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CONTRACT NO: DAMD17-90-C-0087

TITLE: ULTRASOUND ASSISTED ASSAY FOR TOXINS IN BLOOD
SBIR 90.I (A90-180)

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Ultrasound Assisted Assay for Toxins in Blood
SBIR 90.I (A90-180)

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Work was carried out to determine the feasibility for a spectroscopic analysis of saxitoxin in whole blood samples. Modifications of standard chemical assays were to be used in conjunction with TRA's ultrasonic blood separation technology to form an analytical detection system that is suitable for field use by minimally trained personnel. The chemical assay method was successfully to generate a fluorescent saxitoxin derivative using solid oxidants in a stable dry formulation. Direct detection of low levels of this analyte in plasma was precluded by interference from the plasma matrix.

Chemical Assay, Oxidants, Spectroscopic Detection, RAI,
SBIR Phase I, Ultrasound, Fluorimetry.

Unclassified Unclassified Unclassified Unlimited

APPENDIX B
DOD No. 90.1

U.S. DEPARTMENT OF DEFENSE

SMALL BUSINESS INNOVATION RESEARCH (SBIR) PROGRAM
PHASE 1—FY 1990
PROJECT SUMMARY

Topic No. DAMD17-90-0087 Military Department/Agency Army

Name and Address of Proposing Small Business Firm

Technical Research Associates, Inc.
410 Chipeta Way, Suite 222
Salt Lake City, Utah 84108

Name and Title of Principal Investigator

Charles D. Baker, President

Proposal Title

ULTRASOUND ASSISTED ASSAY FOR TOXINS IN BLOOD

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

Work was carried out to determine the feasibility for a spectroscopic analysis of saxitoxin in whole blood samples. Modifications of standard chemical assays were to be used in conjunction with TRA's ultrasonic blood separation technology to form an analytical detection system that is suitable for field use by minimally trained personnel. The chemical assay method was successfully to generate a fluorescent saxitoxin derivative using solid oxidants in a stable dry formulation. Direct detection of low levels of this analyte in plasma was precluded by interference from the plasma matrix.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

These results suggest that the feasibility of saxitoxin analysis via chemical treatment of whole blood samples should be markedly enhanced by the simplification of the liquid (plasma) environment. A plausible scheme to do this is described.

List a maximum of 8 Key Words that describe the Project.

Chemical Assay, Oxidants, Spectroscopic Detection

PROJECT OBJECTIVES

The long-term objective of this project was to successfully combine ultrasonic blood separation technology developed at TRA with toxin assay methods modified for use in whole blood to provide a rapid detection system suitable for field use. Phase I focussed on establishing the feasibility of determining saxitoxin in whole blood samples. The detailed objectives of the Phase I proposal were:

MODIFICATION OF CHEMICAL ASSAY FOR SAXITOXIN. The best known chemical assay for saxitoxin involves conversion of saxitoxin to a fluorescent analyte in water. Because this method uses basic conditions and hydrogen peroxide, it is necessary to modify the assay for use near physiological (blood) pH with stable oxidizing systems that (a) generate hydrogen peroxide in situ or (b) directly convert saxitoxin to the fluorescent analyte.

DEVELOPMENT OF FORMULATIONS FOR COATING SAMPLING TUBES. Coating formulations containing saxitoxin oxidant systems would be developed for application to sample (hematocrit) tubes. The system would then be used for generating the saxitoxin metabolite in whole blood samples.

IMPLEMENTATION OF CHEMICAL ASSAY IN PLASMA, AND DETERMINATION OF DETECTION LIMITS AND EFFECTS OF ULTRASOUND. The best assay method would be evaluated in plasma. Concurrently, detection limits and a linear detection range of the fluorescent analyte would be determined in plasma. The effects of ultrasound on the rate of saxitoxin oxidation in plasma, and on the detection of analyte would also be determined.

DETERMINATION OF HEMATOCRIT AND SAXITOXIN CONCENTRATION IN WHOLE BLOOD. Saxitoxin would be assayed in whole blood using the chemical assay formulation and the TRA ultrasound blood separation technology. This procedure will be optimized to determine the best instrument configuration and testing procedures for rapid analysis.

COMPARISON OF DATA WITH LITERATURE AND SUBMISSION OF FINAL PHASE I REPORT.

WORK CARRIED OUT

The work carried out on this contract is summarized in the Task chart in Figure I. Tasks 2c, 3c-5b were not carried out due to unanticipated experimental results and subsequent difficulties that are described in detail in the Results section of this report.

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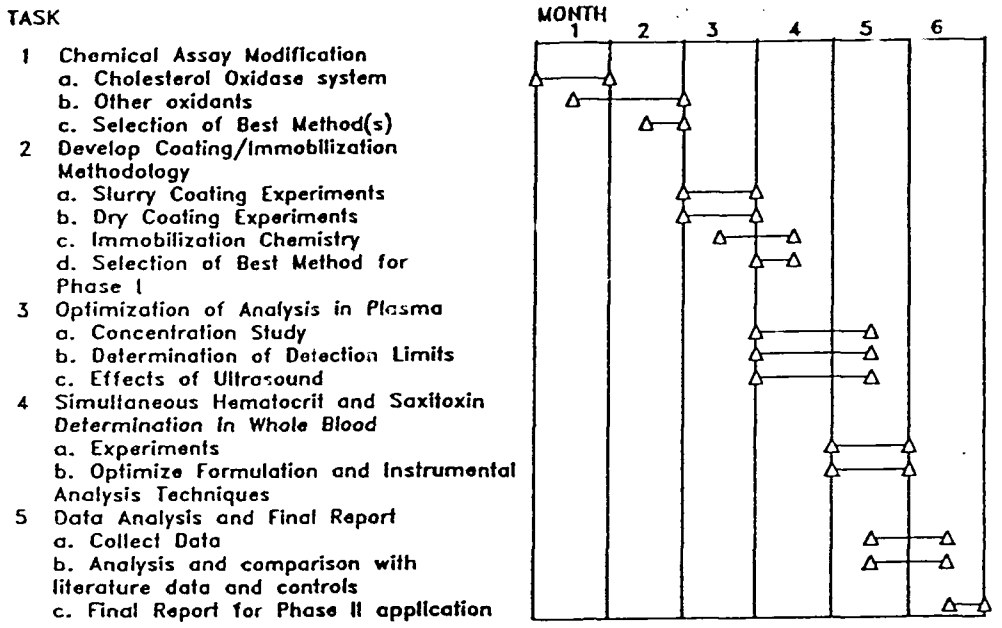


FIGURE 1

TASK 1. CHEMICAL ASSAY MODIFICATION Saxitoxin (obtained from Calbiochem in 5 millimolar (mM) acetic acid solution) was converted to a fluorescent purine analyte according to the procedures of Bates, Kostriken and Rapoport (1,2). The reaction sequence is shown in Figure 2. The reaction involved treatment of 0.35-0.75 milliliter (mL) samples containing 57 micrograms/milliliter (μg/mL) saxitoxin with hydrogen peroxide (HOOH) under basic (pH > 10) conditions at ambient temperature (ca. 20-25 ^{o}C). After 30-40 minutes the pH of the reaction mixture was adjusted to 5.5 with acetic acid. The purine product was analyzed at pH 5.5 for peak position and intensity by UV-Visible (UV-Vis) spectroscopy in a Milton Roy Spectronic 1001 Plus Spectrometer with data acquisition software. Samples and reference solutions were placed in quartz microcells for UV-Vis analysis. After satisfactory characterization of the UV-Vis spectrum of the analyte, the spectral data was used as a reference to evaluate the success of other saxitoxin reactions.

