University of Wisconsin Medical Center
Research for Determination of Immunosuppressants in Whole Blood

Background: Gary Lensmeyer of the University of Wisconsin Medical Center (UWMC) is responsible for exploring new technologies for his laboratory. During Monica Howland's (MicroLiter) employment with 3M she worked with Mr. Lensmeyer to evaluate and assist in development of the Empore line of SPE membranes.

MicroLiter contracted with UMWC to evaluate Instrument Top Sample Prep (ITSP) by developing a method that would typically be used in his lab. UWMC specializes in transplantation of organs. Immunosuppressants, prescription pain killers, and Vitamin D were the principal high volume assays that were run daily in his lab.

Mr. Lensmeyer chose Immunosuppressants as the method he would transfer to our ITSP.

Terms of the UWMC Contract: Mr. Lensmeyer was asked to independently evaluate ITSP to give us good, bad and indifferent opinions relating to the device. While we thought he would have a positive experience we did not ask him to produce data that offered only the benefit of the device.

Because of conflicts in scheduling MicroLiter's HTC PAL, the research was conducted in two phases. The first phase would be development of the method on the HTC PAL. The second phase would be to run the device against current laboratory operations.

Due to the critical nature of the work being performed in his lab, the Lab Manager, Anita Iwanski, would not allow the instrument to be attached on-line with the Agilent LC/MS that was currently running the assay.

Mrs. Anita Iwanski, Lab Manager made it clear that there was no money available for purchase of the system until July 2010.

The Work Product: Mr. Lensmeyer's current method utilized Phenomenex Strata

SDB-L sorbents and we were asked to use this material for a true apples-to-apples comparison. We asked for and obtained a sufficient quantity to begin our study. Initially 10mg bedmass weights were prepared. Mr. Lensmeyer was given 100 devices in advance of the delivery of the instrument to try and produce a manual method that we could program into the PAL. He was able to recreate the method, supplied us the conditions and we wrote the automation in advance of the delivery of the instrument.

On the day we installed the instrument, Mr. Lensmeyer immediately prepared solvents and supernatants and our first test run of the instrument prepped samples that gave Mr. Lensmeyer baseline data with which to start. The very first sample produced data that would have been acceptable to the lab to provide the physician data. After reviewing the method Mr. Lensmeyer determined that there were wash cycles that needed to be added to the method and those were added while we were there. The installation took a day and a half to satisfy Mr. Lensmeyer's approval of the install with a half day required only to replace a Fast Wash Station that was thought to be defective.

During the first month Mr. Lensmeyer ran samples using a combination of control and patient samples to optimize the method. During this time we provided per his request 10mg, then 3, 5, and 15mg bedmass weights to see which weight actually performed best. 15mg bedmass was determined to be his optimum volume.

Phase II was to actually test the device in a real time situation. Because of the positive results of the initial data, Mr. Lensmeyer asked for and received permission to run samples in-line during limited periods of phase II. In preparation for the event the PAL was installed on a metal lab cart so that the system could be plumbed during the research period and removed on a daily basis to perform real-time samples. Unfortunately, Mr. Lensmeyer's LC/MS system had an antiquated version of ChemStation and the installation was not a success.

In the report Mr. Lensmeyer reports that there was a hardware issue during the end of the research. It appeared that the Fast Wash Station had been bumped and the X, Y axis disturbed to the extent that Z axis error messages occurred. While we worked out one location over the phone with him there were actually three locations that were affected. Mr. Lensmeyer believed that had enough data to write his report and discontinued use of the PAL. It is our belief that had the PAL been installed in a proper setting the automation would have been robust.

From here the report speaks for itself regarding the data however there are some hardware conclusions that we believed needed additional investigation.

OpAns, LLC Continuation of the Research: Mr. Lensmeyer's method was performed on an Agilent 1100 LC/MS system. Because of the sensitivity, Mr. Lensmeyer's method requires a 1mL syringe to prep samples for his lab while his injection volume was only 5uL's, out of the range of the 1mL syringe's 100uL minimum injection. In a real-world application this could be overcome by eluting into an open well and mixing steps could be utilized to make sure the 5uL injection had adequate amounts of the desired compounds. While you can overfill the sample loop to use this syringe, the time that was required was not as desirable as offering an injection that was closer to the injection volume of the method.

