OPTIMIZATION OF AN IMMOBILIZED ENZYME SYSTEM FOR CONJUGATED BILE ACIDS

BY

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Submitted in Partial Fulfillment of the Requirements
for the Degree of
Master of Science
in the
Chemistry
Program

YOUNGSTOWN STATE UNIVERSITY

August, 1995

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ABSTRACT

OPTIMIZATION OF AN IMMOBILIZED ENZYME SYSTEM FOR CONJUGATED BILE ACIDS

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The optimization of a reversed phase high performance liquid chromatography system incorporating an immobilized enzyme was investigated. The immobilized enzyme, cholylglycine hydrolase (CGH), was used to generate species that were detected using fluorescence. A separation of a standard containing 10 bile acids was achieved after signal to noise optimization of the system. The 10 conjugated bile acids were hydrolyzed after the separation by the immobilized CGH to yield free bile acids and the amino acids taurine and glycine. The amino acids were then reacted to give o-phthalaldehyde derivatives and detected by fluorescence. Emission and excitation wavelengths were set at 448 nm and 341 nm respectively. Bile samples from humans were also analyzed using the optimized system.

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LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS OR REFERENCE
mL	Milliliter	1×10^{-3} liter
μL	Microliter	1×10^{-6} liter
mg	Milligram	1×10^{-3} gram
mm	Millimeter	1×10^{-3} meter
nm	Nanometer	1×10^{-9} meter
M	Molarity	moles per liter
L	Liter	--
g	Gram	--
mg/mL	Milligram per Milliliter	--
α	Alpha	--
ϵ	Epsilon	--
°C	Celsius Temperature	--
HPLC	High Performance Liquid Chromatography	
NMR	Nuclear Magnetic Resonance	--
UV	Ultraviolet	--
%	Percent	--
i.d.	Internal Diameter	--
C_{18}	Octadecyl	--
S	Signal	--
N	Noise	--
GUDA	Glycoursodeoxycholic Acid	--
TUDA	Tauroursodeoxycholic Acid	--
GLCA	Glycolithocholic Acid	--
TLCA	Taurolithocholic Acid	--

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CHAPTER I

INTRODUCTION

Bile acids are the metabolic products of cholesterol needed for the normal digestion of lipids. They are synthesized in the polygonal cells of the liver and pass through the hepatic and cystic ducts to the gall bladder, where they are stored. Eighty percent of the body's cholesterol is converted by the liver into various bile acids that then conjugate with amino acids such as glycine and taurine to produce bile salts[1]. Bile salt is a more accurate term than bile acid because all bile acids exist as salts at physiological pH. Bile salts are colorless, crystalline solids with a bitter taste[2]. They have variable solubilities in water. The sodium salts tend to be rather soluble, while the free acids are not. An increase in solubility is seen when the bile acids are conjugated with taurine[2]. All of the bile acids tend to be quite soluble in alcohol. In this work, the terms bile acids and bile salts will be used interchangeably.

Due to their structures, bile salts display both hydrophilic and hydrophobic characteristics. The polar side chain of a bile salt interacts strongly with water by hydrogen bonding interactions, whereas the non-polar sterol group interacts with hydrophobic lipids through London Dispersion interactions. Due to the amphipathic nature of bile salts they are able to emulsify triacylglycerols (fats)

and esters of long-chain fatty acids, making these compounds available for hydrolysis by intestinal lipases and esterases[3]. All of the common bile salts possess a 3α-hydroxyl group and one or more hydroxyl groups at positions 6, 7, and 12.

Figure 1 illustrates the biosynthesis of bile salts from cholesterol. The OH groups may be sulfated or glucoronidated. The side chain may be unconjugated or conjugated with taurine, glycine or other amino acids[1]. These modifications can greatly alter the polarity of both the steroid nucleus and the side chain.

The most abundant bile acids are cholic acid and chenodeoxycholic acid. They are called primary bile acids. However, 20% of bile acids are classified as secondary. They are derived from primary bile acids by the action of bacteria in the intestine. The primary bile acids undergo dehydroxylation at the 7α-position to form deoxycholic and lithocholic acids. Bacteria can also cause other alterations to the primary bile acids and form additional secondary bile acids, however these are not absorbed and recirculated, as are deoxycholic and lithocholic acids, and therefore have no physiological purpose in bile[2].

When bile acids leave the liver cells and enter the biliary tract, they transform from their free, or unconjugated state to a conjugated form. In this form the bile acid has added an amino acid, glycine or taurine, by

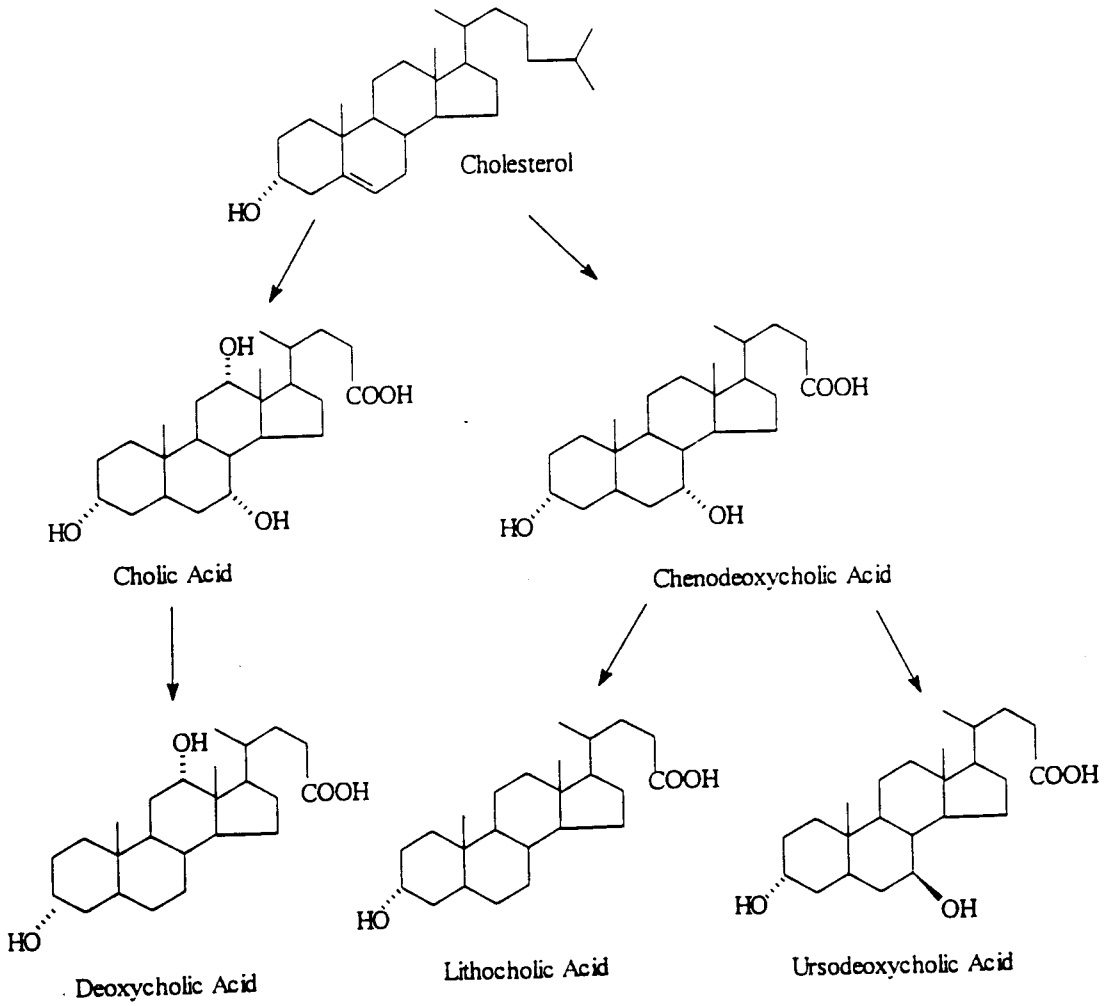


Fig. 1. Biosynthesis of bile salts from cholesterol

amide linkage at the carboxyl side chain. This linkage provides resistance to natural peptidases and is not easily hydrolyzed[4].

There are six bile acids present in humans, if trace materials are ignored. These are formed by conjugation of the three major bile acids, cholic, chenodeoxycholic, and deoxycholic acids to glycine and taurine. The six are listed in Table 1[2].

TABLE 1

BILE ACIDS IN HUMANS

Bile Acid	Abbreviation
Glycocholate	GCA
Taurocholate	TCA
Glycochenodeoxycholate	GCDA
Taurochenodeoxycholate	TCDA
Glycodeoxycholate	GDCA
Taurodeoxycholate	TDCA

Bile acids have four main functions. First, they aid in bile secretion by promoting excretion of water, cholesterol and biliary pigments. Second, they aid in lipid digestion-absorption by emulsifying fats. This process also assists pancreatic lipolysis and mixed micelle formation. The micelles help in solubilizing products of lipolysis as well as other insoluble lipids. The third function of bile acids is related to the small intestine. The acids release hormones, control synthesis of mucosa, and may also prevent growth of bacteria. Last, the acids function in the colon to help prevent undue dehydration of the feces[5].

It has been reported that people with hepatic diseases show an increase or decrease in bile acid composition in the bile, depending upon their condition. For example, patients with ileal disorders have "...grossly disturbed bile salt metabolism...", diarrhea and sometimes steatorrhoea[2]. These are said to be caused by dysfunctions of the terminal ileum. Due to a loss of active transport sites in the terminal ileum, a significant amount of the bile salts pouring into the colon are not reabsorbed. The loss of active transport sites is most likely due to the action of the salts, which inhibit the absorptive functions and increase its motility of the terminal ileum. Patients with this disorder tend to have an increase in the ratio of glycine-conjugated to taurine-conjugated bile salts (G/T ratio).

Detection of this disorder can be done in several ways. One is a measurement of the bile radioactivity after a radioisotopically labelled bile salt has been administered. There can also be a screening test for terminal ileopathy in which a measurement of the G/T ratio is done. The simplest test is a breath test. After the ingestion of glycine 14-C labelled glycolate, measurements of radioactive CO_2 in expired breath are performed. An increase in radioactive CO_2 in the breath in the first 5-6 hours is recorded by patients with ileal disorders as well as those with bacterial contamination of the small bowel. This tends to

be a rather inexpensive screening test, but is only semi-quantitative.

Another disorder that results in a disturbance of bile salt metabolism is cholestasis, or blockage of the bile ducts leading from the gall bladder to the intestine. In this disorder, the bile salts accumulate in the blood because of their failure to enter the intestine. It is possible that lithocholate plays a key role in cholestasis. It has been reported that this bile salt and its conjugates are responsible for producing this disorder in experimental animals[1]. There are several theories on how lithocholate accumulates to cause this condition. Some researchers feel that lithocholate in the liver cells damages the smooth endoplasmic reticulum (SER), which then produces more lithocholate. This damage causes a decrease in the synthesis of other bile salts. The additional lithocholate present causes inflammation and fibrosis of small bile ducts, which will eventually aggravate and prolong the condition[5].

The measurement of serum bile acids has been reported to be a more sensitive indicator of liver disease than the usual liver function tests[6]. This would indicate that bile acid analysis may be helpful in the treatment and diagnosis of many hepatic diseases.

Concentrations of individual bile acids can be measured by various means such as radioimmunoassay, gas

chromatography, gas chromatography-mass spectrometry, refractive index, and HPLC with UV[6].

IMMOBILIZED ENZYMES

Enzymes are the biocatalytically active substances upon which the metabolism of all living organisms are based. They lower the energy of activation of reactions, which in turn speeds up the overall reaction rates. Enzymes themselves do not appear in the products of the reaction and therefore their action resembles that of inorganic catalysts.

Enzymes tend to give way to a much greater decrease in activation energy than can be obtained using inorganic catalysts[7]. Also, conditions such as low temperatures (relative to inorganic catalysts), atmospheric pressure rather than elevated pressure, and physiological pH values are optimum for enzyme catalyzed reactions to occur. Enzymes tend to be more specific than inorganic catalysts and many enzymes usually catalyze only one reaction. This is highly beneficial in that many side reactions are avoided during enzyme catalysis[7].

All enzymes are proteins. Coenzymes or cofactors are low molecular weight, non-protein substances that enable some enzyme proteins to develop their catalytic activity. Prosthetic groups are groups that are tightly bound to the enzyme protein[8].

When an enzyme has its mobility restricted (as in solution) by chemical or physical means, it is said to be immobilized. There are several ways to restrict the mobility of an enzyme, such as binding the enzyme molecules to one another or to some type of carrier substance, or entrapping them in the network of a polymer matrix[7]. In order to categorize a system as immobilized it generally must have some type of human induced hindrance.

Immobilization is done mainly for research purposes or for use in technical processes for industry. There are several advantages in the use of the immobilization process. It makes possible the use of heterogeneous rather than homogeneous catalysis. Because a homogeneous solution is not present, the problems associated with having to separate the dissolved native biocatalyst from the products of the reaction is eliminated[7]. An added advantage is that the reaction can also proceed continuously.

Another advantage to immobilization is the fact that it is possible to achieve and maintain higher enzyme activity in smaller volumes than is possible using soluble enzymes. This in turn, gives a higher product volume and also limits the time that the products and substrates are exposed to the reaction conditions. The immobilized enzyme usually has a longer half-life, predictable decay rates, and can be used repetitively[8]. Because enzymes tend to be so expensive, any

procedure that can extend the useful life of an enzyme is considered beneficial.

It can also be seen that by immobilizing the enzyme, the conversion of substrate to product can be more readily controlled. A reaction in which a soluble enzyme is used requires destruction of the enzyme or a change in the reaction environment in order to stop the process. In the case of immobilized enzymes, the reaction can be altered either by changing the flow rates of the substrate solution through the immobilized enzyme matrix or by simply removing the enzyme from the substrate solution[9].

If an immobilized enzyme is to be used in a reaction procedure it is desirable that it have properties that are of higher quality than that of its original form. Unfortunately, many times the immobilization process can cause major alterations in the pH and temperature relationships of an enzyme as well as affect its stability.

There are many methods of immobilization. The first which will be discussed is cross-linking. In this process bi-functional, or multi-functional, reagents help join individual enzyme molecules to one another. As they are joined together, very high molecular weight and usually insoluble enzyme molecule aggregates are formed[9].

Cross-linking involves the formation of new covalent bonds. Thus enzymes that undergo this type of

immobilization tend to undergo conformational changes, often with a resultant activity loss[7].

Adsorption of the enzyme molecules to an insoluble support material is the oldest and simplest method for immobilization. In the adsorption process, van der Waals forces hold the enzyme to the surface of the carrier substrate. There are usually other forces that help hold the enzyme onto the surface such as hydrophobic interactions, hydrogen bonds, and ionic bonds. It is usually difficult to tell which interaction, or group of interactions, are taking place when individual enzymes are undergoing an adhesion mechanism. Therefore the term adsorption is used in a very loose sense.

Another method of immobilization is covalent binding. In this process, shared electron pairs link the enzyme to the insoluble carrier. A tight correlation is thus made between the enzyme molecule and the carrier. This process is typically used for native enzyme molecules and not for whole cells, since a single link is not sufficient for coupling larger biocatalysts[9].

Functional groups that can be used for covalent binding include carboxyl, sulfhydryl, hydroxyl, and the α- and ϵ-amino groups of amino acids that are a part of the enzyme primary structure. Some functional groups can react directly with suitable functional groups of the carrier, while others must be "activated" before causing a reaction

between the enzyme molecule with the carrier. A good example of the second type is the hydroxyl group. To lessen the risk of altering the structure and therefore the activity of the enzyme, activation of the groups intended for binding is best done on the carrier rather than on the enzyme[7].

There are many carriers that are used for immobilization by covalent binding. They can range from inorganic and organic substances to synthetic polymers. Some examples are alumina, activated charcoal, and synthetic resins.

Another well known carrier is porous glass of controlled porosity. This inorganic carrier contains many -OH groups (silanols) on its surface. The surface is activated for immobilization when it is reacted with organo-silanes such as aminoalkylethoxysilanes. Once activated, the glass is able to take part in subsequent reactions. The reaction in Figure 2 illustrates the activation of porous glass by treatment with an aminoalkylethoxysilane[9]. Alkyl amine groups are thus contributed to the glass so the glass can be coupled to an enzyme with the aid of bifuctional reagents. Glutaraldehyde, the most popular reagent, is shown in Figure 3 in the coupling of the enzyme to glass.

