PROGRESS REPORT

TITLE:

High Performance Liquid Chromatographic Analysis of Cyclosporin A in Human Blood
(1-86 -0017)

PRINCIPLE INVESTIGATOR:

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Two totally different liquid chromatographic procedures for the measurement of cyclosporin A were developed and implemented for routine service at TCH Clinical Chemistry laboratory. The first method was a liquid phase extraction procedure which employed Methyl-t-butyl ether as extraction solvent. The interferent compounds in the whole blood were removed under acidic and basic conditions. The lipid components were then removed by the addition of heptane. This procedure has the advantages of less cost for consumables but is tedious and time-consuming (about 2.5 hrs for sample processing time or 20 minutes for CAP work units). The precision (day to day) is 8.3% for 300 ng/ml of cyclosporin A. The second procedure was a modification and adaptation of a method developed by Moyer et al (Clinical Biochemistry 19: 83-89, 1986). It employed double solid phase cartridges (C18 and silica) to remove contaminants and a short LC column packed with C1 reversed phase to resolve cyclosporin A from internal standard and other compounds. Although it costs a little bit more for consumables, the procedure is simple, accurate (day to day CV=4.9%), sensitive (to 50 ng/mL) and fast (5.7 minutes for CAP work unit). Correlation of the two procedures are excellent. Investigation of the therapeutic range for bone marrow transplantation is in progress. Therefore, I am requesting that the support for this project be continued until the end of 1987.

The two procedure manuals used in TCH Clinical Chemistry laboratory are attached.

Cyclosporine - Liquid Extraction

Cyclosporine is a small cyclic peptide which has immunosuppressive properties and is used to prevent organ rejection following transplantation surgery. Its mode of action appears to be blocking the T-lymphocyte functions. Cyclosporine's effects are reversible soon after withdrawal of the drug, requiring patients to receive cyclosporine throughout their lifetime. The blood concentration of cyclosporine must be monitored because patients vary widely in their ability to absorb and metabolize the drug. Insufficient levels allow host sensitization to the graft and high levels are nephrotoxic and have other side effects.

Principle:

After the addition of an internal standard (Cyclosporine D), cyclosporine is extracted from acidified whole blood, with methyl-t-butyl ether. The ether is then rinsed with base to remove further contaminants, dried, and reconstituted. A final rinse with heptane reduces residual contaminants, before the sample is injected onto a reversed-phase HPLC column where the cyclosporine is separated from its metabolites and the internal standard. The peak height ratio of cyclosporine to cyclosporine D is used to calculate the concentration of cyclosporine.

Patient Preparation:

Trough level preferred.

Collection and Handling of Specimen:

Collect 2 mL whole blood in a purple top tube (EDTA). DO NOT SEPARATE. Store whole blood at refrigerator temperature.

Type of Specimen and Minimum Quantity:

A minimum of 1.6 mL whole blood is required to assay the sample in duplicate.

Reagents:

1. Mobile Phase:

51% acetonitrile (Burdick and Jackson), 30% methanol (Burdick and Jackson), 19%

nanopure H_2O. Degas by sonication. Concentrations of acetonitrile and water may need to be adjusted as column ages (acetonitrile, water) or from column to column.

2. 180 mM HCl: Dilute 15.5 mL of concentrated Ultrex hydrochloric acid (J.T. Baker 4800-1) to 1.0 liter with nanopure water. Transfer to 5.0 mL "Dispensette" bottle and store at room temperature.

3. 10 M NaOH (stock solution): Dissolve 40 g of NaOH in 100 mL of nanopure water. Store in plastic bottle at room temperature.

4. 8 mM NaOH: Dilute 800 µL of 10 M NaOH (stock solution) to 1.0 liter with nanopure water. Transfer to 5.0 mL "Dispensette" bottle and store at room temperature.

5. Methyl-t-butyl-ether. (Burdick and Jackson). Pour directly from bottle into 10 mL "Repipet" dispenser bottle.

6. 76 mM $(NH_4)_2\ SO_4$ for reconstitution solution: Dissolve 5.02 g ammonium sulfate in 500 mL nanopure water. Store at room temperature.

7. Reconstitution Solution: Combine 10 mL acetonitrile (Burdick and Jackson), 10 mL methanol (Burdick and Jackson) and 30 mL 76 mM $(NH_4)_2\ SO_4$. Store at room temperature.

