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Evaluation of Blood Lysis Procedures prior to Automated Sample Preparation for Immunosuppressant Assay by LC-MS/MS

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Introduction

CLAM-2000 (Shimadzu Corp., Japan) fully automates blood or other samples pre-treatment prior to LC-MS analysis (Fig.1). The whole blood has cell debris, fibrin clots and so on. It is desirable to remove such particulates for accurate sample dispensing by centrifugation. However, this poses a challenge to the measurement of immunosuppressants (ISP) in whole blood samples since large proportion of ISP are bound to cytoplasmic proteins in erythrocytes[1)]. Thus, it is mandatory to release erythrocytes prior to any centrifugation of the blood. Current practice is to freeze and thaw samples, which is not convenient for emergency analysis. To address this, several protocols of hemolysis were tested. Evaluation of the protocol was based on lysis efficiency, immunosuppressant recovery and time consumption.

Figure 1. Fully automated sample preparation module CLAM-2000 and triple quadrupole mass spectrometer LCMS-8060.

Methods and Pretreatment

Individual blood sample from healthy volunteer was spiked with tacrolimus (MW: 804.0), sirolimus (MW: 914.2), everolimus (MW: 958.2), and cyclosporine A (MW: 1202.6) and incubated for 30 minutes at room temperature. Then aliquots of each sample were subjected to all lysis protocols. Lysis efficiency was estimated by measuring hemoglobin absorbance in supernatant using a UV-Visible spectrophotometer (UV-1280. Fig.2).

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Lysis Protocols

Lysis reagents were prepared as following. Ammonium chloride mixture was prepared with ammonium chloride, NaHCO3 and EDTA in water. HCl solution was diluted HCl at 2 mol/L in water. Each lysis protocols were performed prior to centrifugation

- Control (1 mL)
- Ammonium Chloride mixture (500 µL) + blood (250 µL)
- HCl solution (100 µL) + blood (1 mL)
- Blood sample (1 mL) with the ultrasound (5 min)
- Blood sample (1 mL) with the ultrasound (10 min)
- Blood sample (1 mL) with the ultrasound (20 min)
- Blood sample (1 mL) with freeze/thaw -20°C (30 min)
- Blood sample (1 mL) with freeze/thaw -80°C(30 min)
- Blood sample (0.2 mL) with freeze/thaw -20°C (30 min)
- Blood sample (0.2 mL) with freeze/thaw -80°C (30 min)

Figure 2. UV-Visible Spectrophotometer UV-1280.

Pretreatment for UV-visible spectrophotometer

Each lysis blood samples (8 µL) are mixed with Drabkin's reagent (2 mL) which has a role of quantitative, colorimetric determination of hemoglobin concentration in whole blood with 540 nm band.

Pretreatment for LC/MS

13C, D2-tacrolimus, D3-sirolimus and D4-everolimus were used for ISTD. Zinc sulfate mixture was prepared with ACN, MeOH, zinc sulfate and ammonium formate. A blood sample (50 µL) was mixed with ISTD (25 µL) and zinc sulfate mixture (350 µL). After centrifugation, ISP recovery was measured using an online SPE-LC-MS/MS method. Supernatant obtained in each tested condition was automatically prepared using CLAM-2000. This included addition of internal standards, protein precipitation, filtration and transfer to the LC autosampler. Acquisition was performed in MRM mode using a triple quad mass spectrometer (LCMS-8060).

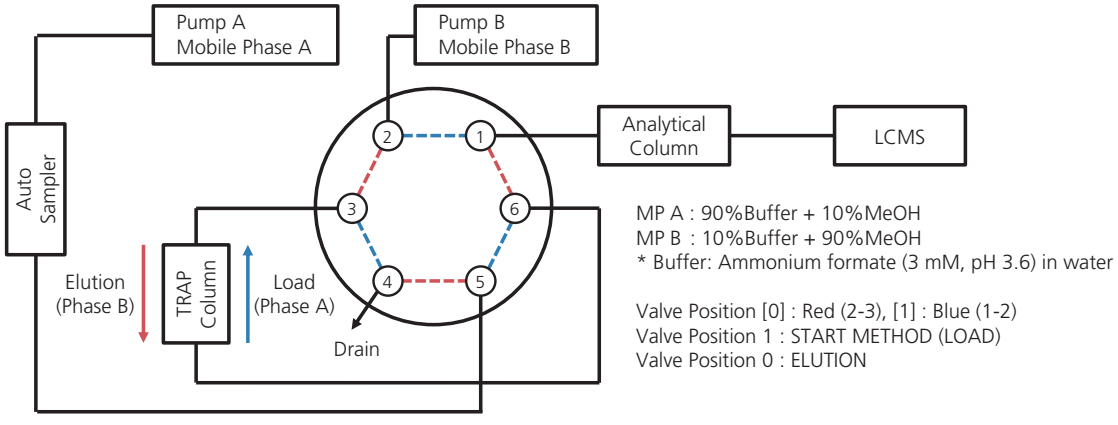

Figure 3. Flow diagram for online SPE

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Table 1. LC and MS conditions

