

DEVELOPMENT OF A SENSITIVE, QUANTITATIVE  
HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY  
FOR THE MEASUREMENT OF DIGOXIN IN PATIENT GROUPS  
WITH HIGH LEVELS OF DIGOXIN-LIKE IMMUNOREACTIVE SUBSTANCES

By

LEANNE EMBREE

B. Sc. (Pharm), University of B.C.,  
Vancouver, B.C., Canada, 1982

M. Sc., University of B.C.,  
Vancouver, B.C., Canada, 1985

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES  
Faculty of Pharmaceutical Sciences  
Division of Pharmaceutical Chemistry

We accept this thesis as conforming  
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

January 1989

© Leanne Embree

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pharmaceutical Sciences

The University of British Columbia  
1956 Main Mall  
Vancouver, Canada  
V6T 1Y3

Date January 30, 1989

### Abstract

Digoxin is the most commonly used digitalis glycoside for the treatment of congestive heart failure and certain disturbances of cardiac rhythm. The low therapeutic index observed for digoxin and the clinical significance of digoxin therapy have necessitated the development of sensitive analytical methods for the quantitation of digoxin in biological samples. Digoxin may be analysed by several methods including immunoassays, chromatographic procedures and various biological and chemical methods.

Immunoassays, both radioimmunoassay (RIA) and fluorescence polarization immunoassay (FPIA) procedures, are used in the clinical laboratory because of their speed, precision, sensitivity and relatively low cost. However, reaction of the digoxin antibodies used in the immunoassay methods with digoxin metabolites, endogenous compounds such as digoxin-like immunoreactive substances (DLIS), and other drugs that may be co-administered with digoxin continues to be a major problem.

The lack of specificity of the immunoassay methods for digoxin has led to difficulties in interpretation of assay values. Attempts to compensate for this lack of specificity have included the use of chromatographic systems as elaborate sample handling methods prior to immunoassay. However, since an immunoassay was used for detection of

digoxin in these techniques, the specificity may still be questionable.

A sensitive and specific assay for digoxin using physico-chemical methods for measurement is therefore needed. A method was developed using pre-column derivatization of digoxin and its metabolites with 3,5-dinitrobenzoyl chloride followed by HPLC analysis with electrochemical detection. A maximum sensitivity of 0.883 ng of 3,5-dinitrobenzoyl digoxin (0.394 ng digoxin) was observed using dual electrode detection in the redox mode. Although resolution between derivatized digoxin and its metabolites was obtained, the low yield of the digoxin derivative and the formation of metabolites when small (ng) samples were derivatized made this method unsuitable for evaluating patient samples.

A high-performance liquid chromatographic (HPLC) assay using post-column derivatization of digoxin, which separated digoxin from its metabolites and some commonly co-administered drugs, was developed. Post-column (PC) derivatization of digoxin with concentrated hydrochloric acid and dehydroascorbic acid, followed by fluorescence detection, allowed for quantitation within the therapeutic range of digoxin.

Steroids which have been reported to cross-react with digoxin antisera were assayed using the HPLC-PC method developed in this study. The steroid samples either did not

elute from the HPLC system or did not produce a fluorescent product under these conditions.

Serum samples from digitalized patients were evaluated using both the HPLC-PC and the FPIA methods. When compared to the HPLC procedure, the FPIA assay results gave, on average, higher digoxin levels. This may have been due to the inclusion of digoxin metabolites or endogenous compounds with the FPIA assay.

Serum samples from undigitalized patient groups where high DLIS levels have been reported were also evaluated. These included umbilical cord blood samples and samples from hypertensive patients, renal failure patients and hepatic failure patients. Comparison of the HPLC-PC and FPIA methods demonstrated that the HPLC-PC assay gave fewer false positive results than the FPIA.

The HPLC-PC assay developed for analysis of digoxin was unaffected by the presence of digoxin metabolites, numerous steroids, co-administered drugs and endogenous compounds, most of which have been reported to give false positive results with the FPIA.

## TABLE OF CONTENTS

Chapter	Page
Abstract	ii
List of Tables	xii
List of Figures	xiv
Symbols and Abbreviations	xvi
Acknowledgement	xix
Introduction	1
1. Literature Survey	4
1.1 Pharmacodynamic Properties	4
1.1.1 Cardiovascular Actions	4
1.1.1.1 Contractility	4
1.1.1.2 Conduction	5
1.1.2 Mechanism of Action	5
1.1.3 Toxicity	6
1.1.4 Pharmacokinetics	8
1.1.4.1 Absorption	9
1.1.4.2 Distribution	11
1.1.4.3 Metabolism And Elimination	13
1.1.5 Chemistry	15
1.1.6 Structure-Activity Relationships	16
1.2 Digoxin Therapy	19
1.3 Digoxin-Like Immunoreactive Substance(s)	20
1.4 Digoxin-Drug Interactions	27
1.4.1 Digoxin-Quinidine	27

1.4.2	Other Drug Interactions	29
1.5	Digoxin Analysis	32
1.5.1	Immunoassays	33
1.5.2	Biological Assays	37
1.5.3	Chemical Methods	38
1.5.4	Chromatographic Techniques	40
1.5.5	HPLC-RIA	43
1.6	HPLC-EC	44
2.	EXPERIMENTAL	49
2.1	Supplies	49
2.1.1	Chemicals	49
2.1.2	Solvents	50
2.1.3	Extraction Supplies	50
2.1.4	Post-Column Derivatization Supplies	51
2.1.5	Commercial Radioimmunoassay Kits	52
2.1.6	Commercial Fluorescence Polarization Immunoassay Kit	53
2.1.7	Filtration of HPLC Mobile Phase	53
2.2	Equipment	53
2.2.1	HPLC Equipment	53
2.2.2	Electrochemical Detector	54
2.2.3	Fluorescence Detector Flow Cell	56
2.2.4	Post-Column Reactor and Pressure Chamber	56
2.2.5	Extraction Equipment	56
2.2.6	Radioimmunoassay Equipment	59

2.3	Pre-column 3,5-Dinitrobenzoyl Chloride	
	Derivatization Procedures	59
2.3.1	For Gram Quantities of Digoxin	59
2.3.2	For Milligram Quantities of Cardiac Glycosides	59
2.3.3	For Nanogram Quantities of Digoxin	60
2.4	Preparation of Solutions	60
2.4.1	Reagents For 3,5-DNBCl Derivatization	61
2.4.1.1	3,5-DNBCl in Pyridine	61
2.4.1.2	Sodium Bicarbonate (5%) with 4-DMAP	61
2.4.1.3	Dilute Hydrochloric Acid	61
2.4.2	Reagents For Fluorogenic Derivatization	61
2.4.2.1	Ascorbic Acid Solution	61
2.4.2.2	Dilute Hydrogen Peroxide Solution	62
2.4.2.3	Dehydroascorbic Acid	62
2.4.2.4	Hydrogen Peroxide with Phosphoric Acid	62
2.4.3	Sodium Acetate Buffer (0.1 M, pH 4.6)	62
2.4.4	Mobile Phase	62
2.4.5	Standard Solutions	63
2.4.5.1	Digoxin in Ethanol	63
2.4.5.2	3,5-Dinitrobenzoyl Digoxin in Methanol	63
2.4.5.2	Digitoxigenin in Ethanol	63
2.4.5.3	Dihydrodigoxin in Ethanol	64
2.4.5.4	Steroid Samples in Methanol	64
2.4.5.5	Preparation of Mexiletine Hydrochloride in 50% Ethanol	65



2.4.5.6	Preparation of Plasma Samples for RIA Analysis	65
2.4.5.7	Preparation of Serum Samples Spiked with Digoxin Metabolites for FPIA Analysis	66
2.5	Preparation of Solvents for Serum Extraction	66
2.5.1	Isooctane/Dichloromethane (20/5)	66
2.5.2	Extraction Solvent	66
2.5.3	Preparation of Protein Precipitation Reagents	66
2.5.3.1	Zinc Sulfate Heptahydrate (5%)	66
2.5.3.2	Barium Hydroxide Octahydrate (0.3 N)	67
2.5.3.3	Cupric Sulfate Pentahydrate (10%)	67
2.5.3.4	Sodium Hydroxide (12%)	67
2.6	RIA Analysis and Cross-Reactivity Calculations	67
2.7	HPLC With Electrochemical Detection	68
2.7.1	Optimization of Electrochemical Conditions	68
2.7.2	Mobile Phases for HPLC with Electrochemical Detection	69
2.8	HPLC Post-Column Derivatization Assay	69
2.8.1	Assembly of Steel and PVC Pressure Vessels	69
2.8.2	Optimization of HPLC Post-Column Derivatization	70
2.8.2.1	Fluorogenic Derivatization with Hydrogen Peroxide, Phosphoric Acid and Concentrated HCl	70

2.8.2.2	Evaluation of the Fluorescence Emission Filters with Dehydroascorbic Acid and HCl Derivatization	71
2.8.3	Final HPLC-PC Assay Procedure	71
2.9	Serum Extraction Procedure	71
2.9.1	Protein Precipitation Methods	71
2.9.2	Centrifree and Centriflo Filtration	73
2.9.3	Solvent-solvent Extraction	73
2.9.4	Final Procedure for Extraction of Digoxin in Serum	73
2.10	Recovery and Precision of Extraction	74
2.11	Calibration Curve	76
2.12	Specificity	76
2.12.1	Steroids	76
2.12.2	Co-Administered Drugs	76
2.13	Quality Control Procedure	77
2.14	Analysis of Digitalized Patient Serum Samples	77
2.15	Analysis of Serum from Undigitalized Patient Groups Where High DLIS Levels have been Reported	77
2.15.1	Hypertension	77
2.15.2	Renal Failure	77
2.15.3	Hepatic Failure	78
2.15.4	Umbilical Cord Blood Samples	78
3.	Results and Discussions	79
3.1	Radioimmunoassay of Digoxin and Its Metabolites	79
3.2	Fluorescence Polarization Immunoassay of Digoxin Metabolites in Serum	82

3.3	Pre-Column 3,5-Dinitrobenzoyl Derivatization of Cardiac Glycosides with HPLC-EC Analysis	84
3.3.1	Ultraviolet Detection	84
3.3.2	Detection With a Single Glassy Carbon Electrode	87
3.3.3	Dual Electrode Detection	90
3.4	Post-Column Fluorogenic HPLC Assay	97
3.4.1	Pressure Vessel	97
3.4.2	Optimization of Post-Column Reactor Conditions	98
3.4.3	Maximum Sensitivity	101
3.4.4	Evaluation of Digoxin and its Metabolites	103
3.5	Analysis of Biological Samples Using The HPLC Fluorogenic Assay	105
3.5.1	Optimization of Serum Extraction Method	105
3.5.1.1	Protein Precipitation Methods	105
3.5.1.2	Removal of Endogenous Interference	107
3.5.2	Recovery and Precision	109
3.5.3	Calibration Curve	112
3.5.4	Comparison of HPLC-PC and FPIA Methods in Spiked Serum Samples	114
3.5.5	Specificity	119
3.5.5.1	Steroids	119
3.5.5.2	Co-administered Drugs	119
3.6	Analysis of Digoxin in Digitalized Patient Serum	120

3.7	Analysis of Serum from Undigitalized Patient	
	Groups Where High DLIS Levels Have Been Reported	122
3.7.1	Hypertensive Patients	122
3.7.2	Renal Failure Patients	124
3.7.3	Hepatic Failure Patients	125
3.7.4	Umbilical Cord Blood	128
4.	Summary and Conclusions	130
5.	References	133

## List of Tables

Table		Page
I	Summary Of Digoxin Pharmacokinetics	13
II	Percent Cross-Reactivity on a Molar Basis for Digoxin Metabolites With Commercial Digoxin RIA Kits	80
III	Evaluation of Digoxin Metabolites in Serum Using the FPIA for Digoxin Analysis	83
IV	HPLC Retention Times of 3,5-DNB Derivatives	85
V	Resolution of 3,5-DNB Digoxin and 3,5-DNB Dihydro- digoxigenin Using HPLC-EC with Various Mobile Phases	88
VI	Effect of Varying the Aqueous Portion of the Mobile Phase on Peak Height Using HPLC-PC Fluorescence Assay	99
VII	Effect of Emission Filters on Peak Height of Digoxin Using HPLC-PC Fluorescence Assay	100
VIII	Effect of HCl Flow Rate on Peak Height of Digoxin Using HPLC-PC Fluorescence Assay	101
IX	Recovery of Digoxin from Spiked Serum Samples	111
X	Precision of Digoxin Assay	112
XI	Comparison of the HPLC-PC and FPIA Methods for Digoxin Analysis in Digitalized Patients	121
XII	Comparison of Undigitalized Hypertensive Patient Samples Evaluated by the HPLC-PC and FPIA Methods	123

XIII	Hepatic Failure Patient Samples Evaluated by the HPLC-PC and FPIA Methods	126
XIV	Mixed Umbilical Cord Blood Samples Evaluated by the HPLC-PC and FPIA Methods	128

## List of Figures

Figure	Page
1 The Chemical Structure of Digoxin	16
2 Reaction Involved in Immunoassay Procedures	33
3 Schematic of Noise Reducer	55
4 Schematic of HPLC-EC System	57
5 Schematic of HPLC-PC Fluorogenic System	58
6 Diagram of HPLC-PC Pressure Vessel With Fittings	72
7 Flow Diagram for Serum Extraction Procedure	75
8 Structure of 3,5-DNB Digoxin	85
9 Chromatogram of 3,5-DNB Digoxin Derivatized in Milligram Quantities	86
10 Chromatogram of Digoxin and its Metabolites as Their 3,5-DNB Derivatives using Electrochemical Detection	89
11 Chromatogram of 125 ng of 3,5-DNB Digoxin Purified by Double Recrystallization	92
12 Chromatogram of 80 ng of Digoxin Derivatized in Milligram Quantities	93
13 Chromatogram of Digoxin and Its Metabolites by HPLC-PC	104
14 Chromatograms of Digoxin and Its Metabolites in Ethanol and Blank Serum Containing Interfering Peak	108
15 Chromatogram of Blank Serum and Serum Spiked with 1.5 ng Digoxin and Internal Standard	110
16 Calibration Curve for HPLC-PC Digoxin Assay	113

17	Correlation Between HPLC-PC and FPIA Methods For Digoxin Analysis	115
----	--	-----



## Symbols and Abbreviations

3,5-DNBCl	3,5-Dinitrobenzoyl chloride
4-DMAP	4-Dimethylaminopyridine
ACN	HPLC grade acetonitrile
ANP	Atrial natriuretic peptide
ACU	U.B.C. Health Sciences Centre Acute Care Unit
ATP	Adenosine Triphosphate
B.C.	British Columbia
C	Centigrade
CA	California
Cl <sub>Cr</sub>	Creatinine clearance
cm	Centimeter
C.V.	Coefficient of Variation
Da	Daltons
DLIS	Digoxin-like immunoreactive substance(s)
EC	Electrochemical
EMIT	Enzyme-multiplied immunoassay
EtOH	Absolute ethanol
FIA	Fluorescence immunoassay
FPIA	Fluorescence polarization immunoassay
g	gram
GFR	Glomerular filtration rate
GLC	Gas-liquid chromatography
HCl	Concentrated hydrochloric acid
HPLC	High-performance liquid chromatography

HPLC-EC	High-performance liquid chromatography-electrochemical detection
HPLC-PC	High-performance liquid chromatography post-column derivatization
I.D.	Internal diameter
IL	Illinois
in.	Inch
IPA	HPLC grade isopropanol
lb.	Pound
M	Molar
MA	Massachusetts
MD	Maryland
MeOH	HPLC grade methanol
MI	Michigan
min.	Minutes
MO	Missouri
Na <sup>+</sup> ,K <sup>+</sup> -ATPase	Sodium-potassium dependent ATPase
ng	Nanogram
NJ	New Jersey
NV	Nevada
NY	New York
Ont.	Ontario
PC	Post-column
PTFE	Polytetrafluoroethylene
PVC	Polyvinylchloride
Que.	Quebec
r	Correlation coefficient

r.c.f.	Relative centrifugal force (gravities)
RIA	Radioimmunoassay
S.D.	Standard deviation
TLC	Thin-layer chromatography
TX	Texas
U.S.A.	United States of America
V	Volts
WI	Wisconsin
$\mu$ L	Microliter
$\mu$ m	Micrometer

### Acknowledgement

I wish to thank my supervisor, Dr. Keith McErlane, for his valuable guidance throughout the course of this study. I am also grateful to the other members of my graduate committee, Drs. Frank Abbott, Helen Burt, Charles Kerr and James Orr for their helpful advice. Furthermore, Dr. James Axelson is acknowledged for contributing significant ideas for consideration.

I am grateful to Drs. C. Kerr, J. Price, U. Steinbrecher, G. Sandor and S. Stordy for their assistance in obtaining clinical samples.

Financial support from the Canadian and British Columbia Heart Foundations is gratefully acknowledged.

I would particularly like to thank Roland Burton, Trine Henriksen, Radana Vaughan and Dale Embree for their technical assistance.

I am indebted to Dr. Allan Goodeve for numerous discussions and proofreading of this document.

Finally, I would like to thank my husband, Dale, for his devotion and encouragement.

## INTRODUCTION

Digitalis glycosides form one of the most beneficial group of drugs available to aid the failing heart. They are the drugs of choice for the treatment of congestive heart failure and certain disturbances in cardiac rhythm. Although digoxin is the only digitalis glycoside available for clinical use in Canada and has been employed medically since about 1937, problems with therapy still exist due to its low therapeutic index. At digoxin concentrations greater than 3 ng/mL in serum, there is a high incidence of toxicity. However, physiological conditions and inter-patient variation in response may lead to toxicity below this value. In an effort to avoid manifestations of toxicity while maintaining the desired therapeutic effect, digoxin serum concentrations are maintained between 0.5 and 2.0 ng/mL. These low concentrations have necessitated the development of extremely sensitive assay techniques.

Digoxin has been analysed by several methods including radioimmunoassay, enzyme-multiplied immunoassay, fluorescence polarization immunoassay, high-performance liquid chromatography, and various biological and chemical methods. None of the methods developed to date can compete with the immunoassays in terms of speed, precision, sensitivity and cost; therefore immunoassay methods are routinely used in clinical laboratories. However, one of the major problems with the immunoassay methods for digoxin

analysis is that of cross-reactivity of the digoxin antibodies with digoxin metabolites, other drugs that may be co-administered with digoxin and endogenous compounds such as digoxin-like immunoactive substance(s).

The lack of specificity of the immunoassay methods used for digoxin analysis has prompted questions of its reliability. In order to avoid the possibility of over-estimation of digoxin due to interference from endogenous substances and other drugs, investigators have introduced chromatographic methods prior to the immunoassay. However, these methods generally require elaborate sample handling protocols and still use antibodies with questionable specificity.

A sensitive and specific assay for digoxin using more reliable methods for measurement is therefore needed. High-performance liquid chromatographic (HPLC) methods have been successful in separating digoxin from most of its metabolites but lack the sensitivity required for biological samples (Bockbrader 1984; Desta 1987; Fujii 1983; Gfeller 1977; Nachtmann 1976a, 1976b). Other HPLC methods do not have sufficient resolution of digoxin from dihydrodigoxin for quantitation (Reh 1985).

An HPLC assay for digoxin at therapeutic concentrations in plasma using post-column fluorogenic derivatization without interference from a number of commonly co-prescribed drugs and the metabolites of digoxin has been reported (Kwong 1986a, 1986b).

The specific aims of the project were as follows:

1. to ascertain the amount of interference from the metabolites of digoxin in radioimmunoassay kits commercially available.

2. to investigate the possibility of increasing the sensitivity of the HPLC post-column fluorogenic assay (Kwong 1986a, 1986b) by derivatization of digoxin prior to chromatographic analysis (Fujii 1983) coupled with electrochemical detection.

3. to improve the extraction procedure and HPLC post-column fluorogenic derivatization method (Kwong 1986a, 1986b) to allow faster sample processing and eliminate possible interference from endogenous compounds or co-prescribed medication.

4. to evaluate serum samples from patients with pathological conditions where endogenous digoxin-like immunoreactive substance(s) have been reported.

## 1. LITERATURE SURVEY

Digitalis glycosides are the most valuable drugs available for the clinical management of congestive heart failure. Since Withering (1937) first documented the effectiveness of digitalis preparations in the therapy of certain forms of dropsy, attention has been directed towards elucidation of their mechanism of action and cellular mechanisms that affect their efficacy. In spite of these efforts, serious problems with therapy still exist. Digoxin, the cardiac glycoside available in Canada, is widely used in the treatment of congestive heart failure and certain disturbances of cardiac rhythm.

### 1.1 Pharmacodynamic Properties

#### 1.1.1 Cardiovascular Actions

The main action of digoxin and other digitalis glycosides is the ability to increase the contractile force of the beating heart (Haustein 1983; Hoffman 1980). The conduction, refractoriness and automaticity of the heart are also affected. Therapeutic use of digoxin is based on changes in contractility and conduction.

##### 1.1.1.1 Contractility

The clinical and hemodynamic changes observed in the treatment of congestive heart failure are the result of a



direct positive inotropic effect of digoxin on the myocardium (Blaustein 1985; Doherty 1975; Haustein 1983). This increase in contractility leads to an increased cardiac output, decreased heart size, decreased venous pressure and blood volume, diuresis and relief of edema in patients with heart failure (Haustein 1983; Hoffman 1980).

#### 1.1.1.2 Conduction

The direct effects of digoxin on electrical activity are strongly dependent on the physiological condition of the heart. Also, differences in electrical response to digoxin were found between muscle fiber types (Haustein 1983; Hoffman 1980). Generally, high digoxin levels are thought to depress the conduction velocity and to delay or block the atrioventricular nodal conduction (Endou 1982). Low doses of digoxin indirectly decrease the rate at which atrial impulses can be transmitted to the ventricles and prolong the refractory period of the atrioventricular node (Hoffman 1980).

#### 1.1.2 Mechanism of Action

Therapeutic concentrations of digitalis glycosides selectively inhibit the plasma membrane sodium pump (the  $\text{Na}^+, \text{K}^+$ -ATPase) in a variety of cell types including cardiac and vascular smooth muscle cells, neurons and renal tubule cells. This inhibition of outward sodium transport leads to an increase in the sodium concentration inside the cell.

These cells also have sodium-calcium exchanger mechanisms which decrease the intracellular sodium by enhancing the calcium influx or reducing the calcium efflux or both. The resultant rise in intracellular calcium concentration results in more calcium being available to the contractile elements and therefore to a positive inotropic effect (Akera 1985; Blaustein 1985; Hoffman 1980; Repke 1984; Smith 1984a). These effects can account for the cardiotonic activity of digoxin as well as effects on the kidneys, nervous tissue and vascular smooth muscle.

### 1.1.3 Toxicity

It has been noted that 15 to 20% of hospitalized patients receiving digoxin exhibit symptoms of toxicity, and that the mortality rate of such patients is from 7 to 50% (Doherty 1975). At digoxin concentrations greater than 3 ng/mL plasma (Aronson 1983), there is a high incidence of toxicity. However, physiological conditions and inter-patient variation in response may lead to toxicity below this value. In an effort to avoid manifestations of toxicity, the therapeutic concentration in plasma is maintained between 0.5 and 2.0 ng/mL. Depending on patient parameters, such as renal function and age, maintenance doses of digoxin are usually 0.125 mg to 0.50 mg daily.

The cardiac and non-cardiac manifestations of digoxin toxicity have been recently reviewed (Antman 1985; Aronson 1983; Haustein 1983). Disorders of the gastrointestinal

tract due to central nervous system effects (nausea, vomiting, anorexia and diarrhea) and cardiac rhythm disorders are usually the earliest side effects observed (Antman 1985; Aronson 1983; Haustein 1983; Mason 1981). The central nervous system effects of digitalis are numerous and include headache, weakness, drowsiness, depression, dizziness, vertigo, ataxia, confusion, hallucinations, psychosis, neuralgia, seizure, stupor and coma (Antman 1985; Haustein 1983). Visual symptoms such as blurring, dimness, flickering or flashing lights, and color disturbances with yellow vision are most common (Aronson 1983) and may be the earliest symptoms of toxicity (Closson 1983). At therapeutic plasma levels, digoxin may produce a progressive deterioration in auditory verbal learning and short-term memory (Tucker 1983). Various arrhythmias, blockage in conduction, and worsening heart failure are cardiac evidence of toxicity (Antman 1985; Aronson 1983; Haustein 1983; Mason 1981; Takayanagi 1986). Recently, the mechanisms and manifestations of digitalis toxicity have been reviewed (Smith 1984a, 1984b, 1984c). The signs and symptoms of digitalis toxicity are usually non-specific, making diagnosis difficult.

Digoxin-specific antibody fragments have been successfully used for the treatment of digitalis intoxication (Cohen 1982; Friedman 1983; Murphy 1982; Rozkovec 1982; Zucker 1982). Agents that decrease absorption of digoxin from the gastrointestinal tract such

as cholestyramine and activated charcoal, have also been used in treating digoxin toxicity (Hoffman 1980).

Tissue digoxin concentrations may not be useful in the determination of digoxin toxicity. Postmortem digoxin levels in cases of digitalis toxicity ranged from 43 to 283 ng/mL while those from cases where toxicity was not the cause of death were from 0 to 463 ng/mL (Aderjan 1979; Andersson 1975; Margot 1983). Using immunoassay methods for digoxin analysis, postmortem blood samples appear to have elevated digoxin levels when compared to antemortem data (Aderjan 1979; Hastreiter 1983; Kim 1975; Margot 1983; McKercher 1986; Vorpahl 1978). Similar results have been found using a rubidium uptake assay (Andersson 1975). The interpretation of postmortem digoxin blood concentrations must take into consideration the time of blood collection after death and the sampling site (Margot 1983) since these factors affect the concentrations observed.

#### 1.1.4 Pharmacokinetics

Large intersubject variation in the pharmacokinetic behavior of digoxin (Aronson 1983; Clark 1974; Gault 1979; Kramer 1974; Luchi 1968; Movselli 1977; Rietbrock 1981, 1985) and interference with the most common digoxin assay method by other compounds (Butler 1978, 1979; DiPiro 1980; Holtzman 1974; Kramer 1978; Kubasik 1974a, 1974b; Lichey 1979; Lindenbaum 1975; Malini 1982; Muller 1978; Osterloh 1982; Pudek 1983a, 1983b; Ravel 1975; Scherrmann 1980;

Schreiber 1981a, 1981b, 1981c; Silber 1979; Smith 1973) make it difficult to generalize about digoxin pharmacokinetics. Due to the inpatient and outpatient variation in the pharmacokinetics as well as problems with the immunoassays of digoxin, nomograms for determination of a dosage regimen have not been popular. Recently, however, it was suggested that a nomogram (Bjornsson 1986) may be useful for adjusting the dosage of digoxin and other cardiovascular drugs that are partially eliminated by the kidneys in patients with reduced renal function.

#### 1.1.4.1 Absorption

Orally administered digoxin is adequately absorbed from the gastrointestinal tract, even in vascular congestion of the gastric mucosa, hypoxia and diarrhea that may exist in the cardiac patient (Smith 1984a). While it has been suggested (Gault 1977; Lindenbaum 1981; Loo 1977; Magnusson 1982a, 1982b; Sonobe 1980) that digoxin can be degraded by acid and/or enzymes to digoxigenin and its mono- and bis-digitoxosides and that this may occur in the gastrointestinal tract, the levels of the degradation products in plasma cannot be differentiated from those metabolically formed. Thus, firm conclusions regarding the *in vivo* degradation have not been established. It has been found that a lowered gastric pH leads to lower plasma digoxin levels and this has been assumed to be due to the sensitivity of these compounds to acid (Hossie 1977;

Sternson 1978). Some investigators (Brown 1978; Greenblatt 1976) regard the lowering of plasma digoxin levels with hyperacidity to be sufficient reason to monitor these patients more closely. It has also been suggested that the intestinal flora may be responsible for the conversion of digoxin to dihydrodigoxin (Dobkin 1983; Lindenbaum 1981). The effect of food ingestion prior to drug therapy has also been reported (Greenblatt 1976; Wagner 1974) to result in large discrepancies in digoxin plasma levels.

The bioavailability of digoxin in a capsule form (Malini 1983) was about 20% higher than that of tablets, indicating that the dosage formulation of digoxin may also affect absorption. Soft gelatin capsules of digoxin as a solution gave 90 to 100% absorption (Johnson 1986). Johnson *et al.* (1986) also noticed no significant variability in digoxin trough concentrations nor in urinary excretion between tablet and capsule formulations. They further noted an inverse ratio of metabolism with bioavailability. This was considered to be due to colonic bacteria, although this aspect was not studied in detail.

Plasma levels of digoxin have also been found to vary when other drugs and substances are administered (Haustein 1983; Manninen 1981). Arterial blood pH in the range 7.25 to 7.50 was found to be directly correlated with digoxin plasma levels at steady state (Catenazzo 1985). These data (Catenazzo 1985) may explain the strongly reduced activity of digoxin in patients with respiratory acidosis.

Permeation of digoxin through mouse and human skin (Cairncross 1985) studied using an *in vitro* system indicates that it may be possible to develop a clinically efficient transdermal therapeutic system.

#### 1.1.4.2 Distribution

After oral administration, the serum digoxin concentration reaches a peak between 45 minutes and 3 hours (Doherty 1975; Hoffman 1980). The distribution of digoxin to central nervous tissue is thought to produce the neurological signs of toxicity such as drowsiness, disorientation, hallucinations and visual disturbances (Aronson 1980; Haustein 1983; Rietbrock 1981). It has also been suggested that the resistance to the arrhythmogenic and vasoconstrictor effects of digoxin may involve the hypothalamus (Otsuka 1982).

Digoxin is also distributed to other tissues: about 65% is found in the liver, 4% in the heart, 3% in the brain and 1.5% in the kidneys (Aronson 1980). Serum protein binding of digoxin, principally to albumin, varies from 10 to 40% (Kramer 1974; Movselli 1977; Smith 1984a) and appears to be unaffected by concentration (Aronson 1980). Digoxin is also bound to  $\beta$ -lipoproteins (Brock 1976) but with a lower affinity than digitoxin. In comparison to albumin, the  $\beta$ -lipoprotein concentration in blood is so small that the concentration of lipoprotein-bound digoxin to the total bound is minimal. Thus, it has been suggested, the fraction

of unbound digoxin in serum can be predicted solely from the albumin concentration and pH (Brock 1976).

A model with at least two kinetically distinct compartments can be used to describe digoxin's disposition (Keys 1980; Nyberg 1974; Rietbrock 1981) where the central compartment represents the blood and well-perfused body fluids and tissue and the peripheral compartment represents the slowly-perfused body space. A recent study suggests that the data best fit a non-linear, two-compartment model with a deep tissue compartment (Kramer 1979). Digoxin may also undergo significant biliary excretion (Reissell 1982) and enterohepatic circulation or intestinal secretion (Reissell 1982; Schafer 1985). This area is still disputed in the literature.