There are several good characteristics to porous glass such as its "...precise control that may be exercised over its pore morphology[7]." One problem with porous glass is

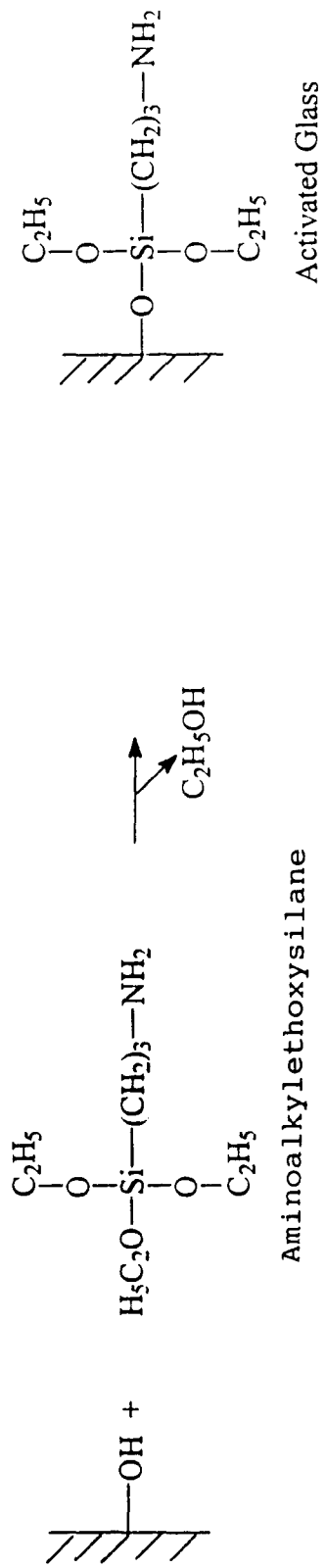


Fig. 2. Activation of glass with aminoalkylethoxysilane

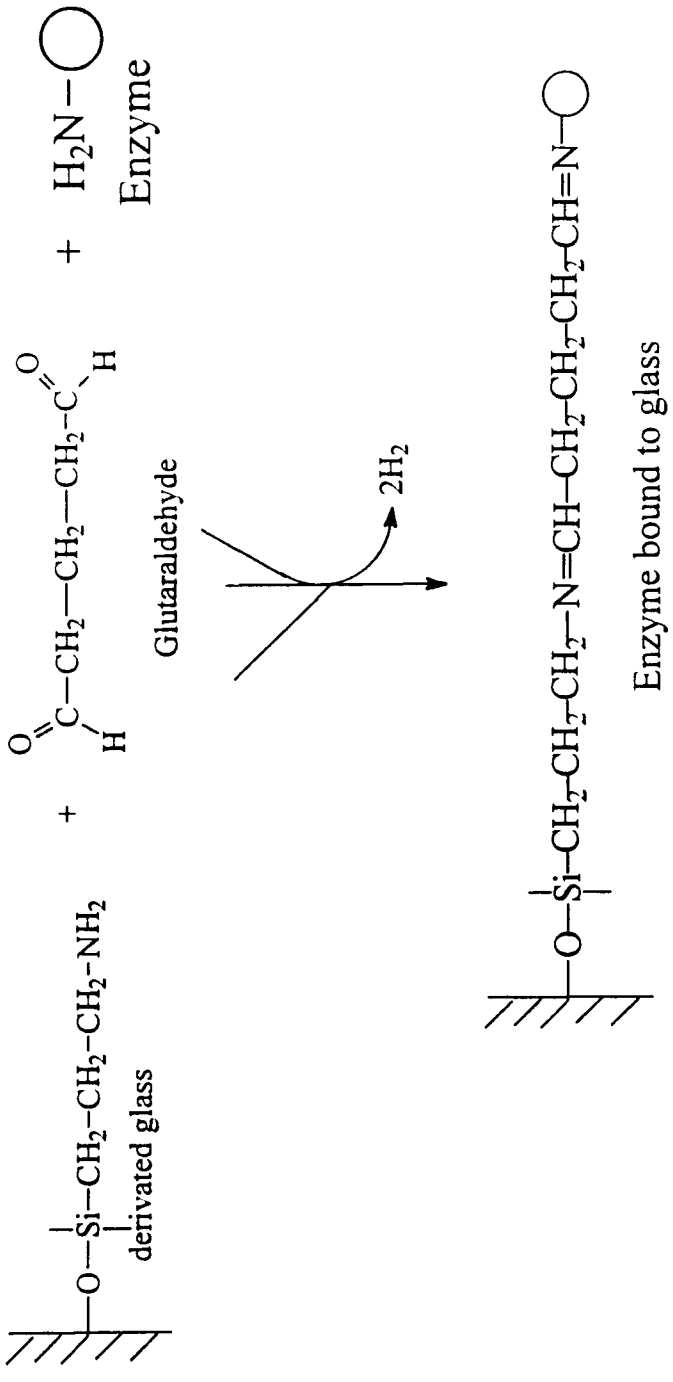


Fig. 3. Coupling of enzyme to glass by means of glutaraldehyde

that it has higher than normal solubility because of its large surface area. Solubility of the glass usually increases by a factor of 1.5 for each 10 degree rise in temperature. Strongly acidic conditions are usually best because solubility is pH dependent, being much less soluble in acidic solution[9].

POST COLUMN REACTION DETECTORS FOR HPLC

High performance liquid chromatography has been very a useful tool in the quantitation of drugs and their metabolites in a variety of matrices that include biological fluids and pharmaceutical dosage forms. Chemical derivations of an analyte can help to improve and facilitate analysis under certain conditions. This is called derivation and has been used widely in other applications such as mass spectroscopy, nuclear magnetic resonance, and gas-liquid and thin-layer chromatography[10].

The use of derivation in HPLC has been widely studied and can be performed in two different ways. The first is termed pre-column derivation. This is usually done to enhance the stability of an analyte, improve separation of an analyte from the sample medium, and to improve detectability by increasing the response of an analyte to the detector.

The second type, post-column derivation, is carried out on-line in a flowing stream using a reaction detector. The primary advantage of using post-column derivation over pre-

column is that there is not the threat of artifact formation during reaction and the reaction does not need to reach completion. A disadvantage to post-column is that the mobile phase has a significant influence on the reaction medium. It is highly unlikely that the optimum chromatographic eluent is the best reaction medium and there are thus major compromises that must be made to alleviate this problem[11].

Among the relevant factors to be considered in developing a post-column reactor are reaction kinetics and physical design of the reactor itself. Fast kinetics are ideal since long retention times lead to significant band broadening and decrease chromatographic resolution.

Tubular reactors are said to be the simplest, and are suitable for reactions with fast kinetics. These reactors consist of straight or helically coiled tubes constructed of materials such as glass, Teflon^{R}, and steel[11]. Bed reactors are used in reactions that tend to have residence times of 30 seconds to several minutes. In reactions with slower kinetics (residence times of 4 minutes and longer) a segmented stream reactor is used in order to minimize band-broadening.

When using tubular reactors, the column effluent is mixed with the reagents in order to produce, for example, a fluorescent product. It is important that the reagent itself does not interfere with the detection signal of the

derivatives. Reagents for post-column derivation should have little or no native fluorescence and should react rapidly.

There are several fluorigenic reagents that are ideal for post-column derivation. Two examples are fluorescamine (Fluram^{R}) developed by Undenfriend[11] and o-phthalaldehyde (OPA) originally proposed by Roth[11]. These reagents yield highly fluorescent derivatives and are selective for primary amines. Some reactions are completed in as quickly as a few seconds, while others take up to a few minutes. This reaction time is related to the reaction environment and also to stearic factors. Fluram^{R} tends to react more slowly than OPA and the reactions tend to reach a plateau after several minutes residence time in the reactor[11].

OPA produces highly fluorescent derivatives when it reacts with amino acids in an alkaline medium in the presence of 2-mercaptoethanol, a reducing agent. Figure 4 illustrates this reaction. Derivatives of OPA may be excited at 340 nm and emit at 445 nm. The reaction is carried out at room temperature and is applicable to the determination of amino acids in effluents from column chromatography. OPA is soluble and stable in water, unlike Fluram^{R}. It also has a tenfold greater sensitivity than Fluram^{R} and is much less expensive[10].

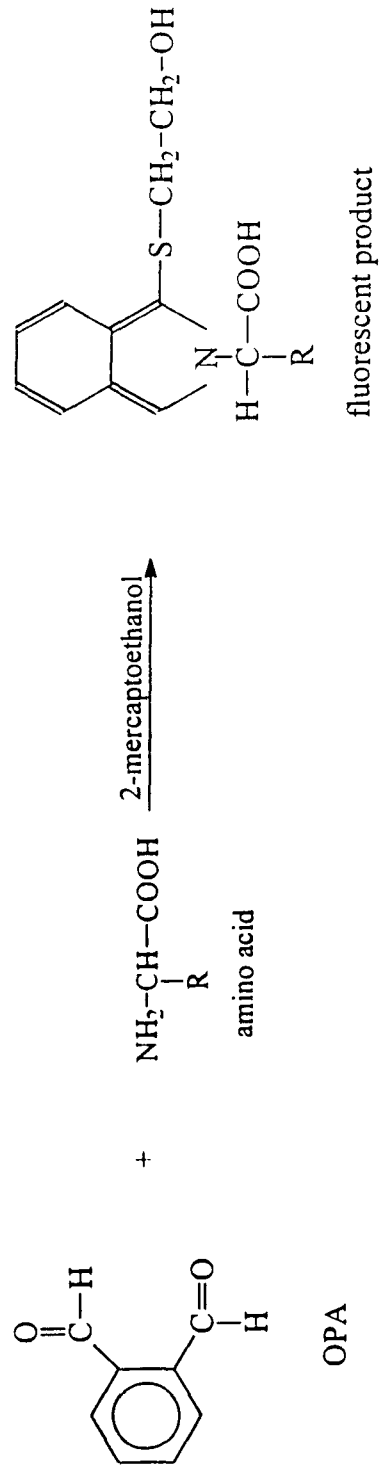


Fig. 4. Reaction of OPA with amino acids in the presence of 2-mercaptoethanol

CHOLYLGLYCINE HYDROLASE

Cholylglycine hydrolase is an enzyme that can be purified from the bacterium Clostridium perfringens[12]. It is inhibited by sulfhydryl reagents and metal ions such as mercury and zinc[13]. This enzyme catalyzes the hydrolysis of the carbon-nitrogen bond in taurine and glycine bile acid conjugates[14]. The enzyme has an optimum pH between 5.6-5.8. It is also said to have a higher activity in acetate buffers than in phosphate buffers[14]. Free bile acids and either glycine or taurine are produced in equal amounts by the hydrolysis reaction.

CHAPTER II

HISTORICAL

Shaw & Elliot reported the first separation of bile acids using HPLC. A Corasil II column was used to resolve all of the major conjugated bile acids[15].

Goto et al. reported a method for simultaneous determination of unconjugated and conjugated bile acids in human bile, which involved the group separation followed by resolution of bile acids of each group by HPLC on a μ-Bondapak C_{18} column using 0.3% ammonium carbonate (pH 7.8)/acetonitrile as a mobile phase. Difficulties arose with the resolution of ursodeoxycholate and cholate. There were also difficulties in finding an appropriate mobile phase pH[16].

The use of cyclodextrin (CD) as a mobile phase additive in the HPLC of bile acids was presented by Shimada et al. The separation of bile acid pyrenacyl esters by inclusion chromatography was performed. The addition of the CD in reversed phase HPLC gave more satisfactory separation of free or conjugated bile acid pyrenacyl esters in a shorter time[17].

It has been reported that the use of fluorophores, which are reactive toward the carboxylic function, such as bromoacetylpyrene, have been successfully used in the HPLC fluorescense determination of bile acids. 2-bromoacetyl-6-methoxynaphthalene has been used in the HPLC analysis of

aliphatic carboxylic acids as a labelling agent. Gatti et al. used this fluorophore in their investigation of the derivation of free and glycine-conjugated bile acids. This derivation was conducted in an aqueous medium in the presence of an ion-pair reagent, tetrahexyl ammonium bromide. This method proved to be adequate for the determination of ursodeoxycholic acid and chenodeoxycholic acid in their dosage forms and for the analysis of the major bile acids in human bile[18].

Scalia and Pazzi performed the HPLC assay of conjugated bile acids. The injection loop was replaced by a small pre-column. Sample pre-treatment was done on the pre-column which was then connected on-line with the analytical column. This alleviated the time consuming clean-up procedures required by off-line techniques. It also prevented possible errors due to multiple sample manipulations previously required[19].

Fluorescence labeling has been used to determine free and conjugated bile acids in serum, feces, and other media. Güldühina et al. used the fluorescence substance 4-bromomethyl-7-methoxycoumarin (BMC) which reacts with carboxylic groups. Fifteen bile acids were analyzed as methoxycoumarin derivatives using HPLC. BMC is commercially available in analytical grade quality[20].

In 1993, Sakakura et al. used a combination of HPLC separation with post-column enzymatic reaction and

fluorescence detection to measure 3α-hydroxylate bile acid levels in serum with increased sensitivity and specificity. The enzyme used was 3α-hydroxysteroid dehydrogenase (3α-HSD). This technique was used for the simultaneous determination of 26 bile acids in rat bile and serum. Bile acid metabolism in human hepatobiliary diseases has been widely studied using rats. This method however, is not suitable for individual bile acid separation in humans because rats have peculiar bile acids such as α-muricholic acid and ß-muricholic acid[6].

In 1983, Tazawa et al. performed direct measurement of urinary bile acids of infants using HPLC with an immobilized enzyme column. The enzyme used in the immobilization process was 3α-HSD. Non 3α-hydroxy bile acids were determined using an ultraviolet spectromonitor while 3α-hydroxy bile acids were determined using a fluorescence spectromonitor[21].

Hasegawa et al. reported an improved method using the same enzyme. Originally, the method was a flow system using enzyme solution, which was quite expensive. In the improved method a new HPLC column and an immobilized enzyme column were used. The reaction was done at alkaline pH which is optimum for the activity of the enzyme. The coenzyme NAD^+ was reduced to NADH by the immobilized enzyme 3α-HSD in the presence of bile acids as substrate eluted from the HPLC column.

A measurement of the fluorescence intensity of the NADH was then recorded[22].

Kamada et al. incorporated the use of the immobilized 3α-HSD enzyme with an electrochemical detector in the determination of free and conjugated bile acids. Bile was fractionated and applied to an HPLC system which consisted of a Radial-Pak A column and the enzyme column. NADH generated was detected using voltammetry after mixing with phanazine methosulfate solution[23].

A single step was used in the separation of bile acids reported by Campbell et al[24]. The system consisted of a C_{18} Spherisorb column, immobilized 3α-HSD column reactor and a spectrofluorimetric detector. The reaction was carried out with adequate sensitivity for the analysis of serum samples at neutral pH. This increased the lifetime of the enzyme column. The use of 3α-HSD enzyme was also reported by Swobodnek et al[25]. In their process, conjugated bile acids in human serum were analyzed using HPLC with an immobilized 3α-HSD post-column reaction and off-line spectrofluormetric detection.

Watanabe et al. used an immobilized 3α-HSD column to separate the 15 major bile acids using HPLC. A Sep-Pak C_{18} solid-phase extraction cartridge and a Sephadex PHP-LH-20 column were used to fractionate bile acids in human bile into free, glycine, and taurine conjugated bile acids. After fractionation, each acid was passed through an HPLC

system which consisted of a Zorbax C_{18} column and a 3α-HSD immobilized enzyme column[26].

CHAPTER III

STATEMENT OF PROBLEM

HPLC has been widely used in the analysis of bile samples. The methods used previously lacked sensitivity and specificity to clearly identify the analyte peaks.

Post-column immobilized reactors have been incorporated with HPLC analysis to improve sensitivity and specificity. Earlier studies relied on immobilization of an α-hydroxysteroid dehydrogenase. The problem with the use of this enzyme was the expense of both the enzyme and consumable reagents associated with the reaction. A method was developed in which the use of immobilized cholylglycine hydrolase was incorporated into the system. This enzyme proved to be less expensive, and did not require the consumable reagents needed for the α-hydroxysteroid dehydrogenase.

This investigation extended the previous research done involving the use of immobilized CGH. Optimization of the system was investigated along with the analysis of bile samples.

CHAPTER IV

MATERIALS AND APPARATUS

Materials

The bile acids and the enzyme cholylglycine hydrolase were purchased from Sigma Chemical Company, St. Louis, MO. Ascorbic acid was purchased from Fisher Scientific, Pittsburgh, PA. All other chemicals used in this research study were reagent grade or the best grade available.

Apparatus

Optimization of the System

In order to be useful for trace analysis, it was necessary to optimize the analytical system for maximum signal to noise ratio. For this optimization, data was collected using a Hewlett Packard HP 3396 Series II Integrator. A model RF-535 fluorescence HPLC monitor (Shimadzu, Japan) was used for detection. Reagent solutions were pumped using three Beckman Model 110B Solvent Delivery Module systems (Beckman Instruments, Irvine, CA). A model RF 5000 Spectrofluorophotometer (Shimadzu, Japan) was used for determination of the optimum excitation and emission wavelengths for the fluorescent derivatives used in this study. A schematic diagram of the system used for the optimization determinations is shown in Figure 5.

