA	EVE	SIR	TAC
QC0.5	2.5-6.7	2.9-11.7	4.1-17.4	3.2-9.7	2.7-10.0	5.6-9.8	4.5-8.0	3.5-9.5
QCI	1.4-3.4	2.4-6.6	1.5-4.5	2.4-5.9	1.1-3.2	2.8-5.8	1.9-6.1	2.3-4.9
QCII	1.4-3.4	2.4-6.6	1.5-4.5	2.4-5.9	1.1-3.0	2.5-5.8	1.9-6.1	2.3-4.9
QCIII	1.8-2.9	2.6-4.5	4.4-5.1	2.1-6.3	1.3-2.6	1.5-4.8	2.5-4.2	2.8-4.9
QCIV	1.9-4.0	1.6-3.7	1.8-4.1	2.9-5.1	1.4-4.0	1.7-3.6	2.4-5.4	1.4-5.5

Table 9. Inter–Assay Accuracy (%)

	System 1				System 2			
Level	CYA	EVE	SIR	TAC	CYA	EVE	SIR	TAC
QC0.5	100.3	101.7	107.5	97.7	102.6	98.5	96.9	99.5
QCI	101.3	102.2	102.0	102.5	102.3	98.9	100.5	103.2
QCII	110.1	102.3	105.9	99.7	109.8	97.9	104.1	100.3
QCIII	106.5	102.4	101.9	101.2	105.3	99.2	102.4	100.7
QCIV	100.6	105.2	98.9	101.3	98.8	100.2	99.4	101.7

Table 10. Inter–Assay Precision (CV%)

	System 1				System 2			
Level	CYA	EVE	SIR	TAC	CYA	EVE	SIR	TAC
QC0.5	5.9	9.1	14.8	9.9	7.6	11.3	9.5	6.5
QCI	2.9	4.3	6.4	4.4	4.5	3.8	4.3	3.8
QCII	2.9	4.1	4.0	4.3	4.2	4.3	4.3	4.0
QCIII	3.4	3.8	5.2	4.6	4.0	4.5	3.7	3.5
QCIV	3.4	2.9	3.4	3.6	3.7	3.4	3.7	3.9

Carry-over

The carry-over was investigated by comparing the response of a blank sample to the response of a proceeding high calibrator and to the average response of LLOQ samples in each batch. The results are presented in Table 9.

Table 9. Carry-over presented as percent of response from preceding highest calibrator and percent of response at LLOQ

	% of preceeding High Calibrator								% of average response at LLOQ							
	System 1				System 2				System 1				System 2			
Batch	CYA	EVE	SIR	TAC	CYA	EVE	SIR	TAC	CYA	EVE	SIR	TAC	CYA	EVE	SIR	TAC
1	0.01	0.18	0.21	0.04	0.03	0.02	0.26	0.05	1.4	1.5	17.2	2.9	6.4	1.9	19.8	3.8
2	0.00	0.01	0.22	0.04	0.02	0.06	0.29	0.07	1.5	1.1	20.2	4.0	3.6	4.5	19.5	5.2
3	0.01	0.13	0.14	0.05	0.02	0.06	0.29	0.05	3.4	12.7	12.4	3.8	3.7	4.9	23.8	3.9
4	0.01	0.03	0.33	0.05	0.02	0.05	0.35	0.04	4.0	2.5	24.6	4.5	4.6	5.2	29.5	3.8
Mean	0.01	0.09	0.23	0.05	0.02	0.05	0.30	0.05	2.6	4.5	18.6	3.8	4.6	4.1	23.2	4.2

DISCUSSION

The results from the analytical validation shows that this method is accurate and robust in the calibrated range and suitable for clinical research studies.The data for accuracy and precision are well within the limits of the EMA guidelines for bioanalytical methods[2]. However, relatively high signal in blank samples was found for Sirolimus. This signal, which tends to be slightly higher than 20% of the response at LLOQ, is seen in all blank samples and is not a result from carry-over. The signal is at least partly due to the presence of unlabelled Sirolimus in the internal standards of Sirolimus and Everolimus. It resulted in a negative impact of the results at the lowest level, and a bias >20% was found in one out of ten batches (22.2 %). For Cyclosporin A, the bias for samples at level II was >15% (15.9%) in one batch out of ten. At this level the average bias was 10% and with a CV of 1.4-5.6%, an error in preparation of the sample is suspected.

CONCLUSIONS

The presented method is fast, robust, accurate, precise and sensitive. The extra sensitivity of this method makes it especially suitable for clinical research studies. The investigated parameters comply with EMA guidlines for bioanalytical validation.[2]

REFERENCES

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2. Guideline on bioanalytical method validation, EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2.

TRADEMARKS/LICENSING

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