TECHNICAL BULLETIN

ASSAY TECHNIQUES FOR DETECTION OF EXPOSURE TO SULFUR MUSTARD, CHOLINESTERASE INHIBITORS, SARIN, SOMAN, GF, AND CYANIDE

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CHAPTER 1
INTRODUCTION

1–1. Purpose

This technical bulletin provides analytical techniques to identify toxic chemical agents in urine or blood samples. It is intended to provide the clinician with laboratory tests to detect exposure to sulfur mustard, cholinesterase inhibitors, sarin, soman, GF, and cyanide.

1–2. References

Referenced publications and selected bibliography are listed in appendix A.

1–3. Explanation of abbreviations and terms

Abbreviations and special terms used in this technical bulletin are explained in the glossary.

CHAPTER 2

VERIFICATION OF SULFUR MUSTARD EXPOSURE—MEASURING THIODIGLYCOL IN URINE BY GAS CHROMATOGRAPH/MASS SPECTROMETER

2–1. Background

Sulfur mustard (HD) exposure can be verified with an assay developed at the Medical Research Institute of Chemical Defense (MRICD).[1] In general, mustard cannot be simply assayed from urine because of its reactive nature. Thiodiglycol (TDG) (2,2′-thiodiethanol) is one of the in vivo degradation products of bis (2-chloroethyl) sulfide (HD)[2,3] and can be used to confirm an exposure. TDG is itself subject to chemical and enzymatic transformations. A recent TDG assay demonstrated the existence of control urines with less than 1 nanogram (ng)/milliliter (ml).[4] However, analyte recoveries were only 50 to 70 percent. In the method presented here, detection of TDG after derivatization with heptafluorobutyric anhydride (HFBA) is achieved by using a gas chromatograph (GC) coupled with a mass selective detector (MSD). The lowest quantifiable concentration is 5.0 ng/ml. Thiodipropanol (TDP) is used as a stabilizer and octa-deuterated thiodiglycol (d_8–TDG) as an internal standard. Through the use of spiked urine standards and the internal standard, a linear regression plot is used to determine TDG concentrations in urine samples.

2–2. Materials and methods

a. Materials. Urine specimens are collected from individuals suspected of exposure to mustard. Control urines should have less than 1 ng/ml TDG before use in standard preparations. Internal standard, d_8–TDG, was obtained from Ash Stevens Incorporated (Detroit, MI). Other materials were obtained commercially. A listing of chemicals and vendors is presented in table 2–1.

b. Supplies and equipment. The procedure requires the use of various pieces of equipment and some common supplies which are listed as follows.

(1) Nitrogen and helium gases, UHP grade (Matheson Gases and Equipment, Montgomeryville, PA).

(2) Polypropylene microcentrifuge tube (Elkay Products Incorporated, Shrewsbury, MA).

(3) N-Evap™ (Organomation Association Incorporated, South Berlin, MA), an evaporator equipped with oil bath and gas nozzles.

(4) Centra-M™ centrifuge (IEC Company, Needham Heights, MA), rotor speed 13,200 revolutions per minute (rpm)/centrifugal force 15,600 × g.

(5) Mixer (Thermolyne Maxi Mix™; Thermolyne Corporation, Dubuque, IA).

(6) GC/MSD, 5890/5970B (Hewlett-Packard, San Fernando, CA).

(7) DB–5 bonded-phase capillary column (J and W Scientific, Folsom, CA), 20 meters, 0.18 millimeter (mm) inner diameter (I.D.), 0.40 micrometer (μm) film thickness.

(8) Pipettes and tips (Rainin Instrument Incorporated, Woburn, MA).

(9) Polyethylene scintillation vials (20 ml capacity).

WARNING

The chemicals involved in these procedures are toxic. Follow all safety precautions listed below.

c. Hazards involved. TDG, d_8–TDG, TDP, and HFBA will be handled in a fume hood. The derivatizing reagent, HFBA, is extremely reactive and toxic. Care must be taken when handling this reagent. Gloves, safety glasses, and a lab coat will be worn when handling chemicals or urine samples. Any further concerns are addressed adequately by the Material Safety Data Sheets.

d. Safety requirements.

(1) *Ventilation.* A general chemical fume hood will be used.

(2) *Clothing.* A standard lab coat, a pair of safety glasses, and latex gloves are required.

(3) *First aid and fire fighting equipment.* There will be standard first aid support and an

[1] Jakubowski, E.M., C.L. Woodard, N.M. Mershon, and T.W. Dolzine. "Quantitation of Thiodiglycol in Urine by Electron Ionization Gas Chromatography-Mass Spectrometry," *J. Chromatog.* 528 (1990), pp. 184–190.

[2] Davison, C.D., R.S. Roman, and P.K. Smith. "Metabolism of Bis-β-Chloroethyl Sulphide (Sulphur Mustard Gas)," *Biochem. Pharmacol.* 7 (1961), pp. 65–74.

[3] Roberts, J.J. and G.P. Warwick. "Studies of the Mode of Action of Alkylating Agents—VI. The Metabolism of Bis-2-Chloroethyl Sulphide (Mustard Gas) and Related Compounds," *Biochem. Pharmacol.* 12 (1963), pp. 1329–1334.

[4] Black, R.M. and R.W. Read. "Detection of Trace Levels of Thiodiglycol in Blood, Plasma, and Urine Using Gas Chromatography-Mass Spectrometry-Electron-Capture Negative Ion Chemical Ionization," *J. Chromatog.* 449 (1988), pp. 261–270.

eyewash station nearby. A standard chemical fire extinguisher will be placed near the work area in case of fire.

e. Preparation of solutions.

(1) *TDP.* In a plastic scintillation vial or other suitable plastic container, weigh out between 12 and 16 milligrams (mg), then add water until a final concentration of 1 mg/ml is reached. This solution is stable and can be used over a period of 3 months if stored under refrigeration.

(2) *D_8–TDG.* In a plastic vial weigh out approximately 16 mg and dilute in water to a final concentration of 1 mg/ml. The stability data of TDG in aqueous media are not available. The stock solution of 1 mg/ml should be kept at −70 °C for long-term storage. The working solution of 0.1 mg/ml should be made fresh daily.

(3) *Enzyme type H–1, β-glucuronidase.* In a plastic vial weigh out 250 mg and add 10 ml of water. Gentle mixing in a closed vial is preferred in order to prevent foaming. This solution may be used for up to 1 week if kept frozen after each use.

(4) *TDG.* In a plastic vial weigh out approximately 16 mg and dilute in water to a final concentration of 1 mg/ml. The stability data of TDG in aqueous media are not available. The stock solution should be made fresh daily or kept at −70 °C for short-term storage.

f. Preparation of the standards and samples.

(1) *Preparation of the TDG standards.*

(a) Pipette 0.1 ml of 1 mg/ml TDG solution and add 9.9 ml water. This gives a concentration of 10 micrograms (μg)/ml.

(b) Pipette 5.0 ml of 10 μg/ml TDG and add an additional 5 ml of water in a separate vial. Label this 5 μg/ml.

(c) Pipette 1.0 ml of 10 μg/ml TDG and add an additional 9 ml in a separate vial. Label this 1 μg/ml.

(d) Pipette 1.0 ml of 5 μg/ml and add an additional 9 ml in a separate vial. Label this 500 ng/ml.

(e) Pipette 1.0 ml of 1 μg/ml and add an additional 9 ml in a separate vial. Label this 100 ng/ml.

(f) Pipette 1.0 ml of 0.5 μg/ml and add an additional 9 ml in a separate vial. Label this 50 ng/ml.

(g) Pipette 1.0 ml of 0.1 μg/ml and add an additional 9 ml in a separate vial. Label this 10 ng/ml.

(h) Label the microcentrifuge tubes that are to be used to contain the standards as 0, 1, 5, 10, 50, 100, and 500 ng. Pipette 1.0 ml of a control urine into each tube. The control urine should contain less than 1 ng/ml of TDG. If water is used instead of urine, a non-linear standard curve will be produced.

(i) Pipette 0.1 ml of 5 μg/ml into the tube labeled 500 ng.

(j) Pipette 0.1 ml of 1 μg/ml into the tube labeled 100 ng.

(k) Pipette 0.1 ml of the 500, 100, 50, 10 ng/ml solutions to the tubes labeled 50, 10, 5, and 1 ng respectively.

(2) *Preparation of the samples.* Label the microcentrifuge tubes that are to be used for the samples appropriately. Pipette 1 ml of the urine sample to each tube.

