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McManamon, Timothy Gerard

AN ASSAY FOR THE MEASUREMENT OF GLYCOLATE IN SERUM FOLLOWING ETHYLENE GLYCOL POISONING

The Ohio State University PH.D. 1985

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AN ASSAY FOR THE MEASUREMENT OF GLYCOLATE IN SERUM FOLLOWING ETHYLENE GLYCOL POISONING

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Timothy Gerard McManamon, B.A.

* * * * *

The Ohio State University

1985

Reading Committee:

Keith E. Richardson, Ph.D.

Martha I. Walters, Ph.D.

Gerald P. Brierley, Ph.D.

Howard A. I. Newman, Ph.D.

Carl E. Speicher, M.D.

Approved by

Keith E. Richardson

Adviser, Department of Physiological Chemistry

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VITA

January 30, 1959.	Born - Phoenix, Arizona
1981.	B.A., University of Arizona; Tucson, Arizona
1981-1892	St. Joseph's Hospital and Medical Center, Phoenix, Arizona
1982-1983	Teaching Associate, Department of Chemistry, The Ohio State University
1983.	Clinical Chemistry Laboratory, The Ohio State University
1984.	Research Associate, Department of Pathology, The Ohio State University
1984-1985	Teaching Associate, Department of Physiological Chemistry, The Ohio State University

PUBLICATIONS

McManamon TG, Walters MI. A sensitive, specific, and rapid spectrophotometric method for the determination of carboxyhemoglobin. Clin Chem 30, 976 (1984). Abstract Presented at the AACC National Convention July, 1984.

FIELDS OF STUDY

Major Field: Clinical Chemistry

Studies in Methods Development. Dr. K. E. Richardson, Dr. M. I. Walters

Studies in Metabolism of Ethylene Glycol. Dr. K. E. Richardson

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ABBREVIATIONS

ADH	Alcohol Dehydrogenase
BUN	Blood Urea Nitrogen
CO_2	Carbon Dioxide
EG	Ethylene Glycol
GAO	Glycolic Acid Oxidase
GC	Gas Chromatograph
HCO_3^-	Bicarbonate
HPLC	High Performance Liquid Chromatography
H_2SO_4	Sulfuric Acid
LD	Lactate Dehydrogenase
mEq	Milliequivalent
mOsm	Milliosmole
MS	Mass Spectrophotometer
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
TLC	Thin Layer Chromatography

INTRODUCTION

Ethylene glycol is used to alter the vapor pressure of a liquid thus lowering the melting point (freezing point) and raising the boiling point. The major use of this chemical is as an antifreeze for automobiles. In 1984, 4.84 billion pounds of ethylene glycol were produced in the United States making it the 28th most abundantly produced chemical (Webber).

This very useful chemical although itself non-toxic is metabolized to several toxic intermediates following ingestion. These intermediates include glycolaldehyde, glycolate, glyoxylate, and oxalate. Glycolate has been demonstrated to be the toxic intermediate in ethylene glycol poisoning (Chou and Richardson). The results of such poisonings are severe metabolic acidosis, followed by cardiac, central nervous system, and renal damage. The initial step in the oxidation of ethylene glycol is catalyzed by alcohol dehydrogenase. This is the same enzyme which catalyzes the oxidation of ethanol. The current basis of treatment is to inhibit the metabolism of ethylene glycol by competitive inhibition with ethanol. In order for this treatment to be successful it must be initiated within 6 hrs

of the time the EG was ingested and the amount of EG must not be excessive.

Research is currently being conducted to develop a treatment regimen for patients who have ingested large quantities of ethylene glycol and for those whose treatment has been delayed. The research is being directed toward the elimination of the accumulated glycolate. A clinical assay to measure glycolate would be required to identify patients in need of this therapy and to monitor the effectiveness of such therapy. the frequency of ethylene glycol poisoning is quite low; therefore, it would be advantageous to develop this assay in such a way as to be compatible with a currently available method for measuring ethylene glycol. Furthermore, since ethanol is commonly measured in the clinical laboratory it was decided to develop the assay using a gas chromatographic column which was known to separate ethanol and ethylene glycol. This would allow a clinical laboratory to have the capability of measuring glycolate without investing in equipment and supplies which would rarely be used. In the course of this research an ultrafiltration technique was employed in preparing the sample for analysis. This ultrafiltration was then applied to the measurement of ethanol as an extension of this research.

Chapter I

LITERATURE REVIEW

Ethylene glycol (1,2-ethanediol) is used in commercial products such as antifreezes, deicers, detergents, paints, lacquers, polishes, inks, fibers, cosmetics, solar collection systems, and as a hydraulic brake fluid (Scherger, et al.; Smith). Antifreeze and deicers contain 70 to 95% ethylene glycol (EG). Ethylene glycol has a specific gravity of 1.12, a boiling point of 197°C, and is relatively nonvolatile (Smith). Ethylene glycol is a colorless, odorless, water-soluble liquid which has a sweet taste and produces a warm sensation when swallowed (Frommer and Ayus). Since the antifreeze tastes good, there is no inherent deterrent to consumption; therefore, large quantities can easily be consumed (Scherger, et al.). The opalescent blue color added to antifreeze is supposed to identify it as nonpotable (Smith); however, this is not an effective deterrent.

Initially, because of the work of Bachem, Page, and Hanzlik et al. it was believed EG was a harmless chemical. These individuals ingested varying amounts of EG without noticeably harmful effects. Hanzlik et al. strongly recommended the use of EG as a replacement for glycerol in medications. As early as 1930, there was some concern expressed

as to the safety of EG (anonymous). Ethylene glycol toxicosis became well accepted in 1937, when at least 76 persons died after ingesting "elixir of sulfanilamide Massengill" which contained diethylene glycol (Geiling and Cannon). These poisonings prompted Holck to investigate the toxicity of ethylene glycol as well as diethylene glycol and both were found to be poisonous to animals. Subsequently, EG and diethylene glycol were abandoned as medicinal additives. Ethylene glycol poisoning, however, has persisted as a problem because of accidental or intentional ingestion of antifreeze. Some 40 to 60 individuals die each year from ingesting this chemical, and it is the most common poisoning in dogs and cats (Thrall et al.).

The lethal dose of EG in humans is about 100 ml (Jacobsen, et al 1982a) (approximately 1.5 mL/Kg body weight). However, a case has been reported (Vites, et al.) in which a victim ingested 500 mL and recovered following aggressive treatment which was initiated 6 hours after the ingestion. In dogs the lethal dose is as high as 4-5 mL/Kg body weight (Hewlett, et al.).

METABOLISM OF ETHYLENE GLYCOL

Ethylene glycol is a gastric irritant (Thrall, et al.) which is rapidly absorbed from the gastrointestinal tract (Scherger, et al.). The volume of distribution of EG is 0.7-0.8 L/Kg human body weight (Peterson, CD, et al., Jacobsen, et al 1982a) with a half life of approximately three hours

(Peterson, CD, et al., Brown et al.). The peak serum concentration is reached between one and four hours and EG is undetectable after 24 hours (Brown et al.).

Ethylene glycol itself is non-toxic but is metabolized to a number of toxic intermediates (Chou and Richardson). In 1961, Gessner et al. proposed the pathway shown in fig. 1 for the metabolism of EG. The first step in this pathway is the oxidation of EG to glycolaldehyde by alcohol dehydrogenase (ADH) which is present in the liver (Coen and Weiss). Catalase may also play a role in this first metabolic step of EG oxidation. The glycolaldehyde is then oxidized to glycolate by aldehyde dehydrogenase or aldehyde oxidase. The glycolate is oxidized to glyoxylate by glycolic acid oxidase (GAO) and lactate dehydrogenase (LD). There are a number of pathways by which glyoxylate can be oxidized. These include conversion to CO_2 by 2-oxoglutarate:glyoxylate carboligase (Koch and Stokstad); to oxalate by xanthine oxidase, LD, or GAO (Sawaki et al.; Richardson and Tolbert); or to glycine by alanine:glyoxylate aminotransferase and ornithine:glyoxylate aminotransferase glutamate oxalate transaminase (Thompson and Richardson; Periano and Pitot). At low doses the majority of a radioactive dose of ethylene glycol is eliminated as CO_2 whereas at higher doses a greater portion of the radioactivity is found in the urine (Gessner et al.) indicating that at higher doses the enzyme catalyzing the CO_2 production becomes saturated. There are

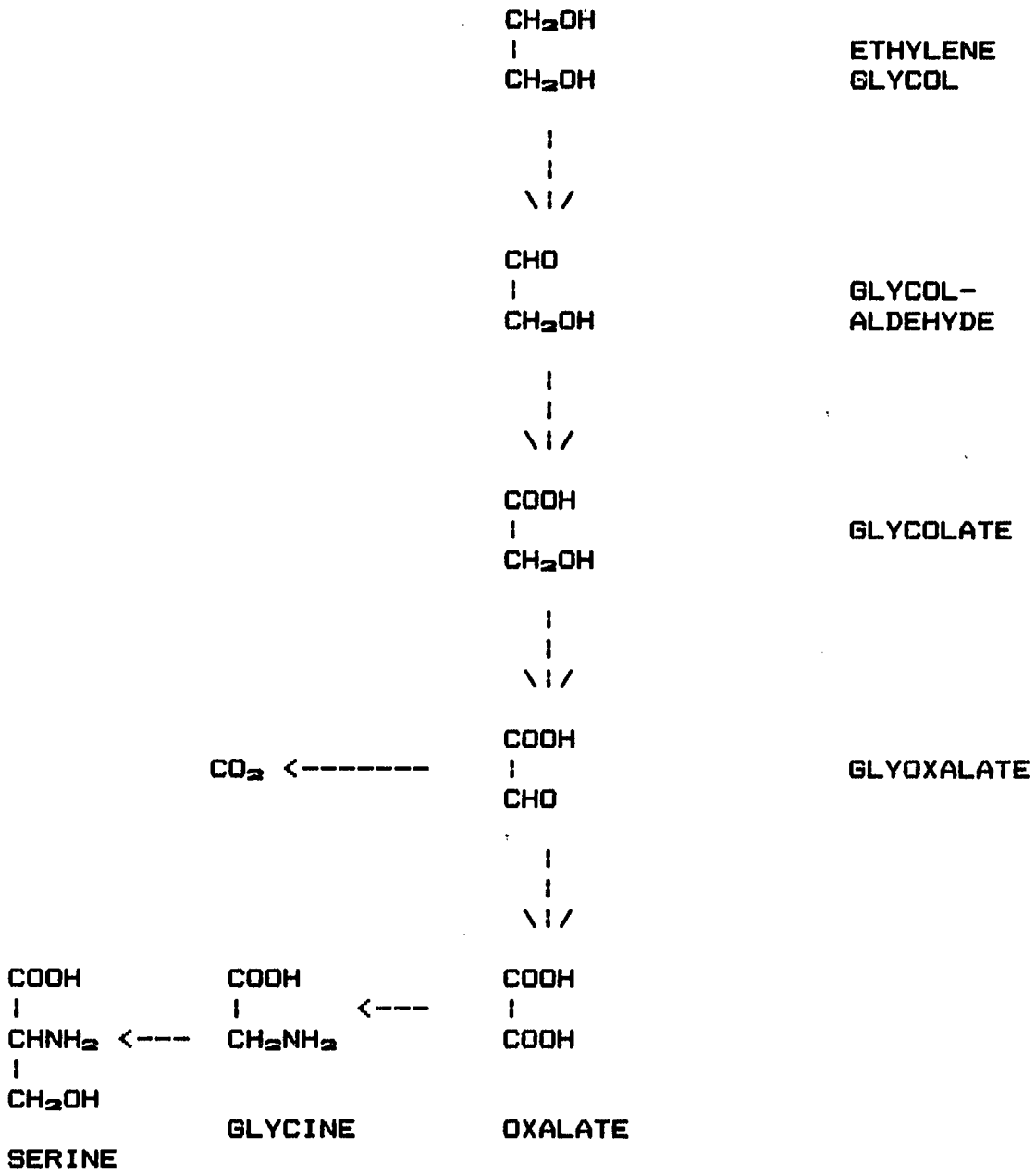


FIGURE 1

The pathway for the oxidation of ethylene glycol (Gessner).

two rate-limiting steps in the metabolism of EG. The first is the initial oxidation by ADH, and the second is the conversion of glycolate to glyoxylate by GAO (Chou and Richardson). There is a direct correlation between the concentration of glycolate in urine and mortality in rats (Chou and Richardson). Glycolate accumulation mediates the toxic effects of ethylene glycol. Richardson demonstrated that rats force fed ethylene glycol and glycolate following partial hepatectomy had a lower mortality rate than rats with intact livers (table 1). The toxicity of glyoxylate, however, increased following partial hepatectomy. The rats which have undergone partial hepatectomies have decreased liver metabolism; therefore, less of the administered substance is metabolized than in rats having intact livers. If the substance administered is itself toxic, the mortality rate will increase following partial hepatectomy because the compound is present longer at a higher concentration; thus, it has a greater toxic effect. This is the case with glyoxalate. However, if the compound is metabolized to a toxic substance, then less of the toxic substance is formed in the partially hepatectomized rats with a decreased toxic effect. These observations indicate that there are two possible mechanisms for the toxicity of ethylene glycol: (1) the metabolites are responsible for the toxicity, as indicated by the decreased toxicity of ethylene glycol and glycolate following partial hepatectomy; (2) the liver serves to

TABLE 1

Mortality rates of rats fed ethylene glycol or its metabolites following partial hepatectomy (Richardson).

	Portion of Liver Removed		
Treatment	0	1/3	2/3
None	0	0	0
Ethylene Glycol	53%	27%	13%
Glycolate	27%	7%	13%
Glyoxalate	20%	40%	73%

detoxify the glyoxylate; therefore, when a portion of the liver is removed the ability to remove the glyoxylate is decreased resulting in increased toxicity. Farinelli and Richardson observed that in totally hepatectomized rats the production of oxalate is reduced to essentially zero but the oxidation of EG to CO_2 is only decreased by 45%. This would indicate the possibility of an alternative pathway for the oxidation of EG to CO_2. Since glycolate and glyoxylate can be converted to oxalate in the hepatectomized rat these intermediates would not be involved in this alternative pathway.

The toxicity of ethylene glycol varies between species. EG is more toxic to species which excrete a greater portion of the dose as oxalate in the urine, whereas EG it is least toxic to those which excrete more of the dose as CO_2. Rabbits and guinea pigs are resistant to EG poisoning, cats are much more prone to EG poisoning, and rats are intermediate between the two extreme responses (Gessner et al.).

Other alterations in normal metabolism following EG poisoning include a decreased storage of liver glycogen in rats due to increased glycogenolysis with increased activity of glycogen phosphorylase (Rajagopal and Ramakrishnan). Furthermore, there is decreased glycogen synthetase activity in the liver so that there is less glycogen produced, an increase in blood glucose concentrations partly due to a decrease of hexokinase activity in the liver, and increased

metabolism of glucose via the hexosemonophosphate shunt.

METABOLISM OF ETHANOL

Ethanol is metabolized by a pathway similar to that of EG. Alcohol dehydrogenase, present in the cytosol of hepatocytes, catalyzes the conversion of ethanol to acetaldehyde. NAD serves as the acceptor of reducing equivalents in this reaction. The production of NADH generates an excess of reducing equivalents in the cytosol which leads to increased concentrations of lactate (Jorfeldt and Juhlin-Dannfelt). These excess reducing equivalents are transported into the mitochondria. The normal source of reducing equivalents for mitochondria is the tricarboxylic acid cycle; however, in the presence of reducing equivalents from ethanol metabolism, the mitochondria use the reducing equivalents from ethanol rather than from the oxidation of two carbon fragments from fatty acids via the tricarboxylic acid cycle. The main source of energy for the liver, fatty acids, is therefore, not utilized in the presence of ethanol. This in turn results in the deposition of fat in the liver from dietary and endogenous sources. Over a long term period this accumulation of fat causes the development of alcoholic fatty liver, which is the first stage of alcoholic liver disease. The K_m, the concentration a half maximal velocity, of ADH for ethanol is 0.4 mmol/L whereas the K_m for EG is 53 mmol/L (Holman). This difference is the basis for treating EG poisoning with ethanol because the difference in K_m

allows for the competitive inhibition of EG metabolism by ADH. The affinity of the enzyme for ethanol is so much greater than for EG that the ethanol is metabolized instead of the EG.

The second step of both ethanol and EG metabolism is catalyzed by aldehyde dehydrogenase, which is a mitochondrial enzyme. In the case of ethanol, this enzyme catalyzes the oxidation of acetaldehyde to acetate. As with ADH, aldehyde dehydrogenase is NAD linked and the reaction results in furthering the excess of reducing equivalents present in the mitochondria. In addition, acetaldehyde is a highly reactive compound which binds to proteins resulting in hepatotoxicity (Nomura and Lieber).

Acetate is the end product of ethanol metabolism in the liver. Acetate has been demonstrated to increase cardiac output, myocardial contractility, and coronary blood flow (Liang and Lowenstein). In addition, acetate inhibits lipolysis which leads to a decrease in circulating free fatty acids. Since free fatty acids are a major fuel source for peripheral tissues this decrease can have significant metabolic consequences.

