



## Biochemical Diagnostics, Inc.

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### DETECTABUSE® "NO VACUUM" GRAVITY GV-65 / GV-65C METHOD FOR THE ANALYSIS OF HYDROLYZED OR "FREE" EXTENDED OPIATES IN URINE, SERUM OR ORAL FLUIDS BY GC/MS

Please see Notes and Supplemental Information before proceeding

#### SAMPLE PREPARATION

##### Acid Hydrolysis of Urine

1. Pipet 1.0 mL sample into a 16x100 mm pressure rated glass tube with a Teflon® lined screw cap.
  2. Pipet 0.2 mL concentrated HCl into each tube.
  3. Add appropriate amount of internal standard(s) to each sample tube. (See Note 3 at the end of the Sample Preparation section)
  4. Add 250ul of 10% Methoxyamine
  5. Vortex.
  6. Cap tubes and heat in autoclave or heating block.
    - a. Autoclave at 150°C for 15 min. at 15 PSI.
    - b. Heating block at 120°C for at least 30 min.
- Complete hydrolysis requires at least 2 hours.
7. Cool tubes.
  8. Add 1.0 mL of 12% aqueous Potassium Hydroxide (KOH) per mL of sample and mix. 12 g KOH (Assay 87%) is added to a 100 mL flask which is then carefully filled with deionized water. (See Precaution at the end of this section)
  9. Add 1.0 mL of a 20% solution of monobasic Potassium Phosphate solution per mL of sample to each column and mix (20 grams/100 mL H<sub>2</sub>O prepared monthly). Final pH should be between 2.0 and 3.0. If necessary, adjust the pH with a few drops of either 1N KOH or 1N HCl.
  10. Samples should be centrifuged for 3 min. at 3000 RPM.

**Precaution:** Adsorption must be done at an acidic pH of approximately 2 - 3. The 1.0 mL of KOH and buffer added in Steps 8 and 9 ordinarily sets up the optimum pH for adsorption. If however, the pH is too high, (i.e. greater than 3.5) drug recoveries will decline with increasing pH. If the pH is too low, the column washes will not remove all of the hydrolysis acid remaining on the column. The excess acid may cause the basic elution solvent to become weakly basic or acidic which will lead to partial or complete hindrance of drug elution. A non-bleeding pH paper can be used to test the pH of the eluate by wetting the paper with the solvent followed by a water rinse which will enable the color reaction to take place. This should only have to be done for the first run to assure that pH conditions are correct throughout the run. If you encounter any problems with this procedure please contact us for assistance.

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#### SAMPLE PREPARATION

##### Enzymatic Hydrolysis of Urine

1. Pipette 1.0 – 2.0 mL of sample for 300 ng opiate cutoff or Pipette 0.5-1.0 mL of sample for 2000 ng opiate cutoff.
2. Add appropriate amount of internal standard(s) to each sample tube. (See Note 3 at the end of the Sample Preparation section)
3. Add approximately 5000 units of Beta-Glucuronidase, (e.g. Helix Pomatia) per mL of sample. Add 0.5 mL of 0.2M Acetate Buffer pH 5.
4. Mix gently and incubate at 55°C for 2 hours.
5. Adjust pH to 2 with ≈ 0.1-0.2 mL of 10% HCl.
6. Add 250 uL of 10% methoxyamine and incubate for 20 min. at room temperature.
7. Centrifuge for 3 minutes at 3500 RPM.

#### SAMPLE PREPARATION WITHOUT HYDROLYSIS "Measurement of Free Opiates"

##### Measurement of Free Opiates in Urine

1. Pipet 1.0 – 2.0 mL sample into a 16 x 100 mm disposable glass tube.
2. Add appropriate amount of internal standard to each tube.
3. Add 1.0 mL of 1% HCl in H<sub>2</sub>O and mix.
4. Centrifuge for 3 minutes at 3000 RPM.

##### Measurement of Free Opiates in Serum or Oral Fluid

1. Pipet serum or oral fluid sample (typically 0.1 - 2.0 mL) into a 13 x 100 mm borosilicate test tube.
2. Add appropriate amount of internal standard into each tube.
3. Add 1.0 mL of 1% HCL per mL of sample and mix.
4. If Cloudy or precipitated centrifuge for 3 minutes at 3000 RPM.

#### COLUMN CONDITIONING –ALL LIQUIDS FLOW BY GRAVITY

(Follow Column Conditioning procedure for EITHER GV-65 or GV-65C columns.)