(3) *Enzyme digestion.* Pipette 0.1 ml each of enzyme reagent, d_8−TDG 0.1 mg/ml, and TDP 1 mg/ml solutions to each tube. Vortex the tubes and let them sit at room temperature for 1 hour.

(4) *Derivatizing the samples.*

(a) Add 40 microliters (μl) of 1.0 normal solution (N) hydrochloric acid (HCl) to each tube and check the pH with pH paper. Continue to add HCl in 10 μl increments until the pH is in the range of 2 to 3. If the pH falls below 2, use 1.0 N sodium hydroxide (NaOH) to adjust the pH.

(b) Place the tubes in the oil bath at a temperature of 90 °C. A gentle flow of nitrogen gas should be used to aid in the evaporation to complete dryness (approximately 1 hour). Remove the tubes from the oil bath.

(c) Add to each tube 1 to 2 molecular sieves.

(d) Pipette 400 μl HFBA and 400 μl ethyl acetate into each tube and allow the reaction to proceed at room temperature for 1 hour. Vigorously agitate each tube every 15 minutes. The sides of the tube can be scraped with a pipette tip to promote thorough derivatization.

(e) Centrifuge for 15 minutes and decant off the supernatant to a new tube.

(f) Add to the residue 200 μl each of HFBA and ethyl acetate. Vortex and derivatize again at 60 °C for 30 minutes.

(g) Centrifuge for 15 minutes. Decant the supernatant off to be combined with the supernatant from first derivatization.

(h) Evaporate the combined supernatant to dryness at 90 °C under nitrogen (about 30 minutes).

(i) Add 100 μl ethyl acetate to each tube and vortex. Centrifuge each tube for 10 minutes.

(j) Inject 1 to 2 μl of the supernatant to the GC/MSD.

g. GC/MSD parameters.

(1) The GC parameters are as follows:

(a) The injector port temperature is 220 °C.

(b) The transfer line temperature is 265 °C.

(c) The oven temperature is programmed as: initial temperature is kept at 45 °C for 1.1 minute, increased to 110 °C at a rate of 40 °C/minute, then to 125 °C at 3 °C/minute, and finally increased to 265 °C at 40 °C/minute and kept constant at 250 °C for 1 minute.

(d) The total inlet flow is set at 50 ml/minute.

(e) The split delay time is set at 0.20 minutes.

(f) The column head pressure is set at 12 pounds per square inch.

(g) The septum purge is set at 2 ml/minute.

(2) The MSD parameters are listed below.

(a) Data acquisition is set for selected ion monitoring.

(b) The solvent delay is set at 8 minutes.

(c) From 8.0 to 10.0 minutes, the ions mass to charge ratio (m/z) 300 and 301 representing TDG and m/z 307 and 309 representing d_8 – TDG are monitored. The dwell time for each ion is set at 20 milliseconds (msec), resulting in a cycling time of 7.2 cycles/second.

(d) From 10.0 to 11.0 minutes, the ions m/z 328 and 542 representing TDP are monitored. The dwell time for each ion is set at 20 msec, resulting in a cycling time of 10.0 cycles/second.

h. Analysis of chromatogram.

(1) *Calculations.*

(a) After injecting the samples, the data editor will be used to integrate the peaks in the chromatograph. Retention times of 9.38, 9.42, and 10.5 minutes have been obtained for d_8 – TDG, TDG, and TDP under these conditions.

(b) Integration of the located peak is enhanced by narrowing the time window. When integrating, the threshold is set at 2 or lower for best results. When the integration is complete, the peaks at m/z 300 and m/z 301 will be within 0.04 minutes of the m/z 309 peak.

(c) The area count is used in analyzing the data. The peak area ratio is calculated by dividing the sum of the m/z 300 and m/z 301 areas by the area of the m/z 309 peak. This gives a peak area ratio which when plotted against concentration will yield a positive slope.

(2) *Sensitivity and linearity.* The range of sensitivity for quantitation is from 5.0 ng/ml to 500 ng/ml using a standard curve with points plotted at 0, 1, 5, 10, 50, 100, and 500 ng/ml. Linearity is found by using linear regression analysis in Lotus 123™. An r^2 value of at least 0.995 must be obtained before the curve can be used with confidence.

(3) *Precision and accuracy.* This assay was found to be accurate within a range of 5.0 ng/ml to 500 ng/ml. The standard curve is non-linear below 10 ng/ml. Precision varies with concentration ranging from 10 percent relative standard deviation (RSD) (percent RSD = standard deviation (SD)/mean × 100) at 10 ng/ml to 2 percent at 500 ng/ml.

i. Quality control.

(1) Tune the instrument each day prior to use.

(2) Change the septum in the injector port every day.

(3) Inject the samples first and then the standard curve starting with the blank and then proceeding from the lower to the higher concentrations.

(4) The source in the MSD should be cleaned periodically.

(5) Carrier gas should be flowing through the column at all times.

2–3. Results and discussion

a. Derivatization of TDG was necessary for two reasons. First, due to a low molecular weight (122 atomic mass unit) its presence would be obscured by the natural components of urine. Secondly, the polar functional group of TDG causes poor chromatographic peak shape, low sensitivity, and a low relative abundance of molecular ion. Detection limits for underivatized TDG in ethyl acetate were between 500 to 1000 ng on column. TDG can easily be esterified with acyl chlorides other than many anhydrides as was demonstrated by Black and Read.[4] The derivatized esters varied in their stability and usefulness in assays. For example, both the trifluoro and heptafluoro derivatives were prepared and analyzed by GC/mass spectrometer (MS) at MRICD. The HFBA derivative was more stable than the trifluoromethyl anhydride (TFA) derivative. The HFBA derivative produced analytically useful fragments at high molecular weights ranging from m/z 241 to m/z 301. Fragments at m/z 300 and 301 represented the loss of one $-OCOC_3F_7$ group leaving most of the analyte molecule intact. Therefore the identity of the HFBA derivatived analyte was confirmed by the char-

[4] Black, R.M. and R.W. Read. "Detection of Trace Levels of Thiodiglycol in Blood, Plasma, and Urine Using Gas Chromatography-Mass Spectrometry-Electron-Capture Negative Ion Chemical Ionization," *J. Chromatog.* 449 (1988), pp. 261–270.

acteristic fragments. However, the molecular ion of both the HFBA and TFA derivatives had low relative abundances (i.e., for the HFBA derivative the M+ was only 5 to 10 percent of the base at m/z 241) as was also noted by Black and Read. HFBA was still the derivative of choice in the analysis of TDG by electron ionization positive ion detection despite the low molecular ion abundance, because the m/z 300 and 301 fragments were structurally informative and possessed high relative abundances of 70 percent and 50 percent, respectively. Analogous fragmentations of the deuterated internal standard produced ions at m/z 307 and 309. The retention time of the bis (heptafluorobutyl derivative) was approximately 9.4 minutes with the deuterated internal standard eluting 0.04 minutes earlier.

b. TDP was used as a stabilizer to decrease binding effects of the analyte.

c. Because of its polar and very hydrophilic nature, TDG has been very difficult to extract from biological matrices. The strategy at MRICD was to derivatize the sample with excess HFBA after initial acidification and drying steps. The derivatived TDG was then more lipophilic and would extract into ethyl acetate. Amines and amino acids would form salts that would be insoluble in organic solvents. Samples were first made acidic (pH 2 to 3) to both stabilize the TDG during the drying step and to increase amine salt formation. Moderately acidic solutions of TDG are stable to many reactions even at temperatures in excess of 100 °C.[5] The extent of derivatization was measured to be 95 percent disubstituted, 4 percent monosubstituted, and 1 percent unreacted. Using this sample preparation scheme it was possible to quantify TDG from 5 to 500 ng in spiked rat or human urine.

d. This assay method was validated in the analysis of urine samples collected from rats after subcutaneous injection and guinea pigs after vapor exposure of neat mustard. Mustard at 750 and 450 µg/kilogram (kg) was injected into rats and produced no overt symptoms. Urine was collected and analyzed from both exposure groups and from a control group. All urines were collected over TDP. Results for the 24, 48, and 116 hour samples are shown in table 2–2. These results were gathered before the deuterated internal standard was available; therefore TDP was the internal standard. The control groups did not contain measurable amounts of TDG. Trace levels of TDG (1 to 15 ng/ml) could be measured for up to a week post-exposure.

e. Guinea pig vapor exposure produced TDG levels of 34.4 to 297 ng/ml.

f. Thirty volunteer human urine samples were collected and assayed. Results indicated levels were below the detection limits of the assay. These results agreed with Black and Read.[6] Despite the limited survey of human control levels, the verification of mustard exposure is possible if samples can be stabilized and analyzed fast enough to ensure integrity.

