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14. ABSTRACT
Verification of exposure to chemical agents is a requirement of the intelligence, depot, and demilitarization communities to confirm claims of exposure to chemical warfare agents. Improving current analytical methods and developing novel methods to verify chemical agent exposure are vital, considering the increased threat of chemical agent use and therefore the possibility of large numbers and types of biomedical samples that a relatively small number of labs must analyze. The current study focuses on development of an analytical method to determine past exposure to soman (GD) by identifying GD adducts of human hemoglobin (Hb). Although previous studies have indicated that GD does bind to the tyrosine residue in human albumin, no such adduct was found for GD and human hemoglobin.

15. SUBJECT TERMS
nerve agent, soman, organophosphate, hemoglobin, chemical warfare agent, liquid chromatography, HPLC

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Background

Recent events, such as the use of sulfur mustard by Iraq in the Iran-Iraq conflict (Benschop et al., 1997), the use of sarin in a terrorist attack in Tokyo (Croddy, 1995), and the search for viable causes of the "Gulf War Syndrome" (Noble, 1994), validate the need for confirmation of chemical warfare agent exposure through analysis of biomedical samples. In the previous decade, a number of analytical techniques to verify exposure of chemical agents were proposed with great success (Table 1). Yet some areas in the forensic investigation of chemical agent exposure still need to be explored. Improving current analytical methods and developing novel methods to verify chemical agent exposure are vital, considering the increased threat of chemical agent use and therefore the possibility of large numbers and types of biological samples that a relatively small number of labs must analyze.

Table 1 outlines some analytical methods for verifying past exposure to the chemical warfare agents sulfur mustard (HD), sarin (GB), soman (GD), and VX. Some potential agent-blood interactions that may be investigated to produce novel analytical methods are outlined in Table 1 by specifying that no analytical method that exploits the binding of a chemical agent with an individual component of blood was found in the literature (ND – no data). Most of the methods referenced utilize liquid chromatography tandem mass spectrometry (LC/MS) to determine past exposure. With the advent of triple quadrupole mass spectrometers, LC/MS has become even better able to identify the large protein adduct fragments that are typically targeted as indicators of past exposure. These analytical methods do an excellent job retroactively of determining whether someone has been exposed to a specific chemical warfare agent, yet there are some targets that have yet to be investigated, specifically GD-hemoglobin adducts, that may prove even more useful in verification of exposure.

Introduction

As mentioned above, there are potentially superior markers of chemical agent exposure that have yet to be explored in the literature. This study initiated research into one of these potentially useful markers: GD binding with hemoglobin. As seen in Table 1, butyrylcholinesterase and aqueous hydrolysis products of GD were the main focus of previous studies to verify past GD exposure. Yet evidence for alternative binding sites other than the cholinesterases for GD is present in the literature. Although most previous authors have limited their search for markers to the binding of organophosphorus nerve agents with cholinesterases in plasma, Black et al. (1999) found that GB and GD bind to the tyrosine residue in albumin. Also, even though GD should not be "reactivated" from cholinesterases because of rapid aging, Adams et al. (2004) found that GD was liberated from the red blood cells of exposed monkeys. Moreover, the amount of GD released was greater than the calculated amount of cholinesterase naturally present in the red blood cells. From Adams' studies, there appears to be an additional site for GD binding in red blood cells besides cholinesterases. Yet even though it appeared the interaction of GD with Hb was an excellent target for a novel analytical method to verify past exposure, it was found in this study that human hemoglobin exposed to GD did not produce any suitable analytical markers to verify past exposure to GD. The specifics of this methodology and the results are discussed below.

Materials and Methods

Materials

The derivatizing agents o-phthalaldehyde (OPA; 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid ampoules) and 9-fluorenylmethyl chloroformate (FMOC; 2.5 mg/mL in acetonitrile ampoules), and the borate buffer (0.4 M in water, pH 10.2), along with the amino acid standard (250 pmol/mL in 0.1 M HCl ampoules), were acquired from Agilent Technologies (Palo Alto, CA). Human hemoglobin, Pronase E, and HPLC grade acetonitrile were procured from Sigma-Aldrich (St. Louis, MO). Soman (methylphosphonofluoridic acid 1,2,2-trimethylpropyl ester, pinacolyl methylphosphonyl fluoride, or GD) was acquired from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD).

