The Establishment of a Therapeutic Monitoring Service for Digoxin

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A thesis submitted in part fulfillment for the Degree of M.Sc.

Based on research carried out at

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September, 1990

ACKNOWLEDGEMENTS

I would like to thank all who helped me with this thesis especially my supervisor Dr. Richard O'Kennedy, Dublin City University; Dr. Dermot Curran, Laboratory Director, Mr. Pat Hall, Chief Technologist and his staff at James Connolly Memorial Hospital, Blanchardstown.

I would also like to thank Ms. Barbara Drew who so competently typed this script.

DECLARATION

I hereby declare that this thesis, being in partial fulfillment of the requirements for M.Sc. course, is my own work.

Signed: John Carbury

ABBREVIATIONS

A - V	Atrioventricular
ATP-ase	Adenosine Triphosphatase
CEDIA	Cloned Enzyme Donor Immunoassay
cv	Co-efficient of Variation
DLIFs	Digoxin-like Immunoreactive Factors
DNA	Deoxyribonucleic Acid
EAs	Enzyme Acceptors
EDs	Enzyme Donors
EIA	Enzyme Immunoassay
EMIT	Enzyme Multiplied Immunoassay Technique
FPIA	Fluorescence Polarization Immunoassay
GLC	Gas Liquid Chromatography
Hb	Haemoglobin
HCL	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
mRNA	Messenger Ribonucleic Acid
n	Number of Samples
NAD^+	Nicotine Adenine Dinucleotide
NADH	Reduced Nicotine Adenine Dinucleotide
p	Probability
r	Correlation Coefficient
^{86}Rb	Rubidenium 86
RIA	Radioimmunoassay
RT	Room Temperature

Rf	The Ratio of the Distance the Solute has moved to the Distance the Solvent Front has moved
s	Slope of Regression Line
SD	Standard Deviation
TCA	Trichloroacetic Acid
TLC	Thin Layer Chromatography

CONTENTS

ABSTRACT

The aim of this study was to evaluate two enzyme immunoassays for the estimation of serum and salivary digoxin. The methods under review were the Enzyme Multiplied Immunoassay Technique and Cloned Enzyme Donor Immunoassay assays. Within and between assay precision for both immunoassays was good giving co-efficient of variations of less than 6.1%. Analytical recoveries ranged from 96-103%. Sensitivity was 0.28 nmol/l of digoxin, approximately. Results from serum specimens from 52 patients using both methods and a RIA method from an external laboratory (Beaumont Hospital) were compared. A correlation of 1.008 was obtained for the EMIT and RIA methods, while 0.985 resulted from the EMIT and CEDIA methods. The RIA and CEDIA methods gave a correlation of 0.94. Haemoglobin, bilirubin, lipaemia, and matrix effects, i.e. (protein concentration) did not interfere with the estimation of digoxin by the EMIT method. In the CEDIA method, high concentrations of protein resulted in falsely low digoxin concentrations, whereas low protein concentrations gave falsely high digoxin concentrations. Haemoglobin, bilirubin, and lipaemia caused interferences with the assay which was concentration dependent. Digoxin-like Immunoreactive Factors were greatly reduced or eliminated by the EMIT technique but interfered with the CEDIA method resulting in "apparent digoxin concentrations" as high as 1.18 nmol/l.

A further study was performed to investigate the possible use of the EMIT and CEDIA methods for salivary digoxin estimations. Accuracy, precision, sensitivity and linearity were found to be comparable with that of the serum digoxin methods. No procedural modification was required for the EMIT method, while minor modifications were needed for the CEDIA.

The mean digoxin saliva/serum concentration ratio in 20 hospital patients using the EMIT method was 0.67 and the correlation was 0.96. The mean ratio using the CEDIA method was 0.62 with a correlation of 0.93. These results were obtained when serum and saliva samples were taken simultaneously. In each method, DLIF concentration less than the sensitivity range was obtained in saliva from patients in renal and hepatic failure and third trimester pregnancy.

On the basis of all the factors assessed, the EMIT is the most suitable for routine use in the clinical chemistry laboratory.

PART 1

Chapter 1

INTRODUCTION

Digoxin is an orally administered cardioactive drug used in the treatment of chronic heart disease. Its rapid absorption and elimination makes it clinically desirable. However, the pharmacokinetics of the drug may also cause over-digitilization necessitating immediate diagnosis and treatment.

Various assay methods have been developed for serum digoxin estimation and are now in routine use in most clinical chemistry laboratories. Two enzyme immunoassays, CEDIA and EMIT were evaluated in this study. Since the concentration of certain drugs in saliva is related to their concentration in plasma it was decided to evaluate both assays for the measurement of salivary digoxin. Such a method offers a non-invasive alternative to the direct measurement of plasma digoxin concentrations.

From the literature search which forms Part 1 of this thesis, there are no reports of comparability studies involving these two assays. Part 2 consists of research work involved in the evaluation of the EMIT and CEDIA methods for digoxin measurement.

In order to appreciate fully the value of digoxin monitoring and the principles of cardiac glycoside therapy, a basic understanding of the anatomy, physiology and pathology of the human heart is essential.

1.1 *ANATOMY:*

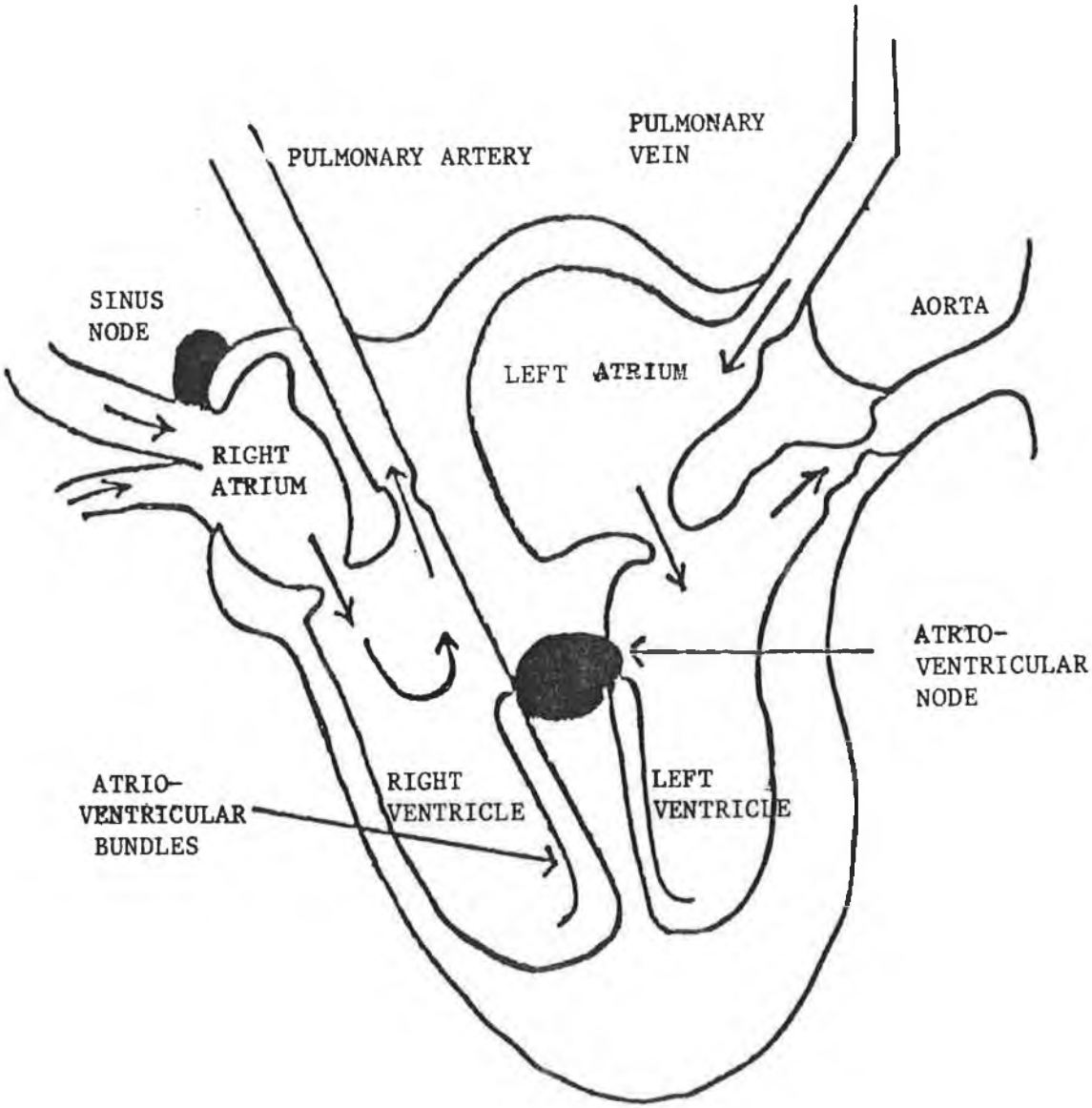
(a) *GENERAL:*

The heart acts as the pump for the circulatory blood system. Figure 1 shows the heart, nervous control system, and the circulatory system. It lies in the thorax behind the sternum with its apex on the diaphragm extending to the left for three and a half inches [1-4].

The heart is surrounded by the pericardium which is an inextensible loose fitting fibrous sac. Lining the pericardium is the serous pericardium which consists of two layers, the parietal layer which lines the inside of the fibrous pericardium and the visceral layer which is the outer surface of the heart. A small space containing a few drops of pericardial fluid lies between these two layers. This allows the heart to beat in the thorax with the minimum of friction.

The pericardium functions by limiting the maximum size of the chambers of the heart, thus preventing stretching of the cardiac fibres due to overfilling of the atria. The pericardium is attached to the diaphragm and when the heart beats it behaves as if the apex was relatively fixed. Thus, when the ventricles contract, instead of the apex moving upwards towards the base, the base and particularly the A-V ring descends towards the apex. This has the effect of increasing the size of the atria at the same time as blood is ejected from the ventricles.

Figure 1: The heart.



(b) *STRUCTURE:*

The wall of the heart consists of three layers of tissue: (i) myocardium, (ii) visceral and (iii) endocardium. The myocardium is the main tissue. The visceral layer covers the myocardium and lining the inside of the myocardium is the endocardium. On the inside surface the myocardium is raised into ridge-like projections called papillary muscles. The heart muscle consists of cells known as fibres which are cylindrical in shape with central nuclei and faint striations. These muscular fibres are arranged in a complex manner, but in such a way that when they contract they tend to squeeze the blood in a forward direction into the next opening through which the blood has to pass. At the same time, some of the fibres of the two atria are continuous with each other and some of the right and left ventricle fibres are also continuous so that the two atria contract simultaneously and the two ventricles contract together.

(c) *CHAMBERS:*

The heart contains four chambers, two upper and two lower called atria and ventricles, respectively. The atria have relatively thin walls as they have to pump blood into the ventricles only. The wall of the right ventricle is thicker than that of the atria because it has to pump blood to the lungs. The wall of the left ventricle is thicker than that of the right ventricle because it pumps blood to the systemic circulation.

The muscle of the atria is entirely separate from the muscle of the ventricle except at one point. This point of communication is

artery thrombosis, and if the patient survives, the part of the myocardium supplied by the affected branch will become permanently deprived of its blood supply and the muscle will be replaced by a fibrous tissue scar. If this is extensive it will weaken the pumping power of the heart.

(f) *THE CONDUCTION SYSTEM:*

Certain tissues in the heart are concerned with the initiation and propagation of the heart beat. They include the S-A node, the A-V node, the A-V bundle and Purkinje fibres.

(i) *S-A Node:*

This is a small mass of modified cardiac muscle situated at the junction of the superior vena cava and the right atrium. These fibres normally initiate the heart beat, for this reason the S-A node is called the pacemaker. Nerve cells and fibres from the right vagus nerves and sympathetic nerves are also present.

(ii) *A-V Node:*

This is a specialised mass of cardiac muscle situated in the septum between the two atria. Atrial muscle fibres unite with the fibres in the A-V node. This node is identical in structure with the S-A node. The A-V node is supplied by the left vagus nerve and sympathetic nerve.

(iii) *The Bundle of His and Purkinje Fibres:*

These modified cardiac muscle fibres originate in the A-V node, pass down the interventricular septum and terminate in the walls of the

ventricles. The Bundle of His contains sympathetic and vagus nerve fibres and many blood vessels.

(g) *THE NERVE SUPPLY:*

Sympathetic fibres and parasympathetic fibres unite to form the cardiac plexus. Muscle fibres enter the heart from the cardiac plexus along with the coronary arteries. The sympathetic fibres which strengthen and accelerate the heart while those from the vagus slow the heart beat.

1.2 *PHYSIOLOGY:*

HEART BEAT:

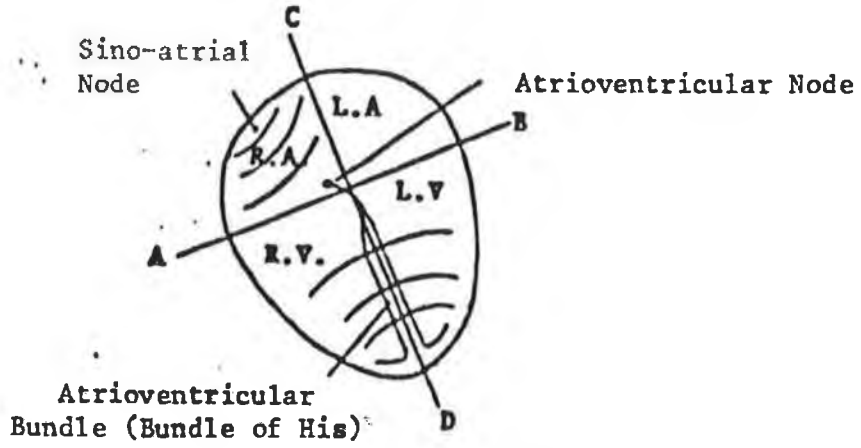
The heart beat originating as a contraction wave at the S-A node, spreads rapidly through the artrial muscle causing both atria to contract simulataneously. The blood in the atria is forced through the atrio-ventricular valves into the ventricles.

Rings of cardiac muscle around the entry of the superior and inferior vena cava and the pulmonary veins close off the veins with a sphincter-like action so that blood does not flow back into the veins when the atria contract.

The spread of the contraction wave through the cardiac muscle ceases at the fibrous septum between the atria and the ventricles which contains the four heart valves.

The only pathway through this non-conducting septum is from the A-V node down the atrio-ventricular bundle (Bundle of His). The contraction wave enters the ventricles near the apex and spreads upwards towards the base. The blood in the ventricles is forced upwards through the base of the heart and out through the aortic and pulmonary values. Figure 2.

Figure 2: Spread of the cardiac impulse from the pacemaker through A-V node and down the Bundle of His. A-B is the septum between the atria and ventricles. C-D is the spetum between the chamber.



1.3 *PATHOLOGY:*

No single simple classification of heart disease is possible. For this study the causes of heart disease may be divided into two categories:

(i) Mechanical e.g. valve defect or hypertension

(ii) Nervous/Electrical e.g. ectopic nodes

The symptoms of heart disease are many including chest pain, loss of breath, fainting etc. The same symptoms may arise from either a mechanical or nervous/electrical cause, Table 1.

Table 1: Shows some heart disorders and their causes.

DISORDER	CAUSES
Atrial Fibrillation	Congestive heart disease
Atrial Flutter	Rheumatic heart disease
	Mitral stenosis
	Ischaemia
	High blood pressure
	Atrial septal defect
	Electrolyte imbalance
	Increase or decrease K^+
	Increase or decrease Ca^{2+}
Ventricular Fibrillation	Ischaemia
	Infarction
	Electrolyte imbalance
	Pulmonary embolism
	Electrocution

(a) *Acute Myocardial Infarction:*

This is the damaging or death of an area of heart muscle resulting from a reduction or blockage in the blood supply to that area. This is frequently caused by a thrombus in the coronary arteries.

(b) *Congestive Heart Disease:*

This often occurs as a result of structural defects in the myocardium. This may be caused by blockage of an arteriole supplying a small area of the right atrium. Consequently, the remaining heart muscle in the right antrium is overworked and loses its efficiency causing insufficient cardiac output to maintain adequate circulation. A backing up of blood in the veins leading to the heart often accompanied by accumulation of fluid in various parts of the body results.

(c) *Atrial Arrhythmias:*

These arise from loss of control of the sinus node over atrial contraction which becomes autonomous. The condition may or may not involve partial ventricular involvement.

(d) *Ventricular Arrhythmias:*

These are extremely serious as they are accompanied by total loss of cardiac output.

(e) *Atrial Flutter:*

This condition has a regular pulse at 125-160/min with atrio-ventricular block.

1.4 *HISTORY OF DIGOXIN:*

The control of abnormal cardiac rates or rhythms (arrhythmia) by medication dates back to the 18th century. William Withering [5] an English doctor introduced digitalis, an extract of the foxglove plant, to treat cardiac patients. He first became involved with the foxglove in 1775 when he was consulted about a family recipe for the treatment of dropsy (oedema) kept a secret by an old woman in Shropshire. The medicine consisted of twenty or more herbs and he recognised that the foxglove was the active substance present. He standarised the dose by using leaves from the plant only when it was in full bloom and administered it as a powder or infusion.

Withering's use of digitalis from 1775 to 1784 provided him with a complete description of its toxic effects. "Sickness, vomiting, giddiness, purging, confused vision, objects appearing green and yellow, increased secretion of urine with frequent motions to part with it and sometimes inability to retain it, slow pulse even as low as 35 in a minute, cold sweats, convulsions and death". Later as he became more familiar with the drug he modified its use "let it (digitalis) be continued until it either acts on the kidneys, the stomach or the bowels, let it be stopped at the first appearance of any of these effects".

Withering was impressed with its diuretic effect but he also noticed its effect on the heart.

Today digitalis is prescribed as digoxin which was first isolated from the white foxglove (digitalis lanata) in 1930 [6] and was accepted as a new drug in Martindale's Pharmacopoica of 1932. Another cardiac glycoside, digitoxin, was found in the purple foxglove (digitalis purpurea).

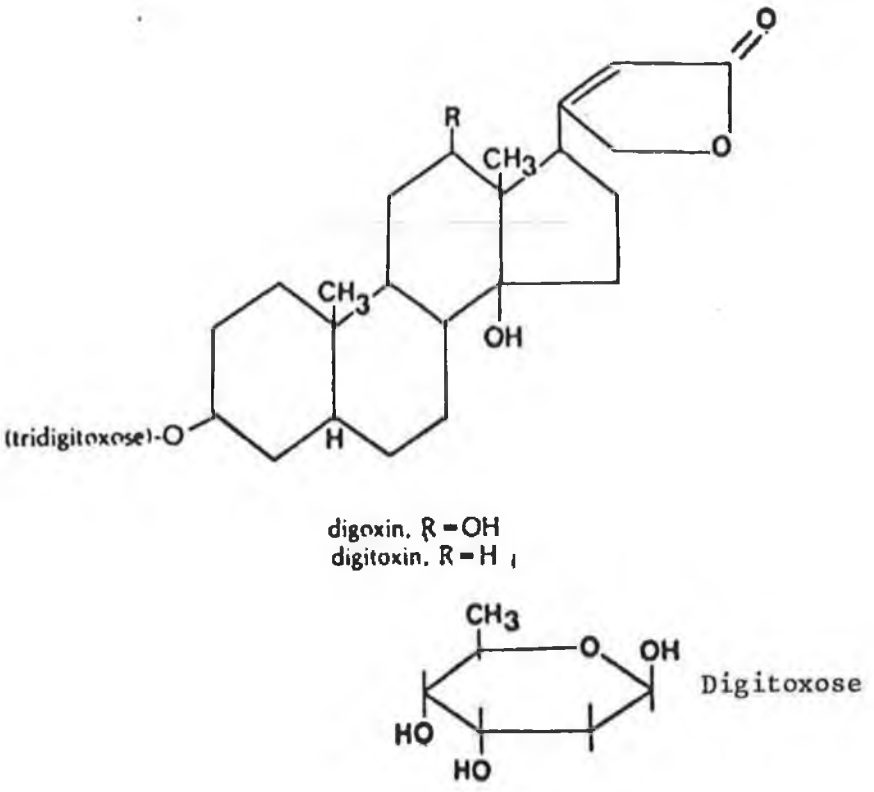
Subsequent to Withering's introduction of the drug a number of people misused the drug for nearly a century and its therapeutic value was doubted. It was used for many disorders often in toxic doses. In the early twentieth century its value in the treatment of atrial fibrillation was rediscovered. Since its extraction in the 1930s, digoxin is the most widely used form of cardiac glycoside in the British Isles [7]. However, in several European countries, a variety of other related glycosides, including digitoxin, lanatoside C and peruvoside, and semi-synthetic glycosides, such as β-methyldigoxin are used. The greater popularity of digoxin over digitoxin is due to the fact that its therapeutic effect disappears in two days to one week following withdrawal of the drug, while the effect of digitoxin may persist for up to three or four weeks. This difference in metabolic behaviour is of great importance in the case of the toxic patient with serum digoxin greater than 2 nmol/l. Another reason for its popularity is due to the accumulation of large amounts of knowledge on its pharmacokinetics starting in the 1960s [8] and being pursued right up to the present time.

1.5 *CHEMISTRY:*

Digoxin in a white crystalline powder with a melting point of 240°C. It is not very soluble in chloroform, ethyl acetate, acetone or water but is soluble in 80% alcohol solution when diluted 1 part to 122. It is soluble in pyridine when diluted 1 part in 4.

Figure 3 shows the chemical structure of digoxin and the related glycoside digitoxin. These compounds are composed of an aglycone, digoxigenin attached to three glycoside (digitoxose) moieties. The digoxigenin consists of a steroid moiety with an α-β-unsaturated lactone ring coupled at C 17 of the steroid. The sugars are attached via the C3 hydroxyl groups. The type of sugar molecules present determine water and lipid solubility. The aglycone component imparts pharmacological activity to the drug. They are chemically related to bile salts, sterols, sex and adrenocortical hormones. The number and position of the OH groups on the digoxin molecule determines the protein binding capacity, its distribution and its excretion rate in the body. Saturation of the lactone ring results in major or total loss of drug action.

Figure 3:



Chemical Structure of Digoxin and Digitoxin.

1.6 *MECHANISM OF ACTION OF DIGOXIN:*

Digoxin is used in the treatment of congestive heart failure, in the control of atrial fibrillation and flutter, and of supraventricular tachycardia [9]. By far the most important of these uses is in congestive heart failure.

Digoxin exerts its beneficial effect on the failing heart by increasing the force of systolic contraction (i.e. digoxin has a positive inotropic effect) [10]. This occurs due to a specific interaction of the drug with the plasma membrane-bound ion transport system known as the Na^+/K^+ ATP-ase. This enzyme is responsible for the maintenance of the sodium and potassium concentration gradients across the plasma membrane of cardiac fibres [11]. These ion gradients are essential to the mechanism by which electrical impulses are generated and conducted through the heart. By pumping sodium ions to the outside of the cell and potassium to the inside, the transport ATP-ase causes intracellular ion concentrations of approximately 150 mmol/l for K^+ and 10 mmol/l for Na^+ in contrast to extracellular concentrations of approximately 5 mmol/l for K^+ and 140 mmol/l for Na^+. Although this process is electrogenic it results in the nett removal of positive charges from the cell (3 Na^+ are transported to the outside for each 2 K^+ ions which enter the cell), the potential difference across the neuronal plasma membrane under resting conditions (the resting potential) is mainly due to the passive diffusion of potassium ions out of the cell along their concentration gradient, the cell membrane being far more permeable to K^+ than to Na^+ under resting conditions.

The magnitude of the resting membrane potential in most cardiac muscle fibres is approximately -90 millivolts inside relative to outside, although the fibres of the S-A node have a resting potential of only -55 to -60 millivolts. This feature of the S-A fibres makes them more susceptible to self-excitation, a property which is related to their role in controlling the rate of beat of the entire heart.

Cardiac fibres are electrically excitable, that is they can convert a small depolarisation (decrease in membrane potential) into a fulminant but rapidly self-terminating reversal of the membrane potential which is known as an action potential. In order that an action potential is generated, it is necessary that the small depolarisation should achieve a certain minimum or threshold voltage below which no action potential is elicited and at or above which the events leading to the evocation of an action potential occur.

Figure 4 shows a number of ion transport systems in the membranes of the myocardial cell. Important factors in the mechanism of action of digoxin and other cardiac glycosides are:

(1) The Na^+ and K^+-ATP-ase or sodium pump. This contains the binding site for cardiac glycosides, which inhibit its ion transport activity resulting in an increase in the intracellular sodium concentration which the pump normally works to reduce after an action potential has passed. This increased intracellular sodium concentration seems to activate

(2) the reversible sodium calcium exchanger, which exchanges intracellular sodium ions for the uptake of calcium ions into the cell in a ratio of 3 Na^+/1 Ca^{2+}. Thus the inhibition of the sodium pump by digoxin results in an increase in intracellular sodium. The sodium then exchanges with extracellular calcium via the sodium calcium exchanger stimulating cardiac contraction. Also shown in Figure 4 are

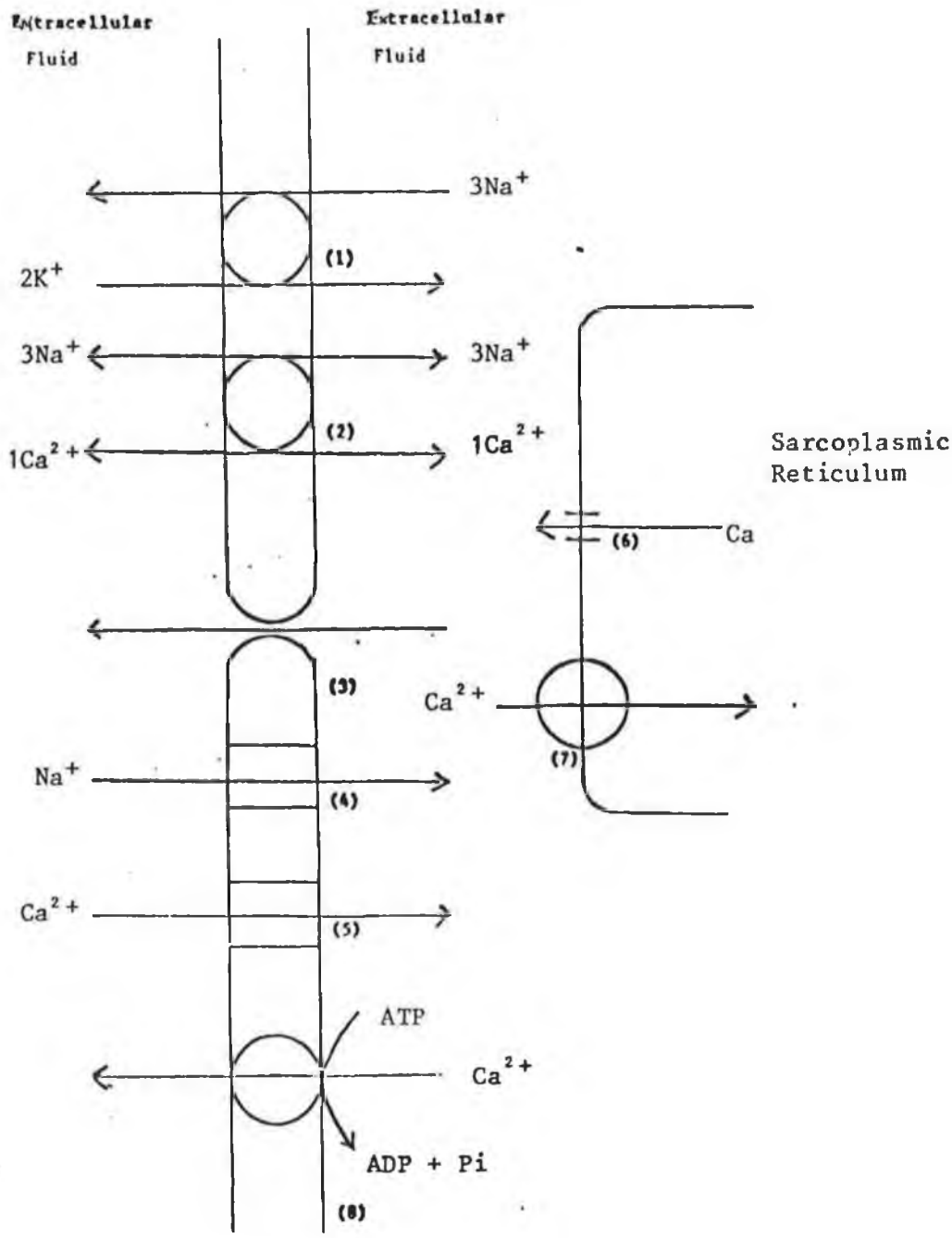
(3) the K channel through which K^+ ions leak out of the cell resulting in the resting membrane potential

(4) the voltage sensitive sodium channel whose opening in response to an impulse beginning in the S-A node results in depolarisation of the membrane due to an influx of sodium ions. This depolarisation leads to the opening of

(5) the slow calcium channel through which calcium normally enters the cell on stimulation. The influx of calcium ions causes the release of further calcium ions from the sarcoplasmic reticulum through its release channel

(6) the muscle cell is relaxed after stimulation by the removal of calcium into the sarcoplasmic reticulum or out of the cell by the ATP-dependent calcium pumps (7) and (8) respectively.

Figure 4: Some of the ion transport systems in the membranes of the myocardial cell.



If the threshold depolarisation is exceeded specific voltage-sensitive membrane proteins become open channels selectively permeable to sodium ions. Sodium enters the cell down its electrochemical gradient through these channels until the membrane potential is reversed. The voltage-sensitive sodium channels then close and assume an inactive conformation which cannot respond to another depolarisation for a length of time which is known as a refractory period. After this time, during which the membrane is resistent to stimulation, the channels resume their original closed but voltage-sensitive conformation. Sodium ions that enter the cell through the voltage-sensitive channels, travel to neighbouring portions of the membrane, depolarising them sufficiently to exceed the threshold voltage and initiate an action potential in them by causing a rapid influx of sodium through the voltage-sensitive channels. In this manner electrical signals are conducted along the fibre.

Coupling of electrical excitation of the fibre to contraction is mediated by an increase in the intracellular concentration of calcium ions (Figure 4) which, by binding to the protein troponin removes the inhibition of interaction of the contractile filaments of actin and myosin. In the relaxed muscle this inhibition is caused by troponin and another protein, tropomyosin, which is thought to cover the active sites on the actin filaments.

In the case of cardiac muscle fibres the source of this calcium is mainly extracellular but there may also be a contribution from the intracellular stores of the sarcoplasmic reticulum. This contrasts

with the situation in skeletal muscle which utilises intracellular calcium stores to release calcium into the sarcoplasmic fluid. Cardiac muscle, unlike skeletal muscle, may utilise the sodium ions gained during excitation to contribute to the increase in intracellular Ca^{2+} . It does this by means of the Na^{+}/Ca^{2+} exchange mechanism of the plasma membrane, exchanging internal Na^{+} ions for extracellular calcium. This system could operate, in addition to the voltage- dependent calcium channels of the plasma membrane, as a mechanism whereby the intracellular calcium concentration is increased.

Although the changes in sodium and potassium concentration in the cell caused by each impulse are minute and many action potentials can be conducted before the loss of transmembrane ion gradients affect the excitability of the cell, it becomes necessary with time to re-establish these transmembrane sodium and potassium concentration gradients. This is the function of the Na^{+}/K^{+} ATP-ase and it is this mechanism which is inhibited by digoxin.

A possible explanation of the positive inotropic effect of digoxin based on its inhibition of the Na^{+}/Ca^{2+} ATP-ase, involves the activation of the reversable plasma membrane Na^{+}/Ca^{2+} exchange system by the increased intracellular sodium concentration, with the resultant increase of intracellular calcium causing increased contractile activity.

Another interesting mechanism by which digitalis can modulate myocardial contractile force, at least under experimental conditions

in vitro is stimulation of the release and blockage of reuptake of noradrenaline from adrenergin (sympathetic) nerve terminals in intact myocardium.

Since the sodium-potassium ATP-ase is electrogenic and transports 3 Na^+ ions out of the cell for every 2 K^+ ions transported into the cell it tends to increase the membrane potential of excitable cells. It also prolongs the period of hyperpolarisation which follows each action potential and prevents re-excitation and the generation of another action potential. Thus, digoxin would be expected to shorten the time between action potentials, i.e. decrease the refractory period of excitable cells. What has been observed is that digoxin does decrease the refractory period in heart muscle cells, but in contrast to this effect, digoxin (and other cardiac glycosides) increase the refractory period and slow the conduction of action potentials in specialised cardiac conducting tissue such as the S-A node (in which the normal rhythmic self-excitatory impulse is generated) and the A-V node (in which the impulse from the atria is delayed before passing into the ventricles). These effects on the S-A and A-V nodes are mediated by the potentiating effect of digoxin on the vagal nerves. This is probably caused by the inhibition of monovalent cation transport in the nerves but detailed mechanisms are not well understood. Increased vagal activity causes the release of the neurotransmitter acetylcholine which has two major effects on the heart. First it decreases the rate of rhythm of the S-A node and second, it decreases the excitability of the A-V junctional fibres between the atrial musculature and the A-V node, thereby, showing transmission of the cardiac impulse into the ventricles.

