DETERMINATION OF CONJUGATED BILE ACIDS USING HPLC AND IMMOBILIZED CHOLOYLGLYCINE HYDROLASE

BY

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Submitted in Partial Fulfillment of the Requirements

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ABSTRACT

DETERMINATION OF CONJUGATED BILE ACIDS USING HPLC AND IMMOBILIZED CHOLOYLGLYCINE HYDROLASE

Xi Li
Master of Science
Youngstown State University, 1991

A new HPLC method for the determination of glycine and taurine conjugated bile acids was investigated. The system was based on a postcolumn enzyme reactor using immobilized choloylglycine hydrolase (CGH) linked to fluorescence detection. Conjugated bile acids were separated using HPLC and then hydrolyzed by the immobilized CGH. The hydrolysis yielded free bile acids and the amino acids glycine and taurine. The liberated amino acids were subsequently derivatized to fluorescent o-phthalaldehyde derivative that was monitored with a high degree of specificity and sensitivity by a fluorescence detector. The separation of 8 conjugated bile acids was performed by using this system. The quantitation of glycocholate and taurocholate was demonstrated. Detection limits for these two bile acids were determined.

I would like to extend my sincere appreciation to Dr. James Mike for his guidance and encouragement throughout this research, and his critical reading of the manuscript. I would also like to thank Dr. Daryl W. Mincey and Dr. Leonard B. Spiegel for their time spent in the review of this manuscript.

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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
μg	Microgram	1×10^{-6} gram
mm	Millimeter	1×10^{-3} meter
nm	Nanometer	1×10^{-9} meter
Å	Angstrom	1×10^{-8} meter
μmole	Micromole	1×10^{-6} mole
pmole	Picomole	1×10^{-12} mole
mol/L	Molarity	moles per liter
L	Liter	--
g	Gram	--
v	Volume	--
w	Weight	--
min	Minute	--
^{o}C	Degrees Celsius	--
HPLC	High Performance Liquid Chromatography	--
UV	Ultraviolet	--
%	Percent	--
I.D.	Internal Diamter	--
C_8	Octyl	--
C_{18}	Octadecyl	--
S	Signal	--
N	Noise	--

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CHAPTER I

INTRODUCTION

Bile Acids

Bile acids are formed in the liver from cholesterol and are secreted into the bile. They are the major metabolites of cholesterol and act as detergents to facilitate the digestion of lipids. In human bile, the most abundant bile acids are cholic (3,7,12-trihydroxycholanic), chenodeoxycholic (3,7-dihydroxycholanic), and deoxycholic (3, 12-dihydroxycholanic) acids. Cholic and chenodexycholic acids, synthesized in the liver, are classified as primary bile acids. Deoxycholic acid is classified as a secondary bile acid, which is derived from the primary bile acids by the action of intestinal bacteria, and is reabsorbed into the blood through the enterohepatic circulation. These bile acids exist mainly as conjugates with the amino acids glycine or taurine (i.e., glycocholic acid, taurocholic acid, etc.), with the glycine conjugates usually predominating. The conjugation process occurs in the liver. After their formation in the liver, the conjugated bile acids are stored in the gallbladder and subsequently secreted into the intestine through the bile duct. In the intestine, the bile acids aid in emulsifying fats, oils, and fatty acids and thus promote both their contact with enzymes catalyzing hydrolysis and their absorption through the intestinal wall[1,2]. The 10 most common conjugated bile acids are given in Table 1. The structures of some bile acids are shown in Figure 1.

In health, only small quantities of bile acids are found in the systemic circulation. However, in liver diseases, or other related intestinal disorders, disturbances in synthesis and clearance of bile acids by the liver and their absorption by the intestine can change the level and pattern of serum

bile acids. Therefore, it is possible that bile acid analysis may be useful in the diagnosis and treatment of liver diseases and in the evaluation of

TABLE 1 CONJUGATED BILE ACIDS

Name	Class	Abbreviation
Glycocholic	Primary	GCA
Taurocholic	Primary	TCA
Glycodeoxycholic	Secondary	GDCA
Taurodeoxycholic	Secondary	TDCA
Glycochenodeoxycholic	Primary	GCDA
Taurochenodeoxycholic	Primary	TCDA
Glycoursodeoxycholic	Secondary	GUDA
Tauroursodeoxycholic	Secondary	TUDA
Glycolithocholic	Secondary	GLCA
Taurolithocholic	Secondary	TLCA

intestinal function[3-5]. It has been observed that glycocholic acid and taurocholic acid levels in serum are increased from normal values during cholestasis[6]. In contrast to this, a decrease of glycocholic acid and taurocholic acid and an increase of free chenodeoxycholic acid and glycochenodeoxycholic acid have been found in the serum of patients with liver cirrhosis[6]. It has been reported that the measurement of serum bile acids is a more sensitive indicator of liver disease than conventional liver function tests[7,8]. Furthermore, it has been suggested that bile acid analysis may be used as a screening test for ileal disease because of the evidence that patients with ileal dysfunction typically have an elevated ratio of glycine to taurine conjugates[9].

All known bile acids contain a carboxyl or sulfonic acid group. Like other weak acids, they are ionized: $HA \rightleftharpoons H^+ + A^-$, where HA is the acid form and A^- is the salt form. Their tendency to ionize or the strength of the acids is indicated by their pK_a values. The pK_a values of free bile acids and glycine conjugates are in the ranges of pK_a=5-6.5 and pK_a=4-5, respectively[1]. The pK_a values of taurine conjugates are difficult to predict

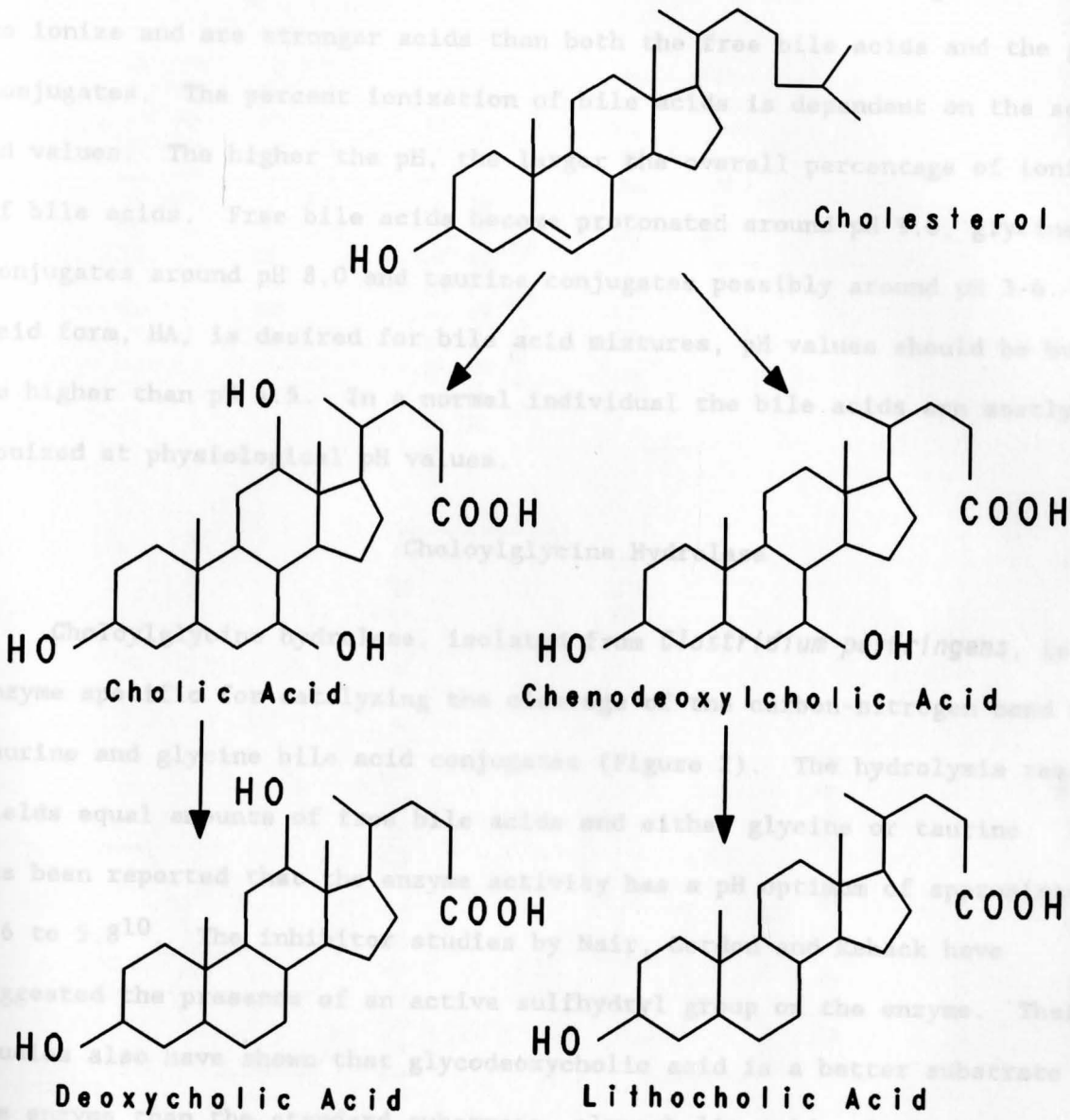

Fig. 1. Structures of bile acids.

due to the strongly acidic nature of the conjugated taurine. However, they can be predicted to fall in the range of pK_a=-1.5 to +1.5, which are estimates from the pK_a for free taurine. Therefore, taurine conjugates have greater tendency to ionize and are stronger acids than both the free bile acids and the glycine conjugates. The percent ionization of bile acids is dependent on the solution pH values. The higher the pH, the larger the overall percentage of ionization of bile acids. Free bile acids become protonated around pH 9.0, glycine conjugates around pH 8.0 and taurine conjugates possibly around pH 3-4. If the acid form, HA, is desired for bile acid mixtures, pH values should be buffered no higher than pH 4.5. In a normal individual the bile acids are mostly ionized at physiological pH values.

Choloylglycine Hydrolase

Choloylglycine hydrolase, isolated from *Clostridium perfringens*, is an enzyme specific for catalyzing the cleavage of the carbon-nitrogen bond in taurine and glycine bile acid conjugates (Figure 2). The hydrolysis reaction yields equal amounts of free bile acids and either glycine or taurine. It has been reported that the enzyme activity has a pH optimum of approximately 5.6 to 5.8[10]. The inhibitor studies by Nair, Gordon and Reback have suggested the presence of an active sulfhydryl group on the enzyme. Their studies also have shown that glycodeoxycholic acid is a better substrate for the enzyme than the standard substrate, glycocholic acid. It is known that taurocholic acid is hydrolyzed at approximately 1/3 the rate found for the hydrolysis of glycocholic acid using choloylglycine hydrolase[11].

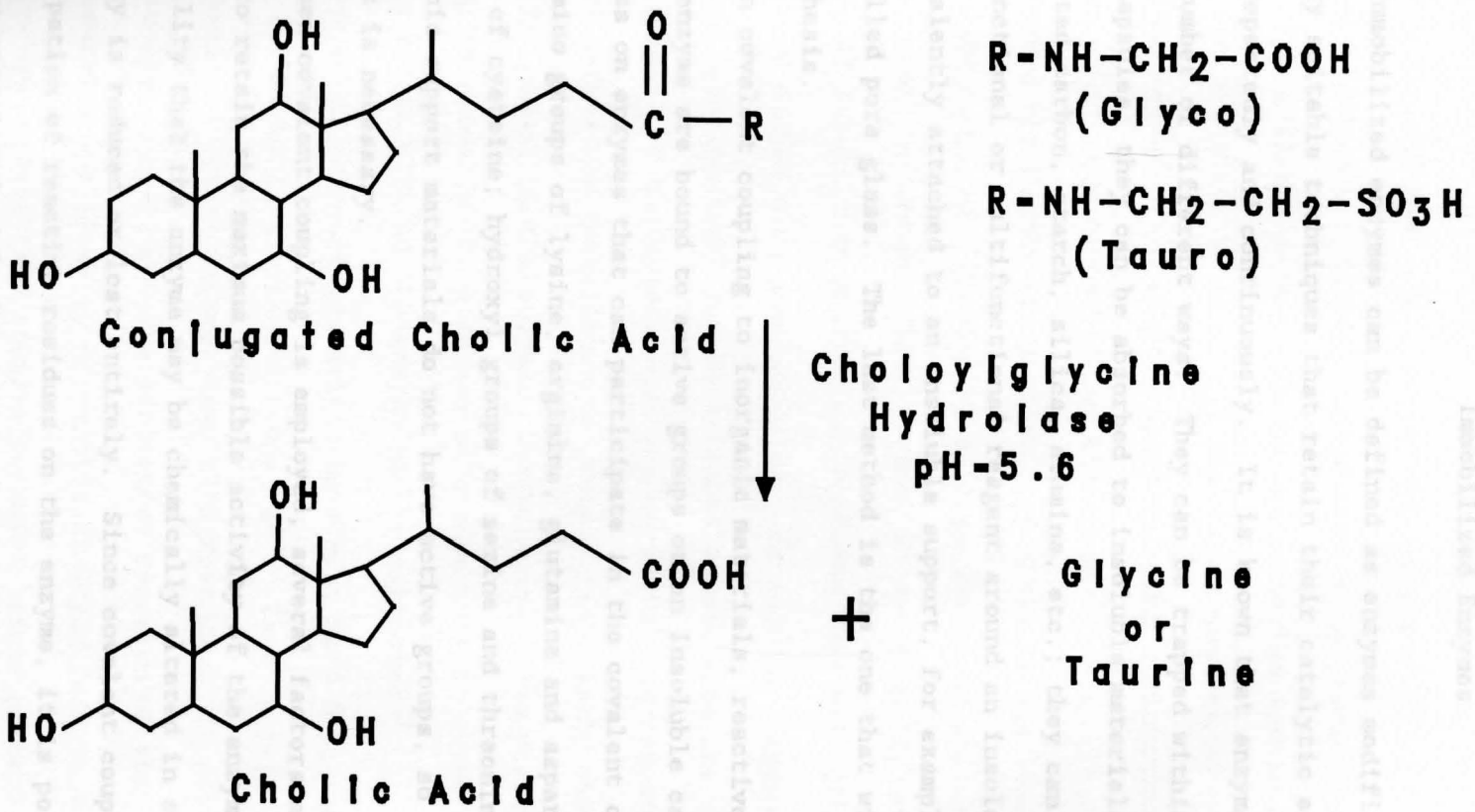

Fig. 2. Hydrolysis reaction of conjugated bile acid with choloylglycine hydrolase.

Immobilized Enzymes

Immobilized enzymes can be defined as enzymes modified to an insoluble form by suitable techniques that retain their catalytic activities and can be used repeatedly and continuously. It is known that enzymes can be immobilized in a number of different ways. They can be trapped within gels or microcapsules; they can be absorbed to insoluble materials, for example, activated carbon, starch, silica, alumina, etc.; they can be cross-linked with a bifunctional or multifunctional reagent around an insoluble matrix; they can be covalently attached to an insoluble support, for example, silica or controlled pore glass. The last method is the one that will be discussed in this thesis.

In covalent coupling to inorganic materials, reactive amino acid residues on an enzyme are bound to active groups on an insoluble carrier. Reactive residues on enzymes that can participate in the covalent coupling include the free amino groups of lysine, arginine, glutamine and asparagine; sulfhydryl groups of cysteine; hydroxyl groups of serine and threonine; and so on. Most inorganic support materials do not have active groups, so that an activation process is necessary.

