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Extraction of Butyrylcholinesterase (BuChE) and
Organophosphorus Nerve Agent (OPNA)-BChE
Adducts from Blood and Plasma Samples and
Analysis by Liquid
Chromatography-High-Resolution Mass
Spectrometry (LC-HRMS)

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Title: Extraction of Butyrylcholinesterase (BuChE) and Organophosphorus Nerve Agent (OPNA)-BuChE Adducts from Blood and Plasma Samples and Analysis by Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS)	

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Introductory Notes/Comments:

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Approval, Concurrence or Review:	Name & Title/Position	Date
<input checked="" type="checkbox"/> Approval <input type="checkbox"/> Review <input type="checkbox"/> Concurrence	<i>Audrey Williams</i> , Audrey Williams, Lakeshore PI	6.30.14
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1. SCOPE AND APPLICATION:

The following procedure describes the extraction of butyrylcholinesterase (BuChE) and organophosphorus nerve agent (OPNA)-BuChE adducts from blood and plasma samples and subsequent analysis by Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS) to determine OPNA (e.g., sarin [GB], soman [GD], and VX) signatures present in a blood/plasma sample. Positive identification of OPNA-BuChE adducts in blood/plasma may signify exposure to OPNAs.

2. SUMMARY OF METHOD:

Blood or plasma samples containing OPNA-BuChE adducts are processed using antibody-coated Immunomagnetic Separation (IMS) beads to extract and pre-concentrate the BuChE from the matrix. The IMS beads are incubated in the human serum/plasma sample and specifically bind the BuChE (both native and OPNA-adducted). The purified BuChE is then enzymatically digested into peptides using pepsin, which yields nine amino acid peptides (nonapeptides) that are specific for the BuChE adduction site. Each OPNA-nonapeptide adduct has a unique chemical structure that is separated and measured using LC-HRMS analysis.

3. INTERFERENCES:

Work deliberately in order to prevent cross-contamination among samples. Due to possible unknown substances within blood/plasma, interferences may be present in samples. Monitor chromatographic separations for interferences or decreased resolution or sensitivity. If degraded method performance is observed, take corrective actions as necessary to restore optimum resolution and sensitivity.

4. SAFETY:

- 4.1. This method does not address all safety issues associated with its use. The laboratory is responsible for maintaining a safe work environment and a current awareness file of OSHA regulations regarding the safe handling of the chemicals listed in this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in these analyses.
- 4.2. Blood is a human body fluid and may contain bloodborne pathogens and as such, must be handled under appropriate BSL-2 conditions. Laboratory personnel working with blood and other derivative materials (such as plasma) should refer to the OSHA Bloodborne Pathogen Standard for specific required precautions.
- 4.3. Standard precautionary measures should be used for handling all solvents included in this method. Suspect blood/plasma samples should be treated as if they contain a high concentration of OPNAs until it is proven otherwise.

5. APPARATUS:

- 5.1. Agilent 1290 Infinity HPLC coupled to an Agilent 6530 Q-TOF LC/MS detector with a Dual Agilent Jet Stream Electrospray Ionization source

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- 5.2. Agilent MassHunter Data Acquisition software (version B.05.01) and Q-TOF Quantitative Analysis software (version B.05.00)
- 5.3. DynaMag-15 Magnet (Life Technologies, 12301D)
- 5.4. Nutating Mixer (VWR, 82007-202)
- 5.5. DynaMag-2 Magnet (Life Technologies, 12321D)
- 5.6. Eppendorf 5810R Centrifuge (022625004) with FA-45-48-11 Rotor (5820760001)
- 5.7. Thermomixer R (Eppendorf, 22670107) with 1.5 mL block (Eppendorf, 22670522)
- 5.8. Vortex (VWR, 14005-824)
- 5.9. Easypet Serological Pipette (Eppendorf, 4430000026)
- 5.10. Pipet-Lite XLS LTS 2-20 μ L (Rainin, L-20XLS)
- 5.11. Pipet-Lite XLS LTS 20-200 μ L (Rainin, L-200XLS)
- 5.12. Pipet-Lite XLS+ LTS 100-1000 μ L (Rainin, L-1000XLS)

