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Accelerated and robust monitoring for immunosuppressants using triple quadrupole mass spectrometry

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Natsuyo Asano[1], Tairo Ogura[1], Kiyomi Arakawa[1]
1 Shimadzu Corporation. 1, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604–8511, Japan

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Introduction

Immunosuppressants are drugs which lower or suppress activity of the immune system. They are used to prevent the rejection after transplantation or treat autoimmune disease. To avoid immunodeficiency as adverse effect, it is recommended to monitor blood level of therapeutic drug with high throughput and high reliability. There are several analytical technique to monitor drugs, LC/MS is superior in terms of cross-reactivity at low level and throughput of analysis. Therefore, it is important to analyze these drugs in blood by using ultra-fast mass spectrometer to accelerate monitoring with high quantitativity. We have developed analytical method for four immunosuppressants (Tacrolimus, Rapamycin, Everolimus and Cyclosporin A) with two internal standards (Ascomycin and Cyclosporin D) using ultra-fast mass spectrometer.

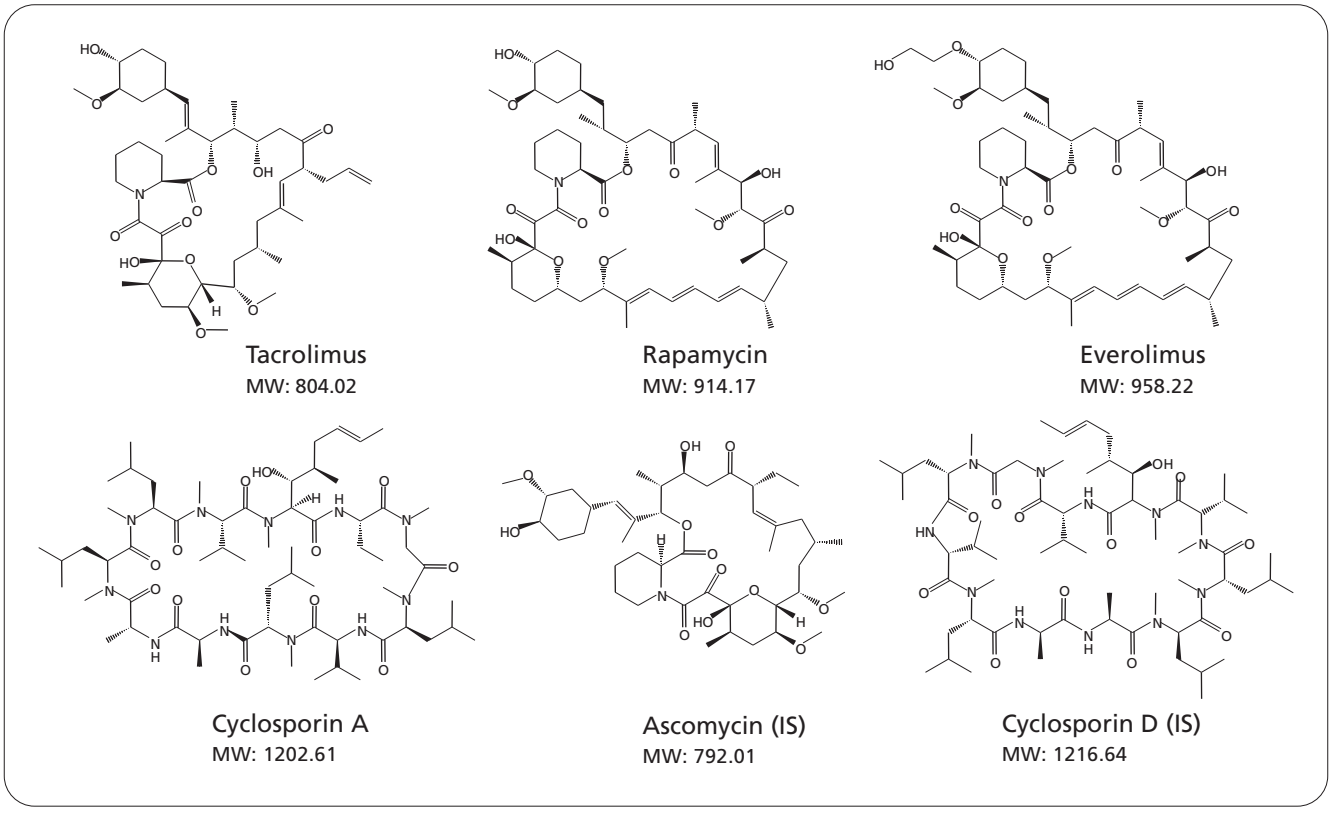

Figure 1 Structure of immunosuppressants and internal standards (IS)

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Methods and Materials

Standard samples of each compound were analyzed to optimize conditions of liquid chromatograph and mass spectrometer. Whole blood extract was prepared based on liquid-liquid extraction described bellow.

2.7 mL of Whole blood and 20.8 mL of Water
↓
Vortex for 15 seconds
↓
Add 36 mL of MTBE/Cyclohexane (1:3)
↓