When covalent coupling is employed, several factors must be considered in order to retain the maximum possible activity of the enzyme. One factor is the possibility that the enzyme may be chemically altered in such a way that its activity is reduced or lost entirely. Since covalent coupling requires the participation of reactive residues on the enzyme, it is possible that residues directly associated with the active site of the enzyme could be involved in the coupling reaction. This could lead to inactivation of the enzyme and the blocking of the active site. Another factor that must be considered during the coupling is the possibility that the active conformation could be changed

and/or conformational changes in the enzyme could be restricted. When the enzyme is immobilized onto a solid, the enzyme's structure is held in one position if there are multiple attachment points between the enzyme and the solid. The active site may thus be buried or blocked so that the substrate cannot reach the active site. If the enzyme depends on conformational changes for regulation of its catalytic activity, such a situation could lead to loss of these allosteric properties of the enzyme[12,13].

Several approaches are available to minimize the loss of enzyme activity in covalent coupling. Presently, a large number of different methods of covalent coupling are possible, so that if one coupling mode does not work well, it is likely that another can be found that will give the desired immobilized enzyme with good activity. In addition, appropriate conditions of pH and ionic strength may be selected to maximize the chances for bonding active enzyme. 2-Mercaptoethanol may be useful for reducing the loss of enzyme activity during the immobilization process, especially when there are active sulfhydryl groups on the enzyme. Furthermore, it is possible to perform the coupling reaction in the presence of a substrate or a reversible inhibitor so that the active site on the enzyme may be protected[13].

Many covalent coupling methods have been used for a number years. One popular technique is alkylamine coupling[14]. In this method, 3-aminopropyl-triethoxysilane (other silanes have also been used) is attached to the surface of the inorganic carrier. The activation reaction of the alkylamine derivative with glutaraldehyde leads to a reactive aldehyde derivative on the carrier. The activated carrier then couples to amino groups present in lysine or arginine residues on the protein to yield the immobilized enzyme (Figure 3).

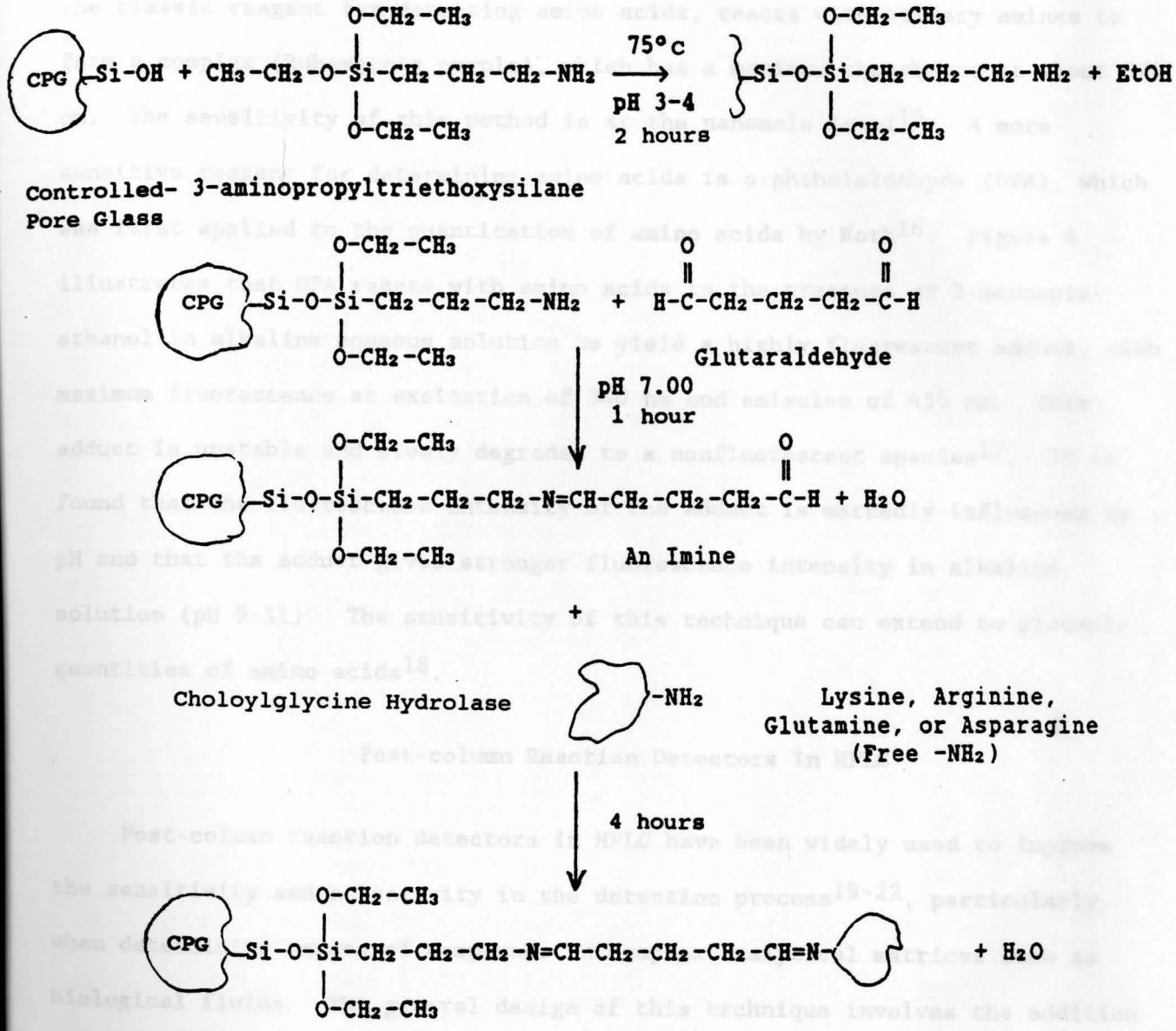

Fig. 3. Immobilization of Choloylglycine Hydrolase.

Amino Acid Detection

Many reagents have been used for the detection of amino acids. Ninhydrin, the classic reagent for detecting amino acids, reacts with primary amines to form a complex (Ruhemann's purple), which has a maximum absorbance at about 570 nm. The sensitivity of this method is at the nanomole level[15]. A more sensitive reagent for determining amino acids is o-phthalaldehyde (OPA), which was first applied to the quantitation of amino acids by Roth[16]. Figure 4 illustrates that OPA reacts with amino acids in the presence of 2-mercapto-ethanol in alkaline aqueous solution to yield a highly fluorescent adduct, with maximum fluorescence at excitation of 340 nm and emission of 455 nm. This adduct is unstable and slowly degrades to a nonfluorescent species[17]. It is found that the fluorescence intensity of the adduct is markedly influenced by pH and that the adduct gives stronger fluorescence intensity in alkaline solution (pH 9-11). The sensitivity of this technique can extend to picomole quantities of amino acids[18].

Post-column Reaction Detectors In HPLC

Post-column reaction detectors in HPLC have been widely used to improve the sensitivity and selectivity in the detection process[19-22], particularly when determining traces of compounds in complex analytical matrices such as biological fluids. The general design of this technique involves the addition of reactants to the column effluent through mixing tees by means of pumps; a reactor(s) placed between the tee and the detector allows the chemical reaction(s) to occur. Three different types of reactors have been employed: tubular reactors; packed-bed reactors; and segmented-stream reactors. The tubular type is the simplest post-column reactor and consists of stainless steel or Teflon tubing. This type of reactor is useful for fast reactions up

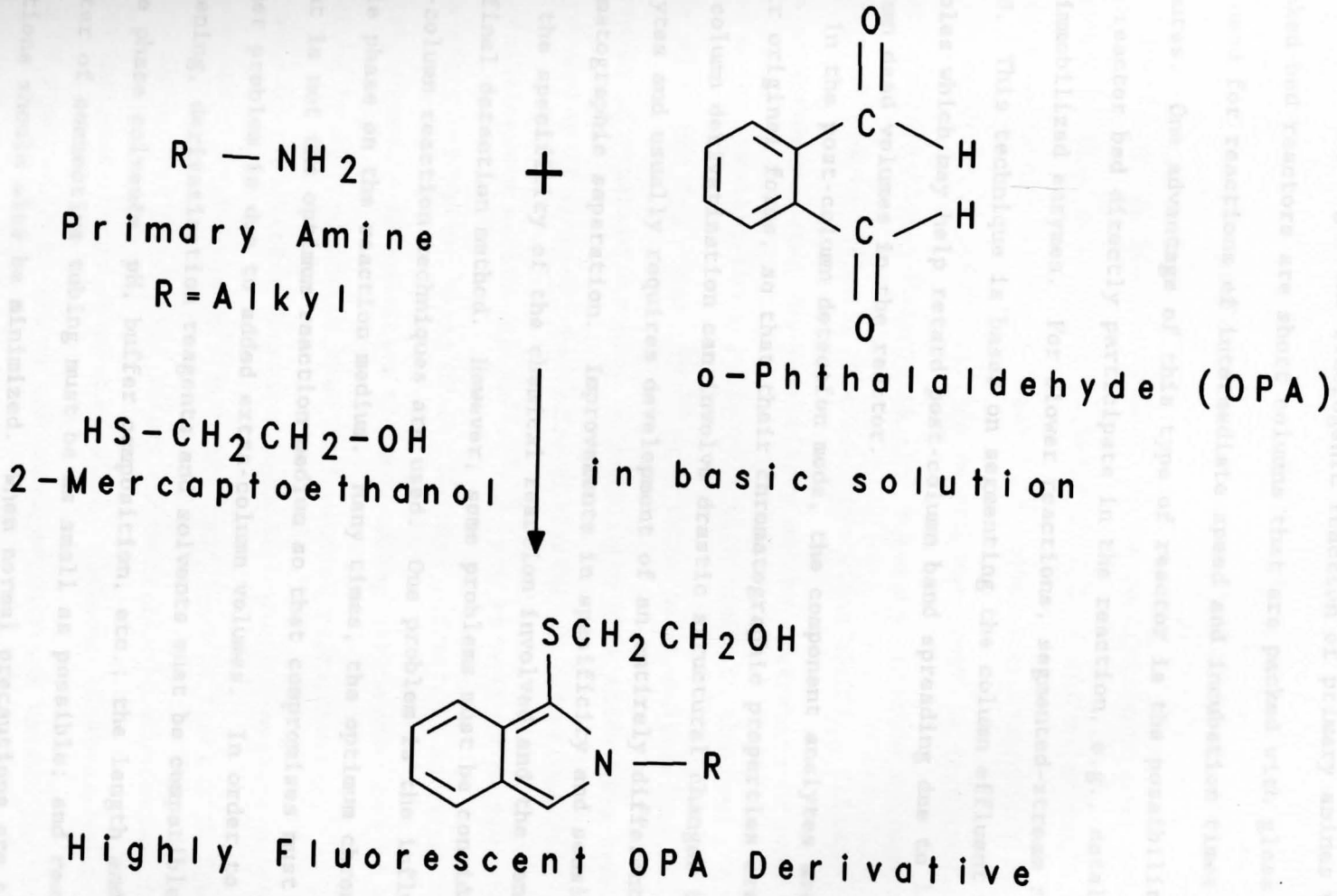

Fig. 4. o-Phthalaldehyde derivatization with amino acid.

to 30 seconds, e.g., the fluorogenic reaction of primary amines with OPA. Packed-bed reactors are short columns that are packed with glass beads and can be used for reactions of intermediate speed and incubation times up to about 4 minutes. One advantage of this type of reactor is the possibility of making the reactor bed directly participate in the reaction, e.g., catalysis reactions of immobilized enzymes. For slower reactions, segmented-stream reactors can be used. This technique is based on segmenting the column effluent with air bubbles which may help retard post-column band spreading due to large extra-column dead volumes in the reactor.

In the post-column detection mode, the component analytes are separated in their original forms, so that their chromatographic properties are unchanged. Pre-column derivatization can involve drastic structural changes to the analytes and usually requires development of an entirely different chromatographic separation. Improvements in specificity and sensitivity arise from the specificity of the chemical reaction involved and the sensitivity of the final detection method. However, some problems must be considered when post-column reaction techniques are used. One problem is the influence of the mobile phase on the reaction medium. Many times, the optimum chromatographic eluent is not the optimum reaction medium so that compromises must be made. Another problem is due to added extra-column volumes. In order to reduce band broadening, derivatization reagents and solvents must be compatible with the mobile phase solvents, pH, buffer composition, etc.; the length and internal diameter of connective tubing must be as small as possible; and reagent pump pulsations should also be minimized. When normal precautions are taken, post-column derivatization techniques are very powerful for solving problems in detection sensitivity and specificity.

CHAPTER II

HISTORICAL

Analysis of Conjugated Bile Acids By HPLC

The first application of HPLC to the separation of bile acids was reported by Shaw and Elliott in 1976[23]. All of the major conjugated bile acids were resolved using a Corasil II column and a recycling mode. However, in the analysis of human bile the method was found to lack specificity. In later investigations, Okuyama et al[24] separated taurine conjugates in biological samples using a reverse-phase μ-Bondapak C_{18} column. Baker et al[25] have also demonstrated the possibility of the separation of conjugated bile acids using straight-phase Perisorb A with gradient elution. Other methods using μBondapak C_{18} columns[26-30] or a Fatty Acid analysis column[27,31] to successfully separate mixtures of conjugated bile acids have been reported.

A number of methods, including differential refractometry[27,32] and UV spectrophotometry at 193-210 nm[27,28,33], may be used to detect conjugated bile acids after separation using HPLC. Neither refractive index nor UV spectrophotometry is sensitive for monitoring bile acids, but of the two, UV detection is more sensitive than differential refractometry.

Some derivatization reagents, such as 1-p-nitrobenzyl-3-p-tolyltriazene[24] and p-bromophenacyl bromide[33] have been employed to react with free and glycine conjugated bile acids before chromatographic separation, in order to improve the sensitivity of UV detection. Although these derivatives show strong UV absorbance at 254 nm and these methods are useful for the assay of bile acids in bile, their sensitivities are still insufficient to determine the small amounts of bile acids present in human serum samples. Taurine conjugated bile acids could not be determined using these reagents since these reagents react only with carboxyl groups.

It has been reported that fluorescence derivatization methods may possess the required sensitivity for low level bile acid analysis[34-36]. Okuyama and co-workers[34] performed the analysis of free bile acids and glycine conjugates based on the esterification of the carboxylic group of bile acids with the fluorescence derivatizing reagent 4-bromomethyl-7-methoxylcoumarin. These fluorescent derivatives of bile acids were separated using HPLC and detected with a fluorescence detector. The detection limit for individual bile acids was estimated to be about 20-30 pmoles.

Kamada et al[35] demonstrated separations of free and conjugated bile acids in serum and bile by the means of fluorescence HPLC. Free and conjugated bile acids extracted from serum or bile were fractionated on a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column. Free and glycine conjugates were derivatized with 1-bromoacetylpyrene. Taurine conjugates were hydrolyzed by choloylglycine hydrolase and then esterified using the same reagent. Derivatized bile acids were separated on a Radial-Pak A column and monitored using a fluorometer. Detection limits of 5 pmoles for glycine conjugated bile acids and 10 pmoles for taurine conjugated bile acids were reported. Ikawa et al[36], using a similar method, determined bile acids in human feces.