[LC] NexeraX2 System	
Column Temp.	: 65 °C
Analytical Column	: Inertsil ODS-3 (5 um 2.1*50 mm)
Trap Column	: MAYI C8 10x4.6 mm
Time Program	: Isocratic
Injection Volume	: 5.0 µL
[MS] LCMS-8060	
Ionization	: ESI Positive
Nebulizer Gas	: 3 L/min
Interface temperature	: 200 °C
Desolvation Line	: 150 °C
Heat Block temperature	: 200 °C
Heating Gas	: 10 L/min
Drying Gas	: 10 L/min

Table 2. MRM transitions for ISP

ISP	transition	CE	Internal Standard
Tacrolimus	821.30 > 768.50	22	13C,D2-Tacrolimus
Sirolimus	931.30 > 864.50	18	D3-Sirolimus
Everolimus	975.30 > 908.40	20	D4-Everolimus
Cyclosporine A	1219.70 > 1202.80	21	-

Results

Lysis efficiency evaluated by UV-Visible spectrophotometer

Absorption value was measured with each lysis protocols (Fig. 4). Absorption value with each lysis protocols was divided by reference which includes RBC for absorption rates. High absorption rates means that there are a lot of hemoglobin which is generated by release of erythrocytes. Freeze/thaw with -80ºC at both volume were efficient for lysis. Freeze/thaw with -20ºC was also efficient. However freezing time is not sufficient for 1.0 mL. In the case of using 0.2 mL, sample pipetting influence the reproducibility (The standard deviation was very high). Ammonium chloride took the place of -80ºC method, because the blood was diluted 3 times with ammonium chloride mixture.

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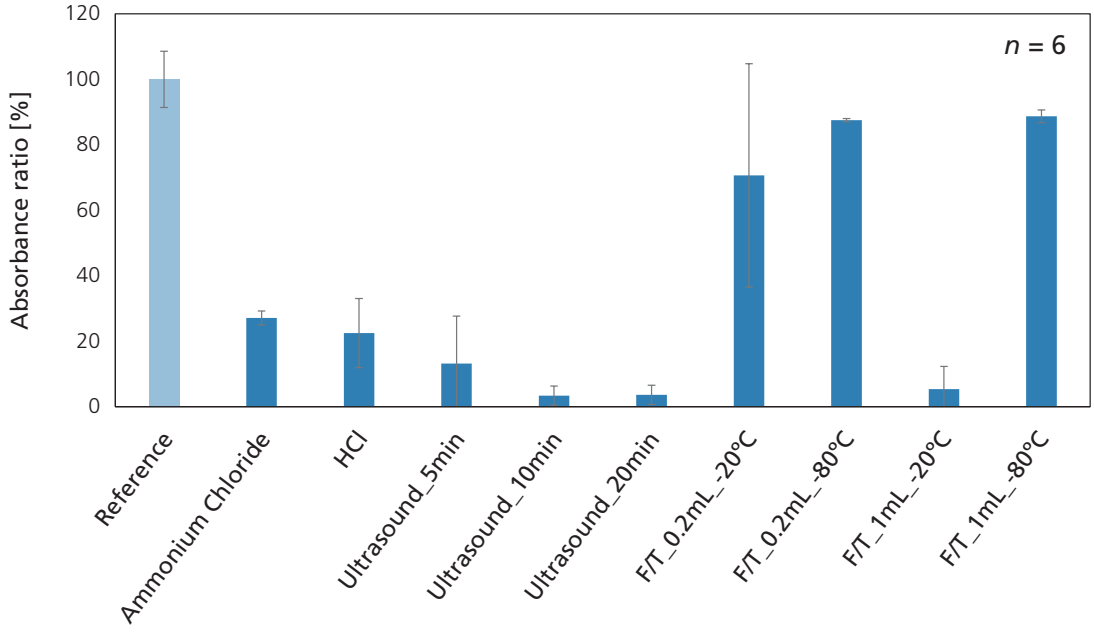

Figure 4. Absorption ratio with each lysis protocols. Freeze/thaw with -80°C was effective lysis procedure. -20°C was also efficient at the volume of 0.2 mL. However, the pipetting technique influenced the number of hemoglobin in such a small volume. Ammonium chloride was also effective method. The reason why is that it was diluted 3 times.