Table 2–1. List of supplies and suggested vendors

Chemical description	Vendor	Catalog no.
3,3′-thiodipropanol 98%	Aldrich	20,534–6
2,2′-thiodiethanol 99%	Aldrich	16,678–2
Ethyl acetate 99.5%	Aldrich	15,485–7
Enzyme type H–1 β-glucuronidase	Sigma	G–0751
Hydrochloric acid 1.0 N	Aldrich	31,894–9
Molecular sieves (5A)	Sigma	M–1510
Heptafluorobutyric anhydride (HFBA)	Sigma	H–1006
Sodium hydroxide 1.0 N	Sigma	930–65

[5] Reid, E.E., ed. *Organic Chemistry of Bivalent Sulphur, Vol. II,* Chemical Publishing Co., N.Y. (1960).

[6] Black, R.M. and R.W. Read. "Detection of Trace Levels of Thiodiglycol in Blood, Plasma, and Urine Using Gas Chromatography-Mass Spectrometry-Electron-Capture Negative Ion Chemical Ionization," *J. Chromatog.* 449 (1988), pp. 261–270.

Table 2–2. Thiodiglycol levels in rat urine after mustard exposure

HD dose (μg/kg)	TDG concentration (ng/ml)		
	24 hours	48 hours	116 hours
750	196±159	116±76.6	17.2±13.2
450	70.5±19.5	60.6±38.0	6.1±1.4
0	<1	<1	<1

CHAPTER 3

VERIFICATION OF NERVE AGENT EXPOSURE—MONITORING BLOOD CHOLINESTERASE ACTIVITY WITH THE TEST-MATE™ OP KIT

3–1. Background

a. Various pesticides, drugs, and chemical agents inhibit cholinesterase (ChE) activity. Determining depression of ChE activity provides an indication of exposure to nerve agents. Plasma ChE recovers in 30 to 40 days and red blood cell (RBC) acetylcholinesterase (AChE) recovers in 90 to 100 days after exposure to organophosphorus nerve agents.[1] It is recommended that the Test-Mate™ OP Kit (EQM Research Incorporated, 2585 Montana Avenue, Cincinnati, OH 45211) be used to establish ChE levels when exposure to nerve agents is suspected.

b. The chemistry employed in the ChE activity determination is that described by Ellman[2] in which thiocholine, the product of substrate acetylthiocholine (ATCh) hydrolyses, is detected by reacting with 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB). This kit can determine RBC AChE and plasma butyrylcholinesterase (BChE) activities within minutes, requiring 10 µl of blood per determination.

3–2. Materials and methods

a. Equipment. The equipment and reagents for the Test-Mate™ OP Kit are contained in a plastic, airtight case measuring 26x23x16 centimeters (cm). The kit consists of the following items:

(1) Alcohol swabs.
(2) Battery, 9 volt.
(3) Biohazard disposal bags.
(4) Biopsy punch.
(5) Blood lancets.
(6) Buffer dropper.
(7) Capillary bulb assembly.
(8) Capillary pipettes.
(9) Erythrocyte AChE reagent.
(10) Gauze pads.
(11) Vinyl gloves.
(12) Graduated cuvette, 2 ml.
(13) Plasma ChE reagent.
(14) Sample buffer.
(15) Stirring paddle.
(16) Test-Mate™ OP blood analyzer.
(17) Transfer pipettes.
(18) Test rack for droppers, paddle and cuvette.
(19) Waste bottle.
(20) Water dropper.
(21) Water reservoir bottle.
(22) Water dispenser.

b. Sample collection. Blood samples are drawn from a pricked finger tip into a 10 µl, heparinized microcapillary tube. If analysis can not be done immediately, blood should be drawn into Vacutainers™ containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant and kept refrigerated or on ice. Tests should be performed within a week for stored samples.

c. Reagents. The reagents in the Test-Mate™ OP Kit are either in a concentrated aqueous form or lyophilized in 96-well microplates. The reagent concentrations reported here are those in the total reaction mixture.

(1) In the erythrocyte AChE assay are:
(a) ATCh, 1 millimole (mM).
(b) Potassium phosphate, 20 mM, pH 7.4.
(c) Triton X-100, 0.1 percent.
(d) DTNB, 0.3 mM.
(e) 10-(α-diethylaminopropionyl)-phenothiazine hydrochloride (Astra 1397), 21 µM.

(2) In the plasma BChE assay are:
(a) Butyrylthiocholine (BTCh), 2 mM.
(b) Potassium phosphate, 20 mM, pH 7.4.
(c) Triton X–100, 0.1 percent.
(d) DTNB, 0.3 mM.

d. Analyzer.

(1) The Test-Mate™ blood analyzer is a battery powered colorimeter with an LED light source and 450 nanometer (nm) filter. It is slightly larger than a hand-held scientific calculator. On the upper face of the colorimeter are function keys, a liquid crystal display screen and the cuvette compartment. The function keys are used to initiate

[1] Grob, D. and A.M. Harvey. "The Effects and Treatment of Nerve Gas Poisoning," *Amer. J. Med.* 14 (1953), pp. 52–63.

[2] Ellman, G.L., K.D. Courtney, J. Andres, Jr., and R.M. Featherstone. "A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity," *Biochem. Pharmacol.* 7 (1961), pp. 88–95.

successive steps in the procedure and to recall the results at the end of analyses.

(2) The LED screen displays instructions prompting the user through the analyses procedure and displays the results at the conclusion. In addition, the results are compared to normal mean values preprogrammed in the colorimeter computer and displayed as a percent of the normal mean value.

WARNING

To avoid the transmission of disease, follow all safety precautions listed below.

e. Safety. Precautions must be taken to eliminate hazards either to the operator or the person being tested. The following rules must be followed during the testing procedure:

(1) Do not reuse blood lancets or capillaries.

(2) Put used lancets and capillaries in a hazard bag.

(3) The operator must wear gloves and safety glasses.

f. Erythrocyte AChE activity analyses. Remove the Test-Mate™ components from the carrying case and lay them out, within easy reach, in front of you on a flat surface. In the field it could be the lid of a car trunk, the back of a pickup truck, a flat stone, etc. Before going out in the field, fill the water reservoir bottle with deionized or distilled water and pack an extra battery. Procedures are as follows:

(1) *Press the "Power" key.* The display screen indicates a 15 second warmup countdown.

(2) *Press the "Mode" key.* Press until "AChE mode" is displayed.

(3) *Press the "Test" key.* The display will read "Add buffer" followed by "Insert cuvette" and "Press test." Add four drops of buffer to the cuvette, add water to the 2 ml mark, using the water dispenser, and mix with the stirring paddle. Insert the cuvette into the colorimeter.

(4) *Press the "Test" key.* A 10 second "Blanking" is displayed ending in a "beep." "Remove cuvette" followed by "Press test" is displayed.

(5) *Press the "Test" key.* The display reads "Add blood" followed by "Insert cuvette" and "Press test."

(6) *Wipe the fingertip of the person being tested with the alcohol swab.* Twist off the cap of the blood lancet and prick the finger at its inside tip, off-center. Wipe off the first drop of blood with the gauze pad and squeeze out a second drop. Fill the capillary tube with blood and dispense into the buffer filled cuvette. Mix the solution with the stirring paddle and insert the cuvette into the sample chamber.

(7) *Press the "Test" key.* A 30 second "Reading" is displayed followed by a "beep." During this time Hgb has been determined. The display reads "Remove cuvette" followed by "Press test."

(8) *Press the "Test" key.* The display reads "Get ready" followed by "With reagent" and "Press test." Remove the plastic covering of one of the reagent wells of the microplate with the biopsy punch. Add four drops of water with the water dropper to the well and dissolve the reagent by aspirating it three times using the transfer pipette. Fill the transfer pipette with the dissolved reagent.

(9) *Press the "Test" key.* The display reads "AT BEEP" followed by "Add reagent." At the "beep" add the reagent in the transfer pipette to the diluted blood-filled cuvette and mix the contents with the stirring paddle. The display reads "Insert cuvette" followed by "Press test." Insert the cuvette into the sample chamber.