During the distribution phase of digoxin, rapid removal from the central compartment is observed. Extensive distribution of digoxin in tissue is indicated by a large steady state volume of distribution. Using tritiated digoxin, the distribution half-life was 60 minutes (Doherty 1975). The reported steady-state volume of distribution of digoxin was extremely variable: between 5.1 and 8.1 L/kg in healthy subjects, 5.0 L/kg in patients with cardiac failure and between 3.3 and 4.4 L/kg in patients with renal failure (Rietbrock 1981). Morphological changes in the heart due to cardiac disease may alter digoxin binding and produce a change in the volume of distribution (Keys 1980). The volume of distribution appears to vary with renal function



but large variation exists in individuals with comparable renal function (Keys 1980). A decrease in the number of digitalis receptors has been observed in renal failure patients (Malini 1985), which may explain the decrease in activity of digitalis glycosides in chronic renal failure. A summary of the pharmacokinetic data for digoxin is shown in Table I.

Table I. Summary Of Digoxin Pharmacokinetics<sup>1</sup>

Pharmacokinetic Parameter

Availability (Oral)	70 $\pm$ 13%
Urinary Excretion	60 $\pm$ 11%
Bound in Plasma <sup>2</sup>	25 $\pm$ 5%
Volume of Distribution (L/Kg) <sup>3,4</sup>	7 $\pm$ 30%
Half-life (Hours) <sup>4</sup>	39 $\pm$ 13
Time to Maximum Concentration (Hours)	2 to 3
Time to Maximum Effect (Hours)	4 to 6

<sup>1</sup> From Gilman *et al.* (1988).

<sup>2</sup> Decreases with uremia.

<sup>3</sup> Decreases with hypothyroidism and increases with hyperthyroidism.

<sup>4</sup> Decreases with hyperthyroidism and increases with uremia, congestive heart failure and hypothyroidism.

#### 1.1.4.3 Metabolism And Elimination

Metabolism of digoxin occurs mainly in the liver (Abel 1965) and the drug is excreted largely by the kidneys via glomerular filtration and tubular secretion (Steiness 1982). Although many reports have evaluated the metabolic turn-over of digoxin, this area is still one of considerable dispute. Some investigators have suggested that only a small

proportion of digoxin was metabolized (Ashley 1958; Beerman 1972; Doherty 1970; Gibson 1980; Marcus 1966), while others have shown that 57 to 60% (Clark 1974; Luchi 1968) was excreted as a metabolic product, largely dihydrodigoxin. A recent report (Aronson 1980) noted a large interpatient variation in metabolite excretion such that from 20 to 55% of the drug was metabolized in a few patients, but the majority excreted the drug 80% unchanged. Using orally administered tritiated digoxin, Gault *et al.* (1979) reported that, with normal renal function, digoxin was excreted 54.5% unchanged, 2.0% as digoxigenin bisdigitoxoside, 0.8% as digoxigenin monodigitoxoside, 0.25% as digoxigenin and 0.3% as dihydrodigoxin. Subjects with minimum renal function (Gault 1979; Gibson 1980) excreted only trace levels of metabolites. Clark and Kalman (1974) speculated that the variability in digoxin metabolism and the lack of specificity of the RIA for digoxin would increase the complexity of evaluating RIA results for patient samples.

The metabolism of digoxin by cleavage of the digitoxose residues produces an increase in lipid solubility while conjugation reactions would cause the lipid solubility to decrease. Lage and Spratt (1966) reported that stepwise cleavage of the digitoxose residues progressively decreased the cardioactivity. Reduction of the lactone ring also substantially decreased the activity (Keys 1980) with the cardioactivity of dihydrodigoxin being only 1/20 that of digoxin (Rietbrock 1981). Epimerization of digoxigenin and

conjugation reactions with sulfate and glucuronide resulted in a loss of almost all activity (Keys 1980). Using adult male mice, the potency ratios of digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin and 3-epidigoxigenin compared to digoxin were found to be 1/1.33, 1/1.47, 1/4.84 and 1/6.45 respectively. With respect to digoxin, the potency ratio for dihydrodigoxigenin (less than 1/46) (Brown 1962) has also been reported.

#### 1.1.5 Chemistry

Digoxin, a naturally occurring cardioactive steroid, has three structural components; a series of sugar residues, a steroid nucleus and a five-membered unsaturated lactone ring. Removal of the sugar residues leaves the genin or aglycone. This glycoside is found in the leaves of the foxglove species *Digitalis lanata* (Haustein 1983) which contain a number of other digitalis glycosides (digitoxin, gitoxin, diginatin and gitaloxin). The aglycone of digitalis glycosides consists of a cyclopentano-perhydrophenanthrene nucleus with a five-membered unsaturated lactone ring attached in the 17- $\beta$  position. Methyl groups are attached at carbons 10 and 13 ( $\beta$  position), a hydroxyl group at carbon 14 ( $\beta$  position) and the five-membered lactone ring ( $\beta$  position) is attached at carbon 17 in all digitalis aglycones (Haustein 1983). In addition, the aglycone for digoxin, digoxigenin, has a hydroxyl group at carbon 12.

Naturally occurring digitalis glycosides have a sugar component attached at position 3 via a glycosidic linkage (Myerson 1967). These sugars are usually six-membered rings in the preferred chair conformation and are linked together by  $\beta$ -glycosidic 1,4-linkages. For digoxin, a three sugar chain of digitoxose residues is joined to the aglycone at position 3. The chemical structure of digoxin is shown in Figure 1.

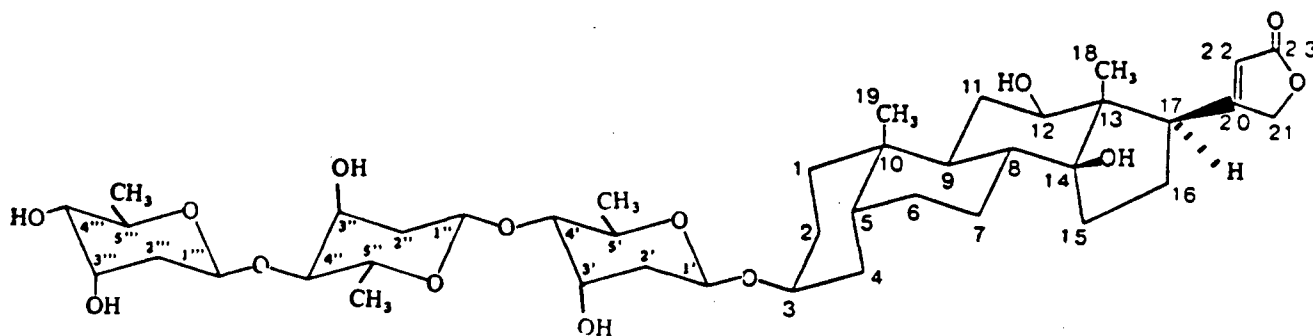


Figure 1. The Chemical Structure of Digoxin

#### 1.1.6 Structure Activity Relationships

Despite the length of time the cardiac glycosides have been in use, problems due to the low therapeutic index and inter- and intra-patient variability are still present. Numerous studies have examined the chemistry and structure-activity relationships of these glycosides. For

cardioactivity to be present, the glycosides must have the following (Guntert 1981; Haustein 1983):

- i) a cis-cis junction of the C and D rings of the genin moiety,
- ii) an unsaturated lactone moiety attached at carbon 17 in the  $\beta$  configuration,
- iii) a sugar component attached to the 3- $\beta$ -hydroxyl on the genin via a glycosidic linkage.

The C and D rings of cardiac glycosides are usually fused cis with the hydroxyl at position 14 in the  $\beta$  position. Comparison of 14- $\alpha$  aglycones (Guntert 1981) with the corresponding 14- $\beta$  compounds indicate that activity is lost when rings C and D are fused in the trans position. Rings C and D are thought to undergo Van der Waals and/or hydrophobic interactions with the proposed receptor (Thomas 1980) and changing the configuration leads to a decrease in binding and therefore a decrease in activity.

An unsaturated lactone ring composed of four or five carbon members attached to the genin at carbon 17 in the  $\beta$  configuration is almost essential for cardiac activity. A change in configuration of this side-chain yields inactive compounds (Guntert 1981; Saito 1970; Tamm 1963). Replacing the lactone ring with open-chain analogues (Fullerton 1976; Gelbart 1978; Smith 1982; Thomas 1974a, 1974b) indicated that there are both steric and electronic requirements for the carbon 17 side-chain. Recently, Griffin *et al.* (1986) suggested that there was an additional receptor binding site for digitalis glycosides since 16- $\beta$  formate and acetate

esters showed increased activity with respect to the parent compound.

Pharmacological activity resides in the aglycone but the sugars attached at position 3 modify the water and lipid solubility and potency of the resulting glycoside (Tamm 1963). Progressive removal of the sugar moieties produced a decrease in activity (Keys 1980). Removal of the last sugar greatly reduced activity (Keys 1980; Luchi 1965; Repke 1963; Thomas 1980). Metabolism before and after absorption from the gastrointestinal tract can lead to the aglycone with a free hydroxyl group at carbon 3 (Gault 1977; Repke 1963). Formation of the 3-dehydro-genin followed by 're-hydrogenation' may yield either the 3  $\beta$ -hydroxyl-genin or the 3  $\alpha$ -hydroxyl-genin. This epimerization at carbon 3 is prevented when a minimum of one sugar residue is present (Luchi 1965; Repke 1963). Part of the decrease in activity observed when the last sugar residue is removed from the genin may be due to epimerization to the 3  $\alpha$ -hydroxyl form which is almost inactive (Guntert 1981). Binding studies with  $\text{Na}^+, \text{K}^+$ -ATPase (Akeru 1981; Takiura 1974; Wallick 1974; Yoda 1974) suggest that both the genin and the sugar component contribute to binding. Conflicting results have been reported concerning the requirements for the oxygen function at carbon 3. Using an isolated frog's heart preparation, Saito *et al.* (1970) found that the oxygen was not required for activity. Inhibition studies with  $\text{Na}^+, \text{K}^+$ -

ATPase (Witty 1975), however, show that the oxygen at position 3 is required for full activity of the aglycone.

Replacement of the digitoxose residues with various branched groups (Siebeneick 1978) gave compounds with lower  $\text{Na}^+, \text{K}^+$ -ATPase inhibitory activity. Aminosugar derivatives were approximately ten times more potent inhibitors than the parent glycoside (Caldwell 1978). Naturally occurring amino-deoxyglyco-cardenolides (Choay 1978) have also been identified.

## 1.2 Digoxin Therapy

Digitalis glycosides are indicated for the treatment of arrhythmias and congestive heart failure. These compounds were considered the best agents for control of the ventricular rate in atrial fibrillation or flutter (Doherty 1985). The calcium antagonist verapamil was also effective in slowing the ventricular rate in atrial fibrillation (Klein 1986). Presently, both digitalis glycosides and verapamil are drugs of choice in the treatment of atrial fibrillation. For congestive heart failure, the digitalis glycosides are useful in either right, left or combined ventricular failure (Doherty 1985).

Reports comparing the clinical response of digoxin therapy with its associated plasma levels, have indicated that 30% of the patients studied did not improve their cardiac efficiency in a predictable manner (Kramer 1979). Whether the latter group was unimproved due to inadequate

therapy or due to an inability to titrate the drug administration was not clear. Also, problems with the digoxin plasma assay have been suggested in this study.

There is great controversy over the prolonged treatment of congestive heart failure in specific patient groups with digitalis glycosides (Applefeld 1986; Doherty 1985; Yusuf 1986). Long-term therapy with digoxin has been reported (Spector 1979) to benefit only a small number of patients, but at present it is not considered good therapy to withdraw the drug once adequate control has been achieved. The effects of digoxin therapeutic withdrawal are underscored by a study (Bowman 1983) in which patients who were at reduced risk had their digoxin therapy discontinued. While only 14% of the patients studied deteriorated, the authors suggested that only those patients who had little indication for digoxin therapy should be considered for discontinuation of therapy. A more recent review of the literature in this area (Applefeld 1986) indicated that caution should be exercised with stopping digoxin therapy in those patients with moderate heart failure and that close monitoring of cardiac function is essential.

### 1.3 Digoxin-Like Immunoreactive Substance(s)

Endogenous factors, distinct from aldosterone, that play a role in sodium excretion and extracellular fluid regulation, have been reported (Wilkins 1985). These factors are secreted in response to hypervolemia, circulate



in plasma and stimulate natriuresis in order to restore sodium and water balance. Recently, an atrial natriuretic peptide (ANP), which has potent natriuretic activity, has been found in the mammalian atria (Lang 1985; Mills 1984; Wilkins 1985). A second endogenous factor that inhibited the  $\text{Na}^+, \text{K}^+$ -ATPase enzyme to block sodium transport in the renal tubules (De Wardener 1977, 1982a; Wilkins 1985) also affected the sodium and water balance. Since both digoxin and this second factor inhibit the activity of  $\text{Na}^+, \text{K}^+$ -ATPase and react with digoxin antibodies, the endogenous digitalis-like factor was termed endoxin (Diamandis 1985; Schreiber 1982). Some researchers (De Wardener 1982a; Diamandis 1985; Hnatowich 1984; Wilkins 1985) have called this factor digoxin-like immunoreactive substance(s) or DLIS.

The properties of ANP and DLIS have recently been compared (Wilkins 1985). The structure of DLIS is unknown but thought to be less than 500 Da in molecular mass. DLIS inhibits  $\text{Na}^+, \text{K}^+$ -ATPase (ANP does not), causes vasoconstriction (ANP causes vasodilation), is excreted in the urine and may be produced in the hypothalamus (ANP is produced in cardiac atria).

DLIS has been found in tissues and biological fluids of normal adult subjects who never received digoxin (Balzan 1984; Clerico 1985; Diamandis 1985; Hamlyn 1982; Klingmuller 1982; Valdes 1983a; Vinge 1988). This substance has been found in umbilical cord blood and placental homogenates (Besch 1976; Diamandis 1985; Kelly 1981; Ng 1985; Pudek

1983a, 1983b; Scherrmann 1986a, 1986b; Yatscoff 1984), neonatal serum (Clerico 1985; Heazlewood 1984; Koren 1984; Pudek 1983a, 1983b; Valdes 1983b; Yatscoff 1984), sera from pregnant women (Barbarash 1984; Boink 1977; Graves 1984; Gusdon 1984; Longerich 1988; Phelps 1988) and amniotic fluid (Drost 1977; Valdes 1983b). Gonzalez *et al.* (1987) and Koren *et al.* (1988) report that DLIS levels in umbilical cord blood (both venous and arterial blood) were significantly greater than in maternal venous blood. Also, DLIS levels in high-risk pregnancies were significantly higher than in normal pregnancies (Koren 1988). Phelps and co-workers (1988) found DLIS to appear in maternal serum and levels increased with increasing gestational age but that there was no significant difference between DLIS levels in patients with and without preeclampsia. Although no causal relationship between DLIS and preeclampsia has been found, it may still have an etiologic role in development of this condition (Phelps 1988).

DLIS has been reported in bile and meconium (Kieval 1988; Pudek 1984) and in patients with renal impairment (Bourgoignie 1972; Craver 1983; D'Arcy 1984; Graves 1983a, 1983b; Kramer 1985b; Oldfield 1985; Yatscoff 1984) and in healthy subjects who have been salt loaded (Kramer 1985a).

The serum from patients with hepatic failure has also been reported to contain DLIS (DiPiro 1980; Greenway 1985; Nanji 1985, 1986; Yang 1988). Initially, a 2 to 3 fold increase in DLIS levels was found in patients with alcoholic

cirrhosis (DiPiro 1980). DLIS levels up to 0.8 ng/mL were reported in patients with infectious hepatitis, acute fatty liver of pregnancy and metastatic liver disease (Greenway 1985; Nanji 1985). Others, however, failed to find elevated DLIS levels in patients with alcoholic and metastatic liver diseases (Frewin 1986).

Recent reports suggest that DLIS, or a fraction thereof, may be the natriuretic hormone and involved in hypertension (Buckalew 1984; Cloix 1987; De Wardener 1982a, 1982b; Grantham 1984; Wilkins 1985) and may also be present in normotensive subjects (Cloix 1987; Hamlyn 1982).

Peak serum DLIS levels in pre-term and term infants correlated with the excretion of sodium (Ebara 1986a), indicating that DLIS may be among the pathological factors involved in hyponatremia in pre-term infants.

The source, structure and composition of DLIS are the subjects of a number of investigations. The adrenal glands have been suggested as a source of DLIS (Pernollet 1986; Schreiber 1981a, 1981b, 1981c; Shilo 1987) as has the fetal adrenal cortex (Pudek 1983b). One manuscript (Diamandis 1985) noted that progesterone and cortisone are present in HPLC fractions of cord blood, placenta and maternal blood and that the chromatographic elution volumes of these fractions were particularly reactive with RIA methods of digoxin analysis. Longerich *et al.* (1988) reported the identification of cortisol and progesterone in a portion of the plasma DLIS in pregnant women. While these substances

in themselves are unlikely to be the DLIS material, their high reactivity causes further difficulties with the validity of the RIA procedure. Dehydroepiandrosterone-sulfate, cortisone, cortisol, deoxycortisone,  $\Delta^4$ androstenedione, progesterone and glycochenodeoxycholic acid cross-reacted with digoxin antisera and had HPLC retention times similar to DLIS-containing fractions (Matthewson 1988).

Dasgupta and co-workers (1987, 1988) established that part of the DLIS found in haemodialysis patients was a phospholipid. Fast atom bombardment mass spectra of this compound (DLIS-2) (Dasgupta 1988) supported the presence of a phosphoserine group in the molecule. Another group, using mass spectrometry of HPLC fractions of plasma from a patient with renal and liver impairment, reported the presence of bile acids in the fractions containing DLIS (Toseland 1988).

Using reversed-phase HPLC, DLIS has been separated into several immunoreactive fractions which indicated that DLIS is not a single substance (Diamandis 1985). DLIS has been suggested to be dehydroepiandrosterone (Vasdev 1985), however, this has been disputed (Pudek 1983a). Recent manuscripts (Braquet 1986a, 1986b; Fagoo 1986) noted that enterolactone which contains a lactone ring similar to that of digoxin, may contribute to the digitalis-like activity of these samples and mono and diglycerides have also been implicated (Soldin 1986a).

Valdes and co-workers (1985a, 1985b) reported the protein binding of endogenous digoxin-immunoreactive factors

in human serum and urine. These factors are water soluble, heat stable and neutral in molecular charge. Serum DLIS has an apparent molecular mass of 200 Da while that from urine appears to be 400 Da (Valdes 1985a, 1985b). Urinary DLIS has a higher affinity for digoxin antisera and less resistance to acid hydrolysis than that isolated from serum. It has been suggested (Valdes 1985a, 1985b) that the urinary DLIS may represent a conjugated metabolite of the factor found in serum. In addition, serum DLIS appears to be bound noncovalently to serum protein (Valdes 1985a, 1985b). Normally over 90% of the total endogenous immunoreactivity in serum is tightly but reversibly bound to protein and is therefore not detected by direct measurement with conventional RIA methods (Valdes 1985a, 1985b). The remaining serum DLIS is weakly protein bound or unbound (Valdes 1985a, 1985b). Valdes (1985a, 1985b) suggested that the increase in DLIS levels, seen in patients with renal failure, in neonates and pregnant women, was due to an increase in the amount of weakly protein bound DLIS rather than an increase in total DLIS. Other reports have indicated that an increase in serum DLIS levels may be at least partly due to a decrease in its removal via the kidney (Clerico 1988a) and that the urinary excretion can be positively correlated with physical activity (Clerico 1988a, 1988b).

The presence of sufficient DLIS in patient samples, particularly neonates, seriously compromises the accuracy

and interpretation of the results from immunoassay methods. Several investigators (Görtner 1985; McCarthy 1985; Ng 1985; Pudek 1985; Scherrmann 1986a; Witherspoon 1987) have tested commercial kits and have recommended particular brands as having lower reactivity to DLIS. Alterations in the incubation conditions with commercial RIA methods also effected the apparent digoxin levels in the presence of DLIS (Smith 1987; Yannakou 1987). Ultrafiltration of serum, which has been shown to remove approximately 90% of the DLIS present (Christenson 1987; Graves 1986), may be used to minimize the interference with digoxin immunoassays. The use of fluorescence polarization immunoassays for digoxin may reduce interference from DLIS (Yatscoff 1984) but will not eliminate it entirely (Bianchi 1986; Soldin 1986b). While the above procedures may appear to be a solution to the problem, batch-to-batch variances in immunoassay kits make this an unreliable solution over time.

In addition to the presence of DLIS, a digitoxin-like immunoreactive substance was detected in amniotic fluid and cord blood (Ebara 1986b). A significant correlation between the levels of DLIS and digitoxin-like immunoreactive substance was also found.

The physiological role of DLIS has yet to be entirely investigated (Clerico 1987). High levels of DLIS, whether due to excess production or reduced excretion, may produce effects on the heart and the autonomic nervous system similar to those seen with digitalis toxicity (Kieval 1988).

Using DLIS isolated from human bile and canine Purkinje fibers, Kieval *et al.* (1988) demonstrated cardiotoxicity similar to ouabain.

#### 1.4 Digoxin-Drug Interactions

The clinical management of patients on digoxin is complicated by the variability of digoxin pharmacokinetics and problems with the assay methods. Furthermore, a low therapeutic index has been reported for digoxin, with the therapeutic range being 0.5 to 2.0 ng/mL serum. The interaction of digoxin with other agents which may produce small changes in digoxin disposition may lead to toxic or sub-therapeutic plasma digoxin concentrations. Since digoxin toxicity and untreated congestive heart failure or arrhythmias may be fatal, it is essential that these patients be carefully observed and appropriate changes in their digoxin regimen be made when other drugs are co-administered.

##### 1.4.1 Digoxin-Quinidine

Multiple drug therapy is often used to control edema or arrhythmias that may accompany congestive heart failure. Unfortunately, many drug interactions have been found between digoxin and other co-therapeutic agents. The most noted drug interaction of digoxin is with quinidine, a widely used antiarrhythmic agent. One of the first reports of a problem with this combination was in 1932 (Gold 1932)

when an increased risk of drug-induced arrhythmias in dogs was noticed. In guinea-pigs, quinidine was found to inhibit the distribution of digoxin (Okudaira 1986) by decreasing both the ATP-dependent binding in heart, muscle and liver and the ATP-independent binding in heart. Also, quinidine inhibited the intestinal secretion of digoxin in guinea pigs (Schafer 1985).

Further studies of this interaction in healthy volunteers (one intravenous digoxin dose) and cardiac patients (at steady state digoxin levels) have suggested that more than one mechanism was involved. Quinidine decreased the total body clearance and non-renal clearance of digoxin in healthy volunteers (Ochs 1981) and cardiac patients (Schenck-Gustafsson 1981a, 1982). It also inhibited renal tubular secretion in patients thus producing a decrease in the renal clearance of digoxin (Schenck-Gustafsson 1982). Recently, Fichtl *et al.* (1983) reported that serum digoxin levels increased to about the same amount irrespective of the degree of renal impairment. The relative contributions of impaired renal and non-renal clearances, and re-distribution of digoxin to the interaction with quinidine are unknown at the present time. Schenck-Gustafsson *et al.* (1981b) reported a reduction of the ratio of skeletal muscle to serum digoxin levels in cardiac patients which contributed to the quinidine-induced decrease in the apparent volume of distribution for digoxin. Steady-state serum digoxin concentrations after quinidine



administration correlated well with serum digoxin concentrations before quinidine therapy in cardiac patients (Friedman 1982), allowing for the estimation of digoxin levels that will be reached after quinidine administration.

#### 1.4.2 Other Drug Interactions

Many other drugs interact with digoxin to produce a change in the plasma levels of digoxin or alterations of its inotropic effect in humans. Burgess and Crane (1986) observed a quantitatively similar positive inotropic response for food and intravenous digoxin in volunteers. This inotropic response exhibited potentiation when digoxin and food were administered together. Quinine has been reported to impair the extrarenal clearance of digoxin which caused an increase in plasma digoxin levels (Pedersen 1985a). Diazepam produced an increase in plasma digoxin concentrations (Castillo-Ferrando 1980) while another benzodiazepine, alprazolam, did not significantly alter digoxin clearance (Ochs 1985).

Cholestyramine, a basic anion exchange resin that is not absorbed by the gastrointestinal tract, has been noted to bind digoxin and prevent absorption (Hoffman 1980), thereby reducing plasma digoxin levels by 69% from those of control subjects (Brown 1976). Activated charcoal has also been observed to decrease the absorption of digoxin from the gastrointestinal tract (Reissell 1982). Metoclopramide, a gastric motility modifier, has been found by one group of

investigators to lower plasma digoxin levels (Lindenbaum 1971), while a more recent study (Johnson 1984) noted that metoclopramide reduced both the time to reach peak plasma levels and the absorption when tablet formulations were used. However, the overall effect on digoxin absorption by metoclopramide was minimized when capsules (containing digoxin in solution) rather than tablets were administered (Johnson 1984).

The serum concentration of certain electrolytes influence myocardial sensitivity to digoxin. Hypokalemia has been shown to result in cardiac toxicity when serum digoxin concentrations were in the therapeutic range (Keys 1980). Some of the diuretics that cause hypokalemia (thiazides, furosemide and ethacrynic acid) may affect digoxin toxicity due to this electrolytic effect (Hoffman 1980). The renal excretion of digoxin, however, was not affected by furosemide (Brown 1976; Malcolm 1977; Tilstone 1977).

Spironolactone reduces the volume of distribution, renal tubular secretion and non-renal clearance of digoxin (Bussey 1982; Waldorff 1978). Caution was also suggested when combining trimethoprim with digoxin (D'Arcy 1985). Phenytoin was found to significantly increase the total digoxin clearance suggesting that, during co-administration, the digoxin serum concentrations should be monitored and if necessary, the digoxin dose increased (Rameis 1985).

The antianginal and antiarrhythmic agents verapamil, nifedipine and amiodarone cause an increase in the serum digoxin concentration in cardiac patients and healthy volunteers (Belz 1981a, 1983; George 1982; Klein 1982; Pedersen 1981, 1982, 1985b; Venkatesh 1985, 1986). The calcium antagonist, bepridil, also produced an increase in the serum digoxin concentrations in healthy subjects (Belz 1986). Digoxin intoxication in patients treated with amiodarone has been reported (Ben-Chetrit 1985).

In rats, Koren *et al.* (1983) found that verapamil produced no change in tissue uptake of digoxin, which suggested that an inhibition of digoxin elimination occurred. The tissue/serum ratios of digoxin concentrations in rat myocardium, skeletal muscle, and brain were decreased when amiodarone or its major metabolite, desethylamiodarone, were added to the regimen (Venkatesh 1985, 1986). However, amiodarone had no effect on the kinetics of a single dose of digoxin in the rabbit (Buss 1985).

The effect of disopyramide, another antiarrhythmic agent, on serum digoxin levels has been studied with conflicting results. Some investigators (Leahey 1980; Wellens 1980) have found no change in the serum digoxin concentration in cardiac patients when therapeutic levels of disopyramide were co-administered with digoxin. Relatively high levels of disopyramide (mean of 5.05  $\mu\text{g/mL}$  compared to therapeutic concentrations of 2.8 to 3.2  $\mu\text{g/mL}$  for atrial arrhythmias) (Gilman 1980) produced a 15% increase in serum

digoxin concentration but was thought to be of limited clinical significance (Manolas 1980). The antiarrhythmic ethmozine produced no statistically significant change in serum digoxin concentrations of cardiac patients with normal renal function (Kennedy 1986).

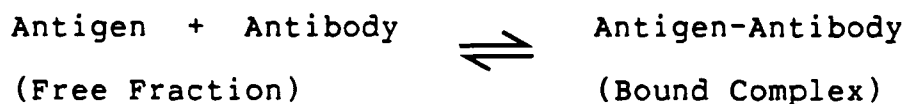
### 1.5 Digoxin Analysis

The low plasma levels of digoxin and its metabolites have prompted the development of extremely sensitive assay procedures. Since a large variability in the metabolism and excretion of digoxin has been reported, it is imperative that any assay procedures for the measurement of digoxin be specific for the intact drug substance. Digoxin may be analysed by several methods including radioimmunoassay (RIA) (Butler 1978, 1979), enzyme-multiplied immunoassay (EMIT) (Brunk 1977; Butler 1978; Eriksen 1978; Linday 1983; Rosenthal 1976; Sun 1976), fluorescence polarization immunoassay (FPIA) (Butler 1978; Erickson 1984; Rawal 1983), high-performance liquid chromatography (HPLC) (Beasley 1983; Davydov 1982; Desta 1982a, 1982b, 1987; Eriksson 1981a; Kwong 1986a, 1986b), and various biological and chemical methods (Haustein 1983; Lowenstein 1965; Simson 1962; Stewart 1981). None of the methods developed to date can compete with the immunoassay methods in terms of speed, precision, sensitivity and cost. As a result these methods are most frequently used in clinical laboratories. FPIA methods have replaced RIA procedures in many clinical

laboratories since the FPIA system provides more rapid analysis (20 min.), greater precision and recovery of digoxin, the reagents have a longer shelf-life and radioactive isotopes are not required (Erickson 1984).

#### 1.5.1 Immunoassays

The analytical procedure based on the reaction between an antigen and an antibody to the antigen is termed an immunoassay. This reaction obeys the Law of Mass Action and is shown in Figure 2.



**Figure 2. Reaction Involved in Immunoassay Procedures**

Immunoassays use a labelled antigen to increase the sensitivity of the assay. Competitive binding of the labelled and non-labelled antigen with the antibody form labelled and non-labelled bound complexes. The labelled antigen in either the free fraction or the bound fraction is then determined. By comparison to a series of samples of known concentration of analyte (assayed at the same time), the concentration of analyte in an unknown sample can be determined.

Radioisotopes, enzymes, coenzymes, red blood cells, latex particles and metals as well as fluorescent,

bioluminescent and chemiluminescent molecules have been used for labelling antigens. For digoxin analysis, radioactivity, enzymes and fluorescent molecules are the most common labelling methods (Butler 1978).

One of the major problems with immunoassay procedures for digoxin analysis has been the cross-reactivity of the antibodies with the metabolites of digoxin and other endogenous compounds. This may lead to erroneous results, particularly in view of the fact that DLIS is present in many patient groups and that digoxigenin and its mono- and bis-digitoxosides are known to have cardiac activity, although at substantially lower potencies than digoxin (Aronson 1980). Naturally occurring and synthetic steroids also exhibit some cross-reactivity with digoxin antibodies isolated from the sera of animals when only relatively short periods of immunization with the digoxin conjugate are used (Butler 1979; Pudek 1983a; Schreiber 1981a, 1981b, 1981c; Smith 1970, 1973).

