



EDGEWOOD CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1249

EX VIVO MEASUREMENT OF ENZYME STABILITY IN HUMAN PLASMA: A POTENTIAL SCREENING METHOD FOR IN VIVO STABILITY

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MAY 2014

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) XX-05-2014		2. REPORT TYPE Final		3. DATES COVERED (From - To) Jun 2013 - Dec 2013	
4. TITLE AND SUBTITLE Ex Vivo Measurement of Enzyme Stability in Human Plasma: A Potential Screening Method for In Vivo Stability				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Guelta, Mark A.; Harvey, Steven P.; and Dixon, Melissa M.				5d. PROJECT NUMBER R.0005816.55.7.1/30003096	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Director, ECBC, ATTN: RDCB-DRB-C, APG, MD 21010-5424				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-1249	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Defense Threat Reduction Agency, 8725 John J. Kingman Road, MSC 6201, Fort Belvoir, VA 22060-6201				10. SPONSOR/MONITOR'S ACRONYM(S) DTRA	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT-LIMIT 200 WORDS Previously, we assayed several phosphotriesterase (PTE) variants with increased activity on G-type chemical nerve agents and observed increased activity on the most toxic enantiomers. The purpose of this study is to determine the feasibility of the use of ex vivo plasma assays as a means of estimating the stability of enzymes that might be candidates for in vivo treatment. Activity measurements using wild-type (WT) PTE and several of its mutants on soman and cyclosarin after incubation in human plasma indicate that WT PTE and the YT mutant of that enzyme have comparable stability in plasma.					
15. SUBJECT TERMS Phosphotriesterase (PTE) Enzyme stability WT PTE VRN-VQFL Organophosphorus hydrolase (OPH) YT L7ep-2a Enzyme					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	18	19b. TELEPHONE NUMBER (include area code) (410) 436-7545

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PREFACE

The work in this report was authorized under Project No. R.0005816.55.7.1/30003096. This work was started in June 2013 and completed in December 2013.

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The text of this report is published as received and was not edited by the Technical Releases Office, U.S. Army Edgewood Chemical Biological Center.

This report has been approved for public release.

Acknowledgments

The authors thank Dr. Frank M. Raushel (Texas A&M University, College Station, TX) for the kind contribution of enzymes used in the study.

Also, the authors gratefully acknowledge the Defense Threat Reduction Agency (Fort Belvoir, VA) for sponsorship of this work.

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EX VIVO MEASUREMENT OF ENZYME STABILITY IN HUMAN PLASMA: A POTENTIAL SCREENING METHOD FOR IN VIVO STABILITY

1 INTRODUCTION

Catalytic enzymes offer the potential to protect against organophosphate poisoning by detoxifying these compounds in the blood stream, before they can enter the neuromuscular junction and bind acetylcholinesterase. A useful enzyme must exhibit high activity (k_{cat}/K_m greater than $10^7 \text{ M}^{-1} \text{ min}^{-1}$), sufficient stability, and activity on the toxic enantiomers of each relevant substrate (typically the P (-) or Sp enantiomer). Although candidate enzymes have been frequently characterized with respect to their activity *in vitro*, the more relevant question concerns their activity and their stability *in vivo*. However, animal experiments with nerve agents, are costly, and human experiments are not feasible. *Ex vivo* studies with human blood or plasma offer an approach to study some of these parameters.

The purpose of this study was to determine the feasibility of the use of *ex vivo* plasma assays as a means of estimating the stability of enzymes that might be candidates for *in vivo* treatment.

2 METHODS

G-agent substrates used in the study were Chemical Agent Standard Analytical Reference Material (CASARM) obtained from ECBC stocks. Phosphotriesterase (PTE) enzymes were a kind gift from Drs. Andrew Bigley and Frank Raushel at Texas A&M University and were made as described^{1,2}.