Ion-pair high performance liquid chromatography for the analysis of conjugated bile acids has also been investigated. Wildgrube et al[37] separated conjugated bile acids in human bile using an Ultrasphere C_{18} Ion Pair column and tetrabutylammonium phosphate, an ionic alkyl compound, which was added to the mobile phase. Quantitation was made using UV absorption at 214 nm. Later, using a similar method, they determined the biliary pattern of conjugated bile acids in human, dog, and rabbit[38].

Post-Column Immobilized Enzyme Detection For Bile Acids In HPLC

In 1979, Takeda and co-workers[39,40] reported a highly sensitive and selective fluorescence HPLC method for determination of bile acids using soluble 3α-hydroxysteroid dehydrogenase (3α-HSD) and the enzyme cofactor NAD^+. This method was based on the fact that 3α-hydroxy bile acids in samples could be measured quantitatively using fluorimetric determination of the amount of NADH generated from NAD^+ and bile acids in the presence of the 3α-HSD enzyme[41,42]. However, the assay consumed considerable amounts of expensive enzyme. Okuyama et al[6] first developed a modification of this method using an immobilized 3α-HSD enzyme column instead of the soluble enzyme. In this system, bile acids in the eluate from a μ-Bondapak/phenyl column reacted with NAD^+ in the 3α-HSD enzyme post-column reactor to produce NADH, which was monitored using a fluorometer. They succeeded in simultaneously separating the individual bile acids including free, glycine and taurine conjugated bile acids in serum, except glycodeoxycholic acid and taurochenodeoxycholic acid.

Hasegawa et al[43]performed the simultaneous determination of fifteen different serum bile acids using HPLC combined with a 3α-HSD enzyme post-column reactor. The system was operated under alkaline conditions that were optimum for the activity of 3α-HSD. They found that enzyme columns could be used for more than 300 analyses without a significant change in their operating characteristics.

Takeuchi and co-workers[44] demonstrated that the sensitivity of detection for bile acids was greatly increased by pre-mixing NAD^+ with the mobile phase using a single pump in the micro HPLC system with an immobilized 3α-HSD post-column reactor. In later studies, they modified the system using a micro pre-column concentration method to simplify pretreatments for the analysis of bile acids in serum[45]. When the enzyme post-column reactor was used repeatedly,

the peak heights of bile acids gradually decreased. The detection limit was reported as 0.13-0.28 pmole with a signal-to-noise ratio of 2.

Tazawa et al[46] determined urinary bile acids of noncholestatic and cholestatic infants using HPLC connected to a 3α-HSD immobilized enzyme column. Non-3α-hydroxy bile acids were detected using an ultraviolet detector, and 3α-hydroxy bile acids using a fluorescence detector.

Watanabe, Arima and Nagashima[47] performed separations of the major 15 bile acids using HPLC. Bile acids in human bile were fractionated into free, glycine, and taurine conjugated bile acids using a Sep-Pak C_{18} solid phase extraction cartridge and a Sephadex PHP-LH-20 column. Each fraction was then passed through an HPLC system consisting of a Zorbax C_{18} column and a 3α-HSD immobilized enzyme column. The analysis could be repeated on one 3α-HSD column without loss of sensitivity for over 400 injections.

Nakayama[48] also reported that HPLC with fixed 3α-HSD detection was suitable for the analysis of major bile acids in human serum. Campbell et al[49] demonstrated the separation of bile acids in a single step with adequate sensitivity for analysis of serum samples. A C_{18} Spherisorb column, an immobilized 3α-HSD post-column reactor and spectrofluorimetric detection were used. The reaction was carried out at neutral pH so that the lifetime of the enzyme column was increased.

Other high performance liquid chromatographic methods using 3α-HSD enzyme for analysis of bile acids have been developed. Swobodnik et al[50] analyzed conjugated bile acids in human serum using HPLC with an immobilized 3α-HSD post-column reaction and off-line spectrofluorimetric detection. Eight glycine and taurine conjugates were determined.

Kamada et al[51] investigated the use of eletrochemical detection of reduced NAD^+, rather than fluorimetric detection. Bile acids extracted from

serum or bile were fractionated and applied to the HPLC system consisting of a Radial-Pak A column and a 3α-HSD enzyme column. NADH generated was detected using voltammetry after mixing with phenazine methosulphate solution. Free and conjugated bile acids were determined, and each bile acid was measurable at the 20 pmole level.

Kawasaki, Maeda, and Tsuji[52] studied the separation of bile acids in serum using a different HPLC method with fluorescence detection. Free bile acids and their conjugates were converted to 3-oxo-bile acids in an immobilized 3α-HSD reactor column with NAD^+. 3-oxo-bile acids were extracted with a Sep-Pak C_{18} cartridge, derivatized with dansyl hydrazine and then separated by high performance liquid chromatography on a reversed-phase column. Derivatives were monitored using fluorimetric detection at 365 nm (excitation) and 520 nm (emission).

It has been found that the α-hydroxysteroid dehydrogenase is the only enzyme that has been used in HPLC for the analysis of bile acids. It is believed that references given for the use of immobilized enzyme detection for bile acids in HPLC are a complete list of that work to date.

CHAPTER III

STATEMENT OF THE PROBLEM

The analysis of bile acids in biological samples is preferentially done using high performance liquid chromatography (HPLC). Most HPLC methods, however, tend to lack sufficient sensitivity and specificity to allow clear identification of the analyte peaks, especially in the presence of the many interferences typically present in biological samples.

Improvements in sensitivity and specificity have been obtained using post-column immobilized enzyme reactors. These methods have mostly relied, however, upon immobilization of an α-hydroxysteroid dehydrogenase with subsequent fluorimetric[51,52], or bioluminescence detection of NADH[53,54]. Somewhat expensive enzymes and expensive consumable reagents such as NAD^+ or bioluminescence reagents must be used for these methods. It is thus desirable to develop a sensitive and specific HPLC method that uses inexpensive enzyme and reagents for determination of conjugated bile acids.

The use of immobilized choloylglycine hydrolase in a post-column reactor in this investigation may have satisfied these needs. Choloylglycine hydrolase, an inexpensive enzyme, specifically catalyzed the hydrolysis of the amide linkage in bile acid conjugates to yield free bile acids, and either glycine or taurine. The liberated amino acids were subsequently derivatized to fluorescent products with o-phthalaldehyde and were detected with high sensitivity using a fluorescence monitor.

CHAPTER IV

MATERIALS AND APPARATUS

Materials

All chemicals used in this research study were of analytical quality, or of the highest quality available.

Choloylglycine hydrolase (EC 3.5.1.24) and all bile acids were obtained from Sigma Chemical Company, St. Louis, MO.

Apparatus

Enzyme Activity Determination

The activity for soluble and immobilized choloylglycine hydrolase was determined by incubation at 37°C in a shaking incubator. Colorimetric analysis was performed using a Model 8452A Diode Array Spectrophotometer (Hewlett Packard Inst.).

Immobilized choloylglycine hydrolase activity was also determined by packing the immobilized enzyme into a stainless steel column (4.6 mm I.D. x 50 mm), equilibrating at 37°C in a water bath, followed by spectrofluorometry using a Model 430 spectrofluorometer (Turner Inst.).

High Performance Liquid Chromatography

Samples were analyzed using a Model LC/9533 Ternary Gradient Liquid Chromatography System (IBM Instruments Inc.). The two types of detectors used were a Model LC/9523 ultraviolet/visible absorbance detector (IBM Instruments Inc.), and a Model RF-535 fluorescence HPLC monitor (Shimadzu.).

An Econosphere C_8 column measuring 4.6 mm I.D. x 150 mm with a particle size of 5 μm (Alltech Associates Inc.) was used for separation of the conjugated bile acids. A 4.6 mm I.D. x 50 mm stainless steel column packed

with the CPG-immobilized choloylglycine hydrolase was used as the post-column enzyme reactor. Reagents were pumped using a Model 110A pump (Beckman Inst.) and a Model 6000 pump (Waters Chromatography). The system used for HPLC analysis and the post-column enzyme reactor is shown in Figure 5.

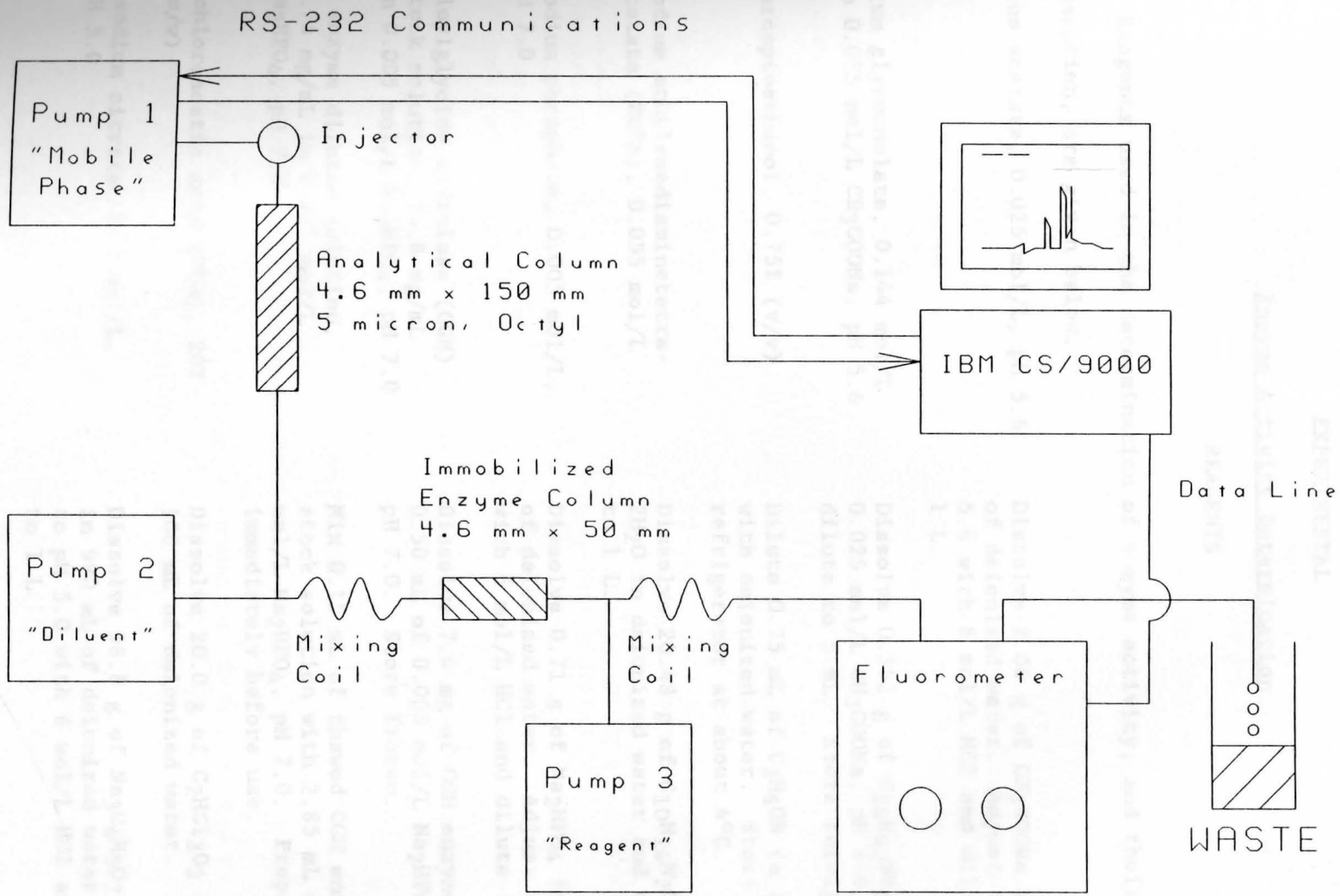

Fig. 5. Scheme of the analytical system.

CHAPTER V

EXPERIMENTAL

Enzyme Activity Determination

REAGENTS

Reagents used in the determination of enzyme activity, and their preparation, are given below.

Sodium acetate, 0.025 mol/L, pH 5.6	Dissolve 2.06 g of CH_3COONa in 990 mL of deionized water. Adjust to pH 5.6 with 6 mol/L HCl and dilute to 1 L.
Sodium glycocholate, 0.144 mol/L in 0.025 mol/L CH_3COONa, pH 5.6	Dissolve 0.352 g of $C_{26}H_{42}NO_6Na$ in 0.025 mol/L CH_3COONa, pH 5.6, and dilute to 5 mL. Store refrigerated.
2-Mercaptoethanol, 0.75% (v/v)	Dilute 0.75 mL of C_2H_6OS to 100 mL with deionized water. Store in refrigerator at about 4°C.
Disodium ethylenediaminetetra-acetate (EDTA), 0.055 mol/L	Dissolve 20.48 g of $C_{10}H_{14}N_2O_8Na_2 . 2H_2O$ in deionized water and dilute to 1 L.
Disodium phosphate, 0.005 mol/L, pH 7.0	Dissolve 0.71 g of Na_2HPO_4 in 990 mL of deionized water. Adjust to pH 7.0 with 6 mol/L HCl and dilute to 1 L.
Choloylglycine hydrolase (CGH) stock solution, 14.8 mg/mL in 0.005 mol/L Na_2HPO_4, pH 7.0	Dissolve 7.4 mg of CGH enzyme in 0.50 mL of 0.005 mol/L Na_2HPO_4, pH 7.0. Store frozen.
CGH enzyme diluted solution, 0.74 mg/mL in 0.005 mol/L Na_2HPO_4, pH 7.0	Mix 0.15 mL of thawed CGH enzyme stock solution with 2.85 mL of 0.005 mol/L Na_2HPO_4, pH 7.0. Prepare immediately before use.
Trichloroacetic acid (TCA), 20% (w/v)	Dissolve 20.0 g of $C_2HCl_3O_2$ in 100 mL of deionized water.
Trisodium citrate, 0.2 mol/L, pH 5.0	Dissolve 58.8 g of $Na_3C_6H_5O_7 . 2H_2O$ in 990 mL of deionized water. Adjust to pH 5.0 with 6 mol/L HCl and dilute to 1 L.
Stannous chloride, 0.16% (w/v) in 0.2 mol/L trisodium citrate, pH 5.0	Dissolve 0.192 g of $SnCl_2 . 2H_2O$ in 100 mL of 0.2 mol/L trisodium citrate, pH 5.0.

n-Propanol solution, 50% (v/v)	Mix 250 mL of n-propanol with 250 mL of deionized water.
Ninhydrin solution, 4% (w/v) in ethylene glycol monomethyl ether	Dissolve 4.0 g of $C_9H_6O_4$ in 100 mL of $C_3H_8O_2$, which should be peroxides free. See Appendix A.
Ninhydrin color reagent	Combine in equal volumes of 0.16% $SnCl_2$, 50% n-propanol, and 4% ninhydrin. Prepare fresh.
Boric acid, 0.40 mol/L, pH 9.7	Dissolve 24.8 g of H_3BO_3 in 800 mL of deionized water. Addition of a small amount of concentrated KOH can help the dissolution. Add an additional 190 mL of deionized water. Adjust to pH 9.7 with concentrated KOH and dilute to 1 L.
o-Phthalaldehyde (OPA) fluorescence reagent	Dissolve 80 mg of $C_8H_6O_2$ in 1 mL of 95% (w/v) ethanol and mix with 100 mL of 0.40 mol/L boric acid, pH 9.7, and 0.2 mL of 2-mercaptoethanol.
Glycine solution, 1.0×10^{-3} mol/mL	Dissolve 75.5 mg of $C_2H_5NO_2$ in 1.0 mL of deionized water.
Glycine solution, 1.0 μmole/mL	Dilute 0.10 mL of 1.0×10^{-3} mol/mL glycine to 100 mL.
Glycine solution, 2.0 μmole/mL	Dilute 0.20 mL of 1.0×10^{-3} mol/mL glycine to 100 mL.
Glycine solution, 3.0 μmole/mL	Dilute 0.30 mL of 1.0×10^{-3} mol/mL glycine to 100 mL.