#### Column Conditioning and Activation of Cation Function using GV-65 Columns

1. Wash column with 1.0 mL of Methanol.

2. Add 1.0 mL of a Sodium Bisulfite solution to each column.

*Prepare by dissolving 5 grams of Sodium Bisulfite in 100 mL of a (1:1) mixture of H<sub>2</sub>O:0.25M Phosphate Buffer, pH 6.0.*

*Prepare monthly. (Store refrigerated)*

3. Wash column with 1 mL deionized water.

4. Proceed to Sample Extraction within 20 min. of column conditioning.

### Column Conditioning using GV-65C Columns

**Note:** *The GV-65C column is manufactured with the cation exchanger and does not require the addition of sodium bisulfite.*

1. Wash column with 1.0 mL of Methanol.

2. Wash with 1.0 mL of deionized water.

3. Proceed to Sample Extraction within 20 min. of column conditioning.

### SAMPLE EXTRACTION

1. Decant samples onto columns.

2. Wash columns with 3.0 mL of 0.01% HCl.

3. Wash columns with 2.0 mL of Methanol.

4. Add 1.0 mL Ethyl Acetate.

### 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 16 x 100 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-Butylchloride: Ethyl Acetate (80:20) with 4% Triethylamine (TEA) to each column.

4. Dry under N<sub>2</sub> or argon at 40°C.

### Anhydride Derivatization - Using Propionic Anhydride, 99+% (Aldrich Chemical Company)

1. To each dried extract add 50 µL Pyridine, vortex mix, then add 50 µL Propionic Anhydride.

2. Mix and incubate the mixture @ 100°C for 45 min.

3. Allow the mixture to come to room temperature. Add 1.0 mL of Hexane. Mix.

4. Dry under argon or nitrogen @ 65°C.

5. Add 100 uL Acetonitrile Transfer to a vial and cap.

### Using MBTFA

1. To each dried extract add 75 uL N-Methyl-bis(trifluoroacetamide) (MBTFA).

2. Mix the tube contents, cap and incubate at 65°C for 30 mins.

3. Transfer contents into 100 uL reaction vials and seal.

4. Inject 1.0-2.0 uL

### MSD SIM PROGRAM - Propionic Anhydride

Drug	Ions Monitored
Hydrocodone-D-6	334, 303
Hydrocodone	328, 297, 329
Codeine-D6	261, 362, 285
Codeine	355, 282, 356
Oxycodone-D6	406, 407
Oxycodone	400, 343, 401
Hydromorphone D-6	376, 320
Hydromorphone	370, 314, 283
Morphine-D6	403, 347
Morphine	324, 341, 397
Oxymorphone D-3	445, 389
Oxymorphone	442, 386, 387

### MSD SIM PROGRAM - MBTFA

Drug	Ions Monitored	Retention Time
Oxymorphone – D3	<u>496</u> , 399, 383	6.90 min.
Oxymorphone	<u>493</u> , 396, 380	6.93 min.
Morphine-D3	<u>367</u> , 480, 314	7.59 min.
Morphine	<u>364</u> , 477, 311	7.60 min.
Oxycodone-D6	<u>417</u> , 320, 304	7.88 min.
Oxycodone	<u>411</u> , 314, 298	7.89 min.
Dihydrocodeine-D6	403, 290, 306	7.95 min.
Dihydrocodeine	397, 284, 300	7.99 min.
Codeine-D3	<u>285</u> , 398, 269	8.10 min.
Codeine	<u>282</u> , 395, 266	8.11 min.
Hydromorphone-D3	<u>384</u> , 328, 355	8.42 min.
Hydromorphone	<u>381</u> , 325, 352	8.43 min.
Norcodeine	477, 305, 351	9.15 min..
Hydrocodone-D3	<u>302</u> , 242, 259	9.27 min.
Hydrocodone	<u>299</u> , 284, 270	9.28 min.

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

*This method is a preliminary procedure for investigational use only. Although it has performed well in our laboratory, your laboratory must validate it before it is used to report patient values. We would appreciate your comments on its performance and welcome your suggestions for improvements or enhancements.*

**Notes:**

1. **ACID HYDROLYSIS** – Sample volume: If the sample volume is increased all of the sample preparation solutions volumes must be adjusted proportionally.
2. **SAMPLES AND WASHES** – Allow all samples and washes to gravity flow completely through the resin bed before adding the next liquid.
3. **INTERNAL STANDARDS** – When preparing the Internal Standard, the quantity added per mL of sample should approximate the cutoff value of the compound(s) being tested for. The Internal Standard can almost always be prepared in an aqueous matrix. If prepared in an organic solvent the solvent must not exceed 5% of the final prepared sample.
4. **TURBID SAMPLES** may need to be centrifuged.
5. **RINSE SOLVENTS** should be delivered to the top part of the column wall to better rinse the aqueous residue from column walls.
6. **ELUTION SOLVENTS** with the TEA should be made fresh daily.
7. **POLAR SOLVENTS** used (e.g. acetonitrile and ethyl acetate) may absorb moisture. Flush bottles with nitrogen, keep stock bottles full or use sodium sulfate to minimize moisture.
8. **AIR TRAPPED** within the column bed or frits may prevent the liquids from eluting freely by gravity flow. Tapping the column mounting plate onto the vacuum box should initiate flow.
9. **IDEAL FRAGMENTS** should be determined by full scans of neat, derivatized standards.
10. **RECOMMENDED CAPILLARY COLUMNS** for adequate partitioning of all opiates would be proprietary columns from Phenomenex and Varian, ZB-DRG1 and FV-200 ms.