The effects of acetylcholine are mediated by an increase in the permeability of the fibre membranes to potassium which allows rapid leakage of potassium to the exterior, resulting in hyperpolarisation which as mentioned above makes excitable cells much less excitable. The vagus nerves which supply the heart are distributed mainly to the S-A and A-V nodes, to a lesser extent to the muscle of the two atria and even less to the ventricular muscle. This may explain the contrasting effects of digoxin on the refractory period of working heart muscle and that of the specialised conducting fibres.

These actions of digitalis are used to advantage in the management of superventricular tachyarrhythmias, including atrial fibrillation or atrial flutter, both of which involve abnormal rapid contractions of the atrial muscle. By prolonging the refractory period of the A-V node, causing many of the atrial impulses which enter the node to be extinguished within it, digoxin reduces the ventricular rate. In the case of atrial flutter, a more serious condition than fibrillation, the abnormal contractions in the atrium are more co-ordinate, and by its non-uniform effects on the refractory period of atrial muscle, digoxin can convert atrial flutter to atrial fibrillation.

Digoxin has a slowing effect on the heart rate by its action on the S-A node, but it should be noted that digoxin's slowing action on the failing heart may be secondary to its improvement in circulation i.e. digoxin causes a reduction in compensatory tachycardia.

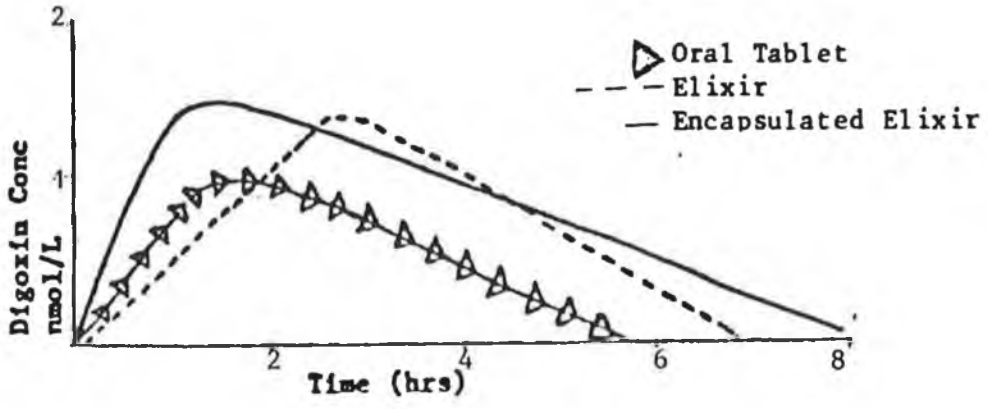
At toxic levels, digoxin can also trigger arrythmias by generating ectopic beats or by blocking A-V transmission resulting in dropped beats. Other arrythmias may result from increased vagal activity.

1.7 *PHARMACOKINETICS:*

(a) *ABSORPTION:*

The degree to which digoxin is absorbed and made available to the systemic circulation varies markedly from one preparation to the next and many reports of toxic digoxin concentrations can be due to a patient changing from one formulation to the next [12-14]. The marked difference in digoxin concentrations is due to the different dissolution rates of the tablet given [15].

Figure 5: The time curve of the serum concentration of the three different preparations of digoxin given orally to one patient.



Studies show that a mean of 67% of digoxin administered as an oral tablet is absorbed from the gastro-intestinal tract in 60-90 minutes after administration and the maximum therapeutic effect is apparent in 3-6 hours [16]. Absorption of digoxin after oral administration of an elixir accounts for only 80% of the administered dose.

Absorption from intramuscular injection sites is variable and incomplete. Intramuscular injection should be avoided as this can cause pain and tissue necrosis [16]. The absorption of encapsulated elixir is 90-100%.

After oral administration of digoxin the blood concentration rises, the rate at which it increases depends on the absorption. During the absorption phase a time is reached when the rate of digoxin output equals digoxin input. At this point a maximum concentration of digoxin is reached in the serum. Subsequently, the concentration falls as a result of the output of digoxin being greater than the input. The area under curve A or B is a measure of the degree of absorption (i.e. the bioavailability).

A reduction in digoxin absorption may also be caused by a number of factors including drug - drug interactions and malabsorption syndromes [16] (Table 2). Malabsorption of digoxin may be caused by motility- stimulating drugs such as metoclopramide. Increased absorption of digoxin may occur as a result of anti-cholinergics which decrase intestinal motility [17]. In approximately 10% of patients antibiotics may increase its absorption by preventing its hydrolysis by intestinal bacteria. Although these and other diseases account for some altered digoxin absorption it has been found that some normal individuals have absorbed as little as 48% of orally administered digoxin tablets [18].

Table 2: Factors influencing absorption of digoxin tablets.

FACTOR	EFFECT
Reduced gastro-intestinal motility	Increased absorption
Fast dissolution rate	Increased absorption
Increased gastro-intestinal motility	Decreased absorption
Sprue	Decreased absorption
Hyperthyroidism	Decreased absorption
High fibre diet	Decreased absorption
Slow dissolution rate	Decreased absorption
Prior food intake	No effect

(B) *DISTRIBUTION:*

After absorption of the drug into the systemic circulation it is distributed to the various body fluids and tissues. The distribution of digoxin after intravenous injection or infusion of the drug can be described by a model which consists of two pharmacokinetically distinct compartments [19]. The first rapid decrease in concentration is due to the dilution effect of the circulation and requires a few minutes to complete with a half-life of 2 minutes. The second stage is the distribution phase (α) during which digoxin reaches an equilibrium between the central and peripheral compartment [20]. The serum digoxin concentration reflects the cardiac activity during this stage. The central compartment can be assumed to represent plasma and highly perfused organs such as liver and kidneys. The peripheral compartment represents the deeper tissues, especially skeletal muscle and myocardium. The largest concentration of digoxin is stored in skeletal muscle even though digoxin concentration is less in skeletal muscle than in myocardium during maintenance therapy. The ratio of the plasma and heart concentration of digoxin lies between 1:30 and 1:200 [21-22].

Digoxin is widely distributed in the tissues as shown by the large apparent volume of distribution of about 6*l*/kg. The extent to which the drug is distributed in the body at equilibrium (i.e. when output is equal to input) is called the apparent volume of distribution, and thus is defined as the volume of body water which would be required to contain the total body digoxin concentration at the concentration present in plasma. The apparent volume of distribution varies from

about 3-10 *l*/kg body weight [23]. This shows the extent of digoxin binding to tissues.

In renal failure the apparent volume of distribution of digoxin can be reduced [24], with a 33% to 50% reduction in patients with severe renal impairment. It is also altered in thyroid disease, being decreased in hyperthyroidism and increased in hypothyroidism. The time required to complete the distribution phase after oral digoxin tablet administration is between 8-12 hours [25]. When serum digoxin concentrations are estimated for evaluating cardiac response the blood sample should be drawn at least eight hours and, and preferably, eighteen hours after drug administration. Samples taken prior to this will show falsely elevated serum concentrations. Once the distribution phase is complete drug elimination from the body begins to occur.

(c) *METABOLISM:*

Until recently it was assumed that digoxin was not significantly metabolised [26]. Today it is accepted that extensive metabolism can occur. The metabolism of digoxin may include saturation of the lactone ring to form a low cardioactive compound called dehydrodigoxin. It has been suggested that bacteria present in the bowel are responsible for this reaction and it may be reduced in people on antibiotics. Consequently, the route of administration will govern the extent of conversion to these compounds. The metabolism can also involve the stepwise removal of three sugar moieties to form digoxigenin followed by epimerization of the 3-

β-hydroxyl to the 3-α position and conjugation to give 3-epi-glucuronide and 3-epi-sulphate [27-28]. These are shown in Figure 6.

Metabolism of digoxin to either the dihydrometabolites or the digoxigenin conjugates renders digoxin cardioinactive, however, both the mono and di-sugars are cardioactive. There is wide interindividual variations in the metabolism of digoxin with some individuals demonstrating a large biotransformation of the drug [29]. A study involving both an RIA and combined RIA chromotography technique used serum from nineteen patients on digoxin. The results show that metabolites accounted for between 1-99% of the radioactivity measured. Thus, the method chosen for digoxin estimation must be specific.

Figure 6: Digoxin Metabolism

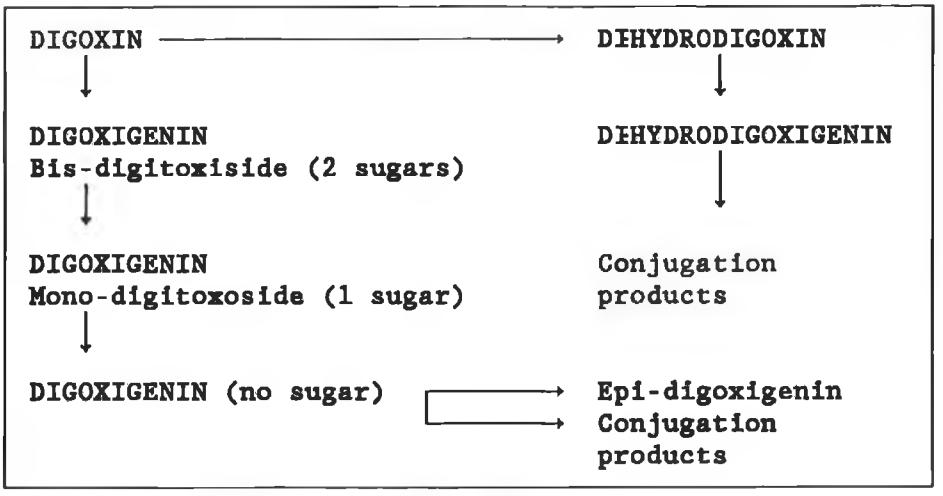
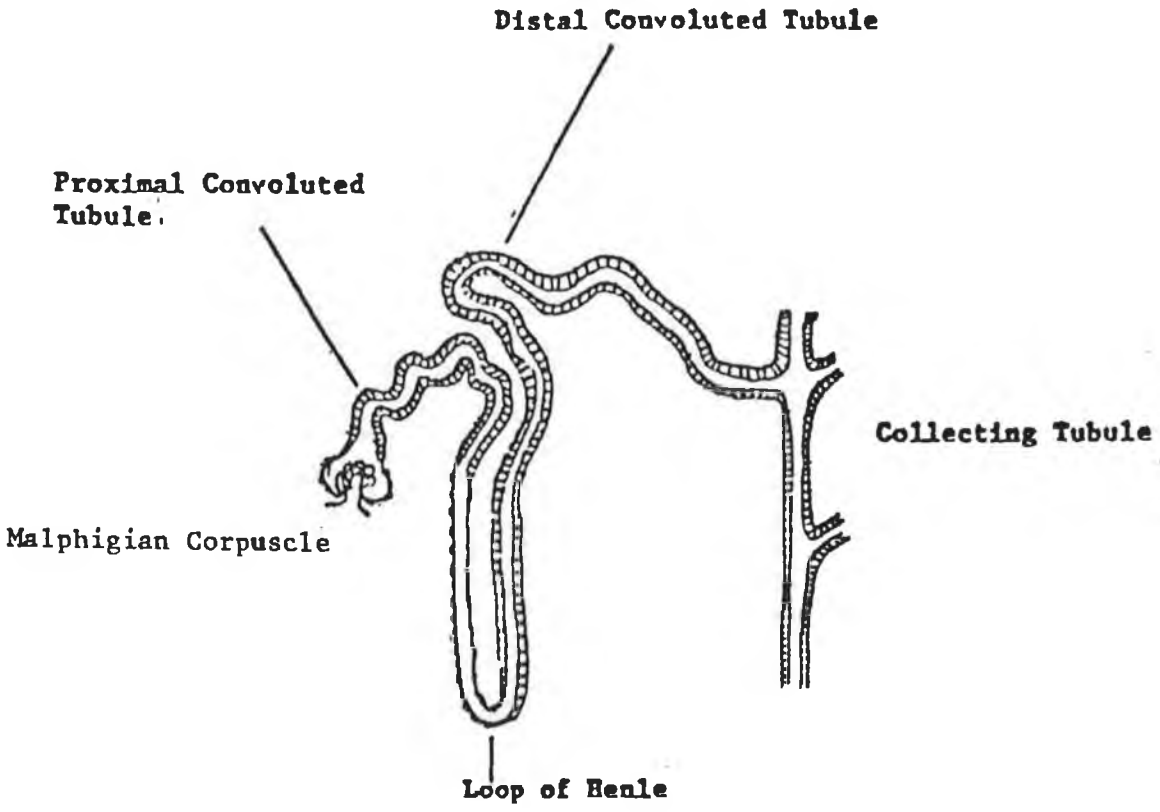


Figure 7: The Nephron.



(d) *SECRETION:*

Digoxin is excreted by the kidneys both as the unchanged digoxin and its metabolites. Digoxin which circulates free (unbound) is filtered by the renal glomerulus (Figure 7).

Some of the digoxin in the glomerular filtrate is passively reabsorbed by the proximal tubule and there is also some secretion of digoxin by the cells in the distal tubules into the glomerular filtrate [30-31]. In some cases the amount of digoxin absorbed equals that secreted so that in these individuals the renal clearance is equal to the rate of glomerular filtration of the fraction of digoxin which is unbound. Patients with renal failure have decreased renal digoxin clearance and in many cases the renal clearance of digoxin can be calculated from creatinine clearance [32]. However, in some patients with congestive cardiac failure and prerenal azotemia, renal excretion of digoxin is reduced due to an increase in proximal tubular reabsorption from the glomeruler filtrate [33].

Drugs such as quinidine and spironolactone inhibit renal clearance of digoxin. Quinidine [34] inhibits distral tubular secretion of digoxin, resulting in decreased renal clearance. It also decreases the apparent volume of distribution of the drug. This causes an increase in plasma digoxin leading to an increased incidence of digoxin toxicity. Spironolactone also inhibits renal tubular secretion of digoxin by approximately 25%.

Complete elimination of digoxin from the plasma involves both renal excretion and hepatic metabolism. Some years ago it was believed that digoxin was eliminated primarily by the kidneys (60-80%). However, recent investigations have demonstrated that dihydrometabolites can account for a major part of digoxin excretion. It has been shown that metabolism accounts for 75% of total elimination [24] in patients with severe renal impairment.

(e) *HALF-LIFE:*

The half-life of digoxin in patients with normal renal function reportedly varies between 26 and 45 hours [35] and is prolonged to 92 hours in anuria. This means that in a patient with renal failure it will take longer to achieve a steady state during maintenance therapy and longer for excretion of digoxin than in patients with normal renal function. Pharmacokinetic variables related to digoxin are summarised in Table 3.

Table 3: Some pharmacokinetic variables related to digoxin.

Oral Dose Absorbed %	48-85
Protein Bound %	20-25
Half-Life Adults	26-45 Hours
Children	11-50 Hours
Time To Reach Peak Plasma Concentration	1.5-5 Hours
Time To Reach Steady State (Adults)	7-11 Days
Time To Reach Steady State (Children)	2-10 Days
Apparent Volume of Distribution	3-10 Kg/L
Effective Blood Concentration	1-2.5 nmol/L
Toxic Blood Concentration	> 2.5 nmol/L

1.8 *INDICATIONS FOR MEASURING PLASMA DIGOXIN:*

Digoxin may produce either therapeutic, toxic or lethal effects according to the dose administered. The therapeutic effect is seen as an improvement in the efficiency of myocardial contraction without requiring a simulaneous increase in oxygen. The toxic and lethal effects of digoxin show themselves as ventricular arrhythmias which are increasingly severe and may possibly be fatal with increasing dosage.

Proper management of digoxin dosage and avoidance of toxic conditions is a complex and difficult task. The response of an individual patient may change over a period of maintenance dosage. Changes in response to digoxin therapy arise from two causes:

(a) *CHANGES IN PATIENTS' SENSITIVITY:*

In thyroid disease plasma digoxin concentrations are altered by two different mechanisms - by a change in the apparent volume of distribution and by a change in renal elimination. Thus, in hyperthyroidism the apparent volume of distribution is increased and renal clearance is increased. These effects result in lower plasma digoxin concentrations both after a loading dose and at steady state. In some patients with renal failure the apparent volume of distribution is reduced and the extent of reduction seems to be related to the degree of renal impairment. An increased sensitivity of digoxin can be noted in states of hypokalaemia, hypomagnesaemia and hypercalcemia which make establishment of the true therapeutic

concentration of digoxin difficult. Digoxin concentration may be affected due to interference by co- administration of other drugs such as quinidine, aspirin, quinine and acetominophen. Serum levels of digoxin have been shown to be increased significantly by the first three of these drugs. Serum creatinine levels were not affected by administration of these drugs. It appears likely that the increases are due to a reduction in digoxin clearance.

(b) *MODIFIED ABSORPTION AND EXCRETION OF THE DRUG:*

This may result in sudden changes in circulatory levels of digoxin during a maintenance regimen. In patients undergoing sudden alterations in renal functions over or underdosing may occur. In this situation the serum digoxin along with clinical correlation with the desired therapeutic effect is desirable. Modified absorption may also be due to variable bioavailability of digoxin from different pharmaceutical drug preparations. Gastrointestinal problems may affect absorption of orally administered digoxin. Rapid gastrointestinal tract transit may result in a low serum digoxin concentration whereas delayed gastric emptying may result in better absorption of the drug due to more thorough dissolution.

It has been estimated that between 11-36% of patients assumed to be on appropriate maintenance doeses of digoxin were found to be underdigitalized. One reason for this may be due to lack of compliance. The serum digoxin concentration will help to establish whether the patient is failing to take his medication properly. Patient compliance is a major determinant of serum digoxin

concentration [36]. If a patient who claims to be taking his medication shows a low serum concentration of the drug the doctor is alerted to the need for further studies or perhaps further discussion about compliance with the patient.

The ratio of toxic dose to therapeutic dose for a drug is known as the therapeutic index. For digoxin this index is small (approximately 2 to 3). There also exists the possibility that a dose which is therapeutic for one patient may be toxic for another. Unfortunately the symptoms and signs of digoxin toxicity such as headache, nausea and bradycardia are not specific. A reduction in symptoms and signs when digoxin is stopped helps to establish the diagnosis of digoxin intoxication.

Chapter 2

DIGOXIN CONCENTRATION MONITORING

2.1 *INTRODUCTION:*

There are a large number of methods available for the measurement of digoxin in plasma. Over the last number of decades digoxin methodologies have changed with advances in instrument technology. Some of these methods have also been modified for salivary digoxin measurement.

Prior to 1949 the effect of digitalis activity on intact animals such as cats and guinea pigs was used to determine the minimum lethal dose required to induce vomiting. These methods were insensitive and were used only for assaying tablet content of the drug [37].

Since 1949, many different methods have been described. Although there is a wide choice most assays can be assigned to one of three groups:

2.2 Chromatographic techniques which include T.L.C., G.L.C. and H.P.L.C.

2.3 Bioassays

2.4 Immunoassays.

2.2. *CHROMATOGRAPHIC TECHNIQUES:*

2.2.1 *Thin Layer Chromatography:*

T.L.C. involves a thin layer of silica gel uniformly spread onto glass plates, plastic sheets or other suitable inert materials [38]. The sample to be analysed is concentrated in a suitable solvent and added as a concise spot or streak near an edge of the plate which is then placed in a closed glass container with the lower edge of the plate in contact with the mobile phase. Although the major factor in determining the chromatographic properties of digoxin is its molecular structure, important modifications are brought about by changes in the composition of the solvent system. This results in changing the retention values of the components in the mixture.

The next stage in T.L.C. is to detect the position to which the spots have run. This may be achieved by using standards, dyes, or Rf values. The ratio of the distance that a specimen spot has moved from the point of application to the solvent front is called the Rf value. One major difficulty is the quantitative assessment of the spots. Quantitation of components separated from the mixture can be carried out on the plate or after the spots containing the components have been scraped from the plates and eluted from the absorbent. Quantitation in situ can be performed using a densitometer.

Since T.L.C. on silica gel was first used to separate cardioactive glycosides [39] numerous T.L.C. systems have been published. It was recognised that separation of complex mixtures of cardioactive glycosides could not be achieved by conventional T.L.C. [40] and

improved resolution was obtained by using two dimensional T.L.C. Further improvements were introduced through the use of a continuous development technique which involved a trough filled with absorbent material attached to the top edge of the T.L.C. plate to take up the mobile phase [41].

In 1972 [42] a method was described which separated digoxin, digitoxin and their metabolites by paper chromatography and then by T.L.C. The spots were identified by their Rf values and then further characterised by G.L.C. equipped with an electron capture detector after making volatile derivatives. This has a sensitivity of 25 picomoles/l of digoxin in plasma.

T.L.C. is a qualitative test for digoxin. Quantitation from the T.L.C. plates is limited by the amount of digoxin which can be isolated, the degree of contamination with other components and the loss of the component because of incomplete elution from the absorbent. T.L.C. systems are simple and economical in that they require no expensive equipment outlay. However, the drawback to this method lies in the technical capability and experience needed to interpret the chromatograms produced as the plate is sprayed with various reagents. The results depend upon subjective interpretation and the metabolites of digoxin present in the sample may produce interference.

T.L.C. offers advantages in terms of cost over all other chromatographic processes. However, it will remain an unsuitable method for digoxin analysis until such time as the procedure is automated.

2.2.2 *Gas Liquid Chromatography:*

G.L.C. is a process in which a mixture of compounds in volatilized form is separated into its constituent components by using a mobile phase over a stationary phase [43]. Therefore, the chromatography has to be performed at high temperatures which are maintained by placing the column in an oven. The mobile phase is an inert gas (hydrogen or helium) that carries the sample extract over a liquid stationary phase in the column. The stationary phase is packed into a column in which one end is connected to an injector where the sample and inert gas are introduced. The other end of the column is connected to the detector.

The chromatographic separation is achieved by diffusion, partitioning, or adsorption between the mobile gaseous and the stationary liquid phases. The retention time for digoxin is the time it takes for the peak to emerge after injection. The retention time of digoxin can be increased or decreased by adjusting the temperature of the oven or by using liquid phases of differing polarity.

Most G.L.C. systems for digoxin use an electron capture detector which responds proportionately to the concentration of digoxin in the sample. Mass ionization detectors are very sensitive but their cost and maintenance requirement prohibit their use in most clinical laboratories. Nitrogen phosphorous detectors are also very sensitive and can detect picomole levels of the drug.

As most digoxin levels are performed using serum or plasma an extraction procedure is necessary so as to separate the drug from the

supporting matrix. This has the advantage of separating digoxin from its metabolites. The specificity of most chromatographic methods makes them suitable for digoxin estimation. However, because of the low concentration of digoxin in serum, sensitivity may still cause problems. In order to improve sensitivity large sample volumes or derivitization is required.

In 1971 a GLC method [42] was introduced in which the serum was extracted using methylene chloride. The extract was derivitized with heptafluorobutyric anhydride followed by T.L.C. which was then subjected to G.L.C. using an electron capture detector. Tritiated digoxin was used as an internal standard. The method required 5ml of plasma and up to five hours of analysis time and consequently, was not very suitable for routine laboratory work except for use in research. The sensitivity was 0.52 nmol/L with a CV of 11% on a specimen containing a digoxin concentration of 3.5 nmol/L. The advantages and disadvantages are summarised in Table 4.

In general G.L.C. with its present refinements has much to offer in therapeutic drug monitoring. It is however, not the method of choice for digoxin measurement.

Table 4: Some characteristics of the G.L.C. technique.

ADVANTAGES	DISADVANTAGES
Good precision	Extraction and chromato-graphy required
Stat analyses	Large sample size
Cheap reagents	Not suitable for paedaitric samples
Semi-automation	Sequential analyses
Very specific	Gas supplies required
Very sensitive	High degree of expertise required
	Equipment expensive

2.2.3 *High Performance Liquid Chromatography:*

In H.P.L.C. [44] digoxin and its metabolites in a specimen are separated on the basis of the different molecular functional groups of each compound by selective interaction between the two phases. Reverse phase H.P.L.C. is the most widely used form of H.P.L.C. for digoxin estimation and it requires a non-polar stationary phase. The most popular reverse phase column packing is the octadecyl type in which a C_{18} hydrocarbon is covalently bonded to silica particles and packed into a narrow tubular steel or plastic column.

Separation depends upon the affinity of the compound with the two phases. Compounds with strong affinities to the stationary phase will emerge after those having affinity towards the mobile phase. The mobile phase consisting of a mixture of solvents feeds into a pumping system capable of delivering uniform pulse-free flow to the chromatographic column. The injector is installed between the pump and the column. This does not disturb the flow system. The other end of the column is connected to a short flow-through cuvette in a detector. The detector uses either uv light, fluorescence or chemical ionisation analysis. The signal is recorded on a strip chart recorder or an electronic integrator. Qualitative and quantitative analysis is performed by measuring the height or area of the peak recorded. Good sample preparation is very important in H.P.L.C. so as to avoid column plugging. In 1975 [45] a H.P.L.C. method for the separation of digoxin and its metabolites was described. This involved an isocratic system using mixtures of acetonitrile and water as mobile phase for the separation of digoxin, bis- and monodigitoxosides and digoxigenin. A gradient system was

also used. Separation of the compounds was accomplished in less than thirty minutes. The sensitivity of the method used in conjunction with a uv detector (220 nm) was 5.2-26 nmol/L thus this method was unsuitable for therapeutic drug monitoring in which the therapeutic range for serum digoxin is 1.3-2.6 nmol/L.

Since H.P.L.C. is performed at ambient temperature the composition of digoxin and its metabolites is not a problem. Thus each of the compounds can be collected separately as they elute from the column. Once the compounds have been separated they may then be subjected to quantitation by other more sensitive methods.

In 1980 such a method involving H.P.L.C. and RIA was used to measure digoxin [46]. Digoxin was extracted using ^{3}H digoxin as an internal standard. This method only required 1 ml of plasma and CVs of 6-10% were obtained in plasma samples containing 0.65 and 3.25 nmol/L of digoxin compared with a CV of 4% for RIA. In 1985 a method was described which involved small column extraction of serum, combined with H.P.L.C. and RIA of the eluted fractions [47]. Using digoxin standards of 1.3, 0.65 and 0.13 nmol/L analytical recoveries of 95%, 93% and 84%, respectively, were achieved. The method gave CVs of 4-6% for sera with values of 0.65-1.3 nmol/L. The limitations of the method was that dihydrodigoxin eluted simultaneously with digoxin. Finding a suitable internal standard for the method was also difficult. It was costly on account of the fact that ten one ml fractions of the H.P.L.C. were required to define the peak for digoxin and determine the concentrations in each fraction by RIA. None of the endogenous DLIFs or drugs showed a peak with the retention time of digoxin.

Pre-H.P.L.C. column derivitization of digoxin with chemicals to increase uv absorbance or for fluorescence detection has been useful in explanations of drug metabolism and excretion, but detection limits are still too high for the picomole amounts of digoxin that are present in reasonable volumes of serum [48-50]. Post H.P.L.C. column reaction with hydrochloric acid to produce a fluorescent species has permitted quantification in serum sample volumes of 3ml [51] and 0.5ml [52] but requires elaborate instrumentation. Some H.P.L.C. methods are shown in Table 5. In cases in which patients have renal failure or liver dysfunction, in neonates or third trimester pregnant women, digoxin immunoassays usually yield unreliable results. The use of chromatographic analysis in these cases, combined with an immunoassay method will give improved results [47].

In general H.P.L.C. procedures are labour intensive and time consuming involving an extraction and chromatographic step. The large sample size requirement may not make it suitable for paediatric analysis. In order to introduce such a method into a small laboratory a great amount of time in training laboratory staff and expenditure in new equipment is required.

Table 5: Evaluation results using different detection systems in combination with H.P.L.C.

DETECTION PRINCIPLES	PRECISION	SENSITIVITY	SEPARATION	REF
RIA	0 - 10%	0.65 nmol/L	Extraction	46
Fluorescence	4 - 6%	0.29 nmol/L	Extraction and Derivitization	51
Fluorescence	5 - 7%	0.29 nmol/L	Extraction and Derivitization	52
RIA	4 - 6%	0.29 nmol/L	Extraction	47

2.3 *BIOASSAYS:*

A bioassay is normally used for measuring drug levels in which the drug itself may not be in pure form. As the drug may exist in impure form bioassays can be used to measure the actual response of the drug.

ERYTHROCYTIC ^{86}Rb UPTAKE INHIBITION ASSAY:

This bioassay for digoxin measures the inhibition of red cell rubidenium 86 uptake into the red cell [53]. The method has been used both in research and as a routine method for digoxin, digitoxin and cardioactive metabolite estimations. The assay depends on the fact that digoxin inhibits Na^+/K^+ ATP-ase on the red cell membrane and consequently the transport of ^{86}Rb into the red cells. The drug is extracted from plasma using methylene chloride and the extract incubated with human red cells. The ^{86}Rb uptake is quantified using a standard digoxin calibration curve. Some of the limitations of the method are shown in Table 6. However, as the assay measures total cardioactive glycosides (that is digoxin and its metabolites which inhibit Na^+/K^+-ATP-ase) more useful information is obtained than that for immunoassays which use antibodies with different specificities and varying degrees of cross- reactivity for digoxin, its metabolites and DLIFs. However, comparisons between ^{86}Rb technique and RIA methods have shown good correlation. In one study of 100 patients good correlation between 72 patients was found [54] and a valid reason for discrepancy between 22 of the 28 remaining cases existed.

Table 6: Limitations of the Erythrocyte ^{86}Rb uptake inhibition assay.

(a) Extraction required
(b) Sample volume - 1 ml
(c) Measures digoxin and digitoxin
(d) Time consuming - up to seven hours
(e) Interference due to Na^+/K^+ - ATP-ase inhibitors
(f) Washed erythrocytes required
(g) Sensitivity 3.9 nmol/L
(h) Precision 6-10%

In another study of 27 patients [55] there was a good correlation (n=0.83) between 23 of the cases with the remaining 4 showing wide discrepancy.

In 1972 a receptor assay was developed [56]. This method used displacement of ^{3}H-ouabain from ATP-ase rich cardiac muscle homogenates from guinea pigs to estimate digoxin in a competitive protein binding assay. This technique assayed cardioactive glycosides present in the serum. The method had the advantage of measuring digoxin and its metabolites relative to their cardioactivity. It did not distinguish between cardiac glycosides so it was important to know which digitalis preparation the patient was on. Otherwise a maintenance digitoxin level (13-39 nmol/L) might be interpreted as a toxic concentration for serum digoxin. Table 7 shows some of the characteristics of the method.

Recently a study using ATP-ase receptors from dog kidney were used but it had limitations similar to the last method [57]. In a receptor technique recently developed ATP-ase receptors from human heart were used [58]. This method gave very good precision and sensitivity of 7 nmol/L. This may be due to the greater concentration and affinity of ATP-ase receptors in cardiac muscle. The cardioinactive metabolites do not participate in the reaction and the cardioactive metabolites are detected relative to their cardioactivity.

Table 7: Some characteristics of the receptor assay.

(a)	Extraction and competitive protein binding assay
(b)	Sample size 5ml
(c)	Sensitivity 0.26 nmol/L
(d)	Exact specificity not found
(e)	Measures ouabain, digoxin and digitoxin
(f)	Lability of enzyme a problem
(g)	As convenient as immunoassay methods except for sample size and complicated extraction step

A sample pretreatment step using sep-pak C_{18} cartridges is required to remove DLIF. However, the method has not been fully evaluated yet.

2.4 *IMMUNOASSAYS:*

The first immunoassay for a drug was described for digitoxin in 1968 [59]. Since then several types of immunoassays depending on the detection system used have been described.

In this review, the following types of immunoassays applicable to serum digoxin determination are discussed.

2.4.1 Radioimmunoassays

2.4.2 Enzymeimmunoassays

2.4.3 Fluorescenceimmunoassay

The various assays may be further classified on the basis of (1) which reactant is to be measured; (2) which reactant is labelled (antigen or antibody); (3) whether the reaction is competitive or non-competitive; and (4) whether the assay is homogenous or heterogenous.

Homogenous assays do not require a separation step to distinguish bound from unbound digoxin. The property of the labelled molecule is regulated by the antibody - antigen reaction such that separation of free and bound label is not required. Heterogenous assays for digoxin require a separation step to separate bound labelled digoxin from free labelled digoxin.

2.4.1 *Radioimmunoassays:*

The RIA method [60] for digoxin was developed in 1969 by building on the work of Barson and Yalow [61] who prepared digitoxin antibody. Digoxin is a small molecule and, therefore, rarely able to induce antibody formation by itself; for this reason, it was conjugated to bovine serum albumin [60]. The conjugate is formed by periodate oxidation of the vicinal hydroxyl groups of the enol sugar to form aldehyde groups which react with the amino groups of bovine serum albumin. Thus, the conjugate linkage is through the carbohydrate moiety of digoxin. The antibody induced by this conjugate is mainly directed against the steroid part of digoxin. Consequently, digoxigenin, bis- and monodigitoxide and digoxigenin all react with the antibody, whereas dihydrodigoxigenin and dihydrodigoxin, each with C 22 reduced, exhibit little or no cross-reactivity. Indeed for some antisera the carbohydrate deficient metabolites are more potent antigens than is digoxin itself, the additional carbohydrate units of digoxin in some way reducing the antibody binding capacity. The cross-reactivity of the metabolites of digoxin with the anti-digoxin antibodies in immunoassays is well known and for most commercial anti-digoxin sera the degree of cross-reactivity is stated. Cross-reactivity occurs with other medications such as spironolactone, hormones such as progesterone, testosterone, dihydroxye-piandrosterone, and compounds with other steroidal configurations. Interferences, caused by peptides, fatty acids and DLIFs, (Chapter 3), have been demonstrated.