(10) *Press the "Test" key.* The display shows an 80 second pre-incubation countdown followed by a 50 second reading phase ending in a "beep." The analysis of erythrocyte AChE is concluded. The display reads "Remove cuvette" followed by "Press test." Remove the cuvette and pour the solution into the waste bottle. Use the water dispenser to rinse the cuvette.

(11) *Press the "Test" key to initiate results recovery.* After the first result is displayed, keep pressing the "Disp." key to recover the rest of the data.

g. Plasma BChE activity analyses. Procedures are as follows:

(1) *Press the "Power" key.* The display screen indicates a 15 second warmup countdown.

(2) *Press the "Mode" key until "PChE mode" is displayed.* From this point on, the procedure is the same as for AChE activity determination except that there is no Hgb assay.

3–3. Results and discussion

The Test-Mate™ kit uses blood as sample for both RBC AChE and plasma BChE activity measurement by using two different modes to distinguish the two enzymes. In the RBC AChE procedure, a specific inhibitor of BChE, Astra 1397,[3] has been incorporated into the lyophilized reagent, eliminating interference due to the plasma enzyme. In the plasma BChE activity analysis, the substrate

[3] Augustinsson, K.B. "A Titrometric Method for the Determination of Plasma and Red Blood Cell Cholinesterase Activity Using Thiocholine Esters as Substrates," *Scan. J. Clin. and Lab. Investigation* 7 (1955), pp. 284–290.

ATCh has been replaced by BTCh, which is hydrolyzed at a much faster rate by BChE than AChE.

a. Reproducibility. A blood specimen was analyzed repetitively six times to establish the reproducibility of the kit. The results are shown in table 3–1.

b. Intrapersonal variability of ChE activity. It is desirable that the pre-exposure baseline values of ChE are available for each individual to serve as control. Of the two enzymes, RBC AChE and plasma BChE, the former has the least intrapersonal variability[4] and therefore is the enzyme of choice to be monitored for possible exposure. The kit provides RBC AChE results in two units; U/ml and U/gHgb. The RBC AChE expressed as U/ml will increase or decrease with the Hgb level which will vary due to blood transfusion, hemorrhage, or blood sampling techniques. Therefore, the RBC AChE value expressed as U/gHgb is recommended. A previous study indicated that the intrapersonal variability of RBC AChE (U/ml RBC) within a year is around 8 percent.[4] With the addition of 1 percent variability of the method, a total of 9 percent variation would be expected. When a test value shows a drop of more than 9 percent from the baseline value, exposure to nerve agent would be suspected.

c. Interpersonal variability of ChE activity. If a baseline value is not available, possible RBC AChE inhibition has to be estimated from the population mean or normal range. Thirty U.S. Army personnel volunteered to participate in the Test-Mate™ kit evaluation study. AChE activity in the blood obtained from a finger stick and BChE activity obtained from blood collected in an EDTA Vacutainer™ were determined on groups of six volunteers per week for 5 weeks. Five volunteers were females and twenty five were males. As shown in table 3–2, RBC AChE values ranged from 22.5 to 33.3 U/gHgb. A test value below 22.5 U/gHgb will indicate a possibility of exposure to nerve agents.

Table 3–1. Reproducibility of the Test-Mate™ kit

	Blood BChE U/ml	Blood AChE U/ml	Blood Hgb g/dl	Blood AChE U/gHgb
MEAN	2.73	4.75	16.2	29.3
SD	0.10	0.07	0.3	0.3
CV%	3.70	1.50	1.9	1.0

Table 3–2. Cholinesterase activities of the volunteer population

	Blood BChE U/ml	Blood AChE U/ml	Blood Hgb g/dl	Blood AChE U/gHgb
N	30.00	30.00	30.0	30.0
MEAN	2.51	4.36	15.6	27.9
SD	0.46	0.41	1.3	2.7
CV%	18.30	9.40	8.3	9.7
MEAN − 2SD	1.59	3.54	13.0	22.5
MEAN + 2SD	3.43	5.18	18.2	33.3

[4] Sidell, F.R. and A. Kaminskis. "Temporal Intrapersonal Physiological Variability of Cholinesterase in Human Plasma and Erythrocytes," *Clin. Chem.* 21 (1975), pp. 1961–1963.

CHAPTER 4

VERIFICATION OF NERVE AGENT EXPOSURE—MEASURING ALKYLMETHYLPHOSPHONIC ACIDS IN URINE BY GAS CHROMATOGRAPH/MASS SPECTROMETER

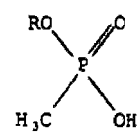
4–1. Background

In animals exposed to the toxic organophosphorus nerve agents, substantial amounts of the parent compounds are hydrolyzed to their corresponding phosphonic acids, the rest are covalently bound to enzymes and tissue proteins.[1,2,3] Analytical procedures for quantifying the hydrolyzed phosphonic acids in environmental samples have been reported by many analysts.[4,5,6,7,8] For more complex matrices such as biological samples, there has not yet been a method reported to detect these polar acids for verification of exposure. The method described in this chapter is a GC/MS method for the detection of the metabolites of three toxic organophosphorus compounds in urine (sarin, soman, and GF), extracted from a published report.[9] Urinary excretion of the metabolite is the primary elimination route for these three compounds. The major differences among them are primarily the extent and rate of excretion. Nearly total recoveries of the given doses for sarin and GF in metabolite form were obtained from the urine in rats dosed subcutaneously.[10] Soman was excreted at a slower rate with a recovery of only 62 percent. The acid metabolites can be detected in urine for 4 to 7 days after exposure in rats.

4–2. Materials and methods

a. Materials.

(1) Isopropyl methylphosphonic acid (IMPA) and pinacolyl methylphosphonic acid (PMPA) were synthesized by personnel at U.S. Army Chemical Biological Defense Agency, Aberdeen Proving Ground, MD and their methyl deuterated analogs (d_3–IMPA and d_3–PMPA) by Chemsyn Science Laboratories (Lenexa, KS). Cyclohexyl methylphosphonic acid (CMPA) was obtained by hydrolyzing GF in base as described in the literature.[1] Their respective structures are shown in figure 4–1.



COMPOUND	R
IMPA	isopropyl
PMPA	pinacolyl
CMPA	cyclohexyl

Figure 4–1. Chemical structures of IMPA, PMPA, and CMPA

(2) D_3–PMPA was used as an internal standard for both PMPA and CMPA, and deuterated analog for IMPA. Other chemicals and their respective sources are the following:

(a) Concentrated HCl, powdered anhydrous potassium carbonate, sodium chloride (NaCl) (Fisher Scientific, Pittsburgh, PA).

(b) 100 mg Bond-Elut™ C18 cartridges (Analytichem International, Harbor City, CA).

(c) 1.9 ml microfuge tube (Elkay Products Incorporated, Shrewsbury, MA).

(d) Methanol and methylene chloride (Burdick and Jackson Labs, Muskegon, MI).

(e) 18-crown-6 ether (Eastman Kodak Company, Rochester, NY).

[1] Harris, L.W., L.M. Braswell, J.P. Fleisher, and W.J. Cliff. "Metabolites of Pinacolyl Methylphosphonofluoridate (Soman) after Enzymatic Hydrolysis *in Vitro*," *Biochem. Pharmacol.* 13, (1964), p. 1129.

[2] Reynolds, M.L., P.J. Little, B.F. Thomas, R.B. Bagley, and B.R. Martin. "Relationship Between the Biodisposition of [3H] Soman and its Pharmacological Effects in Mice," *Toxicol. Appl. Pharmacol,* 80, (1985), p. 409.

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[6] Bossle, P.C., J.J. Martin, E.W. Sarver, and H.Z. Sommer. "High Performance Liquid Chromatography Analysis of Alkyl Methylphosphonic Acids by Derivatization," *J. Chromato.* 267, (1983), p. 209.

[7] Wils, E.R.J. and A.G. Hulst. "Determination of Organophosphorus Acids by Thermospray Liquid Chromatography-Mass Spectrometry," *J. Chromato.* 454, (1988), p. 261.

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[9] Shih, M.L., J.R. Smith, J.D. McMonagle, T.W. Dolzine, and V.C. Gresham. "Detection of Metabolites of Toxic Alkylmethylphosphonates in Biological Samples," *Biol. Mass Spec.* 20, (1991), p. 717.

[10] Shih, M.L., J.D. McMonagle, T.W. Dolzine, and V.C. Gresham. "Metabolite Pharmacokinetics of Soman, Sarin, and GF in Rats and Biological Monitoring of Exposure to Toxic Organophosphorus Agents," *J. Appl. Tox.* 14, (1994), p. 195.