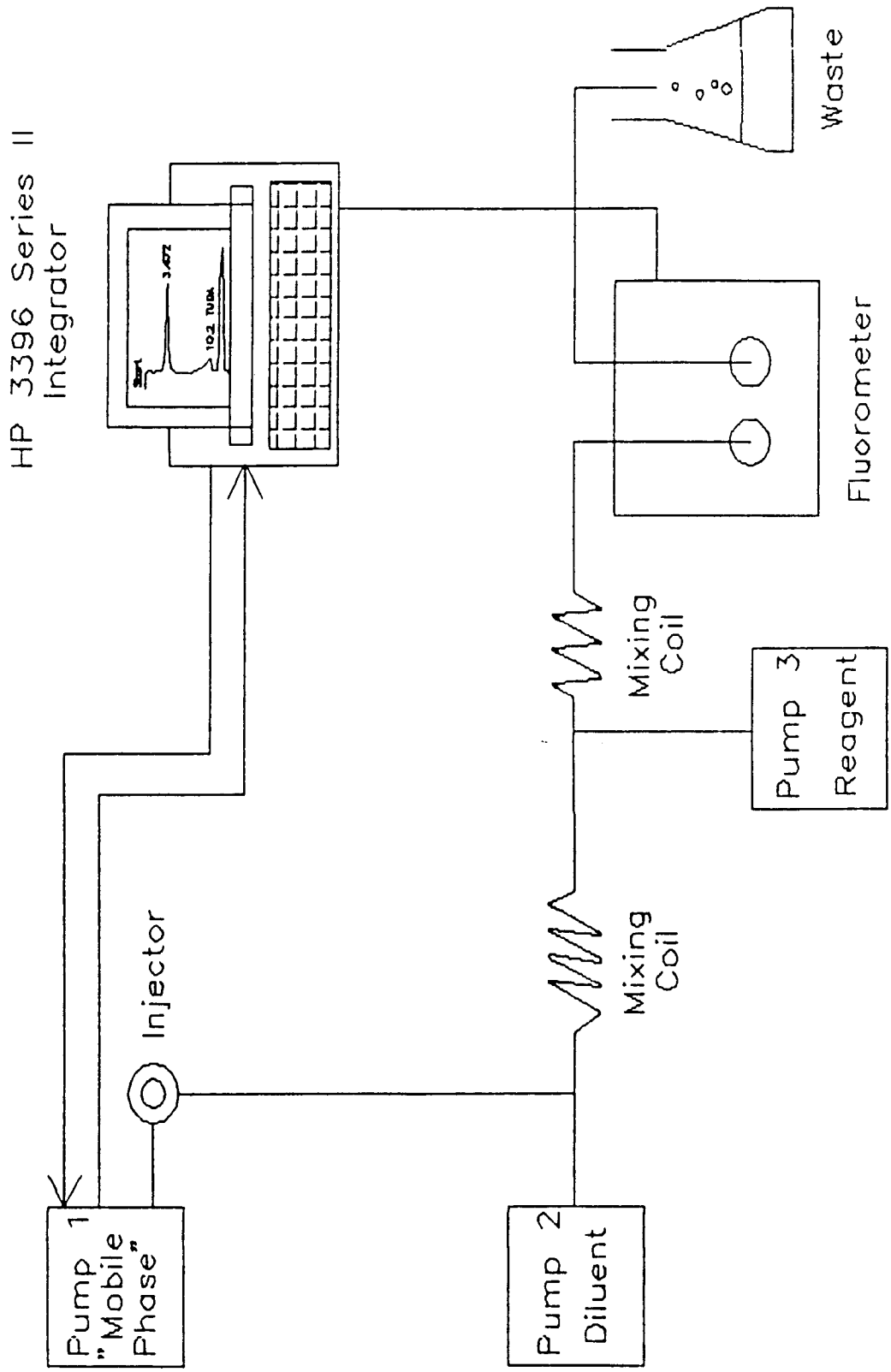


Fig. 5. Scheme of system used for optimization analysis

High Performance Liquid Chromatography

The system used for analyzing samples was similar to the system used for optimization of signal to noise ratio. The major difference was two columns that were added to this system for the separation and analysis of bile acids. An Adsorbosphere C_{18} analytical HPLC column measuring 4.6 mm i.d. x 250 mm, with a particle size of 5 μm (Alltech Associates Inc.) was used for the separation of the bile acids. Secondly, a 4.6 mm i.d. x 50 mm stainless steel column packed with the CPG-immobilized cholylglycine hydrolase was used as the post-column enzyme reactor. A schematic diagram of the system used for the HPLC analysis is shown in Figure 6.

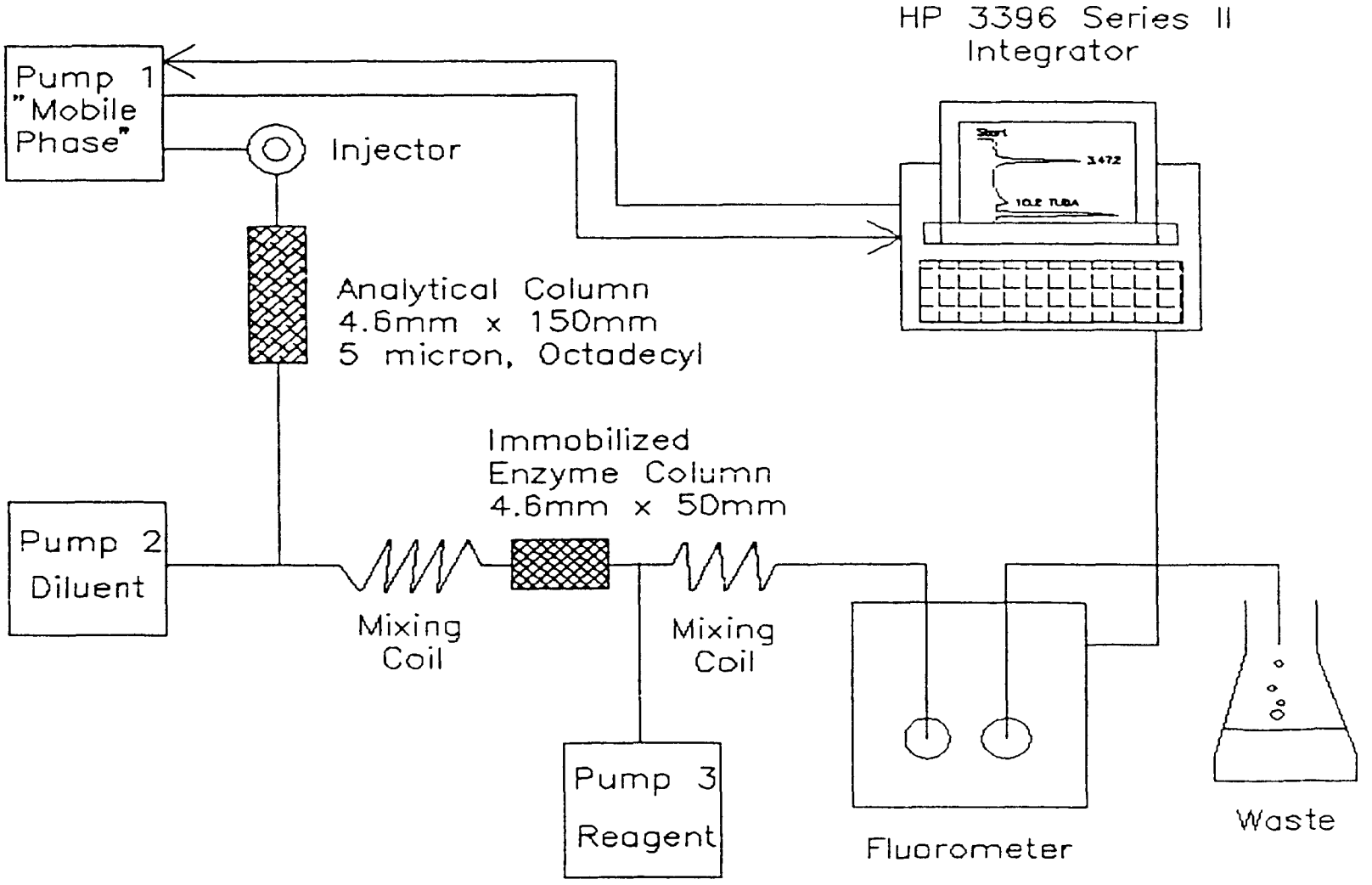


Fig. 6. Scheme of the analytical system

CHAPTER V

EXPERIMENTAL

Optimization of the System

Reagents

Reagents used for the determination of the ideal parameters of the system, and their preparation, are listed below:

Potassium phosphate, 0.05M, pH 4.5 -

Dissolve 3.402 g of KH_2PO_4 in 500 mL of deionized water. Adjust to pH 4.5 with 85% phosphoric acid and 6.0 M NaOH.

EDTA, 0.05 M -

Dissolve 18.61 g of $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$ in 1.0 L of deionized water.

Sodium Acetate, 0.05 M-

Dissolve 4.10 g of $NaC_2H_3O_2$ in 1.0 L of deionized water.

Buffer solution, 0.05 M EDTA, 0.05 M sodium acetate -

Mix 990 mL of EDTA and 990 mL of sodium acetate. Dilute to 2 L.

Diluent, 0.05 M EDTA, 0.05 M sodium acetate, 0.5% ascorbic acid (crystal), pH 4.5-

Dissolve 5.0 g of ascorbic acid in 500 mL of EDTA/sodium acetate buffer solution. Adjust to pH 5.0 with 6.0 M HCl and 6.0 M NaOH.

Sodium Carbonate, 0.40 M-

Dissolve 84.7 g of Na_2CO_3 in 2.0 L of deionized water.

OPA Reagent-

Dissolve 80 mg of OPA in 1.0 mL of 95% ethanol and mix with 100 mL sodium carbonate, and 1.0 mL of 2-mercaptoethanol. Adjust to pH 11.0 with 6.0 M HCl and 6.0 M NaOH.

Glycine stock solution -

Dissolve 1.0 mg of glycine in 100 mL of 65% 0.05 M KH_2PO_4 and 35% methanol, pH 4.5.

Glycine derivative-

1.0 mL of glycine stock solution was diluted to 50 mL with 65% 0.05 M KH_2PO_4 and 35% methanol, pH 4.5.

Taurine stock solution-

Dissolved 1.0 mg of taurine in 100 mL of 65% 0.05 M KH_2PO_4 and 35% methanol, pH 4.5.

Taurine derivative-

1.0 mL of taurine stock solution was diluted to 50 mL with 65% 0.05 M KH_2PO_4 and 35% methanol, pH 4.5.

PROCEDURE

Optimization of the System

The system used for the studies of optimum signal to noise ratio is shown in Figure 5. Pump 1 was used to pump a chromatographic mobile phase consisting of 65% HPLC Reagent grade methanol and 35% 0.05 M KH_2PO_4, pH 4.5. The mobile phase flow rate and composition was kept constant during all optimization studies since these variables were expected to change little, if at all, in the final analytical system. Pump 2 was used to pump the OPA fluorogenic reagent and Pump 3 was used to pump the diluent solution. Injection volumes were maintained at 20 μl, since this would be the size of the sample injected during use of the system for bile acid analysis. Fluorescence detection was done using a fluorescence detector with an excitation wavelength of 341 nm and emission wavelength of 448 nm.

Optimization of the system was done by injecting samples of glycine and taurine individually to obtain signal to noise ratios. The procedure was started by turning on the pumps and ensuring that they were operating correctly and at the proper flow rates. The diluent pump (Pump 3) was turned on first followed by the OPA reagent pump (Pump 2) and lastly the mobile phase pump (Pump 1). The order of turning on the pumps had no significance for this optimization study but would become very important for the final analytical system. As such, flow order was maintained during optimization for consistency.

Once proper flow of the system was obtained, 20 μL samples of glycine and taurine were individually injected. Glycine and taurine concentrations were 2.0×10^{-3} M. Data was collected at several attenuation levels to ensure adequate measurement of both signal and noise levels on the integrator read out for each parameter studied.

CALCULATIONS

The signal obtained from each parameter study was compared to the noise in order to find the signal to noise ratio. It is important to note that the noise was measured at a lower attenuation value than the signals, with a factor of two between each attenuation level. The noise therefore had to be adjusted accordingly. Signal was considered to be the height of the sample peak, from the baseline to the crest of the peak, while the noise was measured as the

width of the baseline from top to bottom, where no signal was present. A ratio of these figures was then calculated.

IMMOBILIZATION OF CHOLYLGLYCINE HYDROLASE PREPARATION

Reagents

Nitric Acid, 5%-

Dilute 5 mL of concentrated HNO_3 to 100 mL with deionized water.

3-aminopropyltriethoxysilane, 10%-

Mix 2 mL of $C_9H_{23}NO_3Si$ with 18 mL of deionized water.

Disodium Phosphate, 0.05 M, pH 7.0-

Dissolve 7.1 g of Na_2HPO_4 in 1.0 L of deionized water. Adjust to pH 7.0 with 6.0 M HCl.

Glutaraldehyde, 2.5% in 0.05 M disodium phosphate, pH 7.0-

Dilute 2.5 mL of 25% $C_5H_8O_2$ to 25 mL with 0.05 M Na2HPO4, pH 7.0. Prepare fresh for each use.

Disodium phosphate, 0.05 M, pH 7.0, containing 0.05% ascorbic acid-

Dilute 0.825 g of ascorbic acid in 100 mL of 0.05 M Na_2HPO_4, pH 7.0.

Cholylglycine hydrolase enzyme solution-

Dissolve 20 mg of CGH enzyme in 0.05 M Na_2HPO_4, pH 7.0 containing 0.5% ascorbic acid.

PROCEDURE

Two procedures were used for the immobilization of the enzyme. The first was silanization of controlled pore glass (CPG). In this method, following Weetal[27], 1.0 g of CPG was weighed out into a 50 mL beaker and cleaned by

soaking in a 5% HNO_3 solution for one hour at 80-90°C. After heating, the CPG was filtered using a Büchner funnel and washed thoroughly with deionized water to get rid of the HNO_3. The cleaned CPG was placed in a 50 mL beaker to which 18 mL of deionized water and 2 mL of 3-aminopropyltriethoxysilane was added. This material was then adjusted to pH 3.5 with 6.0 M HCl, and 6.0 M NaOH if needed. After adjustment of the pH, the material was placed in a water bath maintained at 75°C for 2 hours. The final material was removed from the water bath, filtered on a Büchner funnel and rinsed with 20 mL of deionized water. The material (aminated CPG) was then placed in an oven at 115°C overnight.

Twenty-five mL of 2.5% glutaraldehyde was added to 1.0 g of the aminated CPG. This activated the glass for enzyme attachment by adding carbonyl groups to the glass. The reaction mixture was allowed to stand at room temperature for at least 60 minutes. A color change was observed from white to magenta almost immediately after the glutaraldehyde solution was added. The excess glutaraldehyde was removed by washing the activated CPG with 1 L of deionized water on a Büchner funnel. Twenty mg/mL of CGH enzyme solution containing 0.5% (w/v) ascorbic acid was added to the activated glass. The ascorbic acid was used to prevent oxidation of essential thiol groups on the enzyme's active sites. The reaction was allowed to proceed for at

least 2 hours at room temperature. The CPG-immobilized CGH was then stored in a refrigerator at 4°C.

The other method of immobilization used in this research consisted of using aminopropyl glass. This method was identical to the previous method except the silanization steps were excluded. The aminopropyl glass could be activated with no prior treatment.

HPLC Analysis of Bile Acid Standards

Reagents

Reagents used in this investigation were the same as the reagents used in the optimization study except a pH of 4.0 was used for the potassium phosphate buffer.

PROCEDURE

Three different bile acid standard concentrations were analyzed using the optimized HPLC system. The samples were made from a stock solution. The stock solution contained ten bile acids dissolved in 25 mL of methanol. The three standards were diluted to the desired concentrations using methanol. The immobilized enzyme was packed into a stainless steel column using diluent solution.

The system used in this analysis is illustrated in Figure 6. An Adsorbosphere C_{18} column and a CPG-immobilized CGH enzyme column was used. Pump 1 carried the mobile phase which consisted of 65% methanol and 35% KH_2PO_4, pH 4.0. Pump 2 pumped the diluent. This was needed to

dilute the concentration of methanol in the eluent. A lower concentration of methanol was needed so the enzyme would not be denatured. The ascorbic acid that was dissolved in the diluent solution prevented oxidation of thiol groups in the active site of the enzyme. Pump 3 contained the OPA reagent. A stainless steel mixing coil was placed between the analytical column and the enzyme column to allow sufficient mixing of the eluent and diluent solutions. A Teflon^{R} reaction coil was placed between the enzyme column and the detector to allow ample time for derivation of the OPA reagent with the glycine and taurine that were eluted from the enzyme reaction.

The order in which the pumps were activated was very important. Pump 2 was turned on first, followed by Pump 3, then Pump 1. This was done to ensure that the enzyme was not denatured by direct contact with the mobile phase. Flow rates for Pumps 2 and 3 were 1.5 mL/min. A flow rate of 0.7 mL/min was maintained for Pump 1. The enzyme was kept at a constant temperature of 37°C by placing it in a water jacket that circulated heated water around the column. Standard samples were injected in 20 μL volumes. A fluorescence detector set at an excitation of 341 nm and an emission of 450 nm was used for detection and data recorded using an integrator.