GD Exposure and Enzymatic Digestion

Human hemoglobin was dissolved in HPLC grade H_2O (10 mL at 40 mg/mL). An aliquot of GD was added to the hemoglobin (1 mL of 2 mg/mL GD in saline); the reaction vessel was capped tightly and shaken for twenty-four hours. The solution was transferred to larger reaction vessels (50 mL centrifuge tubes), and globin-HCl was precipitated from the human hemoglobin with 30 mL of 1% HCl-acetone (Black et al., 1997). The solution was then centrifuged, and the precipitate was washed three times each with acetone, then diethyl ether. The precipitate was then allowed to air-dry overnight. The adducted hemoglobin was digested with enzymes (Pronase E) to break the Hb into its primary amino acids. Globin, 350 mg, was dissolved in 0.004 M KH_2PO_4 (35 mL). The pH was adjusted to 7.8 with addition of 0.1 M KOH, 80 mg of enzyme was added to the reaction vessel and the pH was again adjusted to 7.8 with 0.1 M KOH.

The reaction vessel was incubated at 37°C for 7 hours (adjusting the pH to 7.8 every 30 minutes, or as needed) in a water bath. The solution was removed from the water bath and acidified to pH 7.0 with H_3PO_4. The solution was then centrifuged at 3000 rpm for 5 minutes, and the supernatant was removed and freeze dried for future analysis.

Chromatographic Conditions

The freeze-dried samples were reconstituted directly prior to analysis with phosphate buffer (0.1 M, pH 7.5) to produce approximately 40 mg/mL. The solutions were vortexed and filtered with a 0.2 μm nylon syringe filter prior to analysis. The digested Hb was analyzed with a Hewlett-Packard 1100 series liquid chromatography system with dual detectors: a Jasco FP-920 fluorescence detector (excitation: 340 nm; emission: 450 nm for OPA derivatives; gain: 10) and an Agilent G1315A diode array detector (UV signal: 338 nm; reference wavelength: 390 nm). A Zorbax Eclipse-AAA 3 x 150 mm C18 column with 3.5 μm particle size was used to separate the amino acid samples. Mobile phase A consisted of 95:5 mixture of ammonium acetate buffer (pH 7.8) and acetonitrile and mobile phase B consisted of a 95:10 mixture of acetonitrile:water. Mobile phase A and B were mixed at a gradient in %B of 0% for 2 minutes, 30% at 25 minutes, 100% at 27 minutes held for 1 minute, and 0% at 29 minutes held for 1 minute. The method utilized a fully automated sample derivatization that mixed 2.5 μL of borate buffer (pH 9.2), 0.5 μL sample, and 0.5 μL OPA. Then 0.5 μL FMOC was added to derivatize secondary amino acids, 32 μL of H_2O was used to dilute the sample, and the entire reaction mixture was injected. The column temperature was maintained at 40°C with a mobile phase flow rate of 0.5 mL/min.

Results and Discussion

Previous studies have shown that nerve agents bind to a serine residue in butyrylcholinesterase (Fidder et al., 2002) and soman binds to a tyrosine residue in albumin (Black et al., 1999). Therefore, the targets of the GD were expected to be the tyrosine and serine residues in human hemoglobin. Several chromatographic modes and conditions were attempted for analysis of the individual amino acids that make up human hemoglobin. An amino acid standard, with fifteen primary amino acids, was used to evaluate and refine the chromatographic methods. An LC/MS/MS was used to analyze the non-derivatized amino acids, and an HPLC with a diode array and a fluorometric detector was used to analyze samples derivatized with FMOC and OPA. Although several chromatographic conditions and stationary phases were used to create an LC/MS/MS method to directly analyze the amino acids, these studies terminated because of a lack of resolution of the amino acid peaks. Also, initial attempts to develop an HPLC method to separate individual amino acids were very successful when using a method that involved derivatization with OPA and FMOC. Although it is likely that an LC/MS/MS method could have been developed if the right conditions were attempted, the success of the OPA/FMOC derivatization precluded further attempts to analyze non-derivatized amino acids. As seen in Figure 1, the final HPLC method adequately resolved 13 of the 15 amino acids in the standard and fully resolved the two amino acids of primary interest: tyrosine and serine.

