Chapter 6

SUMMARY
The present work was undertaken to develop a cost effective enzymic colorimetric method for determination of bile acid in serum and bile by employing 3α-hydroxysteroid dehydrogenase and diaphorase immobilized onto insoluble support. The use of immobilized enzymes in routine analysis is expected to reduce the cost of procedures, as these can be reused. To develop such method, commercially available 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* and diaphorase from *Clostridium* spp. were immobilized individually on alkylamine glass beads and arylamine glass beads (pore diameter 55nm) through glutaraldehyde coupling and diazotization respectively.

3α-Hydroxysteroid dehydrogenase onto alkylamine and arylamine glass beads gave a conjugation yield of 1.2mg/g and 1.14mg/g supports and 99% and 52.6% retention of initial specific activity of free enzyme respectively. The enzyme showed an increase in optimum pH from pH 9.0 to 9.6 but no change in incubation temperature for maximum activity (25°C) after immobilization on both types of glass. There was a decrease in energy of activation (Ea) from 11Kcal/mol to 2.7 Kcal/mol and 1.96 Kcal/mol, time
of incubation from 15min to 2min and 5min and $K_m$ for taurodeoxycholic acid from 11.19mM to 5.68mM and 0.34mM and $V_{max}$ from 4.76 $\mu$molNADH/min to 1.75 $\mu$molNADH/min and 1.21 $\mu$molNADH/min after immobilization on alkylamine and arylamine glass respectively.

Among the metal chelators tested in immobilized 3α-hydroxysteroid dehydrogenase assay such as EDTA, sodium thiocyanate, sodium azide, diethyldithiocarbamate, sodium salicylate, 8-hydroxyquinoline and sodium pyruvate, only sodium pyruvate at 1.9mM concentration caused slight stimulation of both alkylamine and arylamine conjugated enzyme. Among the coenzymes tested, FAD and FMN caused 50% stimulation of both alkylamine and arylamine conjugated enzyme. The storage stability of 3α-hydroxysteroid dehydrogenase in reaction buffer at 4°C also increased after immobilization.

Diaphorase immobilized onto alkylamine and arylamine glass beads gave a conjugation yield of 0.19mg/g and 0.23mg/g support & 70% and 71.7% retention of initial specific activity of free enzyme. The enzyme showed an increase in optimum pH from 7.5 to 9.6 and 9.5 but decrease in incubation temperature from 35°C to 30°C and 25°C, energy of activation ($E_a$) from 12Kcal/mol to 4.0Kcal/mol and 5.49 Kcal/mol after immobilization on alkylamine and arylamine glass respectively. The time
of incubation for maximum activity was decreased from 10min to 5min for alkylamine conjugated diaphorase but same for arylamine conjugated enzyme. $K_m$ for NADH was increased from 0.41mM to 1.36mM for alkylamine and 1.03mM for arylamine conjugated enzyme $V_{max}$ also increased from 3.03μmolNADH/min to 3.73μmolNADH/min and 3.44μmolNADH/min after immobilization onto alkylamine and arylamine glass beads respectively.

3α-Hydroxysteroid dehydrogenase and diaphorase immobilized individually onto alkylamine and arylamine glass beads were mixed together in 1:2 ratio. The mixture of alkylamine and arylamine glass bound enzymes showed an increase in optimum pH from pH 7.0 to 9.6 and 9.5 respectively. There was a decrease in both incubation temperature from 37°C to 20°C and 30°C and energy of activation ($E_a$) from 6.29Kcal/mol to 5.49Kcal/mol and 4.64Kcal/mol for mixture of alkylamine and arylamine conjugated enzymes respectively. The time of incubation for maximum activity was 15 min for mixture of both alkylamine and arylamine as well as arylamine glass bound enzymes. $K_m$ value for bile acid was decreased from 10.3mM to 2.46mM and 2.65mM. $V_{max}$ also decreased from 5.55 μmolNADH/min to 3.70 μmolNADH/min and 1.66 μmolNADH/min for mixture of alkylamine and arylamine conjugated enzymes respectively. The
mixture of alkylamine and arylamine conjugated 3α-hydroxysteroid dehydrogenase and diaphorase lost 50% of its initial activity after its regular use (300 uses) over a period of 24 weeks (6 months), while the solution of mixture of 3α-hydroxysteroid dehydrogenase and diaphorase was stable only for week, when stored in reaction buffer at 4°C. These results indicate an increase in storage stability of enzyme after immobilization.

