FLUORIDE ION REGENERATION OF SARIN (GB) FROM MINIPIG TISSUE AND FLUIDS FOLLOWING WHOLE-BODY GB VAPOR EXPOSURE

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ABSTRACT

A method has been developed for the analysis of a Sarin (GB) nerve agent biomarker in the tissue and biological fluids of minipigs that is very sensitive and selective. The GB biomarker was the fluoride ion regenerated GB and was found in minipig samples such as red blood cells, brain, liver, lung, and adipose tissue after whole body inhalation exposure. Regenerated GB in the samples was analyzed by GC-FPD after homogenization, addition of fluoride, pH adjustment and C18 solid-phase extraction. Samples were concentrated by injecting 2-200 uL of extract on a TenaxÒ -TA sorbent tube along with 100 pg of decadeuterated diethyl ethyl phosphonate as the internal standard followed by thermal desorption on to the GC column. The method detection limit was 10.5 pg of agent on column. Highest levels were seen in the lungs (22.8 ng/g) and blood (18.8 ng/g) as expected after an inhalation exposure. Regenerated GB levels in red blood cells were approximately 10 times greater than in the plasma. Outside of the lungs and blood certain sections of the brain had some of the highest level of regenerated GB such as the caudate nucleus (5.46 ng/g). Other significant depots of regenerated GB included saliva (137 ng/g), nasal discharge (72 ng/g), and lachrymal discharge (186 ng/g). Limited amounts of free GB were also isolated from saliva (6.91 ng/g) and nasal discharge (0.65 ng/g).

INTRODUCTION

The post-exposure formation and quantification of substituted alkylphosphonofluoridates has been demonstrated for sarin, soman, and VX in serum, plasma, and whole blood of several species including rat and human serum after adjustment to pH 4 with acetate buffer and in the presence of fluoride ion.[1,2,3,4] The basis of this phenomenon is assumed to be that nerve agents covalently bound to the active site of enzymes such as acetylcholinesterase and butyrylcholinesterase undergo a fluoride ion nucleophilic substitution reaction under suitable conditions to regenerate the free nerve agent. In the case of rat serum/plasma there is enough endogenous fluoride ion to generate some soman and sarin in post exposure samples with acidification to pH 4.[1,2] Fluoride ion has been added to human blood samples along with acidification to regenerate the nerve agent sarin (GB) from the Tokyo subway terrorist incident.[3] Currently, only sarin, soman, and VX have been addressed by this technique in plasma.. Other

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physiological fluids and tissues may be amendable to the production of fluoride-substituted products. Isolation of this type of biomarker from tissues and non-plasma fluids has not previously been reported.

The objective of this work was to explore the possible recovery of free GB and regenerated GB from post whole body inhalation exposure minipig samples such as erythrocytes, brain, saliva, liver, kidney, lungs, and muscle.

EXPERIMENTAL METHODS

Potassium fluoride (CAS No. 7789-23-3) and sodium sulfate (anhydrous, CAS No. 7757-82-6) from Aldrich Chemical Company (Milwaukee, Wisconsin). Waters Sep-Pak® (Waters Associates, Millipore Corp., Milford, MA) 200 mg C_{18} solid-phase extraction (SPE) cartridges were used for extractions. Ethyl acetate and 2-propanol were pesticide grade from Aldrich Chemical Company (Milwaukee, Wisconsin). Aqueous acetate buffer (pH 3.5) was prepared from acetic acid (0.189 M) and sodium acetate (10.8 mM)

The internal standard was synthesized by reacting ethylphosphonic dichloride (98%, CAS No. 1066-50-8, Aldrich, Milwaukee, WI) and Ethyl-$^2H_5$-alcohol (99.5%, CAS No. 1859-08-1, Aldrich, Milwaukee, WI) in the presence of the tertiary amine N, N-diisopropylethylamine (99%, CAS No. 7087-68-5, Aldrich, Milwaukee, WI) in a 1:2:2 molar ratio, respectively, in acetonitrile.