8. Internal Standard (Cyclosporine D).
   Stock Solution: 1 mg/mL in methanol. Dissolve 5 mg in 5 mL methanol (Burdick and Jackson). Store at -70° C in plastic tube. Stable at least one year.
   Working Solution: 5 µg/mL in methanol. Dilute 1.0 mL of stock solution to 200 mL methanol (Burdick and Jackson). Store at 4°C. Stable at least one year.

9. Cyclosporine Stock Standard.
   1 mg/mL in methanol. Dissolve 10 mg in 10 mL of methanol (Burdick and Jackson). Stable at least 9 months.

10. Mixture: Dilute 150 µL Cyclosporine Stock (1 mg/mL) and 25 µL Cyclosporine D Stock (1 mg/mL) to 50 mL with reconstitution solution. Inject 50 µL to test chromatographic conditions.

11. Whole Blood Pool for preparation of Standards and Controls.

1. Obtain outdated packed cells and plasma from blood bank. 500 mL whole blood hemosylate is required for each level of standard or control which is being prepared. (NOTE: If whole blood is available from the blood bank it may be carried through the procedure in a similar manner, eliminating step 5).

2. Pool cells in large plastic container and freeze overnight. Thaw in 37° C waterbath.

3. Centrifuge hemolysate at 3000 rpm for 10 minutes.

4. Filter the supernatant through Whatman #1 filter paper.

5. Pool and filter (Millipore 5 µ filter) an adequate volume of plasma to combine with the red cell hemolysate to give 60/40 (plasma/red cell) ratio. Mix by stirring 30 minutes on magnetic stirrer.

6. Label the appropriate 500 mL volumetric flasks and fill with whole blood hemolysate allowing for dilutions.

7. Add stock cyclosporine (1 mg/mL) in the following volumes:

Concentration	Volume Stock Cyclosporine/500 mL
500 ng/mL Std.	250 µL
300 ng/mL Std.	150 µL
100 ng/mL Std.	50 µL
250 ng/mL Control	125 µL

8. Dilute to volume with whole blood hemolysate.

9. Stir on magnetic stirrer for one hour.
10. While stirring continuously, aliquot 5.0 mL into glass screw-top vials.
11. Store at -70° C. Stable at least 9 months.

Equipment:

1. Column: Waters' μ-Bondapak C-18. Washed with methanol for at least 1 hr (flow rate of 1 ml/min) before use.

2. Delivery System: Varian VISTA 5500 LC.

3. Injector: Rheodyne Model 7125.

4. Detector: Varian UV 200.

5. Data System: Varian CDS 402.

6. Recorder: Omni Scribe. 1mV full scale. 0.25 cm/minute.

Procedure

Standards:

Stored in Revco -70°C freezer. After thawing, a tube may be used for up to 2 weeks with storage at 4°C.

Control:

Stored in Revco -70° C freezer.
Boxes W_3, W_4. X_1, X_2, X_3.
Pink bullet tubes.

Critical Points:

1. Turn on lamp and start pump before starting extraction. To check conditions mixture must be injected with autosampler.

2. Use tray-mounted Eppendorf pipet tips throughout the procedure.

3. All tubes used in the extraction msut be pre-rinsed with methyl-t-butyl ether.

4. Shaking time is critical. 15 minutes assures adequate extraction, longer time may cause foaming in some samples. If foaming occurs rim and re-spin.

5. Do not stop during the reconstitution step. The internal standard is lost into the heptane so the entire batch must be treated in exactly the same manner.

6. Injections must be carefully timed to avoid interference from peaks in standards and patients which elute at approximately 17 and 46 minutes.

7. Additional samples cannot be added to the run without extracting the 400 and 600 standards and the control. The control can be injected first and if it is within range, the patient sample can be injected. If the control is out of range, inject the standards to calculate the control and patient sample values.

I. Extraction

1. Rinse 2 screw-top tubes (CMS 251-884) each standard control, and patient with methyl-t-butyl ether.

2. Pipet the following into the rinsed tubes:

800 µL	whole blood (Eppendorf pipet)
2.0 mL	180 mM HCl (dispenser)
200 µL	5 µg/mL cyclosporine D (Eppendorf pipet)
10 mL	methyl-t-butyl ether (4 mL dispenser, 2X).