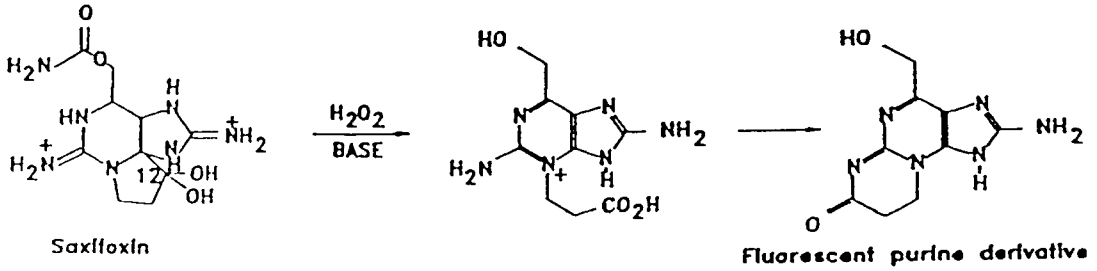


FIGURE 2

Task 1a. Cholesterol oxidase system. The well-known cholesterol oxidase/cholesterol system (3) was used to generate hydrogen peroxide in the presence of saxitoxin. Preliminary experiments were done to establish the best concentrations of cholesterol oxidase and cholesterol for generating sufficient hydrogen peroxide for reaction with saxitoxin at pH= 7. Hydrogen peroxide was detected in the preliminary experiments colorimetrically using a peroxidase-phenol(4-hydroxybenzenesulfonic acid)-4-aminoantipyrine reagent formulation. The cholesterol oxidase/cholesterol reaction was then carried out in the presence of saxitoxin (57 μg/mL) at ambient temperature in a capped microvial. Formation of the purine analyte was monitored periodically by UV-Vis spectroscopy.

Task 1b. Other oxidants. Another enzymatic peroxide generator system, glucose oxidase/glucose was reacted in the presence of saxitoxin. This system was chosen for study because of the potential for precisely controlling the ratio of enzyme to substrate prior to reaction in plasma (the cholesterol oxidase system was chosen to take advantage of the cholesterol already present in human blood). A preliminary study was conducted to optimize the (glucose/glucose oxidase) ratio for rapid hydrogen peroxide generation at pH= 7. Hydrogen peroxide generation was detected colorimetrically in the manner described in Task 1a. The glucose oxidase/glucose reaction was then carried out in the presence of saxitoxin, and purine formation was monitored by UV-Vis spectroscopy.

Table 1 lists several inorganic and organic oxidants that were reacted with solutions of pure saxitoxin diacetate. Generally accepted oxidation mechanisms are listed for each reagent. These oxidants were selected for (a) possible long-term stability in a dry formulation, and (b) potential for oxidizing saxitoxin to the purine analyte via different mechanistic pathways. Figure 3 shows saxitoxin equilibria. At physiological pH the equilibria is dominated by the ketal forms (tetrahedral carbon at C-12) with a very small percentage of the keto form (trigonal carbon at C-12) present. It is known that basic conditions dramatically accelerate the hydrogen peroxide oxidation of saxitoxin. This is due to both a greater population of the keto form and an increase in the concentration of the highly nucleophilic peroxide anion. Therefore it is necessary to survey reagents that could oxidize the ketal forms of saxitoxin in order to maximize the oxidation rate under neutral or physiological conditions.

The reactions were periodically monitored by UV-Vis. Where necessary, the product spectra were subtracted from appropriate background spectra for each reaction to obtain the intensities of the saxitoxin analyte peak.

TABLE 1

Oxidant	Known Oxidation Mechanisms[a]	Supplier
Iodosylbenzoic acid	Oxygen Atom Transfer	Aldrich
Monoperoxyphthalic Acid	Bayer-Villager[b]	Aldrich
Sodium Metaperiodate	Glycol Oxidative Cleavage Oxidative Addition Oxygen Atom Transfer	Aldrich
Sodium Iodate	Oxidative Addition Oxygen Atom Transfer Electron Transfer	Fisher
Potassium Peroxomonosulfate (OXONE REAGENT)	Bayer-Villager[a] Oxygen Atom Transfer Oxidative Decomposition	Aldrich
Hydroxylamine-O-Sulfonic Acid	Oxidative Addition	Aldrich

[a]Names are commonly accepted descriptions of chemical oxidation mechanisms. [b]Oxygen insertion into ketone or aldehyde carbonyl compounds to form esters.

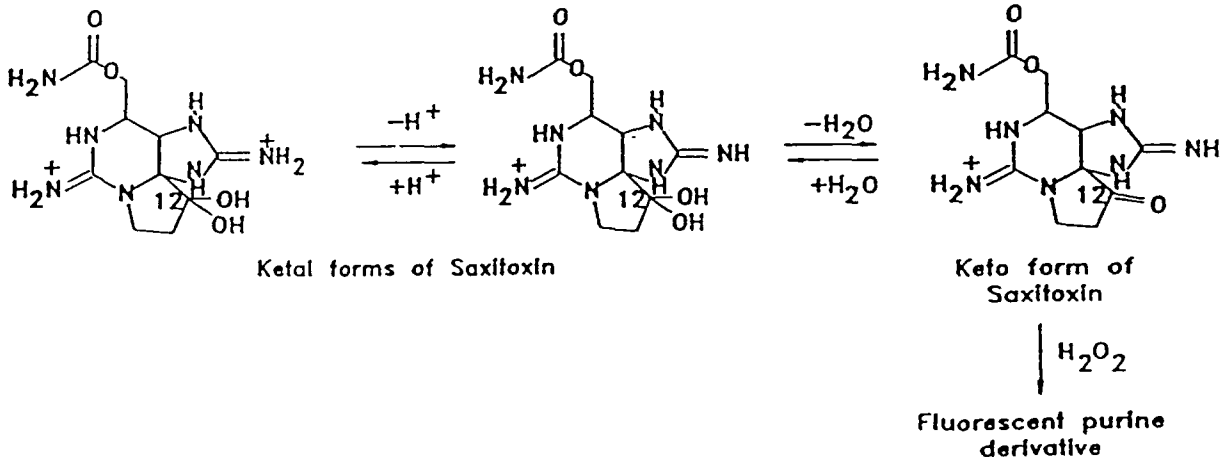


FIGURE 3

Task 1c. Selection of best method. The oxidation procedures that gave the analyte in good yield reproducibly with good stability over a 1 hour period were selected for Task 2.