PROCEDURES

SOLUBLE CGH ENZYME ACTIVITY DETERMINATION

Soluble CGH enzyme activity was determined referring to the ninhydrin method from Sigma Chemical Company[11]. The following reagents were added to a test tube and mixed:

0.20 mL of 0.025 mol/L CH_3COONa pH 5.6;
0.20 mL of 0.144 mol/L sodium glycocholate;
0.20 mL of 0.75% 2-mercaptoethanol;
0.20 mL of 0.055 mol/L EDTA;
1.00 to 0.10 mL of deionized water, according to needs;
0 to 0.90 mL of anhydrous methanol, according to needs.

The test tube was placed in an incubating water bath and allowed to equilibrate to 37°C. Diluted CGH enzyme solution (0.20 mL) equilibrated at 37°C was added to the test tube. The total volume of mixture was 2.00 mL in each sample. The reagent mixture was incubated for exactly 5 minutes. The reaction was stopped by adding 2.0 mL of 20% TCA. The mixture was then centrifuged for 10 minutes. The supernatant solution of test sample was then ready for assaying the amount of glycine liberated during the incubation. A reagent blank was also prepared, as above, except that 20% TCA was added before adding the CGH enzyme.

For determining the amount of glycine hydrolyzed, 0.20 mL of the supernatant solution of test sample (or blank) was added to 2.0 mL of ninhydrin color reagent. Both test tubes were heated in a boiling water bath for 20 minutes and cooled to room temperature. 10.0 mL of 50% n-propanol then was added to each tube. The absorbance was measured at 570 nm using the blank as the reference.

IMMOBILIZED CGH ENZYME ACTIVITY DETERMINATION

The procedure used was similar to that described for determination of soluble CGH using ninhydrin. The following amounts of reagents were mixed in a test tube:

0.20 mL of 0.025 mol/L CH_3COONa, pH 5.6;
0.20 mL of 0.144 mol/L sodium glycocholate;
0.20 mL of 0.75 % 2-mercaptoethanol;
0.20 mL of 0.055 mol/L EDTA;
0.10 mL of deionized water.

The test tube was placed in an incubating water bath to allow equilibration to 37°C. The reagents were then added to 31 mg of CPG-immobilized CGH enzyme, also equilibrated at 37°C. Nitrogen gas at a low flow rate was allowed to pass into the mixture through a Pasteur pipet so that the mixture was stirred and the immobilized enzyme stayed suspended in the surrounding solution. The mixture was allowed to incubate for exactly 5 minutes with nitrogen gas bubbling and was stirred the whole time. The remaining steps were as described above for assay of the soluble enzyme.

GLYCINE STANDARD CURVE PREPARATION

The standard curves for the ninhydrin assays were determined by running a set of glycine standards as follows:

	Blank	Sample
Deionized water	0.20 mL	--
Glycine standard solution, (either 1,2,3 μmole/mL)	--	0.20 mL
Ninhydrin color reagent	2.0 mL	2.0 mL

Reagents in both test tubes were mixed, heated in a boiling water bath for 20 minutes, and cooled to room temperature. 10.0 mL of 50% n-propanol was added to each tube. The absorbance at 570 nm for each standard was obtained using the blank as the reference.

IMMOBILIZED CGH ENZYME ACTIVITY DETERMINATION USING o-PHTHALALDEHYDE (OPA)

Immobilized CGH enzyme activity was determined by slurry packing approximately 0.5 g of CPG-immobilized CGH into a 4.6 mm I.D.x 50 mm stainless steel column using 0.05 mol/L Na_2HPO_4, pH 7.0. The column was equilibrated in a 37°C water bath. 100 μL of sodium glycocholate was injected into the column and eluted with different concentrations of 0.05 mol/L Na_2HPO_4, pH 7.0, and methanol at 1 mL/min. Fractions were collected between 24 to 60 seconds and

assayed for glycine using OPA. 0.20 mL of each fraction was added to 6.0 mL of OPA fluorescent reagent, mixed and allowed to stand for 5 minutes. Fluorescence intensity was determined at an excitation of 340 nm and emission of 455 nm using 6.0 mL of OPA fluorescent reagent as a blank reference.

CALCULATIONS

According to definition, one unit of choloylglycine hydrolase activity can hydrolyze 1.0 micromole of glycocholic acid to glycine and cholic acid in five minutes at pH 5.6 at 37°C[11].

The micromoles of glycine liberated in the 0.20 mL aliquot of the supernatant were obtained from the glycine standard curve in Figure 6. For soluble CGH enzyme, this value was multiplied by 20 to obtain the micromoles glycine hydrolyzed per 5 minutes in the incubation mixture. For immobilized CGH enzyme, this value was multiplied by 19/2. The factor of 20 was obtained from the fact that the total volume of the mixture was 4.0 mL: 2.0 mL of reagent mixture and 2.0 mL of TCA. The factor of 19/2 was obtained by assuming that the volume of 31 mg of CPG-immobilized CGH enzyme was negligible, and the total volume of the mixture was 1.9 mL: 0.9 mL of reagent mixture and 1.0 mL of TCA.

Immobilized CGH Enzyme Preparation

REAGENTS

Reagents used in the immobilized CGH enzyme preparation are as follows:

Nitric acid, 5% (w/v)	Dilute 5 mL of concentrated HNO_3 (68% (w/v)) to 100 mL with deionized water.
3-Aminopropyltriethoxysilane, 10% (v/v)	Mix 2 mL of $C_9H_{23}NO_3Si$ with 18 mL of deionized water. Prepare fresh.

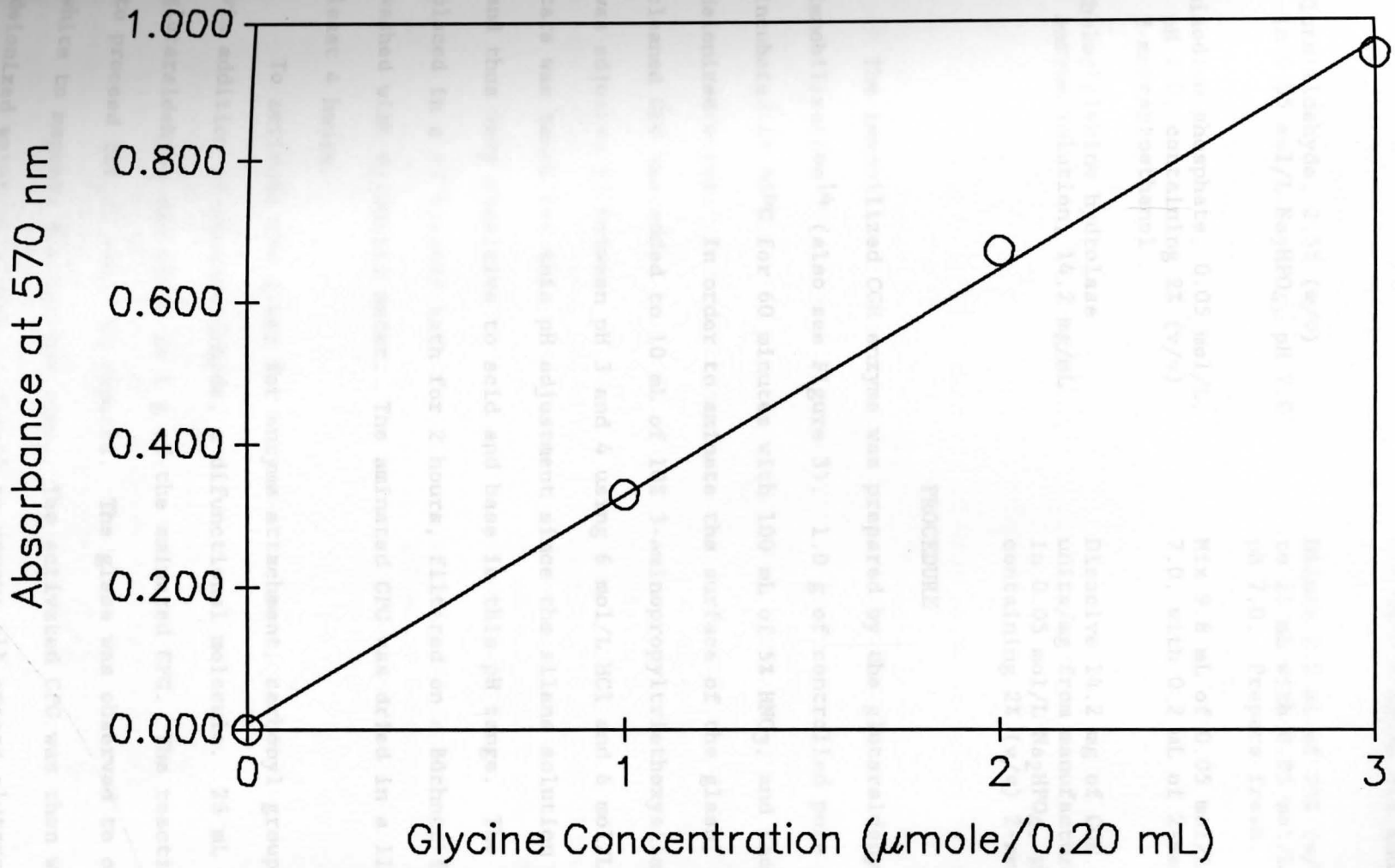

Fig. 6. Glycine standard curve using ninhydrin.
Regression equation: Y=(9.5 x 10E-3) + 0.32X

Disodium phosphate, 0.05 mol/L, pH 7.0	Dissolve 7.1 g of Na_2HPO_4 in 990 mL of deionized water. Adjust to pH 7.0 with 6 mol/L HCl and dilute to 1 L.
Glutaraldehyde, 2.5% (w/v) in 0.05 mol/L Na_2HPO_4, pH 7.0	Dilute 2.5 mL of 25% (w/v) $C_5H_8O_2$ to 25 mL with 0.05 mol/L Na_2HPO_4, pH 7.0. Prepare fresh.
Disodium phosphate, 0.05 mol/L, pH 7.0, containing 2% (v/v) 2-mercaptoethanol	Mix 9.8 mL of 0.05 mol/L Na_2HPO_4, pH 7.0, with 0.2 mL of 2-mercaptoethanol.
Choloylglycine hydrolase enzyme solution, 14.2 mg/mL	Dissolve 14.2 mg of CGH enzyme: 42 units/mg from manufacturers literature, in 0.05 mol/L Na_2HPO_4, pH 7.0, containing 2% (v/v) 2-mercaptoethanol.

PROCEDURE

The immobilized CGH enzyme was prepared by the glutaraldehyde method of immobilization[14] (also see Figure 3). 1.0 g of controlled pore glass (CPG) was incubated at 85°C for 60 minutes with 100 mL of 5% HNO_3, and then rinsed with deionized water. In order to aminate the surface of the glass, 1.0 g of the cleaned CPG was added to 10 mL of 10% 3-aminopropyltriethoxysilane and the pH was adjusted to between pH 3 and 4 using 6 mol/L HCl and 6 mol/L NaOH. Great care was taken for this pH adjustment since the silane solution was unbuffered and thus very sensitive to acid and base in this pH range. The reactants were placed in a 75°C water bath for 2 hours, filtered on a Büchner funnel, and washed with deionized water. The aminated CPG was dried in a 115°C oven for at least 4 hours.

To activate the glass for enzyme attachment, carbonyl groups were added via addition of glutaraldehyde, a difunctional molecule. 25 mL of 2.5% glutaraldehyde was added to 1 g of the aminated CPG. The reaction was allowed to proceed for at least 60 minutes. The glass was observed to change from white to magenta during this time. The activated CPG was then washed with 2 L deionized water on a Büchner funnel to remove all excess glutaraldehyde. 14.2

mg/mL of CGH enzyme solution containing 2% (v/v) 2-mercaptoethanol was added to the activated CPG. Addition of 2-mercaptoethanol to the enzyme solution was required to prevent oxidation of essential thiol groups in the enzyme active site. This mixture was allowed to set for 4 hours. The CPG-immobilized CGH enzyme was washed with deionized water and stored in 0.05 mol/L Na_2HPO_4, pH 7.0 in a refrigerator at 4°C.

HPLC Analysis of Bile Acids

REAGENTS

Reagents used in HPLC analysis of bile acids are given below:

Monopotassium phosphate, 0.1 mol/L, pH 4.5	Dissolve 13.6 g of KH_2PO_4 in deionized water and dilute to 1 L.
Monopotassium phosphate, 0.1 mol/L, pH 3.0	Dissolve 13.6 g of KH_2PO_4 in 990 mL of deionized water. Adjust to pH 3.0 with 6 mol/L HCl and dilute to 1 L.
Monopotassium phosphate, 0.1 mol/L, pH 3.5	Dissolve 13.6 g of KH_2PO_4 in 990 mL of deionized water. Adjust to pH 3.5 with 6 mol/L HCl and dilute to 1 L.
Monopotassium phosphate, 0.1 mol/L, pH 5.0	Dissolve 13.6 g of KH_2PO_4 in 990 mL of deionized water. Adjust to pH 5.0 with 6 mol/L NaOH and dilute to 1 L.
Monopotassium phosphate, 0.1 mol/L, pH 5.5	Dissolve 13.6 g of KH_2PO_4 in 990 mL of deionized water. Adjust to pH 5.5 with 6 mol/L NaOH and dilute to 1 L.

SAMPLES

The samples prepared for HPLC analysis were the sodium salts of conjugated bile acids. They were prepared by dissolving the bile acid in anhydrous methanol. In all cases, the concentrations of the samples were 1 mg/mL methanol of each bile acid.

HPLC ANALYSIS

A mixture containing 1 mg of each of the conjugated bile acids dissolved in 1.0 mL methanol was separated using an Econosphere C_8 column with a mobile phase containing 60% anhydrous methanol and 40% 0.1 mol/L KH_2PO_4 (v/v). A flow rate of 1.5 mL/min was used. A UV detector set to 254 nm was used as a monitor. To optimize the separation, mobile phases with pH values between 3.0 and 5.5 were examined in this work. Injection volumes were 10 μL of samples.

HPLC Analysis of Bile Acids with a Post-column Enzyme Reactor

REAGENTS

Reagents used in HPLC analysis of bile acids with a post-column enzyme reactor are given below:

Monopotassium phosphate, 0.1 mol/L, pH 4.5	Dissolve 13.6 g of KH_2PO_4 in deionized water and dilute to 1 L.
EDTA, 0.1 mol/L	Dissolve 37.2 g of $C_{10}H_{14}N_2O_8Na_2.2H_2O$ in deionized water and dilute to 1 L.
Disodium phosphate, 0.05 mol/L, pH 7.0	Dissolve 7.1 g of Na_2HPO_4 in 990 mL of deionized water. Adjust to pH 7.0 with 6 mol/L HCl and dilute to 1 L.
Diluent, 0.05 mol/L EDTA, 0.025 mol/L Na_2HPO_4, pH 7.0, 2% (v/v) mercaptoethanol	Mix 98 ml of 0.1 mol/L EDTA and 98 mL of 0.025 mol/L Na_2HPO_4, pH 7.0, with 4 mL of 2-mercaptoethanol.
Boric acid, 0.40 mol/L, pH 11.0	Dissolve 24.8 g of H_3BO_3 in 800 mL of deionized water. Addition of a small amount of concentrated KOH can help the dissolution. Add an additional 190 mL of deionized water. Adjust to pH 11.0 with concentrated KOH and dilute to 1 L.
OPA fluorescence reagent	Dissolve 80 mg of OPA in 1 mL of 95% (w/v) ethanol and mix with 100 mL of 0.40 mol/L boric acid, pH 11.0, and 0.2 mL of 2-mercaptoethanol.