There are two other metabolic pathways for the oxidation of ethanol; the microsomal ethanol-oxidizing system and peroxidation by catalase. The microsomal ethanol-oxidizing system converts ethanol to acetaldehyde by a cytochrome P-450 mediated reaction in the smooth endoplasmic

reticulum. The acceptor of the reducing equivalents in this pathway is NADP (Lieber and DeCarli, Ohnishi and Lieber). As with the other two pathways the peroxidation by catalase yields acetaldehyde with water serving as the acceptor of the reducing resulting in the production of hydrogen peroxide. This latter pathway is not believed to be functionally important in vivo (Bartlett).

CLINICAL MANIFESTATIONS

The clinical findings in EG poisoning are divided into three stages based on the organs involved (Moriarty and McDonald, Frommer and Ayus). Stage one is the effect on the central nervous system with manifestations occurring 30 minutes to 12 hours following ingestion. The symptoms during stage one include the appearance of inebriation without the odor of alcohol on the breath, slurred speech, somnolence, opthalmoplegias, nystagmus, strabismus, papilledema, depressed reflexes, and generalized or focal seizures. With large doses, patients may become comatose. Nausea and vomiting may also be present (Frommer and Ayus; Scherger, et al.). In addition, Willis et al. reported the following signs and symptoms: thirst, salivation, anorexia, drowsiness, depression of the sensorium, ataxia, spastic miosis, and electroencephalographic changes. During this stage there is usually a massive leukocytosis (10,000-40,000/mm^3) (Frommer and Ayus) and, occasionally, a pseudomeningitis characterized by white blood cells in the spinal

fluid and xanthochromia (Smith). These symptoms are most severe 6 to 12 hours after ingestion, the time span when EG metabolites are at their maximum concentrations (Frommer and Ayus).

During stage 2, the patient exhibits tachypnea, tachycardia, mild hypertension, and cyanosis. Congestive heart failure and pulmonary edema may also occur, usually with a fatal outcome. The etiology of these changes in the cardiovascular system is not clear. Microscopically, there are widespread petechial hemorrhages in the pleura, heart, and pericardium (Frommer and Ayus).

In stage 3, the renal sequelae of EG appears which includes flank pain and varying degrees of renal impairment including anuria, proteinurea, hematuria, pyuria, and azotemia. There is also deposition of calcium oxalate crystals in the kidneys. About 45% of the humans (Jacobsen et al. 1982b) and dogs poisoned with EG have oxalate crystals appearing in their urine, while only 18% of poisoned cats have crystalluria (Thrall et al.). The predominant crystal type is the calcium oxalate monohydrate species known as the Whewellite or 'hemp seed' form (Godolphin et al., Jacobsen et al. 1982b). These short prismatic crystals are similar in appearance to hippurate, which can be misleading because oxalate crystals are usually the dihydrate species which are tetragonal dipyramidal in shape. One needs to be aware of this when examining the urine so that oxalate crystals are

not mistaken for hippurate.

It is believed that the renal tubular damage is not the result of the oxalate crystals but is caused by the metabolites: glycolaldehyde, glycolate, and glyoxylate. Roberts and Seibold have demonstrated that monkeys intoxicated with small amounts of EG develop mild glomerular damage and azotemia despite the absence of crystals. In addition, they demonstrated that glycolaldehyde, glycolate, and glyoxylate all cause significant renal damage. Renal damage usually appears 2 or 3 days after ingestion of EG. Renal function returns to normal in the majority of patients (Frommer and Ayus).

A suspected case of chronic poisoning was reported in 1950 following the exposure of a group of women to vapors which were believed to be EG. These women had symptoms of nystagmus and recurrent attacks of unconsciousness (Rajagopal et al.). Wills et al., however, exposed prisoners to EG vapors for 22 hours per day for 30 days and found no harmful effects. Ethylene glycol is not absorbed through the skin (Smith); therefore, if chronic exposure has an effect it must be via the respiratory system. Wills et al. determined that before a person could absorb enough EG through the respiratory system there would be severe pain in the tracheobronchial tree which would cause the person to leave the area.

LABORATORY FINDINGS

The laboratory findings in ethylene glycol toxicity include severe metabolic acidosis with a pH often below 7.0 and HCO_3^- concentrations as low as 7 mEq/liter (Clay and Murphy). This acidosis is not readily corrected by the administration of HCO_3^-. Serum electrolytes other than bicarbonate remain within the reference range until kidney damage has occurred (Clay and Murphy).

The cardinal feature of EG toxicity is the presence of both an anion and osmolal gap. Sodium and potassium normally account for about 95% of the serum cations, while chloride and bicarbonate account for approximately 85% of the anions. Therefore, when these electrolytes are measured and the sum of the anions is subtracted from the sum of the cations, there is a slight difference which is referred to as the "anion gap". This gap is normally in the range of 15 mEq/L; however, it is increased when an unmeasured anion is present in excess as is the case in EG poisoning when glycolate accumulates. In EG poisoning the anion gap is typically greater than 30 mEq/L (Jacobsen et al. (1982c)). In addition, the anion gap is raised because the excess H^+, given up by the glycolic acid, reacts with the HCO_3^- to form carbonic acid, is rapidly converted to CO_2, and eliminated by the lungs. This loss of bicarbonate leads to a further increase in the anion gap (Brown et al.).

Osmolality is a measure of the number of particles in a given weight of solvent. In normal individuals the osmolality can be accounted for by the sodium, urea nitrogen, and glucose concentrations, and can be calculated by the following formula:

$$\text{Serum osmolality} = (2 \times Na) + BUN/2.8 + glucose/18. \quad \text{[Equation 1.1]}$$

The plasma osmolality is measured by freezing point depression, where each osmole of solute depresses the freezing point by 1.86C° (Smith). The reference range for serum osmolality is 280 to 295 mOsm/Kg water and the difference between the measured and calculated serum osmolality (osmolal gap) is normally less than 10 mOsm/Kg water (Brown et al.). In EG poisoning, the osmolal gap is between 20 and 50 mosmol/Kg water with a mean of 35 mosmol/Kg water. The osmolal gap for methanol poisoning is much greater, being between 37 and 127 mosmol/Kg water with a mean of 81 mosmol/Kg water (Jacobsen et al. (1982c)).

Rajagopal et al. have demonstrated a decrease in bone calcium and phosphorus when rats were given EG for a period of one week period. They reported increased urinary excretion of calcium and decreased phosphorus excretion. In accord with these findings, serum calcium was decreased and phosphorus was increased. They theorized that oxalate, produced by the metabolism of EG, precipitated as calcium oxalate and was excreted in the urine, thus increasing the

urinary calcium. This in turn lead to a lowering of the serum calcium which resulted in the release of parathyroid hormone which mobilizes calcium and phosphorus from bone and decreases the excretion of calcium while increasing the excretion of phosphorus in the urine; however, this effect on the kidneys was obliterated by the loss of calcium as calcium oxalate. The theory that bone resorption is occurring is supported by an increase in the amount of hydroxyproline, an indicator of bone resorption and calcium mobilization, excreted in the urine.

Post mortem findings in EG poisoning include generalized congestion of the airways and superficial ulceration of the lower esophagus. The stomach contains thick greyish black mucoid fluid adherent to the mucosa (Bowen et al.). The brain reveals a pattern of diffuse, uniform petechial hemorrhages throughout both hemispheres and the cerebellum with evidence of mild cerebral edema (Moriarty and McDonald). Moriarty and McDonald reported the following tissue concentrations of EG at autopsy: blood 32 mg/dL, urine 56 mg/dL, stomach contents 34 mg/dL, intestinal contents 21 mg/dL, brain 26 mg/dL, kidney 17 mg/dL, liver 15 mg/dL, pancreas 13 mg/dL, and bile 9.4 mg/dL.

Histological findings in the central nervous system following death show a mild inflammation reaction on the subarachnoid space with polymorphonuclear cells and an occasional calcium oxalate crystal. In the cardio-vascular

system, the myocardial fibers appear normal with very few envelop shaped oxalate crystals in the small interstitial blood vessels. The lungs reveal patchy collapse and congestion of the lung parenchyma, with oxalate crystals in several medium sized vessels. The striking feature of EG poisoning is a very wide distribution of oxalate crystals throughout the renal parenchyma. These crystals are massed together as sheaves, conglomerated together within the lumen of the tubules, or attached to the epithelial linings which are compressed without necrosis (Bowen et al.).

GLYCOLATE CONCENTRATION FOLLOWING ETHYLENE GLYCOL POISONING

Hewlett et al. poisoned six dogs with EG in order to determine the concentration of glycolate after EG poisoning. Blood and urine samples were collected at periodic intervals following the poisonings. The highest serum concentration of glycolate found in this study was 0.9 mg/ml in a dog given 12 ml/Kg of antifreeze. Glycolic acid excretion was highest in the first 6 hours being approximately 16 mg/hr. They reported that serum glycolate concentrations greater than 0.3 mg/mL may be fatal to dogs. They demonstrated that glycolate was detectable in the dog's serum within hours of EG poisoning and remained elevated for several days. Clay and Murphy demonstrated a rise in glycolate concentration to approximately 15 mEq/L (1.1 mg/mL) over a 16 hour time span following the administration of EG to monkeys. In addition, they were able to demonstrate that glycolate was present in

the blood and urine of a human poisoned by EG; however, they were unable to quantitate the concentration present.

Kasidas and Rose have reported a reference mean for glycolate from a group of 12 men and 12 women of 0.012 mg/mL. The mean of the men was slightly higher than of the females, 0.014 and 0.011 mg/mL respectively.

TREATMENT

Treatment of EG poisoning involves three strategies: decreasing the absorption, inhibiting the metabolism, and/or removing the toxic intermediates. The absorption can be decreased by gastric lavage, induction of emesis, administration of charcoal, or cathartics (Scherger et al.). The metabolism of EG can be inhibited by administering a competitive inhibitor of ADH, such as ethanol (Peterson et al., Wacker et al.) or pyrazole (Van Stee et al., Chou and Richardson). An ethanol concentration of 15 mmole/L (0.07 g/dL) is sufficient to inhibit metabolism of up to 25 mmole/L (0.16 g/dL) of EG (Jacobsen et al 1982a). To achieve an ethanol concentration of 0.1 g/dL Freed et al. suggests giving a conscious 70 Kg male patient 40 g of ethanol as three 1 oz (29.6 mL) shots of 40% whiskey. Peterson CD, et al. suggest a maintenance dose of no more than 20% ethanol, to avoid gastritis, every hour and adjusted according to the blood alcohol concentration.

Pyrazole, another potent inhibitor of ADH, is a hepatotoxic agent (Van Stee et al.). Van Stee contends that the

use of pyrazole in conjunction with fluid, vitamin, and steroid supportive therapy is more effective in treating EG poisoning than ethanol because successful treatment can be started as late as six hours after ingestion.

In the presence of ethanol or pyrazole, the amount of EG metabolized is decreased; thus, the amount of toxic intermediates formed is decreased (Chou and Richardson). In addition, the development of the metabolic acidosis is inhibited under these circumstances (Clay and Murphy). These findings would indicate that the metabolic acidosis is caused by one of the metabolites namely glycolic, glyoxylic, or oxalic acid (Clay and Murphy). Clay and Murphy have demonstrated that only glycolic acid concentrations correlate with the degree of metabolic acidosis. In addition, the oxidation of EG by ADH would increase the cytoplasmic NADH/NAD ratio which could cause an increase in the concentration of a number of acids which require NAD for the enzymatic oxidation. One example of this is lactate which is known to be somewhat increased in patients intoxicated with EG (Clay and Murphy).

Holman et al. have demonstrated that propylene glycol, 1,2-butanediol, and 1,3-butanediol are effective antidotes to EG poisoning in the rat. 1,2-butanediol is not used because it has a significant delayed toxicity. Ethanol and pyrazole have lower therapeutic ranges than propylene glycol, 1,2-butanediol, and 1,3-butanediol.

Jacobsen et al. (1982a) demonstrated that EG is highly dialysable and have reported good results using hemodialysis in the treatment of EG poisoning. They advocate the use of hemodialysis in conjunction with ethanol therapy in all patients with EG poisoning. This treatment not only prevents the metabolism of EG but also removes it from the body more rapidly than the kidneys can. The half-life of EG is reduced from 17 hours when ethanol is used alone to 2.5 hours when hemodialysis is combined with ethanol therapy. (Peterson CD, et al.). Toxic intermediates as well as the EG can be removed by dialysis. The dialysis fluid can be supplemented with ethanol so that the alcohol concentration of the blood is not depleted.

QUANTITATION OF GLYCOLATE

Chalmers and Watts reported a GC procedure for the quantitation of various acids in urine, one of which was glycolic acid. This procedure requires that the acids first be extracted from the urine by column chromatography. The acids are derivatized with O-ethyl oximine which requires a 4 hour incubation. The extract is then freeze-dried over night and the residue is trimethylsilated and injected onto the GC. They report recoveries for glycolate of between 99 and 115%. Chalmers and Watts advocate confirming all positive results for urine glycolate be confirmed by mass spectrometry. The authors suggest that this procedure may also be applied to deproteinized plasma. The intended use

of this procedure is for the screening of inborn metabolic errors in humans. When used in such a manner the time involved in performing this laborious procedure is of little consequence and the need for absolute confirmation of the result by GC/MS is obvious based on the consequences of a missed diagnosis. However, this procedure would not be feasible for use in an emergency toxicology laboratory for confirming EG poisoning.

Niederwieser et al. reported a colorimetric method for the quantitation of glycolic acid in urine for use in the diagnosis of primary hyperoxaluria. In this procedure the urine is first passed through two ion exchange columns. The eluate is acidified and evaporated to a syrupy residue. The Chromotropic acid reagent is added, the solution boiled, and then diluted with distilled water. The absorbance is then measured at 578 nm. This procedure is quite acceptable for it's stated use because the use of two ion exchangers eliminates the majority of the interfering substances present in urine. This procedure has not been adapted to serum or plasma.

An enzymatic procedure for measuring glycolate has been developed by Kasidas and Rose. In this procedure, GAO isolated from spinach leaves is used to catalyze the oxidation of glycolic acid to glyoxylic acid and hydrogen peroxide. The hydrogen peroxide is then measured by use of the Trinder reaction using horseradish peroxidase. The enzyme

also catalyzes the oxidation of lactate; therefore, the lactate concentration in the plasma must be measured by another method and subtracted from the glycolate result. They report a recovery of added glycolate of 100%. This procedure can be performed in a much shorter time than those mentioned above but the cross reactivity with lactate is a disadvantage.

Bais et al. applied this enzymatic method to a centrifugal analyzer and to a Technicon Auto Analyzer and report that ascorbate, oxalate, ethylene glycol, glyoxylate, and glycolaldehyde all interfere. This method would be unacceptable for measuring glycolate following an EG poisoning and would be of questionable value for measuring glycolate in hyperoxaluria which is the stated objective of the authors.

Hewlett et al. described HPLC and GC/MS procedures for measuring glycolate in urine and serum. In these procedures the glycolate is extracted from acidified serum or urine with methyl ethyl ketone, the solvent is evaporated, and the glycolate redissolved in ethyl acetate. The extract is derivatized with O-p-Nitrobenzyl-N,N'-diisopropylisourea and requires a 2 hour incubation. The linear range is reported to be 0.01-6.0 mg [sic]. The retention time for glycolate was 14 min. They use the HPLC procedure to screen for glycolate and then confirm all positive results with GC/MS. The disadvantages of this procedure are the long incubation

time following the extraction and the need to confirm all positive results by GC/MS.

As stated in the introduction, the objective of this research project was to develop a simplified clinical assay for the quantitation of glycolate which is compatible with a currently available method for measuring EG. There are a number of qualitative and quantitative methods for determining EG (Doedens). These include thin layer chromatography (Mayline), spectrophotometry (Rajagopal and Ramakrishnan; Russell et al.), enzymatic assays (Eckfeldt and Light), gas chromatography (Porter and Auansakul; Bost and Sunshine; Peterson and Rogerson; Robinson and Reive) and high performance liquid chromatography (Gupta et al.). The GC method of Bost and Sunshine is the most promising for measuring glycolate. There are several reasons for this: first, it is a simple method, second, the same column can be used to measure alcohols which must also be monitored following EG poisoning, and third, Gastrin et al. used a similar column to measure spinal fluid lactate, which has a chemical structure similar to glycolate.

Chapter II

ASSAY DEVELOPMENT

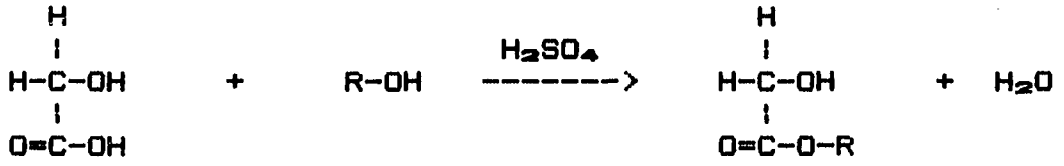
MATERIALS AND METHODS

The gas chromatographic column used in these experiments was an 8 foot stainless steel column containing Carbopack B, 60-80 mesh, coated with 5% Carbowax 20M, Clinical Packing 1-1766 - Blood Alcohol, (Supelco, Inc., Bellefonte, PA 16823). This is the same type of column that Bost and Sunshine used to measure ethylene glycol and Anthony et al. used to measure alcohols. In addition, this column is similar to the FFAP column used by Gastrin et al. which is Carbowax Carbopack treated with nitroerephtalic acid; therefore, it was possible that glycolate which has a similar structure to lactate (figure 2) could also be measured using this column.

