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Extraction of Phosphonic Acids from Urine Samples and Analysis by Gas Chromatography with Detection by Mass Spectrometryand Flame Photometric Detection

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6.3. Agilent HP-5MS UI (equivalent to (5%-Phenyl)-methylpolysiloxane), 30 m x 0.25 mm x 0.25 µm – used for first dimension of GCxGC TOF-MS analysis.
6.4. Restek Rxi®-17Sil MS (fused silica), 1 m x 0.18 mm x 0.18 µm – used for second dimension of GCxGC TOF-MS analysis.
6.5. Phenomenex Strata SAX Solid Phase Extraction Cartridges (500 mg, 3 mL).
6.6. Methanol, HPLC Grade.
6.7. Acetonitrile, HPLC Grade.
6.8. Acetic acid, ACS Grade.
6.9. Trifluoroacetic Acid (Aldrich, 99%)
6.10. Triethylamine (Aldrich, 99.5%)
6.11. Pentafluorobenzyl bromide (Aldrich, 99%)
6.12. Water, HPLC grade
6.13. Pooled human urine (Innovative Research)
6.14. Protonated and deuterated phosphonic acids (Cerilliant; see Section 8)

7. SAMPLE COLLECTION, PRESERVATION AND HANDLING:

Samples should be frozen at -80°C as soon as possible after collection and brought slowly to room temperature immediately before analysis. Method development samples are spiked with the protonated and deuterated phosphonic acids in house. All handling and transportation will be in accordance with the LLNL chemical warfare agent and BSL-2 safety procedures.

8. STANDARDS AND CONTROLS:

8.1. Protonated phosphonic acids: methyl phosphonic acid (MPA, ERM-038), ethyl methylphosphonic acid (EMPA, ERE-024), isopropyl methylphosphonic acid (IMPA, ERI-015), pinacolyl methylphosphonic acid (PMPA, ERP-083) (Cerilliant standards)
8.2. Isotopically labelled phosphonic acids as internal standards: $^{13}CD_3$-Methylphosphonic acid (CDLM-6100-1.2) D_5-ethyl methylphosphonic acid (DLM-6098-1.2), D_7-isopropyl methylphosphonic acid (ERI-017), $^{13}C_6$-pinacolyl methylphosphonic acid (CLM-6620-1.2) (Cerilliant standards)

9. CALIBRATION:

9.1. Perfluorotributylamine (PFTBA) mass spectrometer tune solution.

10. SAMPLING:

This method was developed using pooled urine samples prepared by the following spiking procedure:

10.1. Aliquot a known volume of urine into a glass vial.
10.2. Known amounts of phosphonic acids were spiked in the urine sample.
10.3. Sample vortexed to thoroughly combine sample before extraction.

11. OTHER QUALITY ASSURANCE CONSIDERATIONS:

A derivatization reagent blank should be run with each set of samples, as well as a method blank, if an uncontaminated urine sample is available. Samples should be extracted and analyzed in triplicate, if enough sample is obtained.

12. PROCEDURE (Step-by-Step Directions)

12.1. Preparation of Calibration Standards

- 12.1.1. Prepare separately two mixtures of working stock solutions; one of the protonated phosphonic acids and one of the deuterated internal standards.
- 12.1.2. Using individual vials, prepare a 7-point standard curve of phosphonic acids bracketing the analytical range [i.e. 1, 5, 10, 50, 100, 175, 200 ppb] by adding appropriate amounts of phosphonic acids working stock.
- 12.1.3. Add same amount of deuterated internal standard to each individual calibrant.
- 12.1.4. Evaporate solvent completely.
- 12.1.5. Add 300 µL of acetone and vortex for 60 seconds.
- 12.1.6. Add 5 µL of triethylamine and 5 µL of pentafluorobenzyl bromide. Vortex for 60 seconds and heat for one hour at 70 °C.

12.2. Sample Preparation

- 12.2.1. Add deuterated internal standard mixture to a clean vial.
- 12.2.2. Evaporate the solvent.
- 12.2.3. Add 1 mL of 0.05 M acetic acid in water to sample vial and vortex for 60 seconds.
- 12.2.4. Add 1 mL of urine sample and vortex for 60 seconds.

