Detection of Chemical Weapon Nerve Agents in Bone: Expanding the Post-Incident Interval for Verifying Nerve Agent Exposure using Biomatrices

Katie M. Rubin, PhD and Bruce A. Goldberger, PhD

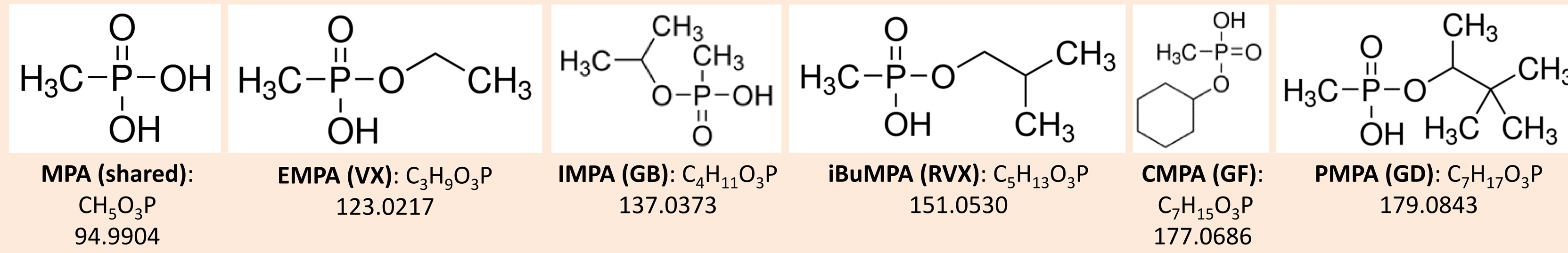
INTRODUCTION

- Use of chemical weapon nerve agents is banned by international convention and widely considered a human rights violation.
- For forensic and judicial purposes, verification of nerve agent exposure from biomatrices is needed to unambiguously identify the use of nerve agents following attacks.
- Because the regions in which suspected violations occur are often too dangerous for investigators to access in the immediate incident-aftermath, traditional biomatrices (such as urine and serum) from survivors may no longer contain markers of exposure at the time of collection; these matrices may no longer be available for the dead.
- It was recently hypothesized that the free alkyl methylphosphonic acid (aMPA) metabolites of the agents may adsorb onto bone mineral surfaces[1], rendering bone an alternative postmortem matrix for detection of *in vivo* nerve agent exposure.
- To test this hypothesis, we developed and validated the first (to the authors' knowledge) method for the isolation of aMPAs from bone.

LC-QToF-MS and LC-QqQ-MS/MS METHOD DEVELOPMENT

Text in blue indicates multiple variants of the parameter were experimentally tested and permutated prior to selection of the optimal condition

ANALYTES:



INSTRUMENTS:
- Agilent Technologies 1290 Infinity II Liquid Chromatography (LC) system and Agilent Technologies 6545B Quadrupole Time of Flight Mass Spectrometer (QToF-MS)
  - Ideal for identifying interfering ion species from a relatively unexplored matrix
- Dionex UltiMate LC system and Thermo Scientific TSQ Quantiva Triple Quadrupole Mass Spectrometer operated in tandem MS mode (QqQ-MS/MS)
  - Used to achieve lower limits of detection (LODs); low LODs are crucial due to the very low levels of aMPAs expected to be in bone following *in vivo* exposures

CHROMATOGRAPHY: Reverse phase
LC COLUMN: Waters Acquity 2.1 x 100 mm (1.8 µm) High Strength Silica T3 column with matching guard columns
COLUMN TEMP: 25°C
MOBILE PHASES:
- Mobile Phase A: 0.5% acetic acid (AA) in 18 mega-ohm water (**Figures 1 and 2**)
- Mobile Phase B: MeOH

GRADIENT: At time 0 min: 98% A, 4.45 min: 0% A, 5.45 min: 0% A, 5.55 min: 98% A, 10 min: 98% A
FLOW RATE: 0.350 µL/min
INJECTION VOLUME: 10 µL