TASK 2. DEVELOP COATING/IMMOBILIZATION METHODOLOGY.

Tasks 2a and 2b. Slurry coating experiments and dry coating experiments. Microhematocrit tubes were coated using slurry mixtures, concentrated solutions and dry powderered sodium metaperiodate in order to determine the best conditions for consistently producing even coatings for saxitoxin analysis.

Task 2d. Selection of best method for Phase I.

TASK 3. DETERMINATION OF UV-VIS AND FLUORIMETRIC DETECTION PARAMETERS.

Tasks 3a and 3b. The UV-Vis and fluorimetric behavior of the saxitoxin oxidation product was determined in plasma. Additionally, the spectroscopic behavior of purine and cytosine were investigated in PBS in order to determine possible UV interference and fluorescence detection sensitivity. Because these compounds are readily available and relatively inexpensive they were also used to estimate the linear concentration range for saxitoxin detection.

RESULTS OBTAINED

TASK 1. Table 2 compares the hydrogen peroxide oxidation of saxitoxin with oxidation by enzymatically generated hydrogen peroxide from the cholesterol oxidase/cholesterol and glucose oxidase/glucose systems. The ratio of cholesterol oxidase/cholesterol and the ratio glucose oxidase/glucose were determined in preliminary experiments. The molar amounts of cholesterol and glucose in Table 2 are roughly equivalent to that of hydrogen peroxide in the reference reaction.

After 40 minutes the yields of purine analyte from the enzymatic reactions are $\leq$ 6% of the analyte yield from the nonenzymatic reference reaction. Control experiments demonstrated that the cholesterol oxidase/cholesterol system slowly generated hydrogen peroxide. The glucose oxidase/glucose system, however, generated peroxide quickly. Therefore, the enzymatic results in the presence of saxitoxin indicate that too slow generation of hydrogen peroxide, as well as inhibition by saxitoxin or (more likely) the lower pH retards the peroxide oxidation reaction of saxitoxin. When the cholesterol oxidase/cholesterol reaction was allowed to stand for 1 week at ambient temperature, the product corresponded to only a 40% yield (after correction for background absorbance). The nonenzymatic reference reaction was virtually unchanged from the t=40 minute value.

TABLE 2

OXIDATION OF SAXITOXIN WITH HYDROGEN PEROXIDE SYSTEMS[a]

	Hydrogen Peroxide[b]	Cholesterol Oxidase/Cholesterol[c]	Glucose Oxidase/Glucose[c]
Concentration of oxidant or substrate[d]	1 mg/mL	14 mg/mL	5 mg/mL
Enzyme concentration	-	0.27 mg/mL	0.64 mg/mL
Reaction solvent	0.5 M NaOH	PBS[e]	PBS[e]
Reaction pH	> 10	7	7
Product pH	5.5[f]	7	7
Absorbance at time = 40 minutes[g]	1.6	0.06	0.05
Purine % yield[h]	100	3.7	3.3

[a]Saxitoxin concentration 54-57 μg/mL. [b]Fisher Biotech grade, 50%, diluted to appropriate concentration. [c]From Sigma Chemical Company. [d]Hydrogen peroxide or substrate in enzymatic peroxide generator system. [e]Phosphate Buffered Saline. [f]After reaction for 40 minutes, followed by addition of acetate buffer. [g]Background signals subtracted to give correct absorbance values. [h]Assuming complete reaction in the nonenzymatic hydrogen peroxide system.

organic and inorganic reagents listed in Table 1. These results are compared to the reference reaction of saxitoxin with hydrogen peroxide. As shown in the table, sodium metaperiodate was the only reagent that gave substantial saxitoxin conversion during the standard 40 minute reaction period. The fact that saxitoxin reacted at pH= 7 is very encouraging, and it suggests that a different oxidation mechanism occurs. Periodic acid has been used in the past to degrade saxitoxin to smaller heterocyclic products (4). Moreover, while periodate is not explicitly mentioned as a mild degradation reagent for saxitoxin, it is very likely that this technique has been considered by other investigators (5). Because of signals by periodate anion, the purine analyte peak is partially masked in the UV-Vis spectrum. Simple spectral subtraction of the background provides a clean analyte signal, as shown in Figure 4. Figure 5 shows that the metaperiodate reaction is virtually complete in 5 minutes, and the product is reasonably stable over 40 minutes.

It was discovered after this work was completed that the concentration of OXONE was only one-third of the proper stoichiometry required for saxitoxin oxidation. Therefore OXONE is the limiting reagent. In view of this the analyte yield in Table 3 is nearly quantitative. Because of the high reactivity and instability of aqueous solutions of OXONE (6), and because of the known reactivity of OXONE with halide ions (7) and with heterocyclic compounds under basic conditions, a study was done to determine the effect of sodium chloride and base on the course of the reaction of OXONE with saxitoxin. From Table 3 and Figure 5 it is clear that saline and base have a marked effect on the reaction. Basic conditions not only retard the buildup of analyte, but also promote the slow loss of signal. This may be due to the accelerated autodecomposition of OXONE to reactive oxygen products under basic conditions. The absence of saline causes a more gradual buildup of analyte. The reaction in PBS follows a course that is similar to the periodate reaction. It should be noted that addition of periodate to OXONE reactions after 40 minutes result in a large and immediate buildup of analyte signal, thus demonstrating that saxitoxin is still present after OXONE is consumed.

Periodate is therefore clearly the best oxidant in the Phase I portion of this work. No other work was done to determine if saxitoxin inhibited enzymatic generation of hydrogen peroxide.

TASK 2. It was found that slurry coating of periodate on the inside of microhematocrit tubes did not give reproducible coatings with even dispersions. The best coating technique was found to be a cycle of filling tubes with saturated periodate solution, drying at 80 ^{o}C in an oven overnight, followed by repeating the coating and drying process two more times.

TABLE 3.