A more expensive solution to this is one that occurs in GC applications. In this instance the requirement of a second rail on the CTC PAL to offer a syringe in a sufficient volume to mange the small injection could be installed on the existing system.

OpAns, LLC has been asked to review the data and concluded that LC/MS/MS could be utilized to scale the application down to at least the range of a 100uL syringe. This work is in process at the moment. Dr. Eaton, coming from an equivalent lab in Texas believes (as do we) that any new instrument being purchased by a lab would set a minimum standard of LC/MS/MS. While OpAns was provided the Strata SDB-L 15mg devices to produce new baseline data, they also believe that LC/MS/MS applications could use more mainstream sorbents such as C8 or CN to scale the application down to the 100uL target.

Once this data is complete we will combine the data into one report that we believe will give a wide range of options to choose from when converting to ITSP from existing applications.

Abstracts have been submitted to ASMS for possible publication at the Annual Meeting.

Development and Validation of a Method for Extraction of Immunosuppressive drugs from Whole Blood using the MicroLiter ITSP extraction device on a CTC Analytics PAL

Introduction

Cyclosporine, sirolimus and tacrolimus are fungal-based drugs that are used extensively to suppress rejection of transplanted organs in patients. These drugs have a narrow therapeutic window and thereby require close monitoring of blood concentrations to achieve effective levels and to prevent toxicity. In 2004 we developed and published (Ther Drug Monit 2004; vol. 27(1): pp 1-7) a liquid chromatography/mass spectrometry (LC/MS) method (See Appendix A) for the three drugs. A solid-phase, semi-automated extraction process on the Gilson XL4 was incorporated prior to LC/MS analysis.

In this report, we describe an alternative way of extracting the three drugs from blood. Here, MicroLiter's ITSP with CTC Analytics PAL handler was used in place of the Gilson XL4. The extraction protocol was essentially the same for both extraction units except, the ITSP used smaller reagent volumes and a different sorbent weight of SDBL (Phenomenex).

Procedure

Following the protocol described in the published method, we precipitated whole blood proteins with 750 uL of precipitating reagent and 250 uL whole blood (EDTA). After a 10 minute incubation at room temperature, the sample was centrifuged (10 min). The clear supernatant was decanted into a test tube containing 500 uL water. This mixture was placed on the ITSP/PAL unit. Briefly, the ITSP probe picks up a sorbent cartridge (SDBL, 15 mg), places it on the dock and primes the sorbent with 0.50 mL acetonitrile followed by 0.50 mL 10% acetonitrile in water. A 1.0 mL portion of the diluted sample supernatant is picked up by the probe/syringe and applied to the sorbent cartridge. The sorbent cartridge is washed with 0.50 mL of 30% acetonitrile in water. The probe/syringe is rinsed with 0.50 mL of 10% acetonitrile in water. Next, the probe/syringe loads 0.6 mL of eluting solution (acetonitrile) and then picks up the sorbent cartridge containing the retained drugs. The probe/syringe with attached sorbent cartridge moves to the elution area and forces the liquid through the cartridge into an empty vial. An aliquot of the eluate is injected into the LC/MS for analysis. In this study, we placed the vial with eluate directly onto the sample tray of the LC/MS. The program details of the ITSP/PAL along with typical LC/MS chromatograms are located in Appendix B.

Studies and Results

With-run Precision

Three whole-blood control products (Utak 2, Utak 3, BioRad Lyphochek 1) that contain cyclosporine, tacrolimus and sirolimus in a range of concentrations were assayed in single run in replicate (n=20). Results are listed in Appendix C. In summary the final results were:

Lyphochek 1	**Tacrolimus**	**Sirolimus**	**Cyclosporine**
Mean ng/mL	3.49	4.03	73.69
Std. Dev. ng/ml	0.13	0.23	1.39
CV (%)	3.6 %	5.7 %	1.9 %
Utak 2			
Mean ng/mL	15.07	18.09	515.5
Std. Dev. ng/ml	0.38	0.49	5.19
CV (%)	2.5 %	2.7 %	1.0 %
Utak 3			
Mean ng/mL	22.52	28.19	1196.3
Std. Dev. ng/ml	0.46	1.01	13.29
CV (%)	2.1 %	3.6%	1.1 %

Conclusion. Results are excellent. Generally CV's < 10% are acceptable.