At the end of the day, the enzyme column was flushed

with 20 mL of a 0.75% 2-mercaptoethanol solution in diluent buffer and placed in a refrigerator. Pumps 2 and 3 were flushed with 30 mL of deionized water to remove buffer solutions. Pump 1 was flushed with methanol to remove buffer solutions and rinse the analytical column.

Bile Analysis

Reagents

Reagents used for bile analysis were the same as the reagents used in the bile acid standard analysis study.

PROCEDURE

Bile samples were stored in a freezer at -20°C until further preparation was needed. The bile sample was placed in a beaker of ice and allowed to sit at room temperature until the sample was completely thawed. Once the sample was thawed it was centrifuged for ten minutes. One hundred μL of bile was placed in a test tube with 100 μL of methanol then centrifuged for ten minutes. Five μL of the bile/methanol mixture was injected into the system. System parameters were the same as for the bile acid standards.

CHAPTER VI

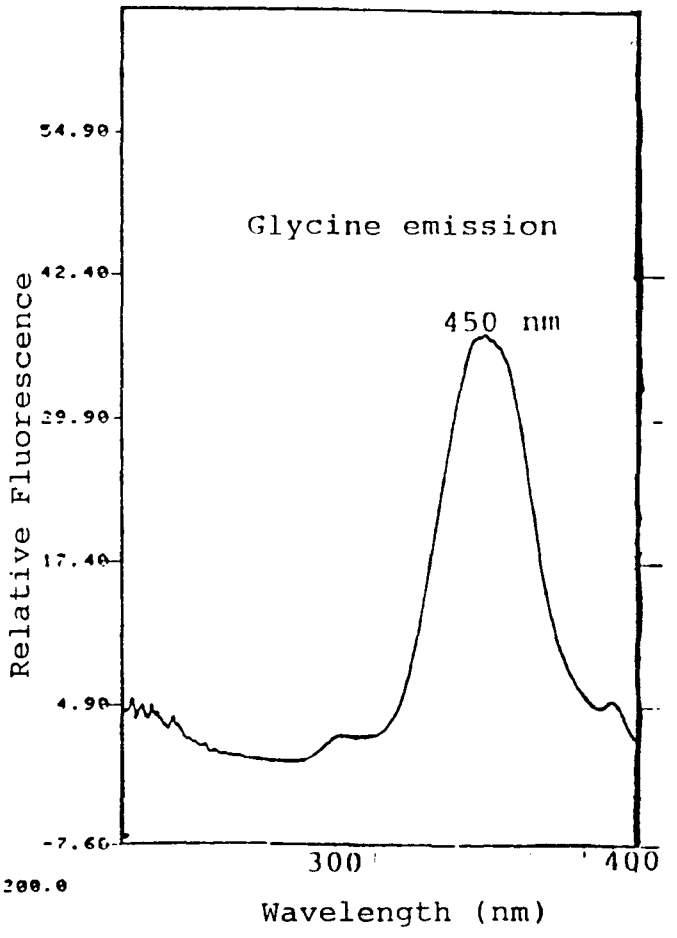
RESULTS AND DISCUSSION

Optimization of the System

Optimization of the analytical system was done to find the ideal system parameters. Several parameters were investigated, including: reagent and diluent solution pH and concentration, pump flow rates, and reaction coil volumes. Direct injections of glycine and taurine were done to determine the signal to noise ratios for each parameter studied. Optimization was determined as the pH, flow rate, etc. which gave the highest ratio.

Optimum excitation and emission wavelengths were determined for the taurine and glycine derivatives using a spectrofluorophotometer. Figures 7 and 8 show the glycine and taurine spectral data of this analysis.

Diluent pH was the first parameter investigated. It is important to note that the mobile phase flow rate and composition were kept constant during these investigations. The composition of the mobile phase was 65% 0.05 M KH_2PO_4 at pH 4.50 and 35% methanol. pH values of 4.0, 5.0, and 6.0 were tested for the diluent solution. During the initial investigation, the pH of the diluent solution was adjusted before the addition of ascorbic acid. It was later found that the ascorbic acid had a significant effect on the pH of the solution. Therefore, in subsequent studies, the pH was adjusted after the addition of ascorbic acid. A pH



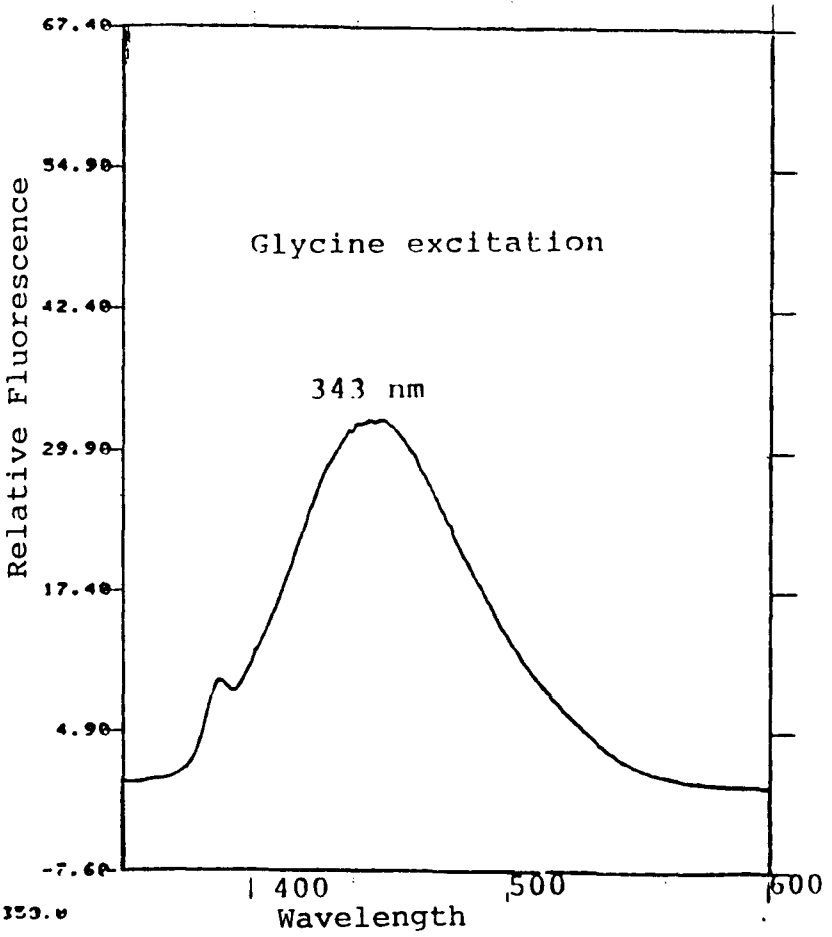
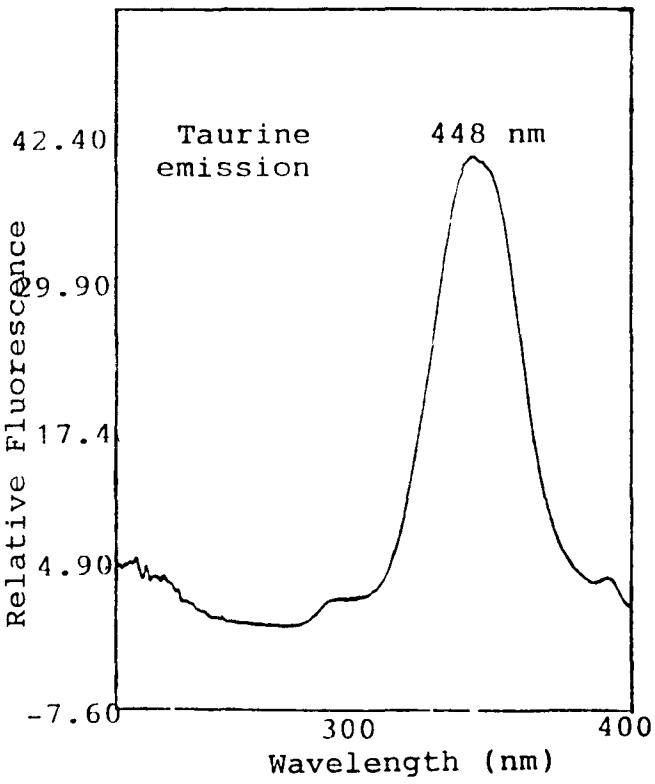


Fig. 7. Emission and excitation of glycine derivative



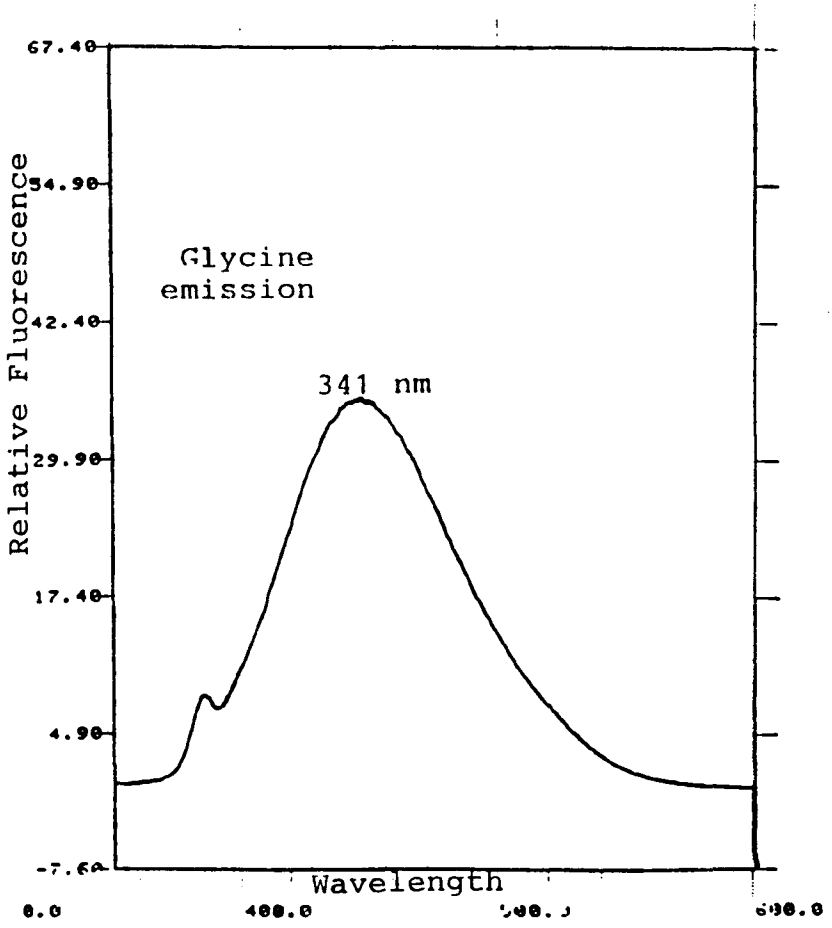


Fig. 8. Emission and excitaion of taurine derivative

value of 5.0 was found to be the optimum pH for the diluent solution according to S/N ratios. The analysis for optimization of signal to noise variation in diluent pH is shown in Figure 9.

OPA reagent pH was the next parameter studied. Solutions with pH values of 10, 10.5, and 11.0 in carbonate buffer were prepared. These pH values were chosen because previous studies indicated that the optimum pH region for OPA was between 9 and 11. The addition of 2-mercaptoethanol had a significant effect on the pH, therefore pH was adjusted after its addition to the OPA reagent solution. The addition of OPA seemed to have no effect on the pH, but was added before pH adjustment. The optimum pH value for the OPA reagent for S/N was found to be 11.0. The results of OPA reagent pH study are shown in Figure 10.

Once the ideal pH of the reagent and diluent solutions was determined, concentrations of chemicals added to the buffer solutions were investigated. Ascorbic acid concentrations of 0.5%, 1.0% and 2.0% in diluent solution were prepared. These values were chosen because in prior studies, an ascorbic acid concentration of 1.0 % was used[13]. The study focused on values in that range. Figure 11 shows the relationship between S/N ratio and ascorbic acid concentration. The 1% ascorbic acid concentration proved to be ideal for this system.

