



# Biochemical Diagnostics, Inc.

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## DETECTABUSE® GRAVITY SERIES GV-65 METHOD FOR THE ANALYSIS OF URINARY ANABOLIC STEROIDS AND METABOLITES BY GC/MS

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### **SAMPLE PREPARATION** - (Please see Notes and Supplemental Information before proceeding)

1. Add 3.0 mL of urine to a 16 x 100 mm disposable borosilicate glass tube.
2. Add the appropriate deuterated standards to each sample.

**Note:** When adding an internal standard dissolved in an organic solvent to a urine or blood sample, the solvent volume must not exceed 3% of the buffered sample volume. Higher solvent concentrations may produce extraction losses.

### **SAMPLE HYDROLYSIS**

1. Add 0.5 mL of 0.2M Acetate Buffer, pH 5.0 to each prepared sample. (pH should be 4.5 - 5.0)
2. Add 10,000 units of Beta-Glucuronidase, Sigma type H-2S from Helix Pomatia (or equivalent) to each sample.
3. Mix gently and incubate at 55°C for two hours or 37°C for 4 hours. Complete hydrolysis is also achieved in 16 hours at room temperature (15 - 30°C).
4. Add 3.0 mL of 0.25M Phosphate Buffer, pH 9.1.
5. Centrifuge for 3 minutes at 4000 RPM.

**HARDWARE SETUP** - (Please refer to the Detectabuse Hardware Setup Instructions)

### **COLUMN CONDITIONING**

1. Wash column with 1.0 mL of Methanol. Allow to flow by gravity.
2. Proceed to Sample Extraction within 30 min. of column conditioning. For longer periods follow the Methanol wash with 3.0 mL of 0.25M Phosphate Buffer, pH 9.1 to prevent column from drying out.

### **SAMPLE EXTRACTION** - (Please see Notes at end of this section before proceeding)

1. Pour samples onto preconditioned columns. Allow to flow by gravity. Samples will flow through the columns at a rate of 1-2 mL/min.
2. Add 2.0 mL of 0.1 N NaOH. Allow columns to flow by gravity.
3. Add 3.0 mL of deionized H<sub>2</sub>O. Allow to flow by gravity.
4. Add 2.0 mL of Water:Methanol (80:20). Allow the columns to flow by gravity.
5. Dry the columns by applying vacuum adjusted to at least 7" Hg for 5 minutes (Test by momentarily placing the heel of hand over the column top. A strong pull should be felt through the column).

**Note:** If liquids do not elute freely by gravity flow, there is probably air trapped within the column bed or frits. Tapping the column mounting plate onto the vacuum box should initiate flow. Any columns that have not emptied within 5 or 6 min. may be induced with a low vacuum from a small vacuum pump.

### **SAMPLE ELUTION**

1. Sample elution is done outside of the vacuum box.
2. Place the column mounting plate on the elution rack loaded with an appropriate number of 12 x 75 mm or 15 x 85 mm borosilicate glass test tubes. Make sure that the hole pattern on the plate matches the hole pattern on the rack.
3. Add 1.5 mL of n-Butyl Chloride to each column and allow solvent to flow through the column by gravity into test tubes.
4. Dry under N<sub>2</sub> or argon at 55°C.

**Note:** If a sample does not elute freely by gravity flow, there is either air trapped within the column bed or frits or aqueous phase remaining on the column because of weak vacuum during the column drying step. In most cases, tapping the column will initiate flow. If this does not do the job, use a rubber bulb to gently push a few drops of elution solvent and trapped air into the collection tube. Allow the remainder of solvent to flow by gravity.

## DERIVATIZATION

1. To each dried extract add 70  $\mu$ L Acetonitrile, vortex mix, then add 15  $\mu$ L Trimethylsilylimidazole (TMSI) and 15  $\mu$ L MBTFA. Mix the tube contents, flush with nitrogen or argon and cap the tube or transfer contents into 100  $\mu$ L reaction vials and seal.
2. Inject 2.0  $\mu$ L.

**Note:** TMSI is a powerful reagent for the silylation of hindered hydroxyl groups. MBTFA will selectively acylate amines (such as Stanozolol) in the presence of hydroxyl and carboxyl groups that have been protected by silylation.

**SUPPLEMENT** - When using an automated robotic system all liquids may be allowed to flow unassisted through the column or may be pulled through the column with vacuum or pushed through with positive pressure.

Assisted flow parameters may be set as follows:

**Column Conditioning** - Pass through column in approximately 20 seconds ( $\pm$  20%).

**Sample, Sample Washes and Elution Solvent** - Pass through column in approximately 60 seconds ( $\pm$  20%).

**Column Drying Steps** – Use 12 – 15 PSI positive pressure for 40 seconds or vacuum set at 15" Hg for 30 seconds (These drying parameters are for individual columns).

## GC/MS ANALYSIS

GC/MS: Hewlett-Packard equipped with Mass Selective Detector  
GC Column: H.P. Ultra 2 Capillary Column (or equivalent), 15 m x 0.25 mm, 0.25  $\mu$ m film thickness.  
Acquisition Mode: SIM  
Injector Temp.: 280°C  
Detector Interface Temp.: 305°C  
Temperature Program:  
Initial: 150°C, program at 25°C/min. to 300°C  
Final Time: Hold for 1.5 min.  
Equil. Time: 1.0 min.  
Splitless  
He Flow: 1.0 mL/min. @ 200°C  
Septum Purge: 2.0 mL/min.  
Purge Off Time: 1.0 min.  
Solvent Delay: 4.0 min.  
Dwell: 20  
Start Acq.: 4.0 min.  
Stop Run: 7.5 min.

## MSD PROGRAM

Steroid	Ions Monitored	Retention Time (min.)
Androsterone	271, <u>272</u> , 347	5.22
Etiocholanolone	<u>246</u> , 272, 362	5.46
Dehydroepiandrosterone	270, <u>304</u> , 360	5.51
Epitestosterone	226, 270, <u>360</u>	5.66
Nandrolone	<u>256</u> , 331, 346	5.69
Androstenedione	201, 244, <u>286</u>	5.77
Testosterone	226, 270, <u>360</u>	5.90
Stanozolol	<u>143</u> , 144, 481	6.94

*This method is a preliminary procedure for investigational use only. Although it has performed well in our laboratory it must be validated by your laboratory before it is used to report patient values.*

*We would appreciate your comments on it's performance and welcome your suggestions for improvements or enhancements.*