Between-Run Precision

Three whole-blood control products (Utak 2, Utak 3, BioRad Lyphochek 1) that contain cyclosporine, tacrolimus and sirolimus in a range of concentrations were assayed in 20 different analytical runs for each control product. Results are listed in Appendix D. In summary the final results were:

ITSP:

Lyphochek1	**Tacrolimus**	**Sirolimus**	**Cyclosporine**
Mean ng/mL	3.63	3.86	78.22
Std. Dev. ng/ml	0.14	0.30	3.34
CV (%)	3.8 %	7.8 %	4.3 %
Utak 2			
Mean ng/mL	15.45	18.47	526.8
Std. Dev. ng/ml	0.29	0.63	6.82
CV (%)	1.9 %	3.4 %	1.3 %
Utak 3			
Mean ng/mL	23.56	29.85	1228.63
Std. Dev. ng/ml	0.58	1.15	20.02
CV (%)	2.5 %	3.8 %	1.6 %

Here are between-run precision for the Gilson XL4 extraction

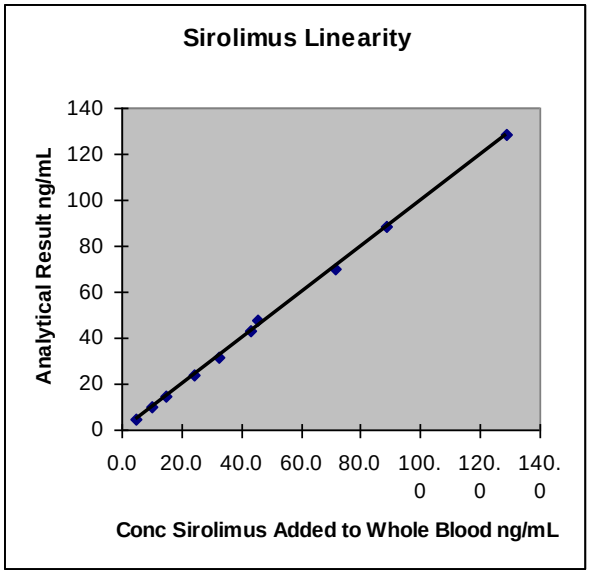
Lyphochek 1	**Tacrolimus**	**Sirolimus**	**Cyclosporine**
Mean ng/mL	3.54	3.70	72.13
Std. Dev. ng/ml	0.153	0.263	1.94
CV (%)	4.3 %	7.1%	2.7 %
Utak 2			
Mean ng/mL	15.11	18.59	526.8
Std. Dev. ng/ml	0.463	0.895	10.47
CV (%)	3.1 %	4.8 %	1.9 %
Utak 3			
Mean ng/mL	23.32	30.01	1231.1
Std. Dev. ng/ml	0.839	1.909	28.76
CV (%)	3.6 %	6.3 %	2.33 %

Conclusion. Results are excellent. Generally CV's < 10% are acceptable.

Linearity

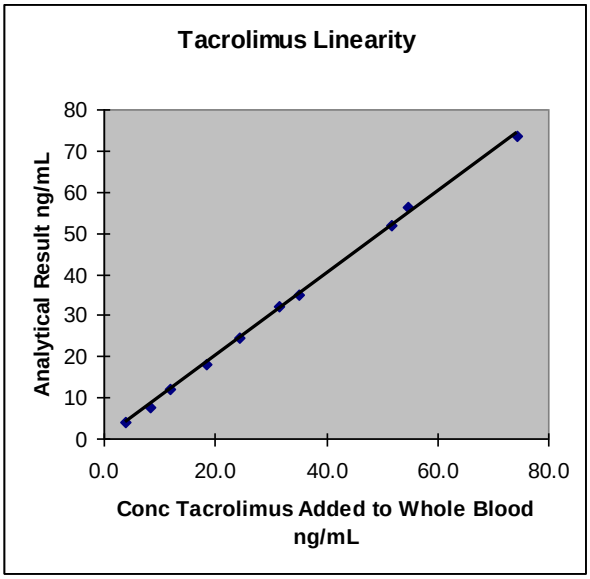
Drug-free whole blood was supplemented with a range of concentrations of the three drugs. The samples were assayed and graphs of the analytical result versus the supplemented concentration were created. This study was performed in two separate runs on different days with a different set of concentrations each day. Data is listed and results are listed in Appendix E.