The assay was initially developed with aqueous solutions of glycolate. These samples were derivatized using methanol and sulphuric acid (figure 3). The solution was then heated in a water bath, and the methyl ester extracted with chloroform and injected onto the GC column. From these experiments the chromatographic conditions listed in table 2

FIGURE 2

Structural formulas showing the similarity of glycolate and lactate.



R = CH_3- or CH_3-CH_2-

FIGURE 3

Derivitization reaction used in the analysis of glycolate.

TABLE 2

Chromatographic conditions used in the assay of glycolate.

Oven temperature:	150°C
Detector:	Flame ionization
Detector temperature:	220°C
Injector temperature:	220°C
Carrier gas:	Helium
He flow rate:	25 mL/min
Air flow rate:	200 mL/min
H_2 flow rate:	30 mL/min
Attenuation:	16 (0-2.00 min) 4 (2.01-6.00 min)

were determined.

CHOICE OF ALCOHOL

The alcohols: methanol, ethanol, n-propanol, and n-butanol were evaluated for use in the esterification reaction. In these experiments, a 1.5 mg/mL aqueous solution of glycolate was analyzed using the conditions in table 2. Methanol yielded a peak of 540 height counts at 3.3 min. Ethanol yielded a peak of 1220 height counts at 5.76 min. n-Propanol yielded a peak of 563 height counts at 11.5 min. n-Butanol, as would be expected, was not miscible with the aqueous components; therefore, this sample was not injected. Since the highest peak was obtained with ethanol this alcohol was chosen for the esterification reaction.

CHOICE OF SOLVENT

Chloroform, ethyl acetate, carbon disulfide, and tetrahydrofuran were evaluated for use as solvents for the extraction of the derivative following derivitization. Chloroform yielded a large solvent peak which returned to baseline before the glycolate eluted. Ethyl acetate also yielded a large solvent peak but there was a second peak which eluted in the region where glycolate eluted. For this reason, ethyl acetate was not used as the solvent. When carbon disulfide was used, no peaks were detected for glycolate. Tetrahydrofuran presented two problems. First, the tetrahydrofuran was miscible with the aqueous phase and secondly when tetrahydrofuran was injected onto the column directly

there was a large rise in baseline starting at approximately 6 min. Chloroform, which was the solvent used by Gastrin et al. for the extraction of lactate, was the only solvent of those tested which was acceptable for extracting the glycolate derivative.

INTERNAL STANDARDIZATION

An internal standard is commonly used in chromatographic procedures to correct for any inconsistencies in the derivitization, extraction, or injection processes. Ideally the internal standard must undergo the same reactions as the analyte species and interact with the column in a similar fashion; therefore, the choice of compounds for use in this assay are limited to those compounds which have a carboxylic group in order to be esterified and either a hydroxyl or an amine group to interact with the column. The possibilities tested were beta-hydroxypropionic acid, beta-alanine, and alpha-hydroxybutyric acid. None of these compounds were satisfactory for the following reasons.

Beta-hydroxypropionic acid eluted from the column at approximately 8 min when esterified with methanol. It was determined; however, that some of the beta-hydroxypropionic acid was retained on the column and carried over to the next analysis. Injecting water onto the column at 200°C following several injections of the beta-hydroxypropionic acid resulted in numerous peaks eluting. In addition, injecting water following an injection of derivatized

beta-hydroxypropionic acid resulted in the elution of a peak corresponding to the internal standard of significant height; therefore, this compound would not work as an internal standard because of the carryover.

Beta-alanine was not detected as eluting from the column.

Alpha-hydroxybutyric acid eluted from the column at about 13 min when derivatized with ethanol. There was no evidence of carryover. A linearity study was conducted and there was a linear relationship between the peak height ratio and concentration. However, when a precision study was conducted using aqueous as well as calf serum based standards the alpha-hydroxybutyric acid peak was far more variable than the glycolate peak. When the use of an internal standard was eliminated and the precision study was conducted the precision was acceptable and better than when the internal standard was being used.

PREPERATION OF PROTEIN FREE FILTRATE

After the assay was initially developed using aqueous samples, the use of serum samples was attempted. As would be expected when the H_2SO_4 was added to the calf serum there was a large amount of protein precipitation which prevented further steps in the esterification. Initially the sample was centrifuged to remove the precipitated protein but the volume of supernatant was not sufficient to perform the esterification; therefore, it was necessary to remove the

serum proteins prior to the esterification process. Both chemical and mechanical means were evaluated to prepare this filtrate.

The chemical methods which were attempted were precipitation of the proteins with trichloroacetic acid and phosphotungstic acid. The trichloroacetic acid produced a clear filtrate; however, the trichloroacetic acid caused interference with the chromatography. This was demonstrated by analyzing a blank water sample after an appropriate amount of trichloroacetic acid was added and esterified. This sample resulted in a elevated baseline. Precipitation with phosphotungstic acid caused a large loss of sensitivity possibly the result of the large volume of aqueous phase relative to the organic phase which resulted in decreased partitioning of the glycolate into the organic phase.

The two mechanical devices which were evaluated were the Millipore Calcium Free Filters and the Amicon Micropartition System. Both of these devices were found to be acceptable in that both produced an ultrafiltrate which when esterified produced a chromatogram similar to the aqueous samples. The Millipore Calcium Free Filters are no longer being produced; therefore, the Amicon Micropartition System was used in all experiments using serum samples.

SUMMARY OF THE GLYCOLATE PROCEDURE

After conducting the above experiments the following procedure was used for the remainder of the experiments.

Into a 5 mL conical test tube, 200 uL of protein free ultrafiltrate or aqueous standard was pipetted. To this was added 50 uL of 50% H_2SO_4 and 200 uL of absolute ethanol. The contents of the tube were mixed with a vortex and heated in a heating block at 100°C for 5 min and cooled for 5 min at room temperature. Following this, 100 uL of chloroform was added to the tube which was then capped with a ground glass stopper and mixed with a vortex for 10 sec. The tube was centrifuged for 5 min at 2500 RPM and the aqueous layer was removed and discarded. Three microliters of the chloroform layer was injected onto the GC. An example of the resulting chromatograph is given in figure 4. The peak at 3.087 min was glycolate and the peak at 3.504 min was the endogenous lactate present in the calf serum.

COLUMN EFFICIENCY

Column efficiency (Willard) was calculated based on the chromatogram in figure 4. The number of theoretical plates was calculated as follows:

$$N_{glycolate} = 5.54\ (V_R'/W_{1/2})^2 \qquad \text{[Equation 2.1]}$$

where: $N_{glycolate}$ is the number of theoretical plates for the separation of glycolate.

V_R' is the retention volume or retention time.

$W_{1/2}$ is the width of the peak at half height in terms of volume or time depending on the units V_R' are expressed.

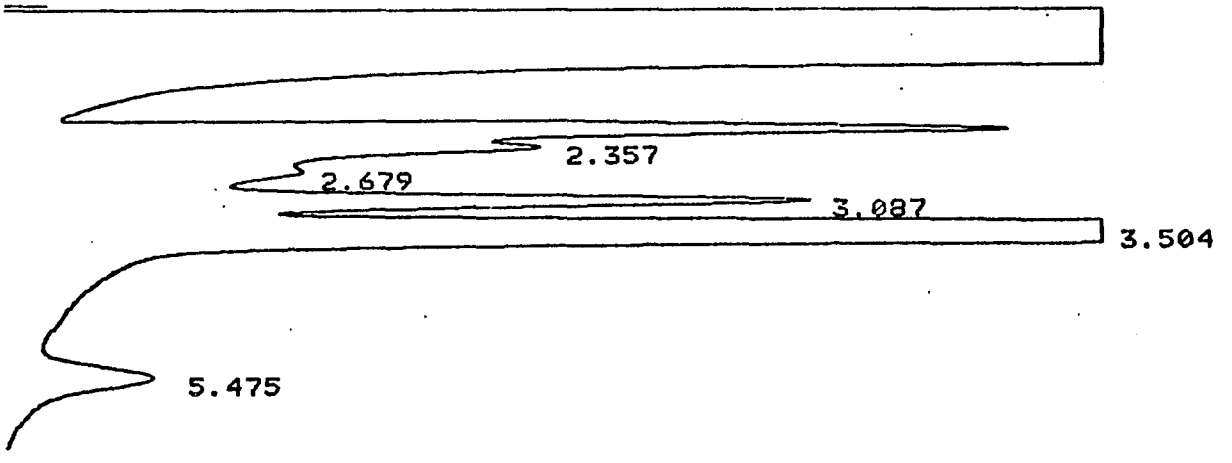


FIGURE 4

An example of the chromatogram of calf serum containing 0.3 mg/mL of glycolate. The peak at 3.087 min. is due to glycolate and the peak at 3.504 min. is due to endogenous lactate in the calf serum.

$N_{glycolate}$ = 5.54 ((3.087 min)(60 sec/min)/9.95 sec)^2

$N_{glycolate}$ = 1919.7

The height equivalent of the theoretical plate was calculated as follows:

$H=L/N_{glycolate}$ [Equation 2.2]

Where: H is the height equivalent of the theoretical plate.

L is the length of the column in mm.

H = (8 ft)(304.8 mm/ft)/1919.7

H = 1.27 mm.

The resolution between the glycolate and lactate peaks was calculated as follows:

$R = (V_{R,2} - V_{R,1})/(0.5\ (W_2 + W_1))$ [Equation 2.3]

Where: R is the resolution between the two peaks.

$V_{R,2}$ is the retention volume of the lactate peak.

$V_{R,1}$ is the retention volume of the glycolate peak.

W_2 is the width of the lactate peak at the baseline.

W_1 is the width of the glycolate peak at the baseline.

R = (35.04 mm - 30.87 mm)/(0.5 (14 mm + 8 mm)

R = 0.38

LINEARITY

Initially a linearity study was conducted using aqueous solutions of glycolate ranging in concentration from 1.50 mg/mL to 0.19 mg/mL (figure 5). The procedure shows acceptable linearity to at least 1.15 mg/mL. Least squares analysis resulted in a slope of 1088 and a y intercept of - 111 with a correlation coefficient of 0.99.

A linearity study was then conducted using serum based samples from 0.30 to 1.5 mg/mL. The procedure was linear to at least 0.90 mg/mL glycolate (figure 6, 7). Analysis by least squares of all the points resulted in a slope of 1629 and a y intercept of -327 with a correlation coefficient of 0.96 while least squares analysis of the linear points resulted in a slope of 1431 and a y intercept of -195 with a correlation coefficient of 1.0.

STANDARDIZATION

For analysis of patient and animal samples the assay was calibrated with standards which were prepared from a stock solution of 15 mg/mL glycolate. This solution was prepared by weighing 0.375 g of glycolate into a 25 mL volumetric flask and diluting to volume with blank calf serum (Colorado Serum Co).

Working standards were prepared in 50 mL quantities in the following concentrations: 0.90, 0.60, 0.30 mg/mL. These samples were analyzed in the calibration mode using the external standard method of the Varian 401 data system

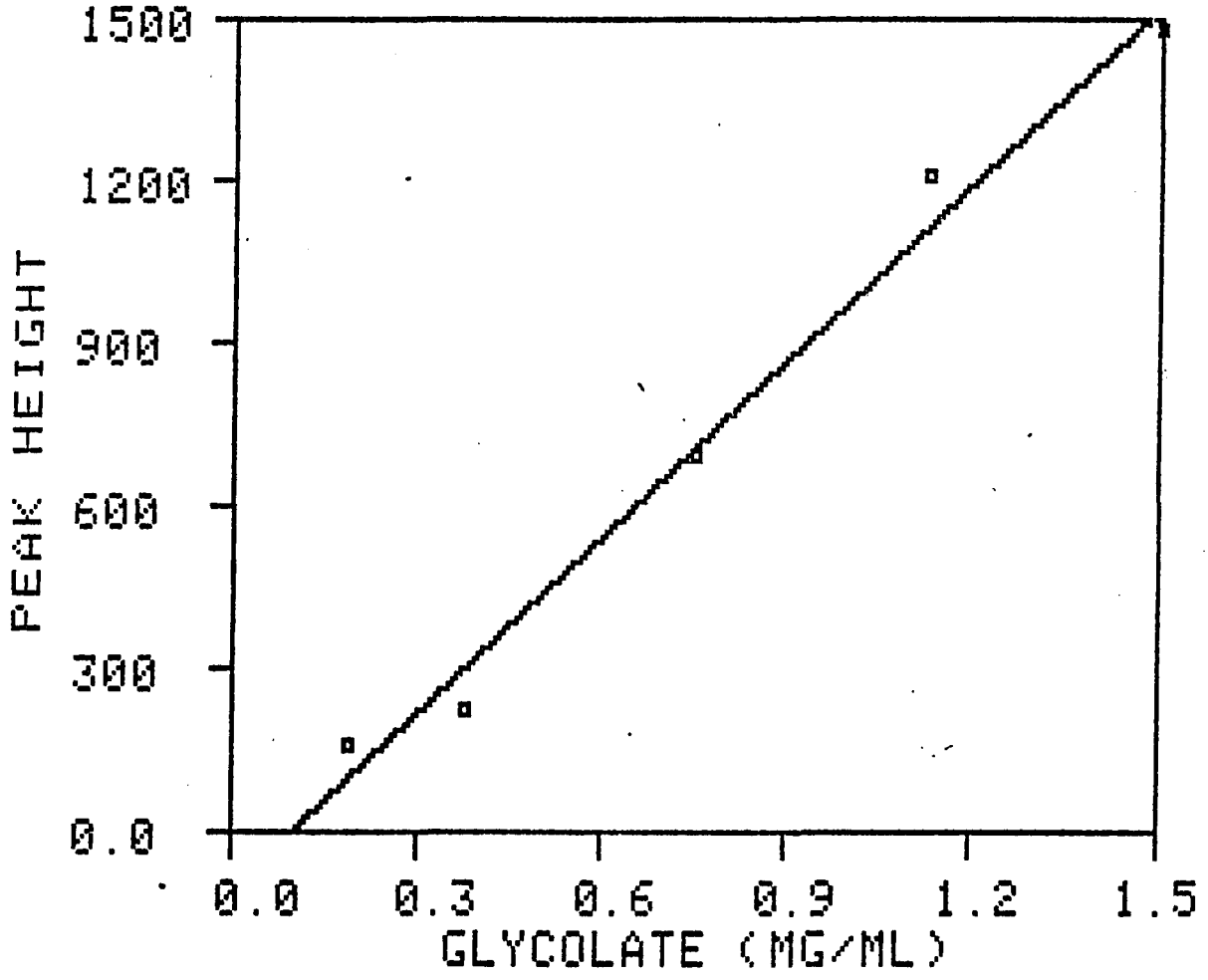


FIGURE 5

A plot of peak height vs concentration of glycolate in aqueous solutions showing linearity to at least 1.15 mg/mL.

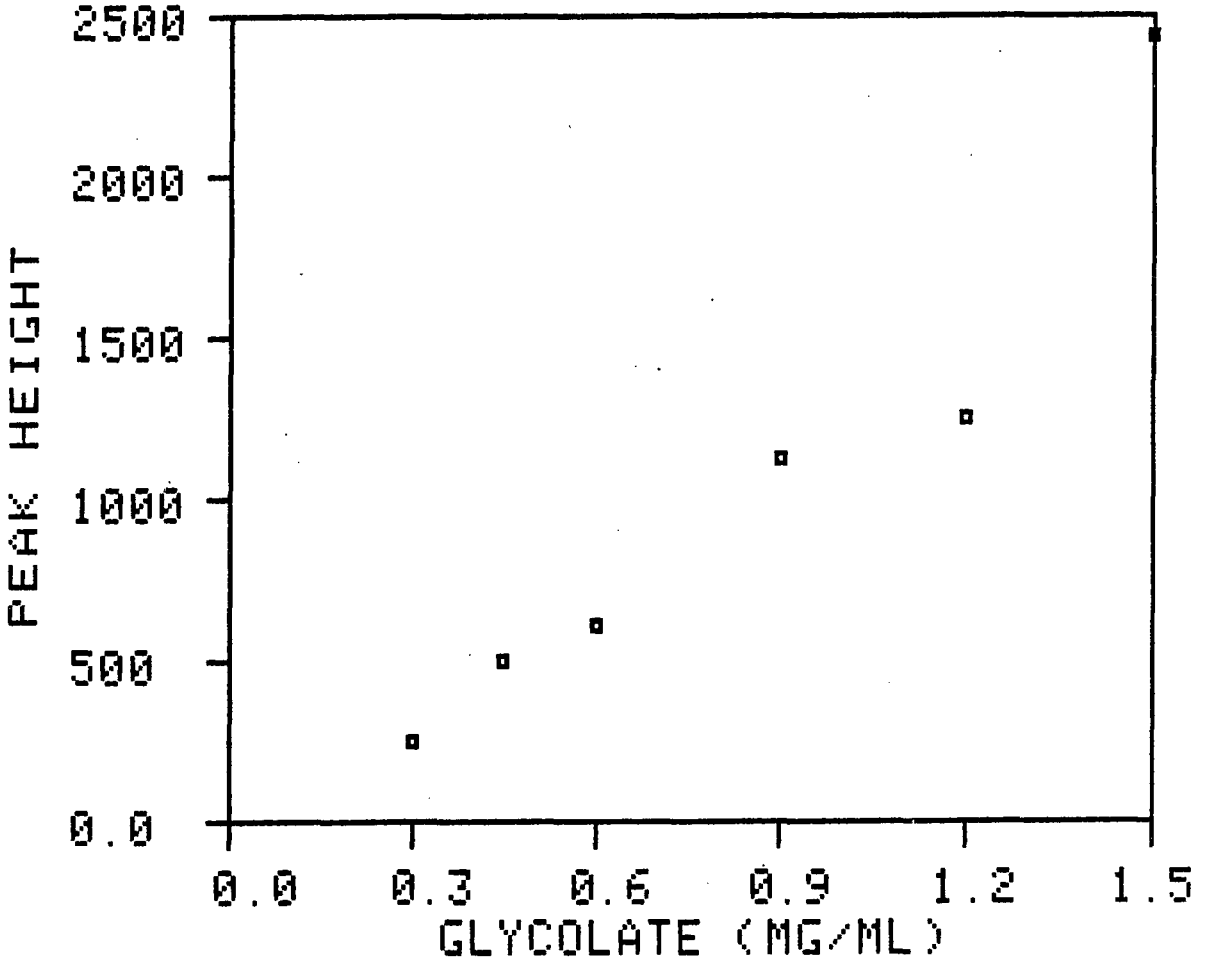


FIGURE 6

A plot of peak height vs concentration of glycolate in calf serum showing linearity to at least 0.90 mg/mL glycolate.

