TP-143

Simultaneous analysis of immunosuppressive drugs in whole blood samples using LC-MS/MS

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1. Introduction

- Immunosuppressants are prescribed to organ transplant patients to control rejection; however, too much drug administration can cause complications such as infections. Therefore, it is important to measure drug concentrations in blood for dose control.
- LC-MS/MS is a powerful tool for the major immunosuppressants analysis; however, the different methods are required for the group of tacrolimus, everolimus, sirolimus and cyclosporin A vs. the other group of mycophenolic acid (MPA) and mycophenolic acid beta-D-glucuronide (MPAG), respectively, due to the differences in whole blood concentrations.
- For efficient operation, we developed a single method for the simultaneous analysis of a total of 6 compounds, immunosuppressants and their metabolites, using LC-MS/MS. The validation analysis of their quantification in whole blood samples were evaluated.

2. Methods

- For standard samples of tacrolimus, everolimus, sirolimus and cyclosporin A with each internal standards(IS), DOSIMMUNE™ (Shimadzu Corporation), a reagent kit for the quantification of immunosuppressant drugs in whole blood was used. For IS of MPA and MPAG, DOSIMYCO™ (Shimadzu Corporation) were also used. Independent standard for MPA and MPAG were purchased from Alsachim, Illkirch-Graffenstaden, France. Six normal human control whole bloods (EDTA-2K added) were purchased from BizCom Japan, Inc., Tokyo, Japan.
- LC-MS/MS measurements were performed using Nexera™ X3 coupled with LCMS-8050 (Shimadzu Corporation). Columns included in DOSIMMUNE kit were used for trap and analytical columns. The HPLC conditions were set with reference to the analytical conditions specified in DOSIMMUNE kit. All compounds were detected in ESI mode.

Table 1 Concentration of each compound in calibrators containing whole blood matrix (Converted to concentration in whole blood)

LEVEL	1	2	3	4	5	6
Tacrolimus(μg/L)	1.73	4.86	9.27	13.96	23.3	33.09
Everolimus (μg/L)	2.04	4.88	9.2	14.77	24.84	36.88
Sirolimus (μg/L)	1.93	5.07	9.76	15.09	24.3	35.4
Cycrosporin A (μg/L)	26.07	106.5	453.7	1027	1574	1866
MPA (mg/L)	0.1	0.5	5	10	25	50
MPAG (mg/L)	1	5	25	50	125	250

3. Results

- Standard solutions of MPA and MPAG were dried up in a microcentrifuge tube using a centrifugal evaporator. The calibrator reagent in the DOSIMMUNE kit, which contains tacrolimus, everolimus, sirolimus and cyclosporin A, was added into the tube. IS and extract solution were also dispensed, and the supernatant was collected after mixing and centrifugation as samples for calibration curves(Fig. 1). Concentration of each compound in calibrators are shown in Table 2.
- QC samples for MPA and MPAG were prepared by adding standard solutions to human whole blood. Control reagents in the DOSIMMUNE kit were used as QC samples for tacrolimus, everolimus, sirolimus and cyclosporin A.
- QC sample, IS and extract solution were dispensed into a micro centrifuge tube and the supernatant was collected after mixing and centrifugation as samples for QC quantification.
- DOSIMMUNE kit specifies mobile phases and columns for 4 immunosuppressants such as tacrolimus. In this study, we used the mobile phase and columns same as DOSIMMUNE kit. The gradient program was modified for the purpose of suitable elution and adjusting the retention times of MPA and MPAG.
- Using ESI negative mode for MPA and MPAG, the dynamic ranges of them shifted to the compatible range for quantification with another immunosuppressants such as tacrolimus. We confirmed that it is possible to conduct simultaneous analysis of 6 compounds using the method developed in this study(Table 2).

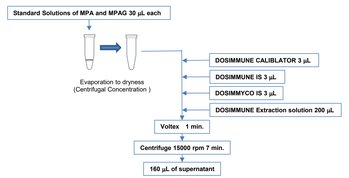

Fig. 1 Pretreatment procedure for calibrators containing whole blood matrix

Table 2 LC-MS condition

■ LC conditions

Trap eluent	: DOSIMMUNE MOBILE PHASE A
Flow rate(Trap eluent)	: 2 mL/min
Mobile phase A	: DOSIMMUNE MOBILE PHASE A
Mobile phase B	: DOSIMMUNE MOBILE PHASE B
Flow rate(Mobile phase)	: 0.8 mL/min
Trap column	: DOSIMMUNE Trap Column
Analytical column	: DOSIMMUNE Analytical Column
Valve position	: 0
Time program	: Shown in the right table
Column temp.	: 65 ℃
Injection vol.	: 20 μ L

Time (min)	Command	Value
0	B.Conc(Mobile phase)	60
0.25	Valve Position	1
0.25	T.Flow(Trap eluent)	2
0.26	T.Flow(Trap eluent)	0.02
1	B.Conc(Mobile phase)	60
1.25	B.Conc(Mobile phase)	100
1.5	Valve Position	0
1.5	T.Flow(Trap eluent)	0.02
1.51	T.Flow(Trap eluent)	2
2.5	B.Conc(Mobile phase)	100
2.51	B.Conc(Mobile phase)	60
3	Stop	

Valve position : 0
Trap eluent - Trap column - Waste

Valve position : 1
Mobile phase - Trap column – Analytical column - MS

■ MS conditions

Ionization	: ESI-Negative min for MPA, MPAG (0 - 1.25 min) ESI-Positive for for Everolimus, Sirolimus, Tacrolimus and Cycrosporin A (1.25 – 3.00 min)
Nebulizing gas flow	: 3 L/min
Heating gas flow	: 10 L/min
Interface temp.	: 200℃
Drying gas flow	: 10 L/min
DL temp.	: 150℃
HB temp.	: 200℃