Composite graphs of the data are presented here:



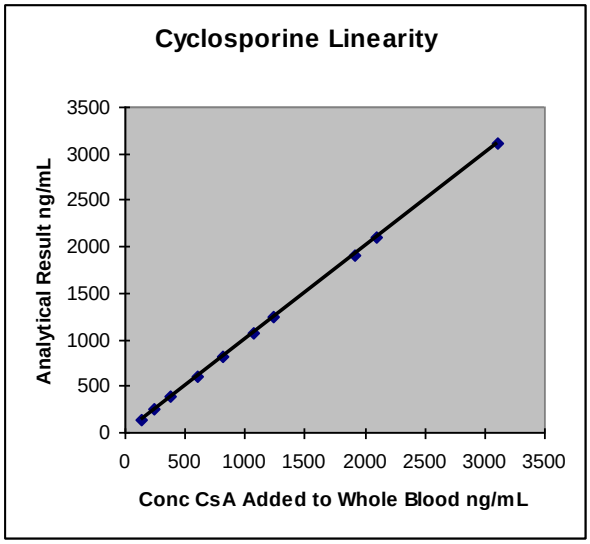
Sirolimus

n = 10
r = 0.9997
y = 1.00x + .005 ng/mL
Sy/x = 1.00 ng/mL



Tacrolimus

n = 10
r = 0.9996
y = 1.00x + .014 ng/mL
Sy/x = 0.611 ng/mL



Cyclosporine

n = 10
r = 1.0000
y = 0.999x + 0.245 ng/mL
Sy/x = 0.512 ng/mL

Gilson XL4 Linearity

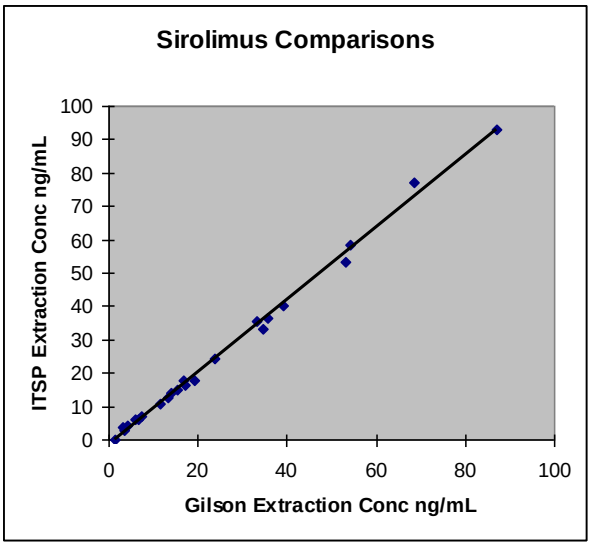
Sirolimus	**Tacrolimus**	**Cyclosporine**
n = 12	n = 12	n =12
r = 0.9999	r = 0.9998	r = 0.9998
y = 1.01x + .04 ng/mL	y = 1.007x + 0.26 ng/ml	y = 0.993x + 4.02
Linear from 1 to at least 80 ng/mL	Linear from 1 to at least 80 ng/mL	Linear from 25 ng/mL to at least 2000 ng/mL

Conclusion: Linearity is acceptable when graphs are evaluated visually and regression data are examined.

