Chapter-3

MATERIALS AND METHODS
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3.1 SOURCE OF CHEMICALS AND BIOCHEMICALS

Zirconia coated alkylamine and arylamine glass beads (Pore diameter 55nm) were gift from Prof. H.H. Weetall, Environment Protection Group, Las Vegas, USA. 3α-hydroxysteroid dehydrogenase (3α-HSD) from Pseudomonas testosteroni (10U/0.72mg), taurodeoxycholic acid, glutaraldehyde (25%) were purchased from Sigma Chemical Co. St. Louis, U.S.A. Nitrobluetetrazolium (NBT) chloride salt, NADH, NAD⁺, Triton X-100 and diaphorase from Clostridium spp. (33U/1mg) were purchased from SISCO Research Laboratory Pvt. Ltd., Mumbai. All other chemicals were of analytical reagent grade.

3.2 INSTRUMENTATION AND EQUIPMENT USED

The following instruments and laboratory items were used in the present study:

Spectronic-20 (Milton and Roy Co. USA), temperature controlled water bath shaker (N.S.W. New Delhi), digital pH meter (335 D, Systronics, Ahmedabad), deep freezer (Westfrost, Denmark), adjustable volume pipette (1000 μL) (Tarson, Kolkata), refrigerated centrifuge (Remi C-24) and
electronic balance (Dhona 100 DS, Kolkata). All the glassware used in the study were from M/s Borosil Glass Works Ltd., Mumbai.

3.3 ASSAY OF FREE 3α–HYDROXY STEROID DEHYDROGENASE

The assay of free/native 3α–hydroxy steroid dehydrogenase was carried out as described by Staver et al (1985) with modification.

3.3.1 Principle:

It is based on two steps. In the first step, 3α–hydroxysteroid dehydrogenase catalyses the oxidation of 3α–hydroxy bile acid by transferring its H⁺ to NAD⁺ to form 3-keto bile acid and reduced NAD⁺. In the second step reduced NAD⁺ reacts with NBT (chromogen) catalyzed by diaphorase to form formazan (chromophore) which absorb at 540nm.

i) 3α – Hydroxy bile acid + NAD⁺ ——■ ——> 3 – Keto bile acid + NADH+H⁺

ii) NADH +H⁺ + Chromogen (NBT) ———Diaphorase——> Chromophore(formazan)+NAD⁺

λ max = 540nm

3.3.2 Preparation of colour reagent

Solid diaphorase (20U), 0.15g NAD⁺ and 0.05g nitrobluetetrazolium chloride (NBT) were dissolved in 40 ml 0.065M sodium phosphate buffer pH 7.0, its pH was adjusted to pH 7.0 and final volume was made upto 90 ml with distilled water. Solution of colour reagent was half divided into two separate bottles and thus, 45ml of the solution was used for preparation of
blank. The stock solution was transferred into two bottles, 45 ml in each. It was stored at 4°C and prepared fresh everyday.

3.3.3 Preparation of bile acid solution

Bile acid solution (200 μmol/L) was prepared by dissolving 104.34 mg commercially available taurodeoxycholic acid in 1 litre of bovine serum.

3.3.4 Preparation of stop solution

Conc. HCl (38%) (8.3 ml) and 7.5 g Triton X-100 were added to approximately 50 ml distilled water and the final volume was made up to 100 ml with distilled water.

3.3.5 Assay of 3α-hydroxysteroid dehydrogenase

3α-Hydroxysteroid dehydrogenase enzyme powder (0.42 mg) was dissolved in 1 ml 0.1 M sodium carbonate bicarbonate buffer, pH 9.5. The assay was carried out in a 25 ml conical flask wrapped with black paper. The reaction mixture contained 0.1 ml dissolved enzyme and 0.5 ml of color reagent. The reaction was started by adding 0.2 ml bile acid solution. After incubation at 25°C for 15 min, 0.5 ml stop solution was added to the stop the reaction. The blank was prepared as described for test except the omission of diaphorase from colour reagent (0.075 g NAD⁺ and 0.025 gm nitroblueetetrazolium chloride dissolved in 20 ml of 0.065 M sodium phosphate buffer (pH 7.0). Colourless to blue colour was noted in test
solution and $A_{540}$ of reaction mixture was read in Spectronic-20 against the blank. The amount of NADH generated in the reaction was extrapolated from standard curve of NADH between NADH concentration and $A_{540}$ (Fig.2).