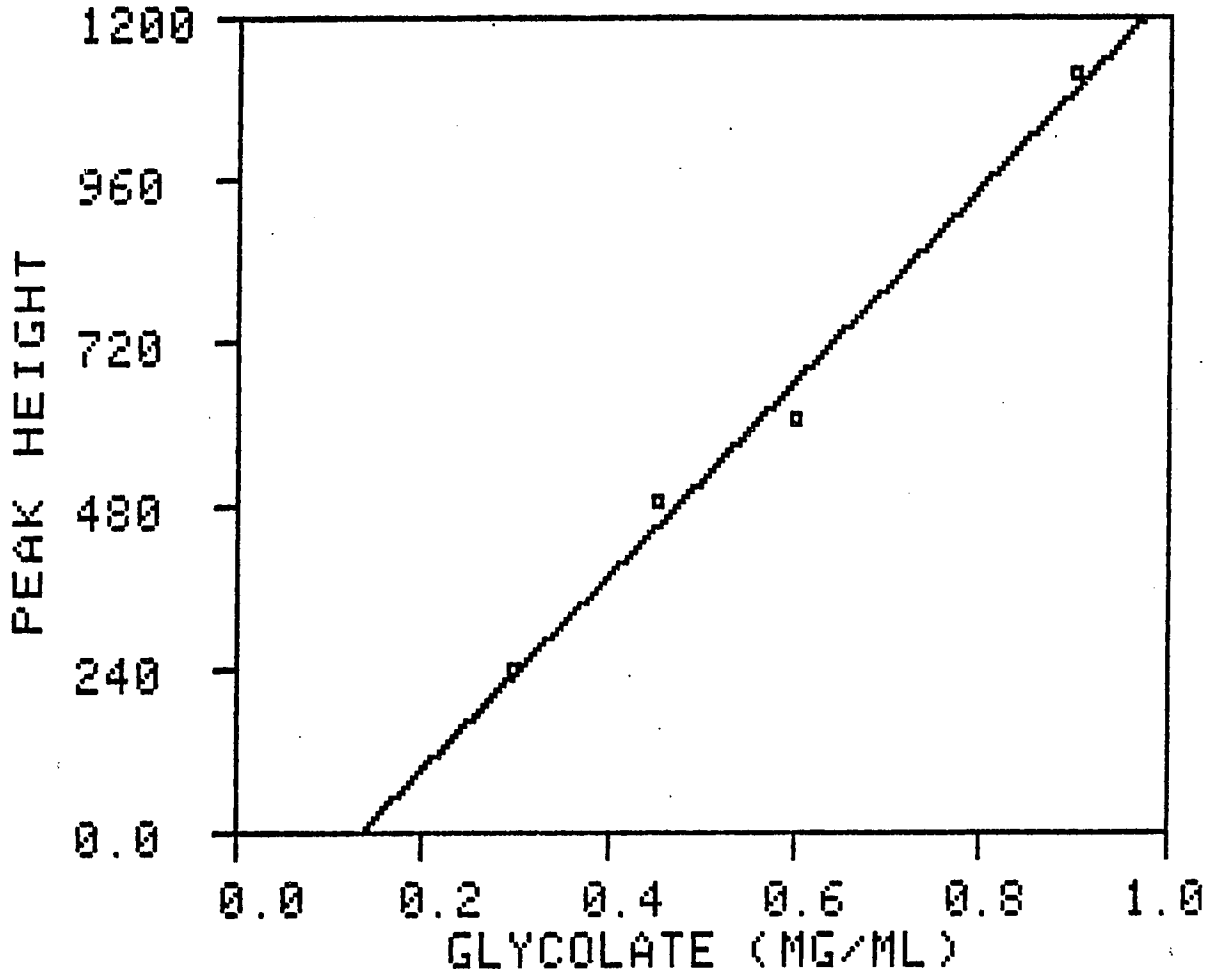


FIGURE 7

A plot of peak height vs concentration of glycolate in calf serum over the linear range of the assay.

(Appendix A). The results of unknown samples were calculated using the average result factor calculated by the 401 data system (Appendix A and B). These standards were used in all experiments unless otherwise noted.

Figure 8 is a plot of the working standards from a typical analysis. The least squares data for this plot are slope of 5728, y intercept of 81, and correlation coefficient of 0.9999.

LIMIT OF DETECTION

The lower limit of detection was determined by diluting the 0.30 mg/mL glycolate standard to concentrations of 0.03, 0.015, and 0.009 mg/mL of glycolate and analyzing as described above. In order for the 401 data system to recognize a peak, the signal to noise ratio must be greater than 2. The 0.015 mg/mL standard was the lowest standard which gave a peak height sufficient to allow the 401 Data System to calculate a valid result.

PRECISION

A preliminary precision study was conducted using aqueous calibration standards which had been prepared from a 30.0 mg/mL stock solution of glycolate. The concentrations of the standards were 1.13, 0.75, 0.38 mg/mL. The result factors were averaged in all cases and used for the calculation of the unknowns. In each analysis, 5 aqueous samples of 0.38 mg/mL glycolate were analyzed to determine within run precision. The study consisted of five analyses. The

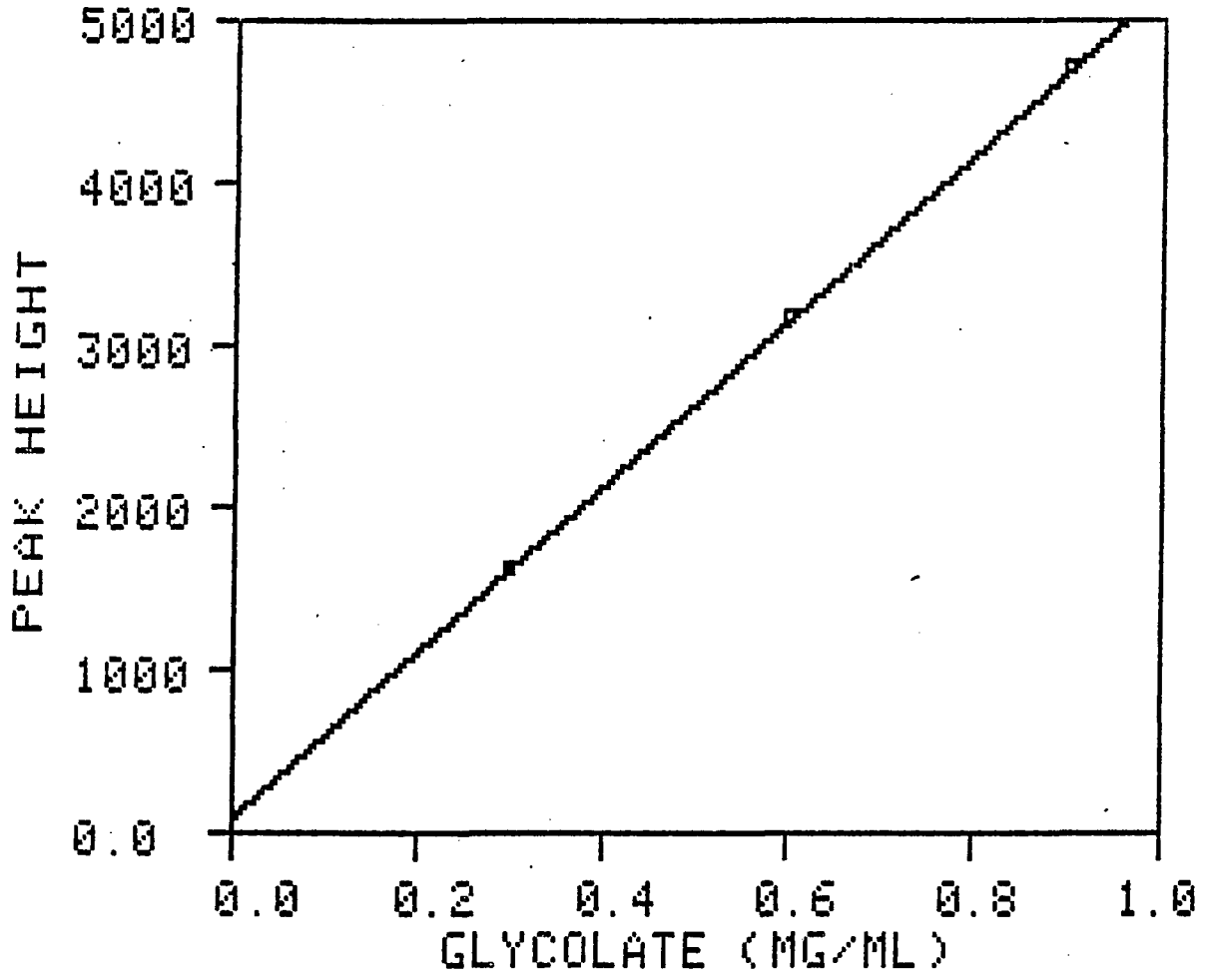


FIGURE 8

A calibration curve from a typical analysis of glycolate.

value obtained for the first sample analyzed from each group was used to assess the between run precision. The results of this study are given in table 3. It is generally accepted that a coefficient of variation below 10% is acceptable for chromatographic analyses. As indicated in table 3, the coefficients of variation were below 10%; therefore, a precision study was conducted using serum.

The precision study was conducted by calibrating with serum standards as described in the Standardization section. Two 50 mL quantities of approximately 0.50 mg/mL and 0.24 mg/mL glycolate were prepared from a 15 mg/mL stock solution of glycolate in calf serum. This was a different stock than that used for the calibration standards. As with the preliminary study 4 samples of the 0.50 and 0.24 mg/mL solutions of glycolate were analyzed on each of five days to determine the within run precision. The data for the between run precision is from the quality control specimens which were analyzed during each analysis. Because of bacterial contamination two preparations of controls were used. The data from each of these preparations is given in table 4.

RECOVERY

A recovery study was conducted by spiking blank calf serum with aqueous standards as indicated in table 5 (Westgard). These samples were analyzed as described above. The mean recovery for the sample spiked with 0.25 mg/mL

TABLE 3

Preliminary precision study using aqueous glycolate standards for calibration and 0.38 mg/mL aqueous samples of glycolate for determining precision.

	Ranges for Within Run (mean)	Between Run
Mean	0.34-0.40(0.37)	0.38
Standard Deviation	0.00-0.036(0.016)	0.025
CV	0.0-8.9(4.4%)	6.5%
n	5	5

TABLE 4

Precision study using calf serum standards for calibration and 0.50 and 0.24 mg/mL glycolate samples.

	Range of Within Run (mean)	Between Run A	Between Run B
Mean	0.52-0.62(0.56)	0.56	0.47
Standard Deviation	0.014-0.033(0.023)	0.048	0.034
CV	2.3-5.7(4.3%)	8.4%	7.3%
n	5	7	10
Mean	0.20-0.22(0.21)	0.22	0.25
Standard Deviation	0.0096-0.019(0.013)	0.012	0.015
CV	4.4-9.3(6.3)	5.6%	6.1%
n	5	7	10

A and B are from two preparations of control which were made consecutively.

TABLE 5

Recovery study using standards which had not been diluted to correct for a matrix effect.

Blank tube: 2.0 mL Calf Serum + 0.1 mL DDI H_2O.
Spike A: 2.0 mL Calf Serum + 0.1 mL 5.22 mg/mL glycolate.
Spike B: 2.0 mL Calf Serum + 0.1 mL 10.44 mg/mL glycolate.

Concentration Added	Result (mg/mL)	%Recovery	Mean Recovery
0.00	0.00	XXXXXXX	XXXXXX
0.25	0.19	76%	
0.25	0.17	68%	
0.25	0.18	72%	
			72%
0.50	0.36	72%	
0.50	0.37	74%	
0.50	0.38	76%	
			74%

glycolate was 72% and for the 0.50 mg/mL was 74%. Since the standards were treated the same as the samples and the quality control samples were within two standard deviations of the mean, the low recovery was possibly due to a matrix effect, i.e. the difference between the relative amounts of the constituents of the calibration standards and the recovery samples resulted in the alteration of the measured result.

To eliminate the possibility of a matrix effect the recovery study was repeated except that 0.1 mL of water was added to each of the standards to correct for any matrix effect (table 6). The mean recovery for both the 0.25 mg/mL and the 0.50 mg/mL samples was 100%. This would indicate that there had been a matrix effect present.

The calculations used to determine the percent recovery were:

$$CA = CS\ (VS/TV) \qquad \text{[Equation 2.4]}$$

Where CA = Concentration Added

CS = Concentration of the Standard

VS = Volume of the Standard Added

TV = Total Volume.

$$\%R = RE/CA \qquad \text{[Equation 2.5]}$$

Where %R = Percent Recovery

RE = Result.

TABLE 6

Recovery study using standards which were diluted (2.0 mL calf serum:0.1 mL DDI H_2O) to correct for any matrix effect.

Spike A: 2.0 mL Calf Serum + 0.1 mL 5.22 mg/mL glycolate.
Spike B: 2.0 mL Calf Serum + 0.1 mL 10.44 mg/mL glycolate.

Concentration Added	Result (mg/mL)	%Recovery	Mean Recovery
0.25	0.24	96%	
0.25	0.26	104%	
			100%
0.50	0.50	100%	
0.50	0.50	100%	
			100%

INTERFERENCE

The compounds listed in table 7 were tested in aqueous samples for interference and none were found to coelute or alter the peak height of the glycolate derivative. The relative retention time, the retention time of the test substance divided by the retention time of glycolate, of the compounds which were detected as eluting from the column are also given in table 7.

REFERENCE PATIENTS

Ten serum samples were randomly selected from specimens received by the routine Chemistry Laboratory. These samples were analyzed as described above. A typical chromatogram of one of these samples is given in figure 9. No glycolate was detected in these patient samples.

CONTRIVED SPECIMENS

A stock solution of 0.96 mg/mL was prepared using pooled human serum as the solvent. Aliquots were diluted with additional pooled serum to arrive at concentrations ranging from 0.12 mg/mL to 0.96 mg/mL. The glycolate concentration was measured in each of these 8 aliquots. Figure 10 is a plot of the measured result versus the expected result. Least squares analysis of the data resulted a slope of 0.97, a y intercept of 0.006, and a correlation coefficient of 0.997.

TABLE 7

Compounds which were tested as interferents in the glycolate assay. The relative retention with respect to glycolate is given for those compounds which were detected as eluting from the column.

	Relative Retention Time
Acetaldehyde	***
Glyoxal	***
Glycine	***
Formate	***
Alanine	***
Glyoxylate	0.6
Oxalate	1.9
Lactate	1.4
Beta-hydroxypropionate	1.4
Alpha-hydroxybutarate	2.4

*** Not detected

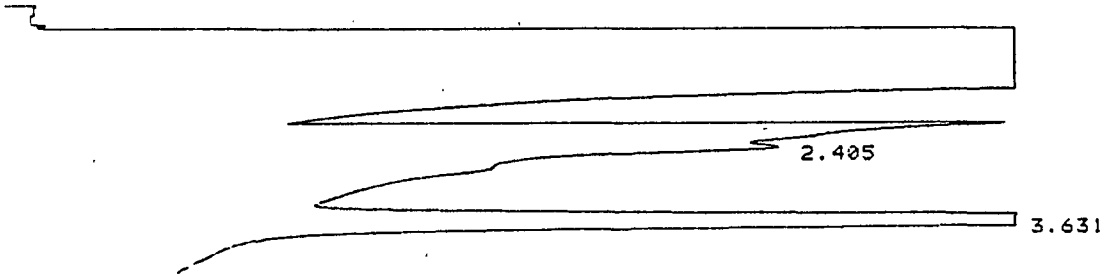


FIGURE 9

A typical chromatogram from a randomly selected patient. No glycolate was detected in any of the randomly selected patients.