The agents GD (soman) and GF (cyclosarin) used in the study represented in Figure 1 both contain fluoride which is released during hydrolysis. Enzymatic activity of these substrates can be measured by monitoring the fluoride concentration in solution after subtracting the spontaneous hydrolysis rate.

Fluoride concentration was measured using a VWR Symphony probe and collected on a WTW 7350 pH/Ion data logger. Assays were performed in a glass 10 mL double wall reaction cell with circulating water for reaction temperature control.

¹ Tsai, Ping-Chuan, Fox, Nicholas, Bigley, Andrew, Harvey, Steven, Barondeau, David, and Raushel, Frank., Enzymes for the homeland defense: optimizing phosphotriesterase for the hydrolysis of organophosphate nerve agents, Biochemistry published online, August 2012.

² Bigley, A.N., C. Xu, T.J. Henderson, S.P. Harvey and F.M. Raushel, 2013. Enzymatic neutralization of the chemical warfare agent VX: Evolution of phosphotriesterase for phosphorothiolate hydrolysis. *J. Am. Chem. Soc.* 135(28): 10426-10432.

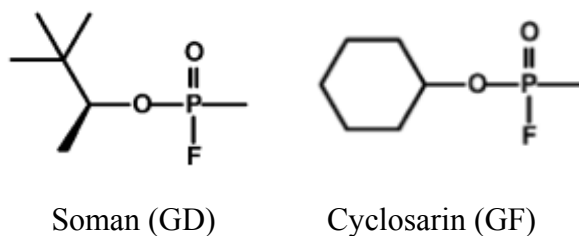


Figure 1. Chemical Structures of GD and GF.

To determine stability, enzymes were inoculated into fresh human plasma and incubated at 37 °C. Changes in rates of enzymatic activity on GD and GF were measured at time points following initial inoculation. Activity of the enzyme-dosed plasma was assayed in 50 mM bis-tris-propane (BTP), pH 7.2 at 37 °C on 1.0 mM substrate. Assays were performed in triplicate at each time point and in several cases time points out to 30-days. Data from multiple runs of each data series were combined.

Three enzymes designed for improved degradation rates and their parent, wild-type (WT PTE), were tested and compared for stability. WT PTE stability was therefore used as a baseline to measure changes in subsequent mutants.

GF was initially chosen as the test substrate as four of the current enzymes of interest show relatively high levels of activity on this substrate. GD was also used as a substrate for two of the four enzymes as two of the enzymes of current interest do not have significant activity on GD. Stability studies were based on initial plasma inoculations of 0.1 mg/mL for GF assays. Initial inoculations of 0.1 and 1.0 mg/mL for PTE mutant H257Y, L303T (YT) and WT PTE respectively were used on GD.

3 RESULTS

3.1 Results on GD

Initial measurements were made of the spontaneous fluoride release from GD in BTP alone and in plasma using the same enzyme concentrations used in the stability assays. Fluoride release was then compared in Figure 2 with WT PTE in BTP and plasma. Protein concentration was 0.01 mg/2.5 mL assay volume.