OXIDATION OF SAXITOXIN WITH ORGANIC AND INORGANIC OXIDANTS[a]

Oxidant	Reaction Solvent	Reaction pH	Product pH	Absorbance after 40 minutes	% Yield	Comments
Hydrogen Peroxide	0.5 M NaOH	> 10	5.5	1.1	100	Reference reaction
Iodosyl-benzoic acid	PBS + NaOH	8	8	0.022	2	Oxidant Insoluble at pH=7
Monoperoxyphthalic acid	PBS	7	7	0.01	1	
Hydroxylamine-O-sulfonic acid	PBS	7	7	0.01	1	
OXONE	PBS	7	7	0.3	30	b
	Phosphate	7	7	0.3	30	b
	Borate	9	9	0.1	10	
Sodium Metaperiodate	PBS	7	7	0.8	80	
Sodium Iodate	PBS	7	7	0.03	3	

[a] Saxitoxin concentration= 57 μg/mL. [b] 100% when corrected for limiting reagent.

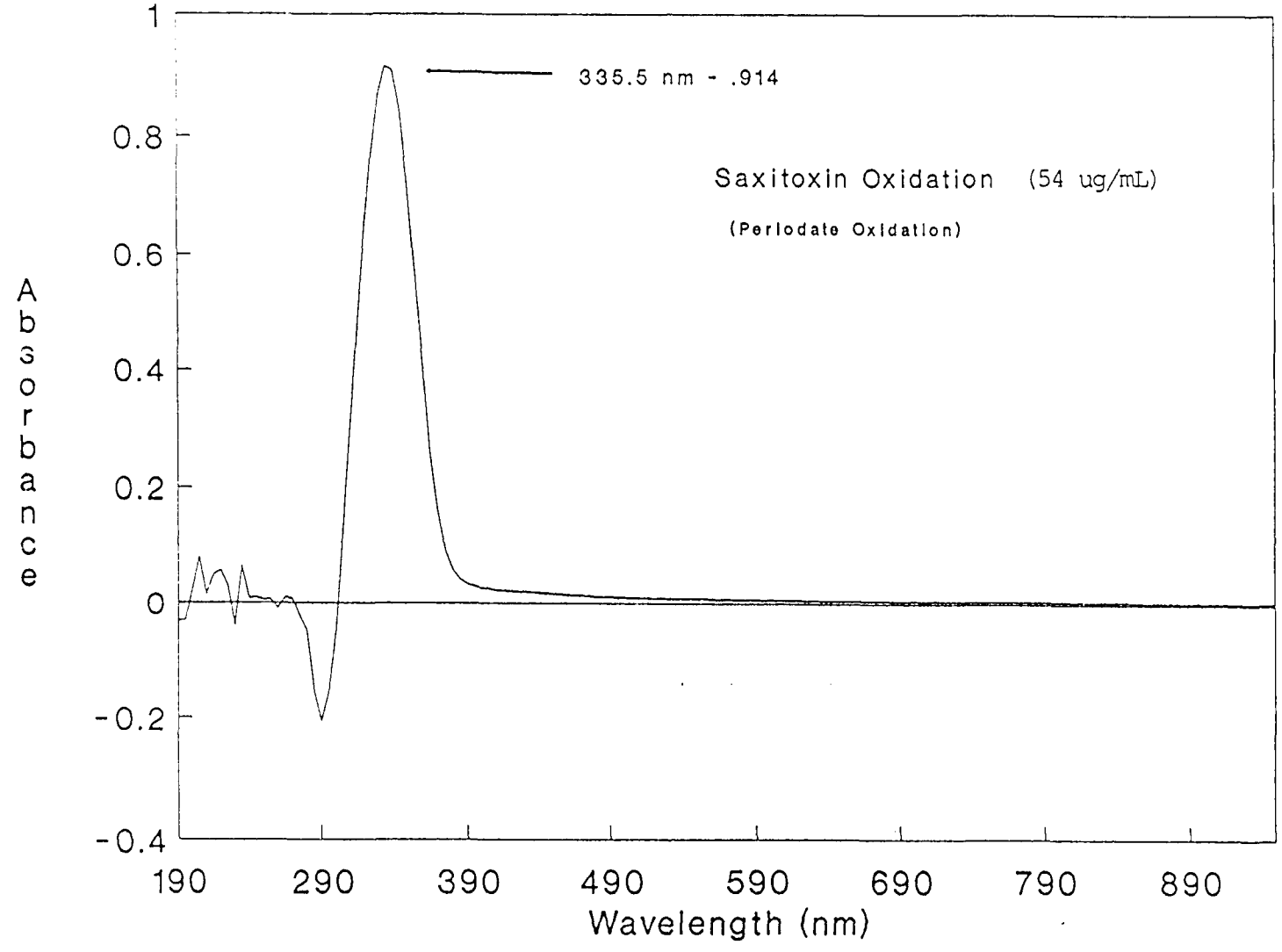

335.5 nm - .914
Saxitoxin Oxidation (54 ug/mL)
(Periodate Oxidation)
Absorbance
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
190
290
390
490
590
690
790
890
Wavelength (nm)