ION SOURCE: ESI
POLARITY: Negative
SCAN TYPE: QToF – MS, Full scan; QqQ – MS/MS, SRM
QToF-MS PARAMETERS: Capillary voltage = 3000V; gas temp = 150°C; sheath gas temp = 375°C; nozzle voltage = 200V for MPA, EMPA, IMPA, and iBuMPA and 800V for CMPA and PMPA; fragmentor = 110V for MPA, EMPA, IMPA, and iBuMPA and 150V for CMPA and PMPA
QqQ-MS/MS PARAMETERS: Spray voltage = -3000V; sheath gas = 50 arb; auxiliary gas = 7 arb; sweep gas = 0 arb; ion transfer tube temp = 300°C; vaporizer temp = 250°C

EXTRACTION SOLVENT: 1.2 M HCl
CLEAN-UP and CONCENTRATION: Solid phase extraction (SPE)
SPE: United Chemical Technologies (UCT) Clean Screen ETG SPE columns (10 mL; 200 mg carbon-based sorbent)
FINAL EXTRACTION METHOD: Demineralize samples by adding 5 mL 1.2 M HCl to 0.5 g powdered bone. React for 1-1.5 hrs. Centrifuge, collect supernatant, and rinse residual powder once with 1 mL HCl; discard collagen pellet. To precipitate salts, add 50 mg $CaCl_2$ and 750 µL NH_4OH to the pooled supernatant and rinse. Mix well and centrifuge. Collect supernatant; rinse salts twice with 3 mL water. Pool supernatant and rinse; discard salt pellet. Re-acidify pooled sample with 12 M HCl. Condition SPE columns with 4 mL 1% FA in MeOH and 4 mL 1.2 M HCl. Load samples. Wash with 8 mL 1.2 M HCl. Elute with 1% FA in MeOH. Evaporate eluate and reconstitute residues in 0.5% AA in water.

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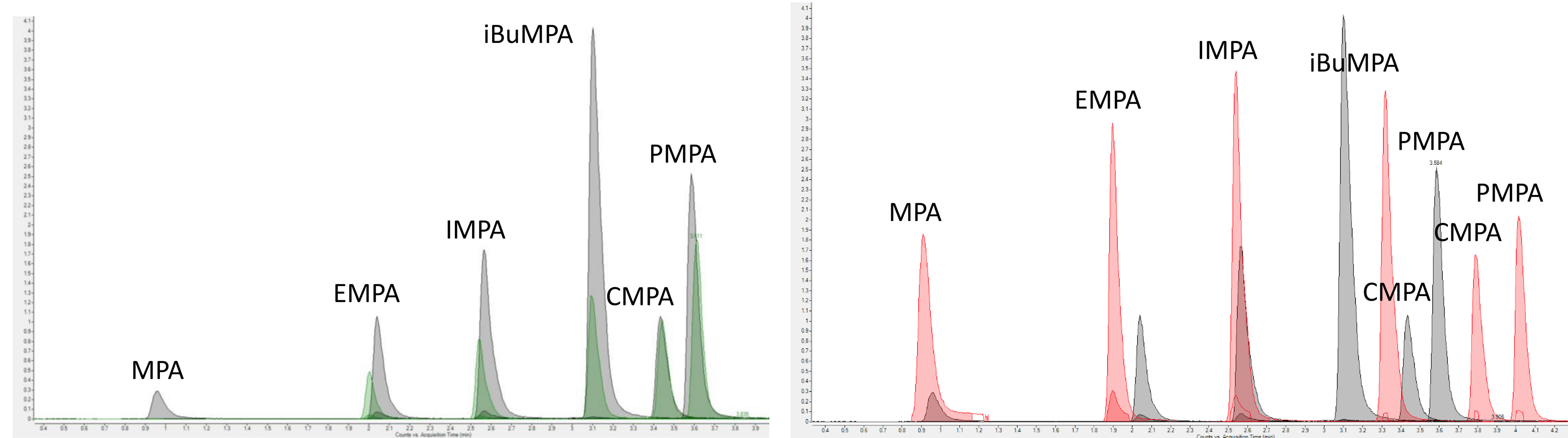

Figure 1: Overlaid QToF EICs for the target analytes prepped in mobile phase A (50 ng/mL each). Green = 0.1% formic acid (FA) in LC-MS grade water as mobile phase A, using a low mass, stable ion tune file. Grey = 0.1% FA in 18 mega-ohm water as mobile phase A, using a low mass, fragile ion tune file.