Vortex for 15 seconds and Centrifuge with 3000 rpm at 20 °C for 10 minutes
↓
Extract an Organic phase
↓
Evaporate and Dry under a Nitrogen gas stream
↓
Redissolve in 1.8 mL of 80 % Methanol solution with 1 mmol/L Ammonium acetate
↓
Vortex for 1 minute and Centrifuge with 3000 rpm at 4 °C for 5 minutes
↓
Filtrate and Transfer into 1 mL glass vial

Table 1 Analytical conditions

UHPLC	
Liquid Chromatograph	: Nexera (Shimadzu, Japan)
Analysis Column	: YMC-Triart C18 (30 mmL. × 2 mmI.D.,1.9 µm)
Mobile Phase A	: 1 mmol/L Ammonium acetate - Water
Mobile Phase B	: 1 mmol/L Ammonium acetate - Methanol
Gradient Program	: 60 % B. (0 min) – 75 % B. (0.10 min) – 95 % B. (0.70 – 0.90 min) – 60 % B. (0.91 – 1.80 min)
Flow Rate	: 0.45 mL/min
Column Temperature	: 65 °C
Injection Volume	: 1.5 µL

MS	
MS Spectrometer	: LCMS-8050 (Shimadzu, Japan)
Ionization	: ESI (negative)
Probe Voltage	: -4.5 ~ -3 kV
Nebulizing Gas Flow	: 3.0 L/min
Drying Gas Flow	: 5.0 L/min
Heating Gas Flow	: 15.0 L/min
Interface Temperature	: 400 °C
DL Temperature	: 150 °C
HB Temperature	: 390 °C

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Result

Immunosuppressants, which we have developed a method for monitoring of, has been often observed as ammonium or sodium adduct ion by using positive ionization. In general, protonated molecule (for positive) or deprotonated molecule (for negative) is more preferable for reliable quantitation than adduct ions such as ammonium, sodium, and potassium adduct. In this study, each compound was detected as deprotonated molecule in negative mode by using heated ESI source of LCMS-8050 (Table 2).

The separation of all compounds was achieved within 1.8 min, with a YMC-Triart C18 column (30 mmL. × 2 mmI.D.,1.9 µm) and at 65 °C of column oven temperature.