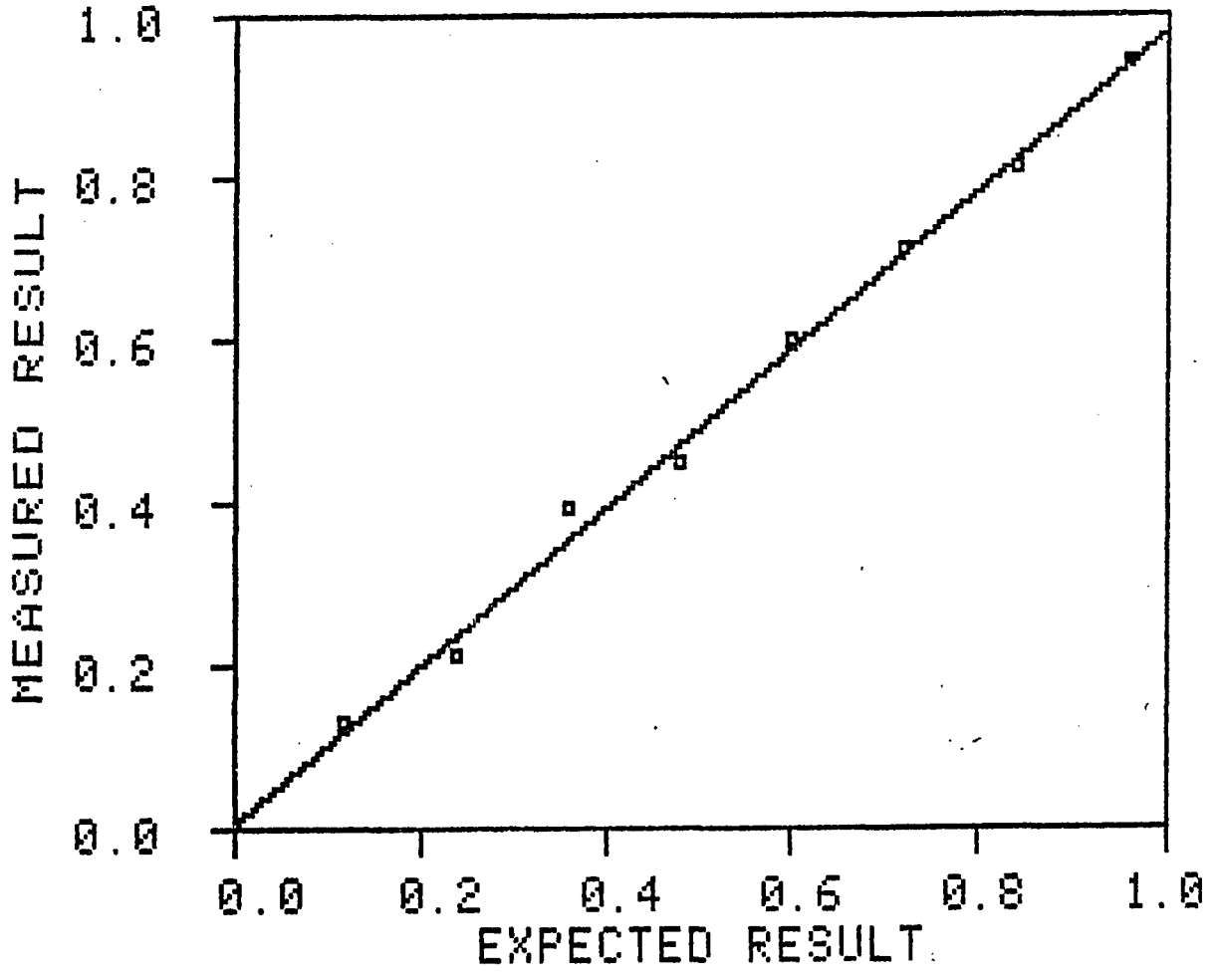


FIGURE 10

A plot of the measured result versus the expected result in a human serum pool with glycolate added. Least squares analysis of the data resulted a slope of 0.97, a y intercept of 0.006, and a correlation coefficient of 0.997.

IN VIVO TESTING

In order to demonstrate that the glycolate assay would detect glycolate following poisoning by EG in an in vivo situation, 8 male Wistar rats weighing between 375 and 400 g were given a 1 mL oral dose of 20 % EG. Following the administration of EG, blood was obtained from the orbital plexus at 0, 2, 6, 8, 12, and 24 hours in six of the rats and at 0, 4, 6, 8, 12, and 24 hours in two rats . The ultrafiltrate prepared from the serum was analyzed for glycolate as described above. In addition, EG was measured in the ultrafiltrate. The results of this study are given in table 8 and a typical graph of the time course is given in figure 11. Appendix C contains figures for each rat which illustrates the variation of results in each rat. The EG and glycolate results have been converted to milimolar concentrations for ease of comparison in figure 11 and those in Appendix C.

To determine if the assay had a potential for use in monitoring therapy, 3 male Wistar rats weighing between 375 and 400 g were each given differing amounts of 10 % ethanol intravenously (IV) via the tail vein. The first rat was given a 1 mL bolus of ethanol. Blood was drawn at 4 hours and the glycolate was determined to be 0.18 mg/mL. The second rat received a continuous IV dose of ethanol at a rate of 1 ml/hr for 4 hrs. Blood was drawn at this time and the glycolate concentration was found to be 0.08 mg/mL. The

TABLE 8

Data from the in vivo study of ethylene glycol poisoning in the rat. Each rat was given 20% EG orally. Glycolate results given in mg/mL and ethylene glycol in g/dL. Numbers in parenthesis are millimoles/liter. Time is in hours.

Time	0	2	6	8	12	24
			Rat 1			
Glycolate	0	0.20 (2.6)	0.22 (2.9)	0.11 (1.4)	0	0
Ethylene Glycol	0	0.06 (9.6)	0.07 (11.0)	0.01 (1.6)	0	0
			Rat 2			
Glycolate	0	0.10 (1.3)	0.05 (0.7)	0.04 (0.5)	0.06 (0.7)	0
Ethylene Glycol	0	0.05 (8.0)	0.05 (8.0)	0.01 (1.6)	0	0
			Rat 3			
Glycolate	0	0.13 (1.7)	0.17 (2.2)	0.14 (1.8)	0.10 (1.3)	0
Ethylene Glycol	0	0.06 (9.6)	0.04 (6.4)	0.02 (3.2)	0	0
			Rat 4			
Glycolate	0	0.25 (3.3)	0.25 (3.3)	0.24 (3.2)	0.06 (0.8)	0
Ethylene Glycol	0	0.02 (3.2)	0.03 (4.8)	QNS	0	0
			Rat 5			
Glycolate	0	0.19 (2.5)	0.24 (3.2)	0.24 (3.2)	0.10 (1.3)	0
Ethylene Glycol	0	0.05 (8.0)	0.05 (8.0)	0.01 (1.6)	0	0

Table 8 continued

			Rat 6			
Time	0	2	6	8	12	24
Glycolate	0	0.23 (3.0)	0.20 (2.6)	0.15 (2.0)	0.05 (0.7)	0
Ethylene Glycol	0	0.06 (9.6)	0.03 (4.8)	0.02 (3.2)	0	0
			Rat 7			
Time	0	4	6	8	12	24
Glycolate	0	0.17 (2.2)	0.16 (2.1)	0.14 (1.8)	0.07 (0.9)	0
Ethylene Glycol	0	0.05 (8.0)	0.03 (4.8)	0.01 (1.6)	0	0
			Rat 8			
Glycolate	0	0.25 (3.3)	0.21 (2.8)	0.13 (1.7)	0	0
Ethylene Glycol	0	0.05 (8.0)	0.02 (3.2)	0	0	0

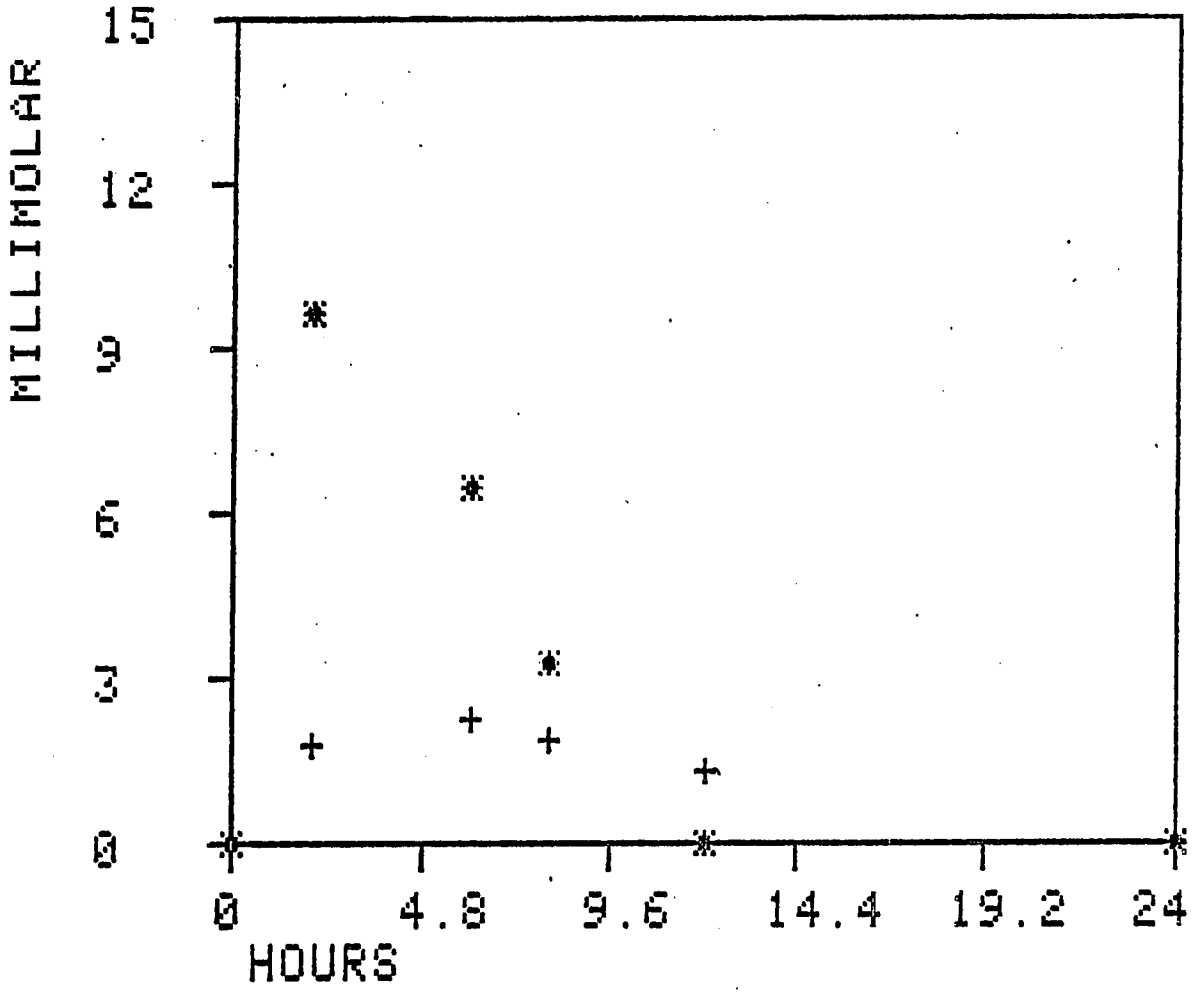


FIGURE 11

An example of the time course of serum ethylene glycol (stars) and glycolate (pluses) concentrations in mili-molarity in a rat following forced ingestion of 1 mL of 20% EG.

third rat received an IV bolus of 2 mL followed by a four hour infusion of ethanol at a rate of 3 mL/hr. The glycolate concentration at that point was undetectable. Blood was also obtained at 6, 8, 12, and 24 hours in this last rat and analyzed for glycolate and EG. Figure 12 is a plot of the data obtained in the third rat.

A FURTHER APPLICATION OF THE USE OF PROTEIN FREE FILTRATES

Ethanol measurement is often requested in cases of ethylene glycol poisoning to monitor treatment. Since there was excess ultrafiltrate available from the glycolate assay, the possibility of using this excess for the measurement of ethanol was explored. In the procedure of Jain, serum is diluted with an aqueous solution of n-propanol which serves as an internal standard. This dilution is then directly injected onto the GC column. The method of Anthony uses the same column but uses head space gas sampling. In this procedure the diluted sample is placed in a sealed vial and heated to vaporize the alcohols. The gases are then aspirated and injected onto the GC column. The advantage of this procedure is that it removes the protein which tends to precipitate on the column resulting in shortened column life. These procedures were modified by preparing an untrafiltrate of serum as was done with the glycolate assay. In this procedure, 100 uL of ultrafiltrate was mixed with 400 uL of 0.04 g/dL n-propanol. The chromatographic

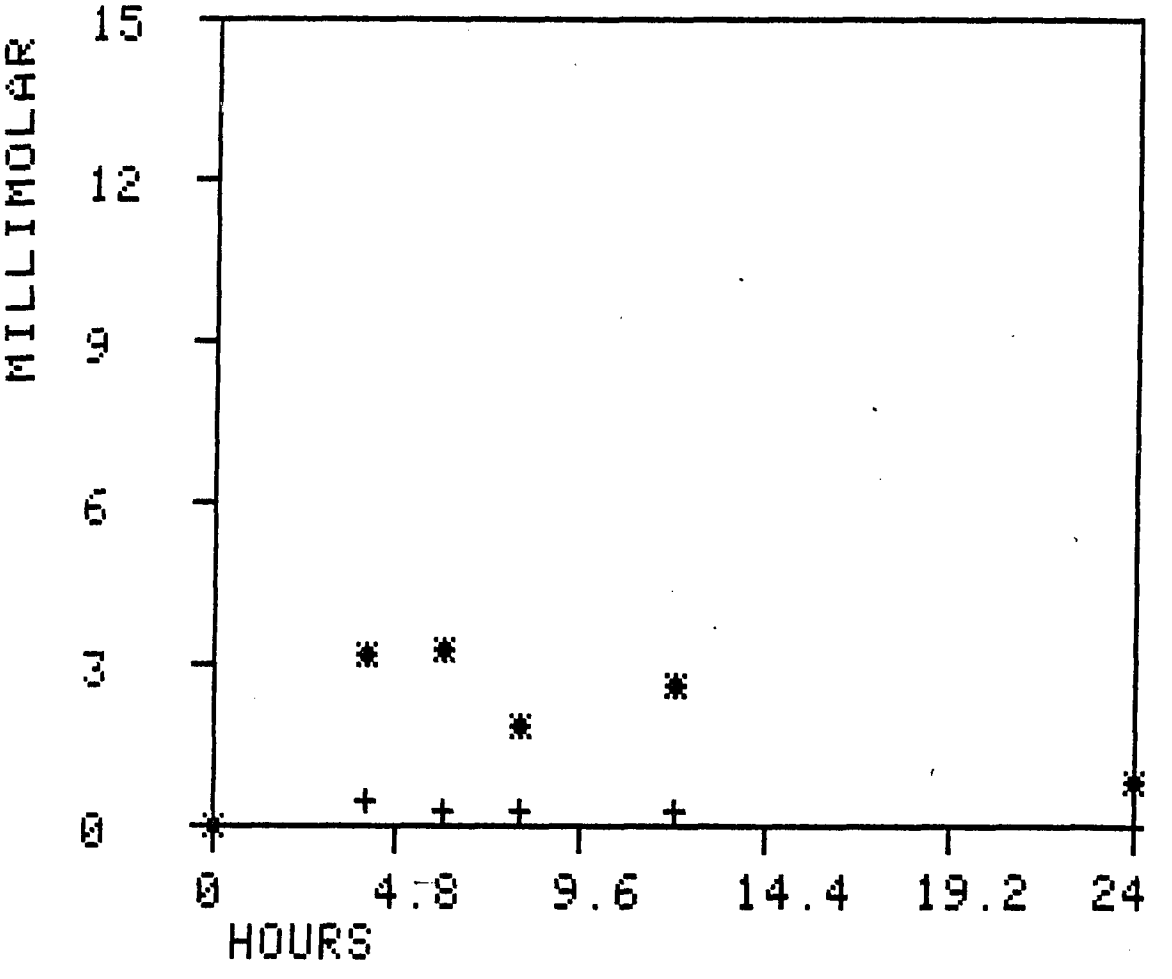


FIGURE 12

A graph of the time course of serum ethylene glycol (stars) and glycolate (pluses) concentrations in milimolarity in a rat following forced ingestion of 1 mL 20% EG. The rat was treated with a 2 mL IV bolus of 10% ethanol followed by a 4 hour infusion at 3 mL/hr.

TABLE 9

Chromatographic conditions used in the measurement of ethanol.

Oven temperature: 85°C

Detector: Flame ionization

Detector temperature: 220°C

Injector temperature: 220°C

Carrier gas: Helium

He flow rate: 25 mL/min

Air flow rate: 200 mL/min

H_2 flow rate: 30 mL/min

Attenuation: 8

conditions are listed in table 9. This procedure will detect methanol, ethanol, isopropanol, and acetone; however, this study will concern itself only with the measurement of ethanol. Figures 13 and 14 are chromatograms of ethanol using the direct injection method and the ultrafiltration method.

The calibration was performed using aqueous solutions of ethanol of the following concentrations 0.02, 0.05, 0.10, and 0.20 g/dL. Figure 15 is a plot demonstrating the linearity of the procedure. A precision study using both the direct injection technique and the ultrafiltration was conducted using calf serum based samples at concentrations of 0.20, 0.10, and 0.05 g/dL of ethanol. The results of this study are given in table 10.

