# Serum bile acid analysis: a rapid, direct enzymatic method using dual-beam spectrophotofluorimetry

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**Summary** The direct quantitative measurement of total bile acids in serum has been achieved using an enzymatic fluorescent method with a dual-beam spectrophotofluorimeter. By use of a 3α-hydroxysteroid dehydrogenase, oxidation of bile acids with NAD is completed in 200 seconds with the observed NADH fluorescence being proportional to the concentration of serum bile acids. This method is rapid (8 minutes per individual sample), has an intrinsic sensitivity of ±1 μM of total bile acids, requires no sample preparation and less than 0.8 ml of serum. Paired data analysis using enzymatic fluorescence and gas–liquid chromatographic methods gives a correlation coefficient (*r*) of 0.99 for 34 samples ranging from 2 to 530 μM.

**Supplementary key words** steroid dehydrogenase

The clinical need for a routine screening method for the determination of total bile acids in serum was established through studies (1) that demonstrated that 2-hr postprandial bile acid measurements were more sensitive than either the standard BSP test or indocyanine green (ICG) (2) disappearance curves for the detection of liver diseases.

Currently, three mthods are available for the determination of serum bile acids. Gas–liquid chromatography (3) provides analysis of mono-, di-, and trihydroxy bile acids, but several preparatory steps (i.e., deproteinization, solvolysis, hydrolysis, extractions) are needed, and usually 2 days are required to analyze approximately 30 samples. Effectively, the GLC method does not lend itself to a rapid analytical screening assay. The procedure using radioimmunoassay (4, 5) for individual bile acids is very sensitive but, again, a number of analytical steps and several specific antibodies for each class of bile acids are required. Such antibodies are not yet commercially available. Specific fluorimetric methods are available (6, 7) for the determination of bile acids in serum; however, these procedures also require several preparatory steps in order to isolate the bile acids and are therefore laborious and time-consuming.

In the following study, we present a rapid enzymatic fluorescence method for the direct determination of total bile acids in serum. This method uses the NAD–NADH reaction with the enzyme 3α-HSD (3α-hydroxysteroid dehydrogenase), a double-beam fluorescent spectrophotometer to cancel nonspecific fluorescence (8), and a standard addition procedure of analysis to compensate for nonspecific serum fluorescence and quenching. The sensitivity of this method for serum samples is ±1.0 μM and each determination requires approximately 8 min and less than 0.8 ml of serum. When identical serum samples were analyzed using GLC and this new method, a linear correlation coefficient of 0.99 was obtained in the range of bile acids from 2 to 530 μM. The values from the enzymatic fluorescence method agreed on the average within 12% with GLC values from 10 to 530 μM.

## Material and methods

*Reagents.* Hydroxysteroid dehydrogenase was obtained from Worthington Biochemicals Corp., Freehold, NJ. The 3α-HSD (1.1.1.50) was supplied as purified powder obtained from adapted cells of mutant *Pseudomonas testosteroni* with activities from 0.5 to 0.8 units per mg. Enzyme solution with an activity of 5 units per ml (1 unit = 1 μmol NADH per min) was prepared with cold Tris-HCL buffer, pH 7.2, containing 1 mM EDTA. The enzyme solution was centrifuged at 40,000 rpm for 60 min, kept in an ice bath when in use, and stored frozen. The solution was stable for at least 1 week. The enzyme powder was stored in a desiccator at −20°C.

β-Nicotinamide adenine dinucleotide (β-$NAD^+$), approximately 98% pure, was obtained from Sigma Chemical Company, St. Louis, MO, and was stored in desiccator at −20°C. A 0.010 M solution was prepared by dissolving 6.33 mg of β-$NAD^+$ per ml of water; this solution, when stored frozen, is stable for at least 2 weeks. The reduced form (β-NADH) was also obtained from Sigma Chemical Company in preweighed, sealed vials. Stock solutions buffered with glycine–hydrazine, pH 9.4, were prepared daily and used for fluorescent standards.