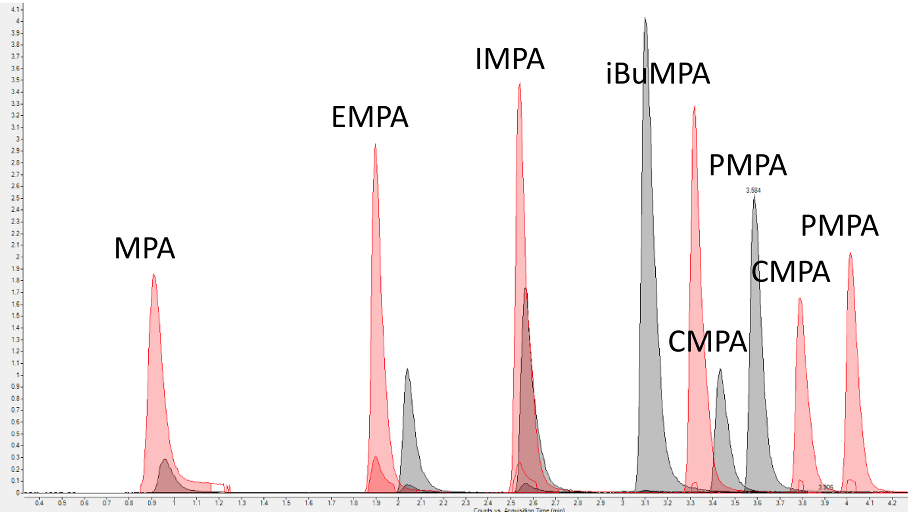

Figure 2: Overlaid QToF EICs for the target analytes prepped in mobile phase A (50 ng/mL each). Grey = 0.1% FA in water as mobile phase A. Red = 0.5% AA in water as mobile phase A.

LC-QqQ-MS/MS METHOD VALIDATION

See **Figure 3**

LODs: MPA = not recoverable; EMPA = 350 pg/g, IMPA = 20 pg/g, iBuMPA = 7.5 pg/g, CMPA = 10 pg/g, PMPA = 5 pg/g
INTERFERENCES: None
Table 1: **MATRIX SUPPRESSION**. Citrate was the primary suppressing species

Concentration	Analyte Suppression (%)					
	MPA	EMPA	IMPA	iBuMPA	CMPA	PMPA
0.5 ng/g	78.7	87.6	50.8	30.7	22.3	11.0
5 ng/g	85.9	88.6	53.3	33.2	25.7	15.4

Table 2: **EXTRACTION EFFICIENCY**

Concentration	Mean Efficiency (%)/RSD (%)					
	MPA	EMPA	IMPA	iBuMPA	CMPA	PMPA
0.5 ng/g	0	100.5/3.26	89.7/2.46	91.4/5.73	90.1/4.90	86.7/2.46
5 ng/g	0	93.0/10.8	93.2/6.51	92.1/9.03	91.7/9.02	90.1/8.33

Table 3: **TOTAL RECOVERY**

Concentration	Mean Recovery (%)/RSD (%)					
	MPA	EMPA	IMPA	iBuMPA	CMPA	PMPA
0.5 ng/g	0	12.8/19.6	44.5/10.4	64.2/12.8	71.5/14.3	77.9/9.03
5 ng/g	0	10.7/14.3	44.2/13.1	63.0/16.7	70.2/15.2	77.6/9.46

CARRYOVER: For PMPA > 500 pg/g only
STABILITY: Stable through 72 hrs at 10°C, with the exception of the low concentration EMPA standard (stable to 48 hrs) and low concentration IMPA standard (stable to 36 hrs)

