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Dose- and Time-Dependent Inhibition of Blood Cholinesterase by Pyridostigmine Bromide in African Green Monkeys

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14. ABSTRACT

Pyridostigmine bromide (PB) is a carbamate drug that can reversibly bind to the enzyme cholinesterase (ChE) and temporarily shield the enzyme from irreversible inhibition by organophosphate nerve agents. PB doses that produce 20-40% inhibition of red blood cell (RBC) ChE have been shown to provide optimal protection against toxicity from nerve agents. As such, PB has been adopted by military forces of several countries as a nerve agent pretreatment to enhance protection provided by standard post-exposure medical countermeasures (atropine, oxime, benzodiazepine anticonvulsant) against nerve agent toxicity. The present study evaluated the dose- and time-dependent changes in blood ChE levels produced by PB in African green monkeys

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Abstract

Pyridostigmine bromide (PB) is a carbamate drug that can reversibly bind to the enzyme cholinesterase (ChE) and temporarily shield the enzyme from irreversible inhibition by organophosphate nerve agents. PB doses that produce 20-40% inhibition of red blood cell (RBC) ChE have been shown to provide optimal protection against toxicity from nerve agents. As such, PB has been adopted by military forces of several countries as a nerve agent pretreatment to enhance protection provided by standard post-exposure medical countermeasures (atropine, oxime, benzodiazepine anticonvulsant) against nerve agent toxicity. The present study evaluated the dose- and time-dependent changes in blood ChE levels produced by PB in African green monkeys. Ten naive adult male African green monkeys (*Chlorocebus aethiops*) were acclimated to placement in restraint chairs. PB was injected intramuscularly in the calf muscle immediately after a baseline blood sample was drawn, and additional samples were obtained 5, 10, 20, 30, 40, 50, 60, 120, 180 and 240 min after PB injection. All animals received 3 different doses of PB (0.010, 0.018, and 0.032 mg/kg) with two weeks between doses, according to a Latin square design. Whole blood (WB) and packed RBC samples were immediately processed and frozen. Whole blood and RBC ChE were assayed utilizing a microplate adaptation of the Ellman assay; WB samples were also assayed by the Walter Reed Army Institute of Research (WRAIR) method. Results were analyzed using a two-factor (dose, time) mixed effects analysis.

The Ellman ChE WB analysis showed dose-dependent maximal inhibitions of 24%, 27% and 31% for the 0.010, 0.018 and 0.032 mg/kg PB doses, respectively, with maximal inhibition at 40, 30 and 20 min after injection, respectively. WB ChE was significantly inhibited relative to baseline as early as 5 min and remained so until the 180 min sample. RBC ChE was maximally inhibited 18%, 25% and 34% by the 0.010, 0.018 and 0.032 mg/kg PB doses, respectively; the 0.032 mg/kg PB dose produced significantly greater inhibition than did the other two doses. Significant RBC inhibition was seen 5 min after injection, maximal inhibition occurred at 30 min for all three doses, and recovery to baseline levels occurred by 120 min. The WRAIR WB method showed a pattern of results similar to the RBC. ChE was maximally inhibited 17%, 26% and 36% by the 0.010, 0.018 and 0.032 mg/kg PB doses, respectively; the 0.032 mg/kg PB dose produced significantly greater inhibition than did the other two doses from 10-60 min after injection. Significant ChE inhibition was seen 5 min after injection and maximal inhibition occurred at 30 min for all doses, but recovery to levels not different from baseline occurred by 120 min for the 0.010 and 0.018 mg/kg PB doses and 180 min for the 0.032 mg/kg PB dose. The data also allowed the development of regression equations to predict the maximal level of ChE inhibition by different PB doses. The dose- and time-dependent profile of blood ChE inhibition seen in African green monkeys is virtually identical to that previously reported for rhesus monkeys and guinea pigs.

INTRODUCTION

Nonhuman primates (NHP) are the animals phylogenetically closest to man. In the context of biomedical research, they are considered to be the animal that physiologically most closely approximates how a drug or toxin would act in man (Miller, 1967; Dixon, 1976; Krasovskii, 1976). The rhesus monkey (*Macaca mulatta*) has traditionally served as the NHP research species of choice for the assessment of nerve agent toxicity and the assessment of the effectiveness of various medical countermeasures. The "choice" of the rhesus monkey, as can best be discerned, was probably a case of historical default. In the late 1940s, animal research into the toxicology of nerve agents and the development of medical countermeasures was first initiated in the US and various allied countries. At that time, rhesus monkeys were readily available and widely used for biomedical research in general. Research on the toxicology of the nerve agents and the assessment of medical countermeasures appears to have simply followed this trend (DeCandole et al., 1953; DeCandole and McPhail, 1957; Johnson et al., 1958; Lipp, 1968, 1973; Adams et al., 1976; Dirnhuber et al., 1979).

