MONITORING SULFUR MUSTARD EXPOSURE BY THE GC-MS ANALYSIS OF THIODIGLYCOL CLEAVED FROM BLOOD PROTEINS

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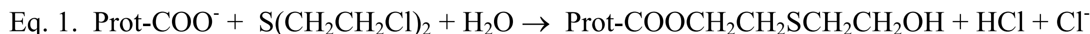
ABSTRACT

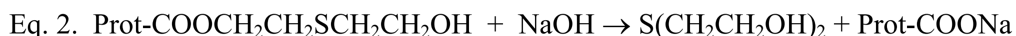
A GC-MS assay of thiodiglycol (TDG) released from blood protein adducts of sulfur mustard (SM) was developed. Exposure of pig blood to SM (24 nM to 400 nM) resulted in a SM concentration-dependent increase in plasma TDG levels from 2.0 pg/mg protein to 38 pg/mg protein. TDG levels in blood samples drawn from monkeys (Chlorocebus aethiops) after a 1.0 mg/kg intravenous SM injection decreased from 220 pg/mg to 10 pg/mg protein during 45 days. TDG levels in plasma samples from pigs after a 2.4 mL (0.4 ml/site, 6 sites) neat SM percutaneous exposure decreased from 60 pg/mg protein to 4 pg/mg protein during 21 days. This assay was precise, reproducible, and relatively simple with the potential to become a routine test for SM exposure.

INTRODUCTION

Several papers have been published recently on assays that enhance analytical methodology to monitor exposure levels of the chemical warfare agent 2,2'-dichlorodiethyl sulfide (sulfur mustard; SM) in biological substrates and have been reviewed.[1] These include the immunoassay of SM bound to DNA,[2] the GC-MS assay for the N-terminal valine adduct of hemoglobin,[3-5] a GC-MS assay of TDG from skin keratin,[5] the LC-MS assay of the cysteine adduct in albumin,[6] and an immunological method for the detection of mustard adducts to skin tissue.[7] We evaluated an alternate method for detecting SM exposure that involved forming and monitoring TDG from blood proteins rather than skin protein.[5] Similar to skin keratin both albumin and globin contain free carboxylic acid groups from aspartic acid and glutamic acid that can be alkylated by the electrophilic SM to give hydroxyethylthioethyl (HETE) esters (Equation 1). These esters can be readily cleaved or hydrolyzed with dilute base to yield TDG (Equation 2). The TDG can be derivatized with pentafluorobenzoyl chloride to give the bis(pentafluorobenzoyl) ester of thiodiglycol (2,2'-thiobisethanol dipentafluorobenzoate, TDGPFB) (Equation 3) and analyzed by a GC-MS negative-ion chemical ionization (NCI) in the selected ion monitoring (SIM) mode.[5,8,9] The key reactions are shown below:



Eq. 1. $\text{Prot-COO}^- + S(CH_2CH_2Cl)_2 + H_2O \rightarrow \text{Prot-COOCH}_2CH_2SCH_2CH_2OH + HCl + Cl^-$



Eq. 2. $\text{Prot-COOCH}_2CH_2SCH_2CH_2OH + NaOH \rightarrow S(CH_2CH_2OH)_2 + \text{Prot-COONa}$

Eq. 3. $S(CH_2CH_2OH)_2 + 2\ C_6F_5COCl \rightarrow (C_6F_5COOCH_2CH_2)_2S + 2\ HCl$

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
18 NOV 2003	**N/A**	**-**

4. TITLE AND SUBTITLE
Monitoring Sulfur Mustard Exposure By The Gc-Ms Analysis Of Thiodiglycol Cleaved From Blood Proteins

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400, USA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

EXPERIMENTAL METHODS

Pig and monkey blood were obtained from experimental animals used in the U.S. Army Medical Research Institute of Chemical Defense Animal Care and Use Committee (IACUC) approved protocols or from a commercial source.

The protein was precipitated from plasma by acetone and then washed with acetone and diethyl ether. For whole blood or red-blood cell samples, acetone with 1% HCl was used for the initial precipitation. After drying, the precipitated protein was treated with 1 M NaOH and heated at 70^0C for 90 minutes. The internal standard octadeuterothiodiglycol (TDGD8) was then added to the homogeneous solution. After neutralization, the sample was extracted with ethyl acetate and the extract dried with sodium sulfate. The TDG and TDGD8 were then derivatized with pentafluorobenzoyl chloride to give TDGPFB and TDGD8PFB, respectively.

