Extraction of Mycophenolic Acid and Mycophenolic Acid Metabolite from Serum Using Supported Liquid Extraction (ISOLUTE® SLE+) Prior to LC-MS-MS Analysis

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Introduction

Immunosuppressant drugs are instrumental in preventing organ and tissue rejection in patients undergoing transplant surgery. Mycophenolic Acid is a common immunosuppressant drug used in patient transplant therapy. The ability to monitor the trough levels in patients to evaluate dosing is important for the administering of the drug. The free drug and its glucoronidated metabolite can be found in patient serum. The ability to extract the free drug and metabolite in patient serum is supported by using a fast and efficient sample preparation extraction method called ISOLUTE Supported Liquid Extraction (SLE+). The free drug and metabolite can be recovered from serum using ISOLUTE SLE+ with high enough efficiency to allow for quantitation at target trough levels.

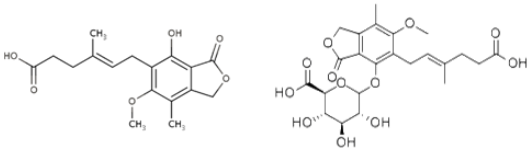

***Figure 1**. Structures of Mycophenolic Acid (MPA) and Mycophenolic Acid Glucoronide (MPAG)*

Experimental

Reagents

Methanol, Formic Acid and Ethyl Acetate were purchased from Sigma-Aldrich Co. (Atlanta, GA.) The immunosuppressant analyte standards (Table 1) were purchased from Cerilliant (Round Rock TX.)

Sample Preparation- MPG and MPGA in Serum

The samples were processed on a 200uL supported liquid extraction 96 well plate format (ISOLUTE SLE+). Several aliquots of negative serum (100uL each) were spiked with MPA and MPAG standard to prepare a series of samples ranging in concentration from 0.1-10 ug/mL. The samples were pretreated with 90uL of an aqueous 20% formic acid solution. The samples were loaded into designated wells. A slight pulse of vacuum or positive pressure was applied to facilitate flow of sample onto sorbent bed. Sample was allowed to sit for 5 minutes. The sample was eluted with two aliquots of ethyl acetate (2 x 500uL) into a collection plate (Figure 2). The sample was dried down and reconstituted in 300uL of aqueous mobile phase.

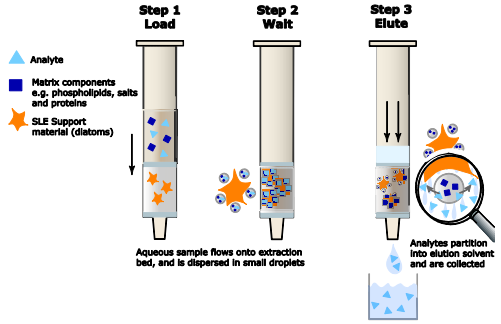

***Figure 1**. Illustration of LOAD-WAIT-ELUTE strategy for ISOLUTE SLE+ (Supported Liquid Extraction)*

Liquid Chromatography-Mass Spectrometry Analysis

An optimized Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analytical method for detection of MPA and MPAG was developed on a Restek Allure Organic Acid column (150mm x 4.6mm, 5µm). A gradient method of 0.1% Formic Acid (**A**) and methanol with 0.1% formic acid (**B**) was developed. The gradient starts at 90% **A** to 2% **A** in 1.5 minutes at 1.0 ml/min flow rate. Detection of the target analyte was achieved using an Applied Biosystems /MDS Sciex 4000 Q-Trap triple quadrapole mass spectrometer (Applied Biosystems, Foster City, CA.) equipped with a Turbo Ionspray® interface. Chromatographic separation was accomplished using the Agilent 1200 Liquid Handling System (Agilent Technologies, Berkshire, UK).