Radioimmunoassay techniques, using a radioisotope label, have been developed for the analysis of digoxin in serum or plasma. Other cardiac glycosides (Besch 1975; Butler 1978; Lenz 1975; Reissell 1982; Weiler 1980) and some of the metabolites of digoxin (Butler 1978, 1982; Eichhorst 1981) have also been assayed by similar radioimmunoassay techniques. The determination of digoxin in urine samples by RIA has also been reported (Christenson 1982).

Antibody specificity is a major problem with the RIA for digoxin. Spironolactone, a synthetic steroid analogue, has produced interference with some RIA kits (DiPiro 1980; Lichey 1979; Schreiber 1981c; Silber 1979). Silber *et al.* (1979) suggested that metabolites of spironolactone (other than canrenone) may be responsible for this interference. Still other reports have indicated that spironolactone did not interfere with the RIA method (Hansell 1979; Muller 1978; Ravel 1975). Furosemide has not been found to interfere with the determination of digoxin using the RIA method (Hansell 1979). In addition to exogenous substances, endogenous substances such as albumin (Holtzman 1974; Lindenbaum 1975) and DLIS (Valdes 1983a, 1983b), as well as bacterial contamination of patient samples (Boone 1977) have caused alterations in apparent digoxin values using the RIA procedure.

Variations in cross-reactivity with digoxin metabolites and endogenous substances has been traced to differences in the commercial sources of the RIA kits (Hansell 1979; Kubasik 1974a, 1974b; Kuczala 1976; Larson 1977; Loo 1977; MacKinney 1975; Smith 1976). Comparing RIA kits using tritium or radioiodine labelled digoxin show that the tritium method was less variable (Kubasik 1975; Pippin 1976; Vemuri 1980). However, radioiodine labelling has some advantages. Iodine-125 is a gamma emitter and counting is faster, more convenient and less expensive than liquid scintillation counting which is necessary with tritium

(Butler 1978). In addition, quenching is not observed with iodine-125 (Kroening 1976).

FPIA is a fluoroimmunoassay (FIA) where a fluorescent tracer is excited with polarized light. The polarization of the emitted light will depend on the extent of random Brownian rotation of the molecules during their excited state (Landon 1981). Since the polarization of the emitted fluorescence is different for antibody-bound and free antigen, FPIA methods do not require a separation step (Dandliker 1964; Jolley 1981; Landon 1981).

The commercially available FPIA for digoxin from Abbott Laboratories is the TDx assay. This assay requires precipitation of serum proteins with trichloroacetic acid (Erickson 1984; Porter 1984) or 5-sulfosalicylic acid (Skogen 1987). The results are significantly influenced by the total protein concentration (Porter 1984) such that increased protein decreases the measured digoxin levels. The use of acid for protein precipitation, however, has been suggested to result in the transformation of digoxin and its metabolites to digoxigenin and/or dihydrodigoxigenin (Gault 1977; Sonobe 1980; Sternson 1978). Therefore with this method, digoxin can not be differentiated from its metabolites present in the serum sample. The TDx FPIA has been reported to be subject to minimal interference from DLIS as compared to the RIA procedure (Yatscoff 1984). However, numerous reports in the literature (Bianchi 1986; Frye 1987; Kanan 1987; Skogen 1987; Soldin 1986b; Weiner



1987) have shown that the TDx assay indicated increased apparent digoxin levels in the presence of DLIS. In addition, it has been suggested that the protein precipitation step used with the FPIA may enhance DLIS interference by disrupting the DLIS-protein interaction and allowing free DLIS to be detected (Skogen 1987; Soldin 1986b).

#### 1.5.2 Biological Assays

Digoxin inhibits the activity of  $\text{Na}^+, \text{K}^+$ -ATPase by binding to the enzyme and ATPase isolated from heart and brain tissues has been used to develop enzyme binding assays for the determination of cardiac glycoside concentrations (Gundert-Remy 1981). However, the metabolites of digoxin also inhibit  $\text{Na}^+, \text{K}^+$ -ATPase activity, with dihydrodigoxin having ten times the binding affinity of digoxin (Gundert-Remy 1981; Marcus 1975). As a result, the ATPase assay procedures, like the immunoassay methods, lack specificity for digoxin.

The rubidium-86 uptake assay is based on the inhibition of rubidium uptake into red blood cells *in vitro* by digitalis glycosides (Belz 1981b; Lowenstein 1965). Determination of digitalis glycosides in plasma using this technique requires approximately 8 hours, hence this assay method is more time consuming than conventional immunoassay methods (Belz 1981b).

A number of test animals, including guinea pigs, frogs, cats and pigeons, have been used in the bioassay of digitalis (Tyler 1976; USP 1985). In the USP assay for digoxin, pigeons are injected with fixed volumes of a diluted digitalis preparation at five minute intervals until the pigeon dies from cardiac arrest (USP 1985). Problems with this bioassay are found since it assumes that toxicity is associated with, and proportional to, the therapeutic effect. In general, bioassays are unable to predict the oral potency of the glycoside being tested and lack specificity (Simson 1962; Tyler 1976). Along with a lack of sensitivity, bioassay methods are impractical for evaluating digitalized patient samples (Simson 1962; Tyler 1976).

#### 1.5.3 Chemical Methods

Digoxin has an ultraviolet absorption maximum at 217 nm with a molar extinction coefficient of 16595 (Flasch 1981). Derivatization procedures, to increase both the intensity of absorption and alter the wavelength of maximum absorbance, have been reported for quantitative analysis of digitalis glycosides (Flasch 1981; Rowson 1952a, 1952b). These methods have not been sufficiently sensitive for the evaluation of biological samples (Flasch 1981) and have not been specific for a particular digitalis glycoside (Eastland 1952; Flasch 1981; Rowson 1952a, 1952b).

The unsaturated carbonyl and the digitoxose sugar residues of digitalis glycosides can be derivatized. In

alkaline solution, picric acid (Baljet reaction), dinitrobenzoic acid (Kedde reaction), tetranitrobiphenyl (Rabitzsch reaction) and several other dinitro aromatic compounds react with the carbonyl function of the lactone ring. Reviews in the literature (Flasch 1981; Rowson 1952a, 1952b) have indicated that, although these reagents were reasonably specific for the lactone ring, they did not differentiate between the various glycosides, aglycones, and metabolites with unsaturated lactone moieties.

Colored derivatives of the digitoxose sugars have been formed by the Keller-Kilani, thiobarbituric acid and xanthyrol reactions (Flasch 1981; Rowson 1952a). Using ferric chloride, acetic acid and sulfuric acid, the Keller-Kilani process removes the digitoxose residues and produces derivatives with absorption maxima at 470 nm and 590 nm. Similarly, the xanthyrol reaction hydrolyses the digitoxose residues with acid, followed by derivatization to a red product (absorption maximum at 520 nm). The thiobarbituric reaction involves oxidation of digitoxose sugars to dialdehydes, opening of the sugar and formation of a colored complex with 2-thiobarbituric acid (maximum absorbance at 532 nm). These reactions are specific for the digitoxose sugars and therefore the aglycone portion of the molecule would not be detected (Flasch 1981; Rowson 1952a). The reported lower limit of detection with these colorimetric procedures was in the  $\mu\text{g}$  range (Flasch 1981).

Fluorescent methods for the analysis of cardiac glycosides lower the detection limit to the ng range. For example, the reaction of digoxin with strong acids such as phosphoric, hydrochloric or trichloroacetic, causes dehydration and hydrolysis to the 14,16-dianhydrogenin (Flasch 1981; Jensen 1952). Since these fluorescent assay methods are specific for the steroid part of digoxin, metabolites of the aglycone would not be differentiated.

Chemical methods of analysis are not specific, but have frequently been used in combination with chromatographic methods to allow isolation and purification of individual glycosides. For example, the sugar and carbonyl color derivatives, as well as the fluorescent derivatives, have been used in combination with paper, thin-layer and liquid chromatographic techniques (Flasch 1981).

#### 1.5.4 Chromatographic Techniques

Gas-liquid chromatographic (GLC) methods of analysis of digoxin have been reported in the literature (Flasch 1981). Formation of trimethylsilyl (Jelliffe 1963) and heptafluorobutyryl (Watson 1972) derivatives prior to GLC analysis have been reported. Flame ionization (Kibbe 1973), mass spectrometry (Flasch 1981) and electron capture (Watson 1972) detector systems have been used in the GLC analysis of digoxin. The GLC assay methods lacked specificity, since digoxin and its metabolites were converted to the aglycone

portion of the molecule during derivatization or extraction (Flasch 1981; Jelliffe 1963; Kibbe 1973; Watson 1972).

Thin-layer chromatographic (TLC) methods of analysis of digoxin, using silica gel, cellulose and reversed-phase plates have been reported (Flasch 1981; Jelliffe 1969; Sabatka 1976). Biological samples have been evaluated using TLC methods but large sample volumes were required and the sensitivity was inadequate for therapeutic monitoring (Flasch 1981).

Numerous reports on the analysis of digitalis glycosides by high-performance liquid chromatography have appeared in the literature. These methods offer an attractive alternative since, unlike GLC methods, digoxin and its metabolites are not degraded by the conditions required. Various types of stationary phases have been used, including silica gel (Bockbrader 1984; Eriksson 1981b; Loo 1977; Nachtmann 1976a, 1976b), ion-exchange phases (Flasch 1981) and partition mode phases (Beasley 1983; Davydov 1982; Desta 1982a, 1982b, 1987; Diamandis 1985; Eriksson 1981b; Gault 1982; Gfeller 1977; Gibson 1980; Kwong 1986a, 1986b; Loo 1981; Pekic 1983; Plum 1986). Both pre-column and post-column derivatization techniques have been used for the analysis of digitalis glycosides in an attempt to increase the sensitivity of HPLC assay techniques. Seiber *et al.* (1981) reviewed the techniques and applications of HPLC for the analysis of digitalis glycosides and related steroids.

Pre-column derivatization techniques using 3,5-dinitrobenzoyl chloride (Bockbrader 1984; Fujii 1983) has been reported. Bockbrader and Reuning (1984) extracted the glycosides from urine, formed the 3,5-dinitrobenzoyl derivative and analysed these using a silica gel stationary phase. The sensitivity was 100 ng/mL in plasma with detection at 254 nm. Fujii *et al.* (1983) derivatized digoxin and its metabolites with 3,5-dinitrobenzoyl chloride and developed a micro HPLC assay system with ultraviolet detection at 230 nm. With this latter procedure, the limit of detection of digoxin was 2 ng. Using pre-column derivatization with 4-nitrobenzoyl chloride followed by separation on a silica gel stationary phase, Nachtmann *et al.* (1976a, 1976b) found a detection limit for digoxin of 11 ng/mL in plasma with detection at 254 nm.

Highly fluorescent derivatives have been formed by post-column derivatization of digoxin and its metabolites. Gfeller *et al.* (1977) developed a procedure for post-column derivatization of digoxin, digoxigenin, lanatoside C and desacetyl lanatoside C with hydrochloric acid and dehydroascorbic acid using an air-segmentation procedure for delivering the reagents. A detection limit of 0.5 ng for desacetyl lanatoside C was found with this system (Gfeller 1977). Further modification of this HPLC-PC air segmentation method (Desta 1987) produced complete resolution of digoxin from dihydrodigoxin but the

sensitivity (10 ng of digoxin) was inadequate for development of a routine clinical assay.

Recent manuscripts (Kwong 1986a, 1986b; Reh 1985) have described the development of HPLC assays for digoxin at therapeutic concentrations in plasma using post-column fluorogenic derivatization. The procedure reported by Kwong and McErlane (1986a) separated digoxin from several drugs frequently co-prescribed with digoxin. Partial resolution of digoxin from dihydrodigoxin was achieved which allowed for a peak height quantitative assay. The minimum detectable quantity was found to be 0.5 ng digoxin/mL in plasma (Kwong 1986b). A post-column fluorogenic derivatization method was also developed by Reh and Jork (1985) but separation of digoxin from dihydrodigoxin and interference from possible co-prescribed drugs were not evaluated.

#### 1.5.5 HPLC-RIA

The separation of digoxin from its metabolites and endogenous steroids using HPLC prior to the analysis of the digoxin fraction using RIA (Gibson 1980; Loo 1977, 1981; Margot 1983; Morais 1981; Plum 1986; Vasdev 1985; Wagner 1983) increased specificity. The HPLC procedures reported separated digoxin from three metabolites; digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside (Gibson 1980; Plum 1986; Wagner 1983). These metabolites have been shown to exhibit high cross-reactivity with

digoxin RIA procedures. While the sensitivity of these HPLC-RIA methods was sufficient to monitor therapeutic concentrations of digoxin in plasma, the collection of timed eluate fractions with subsequent evaporation and RIA measurement may introduce methodological errors.

#### 1.6 HPLC-EC

Electrochemical (EC) detection methods require an electroactive analyte that can be oxidized or reduced. Generally, a constant potential is applied and the current flow can be measured as a function of time (Fleet 1974). Functional groups that have oxidative electroactivity include amines, phenothiazines, phenols, aromatic hydroxyls and catecholamines (Fleet 1974; Volke 1983). Functional groups that exhibit reductive electroactivity include esters, ketones, aldehydes, ethers, diazo compounds and nitro groups (Fleet 1974; Volke 1983).

The combination of HPLC with EC detection (HPLC-EC) for drug measurement has led to very sensitive assay methods (Brooks 1983; Hu 1986; Kreuzig 1981; Leelavathi 1986; Lund 1979; Stewart 1981, 1986; Volke 1983; Wang 1986, 1987). Reviews of EC detection methods and flow cell geometry for HPLC have been prepared by White (1984) and Fleet and Little (1974).

Numerous drugs and biologically active compounds have been evaluated using HPLC-EC in the oxidative mode. Biogenic amines and catecholamines have frequently been assayed by HPLC-EC (Bauersfeld 1986; Davis 1981; Holly 1983;



Krstulovic 1982; Zaczek 1982). Drugs evaluated using oxidative HPLC-EC include theophylline and its derivatives (Greenberg 1979), dopazinol (Mazzo 1986), phenothiazines [trimeprazine (Hu 1986), perphenazine and fluphenazine (Tjaden 1976), chlorpromazine and thioridazine (Svensen 1986)], indoramin (Leelavathi 1986), tricyclic antidepressants [desipramine, imipramine and trimipramine (Wang 1986)], guanethidine (Stewart 1986), atropine (Leroy 1987), physostigmine (Isaksson 1987), codeine (Shah 1987), pentazocine (Shibanoki 1987), anthracycline antibiotics (Riley 1987), erythromycin (Croteau 1987), hydrochlorothiazide (Stewart 1986), and  $\beta$ -receptor antagonists [bopindolol (Humbert 1987) and mepindolol (Krause 1980)].

Reductive HPLC-EC poses numerous problems. The reduction of oxygen and trace metals interfere with EC analysis (Caudill 1985; Lund 1979). This necessitates the use of continuous sparging of the mobile phase with argon or helium gas in order to maintain a de-oxygenated state (Caudill 1985). At potentials more negative than -0.4 V, reduction of dissolved oxygen can significantly increase baseline noise. Passivation of the HPLC pump, injector and associated tubing with 20% nitric acid removes trace metals that can be washed off by the mobile phase creating baseline noise.

Reductive mode HPLC-EC has been used for the analysis of benzodiazepines such as nitrazepam, diazepam and

chlordiazepoxide (Lund 1979). The detection limits were found to depend strongly on the potential used; such that as the potential became more negative the detection limit increased (Lund 1979). Brooks (1983) reviewed the HPLC-EC determination of a large number of benzodiazepines. Using oxidative and reductive electrodes in series, both modes of detection were used to observe chlorpromazine, thioridazine and metabolites from brain tissue (Svendsen 1986). With HPLC and reductive EC, Meering *et al.* (1984) found detection limits of 2 to 4 pg for misonidazole and desmethyl-misonidazole.

The electrochemical reduction of nitro groups has been established by a number of investigators for many years (Fleet 1974; Morales 1987; White 1984). Nitro groups, in particular aromatic nitro groups, are easily reduced in acidic solutions (Morales 1987; Plambeck 1982; Ryan 1984). Mousty *et al.* (1986) reported that p-nitrophenoxyalkyl ammonium salts are reduced in two successive steps in acidic medium (pH 2.7) using potentials from -0.25 to -1.0 V. Many reports in the literature on the analysis of nitro groups using reductive EC have not used flow-through detectors. Using column chromatography and EC detection (0 to -1.15 V), Brilmyer *et al.* (1975) separated ortho and para nitrobenzoic acids in 75% aqueous buffer at pH 6.0. Caudill *et al.* (1985) analysed the trinitrobenzene sulfonic acid derivatives of  $\gamma$ -aminobutyric acid with HPLC-EC at -0.55 V with a mobile phase of 50% aqueous buffer at pH 3.0.

HPLC with electrochemical detection is becoming widely used for the analysis of trace quantities of electroactive compounds. Although the utility of aromatic nitro derivatives for use with reductive HPLC-EC has been suggested (Kissinger 1979), 3,5-dinitrobenzoyl digoxin (Bockbrader 1984; Fujii 1983) or other nitro containing derivatives of digoxin (Nachtmann 1976a) have not been evaluated by reductive EC or HPLC-EC.

Exceptionally low levels of digoxin are used therapeutically. This necessitates using the most sensitive assay methods for evaluating patient samples for digoxin. The expected sensitivity of electrochemical detectors to aromatic nitro groups make investigation of using HPLC-EC for analysis of the 3,5-dinitrobenzoyl derivative of digoxin imperative.

Gfeller *et al.* (1977) developed an HPLC procedure for post-column derivatization of cardiac glycosides using hydrochloric acid. Modifications of this HPLC-PC assay lack either the sensitivity (Desta 1987) or the resolution of digoxin from its metabolites (Reh 1985) required for a specific and sensitive digoxin assay. Problems with reliable delivery of the post-column reagents with one method (Kwong 1986a) have also been observed. Further investigation of post-column derivatization of digoxin to combine sensitivity, resolution from digoxin metabolites, specificity for digoxin and reliable delivery of post-column reagents in one HPLC assay is required. Samples from

patient groups where high levels of DLIS have been reported should also be evaluated by the HPLC assay to ensure that specificity for digoxin is maintained in the presence of endogenous compounds. These were the primary aims of this thesis.

## 2. EXPERIMENTAL

### 2.1 Supplies

#### 2.1.1 Chemicals

Digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, dihydrodigoxin, dihydrodigoxigenin, digitoxigenin and gitoxin were obtained from Boehringer (Mannheim, GFR). R,S,-Mexiletine hydrochloride was purchased from Boehringer Ingelheim Ltd. (Burlington, Ont., Canada). Sodium bicarbonate, L-ascorbic acid, sodium hydroxide (Aristar grade), hydrochloric acid (HCl), hydrogen peroxide (30%), sodium sulfate anhydrous and zinc sulfate heptahydrate were purchased from BDH Chemicals (Toronto, Ont., Canada). Sodium acetate trihydrate (Gold label), glacial acetic acid (Gold label) and 3,5-dinitrobenzoyl chloride (3,5-DNBCl) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). Barium hydroxide octahydrate was purchased from J. T. Baker Chemical (Phillipsburg, NJ, U.S.A.). Cupric sulfate pentahydrate was obtained from Allied Chemical (Morristown, NJ, U.S.A.). 4-Dimethylaminopyridine (4-DMAP), norethindrone, 17 $\alpha$ -ethynyl estradiol, estrone, 6 $\alpha$ -methyl-17 $\alpha$ -hydroxyl-progesterone acetate, estradiol, estrone-3-sulfate, testosterone, 19-nor-testosterone, 17 $\alpha$ -methyl-testosterone, adrenosterone, 5 $\alpha$ -androstane-3,17-dione,  $\Delta^4$ -androstene-3,17-dione,  $\Delta^4$ -androsten-11 $\beta$ -ol-3,17-dione, deoxycorticosterone, 21-

deoxycortisone, estriol, hydrocortisone, 17 $\alpha$ -hydroxypregnenolone, 17 $\alpha$ -hydroxyprogesterone,  $\Delta^5$ -pregnene-3 $\beta$ ,20 $\alpha$ -diol, 5-pregnen-3 $\beta$ -ol-20-one, cortisone, dehydroisoandrosterone, dehydroisoandrosterone-3-sulfate, 5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol, progesterone and Reichstein's Substance S were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 17 $\alpha$ -Estradiol and 17 $\beta$ -estradiol were samples from Ayerst Laboratories (Montreal, Que., Canada).

### 2.1.2 Solvents

Reagent grade absolute ethanol was purchased from Commercial Alcohols Ltd. (Toronto, Ont., Canada). Acetone, methanol and propan-1-ol were reagent grade and obtained from BDH Chemicals (Toronto, Ont., Canada). Isooctane (2,2,4-trimethylpentane) was glass distilled quality and purchased from BDH Chemicals (Toronto, Ont., Canada). HPLC grade water was produced using the Milli-Q Water System (Millipore Corp., Milford, MA, U.S.A.). Pyridine and the remaining HPLC grade solvents were Omnisolv grade from BDH Chemicals (Toronto, Ont., Canada). Prior to use, pyridine was distilled and stored over sodium hydroxide.

### 2.1.3 Extraction Supplies

A Volac pipette controller for volumes of 1 to 20 mL (Scienceware, Pequannock, NJ, U.S.A.) and Repipet Dispenser (Labindustries, Berkeley, CA, U.S.A.) were used for dispensing organic solvents during the extraction procedure.

The filtration unit consisted of a Nylon 66 membrane (0.45  $\mu$ m, 13 mm diameter) filter disc (Rainin Instrument Co., Inc., Woburn, MA, U.S.A.) with a Swinnex 13 Millipore filter holder (Millipore Corp., Milford, MA, U.S.A.) attached to a 5 mL Luer-Lock Multifit B-D glass syringe (Becton Dickinson Canada, Mississauga, Ont., Canada).

Filtration of serum samples prior to extraction was achieved using the Amicon Centrifree micropartition system and the Centriflo ultrafiltration membrane cones (CF25) (Amicon Div., W.R. Grace and Co., Danvers, MA, U.S.A.).

#### 2.1.4 Post-Column Derivatization Supplies

Two 15.2 cm (6 in.) diameter weld neck flanges (Schedule 80 A-53 Grade A steel with 150 lb. flange drilling) and two 15.2 cm (6 in.) diameter blind flanges (Schedule 80 A-53 Grade A steel with 150 lb. flange drilling) (Grinnell Sales Ltd., Vancouver, B.C., Canada) were used for the steel pressure vessel. The gasket material was Teflon Gore-Tex<sup>TM</sup> joint sealant (W.L. Gore and Associates, Inc., Elkton, MD, U.S.A.).

Two 5.1 cm (2 in.) diameter PVC socket weld flanges (Schedule 80 PVC with 150 lb. flange drilling), two 5.1 cm (2 in.) diameter blind flanges (Schedule 80 PVC with 150 lb. flange drilling) and 36.8 cm of 5.1 cm (2 in.) diameter PVC pipe (Schedule 80) (Scepter Mfg. Co. Ltd., Vancouver, B.C., Canada) were used in construction of the PVC pressure vessel. Two 5.1 cm (2 in.) diameter full face 0.32 cm (1/8

in.) Hypalon gaskets (Custom Gaskets, Vancouver, B.C., Canada) were used between the pipe section flanges and the blind flanges. For both the steel and PVC pressure vessels the non-metric equivalent component parts were used. See Figure 6 for diagram of PVC pressure vessel with fittings.

Polytetrafluoroethylene (PTFE) tubing (0.16 cm diameter, 0.3 mm I.D.) was used for the reactor and connection of the column and pressure vessel to the reactor. A three-way PTFE valve (part number 1102 Omnifit Ltd., Cambridge, England) was between the pressure vessel, column and reactor. The PTFE tubing and connectors were obtained from Omnifit Ltd. (Cambridge, England).

#### 2.1.5 Commercial Radioimmunoassay Kits

The six commercial RIA kits used for digoxin analysis were as follows: Digi-Tab RIA (Nuclear-Medical Laboratories Div., Warner-Lambert Technologies Inc., Irving, TX, U.S.A.), Bio-RIA I-125 Digoxin (Institute of Bio-Endocrinology Inc., Montreal, Que., Canada), Amerlex Digoxin RIA Kit (Amersham Corp., Arlington Heights, IL, U.S.A.), Digoxin Solid Phase RIA (Becton Dickinson Immunodiagnosics, Orangeburg, NY, U.S.A.), GammaCoat Digoxin RIA (Clinical Assays Div., Travenol Laboratories Inc., Cambridge, MA, U.S.A.) and Digoxin I-125 Imusay (Abbott Laboratories Diagnostics Div., North Chicago, IL, U.S.A.).



### 2.1.6 Commercial Fluorescence Polarization Immunoassay Kit

The Abbott Laboratories TDx fluorescence polarization immunoassay for digoxin was used (Abbott Laboratories Diagnostics Div., North Chicago, IL, U.S.A.).

### 2.1.7 Filtration of HPLC Mobile Phase

FP Vericel 47 mm 0.45  $\mu$ m membrane filters (Gelman Sciences Inc., Ann Arbor, MI, U.S.A.) were used with the Millipore all-glass filter apparatus (Millipore Waters Associates, Milford, MA, U.S.A.) for filtration of the mobile phase.

## 2.2 Equipment

### 2.2.1 HPLC Equipment

A Beckman Model 100 A dual piston solvent metering system (Beckman Instrument, Inc., Fullerton, CA, U.S.A.) was used as the HPLC pump. The remaining HPLC system consisted of either a model U6K injector (Waters Associates, Milford, MA, U.S.A.) or a model 210 Altex injector (Beckman Instrument, Inc., Fullerton, CA, U.S.A.), a Waters fluorescence detector model 420 AC with a quartz flow cell (1 mm I.D. by 40 mm quartz tubing) (section 2.2.3) and an Altex CR1A Chromatopac Data Processor (Beckman Instrument, Inc., Fullerton, CA, U.S.A.). The noise reducer shown in

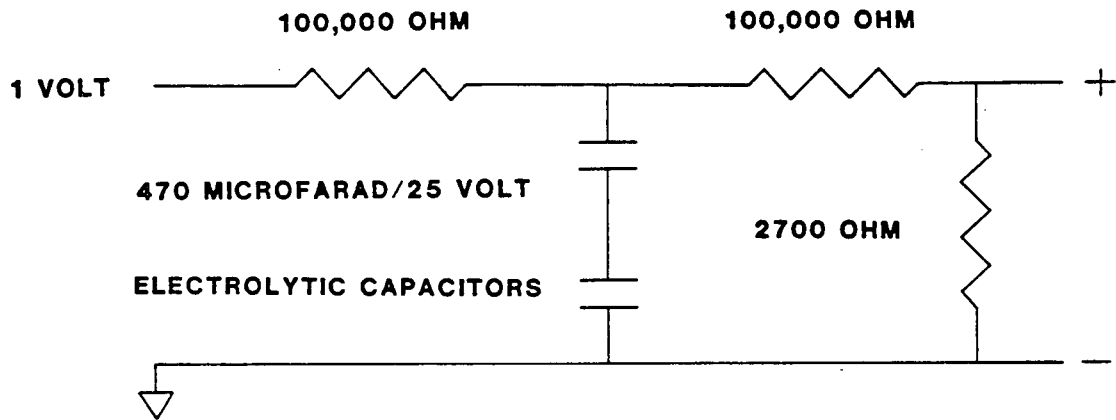
Figure 3 was constructed and used with the Waters 420 fluorescent detector.

A fixed wavelength (254 nm) ultraviolet detector (Beckman model 153 detector, Beckman Instrument Inc., Fullerton, CA, U.S.A.) was used to assay 3,5-dinitrobenzoyl derivatives of digoxin and its metabolites.

A NewGuard holder equipped with a 1.5 cm x 3.2 mm I.D. ODS cartridge (Brownlee Labs Inc., Santa Clara, CA, U.S.A.) was used as a guard column and placed prior to the Spherisorb ODS II (3 $\mu$ ) 15 cm x 4.6 mm I.D. analytical column (Alltech Associates, Deerfield, IL, U.S.A.). Between the injector and guard column, a direct connect column prefilter (Alltech Associates, Deerfield, IL, U.S.A.) was used as an inline filter.

#### 2.2.2 Electrochemical Detector

The Coulochem dual electrode HPLC electrochemical detector model 5100A, guard cell model 5020, dual electrode analytical cell model 5010 and screened wall jet cell model 5012 with a gold electrode were purchased from ESA, Inc. (Bedford, MA, U.S.A.). The guard cell electrode was placed before the injector and used to electrochemically clean the mobile phase before the introduction of samples. The analytical cell (model 5010 or model 5012) was connected directly after the analytical HPLC column as shown in Figure 4.



**Figure 3. Schematic of Noise Reducer**

### 2.2.3 Fluorescence Detector Flow Cell

All stainless steel fittings in the fluorometer flow cell were removed. A 40 mm quartz tube (1 mm I.D.) was positioned in the flow cell block, held in place with epoxy glue and joined to the PTFE tubing via acidflex tubing (part number 116-0538-09, Technicon Instruments Corp., Tarrytown, NY, U.S.A.).

### 2.2.4 Post-Column Reactor and Pressure Chamber

A schematic diagram of the final HPLC post-column (HPLC-PC) fluorogenic system is given in Figure 5. A Beckman model 110 A single piston metering system (Beckman Instrument, Inc., Fullerton, CA, U.S.A.) was used to pump hexane into the pressure vessel to displace concentrated HCl from a steel or PVC pressure chamber (section 2.1.4) to the post-column reactor which consisted of knitted PTFE tubing (0.3 mm I.D.). The reactor was maintained at 79°C by a reaction bath thermostated using a Haake model D1 constant temperature circulator (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.).

### 2.2.5 Extraction Equipment

A Vortex-Genie (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.), a Labquake Shaker (Labindustries, Berkeley, CA, U.S.A.) and an IEC HN-SII Centrifuge (Damon/IEC Division, Western Scientific, Vancouver, BC, Canada) were used in the extraction of biological samples.