12.3. Solid Phase Extraction (SPE) procedure is as follows:

- 12.3.1. Cartridge (Phenomenex Strata SAX cartridge (500 mg/3 mL))
  - 12.3.1.1. Condition with 2 mL of methanol
  - 12.3.1.2. Equilibrate with 2 mL of HPLC DI water
  - 12.3.1.3. Load entire processed sample from above (12.2.4).
  - 12.3.1.4. Wash with 2 mL of HPLC DI water.
  - 12.3.1.5. Wash with 6 mL of methanol.
  - 12.3.1.6. Elute sample using 5 mL of 1% trifluoroacetic acid in acetonitrile.

12.4. Dry captured elutant to complete dryness using a gentle stream of nitrogen gas.

12.5. Add 300 µL of acetone and vortex for 60 seconds.

12.6. Add 5 µL of triethylamine and 5 µL of pentafluorobenzyl bromide.

12.7. Vortex for 60 seconds and heat for one hour at 70 °C.

12.8. Reduce final volume to approximately 100 µL for analysis using gentle stream of nitrogen gas.

12.9. Instrument conditions for GC-MS/MS analysis are as follows:

12.9.1. Carrier gas: Helium (UHP, 99.999% purity)
12.9.2. Analysis mode: Constant Flow
12.9.3. Column flow rate: 1.0 mL/min
12.9.4. Injection volume: 1 µL.
12.9.5. Injection type: Splitless, 0.60 min
12.9.6. Injector temperature: 230 °C.
12.9.7. Oven temperature program:
40°C for 3 min
8 °C/min to 215 °C
20 °C/min to 300 °C
300 °C for 1 min
12.9.8. Transfer line temperature: 270 °C
12.9.9. Mass analyzer: Triple quadrupole
12.9.10. Electron energy: 100 eV
12.9.11. Ionization: chemical ionization
12.9.12. Reaction gas: Methane
12.9.13. Ionisation polarity: Negative
12.9.14. Collision gas: Argon
12.9.15. Collision Energy: 10
12.9.16. Scan range/time: 50-550 m/z in 1 second
12.9.17. Mass resolution: 0.7
12.9.18. Type of MS/MS scan: Product ion scan
12.9.19. Precursor ion(s):
m/z 123: CE 15, Scan Time 1 s
m/z 128: CE 15, Scan Time 1 s
m/z 137: CE 14, Scan Time 1 s
m/z 144: CE 14, Scan Time 1 s
m/z 179: CE 20, Scan Time 1 s
m/z 275: CE 10, Scan Time 1 s
12.9.20. Scan events:
0-20.28 min: 123, 128, 137, 144
20.28-22.31 min: 179
22.31- 38.01 min: 275
12.9.21. Mass spectrometer source temperature: 200 °C
12.9.22. Solvent delay: 3 min

12.10. Instrument conditions for GC-FPD analysis are as follows:

12.10.1. Carrier gas: Hydrogen (UHP, 99.999% purity)
12.10.2. Analysis mode: Ramped Pressure

12.10.3. Ramped Pressure Program:
10 psi for 1 min
0.42 psi/min to 25.0 psi
25.0 psi hold
12.10.4. Nominal initial flow rate: 1.8 mL/min
12.10.5. Injection volume: 1 µL
12.10.6. Injection type: Splitless
12.10.7. Injector temperature: 250 °C.
12.10.8. Oven temperature program:
40 °C for 1 min
10 °C/min to 130 °C
4 °C/min to 170 °C
10 °C/min to 300 °C
300 °C for 4 min
12.10.9. Detector: FPD
12.10.10. Detector temperature: 250 °C
12.10.11. Hydrogen flow: 75 mL/min
12.10.12. Oxidizer: Air
12.10.13. Oxidizer flow: 100 mL/min
12.10.14. Makeup gas: Nitrogen
12.10.15. Mode: Constant column + makeup flow
12.10.16. Combined flow: 15 mL/min