A comparison study between the direct injection and ultrafiltration techniques was conducted using 30 positive specimens received from the Emergency Department for the measurement of alcohol. The results of this study are given in figure 16.

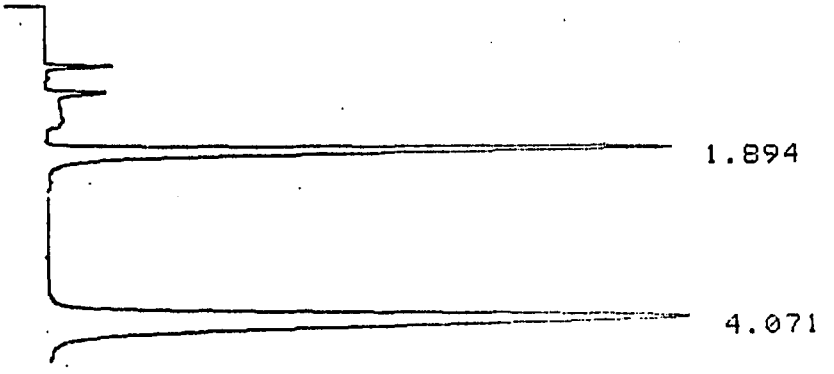


FIGURE 13

A chromatogram of ethanol (1.894), at a concentration of 0.05 g/dL using the direct injection technique. The peak at 4.071 min is the internal standard n-propanol.

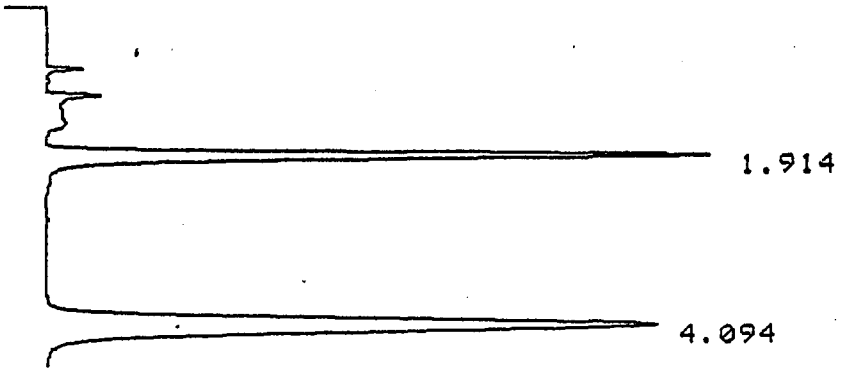


FIGURE 14

A chromatogram of ethanol (1.914), at a concentration of 0.05 g/dL using the ultrafiltration technique. The peak at 4.094 min is the internal standard n-propanol.

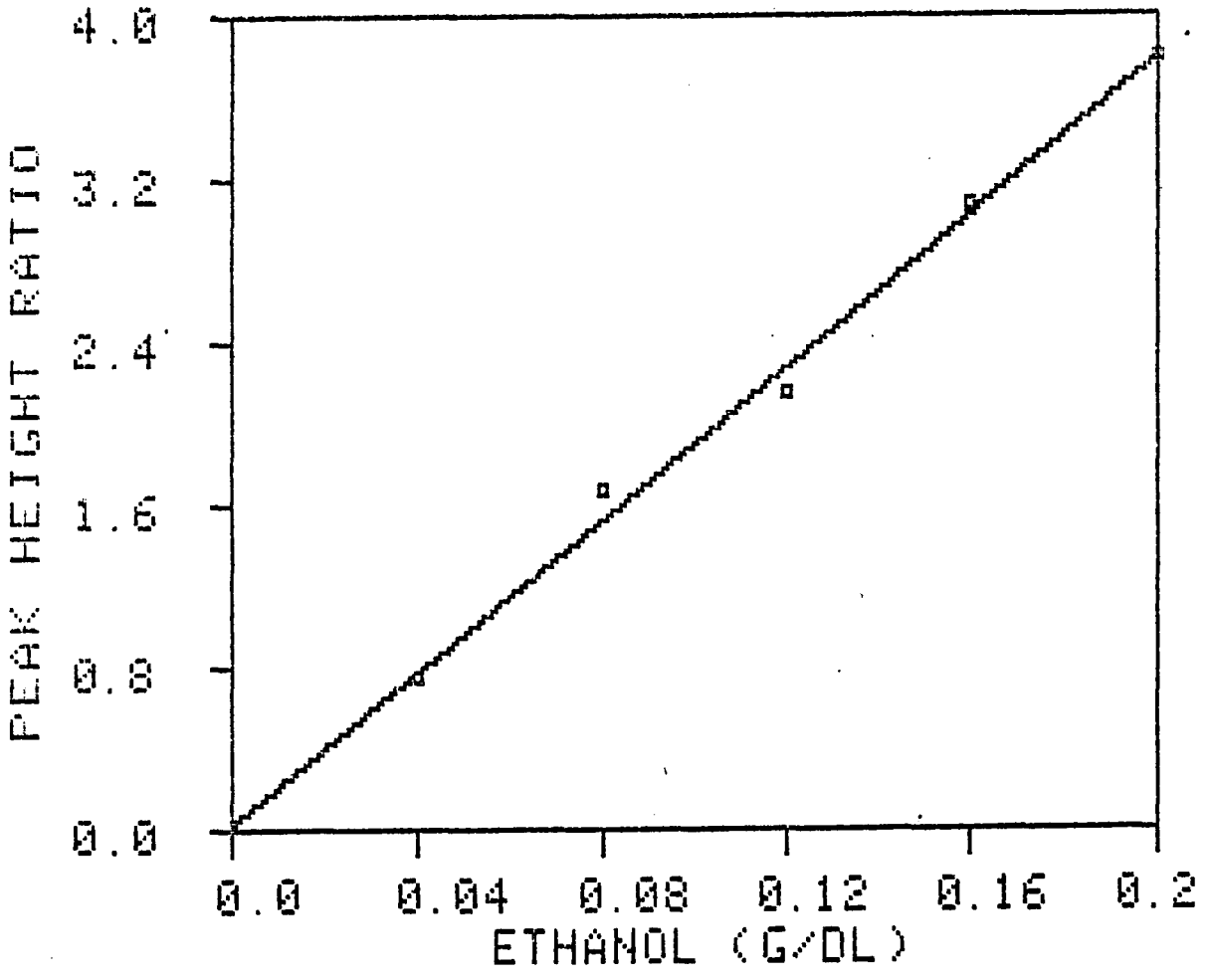


FIGURE 15

A plot of expected concentration vs measured concentration using samples of ethanol prepared in calf serum and analyzed using the ultrafiltration technique.

TABLE 10

Precision study using both the direct injection and the ultrafiltration techniques.

Within Run			
	Direct Injection		
Mean	0.182	0.093	0.054
Standard Deviation	0.0092	0.0048	0.0018
Coefficient of Variation	5.05%	5.21%	3.33%
n	5	5	5
	Ultrafiltration		
Mean	0.199	0.096	0.053
Standard Deviation	0.0054	0.0045	0.001
Coefficient of Variation	2.73%	4.72%	1.89%
n	5	5	5
Between Run			
	Direct Injection		
Mean	0.180	0.094	0.051
Standard Deviation	0.015	0.0058	0.0025
Coefficient of Variation	8.6%	6.2%	4.8%
n	9	9	9

Table 10 continued.

	Ultrafiltration		
Mean	0.196	0.097	0.053
Standard Deviation	0.010	0.0067	0.0042
Coefficient of Variation	5.3%	6.2%	7.9%
n	8	8	9

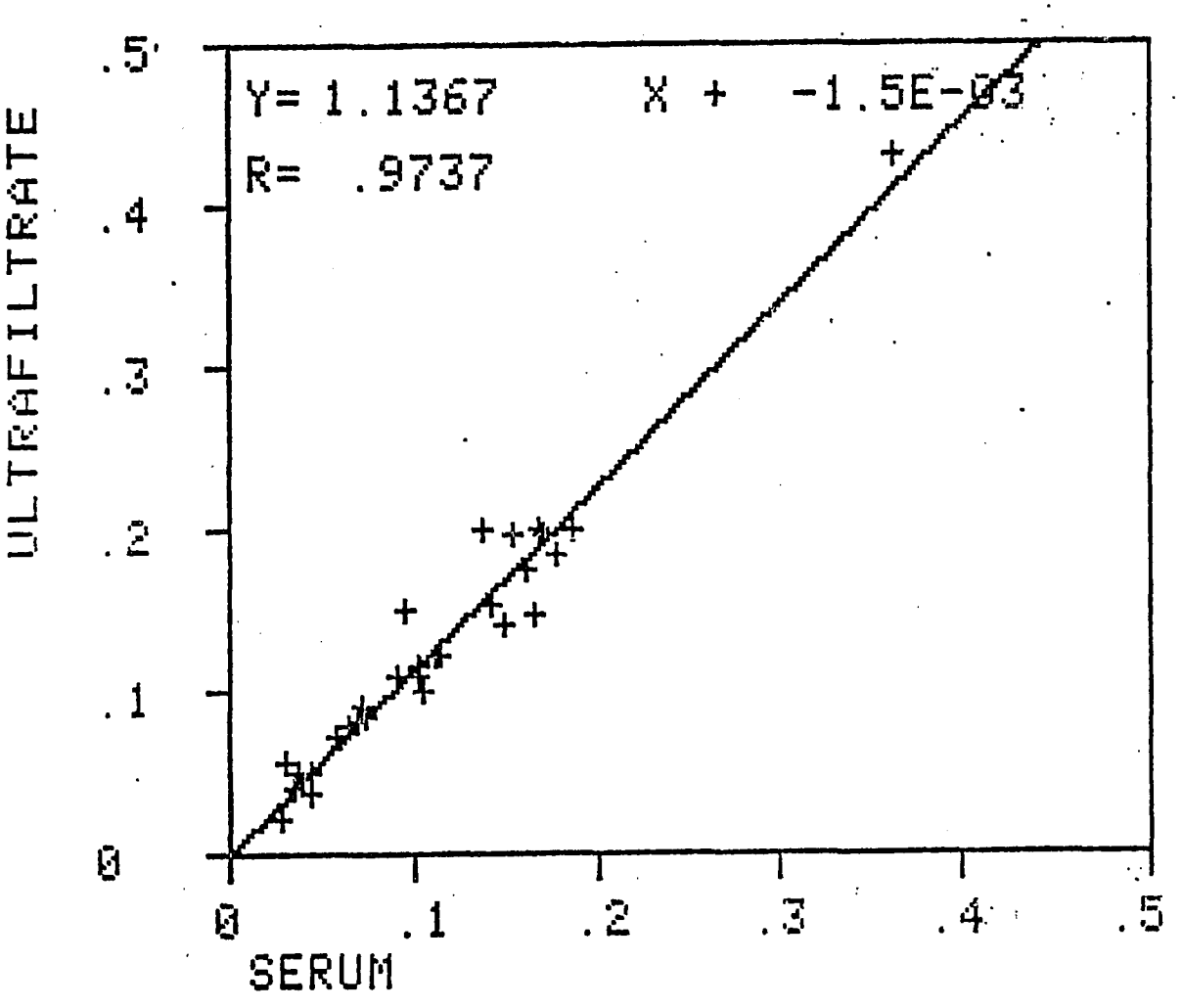


FIGURE 16

A plot of the comparison study between the direct injection and the ultrafiltration techniques. Analysis by least squares gives a y intercept of -0.0102 and a slope of 0.9464 with a correlation coefficient of 0.976. (n= 30).

Chapter III

DISCUSSION

The objective of this research was to develop an assay for glycolate in serum for the purpose of evaluating an ethylene glycol poisoning. The assay developed is simpler than those currently available and is performed on a GC column which can also be used to measure ethanol and ethylene glycol.

Using serum, the glycolate assay is linear to at least 0.90 mg/mL. This was demonstrated by two means. First, by the linearity study using calf serum supplemented with glycolate. Second, by the use of contrived human samples. This second study also demonstrated that there is proportional recovery throughout the linear range because the theoretical difference between each sample was 0.12 mg/mL and the mean difference between the measured and theoretical result was 0.115 mg/mL.

The recovery study revealed the presence of a matrix effect. In the first portion of the recovery study, the method was calibrated with ultrafiltrate samples prepared from whole serum into which sodium glycolate had been weighed. The recovery samples were prepared from whole

serum into which 0.1 mL of an aqueous standard had been added. The glycolate partitioned itself into the aqueous portion of the serum. When the ultrafiltrate was prepared, the glycolate followed the water into the collection portion of the device. Since there was more water in the recovery samples than in the standards, the glycolate in the recovery samples was diluted, resulting in a decrease in recovery. When the same amount of water was added to the standards as was added to the recovery samples, the recovery was 100%. For this reason, the assay must be calibrated with the ultrafiltrate prepared from whole serum supplemented with glycolate. Ethanol on the other hand is partitioned evenly between the ultrafiltrate and the retentate and no concentrating takes place; therefore, the assay can be calibrated with aqueous standards. The contrived samples demonstrated that the glycolate assay could be calibrated with standards in a calf serum matrix for the measurement of glycolate in human serum.

The glycolate assay was developed using a column which eventually deteriorated. This was evident by a slow increase in the retention time of the glycolate peak from 3 to 7 minutes with a simultaneous increase in the peak width. The separation between the glycolate and lactate peaks increased to approximately 1.6 minutes. The column finally deteriorated to a point that the base line became very irregular and the peaks became very wide. At this point,

the column also failed to separate alcohols. The column was replaced and the procedure was kept the same in all respects. The column efficiency calculations based on the chromatogram in figure 4 are with the new column. According to Willard et al. the number of plates should be in the range of 1500 to 3000 and a resolution of 1.5 represents baseline separation. The number of plates (1919.7) is acceptable but the resolution (0.38) is far below the ideal resolution of 1.5.

The precision study, however, indicates that the assay is reproducible even though the resolution is poor. All of the coefficients of variation were below 10%. The use of an internal standard would probably lower these CV's; however, an acceptable internal standard was not found. This lack of an internal standard required careful reproducible injections. The use of an auto sampler would meet the need for this careful injecting. Furthermore, the limit of detection at this resolution is acceptable as evidenced by a lower limit of detection of 0.015 mg/mL which is in accord with the work of Hewlett et al. and Clay and Murphy.

The interference study demonstrated that there was no interference from compounds which might be expected to interfere. The analysis of reference patients demonstrated that there are no substances in human serum which coelute with glycolate.

The purpose of the in vivo study was to demonstrate that the assay would detect glycolate following poisoning from ethylene glycol. There was considerable variation between the glycolate concentrations in the individual rats. This model does not lend itself to pharmacokinetic studies because of the large blood loss resulting from the withdrawal of 2 mL samples, 6 times within a 24 hour period. This in vivo study does, however, illustrate the potential of this assay for confirming EG poisoning because glycolate was detected following the ingestion of EG. The administration of varying amounts of ethanol demonstrated that glycolate decreased as the dose of ethanol increased. This portion of the study demonstrated the potential of this assay as a means of monitoring the effectiveness of ethanol therapy. In the event that a treatment of EG poisoning by removing glycolate is found, this assay would be invaluable as a means of monitoring therapy.

The ethanol portion of this research is relevant because, in addition to measuring ethylene glycol, ethanol is measured to monitor therapy. In the case of EG poisoning, the ultrafiltrate could be prepared from the serum and one aliquot could be used to measure glycolate. Another aliquot of the remaining ultrafiltrate could then be used to measure ethanol. In addition, this modification of the procedures of Jain and Anthony eliminates the need for the use of head space gas sampling while still eliminating the deposition of

protein on the column. The use of ultrafiltrate for the analysis should lengthen column life.

In conclusion, this research has produced a simple assay for glycolate which has acceptable sensitivity and specificity as demonstrated by a lower limit of detection of 0.015 mg/mL and non-interference from compounds of similar structure. In addition, no interference by endogenous substances. This assay could definitely be used to identify patients in need of treatment to remove accumulated glycolate when this treatment becomes available. Furthermore, the effectiveness of this therapy could be monitored by measuring the change in glycolate concentration over time. This assay currently has a clinical use with conventional ethanol therapy because the measurement of glycolate can be used to assess the severity of EG poisoning and to monitor the effectiveness of the ethanol therapy as evidenced from the in vivo studies.

From a research perspective, this assay would be useful in correlating glycolate concentrations in serum following EG poisoning in a laboratory animal with the amount of tissue damage and to help to identify the mechanism by which glycolate exerts its toxic effects.