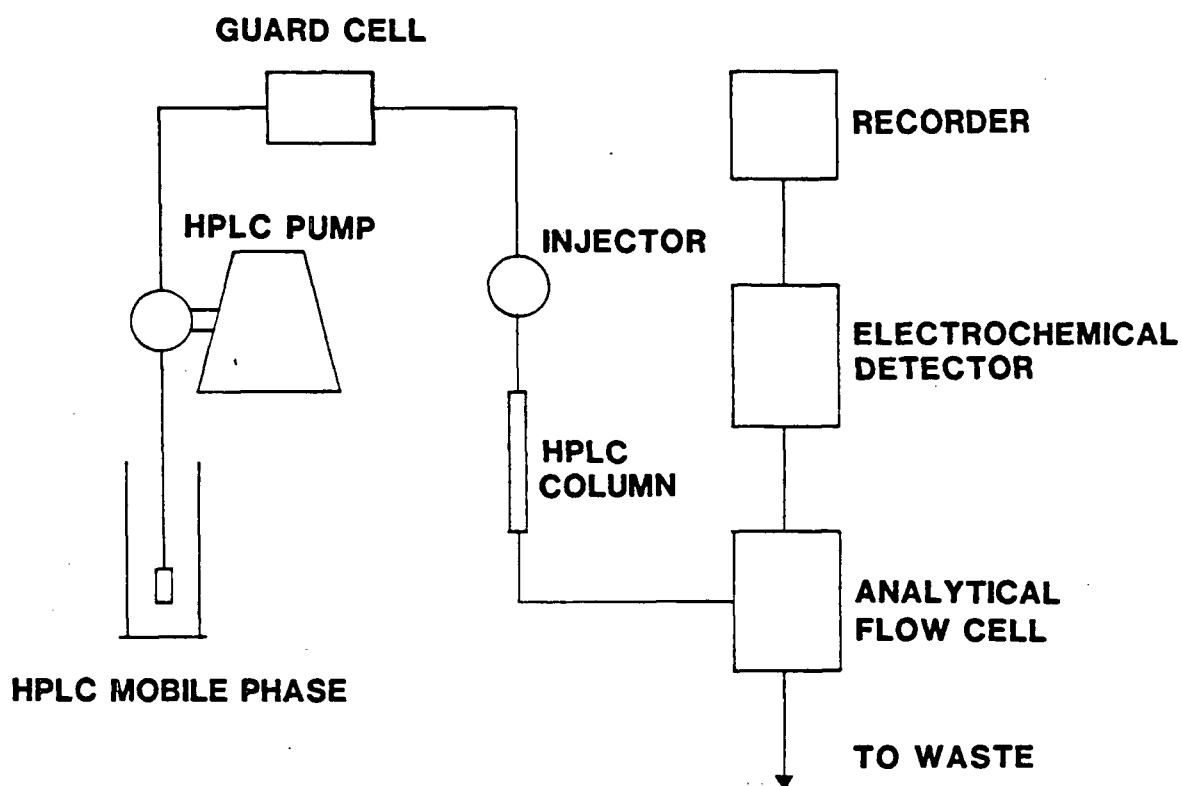


Figure 4. Schematic of HPLC-EC System

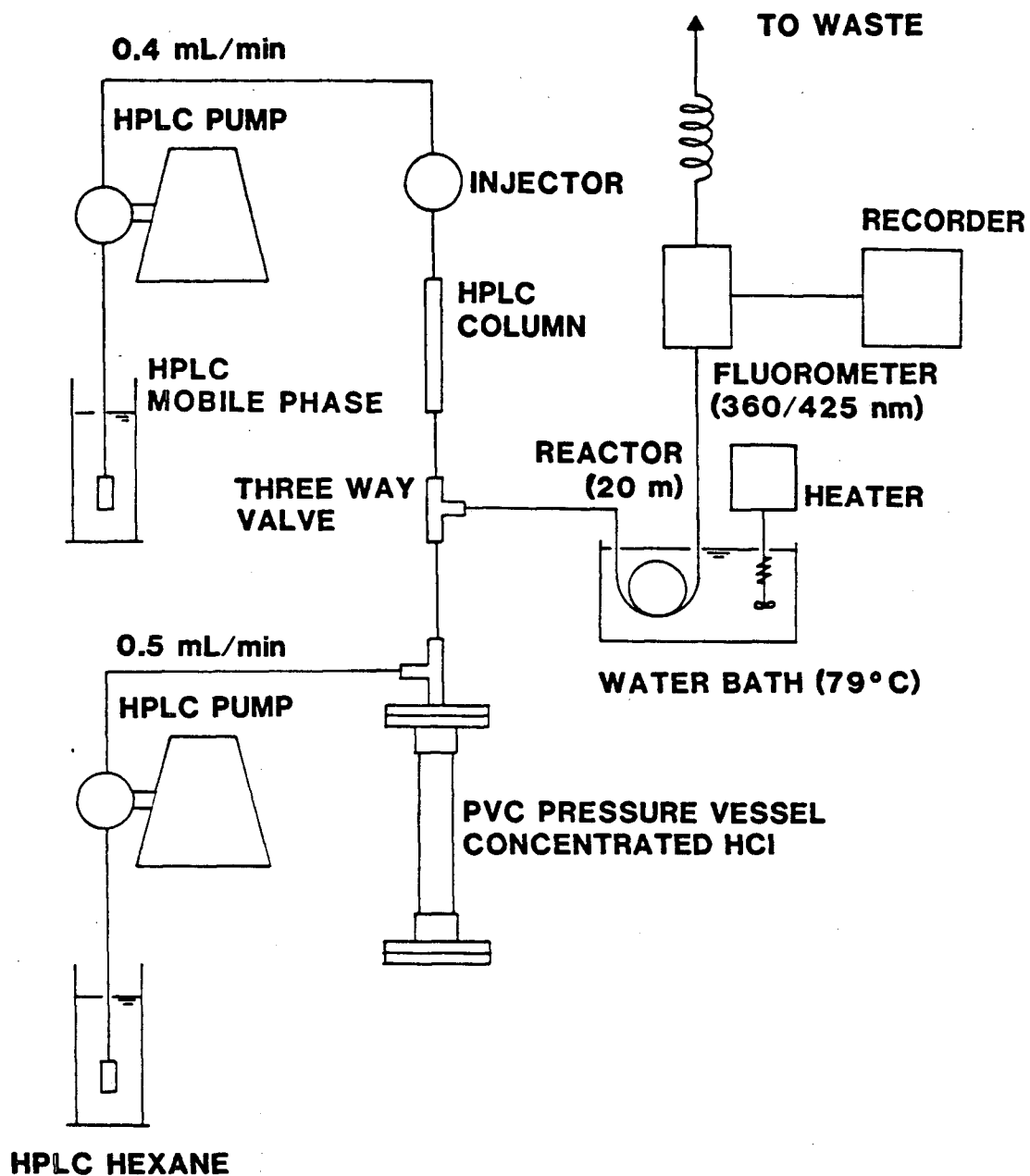


Figure 5. Schematic of HPLC-PC Fluorogenic System

### 2.2.6 Radioimmunoassay Equipment

A Nuclear-Chicago 1185 series automatic gamma counter (Searle Co., Des Plaines, IL, U.S.A.) was used for determining iodine-125 decay with the commercial RIA kits.

## **2.3 Pre-column 3,5-Dinitrobenzoyl Chloride Derivatization Procedures**

### 2.3.1 For Gram Quantities of Digoxin

A solution of 3,5-DNBCl (3.2 g in 30 mL dry pyridine) was added to a solution of digoxin (0.5 g) in 20 mL dry pyridine (molar ratio of 3,5-DNBCl/digoxin (22/1)). The mixture was stirred for 25 minutes at 50°C, poured into 60 mL cold dilute HCl (1% of concentrated) and the precipitate was filtered and washed with water. The crude product was dissolved in 1 L of ethyl acetate/hexane (1/1), washed twice with 250 mL of 5% sodium bicarbonate with 4-DMAP and three times with HPLC water (300 mL). After drying the organic layer with anhydrous sodium sulfate and evaporation *in vacuo*, the residue was purified by double recrystallization in methanol (reagent grade) (mp 199-204°C).

### 2.3.2 For Milligram Quantities of Cardiac Glycosides

3,5-DNBCl (15 mg) was added to a solution of cardiac glycoside (3.0 mg) in 0.2 mL of dry pyridine and the mixture was vortexed for 20 seconds (molar ratio of 3,5-DNBCl/digoxin (20/1)). After reacting for 3 hours at room

temperature, the sample was placed in a 37°C water bath and the pyridine was evaporated under nitrogen. The residue was dissolved in HPLC grade ethyl acetate (1.5 mL) and washed four times with 1 mL of a 5% solution of sodium bicarbonate with 4-DMAP. The organic layer was then washed four times with 1 mL of 1% of concentrated HCl and then four times with HPLC grade water (1 mL). After evaporation of the ethyl acetate with nitrogen gas, the derivatives were reconstituted with the mobile phase and analyzed by HPLC.

### 2.3.3 For Nanogram Quantities of Digoxin

To 0.5 to 10 ng of digoxin in 0.5 mL dry pyridine, 100 ng of 3,5-DNBCl in 10 µL of pyridine was added (molar ratio of 3,5-DNBCl/digoxin from 34/1 to 677/1). To 50 to 80 ng of digoxin, 800 ng of 3,5-DNBCl in 80 µL was added (molar ratio of 3,5-DNBCl/digoxin from 34/1 to 54/1). The samples were allowed to react at room temperature for 2 hours after which the pyridine was evaporated using a 37°C water bath and nitrogen gas. After reconstitution of the residue with ethyl acetate/hexane (1/1), the organic layer was washed four times with 2 mL of 5% sodium bicarbonate with 4-DMAP, 1% of concentrated HCl and HPLC grade water just prior to evaporation under nitrogen. The derivative was then reconstituted with mobile phase and analysed by HPLC.

## **2.4 Preparation of Solutions**



### 2.4.1 Reagents For 3,5-DNBCl Derivatization

#### 2.4.1.1 3,5-DNBCl in Pyridine

3,5-DNBCl (1 mg) was dissolved in and taken to a final volume of 100 mL with dry pyridine (10 ng/ $\mu$ L). The solution was stirred for 20 minutes prior to use.

#### 2.4.1.2 Sodium Bicarbonate (5%) with 4-DMAP

Sodium bicarbonate (5 g) and 4-DMAP (250 mg) were dissolved in HPLC grade water and the solution was taken to a final volume of 100 mL with HPLC grade water. The solution was stirred for 20 minutes.

#### 2.4.1.3 Dilute Hydrochloric Acid

Concentrated HCl (10 mL) was added to 600 mL HPLC grade water, taken to a final volume of 1 L and stirred for 20 minutes.

### 2.4.2 Reagents For Fluorogenic Derivatization

#### 2.4.2.1 Ascorbic Acid Solution

L-Ascorbic acid (250 mg) was weighed out, dissolved, taken to a final volume of 500 mL with HPLC grade water, and stirred for 20 minutes prior to use.

#### 2.4.2.2 Dilute Hydrogen Peroxide Solution

Hydrogen peroxide (1 mL of 30%) was diluted and taken to 200 mL with HPLC grade water, stirred for 20 minutes and used immediately.

#### 2.4.2.3 Dehydroascorbic Acid

Dilute hydrogen peroxide (12.5 mL) was added to the ascorbic acid solution (500 mL) and stirred for 2 hours. The dehydroascorbic acid was stored in a refrigerator until use. New dehydroascorbic acid was prepared every week.

#### 2.4.2.4 Hydrogen Peroxide with Phosphoric Acid

Hydrogen peroxide (40  $\mu$ L of 30%) was diluted and taken to 5 mL with HPLC grade water. Concentrated phosphoric acid was added to the hydrogen peroxide in a 1/1 ratio. This mixture was prepared daily.

#### 2.4.3 Sodium Acetate Buffer (0.1 M, pH 4.6)

Sodium acetate trihydrate (6.8 g) and 3 mL glacial acetic acid were dissolved in 500 mL HPLC water. The solution was taken to a final volume of 1 L with HPLC grade water, stirred for 20 minutes and stored in a refrigerator until use. New buffer solution was prepared every week.

#### 2.4.4 Mobile Phase

The HPLC mobile phase was prepared by mixing the individually measured solvents and then degassing the

mixture by stirring rapidly for 30 minutes and was filtered prior to use. The mobile phases used are described in sections 3.3 and 3.4.

#### 2.4.5 Standard Solutions

##### 2.4.5.1 Digoxin in Ethanol

A stock solution of digoxin was prepared in ethanol (1 mg/100 mL). The stock solution was further diluted to give final concentrations of 5, 7 and 10 ng/10  $\mu$ L (0.5, 0.7 and 1 mL of stock solution taken to 10 mL with ethanol respectively). A 0.5 ng/ $\mu$ L solution (1.25 mL stock solution diluted to 25 mL with ethanol) was used to prepare 1.5, 2 and 3 ng/10  $\mu$ L solutions (3, 4 and 6 mL of 0.5 ng/ $\mu$ L solution diluted to 10 mL with ethanol). Calibration curve serum samples were spiked with 10  $\mu$ L of the final digoxin solutions (1.5, 2, 3, 5, 7 and 10 ng digoxin/3 mL serum).

##### 2.4.5.2 3,5-Dinitrobenzoyl Digoxin in Methanol

A stock solution of 3,5-dinitrobenzoyl digoxin in methanol (1 mg/100 mL or 100 ng/10  $\mu$ L) was further diluted to give a final concentration of 5 ng/10  $\mu$ L (0.5 mL of stock solution taken to 10 mL with methanol).

##### 2.4.5.2 Digitoxigenin in Ethanol

For the fluorogenic HPLC assay, digitoxigenin was used as internal standard. A stock solution of digitoxigenin was

prepared in ethanol (4 mg/100 mL). The stock solution was further diluted to give a final concentration of 80 ng/10  $\mu$ L (10 mL stock diluted to 50 mL with ethanol). Serum samples were spiked with 20  $\mu$ L of this internal standard solution (160 ng).

#### 2.4.5.3 Dihydrodigoxin in Ethanol

One milligram of dihydrodigoxin was accurately weighed and made up to 100 mL with ethanol. An aliquot of this solution (0.5 mL) was diluted to 10 mL with ethanol (5 ng/10  $\mu$ L). To test the specificity of the HPLC post-column fluorogenic assay, 10  $\mu$ L of the final solution was assayed.

#### 2.4.5.4 Steroid Samples in Methanol

One milligram of each of the following steroids was weighed accurately, separately made up to 100 mL in methanol and the indicated amount was assayed by the post-column fluorogenic assay to evaluate the specificity of the fluorogenic assay: norethindrone (680 ng), 17 $\alpha$ -ethynyl estradiol (18 ng), 17 $\alpha$ -estradiol (96 ng), 17 $\beta$ -estradiol (37 ng), estrone (55 ng), 6 $\alpha$ -methyl-17 $\alpha$ -hydroxylprogesterone acetate (25 ng), estriol (55 ng), estrone-3-sulfate (30 ng), testosterone (133 ng), 19-nortestosterone (67 ng) and 17 $\alpha$ -methyltestosterone (60 ng). For the following samples, one milligram of each was weighed accurately, and made up to 10 mL in methanol and approximately 500 ng of each was evaluated using the HPLC-PC assay: andrenosterone, 5 $\alpha$ -

androstane-3,17-dione,  $\Delta^4$ -androstene-3,17-dione,  $\Delta^4$ -  
 androsten-11 $\beta$ -ol-3,17-dione, deoxycorticosterone, 21-  
 deoxycortisone, estriol, hydrocortisone, 17 $\alpha$ -  
 hydroxypregnenolone, 17 $\alpha$ -hydroxyprogesterone,  $\Delta^5$ -pregnene-  
 3 $\beta$ ,20 $\alpha$ -diol, 5-pregnen-3 $\beta$ -ol-20-one, cortisone,  
 dehydroisoandrosterone, dehydroisoandrosterone,  
 dehydroandrosterone-3-sulfate, 5 $\beta$ -pregnene-3 $\alpha$ ,20 $\alpha$ -diol,  
 progesterone and Reichstein's Substance S.

#### 2.4.5.5 Preparation of Mexiletine Hydrochloride in 50% Ethanol

One milligram of R,S-mexiletine hydrochloride was weighed accurately, dissolved in 50% ethanol in HPLC grade water and taken to a final volume of 1 mL. This solution was then evaluated by the HPLC-PC fluorogenic assay.

#### 2.4.5.6 Preparation of Plasma Samples for RIA Analysis

Samples were prepared by dissolving digoxin or the individual metabolites of digoxin in 20% ethanol-water. Serial dilutions of the stock solution (1 mg/100 mL) were made and added to plasma from a healthy, non-digitalized volunteer, to give final concentrations of 0, 0.5, 1.0, 2.0 and 3.0 ng/mL in 2 mL of plasma. For digoxin, 5.0 ng/mL samples were also prepared.

#### 2.4.5.7 Preparation of Serum Samples Spiked with Digoxin Metabolites for FPIA Analysis

Samples were prepared by weighing the individual metabolites of digoxin (1 mg), dissolving them in ethanol and taking the volume to 100 mL with ethanol. Dilutions of this stock solution were prepared so that the final concentration was 1 ng/10  $\mu$ L. Blank serum samples (1 mL) were individually spiked with one metabolite (from 5 to 10  $\mu$ L of the dilute solutions) and the serum samples were assayed by the ACU FPIA.

### **2.5 Preparation of Solvents for Serum Extraction**

#### 2.5.1 Isooctane/Dichloromethane (20/5)

Dichloromethane (5 mL) was added to isooctane (20 mL) and the mixture was stirred for 20 minutes.

#### 2.5.2 Extraction Solvent

Propan-1-ol (2 mL) and dichloromethane (98 mL) were stirred for 20 minutes just prior to use.

#### 2.5.3 Preparation of Protein Precipitation Reagents

##### 2.5.3.1 Zinc Sulfate Heptahydrate (5%)

Zinc sulfate heptahydrate (5 g) was weighed, dissolved and taken to a final volume of 100 mL with distilled water.

#### 2.5.3.2 Barium Hydroxide Octahydrate (0.3 N)

Barium hydroxide octahydrate (23.7 g) was weighed, dissolved and taken to a final volume of 50 mL with distilled water.

#### 2.5.3.3 Cupric Sulfate Pentahydrate (10%)

Cupric sulfate pentahydrate (10 g) was weighed, dissolved in and taken to a final volume of 100 mL with distilled water.

#### 2.5.3.4 Sodium Hydroxide (12%)

Sodium hydroxide (12 g) was weighed, dissolved in and taken to a final volume of 100 mL with distilled water.

### **2.6 RIA Analysis and Cross-Reactivity Calculations**

All samples were assayed in duplicate using one lot of each of the six RIA kits. The presence of ethanol in the samples was evaluated for interference with the RIA procedures. A blank plasma sample was also assayed to determine if any cross-reacting components were present in the plasma used.

Each of the four metabolite-spiked plasma samples was assayed in duplicate. Cross-reactivity with the Digi-Tab RIA was calculated as the ratio between the quantity of digoxin and the quantity of metabolite which caused 50% displacement of the digoxin tracer. For all other RIA kits, cross-reactivity was calculated as the ratio of the digoxin

concentration to the metabolite concentration at 50% inhibition of maximum binding. Since the RIA assay procedure is designed for competitive binding on a molar basis and these metabolites differ greatly in their molecular weight, the percent cross-reactivity was determined on a molar basis.

## 2.7 HPLC With Electrochemical Detection

### 2.7.1 Optimization of Electrochemical Conditions

Using the HPLC system shown in Figure 4 with the 5020 guard cell electrode before the injector and the 5012 screened wall jet cell as the analytical electrode, 3,5-dinitrobenzoyl derivatives of digoxin and dihydrodigoxin were assayed. With a mobile phase of acetonitrile/acetate buffer (20/7), the location of derivatized digoxin was confirmed using ultraviolet detection at 254 nm (Beckman model 153 detector). Recording from detector 1 (glassy carbon electrode from the 5012 cell), gradually decreasing the voltage from 0 to -0.85 V indicated -0.80 V gave the greatest peak height and therefore was used for further experiments.



### 2.7.2 Mobile Phases for HPLC with Electrochemical Detection

The limit of detection for digoxin and resolution of dihydrodigoxin from digoxin was determined for a variety of mobile phases (section 3.2.1) using -0.80 V for detection.

## **2.8 HPLC Post-Column Derivatization Assay**

### 2.8.1 Assembly of Steel and PVC Pressure Vessels

The steel weld neck flanges were welded together back to back. Teflon joint sealant was applied to the exposed end of both neck flanges and the blind flanges were mounted with 1.9 cm (3/4 in.) bolts (National Coarse Thread, 316 stainless steel) (Indufast Fastners Ltd., Vancouver, B.C.).

The ends of the PVC pipe section and the sockets of the PVC flanges were prepared by light sanding. PVC cleaning solvent followed by PVC glue (Scepter Mfg. Co., Ltd., Vancouver, B.C., Canada) were applied to all surfaces to be glued. The socked weld flanges were press fitted onto the ends of the pipe and secured until the glue was set. Excess glue was wiped clean from the internal and external surfaces. Hypalon gaskets were placed between the pipe section flanges and the blind flanges and 1.3 cm (1/2 in.) bolts (National Coarse Thread, 316 stainless steel) (Indufast Fastners Ltd., Vancouver, B.C.) were used to secure the flanges together.

On both the steel and PVC pressure vessels, the blind flanges (to be the top) were drilled and threaded to accept 3/8 in. National pipe thread. Swagelok fittings (Swagelok Tube Fittings, Columbia Valve and Fitting Co., Ltd., North Vancouver, B.C.) were used to produce the inlet for hexane and outlet for HCl as shown in Figure 6.

### 2.8.2 Optimization of HPLC Post-Column Derivatization

Using the Waters U6K injector, the Spherisorb ODS II (3  $\mu$ m) analytical column with an ODS guard column and mobile phase of methanol/ethanol/isopropanol/aqueous (52/3/1/45) at a flow rate of 0.4 to 0.5 mL/min., the cardiac glycoside samples were evaluated. The HPLC effluent was combined with concentrated HCl delivered from a steel or PVC pressure vessel. Hexane was added to the pressure vessel via an HPLC pump at a flow rate of 0.5 to 1.0 mL/min. Using 10 m and 20 m reactors, the post-column derivatization was optimized by comparing peak heights from 9.1 ng of digoxin under the various conditions.

#### 2.8.2.1 Fluorogenic Derivatization with Hydrogen Peroxide, Phosphoric Acid and Concentrated HCl

Using the 10 m reactor and the aqueous portion of the mobile phase as dehydroascorbic acid and hydrogen peroxide with phosphoric acid, peak height was determined for 9.1 ng of digoxin (using 360 nm/425 nm filters).

### 2.8.2.2 Evaluation of the Fluorescence Emission Filters with Dehydroascorbic Acid and HCl Derivatization

With a 360 nm excitation filter and the mobile phase described in section 2.7.3 with all of the aqueous portion as dehydroascorbic acid, emission filters (425, 440 and 460 nm) were evaluated by comparing peak heights from 13 ng injections of digoxin.

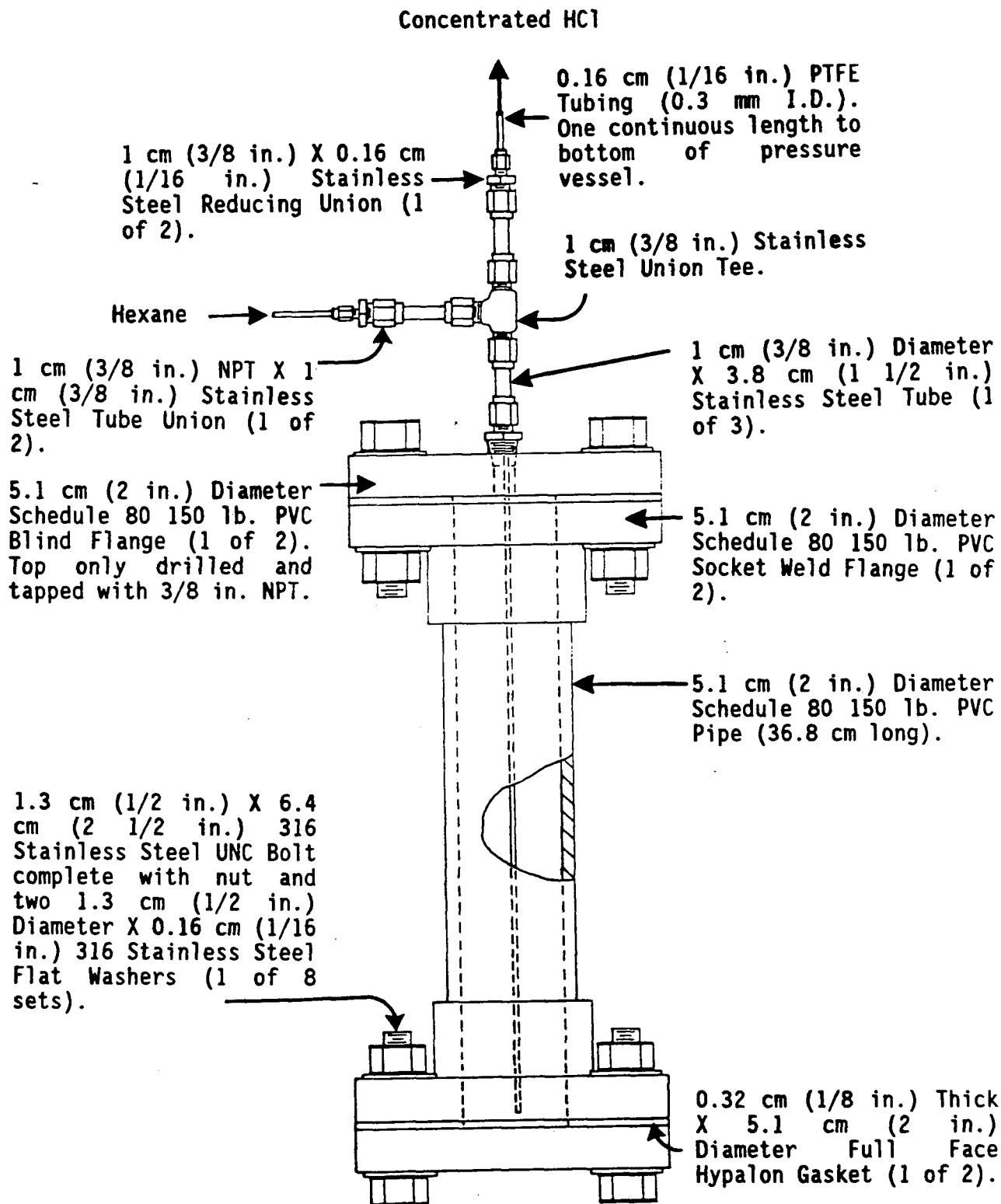
### 2.8.3 Final HPLC-PC Assay Procedure

A schematic of the HPLC-PC assay procedure is shown in Figure 5. The final assay used an HPLC flow rate of 0.4 mL/min. with a hexane flow rate of 0.5 mL/min. and 360 nm excitation and 425 nm emission filters with the fluorometer.

## **2.9 Serum Extraction Procedure**

### 2.9.1 Protein Precipitation Methods

Blank serum samples (3 mL) were precipitated using acetone (3 mL and 6 mL), zinc/barium (0.6 mL of 5% zinc sulfate plus 0.6 mL of 0.3 N barium hydroxide) and cupric sulfate (1 mL of 10% cupric sulfate plus 1 mL of 12% sodium hydroxide) followed by extraction (Kwong 1986a, 1986b).



**Figure 6. Diagram of HPLC-PC Pressure Vessel With Fittings**

### 2.9.2 Centrifree and Centriflo Filtration

Serum samples were filtered by the Centrifree (1 mL serum centrifuged at 1000 r.c.f. for 30 minutes) and the Centriflo (3 mL serum centrifuged at 1000 r.c.f. for 40 minutes) systems followed by an isooctane wash and double extraction (Kwong 1986a). Both serum and water samples were spiked with 9.1 ng of digoxin and prepared using the Centrifree and Centriflo systems as described above. These samples were evaluated using the final HPLC-PC assay procedure.

### 2.9.3 Solvent-solvent Extraction

Using 2 mL of isooctane and isooctane/dichloromethane (20/5) as solvent wash, water samples (3 mL) spiked with 9.1 ng of digoxin were extracted and evaluated using the HPLC-PC assay. Serum (3 mL) containing 1.5 ng of digoxin and blank serum samples were extracted (Kwong 1986a, 1986b) following an isooctane/dichloromethane solvent wash (2 mL of 20/5) and assayed using the HPLC-PC assay.

### 2.9.4 Final Procedure for Extraction of Digoxin in Serum

The frozen serum samples were thawed at room temperature just prior to analysis. For standard curve samples, 3 mL of blank serum was spiked with 10  $\mu$ L of digoxin in ethanol (1.5 to 10 ng/10  $\mu$ L). For all standard curve and patient samples, 20  $\mu$ L of internal standard

solution (160 ng) was added to the serum. After addition of 3 mL acetone, the sample was vortexed 20 seconds, centrifuged 5 minutes at 1500 r.c.f. and the aqueous/acetone supernatant layer was transferred to a clean tube. This solution was then washed with 2 mL of isooctane/dichloromethane (20/5), vortexed 60 seconds, centrifuged 5 minutes at 1500 r.c.f. and the aqueous/acetone layer was partially dried under nitrogen using a 37°C water bath for 20 minutes. The remaining aqueous layer was extracted twice with 10 mL of extraction solvent (dichloromethane/propan-1-ol 98/2) and the combined organic phases were filtered (Nylon 66 membrane) and dried under nitrogen. The residue was resuspended in 100 µL of methanol/water (50/50). Injections into the HPLC-PC were performed using a 100 µL Hamilton syringe (Hamilton Co., Reno, NV, U.S.A.). A flow diagram of this extraction procedure is given in Figure 7.

## 2.10 Recovery and Precision of Extraction

The recovery of digoxin from serum using the final extraction procedure was evaluated. Serum spiked with 1.5, 3 and 10 ng of digoxin were extracted and 10 µL of external standard solution (digitoxigenin in ethanol) was added just prior to analysis. Recovery was calculated by comparing the peak height ratio (digoxin to external standard) for extracted serum samples to that for unextracted samples.