Concentrations of OPA and 2-mercaptoethanol in the OPA

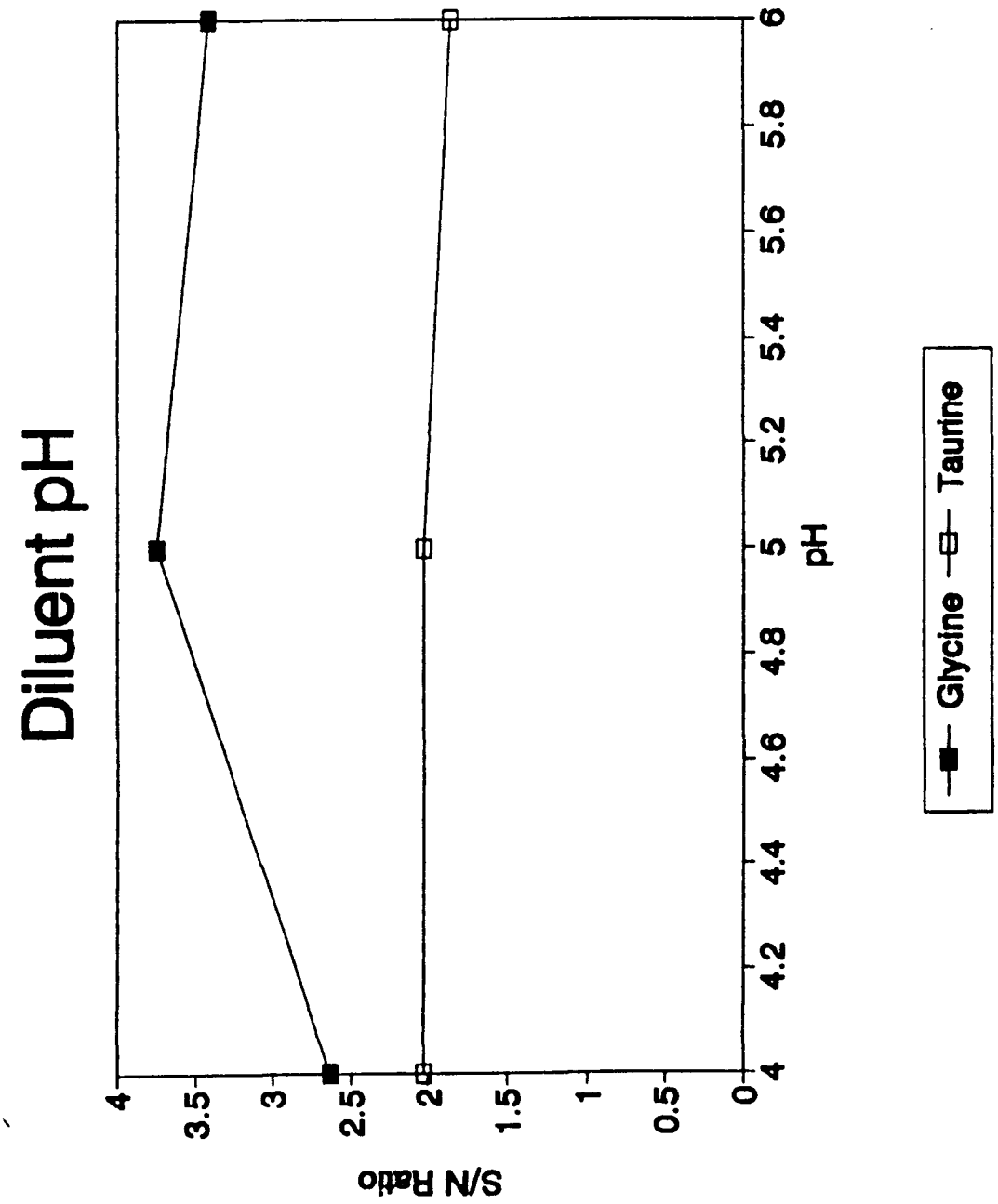


Fig. 9. Optimization of diluent pH

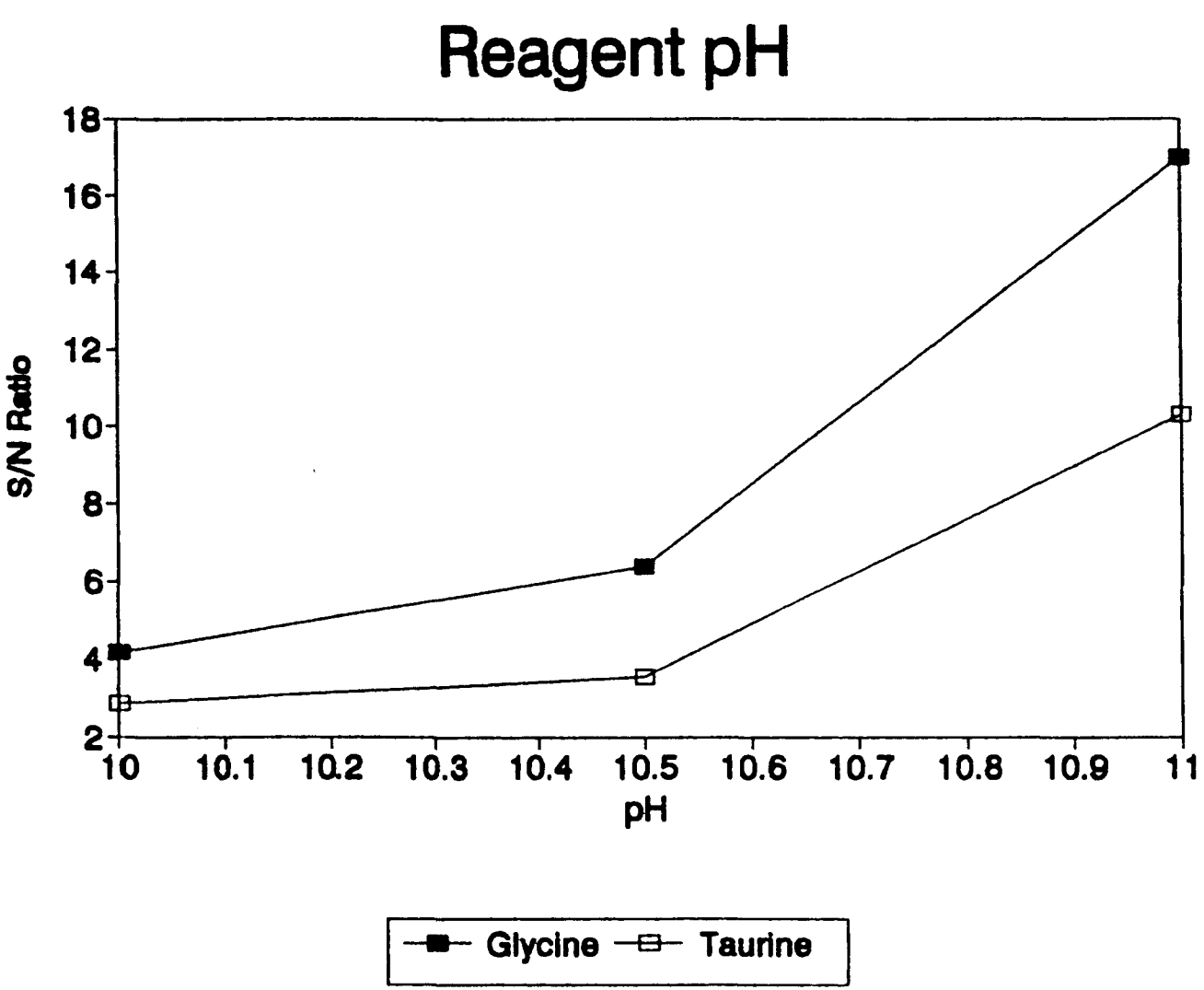


Fig. 10. Optimization of OPA reagent pH

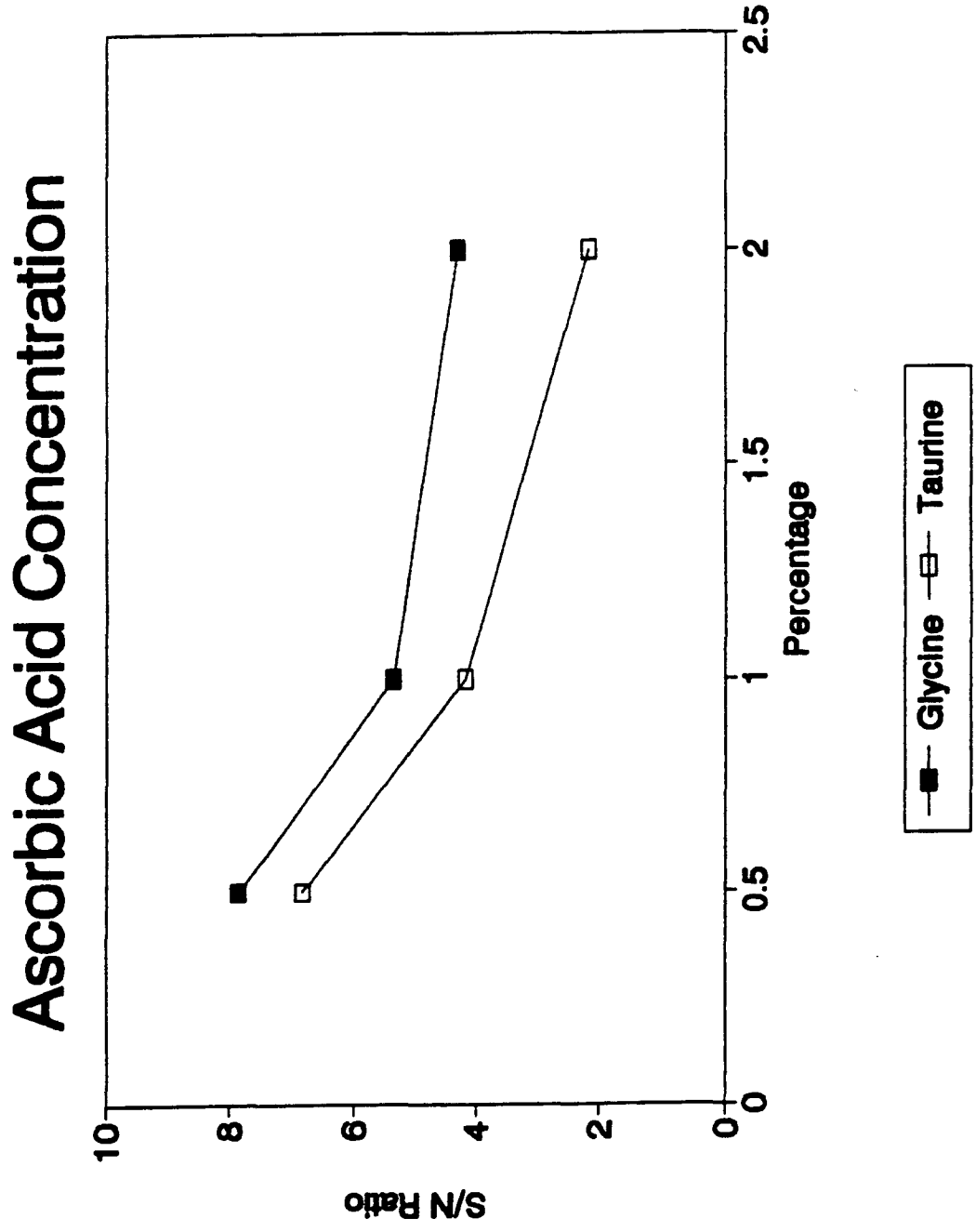


Fig. 11. Optimization of ascorbic acid concentration

reagent were investigated. Three separate OPA reagent solutions were prepared containing 40, 80, and 120 mg of OPA. Eighty mg was found to be the ideal amount of OPA used for reagent preparation. Concentrations of 0.5%, 1.0% and 2.0% of 2-mercaptoethanol in OPA reagent were studied. Figure 12 illustrates the parameter for the OPA concentration. There was no significant difference observed in the 2-mercaptoethanol concentration study (Figure 13) and therefore this parameter investigation was determined not to be important.

Once solution pH and concentration parameters were set, flow rates of the diluent and OPA reagent were investigated. Figures 14 and 15 show that a flow rate of 1.5 mL/min was optimum for both the diluent and OPA reagent. Once solution pH and concentration parameters were set, flow rates of the diluent and OPA reagent were investigated. Both were studied at flow rates of 0.5, 1.0, 1.5, and 2.0 mL/min.

The last parameter investigated was coil reaction volume. The reaction coil was located between the enzyme column and detector and was the area of the system where the OPA derivation reaction would occur. Coil volumes used in the study are listed in Table 2.

