



# Biochemical Diagnostics, Inc.

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## 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 |
|--------------|------------|-------------|---------------|---------|
|--------------|------------|-------------|---------------|---------|

|                       |      |     |      |          |
|-----------------------|------|-----|------|----------|
| <b>Calibrator</b>     | CsA  | 250 | 5000 | Methanol |
| *Prepare 50 mL in     | AM 1 | 250 | 5000 |          |
| vol. flask from 1.0   | AM 9 | 250 | 5000 |          |
| mg/mL stock solutions | AM4n | 250 | 5000 |          |

|                          |     |     |      |          |
|--------------------------|-----|-----|------|----------|
| <b>Internal Standard</b> | CsG | 250 | 5000 | Methanol |
|--------------------------|-----|-----|------|----------|

\*Prepare 50 mL in vol. flask from 1.0 mg/mL stock solutions

#### 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

- 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.
- Dispense 1 mL of negative blood into 5 separate tubes; CAL1, CAL2, CAL3, CAL4, NEG.

- Add drug standard using Hamilton 250 uL 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 (uL) | Final (ng/mL) | CSA   | AM1   | AM9   | AM4n  |
|-----------|-------------|------------|---------------|-------|-------|-------|-------|
| CAL1      | calibration | 10.0       | 50.0          | 50.0  | 50.0  | 50.0  | 50.0  |
| CAL2      | calibration | 20.0       | 100.0         | 100.0 | 100.0 | 100.0 | 100.0 |
| CAL3      | calibration | 50.0       | 250.0         | 250.0 | 250.0 | 250.0 | 250.0 |
| CAL4      | calibration | 100.0      | 500.0         | 500.0 | 500.0 | 500.0 | 500.0 |
| NEG       | none        | 0.0        | 0.0           | 0.0   | 0.0   | 0.0   | 0.0   |

- 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 <sub>4</sub> | Microgenics |

#### Step Action

- Add 4.0 mL (50:50) CH<sub>3</sub>OH/H<sub>2</sub>O (v/v) +2.5% ZnSO<sub>4</sub> lyse reagent to all samples.
- Spike all samples with standard (5000 ng/mL stock) using 100 uL positive displacement pipettor.
- Cap all sample tubes with PTFE-lined screw closures. Vortex for 10-20 seconds.
- Mix samples on end-over-end mixer for at least 15 minutes. (Do not let mix longer than 3 hours.)
- Centrifuge samples 5 minutes, 4500 rpm.

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

| <u>Reagents</u>  | <u>Description</u>                         | <u>Supplier</u>         |
|------------------|--|-------------------------|
| 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<br>Order # 1410072-0 | Biochemical Diagnostics |

| <u>Hardware</u>                 | <u>Catalog #</u> | <u>Supplier</u>         |
|---------------------------------|------------------|-------------------------|
| 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<sub>2</sub>O (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<sub>2</sub>O/CH<sub>3</sub>CN (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.

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