Blood and fluid samples from exposed animals were filtered using a 200 mg C_{18} SPE cartridge (conditioned first with 1.5 mL 2-propanol and then with 1.5 mL acetate buffer) to separate the free from the protein bound nerve agent. The free agent was eluted with 1.5 mL ethyl acetate, collected over sodium sulfate and saved for analysis. For the bound GB assay, separate samples were analyzed in 0.25-mL aliquots by addition of both 0.75-mL acetate buffer and 0.2 mL of potassium fluoride solution. The resulting treated sample was extracted after a fifteen minute incubation time using a 200-mg C_{18} SPE cartridge (conditioned first with 1.0 mL 2-propanol and then with 1.0 mL acetate buffer) and the analytes were eluted with 1.0 mL ethyl acetate. Sodium sulfate was added to dry the extract that was then analyzed by GC-FPD.

Samples were analyzed on either a Hewlett-Packard 5890 GC-FPD or an Agilent 6890 GC-5973 MSD (Newark, DE). Sample inlet was by Tenax® solid sorbent tube (Dynatherm Inc, Oxford, PA) using an ACEM 900 (Dynatherm Inc, Oxford, PA) desorber interfaced to the GC column via butt-connector. The GC column was a 30 m x 25 mm x 0.5 um thickness DB-5 MS (J&W Scientific, Avondale, CA). The ACEM 900 temperature program was as follows: Dry 60 ^oC for 1 minute, Tube Heat 200^oC for 3minutes, cool for 1minute, Trap Heat 275 ^oC for 3 minutes. The GC oven temperature program was as follows: initial 40 ^oC for 2 minutes, ramp to 160 ^oC at 15 ^oC/minute, ramp to 260 ^oC at 40^oC/minute and held for 3 minutes. The MSD was used in the electron ionization mode with selected ion monitoring at m/z 111, 99, and 82. After the sample was desorbed on the GC column the sorbent tube was reconditioned by backflushing using 100 mL/min flow of dry nitrogen at 280-300 ^oC for five to eight minutes to decrease the high boiling point interference from the serum samples. Backflushing of the sorbent tube prevents degradation of the instrument and column producing a stable baseline despite the complex nature of the sample matrix.

A standard curve was established by sequentially injecting five GB standards from 20 ng/mL to 600 ng/mL. Prior to analysis, tubes were spiked with 400 pg of internal standard separately using a flow of nitrogen of approximately 100 mL/min. The peak-area-ratio (PAR) for each standard was plotted against the mass of R-GB injected to yield a linear relationship with a correlation coefficient of greater than 0.99.

RESULTS

Serial blood samples were collected from five minipigs while undergoing whole-body GB vapor exposure at levels close to miosis. Table 1 and figures 1 and 2 illustrate the ability of the "dosemetric" assays to assess internal dose of GB in blood RBC samples resulting from minipig GB vapor exposure. Plasma levels of R-GB were a tenth or less than RBC levels. The plasma of pigs like that of humans does not contain carboxylesterase, which is present in rodents causing higher plasma levels than RBC levels of R-GB after exposure. The data indicate a strong relationship between rate of absorption and exposure level as would be necessary for a dosemetric. Acetylcholinesterase activity shown in figure 3 does not reflect any pattern relative to exposure level. Figure 4 shows that R-GB concentrations are stabile over a 41-day period when the blood is refrigerated.

Figure 5 presents the first experimental evidence that R-GB can be recovered from tissue. After inhalation exposure, the lungs would be expected to yield the highest levels of GB biomarker. Different areas of the brain produced unique R-GB levels. Tissue level experiments were conducted at near lethal levels of GB vapor inhalation exposure in minipigs.

Fluids besides blood had measurable amounts of R-GB and in some cases even free GB was quantified as seen in figure 6. Saliva contains butyrylcholonesterase which can be a depot of R-GB after exposure. Evidence of R-GB around the eye has implications for miosis. Miosis most quickly develops from direct contact of the eye with the nerve agent.