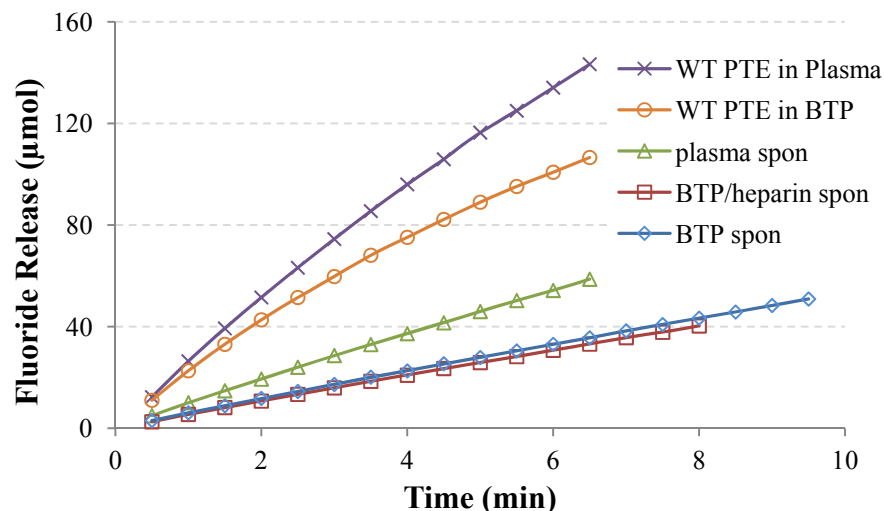


Figure 2. Comparison of fluoride release in BTP and plasma samples with and without WT PTE, using GD as the substrate. “BTP spon ($\diamond$)” is the rate of fluoride released with GD added to BTP alone. “BTP/heparin spon ($\square$)” is GD added to BTP with heparin stabilizer (this was measured to determine any effects of heparin since the plasma was collected in heparinized tubes to prevent coagulation). “Plasma spon ($\triangle$)” is the fluoride release rate when GD was added to plasma in heparinized tubes. “WT PTE in BTP ($\circ$)” is the rate of wild type PTE in BTP without heparin. “WT PTE in Plasma ($\times$)” is the rate of WT PTE in plasma with heparin stabilizer.

Initial rates for WT PTE in plasma versus BTP were 4.964 and 3.670 $\mu\text{mol}/\text{min}/\text{mg}$ respectively. Clearly fluoride release rates are higher for the WT PTE and spontaneous samples in plasma than in buffer. The simplest explanation for the difference may be the presumed presence of native organophosphorus hydrolyzing enzyme activity such as that which would be present in human paraxonase (PON). Heparin alone had no discernible effect on hydrolysis rates, as evidenced by the indistinguishable rates of hydrolysis in BTP with and without heparin.

Similar observations were made regarding the potential contribution of PON in plasma samples over buffer alone using YT on GD as in Figure 3. Enzyme concentration was 0.001 mg/2.5 mL assay volume.

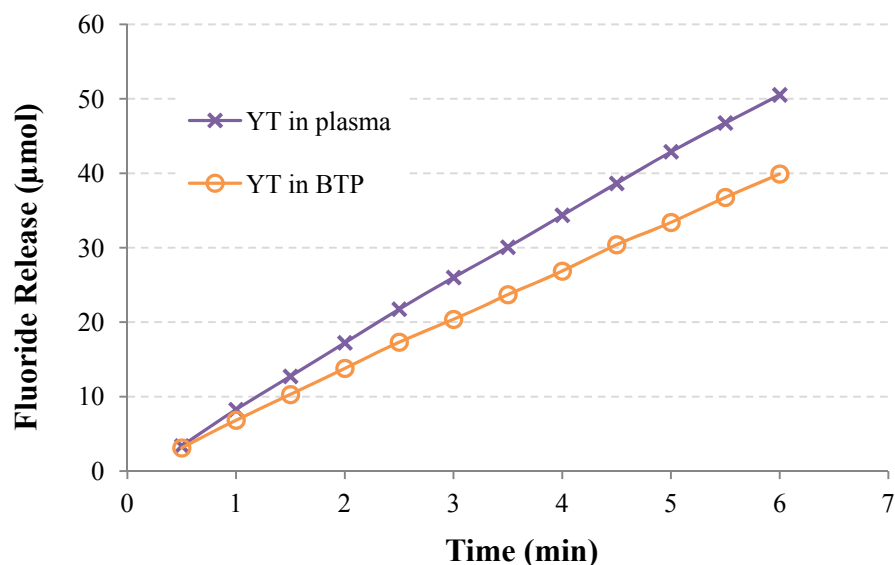


Figure 3. Comparison of Fluoride Release in samples with YT added to BTP (○) and plasma (×) on GD.

