APPLICATION NOTE

Automated and Reproducible Method For Immunosuppressants Analysis in Whole Blood

ToT

INTRODUCTION

Immunosuppressant drug monitoring is commonly utilized in organ transplant rejection prevention and in organ cancers such as kidney, pancreatic, and epithelial cell cancers. The continuous monitoring of these drugs saves lives, therefore a quick and easy sample preparation method prior to analysis is imperative in the hospital setting. Unfortunately, immunosuppressants are highly protein bound, and recoveries with traditional methods can be scarce and lack sensitivity. The method for accurate and robust filtration of four immunosuppressants (Cyclosporin A, Everolimus, Sirolimus, and Tacrolimus) are demonstrated herein.

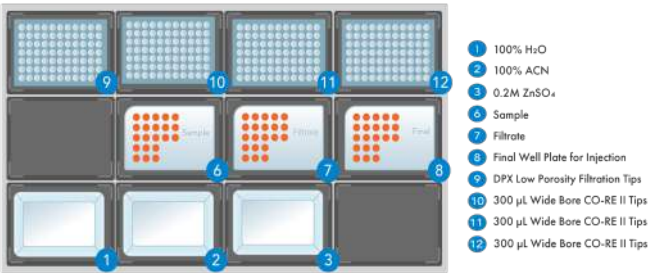

Figure 1. The Hamilton NIMBUS96 deck layout for the automated sample preparation of whole blood samples for immunosuppressant analysis. The method takes 6 minutes to prepare 96 samples.

MATERIALS & METHODS

Analysis was performed on a Shimadzu LC40 paired with a SCIEX 6500+ tandem mass spectrometer (LC-MS/MS). The analytical LC column is a Restek Force Biphenyl, 3 µm, 50 x 2.1 mm LC column (PN 9629352) paired with a Restek Force Biphenyl, 5 x 2.1 mm EXP Guard Cartridge (PN 962950252)[1]. The choice of a biphenyl column allows for optimal separation and more flexibility in sample composition prior to injection; a 10 µL injection volume is used and the LC method is 8.51 minutes. The column is heated to 70°C, and the mobile phases are [A] 0.1% formic acid in HPLC water and [B] methanol (**Figure 1**). The internal standards used are Everolimus-D4 and Cyclosporin A-15N11 (Cerilliant, TX).

The automated method for Tip-on-Tip filtration is displayed in **Figure 1** and **2**. The process involves a MICROLAB® NIMBUS96 automated liquid handler (referred to as ALH), however this can be performed manually or with other automated pipetting platforms. First, 50 µL of 0.2M $ZnSO_4$ in water is added to 50 µL of whole blood in a well plate for the initial step of protein precipitation and mixed thoroughly. Next, a 150 µL aliquot of acetonitrile (ACN) is added and the solution is mixed thoroughly for a fully precipitated sample. The ALH then aspirates the precipitated sample and goes Tip-on-Tip to filter the solution into a well plate.

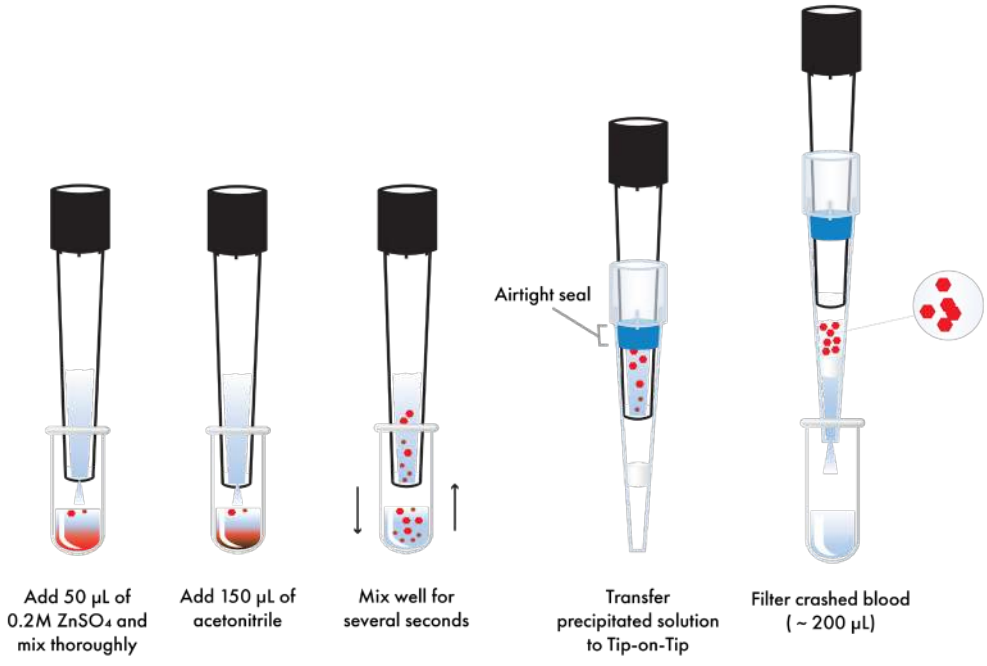

Figure 2. A schematic of the Tip-on-Tip filtration method for immunosuppressants from whole blood. Once sample has been filtered, an aliquot of the filtrate is added to a vial for injection. The sample is then diluted with 125 µL of 20% acetonitrile.