Table 1. Linear Regression Results of R-GB Levels vs Time for 60 Minute GB Inhalation Exposure in Minipigs

	[GB]			
Pig#	mg/m^3	Slope	Intercept	r^2
10	0.20	0.1193	0.0959	0.9800
5	0.17	0.1045	- 0.4348	0.9233
1	0.09	0.0713	- 0.1054	0.9905
3	0.06	0.0536	0.0295	0.9854
2	0.03	0.0323	0.0170	0.9575

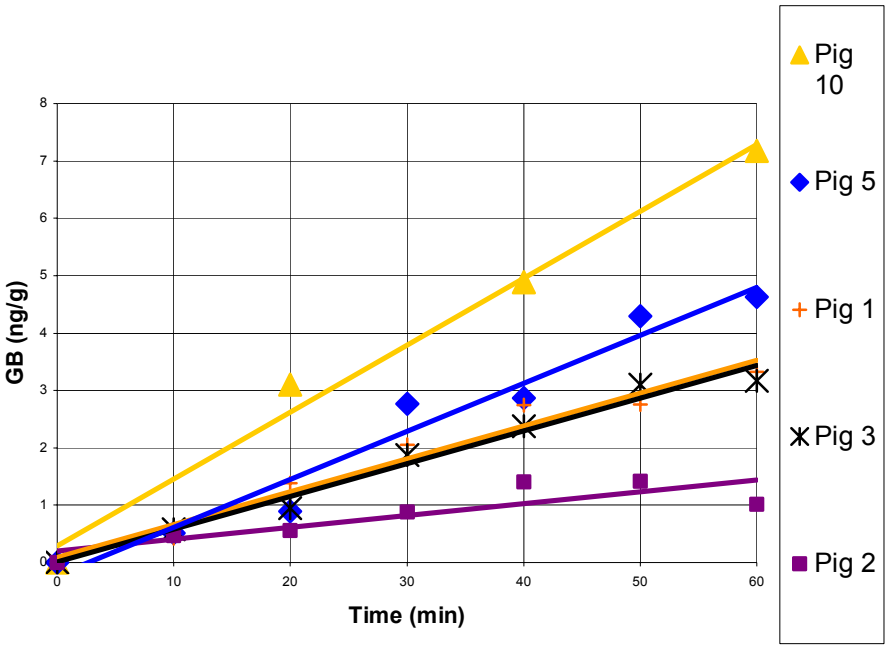

Figure 1. Recoverable GB vs Exposure Concentration: Pig R-GB from RBCs 60 Min Exposure