Degradation rates are calculated as $\mu\text{mol}/\text{min}/\text{mg}$ from change in fluoride present in assay volume per minute per mg of enzyme minus the spontaneous rate for the same assay without enzyme. Initial rate data are listed in Table 1.

Table 1 Average initial rates for all runs at 1 minute after inoculation into plasma for each enzyme tested at 37 °C ($\mu\text{mol}/\text{min}/\text{mg}$).

Substrate	YT	VRN-VQFL	L7ep-2a	WT PTE
GD	106.8			4.7
GF	202.8	69.6	19.516	61.8

*WT PTE on GD plasma inocula = 1.0 mg/mL, all others were 0.1 mg/mL

Rate data from each run was normalized as percent of the initial rate. Data from multiple runs were analyzed and presented as a composite. Statistical outliers were eliminated using the Grubbs test.^{3,4} Data were recorded in Microsoft Excel and trend lines were fitted where appropriate.

The two enzymes, WT PTE and YT, were dosed into plasma at different concentrations, as the rates of YT and WT PTE on GD are nearly an order of magnitude different. YT was

³ Burns, M.J.; Nixon, G.J.; Foy, C.A.; Harris, N. Standardization of Data from Real-time Quantitative PCR Methods Evaluation of Outliers and Comparison of Calibration Curves. *BMC Biotechnol.* **2005**, *5*, 31.

⁴ Grubb's Test for Outliers, 2006. 1.3.5.17, NIST/SEMATECH e-Handbook of Statistical Methods, <http://www.itl.nist.gov/div898/handbook/2006> (accessed December 2013).

inoculated at 0.1 mg/mL, while WT PTE was inoculated at 1.0 mg/mL. Collated data from 3 successive runs of each enzyme are presented in Figure 4.

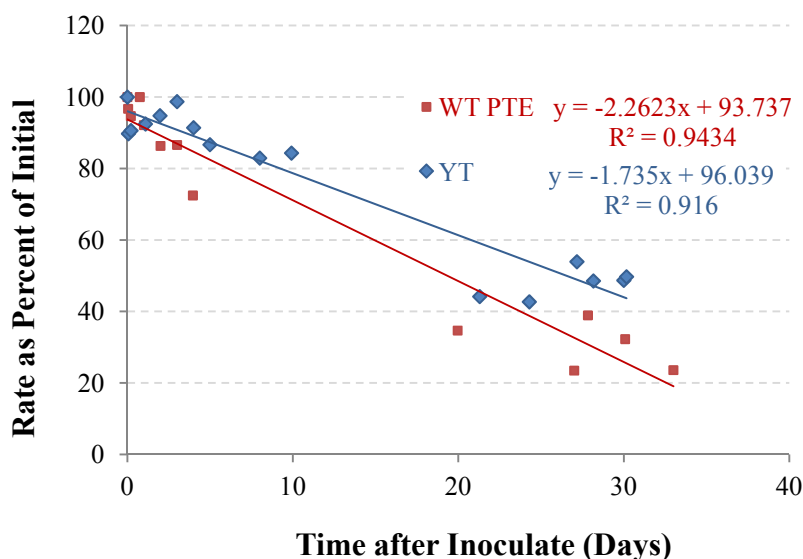


Figure 4. YT (♦) and WT PTE in Plasma (■), Activity levels on GD as a function of time.

The decrease in enzymatic activity for WT PTE and YT each fit well to a linear trend line. YT activity is somewhat more stable than that of WT PTE.

YT lost about 1.7% of its activity per day after inoculation into plasma while WT PTE lost 2.3%.

3.2 Results for GF

In our experience, GF hydrolysis rate measurements are typically somewhat less reproducible than that collected on GD, presumably due to the higher initial free fluoride concentration found in GF samples. Data points were used after initial free fluoride release had equilibrated and before the total substrate fluoride released reached 10%.