12.11. Instrument conditions for GCxGC TOF-MS analysis are as follows:
12.11.1. Carrier gas: Helium (UHP, 99.999% purity)
12.11.2. Analysis mode: Constant Flow
12.11.3. Column flow rate: 1.1 ml/min
12.11.4. Injection volume: 1 µL.
12.11.5. Injection type: Splitless
12.11.6. Injector temperature: 250 °C.
12.11.7. Oven temperature program:
40 °C for 3 min
8 °C/min to 300 °C
300 °C for 3 min (secondary column kept 10 °C above that of primary column throughout program)
12.11.8. Modulator temperature offset: 20 °C, relative to the GC secondary oven temperature
12.11.9. Modulation period: 3.5 sec
12.11.10. Hot pulse time: 0.6 sec
12.11.11. Cool time between stages: 1.15 sec

12.11.12. Transfer line temperature: 290 °C
12.11.13. Mass analyzer: time of flight
12.11.14. Ionization: 70 eV electron ionization
12.11.15. Mass spectrometer: scan mode, m/z 29-600
12.11.16. Scan rate: 200 spectra/s
12.11.17. Mass spectrometer source temperature: 250 °C
12.11.18. Detector Voltage: 1750
12.11.19. Solvent delay: 750 s

12.12. Run each sample by aliquotting for direct injection:
12.12.1. All GC samples can be directly injected.
12.12.2. Run the derivatization reagent blank before running the calibration samples and *each* time before running an actual/authentic sample.

12.13. Data Analysis and Calculation of Recovery and Concentration
12.13.1. To calculate the recovery (%R) of the OPNAs, use the following following formula:

$$\%R = \frac{A_u}{A_{cal}} \times 100$$

where:
A_u = Measured peak area of the *isotopically labelled* phosphonic acid in the urine sample.
A_{cal} = Average measured peak area of the *isotopically labelled* phosphonic acid of the calibration sample set.

12.14. Data Quantitation
12.14.1. Calculate the peak area of each OPNA in each calibration standard.
12.14.2. Calculate the peak area of each internal standard in each calibration standard.
12.14.3. Create a calibration curve using the concentration of each calibrant as the x-axis and *RF* as the y-axis. Calculate *RF* as follows:

$$RF = \frac{A_{OPNA}}{A_{IS}}$$

where:
A_{OPNA} = Measured peak area of the phosphonic acid in the calibration standard.
A_{IS} = Measured peak area of the *labelled* phosphonic acid in the calibration standard.

12.14.4. Calculate RF for each OPNA in each urine sample analyzed, using the corresponding deuterated phosphonic acid. If the appropriate stable isotope-labeled internal standards are not available, RF can be calculated based on the most similar labeled OPNA standard used (for example, MPA may be quantitated by referencing the D_5-EMPA calibration).

12.14.5. Calculate the concentration in the sample using the calibration curve created above.

12.14.6. Correct concentration value for the concentration effect due to sample preparation. This corresponds to a factor of 10x for this current sample prep method.

13. METHOD PERFORMANCE:

13.1. Performance data and related information are provided herein only as examples and guidance. The data do not represent required performance criteria for users of the methods.

14. REFERENCES

14.1. "Solid Phase Extraction and Derivatization of OPNA Metabolites" Centers for Disease Control and Prevention, NCEH/DLS/ERB/LRN-C, Atlanta, GA.

15. POLLUTION PREVENTION

15.1. Pollution prevention encompasses any technique that reduces or eliminates the quantity and/or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

15.2. For information about pollution prevention that may be applicable to laboratories and research institutions consult Less is Better: Laboratory Chemical Management for Waste Reduction available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th St., N.W. Washington, D.C. 20036, http://www.acs.org.

16. WASTE MANAGEMENT

16.1. Waste suspected of contamination with nerve agents and all biological waste should be neutralized before disposal. At LLNL, Document LLNL-MI-417220, "Neutralization Procedure for Chemical Agents and Toxins" should be followed. Decontamination will use commercial bleach to neutralize any agent remaining.

16.2. The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult The Waste Management Manual for Laboratory Personnel available from the American Chemical Society.