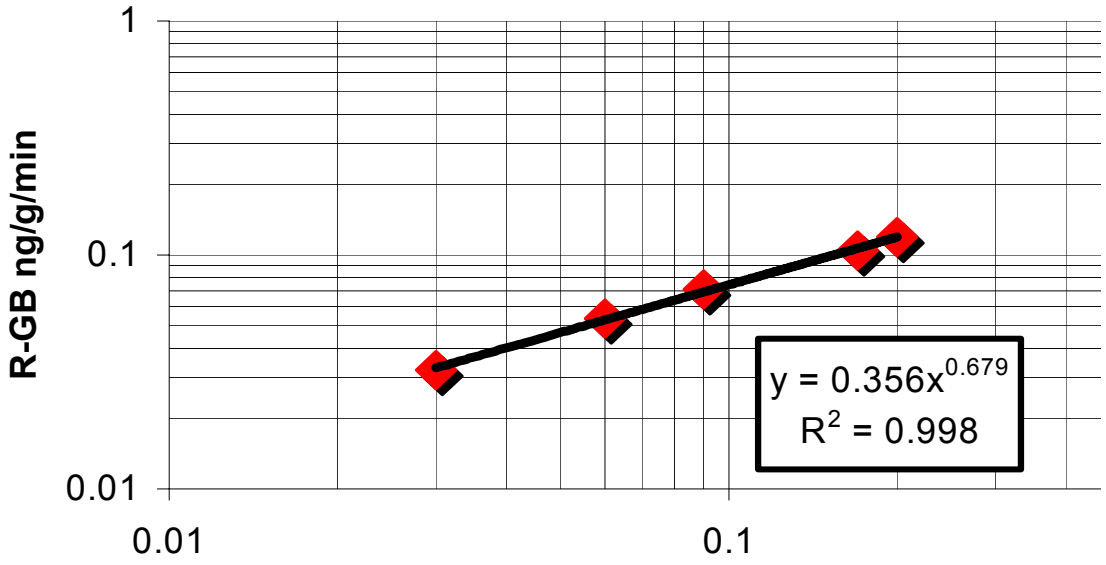

Figure 2. GB Exposure Concentration vs. Slope of R-GB in Blood

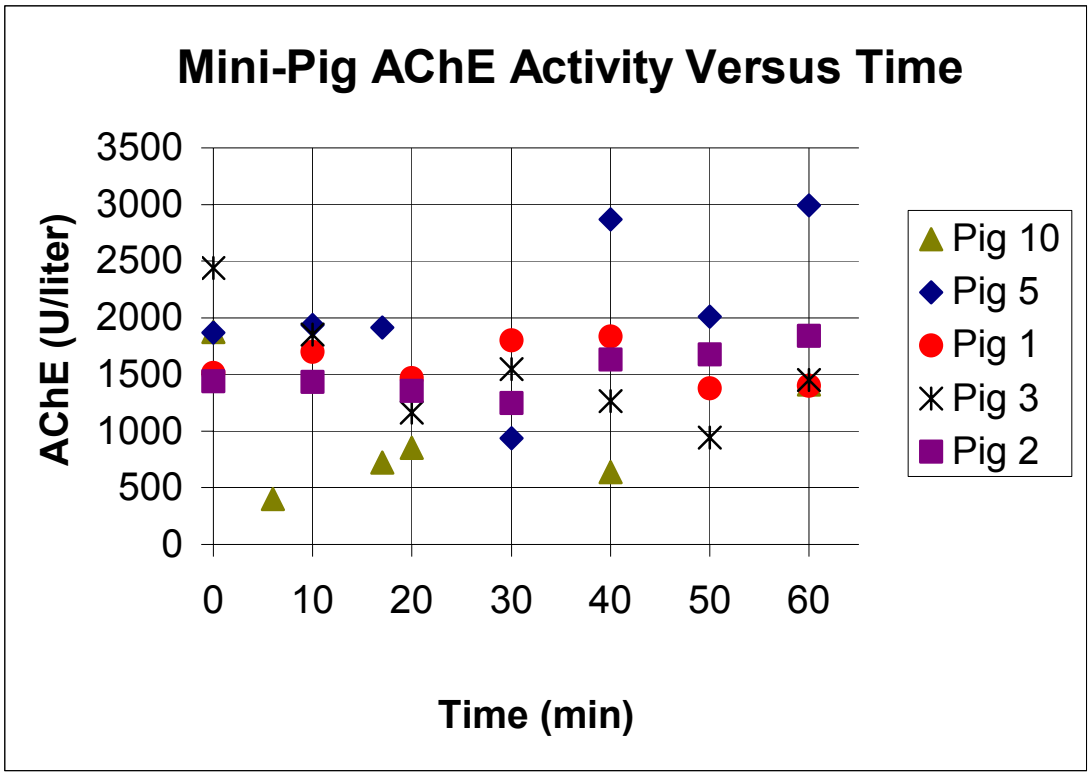

Figure 3. Acetylcholinesterase Activity in Minipig RBC Post Exposure

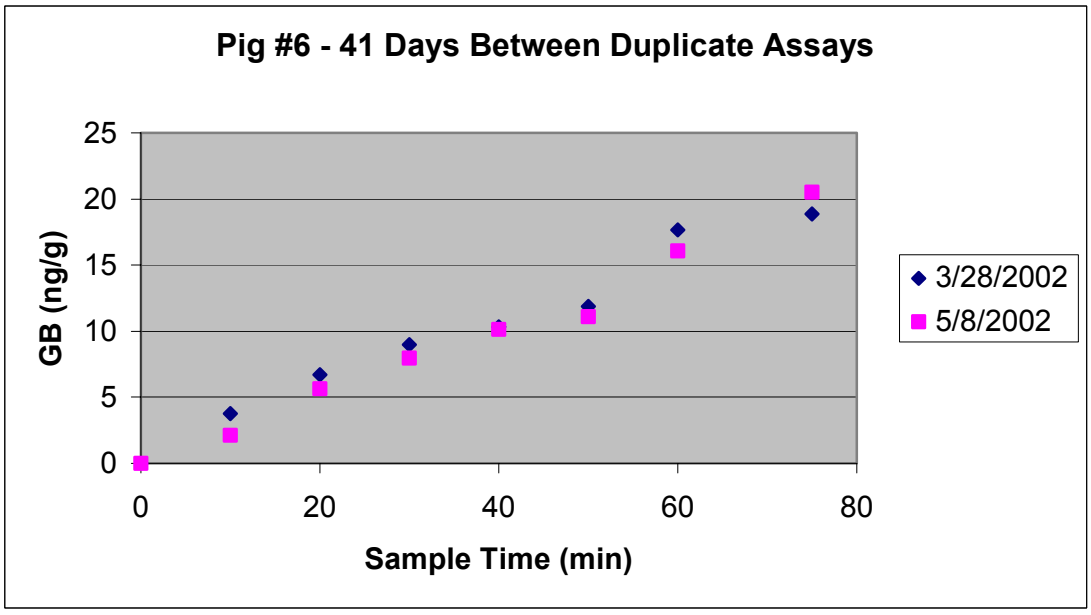

Figure 4. Stability of R-GB in Minipig Blood

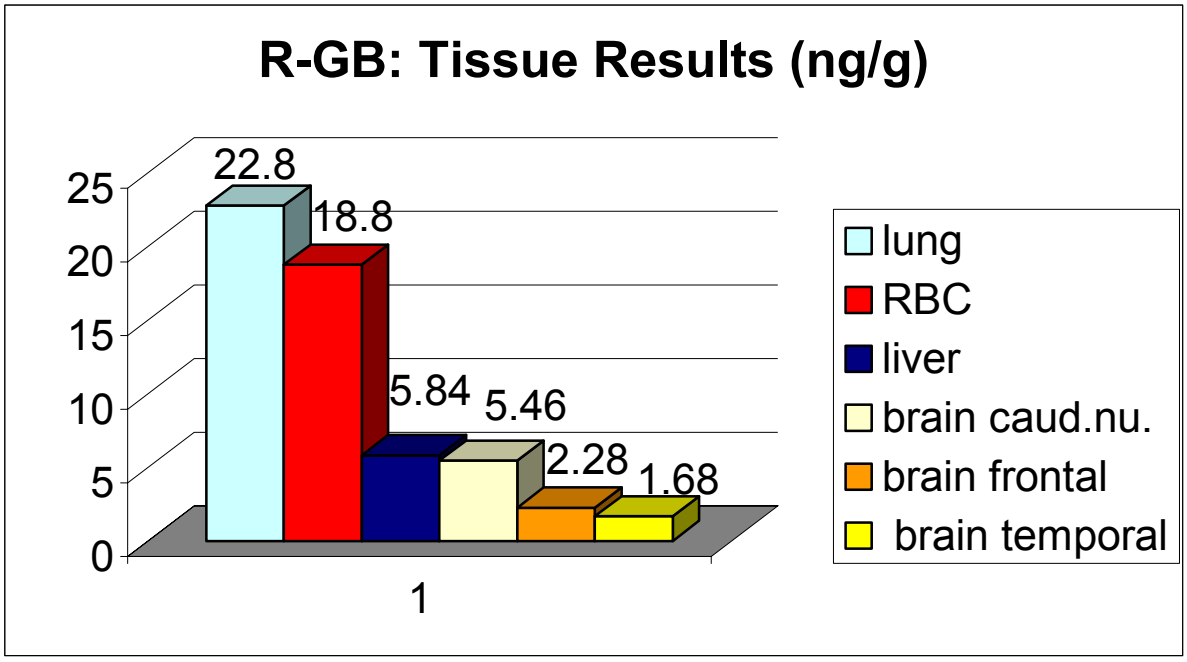

Figure 5. R-GB Levels in Tissue after 60 minutes Exposure to 2 mg/m3 GB

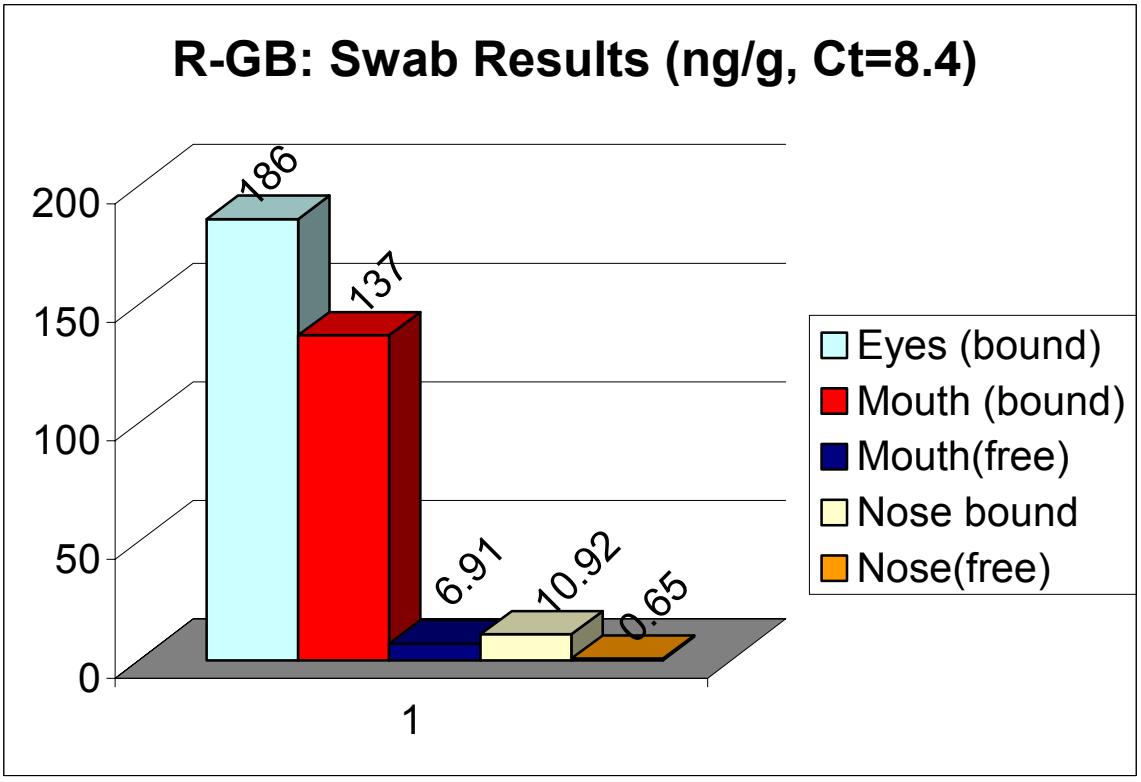

Figure 6. Other Fluids and Sources of R-GB/Free GB

CONCLUSIONS