The enzymes that were used for stability determinations were all dosed at the same concentration, 0.1 mg/mL in plasma and incubated at 37 °C. Collated data for successive runs of YT, VRN-VQFL, L7ep-2a and WT PTE on GF are presented in Figures 5-8.

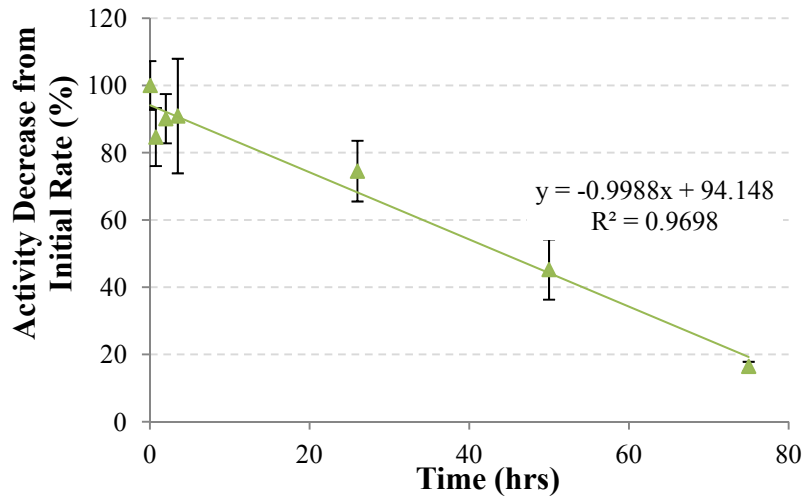


Figure 5. Stability of YT (▲) in plasma, decrease in activity on GF over time.

YT degradation in plasma fits well with a linear trend. Figure 5 represents collated data from 3 successive time point runs.

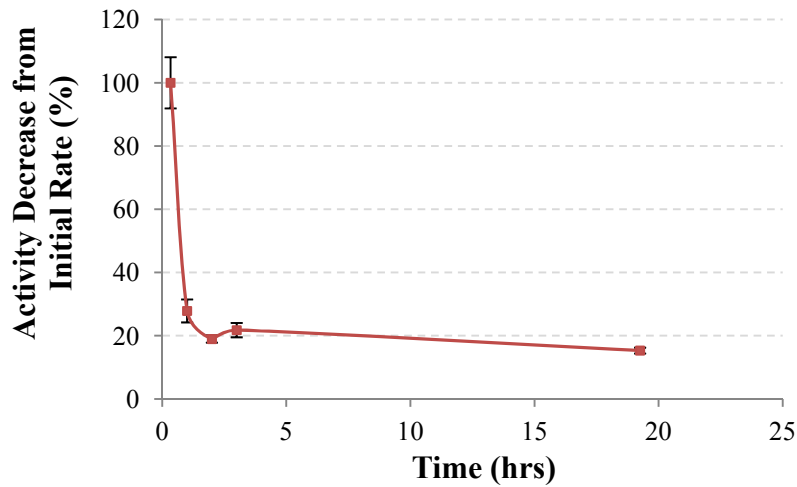


Figure 6. Stability of VRN-VQFL (■) in plasma, decrease in activity on GF over time.

VRN-VQFL in plasma degradation over time is clearly not linear. Figure 6 represents collated data from 3 successive time point runs.