3.3.6 Unit of $3\alpha$-hydroxysteroid dehydrogenase

One unit of $3\alpha$-hydroxysteroid dehydrogenase is defined as the amount of enzyme, required to generate 1 μmole of NADH/min under the standard assay conditions.

3.3.7 Preparation of standard curve of NADH

The different dilutions of NADH containing its 0.4, 0.8, 1.2, 1.6 and 2 μmol/L sodium phosphate buffer, pH 7.0 were prepared in dark. 0.3 ml of these dilutions were taken in 15ml test tubes wrapped with black paper. 0.5ml colour reagent was added to each tube. After incubation at 25°C for 15 min, 0.5ml stop solution was added and $A_{540}$ was read. A standard curve was made by plotting NADH concentration vs. $A_{540}$ (Fig. 2).

3.4 DETERMINATION OF PROTEIN

The protein content in various enzyme preparations was determined by the method of Lowry et al (1951).
Fig 2: Standard curve of NADH prepared in 0.065M Sodium phosphate buffer, pH 7.0.
3.4.1 Preparation of reagents

The following reagents were used in the method:

Reagent A: 2% sodium carbonate in 0.1N sodium hydroxide

Reagent B<sub>1</sub>: 1% copper sulphate in distilled water. It was stored at 4°C.

Reagent B<sub>2</sub>: 2% sodium potassium tartarate. It was stored at 4°C.

Reagent B: B<sub>1</sub>+B<sub>2</sub> in 1:1 ratio. It was prepared fresh just before use.

Reagent C: 50 ml of reagent A+1 ml of reagent B. It was also prepared fresh before use.

Reagent D: 1 part of Folin-Ciocalteau reagent (2N) + 1 part of distilled water.

To prepare 2N Folin-Ciocalteau reagent (FC reagent), a mixture containing 25 mg sodium molybdate (Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O), 100 mg sodium tungstate (Na<sub>2</sub>WO<sub>4</sub>.2H<sub>2</sub>O), 700 ml distilled water, 50 ml of 85% orthophosphoric acid and 100 ml concentrated HCl in 1.5 litre flask was refluxed for 10 hr. After cooling, 150g lithium sulphate, 50 ml distilled water and a few drops of bromine water were added. The mixture was boiled for 15 min without condenser to remove excess of bromine. It was cooled and diluted to 1 litre with distilled water and finally filtered. It was stored at room temperature (30±5°C).
3.4.2 Procedure

The stock solution of bovine serum albumin (Fraction V powder) (BSA) (1 mg/ml) was prepared in distilled water. Different solution of BSA containing 20 µg to 200µg BSA were prepared in a series of test tubes in a total volume of 0.5 ml, 5.0 ml reagent C was added to each tube, mixed well and allowed to stand for 10 min at room temperature. 0.5 ml reagent D was added and mixed immediately with vortex mixer. The mixture was allowed to stand at room temperature (30±5°C) for 30 min. The colour intensity was measured in Spectronic-20 at 750 nm. A standard curve for protein was prepared by plotting amount of BSA vs A₇₅₀ (Fig. 3). Appropriate aliquots of unknown samples were drawn, made up to 0.5 ml, with distilled water and analyzed for protein using the same procedure. The blank contained 0.5 ml distilled water. The colour intensity was measured at 750 nm against blank in a Spectronic-20 and the amount of protein was extrapolated from the standard curve of BSA vs A₇₅₀ (Fig. 3).

3.5 IMMOBILIZATION OF 3α–HYDROXYSTEROID DEHYDROGENASE ON GLASS BEADS

3.5.1 On alkylamine glass beads:

The 3α–hydroxysteroid dehydrogenase was immobilized onto alkylamine glass beads (pore diameter 55nm) through glutaraldehyde
Fig. 3: Standard curve of protein (Bovine serum albumin) using Lowry method.
coupling by the method of Lynn (1975). The reactions involved in the immobilization of enzyme onto alkylamine glass beads are given in Fig. 4.

3.5.1.1 Activation of alkylamine glass beads:

Alkylamine glass beads (pore diameter 55 nm) (100 mg) were activated in a 15ml conical flask by adding 1.0 ml 2.5% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.0) (v/v) and kept for 2 hr at room temperature (30±2°C) with occasional stirring. The excess of glutaraldehyde was decanted, followed by many washing of the beads with 0.05M sodium phosphate buffer (pH 7.0) until the pH of the washing discard was 7.0.

