Biochemical Diagnostics, Inc.
180 Heartland Blvd, Edgewood, NY 11717 ● **Phone (800) 223**-4835
Fax (631) 595-9204 ● **www.biochemicaldiagnostics.com**

DETECTABUSE® GRAVITY SERIES GV-65 METHOD FOR THE ANALYSIS OF CYCLOSPORIN A (CSA) AND METABOLITES AM1, AM9 AND AM4n USING HPLC

DECEMBER 2006

PREPARATION OF STANDARDS:

Stock Solutions:

Cyclosporin A	1 mg/mL	Methanol
Cyclosporin G (Internal Std),	1 mg/mL	Methanol
AM1 (CSA Metabolite),	1 mg/mL	
AM9 (CSA Metabolite),	1 mg/mL	
AM4n (CSA Metabolite),	1 mg/mL	

Working Stds	Components	Spike (uL)*	Final (ng/mL)	Solvent
Calibrator	CsA	250	5000	Methanol
*Prepare 50 mL in vol. flask from 1.0 mg/mL stock solutions	AM 1	250	5000	
	AM 9	250	5000	
	AM4n	250	5000	
Internal Standard *Prepare 50 mL in vol. flask from 1.0 mg/mL stock solutions	CsG	250	5000	Methanol

Controls

Negative Control	Negative Whole Blood
CSA Control	Level 2,3 Biorad or Equivalent

SAMPLE PREPARATION: Nominal sample volume for this procedure is 1.0 mL

Reagents	Description	Catalog #	Supplier
Neg. Control	Neg. Whole Blood		
CsA Control	CsA Whole Blood		
	Control Level 2	562	Bio Rad
	Control Level 3	563	

Step	Action
1.	Dispense 1 mL of test sample into labeled 16 x 100 mm test tube. Dispense (0.5 mL LEVEL 2 control +0.5 mL negative blood) into labeled 16 x 100 mm tube for positive LOW control. Dispense (0.5 mL LEVEL 3 control +0.5 mL negative blood) into labeled 16 x 100 mm tube for positive HI control.
2.	Dispense 1 mL of negative blood into 5 separate tubes; CAL1, CAL2, CAL3, CAL4, NEG.
3.	Add drug standard using Hamilton 250 *u*L gastight syringe to prepare calibrators and control samples following the table below.

Ref: Application developed by Roark Galloway, Microgenics Corp., Fremont, CA

Sample ID	Standard	Spike (*u*L)	Final (ng/mL)			
			CSA	AM1	AM9	AM4n
CAL1	calibration	10.0	50.0	50.0	50.0	50.0
CAL2	calibration	20.0	100.0	100.0	100.0	100.0
CAL3	calibration	50.0	250.0	250.0	250.0	250.0
CAL4	calibration	100.0	500.0	500.0	500.0	500.0
NEG	none	0.0	0.0	0.0	0.0	0.0

4. Vortex mix all samples

SAMPLE HEMOLYSIS: Whole blood hemolysis and salt precipitation of protein is accomplished by the following procedure.

Reagents	Description	Supplier
Lyse reagent	(50:50) Methanol/HPLC water +2.5% $ZnSO_4$	Microgenics

Step	Action
1.	Add 4.0 mL (50:50) CH_3OH/H_2O (v/v) +2.5% ZnSO4 lyse reagent to all samples.
2.	Spike all samples with standard (5000 ng/mL stock) using 100 *u*L positive displacement pipettor.
3.	Cap all sample tubes with PTFE-lined screw closures. Vortex for 10-20 seconds.
4.	Mix samples on end-over-end mixer for at least 15 minutes. (Do not let mix longer than 3 hours.)
5.	Centrifuge samples 5 minutes, 4500 rpm.

SAMPLE EXTRACTION: Solid phase extraction is completed manually using the Biochemical Diagnostics Multi-Prep Workstation with external vacuum.

Reagents	Description	Supplier
Acetonitrile	HPLC grade acetonitrile	Fisher
Water	HPLC Grade	Fisher
SPE Wash Reagent	(60:40) Water/Acetonitrile	Microgenics
Elution Reagent	Ethanol 95-100%	
Extract Wash	HPLC Grade Hexane	Fisher
SPE Column	Detectabuse GV-65 3cc Order # 1410072-0	Biochemical Diagnostics

Hardware	Catalog #	Supplier
10 Place Multi-Prep Workstation	1402210-5	Biochemical Diagnostics
28 Place Multi-Prep Workstation	1402000-1	Biochemical Diagnostics

Step	Action: Solid Phase Extraction
1.	Label one SPE column and 13 x 100 mm screw-top glass collection tube for each corresponding sample and install in collection rack.
2.	Condition each SPE column allowing solutions to pass through by gravity flow: 1 mL acetonitrile, followed by 1 mL H_2O (do not allow column bed to dry). Allow all SPE columns to drain completely before proceeding.
3.	Apply each sample supernate to each corresponding SPE column. If columns do not flow completely in 5 minutes apply minimum vacuum to pass sample through column at 1-2 mL/min.
4.	Wash SPE column (Gravity Flow). 1 mL (60:40) H_2O/CH_3CN (v/v)
5.	Apply full vacuum to dry columns for 30-60 seconds.
6.	Move column mounting plate into position over the tube rack. Align SPE columns with corresponding collection tubes.
7.	Dispense 1.0 mL ethanol into each SPE column to elute cyclosporines (Gravity flow). After initial 1 mL has drained from SPE column, add another 1.0 mL ethanol and collect fraction in same collection tube.
8.	Vortex evaporate samples to dryness.