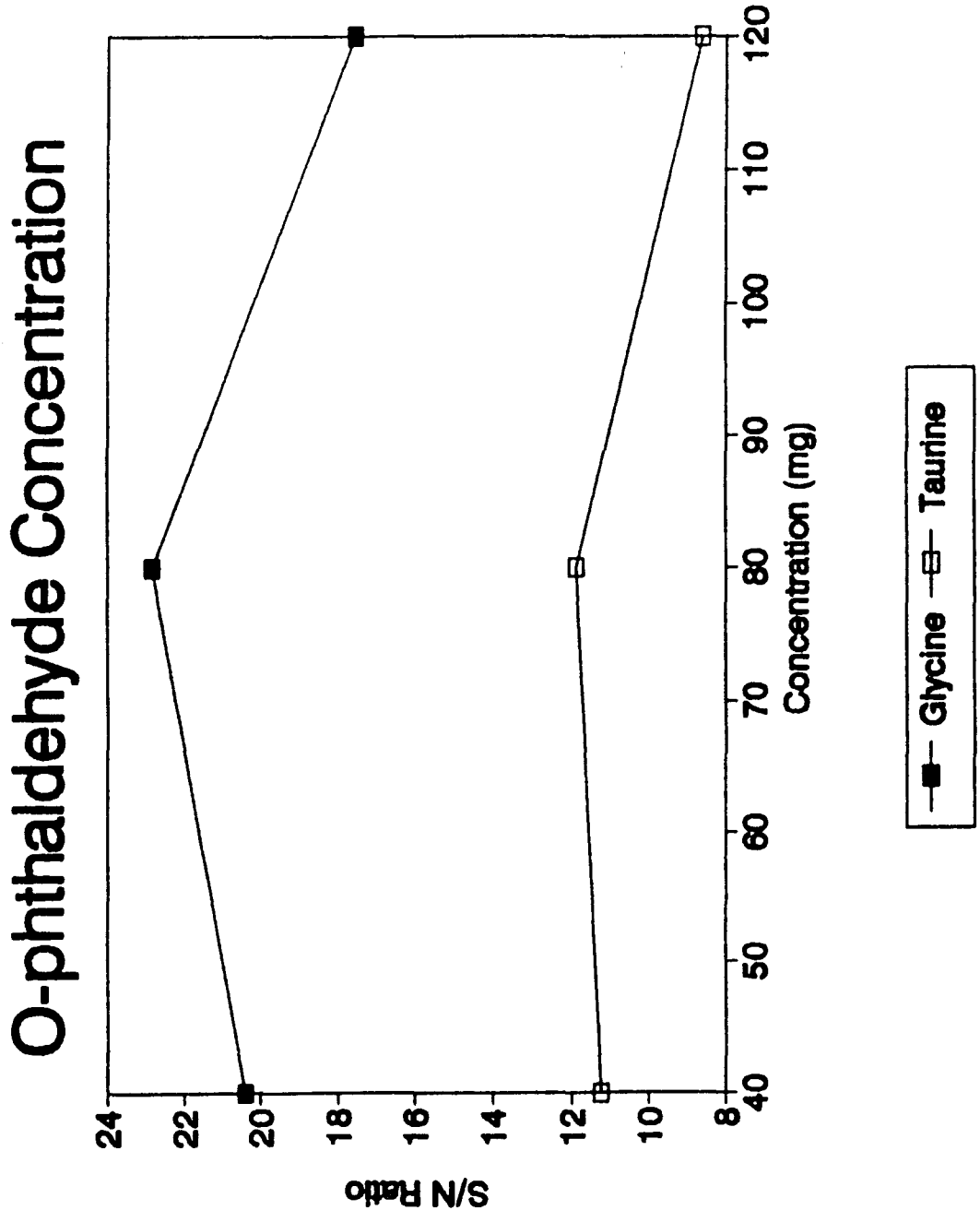


Fig. 12. Optimization of OPA reagent concentration

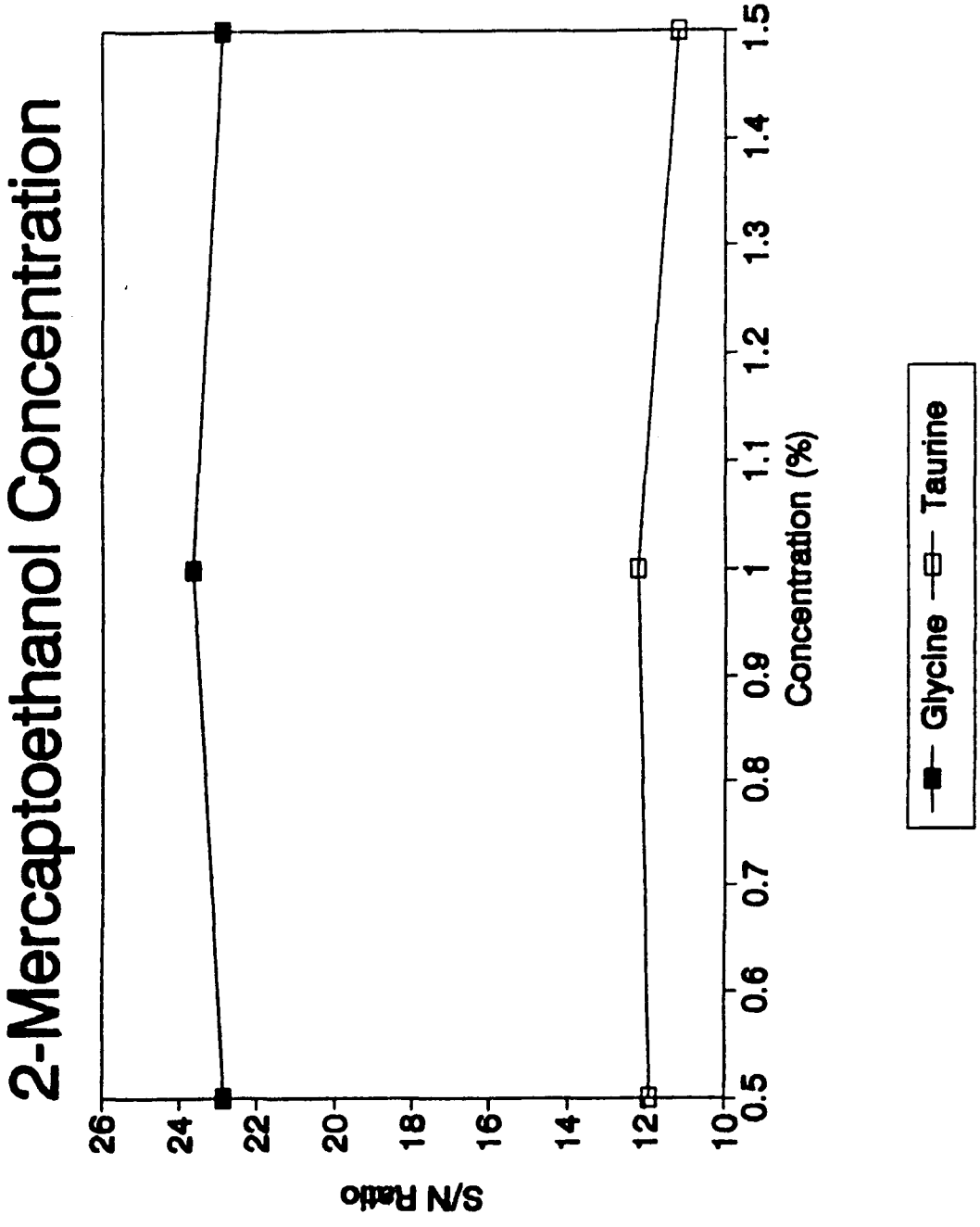


Fig. 13. Optimization of 2-mercaptoethanol concentration

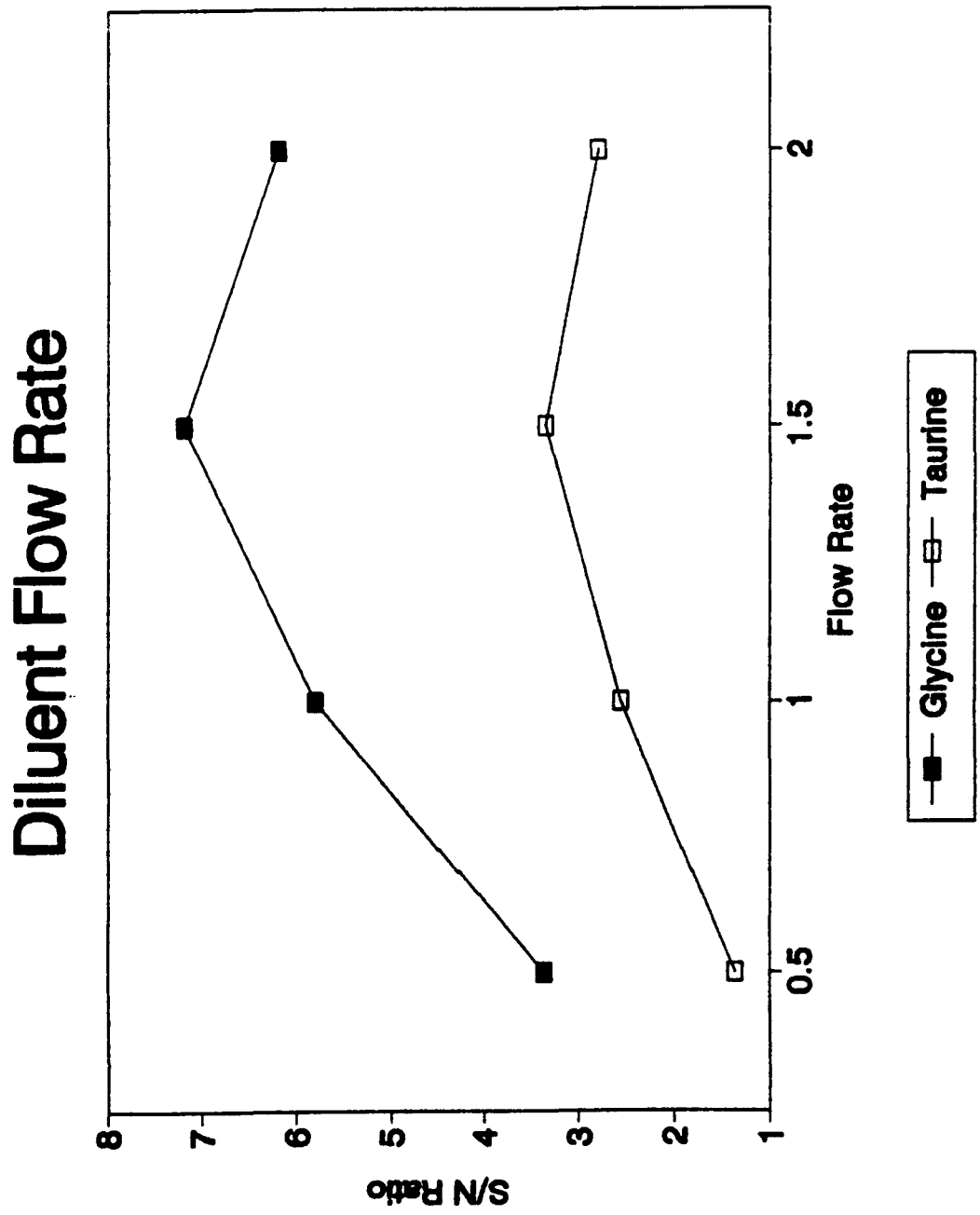


Fig. 14. Optimization of diluent flow rate

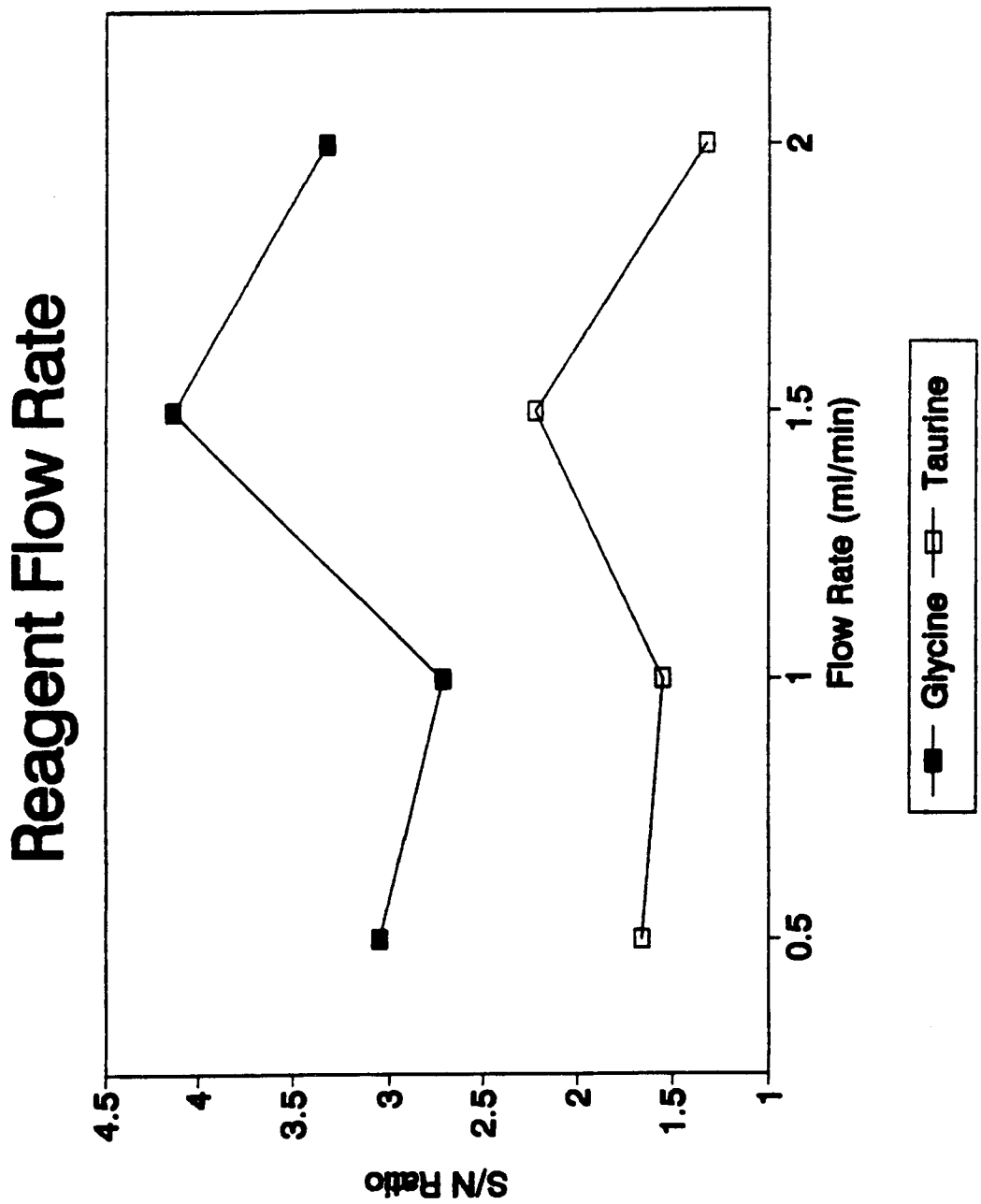


Fig. 15. Optimization of OPA reagent flow rate

TABLE 2

COIL VOLUMES

Coil	Dry Weight (g)	Filled Weight (H_2O) (g)	Volume (mL)
1	16.9340	17.1003	0.1663
2	17.0880	17.2803	0.1923
3	17.0964	17.2640	0.1676

To determine the reaction volume for the coil segment, the coils were all weighed individually for a dry weight then filled with deionized water and reweighed. The difference between these weights was the mass of water in the coil. This mass was also considered to be the volume since water has a density of 1.0 g/mL. A short, coiled length of Teflon^R connecting tube was a zero volume coil. Coils 1 through 3 were added on successively to the zero coil in order to investigate the different reaction coil volumes. Figure 16 shows the results of the coil volume analysis. It was determined that ideal conditions were seen when all three coils were added to the system. A coil volume of 0.5262 mL was obtained by adding together the volumes of all three coils. This meant that a longer delay for derivation was beneficial for the system.

During these investigations it was also observed that the glycine derivatives fluoresced better than the taurine derivatives. It would thus be expected that glyco derivatives would be detected with greater sensitivity than tauro derivatives.

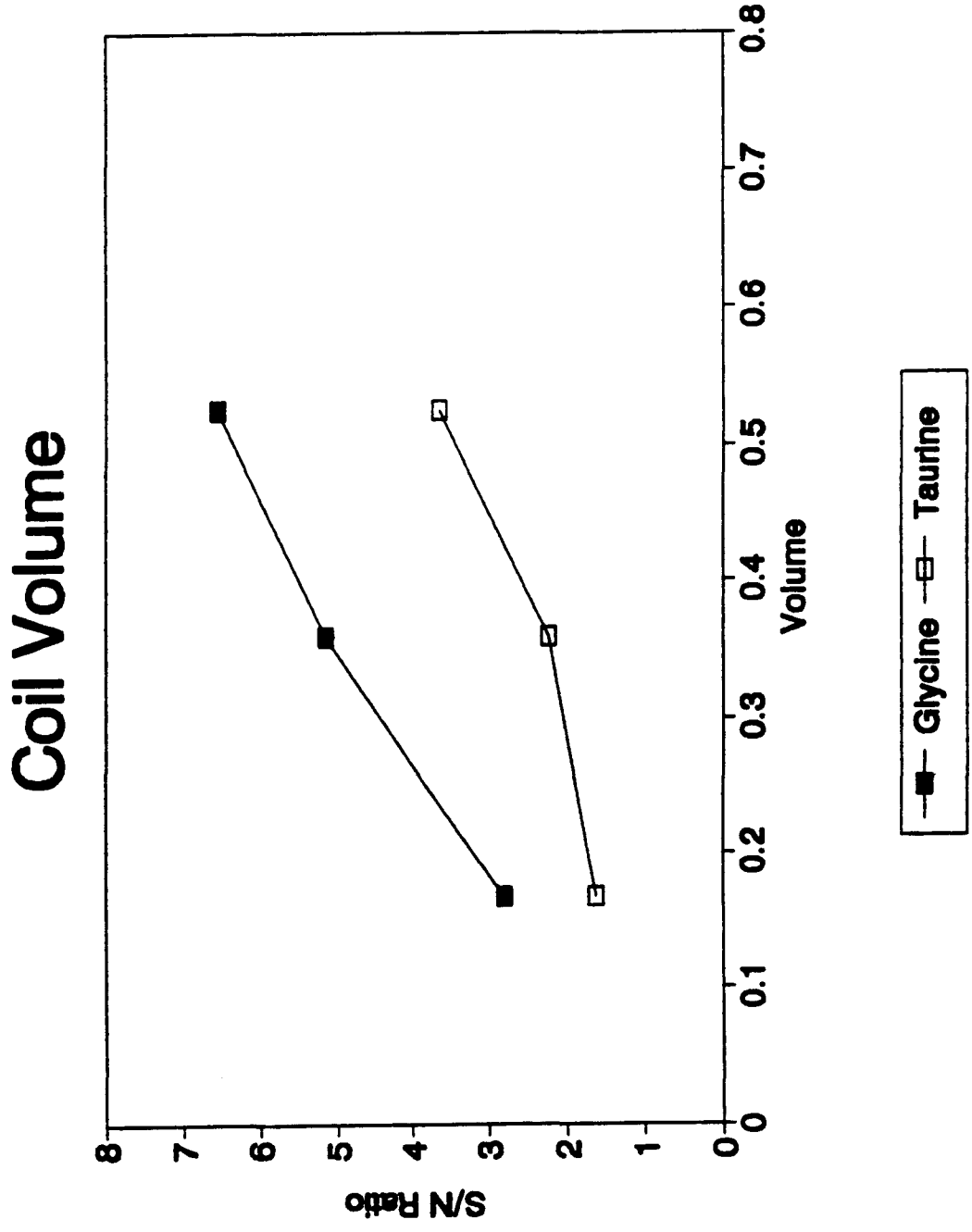


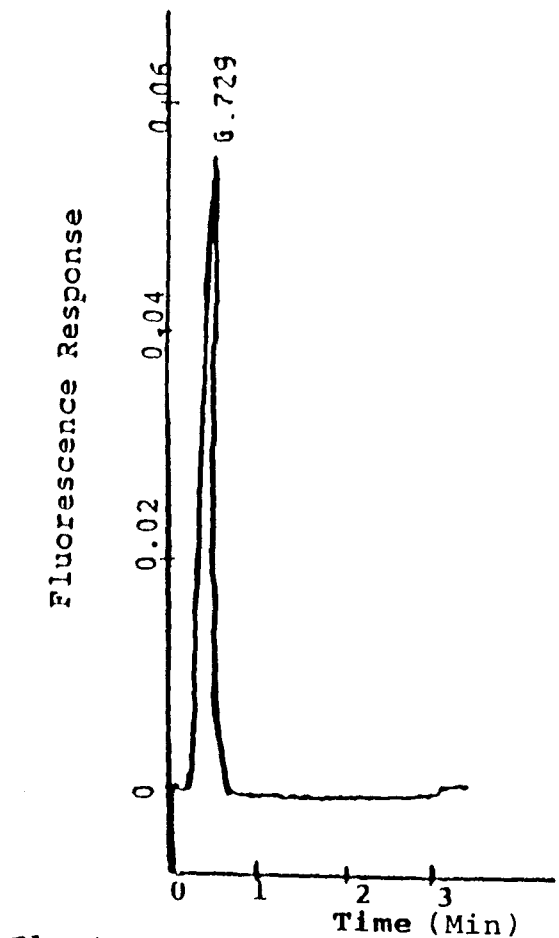
Fig. 16. Optimization of coil volume

Figure 17 shows a chromatogram of the glycine and taurine OPA derivized samples studied for the optimization of the diluent pH. Also included in Figure 17 is the noise level determined for this parameter. The noise level was measured in cm and this value was compared to the height of the signals, also measured in cm, for both the glycine and taurine derivatives. The S/N ratios were plotted versus the diluent pH values investigated (Figure 9).

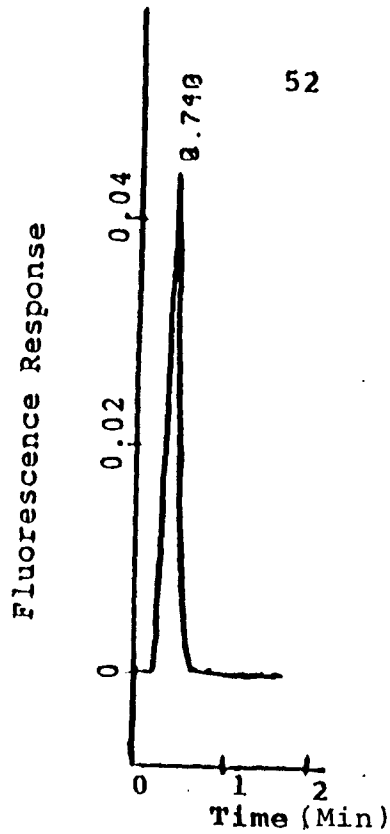
Immobilized Enzyme Concentration

Once all the system parameters had been optimized it was necessary to find the immobilized enzyme concentration on the CPG that gave the best signal for the bile acids being analyzed. Enzyme concentration was studied at an earlier stage of research, but results were not suitable for this study since the reaction system had not yet been optimized and many of the results obtained during that time were not reproducible.

At the early stages of research, an ideal enzyme concentration of 10 mg in the immobilization buffer was determined to be optimum. Analysis of the conjugated bile acid standards was attempted with out success. Peaks were observed, but there was very little reproducibility. Once optimization of the system parameters was performed the enzyme concentration analysis was again studied. Enzyme immobilization concentrations of 5.0, 10.0, and 20.0 mg in



Glycine Derivative



Taurine Derivative

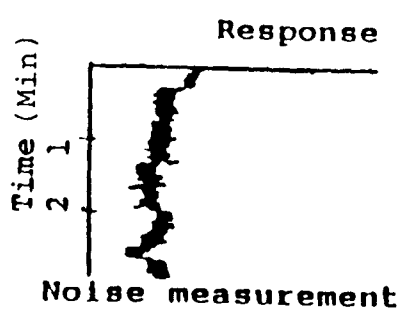


Fig. 17. Diluent pH optimization; pH 4.0

the immobilization buffer were prepared. (See reagent section of HPLC analysis).

Direct injections of glycoursodeoxycholic acid and tauroursodeoxycholic acid were done to determine signal to noise ratios for each enzyme concentration. Thirty-five mg of glycoursodeoxycholate was dissolved in 5.0 mL of methanol. This concentration proved to be too high and 1 mL of this solution was diluted to 10 mL to achieve adequate signal identification. The tauroursodeoxycholic acid sample was prepared by weighing out 17.0 mg of the acid and diluting to 5.0 mL with methanol. One mL of this solution was further diluted to 5.0 mL to ensure the signal remained on scale. Injection volumes were 20 μL. Results of this study are shown in Figure 18. It was found that at higher concentrations of enzyme, better signals were seen. It was determined that an enzyme concentration of 20 mg would be sufficient for this study.