3.5.1.2 Immobilization of 3α-hydroxysteroid dehydrogenase on activated alkylamine glass beads

The solution of 3α-hydroxysteroid dehydrogenase (2.0ml) (0.21 mg/ml) was added to the activated beads in a 15 ml conical flask and kept for 48 hr at 4°C with occasional stirring for coupling. The unbound enzyme was decanted and tested for activity and protein. The beads were washed with 1.0 ml reaction buffer (0.1M sodium carbonate-bicarbonate buffer, pH 9.5) 3-4 times until no activity was detected in the washing. The washing discard was removed from reaction flask carefully with an eppendorf pipette avoiding any loss of glass beads. The enzyme protein bound to glass beads was estimated by determining the loss of protein from the solution during enzyme immobilization using the method of Lowry et al (1951).
Zirconia coated alkylamine glass beads

PRINCIPLE

\[
\begin{align*}
\text{support} & \quad \text{Glutaraldehyde} \quad \text{pH 7.0} \\
\text{----(CH}_2\text{n-NH}_2\text{+OHC (CH}_2\text{)_3-CHO} & \rightarrow \text{----(CH}_2\text{n-N=CH (CH}_2\text{)_3-CHO + H}_2\text{O}}
\end{align*}
\]

\[
\begin{align*}
\text{Enzyme} \quad \text{pH 7.0} & \quad \text{Schiff's base} \\
\text{----(CH}_2\text{n-N=CH(CH}_2\text{)_3-CHO+H}_2\text{N-E} & \rightarrow \text{----(CH}_2\text{n-N=CH (CH}_2\text{)_3-CH=N- E+H}_2\text{O}}
\end{align*}
\]

Fig. 4 Immobilization of 3α-hydroxysteroid dehydrogenase/diaphorase by glutaraldehyde onto activated alkylamine glass beads
3.5.2 On arylamine glass beads

The $3\alpha$-hydroxysteroid dehydrogenase was immobilized onto arylamine glass by diazotization according to the method of Lynn (1975). The reaction involved in the immobilization of enzyme onto arylamine glass beads are given in Fig. 5.

3.5.2.1 Diazotization of arylamine glass beads

Arylamine glass beads (pore diameter 55nm) (200 mg) were taken in a 15 ml conical flask kept in a ice bath upto neck and 2 ml 2N HCl followed by 50 mg of solid NaNO$_2$, were added to the glass beads. The diazotiation reaction was allowed to proceed for about 30 min in the same ice bath. The diazotized beads were washed several times with 0.1M sodium phosphate buffer, pH 7.0 to remove the excess of acid until the pH of the washing was 7.0.

3.5.2.2 Immobilization of $3\alpha$-hydroxysteroid dehydrogenase onto diazotized arylamine glass beads

$3\alpha$-hydroxysteroid dehydrogenase solution (0.21mg/ml) (2.0 ml) was added to 200 mg diazotiazed beads and left for 48 hr at 4°C with occasional stirring. The unbound enzyme was decanted and tested for activity and protein. The beads were washed in reaction buffer several times until no activity was detected in washing.
Zirconia coated arylamine glass beads

Fig. 5 Immobilization of 3a-hydroxysteroid dehydrogenase/diaphorase by diazotization onto activated arylamine glass beads

Fig. 5 Immobilization of 3a-hydroxysteroid dehydrogenase/diaphorase by diazotization onto activated arylamine glass beads
The protein bound to glass beads was estimated by determining the loss of protein from the solution during immobilization using the method of Lowry et al (1951).

3.6 ASSAY OF IMMOBILIZED 3α–HYDROXYSTEROID DEHYDROGENASE

The assay of immobilized 3α–hydroxysteroid dehydrogenase was also based on the same principle as that of native/free enzyme. The assay was carried out in a 15ml conical flask wrapped with black paper and containing 100 mg alkylamine/arylamine glass beads bound to 3α–hydroxysteroid dehydrogenase and 0.2ml of bile acid solution. The reaction was started by adding 0.5 ml of colour reagent. After incubation at 25°C for 15 min under continuous stirring, 0.25 ml stop solution was added. The coloured reaction mixture was withdrawn from flask with the help of an eppendorf pipette carefully avoiding the loss/removal of beads and transferred into a cuvette. The control was also run in the same manner except that glass beads were not coupled to 3α–hydroxysteroid dehydrogenase. A₅₄₀ was read in Spectronic-20 against control and the content of NADH generated in the reaction was measured from the standard curve of NADH (Fig. 2).
3.6.1 Unit of immobilized 3α-hydroxysteroid dehydrogenase

One unit of immobilized 3α-hydroxysteroid dehydrogenase is defined as the amount of enzyme bound to alkyl/arylamine glass beads which generates 1 µmol of NADH per min under standard assay conditions.