3. Cap with teflon-lined screw caps and shake 15 minutes on Eberbach shaker in horizontal position.

4. Spin 5 minutes at 2500 rpm.

5. Transfer ether to prerinsed tubes (CMS 251-884) containing 2.0 mL of 8 mM NaOH (dispenser). Vortex.

6. Spin 5 minutes at 2500 rpm.

7. Transfer ether to prerinsed 16 x 100 mm disposable glass tubes and dry under air using waterbath.

II. Reconstitution

1. Add 100 µL of reconstitution solution (1:1:3 of acetonitrile:methanol:76 mM $(NH_4)_2SO_4$) to each tube and vortex. Let stand 5 minutes.

2. Add 100 µL of heptane (Kodak) to each tube, vortex and immediately spin 5 minutes at 2500 rpm.

3. Remove from centrifuge promptly and immediately transfer 75 µL (Eppendorf pipet) of the bottom layer to an autosampler vial. Load tray in the following order: 600 Std, 400 Std, 200 Std, Control, Patient Samples. Single injections may be made initially. If problems with standard ratio, control value, peak heights, or interferences are observed, inject duplicate. Duplicate samples must be separated at the same time even though single injections are made.

III. Chromatography

Flow: 1mL/min of 100% A
Column Temperature: 70° C

Detector: UV 200 (attached to LC)
Wavelength: 214 nm
Recorder output: 0.005 AU/mV
Time constant: 0.5 sec

Data System: VISTA 402
Channel 4

Programs Cy and END4. Copies attached.

Autosampler: WISP

Injection volume: 50 µL

Run time: 14 minutes. May require adjustment with new column or as column ages to prevent interference of 2 peaks present in standards and samples at approximately 17 and 46 minutes.

IV. Starting Run on Vista 402

1. Use MM to check Cy program. The factor for cyclosporine in Section 3 must be changed to zero daily. Adjust the number of lines in Section 9 if necessary.

2. The programs in AM should be
   1. Cy
   2. End 4

3. Start 402 by entering AS. Channel 4 is used. The program will automatically go from checking to monitor to ready. If it does not advance from checking, ensure that the READY light on the top row of buttons next to the CRT on the pump is lit. If the RUN light is lit, hit RESET. When the data system READY light (bottom row of buttons) comes on, start the WISP by hitting RUN/STOP button.

V. Calculations

1. The data system will calibrate the run based on the 3 standards. Inspect the chromatograms, calibration factors, and control value to determine if manual calculations or further injections are necessary. If run is interrupted, allow interferring peaks to elute (46 minutes after last injection) before making further injections.

2. Inspect each patient chromatogram to determine if duplicate injection is necessary.

3. Results are reported in ng/mL.

4. Linearity is up to 1200 ng/mL. If patient sample concentration is greater than 1200 ng/mL, repeat extraction using a smaller sample volume. It is necessary to repeat the 400 and 600 standards and a control.

VI. Therapeutic Range:

Liver and Heart Transplant: 200-300 ng/mL

Renal Transplant: 100-200 ng/mL

Criteria for immediate notification of supervisor or lab director:

>400 ng/mL

VII. References:

1. Bowers, L., Therapeutic Monitoring of Cyclosporine, Therapeutic Drug Monitoring 7, 107 (1985).

2. Matthews, S.E., Rapid HPLC Assay for Cyclosporine, Clin. Chem. 32, 1056-1057 (1986).

3. Carruthers, S.G., Freeman, D.J., Koegler, J.C., et.al., Simplified Liquid-Chromatographic Analysis for Cyclosporin A, and Comparison with Radio-immunoassay, Clin. Chem. 29, 180-183 (1983).

* Note: This is only used as a back-up procedure.

CNSa 11/18/86

Reviewed by Pharm-N-Cu, Ph.D 11/18/86

Cyclosporine - Solid Phase Extraction

Cyclosporine is a small cyclic peptide which has immunosuppressive properties and is used to prevent organ rejection following transplantation surgery. Its mode of action appears to be blocking the T-Lymphocyte functions. Cyclosporine's effects are reversible soon after withdrawal of the drug, requiring patients to receive cyclosporine throughout their lifetime. The blood concentration of cyclosporine must be monitored because patients vary widely in their ability to absorb and metabolize the drug. Insufficient levels allow host sensitization to the graft and high levels are nephrotoxic and have other side effects.

