Automated SPE-LC/MS/MS Assay for Immunosuppressant Drugs from Whole Blood

Kimberly Eaton[1], Kimberly Gamble[2], Tony Brand[3], M.P. George[3], Ken Lewis [1]
[1]OpAns LLC, Durham, NC; [2]Microliter Analytical Supplies, Inc. Suwanee, GA; [3]Agilent Technologies, Raleigh, NC

Introduction

A quantitative method has been developed for the determination of three immunosuppressive agents, namely cyclosporine, sirolimus and tacrolimus, from whole blood. The drug compounds were extracted using protein precipitation, then subjected to solid phase extraction (SPE) using Instrument Top Sample Preparation (ITSP) followed by reverse phase liquid chromatography. Detection with mass spectrometry was afforded using multiple reaction monitoring (MRM) of the ammonium adducts of the immunosuppressive agents and their respective internal standards.

Background

Over the past several years there have been many reports of quantitative methods for determination of immunosuppressive agents by liquid chromatography-mass spectrometry. Difficulties surrounding these assays are: requirements for an expensive instrument system; a skilled workforce; the need to develop procedures in house; and the requirement to achieve throughput comparable to that of automated clinical analyzers. All procedures require initial protein precipitation of whole blood samples. Practical considerations that influence the quality of LC-MS results include choice of internal standards (**1,2**) and quality of the solvent used for preparation of reagents and mobile phases (**3-5**). Moreover, since electrospray ionization is commonly used for these relatively high molecular weight compounds, LC-MS methods can be prone to the effects of ion suppression from either components in the biological matrices (human whole blood or commercial calibration materials) or sample preparation reagents (e.g., zinc sulfate) (**6**).

The importance of using solid phase extraction (SPE) to produce clean sample extracts has been demonstrated with increased apparent recovery of the drugs an their internal standards and accuracy of commercial calibration materials on a tandem quadrupole LC-MS system (**7,8**). The offline SPE procedure effectively removed compounds present in blood that were co-extracted during protein precipitation and that caused ion suppression. The offline SPE sample preparation procedure, while beneficial, produced large volumes of liquid reagent waste (mLs per sample) and required continuous operator interaction.

More recently some laboratories with tandem quadrupole systems have applied online SPE to the determination of immunosuppressants (**9-11**). Online SPE impacts sample throughput as each sample is subjected sequentially to SPE and then LC-MS. Moreover, the dedicated online SPE extraction cartridge must be cleared of retained compounds and re-equilibrated to the starting condition before another sample is introduced; it also has a finite lifetime.

Either offline or online SPE must be use prior to LC-M S by laboratories with single quadrupole systems because these instruments are inherently less selective than tandem quadrupole instruments (**12-15**).

Herein we present a method the provides the benefits of SPE while minimizing the drawbacks of offline and online methods. Instrument Top Sample Preparation (ITSP) automates the offline SPE process using a commonly available autosampler platform. A separate SPE cartridge is used for each sample and discarded after use; this eliminates the need to remove retained compounds and re-equilibration as for online SPE. While one sample is being analyzed by LC-MS, the next sample is being processed by SPE so that it is ready for injection onto the LC-MS system. This whole parallel process is under computer control. Moreover, with ITSP, only 0.5 mL of liquid reagent is used to process the equivalent of <10 µL of blood. The benefits of increased robustness to the procedure for determination of immunosuppressants is gained using ITSP SPE in combination with an LC-MS system as well as increased sample throughput, freedom for operators to perform other activities and reagent consumption/waste reduced by more than 10-fold.

Analysis Conditions

Instrumentation

LC: Agilent Model 1200 SL
MS: Agilent Model 6420 QQQ

LC Conditions

Solvent A: 2mM ammonium acetate and 0.1% formic acid in water
Solvent B: 2mM ammonium acetate and 0.1% formic acid in methanol
Column: Agilent Eclipse XDB-C8 Rapid Resolution (2.1 x 30 mm, 3.5µ
Column Temp: 60ºC; Injection Vol: 15 uL; Flowrate: 0.5 mL/min
Gradient:

Time (min)	%B
0.0	50
1.0	100 (step)
2.0	50 (step)
3.0	50

MS/MS Conditions

Ionization: ESI (positive ion); Detection: Multiple Reaction Monitoring
Transitions:
Ascomycin (IS): m/z 809.4>756.5
Tacrolimus: m/z 821.4>768.5
Desmethoxy-rapamycin (IS): m/z 901.4>834.6
Sirolimus: m/z 931.4>864.6
Cyclosporine: m/z 1219.7>1203.0
Cyclosporin G (IS): m/z 1233.7>1217.0

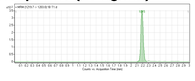
Instrument Top SPE Method

System: CTC Analytics HTC PAL with a 100 µSyringe and 3 cold stack trays
ITSP cartridges: SPE uLplate C8 (10 mg), Product No. 07-C810-20A
Solvent A: Acetonitrile **Solvent B:** 50% Methanol in Acetonitrile
Solvent C: 10% Methanol in Water **Solvent D:** 30% methanol in water

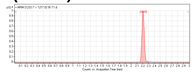
Step	Solvent	Volume	Flowrate
Clean SYR	A	100uL x 2	SYR Max uL/sec
Condition	B	100	20
Condition	C	100	20
Aspirate	Air	25	200
Load	Sample	100	5
Aspirate	Air	25	10
Clean SYR	A	100uL x 2	SYR Max
Wash	D	100	10
Aspirate	Air	25	Air Flush
Elute	B	100	5
Aspirate	Air	25	SYR Max

Low Level Chromatograms

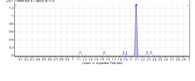
Cyclosporine (10 ng/mL)