Prior to immunization the conjugate was mixed with one of the many adjuvants such as Freund's or acrylamide gel and injected into a

rabbit or goat. Injections were repeated until the animal produced a specific antibody against the digoxin moiety. The antibody was then labelled with an isotope. In the original RIA method ^{3}H, which is a β-emitter, was used. Its use was discontinued due to interference caused by haemolysis, hyperbilirubinemia, hypertriglyceridemia and autoluminescence in the serum of uraemic patients. The interference was due to light photons produced as a result of the effect of β-emissions on the scintillation solvents being either totally or partially absorbed. Instead I^{125} labelled digoxin, which emits gamma rays and is unaffected by colour and chemical quenching, was used.

In general, RIA systems are heterogenous. When applied to digoxin these assays may be (a) competitive; (b) non-competitive (sequential method); (c) solid phase or fluid phase; or (d) antigen labelled.

(a) *Competitive RIA:*

This is a radioimmunoassay where I^{125}- labelled digoxin competes for a fixed time with digoxin in the patient sample for antibody sites. The concentration of the antibody labelled digoxin is inversely proportional to the serum digoxin concentration (Figure 8).

(b) *Sequential Method:*

Unlabelled antigen is first mixed with excess antibody until equilibrium is reached. The next step involves the addition of labelled antigen and after a suitable incubation time the bound and free counts are determined after separation. This results in a higher fraction of serum digoxin binding by the antibody than in a

competitive assay where the ultimate sensitivity limit is dictated by the affinity constant of the antibody.

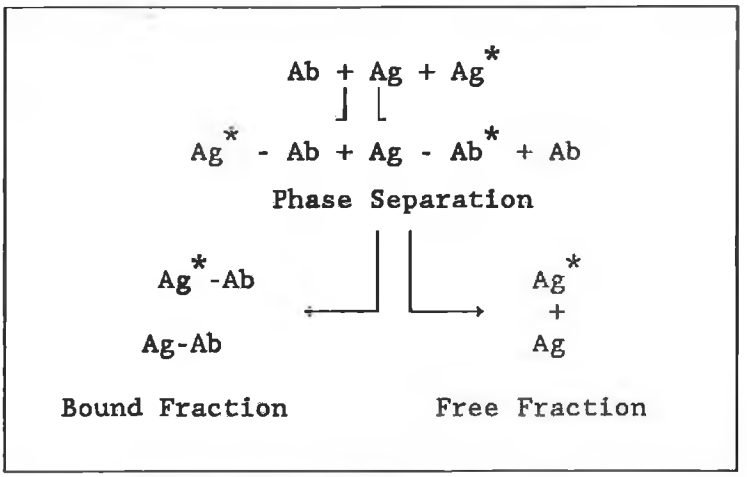


Figure 8: **Principle of RIA. A limited number of antibody binding sites (Ab) a constant amount of radiolabelled antigen (Ag*) and different amounts of antigen (Ag) are incubated and a suitable technique is used to separate the free and bound antibody fractions at equilibrium.**

(c) *Phase Separation Method:*

The phase separation method involves the separation of the free labelled digoxin from the bound using three different techniques:

(i) in the first technique separation is effected by adsorption of the free digoxin. Adsorbents used include charcoal, dextran coated charcoal, ion exchange resin and talc.

(ii) the second type of separation involves precipitation in which the bound antigen is precipiated from the solution by using a protein precipitant such as polyethylene glycol or ethanol. A second antibody can be used to precipitate the bound primary antibody-digoxin complex. This has the disadvantage that it requires longer incubation times and additional steps.

(iii) This method uses solid phase antibodies. The solid phase may be the inside of a tube wall or antibody conjugated by a covalent bond to insoluble inert materials such as glass, or plastic beads. After incubation, the contents of the tube are centrifuged, the supernatant aspirated and counts are performed on the solid phase antibody-antigen complex in the bottom of the tube. The coat-a-count and Pharmacia RIA digoxin kit uses anti-digoxin antibodies coated on the walls of tubes and immobilized on sephadex particles, respectively.

RIA Techniques:

The first RIA method for measuring cardiac glycosides was performed on digitoxin [59]. This method required 5ml of plasma and a prior extraction step. The assay took 36 hours due to the time taken to separate free from bound antibody using second antibody. An improved

method for measuring digoxin was introduced in 1969 [60] which used dextran- coated charcoal to separate the two phases instead of second antibody. This assay could be performed with 1ml of plasma within 1 to 2 hours. Replicate analysis gave a CV of 4% within the digoxin range of 0.65-13.0 nmol/L. The antibody used in this assay gave a cross- reactivity of 10% with digitoxin. Since the therapeutic range of digitoxin is ten times that of digoxin it can also be used to measure it [61]. In 1970 a different type of protein digoxin-conjugate was used to produce antibody which shortened the incubation time to forty minutes [62].

A number of clinical studies were carried out in the early 1970s in which it was confirmed that an overlap existed between toxic patients and those who were free of symptoms. However, the mean values between the two groups were different.

The problems caused by ^{3}H-labelled digoxin when used as a tracer resulted in the introduction of various I^{125}-labelled digoxin derivatives using different conjugation procedures. One such method [63] was described which used dextran-coated charcoal as phase separator. This method had a sensitivity of 0.26 nmol/L and required a sample volume of 50μl. The I^{125}-label using gamma rays shortened the assay time to 10 seconds as compared with 3 minutes for ^{3}H-digoxin using β-radiation. This made emergency assays possible and established this type of immunoassay as the method of choice for measuring plasma digoxin. A number of papers were published which demonstrated the efficacy of digoxin levels in the differential diagnosis of patients thought to have toxic levels, resulting in many laboratories providing a therapeutic drug monitoring service for

digoxin. Subsequent to this a number of different commercial kits for measuring digoxin, some using ^{3}H and others I^{125}-labelled digoxin, were introduced. A number of comparative studies involving RIA kits and other methods for digoxin have been described and are shown in Table 8.

In recent years, RIA has been modified for automation [64] and speed by immobilizing the antibody on a porous pot. The serum sample is mixed with a known amount of labelled digoxin and is then passed through a column containing the immobilized antibody. Free labelled antigen is measured and the bound labelled antigen is released by chaotropic agents and quantified.

In RIA,phase separation can cause problems. The use of antibodies with high dissociation kinetics of antibody-digoxin complex may give erroneous results when used in conjunction with charcoal. This is due to the continuous variation in the equilibrium reaction of the antibody-digoxin complex caused by charcoal adsorption of the free digoxin form. Thus, the bound fraction will decrease with increasing exposure to charcoal. Therefore, it is important that charcoal contact time be kept constant for digoxin standards and unknown.

Solid phase antibodies cause non-specific binding and alteration in reaction kinetics when compared to reactions in an aqueous medium.

Table 8: Comparison of RIA with other immunoassay detection systems.

COMPARISON OF RIA WITH OTHER METHODS							
Method	X	Y	N	S	i	r	Reference
	CA (RIA)	Stratus	108	1.08	0.057	0.968	Dade Data
	NEN (RIA)	Stratus	58	1.02	0.004	0.964	Dade Data
	CA (RIA)	Beckman	180	1.07	0.19	0.93	CC.1985:31:929
	RIA	TDX	84	0.917	0.176	0.96	CC.1988:34:1251
Corning	RIA	TDX	118	0.91	0.149	0.959	Abbott Data

X = RIA

Y = Other Detection Systems

N = Number of Patients

S = Slope

i = Intercept

r = Correlation

In 1987, an RIA method [65] involving a second antibody was described in which sheep anti-digoxin antibody is the primary antibody and donkey anti-sheep antibody is the precipitating agent. This method has the disadvantage that it requires longer incubation times and additional steps. Further advantages and disadvantages of RIA are summarised in Table 9.

Table 9: Characteristics of the RIA method.

(a)	Heterogenous method
(b)	Sample volume less than 0.5ml
(c)	Assay time - 1 hour
(d)	Sensitivity - 0.26 nmol/L
(e)	Shelf life 6-8 weeks
(f)	Radiation hazards
(g)	More complicated technically than EMIT
(h)	Gamma counter required
(i)	Pre-treatment step in some RIA assays only
(j)	Elimination of DLIFs in RIA not as successful as in EMIT [72]
(k)	Specificity - digitoxin metabolites may cause interference with digoxin antibody
(l)	Expensive

2.4.2 *Enzyme Immunoassays:*

EIAs were first used in the detection and quantitation of plasma analytes in 1971 [66]. Since that time numerous papers have been published describing different types of immunoassays.

The catalytic properties of enzymes enable small quantities of digoxin to be measured. The sensitivities of EIAs approach that of RIA. The antibody preparation and immunochemical reactions are similar to RIA only the label and detection method used are different.

EIA for digoxin may be subdivided into:

(a) Heterogenous assay types
(b) Homogenous assay types.

(a) *Heterogenous Enzymeimmunoassays:*

The Du Pont digoxin assay is a heterogenous assay which is based on an affinity column mediated immunoassay technique. In this method patients' serum is preincubated at room temperature, with an antibody enzyme conjugate consisting of rabbit $F(ab')_2$ anti-digoxin antibody covalently linked to β-galactosidase (EC 3.2.1.2.3.). This mixture is sampled by the aca III and put through a column in the test pak containing ouabain, an analog of digoxin. Free antibody-enzyme conjugate is retained, while the bound digoxin antibody-enzyme complex passes through to the testpak reaction chamber. Here the β-galactosidase portion of the complex catalyses the hydrolysis of

ortho-nitrophenyl-β-galactopyranoside to ortho-nitrophenol. The change in absorbance at 405nm is directly proportional to serum digoxin concentration. This method, in common with nearly all immunoassays for digoxin, is affected by DLIFs (Chapter 3). Negative interference in 4 patients' samples were reported with this method when compared with the TDX and Corning RIA method [67]. Further comparisons are shown in Table 10.

Table 10. Comparison of the DuPont ACMIA Method with other digoxin methods.

Method	X	Y	N	S	i	r	Reference
	ACMIA	RIA	29	1.0	-0.21	0.94	CC.1984:30:1012
	ACMIA	TDX	29	0.942	0.07	0.96	DuPont Data
	ACMIA	RIA I	119	0.882	0.23	0.954	DuPont Data
	ACMIA	RIA II	117	0.969	-0.03	0.97	DuPont Data
	ACMIA	RIA III	84	0.924	0.323	0.952	CC.1988:34:1251

X = RIA

Y = Other Detection Systems

N = Number of Patients

S = Slope

i = Intercept

r = Correlation

(b) *Homogenous Enzymeimmunoassay:*

(b.1) *EMIT:*

In 1975 the first homogenous enzymeimmunoassay for digoxin was developed [68] from which the EMIT evolved. The principle method used until then was RIA. The EMIT method was evaluated and the results compared with an RIA technique using 67 samples. A correlation coefficient of 0.979 was obtained between the two methods.

Some of the EMIT evaluations are shown in Table 11. They also studied the effect of haemolysis on the EMIT assay so as to investigate NAD^+ reducing enzymes liberated from the red cell which might cause positive interference of the NAD^+-linked glucose-6-phosphate dehydrogenase. Low digoxin recoveries occured only in the presence of gross haemolysis. Moderate haemolysis caused no interference. Endogenous glucose-6-phosphate dehydrogenase interference is removed because the NAD^+ reacts only with the enzyme supplied in the assay. In the late 1070s two separate studies [69-70] reported good agreement between EMIT and RIA while a third [71] study gave interassay precision of 8% and 4% for EMIT and RIA, respectively, at a serum digoxin concentration of 1.7 nmol/L. However, this latter study gave slightly higher digoxin concentrations than RIA with both patients' sera and controls. In some assays this is caused by the protein concentration, particularly albumin, which can bind both free and labelled digoxin. The bound fraction of both albumin and antibody-labelled digoxin [72] shows a

false positive increase when the albumin and antibody are in solution as with for example charcoal adsorption analysis. Conversely, the bound fraction of radiolabelled digoxin decreases when antibody and albumin are separated as in the solid phase technique. This is caused by albumin combining with labelled digoxin. Errors greater than the 25% in apparent digoxin concentration may occur due to changes in albumin concentration. Other comparison studies involving EMIT are shown in Table 12.

Table 11. Results of an evaluation study on the EMIT method.

Digoxin Concentration	Recovery	CV within day	CV between day
0.65 nmol/L	105%		
3.25 nmol/L	100%		
3.9 nmol/L	100%		
1.47 nmol/L		3.77%	
4.71 nmol/L		2.77%	
1.95 nmol/L			8.42%
4.9 nmol/L			5.52%

Table 12: Comparison of the EMIT method with various immunoassays.

METHOD						
X vs	Y	N	S	i	r	Reference
EMIT	RIA I	155	1.03	0.10	0.98	71
EMIT	RIA 2	170	0.90	0.12	0.97	73
EMIT	RIA 3	153	1.09	-0.07	0.98	73
EMIT	RIA 4	80	0.96	0.02	0.97	73
EMIT	TDX	144	0.95	0.01	0.94	73
EMIT	Corning RIA	138	0.99	0.02	0.98	CC.1986:32:1078
EMIT	CA (RIA)	134	0.88	0.08	0.96	CC.1986:32:1078
EMIT	Immophase RIA	27	0.839	0.581	0.915	Ann Clin. Biochem. 1980: 17:315-318
EMIT	Dac-cel RIA	27	1.00	0.47	0.937	Ann Clin. Biochem. 1980: 17:315-318

X = EMIT

Y = Other Detection Systems

N = Number of Patients

S = Slope

i = Intercept

r = correlation

In 1984 the EMIT method was adapted for automation which resulted in the reduction of the assay time from roughly 30 minutes to 2-5 minutes, thereby increasing the popularity of the method. The use of centrifugal analysers resulted in a precision of approximately 8% in the therapeutic range. Since the antisera of all immunoassays may be able to cross-react with DLIF a pre-treatment step was introduced to eliminate such interferences. This involved the addition of a solid phase hydrophobic-bonded silica gel extraction step into the EMIT assay [73].

When the EMIT assay was compared to six different immunoassay methods, it gave the best results with little or no interference from patient samples known to have relatively high concentrations of DLIF [74]. Therefore, by pre-treatment of the serum with hydrophobic-linked silica gel chromatography it was possible to eliminate or significantly reduce digoxin measurements due to endogenous DLIFs. Further advantages and disadvantages of the earlier EMIT method are summarised in Table 13.

Table 13: Some characteristics of the earlier EMIT technique.

(a)	Homogenous
(b)	Stat capability
(c)	Assay-time - 5 minutes
(d)	Routine enzyme analysers are used
(e)	No radiation hazards
(f)	Shelf life - one year
(g)	Sample size 50μl
(h)	User friendly
(i)	Run with other assays
(j)	Sensitivity 0.65 nmol/L
(k)	Specificity similar to RIA
(l)	Interferences: haemolysis, lipaemia
(m)	Kits expensive
(n)	Enzyme lability
(o)	Incubation - 37^{o}C

(b.2) *CEDIA Enzymeimmunoassay*:

The CEDIA assay is based on a genetically engineered β-galactosidase enzyme. This enzyme has been split into two inactive forms using recombinant DNA techniques known as EA and ED which can combine spontaneously with the formation of active enzyme. The EAs are large polypeptides with deletions which are inactive in solution. The EDs are small polypeptides containing some of the deletions missing from the large polypeptide and are also enzymatically inactive. These inactive forms spontaneously recombine to form active enzyme. The extent of recombination is determined by the binding of anti-digoxin antibodies to the digoxin-labelled peptide which is regulated by the concentration of digoxin in the sample.

Method Evaluation:

The CEDIA method was originally evaluated and correlated against RIA [75] resulting in good agreement having a slope of 1.0 intercept of 0.06 μg/l and correlation of 0.97. More recent comparisons against other digoxin methods are shown in Table 14. No comparison studies against the EMIT method have yet been published.

Table 14: Comparison of CEDIA with other methods for Digoxin measurement.

METHOD						
X	vs Y	N	S	i	r	Reference
CEDIA	TDX	61	1.08	-0.20	0.93	CC.1986:33:1014
CEDIA	CA (RIA)	15	0.99	0.232	0.935	CC.1988:34:1249
CEDIA	RIA	84	0.95	0.115	0.959	CC.1988:34:1251
CEDIA	RIA		1.03-1.07		0.954-0.967	CC.1988:34:1209
CEDIA	RIA	90	1.0	0.06	0.95	CC.1990:36:560-561

X = CEDIA

Y = Other Detection Systems

N = Number of Patients

S = Slope

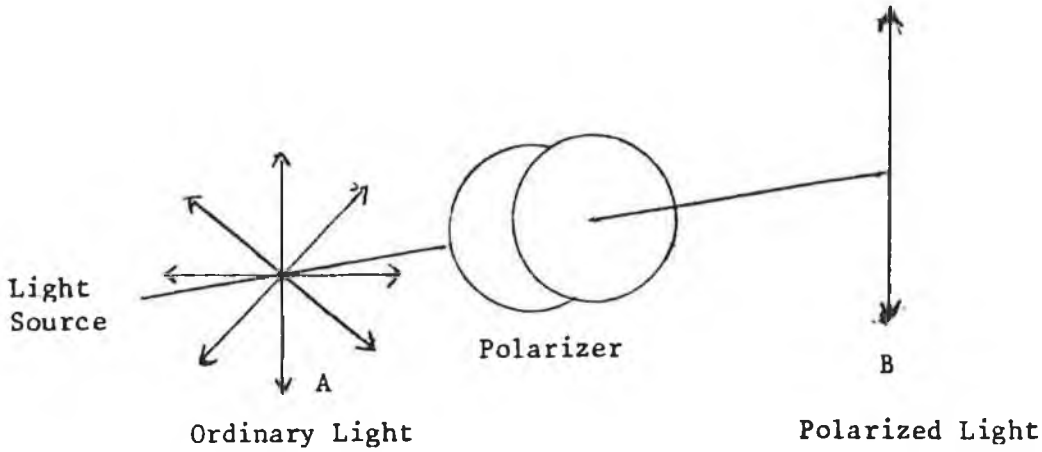
i = Intercept

r = correlation

2.4.3 *Fluorescent Polarization Immunoassay:*

Light waves by conventional sources are oriented randomly Figure 9. plane polarized light is light whose vibrations take place in only one of these possible planes [76]. Ordinary light is turned into plane blue polarized light as in the TDX FPIA method by passing it through a filter and a liquid crystal polarizer.

Figure 9:



When the fluroescein molecule is excited by polarized blue light, the size of the molecule will determine whether polarized green or non-polarized green light is emitted. If the fluorescein molecule is large the rotational relaxation time is longer than the fluorescence decay time causing the emission of plane polarized green light, parallel to the excitation blue polarized light. A small fluorescein molecule, on the other hand, causes the emission of non-polarized green light due to its rotational relaxation time being faster than the fluroescence decay time. When such a small fluorescein molecule is attached to a large molecule as in a flurescein-labelled antigen-antibody complex plane polarized light will be emitted. This

phenomenon is used in TDX immunoassay methods for the quantitation of serum digoxin.

The prinicple of the FPIA TDX method is the competition between the digoxin in the sample and a known amount of fluorescein-labelled digoxin for a limited number of antibody binding sites. The amount of fluorescein- labelled digoxin combining with antibody is inversely proportional to the serum digoxin concentration. Therefore, if the amount of serum digoxin is small the degree of polarization increases. The converse occurs if the analyte in the sample is high.

This method has a pre-treatment step in which an equal volume of sample is mixed with an equal volume of sulphosalicylic acid in methanol to precipitate serum proteins before the immunoassay so as to minimize the native background fluorescence produced by serum proteins, thus resulting in a more favourable signal to noise ratio.

A number of evaluation of the FPIA have been performed. One such method involved comparing FPIA with the Amerlex RIA digoxin assay [77]. Table 15 compares the precision of the two methods. Among the advantages this method has over RIA includes assay time of 14 minutes compared to 3 hours with the Amerlex RIA. It has a shelf-life of one year, as against 4-8 weeks for RIA, and a safer tracer is also used. Comparison studies of FPIA with other methods were generally good (Table 16). However, in some studies the mean serum digoxin concentrations were 10-14% lower than those of RIA with a decrease in serum digoxin by 8% for every 10 g/l increase in serum proteins [78]. These falsely low results were due to the difference in protein

concentration between the standards used for calibration and that of the samples. The protein concentration of the standards used in the kit was 50 g/l and that of the samples normally 60-80 g/l. During the pre-treatment step the magnitude of the binding of serum digoxin is related to the protein concentrations with more digoxin binding to samples containing higher serum proteins than those with lower concentrations causing a reduction in digoxin concentration in the supernatant. By adjusting the FPIA standards to protein concentrations similar to those of the samples resulted in the elimination of this bias and good correlation between FPIA and RIA methods. Therefore, for accurate results using the FPIA technique digoxin standard should be prepared in a matrix with a total protein concentration similar to that of the patients.

Abbott recently modified the TDX [79] method and called it TDX II so as to reduce interference from DLIF. These were almost eliminated in either renal or hepatic failure but appreciable concentrations were measured in patients with both hepatic and renal failure and in neonates.

Table 15: Precision evaluation of the FPIA and Amerlex RIA assay.

METHOD	DIG. CONC. (NMOL/L)	CV% WITHIN RUN	CV% BETWEEN RUN
FPIA	0.79	6.8	8.5
RIA	0.81	2.9	8.5
FPIA	2.12	2.7	4.1
RIA	2.43	3.8	6.0
FPIA	3.16	2.2	3.7
RIA	3.34	4.4	5.1

Table 16: Comparison of the FPIA with other immunoassays.

METHODS						
X vs	Y	N	S	i	r	Reference
FPIA	CA RIA	109	0.902	0.083	0.950	Abbott Data
FPIA	Beckman	172	1.08	0.05	0.94	CC.1985;31;922
FPIA	Stratus	60	1.04	0.10	1.0	CC.1985;31;922
FPIA	RIA	84	0.917	0.176	0.96	CC.1988;34;1251
FPIA	Du Pont	25	1.03	0 .01	0.96	CC.1985;31;928

X = FPIA

Y = Other Detection Systems

N = Number of Patients

S = Slope

i = Intercept

r = correlation

2.5 *SALIVARY DIGOXIN:*

It has been suggested that drug concentration in saliva is a good approximation of the non-protein fraction. Many workers have reported different mean ratios between salivary and serum digoxin (Table 17). The first such study for the determination of digoxin in saliva involved an extraction step followed by RIA of the extract [80]. Since direct measurement of salivary digoxin with RIA gave imprecise results an extraction step was required. The method was then found to be suitable for monitoring salivary digoxin. Most of the other reports [81, 83, 85, 86, 87] have recommended the use of salivary digoxin estimation as an alternative to that of serum digoxin with one author recommending its usefulness in pharmacokinetic studies. Van Der Vijgh [82] had reservations about its use in therapeutic drug monitoring and recommended measuring at least one saliva/serum ratio in each patient. He concluded by stating that serum digoxin monitoring was more reliable than that of saliva. However, two sets of workers [85, 89] have shown inter-individual variation in saliva/serum digoxin ratio. This variability in the saliva/serum digoxin ratio resulted in the rejection of saliva for therapeutic monitoring of serum digoxin by one research group [86].

There are wide inter-individual differences in protein and receptor binding of digoxin in different disease states (e.g. renal failure). It would appear, therefore, that measurement of the free fraction as measured in saliva would be a more effective method for assessing individual therapy if a suitable therapeutic range could be established for a specific disease state.

Table 17: Previous evaluations of saliva/serum digoxin ratios.

INDIVIDUALS STUDIED	SALIVA/SERUM RATIO	S.D.	P	SALIVA/ SERUM r	REF
Hospital patients	0.78	±0.7	<.001	0.99	80
Hospital patients	1.14	±0.48	<.001	0.90	81
Hospital patients	1.7			0.90	82
Healthy adults	1.34	±0.34	<.01-<.05	0.88-0.63	83
Healthy adults	1.25	±0.41	<.001	0.90	84
Hospital patients	1.25	±0.14		0.90	85
Infants	0.66	±0.20	<.001	0.71	86
Hospital patients	0.67	±0.10		0.96	87
Healthy patients	0.55-0.63	±0.06-0.08			87

S.D. = Standard Deviation

P = Probability

r = Correlation coefficient

Chapter 3

DIGOXIN-LIKE IMMUNOREACTIVE FACTORS

As already stated one of the problems associated with serum digoxin assays has been the presence of endogenous factors which gave false positive results. These substances were first reported in 1965 [88-89] using an assay based on the inhibition of rubidenium uptake by human red cells. It was quickly noticed that the discrepancies in assays (as much as 3.9 nmol/L) could not be accounted for by digoxin metabolites [90], interfering drugs or serum protein concentration [91]. In studies performed apparent serum digoxin levels as high as 13 nmol/L [89] have been reported in subjects not on digoxin therapy nor any drug known to interfere with the assay. This may explain the overlap in therapeutic and toxic ranges that occurs in some patients.

The nature of DLIF is still unclear but it appears that it is mostly protein bound (greater than 90% in normal people) and, unless concentrated, is not detectable in serum of normal people using immunoassays. It is a water soluble [92], neutral molecule not possessing carboxylic or primary amino groups with an approximate size of less than 1,000 daltons. A suggested hypothesis [92] is that DLIFs are present in serum in three forms:

(i) tightly but non-covalently bound

(ii) weakly bound

(iii) free form.

DLIFs are divided into two groups:

(a) those that interfere only in digoxin immunoassay e.g. oestrogens, progesterone, lipids and bile salts [90-91].

(b) those that inhibit Na^+/K^+ ATP-ase.

The latter group includes those substances that have been postulated to act as natriuretic hormones in essential hypertension. The observation of a natriuretic factor that appears in the blood after acute volume expansion and inhibits Na^+/K^+ ATP-ase dates back many years. In 1961 blood was cross-circulated between two dogs, in which intravascular volume of one was expanded causing natriuresis in the other [93]. From 1974-1976 a number of studies suggested that this natriuretic factor [93-94] was an inhibitor of Na^+/K^+ ATP-ase and sodium transport. It was shown that this inhibitor of Na^+/K^+ ATP-ase cross-reacted with antibodies to digoxin [95]. In addition, recent studies using serum immunoassays have indicated the presence of digoxin in subjects not receiving the drug during oral salt loading [95] during the third trimester of pregnancy [96], in newborn infants [97] and in patients with renal impairment [98]. Earlier studies showed that red cells from patients with uremia had an increased intracellular sodium concentration and reduced membrane Na^+/K^+ ATP-ase activity. These abnormalities could be induced in normal cells by incubating them in plasma from patients with uremia.

The evidence for an inhibitor of the Na^+/K^+ ATP-ase pump in the plasma of some subjects with low renin hypertension is more recent. This has been demonstrated by a method which involves incubation of

normal red cells in plasma from hypertensive subjects followed by measurement of Na^+/K^+ pump activity. Intravenous injection of antibodies [99] to digoxin has been observed to lower blood pressure in two animals with low renin hypertension. Inhibition of Na^+/K^+ ATP-ase has also been reported at the cell and tissue levels in animals and patients with hypertension, especially low renin hypertension. The natriuretic hormone that appears after acute volume expansion and inhibits Na^+/K^+ ATP-ase appears to be a heat stable small molecule attached to a protein. The hormone may come from the hypothalamus; in fact, experimental lesions of the hypothalamus decreases its level in the blood. This might suggest that the hypothalamus simply influences the secretion of the factor from another source such as the adrenal glands. This would support the idea that it is a steroid similar to digoxin which is known to bind to Na^+/K^+ ATP-ase.

As mentioned, DLIFs have been found in neonates and amniotic fluid even when neither the child nor the mother have been given digoxin. These values ran as high as 1.82 nmol/L and were detected in 90% of infants. A study involving 24 premature infants [100] found that all of them had levels >0.78 nmol/L as measured by the NML RIA kit (range 0.78-6.89 nmol/L). Detectable levels of DLIF may remain in the serum for up to 2 weeks post-partum and reaches a maximum at the 4th day. In 1983 a study of DLIFs in neonates found that within day variation was small, but between day variation was appreciable for 2 weeks post-partum. This suggests that it is not possible to determine an "apparent digoxin concentration" base line due to DLIF prior to commencing digoxin therapy [101].

DLIF increases in serum through pregnancy and reaches a peak in the third trimester. Levels of DLIF in the serum of pregnant women have been shown to range from 0.13 to 0.78 nmol/L with serum concentrations in hypertensive pregnant women reaching higher values during this time which may cause clinical problems. DLIF values decline rapidly in these women after parturition.

Similar high DLIF concentrations have been found in renal or hepatic failure studies. In a study of patients with renal failure but not on digoxin, "apparent digoxin values" as high as 1.3 nmol/L were noted. The "apparent digoxin concentration" was not related to the degree of renal impairment nor the haemodialysis state of the patient [98]. Different immunoassays also varied in "apparent digoxin concentrations" measured from any one specimen. In another study [98] on two groups of patients one in renal failure and the other having normal renal function, variable results were shown between the immunoassays in the renal dysfunction groups when compared with those from the normal renal function group. A study [102] performed on one patient in acute renal failure who was on digoxin showed that the serum digoxin increased for 10 days after the last dose. Different immunoassays gave different digoxin values when used to measure this specimen.

DLIFs have been reduced or eliminated from interfering in digoxin immunoassay measurements using pre-treatment procedures such as:

(i) ultrafiltration

(ii) altering immunoassay incubation times

(iii) combining H.P.L.C. with RIA

(iv) boiling

(v) TCA

In 1986 [103] it was postulated that DLIFs in the serum of normal individuals, patients in renal failure, pregnant women and neonates are mainly highly protein-bound. The increased concentrations of DLIF immunoreactivity with the last three groups are not due to increased concentrations of unbound factors, but rather to changes in protein binding which results in more loosely bound protein DLIF in the serum. This enables digoxin antibodies to remove more DLIF from the protein binding sites. This finding that DLIFs are largely protein-bound suggested that DLIF and their effects on immunoassays can be decreased or eliminated by removing serum proteins. These pre-treatment steps should not disrupt the DLIF protein binding e.g. in protein precipitation with TCA significant amounts of DLIF are left in the samples due to this disruption. In 1987 a method which substituted ultrafiltration for precipitation with sulphosalicylic acid resulted in complete elimination of DLIF using the Abbott TDX FPIA method [104]. By increasing the incubation time from 30-60 minutes DLIFs were reduced by 68% in patients with renal failure, pregnant women and from chord blood. Increasing the incubation time from 30-120 minutes decreased the DLIF by a mean of 67% within a range of 31-100%. The explanation for this phenomenon is thought to be due to the fact that the antibody reaction with DLIF has not reached equilibrium at the end of the stated incubation time. They succeeded in eliminating 89% of DLIFs in a sample using a 20 minute ultrafiltration step. By combining increased incubation and ultra-

filtration [103] DLIF activity was completely eliminated. Boiling the sample resulted in 60% DLIF removal and precipitation with TCA resulted in 33% removal. In 1985 [47] a study involving 50 people on digoxin was performed combining small column extraction of serum with HPLC and RIA of the eluent. It was successful in resolving the digoxin peak from its metabolites with the exception of dihydrodigoxin which co-eluted with digoxin.

The results of these studies show that a very specific method is needed for the accurate measurement of digoxin. In view of this, an investigation of the effects of DLIF and other interferents on the CEDIA and EMIT methods will be of great significance in Part 2 of this thesis.

PART 2

Chapter 4

INTRODUCTION

The clinical importance of measuring serum digoxin for therapeutic drug monitoring has been recognised [60,62,68]. Digoxin is the most widely requested drug in our laboratory and in view of its narrow therapeutic range and virtual total elimination from the body via the kidneys, it is important that it be specifically measured in patients with any degree of renal impairment. This measurement is now routine in many clinical chemistry laboratories, involving a large number of different methods.

Chromatography (e.g. TLC, HPLC) is successfully used in the measurement of most drugs. Optical methods of detection used are generally insufficiently sensitive for digoxin because of its low concentration in serum. While these constraints can be overcome by using large serum sample volumes of up to 5 mls, this renders it unsuitable for neonate samples. When other factors such as quick turn-around time (e.g. for clinics or intravenous infusions), the ability to handle relatively large batches, the need for high precision and the overall cost of setting up and maintaining such a service are considered, the suitability of chromatography by itself is limited.

Studies have shown similar correlations between bioassays and immunoassays. Bioassays are specific for cardioactive glycosides but are limited by problems with sample size, reproducibility and

specificity. The method is cumbersome, time consuming and labour intensive which renders it unsuitable for digoxin estimation.

FPIA is a rapid and sensitive method. The sensitivity and specificity depends both upon the labelled digoxin (e.g. with fluoroscein providing the emitted light) and with the characteristics of the antibody used. The presence of interfering endogenous fluorophore substances in biological samples necessitate the use of an extraction step. As already discussed, this latter step can lead to erroneous digoxin results due to protein concentration differences between the standards used and the serum samples. The measurement of reaction kinetics in these samples requires the use of special instrumentation, thus incurring extra expense.

