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Sample Preparation Report of the Fourth OPCW Confidence Building Exercise on Biomedical Sample Analysis

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Disclaimer

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Sample Preparation Report

of

The Forth Confidence Building Exercise on

Biomedical Sample Analysis

Plasma Sample Preparation Report

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1. Introduction

Following the successful completion of the 3rd biomedical confidence building exercise (February 2013 – March 2013), which included the analysis of plasma and urine samples spiked at low ppb levels as part of the exercise scenario, another confidence building exercise was targeted to be conducted in 2014. In this 4th exercise, it was desired to focus specifically on the analysis of plasma samples. The scenario was designed as an investigation of an alleged use of chemical weapons where plasma samples were collected, as plasma has been reported to contain CWA adducts which remain present in the human body for several weeks (Solano *et al.* 2008).

In the 3rd exercise most participants used the fluoride regeneration method to analyze for the presence of nerve agents in plasma samples. For the 4th biomedical exercise it was decided to evaluate the analysis of human plasma samples for the presence/absence of the VX adducts and aged adducts to blood proteins (*e.g.,* VX-butyrylcholinesterase (BuChE) and aged BuChE adducts using a pepsin digest technique to yield nonapeptides; or equivalent). As the aging of VX-BuChE adducts is relatively slow ($t_{1/2}$ = 77 hr at 37 °C [Aurbek *et al.* 2009]), soman (GD), which ages much more quickly ($t_{1/2}$ = 9 min at 37 °C [Masson *et al.* 2010]), was used to simulate an aged VX sample. Additional objectives of this exercise included having laboratories assess novel OP-adducted plasma sample preparation techniques and analytical instrumentation methodologies, as well as refining/designating the reporting formats for these new techniques.

2. Exercise Scenario

The request for an investigation of an alleged use of chemical weapons was submitted to the OPCW Director-General. A State Party has requested an investigation in an area near their territory where chemical weapons are alleged to have been used. The target area for inspection is described as a small village. An OPCW challenge inspection team was sent to the site and subsequent on-site analysis by the inspection team confirmed the presence of O-ethyl S-2-diisopropylaminoethyl methylphosphonothiolate (VX) in munition fragments and soil samples. It is important to assess whether individuals from the village had been exposed to VX. In this case of Investigation of Alleged Use (IAU), biomedical sampling in response to this event was initiated. Blood samples from 4 individuals living near the munitions fragments were collected, centrifuged into plasma, and sent for biomedical sample analysis. However, due to weather conditions, the transportation of the plasma samples was delayed 10 days. A blank and control sample were prepared at the OPCW Laboratory. Please analyze these 6 human plasma samples for the presence/absence of aged and/or non-aged VX adducts of BuChE and/or albumin.

3. Test Materials

Pooled normal human plasma (anticoagulant: K2 EDTA) was purchased from Innovative

Research (MI, USA). Stock solutions (1000 µg/mL in hexane) of O-pinacolyl methylphosphonofluoridate (soman [GD]) and O-ethyl S-2-diisopropylaminoethyl methylphosphonothiolate (VX) were obtained from Spiez Laboratory, Switzerland. Serum vials (sterile, 5 and 10 mL) and Nalgene bottle top filters (0.45 µm) were purchased from Thermo Scientific. Solvents used were at least of HPLC grade.

4. Preparation of Samples

Thirty sets of plasma samples were prepared at The OPCW Laboratory, Rijswijk, on 20th February 2014. The identities of the samples are shown in Table 1.

The plasma was allowed to thaw in the refrigerator at 4 °C for a period of 2 days. Plasma appeared turbid due to precipitates. Plasma was filtered using Nalgene bottle top filters (0.45 µm). A fresh filter had to be used for about 100 mL of plasma. Plasma blank and P-403 samples were not from filtered plasma, the plasma was only decanted in this case. These two samples were dispensed into individual vials and stored at 4 °C.

The stock solutions were diluted to 10 µg/mL using isopropanol just before spiking. The diluted stock solutions, 10 µg/mL (in IPA) of soman and VX, were used for spiking 150 mL of plasma to obtain the final concentrations listed in Table 1. All spiked samples were mixed thoroughly for 30 minutes at room temperature. The VX-spiked samples were then stored at 4 °C. The soman-spiked samples were allowed to stand at room temperature for 3 hours before all samples were dispensed into individual serum vials (5 mL each). The next day the vials were packed into containers according to the lab codes and kept at 4 °C over the weekend.

Table 1: Sample identities in the 4th OPCW Biomedical Confidence Building Exercise.