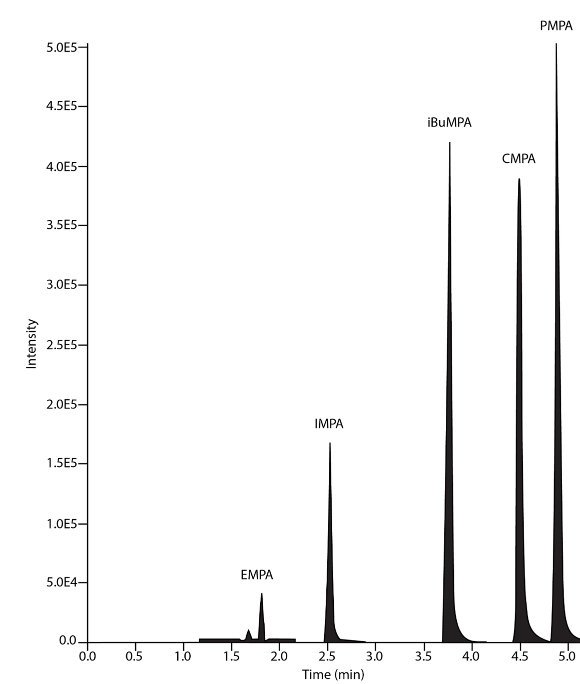

Figure 3: LC-QqQ-MS/MS total ion chromatogram for bone fortified with standards at 5 ng/g. Note that two EMPA peaks are consistently present in fortified bone matrix, though only one peak appears in fortified solvent samples. Also note the high baseline across the EMPA acquisition window.

AUTHENTIC SAMPLES

- The validated method was tested on femoral bone samples from 12 sexually-mature minipigs exposed to VX *in vivo* as part of an unrelated experiment.
- Six pigs received a 0.6 LD_{50} intramuscular dose, while six received a 2 LD_{50} percutaneous dose. Two pigs in the latter group died as a result of their VX exposure. The ten surviving pigs were euthanized 24 hrs after exposure.
- Their removed femora were stored frozen for 0.6-1.5 years, until the time of testing (**Figure 4**).
- Although not detected following intramuscular exposure, EMPA was detected in all animals that underwent percutaneous exposure (**Figure 5**).
  - EMPA concentrations were higher in epiphyseal bone than in diaphyseal bone.
- Detected analyte concentrations, as well as diaphyseal concentration to epiphyseal concentration ratios (D:E), reflected exposure history.
  - Pigs that died from VX : EMPA detected above the method LOD of 350 pg/g in diaphyseal samples and above the positive control of 750 pg/g in epiphyseal samples (D:E = ~1:5).
  - Pigs that did not die from VX: EMPA detected at trace concentrations or possible trace concentrations in both the diaphyseal and epiphyseal samples (D:E = ~1:3).

DISCUSSION/CONCLUSIONS

- Although only confirmed in a subset of samples, the presence of EMPA in the pig femora indicates that aMPAs:
  - 1) likely interact with bone tissue
  - 2) can be isolated and detected from osseous materials postmortem.
- These results suggest the method may one day be used with human bone to detect use of chemical weapons from postmortem biomatrices even well after a suspected attack.
  - Additional studies, assessing the effects of different agents, exposure pathways, and taphonomic variables are needed
- Use of 0.5% AA as a mobile phase modifier instead of 0.1% FA greatly enhanced detection of the more polar analytes, and use of 18 mega-ohm water improved ionization.
- Unlike the commonly used normal phase SPE columns, carbon-based SPE columns allow any volume of aqueous/biological matrix to be tested.
  - Important when detection of low-level exposure indicators is requisite.
  - Although these columns co-retained suppressing species, citrate concentrations should be much lower in other biological matrices than in bone.

Table 4: LODs from published methods for the detection of aMPAs in various biomatrices

Matrix	Reference	LOD (pg/g for solid matrices or pg/mL for liquid matrices)				
		EMPA	IMPA	iBuMPA	CMPA	PMPA
Nails	2	n/a	7500	n/a	n/a	300
Hair	3	n/a	7500	n/a	n/a	150
Serum	4	500	400	400	500	300
Serum	5	110	40	80	80	90
Urine	6	160	240	75	50	30
Urine	5	120	110	90	80	110
Bone	Herein	350	20	7.5	10	5

- Given the low LODs (even in the presence of extremely high matrix suppression; **Table 4**) and the simplicity and adaptability of the sample preparation and instrumental protocols, the presented methods should be tested with other matrices to assess if lower LODs than currently published can be achieved. If so, this may expand the window of detection for attack survivors as well.