6. REAGENTS AND MATERIALS:

- 6.1. Protein G Dynabeads for Immunoprecipitation (Invitrogen, 10004D)
- 6.2. Phosphate Buffered Saline with Tween 20 (PBST) (Sigma, P3563)
- 6.3. BuChE Monoclonal Antibodies; Clone 3E8 (ThermoFisher, HAH-002-01-02)
- 6.4. Triethanolamine (TEA) Buffer Solution (Sigma, T0449)
- 6.5. Dimethyl Pimelimidate Dihydrochloride (DMP) (Sigma, D8388)
- 6.6. Tris Buffered Saline (TBS) (Sigma, T5912)
- 6.7. Pepsin (Sigma, P6887)
- 6.8. Formic Acid, LC/MS-Grade (Fisher, A117)
- 6.9. Acetonitrile, LC/MS-Grade (Fisher, A995)
- 6.10. Water, 18 M Ω from Millipore Milli-Q Advantage A10 system
- 6.11. Pooled Human Plasma (Innovative Research, IPLA-N; K2 EDTA)
- 6.12. Human Whole Blood (Innovative Research, IPLA-WB1; K2 EDTA)
- 6.13. 15 mL Conical Centrifuge Tubes (Falcon, 352097)
- 6.14. 4 mL Glass Vials (Supelco, 27138)
- 6.15. 1.5 mL Microcentrifuge Tubes (Eppendorf, 22364111)
- 6.16. 10 kDa Molecular Weight Cut-Off (MWCO) spin filters (Amicon, UFC501096)
- 6.17. 20 μ L LTS pipet tips, presterilized, filter (Mettler Toledo, RT-L10F)
- 6.18. 200 μ L LTS pipet tips, presterilized, filter (Mettler Toledo, RT-L200F)
- 6.19. 1000 μ L LTS pipet tips, presterilized, filter (Mettler Toledo, RT-L1000F)
- 6.20. 5 mL Disposable Serological Pipettes (Greiner Bio-One, 82050-002)
- 6.21. 10 mL Disposable Serological Pipettes (Greiner Bio-One, 82050-006)
- 6.22. Autosampler Vials (Phenomenex, AR0-9923-13), 250 μ L Inserts (Agilent, 5181-8872), and Caps (Agilent, 5182-0723)
- 6.23. Atlantis T3 C18 column, 100 \AA pores, 3 μ m particles, 2.1 mm x 150 mm (Waters, 186003719)

7. SAMPLE COLLECTION, PRESERVATION AND HANDLING:

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Whole blood should be refrigerated at 4 °C, never frozen, and should be separated into plasma as soon as possible after collection. Plasma should be frozen and stored at -20 °C or colder and be brought slowly to room temperature immediately before analysis. Plasma freeze/thaw cycles should be minimized. Samples should not be heat-sterilized as elevated temperatures will damage the OPNA-BuChE adducts. A minimum of 200 µL of blood/serum/plasma is needed for analysis. Method development samples were generated using human whole blood and pooled human plasma spiked with OPNAs in-house. All handling and transportation was carried out in accordance with the LLNL chemical warfare agent and BSL-2 safety procedures.

8. STANDARDS AND CONTROLS:

8.1. Synthetic GB- and VX-adducted BuChE nonapeptides (The Netherlands Organisation for Applied Scientific Research [TNO], Rijswijk, Netherlands)

9. CALIBRATION:

- 9.1. ESI-TOF Calibrant, ESI – L Low Concentration Tuning Mix (Agilent, G1969-85000)
- 9.2. API-TOF Reference Mass Solution Kit containing purine and HP-0921 (Agilent, G1969-85001)

10. SAMPLING:

This method was developed using human whole blood and pooled human plasma samples prepared by the following spiking procedure:

- 10.1. Aliquot a known volume of blood/plasma into a 4 mL glass vial.
- 10.2. Spike a known amount of OPNA (e.g., GB, VX) into the sample.
 - 10.2.1. OPNA in isopropyl alcohol (IPA) was diluted in Milli-Q water prior to spiking. Final concentration of IPA should be kept below 2% to minimize precipitation of proteins in the sample.
- 10.3. Vortex sample to thoroughly combine.
- 10.4. Incubate overnight at room temperature (~20 °C) to allow adduct formation before extraction

11. OTHER QUALITY ASSURANCE CONSIDERATIONS:

A method blank should be run with each set of samples if an unexposed blood/plasma sample is available. Samples should be prepared and analyzed in triplicate when possible.

12. PROCEDURE (Step-by-Step Directions)

- 12.1. Preparation of BuChE IMS Bead Solution
 - 12.1.1. Vortex to thoroughly resuspend Protein G Dynabeads and transfer 2 mL into a 15 mL tube.
 - 12.1.2. Apply DynaMag-15 magnet and allow beads to aggregate; remove the supernatant.
 - 12.1.3. Add 4 mL of PBST then vortex briefly. Apply magnet and allow beads to aggregate; remove the supernatant. Repeat twice.