Radioimmunoassays are, by nature, heterogenous [60,62]. Since the bound and free labelled drug emits a similar signal, these must be separated to determine serum digoxin. Assays are available which measure either the bound or free labelled drug. Separation of the bound from free labelled drug may be accomplished by adsorption of the free drug onto solid particles (e.g. charcoal) followed by measurement of the radioactivity of the bound labelled drug in the supernatant. Problems may arise due to incomplete adsorption of the free drug and trapping of some bound drug onto the charcoal . These problems may be substantially reduced by using either antibodies bound to solid phases such as the assay tube or magnetic particles. However, solid phase based immunoassays have traditionally been plagued by non- specific binding to the solid phase and by slow reaction kinetics relative to reactants that are free to diffuse in

solution. RIA methods are generally more sensitive than enzyme immunoasssays.

The concentrations in which digoxin can occur in serum are such that the detection need not be very sensitive. Although RIA methods also possess speed, specificity and sensitivity, they have the disadvantage associated with all radioisotopic methods, namely, radiation hazards, decay of radio-labelled reagents, the need for radioactive counting equipment and disposal problems. The shelf-life of most RIA reagents is only 6-8 weeks as against one year for the CEDIA or EMIT methods. Therefore, infrequent use of RIA is uneconomical because of the limited shelf-life of the label.

The CEDIA and EMIT methods chosen for evaluation in this study are homogeneous enzyme immunoassays. In these assays, there is no need to separate bound from free as the optical signal differs from antibody-bound-labelled drug and free labelled drug. Consequently, measurement can take place in a single cuvette. This results in decreased reagent cost, reduces technical labour, avoids some sample handling, permits the use of currently available enzyme analysers, thus facilitating data transfer. More importantly, it can be included in a biochemical screening profile with a single instrument. The two assays chosen are relatively simple and fast to perform by anyone trained to do routine enzyme analyses. This could enable personnel "on call" who cover many areas within the laboratory to perform the test accurately and quickly. Both assays are performed at 37°C and involve two pipetting steps and a number of absorbance readings. Since most assays, including digoxin measurement are

performed at 37°C on the enzyme analyser, there is no time delay for temperature adjustment using these methods.

One problem associated with immunoassays for digoxin is the specificity of the antibody as there maybe interference from non-specific binding due to the digoxin metabolites and endogenous DLIFs. The DLIFs present in certain clinical groups, interfere to varying degrees with most immunoassays. Recent studies have also shown linoleic, arachidonic, linolelaidic and 1-mono- linolenoyl glycerol as compounds likely to contribute to DLIF activity in plasma [105, 58]. This indicates the importance of evaluating each immunoassay for DLIF interference. A suitable pre- treatment step should eliminate or greatly reduce the concentration of DLIF in serum before digoxin is measured with the advantage that a less specific antibody could then be used for accurate measurement of the drug.

The EMIT method uses a pre-treatment procedure involving the addition of a solid phase hydrophobic-bonded silica gel extraction step into the assay. The effectiveness of this step is being investigated in this study. Since the CEDIA method uses no sample pre-treatment step the specificity of the antibody for digoxin must be investigated.

The prinicple of the EMIT method involves an NAD^+-linked glucose-6-phosphate dehydrogenase step. It was important to investigate the possible release of NAD^+ reducing enzymes from the red cell and also the spectrophotometric effect of haemolysis on each method [68].

The total protein concentration of the plasma is about 70-75 g/l.

Albumin is the most abundant protein, representing 55-65% of the total fraction. Holtzman et al [106] studied the effect of different albumin concentrations on the binding of a radiolabelled antigen to antibody. The results showed that the binding of the labelled digoxin to the antibody is very sensitive to variations in albumin concentration. Therefore, using both kits, it was decided to examine this variable. The spectrophotometric effect of bilirubin on each method was also studied.

The use of saliva to determine certain drug concentrations has been shown to give a reliable indication of their concentrations in plasma. It is generally accepted that the non-protein bound or free fraction of a drug exerts the pharmacological effect and that ideally, estimation of this fraction would yield the most useful indication of effective therapy. The extent of protein binding determines the distribution of digoxin between the various fluid compartments and the receptor site. Only non-protein bound digoxin may move from the vascular compartment to the site of action. Interpretion of total drug concentrations become difficult in situations where there is abnormal binding of the drug. In the body, about half the total digoxin is bound to skeletal muscle and approximately 25% of serum digoxin is exclusively bound to serum albumin. However, bound fractions from 18-33% have been reported suggesting that the ratio is not as constant as claimed [25]. In plasma, the ratio of free to total bound drug varies with age and disease state. In the plasma, any variations in the albumin concentration will cause changes in protein binding. Digoxin may also be displaced from albumin by molecules sharing the same binding

site or by drugs administered concomitantly.

Interpretation of total digoxin concentration is also difficult in renal failure where there is some evidence that toxicity may be more likely to occur at plasma digoxin concentrations not normally associated with toxicity. In these situations, salivary digoxin measurements may yield more meaningful results than serum digoxin. In this study, the efficacy of the CEDIA and EMIT assays are investigated for salivary digoxin measurement using saliva/serum ratios and correlations. The modified procedures for each kit are used to investigate the possible presence of DLIFs in saliva.

Chapter 5

MATERIALS AND METHODS

5.1 *MATERIALS:*

5.1.1 *EMIT Column Digoxin Assay:*

(a) EMIT column digoxin assay 250 test kit; Cat. No. 6H019 UL.

(b) EMIT column digoxin calibrators, lyophilised 6 x 3ml ;
Cat. No. 6H109 UL.

(c) Syva solid phase columns, system II, 100 columns;
Cat. No. 6H029 UL.

(d) EMIT column digoxin mobile phase solution, 60% methanol in diluted water; 3 x 100ml
Cat. No. 64039 UL.
EMIT digoxin reagents manufactured by Syva Co., Palo Alto, CA.

(e) CEDIA digoxin assay - CEDIA digoxin assay kit, 100 test size;
Cat. No. 80-300-30.
CEDIA digoxin reagents manufacured by Microgenics Corp., CA.

(f) 1 gram of Digoxin;
Cat. No. 80469 TM - Aldrich Chemical Co., P.O. Box 355, Milwall, W1 53201.

(g) Albuminar-20 (human serum albumin 20% w/v)
Each bottle contains 20g of human serum albumin per 100 ml.
Manufactured by: Pharma GmBH Eschwedge, West Germany.

(h) Buffered phosphate saline pH 7.3 approx. 100 tablets.
Lot No. R000 675 001 - Oxford Ltd., U.K.

(i) Pyridine, 1000ml.

Sigma Chemical Co. Ltd.

(j) 0.1 molar HCL.

5.1.2. *Control Sera:*

Gilford tri-level TDM controls. TDM 701 Ciba Corning Diagnostics.

Level I Lot No. 075701 1.9 ± 0.4 nmol/L

Level II Lot No. 076701 3.1 ± 0.6 nmol/L

Level III Lot No. 077701 4.2 ± 0.8 nmol/L.

5.1.3. *Equipment:*

Cobas Bio Centrifugal Analyser, Hoffman La Roche, Switzerland.

Vac Elut System (vacuum extraction box), Analytichem International, CA, USA.

Vacuum Pump

Vacuum Tubing

Syva Vacuum Receiver

Coulter T-890 and Coulter Mixer, Coulter Electronics Ltd., England.

Water bath, type 586, Grant Instruments Ltd., Cambridge.

Visking Dialysis Tubing type 12 (3/4 inch diameter), Medicell International Ltd., 239 Liverpool Road, London N11LX.

Teflon Cell - Type A, Polypenco Ltd., Engineering Parts Division, P.O. Box 56, 83 Bridge Road East, W.G. City, Hertfordshire, Al 71LA, England.

Oertling pan-balance Mode R-20, 200 g capacity, 100 ml graduated

Vortex Mixer

0.5 - 10 µl	adjustable Oxford pipette
10 - 20 µl	adjustable Oxford pipette
0 - 50 µl	adjustable Oxford pipette
0 - 200 µl	adjustable Oxford pipette
200 - 1000 µl	adjustable Oxford pipette
1 - 5 ml	adjustable Oxford pipette

5.2 *EMIT COLUMN DIGOXIN ASSAY:*

5.2.1. *Reagents:*

Reagents for the measurement of plasma digoxin are supplied in the kit as follows:

(a) Reagent A:

Lyophilised material reconstituted with 3.0 ml of distilled water. It contains:

(1) Gamma globulin fraction from rabbit immunized with digoxin

(2) NAD

(3) Glucose -6-phosphate

(4) 0.055M TRIS HCL, pH 7.5

(5) 0.5% w/v Sodium azide as preservative

(6) Bulking agents and stabilizers

(b) Reagent B:

Lyophilised material reconstituted with 15 ml of distilled water. It contains:

(1) Digoxin covalently bound to glucose-6-phosphate dehydrogenase

(2) 0.55 molar TRIS HCL, pH 7.5

(3) 0.05% w/v Sodium azide as preservative

(c) Buffer Concentrate:

Supplied in liquid concentrate form - volume 13.3 ml. It contains:

(1) 0.83 molar TRIS buffer, pH 8.0

(2) 0.075% w/v Sodium azide as preservative

(3) Bulking agents and stabilizers

5.2.2. *Calibrators:*

6 x 3 ml lyophilised serum based calibrators containing 0.0, 0.64, 1.28, 2.56, 3.84 and 5.12 nmol/L digoxin. Stability is 3 months when stored at 2-8°C.

5.2.3. *Preparation and Storage of Working Reagents:*

(a) Reagent A:

One part of reconstituted Reagent A was mixed with 11 parts of buffer solution. It was stored at 2-8°C and was stable for 3 weeks.

(b) Reagent B:

It was used as reconstituted.

(c) Buffer Solution:

It was diluted to 133ml with distilled water for use. Stability was 12 weeks when stored at room temperature.

5.2.4. *EMIT Extraction Step:*

This procedure is used to separate digoxin from serum, plasma or saliva. Sample was passed through a disposable Syva solid phase column, containing covalently-linked silica gel which adsorbs digoxin [73]. Hydrochloric acid followed by water elutes all other substances except digoxin from the column. The EMIT column digoxin mobile phase solution is used to elute digoxin from the column into a

test tube. The extract is capped, briefly vortexed and the concentration of digoxin determined by the EMIT assay.

5.2.5. *Extraction Procedure:*

(1) The vacuum pump was set at 10-15 in.Hg-sufficient to draw 1ml of water in 15-25 seconds.

(2) The solid phase columns were inserted into ports on the vacuum manifold lid. Any unused holes were plugged.

(3) 350 ml of mobile phase solution was pipetted into each column to "condition" the stationary phase. The vacuum was then applied until the mobile solution had completely washed from the column. The vacuum was then released.

(4) 0.5 ml of either sample, control or calibrator was pipetted into each column. The vacuum was applied to allow the specimen to completely aspirate through the columns. The vacuum may remain on.

(5) 1.0 ml of 0.1 N HCl was added to each column. The vacuum was applied and the solution was aspirated through the columns.

(6) With the vacuum still on, 1.0 ml of distilled water was added to each column and aspirated. The vacuum was maintained for an additional 10 seconds to ensure that no liquid remained in the probes.

(7) The vacuum manifold lid was removed and the probes on the under-side were wiped dry. 10 x 75 mm test tubes (only this size suitable) were labelled for each sample and placed in a rack under each column port. The vacuum lid was then tightly replaced.

(8) 350 ml of mobile phase solution was pipetted into each column. The vacuum was applied until the solution had completely aspirated through the columns into the test tubes. The vacuum was maintained for an extra 10 seconds to ensure complete removal of solution. The vacuum was not left on for more than 1 minute so as to avoid eluent evaporation. The vacuum manifold lid was removed. The tips of the probes were tapped against the test tube walls to recover any remaining solution. Each tube was then thoroughly mixed for 3-5 seconds. The tubes were capped immediately to avoid evaporation. Any eluate stored for greater than 1 hour was mixed. When properly sealed the eluate may be stored for one week at 2-8°C.

5.2.6. *EMIT Assay Principle:*

EMIT is a homogenous enzyme immunoassay system and is quite easily automated. The EMIT method (Figure 10) employs a bacterial (Leuconostoc mesenteroides) enzyme glucose-6-phosphate dehydrogenase covalently linked to digoxin. The linkage is made close to the active site of the enzyme such that combination of the conjugate complex to the antibody inhibits enzyme activity. Free digoxin in the sample reverses the inhibition by competing with enzyme-labelled digoxin for a limited number of antibody binding sites. The enzyme activity of the conjugate is proportional to the concentration of the free drug. The antibody probably inhibits the enzyme activity by inducing or preventing certain conformational changes at the active site of the enzyme. Glucose-6-phosphate dehydrogenase activity is measured spectrophotometrically at 340 nm due to the production of

NADH corresponding to plasma digoxin concentration.

5.2.7. *EMIT Assay Procedure:*

The parameter listing for the EMIT method is shown in Table 18.

Figure 10: Principle of the EMIT Homogenous Assay System.

1. Active enzyme.
2. The addition of the digoxin-specific antibody inhibits the enzyme by inducing or preventing conformational changes necessary for enzyme activity.
3. Digoxin in patients serum modulates enzyme activity. Active enzyme converts NAD^+ to NADH, resulting in an increase in absorbance that is measured at 340nm.

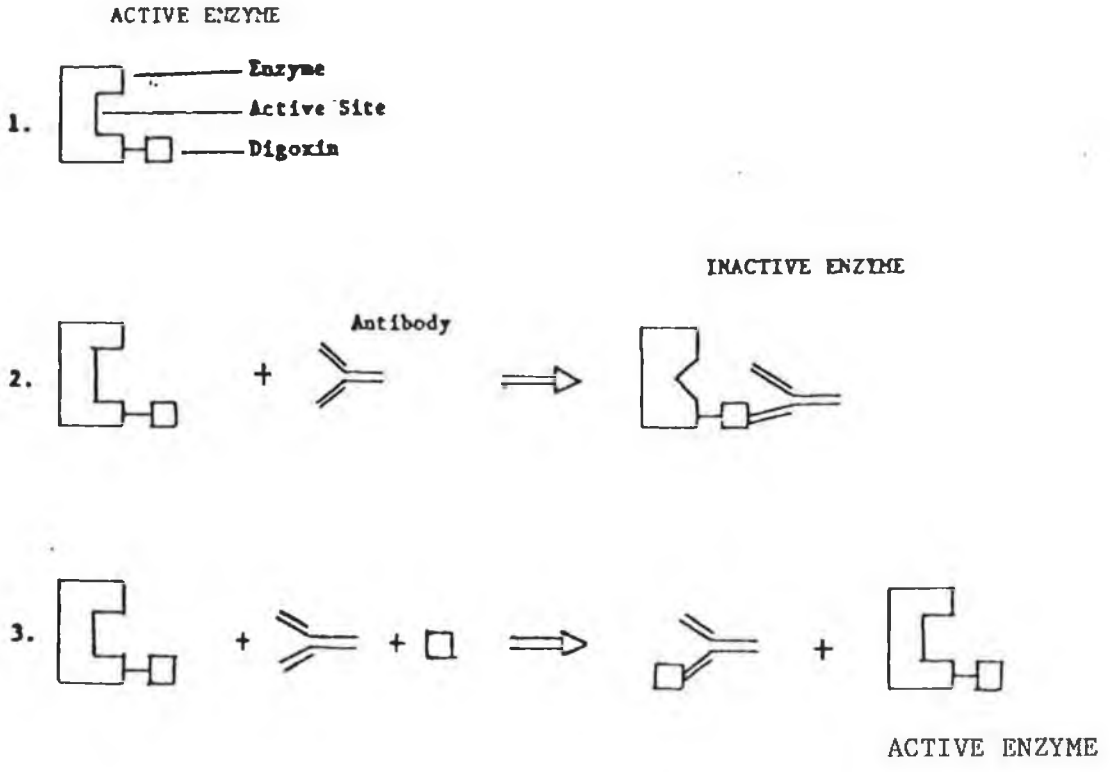


Table 18: Cobas Bio programming for the Syva EMIT column digoxin assay and CEDIA digoxin assay.

α Reaction direction/test name		EMIT	CEDIA 11;44;49;47 (+ Digoxin)
1	Units	14(nmol/L)	14 (nmol/L)
2	Calculation factor	3700	3333.3
3	Standard 1	0.0	0.0
3	Standard 2	0.64	2.56
3	Standard 3	1.28	5.12
3	Standard 4	2.56	
3	Standard 5	3.84	
3	Standard 6	5.12	
6	Limit	0	0
7	Temperature	37°C	37°C
8	Type of analysis	7.3	3
9	Wavelength (nm)	340	420
10	Sample volume µl	30	25
11	Diluent volume µl	60	10
12	Reagent volume µl	115	120
13	Incubation time sec.	100	10
14	Start reagent volume µl	45	75
15	Time of 1st reading sec.	5.0	700
16	Time interval sec.	10	10
17	Number of readings sec.	25	21
18	Blanking mode	0	0
19	Printout mode	0/1	2

5.2.8. *Explanation of Parameters:*

A Cobas Bio centrifugal analyser with standard "Dens" software was used. "Dens" is an acronym for data evaluation for non-linear standard curves. It is possible to construct a new curve, store a curve in memory or correct a stored curve using a single calibrator. The Cobas Bio sample disc contains twenty five sample positions and the cuvette rotor contains twenty-nine cuvettes. This allows analysis of eleven patient samples and three controls in each batch (duplicates).

(a) Type of Analysis 7.3:

This type of analysis enables "Dens" software to process non-linear data reduction of kinetic enzymatic assays with start reagent.

(b) Blanking Mode 0:

This permits water to be used as a blanking solution.

(c) Printout Mode 0:

Printout mode 0 evaluates samples against a stored curve.

(d) Printout Mode 1:

The Cobas Bio automatically calculates a calibration curve using math model no. 1, a four parameter logit function.

Logit function:

$$R = R_o + K_c \frac{1}{1 + \exp[-(a+bc)]}$$

where: R = response (rate of change of absorbance)

c = concentration

a and b = non-linear coefficients

R_o = zero standard response

K_c = scale parameter

(e) Printout Mode 2:

The run is calibrated using math model, no. 2, a five parameter logit fit. Absorbance data was obtained from the instrument by changing "Type of Analysis 7.3" to "3" and by changing the "Printout mode" to "2".

5.3 *CEDIA Digoxin Assay:*

5.3.1. *Reagents:*

Reagents for the measurement of plasma digoxin are provided in vials in each kit as follows:

(a) Enzyme-Donor:

Lyophilised material reconstituted with 10 ml of ED reconstitution buffer. It contains:

(1) ED covalently bound to digoxin

(2) Buffer salts

(3) O-nitrophenyl-β-D-galactopyranoside

(4) 0.5 mg sodium azide as preservative.

(b) Enzyme-Acceptor:

Lyophilised material reconstituted with 16 ml of EA reconstitution buffer. It contains:

(1) Enzyme-acceptor

(2) Digoxin-specific antibody

(3) Buffer salts

(4) 0.8 mg sodium azide as preservative.

(c) Enzyme-Donor Reconstitution Buffer:

(1) Phosphate buffer - volume 11 ml

(2) Stabilizers and 20 mmol/l sodium azide as preservative.

(d) Enzyme-Acceptor Reconstitution Buffer:

(1) Phosphate buffer - volume 17.0 ml

(2) Stabilizers and 20 mmol/l sodium azide as preservative.

5.3.2. *Digoxin Calibrators:*

Serum based calibrators containing 0.0 nmol/l (volume 4.5 ml) 2.56 nmol/l (volume 3.0 ml) and 5.12 nmol/l (volume 3.0 ml) were supplied with each kit. These calibrators were supplied in liquid form and no reconstitution was necessary. All reagents and calibrators were stored at 2-8°C.

5.3.3. *CEDIA Assay Theory:*

The CEDIA immunoassay for the quantitative measurement of digoxin utilises a new concept in enzyme immunoassay [75]. A new homogenous enzyme immunoassay has been developed by the genetic engineering of β-galactosidase (EC 3.2.1.2.3.), an enzyme commonly used in enzyme-linked immunoassays. The lac operon of E. coli consists of (O) operator, (P) promotor and Z, Y, A genes (Figures 11 and 12). The Z gene encodes for a large enzymatically inactive polypeptide consisting of 1021 amino acids which spontaneously associate with other similar polypeptides into a tetrameric form which is the active β-galactosidase. The large polypeptide is formed as a result of the transcription of the DNA in the Z gene to mRNA and the translation of this information into the polypeptide. Using recombinant DNA techniques EAs and EDs were constructed. The EAs are large inactive polypeptides having small deletions or missing sequences in the encoded proteins. The EDs are small inactive polypeptides containing some of the sequences omitted from the EAs. The EAs and the EDs are both enzymatically inactive but spontaneously associate in solution to form fully active tetrameric enzymes similar to natural β-galactosidase. This recombinant DNA technique has been used by

Microgenic Corporation to produce families of EAs and EDs which has allowed the development of CEDIA homogenous assays.

5.3.4. *CEDIA Assay Principle:*

The CEDIA homogenous immunoassay system operates by regulating the spontaneous association of the EDs and EAs through an antibody-antigen reaction. A single digoxin moiety is covalently attached to each ED molecule so that binding by anti-digoxin antibodies inhibits the reassociation of EA and ED fragments. The digoxin in the patients sample competes with the ED-digoxin-conjugate for a limited number of antibody sites. The digoxin concentration in the sample is linearly proportional to the amount of β-galactosidase formed. The amount of active β-galactosidase formed by recombination of EA and ED digoxin components is determined by measuring the rate of chlorophenol red-β-D-galactopyranoside hydrolysis at 570 nm. This is the substrate for the 5 minute test and O-nitrophenyl-β-D galactopyranoside substrate for the 15 minute digoxin test which is measured at 420 nm.

5.3.5. *CEDIA Assay Procedure:*

The parameter listing for the assay is illustrated in (Table 18). The protocol of Henderson et al. which performs a "calibration curve fit" and calculation of results could not be used as the old "Dens" software in the Cobas Bio was not sophisticated enough. Rate of absorbance was used instead for manual calculation by changing the "Type of Analysis 7.3" to "3" and "Printout Mode 5" to "2".

A volume of 100μl of digoxin calibrators, controls and patients' samples were placed in cups in the carousel. The main cavity of the reagent boat was filled with 4 ml of enzyme-acceptor and 2.5 ml of enzyme-donor was placed in the "start" reagent compartment. The reagent boat and sample carousel were loaded and the run started. The appropriate volumes of sample and enzyme-acceptor reagents were added to each cuvette and mixed for 5 seconds by centrifugation. This was followed by a 10 second incubation period during which time an auxillary reading was taken at 420 nm. The auxillary reading is used to give an account of reagent integrity, but is not involved in the calculation of absorbance rate changes. After the incubation time enzyme-donor was added and mixed for 5 seconds followed by a 700 second incubation time. A total of 21 absorbance readings were then taken at 10 second intervals. The instrument compared each absorbance and performed a linear regression. The rate of change of absorbance was calculated by subtracting the lower from the higher absorbance rate.

Figure 11: LAC Operon of E.Coli.

The lac operon consists of the operator, promotor, Z, Y and A genes. The Z gene encodes galactosidase; mutant Z genes encode enzyme-acceptors (EAs) and enzyme-donors (EDs). Both EAs and EDs are enzymatically inactive but, when mixed, spontaneously associate to form enzymatically active β-galactosidase.

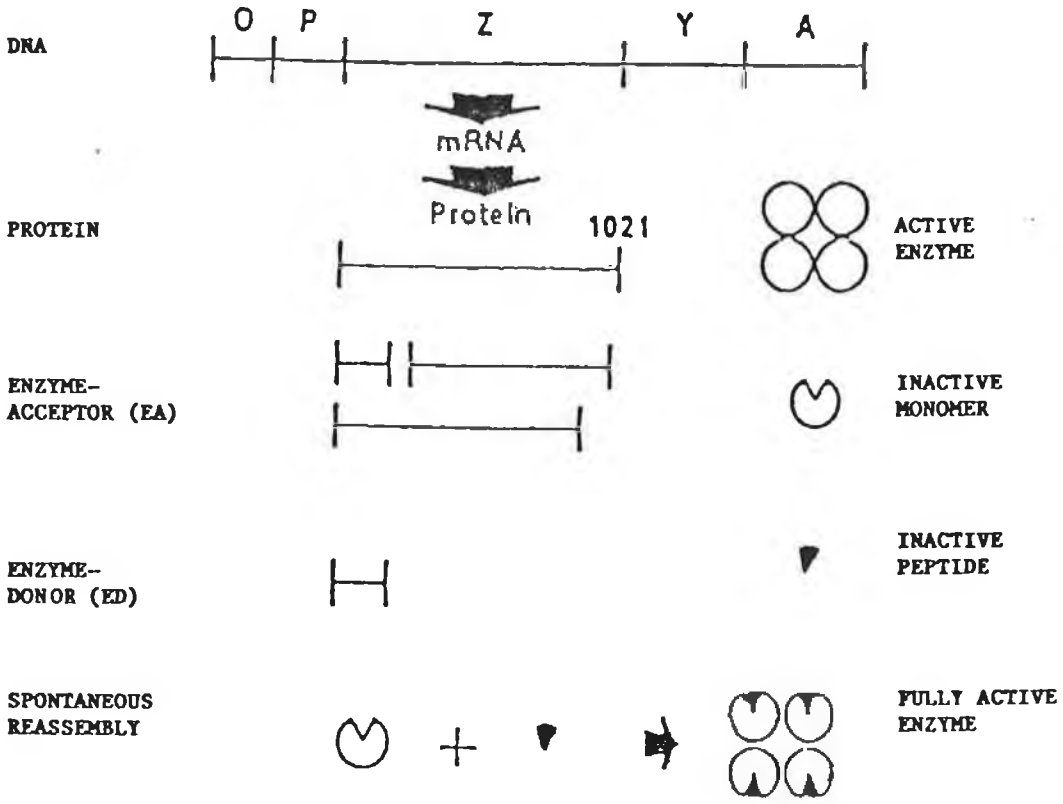
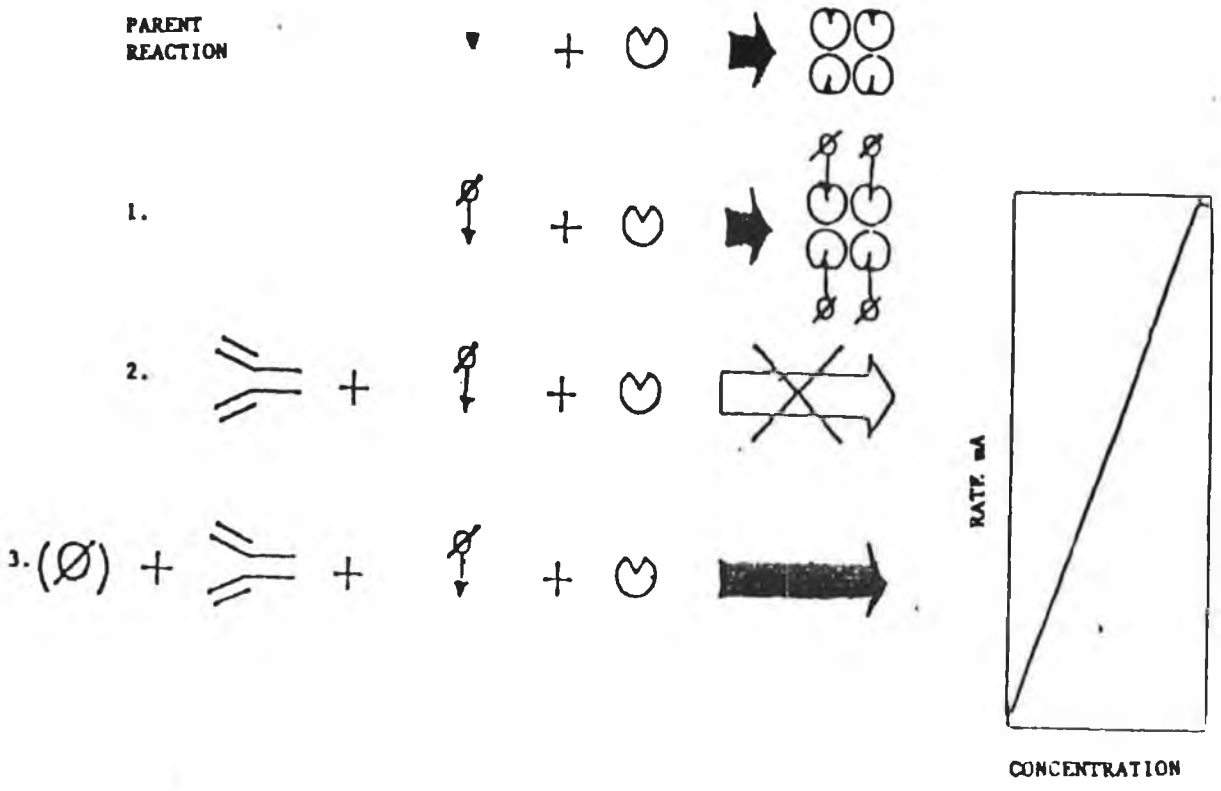


Figure 12: Principle of CEDIA Immunoassays.

Ananalyte (Ø) is attached to an ED such that the analyte-ED conjugate spontaneously recombines with EA to yield active β-galactosidase (1). The addition of analyte-specific antibody inhibits spontaneous enzyme assembly (2). Analyte (Ø) in patients' serum modulates enzyme assembly so that the signal generated by substrate turnover is directly proportional to analyte concentration.



5.4. *SAMPLE COLLECTION:*

Patient samples were taken into vacutainers with no anticoagulant. Samples for therapeutic monitoring of digoxin were drawn from patients at least eighteen hours after oral administration in order to allow time to complete the distribution phase. Samples for maintenance dose estimations were drawn from patients with steady state concentrations. The term steady state refers to a state of equilibrium between the quantity of drug being administered and the quantity eliminated.

(a) Serum was collected from 52 patients for digoxin estimation. Each sample was divided into three equal volumes and stored at -20°C in plastic tubes. One aliquot was sent to the external laboratory for analysis by RIA and the remaining two analysed by the CEDIA and EMIT methods.

(b) The salivary study involved the simultaneous collection of 20 paired salivary and serum samples. They were stored at -20°C.

(c) In order to investigate cross-reactivity by DLIFs in both assays, samples were collected as follows:
Simultaneous serum and saliva samples were taken from 20 adult patients with known renal and hepatic dysfunction. Serum creatinine and liver function tests were performed by routine laboratory analysis. Serum and saliva samples were also collected simultaneously from 12 third trimester pregnant women and sera only from 8 neonates varying in age from 1 to 7 days.

(d) To investigate if a correlation exists between the degree of liver dysfunction and serum DLIF concentrations, samples were collected from 1 adult patient at 2 day intervals over an 8 day

period. The samples were stored at -20°C.

(e) Serum and plasma was collected from patients not on digoxin. These patients showed normal renal, liver and cardiac function as assessed by measuring urea, electrolytes, creatinine, bilirubin, transaminases, alkaline phosphatase, gamma glutamyl transferase, creatine kinase and lactate dehydrogenase. Pools were prepared, aliquoted and stored at -20°C for use in recovery and stability studies. The serum pool was also used as a diluent to investigate possible interference due to haemoglobin, lipids and bilirubin.

(f) Serum samples containing high concentrations of (a) bilirubin, and (b) triglyceride were pooled, aliquoted and stored at -20°C.

(f) A number of blood samples were collected from patients not on digoxin with normal renal, cardiac and liver function tests. These bloods were stored at -20°C for 2 hours approximately. The resulting haemolysed samples were centrifuged, thawed and the serum was pooled and stored at -20°C for subsequent haemoglobin interference studies. A further 8 samples were collected from patients on digoxin. After centrifugation 1.0 ml approx. of non-haemolysed serum was removed and stored at -20°C. The remaining serum from each sample was haemolysed as described above, analysed for haemoglobin using the Coulter Counter and stored at -20°C.

(h) A number of digoxin-free sera and saliva samples were collected from patients with normal renal function and cardiac function tests. Separate saliva and serum pools were prepared and stored at -20°C for use as a diluent in the salivary digoxin study.

Chapter 6

ANALYTICAL STUDIES

6.1 *SERUM DIGOXIN MEASUREMENT:*

6.1.1. *Imprecision Studies:*

These show the agreement between replicate measurements of serum digoxin concentration using the EMIT and CEDIA methods.

(a) Within Run Imprecision:

This was assessed using 20 replicate analyses of the three Gilford controls.

(b) Total Imprecision:

This was assessed over 20 days using daily measurements of the three Gilford controls.

6.1.2. *Sensitivity Study:*

This is a measure of the ability of the CEDIA and EMIT methods to detect low concentrations of digoxin. It has no numerical value but is expressed by the term "detection limit". This is the smallest single result with a state of probability (commonly 95%) which can be distinguished from the zero calibrator. The sensitivity of all the methods was determined by assaying 15 replicates of the zero calibrator. The absorbance readings obtained by each method were used to determine the digoxin concentrations from which the mean + 2 SD was calculated. This defines the sensitivity of the method.