Immobilized CGH Enzyme Preparation

Two preparation techniques were studied for the CGH enzyme. Initially the technique involving silanization of controlled pore glass was adopted. The activity of the enzyme after immobilization seemed to be good and analysis of bile standards began. This again was done before optimization of the system was performed so results of any analysis were not ideal. After several months of using this technique a problem became evident. It seemed that after

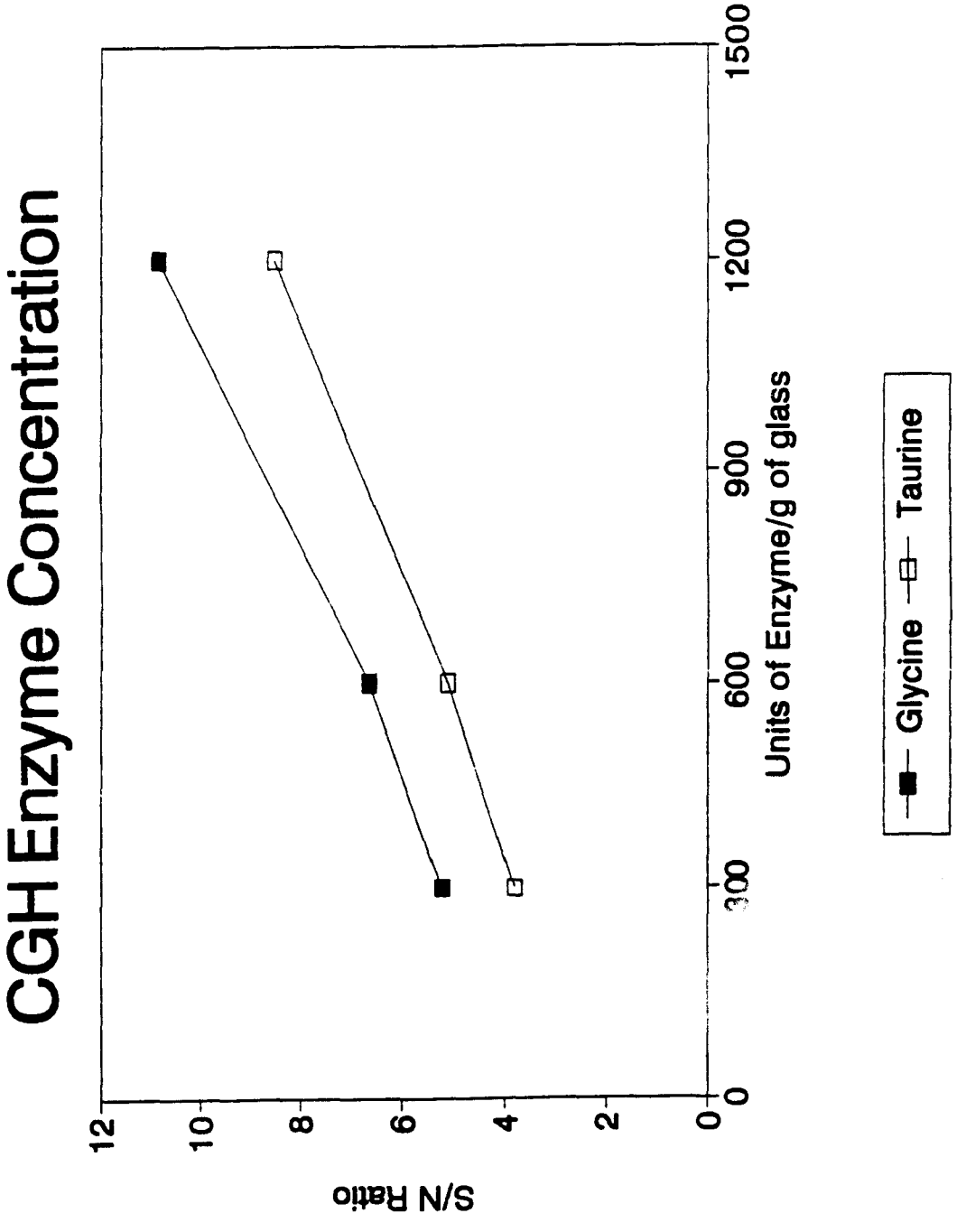


Fig. 18. Optimization of CGH enzyme concentration

amination of the CPG was done, the glutaraldehyde had little effect on the glass. No color change was observed after the addition of the glutaraldehyde, leading to the assumption that the glass was not being activated. A new bottle of glutaraldehyde being used was assumed to be the problem. Another bottle of glutaraldehyde was then used but yielded the same results. New glass was also tested with no success. At this time the procedure involving purchased aminopropyl glass was attempted. This procedure produced the expected color change indicating activation and the enzyme did have activity after the immobilization process was completed. This was the immobilization procedure that was used throughout the rest of the research project. It should be noted that this procedure was also much less time consuming and less expensive than the original immobilization technique.

Although enzyme activity was reported with this immobilization technique, the enzyme tended to denature rather quickly. It was observed that during overnight storage the enzyme lost most of its activity. It was determined that iron from the stainless steel enzyme column jacket was bleeding off the sides and attaching itself onto the thiol-containing active sites of the enzyme. To alleviate this problem the column was rinsed with a 0.75% 2-mercaptoethanol solution in the diluent buffer at the end of the day. The 2-mercaptoethanol would help chelate the

iron and prevent combination with the sulfhydryl groups. Once this storage buffer was used, activity of the enzyme seemed to last longer, but still denatured sooner than desired. This problem was never adequately resolved during this project.

MOBILE PHASE CONDITIONS

Solvent concentrations and pH of the mobile phase were varied to optimize the retention time of the conjugated bile acids. Figure 19 shows the variation of the bile acid retention with pH and solvent concentration. It was found that a 0.05 M concentration of the KH_2PO_4 buffer at pH 4.00 was ideal for the mobile phase. At higher concentrations, the phosphate salt caused a build-up in the mobile phase pump and a large increase in pressure was observed. Lower concentrations of buffer gave inadequate peak separation at the conditions originally used of 65% methanol 35% pH 4.5 0.1 M phosphate buffer. The optimum pH for the 0.05 M phosphate buffer was found to be 4.00. At pH values below the optimum, retention times of the bile acids became too long. At a pH values above 4.5, it became difficult to completely separate the glycine and taurine conjugates. A mixture of 65% 0.05 M KH_2PO_4, pH 4.0 and 35% methanol was used as the mobile phase for analysis of the bile acids. Higher concentrations of methanol caused the enzyme to denature at a rather high rate.

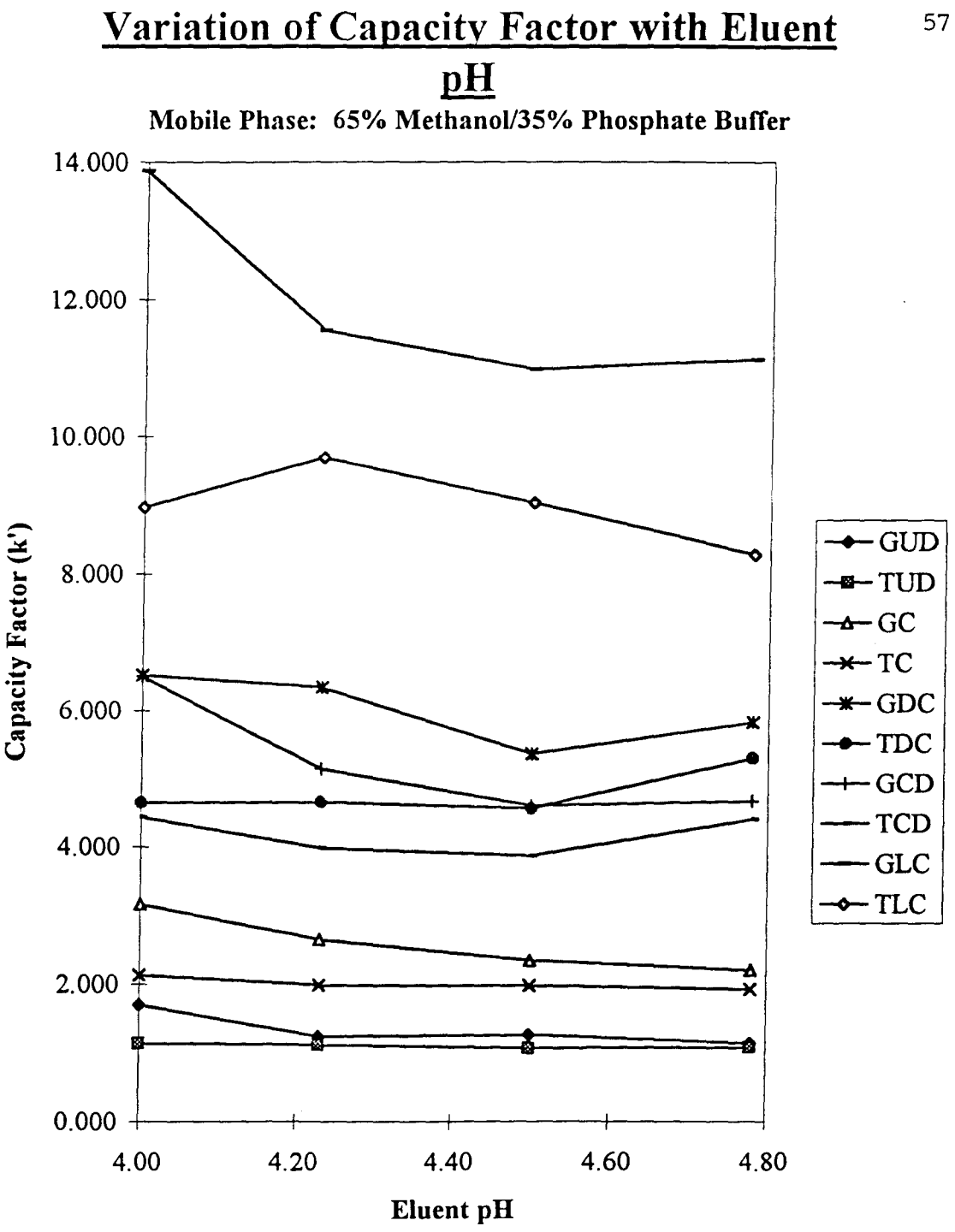


Figure 19: Variation of retention of conjugated bile acids with pH of buffer. 65% methanol and 35% phosphate buffer.

HPLC Analysis of Bile Acid Standards

Once optimization of the system was completed analysis of the bile standards and bile could be done. Figure 6 shows a schematic diagram of the system used for this analysis. A CPG-cholylglycine hydrolase enzyme reactor and an OPA derivation reactor were linked to the HPLC system. Ten bile acids were used to prepare a bile acid stock standard. The bile acids were dissolved in 25 mL of methanol to make a stock solution with concentrations listed in Table 3. From this stock solution three bile acid standards were prepared by diluting standard #1 by a ratio of 0.360, standard #2 by a ratio of 0.507, and standard #3 by a ratio of 0.800. These standards are also listed in Table 3. The standards were individually injected into the system in 20 μL volumes.

Figure 20 shows a chromatogram of the bile standard #1 (a total bile acid concentration of 4.92 mg/mL). A dead volume was observed at around 3.5 minutes for each bile standard analyzed. Only seven peaks were resolved in each of the standards. At the conditions used for this analysis, separation of glycochenodeoxycholate and glycodeoxycholate, and the corresponding taurine conjugates were not observed.

The absence of peaks can be related to several conditions in the analysis. The enzyme conditions were important. From the optimization of the separation, it is likely that separation of the 10 bile acids was occurring on

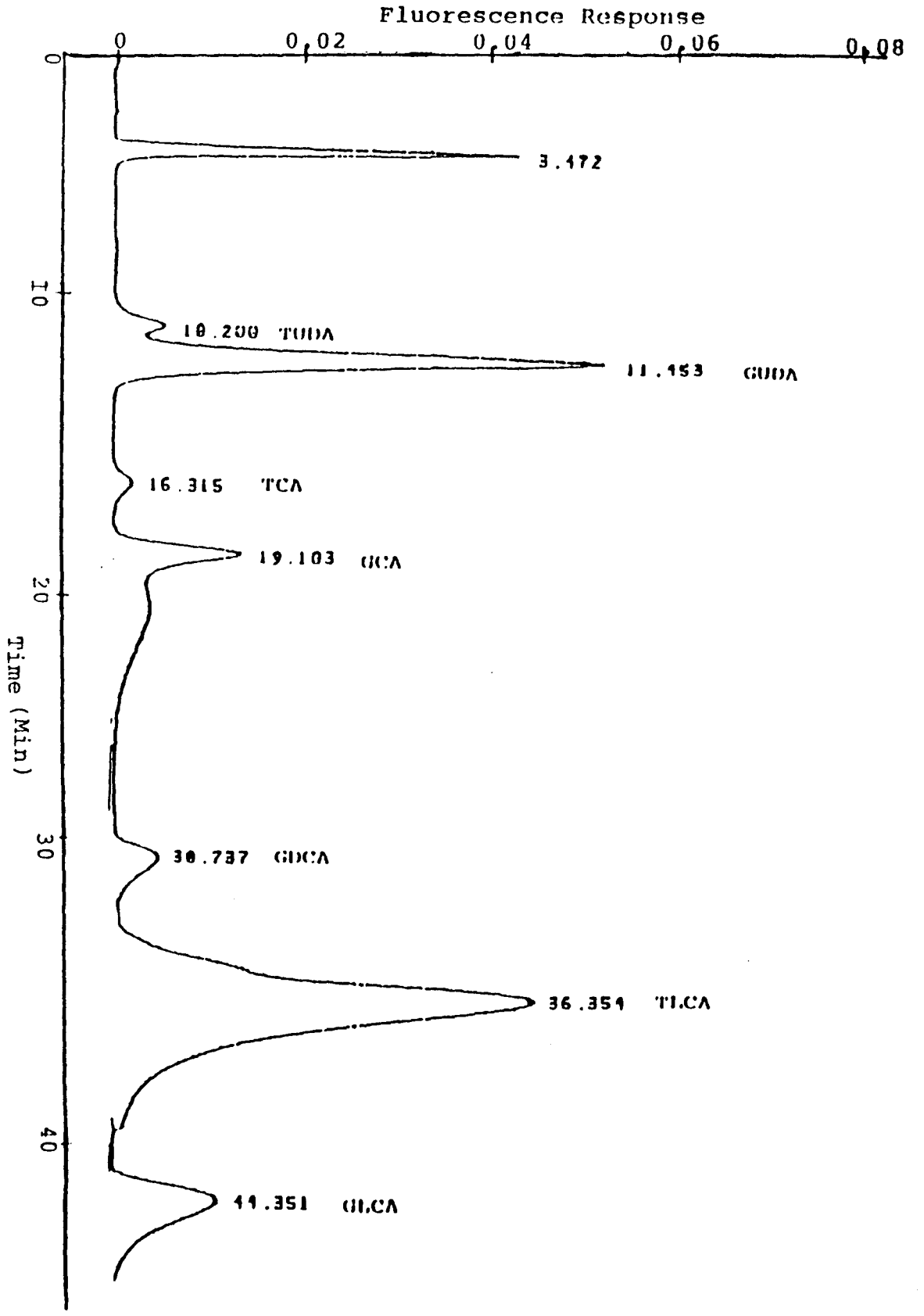


Fig. 20. Chromatogram of the separation of bile standard #1

the HPLC column. The eluent then mixed with the diluent in order to lower the concentration of the methanol that comes in contact with enzyme. At this point free bile acids and either glycine or taurine were produced from the hydrolysis reaction which is catalyzed by the enzyme. The liberated amino acids were derivated to fluorescent products with OPA and detected.

The enzyme reacted differently with the glycine conjugates than with the taurine conjugates. The taurine conjugates were a poor substrate which accounted for its relatively poor response or absence in the analysis.