Glycine buffer, 1 M at pH 9.4, containing hydrazine sulfate (0.4 M) and EDTA (5 mM), was prepared using 75 g of glycine, 52 g of hydrazine sulfate, and 2 g of EDTA dissolved in 500 ml of

Abbreviations: GLC, gas—liquid chromatography; 3αHSD, 3α-hydroxysteroid dehydrogenase; SAM, standard additions method.

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water. The pH was adjusted to 9.4 by adding approximately 200 ml of 5 N NaOH; the solution was then diluted to 1 l and refrigerated.

Bile acids were purchased from Supelco, Inc., Bellefonte, PA. Six stock standards of conjugated bile acids ($1 \times 10^{-3}$M) (glycine and taurine conjugates of cholic, chenodeoxycholic, and deoxycholic acid) were prepared using distilled water. Stock solutions of unconjugated bile acids ($5 \times 10^{-4}$M) were prepared in glycine buffer. The bile acid standards (60 or 600 $\mu$M), used for the standard addition method, were equimolar concentration mixtures of six conjugated bile acids and were prepared by appropriate dilution of the stock solutions with buffer. Stock solution was added to normal plasma to give a final concentration ranging from 15 to 150 $\mu$M of added bile acid. Analysis by both GLC and enzymatic methods gave a coefficient of variation of 10% for the added bile acids corrected for the amount of endogenous bile acid in plasma.

After standardization of the procedure, sera from individuals with and without known liver disease were analyzed by GLC and by the enzymatic method for determination of accuracy and precision.

### Instrument

A Hitachi Perkin-Elmer Model 512 double-beam fluorescence spectrophotometer with a 150 watt xenon lamp was used. All measurements were performed using standard rectangular cells of 1 cm path length, made of fluorescence-free fused silica.

The instrument was modified as follows: (*1*) the R446 (red sensitive multi-alkali photocathode) phototube was replaced by an R212 (S-5) phototube (Hamamutsu Corp., Middlesex, NJ) in order to obtain greater quantum efficiency at 460 nm and (*2*) a constant temperature of 21.5 ± 0.5°C in the sample and reference cells was maintained by using a water bath (Haake Inc., Saddle Brook, NJ, Model FK).

The observed fluorescence can be assumed to be proportional to: F $\alpha$ (I) (e) (C) (L) (QY) ($\Omega$) (d), where (I) is the excitation intensity in photons/cm$^2$ per sec, (e) the extinction coefficient for excitation wavelength in $M^{-1}cm^{-1}$, and (C), the concentration of the fluorescent compound. (L) is the cell path length in cm, (QY) the fluorescent yields, ($\Omega$) the solid angle of detection system of the monochromator, and (d) the quantum efficiency of the phototube. For a concentration of NADH of 1 nM, $e = 6.3 \times 10^3$; L = 1 cm; QY = 0.10; $\Omega = 0.007$; d = 0.2; the fluorescence will thus be proportional to $8.8 \times 10^{-10}$I where (I) is the intensity of the exciting source. For a xenon lamp at 350 nm, $I = 10^{14}$ photons/sec per nM. For a 1% detection accuracy ($10^4$ photons), this fluorescent yield would correspond to approximately 0.1 nM. Since the minimum sensitivity observed was $5 \times 10^{-9}$M, this instrument is very close to the expected limit considering the variability in parameters.

The following instrumental settings were used for analysis: subtract mode; dynode voltage = 750 volts, excitation wavelength = 350 nM; slit width = 20 nm; emission wavelength = 460 nm with a slit width of 20 nm; reference attenuation knob, fully clockwise; medium filter; and a recorder speed of 0.1 cm/sec on the 10 mV scale. The sensitivity scaling factor used to measure fluorescence intensity was usually at the X1 position.