(f) Pentafluorobenzyl bromide (PFBBr) (Pierce Chemical Company, Rockford, IL).

(g) Carbon tetrachloride (Mallinckrodt, Paris, KY).

(h) Nitrogen and helium gases (UHP grade), (Matheson Gases and Equipment, Montgomeryville, PA).

(i) Isobutane reagent gas (99.5 percent), (Specialty Products, Houston, TX).

b. Equipment.

(1) Centra-M™ centrifuge (IEC Company, Needham Heights, MA).

(2) Heating block (Reacti-Therm™, heating/stirring module; Pierce Chemical Company, Rockford, IL).

(3) Oil bath (N–EVAP™ Analytical Evaporator; Organomation Association Incorporated, South Berlin, MA).

(4) Mixer (Thermolyne Maxi Mix™; Thermolyne Corporation, Dubuque, IA).

(5) Hewlett-Packard 5970B MSD and 5890 GC (Hewlett-Packard, Wilmington, DE).

(6) Finnigan Incos 50B MS (Finnigan MAT, San Jose, CA).

(7) DB–17 and DB–5 bonded-phase capillary column (30 meters × 0.25 mm I.D., film thickness 0.25 µm; J and W Scientific, Folsom, CA).

WARNING

The chemicals involved in these procedures are toxic. Follow all safety precautions listed below.

c. Sample collection and safety. The three acid metabolites are stable compounds at neutral pH. The samples should be kept frozen to prevent bacterial growth and thawed at analysis time. If noticeable amounts of sediment or particulate matter are present, the sample should be centrifuged and only the supernatant should be used. PFBBr is a lacrimator and eye irritant. All derivatization procedures should be carried out in a ventilated hood.

d. Preparation of standard solution. Weigh about 50 mg of IMPA, PMPA, CMPA, d_3–IMPA and d_3–PMPA in individual plastic scintillation vials. Add distilled and deionized water to reach a concentration of 10 mg/ml. Dilute each stock solution to 10 µg/ml (10 µl stock solution to 10 ml water). These solutions are stable for at least 3 months if kept refrigerated.

e. Linear standard curve. Spike 1 ml control urine with the prepared standard solutions (10 µg/ml) to make six concentrations of the acids ranging from 20 to 200 ng/ml (e.g., 5 µl of standard solution added to 1 ml urine makes 50 ng/ml).

f. Sample extraction.

(1) Prepare the C18 solid phase cartridge with 2 × 1 ml of methanol and 2 × 1 ml of purified water.

(2) Add 10 µl each of the deuterated internal standard solution (d_3–IMPA and d_3–PMPA, 10 µg/ml) to 1 ml of unknown or spiked urine sample. Acidify the urine sample to approximately pH one by adding concentrated HCl and then pass it through the C18 Bond-Elut™ extraction cartridge.

(3) After rinsing with 1 ml 20 percent NaCl in 0.1 N HCl, elute the extraction cartridge with 1 ml methanol into a 1.9 ml microfuge tube containing 20 mg powdered anhydrous potassium carbonate.

(4) Evaporate the eluant to dryness at 85 °C in an oil bath under nitrogen.

(5) Add 1 ml methylene chloride containing 3 mg 18-crown-6 ether. Add 10 µl PFBBr. Heat the capped tube in a reaction block at 50 °C for 1 hour and vortex the tube every 15 minutes.

(6) Centrifuge the tube for 1 minute. Decant the organic phase to a clean tube and evaporate to dryness at room temperature with nitrogen.

(7) Reconstitute with 100 µl each of carbon tetrachloride and purified water.

(8) Inject 1 µl of the organic layer onto GC/MS for electron impact (EI) analysis.

(9) If necessary, GC/MS analysis using chemical ionization (CI) for positive and/or negative ions can be carried out to further confirm suspected samples.

g. GC/MS.

(1) Helium, the carrier gas, is set at a linear velocity of 35 cm/second (butane injection at 120 °C). The oven temperature is held initially at 60 °C for 1 minute, programmed from 60 °C to 200 °C at 20 °C/minute, held at 200 °C for 4 minutes, and then programmed from 200 °C to 260 °C at 30 °C/minute and held for 5 minutes. Splitless injections of 1 µl are made with the split delay set at 0.5 minute. The injection port temperature is set at 180 °C; split flow 40 ml/minute; transfer line temperature 280 °C and septum purge 2 ml/minute. The EI MS operating conditions are as follows: ion source pressure 1.5×10^{-5} torr; source temperature 200 °C; electron energy 70 electron volt (eV); and electron emission current 220 microamperes (µA). The MS is operated in the selected ion mode. Three fragment ions characteristic for the derivatized compound of interest are monitored (303, 277, 256 for PMPA; 303, 276, 256 for IMPA; 277, 256, and 80 for CMPA; see relative abundance of the ions in table 4–2), along with the m/z ion of 259 for the deuterated internal stan-

dard. The dwell time for each ion is 20 msec resulting in a total scan rate of 6.7 cycles/second.

(2) PMPA is composed of four stereoisomers due to the asymmetric centers at the phosphorus atom and the pinacolyl carbon. Under the chromatographic conditions employed, the stereoisomers are separated into two diastereomer pairs. Quantitation is performed by combining the integrated peak area for each peak. Calibration curves for EI are constructed by plotting the ratio of the peak area of the fragment ion at m/z 256 versus the area of the corresponding m/z 259 fragment ion of the deuterated internal standard. CMPA and IMPA produced only a single peak.

(3) Chemical ionization GC/MS analyses were performed on a Finnegan Incos 50B MS interfaced to a Hewlett-Packard 5890 GC. The GC is fitted with a 30 m × 0.25 mm I.D. DB–5 bonded-phase capillary column, film thickness 0.25 μm. Helium is set at a linear velocity of 37 cm/second (butane injection at 120 °C). The oven temperature is held initially at 40 °C for 2 minutes, programmed from 40 to 200 °C at 20 °C/minute, held at 200 °C for 4 minutes, and then programmed from 200 to 250 °C at 20 °C/minute. Splitless injections of 1 μl are made with the split delay set at 1.5 minutes. Injection port temperature is set at 180 °C; split flow 40 ml/minute; transfer line temperature 240 °C and septum purge 2 ml/minute. The isobutane chemical ionization MS operating conditions are as follows: ion source pressure 1.0 torr; source temperature 115 °C; electron energy 110 eV; and electron emission current 750 μA. The MS is operated in the selected ion mode. In positive CI mode, the MH^+ ion of the derivatized compound is monitored along with its primary fragment ion. In negative CI mode, only the methyl phosphonate anion is observed and monitored (m/z 179 for PMPA, 137 for IMPA, and 177 for CMPA). The scan rate in both positive and negative modes is 0.36 scans/second.

4–3. Results and discussion

a. Solid phase extraction. The recovery from solid-phase extraction was determined for PMPA and IMPA using ^{14}C-labeled compounds. From urine samples the values were 94 percent and 85 percent with coefficients of variation (CV) 1.7 percent and 4.5 percent for PMPA and IMPA respectively (N=6). The hydrophobic pi value of the cyclohexyl group is close to the pinacolyl group. Hence, the recovery for CMPA is expected to be similar to PMPA.

b. Linearity and reproducibility. In spiked urine samples the peak area ratios were linear over concentration ranges from 10 to 200 ng/ml for PMPA, IMPA, and CMPA with a correlation coefficient better than 0.99. The coefficient of variation of repetitive assay was less than 5 percent for urine samples (N=6). The lower quantitation limit was 1 ng/ml in urine for PMPA (CV=5.7 percent), 5 ng/ml for CMPA (CV=10.2 percent), and 10 ng/ml for IMPA (CV=9.6 percent).

c. Chromatograms.

(1) Table 4–1 shows the retention times of the perfluorobenzyl ester of the various phosphonic acids and the deuterated analogs. The retention time increases proportionately as the lipophilicity of the alkyl side chain of the methylphosphonic acid increases. The PMPAs were resolved into two pairs of diastereomer separated by 0.23 minutes. Control human urine (N=10) did not show any interfering peaks at the region where PMPA and CMPA appeared. An interfering peak did appear at the retention time of IMPA when m/z 256 ion was monitored but not 303 ion. For quantitation purposes a correction from the control sample has to be made if possible. If m/z 256 ion was detected in the EI spectra for unknown samples, CI spectra should be performed to confirm the finding.