The GC-MS analysis was accomplished using NCI in the SIM mode. Ions 510 and 511 were monitored for the TDGPFB and ions 518 and 519 for the TDGD8PFB internal standard. A typical chromatogram with peaks for both TDGPFB and TDGD8PFB is shown in Figure 1. Analysis of the purified derivative of TDGPFB indicated that the lower limit of detection of TDG was about 10 pg/ml.

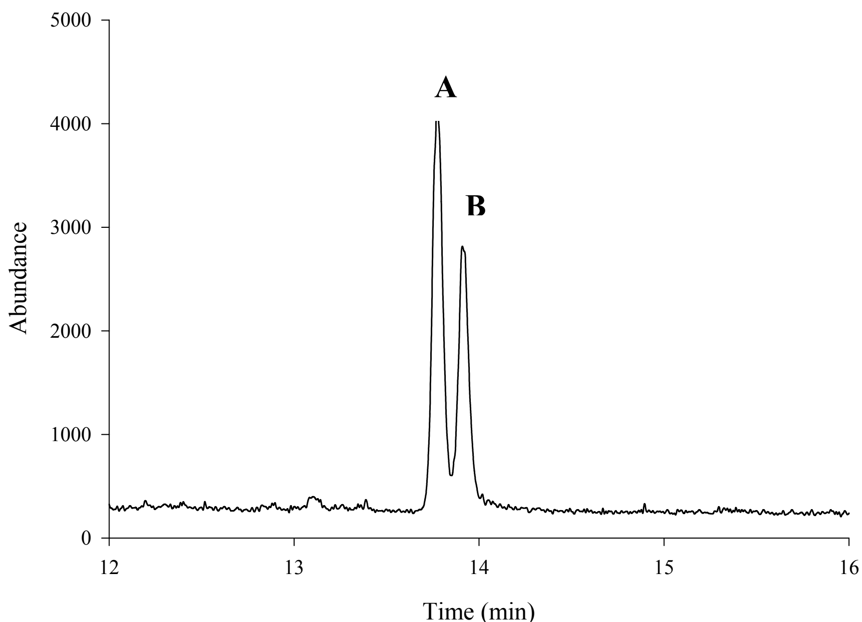

Figure 1. A typical chromatogram showing the relationship between the bis(pentafluorobenzoyl) derivative of TDGD8 (6.5 ng/ml), peak A, and the analogous derivative of TDG (3.9 ng/ml), peak B, analyzed in the NCI-SIM mode on the GC-MS.

RESULTS AND DISCUSSION

The major objective of this study was to demonstrate the sensitivity, precision, retrospectivity, and simplicity of the assay for TDG hydrolyzed from protein-HETE esters following a SM exposure. With numerous "free" carboxylic acid groups associated with aspartic acid and glutamic acid in albumin that can react with SM to form ester linkages, it was reasonable to expect a relatively high quantity of protein-SM adducts compared with the adducts formed at the four terminal valine sites in globin or the single cysteine site in albumin.[1] However, the ester linkages with albumin would be vulnerable to catalytic hydrolysis in the circulatory system, possibly leading to a rapid decrease in the concentration of the protein-HETE ester. A rapid hydrolysis could negate any advantage of multiple reaction sites in albumin.

The results of the analysis for TDG hydrolyzed from the plasma of SM spiked pig blood over a 16-fold range, 400 to 24 nM, are summarized in Figure 2. A linear concentration-exposure relationship ($R^2 = 0.9999$) is consistent with previous reports with assays of other protein-SM adducts.[3-6] The lower limit of detection of 2 pg/mg protein at the 24 nM exposure level indicates a greater sensitivity with this assay than described for the terminal valine assay.[1,3-6] At the lowest level of exposure (24 nM) one TDG molecule was recovered for every one million protein (assume albumin) molecules and an estimated 7% of the SM had become bound to the carboxylate group of proteins in the plasma. Any hydrolysis of the protein-HETE esters in spiked plasma did not appear to interfere significantly with the assay.