SAMPLES

All samples used in this part of the investigation were the sodium salts of bile acids, dissolved in anhydrous methanol. 1 mg/mL and 0.1 mg/mL methanol of each individual bile acid were used in mixtures.

HPLC ANALYSIS WITH A POST-COLUMN ENZYME REACTOR

The system used in this work has been illustrated in Figure 5. An Econosphere C_8 column and a CPG-immobilized CGH enzyme column were used. The mobile phase, pumped by Pump 1, was 60% anhydrous methanol and 40% 0.1 mol/L KH_2PO_4, pH 4.5, (v/v). The diluent, pumped by Pump 2, contained 0.05 mol/L EDTA, 0.025 mol/L Na_2HPO_4, pH 7.0, and 2% (v/v) 2-mercaptoethanol. The diluent was employed to dilute the methanol concentration in the eluent to prevent the denaturation of the immobilized enzyme. 2-Mercaptoethanol in the diluent played the dual roles of preventing oxidation of essential thiol groups in the immobilized enzyme active site, as well as participating in the OPA reaction. A small stainless steel mixing coil was placed between the analytical column and the immobilized enzyme column to allow for efficient mixing of the eluent and diluent solutions. An 1.5 mL knitted Teflon reaction coil was placed between the enzyme column and the detector to allow sufficient reaction time for the derivatization of OPA fluorescence reagent with the glycine or taurine liberated by the enzyme reaction. This reagent was pumped by Pump 3.

Flow rates tried for Pump 1 and Pump 2 were 1.0 mL/min and 2.0 mL/min, respectively. The flow rates tested in Pump 3 were 0.5, 1.0, and 1.5 mL/min. Injection volumes were 10 μL of samples. The enzyme column was equilibrated at 37°C by placing it into a column heater and circulating heated water from a temperature controlled water bath. Detection was by means of a fluorescence

detector at an excitation of 340 nm and emission of 455 nm.

The enzyme column for HPLC analysis was prepared by packing the CPG-immobilized choloylglycine hydrolase (CPG diameter, 1000 Å) into a stainless steel column using 0.05 mol/L Na_2HPO_4, pH 7.0. Each day, before use, the enzyme column was equilibrated in the 37°C water bath and after use, the column was flushed with 15 mL of 0.05 mol/L Na_2HPO_4, pH 7.0 and stored in a refrigerator.

CHAPTER VI

RESULTS AND DISCUSSION

Enzyme Activity Determination

In the use of ninhydrin for assay of glycine in the analysis of CGH activity, it was noted that ethylene glycol monomethyl ether used for the reagent must be free from contaminating peroxides. When the ninhydrin reagent contained peroxides, Ruhemann's purple did not appear in the reaction of ninhydrin and amino acids. The test for peroxides is outlined in Appendix A.

The results of the soluble CGH enzyme activity determination using ninhydrin assay of glycine, which were compared to the glycine standard curve (Figure 6 and Table 2), are given in Tables 3 through 5, and in Figures 7 and 8. The CPG-immobilized CGH activity was also determined using OPA analysis of glycine. These results are shown in Table 6 and Figure 8.

TABLE 2

GLYCINE STANDARD CURVE (NINHYDRIN ASSAY)

Glycine Concentration (μmole/0.20 mL aliquot)	$A_{570\ nm}$
0	0
1	0.331
2	0.675
3	0.952

TABLE 3

SOLUBLE CGH ACTIVITY AT DIFFERENT INCUBATION TIMES (NINHYDRIN ASSAY)*

Incubation Time (min)	$A_{570\ nm}$	μmole Glycine in 0.20 mL Aliquot	Activity (μmole)
0	0	0	0
1	0.0755	0.206	4.12
2	0.170	0.502	10.0
3	0.256	0.770	15.4
4	0.284	0.858	17.2
5	0.301	0.910	18.2

* Methanol concentration : 15% (v/v)

TABLE 4

SOLUBLE CGH ACTIVITY IN METHANOL USING NINHYDRIN

% Methanol (v/v)	$A_{570\ nm}$	μmole Glycine in 0.20 mL Aliquot	Activity (μmole/5 min)
0	0.284	0.858	17.2
15	0.236	0.708	14.2
25	0.0842	0.233	4.66
35	0.0227	0.041	0.82
45	0.0005	0	0

Table 3 and Figure 7 indicate that the activity of choloylglycine hydrolase enzyme was raised with incubation time and increased up to 5 minutes. This was in agreement with the method from Sigma Chemical Company[11], where a 5-minute incubation time was chosen.

Although the activities in Tables 4 and 6 and Figure 8 determined using different methods cannot be compared with their actual values, comparison of relative values is still meaningful. The activity of both soluble and CPG-immobilized CGH enzyme declined with increasing methanol concentration. However, the characteristics of their changes were different. For the immobilized enzyme, its activity linearly reduced with increasing methanol concentration. For the soluble enzyme, its activity was reduced smoothly at the beginning, but when methanol concentration was larger than 15%, its activity decreased sharply. In 25% methanol, the CPG-immobilized enzyme maintained 53% of activity, but the soluble enzyme could only maintain 30%. Immobilization therefore seemed to improve the stability of CGH enzyme in methanol. The results above also indicated that the immobilized enzyme could tolerate moderate (about 20 to 25%) methanol concentrations. When the concentration of methanol exceeded 25%, the enzyme activity decreased greatly and the use of enzyme became difficult. This effect was also noted by Bowers[55].

TABLE 5

CPG-IMMOBILIZED CGH ACTIVITY USING NINHYDRIN

CPG Pore Diameter (Å)	$A_{570\ nm}$	μmole Glycine in 0.20 mL Aliquot	Activity (μmole/5 min)
527	0.0542	0.140	1.33
1870	0.0437	0.107	1.02

TABLE 6

CPG-IMMOBILIZED CGH ACTIVITY IN METHANOL USING OPA*

% Methanol (v/v)	Relative Fluorescence Intensity
0	28.5
5	25.5
10	23.0
15	19.0
20	18.8
30	12.5

* Sodium glycocholate concentration : 0.036 mol/L.

Immobilized CGH Enzyme Preparation

The CGH enzyme immobilization was tried initially without the addition of 2-mercaptoethanol. An immobilized enzyme product with a very low activity was obtained. It was suggested that denaturation of the enzyme had occurred during the immobilization process. The most probable reason for this was that the active thiol groups on the enzyme were oxidized. A reducing reagent, 2% (v/v) of 2-mercaptoethanol, was added to solve the problem. Consequently, a much more active immobilized enzyme was obtained. Therefore, it was believed that the protection of these essential sulfhydryl groups in the active site was required during the immobilization in order to maintain the enzyme in an active state after immobilization.

However, the use of 2-mercaptoethanol may have brought another problem. Instead of coupling with the amino group on the enzyme, it was suggested that

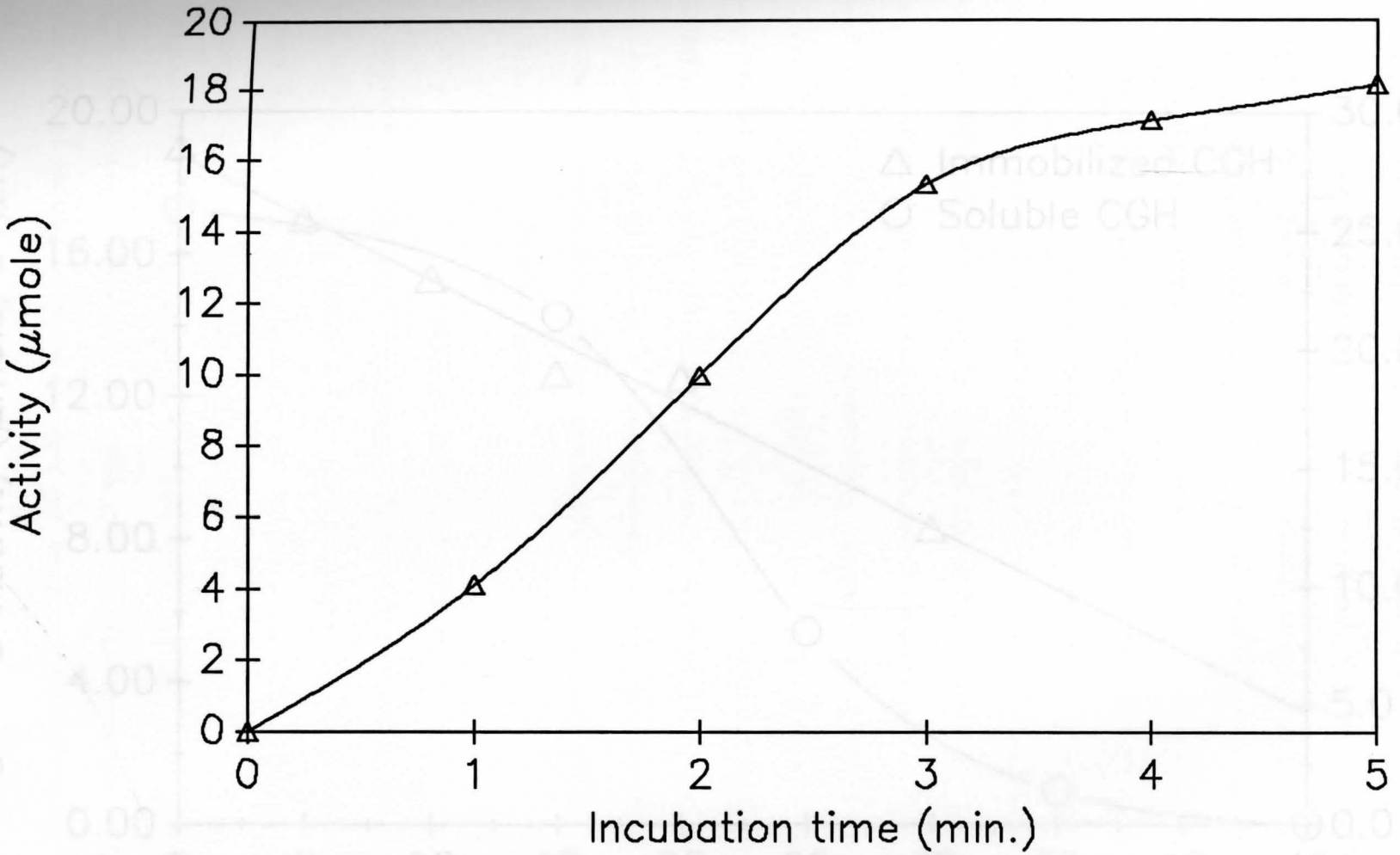

Fig. 7. Soluble CGH activity vs incubation time.

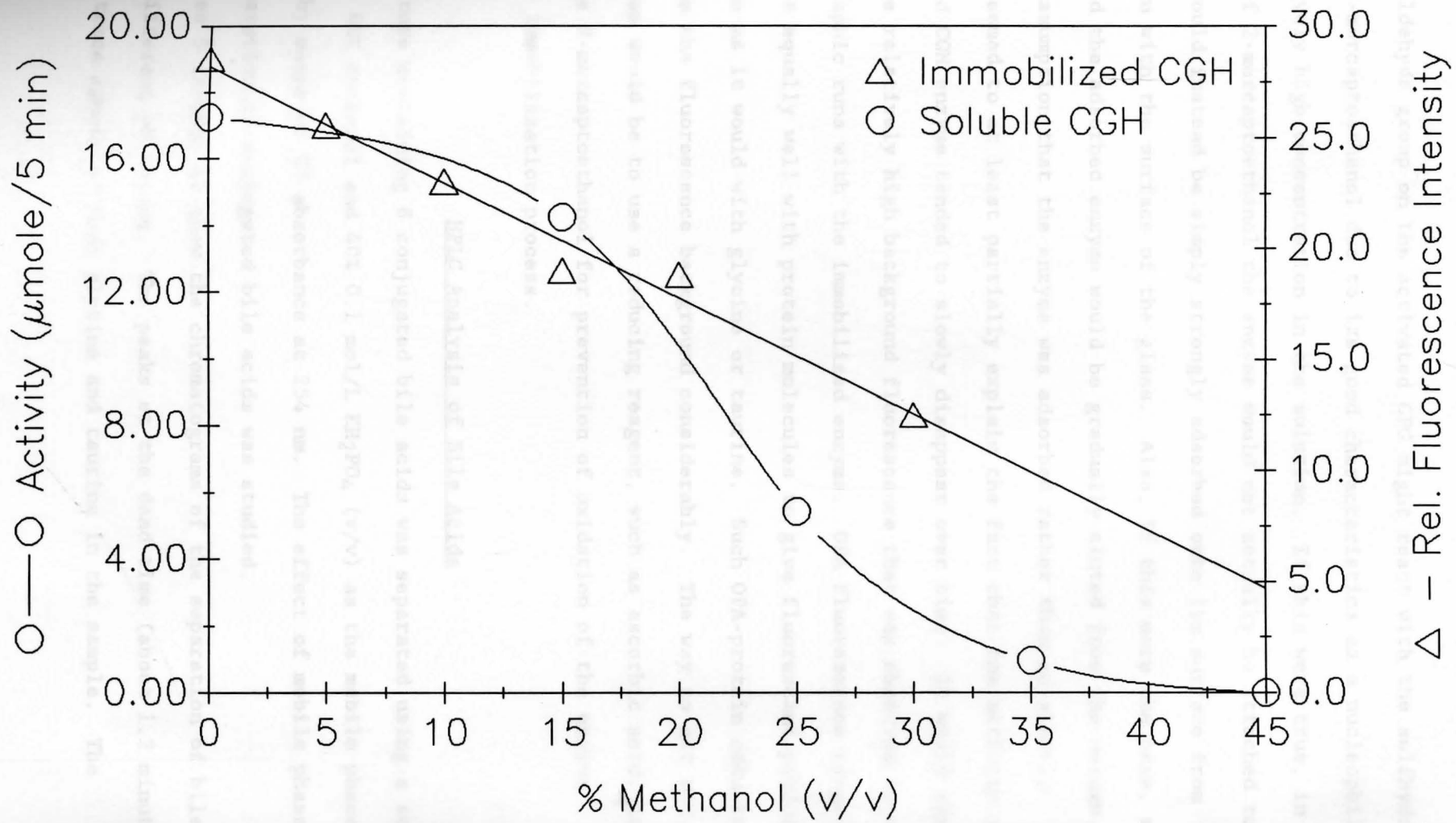

Fig. 8. Effect of methanol on CGH activity.
Sodium glycocholat substrate; 37°C.

the free aldehyde group on the activated CPG might react with the sulfhydryl group on 2-mercaptoethanol due to its good characteristics as a nucleophile and its relatively high concentration in the solution. If this were true, in the presence of 2-mercaptoethanol the enzyme would not actually be attached to the CPG, but would instead be simply strongly adsorbed onto its surface from interaction with the surface of the glass. Also, if this were the case, as time passed the adsorbed enzyme would be gradually eluted from the column.