Trypsin and Pronase E were candidates for enzymatic digestion. Pronase E was determined to be a stronger candidate for digestion because it produced an excellent fingerprint of individual amino acids from the human hemoglobin, whereas trypsin digests produced peptide fragments of the hemoglobin that were more difficult to analyze reproducibly. Therefore, efforts were focused on developing an HPLC method to separate and analyze Hb amino acids following a Pronase E digest and subsequently derivatizing the digests with OPA and FMOC.

Figure 2 shows a chromatographic fingerprint of the enzymatic digest of human hemoglobin exposed to GD from the HPLC method described above. A control sample of non-exposed hemoglobin was also subjected to the enzymatic digest. Figure 2 compares the control hemoglobin (upper traces) and the hemoglobin exposed to GD (lower traces). As seen by the almost identical traces for the exposed and non-exposed hemoglobin for both the diode array and the fluorometric detector, no extra peaks corresponding to a GD phosphonylation site in the GD exposed hemoglobin could be discerned. Therefore, it was concluded that although there may still be a phosphonylation site in hemoglobin, it is not abundant enough to constitute a suitable marker for verification of past exposure to GD.

There are several possible reasons why a suitable marker for GD could not be found from the current study when Adams and coworkers (2004) found that soman could be reactivated from red blood cells (from non-human primates). First, other sinks of GD in the red blood cells, besides hemoglobin, could be responsible for reactivated soman, the most likely being carboxylesterase. Although alternate sinks may account for the reactivated GD in Adams' studies, they do not account for the lack of phosphonylation of Hb by GD in the current study. The simplest explanation may be that fluorometric and optical detection are not sensitive enough to detect the GD-amino acid adduct. If this is the case, LC/MS/MS may provide a solution. Another possible reason may be that although there are several serine and tyrosine residues in Hb, the reactive hydroxyl groups on these residues may not be accessible to soman. Soman could also react with the Hb, but the phosphonylation site may be highly susceptible to hydrolysis of the GD such that it is all removed from the Hb and hydrolyzed to the methyl phosphonic acid under the conditions of this study. In this case, the potential short-lived GD-Hb marker would not be suitable to determine past exposure to GD, when other methods of determining GD exposure are simpler and less time consuming (Adams et al., 2004).

Future Directions

Although no GD-Hb adduct was elucidated under the conditions of this study, there is still the possibility that one may form under slightly different conditions or at concentrations below the sensitivity of the HPLC method used in this study. Development of an LC/MS/MS method more sensitive than the current HPLC method would be the first step in determining whether a GD-Hb adduct does form. Also, we feel that the creation of GD-serine and GD-tyrosine standards would be important for verifying the validity of any LC/MS/MS method that was developed and would also have been helpful in this study. These standards could be used to develop and validate an LC/MS/MS method capable of determining very low concentrations of a GD-Hb adduct.

Table 1. Some analytical methods described in the literature for the verification of past exposure to chemical warfare agents: sulfur mustard, sarin, soman, and VX. All the methods referenced below isolate the analyte from blood.