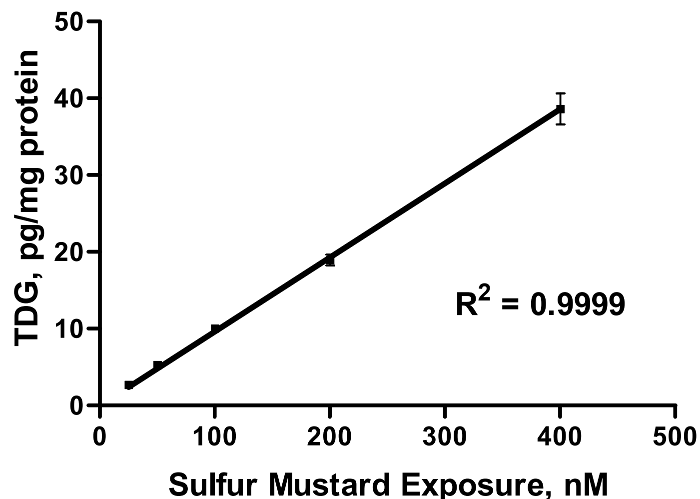

Figure 2. The quantity of TDG determined from the analysis of pig blood spiked with concentrations of SM that varied from 24 nM to 400 nM. $R^2 = 0.9999$. The error bars represent a variation of one standard deviation. CV's ranged from 2 to 12%.

The assay was also evaluated by monitoring two *in vivo* studies, the first involving the direct intravenous injection of SM (1 mg/Kg) in five monkeys, and the second involving a percutaneous exposure of two pigs. The results of analysis of whole blood drawn from the five dosed monkeys shown in Figure 3 indicate that measurable quantities of TDG could be released from proteins for at

least 45 days after the exposure. The rate of loss of the protein-HETE adduct with time was greater than the turn-over rate for the blood proteins (three to four months). The shorter half-life was consistent with additional protein-HETE ester hydrolysis. However, the 45-day period seemed sufficiently long for most diagnostic applications. The smooth exponential decay of the average values of TDG for data from the five monkeys and the relatively small variation of the values at each day represented by the error bars, indicate the precision of the assay. The quantity of SM bound to free carboxylic acid groups in the circulating blood protein was significantly lower, only 0.2 % of the initial SM exposure, than the 7% bound in the spiked blood. Therefore the lower limit of detection of TDG in the blood of living animals would be associated with a low μM SM blood exposure rather than the low nM exposure observed in spiked plasma.

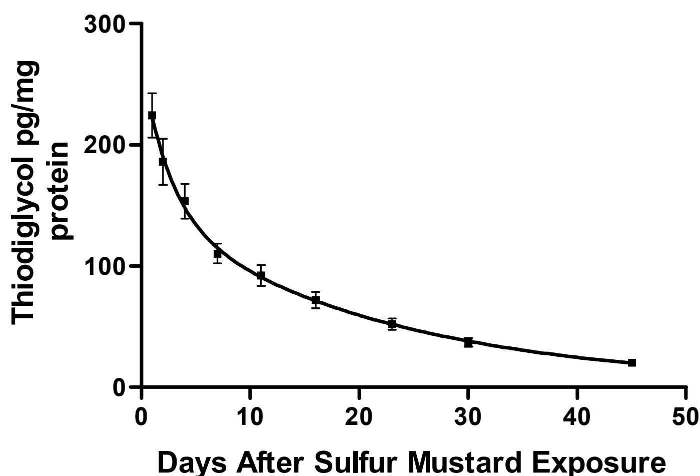

Figure 3. TDG levels obtained from the blood protein of five monkeys following a single intravenous exposure to SM (1mg/kg) and monitored over a 45-day period. Error bars indicate a variation of one standard deviation.

A second separate set of results displayed in Figure 4 and obtained by using both concentrated red blood cells and whole blood from a sixth monkey indicated that the values of TDG from red blood cells were lower than the TDG values obtained from the whole blood of the same animal, particularly during the early time period of three days after exposure. Therefore, TDG values per mg of plasma protein are probably greater than TDG values per mg of globin from the red blood cell. However, the difference is relatively small, indicating that plasma, whole blood, or red blood cells can be used in the assay and yield similar results.