The R-GB method can be used to monitor internal dose in minipigs exposed to GB at miosis levels. Tissue samples such as brain, lung, and kidney can be analyzed for R-GB using the RBC method. Fluids Other than blood contain significant depots of bound and free GB.

FUTURE STUDIES

In the future, attempts will be made to demonstrate the relationship between tissue level and inhalation exposure. Another important aspect to be pursued is the comparison of intravenous exposure to inhalation using R-GB method. Plans are underway to look at R-GB concentrations in eyes post miosis-level exposure since this is primarily a direct exposure result. Ultimately, methods are needed that are not subject to enzyme aging effects.

REFERENCES

1. L.P.A.De Jong, C. Van Dijk, Biochem. Pharmacol.. **33**, 663-669, (1984).

2. H.P.Benschop, E.C.Bijeveld, M.F.Otto, C.E.A.M.Degenhardt, H.P.M.Van Helden, L.P.A.De Jong, Analytical Biochem., **151**, 242-253, (1985).

3. M.Polhuijs, J.P.Langenberg, H.P.Benschop, Toxicol. Appl. Pharmacol., **146**, 156-161, (1997).

4. E.M. Jakubowski, L.S. Heykamp, H.D. Durst, S.A. Thomson, Analytical Letters, 34(5), 727-737 (2001)