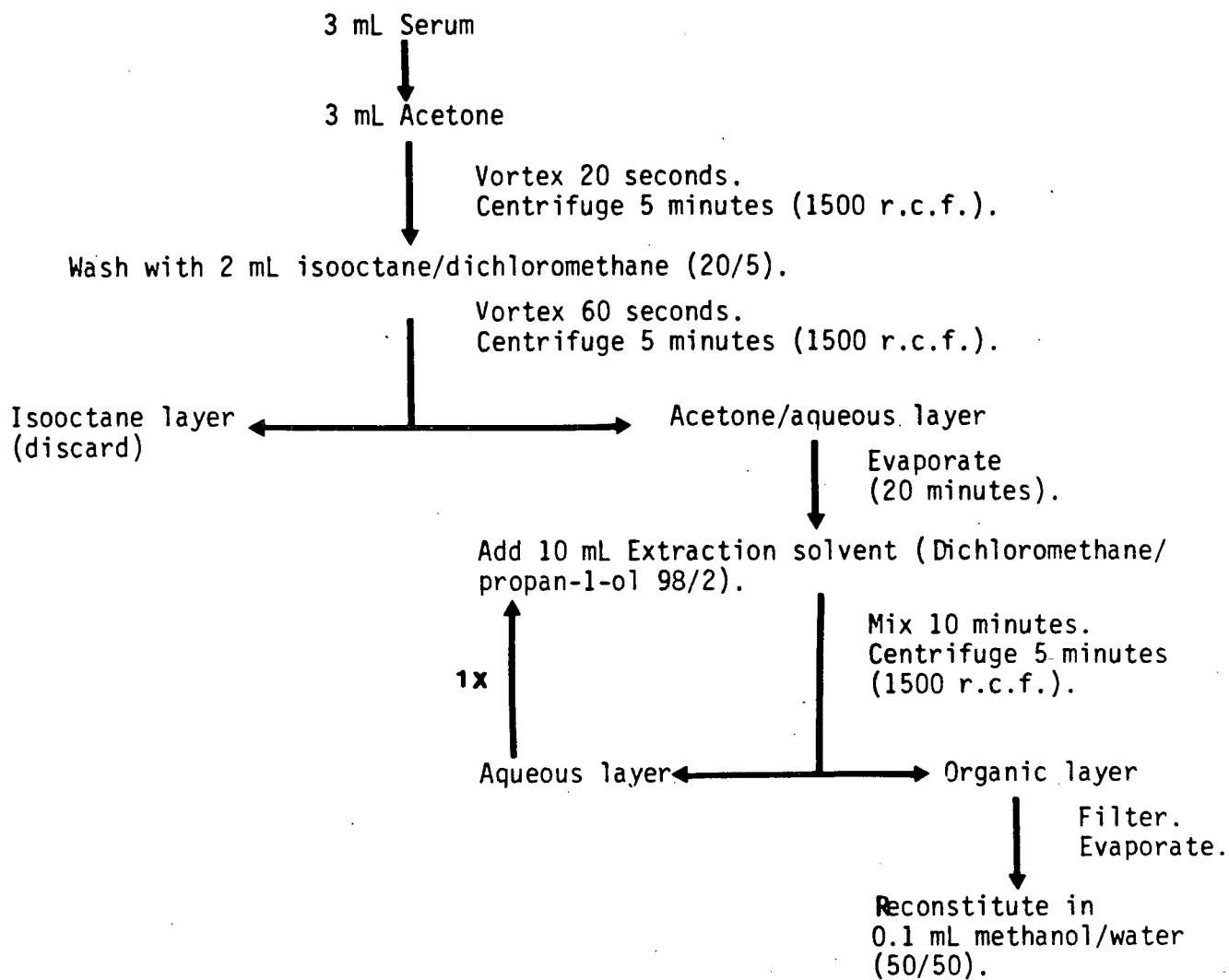


Figure 7. Flow Diagram for Serum Extraction Procedure

Precision of the extraction procedure was determined by repeated extraction of five blank serum samples (3 mL) spiked with 3 ng of digoxin and 20  $\mu$ L of internal standard for five consecutive days. The coefficient of variation for within and between days was calculated.

### 2.11 Calibration Curve

Serum samples (3 mL) were spiked with digoxin in ethanol (section 2.4.5.1), extracted and analyzed using the HPLC-PC fluorogenic assay. The peak height and concentration ratios were then calculated.

### 2.12 Specificity

#### 2.12.1 Steroids

The steroid samples prepared in section 2.4.5.4 were assayed using the post-column fluorogenic HPLC assay.

#### 2.12.2 Co-Administered Drugs

The R,S-mexiletine sample (section 2.4.5.5) was assayed by the HPLC-PC method to establish assay specificity in the presence of this drug.



### 2.13 Quality Control Procedure

Blank serum samples (4 mL) were spiked with 13  $\mu$ L of the digoxin in ethanol solutions (section 2.4.5.1). After mixing, 3 mL samples were removed, extracted and analyzed using the HPLC-PC fluorogenic assay. The remaining sample was then analysed with the FPIA for digoxin at the ACU Laboratory.

### 2.14 Analysis of Digitalized Patient Serum Samples

Serum samples from 25 U.B.C. Health Sciences Centre Acute Care Unit Hospital (Vancouver, B.C.) patients who received digoxin therapeutically were assayed by both HPLC-PC and FPIA methods. The results from the two analytical methods were then compared.

### 2.15 Analysis of Serum from Undigitalized Patient Groups Where High DLIS Levels have been Reported

#### 2.15.1 Hypertension

Serum samples from 5 undigitalized hypertensive patients were obtained (U.B.C. Health Sciences Centre Acute Care Unit Hospital, Vancouver, B.C.) and evaluated by both the HPLC-PC and FPIA methods.

#### 2.15.2 Renal Failure

Serum samples from 20 undigitalized renal failure patients who were on dialysis were obtained from the Willow

Dialysis Unit (Vancouver General Hospital, Vancouver, B.C.) and assayed by both the HPLC-PC and FPIA for digoxin.

#### 2.15.3 Hepatic Failure

Serum samples from 8 undigitalized hepatic failure patients and two samples from one digitalized hepatic failure patient were obtained from U.B.C. Health Sciences Centre Acute Care Unit Hospital (Vancouver, B.C.) and St Paul's Hospital (Vancouver, B.C.) and have been evaluated by both HPLC-PC and FPIA methods.

#### 2.15.4 Umbilical Cord Blood Samples

A total of 17 mixed cord blood samples from 11 patients were obtained from Children's Hospital (Vancouver, B.C.) and were assayed by the HPLC-PC and FPIA methods.

### 3. RESULTS AND DISCUSSIONS

#### 3.1 Radioimmunoassay of Digoxin and Its Metabolites

Samples of digoxin and its metabolites exhibited cross-reactivity values of varying magnitudes, calculated on a molar basis, using the radioimmunoassay kits as shown in Table II. Cross-reactivity of dihydrodigoxin was found to be approximately 46% for the Digi-Tab RIA kit but was too low to be determined for the other five kits using this concentration range. However, these results indicate that dihydrodigoxin, at concentrations within the therapeutic range for digoxin, interferes to a greater extent than reported by this manufacturer (cross-reactivity of 1.8%). Other investigators have found that this metabolite has a cross-reactivity with digoxin antibodies ranging from very low values (Malini 1982) to as high as 30% (Gault 1979; Kramer 1976, 1978; Oge 1978). The cross-reactivity observed here for dihydrodigoxin was greater than that indicated previously in the literature.

Dihydrodigoxigenin, if present, represents a minor fraction of the metabolites of digoxin. However, large quantities of dihydrodigoxigenin have been observed in urine samples from patients with increased digoxin requirements (Luchi 1968). Considerable interference (approximately 22% cross-reactivity) was observed when this metabolite was assayed using the Digi-Tab kit. The cross-reactivity of dihydrodigoxigenin was too low to be estimated with the

other kits at these concentrations. For dihydrodigoxigenin, a cross-reactivity of less than 12% has been reported with digoxin RIA methods (Gault 1982). The interference found here for dihydrodigoxigenin was greater than that reported in the literature.

**Table II. Percent Cross-Reactivity on a Molar Basis for Digoxin Metabolites With Commercial Digoxin RIA Kits.**

	RIA Kits Used					
	A	B	C	D	E	F
Digoxin	100	100	100	100	100	100
Digoxigenin	60	250	95	103	74	95
Digoxigenin Monodigitoxoside	133	152	95	116	113	95
Digoxigenin Bisdigitoxoside	208	159	89	102	103	104
Dihydrodigoxin	46	-	-	-	-	-
Dihydrodigoxigenin	22	-	-	-	-	-

RIA Kits Used: A Digi-Tab RIA  
 B Bio-RIA I-125 Digoxin  
 C Amerlex Digoxin RIA Kit  
 D Digoxin Solid Phase RIA (Becton Dickinson)  
 E GammaCoat Digoxin RIA  
 F Digoxin I-125 Imusay

For digoxigenin, digoxigenin mono- and bis-digitoxoside, the average cross-reactivity was 120% (range 60% to 250%). These metabolites exhibited the greatest range in cross-reactivity for the Digi-Tab RIA (A) and Bio-RIA (B) kits. The other four RIA procedures showed a smaller range and an average cross-reactivity of 99% (range

74% to 116%) for these three digitoxose metabolites. These results agree with previous reports on cross-reactivity of these metabolites with commercial iodine-125 digoxin RIA kits (Valdes 1984).

The results reported here emphasize the lack of specificity of the polyclonal antibodies used in commercial iodine-125 RIA kits for digoxin analysis. Variability in the cross-reactivity was observed between the RIA kits and between the metabolites of digoxin. The presence of an intact lactone ring to interact with the digoxin antibody appears to be necessary for high cross-reactivity. However, removal of the digitoxose sugars does not greatly affect the cross-reactivity. Digoxin polyclonal antibodies are formed against an immunogenic digoxin-protein conjugate with an intact aglycone (Butler 1978), therefore, most digoxin antisera will cross-react extensively to the digoxigenin moiety as was observed.

Along with this lack of specificity, interpretation of RIA results are further complicated since the metabolites of digoxin do not have the same potency as digoxin (Aronson 1980; Iisalo 1977) and there is a large interpatient variability in digoxin metabolism (Aronson 1980; Clark 1974; Gault 1976; Iisalo 1977). Inappropriate changes in patient digoxin therapy may be initiated due to the lack of specificity of the RIA used.

### 3.2 Fluorescence Polarization Immunoassay of Digoxin Metabolites in Serum

The FPIA requires serum proteins to be precipitated with 5-sulfosalicylic acid (Skogen 1987) which has been suggested to result in the transformation of digoxin and its metabolites to digoxigenin and/or dihydrodigoxigenin (Gault 1977; Sonobe 1980; Sternson 1978). Therefore it is anticipated that digoxin will not be differentiated from its metabolites which may be present in patient samples.

Serum samples containing approximately 1.3 nmol/L of each metabolite were assayed by the FPIA. The results from the FPIA shown in Table III ranged from 0.7 to 4.8 nmol/L although no digoxin was present in these samples. Interference from digoxin metabolites is therefore a significant problem with the FPIA assay. The cross-reactivity values reported with the FPIA for digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside are as great or greater than those observed for the six RIA kits evaluated. For dihydrodigoxigenin, the reported cross-reactivity for the FPIA (91%) was considerably greater than that found with the Digi-Tab RIA (22%). The cross-reactivity of the FPIA method with the digoxin metabolites is as great or greater than that observed with the RIA procedure.

The FPIA assay indicated increased apparent digoxin levels in the presence of DLIS (Bianchi 1986; Frye 1987;

**Table III. Evaluation of Digoxin Metabolites in Serum  
Using the FPIA for Digoxin Analysis**

Metabolite	Actual Concentration (nmol/L)	FPIA Assay Results (nmol/L)	Cross-Reactivity Reported by FPIA Manufacturer (%)
Blank Serum	none	none	
Digoxigenin	1.41	1.8	>100
Digoxigenin Monodigitoxoside	1.48	4.8	>100
Digoxigenin Bisdigitoxoside	1.17	3.6	>100
Dihydrodigoxin	1.27	0.8	
Dihydro- digoxigenin	1.70	0.7	91

Kanan 1987; Skogen 1987; Soldin 1986b; Weiner 1987), however less interference from DLIS was reported with the FPIA than the RIA method (Yatscoff 1984). For evaluation of digoxin in patient samples, the FPIA may therefore be better than the RIA when DLIS is present.

The clinical significance of high cross-reactivity of both the RIA and FPIA methods with digoxigenin bisdigitoxoside and digoxigenin monodigitoxoside may be lost since these metabolites retain some potency (Keys 1980). Digoxigenin, dihydrodigoxin and dihydrodigoxigenin have substantially lower potency than digoxin so high cross-reactivity with the RIA and FPIA methods is more significant. Considering the large interpatient variability in digoxin metabolism (Aronson 1980) and that from 57 to 60%

of digoxin may be excreted as metabolic product, mainly dihydrodigoxin (Clark 1974; Luchi 1968), the possibility of patient samples containing high levels of these metabolites with low potency and high cross-reactivity exists. Since the potencies of these metabolites are very low with respect to digoxin, their interference with the RIA and FPIA methods is clinically significant. Over-estimation of digoxin due to interference from its metabolites may lead to inappropriate changes in patient digoxin therapy.

### 3.3 Pre-Column 3,5-Dinitrobenzoyl Derivatization of Cardiac Glycosides with HPLC-EC Analysis

#### 3.3.1 Ultraviolet Detection

With the 3,5-DNB derivatization procedure reported by Fujii (1983), the structure of derivatized digoxin formed is shown in Figure 8. Trace amounts of digoxin, dihydrodigoxin, digoxigenin, dihydrodigoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside as well as digitoxigenin and gitoxin were derivatized. The retention times of 3,5-DNB digoxin and its derivatized metabolites are shown in Table IV. The chromatogram of digoxin derivatized by this procedure (Figure 9) indicates that small amounts of the derivatized metabolites [digoxigenin (5.96 minutes), digoxigenin monodigitoxoside (9.82 minutes) and possibly digoxigenin bisdigitoxoside (17.77 minutes)] are present.



Table IV. HPLC Retention Times of 3,5-DNB Derivatives

Glycoside	Retention time (minutes)
Digoxin	37.23
Digoxigenin	5.64
Dihydrodigoxigenin	5.63
Digoxigenin Monodigitoxoside	9.63
Digoxigenin Bisdigitoxoside	16.90
Dihydrodigoxin	35.81

Chromatographic conditions: Column: Spherisorb ODS II 3 $\mu$ m HPLC (4.6 mm x 15 cm); flow rate: 1.0 mL/min.; mobile phase: acetonitrile/water (20/7); ultraviolet detection wavelength: 254 nm.

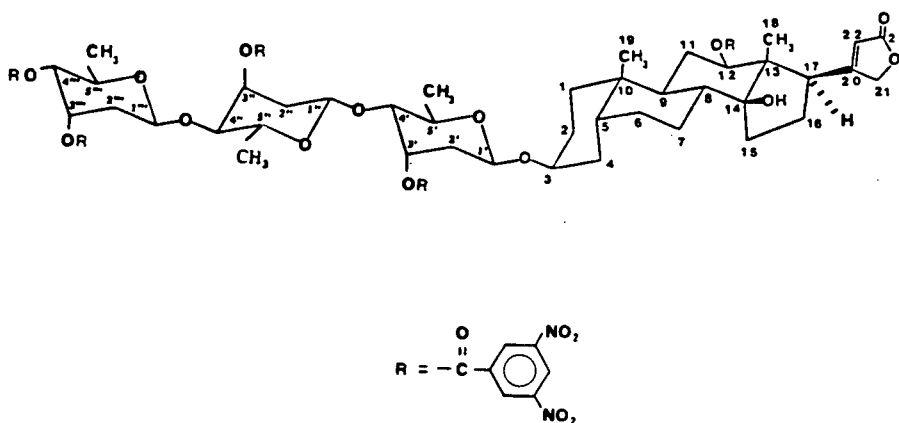
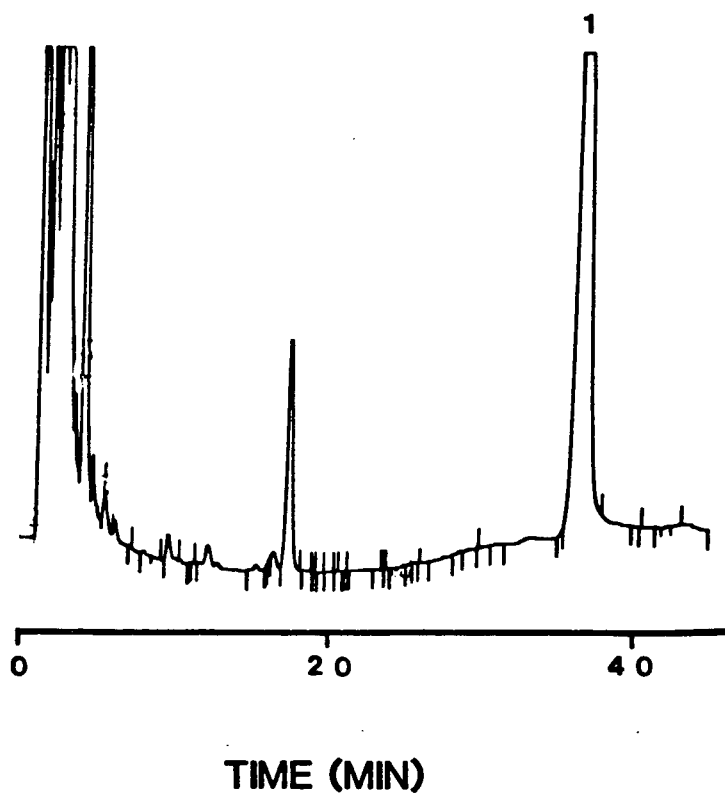


Figure 8. Structure of 3,5-DNB Digoxin



**Figure 9. Chromatogram of 3,5-DNB Digoxin  
Derivatized in Milligram Quantities**

Chromatographic conditions: Column: Spherisorb ODS II 3 $\mu$ m HPLC (4.6 mm x 15 cm); flow rate: 1.0 mL/min.; mobile phase: acetonitrile/water (20/7); ultraviolet detection wavelength: 254 nm; range: 0.005; injection volume: 10  $\mu$ L; chart speed: 0.2 cm/min. Peak identity: 1, 3,5-DNB digoxin.

Comparison to the chromatogram obtained from the blank sample confirms that these peaks are due to breakdown products or contaminants of the digoxin used.

Digoxin was derivatized on a large scale with 3,5-dinitrobenzoyl chloride as described in section 2.3.1 and purified by double recrystallization from methanol. The melting point (observed = 203 °C; expected = 203-205 °C (Fujii 1983)), HPLC retention time with acetonitrile/water (20/7) mobile phase and ultraviolet detection for 3,5-DNB digoxin (observed = 37.23 minutes; expected = 36 minutes (Fujii 1983)), and the order of elution of 3,5-DNB digoxin metabolites, 3,5-DNB gitoxin and 3,5-DNB digitoxigenin (section 2.3.2) confirmed the identity of the recrystallized 3,5-DNB digoxin.

### 3.3.2 Detection With a Single Glassy Carbon Electrode

Preliminary results with an acetonitrile/acetate buffer system (85/15) and reduction using the single glassy carbon electrode of the 5012 screened wall jet cell at potentials from 0 to -0.85 V indicated that a potential of -0.80 V with 0.1 M acetate buffer gave the greatest sensitivity. Decreasing the buffer concentration lead to a loss in peak height. Concentrations significantly greater than 0.1 M are not recommended for use with this detector (ESA Manual). With 0.1 M sodium acetate/acetic acid buffer (pH 4.6), the limit of detection for 3,5-DNB digoxin and resolution of 3,5-DNB digoxin from 3,5-DNB dihydrodigoxin

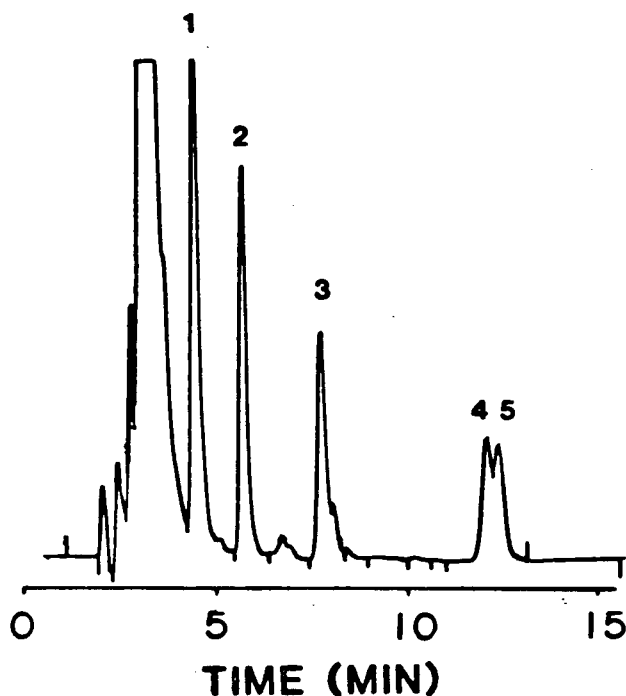
for various mobile phases is shown in Table V. The limit of detection was found to be 2.2 ng of 3,5-DNB digoxin (0.98 ng digoxin) using the recrystallized sample. With similar sensitivity, better resolution between 3,5-DNB digoxin and 3,5-DNB dihydrodigoxin was obtained with a mobile phase of methanol/ethanol/acetonitrile/isopropanol/buffer (40/3/60/2/22). Figure 10 shows a chromatogram of the 3,5-DNB derivatives of digoxin and its metabolites using this mobile phase.

**Table V. Resolution of 3,5-DNB Digoxin and 3,5-DNB Dihydrodigoxin Using HPLC-EC with Various Mobile Phases**

Mobile Phase Composition					Polarity	Resolution of 3,5-DNB Digoxin and 3,5-DNB Dihydrodigoxin	Limit of Detection (3,5-DNB digoxin)
MeOH	EtOH	ACN	IPA	Buffer			
40	3	40	3	15	6.08	none	
40	3	40	3	17	6.16	none	
40	3	40	3	18	6.19	0.86	
38	3	40	3	18	6.194	0.80	
40	3	40	2.75	18	6.20	0.70	
40	3	40	3	18.4	6.21	0.67	8.8 ng
40	3	50	3	20	6.229	0.60	
40	3	60	3	22	6.258	0.50	2.8 ng
40	3	60	2	22	6.276	0.75	2.8 ng
40	3	60	1	22	6.295	0.70	2.2 ng

MeOH      HPLC methanol  
EtOH      absolute ethanol  
ACN      HPLC acetonitrile  
IPA      HPLC isopropanol  
Buffer    0.1 M sodium acetate/acetic acid buffer (pH 4.6)

Chromatographic conditions: Column: Spherisorb ODS II 3  $\mu$ m HPLC (4.6 mm x 15 cm); flow rate: 1.0 mL/min.; guard electrode: 5020 guard cell electrode at -0.85 V pre-injector; detection: 5012 screened wall jet cell recording glassy carbon electrode at -0.80 V.



**Figure 10. Chromatogram of Digoxin and its Metabolites as Their 3,5-DNB Derivatives using Electrochemical Detection**

Chromatographic conditions: Column: Spherisorb ODS II 3  $\mu$ m HPLC (4.6 mm x 15 cm); flow rate: 1.0 mL/min.; mobile phase: methanol/ethanol/acetonitrile/isopropanol/acetate buffer (pH 4.6, 0.1 M) (40/3/60/2/22); guard electrode: 5020 guard cell electrode at -0.85 V pre-injector; detection: 5012 screened wall jet cell recording glassy carbon electrode at -0.80 V. Peak identity: 1, 3,5-DNB digoxigenin; 2, 3,5-DNB digoxigenin monodigitoxoside; 3, 3,5-DNB digoxigenin bisdigitoxoside; 4, 3,5-DNB dihydrodigoxin; 5, 3,5-DNB digoxin.

### 3.3.3 Dual Electrode Detection

To improve the stability and sensitivity of electrochemical detection, a 5010 flow cell, which has two glassy carbon electrodes, was used in the redox mode. The 3,5-DNB digoxin was reduced at the first electrode (-0.80 V) and then the reduced products were oxidized at the second electrode (+0.80 V).

Recording from the oxidative electrode gave a maximum sensitivity of 0.883 ng of recrystallized 3,5-DNB digoxin (0.394 ng digoxin) (mobile phase: methanol/ethanol/acetonitrile/isopropanol/acetate buffer 40/3/60/2/22). Recording the oxidative electrode alone decreased the baseline noise seen in the reductive mode, and lowered the equilibrium time for the system.

Derivatization of milligram quantities of digoxin (section 2.3.2) followed by HPLC analysis described above indicated 3,5-DNB digoxin was formed. HPLC analysis of the residue from trace level derivatization of digoxin (section 2.3.3) indicated no 3,5-DNB digoxin was present. Silanization of all glassware with dimethylchlorosilane in toluene, followed by derivatization and analysis of 1 to 80 ng samples of digoxin by the same procedure (section 2.3.3) showed 3,5-DNB digoxin was formed in the larger samples. There is a significant loss in sensitivity when ng samples are derivatized as compared to analysis of ng quantities of the recrystallized derivative (section 2.3.1) as shown by comparison of Figures 11 and 12. Figure 12 also shows the

high levels of derivatized metabolites formed when silanized glassware was used (3,5-DNB digoxigenin bisdigitoxoside at 7.61 minutes and 3,5-DNB digoxigenin monodigitoxoside at 5.51 minutes). The amount of metabolite formation during derivatization was much greater than previously indicated (Fujii 1983). 3,5-DNB digoxin and the derivatized digoxin metabolites appear to bind to the unsilanized glass, and when ng quantities of digoxin are derivatized this adsorption becomes significant. Also, derivatization was not as complete as expected (Fujii 1983), but the yield was not determined since the sensitivity of the HPLC-EC assay was not sufficient for a quantitative serum digoxin assay.

In acidic aqueous solutions aromatic nitro groups are electrochemically reduced in two successive steps corresponding to 4- and 2-electron transfers respectively (Mousty 1986). The intermediate hydroxylamine formed by the 4-electron transfer can then be further reduced to the amine (Mousty 1986). In neutral or basic media intermediates are formed prior to the hydroxylamine (Mousty 1986). With aromatic 3,5-dinitro groups, the nitro groups are either individually reduced to amines in 6-electron transfers or simultaneously reduced in one 12-electron transfer (Lund 1983). In alkaline solution selective reduction of one nitro group of 3,5-dinitro aromatic compounds was difficult (Lund 1983).

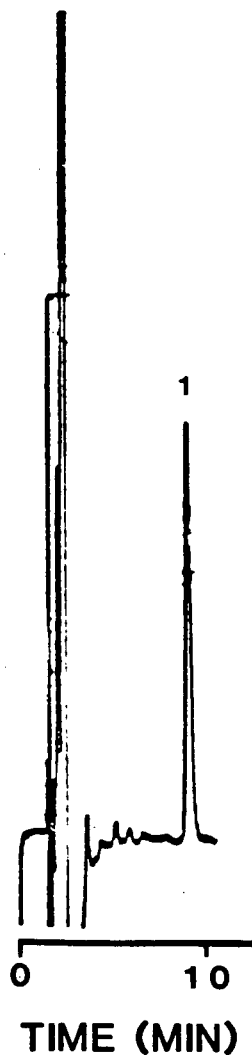


Figure 11. Chromatogram of 125 ng of 3,5-DNB Digoxin  
Purified by Double Recrystallization

Chromatographic conditions: Column: Spherisorb ODS II 3  $\mu$ m HPLC (4.6 mm x 15 cm); flow rate: 1.0 mL/min.; mobile phase: methanol/ethanol/acetonitrile/acetate buffer (pH 4.6, 0.1 M) (40/3/50/3/20); guard electrode: 5020 guard cell electrode at -0.85 V pre-injector; detection: 5010 analytical cell with electrode 1 at -0.80 V and electrode 2 at +0.80 V (recording electrode 2). Peak identity: 1, 3,5-DNB digoxin.





**Figure 12. Chromatogram of 80 ng of Digoxin  
Derivatized in Milligram Quantities**

Chromatographic conditions: Same as for Figure 11. Peak identity: 1, 3,5-DNB digoxin.

With the HPLC-EC system developed for 3,5-DNB digoxin, reduction occurred in the mixture of organic solvents and acetate buffer (pH 4.6) used for the HPLC mobile phase. Under these conditions, it is anticipated that both nitro groups are reduced simultaneously to amines. Whether reduction of one or both nitro groups with this HPLC-EC system occurred was not determined. The possibility of hydroxylamine formation was also not evaluated.

Amperometric detectors apply a constant potential to the detecting electrode and monitor the resultant currents produced as analytes flow past the electrodes. Typically, between 1 and 5% of the analyte is electrolyzed. As the fraction of sample electrolyzed approaches unity, the detector is said to be coulometric. The high surface area of the glassy carbon electrodes used here allows them to function coulometrically. Assuming all the nitro groups on 3,5-DNB digoxin were available for reduction, the number of electrons transferred per mole and therefore the extent of reduction, could be estimated using the peak area obtained from injection of a known quantity of 3,5-DNB digoxin. 3,5-DNB digoxin and 3,5-DNB dihydrodigoxin were partially resolved. Some breakdown of digoxin to its metabolites during derivatization and the maximum sensitivity obtained made it impractical to develop an assay for therapeutic monitoring of digoxin using this HPLC-EC assay. Therefore, the number of electrons transferred and the product formed by reduction of 3,5-DNB digoxin were not determined.

Unexamined alterations to the HPLC-EC assay for 3,5-DNB digoxin that may have led to an increased sensitivity were modifying the surface of the detecting electrode, increasing the time spent in the analytical flow cell and increasing the aqueous portion of the mobile phase. Electrodes with chemically modified surfaces have been developed (Lund 1983) to alter both selectivity and sensitivity. No modifications have been suggested for enhancing sensitivity in the reduction of nitro groups. Also, the flow-through glassy carbon electrode may develop flow related problems if surface modifying materials were added, so this was not evaluated. Without modifying the geometry of the flow cell, the time spent in the detector could be increased by decreasing the mobile phase flow rate. Improved sensitivity may result if the reduction was not complete due to insufficient time spent in the flow cell. This was not attempted since the decrease in chromatographic efficiency produced may result in a decrease in resolution between 3,5-DNB digoxin and 3,5-DNB dihydrodigoxin. Increasing the aqueous portion of the mobile phase would allow addition of chelating agents and possibly a more stable baseline would be formed. This was not evaluated due to the increase in chromatographic retention time and possible decrease in resolution between 3,5-DNB digoxin and 3,5-DNB dihydrodigoxin.

Other derivatization methods for adding nitro groups to digoxin have been reported (Flasch 1981; Nachtmann 1976a,

1976b)). The carbonyl function of the lactone ring of digoxin has been derivatized with dinitrobenzoic acid, tetranitrobiphenyl and several other dinitro aromatic compounds (Flasch 1981). The total number of nitro groups added to digoxin by these reactions is considerably less than with 3,5-DNBCl. Derivatization with 4-nitrobenzoyl chloride (Nachtmann 1976a, 1976b) would also result in fewer nitro groups added to digoxin than with 3,5-DNBCl. The sensitivity of detection is proportional to the total number of nitro groups per molecule of derivatized digoxin so these methods were not evaluated. Derivatization of digoxin with other electroactive agents has not been reported in the literature.

Kadish (1975) describes the use of polarography for the electrochemical determination of both digoxin and digitoxin. The peak reductive potential for digoxin was -2.285 V in isopropanol, with tetrabutylammonium iodide (0.01 M) as electrolyte. Carbon electrodes have a working range from +1.0 V to -0.8 V which can be extended a few tenths of a volt by using the screening electrode to decrease the background noise. This flow through detector would not be functional at -2.285 V and so reduction of underivatized digoxin was not possible.