A cost effective enzymic colourimetric method for determination of bile acid in serum and bile was developed employing mixture of alkylamine glass bead bound 3α-hydroxysteroid dehydrogenase and diaphorase and also the same enzymes bound individually onto arylamine glass beads in 1:2 ratio. The method is based upon the measurement of NADH generated from bile acid of serum/bile by immobilized 3α-hydroxysteroid dehydrogenase using a color reaction, consisting of nitrobluetetrazolium (NBT) chloride salts, NAD⁺ and immobilized diaphorase. The chemical reactions involved in this assay are as follows:

\[ 3\alpha - \text{Hydroxy bile acid} + \text{NAD}^+ \xrightarrow{3\alpha-HSD} 3\alpha - \text{Keto bile acid} + \text{NADH} + H^+ \]

\[ \text{NADH} + H^+ + \text{NBT} \xrightarrow{\text{Diaphorase}} \text{Formazan (Chromophore)} + \text{NAD}^+ \]

\[ (\lambda_{\text{max}} = 540\text{nm}) \]
The linearity of the method was up to 150\textmu mol/L for both mixtures of alkylamine as well as arylamine glass bound enzymes (1:2 ratio). The lower detection limit was 4.8\textmu mol/L for mixture of alkylamine conjugated enzymes and 2.6\textmu mol/L for mixture of arylamine conjugated enzymes. The mean analytical recovery for added taurodeoxycholic acid (50\textmu mol/L and 200\textmu mol/L) was 89.13\% & 80.8\% for serum and 98.95\% & 92.30\% for bile for mixture of alkylamine conjugated enzymes and 95.57\% & 85.46\% for serum and 97.6\% & 91.6\% for bile for mixture of arylamine conjugated enzyme. The within and between batch coefficient of variation (CV) were <1.0\% & <0.2\% for serum and >0.1\% & <1.0\% for bile for mixture of alkylamine conjugated enzyme and <0.2\% & <0.6\% for serum and >0.1\% & <1.0\% for bile for mixture of arylamine conjugated enzymes. Serum bile acid value as determined by the present method, employing mixture of alkylamine conjugated enzymes ranged from 4.8\textmu mol/L to 29.2\textmu mol/L for both apparently healthy females and males respectively. The method employing mixture of arylamine conjugated enzymes had serum bile acid values in the range 2.8\textmu mol/L to 15.9\textmu mol/L for both apparently healthy females and males. Bile acid level in serum of gallstone patients ranged from 31.8\textmu mol/L to 159.3\textmu mol/L for females and 47.7\textmu mol/L to 690.3\textmu mol/L for males and 43.9\textmu mol/L to 204.9\textmu mol/L for females and
from 48.8\(\mu\)mol/L to 439.2\(\mu\)mol/L for male by the method employing mixture of alkylamine and arylamine conjugated enzymes respectively. Bile acid level in bile of gallstone patients ranged from 48.8\(\mu\)mol/L to 585.8\(\mu\)mol/L for females and 58.4\(\mu\)mol/L to 690.3\(\mu\)mol/L for males by the mixture of alkylamine conjugated enzymes and from 34.1\(\mu\)mol/L to 634.4\(\mu\)mol/L for females and from 37.1\(\mu\)mol/L to 743.4\(\mu\)mol/L for males by mixture of arylamine conjugated enzymes. Serum bile acid values as measured by present method (\(y\)) showed a good correlation with those of standard chemical method (\(x\)), \(r_1=0.9544289\) (regression equation, \(y=1.009x+5.039\)) and \(r_1' = 0.923483\) (regression equation, \(y=0.877x+7.23\)) for the method employing mixture of alkylamine and arylamine conjugated enzymes respectively. Bile acid value in bile as measured by the present method (\(y\)) showed a good correlation with those of standard chemical method (\(x\)), \(r_2=0.934001\) (regression equation, \(y=1.593x-38.052\)) and \(r_2' = 0.9677302\) (regression equation, \(y=0.1123x - 7.123\)) by the method employing mixture of alkylamine and arylamine conjugated enzymes respectively.

Among the interference of various metabolites tested in the method, such as bilirubin, sucrose, cholesterol, triglycerides, acetone, urea, uric acid, citric acid, L-ascorbic acid, citrate, pyruvate, haemoglobin, gamma-
globulin, sodium pyruvate, NaCl, KCl, Ca^{2+}; EDTA, bilirubin, urea, uric acid and L-ascorbic acid, NaCl, KCl, Ca^{2+} had inhibitory effect on mixture of individually immobilized 3α-hydroxysteroid dehydrogenase and diaphorase onto alkylamine as well as arylamine glass beads, but sucrose, cholesterol, triglycerides and sodium pyruvate had stimulatory effect on mixture of individually immobilized 3α-hydroxysteroid dehydrogenase and diaphorase onto either alkylamine or arylamine glass beads.

A Sigma kit for 100 serum bile acid determination costs US $323 i.e. 3.23 per assay. In this kit, enzyme components such as 3α-hydroxysteroid dehydrogenase and diaphorase are expensive and less stable which leads to high cost of procedure. The cost of one serum bile acid assay by the present method using mixture of individually immobilized 3α-hydroxysteroid dehydrogenase and diaphorase is US $ 0.304 which is US $ 30.44 for 100 serum samples (including the cost of immobilized enzymes and 4 times washing of immobilized enzymes after every use), which is 3.18 times lower that that of Sigma kit. Thus the present method stands to be economical than Sigma kit method for routine analysis.