Principle:

After the addition of an internal standard (Cyclosporine D), a mixture of methanol/acetonitride is added to a sample of whole blood to precipitate the protein and extract cyclosporine. The organic supernatant is transferred to a C-18 extraction cartridge. After washing the cartridge to remove contaminants, the cyclosporine is eluted with a mixture of ethyl acetate and isopropanol. The eluate is then passed through a silica cartridge. The effluent is evaporated and reconstituted in a small volume. The sample is injected onto a reversed phase HPLC column where cyclosporine is separated from its metabolites and the internal standard. The peak height ratio of cyclosporine to cyclosporine D is used to calculate the concentration of cyclosporine.

Patient Preparation:

Trough level preferred.

Collection and Handling of Specimen:

Collect 2 mL whole blood in a purple top tube (EDTA). DO NOT SEPARATE. Store whole blood at refrigerator temperature.

Type of Specimen and Minimum Quantity:

A minimum of 1.6 mL whole blood is required to assay the sample in duplicate.

Reagents:

1. Acetone, acetonitrile, ethyl acetate, isopropanol, methanol, and hexane are Burdick and Jackson HPLC grade.
2. Acetonitrile/methanol-9:1. 900 mL acetonitrile and 100mL methanol.
3. 70% methanol in H_2O. 700 mL methanol and 300 mL nanopure H_2O.
4. 1% acetone in hexane. 10 mL acetone and 990 mL hexane.
5. Ethyl acetate/Isopropanol 3:1. 900 mL ethyl acetate and 300 mL isopropanol.
6. Reconstitution Solution (1:1:3 ACN/MeOH/H_2O). Mix 2 mL acetonitrile, 2 mL methanol, 6 mL nanopure H_2O. Prepare fresh weekly.
7. Mobile Phase: 36/27/37 ACN/MeOH/H_2O. Combine 360 mL acentonitrile, 270 mL methanol, and 370 mL nanopure H_2O. Mix thoroughly and degas. To adjust for optimum separation, add H_2O in 20 mL increments to slow chromatogram or ACN to speed it up.
8. Internal Standard (Cyclosporine D).

   Stock Solution: 1 mg/mL in methanol. Dissolve 5 mg in 5 mL methanol (Burdick and Jackson). Store at -70% C in plastic tube. Stable at least one year.

   Working Solution: 5µg/ml in methanol. Dilute 1.0 mL of stock solution to 200 mL methanol (Burdick and Jackson). Store at 4°C. Stable at least one year.
9. Cyclosporine Stock Standard.

   1 mg/mL in methanol. Dissolve 10 mg in 10 mL of methanol (Burdick and Jackson). Stable at least 9 months.
10. Mixture: Dilute 150 µL Cyclosporine Stock (1 mg/mL) and 25 µL Cyclosporine D Stock (1 mg/mL) to 50 mL with reconstitution solution. Inject 50 µl to test chromatographic conditions.

11. Whole Blood Pool for preparation of Standards and Controls.
    1. Obtain outdated packed cells and plasma from blood bank. 500 mL whole blood hemosylate is required for each level of standard or control which is being prepared. (NOTE: If whole blood is available from the blood bank, it may be carried through the procedure in a similar manner, eliminating step 5).
    2. Pool cells in large plastic container and freeze overnight. Thaw in 37° C waterbath.

3. Centrifuge hemolysate at 3000 rpm for 10 minutes.
4. Filter the supernatant through Whatman #1 filter paper.
5. Pool and filter (Millipore 5 μ filter) an adequate volume of plasma to combine with the red cell hemolysate to give 60/40 (plasma/red cell) ratio. Mix by stirring 30 minutes on magnetic stirrer.
6. Label the appropriate 500 mL volumetric flasks and fill with whole blood hemolysate allowing for dilutions.

7. Add stock cyclosporine (1 mg/mL) in the following volumes:

Concentration	Volume Stock Cyclosporine/500 mL
500 ng/mL Std.	250 μL
300 ng/mL Std.	150 μL
100 ng/mL Std.	50 μL
250 ng/mL Control	125 μL

8. Dilute to volume with whole blood hemolysate.

9. Stir on magnetic stirrer for one hour.
10. While stirring continuously, aliquot 5 mL in glass screw-top tubes.
11. Store at -70° C. Stable at least 9 months.