### Procedure

Serum is diluted tenfold using glycine buffer, pH 9.4, and 2.0-ml aliquots are placed into both reference and sample cuvettes. Then enzyme solution (0.1 ml) is injected into the sample cell and the recorder is balanced to zero. The reaction is initiated by injecting 0.10 ml of $\beta$-NAD$^+$ (0.01 M) into each cell, and the net change in fluorescence ($F_X$) is recorded at 200 seconds. Next 25 $\mu$l of the standard mixture of bile acids is added to the sample cell and the fluorescence ($F_1$) is recorded at 200 sec. If $F_X$ is less than 12 fluorescence units, 60 $\mu$M of standard bile acids is used; however, if $F_X$ is greater than 12 fluorescence units, 600 $\mu$M is used. If the ratio $F_1/F_X$ is greater than 2.5, the accuracy of the standard addition method is reduced (>8%), and the run should be repeated using either a different serum dilution or a lower concentration of known bile acids. Cells are emptied by aspiration and washed with water. This procedure is repeated for each analysis. The unknown concentration of bile acids is calculated by a graphic method or by solving the following equation

$$C_X = (DF)(C'_s)(F_X)/(F_1 - F_X) \qquad \text{Eq. 1}$$

where DF is the dilution factor corrected for reagent's volume, and $C'_s$ is the final concentration of the added standard in the reaction mixture.

### Results and discussion

The enzymatic fluorometric technique was developed in two stages. The goal of the first stage was to establish and optimize the quantitative aspects of the enzymatic reaction in known aqueous bile acid solutions with respect to both sensitivity and precision in the presence and absence of compounds that could affect fluorescence. The second step was the applicability of the newly developed enzymatic procedure to the determination of bile acids directly in serum.

*A) Studies of bile acids–NAD$^+$–HSD reaction aqueous solution chemical system.* The analytical method for the determination of bile acids is based on the following reaction

$$3\alpha\text{-hydroxy bile acid} + \beta\text{-NAD}^+ \xrightarrow[\text{pH } 9.4]{3\alpha - \text{HSD}} \text{oxo–bile acid} + \text{NADH} \quad \text{Eq. 2}$$

First, we investigated the instrumental parameters required for maximum detection sensitivity of pure NADH in pH 9.4 buffer, using the dual-beam fluorescence spectrophotometer modified for high sensitivity. The optimum wavelengths were 350 nm for excitation and 460 nm for emission. A linear fluorescence response was observed over a concentration range from 0.064 to 32 $\mu$M for pure NADH. The limit of detection for NADH was determined to be $5 \times 10^{-9}$M, which is close to the theoretical predicted value. This observed sensitivity is approximately 1000 times greater than that required for clinical estimation of normal serum bile acid concentration (6).

*Analytic parameters of reaction (Eq. 2).* From a study of the reaction kinetics of bile acids at concentrations up to 70 $\mu$M with NAD$^+$ and HSD, the reaction went to completion in less than 200 sec. The reaction could be initiated by adding either NAD or 3$\alpha$-HSD since the measured fluorescence is the same in each case. Maintenance of constant temperature stabilization in the cells is crucial to the measurements since the fluorescence intensity of NADH increases by about 2% per degree as the temperature decreases. A constant temperature of 21.5°C was chosen as the best compromise between sensitivity and convenience of sample handling.

Since the fluorescence yields from equimolar concentrations of pure NADH and bile acids at all ranges studied were observed to be the same within experimental error, we concluded that the reaction produces an equimolar amount of NADH for each mole of bile acid present. Fluorescence was not observed when the following combinations of reagents were tested: bile acids + HSD or buffer + HSD. However, NAD alone or together with either buffer, or bile acids, or HSD gave a fluorescence equivalent to 0.2–1.0 $\mu$M NADH, which can be attributed to the 0.1% NADH impurity in the commercial NAD compound. These fluorescence contributions are subtracted by balancing the spectrophotometer before the reaction is initiated.

**Table 1** shows the results of typical runs using standard bile acids in aqueous solutions. A mean percent difference of 3.5% was observed over a bile acid concentration range from 6.0 to 60.0 $\mu$M. The calibration curve comparing fluorescence to bile acid concentration had a correlation coefficient of 0.999. Six replicate determinations were made at concentrations of 12.0 and 30.0 $\mu$M. The coefficient of variation for each concentration was 0.8%.