(2) The EI mass spectra of all three derivatized acids shared several characteristics. A molecular ion was absent for all three derivatized acids. The base peak for all three compounds was at m/z 181, a non-specific ion of the PFBBr derivatizing reagent. The relative abundance of the major ions observed in the EI mass spectra are summarized in table 4–2. The ready loss of the entire R group under EI conditions produced two fragmentation pathways designated as class specific. Both pathways produced abundant ions for all three compounds. In the first fragmentation scheme the R group was lost along with proton transfer to the remaining oxygen. In addition, protonation of the $-P=O$ oxygen also occurred. This process was observed for both the derivatized PMPA and CMPA and has been reported previously for related organophosphorus compounds.[11, 12] Unlike the other two compounds, protonation of just one of the oxygens produced a more prominent ion at m/z 276 for the derivatized IMPA. Further fragmentation with loss of the $-$PFB (pentafluorobenzyl) group followed by loss of an OH group produced ions at m/z 97 and 80, respectively. The second class specific pathway resulted from the net loss of the R group along with

[11] Occolowitz, J.L. and G.L. White. "The Mass Spectrometry of Esters of Phosphorous and Phosphonic Acids," *Anal. Chem.* 35, (1963), p. 1179.

[12] Gillis, R.G. and J.L. Occolowitz. In *Analytical Chemistry of Phosphorous Compounds,* ed. by M. Halmann, Wiley, New York, (1972), p. 313.

loss of a single fluorine atom which produced a m/z 256 ion.

(3) Compound specific fragmentation pathways where the R group was only partially fragmented or remained attached in whole to the phosphonyl backbone varied greatly among the three compounds in their relative importance. Derivatized PMPA produced several compound specific ions, but the derivatized CMPA produced virtually none. Loss of a methyl group from −R alkyl chain was observed for the pinacolyl and isopropyl compounds producing ions at m/z 345 and 303 for PMPA and IMPA, respectively. The ion abundance was very small for the pinacolyl group but very prominent for the isopropyl group. An additional compound specific pathway existed for the derivatized PMPA due to the tendency of the pinacolyl moiety to lose an isobutene group. Loss of the isobutene produced an ion at m/z 303 with further loss of the derivatizing group producing an m/z 123 ion. The methyl deuterated label attached directly to the phosphorus atom remained intact in all class and compound specific ions, indicating that the methyl group was not affected by the ionization process.

(4) Under isobutane positive CI conditions, all three derivatized acids produced a strong MH^+ ion with a relative abundance of at least 93 percent and was the base peak for both IMPA and CMPA. Other compound specific ions observed resulted from the loss of the PFB derivatizing group with protonation of both oxygens. This process was observed for CMPA and IMPA but the corresponding m/z 181 ion for PMPA was nearly absent (table 4–3). Isobutane adduct ions were also observed for all three compounds. A prominent class specific m/z 277 fragment ion was also observed for all three compounds. While the protonation of the oxygen under EI conditions also produced an m/z 277 ion, the ready availability of protons under CI conditions appeared to make this a much more predominant pathway and produced the base peak for PMPA.

(5) Monitoring of the compound specific ions in EI and CI spectra along with the retention time difference from the deuterated internal standard allows positive identification of each of these metabolites in alleged victims. The presence of closely separated diastereomer pairs produced a distinctive ion chromatogram for PMPA.

Table 4–1. Retention times of the derivatized acids

Compound	Retention times (minutes)	Difference*
PMPA	9.992, 10.213	0.034, 0.026
d_3–PMPA	9.958, 10.187	
IMPA	8.571	0.024
d_3–IMPA	8.547	
CMPA	13.292	3.300

*The difference in minutes between the retention times of the analyte and its internal standard.

Table 4–2. Electron impact mass spectra

m/z	Proposed structure	Relative abundance (%)		
		PMPA	IMPA	CMPA
A. Compound specific ions				
345	M–CH_3 (PMPA)	1	0	0
303	M–CH_3 (IMPA)/	.....	14	.....
	M–$C(CH_3)_3$ (PMPA)	20	.....	0
123	M–$C(CH_3)_3$–PFB+H (PMPA)	42	0	0
B. Class specific ions				
277	M–R+2H	25	3	15
276	M–R+H	4	10	3
256	M–RF	54	66	32
97	$CH_3P(OH)_3$	23	9	9
80	$CH_3P(OH)_2$	13	44	22

Table 4–2. Electron impact mass spectra (Continued)

m/z	Proposed structure	Relative abundance (%)		
		PMPA	IMPA	CMPA
C. Non-specific ions				
181	PFB	100	100	100
161	PFB–HF	8	10	4

Table 4–3. Positive CI mass spectra

m/z	Proposed structure	Relative abundance (%)		
		PMPA	IMPA	CMPA
A. Compound specific ions				
399	$[M+C_3H_3]^+$ (PMPA)	7	0	0
397	$[M+C_3H_3]^+$ (CMPA)	0	0	6
361	$[M+H]^+$ (PMPA)	93	0	3
359	$[M+H]^+$ (CMPA)	1	0	100
357	$[M+C_3H_3]^+$ (IMPA)	0	1	1
319	$[M+H]^+$ (IMPA)	6	100	9
179	$[M-PFB+2H]^+$ (CMPA)	0	1	72
139	$[M-PFB+2H]^+$ (IMPA)	0	23	4
B. Class specific ions				
277	$[M-R+2H]^+$	100	12	65
256	$[M-RF]^+$	4	3	9
C. Non-specific ions				
181	$[PFB]^+$	2	7	9

CHAPTER 5

VERIFICATION OF CYANIDE EXPOSURE—AN AUTOMATED MICRODISTILLATION ASSAY FOR CYANIDE IN BLOOD

5–1. Background

a. Cyanide (CN^-) is an extremely poisonous and fast acting compound that is rapidly absorbed in the blood. Its primary toxic action is the inhibition of cytochrome oxidase. Although detoxication occurs at a relatively fast rate, frequently the organism is overwhelmed and expires within minutes due to lack of cellular respiration.

b. Various methods have been used for CN^- detection in blood.[1,2] Most of them involve prolonged specimen preparation using diffusion or bubbling procedures, both of which require larger blood volumes to achieve desired sensitivity than the automated fluorometric method described here.[3] The CN^- assay methods provide direct measurement of plasma free CN^- and the stabilization of total CN^- in blood. Samples for both plasma free CN^- and blood total CN^- are assayed directly without prior isolation of CN^-, by a completely automated method requiring only 16 minutes from sampling to readout.

5–2. Materials and methods

a. Reagents for free CN^- assay.

(1) Phosphate buffer, pH 7.4, 0.05 mole (M). Add 250.0 ml of 0.2 M potassium dihydrogen phosphate (KH_2PO_4) and 197.5 ml of 0.2 M NaOH to a 1 liter (L) volumetric flask. Dilute to 1 L with deionized water.

(2) First and second diluents: phosphate buffer, pH 7.4, 0.05 M containing 1.0 ml Brij 35 per L.

(3) Recipient solution: phosphate buffer, pH 7.4, 0.05 M containing 0.1 ml Brij 35 per L.

b. Reagents for total CN^- assay.

(1) First diluent: Triton X–100, 0.5 percent. Dissolve 5.0 ml of Triton X–100 in 900 ml of physiological saline and dilute to 1 L.

(2) Second diluent: Sulfuric acid, 0.5 percent. Add 5.0 ml of concentrated sulfuric acid (H_2SO_4) to 500 ml of deionized water. Dilute to 1 L with deionized water and mix.

(3) Recipient solution: Sulfuric acid, 0.25 percent. Add 2.5 ml of concentrated H_2SO_4 to 500 ml of deionized water. Dilute to 1 L and mix. Add 0.1 ml of Brij 35 and mix.

c. Reagents for free and total CN^- assay.

(1) *Glycine-sodium chloride stock solution.* Dissolve 77.4 grams (g) glycine and 58.6 g NaCl in deionized water. Dilute to 1 L with deionized water and mix. Keep refrigerated.

(2) *Glycine buffer, pH 10.* Add 63.0 ml of stock glycine-sodium chloride solution to 850 ml deionized water. Adjust pH to 10.0 with 1.0 M NaOH. Dilute to 1 L with deionized water. Refrigerate when not in use and prepare fresh weekly.