6.1.3. *Analytical Range:*

This is the concentration range over which no modification is required in the CEDIA or EMIT methods and is determined by a "linearity experiment".

(a) A digoxin solution of 10 μmol/L was prepared by dissolving 7.8125 μg of digoxin in 25 mls of pyridine. This was then diluted to one litre with a solution made up as follows:

(i) 6 tablets of phosphate buffered saline, pH 7.3, dissolved in distilled water.

(ii) 400 ml of human serum albumin 20% (albuminar-20) giving an albumin concentration of 80 g/l.

(iii) 20 mmol/L of sodium azide as preservative prepared by adding 1.3 g to the solution.

(b) A digoxin-free diluent solution was prepared similarly to the above solution. A number of dilutions were then prepared yielding digoxin concentrations from 0.5 - 10 nmol/L (see Table 19).

(c) A digoxin-free diluent solution for saliva was prepared similarly to that for (a) except that 400 ml of albuminar-20 was added resulting in an albumin concentration of 80 g/l along with 6 tablets of phosphate buffered saline, pH 7.3 made up to 1 litre using distilled water.

Table 19: Dilution protocol for preparation of solutions to assess analytical range.

Digoxin Standard 10 (μmol/L)	Diluent	Digoxin Conc. (nmol/L)
μl	μl	
50	950	0.5
100	900	1.0
150	850	1.5
200	800	2.0
250	750	2.5
300	700	3.0
400	600	4.0
500	500	5.0
550	450	5.5
600	400	6.0
700	300	7.0
800	200	8.0
900	100	9.0
1000	0	10.0

The diluent used was that described in Section 6.1.3 (b).

6.1.4. *Recovery Studies:*

These studies show the agreement between the best estimate of the serum digoxin concentration and its true value using the CEDIA and EMIT methods. Accuracy was assessed by spiking the pooled digoxin-free serum with the 10 μmol/L digoxin standard. This gave various concentrations covering the analytical range. Spiked solutions were prepared to a volume of 1 ml as in Table 20.

Table 20: Dilution protocol for digoxin solutions used in the assessment of accuracy

Digoxin Standard conc. (10 μmol/L)	Digoxin-free serum	Digoxin conc. (nmol/L)
μl	μl	
50	950	0.5
100	900	1.0
200	800	2.0
300	700	3.0
400	600	4.0
500	500	5.0

Each of these solutions was measured 10 times and the mean concentration of each was used to calculate the percentage recovery.

6.1.5. *Calibration Curve Stability:*

A calibration curve was established for the EMIT and stored in the instrument using the "DENS" option. The stability of the working reagents as reflected by the stability of the curves was tested at 2 day intervals up to 18 days by measuring the calibrators as samples along with 3 control sera. A calibration curve for the CEDIA was plotted using absorbance readings obtained at 420 nm versus concentration. The stability study
procedure was similar to the EMIT except absorbance values were taken and the concentration read from the manually plotted graph.

6.1.6. *Interference Studies:*

(a) DLIFs:

The samples collected from the various groups of patients (5.4) were used to investigate cross-reactivity in the kits.

(b) Bilirubin:

The pooled sample when analysed gave a bilirubin concentration of 312 μmol/l.

These pools were mixed with a normal digoxin-free pool to give different levels of the potential interferent. Different digoxin concentrations were then added as illustrated in Tables 21-23.

Table 21: Dilution protocol for bilirubin solutions used in interference studies at digoxin concentrations of 2 nmol/L.

Pooled Bilirubin conc. (312 μmol/L)	Digoxin-free serum	Digoxin Std. (40 μmol/L)	Bilirubin conc.
μl	μl	μl	(μmol/L)
900	50	50	280.8
850	100	50	265.2
800	150	50	249.6
750	200	50	234.0
700	250	50	218.4
650	300	50	202.8
600	350	50	187.2
550	400	50	171.6
500	450	50	156.0
450	500	50	140.4
425	525	50	132.6
400	550	50	124.8
350	600	50	109.2
250	700	50	78.0
200	750	50	62.4
175	775	50	54.6
150	800	50	46.8
100	850	50	31.2
50	900	50	15.6

The 40 μmol/L digoxin standard was prepared using 31.25 μg of digoxin and following the procedure for the 10 μmol/L standard [6.1.3 (a)].

Table 22: Dilution protocol for bilirubin solutions used in interference studies at digoxin concentrations of 3 nmol/L.

Pooled Bilirubin conc. (312 μmol/L)	Digoxin-free serum	Digoxin Std. (40 μmol/L)	Bilirubin conc.
μl	μl	μl	(μmol/L)
900	25	75	280.8
850	75	75	265.2
800	125	75	249.6
750	175	75	234.0
700	225	75	218.4
650	275	75	202.8
600	325	75	187.2
550	375	75	171.6
500	425	75	156.0
450	475	75	140.4
425	500	75	132.6
400	525	75	124.8
350	575	75	109.2
250	675	75	78.0
200	725	75	62.4
175	750	75	54.6
150	775	75	46.8
100	825	75	31.2
50	875	75	15.6

Table 23: Dilution protocol for bilirubin solutions used in interference studies at digoxin concentrations of 4 nmol/L.

Pooled Bilirubin conc. (312 µmol/L)	Digoxin-free serum	Digoxin Std. (40 µmol/L)	Bilirubin conc.
µl	µl	µl	(µmol/L)
900	-	100	280.8
850	50	100	265.2
800	100	100	249.6
750	150	100	234.0
700	200	100	218.4
650	250	100	202.8
600	300	100	187.2
550	350	100	171.6
500	400	100	156.0
450	450	100	140.4
425	475	100	132.6
400	500	100	124.8
350	550	100	109.2
250	650	100	78.0
200	700	100	62.4
175	725	100	54.6
150	750	100	46.8
100	800	100	31.2
50	850	100	15.6

(c) *Lipids:*

The pooled sample when analysed resulted in a serum triglyceride concentration of 18.94 mmol/L and a serum cholesterol concentration of 7.49 mmol/L. The procedures used in this study are shown in Tables 24 - 26.

Table 24: Dilution protocol for lipid solutions used in interference studies at digoxin concentrations of 2 nmol/L.

Pooled Serum Trig.18.94 mmol/L Chol.7.49 mmol/L	Digoxin-free Serum	Digoxin Std. (40μmol/L)	Trig. Conc.	Chol. Conc.
μl	μl	μl	(mmol/L)	(mmol/L)
900	50	50	17.05	6.74
850	100	50	16.10	6.37
800	150	50	15.15	6.00
750	200	50	14.21	5.62
700	250	50	13.26	5.24
650	300	50	12.31	4.87
600	350	50	11.36	4.49
550	400	50	10.42	4.12
500	450	50	9.47	3.75
450	500	50	8.52	3.37
400	550	50	7.58	3.00
350	600	50	6.63	2.25
250	700	50	4.74	1.87
150	800	50	2.84	1.12

Table 25: Dilution protocol for lipid solutions used in interference studies at digoxin concentrations of 3 nmol/L.

Pooled Serum Trig.18.94 mmol/L Chol.7.49 mmol/L	Digoxin-free Serum	Digoxin Std. (40μmol/L)	Trig. Conc.	Chol. Conc.
μl	μl	μl	(mmol/L)	(mmol/L)
900	25	75	17.05	6.74
850	75	75	16.10	6.37
800	125	75	15.15	6.00
750	175	75	14.21	5.62
700	225	75	13.26	5.24
650	275	75	12.31	4.87
600	325	75	11.36	4.49
550	375	75	10.42	4.12
500	425	75	9.47	3.75
450	475	75	8.52	3.37
400	525	75	7.58	3.00
350	575	75	6.63	2.25
250	625	75	4.74	1.87
150	675	75	2.84	1.12

Table 26: Dilution protocol for lipid solutions used in interference studies at digoxin concentrations of 4 nmol/L.

Pooled Serum Trig.18.94 mmol/L Chol.7.49 mmol/L	Digoxin-free Serum	Digoxin Std. (40μmol/L)	Trig. Conc.	Chol. Conc.
μl	μl	μl	(mmol/L)	(mmol/L)
900	25	75	17.05	6.74
850	75	75	16.10	6.37
800	125	75	15.15	6.00
750	175	75	14.21	5.62
700	225	75	13.26	5.24
650	275	75	12.31	4.87
600	325	75	11.36	4.49
550	375	75	10.42	4.12
500	425	75	9.47	3.75
450	475	75	8.52	3.37
400	525	75	7.58	3.00
350	575	75	6.63	2.25
250	625	75	4.74	1.87
150	675	75	2.84	1.12

Serum samples from patients A, B and C with increased lipid concentration as determined by routine analysis were also studied for interference studies. The 5.12 nmol/L digoxin standard was used.

Table 27: Dilution protocol for lipid solutions used in interference studies for Patients A, B and C.

Patient	Digoxin Std. (5.12 nmol/L)	Digoxin Conc. (nmol/L)	Trig. Conc. (mmol/L)	Chol. Conc. (mmol/L)
A: Trig. 11.6 mmol/L Chol. 12.8 mmol/L				
µl	µl			
100	300	1.28	2.9	3.2
100	200	3.41	3.87	4.27
200	200	2.56	5.8	6.4
200	100	1.706	7.74	8.54
B: Trig. 14.0 mmol/L Chol. 18.2 mmol/L				
µl	µl			
100	300	1.28	3.5	4.55
100	200	3.41	4.66	6.06
200	200	2.56	7.0	9.1
200	100	1.706	9.31	12.12
C: Trig. 18.2 mmol/L Chol. 10.5 mmol/L				
µl	µl			
100	300	1.28	4.6	2.62
100	200	3.41	6.07	3.50
200	200	2.56	9.1	5.25
200	100	1.706	12.13	7.0

(d) *Haemoglobin:*

The pooled haemolysed samples, when analysed gave a haemoglobin concentration of 700 mg/dl. The dilution protocol used in the haemoglobin study are shown in Tables 28 - 30.

Table 28: Dilution protocol for haemoglobin solutions used in interference studies at digoxin concentrations of 2.0 nmol/L.

Pooled Hb (Conc. 700mg/dl)	Digoxin-free Serum	Digoxin Std. (40μmol/L)	Hb Conc. (mg/dl)
μl	μl	μl	
750	200	50	525
650	300	50	455
550	400	50	385
450	500	50	315
400	550	50	280
380	570	50	266
350	600	50	245
250	700	50	175
150	800	50	105
130	820	50	91
115	835	50	80.5
100	850	50	70.0
90	860	50	63.0
80	870	50	56.0

Table 29: Dilution protocol for haemoglobin solutions used in interference studies at digoxin concentrations of 3.0 nmol/L.

Pooled Hb (Conc. 700mg/dl)	Digoxin-free Serum	Digoxin Std. (40μmol/L)	Hb Conc. (mg/dl)
μl	μl	μl	
750	175	75	525
650	275	75	455
550	375	75	385
450	475	75	315
400	525	75	280
380	545	75	266
350	575	75	245
250	675	75	175
150	775	75	105
130	795	75	91
115	810	75	80.5
100	825	75	70.0
90	835	75	63.0
80	845	75	56.0

Table 30: Dilution protocol for haemoglobin solutions used in interference studies at digoxin concentrations of 4.0 nmol/L.

Pooled Hb (Conc. 700mg/dl)	Digoxin-free Serum	Digoxin Std. (40μmol/L)	Hb Conc. (mg/dl)
μl	μl	μl	
750	150	100	525
650	250	100	455
550	350	100	385
450	450	100	315
400	500	100	280
380	520	100	266
350	550	100	245
250	650	100	175
150	750	100	105
130	770	100	91
115	785	100	80.5
100	800	100	70.0
90	810	100	63.0
80	820	100	56.0

(e) *Protein:*

This was prepared by spiking 1.0 ml of phosphate buffered saline, pH 7.3 with 1.0 ml of albuminar-20 (human serum albumin 20% w/v) which, when analysed resulted in a serum protein concentration of 122 g/L. The phosphate buffered saline, pH 7.3 used in this study was prepared by dissolving 10 phosphate tablets in one litre of distilled water. The dilution protocol used in the study is shown in Tables 31-33.

Table 31: Dilution protocol for protein solutions used in interference studies at digoxin concentrations of 2 nmol/L.

Phosphate Buffered Protein (122 g/L)	Phosphate Buffer, pH 7.3.	Digoxin Std. (40μmol/L)	Protein Conc. (g/L)	Digoxin Conc. (nmol/L)
μl	μl	μl		
915	35	50	111.63	2
900	50	50	109.8	2
850	100	50	103.7	2
800	150	50	97.6	2
750	200	50	91.5	2
700	250	50	85.4	2
650	300	50	79.3	2
600	350	50	73.2	2
550	400	50	67.1	2
500	450	50	61.0	2
450	500	50	54.9	2
400	550	50	48.8	2
350	600	50	42.7	2
250	700	50	30.5	2
150	800	50	18.3	2
100	850	50	12.2	2
50	900	50	6.1	2

Table 32: Dilution protocol for protein solutions used in interference studies at digoxin concentrations of 3 nmol/L.

Phosphate Buffered Protein (122 g/L)	Phosphate Buffer, pH 7.3.	Digoxin Std. (40µmol/L)	Protein Conc. (g/L)	Digoxin Conc. (nmol/L)
µl	µl	µl		
915	10	75	111.63	3
900	25	75	109.8	3
850	75	75	103.7	3
800	125	75	97.6	3
750	175	75	91.5	3
700	225	75	85.4	3
650	275	75	79.3	3
600	325	75	73.2	3
550	375	75	67.1	3
500	425	75	61.0	3
450	475	75	54.9	3
400	525	75	48.8	3
350	575	75	42.7	3
250	675	75	30.5	3
150	775	75	18.3	3
100	825	75	12.2	3
50	875	75	6.1	3

Table 33: Dilution protocol for protein solutions used in interference studies at digoxin concentrations of 4 nmol/L.

Phosphate Buffered Protein (122 g/L)	Phosphate Buffer, pH 7.3.	Digoxin Std. (40µmol/L)	Protein Conc. (g/L)	Digoxin Conc. (nmol/L)
µl	µl	µl		
900	-	100	109.8	4
850	50	100	103.7	4
800	100	100	97.6	4
750	150	100	91.5	4
700	200	100	85.4	4
650	250	100	79.3	4
600	300	100	73.2	4
550	350	100	67.1	4
500	400	100	61.0	4
450	450	100	54.9	4
400	500	100	48.8	4
350	550	100	42.7	4
250	650	100	30.5	4
150	750	100	18.3	4
100	800	100	12.2	4
50	850	100	6.1	4

6.1.7. *Suitability and Stability of Digoxin in Serum and Plasma:*

This study was performed to ascertain:

(a) The suitability of the use of serum or plasma sample for digoxin analysis using the CEDIA and EMIT methods.

(b) The stability of digoxin in serum or plasma at various temperatures since samples were stored for use in this project. Pooled digoxin-free serum and plasma was spiked with digoxin to give a final concentration of 1.0 nmol/L (25 μl of 40 μmol/L stock standard and 950 μl of pooled serum). 10 ml volumes were prepared aliquoted and stored at 2-8°C, room temperature, and -20°C. Replicate analyses were performed on these samples at intervals up to 18 days.

A further study was performed using two serum aliquots from 5 patients on digoxin. One of the aliquots was added to a heparinized vacutainer and the other stored in a tube containing no anticoagulant. Heparinised and serum samples were taken simultaneously from six of the eleven patients.

6.2 *SALIVARY DIGOXIN MEASUREMENT:*

6.2.1. *EMIT Column Digoxin Assay:*

Reagent preparation extraction and assay procedures used were similar to those for serum digoxin (Section 5.2). The parameter listing is shown in Table 34.

6.2.2. *CEDIA Digoxin Assay:*

(a) Reagent Preparation:

The same procedure as for serum digoxin was used.

(b) Assay Procedure:

The sample volume of 25 μl used in the serum digoxin protocol was increased to 50 μl for salivary digoxin estimations. Sample preparation involved the addition of 0.1 ml of digoxin-free serum pool (6.1.3.c) to 0.1 ml of saliva. The parameter listing is shown in Table 34.

Table 34: Cobas Bio parameter listing for salivary digoxin using the CEDIA digoxin assay and EMIT assay.

		CEDIA	EMIT
α Reaction direction/Test name 11;44;49;47 (+ Digoxin)			
1	Units	14 (nmol/L)	14 (nmol/L)
2	Calculation factor	3333.3	3700
3	Standard 1	0.0	0.0
3	Standard 2	2.56	0.64
3	Standard 3	5.12	1.28
3	Standard 4		2.56
3	Standard 5		3.84
3	Standard 6		5.12
6	Limit	0	0
7	Temperature	37°C	37°C
8	Type of Analysis	3	7.3
9	Wavelength (nm)	420	340
10	Sample volume µl	50	30
11	Diluent volume µl	10	60
12	Reagent volume µl	120	115
13	Incubation time sec.	10	100
14	Start reagent volume µl	75	45
15	Time of first reading sec.	700	5
16	Time interval sec.	10	10
17	Number of readings sec.	21	25
18	Blanking mode	0	0
19	Printout mode	2	0/1

6.2.3. *Salivary Calibration Curve:*

(a) EMIT Assay:

The standards used for the calibration curve are shown in Table 35.

(b) CEDIA Assay:

0.1 ml of digoxin-free pooled saliva was added to 0.1 ml of the 0.0, 2.56 and 5.12 nmol/L digoxin standards.

6.2.4. *Sensitivity Studies:*

The zero calibrator (Table 35) was used for the EMIT method. For the CEDIA assay an equal volume of zero calibrator (serum) and digoxin-free saliva were mixed. The sensitivity was determined by assaying 10 replicates of the zero calibrator solution and calculating the concentration of digoxin which gave an identical response to that of 2.5 standard deviations above the zero calibrator.

6.2.5. *Analytical Range:*

A number of standards ranging from 0.0 to 10 nmol/L were used in this study (Table 35). The procedures are similar to the sensitivity studies (6.2.4.).

Table 35: Dilution protocol for preparation of solutions to assess analytical range for salivary digoxin.

Digoxin Standard (10 μmol/L) μl	Digoxin-Free Pooled Saliva μl	Digoxin Conc. (nmol/L)
0	1000	0.0
50	950	0.5
100	900	1.0
200	800	2.0
300	700	3.0
400	600	4.0
500	500	5.0
600	400	6.0
700	300	7.0
800	200	8.0
900	100	9.0
1000	-	10.0

The preparation of the digoxin standard solution is described in Section 6.1.3. (a).

6.2.6. *Imprecision Studies:*

Using the three Gilford controls reproducibility was determined by mixing an equal volume of pooled digoxin-free saliva and controls for both methods.

(a) Within Run Imprecision:

This was determined using 20 replicate analysis of the three Gilford controls.

(b) Total Imprecision:

This was assessed by analysing the three Gilford controls at daily intervals over 20 days.

6.2.7. *Recovery Studies:*

Accuracy was assessed by spiking digoxin-free saliva with 10 μmol/L digoxin standard covering the analytical range of the method. The digoxin standard was prepared as in Section 6.1.3. (a) (Table 36).

Table 36: Protocol for the preparation of digoxin solutions used in recovery studies.

Digoxin Standard (10 μmol/L) μl	Digoxin-Free Pooled Saliva μl	Digoxin Conc. (nmol/L)
50	950	0.5
100	900	1.0
200	800	2.0
300	700	3.0
400	600	4.0
500	500	5.0

Each solution was assayed 5 times and the mean concentration was used to determine percentage recovery.

6.2.8. *Interference Studies:*

A study was performed to investigate the presence of DLIFs in saliva from patients with renal failure, liver dysfunction and in third trimester pregnant women. None of these patients were receiving digoxin therapy.

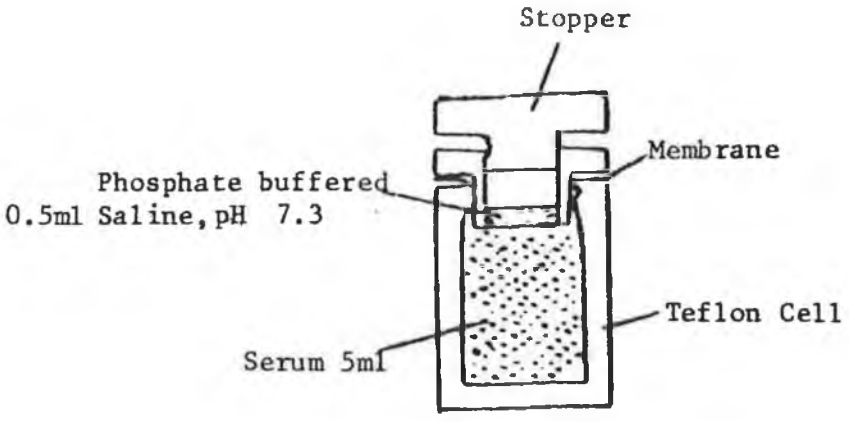
6.2.9. *Assay of Free Serum Digoxin:*

Equilibrium dialysis was used to determine free serum digoxin. Dialysis was carried out in small Type A Teflon cells (Figure 13), designed for the analysis of free thyroxine hormones in serum [107]. The procedure used in free thyroxine estimation involved the pipetting of 5 ml of dialysis buffer into the main chamber of the cell and placing a Visking dialysis membrane across the top secured by an annular clamping ring. A 200 μl volume of test serum was pipetted into the small chamber above the membrane. The cell was stoppered and placed in a water bath for 18 hours at 37°C.

The procedure was reversed for digoxin estimation, with 5 ml of serum being placed in the main chamber. 500 μl of phosphate buffered saline, pH 7.3, was pipetted above the membrane in the small chamber. The cell was then stoppered and placed in a water bath for 16 hours at 37°C with periodic shaking. The dialysate was removed from the small chamber above the membrane. Both dialysate and serum were assayed for digoxin concentration using both the CEDIA salivary and serum digoxin methods, respectively.

This study was performed on eight patients with normal renal, liver and cardiac function tests.

Figure 13:



Type A Teflon cell.

Equilibrium dialysis was used to determine free serum digoxin concentrations.

Chapter 7

ASSAY PERFORMANCE

7.1. *IMPRECISION STUDIES:*

The within batch and total imprecision performances of the EMIT and CEDIA assays follow.

7.1.1. *EMIT Column Digoxin Assay:*

	Within Batch	Total
Gilford Level I:	Mean = 1.85 nmol/L	Mean = 1.92 nmol/L
	SD = 0.05 nmol/L	SD = 0.07 nmol/L
	CV = 2.7%	CV = 3.6%
Gilford Level II:	Mean = 3.0 nmol/L	Mean = 2.91 nmol/L
	SD = 0.09 nmol/L	SD = 0.13 nmol/L
	CV = 3.0%	CV = 4.5%
Gilford Level III:	Mean = 4.4 nmol/L	Mean = 4.64 nmol/l
	SD = 0.21 nmol/L	SD = 0.27 nmol/L
	CV = 4.8%	CV = 5.8%

Number of estimates = 20.

7.1.2. *CEDIA Digoxin Assay:*

	Within Batch	Total
Gilford Level I:	Mean = 1.80 nmol/L	Mean = 1.98 nmol/L
	SD = 0.53 nmol/L	SD = 0.74 nmol/L
	CV = 2.94%	CV = 3.75%
Gilford Level II:	Mean = 3.06 nmol/L	Mean = 3.15 nmol/L
	SD = 0.10 nmol/l	SD = 0.145 nmol/L
	CV = 3.3%	CV = 4.6%
Gilford Level III:	Mean = 4.32 nmol/L	Mean = 4.58 nmol/L
	SD = 0.22 nmol/L	SD = 0.28 nmol/L
	CV = 5.1%	CV = 6.15

Number of estimates = 20.

These results compare well with those of the EMIT. The mean concentrations observed compare well with the target values quoted for the quality control materials (Section 5.1.2.). In general, the two methods are considered reproducible enough for routine use.

7.2. *SENSITIVITY*:

Values of 15 replicates of the zero calibrator.

	EMIT Digoxin Conc. (nmol/L)	CEDIA Digoxin Conc. (nmol/L)
MEAN	0.2	0.22
SD	0.03	0.032
Detection Limit (Mean ± 2 SD)	0.275	0.30

7.3. *ANALYTICAL RANGE:*

	EMIT	CEDIA
Range	0.275 - 6.0 nmol/L	0.3 - 5.12 nmol/l
Response	Logarithmic	Linear

Figures 14-15 show the responses of both methods to digoxin concentrations ranging from 0.64 to 5.12 nmol/l. A rate change of approximately 28 mA was noted for each 1.28 nmol/L of digoxin for the CEDIA method.

7.4. *RECOVERY:*

The accuracy of the recovery of digoxin from samples spiked to concentrations covering the analytical range was assessed. Table 36 shows the mean percentage recovery for each method at concentrations over the analytical range. The figures in parenthesis are coefficients of variations for the ten estimates.

Table 36: The mean percentage recoveries for both methods:

Digoxin conc. (nmol/l)	% RECOVERY EMIT	CEDIA
0.64	96 (5.9)	97 (5.8)
1.28	103 (4.3)	98 (5.7)
2.56	99 (3.5)	101 (4.6)
3.84	102 (3.8)	97 (4.4)
5.12	98 (4.7)	102 (5.5)

Figure 14: Calibration curve for EMIT immunoassay.

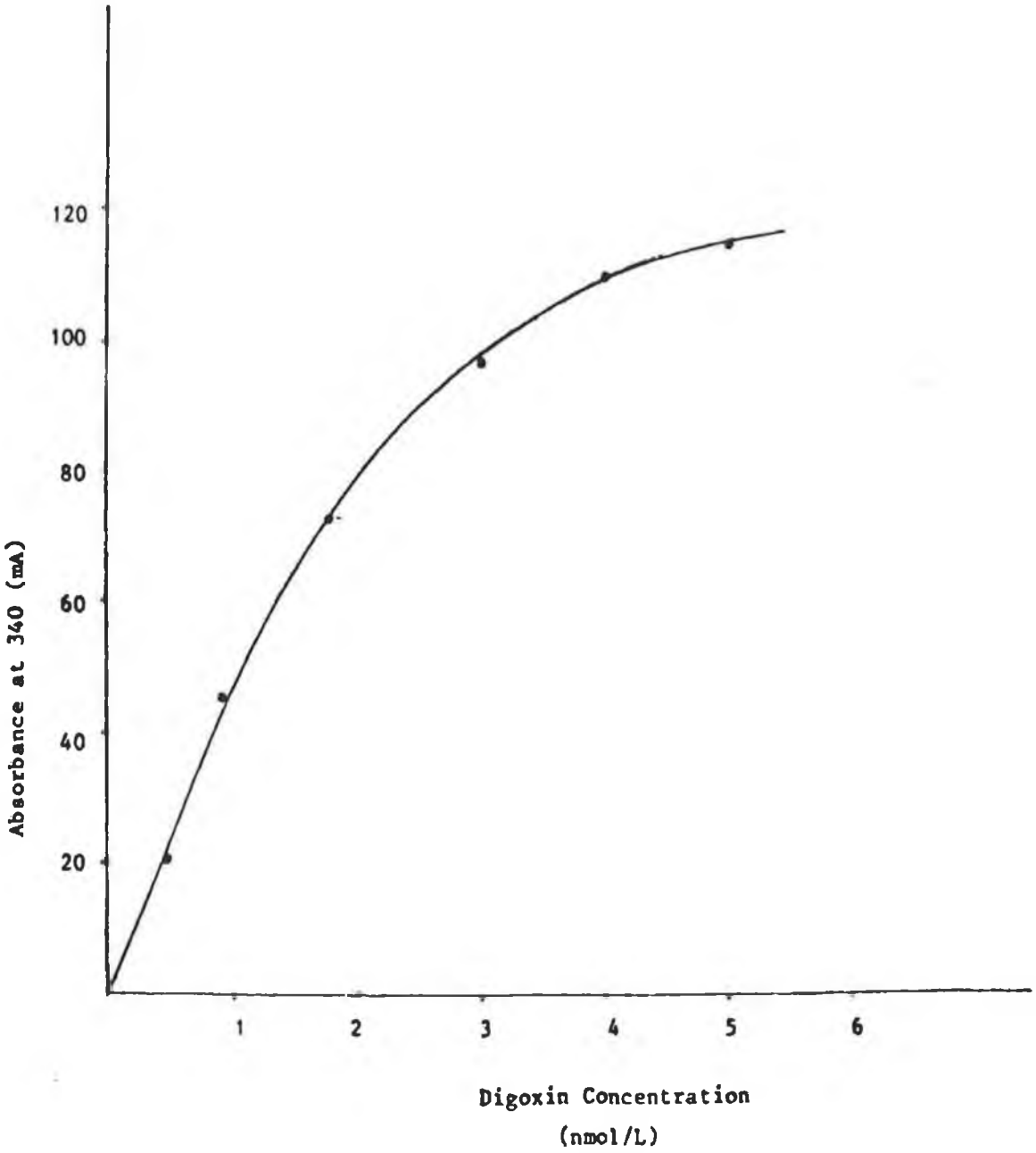
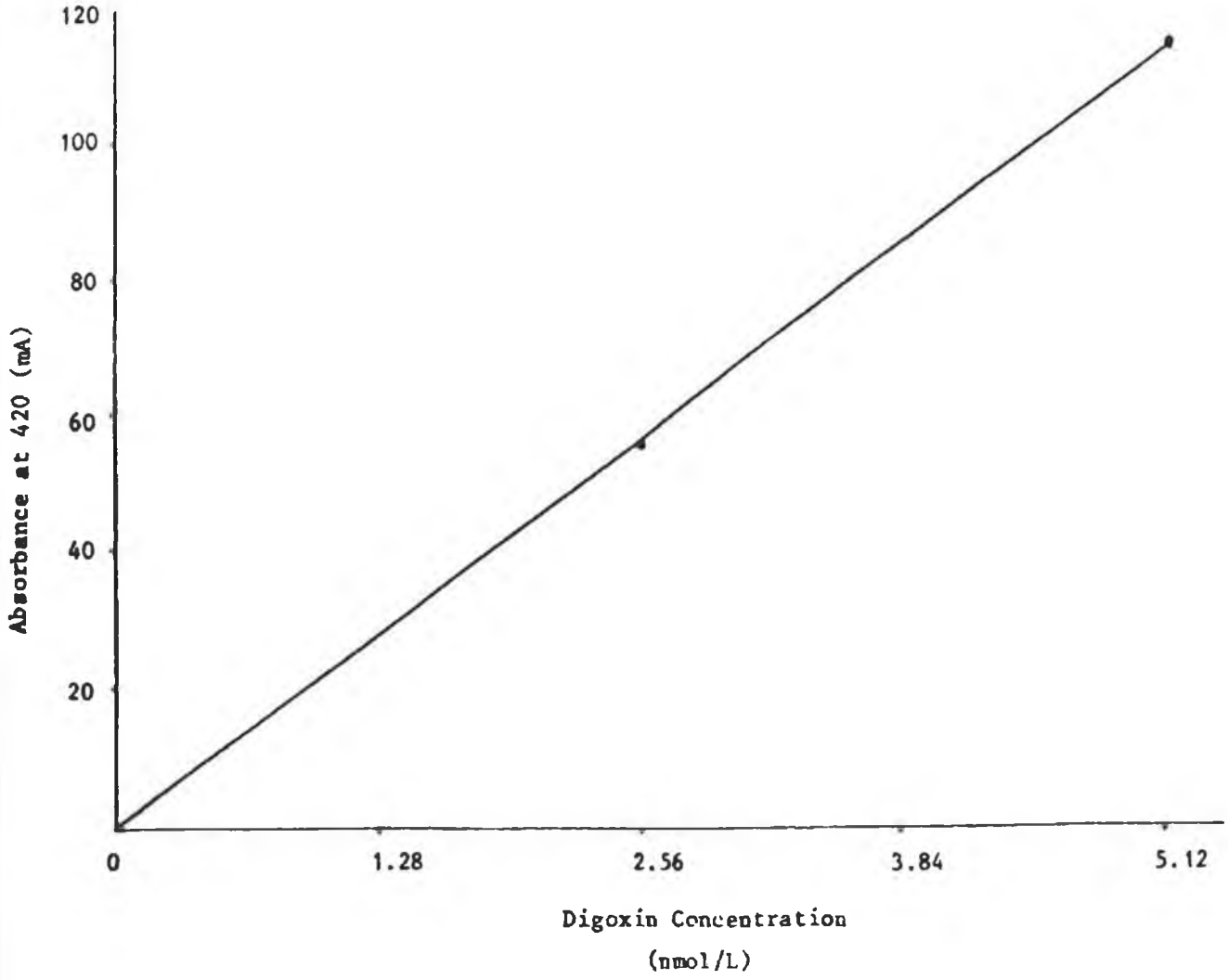


Figure 15: Calibration curve for CEDIA immunoassay.



7.5 *CALIBRATION CURVE STABILITY:*

The stability of the calibration curves is shown in Figures 16 and 17. The calibration curves for the EMIT and CEDIA assays were stable for the first 12 days followed by decreasing digoxin concentration values.