Sample Code	Compound Name	Concentration (ng/mL)
P-401	O-Pinacolyl methylphosphonofluoridate (GD; control sample)	10
P-402	O-Ethyl S-2-diisopropylaminoethyl methylphosphonothiolate (VX)	8
P-403	Blank sample	-
P-404	O-Ethyl S-2-diisopropylaminoethyl methylphosphonothiolate (VX)	5
P-405	O-Pinacolyl methylphosphonofluoridate (GD)	5
P-406	O-Pinacolyl methylphosphonofluoridate (GD)	1

Each participating laboratory was assigned to receive a set of the following samples:

Plasma Sample P-401/XX*	5 mL
Plasma Sample P-402/XX	5 mL
Plasma Sample P-403/XX	5 mL
Plasma Sample P-404/XX	5 mL
Plasma Sample P-405/XX	5 mL
Plasma Sample P-406/XX	5 mL
Plasma Blank/XX	10 mL
Reference Standard 1/XX	1 mL (10 µg/mL O-pinacolyl methylphosphonofluoridate [GD])
Reference Standard 2/XX	1 mL (10 µg/mL O-ethyl S-2-diisopropylaminoethyl methylphosphonothiolate [VX])

*Where XX is the Laboratory code.

The samples were shipped under cold condition using ice packs on 24th February 2014 to the participating laboratories.

5. Sample Dispatch

The prepared samples were shipped from The OPCW Laboratory (Rijswijk, The Netherlands) on 24th February 2014 via FedEx Courier. Five sets of samples with one set of reference standards were shipped to Lawrence Livermore National Laboratory (LLNL), USA, for stability studies. The shipment arrived at LLNL on 26th February 2014 at 10:15 hrs.

6. Analytical Protocols

A. Butyrylcholinesterase Immunomagnetic Separation Bead Preparation:

Butyrylcholinesterase (BuChE) immunomagnetic separation (IMS) beads were prepared by first transferring 2 mL of Protein G Dynabeads for Immunoprecipitation (Invitrogen, 10004D) into a 15 mL tube. A magnet was applied, capturing the beads, and the storage solution was removed. The beads were washed three times with 4 mL of Phosphate Buffered Saline with Tween-20 (PBST; Sigma, P3563). After resuspending in 8 mL PBST, 400 µg BuChE Monoclonal Antibodies (400 µL of a 1 mg/mL solution, ThermoFisher, HAH-002-01-02) was added, and the bead solution was left to incubate overnight (~18 hours), in the dark, at room temperature (~20 °C) with rotation.

The next morning the beads were captured using a magnet and the supernatant was removed. The beads were washed twice with 4 mL triethanolamine (TEA) buffer (Sigma, T0449). 4 mL of 5.4 mg/mL Dimethyl Pimelimidate Dihydrochloride (DMP; Sigma, D8388) in TEA buffer was added to the beads and incubated for 30 minutes at room temperature with rotation. After incubation the beads were captured and the supernatant removed. 4 mL of Tris Buffered Saline (TBS; Sigma, T5912) was added and incubated for 15 minutes at room temperature with rotation. After incubation the beads were captured and the supernatant removed, then

washed twice with 2 mL of PBST. The final BuChE IMS beads were resuspended in 1.9 mL PBST and stored at 4 °C.

B. Plasma Sample Preparation Prior to LC-HRMS Analysis:

100 µL of BuChE IMS beads (20 µg of conjugated BuChE antibody per 100 µL of beads) were removed from their storage solution for each sample. 300 µL of each plasma sample was clarified to remove cells/debris by centrifugation at 10,000 x g for 5 minutes at 20 °C. 200 µL of the clarified samples was added to the IMS beads, vortexed briefly (3 second pulse at 3000 rpm), and incubated at 20 °C for 120 minutes with mixing at 1400 rpm. IMS beads were captured using a magnet and washed three times with 500 µL PBST with brief vortexing between each wash. The BuChE bound to the IMS beads was then resuspended in 75 µL of HPLC-grade water and digested with 10 µL of 2 mg/mL pepsin in 5% formic acid at 37 °C for 30 minutes with mixing at 1000 rpm for 10 seconds every minute. The digestion was stopped and peptides were isolated by centrifugation through a 10 kDa molecular weight cutoff filter. The resulting solution was directly analyzed by LC-HRMS.