(3) *Chelate: Potassium bis (5-sulfoxino) palladium (II).* Dissolve 120 mg chelate in 1 L deionized water and add 1 ml Brij 35. Refrigerate when not in use and prepare fresh weekly. Prepare according to Hanker, et. al.[4]

(4) *Magnesium chloride.* Dissolve 12 g magnesium chloride ($MgCl_2$) $6H_20$ in 1 L deionized water and add 1 ml Brij 35. Refrigerate when not in use and prepare fresh weekly.

(5) *Isotonic saline.* Dissolve 9.0 g NaCl in 1 L deionized water.

(6) *CN^- stock solution, 0.04 M (1040 µg/ml).* Dissolve 196 mg of sodium cyanide (NaCN) and dilute to 100 ml with 0.1 M NaOH. Refrigerate when not in use and prepare fresh monthly.

(7) *CN^- standards.* Dilute appropriate volumes of CN^- stock solution with 0.01 M NaOH. Refrigerate when not in use and prepare fresh weekly.

(8) *4-Dimethylaminophenol (4-DMAP), 0.5 g/dl.* Dissolve 0.05 g 4-DMAP in 10 ml deionized water. Refrigerate when not in use and prepare fresh weekly.

(9) *Brij 35 and Triton X–100.* These surfactants are obtained from Sigma Chemical Company, St. Louis, MO.

[1] Feldstein, M. and N.C. Klendshoj. "The Determination of Cyanide in Biological Fluids by Microdiffusion Analysis," *J. Lab. and Clin. Med.* 44 (1954), pp. 166–170.

[2] Lundquist, P., H. Rosling, and B. Sorbo. "Determination of Cyanide in Whole Blood, Erythrocytes, and Plasma," *Clin. Chem.* 31 (1985), pp. 591–595.

[3] Groff, W.A., Sr., F.W. Stemler, A. Kaminskis, H.R. Froehlich, and R.P. Johnson. "Plasma Free Cyanide and Blood Total Cyanide: A Rapid Completely Automated Microdistillation Assay," *Clin. Toxicol.* 23 (1985), pp. 133–163.

[4] Hanker, J.S., A. Gelberg, and B. Witten. "Fluorometric and Colorimetric Estimation of Cyanide and Sulfide by Dimasking Reactions of Palladium Chelates," *Anal. Chem.* 30 (1958), pp. 93–95.

d. Apparatus. The analytical system consists of the following modules from Technicon Instruments Corporation, Tarrytown, NY.

(1) Sampler IV.

(2) Proportioning pump IV.

(3) Dialyzers, one each, 6-, 12-, and 24-inch lengths with type C membranes.

(4) Silicon oil heating bath at 116 °C.

(5) Fluoronephelometer III with Corning Glass filters excitation-#5970 with maximum transmission at 370 nm and emission-#4308 and #3389 with maximum transmission at 470 nm and sharp cut off below 400 nm.

(6) Flatbed recorder.

(7) An Eppendorf Model #5412 microcentrifuge.

e. Analyses manifold (fig 5–1).

(1) The manifold is constructed using the apparatus listed above.

(2) Standards, plasma, and blood specimens are sampled with one saline wash cup between each at 60/hour resulting in an effective sampling rate of 30/hour. In the free CN^- assay, plasma is mixed with and dialyzed against pH 7.4 phosphate buffer. In the total CN^- assay, the blood is mixed with saline containing Triton X–100 which hemolyzes the erythrocytes and then is acidified with the addition of 0.5 percent sulfuric acid. The sample stream is dialyzed against 0.25 percent sulfuric acid. Additional air is introduced in the recipient stream before distillation.

(3) The distillation assembly is constructed of glass-to-glass fittings joined with heat shrink tubing. After distillation, the liquid and vapor phases are separated in the manifold trap (#116–0110) and a portion of the latter segments the glycine buffer stream where the CN^- is absorbed.

(4) An aliquot of the solution is added to the chelate stream where the CN^- demasks the nonfluorescent potassium bis (5-sulfoxino) palladium (II). The resulting 8-hydroxy-5-quiniline sulfonic acid coorditates with magnesium to form the fluorescent chelate.

f. Sample collection.

(1) Blood should be collected with syringes or Vacutainers™ containing heparin or EDTA. Immediately transfer 250 µl to a 0.5 ml sample cup containing 5 µl of 4–DMAP. Cap, mix and set aside for total CN^- assay. Immediately centrifuge 1 ml of blood for 1 minute at 15,000 rpm, quickly remove plasma and sample for free CN^-. CN^- bound to plasma albumin is unrecoverable.

(2) After the free CN^- analyses are completed, change to the total CN^- reagents and perform the total CN^- assay on the blood specimens previously set aside.

g. Fluorometer adjustment. All modules are powered up and the manifold filled with reagents.

(1) Set Sample Aperture to Position C.

(2) Set Standard Calibration control to 0.00.

(3) Set Baseline control to 0.00.

(4) Set Reference Aperture to Position 3.

(5) Set Function switch to the Reference Position.

(6) Adjust the light pipe to give a 15 chart units reading on the recorder strip chart.

(7) Reset Function switch to No Damping.

(8) Reset Sample and Reference Apertures and Standard Calibration for desired sensitivity (table 5–1).

(9) Adjust baseline to 0 chart units.

h. Standardization. Standards are prepared by diluting the stock CN^- solution with 0.01 M NaOH. The concentration range of standards should cover the expected CN^- range of the samples. The full set of standards is assayed at the beginning and end of the analyses of the unknowns. An intermediate standard should be sampled periodically as well to provide a measure of precision and correction for gain associated with continuous flow methods.

i. Precision and recovery. Appropriate concentrations of CN^- were prepared by adding µl volumes of stock CN^- to 0.01 M NaOH and to heparinized blood containing 20 µl/ml of 4–DMAP. Each CN^- concentration was analyzed 12 times during one working day.

5–3. Results

a. Standardization. Linear curves are obtained for each range of CN^- aqueous concentrations when µM values are plotted versus fluorescence on cartesian coordinate graph paper. Blood CN^- readings fall within the 95 percent confidence limits of the aqueous standards.

b. Precision. The highest coefficient of variation for aqueous CN^- concentrations ranging from 4000 µM to 1 µM is 5.8 percent and occurs at the lowest concentration measured. Fluorescence units were used to establish the precision in table 5–2.

c. Recovery. The correlation of added versus measured CN^- in blood is excellent (r=0.999). Recovery and other statistical data are presented in table 5–3.

d. Stabilizing total CN^- in blood.

(1) CN^- added to blood is rapidly bound to Hgb and to plasma proteins. Subsequent analyses show the loss of recoverable CN^- with time.

(2) Hgb is converted to methemoglobin when 4–DMAP is added to blood.[5] Methemoglobin is further converted to cyanmethemoglobin in the presence of CN^-. In this form the CN^- is stable for at least 3 hours at ambient room temperature and at least for a week when refrigerated.

5–4. Discussion

a. The automated system for the determination of free and total CN^- in blood incorporates dialysis, distillation, absorption, and the production of a fluorescent chelate.

b. The introduction of additional air in the recipient stream before distillation increases the air-liquid surface area and enhances the volatilization of CN^- into the air segmented liquid stream.

c. A minimum amount of the surfactant (Brij 35) is added to the recipient stream to prevent foaming in the manifold trap after distillation. The distillation-absorption assembly is an all glass construction, eliminating contact with plastic tubing, which would result in reaction and loss of CN^-.

d. The wide range of sensitivities is achieved by interchanging dialyzer sizes and adjusting the sample aperture of the fluorometer. Changes in flow rates, predilution of samples, or reagent concentration changes are unnecessary.

e. Thiocyanate and thiosulfate, which interfere in many CN^- methods, do not affect the CN^- measurement.

f. Plasma-free CN^- levels are obtained by combining rapid techniques for plasma separation, to minimize loss of CN^-, followed by rapid automated analysis.

g. In the total CN^- analysis, a fraction of the endogenous Hgb is oxidized to methemoglobin by the addition of 4–DMAP to the blood specimen. The procedure described in this report results in the production of 60 percent methemoglobin within 15 to 30 minutes and stabilizes blood CN^- for at least 3 hours at room temperature.