7.6. *INTERFERENCE STUDIES:*

7.6.1. *DLIF Cross-Reactivity:*

Digoxin-free serum from patients in renal failure, liver disease, third trimester pregnancy and neonates was tested for DLIF interference using the CEDIA and EMIT methods. These groups were selected since they are widely reported as having significant DLIF concentrations that interfere with most digoxin immunoassays. DLIF concentration was measured as "apparent digoxin" in each group.

(a) Renal Failure:

Table 37: DLIF determined as "apparent digoxin" in renal failure.

	Creatinine (μmol/L)	"Apparent Digoxin" (μmol/L)	
		CEDIA	EMIT
Mean	665	0.74	0.024
SD	382	0.24	0.06
Range (min-max)	134-1609	0.0-1.05	0.0-0.2

Figure 18 shows DLIF cross-reactivity as "apparent digoxin" in both assays.

In renal failure the EMIT method detected no DLIF in 16 samples and in the other 4 samples DLIF concentrations did not exceed 0.2 nmol/L. The EMIT greatly reduced or eliminated DLIF in the patients studied. In contrast, the CEDIA had only one sample where DLIF was not detected with 12 samples within the range 0.8 to 1.05 nmol/L. The remaining 7 samples had concentrations between 0.4 and 0.8 nmol/L. In renal disease DLIFs showed a significant interference with the CEDIA method.

(b) Liver Disease:

Table 38: DLIF determined as "apparent digoxin" in liver disease.

	Bilirubin (μmol/L)	"Apparent Digoxin" (nmol/L)	
		CEDIA	EMIT
Mean	70	0.19	0.06
SD	26	0.13	0.09
Range (min-max)	18-146	0.0-0.4	0.0-0.23

Figure 19 shows DLIF interference as "apparent digoxin" in both assays.

DLIFs were completely eliminated from 65% of the samples in the EMIT assay with 0.25 nmol/l being the highest concentration achieved. Using the CEDIA method, DLIF concentrations were significantly lower than those reported for it in renal failure. The highest concentration recorded was 0.4 nmol/L with 5 samples showing no DLIF interference.

Figure 16: Stability of the EMIT calibration curve.

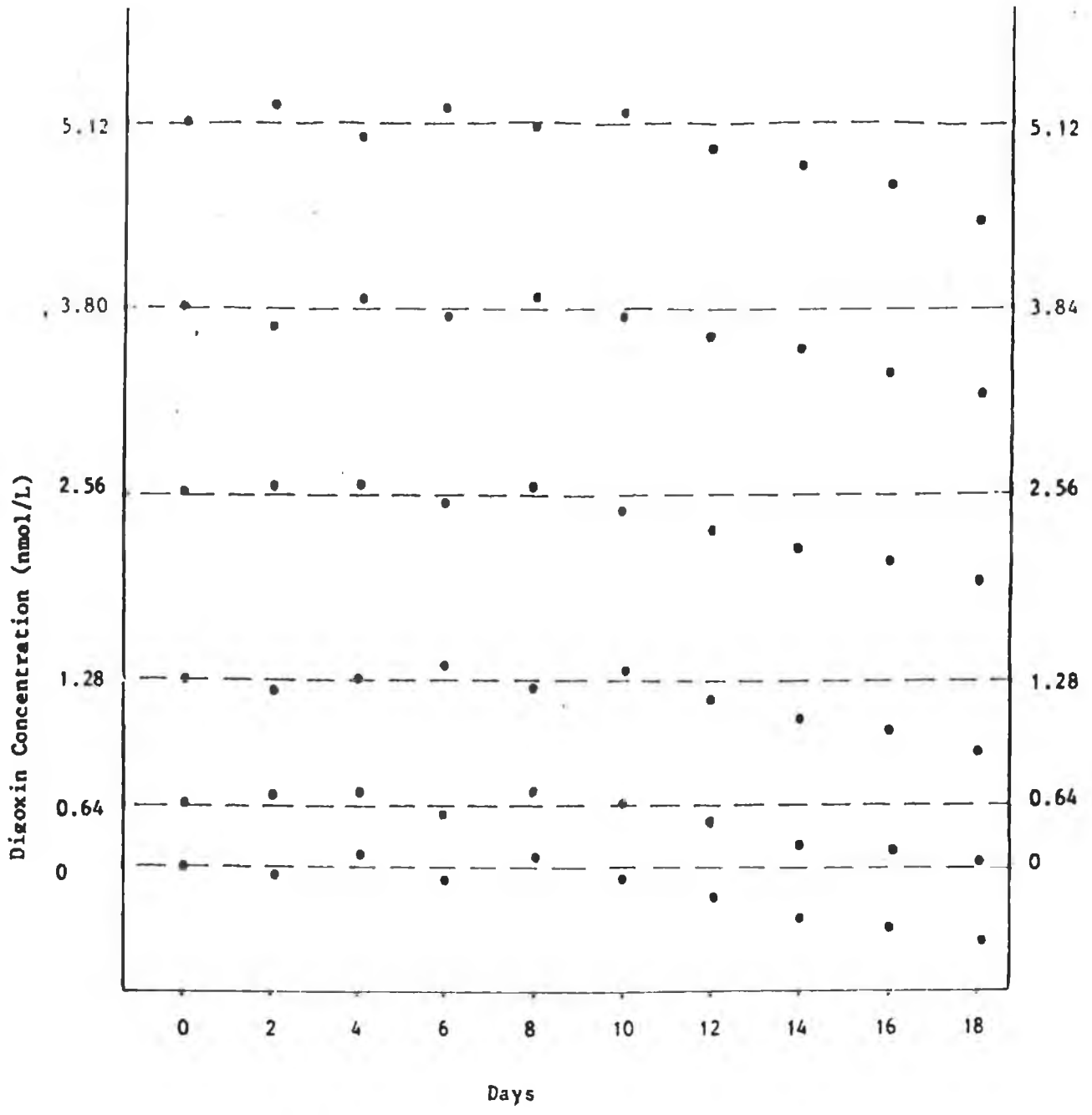


Figure 17: Stability of the CEDIA calibration curve.

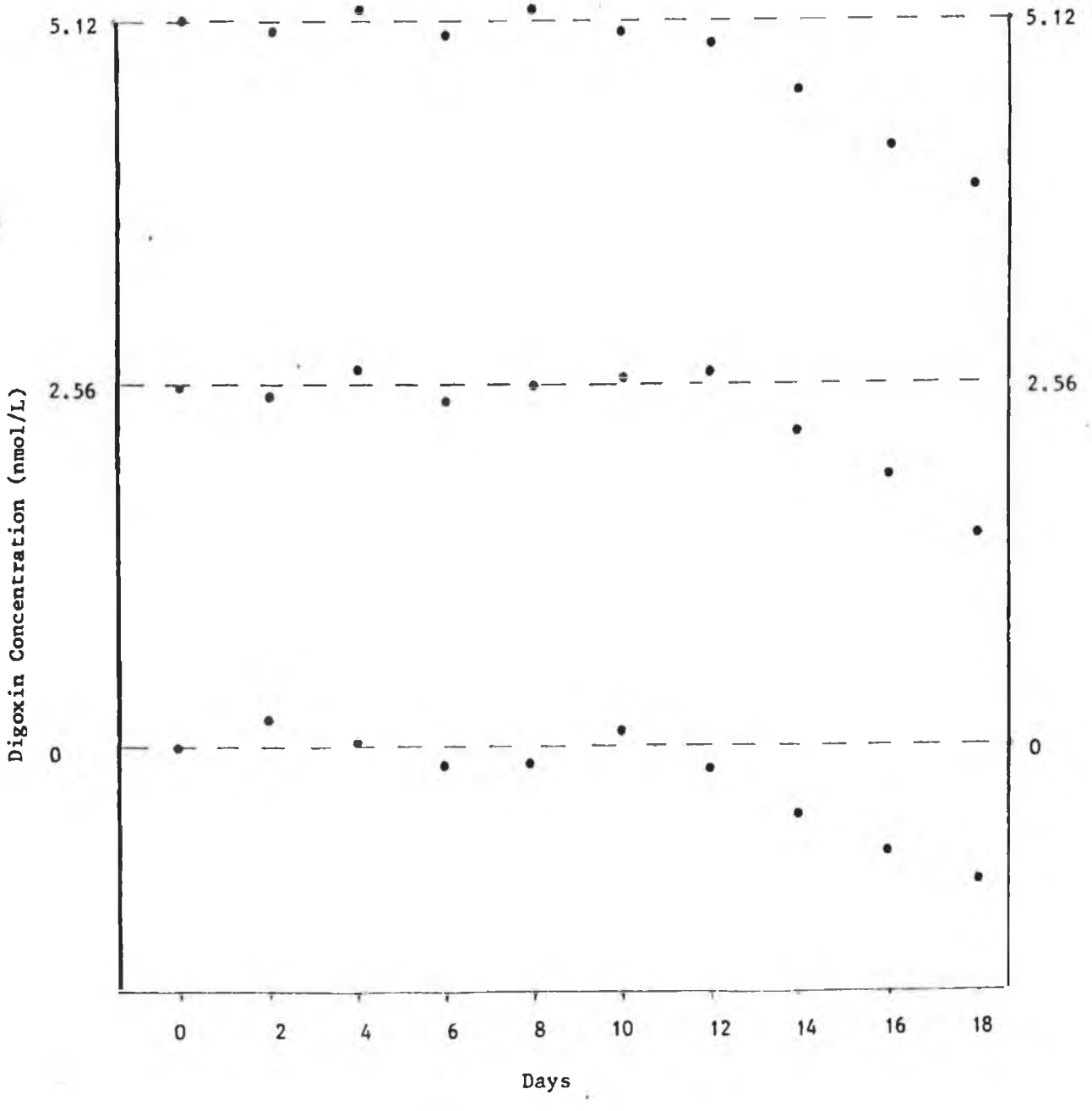


Figure 18: Concentration of DLIFs in Renal Failure.

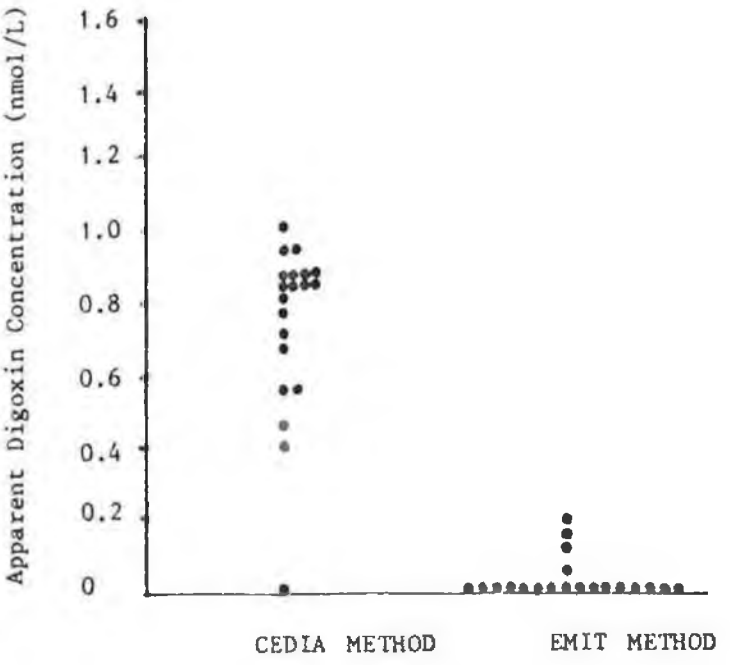
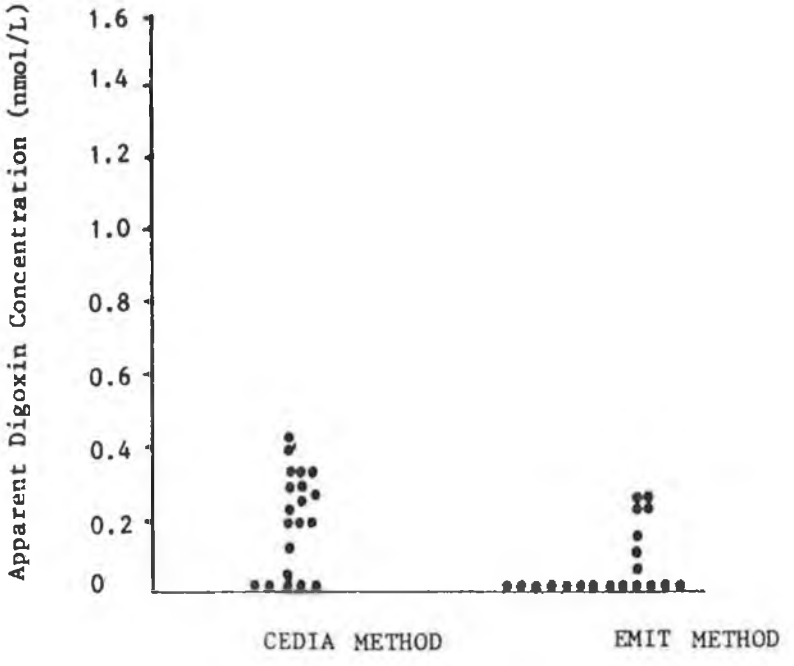


Figure 19: Concentration of DLIFs in Liver Disease.



• = "Apparent digoxin concentrations" for each patient sample.

(c) Third Trimester Pregnancy:

Table 39: DLIF determined as "apparent digoxin" in third trimester pregnancy.

	"Apparent Digoxin" Conc. (nmol/L)	
	CEDIA	EMIT
Mean	0.613	0.06
SD	0.176	0.07
Range (min-max)	0.0-0.77	0.0-0.2

Figure 20 shows DLIF cross-reactivity as "apparent digoxin" in both methods.

In pregnancy, 50% of samples had no DLIF detected using the EMIT and the remaining samples were within the range 0.05 to 0.2 nmol/L. The CEDIA method had 75% of the samples with DLIF concentrations ranging from 0.65 to 0.77 nmol/L with the remaining 3 samples giving concentrations from 0.28 - 0.38 nmol/L

(d) Neonates:

Table 40: DLIF determined as "apparent digoxin" in neonates.

	"Apparent Digoxin" Conc. (nmol/L)	
	CEDIA	EMIT
Mean	0.7	0.12
SD	0.35	0.16
Range (min-max)	0.0-1.18	0.0-0.35

Figure 21 shows DLIF interference as "apparent digoxin" in both methods.

In neonates, over half the samples analysed had no DLIF present and the other samples had concentrations varying from 0.27 to 0.36 nmol/L. In the CEDIA, only one sample had no DLIF detected with 75% of the samples having DLIF concentrations between 0.6 and 1.18 nmol/L.

Figure 20: DLIF determined as "apparent digoxin" in third trimester pregnancy.

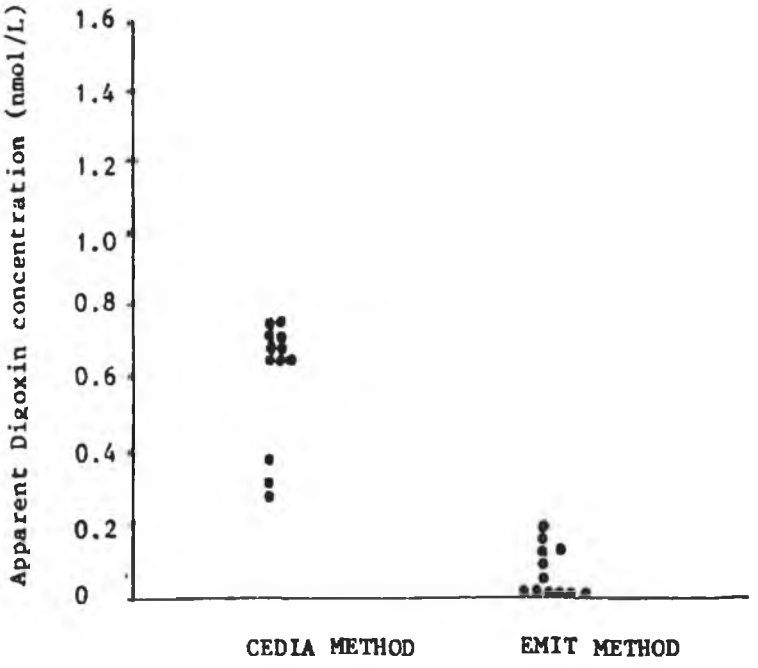
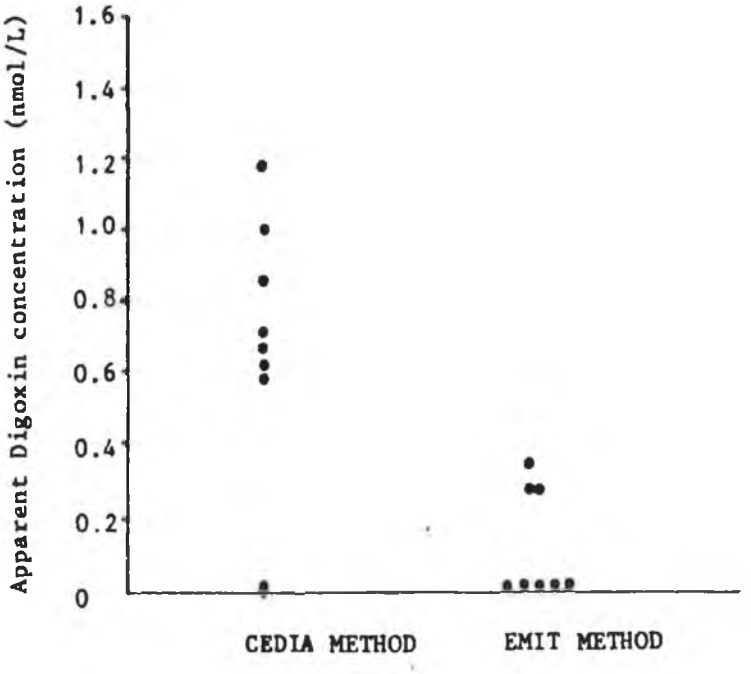


Figure 21: DLIF determined as "apparent digoxin" in neonates.



• = Apparent digoxin concentration for each patient sample

(e) Correlation between varying degrees of liver disease and serum DLIF concentration from a single patient using the CEDIA assay:

Table 41: Shows the results.

Days	Bilirubin Concentration (μmol/l)	"Apparent Digoxin" (nmol/l)
0	106	0.40
2	94	0.43
4	63	0.58
6	41	0.62
8	21	0.60

It is apparent from the above table that no correlation exists. A reduction in serum bilirubin concentration results in increased "apparent digoxin concentration".

7.6.2. *Bilirubin:*

Tables 42-44 show the effect of bilirubin on the measurement of digoxin by the EMIT and CEDIA assays. Digoxin recoveries using the EMIT were good showing similar values to those obtained for the accuracy studies. The results for the CEDIA show that with bilirubin at concentrations greater than 140.4 μmol/L no digoxin recovery was obtained for all concentrations measured. In fact, the absorbance readings obtained were less than those of the zero calibrator. Bilirubin concentrations of 54.6 μmol/L or less showed good recoveries.

Table 42: The effect of pooled bilirubin serum on a digoxin concentration of 2.0 nmol/L with the EMIT and CEDIA methods.

BILIRUBIN CONC. (μmol/L)	DIGOXIN CONC. (nmol/1)	% RECOVERY EMIT	CEDIA
280.8	2	102	0
265.2	2	104	0
249.6	2	95	0
234.0	2	103	0
218.4	2	97	0
202.8	2	94	0
187.2	2	96	0
171.6	2	102	0
156.0	2	95	0
140.4	2	105	0
132.6	2	101	9
124.8	2	99	17.8
109.2	2	95	28.1
78.0	2	97	49.0
62.4	2	99	69.1
54.6	2	101	84.3
46.8	2	98	90
31.2	2	105	96
15.6	2	103	97

Table 43: The effect of pooled bilirubin serum on a digoxin concentration of 3.0 nmol/L with the EMIT and CEDIA methods.

BILIRUBIN CONC. (μmol/L)	DIGOXIN CONC. (nmol/l)	% RECOVERY EMIT	CEDIA
280.8	3	96	0
265.2	3	102	0
249.6	3	104	0
234.0	3	95	0
218.4	3	97	0
202.8	3	94	0
187.2	3	101	0
171.6	3	103	0
156.0	3	100	0
140.4	3	98	0
132.6	3	103	5.1
124.8	3	96	12.6
109.2	3	97	32.1
78.0	3	99	52.0
62.4	3	101	72.0
54.6	3	96	84
46.8	3	103	96
31.2	3	104	97
15.6	3	102	95

Table 44: The effect of pooled bilirubin serum on a digoxin concentration of 4.0nmol/L with the EMIT and CEDIA methods.

BILIRUBIN CONC. (μmol/L)	DIGOXIN CONC. (nmol/l)	% RECOVERY EMIT	CEDIA
280.8	4	96	0
265.2	4	102	0
249.6	4	104	0
234.0	4	99	0
218.4	4	98	0
202.8	4	104	0
187.2	4	95	0
171.6	4	99	0
156.0	4	98	0
140.4	4	97	0
132.6	4	99	12.2
124.8	4	102	20.1
109.2	4	103	42.3
78.0	4	97	68.0
62.4	4	100	77.3
54.6	4	101	83.9
46.8	4	97	98.0
31.2	4	100	96.6
15.6	4	98	97.5

7.6.3. *Lipids:*

The results of these studies are presented in Tables 45-47. Lipids had no clinically significant effect on the determination of digoxin by the EMIT assay. In the CEDIA method, triglyceride concentrations greater than 15.15 mmol/L and cholesterol concentrations greater than 6.0 mmol/l gave no digoxin recoveries and inhibition was such that absorbance readings less than the zero standard were obtained (Table 46). Insignificant interference occured at triglyceride and cholesterol concentrations less than 9.47 mmol/L and 3.75 mmol/L, respectively. For the individual patient samples (Table 48) total recovery and inhibition occured at variable concentrations of triglyceride and cholesterol. For example, in patient C, 94% recovery occured at a triglyceride concentration of 6.17 mmol/L while patient A had 96% recovery at a concentration of only 3.87 mmol/L. A recovery of 90% was observed at a cholesterol concentration of 6.06 mmol/L in patient B while only 63% was reported at 6.4 mmol/L for patient A.

Table 45: The effect of pooled lipaemic serum on digoxin concentration of 2.0 nmol/L using the EMIT and CEDIA methods.

Trig. Conc. (mmol/L)	Chol. Conc. (mmol/L)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	% RECOVERY CEDIA
17.05	6.74	2	102	0
16.1	6.37	2	105	0
15.15	6.00	2	97	0
14.21	5.62	2	98	40
13.26	5.24	2	100	57.6
12.31	4.87	2	102	69.8
11.36	4.49	2	97	81.2
10.42	4.12	2	104	88.4
9.47	3.75	2	96	90
8.52	3.37	2	99	95
7.58	3.00	2	105	96
6.63	2.25	2	97	104
4.74	1.87	2	101	101
2.84	1.12	2	103	100

Table 46: The effect of pooled lipaemic serum on digoxin concentration of 3.0 nmol/L using the EMIT and CEDIA methods.

Trig. Conc. (mmol/L)	Chol. Conc. (mmol/L)	Digoxin Conc. (nmol/ L)	% RECOVERY EMIT	% RECOVERY CEDIA
17.05	6.74	3	96	0
16.1	6.37	3	98	0
15.15	6.00	3	101	0
14.21	5.62	3	95	34
13.26	5.24	3	97	54
12.31	4.87	3	102	66
11.36	4.49	3	96	79
10.42	4.12	3	104	88
9.47	3.75	3	103	93
8.52	3.37	3	99	96
7.58	3.00	3	104	97
6.63	2.25	3	98	96
4.75	1.87	3	101	102
2.84	1.12	3	97	101

Table 47: The effect of pooled lipaemic serum on digoxin concentration of 4.0 nmol/L using the EMIT and CEDIA methods.

Trig. Conc. (mmol/L)	Chol. Conc. (mmol/L)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	% RECOVERY CEDIA
17.05	6.74	4	94	0
16.1	6.37	4	102	0
15.15	6.00	4	97	0
14.21	5.62	4	98	37
13.26	5.24	4	95	49
12.31	4.87	4	99	63
11.36	4.49	4	102	77
10.42	4.12	4	97	86
9.47	3.75	4	104	92
8.52	3.37	4	101	99
7.58	3.00	4	99	95
6.63	2.25	4	103	102
4.74	1.87	4	104	95
2.84	1.12	4	97	103

Table 48: The interference due to lipids in patients A, B and C.

Patient	Chol. Conc. (mmol/L)	Trig. Conc. (mmol/L)	% Recovery EMIT	% Recovery CEDIA
A	3.2	2.90	98	97
	4.27	3.87	97	96
	6.4	5.80	105	63
	8.54	7.74	101	0
b	4.55	3.5	99	97
	6.06	4.66	104	90
	9.1	7.00	103	38
	12.12	9.32	96	0
C	2.62	4.55	94	98
	3.50	6.17	100	94
	5.25	9.10	104	68
	7.0	12.13	95	0

7.6.4. *Haemoglobin:*

These results are shown in Tables 49-51. The EMIT method had excellent recoveries with values ranging from 94% to 104%. For the CEDIA, an average recovery of 94% was observed at a haemoglobin concentration of 70 mg/dl. However, there was no digoxin recovery with haemoglobin concentrations greater than 266 mg/dl. The absorbance values at these haemoglobin concentrations were less than those for the zero calibrator.

In Table 52, the CEDIA method had no absorbance readings recorded on the Cobas Bio printout for haemolysed sera with haemoglobin concentrations from 1.8 to 3.0 g/dl. They were "flagged" instead as "high absorbance". Using the EMIT method, the haemolysed samples for these 8 patients gave similar digoxin concentrations to those of the non-haemolysed samples.

Table 49: The effect of pooled haemolysed serum on a digoxin concentration of 2 nmol/L by the EMIT and CEDIA method.

Hb. Conc. (mg/dl)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	CEDIA
525	2	97	0
455	2	94	0
385	2	100	0
315	2	104	0
280	2	101	0
266	2	95	0
245	2	102	16
175	2	96	53
105	2	95	71
91	2	98	77
80.5	2	97	85
70.0	2	103	92
63.0	2	97	95
56.0	2	101	96

Table 50: The effect of pooled haemolysed serum on a digoxin concentration of 3 nmol/L by the EMIT and CEDIA method.

Hb. Conc. (mg/dl)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	CEDIA
525	3	102	0
455	3	96	0
385	3	95	0
315	3	97	0
280	3	94	0
266	3	99	0
245	3	101	12
175	3	97	43
105	3	102	68
91	3	103	80
80.5	3	100	87
70.0	3	96	94
63.0	3	95	99
56.0	3	98	101

Table 51: The effect of pooled haemolysed serum on a digoxin concentration of 4 nmol/L by the EMIT and CEDIA method.

Hb. Conc. (mg/dl)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	CEDIA
525	4	95	0
455	4	98	0
385	4	104	0
315	4	99	0
280	4	102	0
266	4	101	0
245	4	96	18
175	4	97	57
105	4	99	70
91	4	104	79
80.5	4	102	84
70.0	4	101	96
63.0	4	99	100
56.0	4	100	98

Table 52: Shows results of a study on 8 patients using haemolysed and non-haemolysed serum from each sample where the digoxin concentration was determined.

Patient	Hb Conc. (g/dl)	Digoxin Conc. (nmol/L) Haemolysed CEDIA	EMIT	Non-Haemolysed CEDIA	EMIT
1	2.0	"High Abs"	1.6	1.5	1.7
2	2.5	"High Abs"	2.2	2.2	2.1
3	1.8	"High Abs"	2.4	2.6	2.4
4	2.4	"High Abs"	0.9	0.8	0.8
5	2.2	"High Abs"	1.3	1.1	1.0
6	1.9	"High Abs"	1.4	1.5	1.3
7	3.0	"High Abs"	1.5	1.3	1.4
8	2.8	"High Abs"	2.7	3.0	2.8

7.6.5. *Protein:*

Tables 53-55 show the effect of protein on digoxin estimation by the EMIT and CEDIA assays. In the EMIT method, excellent overall recovery was achieved. For the CEDIA method, very good recovery was attained between 42.7 and 79.3 g/l. Protein concentrations greater than this gave decreased digoxin recovery with 111.6 g/l giving a mean of only 63%. Digoxin concentrations were over-estimated with protein concentrations of 30.5 g/l or below.

Table 53: The effect of protein on a digoxin concentration of 2 nmol/L using the EMIT and CEDIA techniques.

Protein Conc. (g/l)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	CEDIA
111.6	2	96	57.5
109.8	2	98	62.5
103.7	2	101	70.0
97.6	2	95	77.5
91.5	2	104	80.0
85.4	2	97	85.0
79.3	2	102	97.5
73.2	2	103	95.0
67.1	2	99	102.5
61.0	2	96	105
54.9	2	97	100
48.9	2	103	92.5
42.7	2	101	105
30.5	2	100	128
18.3	2	97	140
12.3	2	96	152.5
6.1	2	101	235

Table 54: The effect of protein on a digoxin concentration of 3 nmol/L using the EMIT and CEDIA techniques.

Protein Conc. (g/l)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	CEDIA
111.6	3	101	61.7
109.8	3	95	63.3
103.7	3	100	70
97.6	3	99	78.3
91.5	3	97	83.3
85.4	3	96	86.6
79.3	3	98	98
73.2	3	102	96.6
67.1	3	97	101.7
61.0	3	103	96.7
54.9	3	101	98.3
48.9	3	100	103.3
42.7	3	97	103
30.5	3	96	126
18.3	3	101	135
12.3	3	102	158.3
6.1	3	99	247

Table 55: The effect of protein on a digoxin concentration of 4 nmol/L using the EMIT and CEDIA techniques.