NOTE: Extracts may be stored capped at -20°C up to 3 days

EXTRACT WASH: Sample extracts must be washed with hexane before HPLC analysis.

Reagents	Description	Supplier
Acetonitrile	HPLC grade acetonitrile	Fisher
Methanol	HPLC grade methanol HPLC mobile phase B	Fisher
Water	HPLC grade water	Fisher
	HPLC weak eluate (10:90) Methanol/Water	Microgenics
	HPLC strong eluent (30:70) Methanol/Acetonitrile	Microgenics
Waters 717 vial	96 position amber glass 1 mL vial with cap: Order # C4015-99	National Scientific
Vial Insert	Polyspring glass insert for 1 mL vial: Order #C4015-96A	National Scientific

Step	Action: Extract Hexane Wash
1.	Reconstitute dried extracts: Dissolve in 75 *u*L HPLC strong eluent (D) and vortex Add 75 *u*L HPLC weak eluent (C) and vortex mix.
2.	Add 0.5 mL hexane to sample extracts. Cap all sample tubes.
3.	Vortex samples for 10-15 seconds.
4.	Centrifuge all samples 5 minutes at 4500 rpm.
5.	Carefully remove upper (hexane) layer form sample extract and discard.
6.	Use 200 *u*L pipetor with gel-loading tip set to transfer 125 *u*L sample extract to vial for 717 auto sampler. Vial must have glass poly-spring limited volume insert installed. Cap sample vial.

HPLC ANALYSIS: Set up the prepared samples to analyze by HPLC using the following procedure.
The installed HPLC column must contain a Sepherisorb C8 (250 mm x 4.6 mm) resin.

Reagents	Description	Supplier
HPLC weak eluent (C)	(10:90) Methanol/Water	Microgenics
HPLC strong eluent (D)	(30:70) Methanol/Acetonitrile	Microgenics
HPLC wash eluent (B)	Methanol, HPLC grade	Fisher
HPLC column	Spherisorb C8 250 x 4.6 mm	MACH-MOD

Step	Action: HPLC Setup
1.	Check HPLC waste bottle to make sure contents will not overflow during the sample analysis. Replace waste bottle with empty collection bottle if needed.
2.	Make sure HPLC reservoir (B), (C), and (D) all have
3.	Turn on power switches to all modules, computer and monitor on HPLC system DL-17.
4.	Manually establish flow from the Controller keypad using the following control sequence.

HPLC GRADIENT PROGRAM: All (cyclosporin) samples are analyzed using the following gradient program on the WATERS HPLC system. The two step program allows elution of CsA and metabolites during isocratic mobile phase composition. CsA elutes during Step 5-6 (l2.26-27.50 min.)

The HPLC column temperature is maintained at 70°C during the HPLC run.

CsA GRADIENT PROGRAM

	Time*	Flow**	%A	%B	%C	%D	Curve
1		1.00	0.0	0.0	40.0	60.0	1
2	3.00	1.00	0.0	0.0	40.0	60.0	1
3	3.01	1.00	0.0	0.0	35.0	65.0	1
4	12.25	1.00	0.0	0.0	35.0	65.0	1
5	12.26	1.00	0.0	0.0	30.0	70.0	1
6	27.50	1.00	0.0	0.0	30.0	70.0	1
7	27.51	1.00	0.0	0.0	40.0	60.0	1
8	35.00	1.00	0.0	0.0	40.0	60.0	1

* (min) ** (mL/min)

C= Weak Eluent = l0% CH_3OH/H_2O

D= Strong Eluent = (30:70) CH_3OHCH_3CN

Parameter	Description		Result
Linearity:	Accurate quantitation analyte within specified concentration range	CsA:	25 - 2000 ng/mL
		Metabolites:	25 - 2000 ng/mL
Recovery:	Amount of analyte recovered after extraction process	CsA:	65%
		Metabolites:	53 - 61%

Interferences: Some older samples have shown artifact peaks which interfere with CsG internal standard and will adversely affect quantitation of CsA and metabolites.

No interferences with CsA have been observed with this method.

Some samples will produce several peaks and shoulders around metabolites AM1 and AM9. Manual integration of sample result chromatograms will be necessary to obtain accurate results.

Precaution: This is an experimental procedure which has given good results in our laboratory. The performance of this procedure must be validated by your laboratory before it is used to report patient values.