Incorporation of ISP into erythrocytes by LC/MS/MS

ISP are spiked to the whole blood and separated only spiked and centrifuged sample and reference sample which includes particulates. Where the ISP are located was tested by LC/MS/MS (LCMS-8060) for the two samples (plasma and whole blood). Fig.5 shows the MRM chromatogram of the ISP and internal standard.

Compared with these results, about 90% of tacrolimus, sirolimus, everolimus and about 60% of CSA were located to the erythrocytes.
After the experiment, ISP recovery rate with each lysis protocols were evaluated by LC/MS/MS. The whole blood sample were performed for reference.

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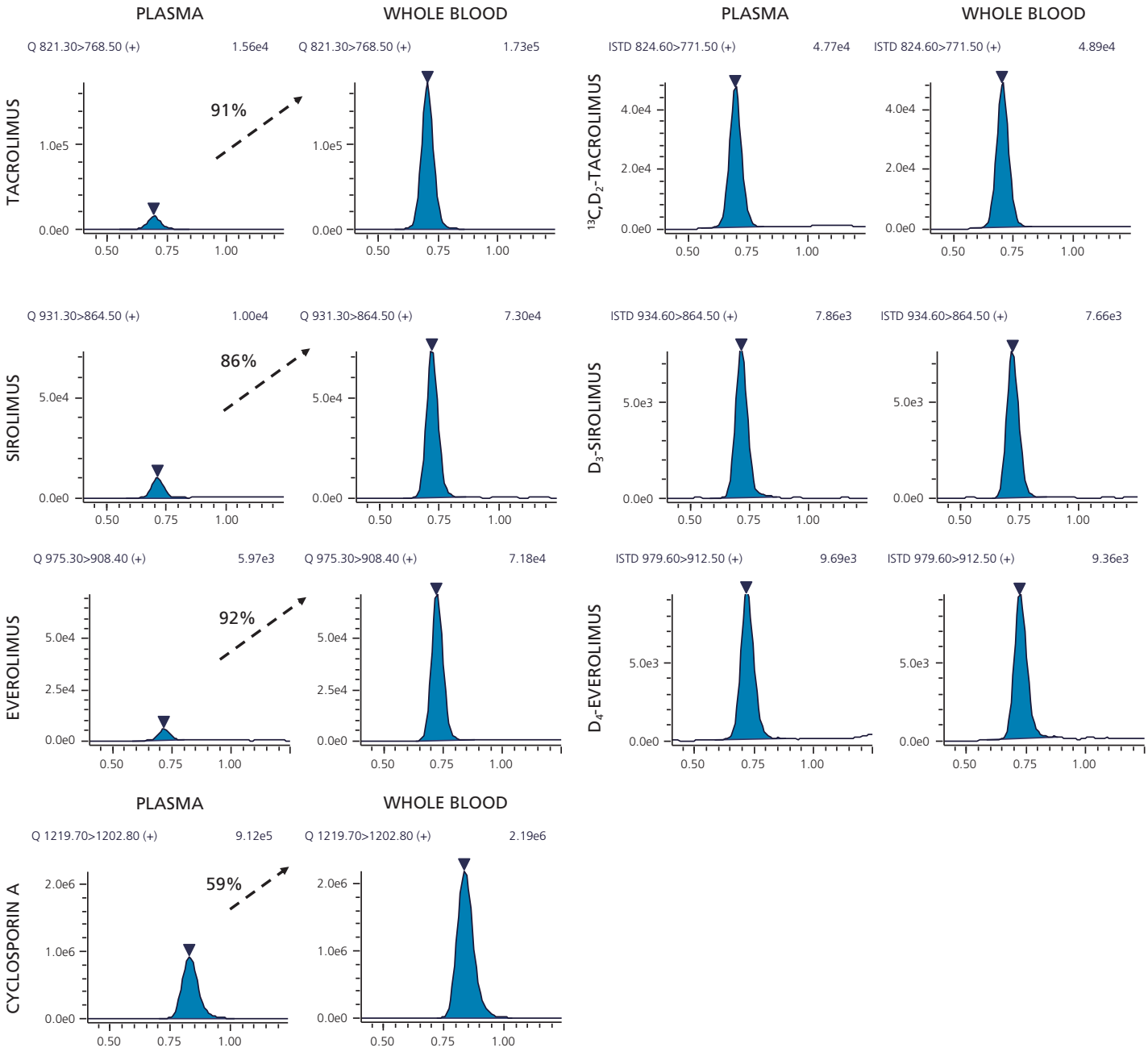