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- 12.1.4. Add 8 mL of PBST and 400 µg of BuChE antibody (400 µL of a 1 mg/mL solution) then vortex briefly.
- 12.1.5. Incubate overnight (~19 hours) at room temperature (~20 °C) on nutating mixer.
- 12.1.6. Apply magnet and allow beads to aggregate; remove the supernatant.
- 12.1.7. Add 4 mL of TEA buffer solution then vortex briefly. Apply magnet and allow beads to aggregate; remove the supernatant. Repeat.
- 12.1.8. Add 4 mL of 5.4 mg/mL DMP in TEA buffer then vortex briefly.
- 12.1.9. Incubate 30 minutes at room temperature on nutating mixer, then apply magnet and allow beads to aggregate; remove the supernatant.
- 12.1.10. Add 4 mL of TBS then vortex briefly.
- 12.1.11. Incubate 15 minutes at room temperature on nutating mixer, then apply magnet and allow beads to aggregate; remove the supernatant.
- 12.1.12. Add 2 mL of PBST then vortex briefly. Apply magnet and allow beads to aggregate; remove the supernatant. Repeat.
- 12.1.13. Add 1.9 mL of PBST then vortex briefly. This is the final BuChE IMS bead solution. Store at 4 °C for up to 3 months.

12.2. Blood/Plasma Sample Preparation

- 12.2.1. Vortex BuChE IMS bead solution until thoroughly resuspend and transfer 50 µL into each 1.5 mL microcentrifuge tube (one per sample).
- 12.2.2. Apply DynaMag-2 magnet and allow beads to aggregate; remove the supernatant.
- 12.2.3. Transfer 200 µL of each blood/plasma sample to a new microcentrifuge tube.
- 12.2.4. Centrifuge sample at 10,000 x G for 5 minutes at 20 °C to separate any lipids/solids.
- 12.2.5. Alternatively, the sample can be spun through a 0.45 µm PVDF filter at 20 °C to separate lipids/solids.
- 12.2.6. Transfer 75 µL clarified sample into tube containing the BuChE IMS beads and vortex briefly.
- 12.2.7. Incubate on Thermixer for 120 minutes at 20 °C with mixing at 1400 rpm.
- 12.2.8. Apply magnet and allow beads to aggregate; remove the supernatant.
- 12.2.9. Add 500 µL PBST and vortex briefly. Apply magnet and allow beads to aggregate; remove the supernatant. Repeat twice.
- 12.2.10. Prepare 2 mg/mL pepsin solution in 5% formic acid in Milli-Q water. Incubate (static) for 30 minutes at 20 °C before use.
- 12.2.11. Add 75 µL of Milli-Q water.
- 12.2.12. Add 10 µL of prepared 2 mg/mL pepsin solution.

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- 12.2.13. Incubate on Thermixer for 30 minutes at 37 °C with mixing at 1000 rpm for 10 seconds every minute.
- 12.2.14. Apply magnet and allow beads to aggregate. Transfer supernatant to 10 kDa MWCO spin filter. Centrifuge at 14,000 x G for 45 minutes at 20 °C.
- 12.2.15. Transfer all of flow through (digested BuChE peptides, ~60 µL) to autosampler vial with insert and cap.

12.3. Preparation of Calibration Standards for Instrument Detection Limit (IDL) Study

- 12.3.1. Prepare blank processed plasma matrix (e.g., 16 blank plasma samples yields 800 µL of processed matrix to use for calibration standards).
- 12.3.2. Prepare a working stock solution of BuChE nonapeptide standard(s) in 0.6% formic acid in Milli-Q water.
- 12.3.3. Using individual vials with inserts, prepare an eight-point standard curve of BuChE nonapeptide standard(s) bracketing the analytical range (e.g., 0, 1, 4, 6, 16, 32, 64, 125, and 250 ng/mL) by adding appropriate amounts of working stock to blank processed plasma matrix.
- 12.3.4. Vortex and store at 4 °C.

12.4. Instrument Conditions for LC-HRMS Analysis

- 12.4.1. Injection volume: 10 µL
- 12.4.2. Mobile phase solvent A: 0.1% formic acid in Milli-Q water
- 12.4.3. Mobile phase solvent B: 0.1% formic acid in LC/MS-Grade acetonitrile
- 12.4.4. Mobile phase gradient program:
 - 12.4.4.1. 95% solvent A for 5 minutes
 - 12.4.4.2. Linear gradient to 10% solvent A over 15 minutes
 - 12.4.4.3. 95% solvent A for 10 minutes (column re-equilibration)
 - 12.4.4.4. Total analysis time: 30 minutes
- 12.4.5. Mobile phase flow rate: 0.5 mL/min
- 12.4.6. Column temperature: 45 °C
- 12.4.7. Ionization: electrospray, positive mode
- 12.4.8. Capillary voltage: 5000 V
- 12.4.9. Nozzle voltage: 750 V
- 12.4.10. Source gas temperature: 300 °C
- 12.4.11. Source gas flow rate: 4 L/min
- 12.4.12. Nebulizer: 55 psig
- 12.4.13. Sheath gas temperature: 250 °C
- 12.4.14. Sheath gas flow rate: 8 L/min
- 12.4.15. Mass scan range: 85-1200 *m/z*