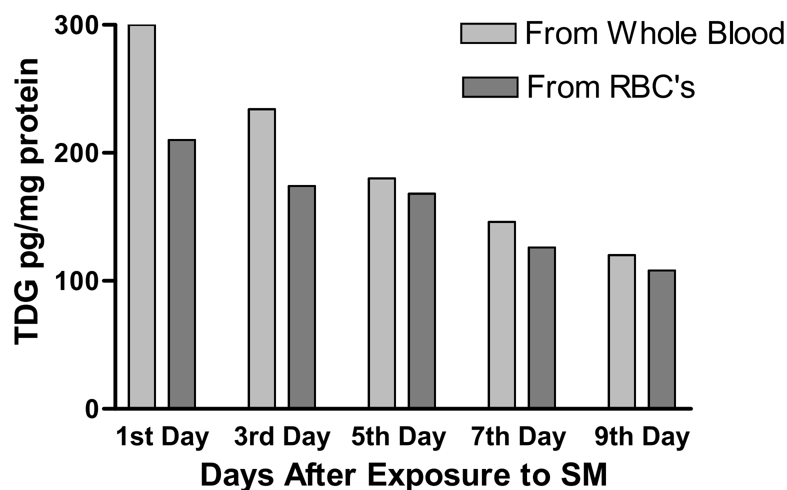

Figure 4. A comparison of quantities of TDG obtained from analysis of either whole blood or concentrated red blood cells after an intravenous exposure of a monkey with SM (1 mg/kg).

The second *in vivo* application of the assay focused on the quantity of TDG recovered from plasma protein after a single 2.4 mL (0.4 mL used at each of six sites) neat SM percutaneous exposure of two pigs. The TDG level was determined five different times over a 21-day period, and the data in Figure 5 represent the average of duplicate samples from both pigs. The initial day one average value of TDG (60 pg/mg protein) decreased to an average of 4 pg/mg protein at day 21. Day 28 values were essentially the same as the background, so only data to day 21 following exposure are plotted. The smooth exponential decay curve and relatively small error bars indicate the precision of the assay.

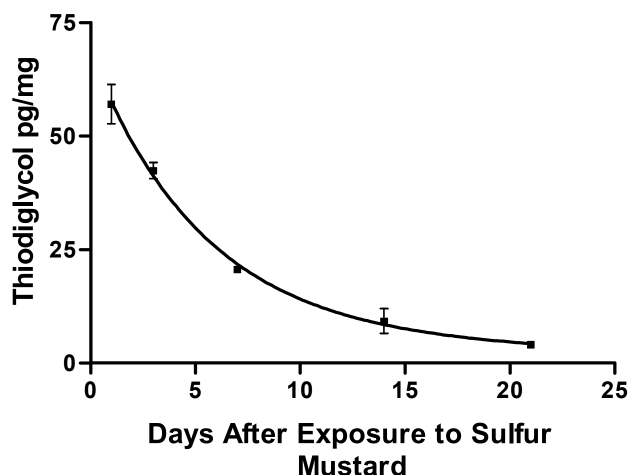

Figure 5. TDG levels obtained from the plasma of two pigs following a percutaneous exposure to neat SM (2.4 mL/pig) and monitored over a 21-day period. Error bars indicate a variation of one standard deviation.

CONCLUSIONS

The assay for the diagnosis of SM exposure by determining TDG liberated from blood proteins was sensitive, precise, reproducible and relatively simple. Plasma protein, whole blood protein, or globin from red blood cells could be used in the assay. TDG from the protein-TDG adducts was detected in blood from live animals for up to 45 days after an intravenous SM exposure and 21 days after a percutaneous exposure indicating an good retrospective assay for SM exposure. Interference from plasma TDG or protein-HETE adducts from sources of TDG in the environment appeared minimal in this study except for values below one pg/mg. The compound TDGD8 served as an excellent internal standard. The assay has the potential to be a routine test of SM exposure and further validation studies are proceeding.

ACKNOWLEDGMENTS

We appreciate the donation of SM exposed swine blood from Dr. John Graham. W. Korte also appreciates partial support from the U.S. Army Faculty Summer Research Program administered by Battelle (Contract No. DAAAHO4-96-C-0086, Report No. TCN 00-080) and the Defense Threat Reduction Agency of the Department of Defense under the auspices of the US Army Research Office Scientific Services Program administered by Battelle (TCN 01136, Delivery Order 802).

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