3.6.2 Reuse of immobilized 3α-hydroxysteroid dehydrogenase

After removal of the reaction mixture, 1.0 ml of reaction buffer (0.065M sodium phosphate buffer pH 7.0) was added to the flask along its side wall with the help of a pipette. The beads were shaken gently for 10-20 seconds in the reaction buffer and allowed to settle down. The buffer was removed carefully with the help of an eppendorf pipette avoiding the removal of beads. This process was repeated 3-4 times. The washed beads were reused in the next assay. The beads were stored in 0.065 M sodium phosphate buffer pH 7.0 at 4°C when not in use.

3.7 ASSAY OF FREE DIAPHORASE

Commercial diaphorase (5mg) was dissolved in 5 ml of 0.065M sodium phosphate buffer, pH 7.5 and stored at 4°C until use. The enzyme assay was carried out in a 15 ml of conical flask wrapped with black paper. The reaction mixture contained 0.3 ml NADH solution (200 µmol/L) and 0.5 ml colour reagent (20U diaphorase and 0.05g nitrobluetetrazolium salt per 40 ml of 0.065 M sodium phosphate buffer, pH 7.0 and 50 ml of distilled
water). After incubation at 25°C for 15 min, 0.5 ml stop solution (Triton X-100; 75g/L in 38% HCl) was added. The control was also run in the same manner except the NADH solution was replaced by water. A$_{540}$ of coloured reaction mixture was read against control. The content of NADH utilized in the reaction was extrapolated from standard curve of NADH (Fig. 2).

3.7.1 **Unit of diaphorase**

One unit of diaphorase is defined as the amount of enzyme, which utilized 1μmole of NADH per min under the standard assay conditions.

3.8 **IMMOBILIZATION OF DIAPHORASE ON GLASS BEADS**

3.8.1 **On alkylamine glass beads**

The enzyme was immobilized onto alkylamine glass beads (pore diameter 55nm) through the process of glutaraldehyde coupling using the method of Lynn (1975). The reactions involved in the immobilization were same as that for 3α-hydroxysteroid dehydrogenase (Fig. 4).

3.8.1.1 **Activation of alkylamine glass beads**

Alkylamine glass beads (pore diameter 55nm) (100 mg) were activated as described earlier for immobilization of 3α- hydroxysteroid dehydrogenase.
3.8.1.2 Immobilization of diaphorase onto the activated alkylamine glass beads

Diaphorase solution (1 mg/ml) (1 ml) was added to the activated glass beads in a 15 ml conical flask and kept for 48 hr at 4°C with occasional stirring for coupling. The unbound enzyme was decanted and tested for activity and protein. The beads were washed with 2.0 ml reaction buffer (65 mmol/L sodium phosphate buffer, pH 7.0) 3-4 times until no activity was detected in the washing. The enzyme protein bound to glass beads was estimated by determining the loss of protein from the solution during enzyme immobilization using the method of Lowry et al (1951).

3.8.2 Immobilization of diaphorase onto arylamine glass beads

The diaphorase was immobilized onto arylamine glass beads by diazotization according to the method of Lynn (1975). The reaction involved in the immobilization of enzyme onto arylamine glass beads is given in Fig. 5.

3.8.2.1 Diazotization of arylamine glass beads

Arylamine glass beads (200 mg) were activated through diazotization as described for immobilization of 3α-hydroxysteroid dehydrogenase on arylamine glass beads.
3.8.2.2 Immobilization of diaphorase onto diazotized arylamine glass beads

Diaphorase solution (1 mg/ml) (2 ml) was added to 200 mg diazotized beads and left for 48 hr at 4°C with occasional stirring. The unbound enzyme was decanted and tested for activity and protein. The beads were finally washed in reaction buffer.