TABLE 3

BILE ACID CONCENTRATIONS USED IN STANDARD SOLUTIONS

Bile Acid	Amount (mg)	Stock	Std1	Std2	Std3
			(mg/mL)		
Glycocholic	34.0	1.36	0.489	0.689	1.090
Taurocholic	39.0	1.56	0.562	0.791	1.250
Glycodeoxycholic	32.0	1.28	0.461	0.649	1.020
Taurodeoxycholic	38.0	1.52	0.547	0.771	1.210
Glycochenodeoxycholic	33.0	1.32	0.475	0.669	1.060
Taurochenodeoxycholic	38.0	1.52	0.547	0.771	1.210
Glycoursodeoxycholic	38.0	1.52	0.547	0.771	1.210
Tauroursodeoxycholic	36.0	1.44	0.308	0.730	1.150
Glycolithocholic	16.0	0.64	0.230	0.324	0.512
Taurolithocholic	37.0	1.48	0.531	0.750	1.184

A large amount of band broadening was observed for all peaks. This band broadening very likely caused an overlap of the taurodeoxycholic acid and the taurochenodeoxycholic acid. Overlapping also occurred between glycodeoxycholic acid and glycochenodeoxycholic acid. Figures 21 and 22 show

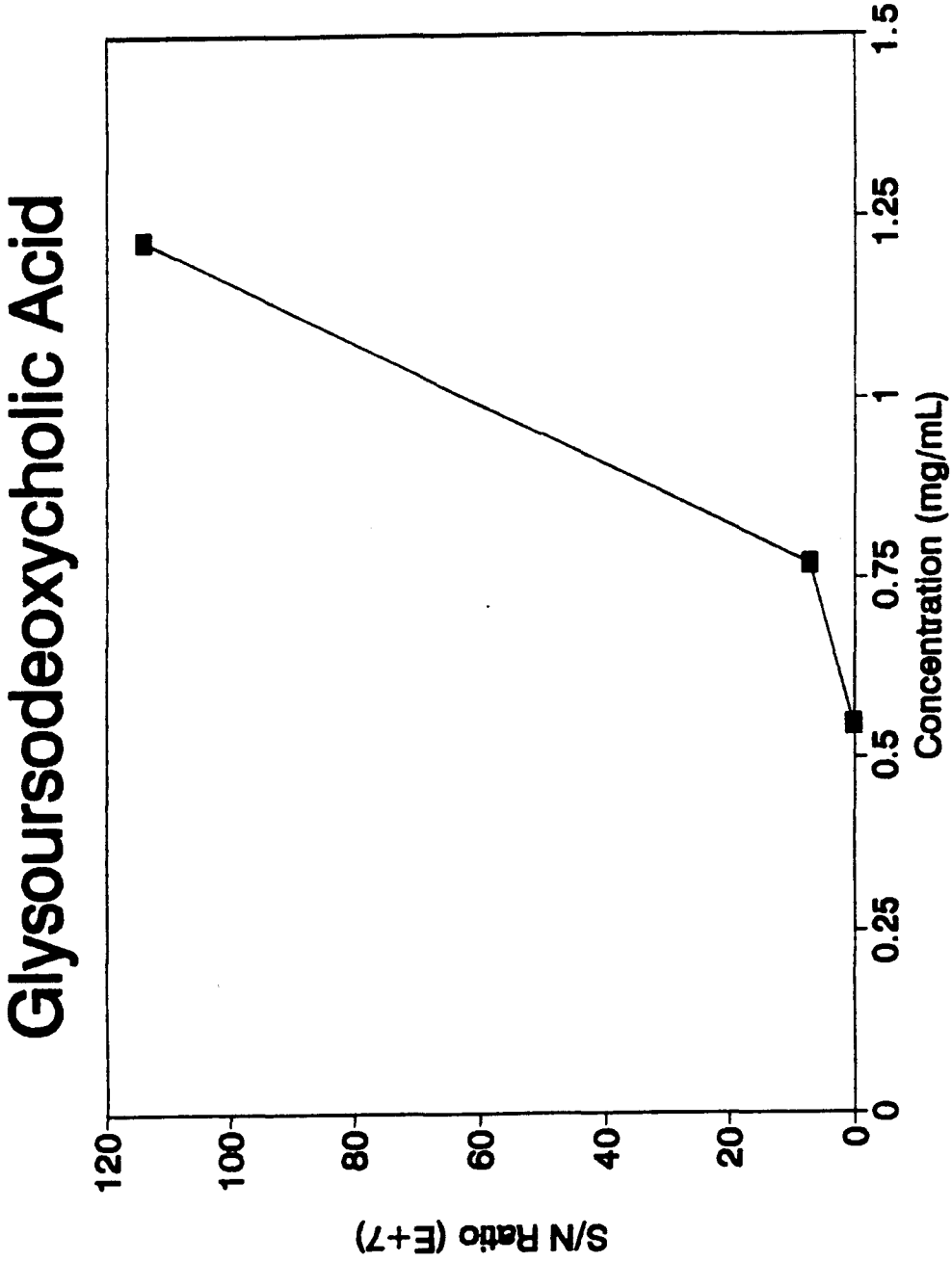


Fig. 21. Calibration curve for glycoursodeoxycholic acid. Bile standards #; 1, 2, and 3

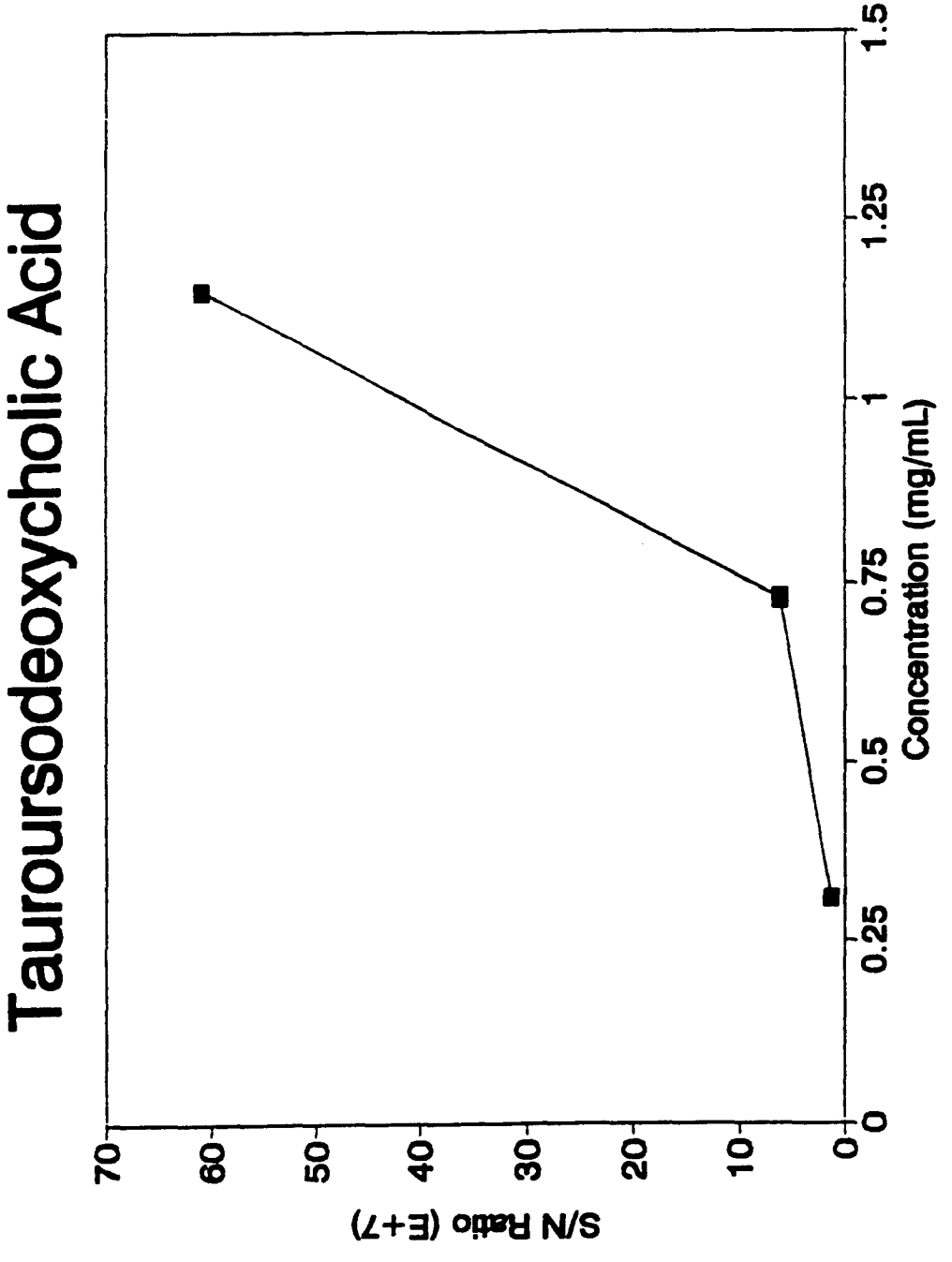


Fig. 22. Calibration curve of taurodeoxycholic acid. Bile standards #: 1, 2, and 3

standard curves of glycoursodeoxycholic and tauroursodeoxycholic derivatives respectively. The graphs indicate that linearity was not achieved. This non-linearity could be related to the enzyme-substrate relationship.

HPLC Analysis of Bile Samples

The system used in this analysis was identical to the schematic in Figure 6, except a guard column was inserted between the mobile phase pump and the HPLC column to prevent contamination of the analytical column pigments present in the bile sample. Two injections of a bile standard were done before the analysis of the sample in order to calibrate the system.

The analysis of samples was limited since the goal of this research was the optimization of the analytical system. Only one sample was analyzed with minimal results. Figure 23 shows the chromatogram of the bile sample. Three peaks were observed, corresponding to glycoursodeoxycholic acid, glycocholic acid, and glycolithocholic acid. These acids are reportedly the most abundant bile acids in human serum which would thus account for their easy detection.

Figure 24 shows a chromatogram of the same bile sample using UV detection instead of fluorescence. In this analysis, post column derivation was also eliminated but all other conditions were similar to the fluorescence analysis.

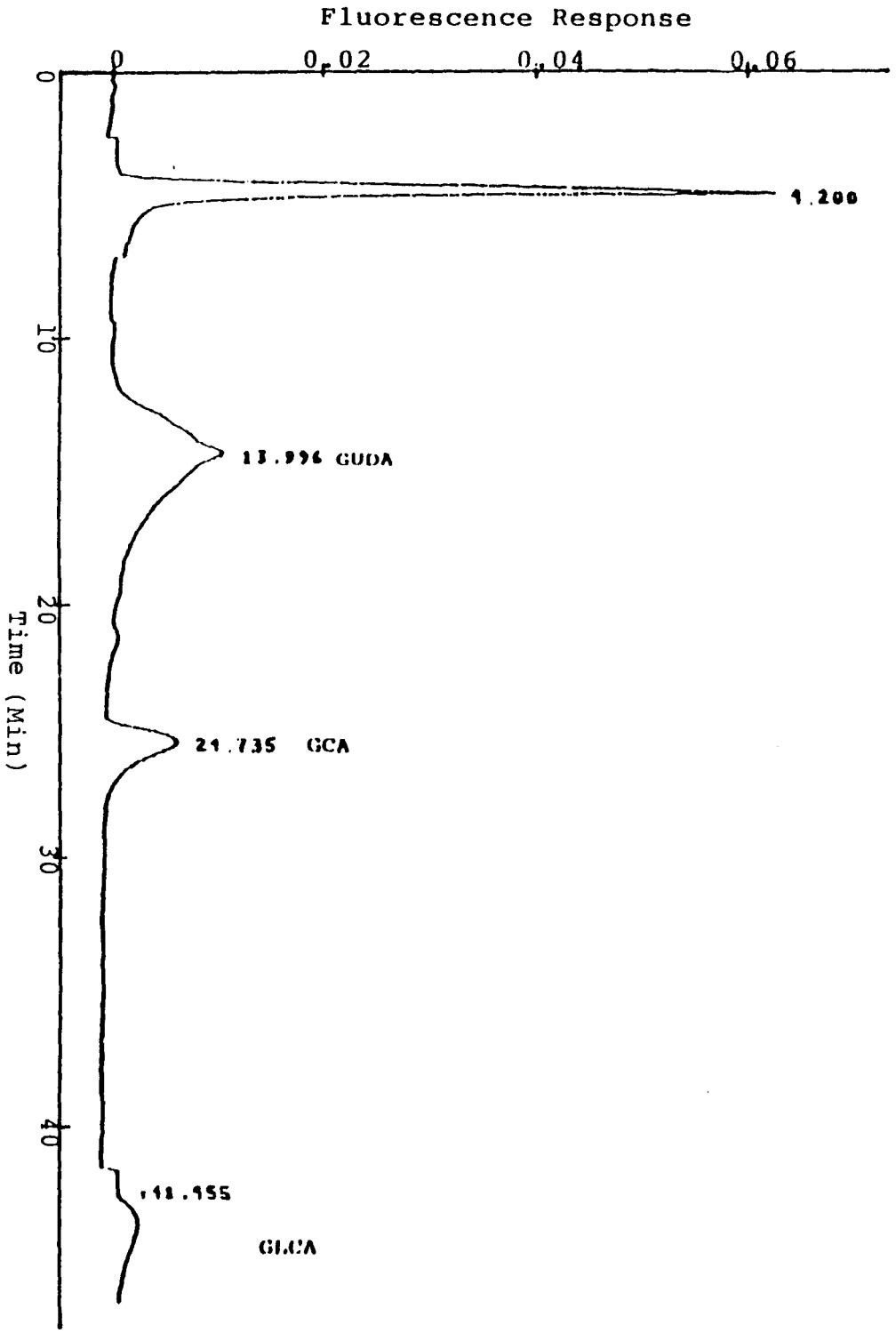


Fig. 23. Fluorescence chromatogram of bile sample #1

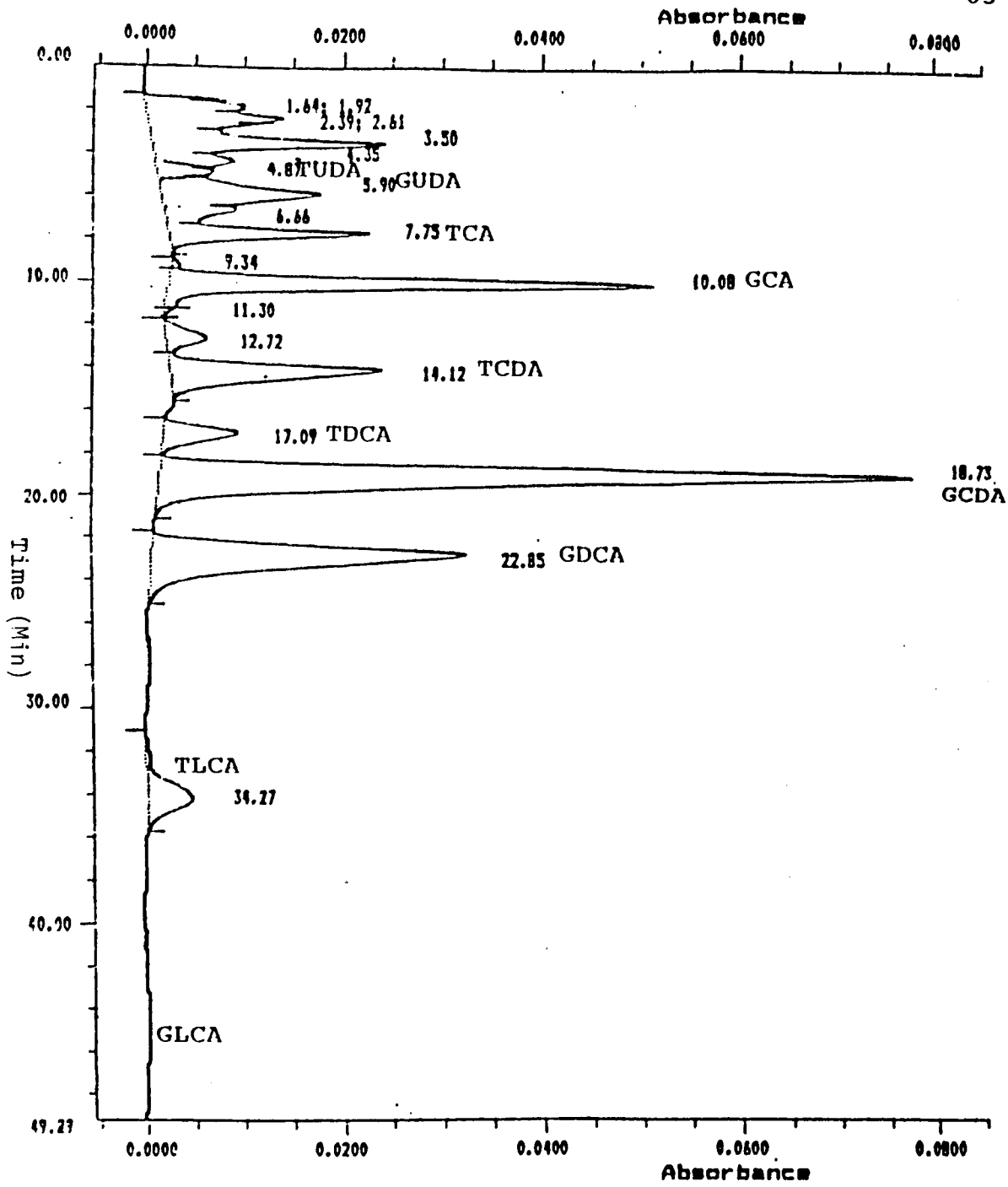


Fig. 24. UV chromatogram of bile sample #1

CHAPTER VII

CONCLUSIONS

From the analysis studied in this research, several conclusions may be reached: (1) optimization of the system improved the separation and detection of the conjugated bile acids. (2) using the aminopropyl glass proved to be less time consuming than the silanization method of immobilization used in previous research. (3) the work done in this analysis is the first successful attempt at separation of bile samples using the immobilized CGH enzyme. Although the separation needs to be improved this analysis shows that it is possible. Further studies of bile sample analysis could allow this technique to be used in the diagnosis and treatment of hepatic diseases.

It should also be noted that there were significant problems with the system. The immobilized enzyme proved to be unstable. The life of the enzyme allowed only 7-10 runs before activity was lost. This was a significant problem because of preparation time and cost of the enzyme. The fact that the enzyme was unstable could account for the non-linear standard curves which were obtained when the standard bile acid samples were analyzed. Non-linear standard curves were most likely related to the enzymes affiliation with the substrate.

CHAPTER VIII

SUGGESTIONS FOR FUTURE RESEARCH

In the future there are several areas of this work that can be further investigated. The most important area is stabilization of the enzyme. This could possibly be done by either cross-linking the enzyme or co-immobilization of the enzyme. The enzyme substrate relationship in the controlled pore glass also needs to be studied in order to understand the non-linearity which was observed in this investigation. There also needs to be further studies of the bile samples to see if the will be more problematic than the bile standards.

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