The assumption that the enzyme was adsorbed rather than covalently attached seemed to at least partially explain the fact that the activity of the immobilized CGH enzyme tended to slowly disappear over time. It would also explain the relatively high background fluorescence that was observed in all chromatographic runs with the immobilized enzyme. OPA fluorescence reagent would react equally well with protein molecules to give fluorescent products in solution as it would with glycine or taurine. Such OPA-protein adducts would raise the fluorescence background considerably. The way to get rid of this problem would be to use a reducing reagent, such as ascorbic acid, to replace the 2-mercaptoethanol for prevention of oxidation of the enzyme during the immobilization process.

HPLC Analysis of Bile Acids

A mixture containing 8 conjugated bile acids was separated using a solvent mixture of 60% methanol and 40% 0.1 mol/L KH_2PO_4 (v/v) as the mobile phase and detection by means of UV absorbance at 254 nm. The effect of mobile phase pH on the separation of conjugated bile acids was studied.

Figures 9 through 11 show the chromatograms of the separation of bile acids at different pH values. The peaks at the dead time (about 1.2 minutes) were from trace amount of free glycine and taurine in the sample. The

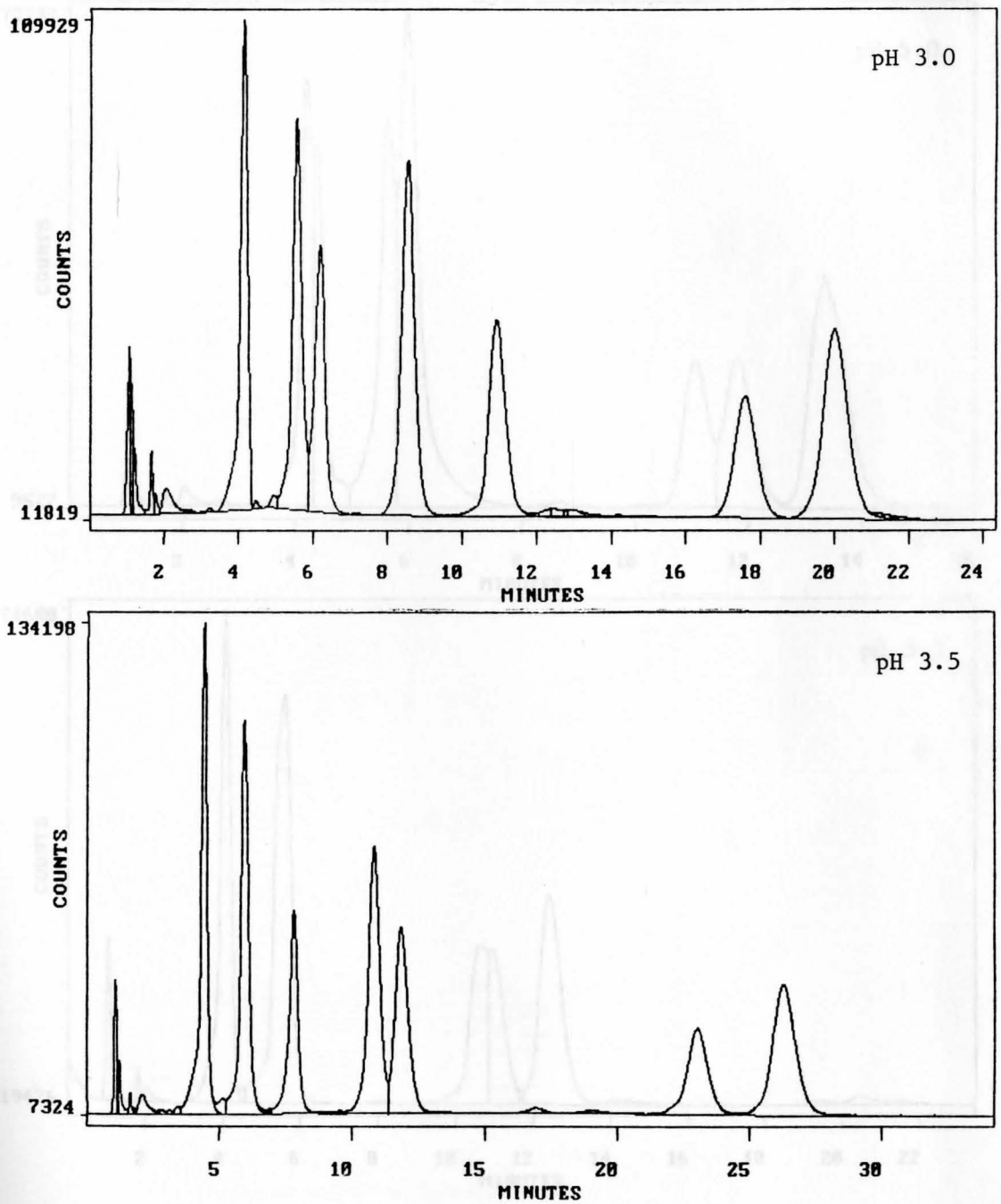

Fig. 9. Chromatograms of the separation of conjugated bile acids at pH 3.0, and 3.5.

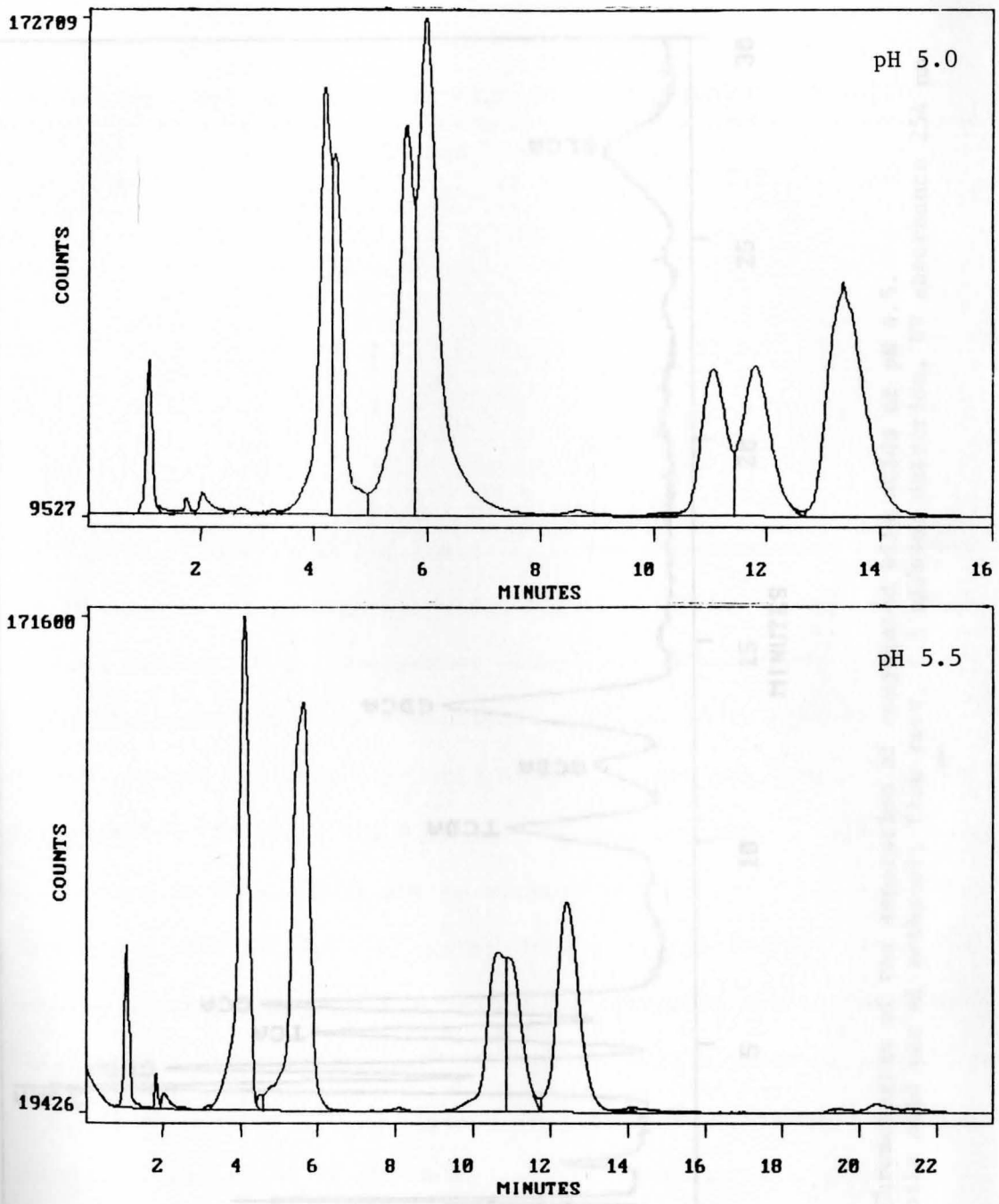

Fig. 10. Chromatograms of the separation of conjugated bile acids at pH 5.0, and 5.5.

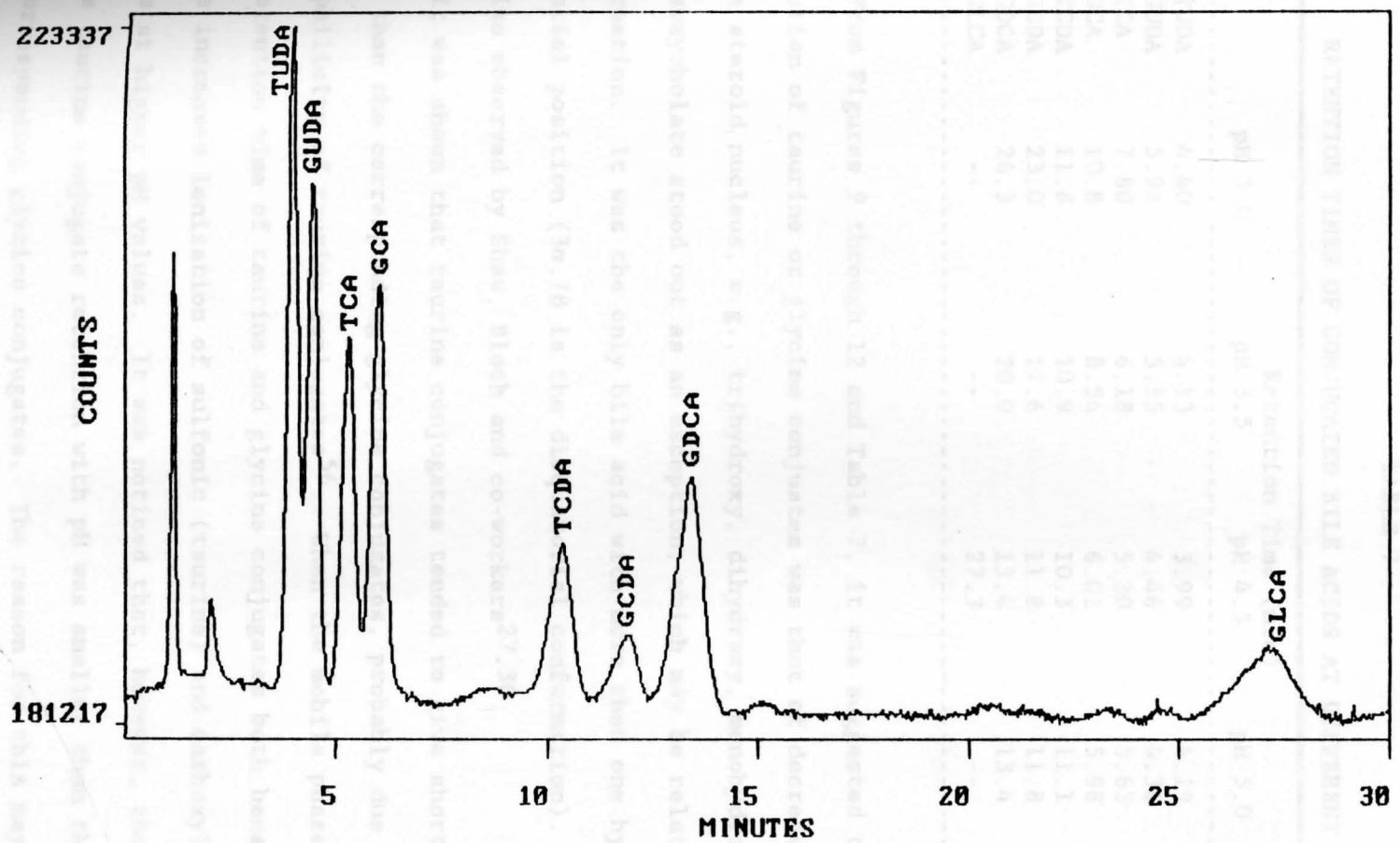

Fig. 11. Chromatogram of the separation of conjugated bile acids at pH 4.5.
1 mg each bile acid per mL methanol; flow rate, 1.5 mL/min; detection, UV absorbance 254 nm.

retention time of each conjugated bile acid is given in Table 7. Figure 12 shows the variation of bile acid retention with pH.

TABLE 7

RETENTION TIMES OF CONJUGATED BILE ACIDS AT DIFFERENT pH VALUES

	Retention Time (min)				
	pH 3.0	pH 3.5	pH 4.5	pH 5.0	pH 5.5
TUDA	4.40	4.13	3.99	4.19	4.06
GUDA	5.94	5.55	4.46	4.36	4.06
TCA	7.80	6.18	5.30	5.63	5.59
GCA	10.8	8.54	6.02	5.98	5.59
TCDA	11.8	10.9	10.3	11.1	10.6
GCDA	23.0	17.6	11.8	11.8	10.9
GDCA	26.3	20.0	13.4	13.4	12.4
GLCA	--	--	27.3	--	--

From Figures 9 through 12 and Table 7, it was suggested that the order of elution of taurine or glycine conjuates was that of decreasing polarity of the steroid nucleus, e.g., trihydroxy, dihydroxy, monohydroxy. But ursodeoxycholate stood out as an exception, which may be related to its conformation. It was the only bile acid with more than one hydroxyl group in equatorial position (3α,7ß is the diequatorial conformation). This behavior was also observed by Shaw, Bloch and co-workers[27,30].