3.9 ASSAY OF IMMOBILIZED DIAPHORASE

The assay was carried out in a 15 ml conical flask wrapped with black paper. The flask contained 0.5 ml colour reagent (0.05 g nitrobluetetrazolium salt per 40 ml of 0.065 M sodium phosphate buffer, pH 7.0 and 50 ml of distilled water) and 200 mg glass beads bound to diaphorase. Reaction was started by adding 0.2 ml NADH solution. After incubation at 25°C for 15 min under continuous stirring, 0.5 ml stop solution (Triton X-100, 75 g/L in 38% HCl) was added. The control was also run in the similar manner except that glass beads were not coupled to diaphorase. A540 was read against control and the content of NADH utilized in reaction was extrapolated from standard curve of NADH (Fig. 2).

3.9.1 Unit of immobilized diaphorase

One unit of immobilized diaphorase is defined as the amount of enzyme bound to glass beads which utilized 1 μmol of NADH per min under standard assay conditions.
3.9.2 Reuse of immobilized diaphorase

After removal of the reaction mixture, 2.0 ml of reaction buffer was added to the flask along its sidewall with the help of pipette. The beads were shaken gently for 10-20 seconds in the reaction buffer and allowed to settle down. The buffer was removed carefully with the help of an eppendorf pipette. This process was repeated 3-4 times avoiding the loss/removal of beads. The washed beads were used in the next assay. The beads were stored in 0.065 M sodium phosphate buffer pH 7.0 at 4°C when not in use.

3.10 ASSAY OF MIXTURE OF IMMOBILIZED 3α–HYDROXYSTEROID DEHYDROGENASE AND DIAPHORASE

It was carried out in a 15 ml conical flask wrapped with a black paper. The flask contained mixture of 100 mg alkylamine/200mg arylamine glass beads bound to 3α–hydroxysteroid dehydrogenase and 100 mg alkylamine/200mg arylamine glass bead coupled to diaphorase and 0.5 ml colour reagent (0.075 g of NAD$, 0.025gm NBT in 20 ml of 0.065M sodium phosphate buffer, pH 7.0 to make final volume upto 45 ml with distilled water). The reaction was started by addition of 0.2ml bile acid solution. Rest of the procedure was same as described for assay of immobilized 3α–hydroxysteroid dehydrogenase.
3.11 KINETIC PROPERTIES OF FREE, ALKYLAMINE AND ARYLAMINE GLASS BOUND 3α–HYDROXysteroid dehydro-GENASE

The following kinetic properties of free and alkylamine & arylamine glass bound 3α–hydroxysteroid dehydrogenase were studied and compared with those of free enzyme.

3.11.1 Effect of pH

To determine the optimum pH of alkylamine/arylamine glass bound 3α–hydroxysteroid dehydrogenase, the pH of reaction buffer was varied from pH 6.0 to 10.0 using the following buffers, each at a final concentration of 0.065M: pH 6.0 to 8.5, sodium phosphate buffer and pH 9.0 to 10.0, sodium carbonate bicarbonate buffer.

3.11.2 Effect of incubation temperature

Effect of incubation temperature on free, alkylamine and arylamine bound 3α–hydroxysteroid dehydrogenase was studied by varying temperature from 15°C to 40°C with an interval of 5°C.

3.11.3 Energy of activation

The activation energy (E_a) of both free and immobilized 3α–hydroxysteroid dehydrogenase was calculated using Arrhenius plot of log v vs 1/T. E_a was calculated from this plot using the following equation
\[ \log k = \frac{E_a}{(2.303R)} \frac{1}{T} + C \]

Where
- \( k \) = Reaction rate constant
- \( C \) = Proportionality constant
- \( R \) = Gas constant = 1.987 K Cal/mol/degree
- \( T \) = Temperature in Kelvin

In case of enzyme reaction, \( K \) is considered equivalent to the initial velocity of enzyme reaction (v)

### 3.11.4 Time course study

The time for maximum activity of free, alkylamine and arylamine glass bound 3α-hydroxysteroid dehydrogenase was determined by incubating the reaction mixture for different time ranging from 2 to 20 min at an interval of 1 min.

### 3.11.5 Effect of substrate concentration and calculation of \( K_m \) and \( V_{max} \)

Effect of substrate concentration on the initial velocity of free, alkylamine and arylamine glass bound 3α-hydroxysteroid dehydrogenase was studied by varying the final concentration of bile acid (Taurodeoxycholic acid) from 6.25 μmol/L to 800 μmol/L in the reaction mixture. \( K_m \) and \( V_{max} \) were calculated from Lineweaver-Burk plot.
3.11.6 Calculation of $K_m$ and $V_{max}$ values

$K_m$ and $V_{max}$ were calculated from Lineweaver-Burk plot, between reciprocals of initial velocity of the enzyme reaction ($v$) and taurodeoxycholic concentration [$S$] by using the following equation.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

3.11.7 Effect of metal chelators

To study the effect of metal chelators on free, alkylamine and arylamine glass bound 3α-hydroxysteroid dehydrogenase, the following metal chelators were added individually to the reaction mixture before starting the assay reaction to give a final concentration of 1.0mM: EDTA, sodium thiocyanate, sodium acids, diethylidithiocabamate, sodium salicylate, 8-hydroxyquinoline and sodium pyruvate.