Equipment:

1. Column: Supelcosil LC-1, 5 cm (Supelco, Inc. #5-8237). Condition new column with methanol for at least 1 hour prior to use (flow rate 0.5 mL/min).
2. Delivery System: Waters 501 HPLC pump.
3. Injector: Rheodyne Model 7125.
4. Column Heater: Fiatron TC-50 controller. Set at 70°C.
5. Detector: Waters Model 441. 214 nm filter.
6. Data System: Waters 740 Data module.
7. Recorder: Omni Scribe 1 mV full scale. 0.25 cm/minute.
8. Meyer N-Evap evaporator. Set at 30-40°C.
9. SMI Multitube Vortexer.
10. Analytichem Vac-Elut.

11. Bond-Elute C-18 extraction cartridge, 6 mL (Catalog #607306 Analytichem)
12. Bond-Elute Si extraction cartridge, 3 mL (Catalog #601303, Analytichem)

PROCEDURE

Standards:

Stored in Revco - 70°C freezer. After thawing, a tube may be used for up to 2 weeks with storage at 4°C.

Control:

Stored in Revco -70°C freezer.

I. Sample Preparation

1. Pipet the following into labeled 16 x 100 mm:
   1.0 mL whole blood (Eppendorf pipet)
   200 µL 5ug/mL CyD (Eppendorf pipet)
   4 mL Acetonitrile/methanol 9:1.
2. Vortex for 5 min and centrifuge for 5 min at 2500 rpm.
3. Decant supernatant into clean 16 x 100 mm tubes.
4. Add 2 mL of nanopure H_2O to each tube and vortex 5 sec.

II. Solid Phase Extraction

1. Prepare one 6mL C_{18} cartridge for each tube by washing with 3 mL Methanol, then 3 mL H_2O. Discard effluent.
2. Pour sample into appropriate column cylinder. Aspirate, discarding effluent.
3. Wash columns with 3 mL of 70% Methanol in H_2O. Discard effluent.
4. Wash columns with 1% Acetone in Hexane. Discard effluent.
5. Remove Vac-Elut top and place rack with 16 x 100 mm tubes inside for sample collection.
6. Replace top assuring needles are inside tubes.
7. Elute cyclosporine with 3 mL Ethyl Acetate/Isopropanol 3:1.
8. Remove rack containing tubes and discard C_{18} cartridges.
9. Place 3 mL Si columns on Vac-Elut and wash with 2 mL Methanol followed by 2 mL 1% Acetone in Hexane. Discard effluent.
10. Remove Vac-Elut top and place rack containing 16 x 100 mm tubes inside. Replace top.

11. Turn vacuum on.

12. *Pour the eluate from the C_{18} cartridges into the Si cartridges and collect* all effluent.

13. Dry effluents under air flow at 40°C.

14. Reconstitute in 100 uL reconstitution solution. Vortex. Let sit 5 minutes. **(DO NOT REDUCE TIME INVOLVED IN THIS STEP).**

15. Transfer samples to injection vials.

III. Chromatography

Flow: 1 mL/min

Column Temperature: 70°C

Detector: Waters 441

214 nm filter

0.01 AUFS

Data System: Waters 740 Data Module

WISP Autosampler: Injection Volume 50 uL

Run Time: 14 minutes

IV. Calculations

1. Calibrate the run with the Waters 740 Data Module measuring peak height of the 300 ng/mL standard and incorporating the origin. Inspect the chromatograms, calibration factor and control value to determine if further standard injections and/or manual calculations are necessary.

2. Inspect each patient chromatogram to determine if duplicate injection or repeat extraction is necessary.

3. Results are reported in ng/mL.

4. Linearity is up to 2000 ng/mL. If a patient sample concentration is greater than 2000 ng/mL, repeat extraction using a smaller sample volume.

V. Therapeutic Range

Liver and Heart Transplant: 200-300 ng/mL

Renal Transplant: 100-200 ng/mL

Criteria for immediate notification of supervisor or lab director:

>400 ng/mL

VII. References:

1. Moyer, T.P., Johnson, P., Faynor, S.M. and Steriott, S., Cyclosporine: A Review of Drug Monitoring Problems and Presentation of a Simple, Accurate Liquid Chromatographic Procedure that Solves These Problems. Clinical Biochemistry 19, 83-89 (1986).

2. Bowers, L., Therapeutic Monitoring of Cyclosporine, Therapeutic Drug Monitoring 7, 107 (1985).

Revised by Chang-Ning Chen, Ph.D. 11/18/86