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12.4.16. Scan rate: 3 spectra/second

12.5. Data Analysis

12.5.1. Calculate the peak areas of each OPNA-adducted BuChE nonapeptide in each calibration standard, blood/plasma sample spiked with OPNA, and unknown blood/plasma sample using the following parameters:

Compound	Formula	[M+H] ⁺	RT (min)
Nonapeptide	C33H49N9O14	796.3472 ± 0.0400	7.00 ± 0.20
VX-adducted nonapeptide	C36H56N9O16P	902.3655 ± 0.0500	8.05 ± 0.20
GB-adducted nonapeptide	C37H58N9O16P	916.3812 ± 0.0200	8.45 ± 0.20

12.5.1.1. Smooth the data using the Gaussian smoothing function with a function width of 15 points and a Gaussian width of 5 points.

12.5.1.2. Use the Agilent Agile integrator to generate peak areas.

12.5.2. Calculate the instrument detection limit (IDL) and limit of quantitation (LOQ) as follows:

12.5.2.1. Create a calibration curve using the concentration of each calibration standard as the x-axis (e.g., 0, 1, 4, 6, 16, 32, 64, 125, and 250 ng/mL), analyte peak area as the y-axis, and a linear curve fit (no curve weighting or forcing through the origin).

12.5.2.2. Calculate the IDL by adding the average peak area of the blank samples to three times the standard deviation of the peak areas of the blank samples and converting this peak area value to concentration using the calibration curve.

12.5.2.3. Calculate the LOQ by adding the average peak area of the blank samples to ten times the standard deviation of the peak areas of the blank samples and converting this peak area value to concentration using the calibration curve.

12.5.3. Calculate the method detection limit (MDL) as follows:

12.5.3.1. Create a calibration curve using OPNA spiking concentration as the x-axis (e.g., 0, 0.1, 0.5, 1, 2.5, and 5 ng/mL), analyte peak area as the y-axis, and a linear curve fit (no curve weighting or forcing through the origin).

12.5.3.2. Calculate the measured concentrations of test samples spiked close to the estimated MDL (e.g., 0.25 ng/mL) and prepared several times through the sample preparation procedure (e.g., $n = 6$) using the calibration curve.

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- 12.5.3.3. Calculate the MDL by multiplying the standard deviation of the measured concentrations of the test samples by the appropriate Student's *t* statistic (*n*-1 degrees of freedom, 99% confidence level, one-tailed).
- 12.5.4. Calculate exposure concentrations of unknown blood/plasma samples using the MDL calibration curve generated using blood/plasma samples spiked with known concentrations of OPNAs.

13. METHOD PERFORMANCE:

- 13.1. Instrument detection limits (IDLs) for nonapeptide standards in digested peptide matrix were 2.6 ng/mL for VX-adducted nonapeptide and 3.6 ng/mL for GB-adducted nonapeptide. Limits of Quantitation (LOQs) were 3.1 ng/mL for VX-adducted nonapeptide and 3.9 ng/mL for GB-adducted nonapeptide.
- 13.2. Method detection limits (MDLs) for agent-spikes into pooled human plasma carried through the sample preparation procedure were 0.17 ng/mL for VX and 0.18 ng/mL for GB.
- 13.3. 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. Centers for Disease Control and Prevention Laboratory Procedure Manual (2013). Unadducted Butyrylcholinesterase (BuChE) and Sarin (GB) and VX Adducts to BuChE in Human Plasma, Serum, and Blood Using Immunomagnetic Separation-Liquid Chromatography-Isotope Dilution-Tandem Mass Spectrometry. Method No. 2523.
- 14.2. Sporty, J. L. S., Lemire, S. W., Jakubowski, E. M., Renner, J. A., Evans, R. A., Williams, R. F., Schmidt, J. G., van der Schans, M. J., Noort, D., Johnson, R. C. (2010). Immunomagnetic separation and quantification of butyrylcholinesterase nerve agent adducts in human serum. *Anal. Chem.* 82:6593-6600.
- 14.3. Carter, M. D., Crow, B. S., Pantazides, B. G., Watson, C. M., deCastro, B. R., Thomas, J. D., Blake, T. A., Johnson, R. C. (2014). Profiling cholinesterase adduction: A high-throughput prioritization method for organophosphate exposure samples. *J. Biomol. Screen.* 19:325-330.

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

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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.