C. LC-HRMS Analysis:

An Agilent 1290 Infinity Binary LC System, including autosampler and thermostatted column compartment, was interfaced to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer for peptide sample analyses. Gradient elution was performed at 45 °C using a 2.1 x 150 mm Waters Atlantis T3 C18 column with 3 µm particles. The mobile phase was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) delivered at a flow rate of 0.5 mL/min. After the 10 µL sample injection, a segmented gradient separated the analytes during a 30 minute run as follows: 95% A for 5 minutes, linear to 10% A over 15 minutes, 95% A for 10 minutes (column re-equilibration). The Dual Agilent Jet Stream Electrospray Ionization source was operated in positive mode using the following parameters optimized for generation of singly charged peptide ions: capillary voltage 5000 V, nozzle voltage 750 V, source gas temperature 300 °C, source gas flow rate 4 L/min, nebulizer 55 psig, sheath gas temperature 250 °C, and sheath gas flow rate 8 L/min. Ultrapure nitrogen was used for all source gases. Agilent MassHunter Data Acquisition software (version B.05.01) was used to acquire data over the mass range 85-1200 *m/z* at a rate of three spectra per second. Agilent MassHunter Q-TOF Quantitative Analysis software (version B.05.00) was used to extract and integrate analyte exact masses listed in Table 2.

Table 2. Analyte exact masses that were extracted and integrated from LC-HRMS data.

Analyte	**Structure**	**Formula**	**$[M+H]^+$**
Nonapeptide	FGESAGAAS	C33H49N9O14	796.3472
Aged adducted nonapeptide	FGES(MPA)AGAAS	C34H52N9O16P	874.3342
VX-adducted nonapeptide	FGES(EMPA)AGAAS	C36H56N9O16P	902.3655
GD-adducted nonapeptide	FGES(PMPA)AGAAS	C40H64N9O16P	958.4281

7. Stability Studies

The stability profiles for samples P-401, P-402, P-404, P-405, and P-406 were investigated for 26 days starting from the day after sample receipt (27th February 2014). All samples were prepared in triplicate and analyzed using LC-HRMS in triplicate (*n* = 9 total) at each time point. Figures 1-4 show the stability profiles of the analytes.

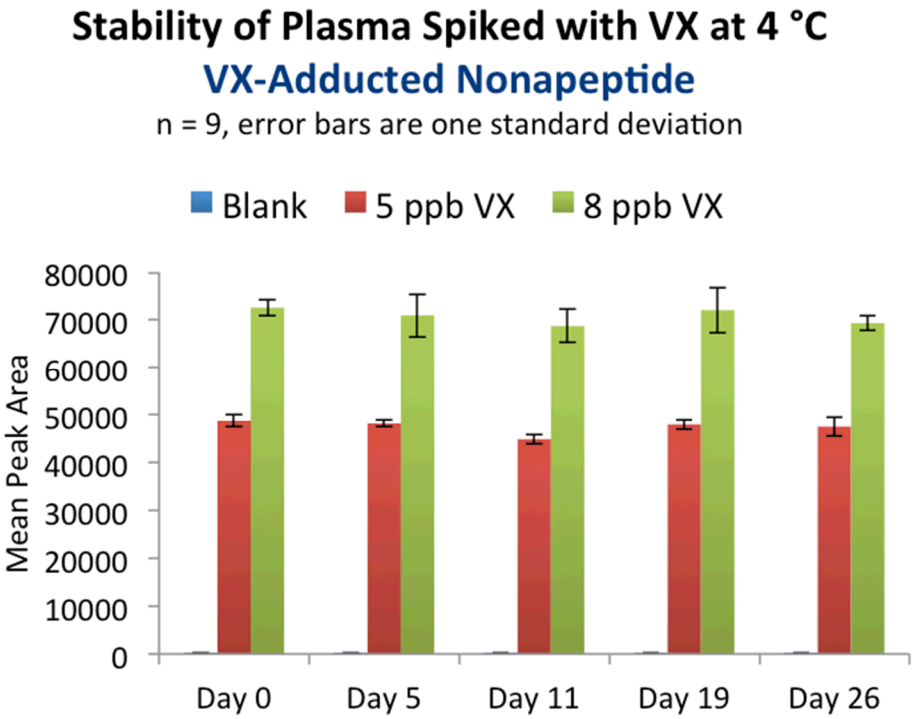

Figure 1: Stability of VX-adducted nonapeptide in samples P-402 and P-404 (plasma spiked with 8 ppb and 5 ppb of VX, respectively) under storage at 4 °C.