Table 5–1. Conditions for measuring three concentration ranges of CN^-

Range	Dialyzer (inches)	CN^- μM	Apertures		Standard calibration
			S	R	
I	6	≦4000	2	4	-0.5
II	12	≦200	3	4	-1.2
III	24	≦10	4	4	-4.0

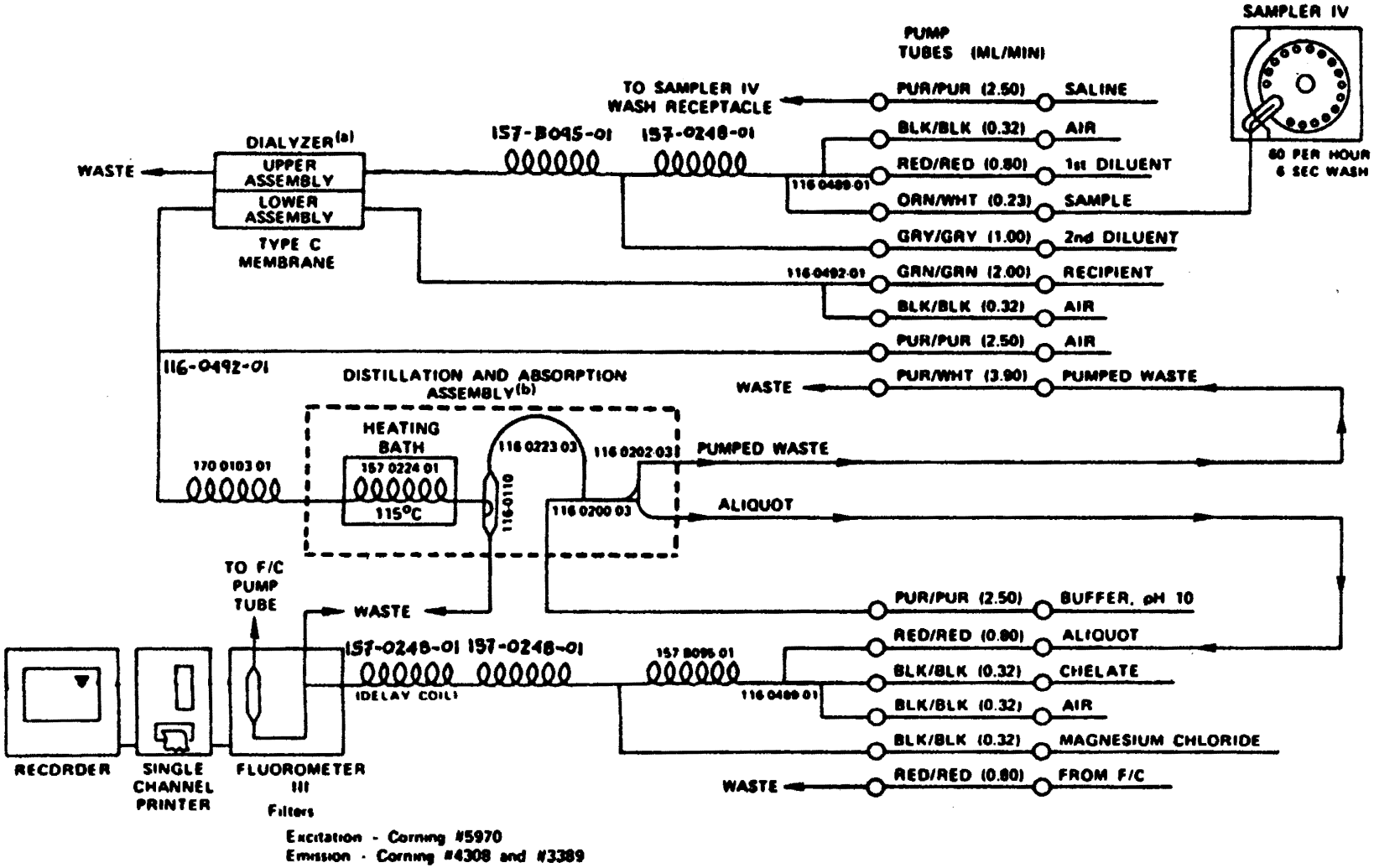
Table 5–2. Precision of CN^- measurements in aqueous solution

Range	CN^- μM	Fluorescence units		
		X	SD	CV%
I	4000	90.79	0.61	0.7
	3000	71.61	0.61	0.9
	2000	49.03	0.60	1.2
	1000	26.03	0.38	1.5
	500	13.35	0.10	0.8
	400	10.75	0.11	1.0
II	200	90.62	0.66	0.7
	100	42.83	1.37	3.2
	50	20.99	0.67	3.2
	40	16.35	0.78	4.8
	20	7.35	0.25	3.4
III	10	89.97	1.59	1.7
	5	45.70	0.97	2.1
	2	14.89	0.48	3.2
	1	6.48	0.38	5.8

[5] Kiese, M. and N. Weger. "Formation of Ferri-hemoglobin With Aminophenols in the Human for the Treatment of Cyanide Poisoning," *Eur. J. Pharmacol.* 7 (1969), pp. 97–105.

Table 5–3. Precision and recovery of CN^- from blood in vitro

Range	Added CN^- (μM)	CN^-	Measured		μM
		X	SD	CV%	Recovered (%)
I	2500	2511.40	48.08	1.9	100.5
	1000	1063.90	18.04	1.7	106.4
	500	513.30	7.94	1.6	102.7
II	200	202.70	2.57	1.3	101.4
	50	50.50	0.84	1.6	101.0
	20	18.64	0.46	2.7	93.1
III	10	10.12	0.38	3.8	101.2
	5	5.10	0.18	3.5	102.0
	2	2.16	0.05	2.3	108.0



(a) DIALYZER PLATES

6 inch plates for total cyanide assay:
Upper assembly PN 177-8077-01C
Lower assembly PN 177-8008-01C

24 inch plates for free cyanide assay:
Upper assembly PN 157-8369-01C
Lower assembly PN 157-8370-01C

(b) DISTILLATION AND ABSORPTION ASSEMBLY

All fittings within dotted lines are connected glass to glass using heat shrinkable tubing as sleeving. (See text and Fig. 2)

Figure 5–1. Flow diagram of manifold for assay of blood total CN^- and plasma free CN^-

APPENDIX A
REFERENCES

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GLOSSARY

Section I
Abbreviations

AChE
acetylcholinesterase

ATCh
acetylthiocholine

BChE
butyrylcholinesterase

BTCh
butyrylthiocholine

C
centigrade

ChE
cholinesterase

CI
chemical ionization

cm
centimeter(s)

CMPA
cyclohexyl methylphosphonic acid

CN^-
cyanide

CV
coefficient of variation

dl
deciliter

DTNB
5,5′-dithio-bis (2-nitrobenzoic acid)

d_3-IMPA
deuterated IMPA

d_3-PMPA
deuterated PMPA

d_8-TDG
octa-deuterated thiodiglycol

4-DMAP
4-dimethylaminophenol

EDTA
ethylenediaminetetraacetic acid

EI
electron impact

eV
electron volt

g
gram(s)

GC
gas chromatograph

GF
cyclohexylmethylphosphonofluoridate

HCl
hydrochloric acid

HD
sulphur mustard

HFBA
heptafluorobutyric anhydride

Hgb
hemoglobin

H_2SO_4
sulfuric acid

I.D.
inner diameter

IMPA
isopropyl methylphosphonic acid

kg
kilogram(s)

KH_2PO_4
potassium dihydrophosphate

L
liter(s)

M
mole

mg
milligram(s)

$MgCl_2$
magnesium chloride

ml
milliliter(s)

mm
millimeter(s)

mM
millimole(s)

MRICD
Medical Research Institute of Chemical Defense

MS
mass spectrometer

MSD
mass selective detector

msec
millisecond(s)

m/z
mass to charge ratio

µA
microampere(s)

µg
microgram(s)

µl
microliter(s)

µm
micrometer(s)

µM
micromole(s)

N
normal solution

NaCl
sodium chloride

NaCN
sodium cyanide

NaOH
sodium hydroxide

ng
nanogram(s)

nm
nanometer(s)

PFB
pentafluorobenzyl

PFBBr
pentafluorobenzyl bromide

PMPA
pinacolyl methylphosphonic acid

RBC
red blood cell

rpm
revolutions per minute

RSD
relative standard deviation

SD
standard deviation

TDG
thiodiglycol

TDP
thiodipropanol

TFA
trifluoromethyl anhydride

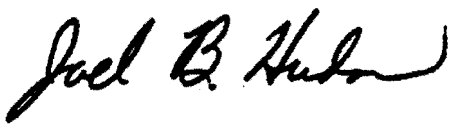
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millimeters of mercury

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unit(s)

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