*B) Studies of bile acids–NAD–HSD reaction in serum: characteristics of reaction.* After determining the conditions for the measurement of bile acids in aqueous media, we examined the applicability of this procedure to the analysis of bile acids in serum. By studying the reactions, serum + $\beta$-NAD$^+$ and serum + HSD against serum in the reference cell, we noted that the serum + $\beta$-NAD$^+$ mixture (blank reaction) gave a slow reaction that did not go to completion in 30 min. By injecting $\beta$-NAD$^+$ into each cell, balancing the spectrometer, and then adding HSD to the sample cell, the bile acid oxidation reaction went to completion in less than 200 sec, with compensation for the non-HSD generation of NADH.

Experiments also showed that a 5-fold dilution of serum in buffer gave appreciably less NADH fluorescence than expected. Studies on forty human sera, diluted 5-fold, were observed to have a quenching effect of approximately 25–75% attributable to bilirubin. Although bilirubin has no background fluorescence, a quenching effect does occur, as shown in **Fig. 1.** A solution of 1 mg/dl of bilirubin absorbs 0.3 absorption units (A.U.) at 340 nm and 1.0 A.U. at 453 nm. Bilirubin is thus capable of quenching both the excitation and emission photons in the NAD–NADH reaction. In studies of the effect of albumin concentration on the fluorescence of the NAD–NADH reaction in buffer solution, it was found that addition of albumin up to a final concentration of 1% did not change fluorescence. If a 5-fold dilution of serum is used, the albumin concentration is less than 1%. Therefore, significant quenching attributable to albumin does not occur.

As a consequence of these findings, it was decided to use routinely a 10-fold dilution of serum to reduce quenching attributable to bilirubin in patients with liver disease.

Background fluorescence of 40 serum samples, diluted 5-fold with buffer, varied between values equivalent to 10–70 $\mu$M NADH. When NADH was added to these sera, the increase in fluorescence was always a linear function of the concentration up to 120 fluorescence units. However, the slope of the line varied from serum to serum depending on the quenching factors in the individual sera, which were not entirely attributable to bilirubin concentration. By use of the subtract mode, it was possible to compensate for the initial fluorescence of the serum but an internal standardization technique was found the most practical and accurate approach to

TABLE 1. Recovery of bile acids added to glycine buffer using enzymatic method and dual-beam spectrophotofluorimetry

| Added | Observed[a] | % Difference |
|---|---|---|
| $\mu M$ | $\mu M$ | |
| 60.0 | 59.84 | −0.27 |
| 42.0 | 41.56 | −1.05 |
| 30.0 | 30.51 | 1.70 |
| 18.0 | 18.97 | 5.38 |
| 12.0 | 11.74 | −2.14 |
| 6.0 | 5.37 | −10.5 |

[a] Means of two determinations, standard mixture of conjugated bile acids, see text.

the intrinsic variations in serum quenching of fluorescence.

*Standard addition method.* Because the fluorescence intensity of the enzymatic reaction with serum depends not only on the bile acid concentration but also on the quenching factor inherent in each individual serum, calibration curves of bile acids in buffer solution or in a pooled serum could not be used to calculate the value of the unknown concentration of bile acids in serum. Therefore, it was decided that an addition of measured volumes of known concentrations of standard bile acid solutions was necessary for the accurate determination of bile acids.

In the standard addition method (SAM), small volumes of standard solutions of bile acids are added to the unknown sample and the fluorescence of the mixture is measured using the double-beam fluorescence spectrophotometer. It is assumed that the added mixture of known bile acids reacts in the same fashion as the unknown bile acids in the sample and therefore that the change in fluorescence is a linear function of concentration.