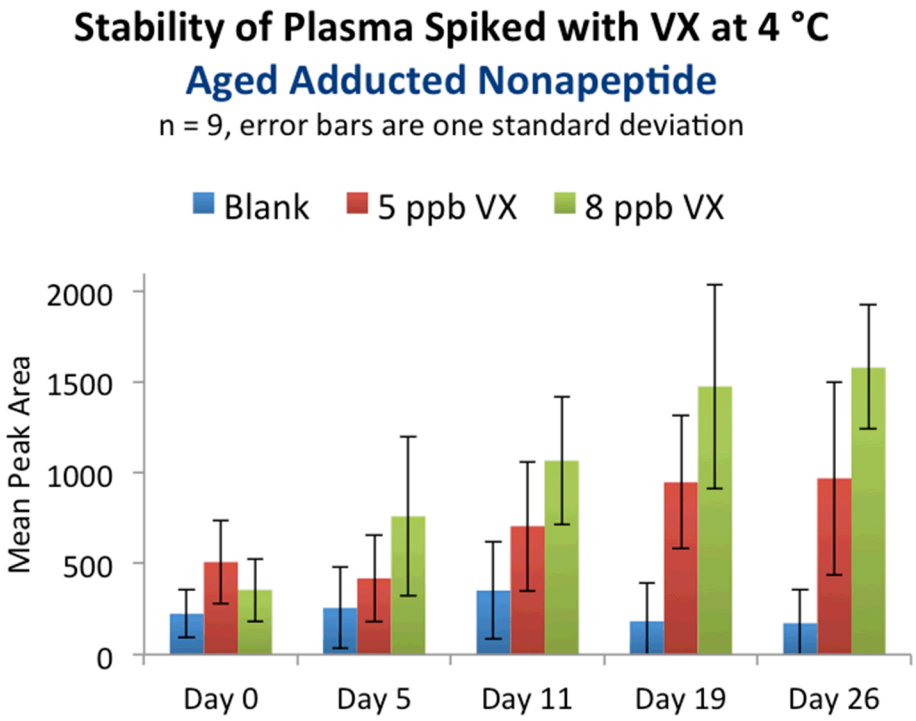

Figure 2: Stability of aged adducted nonapeptide in samples P-402 and P-404 (plasma spiked with 8 ppb and 5 ppb of VX, respectively) under storage at 4 °C.

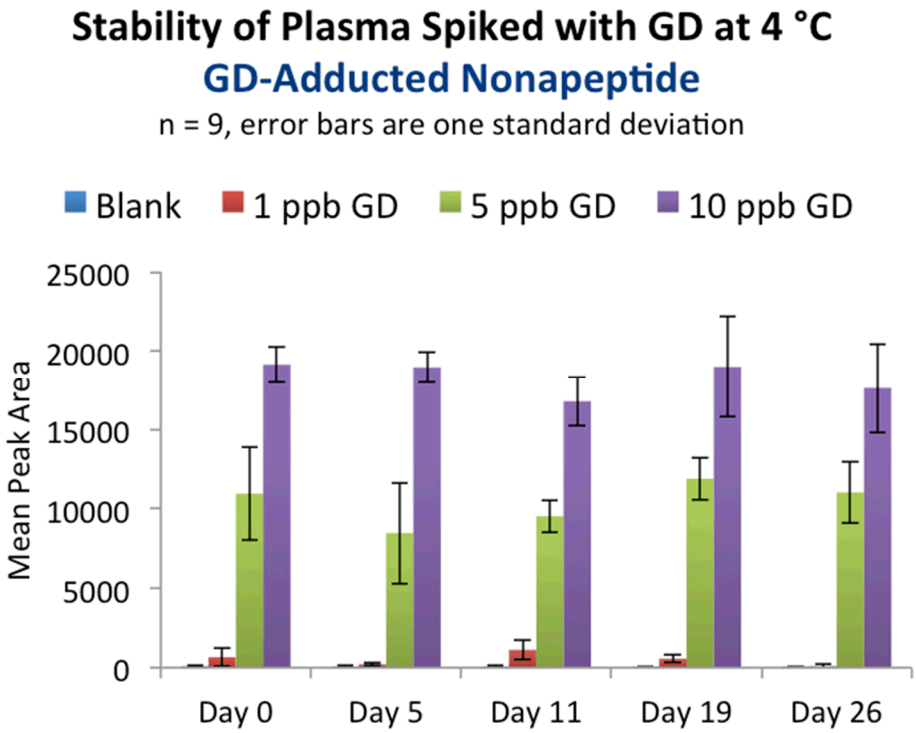

Figure 3: Stability of GD-adducted nonapeptide in samples P-401, P-405, and P-406 (plasma spiked with 10 ppb, 5 ppb, and 1 ppb of GD, respectively) under storage at 4 °C. The signal was near the LOD in sample P-406 (plasma spiked with 1 ppb GD).