India, the major foreign source of rhesus monkeys, stopped exporting these animals for biomedical research purposes in the late 1970s. Subsequently, the increasing demand for these animals, coupled with limited domestic breeding sources, greatly decreased availability while substantially increasing cost. In addition, rhesus monkeys pose a serious health hazard to research and husbandry personnel due to the potential for transmission of Cercopithecine herpesvirus 1 (previously termed B virus), which has exceptional virulence in humans (Artenstein et al., 1991; Davenport et al., 1994; Holmes et al., 1990). Protecting personnel from this virus requires additional personal protective equipment and special medical monitoring, all of which increase the overall husbandry and research costs of using these animals. For these reasons, an alternative monkey species is needed to evaluate medical countermeasures against chemical warfare nerve agents. Two other NHP species have been reported to be used in this regard, the cynomologus monkey (*Macaca fascicularis*) (Lallement et al., 1997, 1998, 1999, 2000; von Bredow et al., 1991) and the common marmoset (*Callithrix jacchus*) (D'Mello and Scott, 1986; Wetherell and French, 1991; van Helden et al., 1992; Busker et al., 1996; Philippens et al., 2000). Both of these species have drawbacks. The cynomologus monkey also carries Cercopithecine herpesvirus 1 and thus poses a health risk and associated protective costs equivalent to the rhesus monkey. The marmoset monkey is a substantially smaller species (body weight 100-850 g) which limits the ability to take repeated blood samples and the availability of sampling sites.

The African green monkey (*Chlorocebus aethiops*) may be an ideal replacement for the rhesus monkey. African green monkeys are Old World monkeys and grow to ~60% of the size of a rhesus. They are considerably less aggressive than rhesus, and well-trained personnel can perform repeated blood sampling from superficial veins. African green monkeys are readily available from a variety of sources for a fraction of the price of a rhesus monkey, and most important, they do not naturally carry Cercopithecine herpesvirus.

Our research group has been working to determine the suitability of the African green monkey to substitute for the rhesus in studies of nerve agent toxicology and effectiveness of medical countermeasures. We have shown that African green monkeys show the same susceptibility to the lethal effects (e.g., LD50 dose, toxic signs) of the nerve agent soman as do rhesus monkeys (Despain et al., 2007). Additionally we have evaluated the dose-dependent

behavioral, pharmacokinetic and cardiovascular effects of the oximes 2-PAM, MMB-4 and HI-6 in African green monkeys (Moffett et al., 2018). PB was recently approved by the Food and Drug Administration (FDA) for military use as a pretreatment when the threat of nerve agent attack is high. Typically, prophylactic dosing with PB is targeted to achieve ~20-30% RBC ChE inhibition (Dunn et al., 1997). The present study was designed to examine the magnitude and time course of blood ChE inhibition in African green monkeys in response to different doses of PB and compares the results to similar published data for rhesus monkeys.

METHODS

Subjects: Ten naïve adult male African green monkeys, weighing 4.5-7.0 kg, served as subjects. Animals were housed individually in 4.3-square-foot stainless steel squeeze-back cages with built-in perches. They were fed commercial certified primate ration by Harlan/Teklad (15%) (W), fresh fruit, and tap water ad libitum. Animal rooms were maintained at 21°C + 2°C, relative humidity of 50 $\pm$ 20%, and a 12 h light (0600-1800):12 h dark (1800-0600) cycle with no twilight.

Experimental Procedure: The animals were fitted with collars that allowed pole capture and chair restraint (Crist Instrument Co., Inc.; Hagerstown, MD) and were individually trained and habituated to chair restraint over several months prior to the initiation of the study. On a study day, animals were seated in a restraint chair, the legs were shaved and a baseline blood sample was obtained. They were then injected intramuscularly (IM) in the calf muscle with one of three doses of PB (0.48 mg/ml in sterile water; Hoffman-La Roche, Nutley, NJ): 0.010, 0.018 or 0.032 mg/kg. All animals received each PB dose according to a Latin square design with at least a two-week recovery period between doses. Blood samples were taken at 5, 10, 20, 30, 40, 50, 60, 120, 180, and 240 min after PB injection.