Initially, the SAM was applied to three different aliquots of the serum sample. One aliquot was used to measure the unknown fluorescence, and different additions of known bile acids to the other two aliquots were made. The results for the enzymatic fluorescence method, calculated by least squares method, were compared to those from GLC. A correlation coefficient of 0.998 was observed for 14 samples from 1.2 to over 500 $\mu$M. Above 10 $\mu$M there was an average agreement of 12% with GLC values, and below 10 $\mu$M, the average agreement was 50%. Repeated GLC analysis of bile acid standards below 10 $\mu$M indicated considerable variation in duplicate analyses. Consequently, suitable standards for evaluation of the enzymatic method are not available at a bile acid concentration of less than 10 $\mu$M. Since fluorescence changes of 0.5 $\mu$M of bile acids can be accurately and reproducibly detected by our method using bile acid standards added to serum,

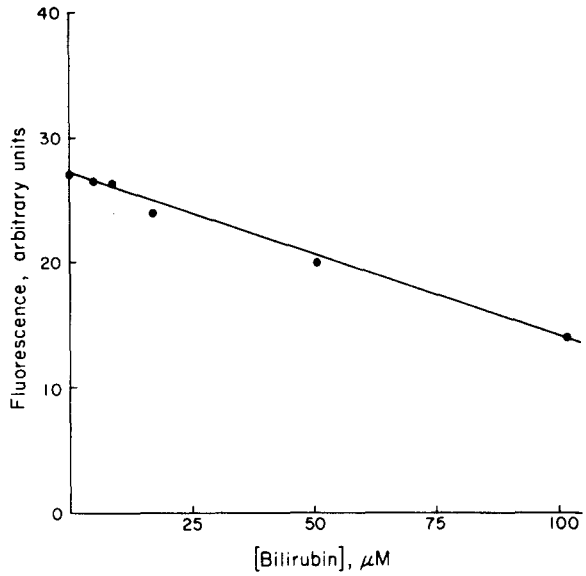


**Fig. 1.** Effect of increasing concentration of bilirubin on the fluorescence of 10 $\mu$M NADH in 1 g% human serum albumin solution.

we conclude that the fluorescence method in individual portions of serum has an accuracy of at least 12% over the concentration range of 1–530 $\mu$M.

There are some disadvantages of the SAM with multiple portions of serum, since large serum volumes are required (2.4 ml of serum) and total analysis time for duplicate samples is approximately 20 min. In order to save sample volume, time of analysis, and costs of reagents, we investigated the use of the SAM in the same sample portion of serum. The feasibility of this approach is based on the fact that the reaction goes to completion in 20–200 sec after

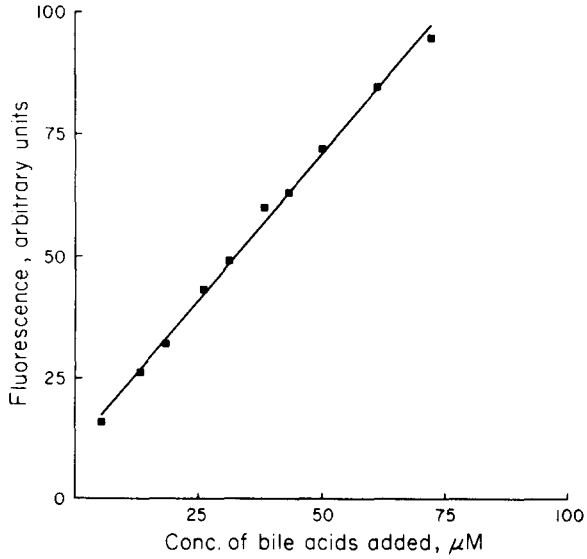


**Fig. 2.** Fluorescence following serial addition of bile acids to the same cell.

TABLE 2. Recovery of bile acids added to normal serum using enzymatic method and dual-beam spectrophotofluorimetry[a]

| Added | Observed[b] | % Difference |
|---|---|---|
| $\mu M$ | $\mu M$ | |
| 72.0 | 69.66 | −3.25 |
| 61.2 | 61.38 | −0.29 |
| 50.0 | 50.78 | 1.56 |
| 43.4 | 43.32 | −0.17 |
| 38.3 | 40.84 | 6.63 |
| 31.3 | 31.73 | 1.37 |
| 26.1 | 27.01 | 3.48 |
| 18.6 | 17.65 | −5.11 |
| 13.3 | 12.93 | −2.78 |
| 5.5 | 4.40 | −20.0 |

[a] Analysis done by adding standard bile acids in tenfold diluted serum in buffer.
[b] Means of two determinations.

each successive addition, since a large excess of NAD and enzyme is present.