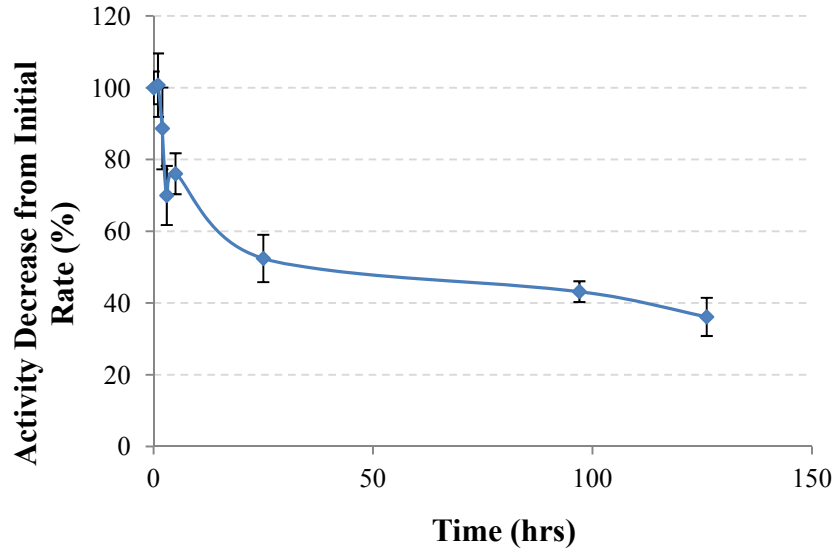


Figure 7. Stability of L7ep-2a (♦) in plasma, decrease in activity on GF over time.

The rate of decrease in L7-ep2a activity on GF in plasma does not appear to be linear. Figure 7 represents collated data from 3 successive time point runs.

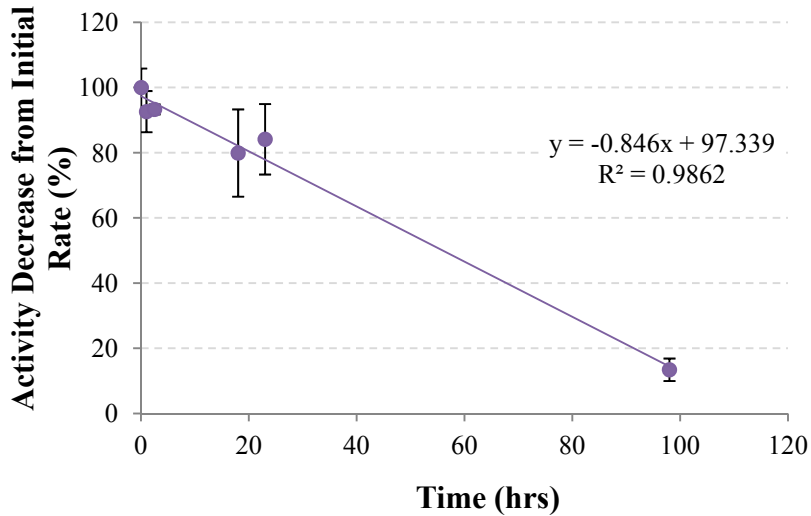


Figure 8. Stability of WT PTE (●) in plasma, decrease in activity on GF over time.

The rate of decrease in activity of WT PTE in plasma on GF follows a linear trend. Figure 8 represents collated data from 4 successive time point runs.

4 SUMMARY

From the experiments described above, it appears generally feasible to measure the stability of various enzymes in human plasma over a period of several weeks. It might be expected that these straightforward and inexpensive assays could serve as an effective screen for mutants that might be stable *in vivo*. While those stable in human plasma may or may not be useful *in vivo*, it is at least reasonable to conclude that those showing poor plasma stability are significantly less promising candidates for *in vivo* trials.

From the same data, some specific conclusions can also be drawn regarding stability of these enzymes in human plasma. WT PTE and YT each demonstrated decreases in activity with time that can be fit to a linear relationship. Data indicate that the both YT and WT PTE have comparable stability (enzyme activity decreases by about 2% per day in each case). YT is slightly more stable on GD, WT PTE more so on GF. VRN-VQFL and L7ep-2a were assayed on GF only as they do not have significant activity on GD. Data indicate that these enzymes are not as stable as WT PTE. From Figures 4 and 5 it can be observed that VRN-VQFL activity decreases very rapidly and L7ep-2a less rapidly.