FIGURE 4

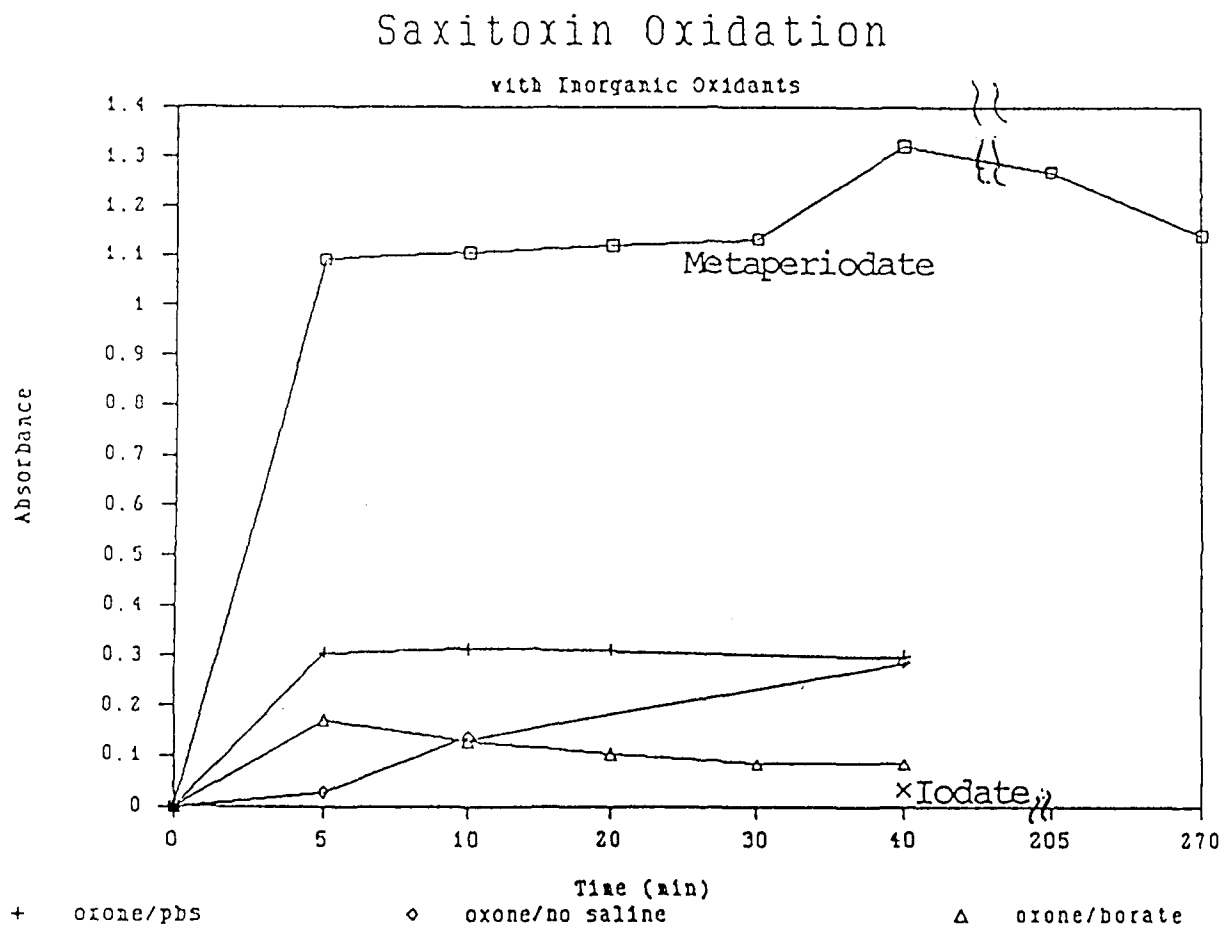

Saxitoxin Oxidation
with Inorganic Oxidants
Absorbance
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Metaperiodate
Iodate
0
5
10
20
30
40
205
270
Time (min)
+ oxone/pbs
◇ oxone/no saline
△ oxone/borate

FIGURE 5

TASK 3. Ultraviolet spectra of purine and cytosine in PBS show that these compounds do not have absorbances at 330-350 nanometers (nm), the region of longest wavelength absorbance for the saxitoxin oxidation product. Therefore these compounds, if present in plasma, would not interfere with the UV-Vis determination of the saxitoxin analyte. Both purine and cytosine have two well-defined peaks in the UV spectra. Linearity studies were carried out at two appropriate wavelengths in each compound. It was found that the linear detection range for both compounds at all wavelengths studied were in the concentration range 7-30 μg/mL. By analogy, the saxitoxin analyte would be expected to behave in the same manner.

At this point the consideration of reasonable lethal saxitoxin dose levels becomes important. Figures 6-8 show a UV-Vis spectrum of plasma from a typical donor, and difference spectra of plasma spiked with two concetrations of the saxitoxin analyte. The analyte was generated prior to spiking the plasma sample. It is clear from Figures 7 and 8 that the saxitoxin analyte signal will be effectively masked by the spectrum of plasma. Figure 8 is most relevant inasmuch as the analyte concentration approximately corresponds to the minimum lethal dose of saxitoxin for humans (8). The uncertainty associated with the minimal peak intensity at 336.8 nm mitigates against reliable UV detection of oxidized saxitoxin in plasma samples.

Figure 9 shows a fluorescence emission spectrum of plasma spiked with 1.5 μg/mL of saxitoxin analyte (excitation wavelength= 330 nm). The near baseline intensity at 380 nm indicates that the plasma environment is not suitable for direct detection of practical concentrations of the saxitoxin analyte. It is well known that plasma contains several proteins that have fluorescent groups. These proteins and other plasma components are most likely masking or quenching analyte fluorescence. Plasma components would also compete with saxitoxin for periodate oxidation. Figure 10 shows a plot of fluorescence versus concentration for plasma spiked with saxitoxin analyte. Despite the linearity of this plot, the fluorescence range is too narrow for obtaining reliable analytical data. It is for this reason that the ultrasound hematocrit method was not applied to this study.

ESTIMATES OF TECHNICAL FEASIBILITY

The ability to oxidize saxitoxin to a fluorescent analyte at neutral pH using inexpensive, widely available and easy-to-store reagents (periodate and OXONE) is a very promising result. Clearly, further work needs to be done to verify that the product is indeed the fluorescent purine derivative that is obtained via alkaline hydrogen peroxide oxidation. The use of periodate as an oxidant has the potential for detecting saxitoxin in the presence of tetrodotoxin, which does not oxidize to a fluorescent with periodate.