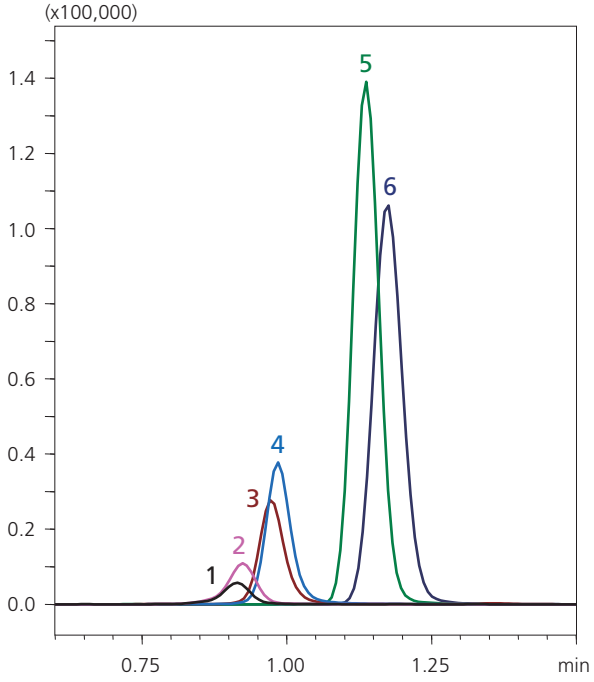

Figure 2 MRM chromatograms of immnosuppresants in human whole blood (50 ng/mL)

Table 2 MRM transitions

Peak No.	Compound	Porality	Precursor ion (*m/z*)	Product ion (*m/z*)
1	Ascomysin (IS)	neg	790.40	548.20
2	Tacrolimus	neg	802.70	560.50
3	Rapamycin	neg	912.70	321.20
4	Everolimus	neg	956.80	365.35
5	Cyclosporin A	neg	1200.90	1088.70
6	Cyclosporin D (IS)	neg	1215.10	1102.60

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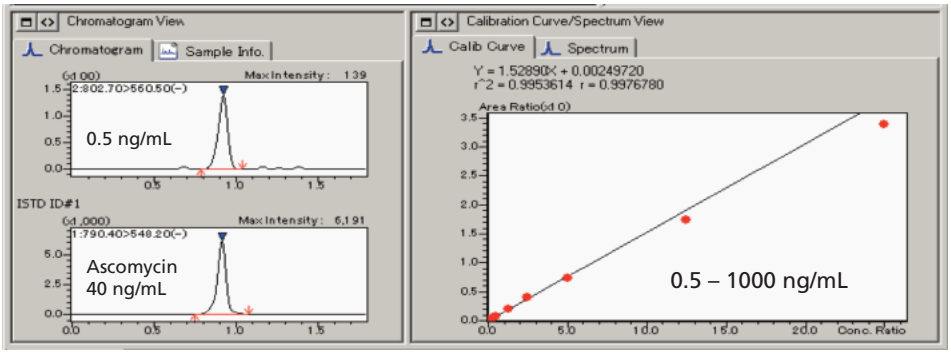
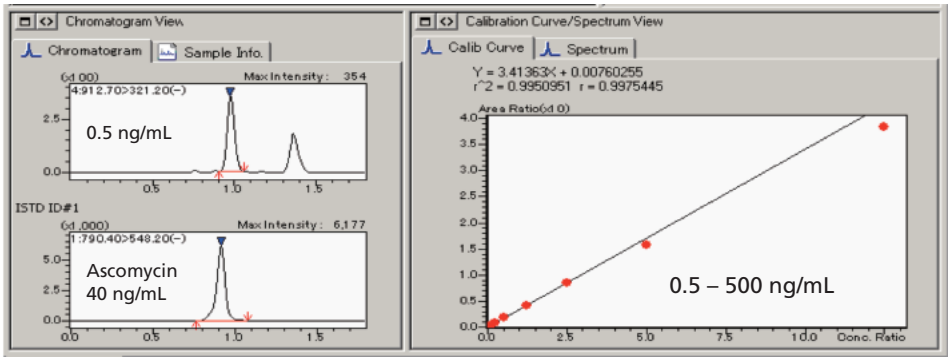
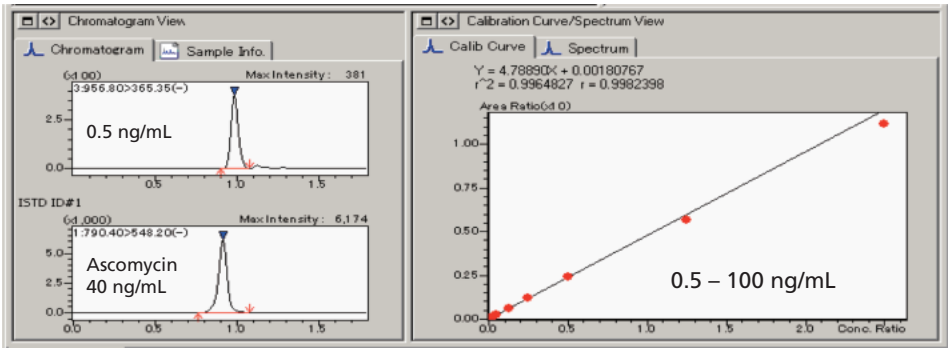
a) Tacrolimus