### 3.4 Post-Column Fluorogenic HPLC Assay

#### 3.4.1 Pressure Vessel

Improvements to previously reported HPLC-PC fluorescence assay methods (Desta 1987; Gfeller 1977; Kwong 1986a, 1986b) are based on altering the method of delivering the post-column reagents. Using a pressure chamber (Reh 1985) for holding the concentrated HCl and pumping hexane into the vessel by an HPLC pump that is capable of operating against the resistance from the post-column reactor, greatly reduces the chromatographic time [from 1 hr (Kwong 1986a, 1986b) to 25 minutes]. Initially steel was used for the pressure vessel. When hexane was added to the vessel, the HCl was displaced and vented from the vessel via a teflon tube that went from the beaker (in the bottom of pressure vessel) to the valve where it joined the HPLC effluent and entered the reactor. Problems occurred with corrosion of the steel and there was the possibility of contaminating the HCl with iron oxides. Using PVC for the pressure vessel eliminated rust and the associated problems. HCl does not corrode PVC so a beaker or separate container inside was not required. Also, the PVC pressure vessel was light weight which allowed for more convenient cleaning and filling with the acid. To date, degradation of the PVC or Hypalon™ gasket material (chlorsulfonated polyethylene) by the hexane or HCl has not been noted.

### 3.4.2 Optimization of Post-Column Reactor Conditions

Concentrated HCl, phosphoric acid and trichloroacetic acid have been used for derivatization of digoxin to fluorescent products (Flasch 1981). Post-column fluorogenic HPLC assays reported for digoxin use concentrated HCl derivatization and dehydroascorbic acid (Desta 1987; Gfeller 1977; Kwong 1986a) or hydrogen peroxide solutions (Reh 1985) for fluorescence enhancement.

The following sequence of reactions for digoxin in concentrated HCl was reported (Flasch 1981): hydrolysis of the glycoside to the genin, formation of the 14-anhydrogenin, reaction to the corresponding 14-anhydro-16-chloro derivative and dehalogenation to the 14,16-dianhydrogenin. Although part of the reaction sequence has been reported, the exact structure of the 14,16-dianhydrogenin formed by the HPLC-PC assay used is unknown.

Dehydroascorbic acid was added as a post-column reagent along with concentrated HCl with previous HPLC-PC assay procedures for digoxin analysis (Desta 1987; Gfeller 1977; Kwong 1986a). The HPLC-PC assay for digoxin reported by Reh and Jork (1985) used a mobile phase of methanol/water/dilute hydrogen peroxide with phosphoric acid and post-column addition of concentrated HCl for digoxin derivatization. This allowed for separation of digoxin from digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, digoxigenin and lanatoside C (Reh 1985). Since it is desirable to resolve digoxin from dihydrodigoxin, the

aqueous portion of a mobile phase previously reported to partially resolve digoxin from dihydrodigoxin (Kwong 1986a) was replaced by the combinations of dilute hydrogen peroxide with phosphoric acid and dehydroascorbic acid shown in Table VI. Maximum peak height was obtained when 45 mL dehydroascorbic acid was used.

**Table VI. Effect of Varying the Aqueous Portion of the Mobile Phase on Peak Height Using HPLC-PC Fluorescence Assay**

Aqueous Portion Of Mobile Phase	Peak Height (cm)
30 water/15 Hydrogen peroxide/phosphoric acid	1.1
25 water/20 Hydrogen peroxide/phosphoric acid	1.9
45 Dehydroascorbic acid	4.9
35 Dehydroascorbic acid/10 water	4.4
35 Dehydroascorbic acid /10 Hydrogen peroxide/phosphoric acid	3.5
25 Dehydroascorbic acid /20 Hydrogen peroxide/phosphoric acid	3.9

Chromatographic conditions: Column: Spherisorb ODS II 3  $\mu$ m HPLC (4.6 mm x 15 cm); mobile phase: methanol/ethanol/isopropanol/aqueous (52/3/1/45); HPLC flow rate: 0.5 mL/min.; Hexane flow rate: 0.5 mL/min.; post-column reactor: 10 m in 79 °C water bath; detection: fluorometer equipped with 360 nm excitation and 425 nm emission filters.

Both 360 nm (Desta 1987; Kwong 1986a; Reh 1985) and 350 nm (Gfeller 1977) excitation filters and emission filters ranging from 415 nm (Gfeller 1977) to 480 nm (Reh 1985) were used with previous methods for post-column derivatization of digoxin. Since the exact structure of the digoxin derivative formed when dehydroascorbic acid was added to the HPLC mobile phase and concentrated HCl was introduced as a

post-column reagent is unknown, the emission filter wavelength was optimized for maximum fluorescence.

With the best mobile phase, 425, 440 and 460 nm emission filters were then evaluated using a 360 nm excitation filter and the results are shown in Table VII. Maximum peak height was found with the 425 nm emission filter.

**Table VII. Effect of Emission Filters on Peak Height of Digoxin Using HPLC-PC Fluorescence Assay**

Emission Filter (nm)	Peak Height (cm) (Average of n=4)
425	9.5
440	5.7
460	4.2

Chromatographic conditions: Same as for Table V with mobile phase: methanol/ethanol/isopropanol/dehydroascorbic acid (52/3/1/45); detection: fluorometer equipped with 360 nm excitation filter.

The peristaltic pumps previously used for delivery of post-column reagents (Desta 1987; Gfeller 1977; Kwong 1986a) severely limited the choice of flow rate and reactor size. These limits were effectively removed by using a second HPLC pump and hexane displacement of HCl from a pressure vessel. The flow rate of HCl and post-column reactor length were optimized together since they both influence the time available for derivatization to occur. Changing the hexane flow rates from 0.5 mL/min to 1.0 mL/min, with the 10 m reactor, gradually reduced the peak height (Table VIII). The shorter time spent in the reactor with the 1.0 mL/min



flow rate did not allow the development of maximum fluorescence. In order to increase the reaction time without reducing the hexane flow rate below 0.5 mL/min, a 20 m reactor was used. With the 20 m reactor, the best sensitivity was obtained with a hexane flow rate of 0.5 mL/min (direct injection of 0.585 ng digoxin with a signal/noise ratio of 4/1). Reduced baseline noise was also observed with the 20 m reactor. When the 20 m reactor was used, digoxin eluted later in the chromatogram (17 minutes compared to 13 minutes) allowing the endogenous peaks from serum eluting in the early part of the chromatogram to be well separated from digoxin.

**Table VIII. Effect of HCl Flow Rate on Peak Height of Digoxin Using HPLC-PC Fluorescence Assay**

HCl (Hexane) Flow Rate (mL/min)	Peak Height (cm) (Average of n=4)
0.44 (0.50)	9.3
0.68 (0.70)	7.6
0.99 (1.00)	5.4

Chromatographic conditions: Same as for Table VI with detection: fluorometer equipped with 360 nm excitation and 425 nm emission filters.

### 3.4.3 Maximum Sensitivity

The maximum sensitivity obtained with the optimized HPLC-PC conditions given above was 0.5 ng digoxin/injection

directly and 1.5 ng digoxin/3 mL serum with extraction as described in section 2.9.4.

For other HPLC-PC digoxin assay methods, the maximum sensitivity values reported were 0.1 ng digoxin/injection (Reh 1985), 0.5 ng digoxin/injection (Kwong 1986a) and 10 ng digoxin/injection (Desta 1987). The HPLC-PC assay described here is less sensitive than that described by Reh and Jork (1985) but is still preferable for evaluating patient samples since partial resolution of digoxin from dihydrodigoxin was obtained. This HPLC-PC method has equal or better sensitivity than the other HPLC-PC methods reporting sensitivity to digoxin (Desta 1987; Kwong 1986a). Removal of the problems associated with using a peristaltic pump for post-column reagent delivery (Desta 1987; Kwong 1986a) make the HPLC-PC system developed here more appropriate for routine use. The sensitivity observed with this HPLC-PC assay may be significantly improved by assessing different fluorometric detectors.

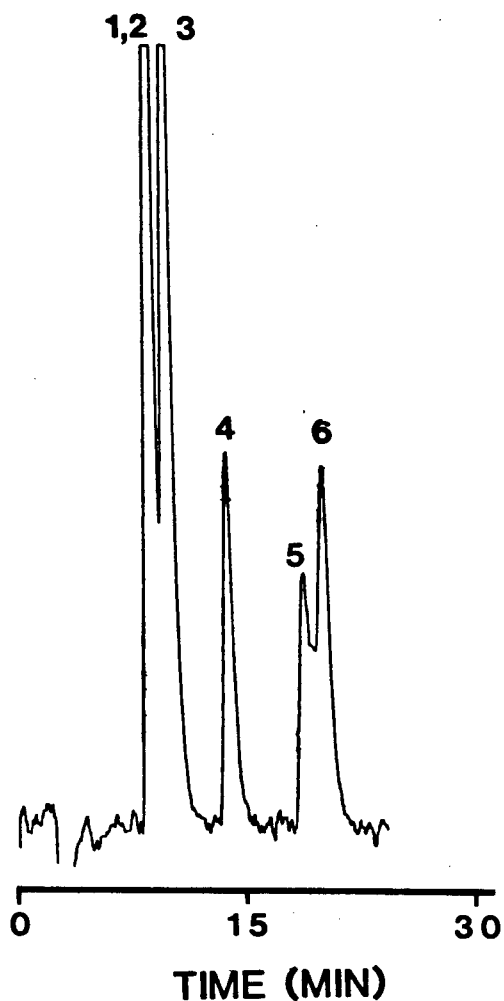
The sensitivity of immunoassay methods and HPLC-RIA procedures for digoxin is considerably greater than that obtained for HPLC-PC methods of analysis. Immunoassay methods alone lack the desired specificity for digoxin and chromatographic separation of digoxin from dihydrodigoxin was not described by most HPLC-RIA methods of digoxin analysis (Gibson 1980; Loo 1977, 1981; Margot 1983; Plum 1986). The HPLC-RIA assay method that partially separates digoxin from dihydrodigoxin does not describe the resolution

obtained (Morais 1981; Wagner 1983). Therefore, the HPLC-PC assay described here is superior for digoxin analysis when adequate sample volumes (3 mL serum) are available.

#### 3.4.4 Evaluation of Digoxin and its Metabolites

Using the final HPLC-PC system, digoxin is baseline resolved from digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside as shown in Figure 13. The resolution between digoxin and dihydrodigoxin ( $R = 0.899$ ) is sufficient for peak height quantitation. Since the HPLC-PC system separates digoxin from all of its metabolites, there is no interference from these metabolites in the analysis of digoxin.

Resolution of digoxin from dihydrodigoxin was not described for HPLC-RIA methods for digoxin analysis (Gibson 1980; Loo 1977, 1981; Margot 1983; Plum 1986). Along with the lack of specificity of the RIA used for digoxin in the presence of DLIS (Valdes 1985a, 1985b), interference from dihydrodigoxin may occur with these HPLC-RIA methods. Since the potential for cross-reactivity of dihydrodigoxin and DLIS with the RIA exists, the HPLC-PC method described in this report is better than the HPLC-RIA methods with respect to specificity for digoxin. The one HPLC-RIA method claiming partial separation of digoxin from dihydrodigoxin does not describe the resolution obtained by their chromatographic system (Morais 1981; Wagner 1983).



**Figure 13. Chromatogram of Digoxin and  
Its Metabolites by HPLC-PC**

Chromatographic conditions: Column: Spherisorb ODS II 3  $\mu$ m HPLC (4.6 mm x 15 cm); mobile phase: methanol/ethanol/isopropanol/dehydroascorbic acid (52/3/1/45); HPLC flow rate: 0.4 mL/min.; Hexane flow rate: 0.5 mL/min.; post-column reactor: 20 m in 79  $^{\circ}$ C water bath; detection: fluorometer equipped with 360 nm excitation and 425 nm emission filters. Peak identity: 1, digoxigenin; 2, dihydrodigoxigenin; 3, digoxigenin monodigitoxoside; 4, digoxigenin bisdigitoxoside; 5, dihydrodigoxin; 6, digoxin.

The post-column fluorogenic derivatization HPLC assays for digoxin reported by Gfeller *et al.* (1977) and Reh and Jork (1985) do not describe separation of digoxin from dihydrodigoxin. Baseline resolution of digoxin from dihydrodigoxin has been reported (Desta 1987) but the sensitivity of this assay for digoxin was not adequate for therapeutic monitoring. The resolution of digoxin from dihydrodigoxin obtained with the HPLC-PC assay described here was slightly less than previously reported ( $R = 0.91$ ) (Kwong 1986a). Although the sensitivity of this assay was sufficient for evaluating patient samples (Kwong 1986a, 1986b) the long chromatographic time (40 minutes) and unreliable delivery of post-column reagents make it unsuitable for routine clinical use.

### 3.5 Analysis of Biological Samples Using The HPLC Fluorogenic Assay

#### 3.5.1 Optimization of Serum Extraction Method

##### 3.5.1.1 Protein Precipitation Methods

Emulsion formation of the aqueous phase and the extraction solvent is a problem found with a previously reported extraction procedure (Kwong 1986a). Reports in the literature indicate that complete protein precipitation is difficult or impossible unless cold acetone is used or unless the sample is heated to coagulate the precipitate

(Henry 1964). A final concentration of at least 63% cold acetone is required for serum or whole blood (1 volume sample plus 9 volumes of 70% acetone) (Henry 1964). The emulsion formed is due to incomplete protein precipitation since increasing the amount of acetone used in this extraction procedure to 6 mL reduced the amount of emulsion between the aqueous phase and the extraction solvent.

Anionic precipitants are commonly used for the removal of proteins (such as picric acid and trichloroacetic acid) (Henry 1964) but would degrade any digoxin present and therefore could not be used with this extraction. Zinc, zinc plus barium, and copper precipitation methods do not use acid so may be used in the extraction of digoxin. Zinc sulfate with barium hydroxide (Henry 1964; Somogyi 1945) and copper sulfate with sodium hydroxide (Henry 1964) methods were both evaluated for protein precipitation, replacing the acetone in this extraction procedure. Emulsion formation with the extraction solvent (dichloromethane/propan-1-ol 98/2) still occurred resulting in lower recovery of digoxin and also made the filtration step more difficult than when acetone was used. Other alterations to the extraction method (section 3.5.1.2) reduced the emulsion formation previously observed. Protein precipitation in the final extraction procedure was achieved by using 3 mL acetone.

### 3.5.1.2 Removal of Endogenous Interference

Using the HPLC-PC assay described in section 2.8.3 and a serum extraction method previously reported (Kwong 1986a, 1986b), serum from one healthy undigitalized female volunteer contained an interfering peak eluting close to the retention time of digoxin as shown in Figure 14.

Although the structure and immunoreactivity of this interfering peak were not evaluated, it may be a fraction of the DLIS observed in normal adult subjects who never received digoxin (Balzan 1984; Clerico 1985; Diamandis 1985; Hamlyn 1982; Klingmuller 1982; Valdes 1983a; Vinge 1988). Attempts to eliminate DLIS from serum have included ultrafiltration with Centrifree micropartition systems (Christenson 1987; Graves 1986). This removed 90% of the DLIS and allowed for greater than 95% recovery of digoxin (Graves 1986). The Centrifree ultrafiltration system has a maximum sample volume of 1.0 mL. Larger serum samples (3 mL) were required here so the Centriflo system (maximum sample volume of 7.0 mL) was also evaluated. Ultrafiltration of serum containing this interfering peak by the Centrifree and Centriflo systems completely removed the interfering compound but did not allow recovery of digoxin from the samples. These results suggest that the interfering peak was relatively large (greater than 25000 Da in molecular weight). Ultrafiltration could not be used for sample purification since digoxin was not recovered.

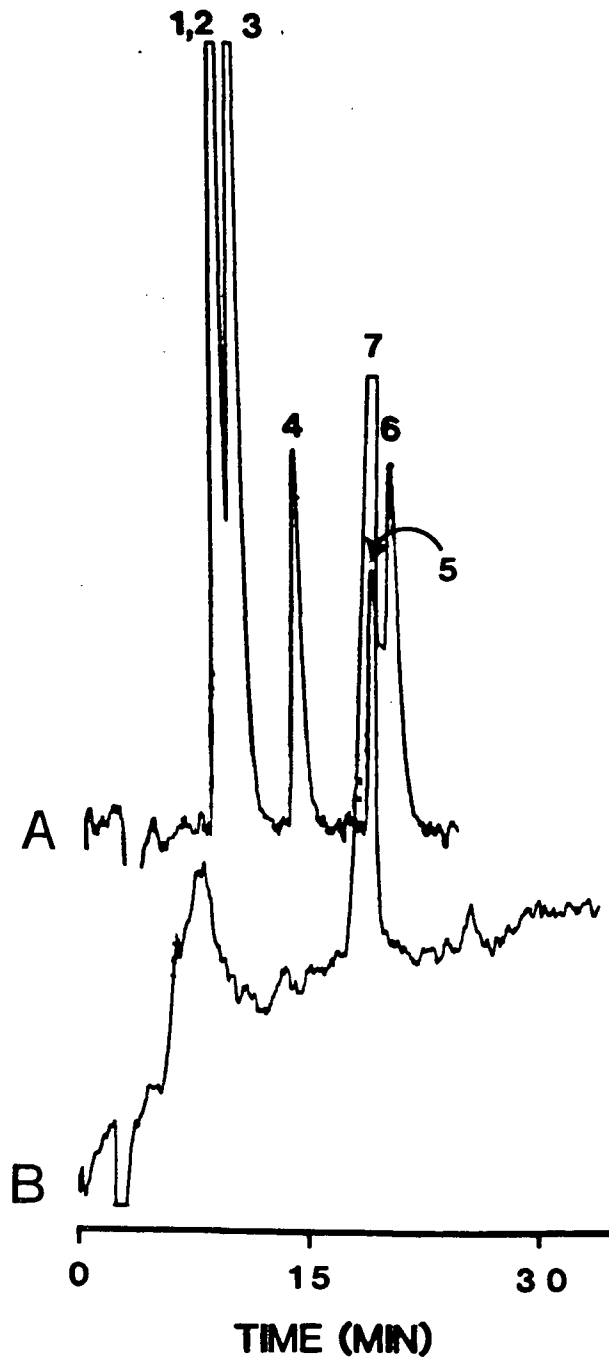


Figure 14. Chromatograms of Digoxin and Its  
Metabolites in Ethanol and Blank Serum  
Containing Interfering Peak

Chromatographic conditions: Same as for Figure 13.

A Digoxin and Its Metabolites in Ethanol

B Blank Serum Containing Interfering Peak

Peak identity: 1, digoxigenin; 2, dihydrodigoxigenin; 3, digoxigenin monodigitoxoside; 4, digoxigenin bisdigitoxoside; 5, dihydrodigoxin; 6, digoxin; 7, interfering peak.

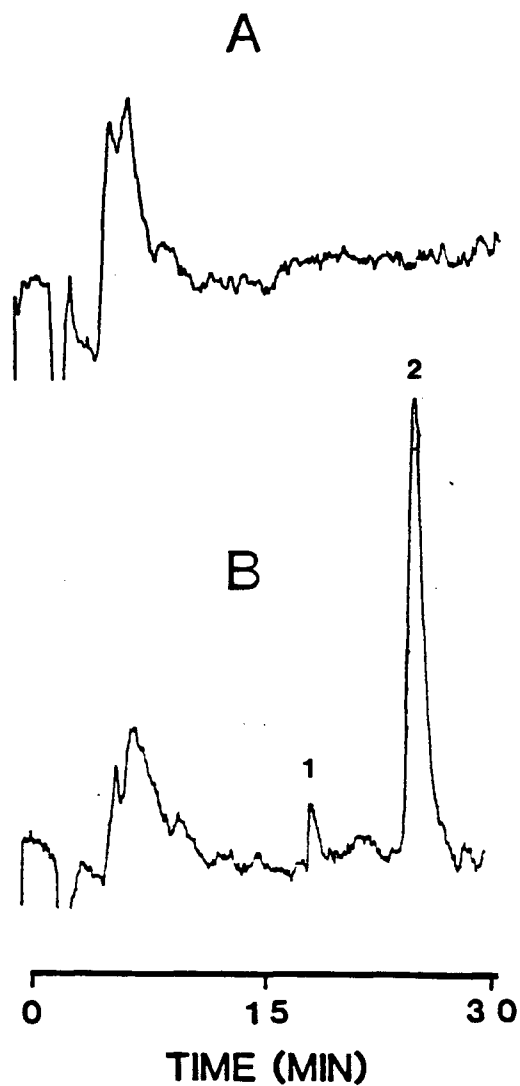


Removal of the interfering peak by modifying the isooctane solvent wash was then evaluated. Recovery of digoxin from water after an isooctane/dichloromethane (20/5) solvent wash and extraction was 8% lower than when isooctane alone was used. Blank serum (3 mL) which contained the interfering peak and a serum sample (3 mL) spiked with digoxin (1.5 ng) and internal standard (160 ng digitoxigenin) were washed with 2 mL of isooctane/dichloromethane (20/5) and extracted. The chromatogram obtained from HPLC-PC analysis (see Figure 15) indicated that the interfering peak was removed and digoxin was recovered when dichloromethane was added to the solvent wash. Serum protein precipitation with 3 mL acetone followed by a 2 mL isooctane/dichloromethane solvent wash resulted in little or no emulsion formed with the extraction solvent.

The final extraction procedure (section 2.9.4) employed 3 mL acetone for protein precipitation and a 2 mL isooctane/dichloromethane (20/5) solvent wash for removal of endogenous compounds that interfere with digoxin analysis.

### 3.5.2 Recovery and Precision

The recovery of digoxin was determined for blank serum spiked with 0.5, 3.0 and 10.0 ng of digoxin. Table IX shows the percent recovery for each concentration.



**Figure 15. Chromatogram of Blank Serum and Serum Spiked  
with 1.5 ng Digoxin and Internal Standard**

Chromatographic conditions: Same as for Figure 13.

Peak identity: 1, digoxin; 2, internal standard  
(digitoxigenin).

A Blank Serum

B Spiked Serum

Table IX. Recovery of Digoxin from Spiked Serum Samples

Concentration (ng digoxin/3 mL serum)	Recovery (%)	C.V. (%)	Number of Samples
1.5	72.0	6.4	7
3.0	78.2	7.2	8
10.0	78.8	6.1	7

Better digoxin recovery was observed here than with a previously reported HPLC-PC assay and similar extraction procedure (70%) (Kwong 1986b). Considerably greater recovery of digoxin (99.7%) using a very different extraction method has been reported (Reh 1985) but is not appropriate for routine use.

The precision of the HPLC-PC assay was determined using digoxin in ethanol rather than repeated injections from one extracted serum sample since the entire extracted serum sample was required for analysis. For the HPLC-PC assay the coefficient of variation for 1.5 and 10 ng injections of digoxin in ethanol was 4.7% (n=4) and 3.3% (n=10) respectively.

The precision of the extraction procedure was determined by repeated extraction of five blank serum samples (3 mL) spiked with 3 ng of digoxin and 20  $\mu$ L of internal standard (160 ng) followed by HPLC-PC analysis and comparison of peak height ratios. The coefficient of variation for within each day is shown in Table X. Analysis of variance for peak height ratio means ( $F_{(3,16)} = 0.84$  (p=0.49)) and for equality of variance ( $F_{(3,16)} = 0.22$

( $p=0.88$ )) verifies that there is no significant difference in the between-day results.

A greater inter-assay coefficient of variation (8%) was reported for a similar extraction procedure (Kwong 1986b). This indicates that in comparison to a previous method (Kwong 1986b) the extraction procedure and HPLC-PC assay described here has a lower spread of values in relation to the mean values.

Table X. Precision of Digoxin Assay

Day	C.V. (%)
1	4.6
2	5.7
3	6.6
4	5.3
mean	5.6

### 3.5.3 Calibration Curve

Serum samples (3 mL) were spiked with from 1 to 3.3 ng digoxin/mL and internal standard, extracted and analyzed using the HPLC-PC fluorogenic assay. The peak height and concentration ratios were then calculated and the calibration curve shown in Figure 16 was prepared using average values from four separate determinations. The correlation coefficient of 0.9876 was low but acceptable for this complex an assay (Falkner 1981) and the y-intercept (0.0273) was not significantly different from zero ( $t$ -ratio = 1.23). Calibration curve correlation coefficients as high

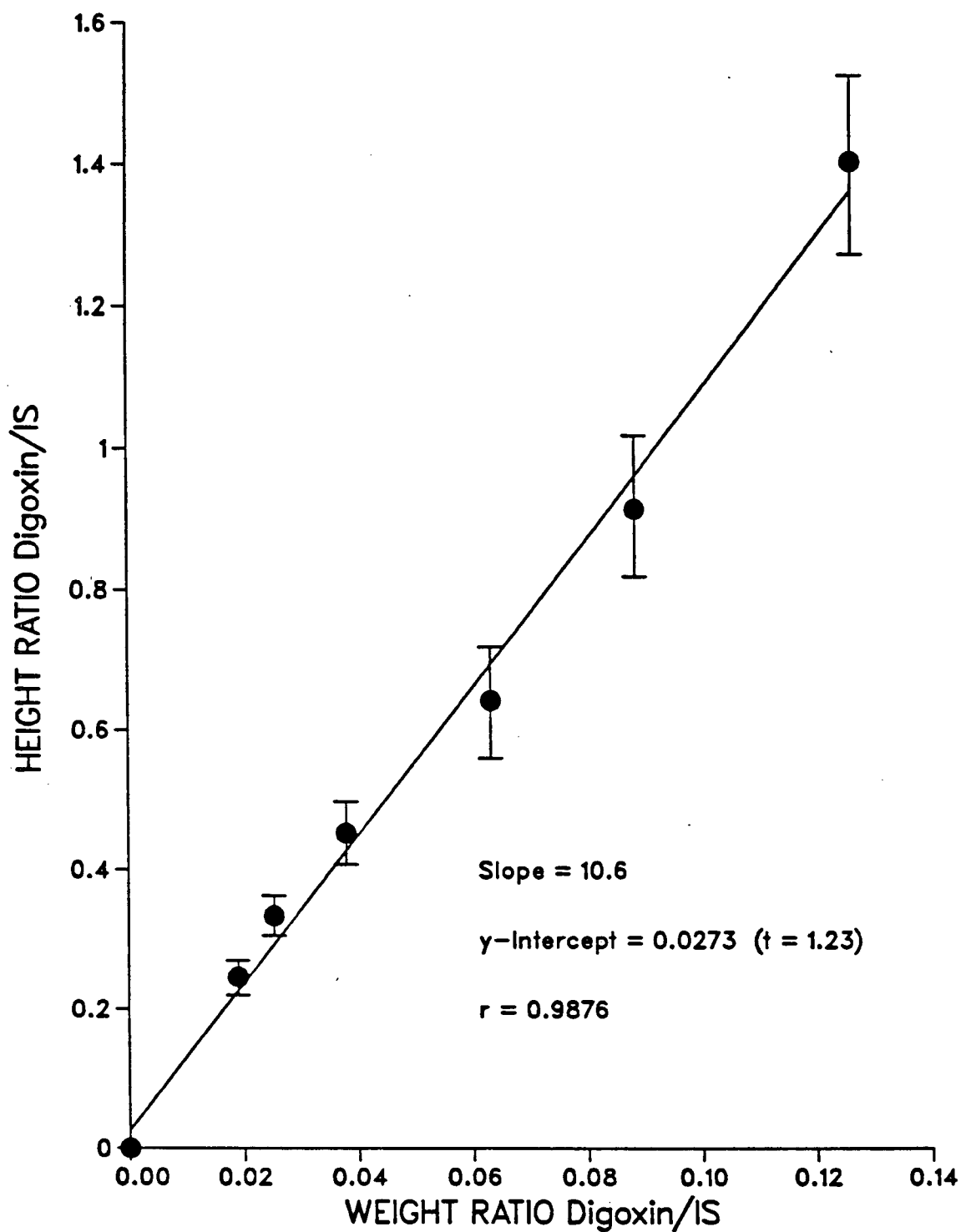


Figure 16. Calibration Curve for HPLC-PC Digoxin Assay

as 0.9999 have been previously reported with HPLC-PC analysis of digoxin (Kwong 1986b). The correlation coefficient observed here indicates that 97.5% of the total variability in the height and weight ratios is accounted for by mutual dependence of these ratios.

#### 3.5.4 Comparison of HPLC-PC and FPIA Methods in Spiked Serum Samples

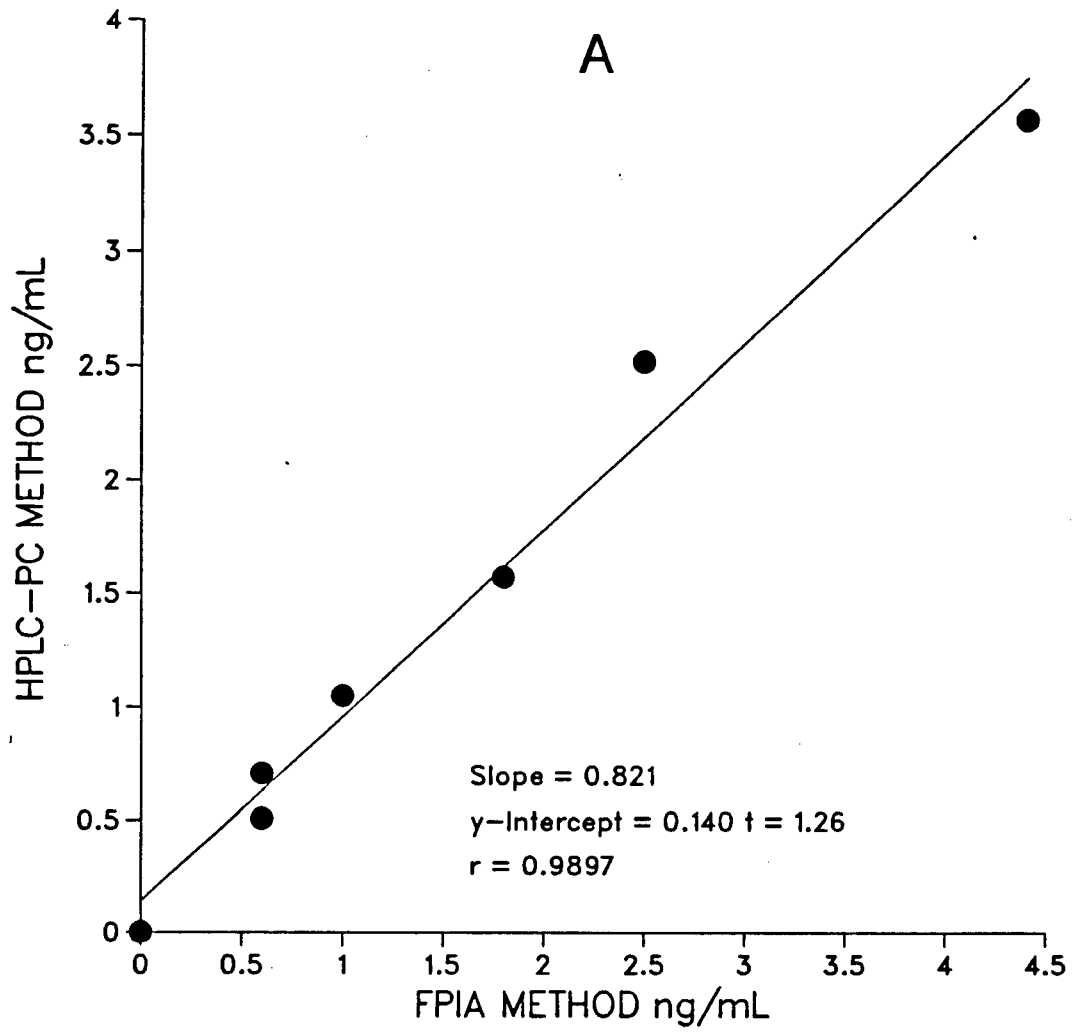
Seven blank serum samples spiked with from 0 to 3.4 ng of digoxin per mL were prepared and assayed by both methods. As shown in Figure 17, the correlation between the HPLC-PC and FPIA methods was 0.9897. This indicates that 98% of the total variability of the two methods is accounted for by mutual dependence. Also, a comparison of the individual methods with the actual digoxin levels shows that the HPLC-PC procedure ( $r = 0.9979$ ) had better correlation with the actual values than the FPIA method ( $r = 0.9895$ ).