CW Agent	Component of Blood	Analyte	Analytical Method	Investigators
Sulfur Mustard (HD)	Aqueous	Thiodiglycol	GC-MS (NCI) after derivatization	Black and Read (1991) Black and Read (1988)
	Albumin	HETE – T5 Fragment	LC/MS/MS	Noort et al. (2000)
		(S-HETE)-Cys-Pro-Phe	Micro-LC/MS	Noort et al. (1999)
	Hemoglobin	N-terminal-valine Alkylated histidine	LC/MS	Black et al. (1997)
		Alkylated histidine	LC/MS	Noort et al. (1997)
		N-terminal-valine Alkylated histidine	LC/MS	Noort et al. (1996)
	DNA	N7-HETE-Guanine	Immunochemical then GC-MS(NCI)	Benschop et al. (1997)
		N7-HETE-Guanine 3-HETE-Adnine	GC-MS and HPLC	Ludlum et al. (1994)
		N7-HETE-Guanine 3-HETE-Adnine O6-HETE-Guanine Di-2-Guanin-7-yl sulfide	LC/MS	Fidder et al. (1994)
Sarin (GB)	Aqueous	Isopropyl methylphosponic acid	LC/MS LC/MS/MS IPD-IC[a]	Smith and Shih (2001) Polhijs et al. (1999) Katagi et al. (1997)
	Albumin	Tyrosine residue	LC/MS	Black et al. (1999)
	Acetylcholinesterase	Amino acid residue	GC/MS after derivatization	Nagao et al. (1997)
	Butyrylcholinesterase	Amino acid residue Serine residue Reactivated Sarin	LC/MS LC/MS GC/MS	Fidder et al. (2002) Black et al. (1999) Polhuijs et al. (1997)[b]
	Hemoglobin	ND[c]	ND[c]	ND[c]
Soman (GD)	Aqueous	Pinacolyl methylphosphonic acid	LC/MS IPD-IC[a]	Smith and Shih (2001) Katagi et al. (1997)
	Albumin	Tyrosine residue	LC/MS	Black et al. (1999)
	Acetylcholinesterase	ND[c]	ND[c]	ND[c]
	Butyrylcholinesterase	Serine residue	LC/MS	Black et al. (1999)
	Hemoglobin	ND[c]	ND[c]	ND[c]
VX	Aqueous	Ethyl methylphosphonic acid	LC/MS IPD-IC[a]	Smith and Shih (2001) Katagi et al. (1997)
	Albumin	ND[c]	ND[c]	ND[c]
	Acetylcholinesterase	ND[c]	ND[c]	ND[c]
	Butyrylcholinesterase	ND[c]	ND[c]	ND[c]
	Hemoglobin	ND[c]	ND[c]	ND[c]

[a] Indirect Photometric Detection – Ion Chromatography.
[b] Polhuijs et al. (1997) assumed sarin was released from butyrylcholinesterase.
[c] No data. A method previously reported in the literature could not be found.

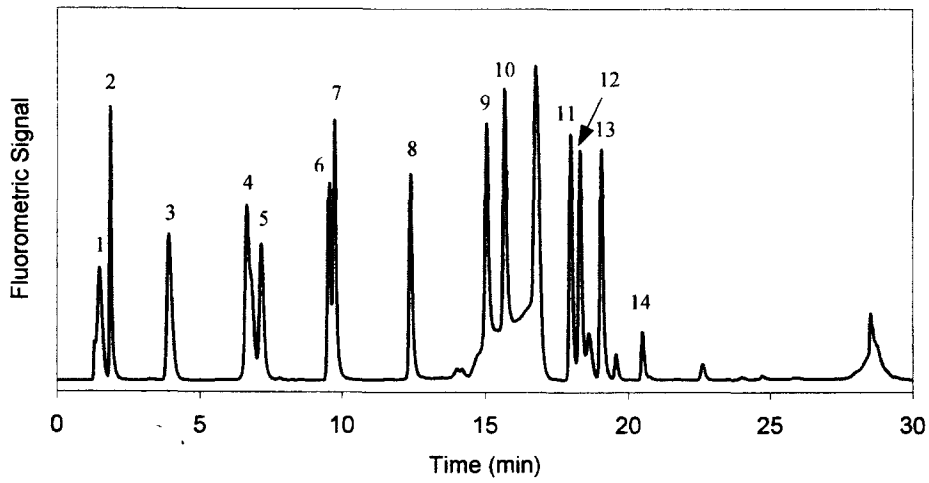

Figure 1. Chromatograph of a 15 amino acid standard. The individual amino acids elute in the order aspartic acid (1), glutamic acid (2), serine (3), glycine/histidine (4), threonine (5), alanine (6), arginine (7), tyrosine (8), valine (9), methionine (10), isoleucine (11), phenylalanine (12), leucine (13), and lysine (14). (Chromatographic conditions are described in the Materials and Methods.)