Protein Conc. (g/l)	Digoxin Conc. (nmol/L)	% RECOVERY EMIT	CEDIA
111.6	4	99	70
109.8	4	101	72.5
103.7	4	102	75
97.6	4	100	80
91.5	4	97	82.5
85.4	4	99	86.2
79.3	4	96	96
73.2	4	95	98.8
67.1	4	101	98.7
61.0	4	103	102.5
54.9	4	101	101
48.9	4	104	102.5
42.7	4	96	104
30.5	4	98	127
18.3	4	100	137.5
12.3	4	97	151
6.1	4	102	231

7.7. *METHOD COMPARISONS:*

Direct comparisons of the methods investigated using results from 52 patients' samples are illustated in Figures 22 to 24. Data derived from linear regression is given as:

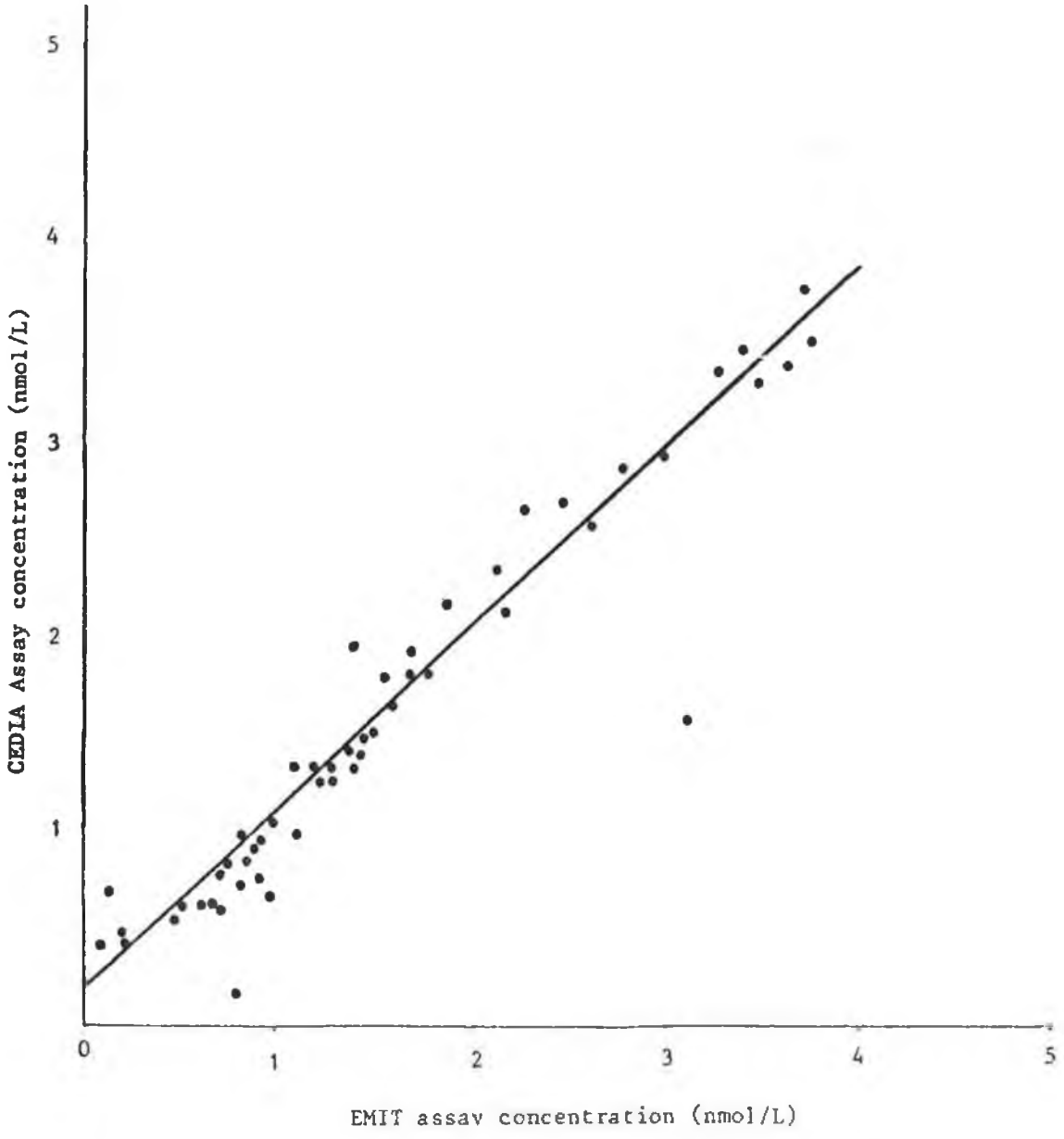
$$y\ (CEDIA) = 0.91\ (EMIT) + 0.17$$

$$y\ (CEDIA) = 0.92\ (RIA) + 0.16$$

$$y\ (EMIT) = 1.008\ (RIA) + 0.009$$

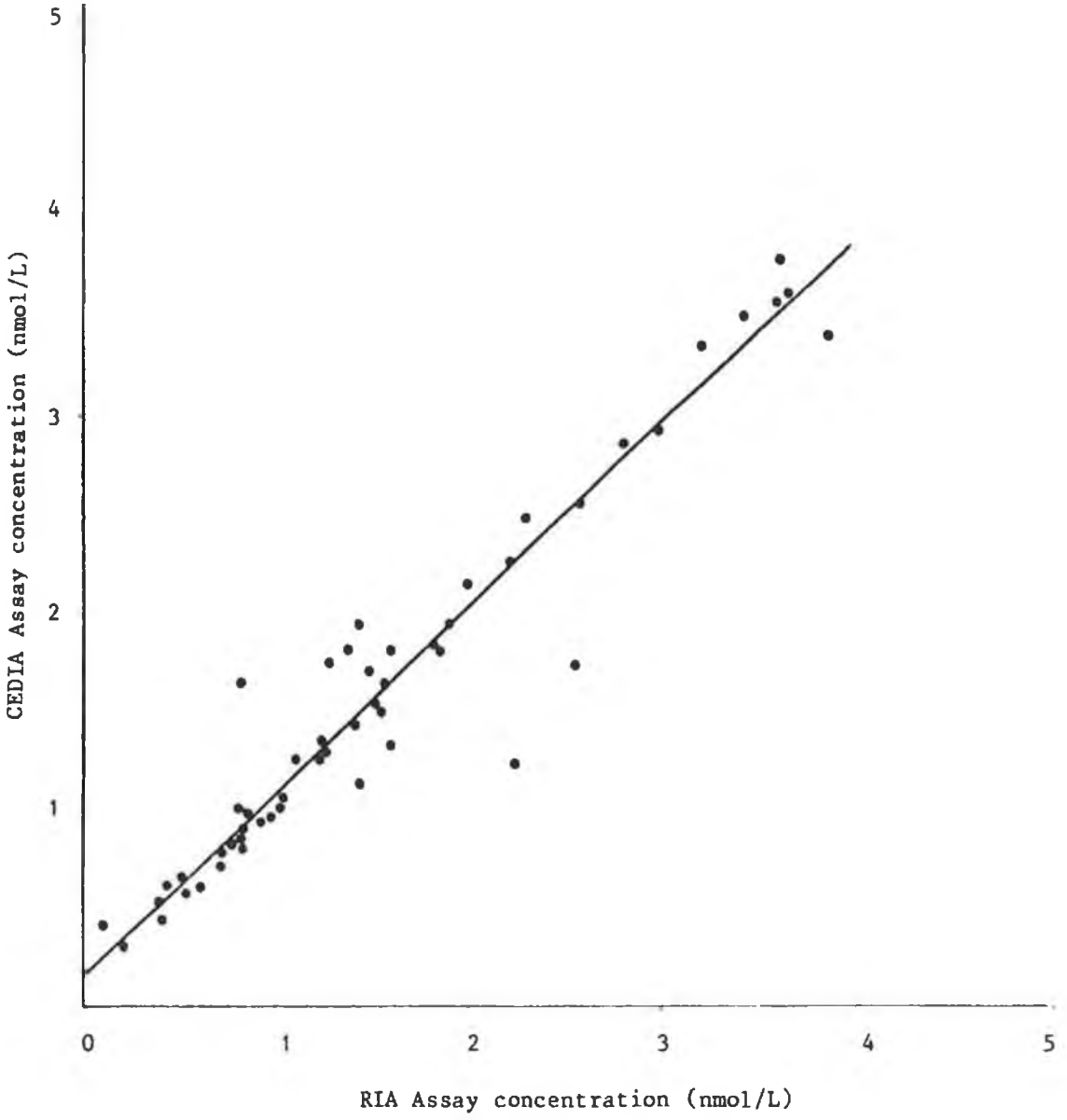
The correlation between all the methods was found to be in good agreement within the range of the calibration curve.

Figure 22: Correlation between digoxin assayed by EMIT and CEDIA enzyme immunoassays.



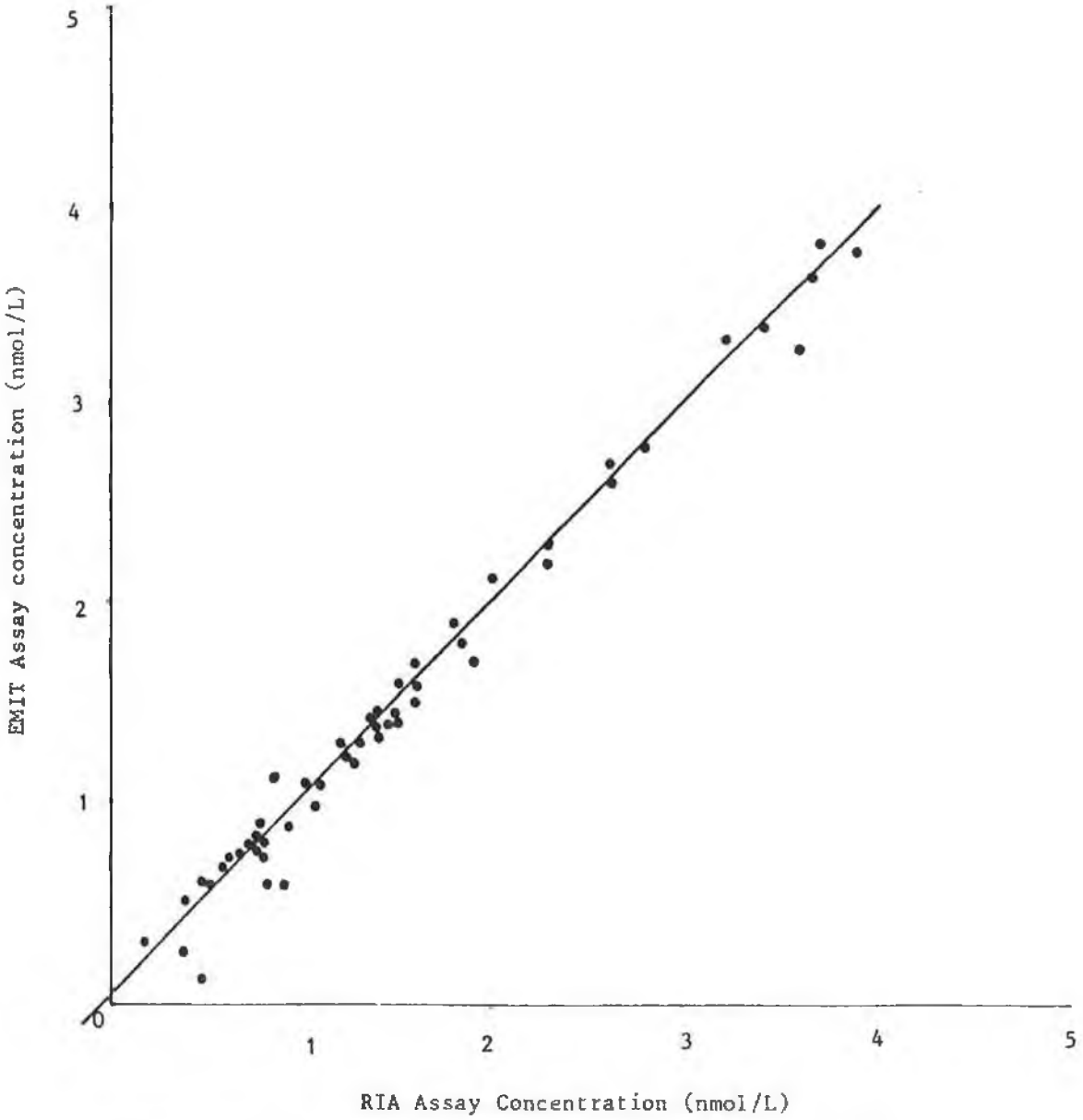
Slope 0.91; Intercept (nmol/L) 0.17; Mean (nmol/L);
EMIT 1.53; CEDIA 1.66; Correlation Coefficient 0.985;
y = 0.91x + 0.17; n = 52.

Figure 23: Correlation between digoxin assayed by RIA and CEDIA methods.



Slope 0.92; Intercept (nmol/L) 0.16; Mean (nmol/L);
CEDIA 1.56; RIA 1.527; Correlation Coefficient 0.94;
y = 0.92x + 0.16; n = 52.

Figure 24: Correlation between digoxin assayed by RIA and EMIT.



Slope 1.008; Intercept (nmol/L) 0.009; Mean (nmol/L);
EMIT 1.53; RIA 1.527; Correlation Coefficient 0.995;
y = 1.998x + 0.009; n = 52.

7.8. *STABILITY OF DIGOXIN IN SERUM AND PLASMA AT -20°C, 4°C AND ROOM TEMPERATURE:*

Figures 25 and 26 show the stabilities of digoxin in plasma and serum over a period of 18 days. The EMIT method gave very good digoxin stability for plasma and serum at the three temperatures studied. The CEDIA showed excellent stability for serum but variable digoxin was noted for the plasma. Significant instability occured during the first eight days, e.g. a drop of 41% was recorded from day 2 to day 4 at -20°C.

The addition of heparin to the serum samples did not appear to have any effect (Table 56). Some of the plasma digoxin results were variable relative to those of the serum.

Figure 25: Stability of Digoxin in serum for EMIT assay.

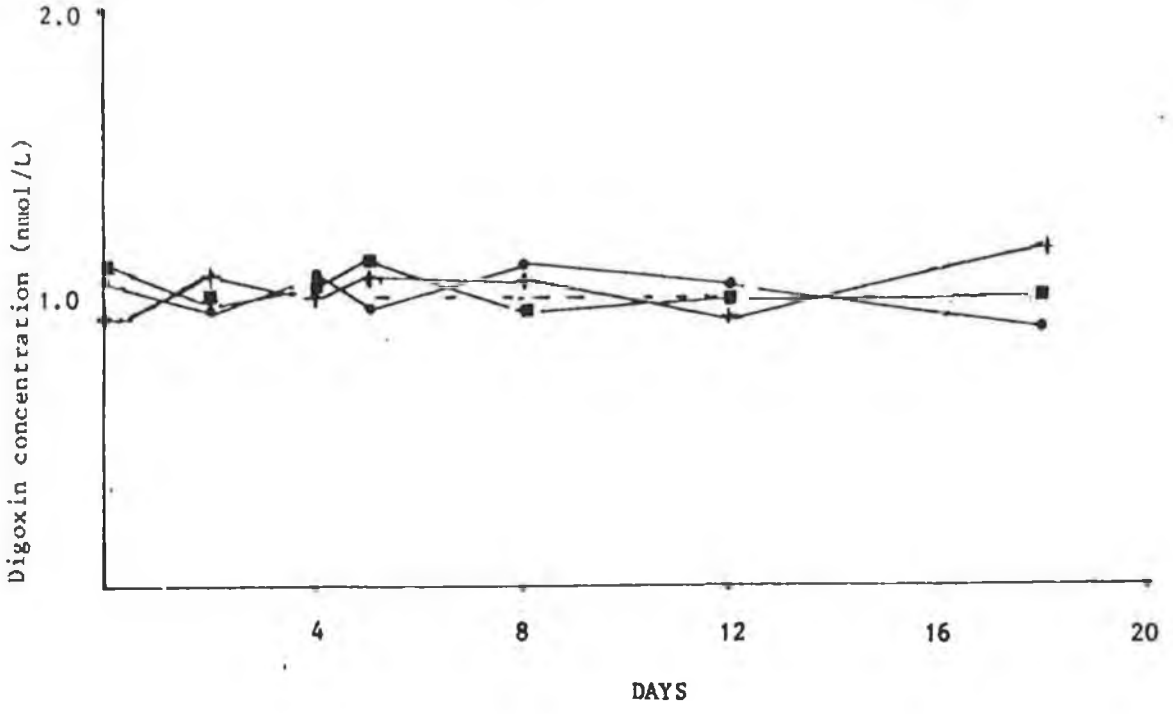


Figure 26: Stability of Digoxin in plasma for EMIT assay.

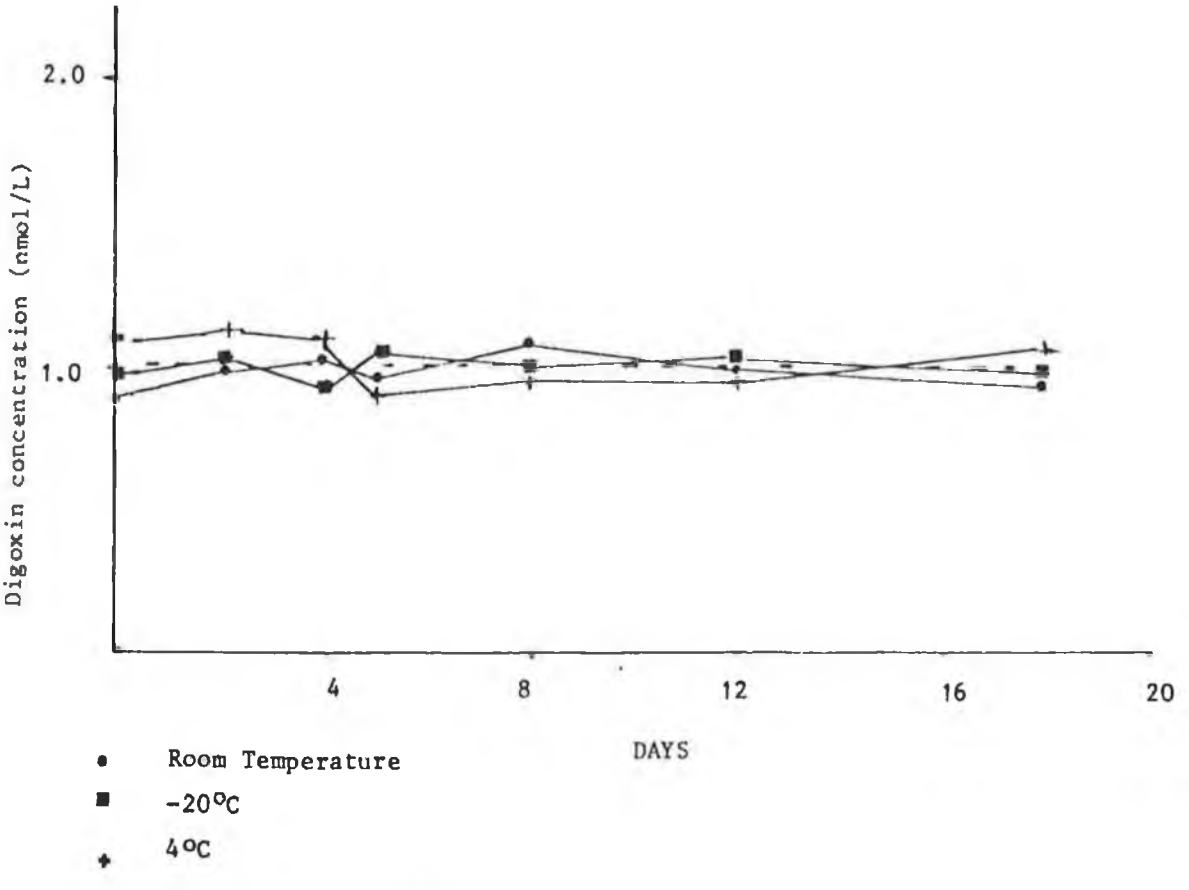


Figure 27: Stability of Digoxin in serum for CEDIA assay.

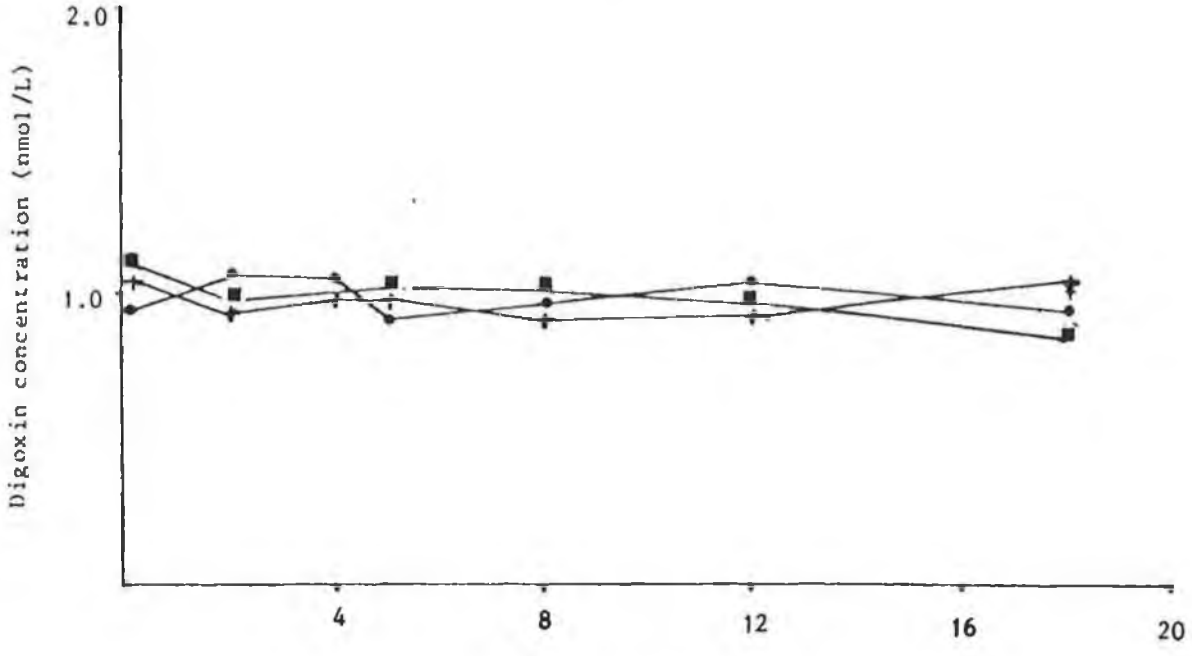


Figure 28: Stability of Digoxin in plasma for CEDIA assay.

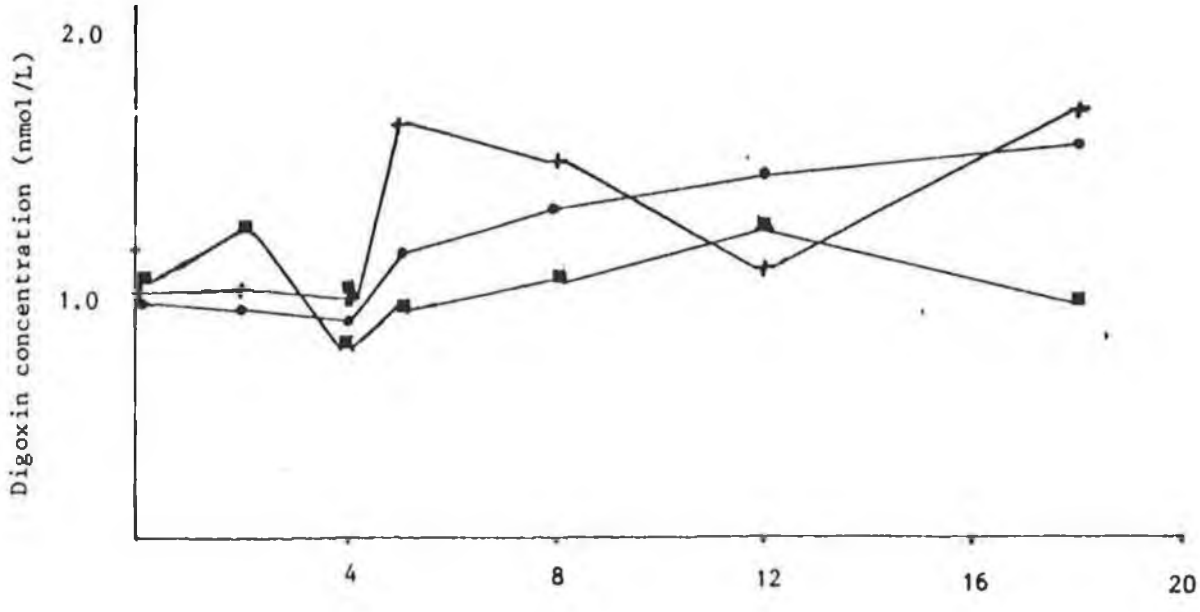


Table 56: The effect of heparin on the CEDIA immunoassay using sera and plasma from eleven patients on digoxin

Patient	Serum Digoxin Conc. (nmol/L)	Heparinised Plasma Digoxin Conc. (nmol/L)	Serum + Heparin Digoxin Conc. (nmol/L)
A	0.4	0.35	0.45
B	0.75	0.24	0.62
C	0.4	0.5	0.475
D	1.325	1.0	0.186
E	0.925	1.05	1.85
F	0.72	0.86	0.80
G	0.67		0.70
H	2.88		2.85
I	1.73		1.66
J	0.44		0.38
K	1.46		1.38

Simultaneous heparinised plasma and serum was taken from 6 patients namely, A, B, C, D, E and F.

7.9. *SALIVARY DIGOXIN MEASUREMENT:*

7.9.1. *Imprecision Studies:*

The within batch and total imprecision performances of the EMIT and CEDIA assays follow:

(a) CEDIA Method:

	Within Batch	**Total**
Gilford Level I:	Mean = 1.85 nmol/L	Mean = 2.03 nmol/L
	SD = 0.07 nmol/L	SD = 0.085 nmol/L
	CV = 3.8%	CV = 4.2%
Gilford Level II:	Mean = 3.28 nmol/L	Mean = 3.39 nmol/L
	SD = 0.134 nmol/L	SD = 0.152 nmol/L
	CV = 4.1%	CV = 4.5%
Gilford Level III:	Mean = 4.26 nmol/L	Mean = 4.5 nmol/l
	SD = 0.187 nmol/L	SD = 0.21 nmol/L
	CV = 4.4%	CV = 4.7%

Number of estimates = 15.

(b) EMIT Assay:

	Within Batch	Total
Gilford Level I:	Mean = 0.89 nmol/L	Mean = 0.96 nmol/L
	SD = 0.33 nmol/L	SD = .038 nmol/L
	CV = 3.7%	CV = 4.0%
Gilford Level II:	Mean = 1.6 nmol/L	Mean = 1.71 nmol/L
	SD = 0.061 nmol/L	SD = 0.072 nmol/L
	CV = 3.8%	CV = 4.2%
Gilford Level III:	Mean = 2.12 nmol/L	Mean = 2.28 nmol/1
	SD = 0.085 nmol/L	SD = 0.10 nmol/L
	CV = 4.0%	CV = 4.5%

Number of estimates = 15.

Digoxin concentration values for EMIT are approximately half that for CEDIA because the Gilford controls were diluted with equal volumes of saliva and serum. The parameter listing for the EMIT on the Cobas Bio, unlike that for the CEDIA, would not accept the 100% increase in sample volume.

7.9.2. *Sensitivity:*

Table 57: Results of 10 replicates of the zero calibrator solution.

	EMIT Digoxin (nmol/L)	CEDIA Digoxin (nmol/L)
Mean	0.231	0.242
SD	0.036	0.042
Detection Limits (mean ± 2.5 SD)	0.32	0.34

7.9.3. *Analytical Range:*

The EMIT method is linear from 0.32 to 5.12 nmol/L and the CEDIA method has an assay range from 0.34 to 5.12 nmol/L. Figure 29 show salivary calibration curve for CEDIA method. The salivary calibration curve for EMIT was similar to that for serum (Figure 14).

7.9.4. *Recovery:*

Table 58: Results of 5 replicates of 0.5, 1.0, 2.0, 3.0, 4.0 and 5 nmol/L of spiked digoxin saliva solutions.

Digoxin conc. (nmol/l)	% RECOVERY	
	EMIT	CEDIA
0.5	95 (5.1)	96 (5.0)
1.0	96 (3.9)	95 (3.8)
2.0	103 (3.7)	97 (3.9)
3.0	98 (4.1)	104 (4.3)
4.0	102 (3.9)	97 (4.4)
5.0	103 (4.3)	104 (4.2)

Shown in parenthesis is the coefficient of variation.

Figure 29: Calibration curve for salivary digoxin measurement using the CEDIA immunoassay method.

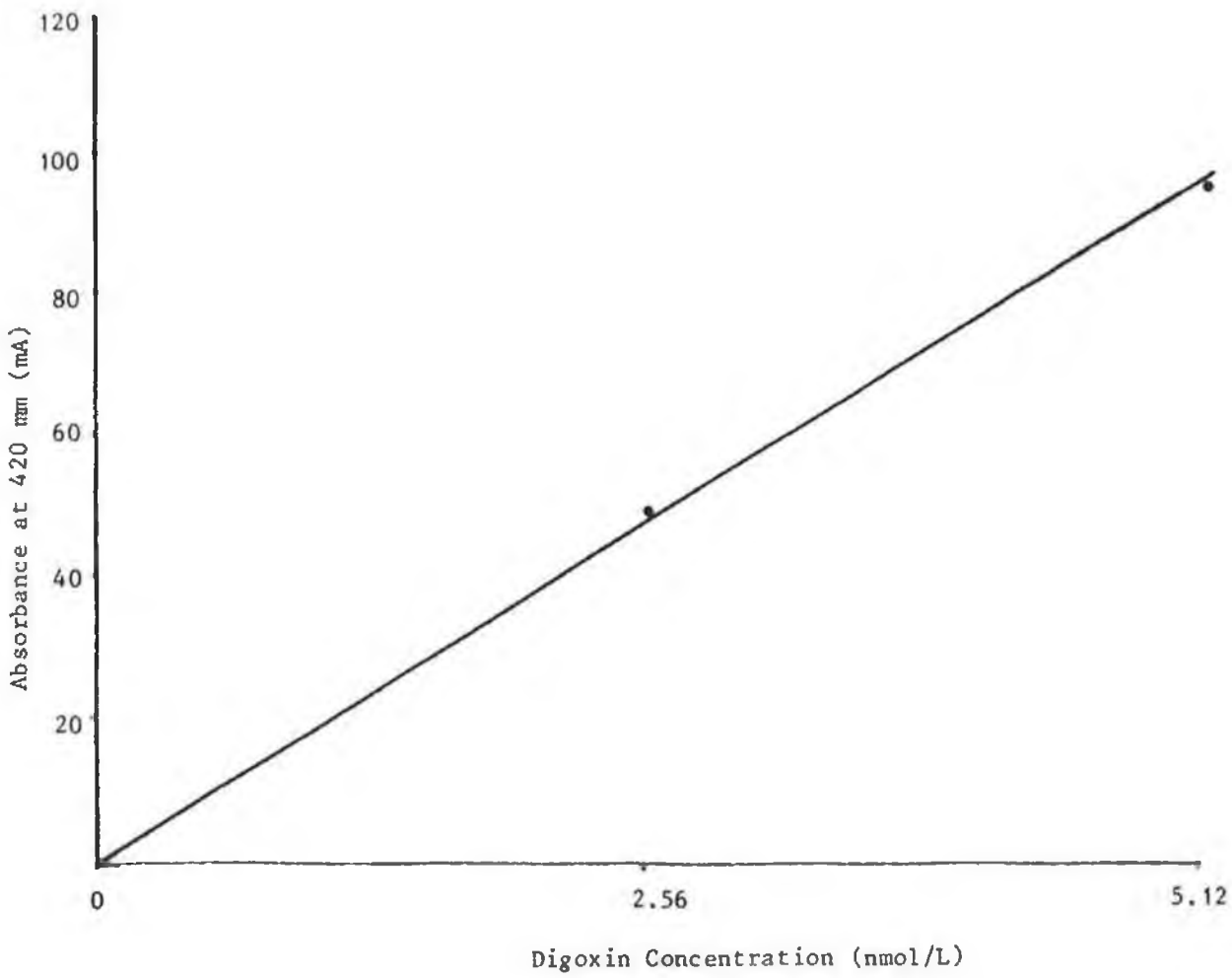


Figure 30 shows salivary DLIF cross-reactivity as "apparent digoxin" in both assays.

In renal failure, the EMIT method detected no DLIF in 16 samples. The remaining 4 samples were within the range 0.08-0.18 nmol/L. In the CEDIA method, 7 samples showed values up to 0.22 nmol/L.

7.9.5. *Cross-Reactivity of DLIFs in Saliva:*

Digoxin-free saliva from patients in renal failure, liver disease and third trimester pregnancy was tested for DLIF interference using the EMIT and CEDIA methods. These groups were selected since they are widely reported as having significant serum DLIF concentrations that interference with most digoxin immunoassays. DLIF concentration was measured as "apparent digoxin" in each group.

(a) Renal Disease:

Table 59: Salivary DLIF values determined as "apparent digoxin" in renal disease (n=20).

	Serum Creatinine (μmol/L)	"Apparent Digoxin" (nmol/L)	
		CEDIA	EMIT
Mean	665	0.05	0.026
SD	382	0.076	0.055
Range (min-max)	134-1609	0.0-0.22	0.0-0.18

(b) Liver Disease:

Table 60: Salivary DLIF determined as "apparent digoxin" in liver disease.

	Serum Bilirubin (μmol/L)	"Apparent Digoxin" (μmol/L)	
		CEDIA	EMIT
Mean	70	0.045	0.03
SD	26	0.072	0.057
Range (min-max)	18-146	0.0-0.23	0.0-0.17

There was no DLIF detection in 75% and 65% of the samples in the EMIT and CEDIA methods, respectively. The remainder of the samples were outside the sensitivity range of both methods.

Figure 31 shows salivary DLIF cross-reactivity as "apparent digoxin" in both assays.

Figure 30: Salivary DLIFs in renal disease.

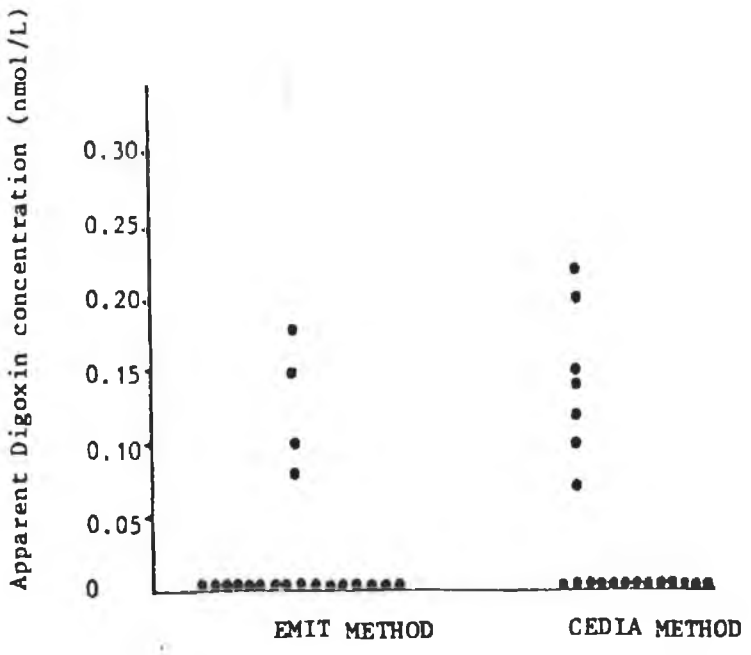
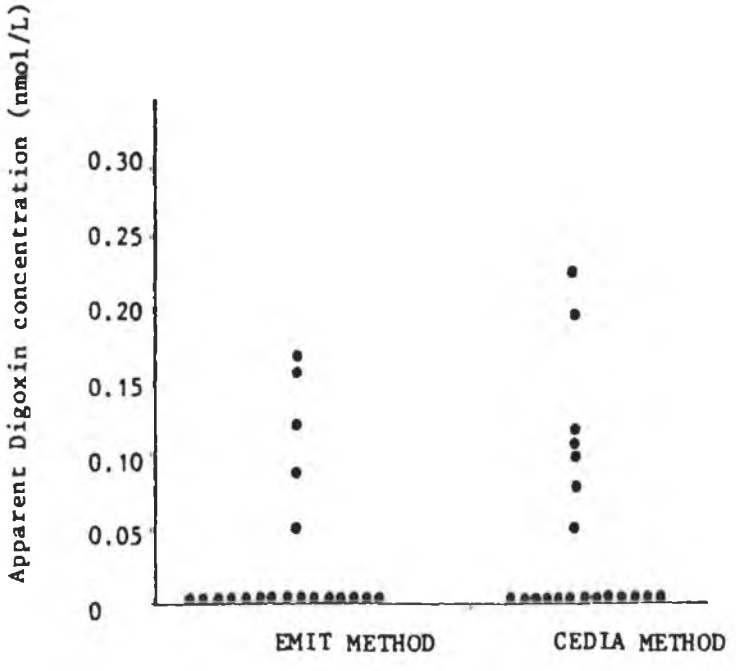


Figure 31: Salivary DLIFs in liver disease.