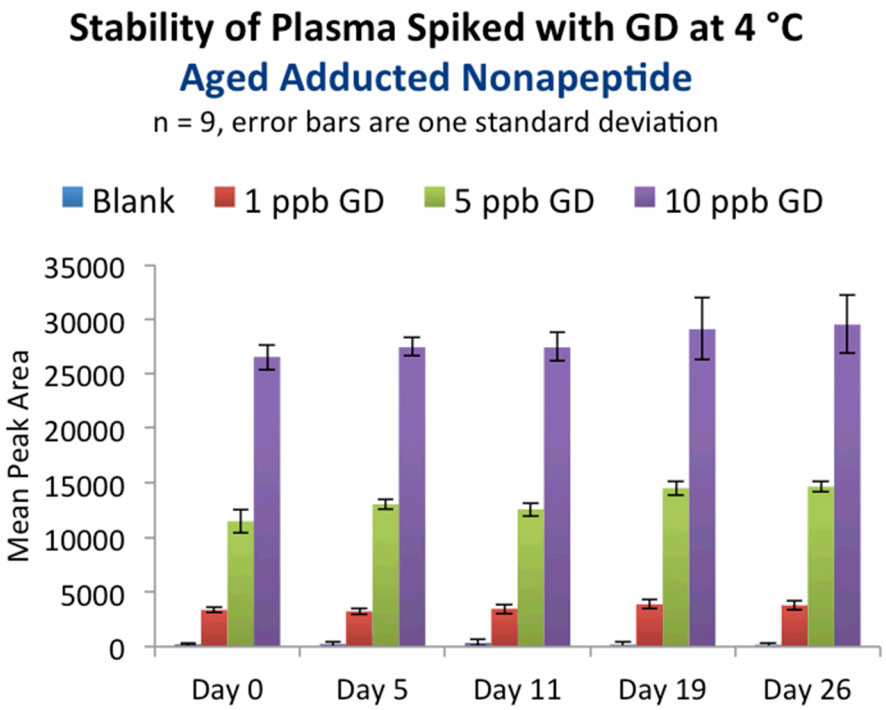

Figure 4: Stability of aged adducted nonapeptide in samples P-401, P-405, and P-406 (plasma spiked with 10 ppb, 5 ppb, and 1 ppb of GD, respectively) under storage at 4 °C.

Analysis of Variance (ANOVA) was used to separate and evaluate sample stability variation from variation due to random errors in sample preparation and analysis. The VX-adducted nonapeptide signature (Figure 1) did not vary significantly during the stability study in the 8 ppb VX sample ($p = 0.13$), but it did have significant inter-day variability in the 5 ppb VX sample ($p < 0.05$). This was due to small random errors (error bars) and the day 11 response being slightly decreased compared to the other time points. The day 26 abundance of VX-adducted nonapeptide was 98% of the abundance on day 0, indicating that the signature was stable for detection throughout the stability study. The aged adducted nonapeptide signature in both of the samples spiked with VX (Figure 2) significantly increased during the study ($p < 0.05$). However, the abundance of the aged adduct is only ~2% of the abundance of the VX-adducted nonapeptide on day 26 for both plasma samples spiked with VX, indicating very slow aging of the VX-adducted BuChE at 4 °C, as expected.

The presence of appreciable amounts of intact GD-adducted nonapeptide in the plasma samples spiked with GD was unexpected, as a review of the literature suggested a three hour incubation at room temperature should completely convert the GD-BuChE adducts to aged BuChE adducts. This result is the subject of ongoing research. The abundance of the GD-adducted nonapeptide (Figure 3) was near the LOD in the 1 ppb GD sample and did not vary significantly during the stability study in the 10 ppb GD sample ($p = 0.10$). There was significant inter-day variability in the 5 ppb GD sample ($p < 0.05$), however, there was no trend and the

abundances on day 0 and day 26 are statistically the same, indicating that the GD-adducted nonapeptide was stable for detection throughout the exercise time period. The aged adducted nonapeptide signature (Figure 4) significantly increased in abundance during the stability study in all three of the plasma samples spiked with GD ($p < 0.05$). Relative to the abundance on day 0, the abundance of the aged adducted nonapeptide on day 26 was 113% for 1 ppb GD, 128% for 5 ppb GD, and 111% for 10 ppb GD. These results show slow aging of GD-adducted BuChE in plasma stored at 4 °C.

In summary, all plasma samples spiked with VX or GD were distinguishable from the blank plasma sample throughout the exercise time period. Minimal aging of VX- and GD-adducts to BuChE was observed, indicating the plasma samples were stable under storage at 4 °C for 26 days.

8. Technical assistance during the exercise period:

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