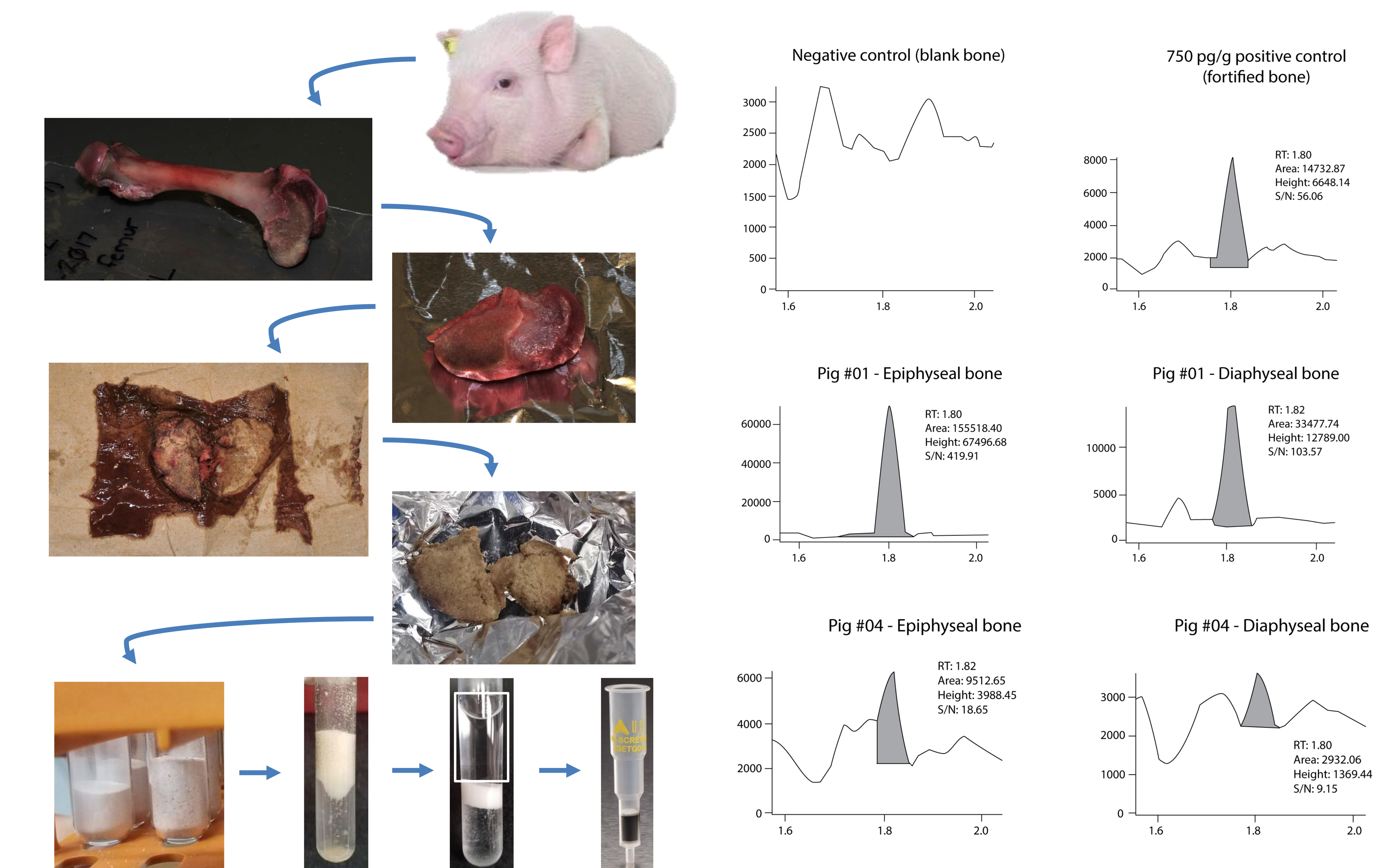

Figure 4: Schematic showing stages of sample preparation – removal of femora from pig, removal of epiphyseal portion (unilateral, distal condyle), removal of marrow from epiphyseal sample, pulverization of bone, demineralization of bone, precipitation of salts, and SPE.

Figure 5: LC-QqQ-MS/MS chromatograms showing examples of results for the controls, high-positive authentic samples (pig #01), and low-positive (trace) authentic samples (pig #04). X-axes show retention time, in minutes; Y-axes indicate absolute intensity.

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