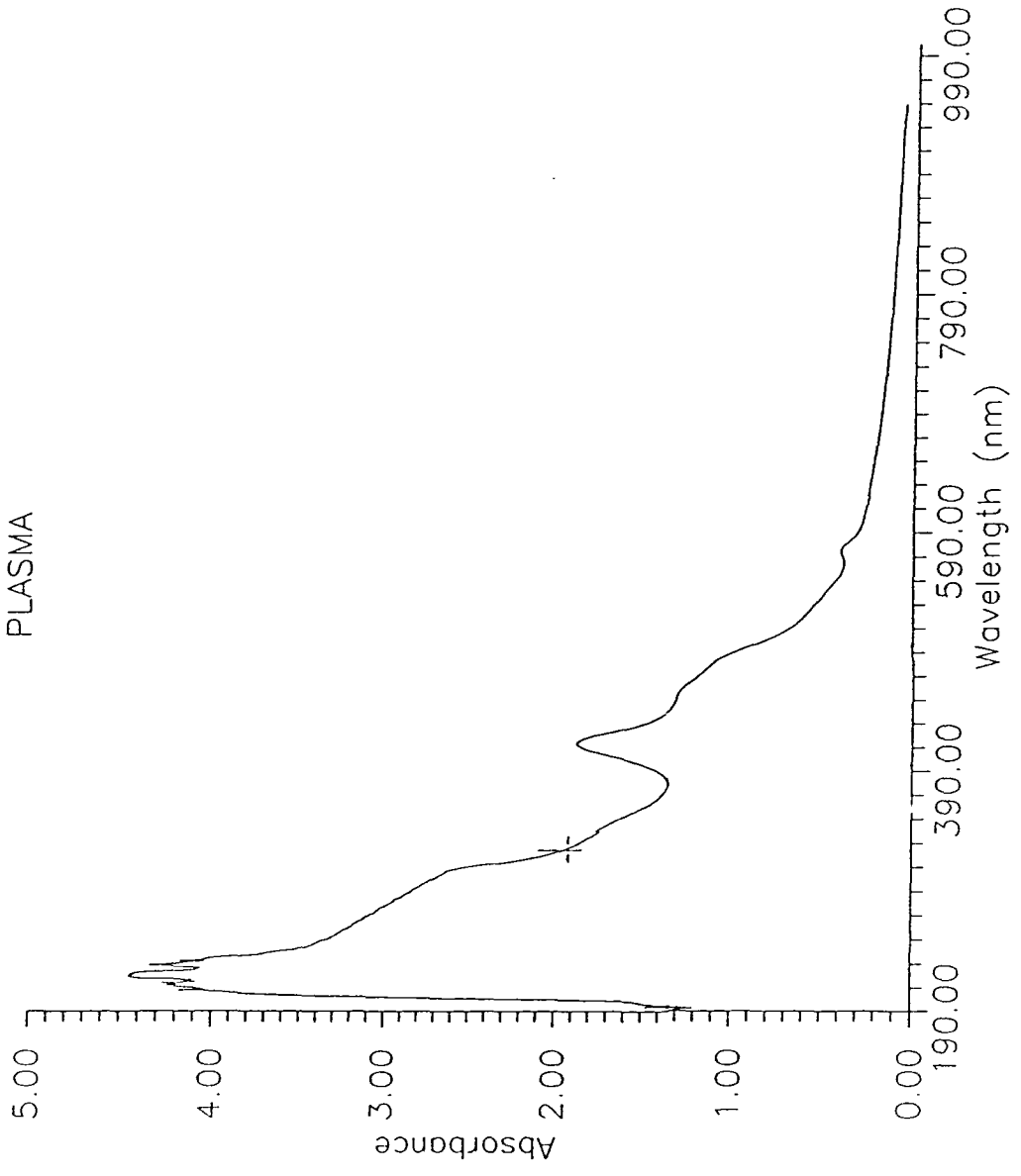

PLASMA
Absorbance
5.00
4.00
3.00
2.00
1.00
0.00
190.00
390.00
590.00
790.00
990.00
Wavelength (nm)

FIGURE 6

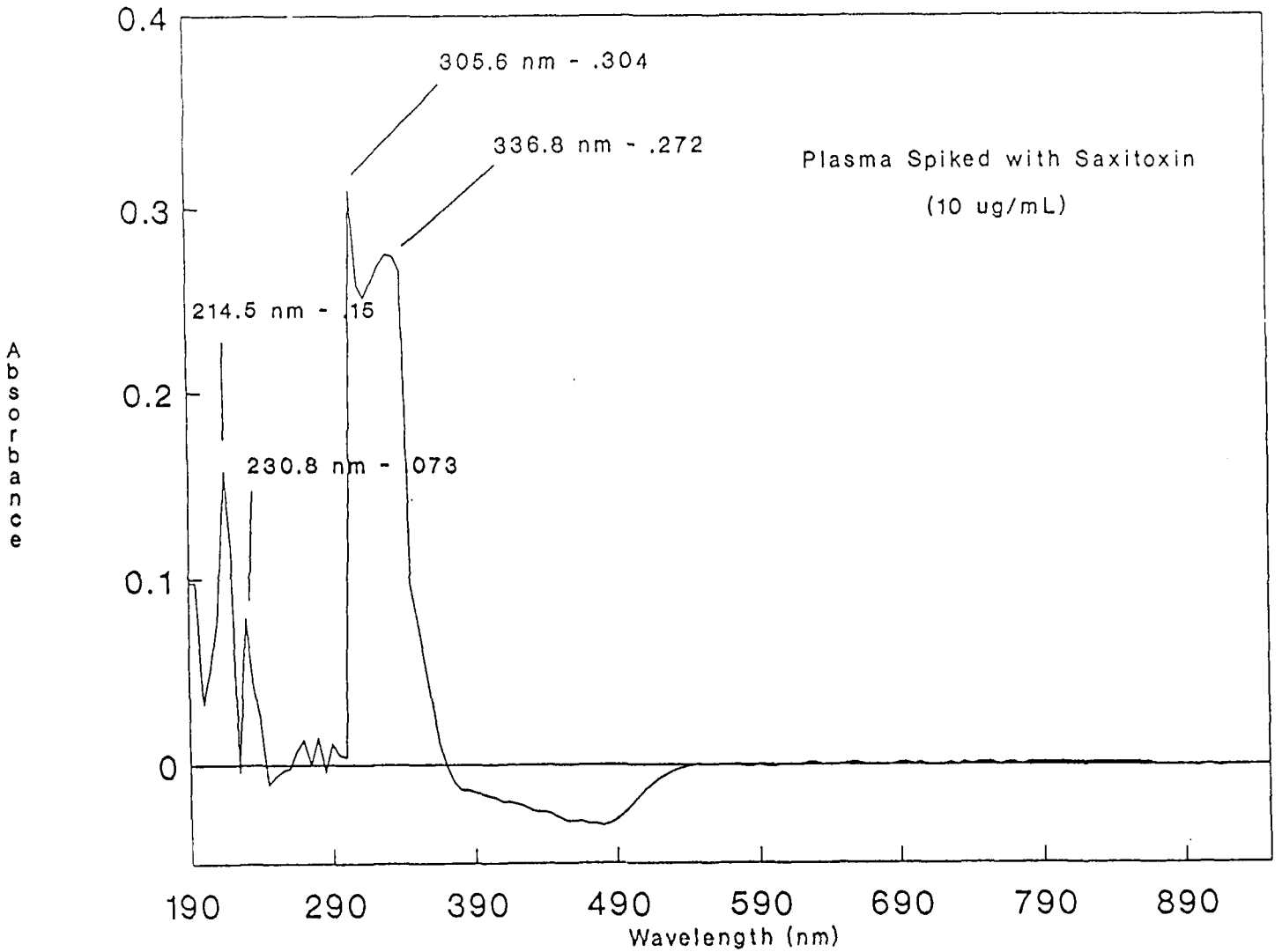

0.4
0.3
0.2
0.1
0
Absorbance
305.6 nm - .304
336.8 nm - .272
214.5 nm - .15
230.8 nm - .073
Plasma Spiked with Saxitoxin
(10 ug/mL)
190
290
390
490
590
690
790
890
Wavelength (nm)