3.11.8 Effect of metal ions

To study the effects of certain metal ions on either the free or immobilized enzyme or the end colour formation, the following compounds were added individually in the reaction mixture at the final concentration of 1.0mM: NaCl, KCl, CuSO$_4$, ZnSO$_4$, CaCl$_2$, MgSO$_4$, MnCl$_2$, CdCl$_2$, FeSO$_4$, NiSO$_4$, BaCl$_2$, HgCl$_2$, SrCl$_2$ and Pb (NO$_3$)$_2$. Each compound was added in the reaction mixture before starting the reaction.
3.11.9 Effect of anions

To study the effect of anions on the activity of both free and alkylamine and arylamine glass bound enzyme, the following compounds were added individually in the assay, each at a final concentration of 1.0mM. NaI, NaBr, NaSO₄, Na₂CO₃, NaHCO₃ and K₂HPO₄. Each compound was added in the reaction mixture before starting the reaction.

3.11.10 Effect of co-enzymes

To study the effect of co-enzymes on free or alkylamine and arylamine glass bound 3α-hydroxysteroid dehydrogenase, the following compounds were added individually in the reaction mixture before starting the assay reaction to give a final concentration of 0.1mM: FAD and FMN.

3.11.11 Effect of other compounds

To study the effect of other compounds on the activity of free or alkylamine and arylamine conjugated 3α-hydroxysteroid dehydrogenase, the following compounds were added individually in the reaction mixture before starting the assay reaction to give a final concentration of 1.0mM: potassium ferricyanide, sodium dithionite and ammonium molybdate.
3.12 KINETIC PROPERTIES OF FREE, ALKYLAMINE AND ARYLAMINE GLASS BOUND DIAPHORASE

The following kinetic properties of free, alkylamine and arylamine bound diaphorase were studied as described for 3α-hydroxysteroid dehydrogenase: Effect of pH, incubation temperature, time of incubation, substrate (NADH) concentrations and calculation of $K_m$ for NADH & $V_{max}$, effect of metal chelators, metals, anions, co-enzymes, metabolites & other compounds.

3.13 KINETIC PROPERTIES OF MIXTURE OF FREE, ALKYLAMINE AND ARYLAMINE GLASS BOUND 3α-HYDROXYSTEROID DEHYDROGENASE AND DIAPHORASE

The following kinetic properties of mixture of free, alkylamine and arylamine glass bound 3α-hydroxysteroid dehydrogenase and diaphorase were studied as described for 3α-hydroxysteroid dehydrogenase and compared with those of free enzymes. Effect of pH, incubation temperature, time of incubation, substrate concentration (bile acid) (taurodeoxycholate), $K_m$ for bile acid & $V_{max}$, effects of metal chelators, metals, anions, coenzymes, metabolites and other compounds (Fig. 6 & 7).

3.14 STORAGE STABILITY

The alkylamine and arylamine glass bound 3α-hydroxysteroid dehydrogenase and diaphorase both individually as well as in mixture were
**Fig. 6:** Flask 1 – Control, containing activated alkylamine glass beads and reaction mixture.

Flask 2 – Test, containing the mixture of alkylamine glass bound 3α-hydroxysteroid dehydrogenase and diaphorase & reaction mixture.
Fig. 7: Flask 1 – Control, containing activated arylamine glass beads and reaction mixture.

Flask 2 – Test, containing the mixture of arylamine glass bound 3β-hydroxysteroid dehydrogenase and diaphorase & reaction mixture.
stored in reaction buffer (0.065mM sodium phosphate buffer, pH 7.0) at 4°C when not in use. The dissolved free enzymes were also stored at 4°C.

3.15 DETERMINATION OF SERUM BILE ACID BY MIXTURE OF ALKYLAMINE/ARYLAMINE GLASS BOUND 3α–HYDROXYSTEROID DEHYDROGENASE AND DIAPHORASE

3.15.1 Collection of blood

One ml blood from apparently healthy individuals (both female and male) of different age groups (children, young and aged persons) and persons suffering from gallstone disease (cholelithiasis) were obtained from local medical institute and hospital.