b) Rapamycin



c) Everolimus



d) Cyclosporin A

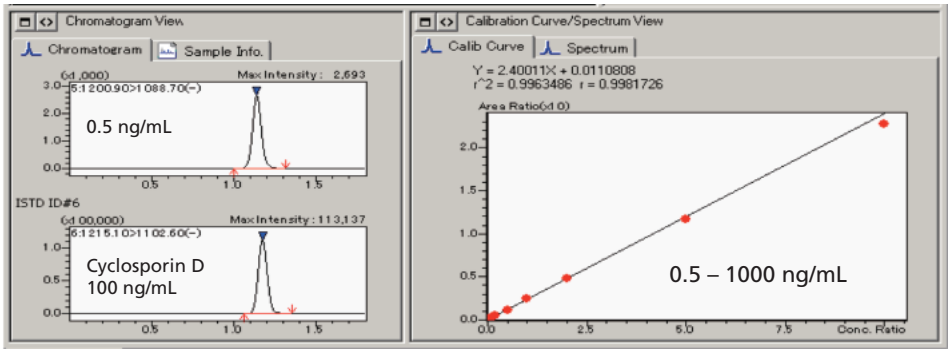


Figure 3 MRM chromatograms at LLOQ and ISTD (left), and calibration curves (right) for four immnosuppresants in human whole blood

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Figure 3 illustrates both a calibration curve and chromatogram at the lowest calibration level for all immunosuppressants analyzed. Table 3 lists both the reproducibility and accuracy for each immunosuppressant that has been simultaneously measured in 1.8 minutes.

Table 3 Reproducibility and Accuracy

Compound	Concentration	CV % (n = 6)	Accuracy %
Tacrolimus	Low (0.5 ng/mL) Low-Mid (2 ng/mL) High (1000 ng/mL)	18.0 13.0 2.87	99.4 99.5 88.7
Rapamycin	Low (0.5 ng/mL) Low-Mid (5 ng/mL) High (500 ng/mL)	6.87 2.88 3.41	95.6 109.3 90.0
Everolimus	Low (0.5 ng/mL) Low-Mid (5 ng/mL) High (100 ng/mL)	10.4 5.11 2.26	95.3 104.4 93.3
Cyclosporin A	Low (0.5 ng/mL) Low-Mid (10 ng/mL) High (1000 ng/mL)	7.31 2.36 2.67	95.1 99.9 94.9

In high speed measurement condition, we have achieved high sensitivity and wide dynamic range for all analytes. Additionally, the accuracy of each analyte ranged from 88 to 110 % and area reproducibility at the lowest calibration level of each analyte was less than 20%.

Conclusions

- Monitoring with negative mode ionization permitted more sensitive, robust and reliable quantitation for four immunosuppressants.
- A total of six compounds were measured in 1.8 minutes. The combination of Nexera and LCMS-8050 provided a faster run time without sacrificing the quality of results.
- Even with a low injection volume of 1.5 µL, the lower limit of quantitation (LLOQ) for all compounds was 0.5 ng/mL.
- In this study, it is demonstrated that LCMS-8050 is useful for the rugged and rapid quantitation for immunosuppressants in whole blood.

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