FIGURE 7

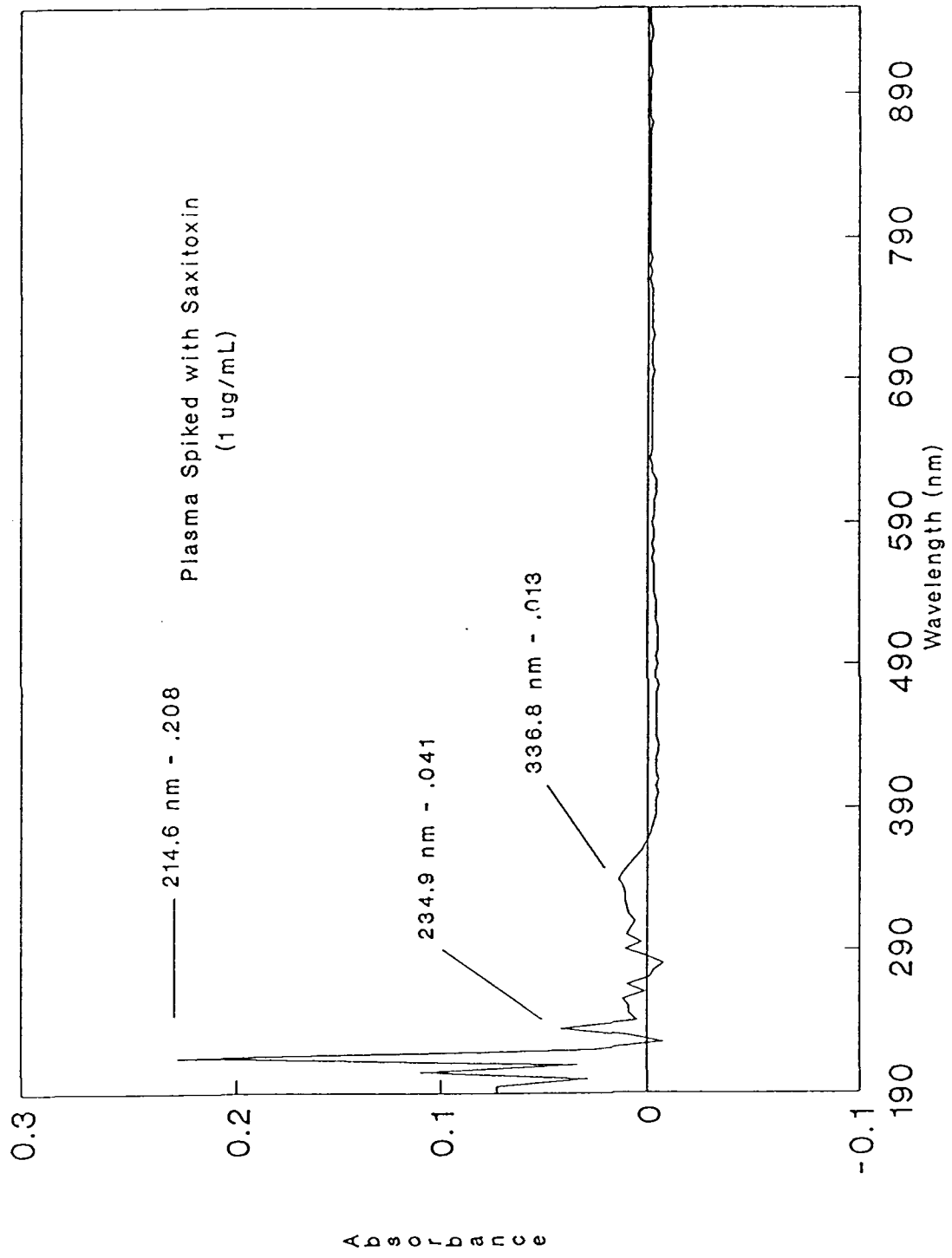

Plasma Spiked with Saxitoxin
(1 ug/mL)
214.6 nm - .208
234.9 nm - .041
336.8 nm - .013
Absorbance
0.3
0.2
0.1
0
-0.1
190
290
390
490
590
690
790
890
Wavelength (nm)