3.15.2 Preparation of serum

Fresh blood (1.0ml) withdrawn intravenously by sterilized syringe and needle was transferred to a vial and allowed to coagulate for 30 min at room temperature. It was centrifuged at 5000rpm for 10 min in a centrifuge. The supernatant was collected stored at -20°C until use.

3.15.3 Preparation of standard curve for bile acid

The standard curve of bile acid was prepared employing mixture of immobilized 3α–hydroxysteroid dehydrogenase and diaphorase onto alkyamine and arylamine glass beads in 1:2 ratio under their optimal assay
conditions. Taurodeoxycholic acid was used standard bile acid. The solution of taurodeoxycholic acid of different concentration was prepared in the concentration range of 6.25µmol/L to 150µmol/L. The rest of the procedure was same as described for assay of mixture of immobilized 3α-hydroxysteroid dehydrogenase and diaphorase. A standard curve between various taurodeoxycholic acid concentrations and A540 was plotted employing the mixture of both alkylamine and arylamine conjugated enzymes (Fig. 8 & 9).

3.15.4 Assay of serum bile acid

The assay of serum bile acid was carried out in a 15 ml conical flask in dark as described for preparation of standard curve except that standard taurodeoxycholic acid solution was replaced by 0.2ml serum sample. The blank contained normal saline in place of serum. A540 was read against the reagent blank and bile acid in serum was calculated from standard curve between taurodeoxycholic acid and A540 employing the mixture of both alkylamine as well as arylamine glass bound enzymes (Fig. 8 & 9). To reuse the glass beads, the beads were washed 3-4 times with 0.065M sodium phosphate buffer pH 7.0 and used in the next assay.
Fig. 8: Standard curve for bile acid concentration using mixture of alkylamine conjugated 3α-hydroxysteroid dehydrogenase and diaphorase.
Fig. 9 Standard curve for bile acid concentration using mixture of arylamine conjugated 3α-hydroxysteroid dehydrogenase and diaphorase.
3.16 DETERMINATION OF BILE ACID IN BILE OF PATIENTS OF GALLSTONE PATIENTS BY MIXTURE OF ALKYLAMINE/ARYLAMINE GLASS BOUND 3α–HYDROXYSTEROID DEHYDROGENASE AND DIAPHORASE

3.16.1 Collection and preparation of bile

One ml bile was removed during laparoscopic cholecystectomy of gallstone patients and transferred it to a glass vial. These were sterilized and stored at 4°C until use.

3.16.2 Assay of bile acid in bile

To determine concentration of bile acid in bile, the same procedure was followed as that for serum bile acid except that serum was replaced by bile. The concentration of bile acid was extrapolated from standard curve between taurodeoxycholic acid concentration and A_{540} employing the mixture of both alkylamine and arylamine glass bound enzymes (Fig. 8 & 9).

3.17 CRITERIA FOR EVALUATION OF METHOD FOR DETERMINATION OF BILE ACID IN SERUM/BILE

The following parameters were studied in order to evaluate both the methods for determination of bile acid in serum and bile employing mixture of individually immobilized 3α–hydroxysteroid dehydrogenase and diaphorase onto alkylamine and arylamine glass beads.
3.17.1 Detection limit

In order to check minimum and maximum limit of the method, the mixture of individually immobilized 3α-hydroxysteroid dehydrogenase and diaphorase was assayed at varying taurodeoxycholic acid concentrations ranging from 6.25 μmol/L to 150 μmol/L under standard assay conditions.

3.17.2 Percent recovery

To determine the reliability of the method, two concentration of taurodeoxycholic acid (50 μmol/L and 200 μmol/L) were added to the serum samples and bile acid content was determined before and after addition of taurodeoxycholic acid into these serum and bile samples. The percent recovery of added taurodeoxycholic acid was calculated.

3.17.3 Precision

To study the reproducibility of the methods, the bile acid content was determined in the six serum and six bile samples repeatedly on the same day (within batch) and in the same samples after their storage at −20°C for serum and 2 to 4°C for bile for one week (between batch). The within and between batch coefficient of variation (CV) were calculated for both the methods.
3.17.4 Accuracy

In order to determine the accuracy of the method employing mixture of individually immobilized enzyme system, the bile acid in 20 serum and bile samples were determined by the chemical method (Carey, 1958) with modification (x) as well as by the present method (y). The values obtained by both the assay systems were correlated using the regression equation.