It was shown that taurine conjugates tended to give shorter retention times than the corresponding glycine conjugates, probably due to the higher hydrophilicity of taurine conjugates[56]. When the mobile phase pH was raised, the retention time of taurine and glycine conjugates both became shorter due to the increased ionization of sulfonic (taurine) and carboxyl (glycine) groups at higher pH values. It was noticed that, however, the relative change of the taurine conjugate retention with pH was smaller than that observed for the corresponding glycine conjugates. The reason for this may be that the pH range studied falls in the protonation pH range of the taurine conjugates (at

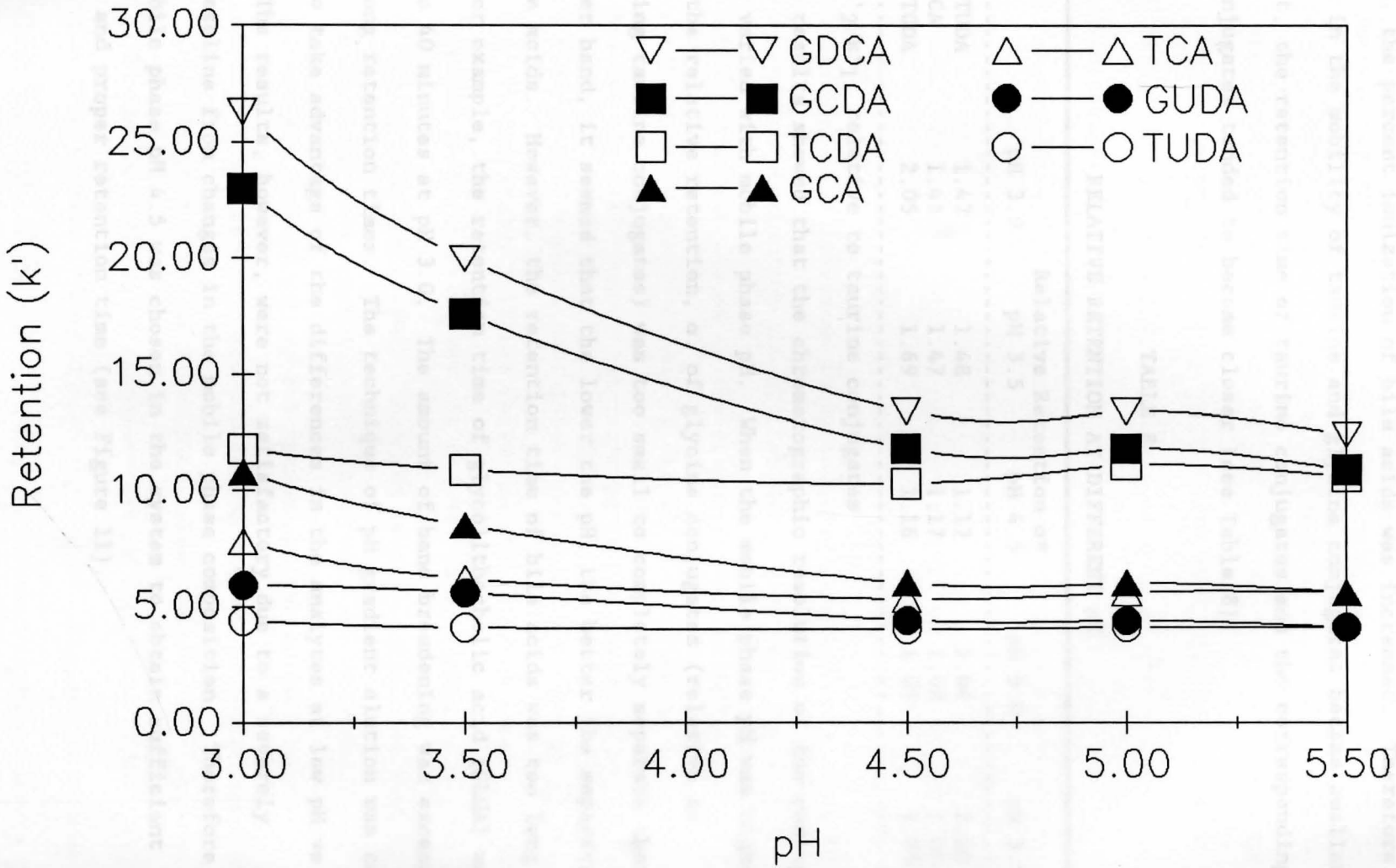

Fig. 12. Variation of bile acid retention with pH.
60% methanol and 40% 0.10 mol/L KH_2PO_4; flow rate, 1.5 mL/min; detection, UV absorbance 254 nm.

pH 3-4) but not glycine conjugates (around pH 8). When the mobile phase pH was raised, the percent ionization of bile acids was increased. Therefore, the difference in the mobility of taurine and glycine conjugates became smaller. As a result, the retention time of taurine conjugates and the corresponding glycine conjugates tended to become closer (see Table 8).

TABLE 8

RELATIVE RETENTION AT DIFFERENT pH

	Relative Retention α*				
	pH 3.0	pH 3.5	pH 4.5	pH 5.0	pH 5.5
GUDA-TUDA	1.47	1.48	1.17	1.06	1.00
GCA-TCA	1.45	1.47	1.17	1.08	1.00
GCDA-TCDA	2.05	1.69	1.16	1.07	1.03

* $\alpha = k'_2/k'_1$, relative to taurine conjugates

These results showed that the chromatographic resolution of the conjugated bile acids varied with mobile phase pH. When the mobile phase pH was higher than 5.0, the relative retention, α, of glycine conjugates (relative to corresponding taurine conjugates) was too small to completely separate them. On the other hand, it seemed that the lower the pH, the better the separation of the bile acids. However, the retention time of bile acids was too long at low pH. For example, the retention time of glycolithocholic acid (GLCA) was longer than 40 minutes at pH 3.0. The amount of band broadening was excessive at these long retention times. The technique of pH gradient elution was tried in order to take advantage of the differences in the analytes at low pH vs. high pH. The results, however, were not satisfactory due to a severely drifting baseline from changes in the mobile phase composition. Therefore, the optimal mobile phase pH 4.5 was chosen in the system to obtain sufficient separation and proper retention time (see Figure 11).

HPLC Analysis of Bile Acids With a Post-Column Enzyme Reactor

A CPG-immobilized choloylglycine hydrolase enzyme reactor and an o-phthalaldehyde derivatization reactor were linked to the HPLC system to improve the specificity and sensitivity of the analysis of bile acids (see Figure 5). The main considerations in this system were the compatibility of the HPLC eluent and the immobilized enzyme, as well as that of the eluent and OPA derivatization.

As mentioned before, the CGH enzyme could only tolerate a concentration of about 20 to 25% methanol. The concentration of methanol in the HPLC eluent, however, was 60%. The diluent at 2 mL/min, therefore, was introduced to the system to lower the methanol concentration to 20% so that the CGH enzyme in the reactor could maintain sufficient activity (without denaturation) to hydrolyze conjugated bile acids eluted from the HPLC column. Moreover, the diluent also acted as a buffer to adjust the pH of the eluent to the optimal pH 5.6 for the CGH enzyme reaction. 2-Mercaptoethanol in the diluent worked as a reducing reagent to prevent the oxidation of thiol groups in the enzyme active site.

The OPA fluorescence reagent containing 0.40 mol/L boric acid, pH 9.7 was initially tried in the system. It was found that the fluorescence yield of the adduct from OPA derivatization was low due to the decreased pH of the OPA reagent after mixing with the eluent. When OPA reagent containing 0.40 mol/L boric acid, pH 11.0 was used, much higher fluorescence was obtained since the OPA derivatization was carried out at its optimal pH region of 9 to 11.

Some authors[18] have reported that OPA reacts with amino acids immediately, but others[16] have observed that the fluorophor formation with OPA required a few minutes. To test the effect of reaction time on fluorescence yield of OPA derivative, the fluorescence intensity was compared by using a reaction coil of 1.5 mL volume and without the coil. Great enhancement in fluorescence yield

was observed when the reaction coil was in place. The results are shown in Figure 13. The reaction coil used was constructed from knitted Teflon tubing, which had a relatively low band broadening effect from secondary-flow phenomena[22,57].

Figure 13 also illustrates the effect of reagent flow rate on fluorescence yield. It seemed that the higher the reagent flow rate, the higher the fluorescence yield. However, the total flow rate of the solute passing through the flow cell had to be considered. Since the flow rates of mobile phase and diluent were 1 mL/min and 2 mL/min, respectively, when 1.5 mL/min of reagent flow rate was used, the sum of solute flow rate was 4.5 mL/min. This was a high flow rate. When the solute at a very high speed passed through the flow cell, it might cause some adverse effects. Therefore, when a good signal to noise (S/2N) ratio (larger than 10) had been attained at 1.5 mL/min, higher reagent flow rates were not tried.

The chromatograms of the separation of a standard mixture of 8 conjugated bile acids using HPLC with immobilized CGH enzyme are given in Figures 14 and 15. They show characteristics similar to those seen in Figure 11. However, apparently, the sensitivity in using CGH enzyme with fluorescence detection was much higher than with UV detection.

Quantitation

In order to demonstrate the applicability of this HPLC method to quantitation, standard curves for glycocholate and taurocholate were determined. 2 μg to 10 μg/mL in methanol of glycocholate and 20 μg to 100 μg/mL in methanol of taurocholate were used for the determination. The injection volumes were 10 μL. Standard curves for peak height versus concentration are presented in Figures 16 and 17. Peak heights were measured from electronic (analog to digital converter) counts from the chromatograms.

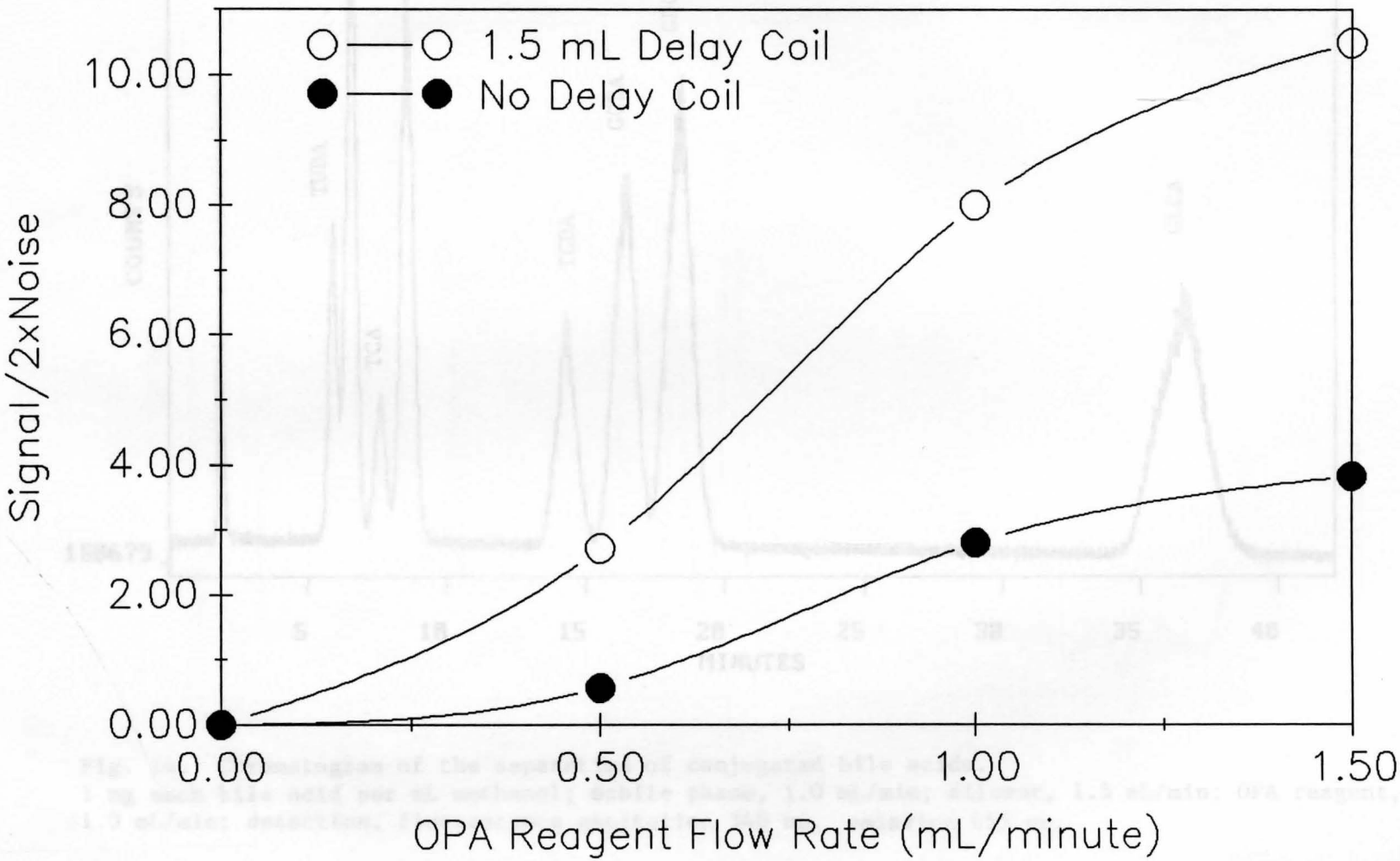

Fig. 13. Effect of reagent flow rate and reaction time on fluorescence yield. Sodium glycocholate substrate; mobile phase, 1.0 mL/min; diluent, 1.5 mL/min.

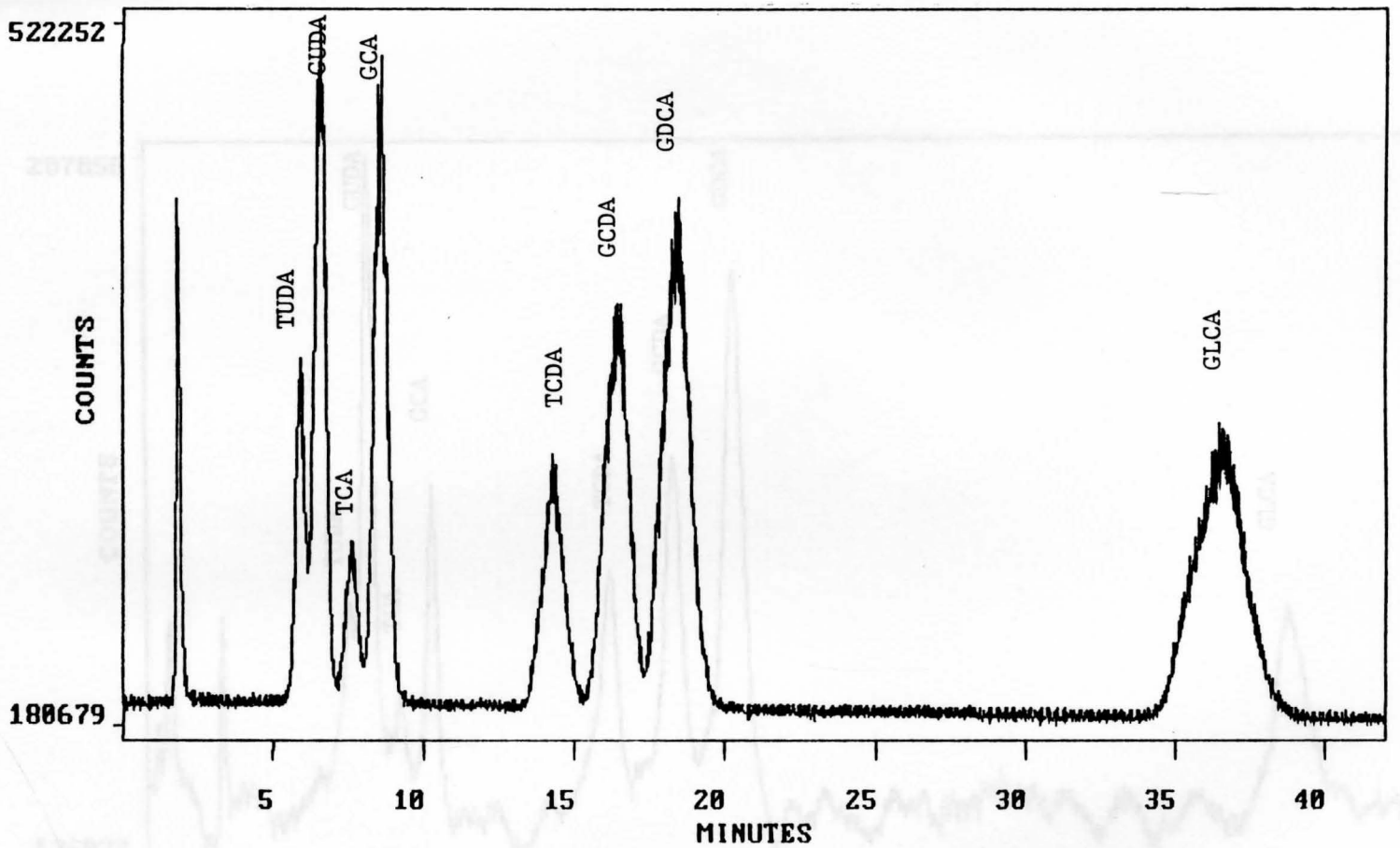

Fig. 14. Chromatogram of the separation of conjugated bile acids.
1 mg each bile acid per mL methanol; mobile phase, 1.0 mL/min; diluent, 1.5 mL/min; OPA reagent, 1.0 mL/min; detection, fluorescence excitation 340 nm, emission 455 nm.