The plot of FPIA versus actual values gave a slope of 1.25 indicating that the FPIA results were higher than the amount of digoxin added. With the FPIA, no digoxin was found in the serum prior to spiking. A possible explanation for these higher than actual FPIA results is that DLIS present in the blank serum was displaced from tight binding sites to unbound or weak binding sights (Valdes 1985a, 1985b). The movement of DLIS from tight binding sites would

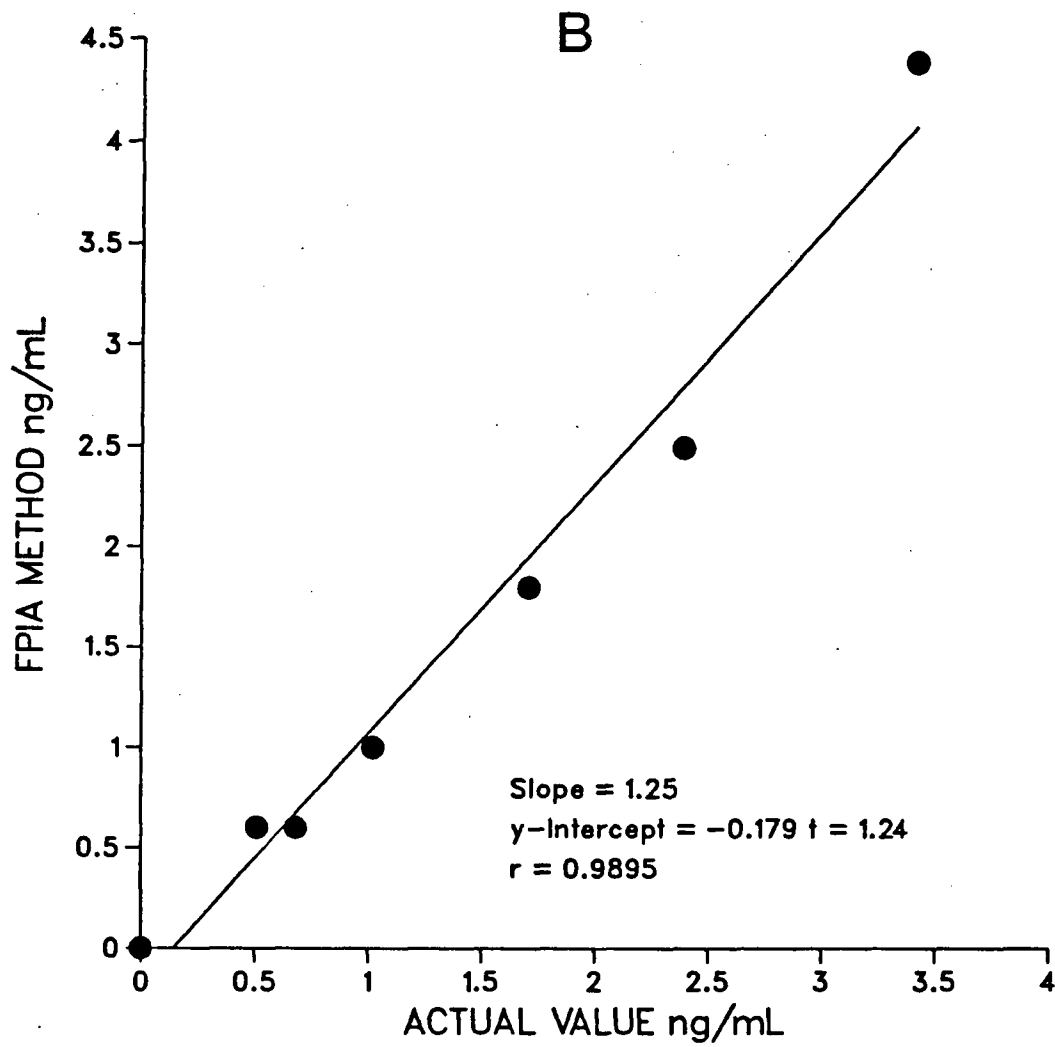
**Figure 17. Correlation Between HPLC-PC and FPIA Methods**

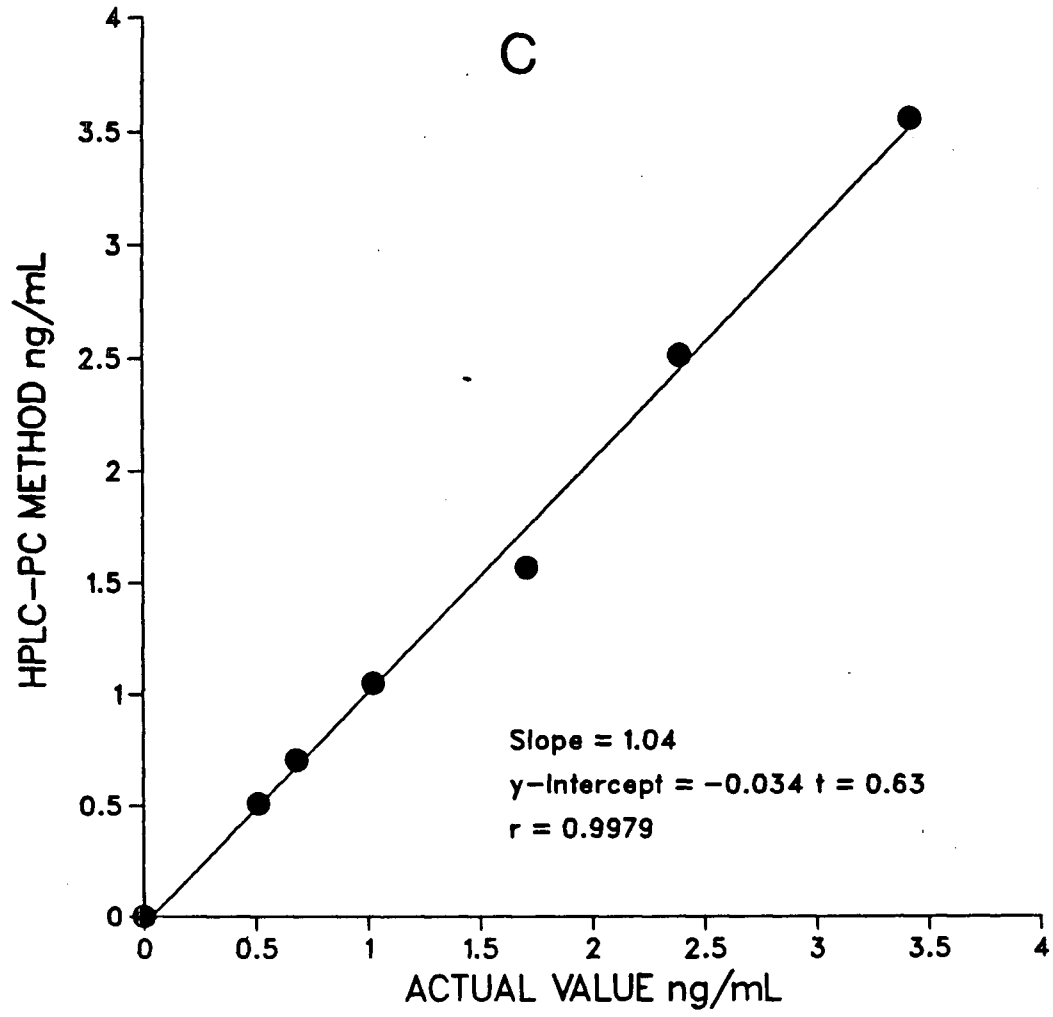
**For Digoxin Analysis**

- A HPLC-PC versus FPIA Method
- B FPIA versus Actual Levels
- C HPLC-PC versus Actual Levels









make it available for detection with the FPIA (Valdes 1985a, 1985b) and therefore may cause higher apparent digoxin levels to be observed.

### 3.5.5 Specificity

#### 3.5.5.1 Steroids

Numerous steroids have been reported to cross-react with digoxin antisera (Diamandis 1985; Longerich 1988; Matthewson 1988). Since cross-reactivity with digoxin antisera was reported, the specificity of immunoassay methods using these antibodies is questionable. Samples of endogenous and synthetic steroids were evaluated to ensure that they did not interfere with the HPLC-PC assay procedure. No fluorescent peaks were observed after direct injection of these steroids in methanol. The steroids evaluated either do not elute from the HPLC system or do not produce a fluorescent product under these conditions and, therefore, would not interfere with the HPLC-PC analysis of digoxin.

#### 3.5.5.2 Co-administered Drugs

It is imperative that drugs which may be co-administered with digoxin be evaluated for interference with the analytical method. Using a similar HPLC-PC assay for digoxin the following drugs were evaluated (Kwong 1986b): spironolactone, furosemide, disopyramide, captopril,

dipyridamol, quinidine, verapamil, propafenone, procainamide and trimethoprim-sulfamethoxazole. Only furosemide and spironolactone yielded a fluorescent response and under the conditions used were chromatographically separated from digoxin (Kwong 1986b). The newer antiarrhythmic agent, mexiletine, was not previously evaluated. Therefore, mexiletine was assayed using the HPLC-PC assay developed here to determine if any fluorescence was produced under these conditions. No fluorescence was found on injection of 7.5  $\mu$ g of mexiletine which would be the maximum expected in 3 mL of serum from patients within the therapeutic range for this drug (Talbot 1973).

### 3.6 Analysis of Digoxin in Digitalized Patient Serum

Serum samples from 25 patients who received digoxin therapeutically were assayed by both HPLC-PC and FPIA methods. The results from both methods are shown in Table XI. Since these methods differ in their sensitivity to digoxin, only the samples within range for both assays were used (n=15). The mean concentration obtained using the HPLC-PC assay was  $0.99 \pm 0.56$  and that found with the FPIA method was  $1.13 \pm 0.73$ . The FPIA assay gave higher average results which is possibly due to the metabolites of digoxin being included in the total assay value. While there were individual discrepancies between the analyses of the sample by the two procedures, it was not possible to assess interference with the FPIA method that would lead to lower

Table XI. Comparison of the HPLC-PC and FPIA Methods  
for Digoxin Analysis in Digitalized Patients

Patient	Digoxin Concentration ng/mL		HPLC-PC/FPIA Ratio
	HPLC-PC	FPIA	
1	1.11	1.2	0.925
2	1.41	1.6	0.881
3	0.69	0.8	0.863
4	0.98	1.0	0.980
5	ND	0.9	-
6	1.01	1.0	1.010
7	0.89	0.9	0.989
8	0.94	1.1	0.855
9	1.07	0.9	1.189
10	0.47	0.6	0.783
11	0.93	1.1	0.845
12	2.80	3.6	0.778
13	ND	0.6	-
14	0.41*	0.5	-
15	0.53	0.9	0.589
16	0.42*	0.6	-
17	0.81	0.9	0.900
18	0.41*	0.6	-
19	ND	0.2	-
20	0.20*	0.2	-
21	0.25*	0.2	-
22	0.41*	0.5	-
23	0.62	0.6	1.033
24	0.69	0.7	0.986
25	ND	0.4	-
mean±S.D.	0.99±0.56	0.86±0.66	0.91±0.14
	(n=15)	(n=25)	(n=15)

\* quantities are below the accepted signal-to-noise ratio of 4:1 and are not included in the mean data.

ND = none detected.

levels found with this method for three of the samples. However, no interference has been shown with the metabolites of digoxin or other drugs assessed using the HPLC-PC assay.

Interference from endogenous substances previously observed with this assay was eliminated by altering the extraction method.

The range of HPLC-PC to FPIA values for these samples was from 0.589 to 1.189. HPLC/immunoassay ratio values have been reported by Loo *et al.* (1981) ( $0.84 \pm 0.13$ ), Gibson and Nelson (1980) ( $0.83 \pm 0.12$  for renal failure dialysis patients;  $1.06 \pm 0.09$  for patients with renal impairment) using HPLC-RIA and RIA assay methods and Kwong (1984) ( $1.00 \pm 0.34$  and  $0.94 \pm 0.30$  from separate hospitals) using HPLC-PC and RIA procedures. Although direct comparison of the HPLC/immunoassay ratio values may not be justified due to the different HPLC and immunoassay methods used, the ratio obtained here with HPLC-PC and FPIA methods is similar to previously reported ratio values.

### 3.7 Analysis of Serum from Undigitalized Patient Groups Where High Levels of DLIS have been Reported

#### 3.7.1 Hypertensive Patients

Serum samples from five undigitalized hypertensive patients were obtained. These samples were evaluated by both the HPLC-PC final assay procedure and the FPIA and the results are shown in Table XII.

**Table XII. Comparison of Undigitalized Hypertensive Patient Samples Evaluated by the HPLC-PC and FPIA Methods**

Patient	Apparent Digoxin Concentration ng/mL	
	HPLC-PC	FPIA
1	ND	ND
2	ND	ND
3	ND	0.1
4	ND	ND
5	ND	ND

ND = none detected.

The hypertensive patients were on the following medications: atenolol, spirapril, enalapril, eltroxin, timolol and pilocarpine. Their blood pressure ranged from 154/104 to 174/110.

Endogenous compounds that cross-react with digoxin immunoassays have been reported in some patients with hypertension. Recent reports suggest that DLIS, or a fraction thereof, may be the natriuretic hormone and involved in hypertension (Buckalew 1985; Cloix 1987; De Wardener 1982a, 1982b; Grantham 1984; Wilkins 1985) and may also be present in normotensive subjects (Cloix 1987; Hamlyn 1982).

The results presented in Table XII do not show significant levels of endogenous compounds that interfere with the FPIA assay. Since the HPLC-PC results also show no interference from endogenous compounds, it appears that the HPLC-PC is as unaffected by endogenous compounds in this

patient group as the FPIA in these samples. DLIS interference with the FPIA method is considerably less than with RIA methods (Greenway 1985; Nanji 1985). Analysis of these samples by an RIA method may have resulted in greater apparent digoxin levels but the limited sample size prohibited duplicate determination by immunoassay. A larger number of patients may have revealed higher apparent digoxin levels with the FPIA, however, these additional patients were not available during the course of this investigation.

### 3.7.2 Renal Failure Patients

Serum samples from 20 undigitalized renal failure patients who were on dialysis at the Willow Dialysis Unit were obtained. All the patients were on the following medications: folic acid, Z-Bec (multivitamins with zinc), antacids (Robalate, Amphojel, and Tums), Basaljel (aluminum hydroxide for binding phosphate in the gastrointestinal tract), Imferon (iron dextran), and vitamin D supplements. Along with these, some patients required other medications (metoprolol, propranolol, sulfisoxazole, phenytoin, indomethacin, allopurinol, acetylsalicylic acid, docusate sodium, isosorbide dinitrate, insulin, triazolam, prednisone, captopril, and hydroxyzine).

The renal failure patient serum samples were evaluated by both the HPLC-PC final assay procedure and the FPIA and digoxin was not detected in any of the serum samples by either method. This indicates that endogenous substances



were not present in sufficient quantities to interfere with either of the assay methods. The HPLC-PC assay is at least as specific as the FPIA for digoxin in this patient group as represented by these samples. Endogenous compounds (DLIS) that interfere with RIA methods have been observed with this patient group (Bourgoignie 1972; Craver 1983; D'Arcy 1984; Gibson 1980; Graves 1983a, 1983b; Kramer 1985b). DLIS has also been observed using FPIA assay methods in renal failure patients (Oldfield 1985; Yatscoff 1984) but the degree of interference is generally less than that found with RIA methods. It is possible that the renal failure patient samples evaluated here contained DLIS at levels too low to be determined using the FPIA. It was not possible to further evaluate these samples with an RIA procedure due to the limited sample size. Analysis of these samples by RIA may have indicated significant levels of DLIS. Evaluation of a larger number of renal failure patient serum samples with the FPIA may have provided samples with higher apparent digoxin levels.

### 3.7.3 Hepatic Failure Patients

Ten serum samples from nine hepatic failure patients were evaluated by both HPLC-PC and FPIA methods. The results are given in Table XIII.

The hepatic failure patients were on the following medications: furosemide, spironolactone, nifedipine, cholestyramine, oxazepam, co-trimoxazole, ranitidine,

cephalexin, codeine phosphate, demerol, domperidone, haloperidol, procainamide, camphorated opium tincture, bisacodyl, valium, clindamycin, gentamycin, cefoxitin, vitamin E, vitamin K, pancrelipase, atropine, heparin, lactulose, thiamine, salbutamol, potassium chloride, calcium gluconate, folic acid and norfloxacin. As required, tylenol, dimenhydrinate, pentazocine, lorazepam and prochlorperazine were administered. Patient nine was the only digitalized patient (0.125 mg daily). The patients were all diagnosed as having alcoholic cirrhosis.

**Table XIII. Hepatic Failure Patient Samples**  
**Evaluated by the HPLC-PC and FPIA Methods**

Patient	Apparent Digoxin Concentration ng/mL	
	HPLC-PC	FPIA
1	ND	0.6
2	ND	0.2
3	ND	0.6
4	ND	0.5
5	ND	ND
6	ND	ND
7	ND	0.2
8	ND	ND
9*	0.62	1.2
	1.06	1.3

\* Digitalized

ND = none detected.

Liver Function Test (normal range)	Patient values
Serum Bilirubin Total (2-23 $\mu$ mole/L)	normal - 344
Alkaline Phosphatase (30-110 U/L)	normal - 610
Aspartate Aminotransferase (5-47 U/L)	63 - 223
$\gamma$ -Glutamyl Transpeptidase (5-55 U/L)	122 - 393
	(one patient at 1856)

Using RIA methods, DLIS has been reported in hepatic failure patient serum samples (DiPiro 1980; Greenway 1985; Nanji 1985, 1986; Yang 1988). As previously described, DLIS interference with the FPIA method is less than with RIA methods in hepatic failure patient samples (Greenway 1985; Nanji 1985).

For the undigitalized patients, the HPLC-PC assay was consistently blank while the FPIA indicated apparent digoxin levels as high as 0.6 ng/mL. This indicates that whatever gave the false positive values with the FPIA did not affect the HPLC-PC assay and that the HPLC-PC method would more accurately estimate digoxin levels in these patients. In hepatic failure patients, Nanji and Greenway (1985) reported apparent digoxin levels of 0.2 ng/mL or less with the FPIA while RIA levels as great as 1.1 ng/mL were observed. The FPIA values of 0.6 ng/mL reported here may therefore represent significantly high levels of DLIS. Analysis of these samples by an RIA method was not possible due to the limited sample size.

For the digitalized patient, the FPIA results were higher than those for the HPLC-PC assay. This difference is possibly due to interference from metabolites or endogenous compounds with the FPIA. The HPLC-PC assay is unaffected by the metabolites of digoxin and endogenous compounds that were present in the undigitalized hepatic failure patient samples. Therefore, the HPLC-PC results probably represent the true digoxin concentration in these samples.

### 3.7.4 Umbilical Cord Blood

A total of 17 mixed umbilical cord blood samples from 11 patients were obtained and assayed by both HPLC-PC and FPIA methods. The results are shown in Table XIV.

**Table XIV. Mixed Umbilical Cord Blood Samples  
Evaluated by the HPLC-PC and FPIA Methods**

Patient	Apparent Digoxin Concentration ng/mL	
	HPLC-PC	FPIA
1	ND	0.4
	ND	0.3
2	ND	0.3
	ND	0.4
	ND	0.4
3	ND	ND
	ND	0.2
4	ND	0.2
5	ND	0.2
	ND	0.2
6	ND	0.3
7	ND	0.4
8	ND	0.2
9	ND	ND
	ND	0.6
10	ND	0.3
11	ND	0.2

ND = none detected.

The medication profiles for the patients involved in this part of the clinical study were not available.

Digoxin-like immunoreactive substances have been found in umbilical cord blood (Besch 1976; Diamandis 1985; Kelly 1981; Ng 1985; Pudek 1983a, 1983b; Scherrmann 1986a, 1986b; Yatscoff 1984). Reports in the literature (Gonzalez 1987; Koren 1988) suggest that DLIS levels in umbilical cord blood

(both venous and arterial blood) were significantly greater than in maternal venous blood.

For the 17 cord blood samples assayed, none indicated digoxin was present at levels above the sensitivity of the HPLC-PC assay (0.5 ng/mL). All but one sample for patient 9 gave FPIA results less than 0.5 ng/mL.

The FPIA has been reported to give lower apparent digoxin levels than the RIA in the presence of DLIS (Greenway 1985; Nanji 1985). Comparison of FPIA and RIA levels reported (Nanji 1985) suggest that even when low DLIS levels are found with the FPIA (0.2 ng/mL or less) that significant interference with the RIA may result (up to 1.1 ng/mL). The low apparent digoxin FPIA values observed here may correspond to relatively high levels with the RIA. It was not possible to evaluate these samples by both the FPIA and RIA methods due to the limited sample size available. Since no interference was found with the HPLC-PC assay, it is at least as unaffected by endogenous compounds as the FPIA in this patient group as represented by these samples.

#### 4. SUMMARY AND CONCLUSIONS

The results obtained for cross-reactivity of the metabolites of digoxin with six RIA kits (Table II) show there is extensive cross-reactivity to the digoxigenin moiety as was expected (Butler 1978; Valdes 1984). Higher cross-reactivity was demonstrated with one RIA to dihydrodigoxin (46%) and dihydrodigoxigenin (22%) than previously reported. Furthermore, interference from digoxin metabolites with the FPIA (Table III) was shown and may be partially due to the protein precipitation step (Erickson 1984; Porter 1984; Skogen 1987) which will cause the digitoxose sugars to be removed from digoxin and its metabolites prior to analysis. The cross-reactivity of dihydrodigoxigenin reported with the FPIA is significantly greater than that observed with the RIA methods.

Pre-column derivatization of digoxin and its metabolites with 3,5-dinitrobenzoyl chloride (Fujii 1983) followed by HPLC analysis with electrochemical detection was demonstrated (section 3.3). To date, analysis of 3,5-DNB digoxin using HPLC with electrochemical detection has not been reported in the literature. A maximum sensitivity of 0.394 ng digoxin was observed using dual electrode detection in the redox mode. Partial resolution between 3,5-DNB digoxin and 3,5-DNB dihydrodigoxin was also reported. Problems encountered with derivatization of small (ng) quantities of digoxin resulted in a significant loss in

sensitivity making this method impractical for use with clinical samples.

A post-column fluorogenic HPLC assay using concentrated HCl and dehydroascorbic acid derivatization was developed. Hexane, delivered by a second HPLC pump, was used to displace the concentrated HCl from a pressure vessel allowing for reliable and relatively pulse-free flow of acid into the post-column reactor. Dehydroascorbic acid was added to the aqueous portion of the HPLC mobile phase. The chromatographic column separated digoxin from its metabolites prior to derivatization (Figure 13) allowing for quantitation of digoxin in digitalized patient samples where metabolites may be present.

Numerous steroids have been reported to cross-react with digoxin antisera (Diamandis 1985; Longerich 1988; Matthewson 1988). Steroid samples evaluated by the HPLC-PC assay in this study either do not elute from the HPLC system or do not produce a fluorescent product under these conditions. This indicates that the HPLC-PC assay developed here would be able to quantitate digoxin in the presence of the steroids tested.

Comparison of the HPLC-PC and FPIA methods for digitalized patient samples shows higher mean digoxin levels by the FPIA assay which is possibly due to the inclusion of digoxin metabolites in the total assay value.

Evaluation of undigitalized patient samples from groups where high DLIS levels have been reported shows that the

HPLC-PC assay gives fewer false positive results than the FPIA. In the presence of DLIS, the FPIA assay has been reported to give considerably lower apparent digoxin levels than the RIA (Greenway 1985; Nanji 1985). For a number of the hepatic failure and umbilical cord blood samples, apparent digoxin values ranging from 0.4 to 0.6 ng/mL were obtained with the FPIA. Taking into consideration the difference in sensitivity to DLIS reported for the FPIA and RIA methods, the false positive values obtained here using the FPIA may represent relatively high levels of interfering compounds. The HPLC-PC assay was unaffected by the endogenous compounds responsible for the false positive FPIA values obtained here. This indicates that the HPLC-PC assay developed here is unaffected by the endogenous compounds giving the false positive FPIA values and therefore would be able to specifically quantitate digoxin in these patient samples.



## 5. REFERENCES

- Abel R.M., Lunchi R.L., Peskin G.W., Conn H.L., Miller C.D.  
*J. Pharmacol. Exp. Ther.* 150:463 (1965).
- Aderjan R., Buhr H., Schmidt G. *Arch. Toxicol.* 42:107 (1979).
- Akera T. *Handbook Exp. Pharmacol.* 56/I:287 (1981).
- Akera T., Brody T.M. *Trends Pharmacol. Sci.* 6:296 (1985).
- Andersson K.E., Bertler A., Wettrell G. *Acta Paediatr. Scand.* 64:497 (1975).
- Antman E.M., Smith T.W. *Annu. Rev. Med.* 36:357 (1985).
- Applefeld M.M., Roffman D.S. *Am. J. Med.* 80(suppl 2B): 40 (1986).
- Aronson J.K. *Clin. Pharmacokinet.* 5:137 (1980).
- Aronson J.K. *Clin. Sci.* 64:253 (1983).
- Ashley J.J., Brown B.T., Okita G.T., Wright S.E. *J. Biol. Chem.* 232:315 (1958).
- Balzan S., Clerico A., De Chicca M.G., Montali U., Ghione S. *Clin. Chem.* 30:450 (1984).
- Barbarash R.A. *Eur. J. Clin. Pharmacol.* 27:125 (1984).
- Bauersfeld W., Ratge D., Knoll E., Wisser H. *J. Clin. Chem. Clin. Biochem.* 24:185 (1986).
- Beasley M.W., Skierkowski P., Cleary R.W., Jones A.B., Kibbe A.H. *J. Pharm. Sci.* 72:505 (1983).
- Beerman B., Hellstrom K., Rosen A. *Clin. Sci.* 43:507 (1972).
- Belz G.G., Aust P.E., Munkes R. *Lancet* 1:844 (1981a).
- Belz G.G. *Handbook Exp. Pharmacol.* 56/I:95 (1981b).
- Belz G.G., Doering W., Munkes R., Matthews J. *Clin. Pharmacol. Ther.* 33:410 (1983).
- Belz G.G., Wistuba S., Matthews J.H. *Clin. Pharmacol. Ther.* 39:65 (1986).

- Ben-Chetrit E., Ackerman Z., Elaikim M. *Am. J. Med. Sci.* 289:114 (1985).
- Besch H.R., Watanabe A.M. *Clin. Chem.* 21:1815 (1975).
- Besch H.R., Huffert S., Lake M., Hurwitz R., Watanabe A.M. *Clin. Chem.* 22:1168 (1976).
- Bianchi P. *Clin. Chem.* 32:2099 (1986).
- Bjornsson T.D. *Clin. Pharmacokinet.* 11:164 (1986).
- Blaustein M.P. *Trends Pharmacol. Sci.* 6:289 (1985).
- Bockbrader H.N., Reuning R.H. *J. Chromatogr.* 310:85 (1984).
- Boink A.B.T.J., Kruyswijk H.H., Willebrands A.F., Maas A.H. *J. Clin. Chem. Clin. Biochem.* 15:261 (1977).
- Boone J., Griffin C., Shaw W. *Clin. Chem.* 23:2180 (1977).
- Bourgoignie J.H.J., Hwang K.H., Espinel C., Klahr S., Bricker N.S. *J. Clin. Invest.* 51:1514 (1972).
- Bowman K. *Acta Med. Scand.* 214:353 (1983).
- Braquet P., Senn N., Robin J.-P., Esanu A., Godfraind T., Garay R. *Pharmacol. Res. Commun.* 18:227 (1986a).
- Braquet P., Senn N., Fagoo M., Garay R., Robin J.-P., Esanu A., Chabrier E., Godfraind T. *C. R. Acad. Sci. (Paris)* 302:443 (1986b).
- Brilmyer G.H., Lamey S.C., Maloy J.T. *Anal. Chem.* 47:2304 (1975).
- Brock A. *Acta Pharmacol. Toxicol.* 38:267 (1976).
- Brooks M.A. *Bioelectrochem. Bioenerg.* 10:37 (1983).
- Brown B.T., Stafford A., Wright S.E. *Br. J. Pharmacol.* 18:311 (1962).
- Brown D.D., Dorman J.C., Abraham G.N., Lewis K., Dixon K. *Clin. Pharmacol. Ther.* 20:395 (1976).
- Brown D.D., Juhl R.P., Warner S.L. *Circulation* 58:165 (1978).
- Brunk S.D., Malmstadt H.V. *Clin. Chem.* 23:1054 (1977).
- Buckalew V.M., Gruber K.A. *Annu. Rev. Physiol.* 46:343 (1984).

- Burgess C.D., Crane J. *Eur. J. Clin. Pharmacol.* 29:685 (1986).
- Buss D.C., Routledge P.A. *J. Pharm. Pharmacol.* 37:669 (1985).
- Bussey H.I. *Am. Heart J.* 104:289 (1982).
- Butler V.P. *J. Pharmacol. Exp. Ther.* 29:103 (1978).
- Butler V.P. in "Radioimmunoassay of Drugs and Hormones in Cardiovascular Medicine," Albertini A., DaPrada M., Peskar B.A. (Editors), North-Holland Biomedical Press, Elsevier, p. 47 (1979).
- Butler V.P., Tse-Eng D., Lindenbaum J., Kalman S.M., Preibisz J.J., Rund D.G., Wissel P.S. *J. Pharmacol. Exp. Ther.* 221:123 (1982).
- Cairncross C., Ackermann C. *Int. J. Pharmaceut.* 27:361 (1985).
- Caldwell R.W., Nash C.B. *J. Pharmacol. Exp. Ther.* 204:141 (1978).
- Castillo-Ferrando J.R., Garcia M., Carmona J. *Lancet* 2:368 (1980).
- Catenazzo G., Camisasca E., Ghirardi P., Marzo A. *Arzneim.-Forsch. Drug Res.* 351:852 (1985).
- Caudill W.L., Bigelow J.C., Wightman R.M. *Neurochem. Res.* 10:319 (1985).
- Choay P., Coraboeuf E., Deroubaix E. *Eur. J. Pharmacol.* 50:317 (1978).
- Christenson R.H., Hammond J.E., Hall J.H., Bustrack J.A. *Clin. Chim. Acta* 120:13 (1982).
- Christenson R.H., Studenberg S.D., Beck-Davis S., Sedor F.A. *Clin. Chem.* 33:606 (1987).
- Clark D.B., Kalman S.M. *Drug Metab. Dispos.* 2:148 (1974).
- Clerico A., Boldrini A., Balzan S., Biver P., Fantoni M. *Biol. Neonate* 47:217 (1985).
- Clerico A. *J. Nucl. Med. Allied Sci.* 31:341 (1987).
- Clerico A., Cambi A., Chicca M.G.D., Cecchini L., Giaconi S. *Clin. Chem.* 34:215 (1988a).