3.17.5 Determination of serum bile acid by Carey’s chemical method

Bile acid in serum and bile of gallstone diseases was determined by the standard chemical method (Carey, 1958).

3.17.5.1 Preparation of reagents:

Following reagents were prepared.

3.17.5.1.1 Salicylaldehyde

3.17.5.1.2 Dilute H₂SO₄: 20 ml of concentrated H₂SO₄ was diluted with 100 ml distilled water while mixing and cooling with tap water.

3.17.5.1.3 Glacial acetic acid

3.17.5.1.4 Ethyl alcohol (95%)

3.17.5.1.5 Stock standard solution: 80 mg of pure taurodeoxycholic acid was weighed and dissolved in 100 ml of 95% ethyl alcohol.

3.17.5.1.6 Working standard: Stock solution was diluted 1:10 ratio with 95% ethyl alcohol.
3.17.5.2 Bile acid determination

Serum (0.1 ml) was added to 100 ml of 95% ethyl alcohol. 10ml of above solution was pipetted in a flask and diluted to 100 ml with 95% ethyl alcohol and mixed thoroughly. 5 ml of above aliquot and standard solution were measured into two glass stoppered tubes. The alcohol was evaporated by heating the tubes in boiling water bath and drying at 100°C for 15 min in an oven to ensure complete removal of alcohol. It was cooled at room temperature. 1ml salicylaldehyde was pipetted into a graduated cylinder and diluted to 35 ml with dilute H₂SO₄. It was mixed by inverting the cylinder several times. It was transferred to a fast draining burette and used immediately. 3 ml of this solution was added to both standard and sample tubes. The tubes were swirled to moisten the residue completely. The stoppered tubes were placed in water bath at 40°C for 15 min. The tubes were removed from the water bath and allowed to stand at room temperature for 5 min. 20 ml of glacial acetic acid was added from a fast draining burette into each tube and shook vigorously for 10 seconds to get a uniform colour and a clean solution. The colour was read exactly 5 min after addition of glacial acetic acid. The colour intensity was read at 680 nm with a blank prepared simultaneously.
3.17.5.3 Calculation:

\[
\frac{T}{S} \times \frac{0.40 \times 10 \times 1000}{0.1 \text{ml (quantity of serum added)}} = \text{Bile acid conc. (\(\mu\text{mol/L}\))}
\]

\(T = \text{Test}\)

\(S = \text{Standard}\)

3.17.6 Interference study

To study the effect of several inorganic and organic substances found in serum in the present method of bile acid determination, the aqueous solutions of the following compounds were added in the reaction mixture individually at their physiological concentration or as indicated: bilirubin, sucrose, cholesterol, triglycerides, acetone, urea, uric acid, citric acid, L-ascorbic acid, citrate, pyruvate, haemoglobin, gamma-globulin, sodium pyruvate, NaCl, KCl, Ca\(^{2+}\) and EDTA. were studied. Each compound was added in the reaction mixture before starting the reaction by adding taurodeoxycholic acid.

3.18 STATISTICAL METHOD USED

Following statistical formula were used to evaluate the data

3.18.1 Standard deviation (S.D.)

\[
(\sigma) = \sqrt{\frac{\Sigma x}{N} - \left(\frac{\Sigma x^2}{n}\right)^2}
\]

where \(x = \text{deviation from mean}\)

\(n = \text{number of samples}\)
3.18.2 Coefficient of variation (CV)

\[ cv = \frac{\sigma \times 100}{\bar{a}} \]

Where \( \sigma \) = standard deviation;
\( \bar{a} \) = mean of series

3.18.3 Standard error (S.E.)

\[ \text{S.E.} = \frac{\sigma}{\sqrt{n}} \]

where \( \sigma \) = standard deviation
\( n \) = number of series

3.18.4 Student 't' test

\[ \frac{\text{Mean control} - \text{Mean experimental}}{\text{Standard error (SE)}} \]

3.18.5 Correlation coefficient (r)

\[ r = \frac{\Sigma xy - \Sigma x \Sigma y / N}{\left\{ n \Sigma x^2 - (\Sigma x)^2 \right\} \left\{ n \Sigma y^2 - (\Sigma y)^2 \right\}} \]

Where \( x \) = values obtained by reference method
\( y \) = values obtained by present method