FIGURE 8

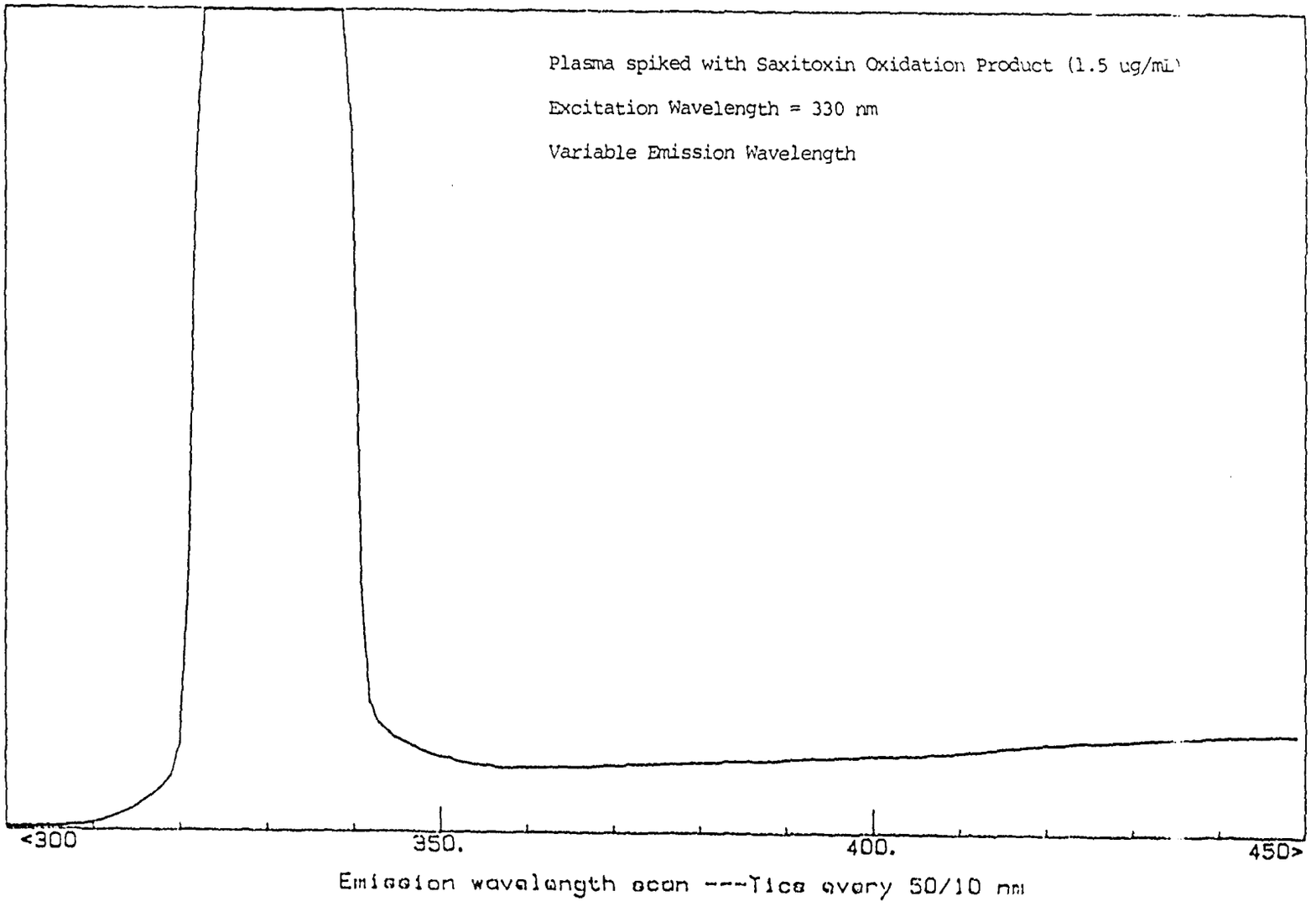
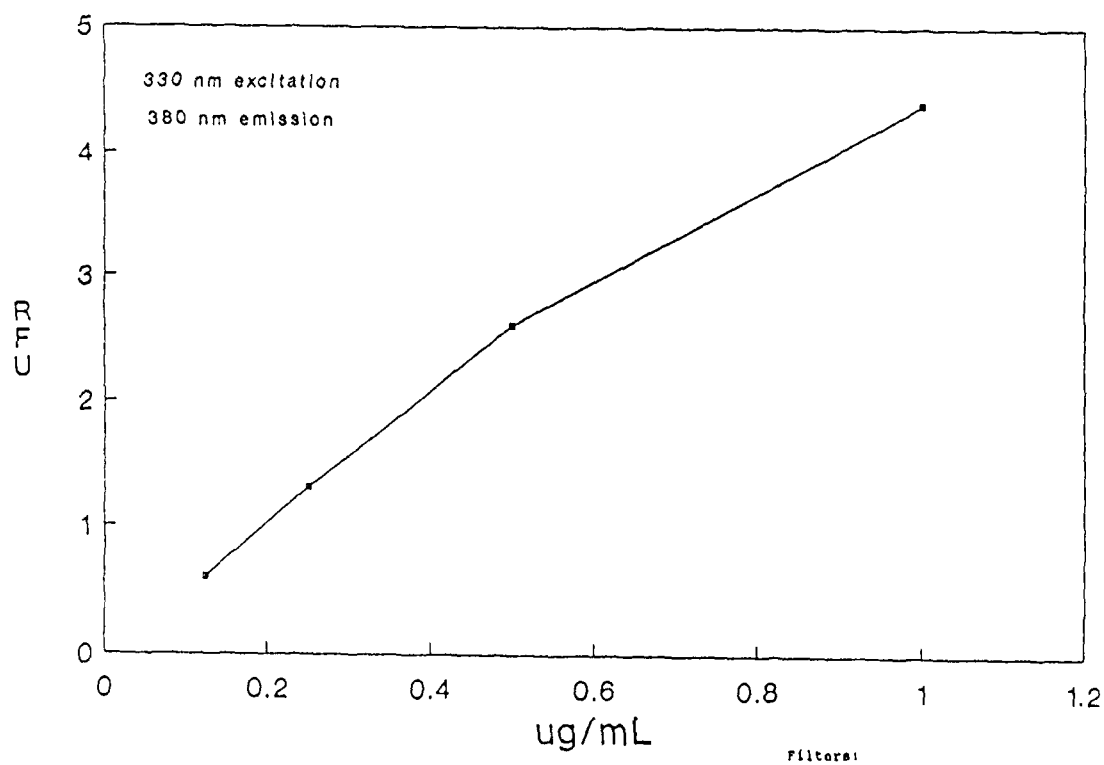

Plasma spiked with Saxitoxin Oxidation Product (1.5 ug/mL)
Excitation Wavelength = 330 nm
Variable Emission Wavelength
<300
350.
400.
450>
Emission wavelength scan ---Tics every 50/10 nm

FIGURE 9



Filters:
7-54 + 34A (excitation)
47D (emission)

ug/mL	RFU			Avg.
1.0	4.2	4.5	4.5	4.4
0.5	2.4	2.7	2.8	2.6
0.25	1.3	1.5	1.2	1.3
0.125	0.7	0.6	0.6	0.6

FIGURE 10

It is obvious that the plasma matrix is too complicated to generate and detect the saxitoxin analyte with good sensitivity. Moreover, the analyte too closely resembles purines and nucleic acid degradation products that naturally occur in plasma. However, the strong binding properties of saxitoxin with various compounds and membranes suggests that saxitoxin might be readily extractable from plasma by these materials. If this is possible, then (in principle) a better procedure for detecting saxitoxin could be devised. This would involve the design of a surface with high saxitoxin affinity. This surface may then be exposed to plasma to bind available saxitoxin (and/or tetrodotoxin). The surface could be removed from the blood and cleaned to remove (or minimize the effect of) fluorescent reagents in plasma. Exposure of this surface to periodate solution could possibly oxidize the bound saxitoxin to the analyte in a much simpler matrix for fluorimetric trace detection. The role of ultrasonic effects on complexation and ligand binding could be assessed in this regard. Filtration of the plasma to remove proteins, as well as devising novel optical detection systems is clearly within the scope of TRA's technical expertise. Unfortunately, these innovations are beyond the time frame of this Phase I feasibility study.

REFERENCES

1. Bates, H.A., Rapoport, H., J. Agr. Food Chem., 39, 237, 1975.

2. Bates, H.A., Kostriken, R., Rapoport, H., J. Agr. Food Chem. 28, 252, 1978.

3. Huang, H., Kuhn, J.W., Guilbault, G.G., Clin. Chem. 21, 1605, 1975.

4. Schantz, E., et al. Can. J. Chem. 39, 2117, 1961.

5. Wong, J.L. Brown, M.S., Matsumota, K., Oesterlin, R. Rapoport, H. J. Am. Chem. Soc. 93, 4633, 1971.

6. Fatope, M.O., Zydowsky, T.M., Floss, H.G. J. Che. Res.(S), 328, 1984.

7. Fortnum, D.H., Battaglia, C.J., Cohen, S.R., Edwards, J.O. J. Am. Chem. Soc. 82, 778, 1960.

8. Buckley, L.J., Ikawa, M., Sasner, J.J. J. Agr. Food Chem. 24, 107, 1976.

SUMMARY (To be pasted on standard government form)

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ESTIMATION OF TECHNICAL FEASIBILITY

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KEYWORDS

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