Blood Sampling and Processing: Blood (~ 1 ml) was drawn from the saphenous vein in syringes coated with EDTA Na. The needle was immediately removed from the syringe, and the blood expressed into a 1.5 ml snap-top vial containing 35 µl of EDTA Na. Blood was immediately processed. For whole blood (WB) samples to be analyzed by a microplate adaptation of the Ellman method (Ellman et al., 1961), 40 µl of WB was added to 960 µl of a 1% Triton-X/saline lysing solution, vortexed and then flash frozen. For WB samples to be analyzed by the WRAIR method (Fester et al., 2004; Haigh et al., 2008), 10 µl of WB was added to 190 µl of distilled water, vortexed and then flash frozen. The rest of the WB sample was then centrifuged at 14,000 RPM for 10 min, the plasma fraction was removed and then 20 µl of packed RBCs was added to 980 µl of a 1% Triton-X/saline lysing solution, vortexed, and then flash frozen. All samples were stored at -80°C until analysis.

ChE Analysis, Ellman Methods: On the day of ChE analysis, samples were thawed, and three 10 µl replicates of the WB and RBC samples were pipetted into a 96-well microplate (UV Star, Greiner, Longwood, FL). Standard curves were established by adding 10 µl ChE from electric eel (Sigma, St. Louis, MO) at 3.75, 7.5, and 15 U/ml. Seventeen (17) µl of deionized water was added to each WB and RBC sample. Following the addition of the water, 200 µl of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; 0.424 M, pH 8.2; Sigma, St. Louis, MO) was added as the chromatophore to each sample well. Each microplate was allowed to shake for 1 min, then

incubated for 10 min at 37°C before being placed in the Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Immediately after, 30 µl of the substrate acetylthiocholine iodide (51.4 mM; Sigma, St. Louis, MO) was added to each well. The samples were shaken/incubated for 1 min and then read at 412 nm at 12 sec intervals for 2 min, and the activity (µmole/ml/min) was determined using Softmax plus 4.0 software (Molecular Devices).

ChE Analysis, WRAIR Assay: On the day of ChE analysis, three 10 µl replicates of the WRAIR WB samples were pipetted into a 96-well microplate (UV Star, Greiner, Longwood, FL). The final assay mixture (total volume 300 µl) also contained 1 mM of acetylthiocholine (ATCh; Sigma, St. Louis, MO) iodide; all wells also contained 0.2 mM of the chromophore 4,4'-dithiopyridine (DTP; Sigma, St. Louis, MO) and 50 mM sodium phosphate buffer, pH 8.0. The phosphate buffer and chromophore were added first, and the microplate was then shaken for 1 min and then incubated for 10 min at 37°C before being placed in the Spectramax Plus 384 microplate reader. Immediately after, the ATCh was added, the samples were shaken/incubated for 1 min and then read at 324 nm at 12 sec intervals for 2 min, and the activity (µmole/ml/min) was determined using Softmax plus 4.0 software (Molecular Devices).

Data Analysis: Results for each method were analyzed utilizing a mixed effects analysis, with dose and time as the main factors (GraphPad Prism 8.3). Significant effects were further evaluated using Tukey's multiple comparison test. In all cases, $p < 0.05$ was considered significant.

RESULTS

The results of all three methods showed time-dependent inhibition of blood ChE by PB treatment in African green monkeys.

Ellman WB Assay: For the Ellman WB assay there was a significant main effect of time ($F_{(4.445, 120)} = 30.97$, $p < 0.0001$), while the dose and dose X time interactions were not significant. The results for the Ellman WB ChE assay are depicted in Figure 1. The 0.010, 0.018 and 0.032 mg/kg PB doses produced maximal ChE inhibitions of 24%, 27% and 31% in WB, respectively, but these levels of inhibition were not significantly different from each other. For all three doses, significant WB inhibition was seen as early as 5 min after PB injection, maximal inhibition occurred from 30–60 min, and recovery of ChE activity to levels not different from baseline occurred by 120 min after injection.

Ellman RBC ChE Assay: For the Ellman RBC assay there was a significant main effect of time ($F_{(6.13, 165.5)} = 32.58$, $p < 0.0001$), while the dose and dose X time interactions were not significant. The results of the Ellman RBC ChE assay are depicted in Figure 2. For all three doses, significant RBC ChE inhibition was seen as early as 5 min after PB injection, maximal inhibition occurred at 40-60 min, and recovery of ChE activity to levels not different from baseline occurred by 120 min after injection.