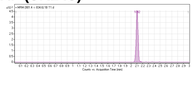
CSG (CSA IS)



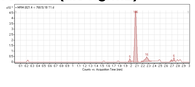
Sirolimus (1.0 ng/mL)



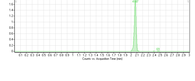
DMRP (SRL IS)



Tacrolimus (1.0 ng/mL)

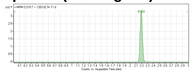


ASCO (TAC IS)

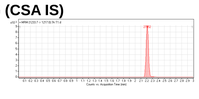


High Level Chromatograms

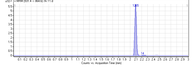
Cyclosporine (1500 ng/mL)



CSG (CSA IS)

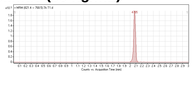


Sirolimus (50 ng/mL)

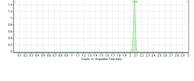


DMRP (SRL IS)

Tacrolimus (50 ng/mL)



ASCO (TAC IS)



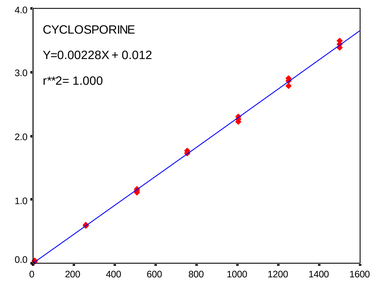
Standard and Sample Preparation

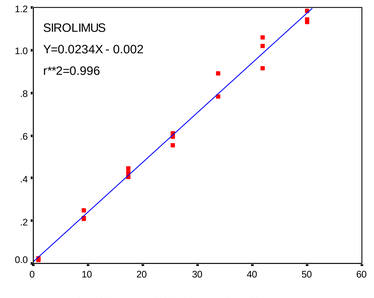
Standards: Cyclosporine, sirolimus and tacrolimus standard and ascomycin internal standard materials were obtained from LC Laboratories. Cyclosporin G and desmethoxy-rapamycin internal standard materials were the kind gift of G. Lensmeyer (U Wisconsin). Cyclosporine and sirolimus working standards were prepared in methanol, tacrolimus in acetonitrile. Fresh human blood was supplemented with drug compounds to provide 10, 25, 100, 500, 1000 and 1500 µg/L for cyclosporine and 1, 2.5, 5, 10, 25 and 50 µg/L for sirolimus and tacrolimus.

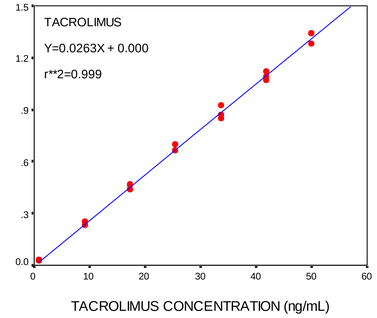
Samples: The drug compounds were extracted form whole blood using an adaptation of a published procedure (**14**). Whole blood aliquots (50 µL) were exposed to 200 µL of 10% methanol in 0.1M ammonium bicarbonate for 20 min. Then 200 µL of 0.1M zinc sulfate and 400 µL of internal standard solution (4µg/L ascomycin, 4 µg/L desmethoxy-rapamycin and 10 µg/L cyclosporin G in acetonitrile) were added sequentially. After 15-min incubation the extracts were centrifuged. Then 120 µL of supernatant was added to 90 µL of water in preparation for SPE using ITSP.

Results

Linearity across the concentration ranges was assessed by analysis in triplicate of seven test samples prepared by proportional dilution of the lowest with the highest whole blood standard (**16**). Deviations from expected were –0.2 2.2% (range –5.3 to +2.6%), cyclosporine; 0.2 7.9% (range –17.9 to +15.7%), sirolimus; 0.0 3.7% (range –4.9 to +9.1%), tacrolimus.







Results (continued)

Functional sensitivity was assessed using single 15 µL injections of 120 replicate samples (**17**). Respectively, percentage coefficient of variation and deviation were 6.1% and +3.5%, cyclosporine (10 µg/L; 9.2% and –3.6%, sirolimus (2.5 µg/L), 15.6% and –7.7%, tacrolimus (1.0 µg/L).

Precision experiments to determine within run and total imprecision (as percentage, %) were performed using three replicates of each of three levels of quality control materials (UTAK Laboratories) across five independent analytical runs (n=15) (**18**). Each run was controlled using a 6-point calibration curve.

	Cyclosporine	
Level	**Within**	**Total**
1 (Low)	4.5	5.3
2 (Med)	3.2	3.8
3 (High)	2.7	3.2
	Sirolimus	
	Within	**Total**
1 (Low)	9.6	9.7
2 (Med)	4.8	7.7
3 (High)	7.4	7.4
	Tacrolimus	
	Within	**Total**
1 (Low)	1.8	2.3
2 (Med)	1.3	3.4
3 (High)	1.9	2.7

Sample-to-sample carryover by the PAL was assessed three times by preparation of a solvent blank immediately following the highest whole blood standard. Carryover of 0.03%, no detectable amount and 0.1% was observed for cyclosporine, sirolimus and tacrolimus, respectively. Similar values were observed for the respective internal standards.

References

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For Further Information

www.OpAns.com
Contact Ken Lewis at KLewis@OpAns.com
OpAns, LLC, 4134 S. Alston Ave. Durham, NC 27713
(919) 323-4300
www.Microliter.com
Contact Kim Gamble at Kim.Gamble@Microliter.com
Microliter Analytical Supplies, PO Box 808, Suwanee, GA 30024
(888)-232-7840