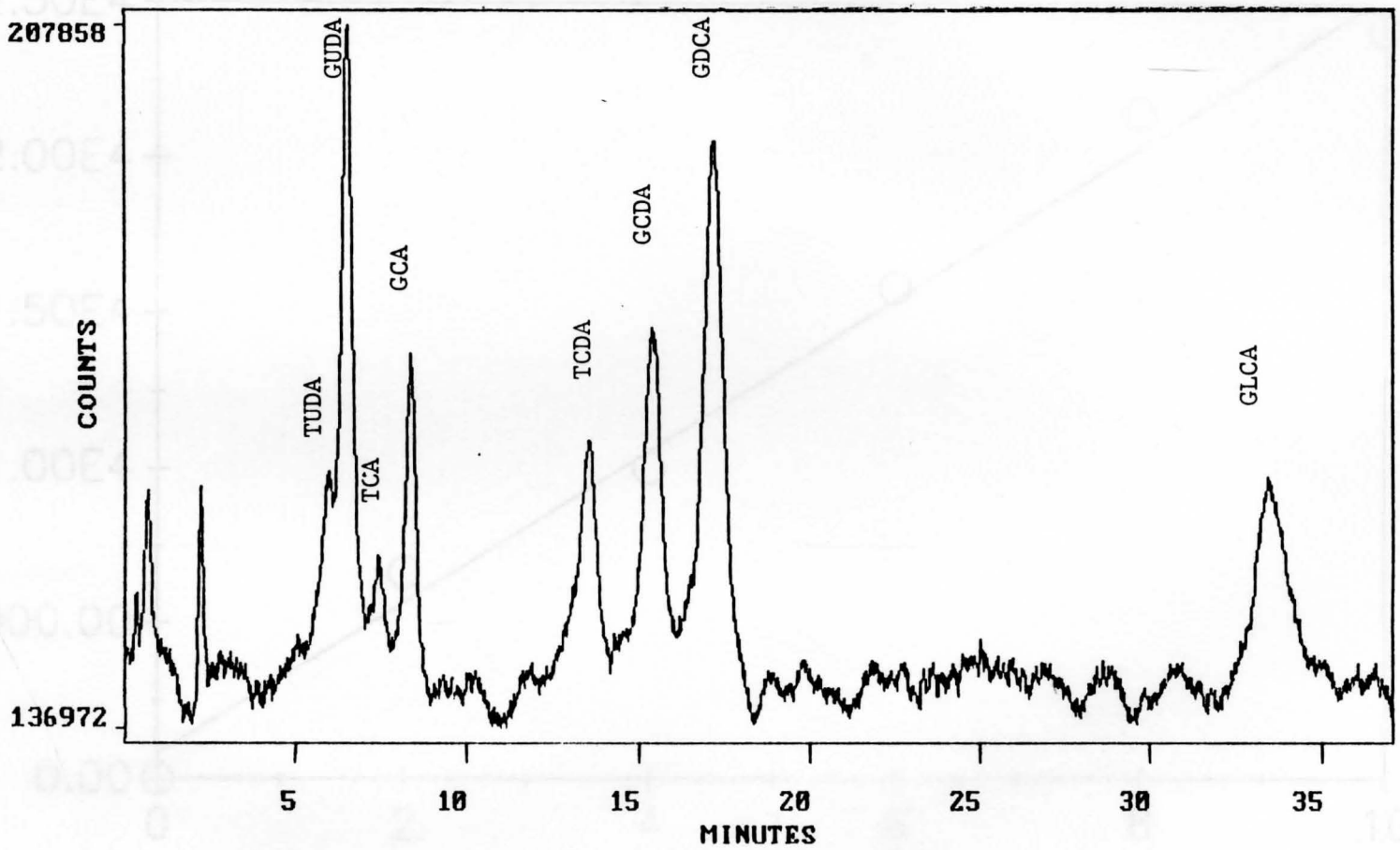

Fig. 15. Chromatogram of the separation of conjugated bile acids.
0.1 mg each bile acid per mL methanol; mobile phase, 1.0 mL/min; diluent, 2.0 mL/min; OPA reagent, 1.5 mL/min; detection, fluorescence excitation 340 nm, emission 455 nm.

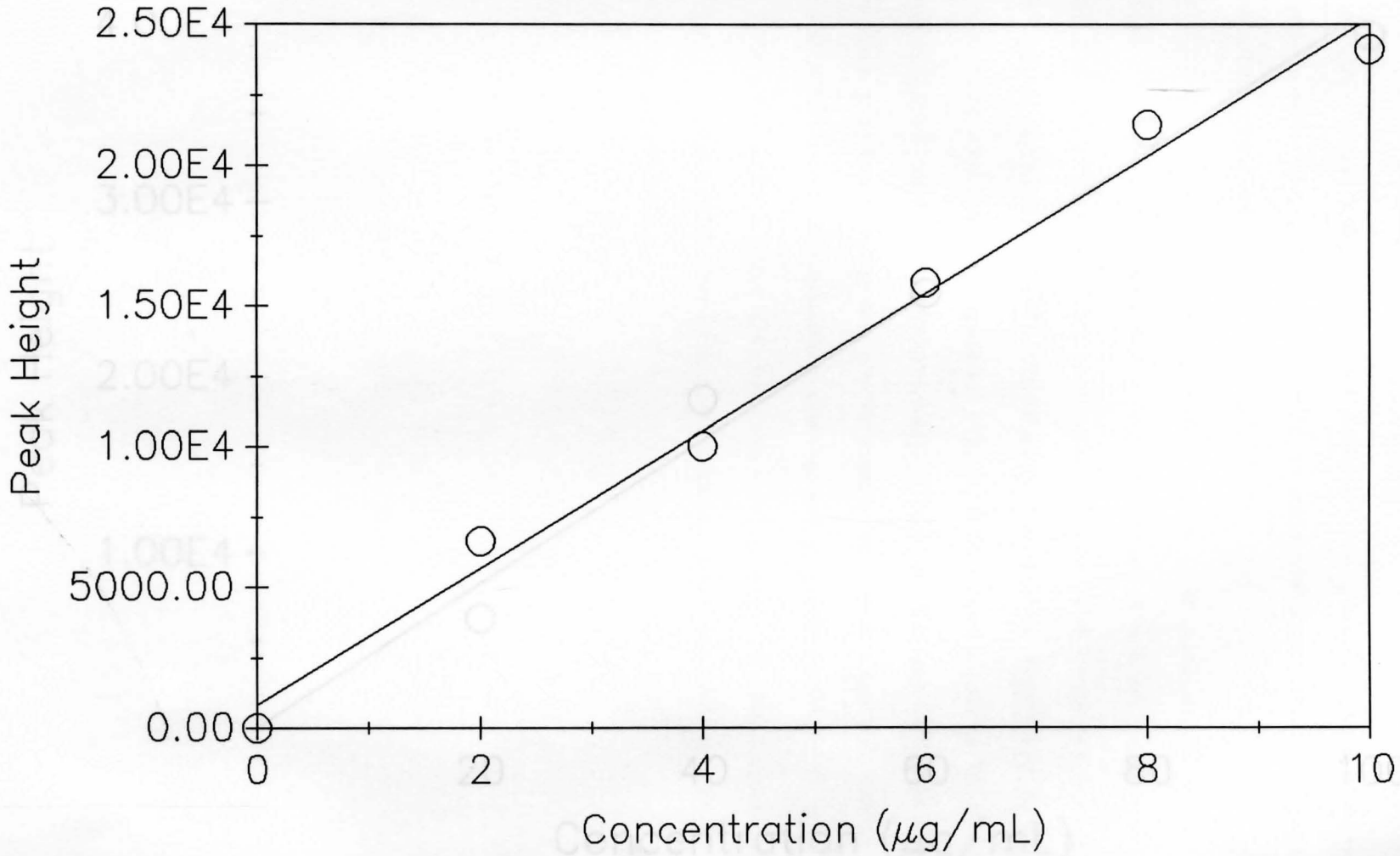

Fig. 16. Glycocholate standard curve.
10 μL injections.

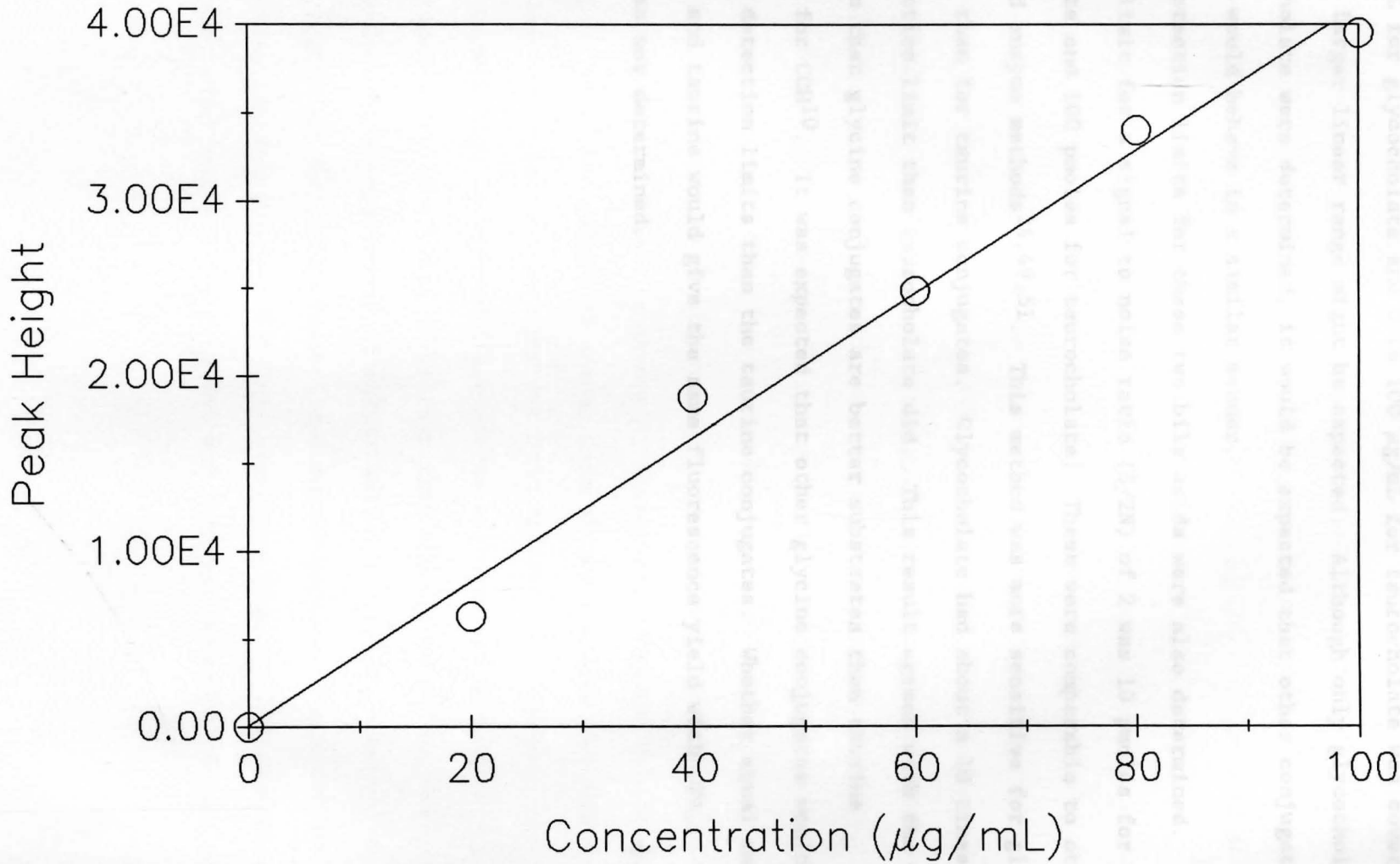

Fig. 17. Taurocholate standard curve.
10 μL injections.

Both standard curves gave a good straight line. The concentration range of 0 to 10 μg/mL for glycocholate and 0 to 100 μg/mL for taurocholate was covered, although a larger linear range might be expected. Although only glycocholate and taurocholate were determined, it would be expected that other conjugated bile acids would behave in a similar manner.

The detection limits for these two bile acids were also determined. The detection limit for a signal to noise ratio (S/2N) of 2 was 10 pmoles for glycocholate and 100 pmoles for taurocholate. These were comparable to other immobilized enzyme methods[36,49,51]. This method was more sensitive for glycine conjugates than for taurine conjugates. Glycocholate had about a 10 times lower detection limit than taurocholate did. This result agrees with the observation that glycine conjugates are better substrates than taurine conjugates for CGH[10]. It was expected that other glycine conjugates would also have lower detection limits than the taurine conjugates. Whether equal moles of glycine and taurine would give the same fluorescence yield with OPA, however, was not determined.

CHAPTER VII

CONCLUSIONS

From this study, the following conclusions may be reached: (1) this method was specific for glycine and taurine conjugates of bile acids. Potential interferences from free amino acids in biological samples were avoided since these were eluted at the chromatographic dead time. (2) The method was sensitive for the analysis of glycine and taurine conjugates of bile acids. Detection limits appear to be comparable to other immobilized enzyme methods and better than UV methods. (3) The work done in this investigation was believed to be the first successful application of analysis of conjugated bile acids using HPLC with immobilized choloylglycine hydrolase. Although more work must be done, such as improvement of the immobilization procedure, optimization of the system, and application of the system to the analysis of biological samples, the separation of the 8 conjugated bile acids serves as a demonstration of the utility of the system. (4) As time passed, the activity of the immobilized CGH tended to gradually disappear. Whether this was due to slow denaturation of the enzyme or actual loss of protein from the column, or was a combination of factors, has not yet been determined. However, the fact that a relatively high background fluorescence was observed seemed to support the suggestion that the elution of protein from the enzyme column was mainly responsible for the disappearance of enzyme activity. (5) This method should prove less expensive than other immobilized enzyme methods. On a per-unit activity basis, CGH was considerably less expensive than the α-hydroxysteroid dehydrogenase. The reagents, such as OPA and buffers, were inexpensive as well.

APPENDIX A

PEROXIDE TESTING

The presence of peroxides in ethylene glycol monomethyl ether can be tested by mixing 1 mL of the solvent with several drops of 10% potassium iodide, KI, and a few drops of 6 mol/L HCl. If yellow color appears, the test is positive and this ethylene glycol monomethyl ether may not be used for preparation of ninhydrin color reagent.

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