• = "Apparent Digoxin concentration" for each patient sample.

(c) Third Trimester Pregnancy:

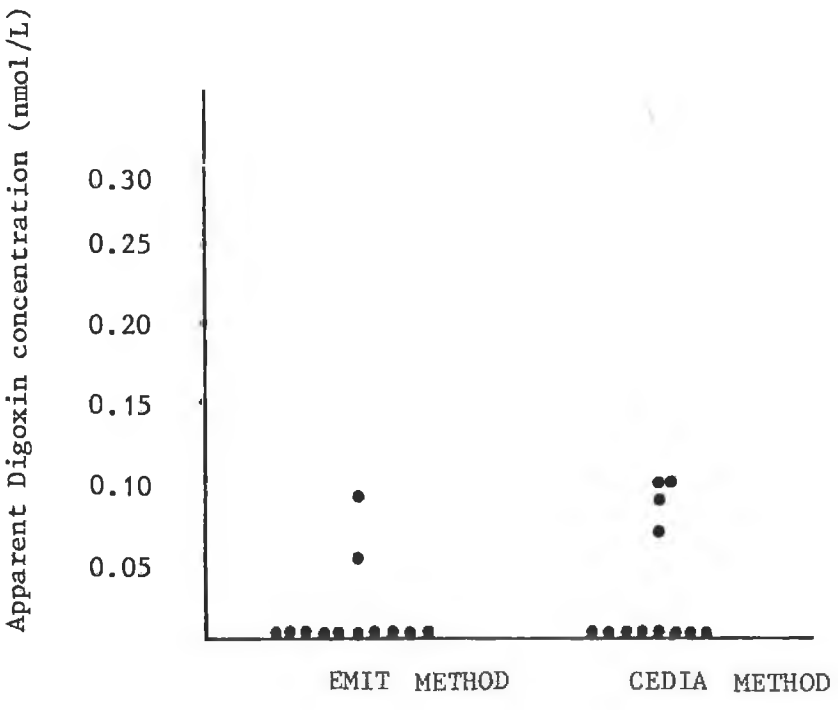
Table 61: Salivary DLIF determined as "apparent digoxin" in third trimester pregnancy (n=12).

	"Apparent Digoxin" Conc. (nmol/L)	
	CEDIA	EMIT
Mean	0.03	0.012
SD	0.045	0.029
Range (min-max)	0.0-0.1	0.0-0.09

In the EMIT method 83% of the salivary samples had no DLIF detected with negligible concentrations in the other samples. The CEDIA had 66.6% of the samples showing no DLIF concentration and the remaining samples were beyond the sensitivity of the method.

Salivary DLIF are presented as "apparent digoxin" in Figure 32.

Figure 32: Salivary DLIFs in Pregnancy



• = "Apparent Digoxin Concentration" for each patient sample.

7.9.6. *Serum/Saliva Ratio of Digoxin for the CEDIA and EMIT Methods:*

The relationship between saliva and serum concentrations of digoxin for both methods is shown in Figures 33 and 34. The values for paired salivary and serum specimens were related by the following linear regression equations (based on $y = mx + c$).

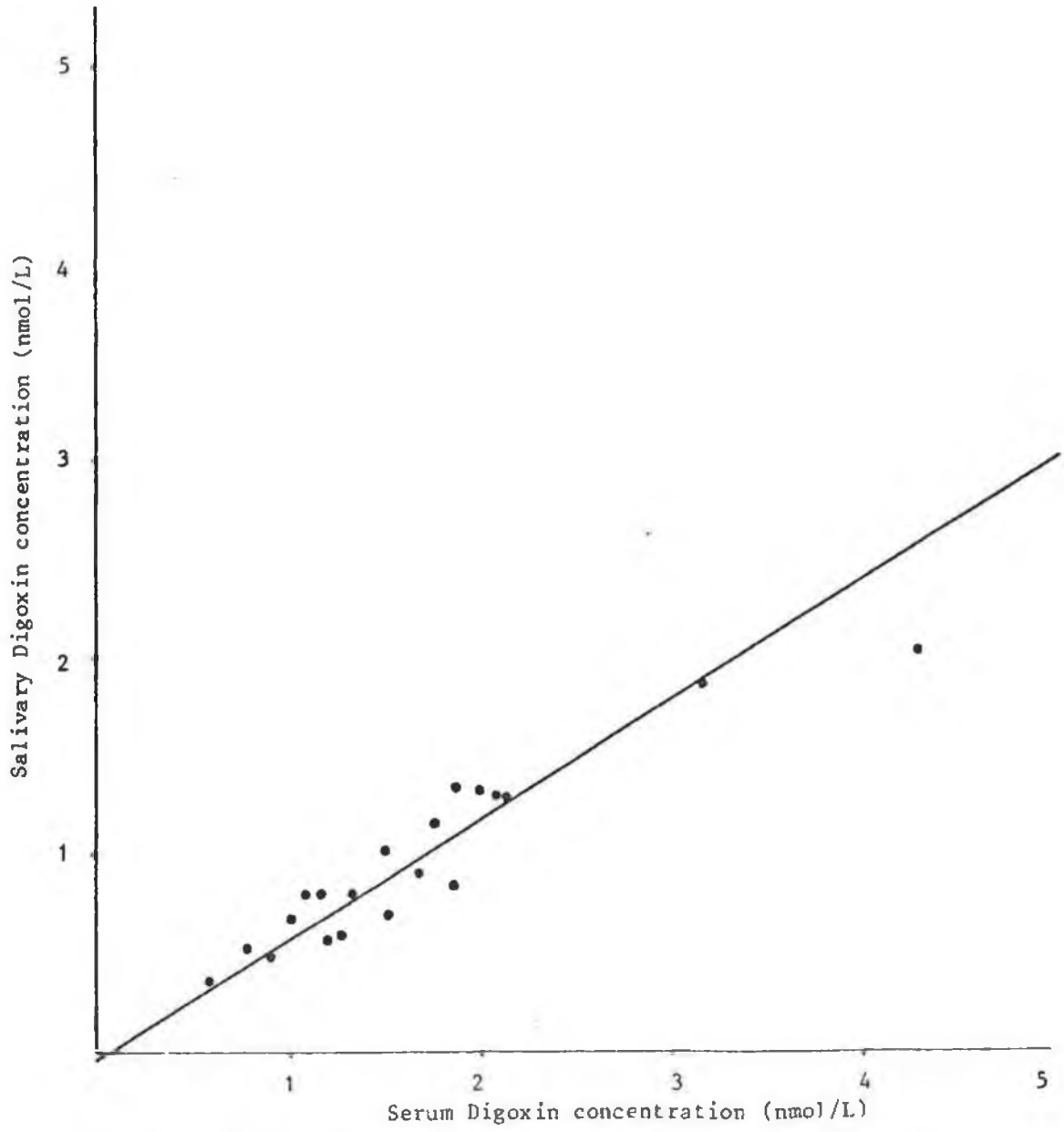
CEDIA:	Salivary Digoxin	=	0.61 (Serum Digoxin)	-	0.04
EMIT:	Salivary Digoxin	=	0.67 (Serum Digoxin)	+	0

The mean saliva/serum concentration ratio (Table 62) is:

CEDIA: 0.62 with a standard deviation of 0.1

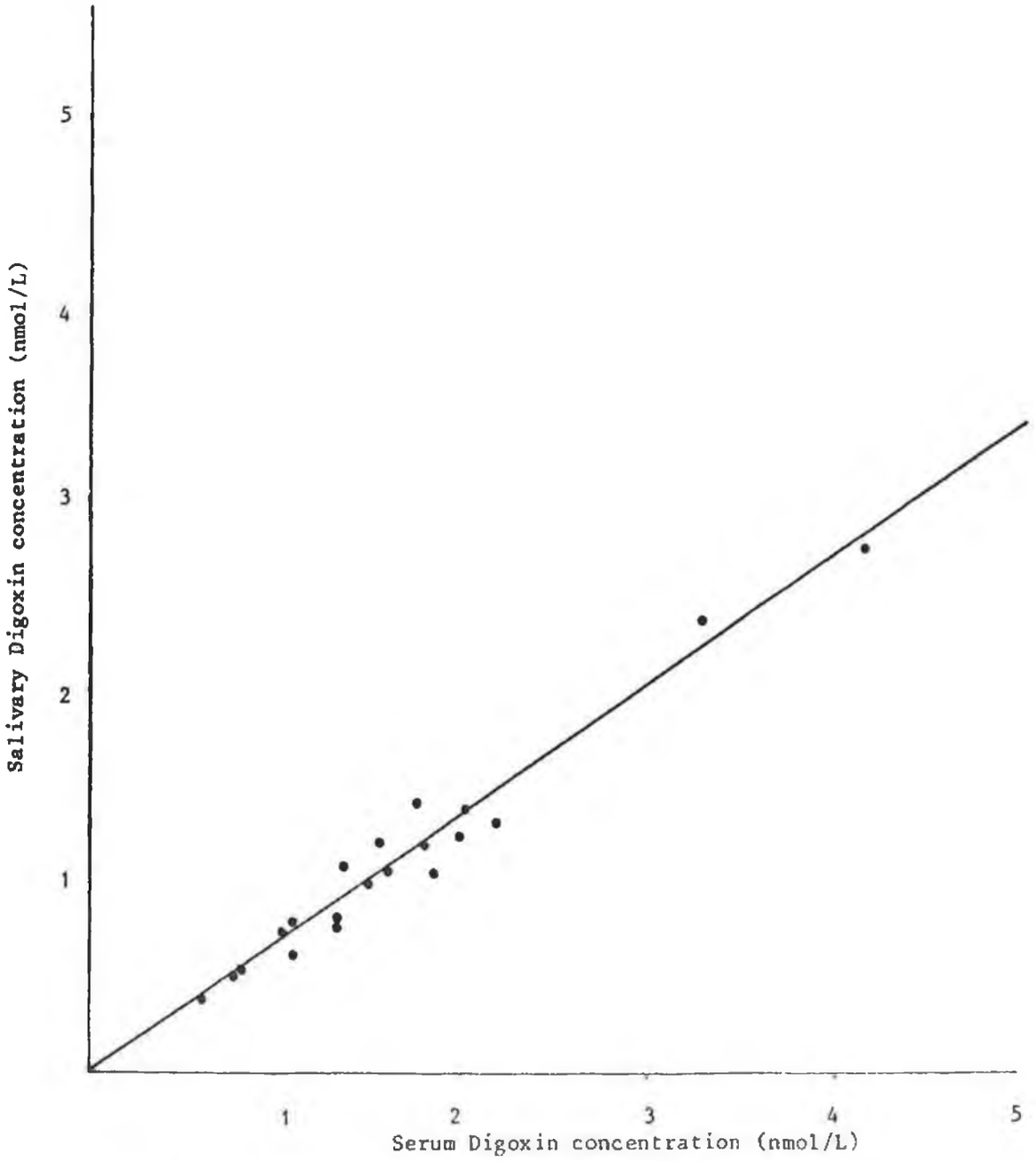
EMIT: 0.67 with a standard deviation of 0.1

Figure 33: Correlation between salivary and serum Digoxin levels using the modified Cedia method.



Slope 0.61; Intercept (nmol/L) - 0.04; Mean (nmol/L);
Serum 1.66; Saliva 0.97; Correlation Coefficient 0.94;
n = 20; y = 0.61x - 0.04.

Figure 34: Correlation between salivary and serum Digoxin levels using the EMIT method.



Slope 0.67; Intercept (nmol/L) 0; Mean (nmol/L);
Serum 1.62; Saliva 1.09; Correlation Coefficient 0.97;
n = 20; y = 0.67x + 0.

7.9.7. *Relationship between Free and Bound Digoxin in Serum:*

Table 62: The following were the results obtained from 8 normal patients on digoxin.

Free Serum Digoxin (nmol/L)	Total Serum Digoxin (nmol/L)	Free Digoxin/Total Digoxin Ratio %
1.06	1.52	0.70
0.82	1.12	0.73
1.61	2.14	0.75
1.07	1.41	0.76
0.57	0.85	0.67
0.45	0.71	0.63
2.5	3.52	0.71
2.04	2.88	0.70

The mean saliva/serum ratio is 0.71 $\pm$ 0.04 (S.D.).

Chapter 8

DISCUSSION

The narrow therapeutic range for digoxin makes it one of the more difficult drugs to administer even in patients with normal renal function. Different immunoassays give different responses to interfering substances. It is important, therefore, that an accurate method for digoxin monitoring is available, and with this in mind, the CEDIA and EMIT methods were evaluated in terms of imprecision, sensitivity, analytical range, curve stability, accuracy and interference.

IMPRECISION:

The coefficients of variation obtained for the EMIT assay are better than those reported by Syva in their technical information sheet for the Cobas Bio [73]. Their data show increased imprecision at normal and high digoxin concentration with within batch CVs of 4.3% at 2.54 nmol/L and 5.9% at 3.29 nmol/L. A mean within batch CV of 3.5% was found in this study in the range of 1.85 to 4.4 nmol/L digoxin. The total imprecision was found to be approximately 4.6% compared to 5% for the manufacturer over the range 2.51 to 3.58 nmol/L digoxin.

The CEDIA assay was found to be precise also and gave CVs which were similar to those for EMIT. The results compared well with those reported by Jacobs [108].

The good precision may be related to the stability of the reagents on storage and to features of the Cobas Bio such as a digitally-controlled syringe pipetting system, closed sample vials, photometric system, longitudinal cuvette arrangement and excellent temperature regulation. The total precision also confirms the stability and suitability of the quality control materials used.

SENSITIVITY AND ANALYTICAL RANGE:

The CEDIA calibration curve was linear from 0.0 to 5.12 nmol/L as illustrated in Figure 15. The linear response occurs because the amount of enzyme activity present is directly proportional to the amount of digoxin. Samples with reaction rates greater than that for 5.12 nmol/L require a predilution step using the zero digoxin calibrator. An average rate change of 28 mA per 1.28 nmol/L was observed. The sensitivity was found to be 0.3 nmol/L which compares well with the 0.2 ng/ml (.26 nmol/L) quoted in the kit insert.

The calibration curve for the EMIT method was non-linear because with increasing digoxin concentration, the glucose-6-phosphate dehydrogenase reacts with digoxin in a logarithmic manner. The kit insert gives the assay range as 0.64-5.12 nmol/L. This study showed that the Cobas Bio can distinguish between digoxin concentrations up to 6.0 nmol/L. This obviates the need for sample predilution and repeat measurement, hence faster turn-around time for samples with digoxin concentrations of approximately 6.0 nmol/L. However, the precision of values outside the assay range was not assessed.

The sensitivity of the EMIT method was 0.28 nmol/L which was better than that for the CEDIA. The higher value was probably matrix related. The information relating to the good sensitivity achieved here would be very useful in the analysis of specimens with digoxin concentrations less than the assay range. Calibrators could be diluted to extend the range.

ACCURACY:

This was assessed by the method of addition. The two methods are almost identical in terms of accuracy with recoveries of 96-102%. The CVs for the recovery studies did not exceed 4% and generally compared well with the imprecision of the assays. The CEDIA literature report recoveries of 100% at 2.14 nmol/L, 97% at 2.56 nmol/L and 98% at 3.08 nmol/L [108]. No further recovery evaluations for either method could be found.

CALIBRATION CURVE STABILITY:

As reagents deteriorate over time, it is necessary to determine stability, thus minimizing expenditure on reagents and calibrators and ensuring adequate assay performance. In this study, both assays were calibrated on day zero after which calibrators were treated as samples. The results for both the EMIT and CEDIA methods were consistent for the first 12 days, thus allowing the analysis of a greater number of samples per batch (Figures 16-17). Suitable controls should be routinely used to assess curve stability.

The "DENS" option on the Cobas Bio has a facility for assessing calibration curve stability based on analysing the zero calibrator only. Using absorbance readings from this standard, the slope and intercept are updated. This can be utilized on a daily basis once verified by appropriate controls. Significant savings can be made for low volume assays in which large amounts of reagents are consumed.

DLIF INTERFERENCE:

DLIFs continue to cause problems in digoxin immunoassays. The use of such assays which suffer from DLIF interference for digoxin monitoring can indicate "apparent digoxin concentrations" greater than the therapeutic range for exogenous digoxin leading to errors in measurement and clinical interpretation. These limitations suggest that each assay and antibody lot should be evaluated for DLIF interference and a selective assay chosen to discriminate between these endogenous factors and digoxin.

In this study, two methods were compared for their ability to eliminate DLIF interference. The CEDIA assay required no pre-treatment of the serum sample, thus relying exclusively on the specificity of the antibody. No previous evaluations of DLIF interference in the CEDIA immunoassay could be found. In the EMIT method, the sample extraction step is used to remove protein and other substances that might interfere. The EMIT method appears to eliminates or reduces DLIF interference in the groups of patients mentioned (Figures 18-21).

From the results obtained, certain observations were made. The data confirms the presence of significant amounts of DLIF in the clinical groups studied. The concentration was dependent on the immunoassay used. The greatest concentrations were measured by the CEDIA method having 55% of its DLIF values within the range of 0.5-1.18 nmol/L of "apparent digoxin" compared with a range of 0-0.35 nmol/L for all of the samples using the EMIT method. For the CEDIA method, only 11.8% of the samples showed no detectable levels of DLIF as against 68.6% for the EMIT. The remaining DLIFs were below the detection limits for the EMIT (i.e. 0.28 nmol/L). Some researchers have omitted these lower detection limits from their work and others have linked their results to sera with no detectable DLIFs.

Valdes [92] reports that DLIF is present in three forms in the serum, tightly protein bound, weakly bound and free. More than 90% of DLIFs is lightly but reversibly bound in serum of normal people. In pregnant women, neonates and renal failure, an increase in the weakly bound fraction occurs rather than an increased total DLIF. The increase in the weakly bound fraction makes the DLIF more available to the immunoassay antibody resulting in increased DLIF concentration. The hydrophobic column pre-assay separation step used in EMIT, separated the weakly protein-bound DLIF from the serum. This protein separation method does not appear to disrupt protein bound DLIF.

It is postulated that DLIFs cross-react with antibodies due to structural similarities with the drugs. It has been suggested that if the same biological response is produced by DLIF and the drug, they may react at the same receptor sites. This structural

similarity at the epitope would then lead to antibody cross-reactivity.

It has been shown that haemolysis, icterus, lipaemia and protein may affect the accuracy of many clinical chemistry analyses. For handling specimens with these potential interferents, the stated limitations of the procedure are often vague, e.g. Cedia insert "The use of grossly haemolysed, lipaemic, icteric specimens is not recommended". The knowledge of the type of bias and the concentration at which the interferent is detectable is important. With a proper analytical system, this data can be used to eliminate unnecessary repeat sampling associated with qualitative methods.

BILIRUBIN:

Bilirubin is present in the plasma in two forms; conjugated, which is the water soluble form, and unconjugated, which is transported bound to albumin. Although the presence of bilirubin conjugated to glucuronic acid has been recognised for some time, recent evidence indicates that bilirubin covalently bound to albumin can also be a significant contributor to jaundice. Bilirubin is known to interfere with a number of analytical methods, e.g. haemoglobin and cholesterol estimation. In this study, the effect of bilirubin on the CEDIA and EMIT assay was investigated.

Bilirubin did not interfere with the EMIT method, (Tables 42-44). The column chromatography pre-treatment step successfully removed bilirubin and other interferents, thus ensuring specificity of the assay.

In the CEDIA assay, the pooled serum studied showed falsely low digoxin recoveries at bilirubin concentrations greater than 46.8 μmol/l approximately, with total loss of recovery at 140.4 μmol/L, (Tables 42-44). This may explain the low concentration of DLIF found in liver disease (Table 38). It may also account for the DLIF concentration pattern found in the correlations study which investigated the relationship between liver disease and DLIF concentration (Table 41).

It is not clear how bilirubin interferes with the assay. It may be that elevated levels of other chemicals due to liver disease cause the interference. Another reason it interferes may be due to the similarity between the colour of bilirubin and that of the substrate product which is yellow. The initial absorbance reading produced is proportional to the bilirubin concentration. In the Cobas Bio absorbance rates for the samples are calculated by subtracting the lower from the higher absorbance readings (Section 5.3.5.). In icteric samples, the absorbance changes recorded are small. This is because the combined absorbance readings of the bilirubin and substrate product exceed the absorbance capability of the spectrophotometer.

LIPIDS:

In recent studies it has been shown that fatty acids cross-react with digoxin antibodies. This particular study examined the interference caused by lipaemia on the two methods using a pooled sample and three lipaemic samples A, B and C.

No lipid interference was encountered in the EMIT method (Tables 45-47). This was due to the extraction of lipid and protein from serum digoxin in the pre-treatment step.

In the CEDIA assay inhibition of digoxin recovery occured (Tables 45-47). The concentration of cholesterol and triglyceride at which the interference occured was highly variable in the three patients and pooled sera studied. Both appear to contribute to the low digoxin recoveries.

Murty et al [109] reported one severly lipaemic sample giving a digoxin concentration 30% lower than that from an RIA method. From my study it is evident that qualitative terms such as "severly lipaemic" is inadequate to describe CEDIA assay limitations. For example, patient A with cholesterol concentrations of 6.4 mmol/L and triglyceride concentrations of 5.8 mmol/L caused inhibition but still cannot be classified as severely lipaemic (Table 48). Consequently, serum triglyceride and cholesterol concentrations are required to define the degree of interference with lipids.

In the CEDIA assay, falsely low digoxin values may result from cholesterol and triglyceride blocking the binding sites on the EA molecule. This inhibits complementation of EA and ED when the ED molecule is added to the reaction mixture, i.e. inhibition of β-galactosidase formation. Another problem encountered with lipaemic samples is the change in viscosity resulting in poor sampling precision due to the high concentration of triglyceride.

HAEMOLYSIS:

The influence of haemolysis on serum digoxin was evaluated using both methods (Tables 49-51). Haemolysis did not interfere with EMIT due to the removal of haemoglobin along with other proteins from digoxin in serum samples by the pre-treatment step.

In the CEDIA method, the presence of haemoglobin exceeding 70 mg/dl caused falsely reduced results and concentrations greater than 266 mg/dl resulted in zero percentage recovery of the 2, 3 and 4 nmol/L digoxin solutions.

In the second study involving paired haemolysed and non-haemolysed samples from each of eight patients on digoxin, the non-haemolysed sera showed comparable results for both methods. The presence of haemolysis in the sera caused no interference with the EMIT but total inhibition of digoxin recovery with the CEDIA (Table 52).

These results for the CEDIA reject those by Multy et al. who reported that haemolysis had no effect. No information was given on the haemoglobin concentration or the procedure used by him. My study showed that haemoglobin concentrations of 80.5 mg/dl gave reductions of 14% approximtely in digoxin with no recovery of digoxin at 266 mg/dl. The use of "haemolysis" in such vague terms as that used by Multy et al can be very misleading and may have serious clinical consequences.

A sample that contains haemoglobin with a concentration greater than 70 mg/dl will inhibit digoxin recovery. This occurs because the

methaemoglobin present in the haemolysed sample exhibits its maximum absorbance in the Sorèt band region 400-440 nm. Since the CEDIA method uses a wavelength of 420 nm for digoxin measurement, spectral interference occurs. The initial absorbance reading is proportional to the methaemoglobin concentration. In grossly haemolysed sera (Table 52) the initial absorbance reading was so high that it exceeded the linearity of the method and thus "flagged" "high absorbance". In specimens with haemoglobin concentrations of, for example, 91 mg/dl the absorbance changes recorded were small. This occured because the combined absorbance values of the methaemoglobin and substrate product were outside the absorbance capability of the spectrophotometer.

PROTEIN:

Burnett **et al**. using an RIA method suggested that the determined digoxin concentrations are, to a certain extent, related to albumin concentrations [110]. This effect of albumin on the value for digoxin has been investigated in this study using both methods.

The EMIT method was not affected by albumin because the hydrophobic column used in the extraction step separated the albumin from the serum digoxin (Tables 53-55). As a result of this, the method is not sensitive to changes in albumin concentration.

For the CEDIA method the determined digoxin concentration was falsely high at low albumin concentrations (less than 40 g/l) (Tables 53-55). The data suggests that with increasing albumin concentration within the range 0-40 g/l approximately, there was an apparent decrease in

digoxin concentration. However, Holtzman et al. suggests that albumin increases the binding of radiolabel to antibody, an apparent contradiction to my findings [106]. This apparent difference is explained by the type of separation method used. In his study, Holtzman used charcoal to adsorb the free digoxin and assayed the albumin with the antibody-digoxin complex. Thus, when the albumin is in solution with the antibody, an apparent increase in the digoxin binding to albumin is observed due to the radiolabel combining with albumin and antibody. On the contrary, when the digoxin-antibody complex is separated from albumin there is a decrease in the apparent digoxin concentration.

The exact mechanism by which albumin i.e. less than 40 g/l approximately, interferes with digoxin measurement is unclear. The data acquired in this study may be partly explained by the fact that 25% approximately of serum digoxin is bound to albumin, and as albumin decreases more digoxin is present in the free state. The unbound digoxin is free to react with the antibody, thus resulting in increased β-galactosidase formation and activity. While the data suggests that the CEDIA assay is sensitive to albumin concentration, this is insufficient to explain the large discrepancies in digoxin concentration encountered. It is also extremely unlikely that low affinity of the antibody was responsible due to the adequate sensitivity of the method. Drug interference can also be eliminated. As already shown, the lack of specificity of the antibody in the CEDIA assay may be a factor.

At high albumin concentration i.e. greater than 79 g/l approximately, decreased recovery of digoxin was observed using the CEDIA method.

This decrease was due to the dilutional effect of the albumin. Falsely elevated results due to hypoalbuminemia can be corrected by adding 40 g/l of albumin to the analyte solution. Pseudo-hypodigoxinemia can be rectified by adding an appropriate volume of isotonic saline to the specimen [111].

In this study, it has been shown that haemolysis, lipaemia, proteins and icterus interfere with the CEDIA method. The use of qualitative terms when describing interference should be avoided because it is difficult to accurately distinguish between various concentrations of lipaemia, haemolysis or icterus. The inaccurate results attributable to these interferences can be avoided through the development of accurate electronic methods which have the ability to identify and reject such samples, or preferably, the development of a pre-treatment step such as that used in the EMIT technique.

METHOD COMPARISON:

Plots of the comparison between the EMIT, CEDIA and external laboratory RIA, with regression analysis data are shown in (Figures 22-24). The agreement between EMIT and RIA methods for analysis of patient samples was considered suitable for routine use. However, there were statistically significant differences between these and the CEDIA. The largest difference was between the CEDIA and RIA methods.

When the RIA and EMIT were compared with CEDIA, the slopes were less than 0.93, indicating that the EMIT and RIA values tended to be lower than the CEDIA values. Comparison of RIA and EMIT showed an

intercept of almost zero but when compared with CEDIA, a positive intercept of approximately 0.16 nmol/L was observed. When the mean values of EMIT and RIA were compared with CEDIA, a negative bias similar to that of the intercept was demonstrated. The higher mean value for CEDIA is probably attributable to the presence of endogenous DLIF in some of the samples augmenting the digoxin results. This supports the assessment already made that DLIF had no effect on the measured digoxin in serum from renal or liver disease, third trimester pregnant women and neonates when using the EMIT assay. This, once again, demonstrates the unsuitability of the CEDIA method for measuring such samples.

STABILITY OF DIGOXIN IN SERUM AND PLASMA:

(Figures 25-28) show that digoxin is stable in serum and plasma for up to 18 days when stored at -20°C, room temperature and 4°C using the EMIT method. Serum digoxin was stable for 18 days using the CEDIA method while plasma digoxin was quite unstable. When stored at 4°C, for example, it showed an increase of +48% approximately at day 5, decreasing to -6% on day 12, and increasing to 48% again on day 18. In view of the good stability of digoxin in serum, it is clear that plasma is unsuitable for digoxin estimation using the CEDIA method.

In this study, heparanised plasma samples were used. In their study, Stromme et al. described how heparin interfered with the methodology for creatine kinase [112]. Heparin reacts with several plasma proteins forming heparin-protein complexes. The precipitation of this complex is dependent on the pH and inorganic ions present in the assay solution. For this reason, he suggests that heparinised plasma

may not be suitable for enzyme assays. However, my study involving eleven patients' sera showed that heparin, when added to these samples, did not interfere with the CEDIA immunoassay. In the heparinised plasma samples some results varied from those of the serum. However, a larger study is required to validate this and if proven, fibrinogen or the clotting factors, should be investigated as the possible cause of these variable results.

SALIVARY DIGOXIN:

The manner in which therapeutic drugs enter saliva is well documented [80]. Transfer of digoxin, which is a non-ionized, relatively fat soluble, neutral drug into saliva appears to be passive and the salivary digoxin level should correlate with the free-digoxin (unbound) in plasma. Salivary sampling is a non-invasive technique, suitable for small children, aged people or where repeated sampling is required. Where appropriate, it can eliminate costs associated with hospitalization and phlebotomy.

In this study, the EMIT and CEDIA kits have been successfully used for salivary digoxin measurement. In adapting the EMIT kit, no procedural modifications were required and the saliva was treated similarly to serum. This was made possible by the pre-assay extraction step which eliminated the incidence of spurious and non-reproducible digoxin results found in direct RIA procedures [80].

In the adaptation of the CEDIA method, equal volumes of albumin diluent (conc. 80 g/l) were added to saliva samples in order to equalize the protein concentration in saliva and serum. Saliva has

low protein concentration of 1-4 g/l. The effect of this low protein concentration has already been demonstrated (Section 7.6.5.). The sample volume was increased from 25 to 50 μl to compensate for the sample dilution.

In the standard curve plot, (Figure 29), the absorbances for the CEDIA salivary modification were lower than those for the serum digoxin. This was probably attributable to the dilutional effect due to increased sample volume. The precision, sensitivity and recoveries for salivary digoxin were comparable to those reported for serum digoxin (Tables 57-58 and Section 7.9.1.).

The EMIT method showed a good linear relationship between digoxin concentrations in serum and saliva with a mean ratio of saliva to serum digoxin of 0.67 with a standard deviation of 0.1 (Figure 34). The CEDIA method had a lower saliva/:serum ratio (0.62) (Figure 33). This was probably due to the viscosity of some of the saliva samples causing decreased sample aspiration by the Cobas Bio and hence, lower results.

Equilibrium dialysis showed that the fraction of free drug in saliva was 0.71 (Table 62), which compares favourably with that for EMIT. As this fraction of the total serum digoxin is free, it appears that it is in equilibrium with saliva. The saliva/serum ratios for EMIT and CEDIA are lower, compared with the values of 1.7, 1.14 and 1.27 reported by other workers [81,82,84]. A number of explanations for ratio differences have been proposed, including specificity of digoxin antibody used and saliva enzymes. Patients with heart failure have impaired parasympathetic function which is independent

of that associated with digoxin therapy. This may alter the composition of saliva. Varability in saliva/serum ratios have been reduced in one study by increasing saliva flow [81]. However, in other studies, the salivary digoxin was found to be independent of flow [80]. Another reason for discrepant results may be due to improper sampling procedure. In the early stages of my study, samples from twelve patients when analysed gave saliva/serum ratios as high as 1.64. This was caused by paired saliva/serum samples not being taken simultaneously.

Using both methods, apparent DLIF concentrations exceeding the sensitivity were shown and because of this, it was difficult to assess their significance (Tables 59-60). However, this may reflect the salivary fraction which is in equilibrium with the 10% approximation of free serum DLIF. The apparent lack of DLIF in saliva renders it more appropriate than serum for use in immunoassays having non-specific digoxin antibodies.

From this investigation, it is evident that the discrepancies reported by various workers may be attributable to unreliable methodology. This was evident in the CEDIA where salivary matrix interfered. This effect was eliminated in the EMIT through the use of an extraction step, thus rendering it suitable for salivary digoxin measurement. The determination of salivary digoxin concentration appears to be an alternative to blood sampling with good reproducibility, accuracy and correlation with serum concentration.

CONCLUSION

This study provides further evidence of the lack of specificity in some present-day digoxin immunoassays. Matrix effects associated with variations in protein as well as interference by DLIFs, lipaemia, haemolysis, bilirubin and anticoagulants used, have been observed in the CEDIA method. THe use of hydrophobic chromatography greatly reduced or eliminated similar interference in the EMIT. The EMIT was also successfully used for salivary digoxin estimation. The saliva/serum ratio was comparable to that obtained by the equilibrium dialysis method but that for the CEDIA was lower due to the matrix effect, i.e. increased saliva viscosity.

The EMIT method is a simple, widely applicable specific method for digoxin estimation. Its use is very appropriate in certain clinical groups where DLIF values are high, such as in patients with renal and hepatic failure, pregnancy and neonates. The EMIT method is the most suitable for measuring serum and salivary digoxin.

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