We observed, as in **Fig. 2**, that fluorescence was a linear function of concentration with a variation of less than 5% for multiple additions of known bile acids to the same cuvette. (**Table 2**).

The unknown serum concentration was determined by three methods. The first involved solving Equation 3 by a least squares method for the unknown concentration with the observed fluorescent value for multiple additions of known volumes of bile acid. The second method, Equation 4, used two standard additions of known bile acids where the total volume of additions was small compared to the volume of unknown plus reagents. The third method involved only one standard addition using Equation 5.

$$F_i = \alpha(C_x'V_u + C_sV_i)/(V_u + V_i) \quad \text{Eq. 3}$$

$$F_i = \alpha C_x' + \alpha C_sV_i/V_u \quad \text{Eq. 4}$$

$$C_x' = (C_s')(F_s)/(F_i - F_x) \quad \text{Eq. 5}$$

$$C_x = DFC_x' \quad \text{Eq. 6}$$

$F_i$ is the measured fluorescence after the $i^{th}$ addition of known bile acids of concentrations $C_s$. $V_u$ is the volume of reagents plus the volume of diluted serum of concentration $C_x'$, and $\alpha$ is the constant of proportionality between observed fluorescence and concentration. $C_s'$ is the final concentration of added standard in the reacting mixture, and C is the unknown concentration in serum.

By Eq. 3, the results of 15 samples greater than 15 $\mu$M showed an agreement of 18% with GLC values. If volume dilution errors, (Eq. 4) approximately 8%, are neglected, an agreement with GLC values of 12% was obtained. For one addition, Eq. 6, with no volume dilution error, an agreement of the same 15 samples with GLC values was 14%.

For samples of serum less than 10 $\mu$M, we observed that the unknown concentration determined by Eq. 3 was significantly different (25%) from the values calculated by Eqs. 4 and 5, yet the fit of the observed fluorescence of the standard additions to Eq. 3 was linear to better than 5%. Since the SAM using separate portions of unknown serum maintained linearity over the concentration range from 1 to 530 $\mu$M, we believe that, at small concentrations of bile acids (less than 10 $\mu$M), the use of multiple additions in the same cuvette is slightly inaccurate because of nonlinear quenching effects or because of other competitive reactions occurring in serum. However, if only one addition is made and Eq. 5 is used for the analysis, this nonlinear effect is minimized, and the same accuracy, 12% for SAM, with the same or different portions of serum is obtained.

In **Fig. 3**, a typical recording of observed reaction curves is shown using the standard addition method in the same sample cuvette.

A comparison of enzymatic fluorescence results with GLC is shown in **Fig. 4** where a correlation coefficient of 0.995 is obtained for sample concentrations from 10 to 350 $\mu$M with an average error of 12% for any observation. The agreement with GLC for 11 measurements less than 10 $\mu$M (**Table 3**) varied by 38% but this is because of the lack of precision and sensitivity of GLC in this concentration range. The en-

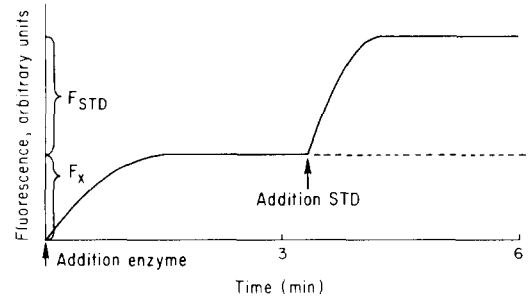


**Fig. 3.** Typical recording of reaction curves for total serum bile acids using the standard addition method.

TABLE 3. Serum bile acid analysis. Comparison of GLC and enzymatic method using dual-beam spectrophotofluorimetry