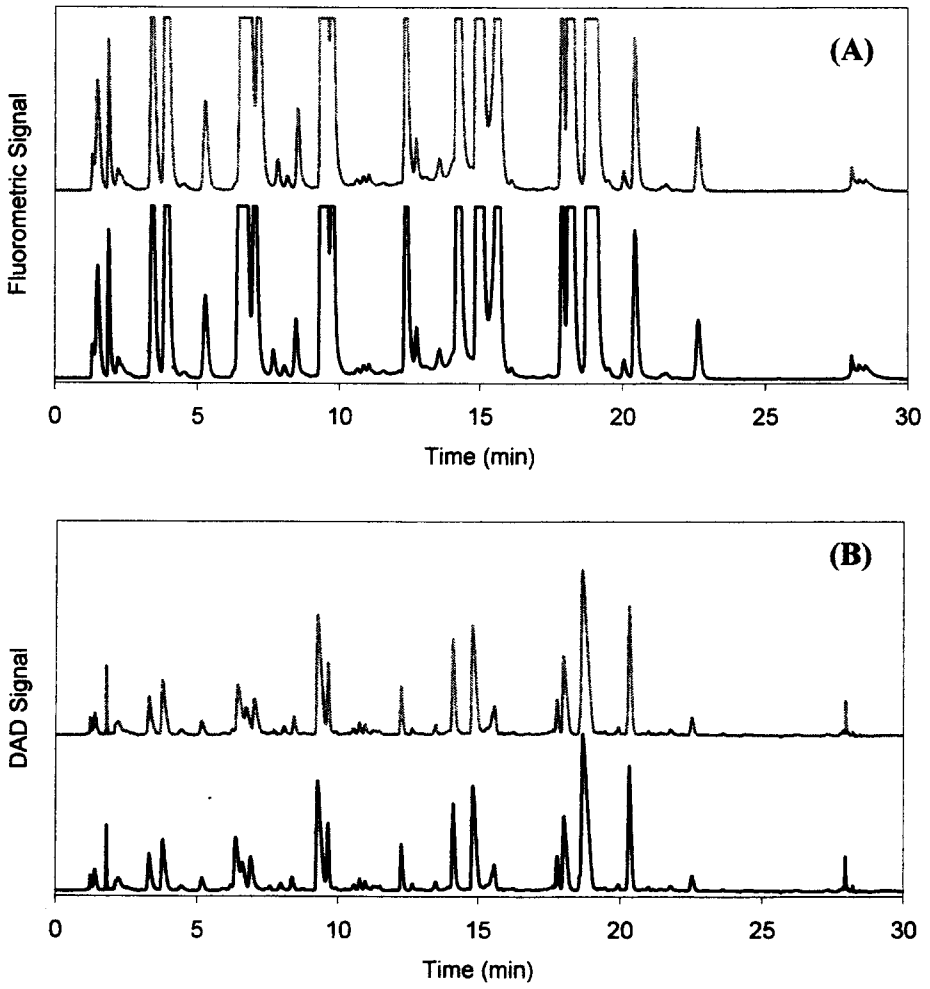

Figure 2. Chromatographs of Pronase E digestions of human hemoglobin. The hemoglobin exposed to soman (upper traces) showed little difference when compared with the control hemoglobin (lower traces) and no extra peaks of interest appeared in the chromatograph of the GD-exposed hemoglobin. Both the A) fluorometric trace (excitation at 340 nm, emission at 450 nm) and the B) UV absorbance trace (338 nm) confirmed the results. (Chromatographic conditions are described above.)

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