RESULTS AND DISCUSSION

The immunosuppressants levels of quantification (LOQs) are determined to be 1 ng/mL for Tacrolimus, Sirolimus, and Everolimus and 10 ng/mL for Cyclosporin A (**Figure 4 and Table 1**). Reference ranges, based on available literature, for these analytes are above the determined LOQs. During a linearity study conducted in correlation with 2 UTAK Control QC's, accuracy values were found between 91.0% and 105% for all compounds (**Table 2**). The low and high QC verified values were 13.3 ng/mL and 23 ng/mL for Tacrolimus, 11 ng/mL and 24.2 ng/mL for Sirolimus, 10.1 ng/mL and 23ng/mL for Everolimus, and 571 ng/mL and 1200 ng/mL for Cyclosporin A. The recoveries are found to range from 93% to 100% for all analytes from DPX filtration and a total recovery from filtration and protein precipitation is found to be 72% to 83%. Most recovery loss is a result of the protein precipitation step. This method provides excellent recovery of all immunosuppressants tested, especially when compared to previously established methods of immunosuppressant analysis.

Analyte	Quantitative Transition (m/z)	Qualitative Transition (m/z)
Cyclosporin A	1224.7 → 1112.7	1224.7 → 10846
Everolimus	980.4 → 389.2	980.4 → 409.2
Sirolimus	936.5 → 409.2	936.5 → 607.4
Tacrolimus	826.2 → 616.3	826.2 → 808.4

Time	Flow Rate	% A	% B
0.5 min	0.4	40	60
3.5 min	0.4	0	100
6.5 min	0.4	0	100
6.51 min	0.4	40	60
8.51 min	0.4	40	60

Figure 3. The LC and MS/MS method used for the analysis of immunosuppressants in whole blood.

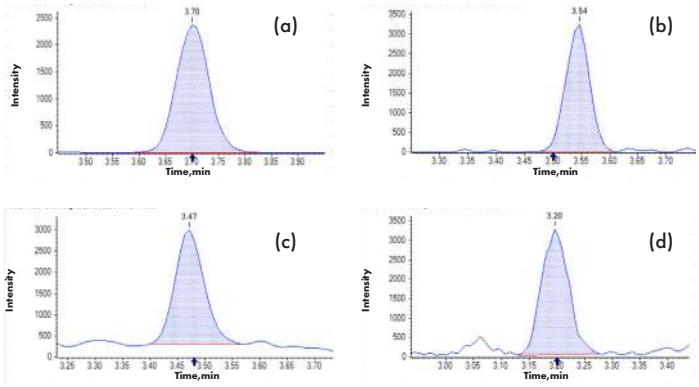

Figure 4. The chromatography of 4 immunosuppressants at the lowest level of calibration and the analyte's corresponding level of quantitation (LOQ). (a) Cyclosporin A, (b) Everolimus, (c) Sirolimus, (d) Tacrolimus.

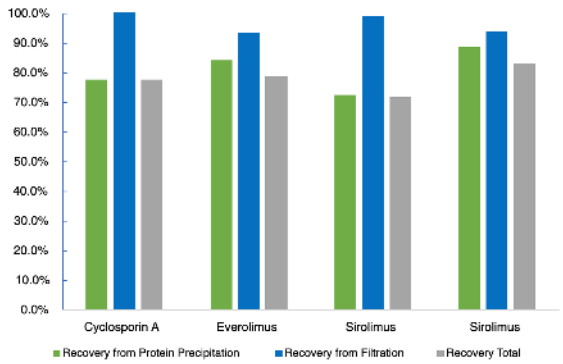

Figure 5. The recovery of immunosuppressants throughout the protein precipitation and filtration steps. Majority of recovery loss is a result of the protein precipitation.

Table 1. The linearity data seen for the four immunosuppressants is displayed.

Analyte	Linear Dynamic Range	R^2	LOQ (s/n)
Cyclosporin A	10 - 1500 ng/mL	0.998	10 ng /mL
Everolimus	1 - 150 ng/mL	0.998	1 ng /mL
Sirolimus	2.5 - 150 ng/mL	0.996	1 ng /mL
Tacrolimus	2.5 - 150 ng/mL	0.997	1 ng /mL

Table 2. The accuracy and precision values for the four immunosuppressants are displayed including inter-day and intra-day precision of analytes from UTAK quality controls.

		Cyclosporin A	Everolimus	Sirolimus	Tacrolimus
LOW QC	Accuracy (%)	103.1	98.1	91.0	104.7
	Inter - day Precision %	6.4	9.0	9.7	7.1
	Intra - day Precision %	12.0	8.6	8.6	13.2
HIGH QC	Accuracy (%)	99.5	93.6	104.2	100.8
	Inter - day Precision %	8.0	3.5	9.3	11.1
	Intra - day Precision %	9.2	7.5	10.2	5.7

CONCLUSION

For highly protein bound analytes such as immunosuppressants in whole blood, an effective sample preparation protocol is necessary to ensure accuracy of patient results. A fast and reproducible method for the analysis of immunosuppressants in whole blood is needed to determine how well a patient has acclimated to a new organ transplant. The use of this DPX Tip-on-Tip method provides a fast and sensitive approach for the analysis of these important drugs.

REFERENCES

1. Liang, S. H., & Carroll, F. (2017). High-Throughput Analysis of Immunosuppressive Drugs from Whole Blood by LC-MS/MS (Lit. Cat# CFAN2666-UNV). Restek. https://www.restek.com/articles/high-throughput-analysis-of-immunosuppressive-drugs-from-whole-blood-by-LC-MSMS