

December 7, 2006

## FOR INVESTIGATIONAL USE ONLY

### MULTI-PREP™ GRAVITY SERIES GVSA-100 METHOD FOR THE ANALYSIS OF HOMOCYSTEINE IN SERUM OR PLASMA USING GC/MS

#### STANDARD PREPARATION

##### External Standards

Homocysteine, and Homocysteine D<sub>4</sub> will gradually oxidize respectively to Homocystine and Homocystine D<sub>8</sub> when stored in solution. Standards which are dried down without extraction must be protected from oxidation.

This method incorporates the use of Stabilizer "A", a proprietary reducing agent, to maintain Homocysteine in a reduced state.

**The working standard stabilizer solution** is prepared daily by the addition of 0.1 mL Stabilizer "A" concentrate to 10 mL of Methanol containing 3% Ammonium Hydroxide . Make sure Stabilizer "A" concentrate and working solution containers are flushed with nitrogen, closed tightly, and refrigerated when not in use.

**1 mg/mL stock solutions of Homocysteine and Homocysteine D<sub>4</sub>** are prepared by dissolution in deionized water.

**The working standard solution** is prepared by diluting the stock standard solution 1:100 with Methanol:H<sub>2</sub>O (90:10).

**Working standards** are pipetted from the working standard solution into 1 mL of working standard stabilizer solution. e.g. a 200 ng standard is prepared by pipetting 20  $\mu$ L of working standard into 1 mL of working standard stabilizer solution. Wait 30 minutes for reduction before drydown of working standards.

##### Extracted Standards

When preparing standards for extraction or internal standardization, it is preferable to start with Homocystine and Homocystine D<sub>8</sub> which will be reduced respectively to Homocysteine and Homocysteine D<sub>4</sub> during the extraction procedure.

The use of these compounds for extraction serve as a check on the overall reduction of serum and plasma target analytes when compared to the reduced external standards.

**1 mg/mL stock solutions of Homocystine and Homocystine D<sub>8</sub>** are prepared in Methanol containing 1% HCL (v/v). Non-extracted working standards or internal standard to be dried down directly, are diluted with Methanol:H<sub>2</sub>O (90:10) and pipetted into working standard stabilizer solution as above.

**Working Standards for extraction or internal standardization** are diluted 1:100 with Methanol:H<sub>2</sub>O

(90:10) solution and added directly to 100  $\mu$ L of serum.

**Values for Total Homocysteine in serum or plasma** vary somewhat from lab to lab but 5 -15  $\mu$  MoL/L is usually considered normal

#### **SAMPLE COLLECTION:**

Blood should be collected and stored on ice. Serum or plasma should be separated within 1 hour of collection and refrigerated (up to 5 days) or frozen until assayed.

#### **SAMPLE PREPARATION:**

1. 100  $\mu$ L of serum or plasma containing 200 ng Homocystine-D<sub>8</sub> in 20  $\mu$ L Methanol:H<sub>2</sub>O (85:15) solution as internal standard.
2. Add 100  $\mu$ L Stabilizer "A" concentrate and mix gently. Incubate at room temperature for at least 2 min.
3. Add 100  $\mu$ L 0.5N Sodium Hydroxide, mix and incubate at 40°C for 30 min.
4. Add 3 mL of 0.1M Tris Buffer, pH 9.0.
5. Centrifuge 3000 RPM for 3 min.

#### **COLUMN PREPARATION:**

Wash each column sequentially with the following solutions, allowing each wash to drain through the column by gravity flow.

1. 3 mL 0.2M Acetic Acid in Acetone
2. 3 mL 10% HCL
3. 3 mL H<sub>2</sub>O

#### **SAMPLE EXTRACTION AND PURIFICATION:**

1. Pour prepared samples onto the columns and allow them to drain by gravity flow.
2. Wash each column as follows:
  - a. 2 mL Acetone:H<sub>2</sub>O 1:1.
  - b. 5 mL H<sub>2</sub>O

#### **SAMPLE ELUTION**

1. Elute with 1.5 mL 0.5M Acetic Acid in Methanol.
2. Dry at 60°C under Nitrogen or Argon. Remove from heat as soon as eluates have dried.

**Elution Solvent Preparation:** Add 2.8 mL of Glacial Acetic Acid to 95 mL of Methanol.

#### **DERIVATIZATION**

1. Add 50  $\mu$ L of n-Butylchloride and 50  $\mu$ L MTBSTFA to each dried eluate and standard. Mix well.
2. Cap tubes and incubate at 75°C for 20 min.

## GC/MS ANALYSIS

5970 MSD

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.: 250°C

Detector Interface Temp.: 300°C

Temperature Program:

Initial: 85°C then temp. program to 170

C @ 12°/min.

Final: 170 - 285°C @ 20°/min.

Equil Time: 0.5 min.

Splitless

He Flow: 1.0 mL/min. @ 150°C

Septum Purge: 2 mL/min.

Purge Off Time: 1 min.

Solvent Delay: 3 min.

Start Acq.: 3 min.

Stop Run: 13min.

## MSD PROGRAM

Drug	Ions Monitored	Retention
Homocysteine	420	11.70 min.
Homocysteine D <sub>4</sub>	424	11.69 min

**Note: Retention time and ion spectra will vary somewhat from instrument to instrument.**

<sup>1</sup> Clinical Chemistry, VOL 39, NO 9, 1993, 1764-1779

**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**