WRAIR WB ChE Assay: For the WRAIR WB ChE assay there were significant effects for time ($F_{(3.255, 87.99)} = 81.94$, $p<0.0001$) and the dose X time interaction ($F_{(20, 270)} = 5.402$, $p<0.0001$); the dose factor was not significant. The results of the WRAIR WB ChE assay are depicted in

Figure 3. ChE was maximally inhibited 17%, 26% and 36% by the 0.010, 0.018 and 0.032 mg/kg PB doses, respectively. From 20-60 min after PB injection the 0.032 mg/kg PB dose produced significantly greater ChE inhibition than the 0.010 mg/kg dose, but not the 0.018 mg/kg dose. For all three doses, significant ChE inhibition was seen 5 min after injection, maximal inhibition occurred from 30-50 min for the 0.010 mg/kg dose, 20-60 min for the 0.018 mg/kg dose and as soon as 5-60 min for the 0.032 mg/kg dose. The 0.010 and 0.018 mg/kg doses recovered to levels not different from baseline by 120 min, while the inhibition produced by 0.032 mg/kg did not recover to levels not different from baseline until 180 min.

Regression Analysis: Linear regressions were performed on all data sets to determine if the level of inhibition desired at 30 min after injection (time of maximal inhibition) could be used to predict the PB dose. To do this, the individual levels of ChE inhibition achieved by each PB dose observed at 30 min after dosing were used in these calculations. The regression for the Ellman WB ChE analysis was significant ($p = 0.02$), but had low power and low predictive ability; dose = 0.0111 + (0.000342 X % inhibition); $R = 0.405$. The regression analysis for the Ellman RBC ChE analysis was significant ($p = 0.001$), had good power (power with alpha = 0.05: 0.912) and reasonable predictive ability: dose = 0.00799 + (0.000458 X % inhibition); $R = 0.563$. The regression for the WRAIR ChE analysis was significant ($p < 0.001$), with excellent power (power with alpha = 0.05: >0.999) and high predictive ability: dose = -0.00295 + (0.000862 X % inhibition); $R = 0.836$. A plot of the WRAIR ChE regression is displayed in Figure 4.

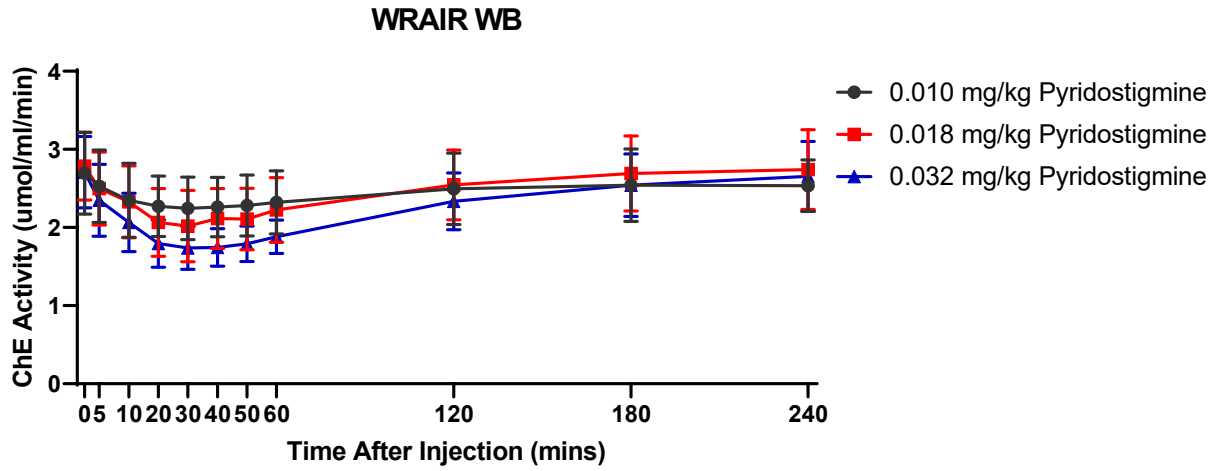

Figure 1. Time-dependent inhibition of African green monkey WB ChE (Ellman assay) by three different doses of PB. Mean $\pm$ SD.