Figure 5. Peak area of ISP and ISTD in a plasma or whole blood. About 90% of tacrolimus, sirolimus and everolimus and about 60% of cyclosporine A were entrapped by erythrocytes.

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ISP recovery rate with each lysis protocols

Area ratio of ISP which was devided by peak area of reference was shown in Fig. 6. There are a correlation between the results of UV spectrophotometer and LC/MS. Freeze/thaw with -80°C was the closest to the reference sample. -20°C was also effective. However, the reproducibility was very low. This is because freezing time was not sufficient. F/T with -80°C was the most efficient pre-treatment method for CLAM-2000. Ammonium chloride was an alternative method. However, it requires sample preparation.

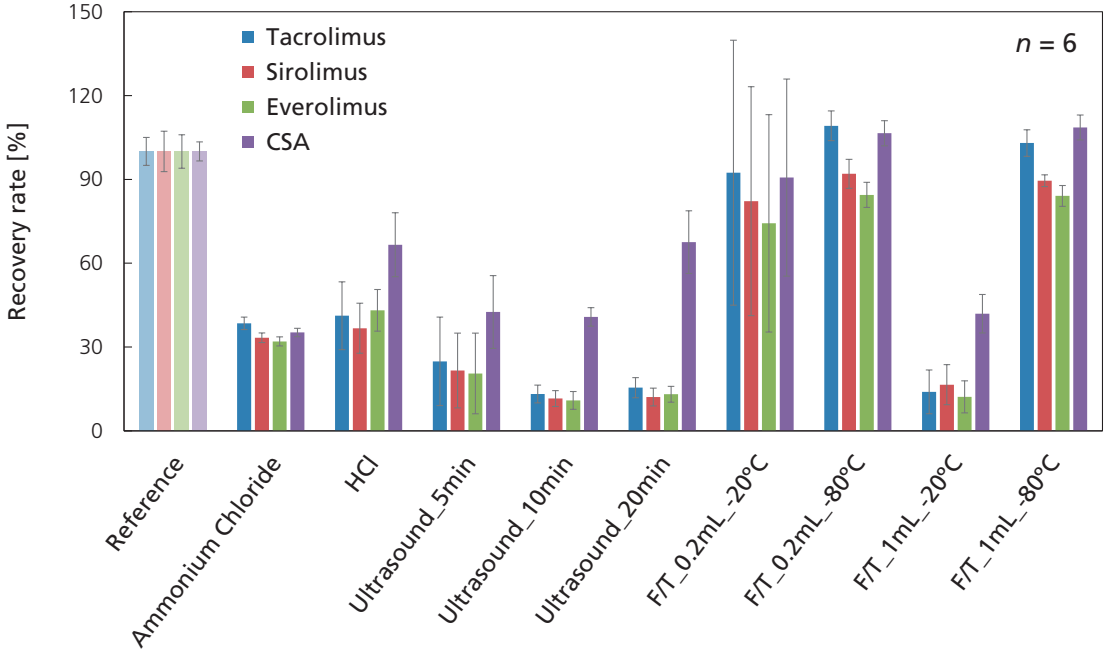

Figure 6. Area ratio of tacrolimus, sirolimus, everolimus, and CSA.
There are a correlation between lysis efficiency and recovery rates of ISP.
(Freeze/thaw with -80°C was the closest to the reference sample)

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Conclusion

- Freeze/thaw with -80°C was efficient for blood lysis and recovery of ISP whatever the volume.
- Freezing time was not sufficient for -20°C in a volume of 1.0 mL or 0.2 mL.
- Ammonium chloride can be used as alternative. However, it induce sample dilution. Thus, this should be taken into account.
- ISP recovery is correlated with lysis efficiency.

Reference

1) Lugia Rossi, et al., *Advanced Drug Delivery Reviews, 2016*, **106**, 73-87

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