APPENDIX A

Method program from the 401 Data System for glycolate.

```
SINGLE CHANNEL METHOD: GLYCOLATE

SECTION 1: BASIC
PAGE 1
  ANALYSIS PARAMETERS
  CHANNEL: 3
  CALCULATION: ES
  AREA/HT: H
  STOP TIME:    6.00
  NUMB EXPECTED PKS:    40
  EQUILIBRATION TIME:   0
  UNRETAINED PK TIME:    0.00
  UNIDENT PK FACTOR: 0.000000
  SLICE WIDTH:   10

PAGE 2
  SAMPLE PARAMETERS
  RUN TYPE: C
  SAMPLE ID: .3
  DIVISOR: 1.000000
  AMT STD: 0.300000
  MLTPLR: 1.000000

PAGE 3
  REPORT INSTRUCTIONS
  WHERE TO REPORT: R
  COPIES: 1
  TITLE: GLYCOLATE
  FORMAT: E
  DECIMAL PLACE: 2
  RESULT UNITS: MG/ML
  REPORT UNIDENT PKS: Y
  REPORT INSTRUMENT CONDITIONS: N

PAGE 4
  PLOT INSTRUCTIONS
  PLOT: Y
  ZERO OFFSET:  5
  ANNOTATION
    RETENTION TIME: Y
    PLOT CONTROL: Y
    TIME TICKS: Y
    TIME EVENTS: N
    PK START/END: N

PAGE 5
  CHART SPEED
    PAGES OR CM/MIN: C
    INIT VALUE:  0.0
   LINE# TIME   CHART SPEED
   1       0.01      1.0

PAGE 6
  PLOT ATTEN
    INIT PLOT ATTEN: 16
   LINE# TIME   PLOT ATTEN
   1       2.00     4
```

```
SECTION 2: TIME EVENTS
PAGE 1
   LINE#  TIME  EVENT   VALUE
   1       0.00  PR        100
   2       0.00  SN          2
   3       0.00  T%        5.0
   4       0.00  WI          4
   5       0.00  II       2.00
   6       0.00  SB         10


SECTION 3: PEAK TABLE
PAGE 1
  STD PK#:  0
  RELATIVE RETEN PK#:  0
  RESOLUTION PK#:  0
  RESOLUTION MINIMUM:   0.0
  FACT%:   5.0
  IDENTIFICATION TIME WINDOWS +/-
  REF
    %: 10
    MIN:   0.00
  NON REF
    %:  5
    MIN:   0.00

PAGE 2
   PK# TIME  NAME         FACTOR   AMOUNT REF GR# MUST LO   MUST HI
   1    3.08 GLYCOLATE  1.325070 1.000000       0.000000 0.000000


SECTION 7: POST RUN
PAGE 1
  FILE NAME:
  SAVE INSTRUCTIONS
    TYPE:
    WHERE TO SAVE: M
  TRANSMIT/REPLOT INSTRUCTIONS
    TRANSMIT RAW DATA: N
    REPLOT WITH BASELINES: N
    RAW DATA LOCATION: U
    TRANSMIT REPORT: N

PAGE 2
  METHOD LINKING INSTRUCTIONS
    METHOD:
    LINK CALC RESULTS: N
  PROGRAM EXECUTION
    PROGRAM:
    PARAMETERS:
    RESERVE PRINTER: Y


SECTION 9: SAMPLE LIST+A/S CONTROL
PAGE 1
  AUTOSAMPLER CONTROL
  INJECT/CALIBRATION: 1
  INJECT/ANALYSIS: 1
  SAMP VOLUME: 1
  VISTA AUTOSAMPLER ONLY
    A/S MODE: MR
    PURGE PULSES: 2
    INJECT TIME:  0.03

PAGE 2
   SAMP#   RACK/VIAL  TYPE SAMPLE ID    DIVISOR   AMT STD   MLTPLR
   1                   C   .3          1.000000  0.300000 1.000000
   2                       .6                    0.600000
   3                       .9                    0.900000
   4                   A   .50 QC                 1.000000
   5                       .24 QC
```

APPENDIX B

VARIAN 401 DATA SYSTEM CALCULATIONS

The calculations below are those used by the Varian 401 data system to calculate results using the external standards mode (Varian Instruments).

Standards

Result Factor = (Concentration /Peak Height) X 10,000

[Equation B.1]

Unknowns

Concentration = (Peak Height X Result Factor)/10,000

[Equation B.2]

APPENDIX C

FIGURES FROM THE IN VIVO STUDIES

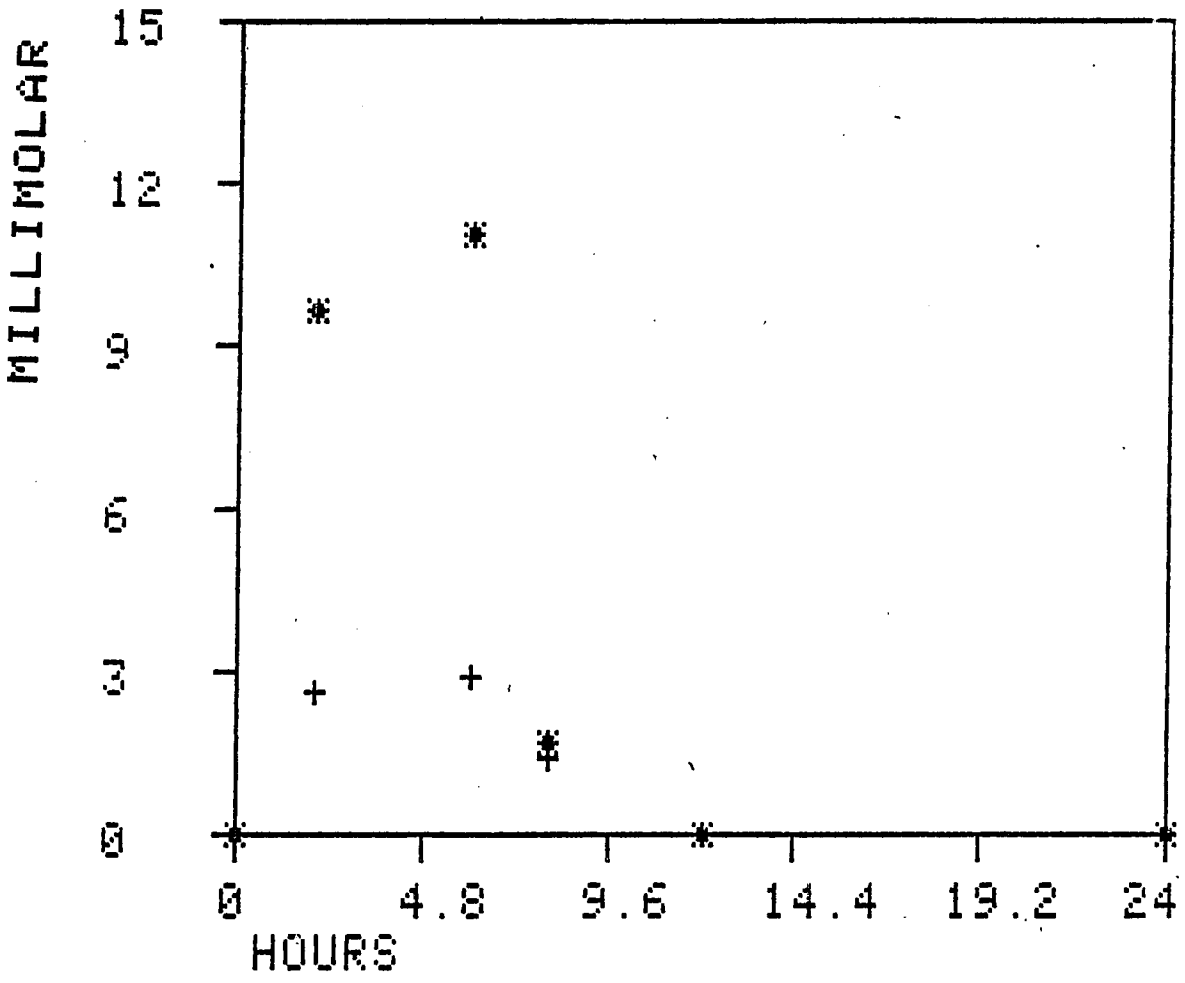


FIGURE 17

Graphical representation of the data from rat 1. Stars represent ethylene glycol and the plus signs glycolate.

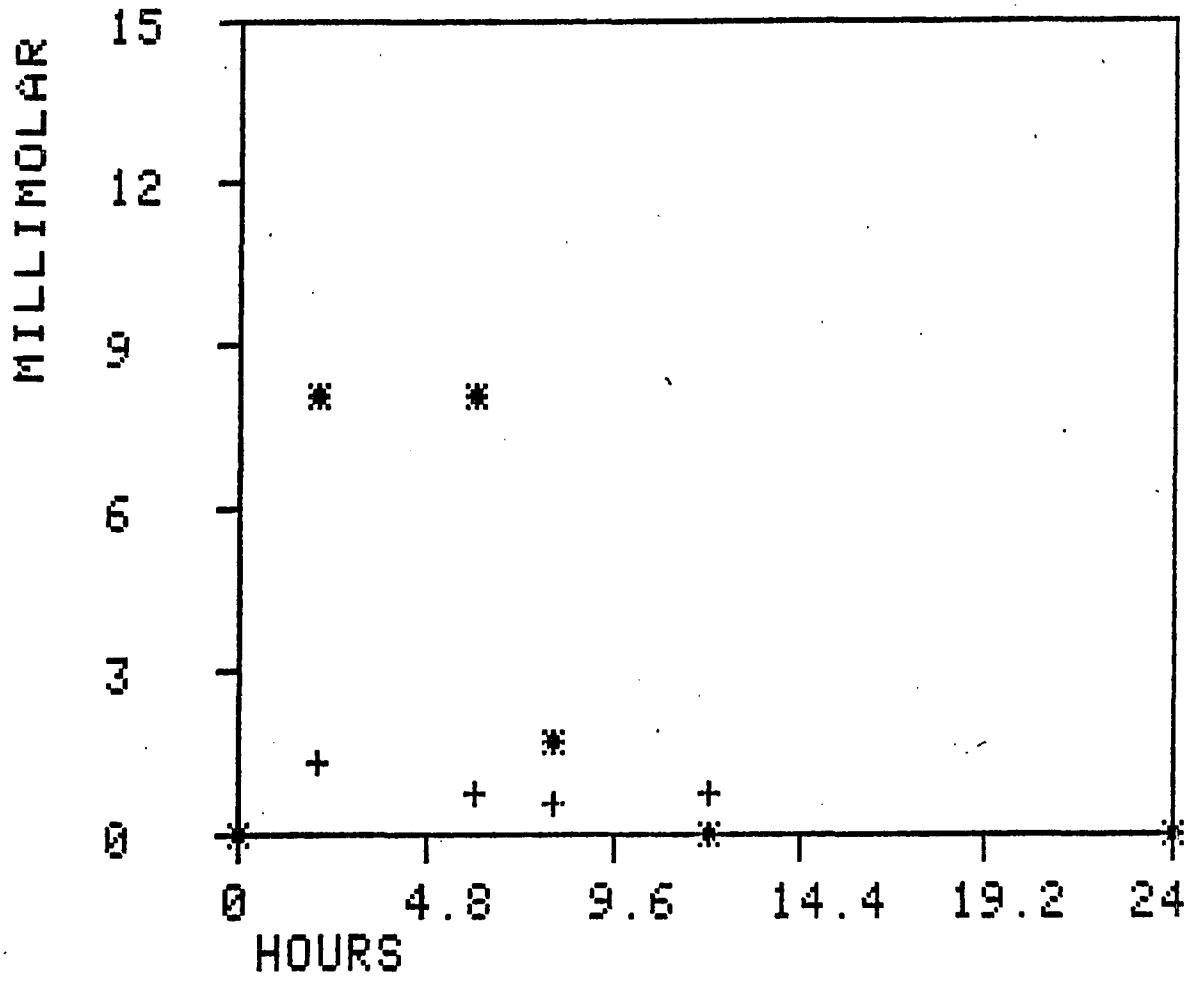


FIGURE 18

Graphical representation of the data from rat 2. Stars represent ethylene glycol and the plus signs glycolate.

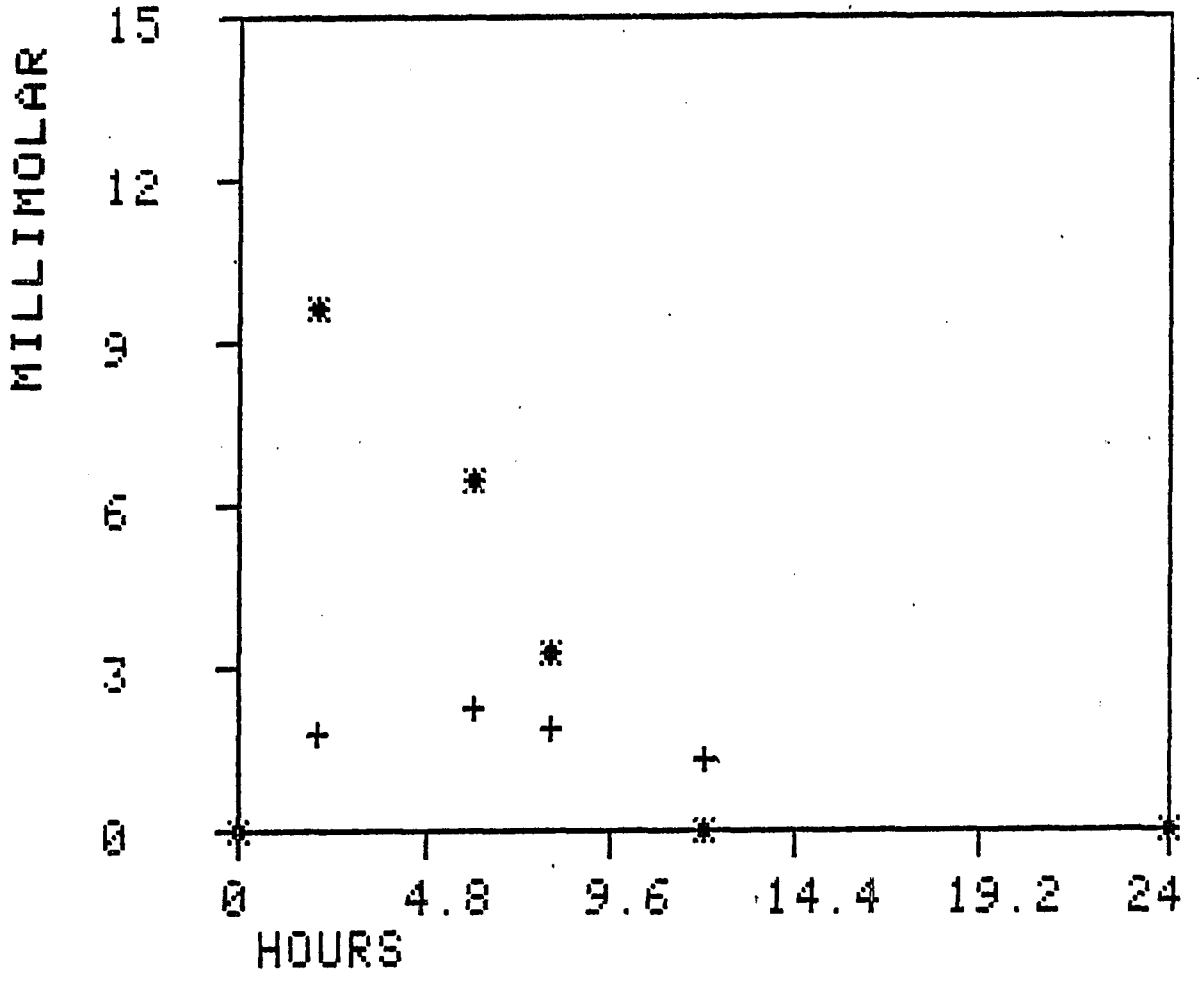


FIGURE 19

Graphical representation of the data from rat 3. Stars represent ethylene glycol and the plus signs glycolate.

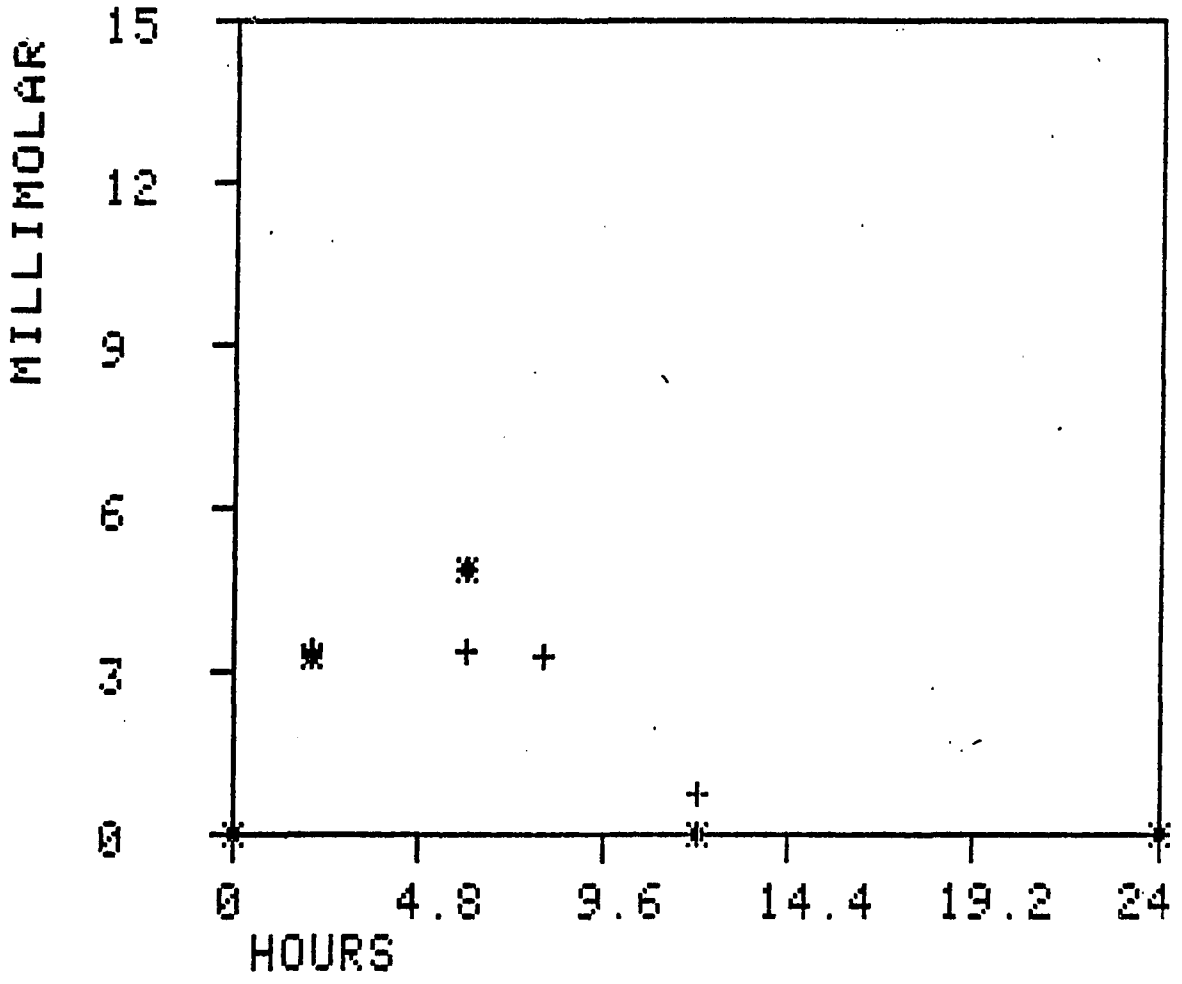


FIGURE 20

Graphical representation of the data from rat 4. Stars represent ethylene glycol and the plus signs glycolate.

