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DETECTABUSE® "NO VACUUM" GRAVITY SERIES GV-65 / GV-65 METHOD FOR THE ANALYSIS OF FENTANYL IN URINE, SERUM OR ORAL FLUIDS BY GC/MS

Revised: January 2009
(See: Column Conditioning – Revised GV-65C conditioning)

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

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

Enzymatic Hydrolysis of Urine
1. Pipette 1.0 mL of sample and 0.1 mL of 0.2M Acetate Buffer, pH 5.0 into a 13 x 100 mm pressure rated borosilicate glass tube with a Teflon lined screw cap (pH should be 4.5-5.0). Add 1000 ng/mL of appropriate deuterated internal standards to each sample tube.
2. Add approximately 5000 units of Beta-Glucuronidase, Sigma type HP-25 from Helix Pomatia, to each sample. Typical preparations contain approximately 100,000 units per mL. In this case 50 *u*L contains 5000 units.
3. Mix gently and incubate at 50°C for 2 1/2 hours or 37°C for 4 hours. Complete hydrolysis is also achieved in 16 hours at room temperature (15 - 30°C).
4. Add 1.0 mL of a 1% HCl in H2O and mix.
5. Centrifuge for 3 minutes at 3500 RPM.

Note: If methoxylamine is used to form the oxime derivative 100 mg should be added per mL of the Acetate buffer.

Sample Preparation Without Hydrolysis "Measurement of Fentanyl"

Measurement of Fentanyl in Urine
1. Pipet 1.0 mL sample into a 13 x 100 mm pressure rated glass tube with a Teflon® lined screw cap.
2. Add 500 ng Fentanyl
3. Add 1.0 mL of 1% HCl in H2O and mix.
4. If cloudy or precipitated centrifuge for 3 minutes at 3000 RPM.

Measurement of Fentanyl 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 50 ng each of appropriate deuterated internal standard into each tube.
3. Add 1.0 mL of 1% HCL in H2O (per mL of sample) and mix.
4. If Cloudy or precipitated centrifuge for 3 minutes at 3000 RPM.

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

COLUMN CONDITIONING – *(Follow Column Conditioning procedure for <u>EITHER</u> GV-65 or GV-65C columns.)*

Column Conditioning and Activation of Cation Function using <u>GV-65</u> Columns.

1. Wash column with 1.0 mL of Methanol. Allow to flow by gravity.
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 H2O:0. 25M Phosphate Buffer, pH 6.0. Prepare monthly (Store Refrigerated).***
3. Proceed to Sample Extraction within 60 min. of column conditioning.

Column Conditioning using <u>GV-65C</u> 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. Allow to flow by gravity.
2. Wash with 1.0 mL of deionized water. Allow to flow by gravity.
3. Proceed to Sample Extraction within 60 min of column conditioning.

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

1. Pour samples onto preconditioned columns. Hydrolyzed samples should be carefully poured to prevent transfer of hydrolysis debris onto the columns. Allow to flow by gravity. Samples will flow through the columns at a rate of 1-2 mL/min.
2. Wash columns with 3.0 mL of deionized water. Allow the columns to flow by gravity.
3. Wash columns with 2.0 mL of Methanol. Allow the columns to flow by gravity.

4. Add 1.0 mL Ethyl Acetate. Allow the columns to flow by gravity.

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.*

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: Ethyl acetate (80:20) with 4% Triethylamine (TEA)* to each column and allow solvent to flow through the columns by gravity into the test tubes.
4. Dry under N2 or argon at less than 50°C.

*** Elution Solvent with 4% TEA** (4 mL TEA is added to 96 mL of n-ButylChloride**:** Ethyl Acetate, 80:20) is stable for approximately one week stored in a glass bottle with a Teflon or polypropylene lined cap. Close bottle tightly when not in use. A white residue begins to appear in the dried down eluate when the TEA begins to deteriorate. Artifacts from this process may interfere with "fast" GC/MS methods.

Note*: If a sample does not elute freely by gravity flow, there is probably air trapped within the column bed or frits. 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 – Following are two of the more commonly used derivatizing schemes for Fentanyl. They are superior to TMS derivatives because the compounds are well separated by either retention time or eliminating the need to form oximes to achieve the separation required for quantitation.

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 the tube contents, flush with nitrogen or argon and cap the tube.
3. Incubate the mixture @ 100°C for 45 min.
4. Allow the mixture to come to room temperature. Add 1.0 mL of Hexane. Mix.
5. Dry under argon or nitrogen @ 65^{0}C.
6. Add 100 uL Ethyl Acetate. Mix and inject 1.0-2.0 uL

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^{0}C for 30 mins.
3. Transfer contents into 100 uL reaction vials and seal.
4. Inject 1.0-2.0 uL

Note: Polar solvents commonly used for derivatization such as Pyridine, Acetonitrile, or Ethyl Acetate will pick up moisture over time. Because moisture will inhibit or prevent derivatization, it is important to keep a supply of solvents used for this purpose stored as protected from moisture as possible. Storing these solvents in a desiccator or flushing with Nitrogen or Argon after use is recommended.

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 (± 20%).
Sample, Sample Washes, and Elution Solvent - Pass through column in approximately 60 seconds (± 20%).

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 µm film thickness
Acquisition Mode: SIM

Temperature Program:
Injector Temp.: 280°C
Detector Temp.: 300°C
Initial: 140°C, program at 20°C/min. to 290°C, Hold for 3 min.
Equil. Time: 1.0 min.
Split Ratio: Splitless
He Flow: 1.0 mL/min. @ 200°C
Septum Purge: 2 mL/min.
Purge Off Time: 1.0 min.
Dwell: 20
Solvent Delay: 5.0 min.
Start Acq.: 5.0 min.
Stop Run: 10.5 min.

MSD SIM PROGRAM - Propionic Anhydride

Drug	Ions Monitored	Retention Time
Nor-Fentanyl	158, 231, 288	6.50 min.
Fentanyl	146, 189, 245	7.70 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.