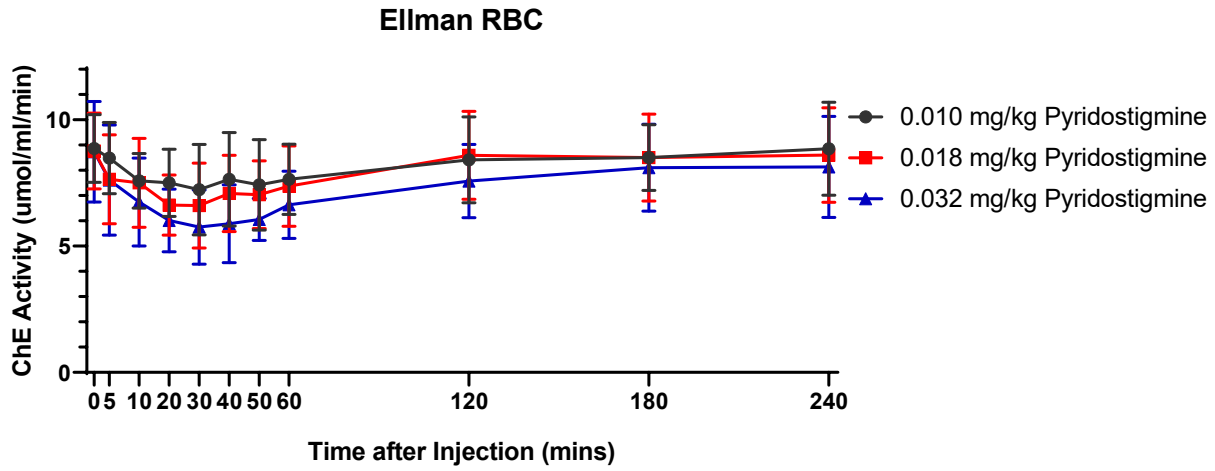

Figure 2. Time-dependent inhibition of African green monkey RBC ChE (Ellman assay) by three different doses of PB. Mean ± SD.

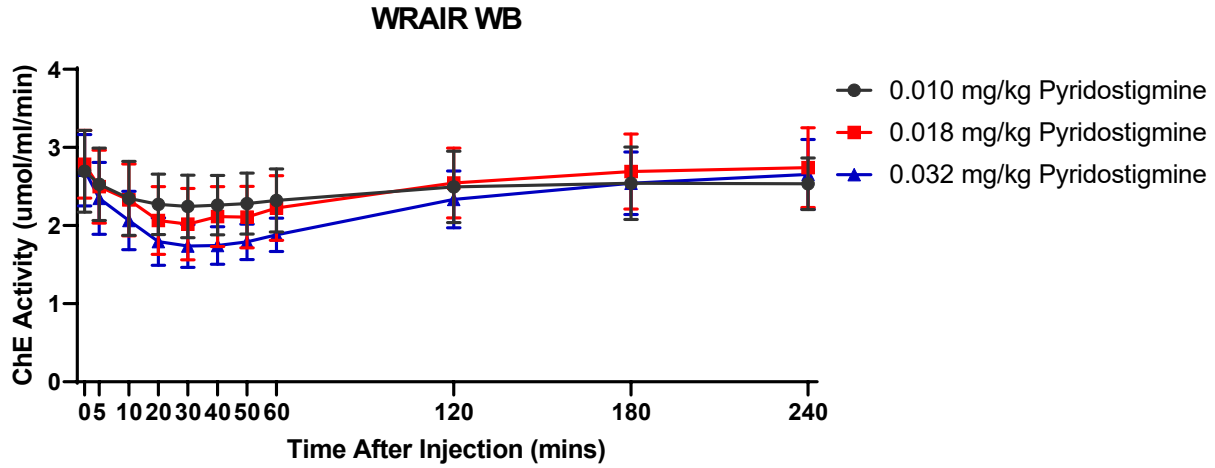

Figure 3. Time-dependent inhibition of African green monkey WB ChE (WRAIR assay) by three different doses of PB. Mean ± SD.