| Concentration Range | Number of Analyses | GLC (Mean) | Enzymatic[a] (Mean) | % Difference (Mean) |
|---|---|---|---|---|
| $\mu M$ | | $\mu M$ | $\mu M$ | |
| 1.2– 9.3 | 11 | 6.3 | 7.3[b] | 38 |
| 12.3– 89 | 12 | 39 | 46 | 15 |
| 128 –530 | 11 | 231 | 244 | 5.5 |

[a] For the purpose of this study the actual GLC concentration was used although the method (ref. 3) loses precision below 6 $\mu$M, a value well within the normal range of 6 ± 3 $\mu$M.
[b] Reproducibility of the enzymatic method is 12%.

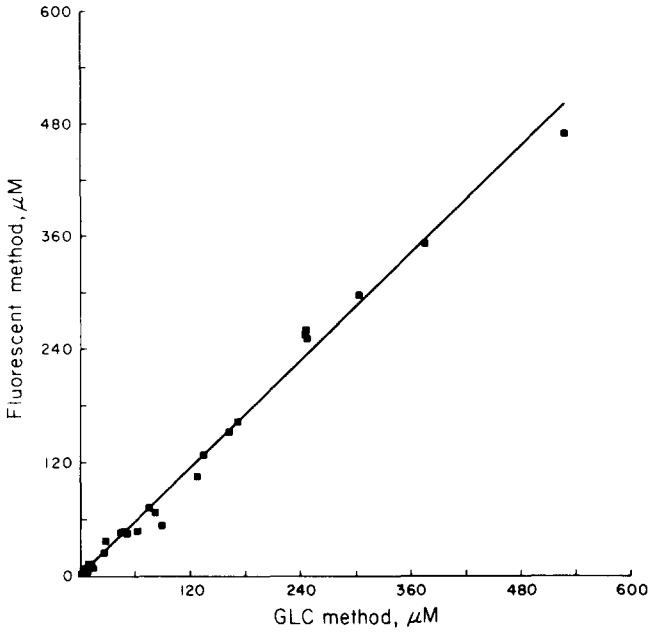


**Fig. 4.** Calculated regression line between enzymatic and GLC methods for serum bile acid analysis. For 34 samples, a correlation coefficient ($r$) of 0.995 was obtained.

zymatic fluorescence method is expected to differ slightly from GLC results for values greater than 10 $\mu$M because some of the serum bile acids may be esterified at the 3$\alpha$-hydroxyl group with sulfuric or glucuronic acids and therefore not be accessible to the enzyme.

In patients with liver disease, esterified bile acids in serum have not been reported to exceed 10% (9). On the other hand, 3$\alpha$-hydroxysteroid hormones will react with 3$\alpha$-HSD and thus tend to give higher apparent bile acid values. Only a fraction of these steroids, less than 10%, is normally unesterified.

A present practical limitation of the proposed method is the restricted range of the spectrophotometer in the background subtraction mode for nonspecific fluorescence. Linearity of fluorescence with concentration is maintained to approximately 120 fluorescence units including that for background fluorescence. This restriction makes it necessary to repeat samples that normally have a high bile acid concentration since the appropriate dilution may not be performed. Also, for a few samples at low concentrations of bile acids, a background fluorescence of up to 80 f.u. may be observed so that the limit of linearity of 120 f.u. may be exceeded, requiring another run at a different dilution.

Currently, only total bile acids are determined. The normal range is 6 ± 3 $\mu$M, a value that compares closely to that reported (6 ± 2) (6) using a column purification step prior to hydroxysteroid dehydrogenase assay. However, there are promising approaches for differentiating the individual classes of bile acids. The use of 7$\alpha$-HSD, and 12$\alpha$-HSD together with the 3$\alpha$-HSD enzyme would allow the direct measurement of the three major classes of bile acids. This would require three measurements in the same sample.

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