Patient Sample Comparisons

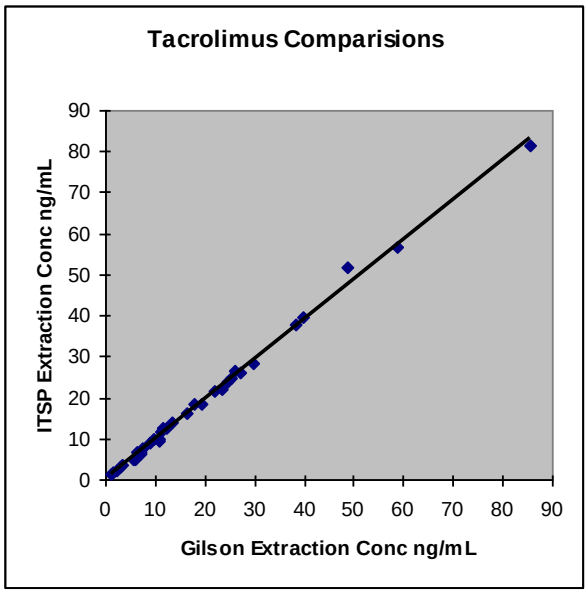
Samples from patients receiving the immunosuppressive drugs and drug-supplemented samples were extracted by both the ITSP/PAL and the Gilson XL4. Results were compared and regression graphs were created. Analytical results can be found in Appendix F.

Composite graphs of the data are presented here:



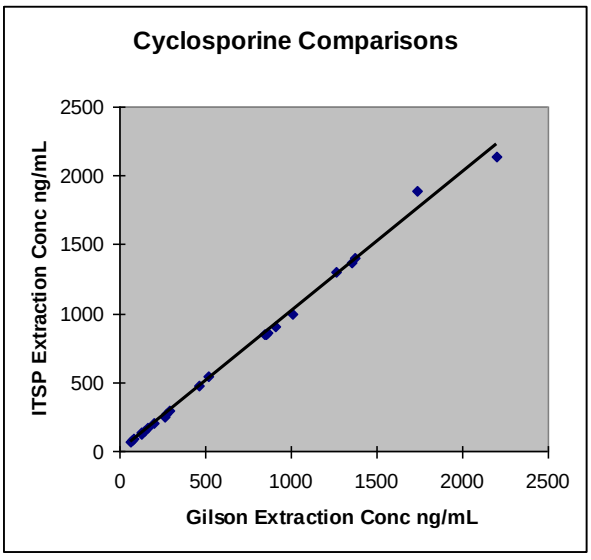
Sirolimus

n = 23
r = 0.9982
y = 1.08x – 1.38 ng/mL
Sy/x = 1.51 ng/mL



Tacrolimus

n = 25
r = 0.9983
y = 0.972x + 0.24 ng/mL
Sy/x = 1.01 ng/mL



Cyclosporine

n = 23
r = 0.9981
y = 1.01x + 1.71 ng/mL
Sy/x = 37.6 ng/mL

Conclusion:
Comparison results are excellent. Results for extractions with Gilson XL4 and the ITSP/PAL are essentially identical.

Low Limit of Quantization (LLQ)

The lyphochek 1 control was diluted with drug-free whole blood to achieve concentrations of approximately 0-1 ng/mL for Tacrolimus and Sirolimus. For cyclosporine concentrations were 10-20 ng/mL for cyclosporine. Each dilution was assayed in replicate (n = 9).

Results:
LLQ is for cyclosporine is 8 -10 ng/mL and for tacrolimus 0.8 – 1.0 ng/mL and sirolimus 1.5 – 1.8 ng/mL. Lower limits of LLQ can be achieved by injecting larger sample volumes.
Conclusion: LLQ for the ITSP extraction and Gilson XL4 extractions are essentially the same.

Comments

1. The operation of the ITSP/PAL instrument for this study was essentially trouble-free. On two occasions, the Z -axis of the probe/syringe became misaligned when a liquid was about to be applied to the sorbent cartridge setting in the dock and when the probe/syringe was attempting dock on the port for the first priming solution. The problem was corrected by optimizing the setting via the PAL software. Both issues occurred during the last week of the study. Also, at times when it was necessary to abort a run, the computer program failed (lost address of probe/syringe location) when a sequence was re-initiated. Rebooting the computer corrected the problem.
2. The benefits of the ITSP/PAL over the Gilson XL4 for the extraction of the immunosuppressive drugs:
    - > 60% decrease in solvent usage
    - decrease in amount of disposable glassware required
    - less amount of sorbent (SDBL) required (15 vs. 25 mg)
    - less manual intervention necessary/less labor
    - can directly inject into LC/MS (This study did not use this feature)
    - “just-in-time” extraction allows easy insertion of stat samples
    - potentially allows just one technologist to process all samples for the three drugs that come into our laboratory during day shift. (Improvement)