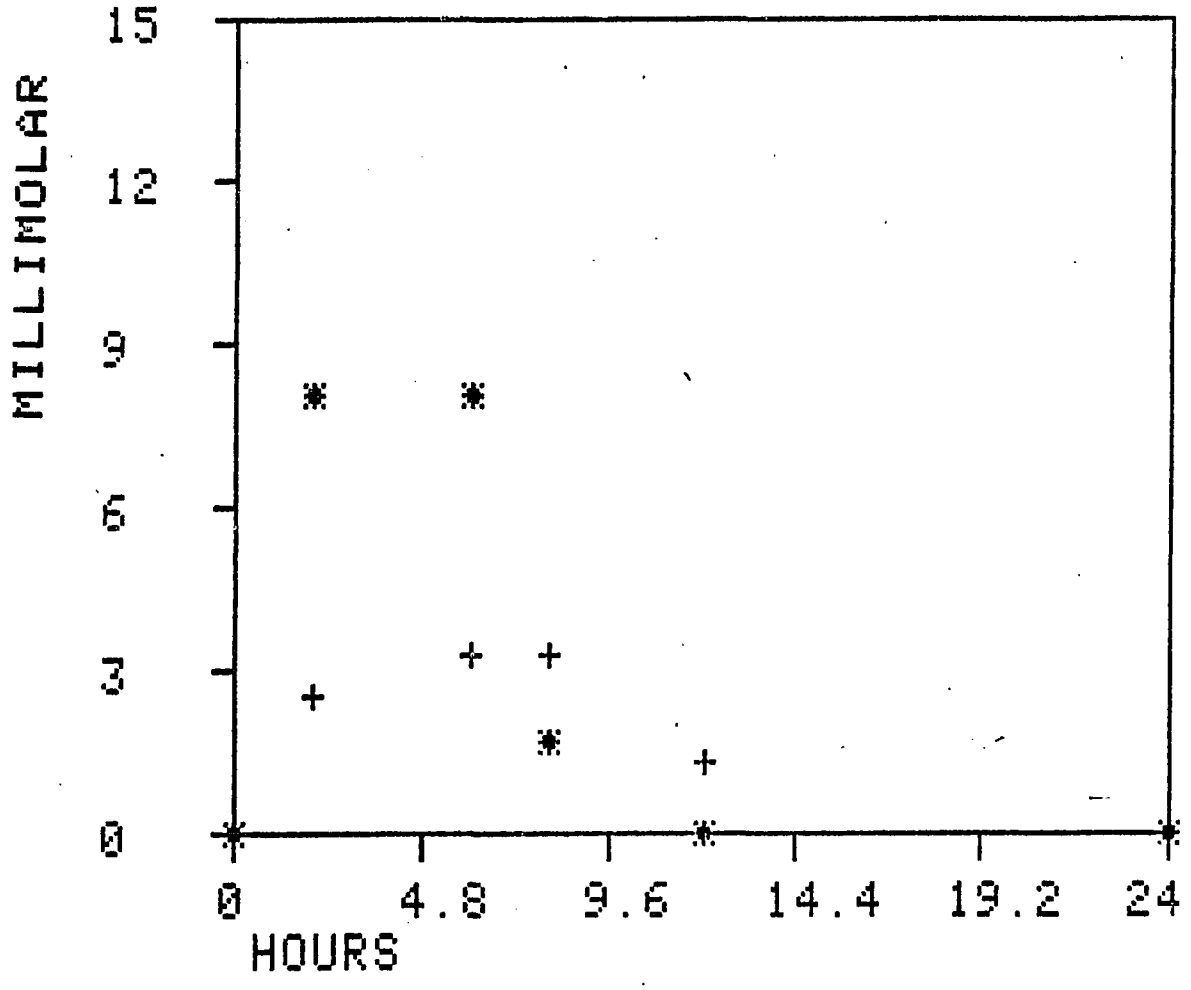


FIGURE 21

Graphical representation of the data from rat 5. Stars represent ethylene glycol and the plus signs glycolate.

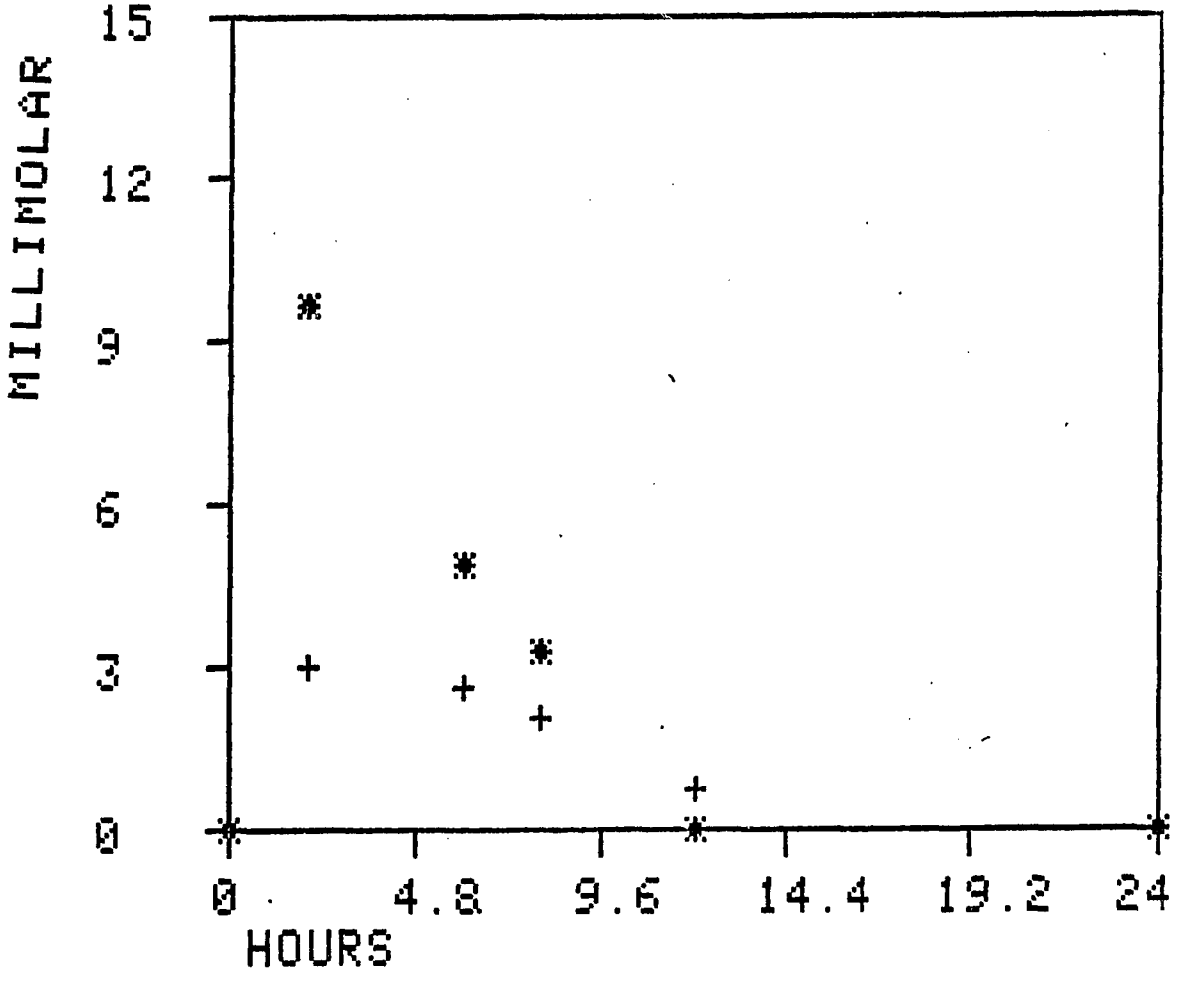


FIGURE 22

Graphical representation of the data from rat 6. Stars represent ethylene glycol and the plus signs glycolate.

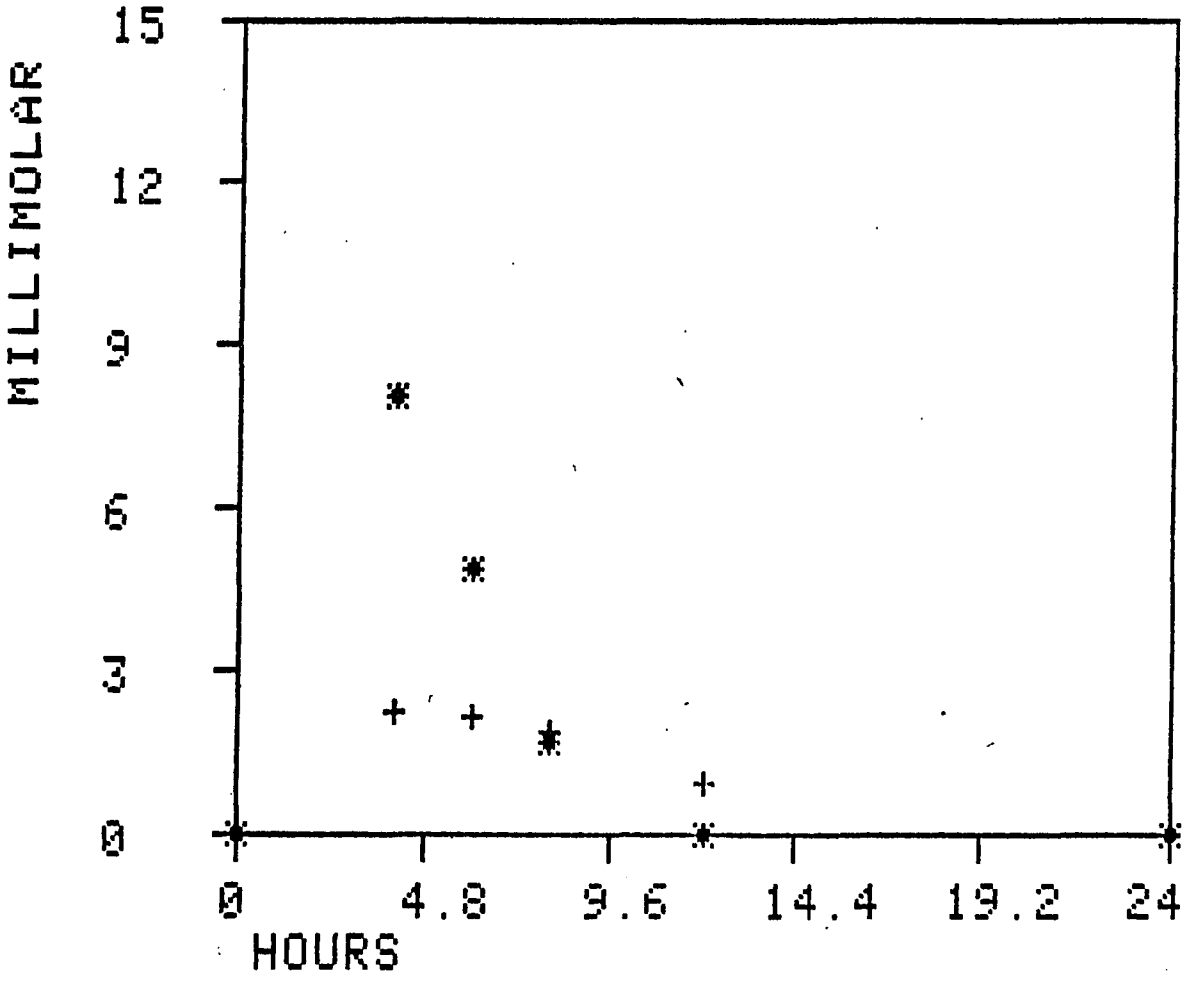


FIGURE 23

Graphical representation of the data from rat 7. Stars represent ethylene glycol and the plus signs glycolate.

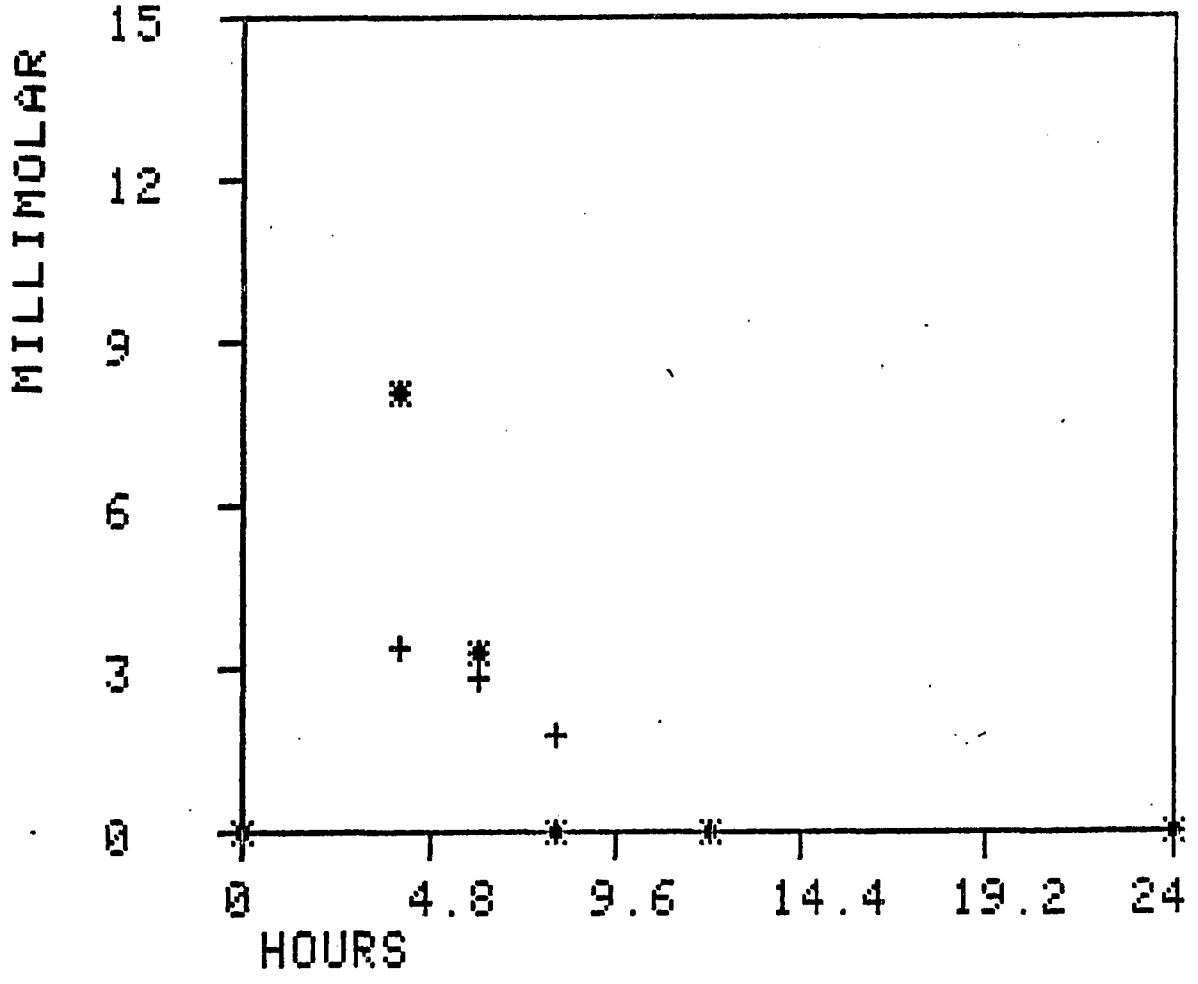


FIGURE 24

Graphical representation of the data from rat 8. Stars represent ethylene glycol and the plus signs glycolate.

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