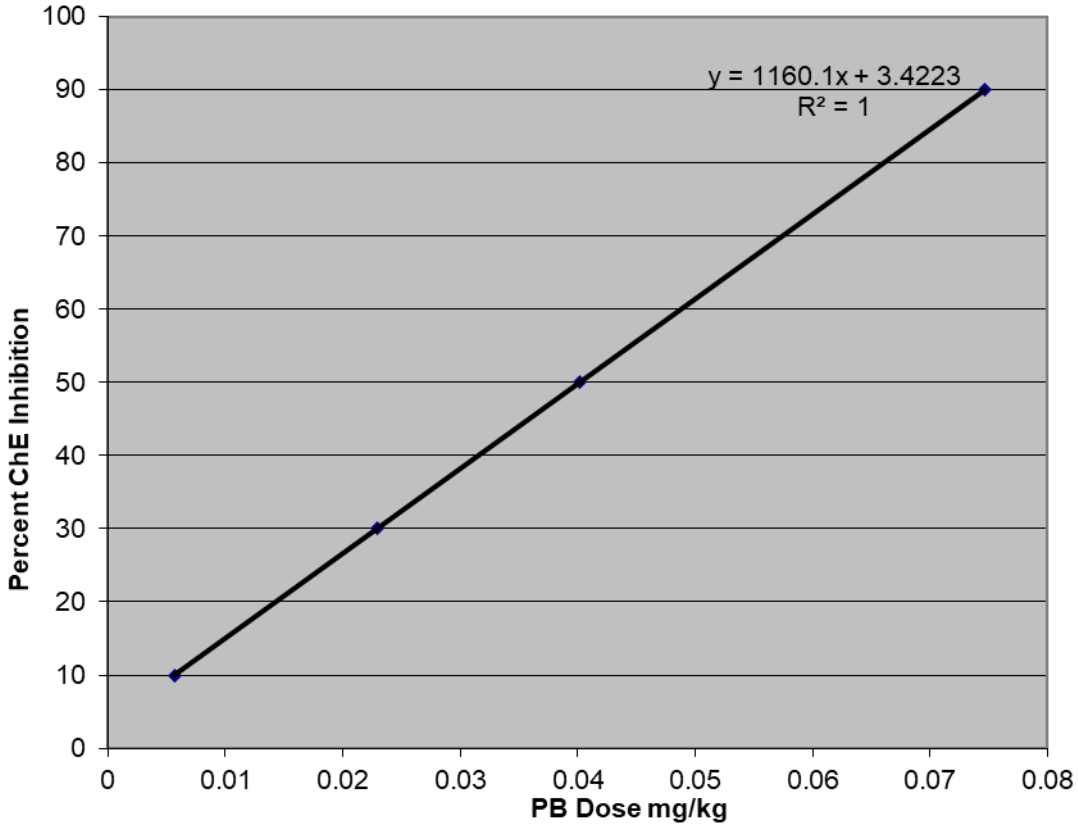

Figure 4. Regression plot using the WRAIR WB ChE data at 30 min to predict percent ChE inhibition by differing doses of PB.

DISCUSSION

The results show that African green monkeys display blood ChE inhibition profiles to IM administered PB comparable to those reported for several other species. Specifically, rhesus monkeys (Olson et al., 1995) and guinea pigs (Lennox et al., 1985) have dose- and time-dependent inhibition profiles that are almost identical to those obtained here with African green monkeys.

Based on the Olson et al. (1995) data, a 0.024 mg/kg IM dose of PB was used in a previous study with rhesus monkeys (McDonough et al., 2002). This empirically produced an average 25% inhibition of RBC ChE at 40 min after injection. According to the Olson et al. (1995) report, the empiric time for maximum ChE inhibition, averaged over different PB dose groups (0.0084-0.027 mg/kg), was 42 min. In contrast, the time for maximum inhibition in the present study was 30 min after injection (Ellman RBC assay, WRAIR assay), although inspection of the data shows no statistically significant differences in inhibition levels at a given dose between 20 and 50 min after injection.

PB doses that produce 20-40% inhibition of RBC ChE have been shown to provide optimal protection against toxicity from nerve agents that age rapidly, like soman (Dunn et al., 1997). The regression equation for data from the Ellman RBC assay predicts doses 0.017–0.026 mg/kg PB would produce 20-40% inhibition of RBC ChE activity in African green monkeys 30 min after injection.

In conclusion, the results show that inhibition of blood ChE by differing doses of PB in the African green monkey is virtually identical to that reported for the rhesus monkey (Olson et al., 1995). Likewise, the toxicity of soman in African green monkeys is identical to that seen with this agent in rhesus monkeys (Despain et al., 2007). Based on these results, PB pretreatment of the African green monkey should afford the same type of protection against the lethal effects of rapidly aging nerve agents such as soman when used in conjunction with comparable therapeutic countermeasures (e.g., atropine, oxime, benzodiazepine anticonvulsant).

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