■ MRM

ESI negative

MPA	: 319.20>191.25	(CE 30 V)
MPA IS	: 323.20>191.25	(CE 23 V)
MPAG	: 495.20>191.30	(CE 37 V)
MPAG IS	: 499.20>191.20	(CE 42 V)

ESI positive

Everolimus	: 975.60>908.55	(CE -17 V)
Everolimus IS	: 981.60>914.70	(CE -17 V)
Sirolimus	: 931.60>864.65	(CE -17 V)
Sirolimus IS	: 935.60>864.50	(CE -18 V)
Tacrolimus	: 821.50>768.55	(CE -22 V)
Tacrolimus IS	: 826.50>773.60	(CE -22 V)
Cycrosporin A	: 1220.00>1202.95	(CE -19 V)
Cycrosporin A IS	: 1232.00>1215.00	(CE -19 V)

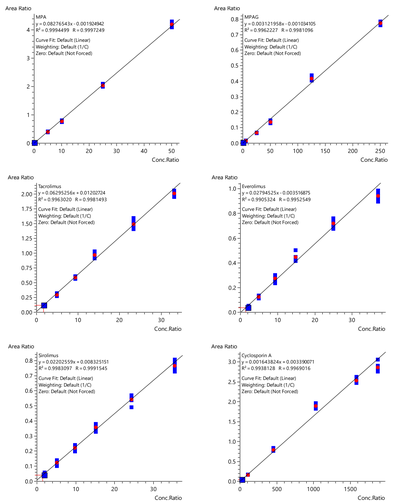

Fig. 2 Calibration curves for each compound

- The calibration curves obtained by 6 repeated measurements of each concentration is shown in Fig. 2. For all compounds, the coefficient of determination was confirmed to be 0.99 or higher.
- As a result of 6 times repeated measurements of each QC sample , it was confirmed that the quantitative values were within the reference range for all compounds. Table 3 shows the quantification results for each compound.

4. Conclusion

- We developed a useful method for quantifying a total of 6 compounds, immunosuppressants and their metabolites, in whole blood using LC-MS/MS.
- As a result of quantitative analysis of 6 compounds in whole blood using this method, it was confirmed that the quantitative values were within the reference range for all compounds.
- The results evaluated in this study are expected to be applied as a rapid analytical method for drug concentration measurement in clinical practice.

Table 3 The reference values and quantification results for each compound.

QC Sample	Compound	Level 1 Conc. (ave.)	1 Range	1 %RSD	Level 2 Conc. (ave.)	2 Range	2 %RSD	Level 3 Conc. (ave.)	3 Range	3 %RSD	Level 4 Conc. (ave.)	4 Range	4 %RSD
Whole Blood 1	MPA (mg/L)	**2.22**	1.7 - 2.3	2.46	**19.5**	17-23	1.92	**40.1**	34-46	1.21	-	-	-
	MPAG (mg/L)	**10.1**	8.5-11.5	2.63	**97.34**	85-115	2.13	**199.6**	170-230	2.3	-	-	-
Whole Blood 2	MPA (mg/L)	**2.07**	1.7 - 2.3	2.93	**18.96**	17-23	1.94	**43.41**	34-46	1.27	-	-	-
	MPAG (mg/L)	**10.9**	8.5-11.5	2.56	**93.28**	85-115	3.3	**180.9**	170-230	2.04	-	-	-
Whole Blood 3	MPA (mg/L)	**2.1**	1.7 - 2.3	0.92	**19.56**	17-23	1.32	**40.73**	34-46	2.49	-	-	-
	MPAG (mg/L)	**9.72**	8.5-11.5	8.56	**98.71**	85-115	3.1	**203**	170-230	1.03	-	-	-
Whole Blood 4	MPA (mg/L)	**1.94**	1.7 - 2.3	3.04	**20.83**	17-23	2.19	**43.72**	34-46	2.46	-	-	-
	MPAG (mg/L)	**9.51**	8.5-11.5	9.32	**97.02**	85-115	5.92	**214.6**	170-230	1.08	-	-	-
Whole Blood 5	MPA (mg/L)	**1.98**	1.7 - 2.3	2.76	**20.03**	17-23	0.86	**40.17**	34-46	0.51	-	-	-
	MPAG (mg/L)	**9.49**	8.5-11.5	5.23	**103**	85-115	1.47	**206.6**	170-230	1.36	-	-	-
Whole Blood 6	MPA (mg/L)	**2.08**	1.7 - 2.3	3.68	**20.46**	17-23	1.32	**39.43**	34-46	2.55	-	-	-
	MPAG (mg/L)	**10.3**	8.5-11.5	5.3	**92.72**	85-115	3.31	**195.8**	170-230	1.68	-	-	-
DOSIMMUNE Control	Tacrolimus(μg/L)	**3.17**	2.69-4.03	6.31	**7.69**	6.17-9.25	5.65	**12.07**	10.14-15.20	3.05	**18.83**	14.96-22.44	3.11
	Everolimus (μg/L)	**3.83**	3.13-4.70	5.57	**9.8**	7.34-11.02	7.86	**14.01**	9.96-14.94	4.06	**21.64**	16.58-24.88	7.69
	Sirolimus (μg/L)	**3.99**	3.06-4.60	5.34	**9.48**	6.80-10.20	3.07	**12.09**	9.54-14.32	4.87	**19.38**	16.74-25.12	6.75
	Cycrosporin A (μg/L)	**40.8**	34.06-51.10	4.69	**169.8**	125.89-188.83	7.1	**822.1**	622.24-933.36	3.42	**1411.8**	1089.84-1634.76	1.44