Scan	Analyte	MRM Transition	Declusering Potential (DP)	Collision Energy (CE)	Cell Exit Potential (CXP)
1	MPA	321.0 > 207.1	40	30	16
2	MPAG-1	321.1 > 207.1	40	30	16
3	MPAG-2	514.2>207.1	40	30	16
4	MPAG-3	497.1>207.1	40	30	16

***Table 1.** MRM transitions for MPA and MPAG.*

Results/Discussion

The MRM transitions were identified for the MPA and MPAG (**Table 1**). It became apparent immediately that the MPAG was fragmenting in the ionization source and losing the glucoronide moiety. The in-source fragmentation of MPAG to MPA was monitored as mass transition MPAG-1 (MRM 321.1>207.1) and yielded a significantly better response than the MPAG-2 and MPAG-3 mass transitions. Due to the loss of the glucoronide ligand, several mass transitions were identified and monitored for the MPAG to verify that the parent compound was present. The MPAG-1 mass transition with in-source loss of glucoronide, makes the MPAG-1 transition a "pseudo" isobar of the parent compound (MPA). To address the isobar issue of using the same transition for MPA and MPAG, chromatographic separation was achieved at baseline resolution for the metabolite and free drug (**Figure 3**).

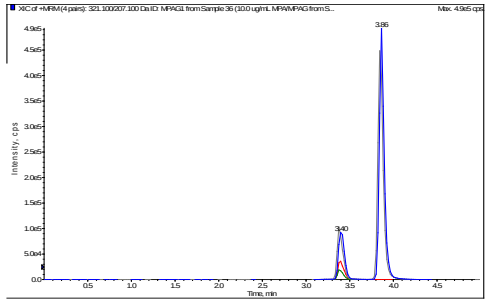

***Figure 3.** Typical extracted ion chromatogram for MPAG (r.t. 3.40 mins) and MPG (r.t. 3.86 mins) at 10ug/mL spiked concentration in serum and extracted using ISOLUTE SLE+.*

The MPAG and MPA were extracted with good efficiency from serum across the targeted spiking concentration of 0.1ug/mL to 10ug/mL. Figure 4 shows the recoveries observed for both of the target analytes across the dynamic concentration range.

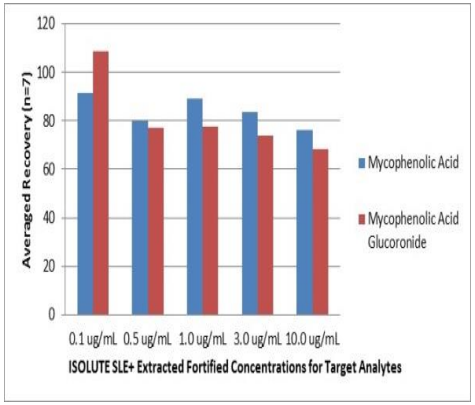

***Figure 4.** Recoveries of MPA and MPAG across dynamic concentration range extracted from spiked serum using supported liquid extraction.*

An evaluation of the performance of protein precipitation as an alternative sample preparation method was evaluated. Protein precipitation is a common sample preparation method utilized in clinical labs analyzing for MPA and MPAG. The recoveries observed for the same dynamic range of spike analytes in serum and extracted using protein precipitation is shown in Figure 5. Overall the recoveries for the MPA and MPAG extracted with protein precipitation were significantly lower. This highlights the effects of possible matrix ion suppression typically not addressed when employing protein precipitation as the sample preparation alternative.

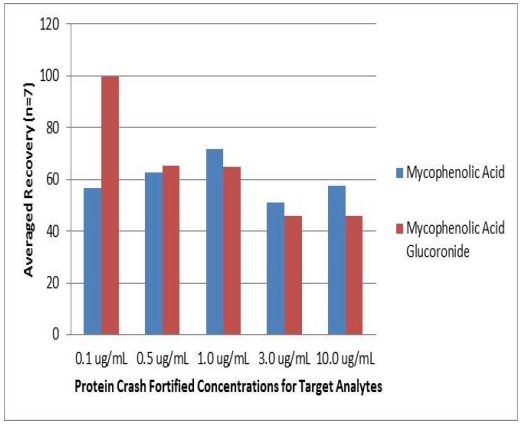

***Figure 5.** Recoveries of MPA and MPAG across dynamic concentration range extracted from spiked serum using protein precipitation.*

Conclusion

- ISOLUTE® SLE+ is a fast sample preparation alternative for biological fluids.
- The extraction of the immunosuppressant mycophenolic acid and its metabolite mycophenolic acid glucoronide was demonstrated using supported liquid extraction (ISOLUTE SLE+) as a viable alternative to protein crash.
- Improved recoveries were observed using supported liquid extraction which can ultimately lead to better quantitation and better sensitivities at lower limits of detection.
- Future work will include determining lower limits of detection for the analytes using ISOLUTE SLE+