- Clerico A., Giampietro O., Gregori G., Chicca M.G.D., Bertoli S., Miccoli R., Navalesi R. *Clin. Chem.* 34:554 (1988b).
- Cloix J.F., Crabos M., Grichois M.L., Meyer P. *Can. J. Physiol. Pharmacol.* 65:1522 (1987).
- Closson R.G. *Arch. Neurol.* 40:386 (1983).
- Cohen S.N. *Pediatrics* 70:494 (1982).
- Craver J.L., Valdes R. *Ann. Intern. Med.* 98:483 (1983).
- Croteau D., Vallee F., Bergeron M.G., LeBel M. *J. Chromatogr.* 419:205 (1987).
- Dandliker W.B., Schapiro H.C., Meduski J.W., Alonso R., Feigen G.A., Hamrick J.R. *Immunochemistry* 1:165 (1964).
- D'Arcy P.F. *Pharmacy Int.* 5:163 (1984).
- D'Arcy P.F. *Pharmacy Int.* 6:289 (1985).
- Dasgupta A., Yeo K.T., Malik S., Sandu P., Ahmad S., Kenny M. *Biochem. Biophys. Res. Commun.* 148:623 (1987).
- Dasgupta A., Malik S., Ahmad S., Kenny M. *Biochem. Biophys. Res. Commun.* 152:1435 (1988).
- Davis G.C., Kissinger P.T., Shoup R.E. *Anal. Chem.* 53:156 (1981).
- Davydov V.Y., Gonzalez M.E., Kiselev A.V. *J. Chromatogr.* 248:49 (1982).
- Desta B., McErlane K.M. *J. Pharm. Sci.* 71:777 (1982a).
- Desta B., McErlane K.M. *J. Pharm. Sci.* 71:1018 (1982b).
- Desta B. *J. Chromatogr.* 421:381 (1987).
- De Wardener H.E. *Clin. Sci. Mol. Med.* 53:1 (1977).
- De Wardener H.E., MacGregor G.A. *Lancet* 26:1450 (1982a).
- De Wardener H.E., Clarkson E.M. *Clin. Sci.* 63:415 (1982b).
- Diamandis E.P., Papanastasiou-Diamandis A., Soldin S.J. *Clin. Biochem.* 18:48 (1985).
- DiPiro J.T., Cote J.R., DiPiro C., Bustrack J.A. *Am. J. Hosp. Pharm.* 37:1518 (1980).

- Dobkin J.F., Saha J.R., Butler V.P., Neu H.C., Lindenbaum J. *Science* 220:325 (1983).
- Doherty J.E., Flannigan W.T., Murphy M.L., Bulloch R.T., Dalrymple G.L., Beard O.W., Perkins W.H. *Circulation* 42:867 (1970).
- Doherty J.E., Kane J.J. *Annu. Rev. Med.* 26:159 (1975).
- Doherty J.E. *Cardiology* 72:225 (1985).
- Drost R.H., Plomp T.A., Teunissen A.J., Maas A.H.J., Maes R.A.A. *Clin. Chim. Acta.* 79:557 (1977).
- Eastland C.J., Lawday D.P., Sellwood E.H.B. *J. Pharm. Pharmacol.* 4:811 (1952).
- Ebara H., Suzuki S., Nagashima K., Shimano S., Kuroume T. *J. Pediatr.* 108:760 (1986a).
- Ebara H., Suzuki S., Nagashima K., Koizumi T., Nishida A., Kanbe Y., Kuroume T. *Pediatr. Res.* 20:28 (1986b).
- Eichhorst O., Hinderling P.H. *J. Chromatogr.* 224:67 (1981).
- Endou K., Sawanobori T., Adaniya H., Hiraoka M. *Jpn. Heart J.* 23(Suppl):95 (1982).
- Erickson K.A., Green P.J. *Clin. Chem.* 30:1225 (1984).
- Eriksen P.B., Andersen O. *Clin. Chem.* 25:169 (1978).
- Eriksson B.M., Tekenbergs L. *J. Chromatogr.* 223:401 (1981b).
- Eriksson F., Carlmark B., Jogestrand T., Sundqvist K. *Scand. J. Clin. Lab. Invest.* 41:457 (1981a).
- Fagoo M., Braquet P., Robin J.-P., Esanu A., Godfraind T. *Biochem. Biophys. Res. Commun.* 134:1064 (1986).
- Falkner F.C. *Biomed. Mass Spectrom.* 8:43 (1981).
- Fichtl B., Doering W., Seidel H. *Int. J. Clin. Pharmacol. Ther. Toxicol.* 21:229 (1983).
- Flasch H., Diembeck W. *Handbook Exp. Pharmacol.* 56/I:27 (1981).
- Fleet B., Little J.C. *J. Chromatogr. Sci.* 12:747 (1974).
- Frewin D.B., Marmanidis H., Lehmann D.R. *Aust. N.Z. J. Med.* 16:241 (1986).

- Friedman H.S., Chen T-S. *Am. Heart J.* 104:72 (1982).
- Friedman P.L., Smith T.W. *Int. J. Cardiol.* 3:237 (1983).
- Frye R., Mathews S.E. *Clin. Chem.* 33:629 (1987).
- Fujii Y., Oguri R., Mitsuhashi A., Yamazaki M. *J. Chromatogr. Sci.* 21:495 (1983).
- Fullerton D.S., Pankaskie M.C., Ahmed K., From A.H.L. *J. Med. Chem.* 19:1330 (1976).
- Gault M.H., Jeffrey J.R., Chirito E., Ward L.L. *Nephron* 17:161 (1976).
- Gault M.H., Charles J.D., Sugden D.L., Kepkay D.C. *J. Pharm. Pharmacol.* 29:27 (1977).
- Gault M.H., Sugden D., Maloney C., Ahmed M., Tweedale M. *Clin. Pharmacol. Ther.* 25:499 (1979).
- Gault M.H., Kalra J., Longerich L., Dawe M. *Clin. Pharmacol. Ther.* 31:695 (1982).
- Gelbart A., Thomas R. *J. Med. Chem.* 21:284 (1978).
- George C.F. *Br. Med. J.* 284:291 (1982).
- Gfeller J.C., Fry G., Frei R.W. *J. Chromatogr.* 142:217 (1977).
- Gibson T.P., Nelson H.A. *Clin. Pharmacol. Ther.* 27:219 (1980).
- Gilman A.G., Goodman L.S., Gilman A. (Editors) "The Pharmacological Basis of Therapeutics," 6th edn., MacMillan Publishing Co., Inc., New York, USA, p. 1684, 733, 735. (1988).
- Gold H., Modell W., Price L. *Arch. Intern. Med.* 50:766 (1932).
- Gonzalez A.R., Phelps S.J., Cochran E.B., Sibai B.M. *Am. J. Obstet. Gynecol.* 157:660 (1987).
- Gortner L., Hellenbrecht D. *Clin. Chem.* 31:155 (1985).
- Grantham J.J., Edwards R.M. *J. Lab. Clin. Med.* 103:333 (1984).
- Graves S., Brown B., Valdes R. *Clin. Chem.* 29:1166 (1983a).

Graves S., Brown B., Valdes R. *Ann. Intern. Med.* 99:604 (1983b).

Graves S.W., Valdes R., Brown B.A., Knight A.B., Craig H.R. *J. Clin. Endocrinol. Metab.* 58:748 (1984).

Graves S.W., Sharma K., Chandler A.B. *Clin. Chem.* 32:1506 (1986).

Greenberg M.S., Mayer W.J. *J. Chromatogr.* 169:321 (1979).

Greenblatt D.J., Duhme D.W., Koch-Weser J., Smith T.W. *Pharmacology* 14:182 (1976).

Greenway D.C., Nanjii A.A. *Clin. Chem.* 31:1078 (1985).

Griffin J.F., Rohrer D.C., Ahmed K., From A.H.L., Hashimoto T., Rathore H., Fullerton D.S. *Mol. Pharmacol.* 29:270 (1986).

Gundert T.W., Linde H.H.A. *Handbook Exp. Pharmacol.* 56/I:13 (1981).

Gundert-Remy U., Weber E. *Handbook Exp. Pharmacol.* 56/I:83 (1981).

Gusdon J.P., Buckalew V.M., Hennessy J.F. *Am. J. Obstet. Gynecol.* 150:83 (1984).

Hamlyn J.M., Schaeffer R.R.J., Levinson P.D., Hamilton B.P., Kowarski A.A., Blaustein M.P. *Nature* 300:650 (1982).

Hansell J.R. *Am. J. Clin. Pathol.* 72:341 (1979).

Hastreiter A.R., Van Der Horst R.L. *Am. J. Cardiol.* 52:330 (1983).

Haustein K.O. *Pharmacol. Ther.* 18:1 (1983).

Heazlewood V.J., Heazlewood R.L., Jellett L.B. *Ann. Intern. Med.* 100:618 (1984).

Henry R.J. in "Clinical Chemistry. Principles and Techniques", Hoeber Medical Division, Harper and Row Publishing, p. 160 (1964).

Hnatowich M., Labella F. *Eur. J. Pharmacol.* 106:567 (1984).

Hoffman B.F., Bigger J.T. in "The Pharmacological Basis of Therapeutics", 6th edn., Gilman A.G., Goodman L.S., Gilman A. (Editors), MacMillan Publishing Co., Inc., New York, U.S.A., p. 729 (1980).

- Holly J.M.P., Makin H.L.J. *Anal. Biochem.* 128:257 (1983).
- Holtzman J.L., Shafer T.B., Erickson R.R. *Clin. Chem.* 20:1194 (1974).
- Hossie R.D., Loo J.C.K., McGilveray I.J., Jordan N. *Can. J. Pharm. Sci.* 12:2 (1977).
- Hu O.Y.P., Gfeller E., Perrin J.H., Curry S.H. *J. Pharm. Pharmacol.* 38:172 (1986).
- Humbert H., Denouel J., Keller H.P. *J. Chromatogr.* 422:205 (1987).
- Iisalo E. *Clin. Pharmacokinet.* 2:1 (1977).
- Isaksson K., Kissinger P.T. *J. Chromatogr.* 419:165 (1987).
- Jelliffe R.W., Blackenhorn D.H. *J. Chromatogr.* 12:268 (1963).
- Jelliffe R.W., Stephenson R.G. *Am. J. Clin. Pathol.* 51:347 (1969).
- Jensen K.B. *J. Pharm. Pharmacol.* 4:783 (1952).
- Johnson B.V., Bustrack J.A., Urbach D.R., Hull J.H., Marwaha R. *Clin. Pharmacol. Ther.* 36:724 (1984).
- Johnson B.F., Lindenbaum J., Budnitz E., Marwaha R. *Clin. Pharmacol. Ther.* 39:306 (1986).
- Jolley M.E. *J. Anal. Toxicol.* 5:236 (1981).
- Kadish K.M., Spiehler V.R. *Anal. Chem.* 47:1714 (1975).
- Kanan R., Chan K.M., Dietzler D.N. *Clin. Chem.* 33:446 (1987).
- Kelly J.G., Naider R.K., O'Malley K. *Br. Med. J.* 282:1241 (1981).
- Kennedy H.L., Sprague M.K., Redd R.M., Wiens R.D., Blum R.I., Buckingham T.A. *Am. Heart J.* 111:667 (1986).
- Keys P.W. in "Applied Pharmacokinetics. Principles of Therapeutic Drug Monitoring", Evans W.E., Schentag J.J., Jusko W.J. (Editors), Applied Therapeutics Inc., San Francisco, USA, p. 319 (1980).
- Kibbe A.H., Araujo I.E. *J. Pharm. Sci.* 62:1703 (1973).



- Kieval R.S., Butler V.P., Derguini F., Bruening R.C., Rosen M.R. *J. Am. Coll. Cardiol.* 11:637 (1988).
- Kim P.W., Krasula R.W., Soyka L.F., Hastreiter A.R. *Circulation* 52:1128 (1975).
- Kissinger P.T., Bratin K., Davis G.C., Pachla L.A. *J. Chromatogr. Sci.* 17:137 (1979).
- Klein H.O., Kaplinsky E. *Am. J. Cardiol.* 50:894 (1982).
- Klein H.O., Kaplinsky E. *Drugs* 31:185 (1986).
- Klingmuller D., Weiler E., Kramer H.J. *Klin. Wochenschr.* 60:1249 (1982).
- Koren G., Soldin S., MacLeod S.M. *J. Cardiovasc. Pharmacol.* 5:443 (1983).
- Koren G., Farine D., Maresky D., Taylor J., Heyes J., Soldin S., MacLeod S. *Clin. Pharmacol. Ther.* 36:759 (1984).
- Koren G., Farine D., Grundmann H., Heyes J., Soldin S., Taylor J., MacLeod S.M. *Dev. Pharmacol. Ther.* 11:82 (1988).
- Kramer H.J., Backer A., Schurmann J., Weiler E., Klingmuller D. *Regul. Pept. Suppl* 4:124 (1985a).
- Kramer H.J., Pennig J., Klingmuller D., Kipnowski J., Glanzer K., Dusing R. *Nephron* 40:297 (1985b).
- Kramer P., Kothe E., Saul J., Scheler F. *Eur. J. Clin. Invest.* 4:53 (1974).
- Kramer W.G., Bathala M.S., Reuning R.H. *Res. Commun. Chem. Pathol. Pharmacol.* 14:83 (1976).
- Kramer W.G., Kinnear N.L., Morgan H.K. *Clin. Chem.* 24:155 (1978).
- Kramer W.G., Kolibash A.J., Lewis R.P., Bathala M.S., Visconti J.A., Reuning R.H. *J. Pharmacokin. Biopharm.* 7:47 (1979).
- Krause W. *J. Chromatogr.* 181:67 (1980).
- Kreuzig F., Frank J. *J. Chromatogr.* 218:615 (1981).
- Kroening B.H., Weintraub M. *Clin. Chem.* 22:1732 (1976).
- Krstulovic A.M., Bertani-Dziedzic L., Bautista-Cerqueira S., Gitlow S.E. *J. Chromatogr.* 227:379 (1982).

Kubasik N.P., Norkus N.S., Sine H.E. *Clin. Biochem.* 7:307 (1974a).

Kubasik N.P., Schauseil S., Sine H.E. *Clin. Biochem.* 7:206 (1974b).

Kubasik N.P., Brody B.B., Barold S.S. *Am. J. Cardiol.* 36:975 (1975).

Kuczala Z.J., Ahluwalia G.S. *Clin. Chem.* 22:193 (1976).

Kwong E. in "Development of a High-Performance Liquid Chromatographic Assay for Digoxin in Plasma Using Post-Column Fluorogenic Derivatization", Vancouver, B.C., Canada, University of British Columbia, Dissertation (1984).

Kwong E., McErlane K.M. *J. Chromatogr.* 377:233 (1986a).

Kwong E., McErlane K.M. *J. Chromatogr.* 381:357 (1986b).

Lage G.L., Spratt J.L. *J. Pharmacol. Exp. Ther.* 152:501 (1966).

Landon J., Kamel R.S. in "Immunoassays for the 80's," Voller A., Barlett A., Bidwell D. (Editors), University Park Press, Baltimore, Maryland, p. 91 (1981).

Lang R.E., Tholken H., Ganten D., Luft F.C., Ruskoaho H., Unger T. *Nature* 314:264 (1985).

Larson J.H., Beckala H., Homberg H.A. *Clin. Chem.* 23:1792 (1977).

Leahey E.B., Reiffel J.A., Giardina E.G.V., Bigger J.T. *Ann. Intern. Med.* 92:605 (1980).

Leelavathi D.E., Soffer E.F., Dressler D.E., Knowles J. *J. Pharm. Sci.* 75:421 (1986).

Lenz P.H., Wien G.H., Toth J.M. *Clin. Chem.* 21:1169 (1975).

Leroy P., Nicolas A. *J. Pharm. Biomed. Anal.* 5:477 (1987).

Lichey J., Rietbrock N., Borner K. *Int. J. Clin. Pharmacol. Biopharm.* 17:61 (1979).

Linday L., Drayer D.E. *Clin. Chem.* 29:175 (1983).

Lindenbaum J., Mellow M.H., Blackstone M.O., Butler V.P. *New Engl. J. Med.* 285:1344 (1971).

- Lindenbaum J. *Clin. Pharmacol. Ther.* 17:296 (1975).
- Lindenbaum J., Tse-Eng D., Butler V.P., Rund D. *Am. J. Med.* 71:67 (1981).
- Longerich L., Brent D.A., Johnson R.L., Vasdev S., Gault M.H. *Res. Commun. Chem. Pathol. Pharmacol.* 59:383 (1988).
- Loo J.C.K., McGilveray I.J., Jordan N. *Res. Commun. Chem. Pathol. Pharmacol.* 17:497 (1977).
- Loo J.C.K., McGilveray I.J., Jordan N. *J. Liq. Chromatogr.* 4:879 (1981).
- Lowenstein J.M. *Circulation* 31:228 (1965).
- Luchi R.J., Conn H.L. *Prog. Cardiovasc. Dis.* 7:336 (1965).
- Luchi R.W., Gruber J.W. *Am. J. Med.* 45:322 (1968).
- Lund H. in "Organic Electrochemistry", Baizer M.M., Lund H. (Editors), Second Edition, Marcel Dekker, Inc., New York, NY, p. 185, 303 (1983).
- Lund W., Hannisdal M., Greibrook T. *J. Chromatogr.* 173:249 (1979).
- MacKinney A.A., Burnett G.H., Conklin R.L., Watson G.W. *Clin. Chem.* 21:857 (1975).
- Magnusson J.O., Bergdahl B., Bogentoft C., Jonsson U.E. *Br. J. Clin. Pharmacol.* 14:284 (1982a).
- Magnusson J.O., Bergdahl B., Bogentoft C., Jonsson U.E., Tekenbergs L. *Biopharm. Drug Dispos.* 3:211 (1982b).
- Malcolm A.D., Leung F.Y., Fuchs J.C.A., Duarte J.E. *Clin. Pharmacol. Ther.* 21:567 (1977).
- Malini P.L., Marata A.M., Stocchi E., Ambrosioni E. *Clin. Chem.* 28:2445 (1982).
- Malini P.L., Stocchi E., Negroni S., Ambrosioni E., Magnani B. *Curr. Ther. Res.* 33:646 (1983).
- Malini P.L., Stocchi E., Feliciangeli G., Buscaroli A., Bonomini V., Ambrosioni E. *Clin. Exp. Pharmacol. Physiol.* 12:115 (1985).
- Manninen V., Nyberg L. *Handbook Exp. Pharmacol.* 56/II:299 (1981).

- Manolas E.G., Hunt D., Sloman G. *Aust. N.Z. J. Med.* 10:426 (1980).
- Marcus F.I., Buckhalter L., Cuccia C., Pavolich S., Kapadia G.G. *Circulation* 34:865 (1966).
- Marcus F.I., Ryan J.N., Stafford M.G. *J. Lab. Clin. Med.* 85:610 (1975).
- Margot P.A., Finkle B.S., Peat M.A. *Proc. West. Pharmacol. Soc.* 26:393 (1983).
- Mason D.T., Lee G. *Handbook Exp. Pharmacol.* 56/II:207 (1981).
- Matthewson B.E. in "Digoxin-Like Immunoreactive Substances in the Neonate", Vancouver, B.C., Canada, University of British Columbia, Dissertation (1988).
- Mazzo D.J., Forbes K.A., Brooks M.A. *J. Chromatogr.* 367:405 (1986).
- McCarthy R.C. *Clin. Chem.* 31:1240 (1985).
- McKercher H.G., Mikhael N.Z., De Gouffe M., Lukaszewski T., Peel H.W. *Res. Commun. Chem. Pathol. Pharmacol.* 52:141 (1986).
- Meering P.G., Baumann R.A., Zijp J.J., Maes R.A.A. *J. Chromatogr.* 310:159 (1984).
- Mills I.H. *Br. Med. J.* 289:210 (1984).
- Morais J.A., Zlotecki R.A., Sakmar E., Stetson P.L., Wagner J.G. *Res. Commun. Chem. Pathol. Pharmacol.* 31:285 (1981).
- Morales A., Richter P., Toral M.I. *Analyst* 112:965 (1987).
- Mousty C., Pouillen P., Martre A., Mousset G. *J. Colloid Interface Sci.* 113:521 (1986).
- Movselli P.L. (Editor) "Drug Disposition During Development", Spectrum Publications Inc., New York, USA, p.394 (1977).
- Muller H., Brauer H., Resch B. *Clin. Chem.* 24:706 (1978).
- Murphy D.J., Bremner W.F., Haber E., Butler V.P., Smith T.W., Gaum W.E. *Pediatrics* 70:472 (1982).
- Myerson R.M. in "Topics in Medicinal Chemistry", vol. 1, Rabinowitz J.L., Myerson R.M. (Editors), J. Wiley and Sons, New York, USA, p. 303 (1967).

- Nachtmann F., Spitzzy H., Frei R.W. *J. Chromatogr.* 122:293 (1976a).
- Nachtmann F., Spitzzy H., Frei R.W. *Anal. Chem.* 48:1576 (1976b).
- Nanji A.A., Greenway D.C. *Br. Med. J.* 290:432 (1985).
- Nanji A.A., Greenway D.C. *J. Clin. Pharmacol.* 26:152 (1986).
- Ng P.K., LeGatt D., Coates J., Collins-Nakai R.L. *Am. J. Hosp. Pharm.* 42:1977 (1985).
- Nyberg L., Anderson K-E., Bertler A. *Acta Pharm. Suec.* 11:459 (1974).
- Ochs H.R., Bodem G., Greenblatt D.J. *J. Clin. Pharmacol.* 21:396 (1981).
- Ochs H.R., Greenblatt D.J., Verburg-Ochs B. *Clin. Pharmacol. Ther.* 38:595 (1985).
- Oge E.K., Polonus P.A. *Clin. Chem.* 24:1086 (1978).
- Okudaira K., Sawada Y., Sugiyama Y., Iga T., Hanano M. *J. Pharm. Pharmacol.* 38:137 (1986).
- Oldfield P.R., Singer B.E., Toseland P.A. *Clin. Chem.* 31:1246 (1985).
- Osterloh J., Herold S., Pond S. *J. Am. Med. Assn.* 247:1596 (1982).
- Otsuka K., Kaba H., Saito H., Seto K., Yanaga T. *Am. Heart J.* 104:649 (1982).
- Pedersen K.E., Dorph-Pederson A., Hvidt S., Klitgaard N.A., Nielsen-Kudsk F. *Clin. Pharmacol. Ther.* 30:311 (1981).
- Pedersen K.E., Dorph-Pederson A., Hvidt S., Klitgaard N.A., Kjaer K., Nielsen-Kudsk F. *Clin. Pharmacol. Ther.* 32:562 (1982).
- Pedersen K.E., Madsen J.L., Klitgaard N.A., Kjaer K., Hvidt S. *Acta Med. Scand.* 218:229 (1985a).
- Pedersen K.E. *Acta Med. Scand.* 697:S1 (1985b).
- Pekic B., Petrovic S.M., Slavica B. *J. Chromatogr.* 268:237 (1983).

- Pernollet M.G., Ali R.M., Meyer P., Devynck M.A. *J. Hypertens.* 4:S382 (1986).
- Phelps S.J., Cochran E.B., Gonzalez-Ruiz A., Tolley E.A., Hammond K.D., Sibai B.M. *Am. J. Obstet. Gynecol.* 158:34 (1988).
- Pippin S.L., Marcus F.I. *Clin. Chem.* 22:286 (1976).
- Plambeck J.A. in "Electroanalytical Chemistry: Basic Principles and Applications" J. Wiley and Sons, New York, U.S.A., p. 301 (1982).
- Plum J., Daldrup T. *J. Chromatogr.* 377:221 (1986).
- Porter W.H., Haver V.M., Bush B.A. *Clin. Chem.* 30:1826 (1984).
- Pudek M.R., Seccombe D.W., Jacobson B.E., Whitfield M.F. *Clin. Chem.* 29:1972 (1983a).
- Pudek M.R., Seccombe D.W., Whitfield M.F., Ling E. *New Engl. J. Med.* 308:904 (1983b).
- Pudek M.R., Seccombe D.W., Whitfield M.F., Jacobson B.E., Wittman B., King J.F., Taylor G. *Clin. Biochem.* 17:200 (1984).
- Pudek M.R., Seccombe D.W., Jacobson B.E., Humphries K. *Clin. Chem.* 31:1806 (1985).
- Rameis H. *Eur. J. Clin. Pharmacol.* 29:49 (1985).
- Ravel R. *Clin. Chem.* 21:1801 (1975).
- Rawal N., Leung F.Y., Henderson A.R. *Clin. Chem.* 29:586 (1983).
- Reh E., Jork H. *Fresenius Z. Anal. Chem.* 322:365 (1985).
- Reissel P., Manninen V. *Acta Med. Scand.* 668:88 (1982).
- Repke K. in "Proceedings of the First International Pharmacological Meeting", vol. 3, Wilbrandt W. (Editor), Pergamon Press, Oxford, England, p. 47 (1963).
- Repke K.R.H., Schonfeld W. *Trends Pharmacol. Sci.* 5:393 (1984).
- Rietbrock N., Woodcock B.G. *Handbook Exp. Pharmacol.* 56/II:31 (1981).

- Rietbrock N., Woodcock B.G. *Trends Pharmacol. Sci.* 6:267 (1985).
- Riley C.M., Runyan A.K. *J. Pharm. Biomed. Anal.* 5:33 (1987).
- Rosenthal A.F., Vargas M.G., Klass C.S. *Clin. Chem.* 22:1899 (1976).
- Rowson J.M. *J. Pharm. Pharmacol.* 4:814 (1952a).
- Rowson J.M., Dyer F.J. *J. Pharm. Pharmacol.* 4:831 (1952b).
- Rozkovec A., Coltart D.J. *Br. Med. J.* 285:1315 (1982).
- Ryan T.H. in "Electrochemical Detectors: Fundamental Aspects and Analytical Applications", Ryan T.H. (Editor), Plenum Press, New York, U.S.A., p. 113 (1984).
- Sabatka J.J., Brent D.A., Murphy J., Charles J., Vance J., Gault M.H. *J. Chromatogr.* 125:523 (1976).
- Saito Y., Kanemasa Y., Okada M. *Chem. Pharm. Bull.* 18:629 (1970).
- Schafer S.G., Schuhmann G., Doering W., Fichtl B. *Chem. Biol. Interact.* 55:203 (1985).
- Schenck-Gustafsson K., Dahlqvist R. *Br. J. Clin. Pathol.* 11:181 (1981a).
- Schenck-Gustafsson K., Jogestrand T., Norlander R., Dahlqvist R. *Med. Intell.* 305:209 (1981b).
- Schenck-Gustafsson K., Juhlin-Dannfelt A., Dahlqvist R. *Clin. Physiol.* 2:401 (1982).
- Scherrmann J.M., Bourdon T. *Clin. Chem.* 26:670 (1980).
- Scherrmann J.M., Sandouk P., Guedeney X. *Clin. Chem.* 32:97 (1986a).
- Scherrmann J.M., Sandouk P., Guedeney X. *Chest* 89:468 (1986b).
- Schreiber V., Gregorova I., Pribly T., Stepan J. *Endocrinol. Exp.* 15:229 (1981a).
- Schreiber V., Stepan J., Gregorova I., Krejcikova J. *Biochem. Pharmacol.* 30:805 (1981b).
- Schreiber V., Stepan J., Starka L. *Physiol. Bohemoslov.* 30:569 (1981c).

- Schreiber V., Pribyl T., Stepan J., Horky K. *Physiol. Bohemoslov.* 31:83 (1982).
- Seiber J.M., Nelson C.J., Benson J.M. in "Steroid Analysis by HPLC. Recent Applications", Kautsky M.P. (Editor), Marcel Dekker Inc., New York, USA, Chromatographic Series 16:41 (1981).
- Shah J., Mason W.D. *Anal. Lett.* 20:881 (1987).
- Shibanoki S., Imamura Y., Itoh T., Ishikawa K. *J. Chromatogr.* 421:425 (1987).
- Shilo L., Dolev S., Shapiro M.S., Shenkman L. *Isr. J. Med. Sci.* 23:294 (1987).
- Siebeneick H.U., Hoffmann W. *J. Med. Chem.* 21:1310 (1978).
- Silber B., Sheiner L.B., Powers J.L., Winter M.E., Sadee W. *Clin. Chem.* 25:48 (1979).
- Simson G. *Am. Heart J.* 64:401 (1962).
- Skogen W.F., Rea M.R., Valdes R. *Clin. Chem.* 33:401 (1987).
- Smith P., Brown L., Boutagy J., Thomas R. *J. Med. Chem.* 25:1222 (1982).
- Smith R.L. *Clin. Chem.* 33:1697 (1987).
- Smith T.W., Butler V.P., Haber E. *Biochemistry* 9:331 (1970).
- Smith T.W., Haber E. *Pharmacol. Rev.* 25:219 (1973).
- Smith T.W. *J. Pharmacokinet. Biopharm.* 4:197 (1976).
- Smith T.W., Antman E.M., Friedman P.L., Blatt C.M., Marsh J.D. *Prog. Cardiovasc. Dis.* 26:413 (1984a).
- Smith T.W., Antman E.M., Friedman P.L., Blatt C.M., Marsh J.D. *Prog. Cardiovasc. Dis.* 26:495 (1984b).
- Smith T.W., Antman E.M., Friedman P.L., Blatt C.M., Marsh J.D. *Prog. Cardiovasc. Dis.* 27:21 (1984c).
- Soldin S.J. *Clin. Chem.* 32:5 (1986a).
- Soldin S.J., Stephey C., Giesbrecht E., Harding R. *Clin. Chem.* 32:1591 (1986b).
- Somogyi M. *J. Biol. Chem.* 160:69 (1945).



Sonobe T., Hasumi S., Yoshino T., Kobayaski Y., Kawata H., Nagai T. *J. Pharm. Sci.* 69:410 (1980).

Spector R. *J. Clin. Pharmacol.* 19:692 (1979).

Steiness E., Waldorff S., Hansen P.B. *Eur. J. Clin. Pharmacol.* 23:151 (1982).

Sternson L.A., Shaffer R.D. *J. Pharm. Sci.* 67:327 (1978).

Stewart G.A. *Handbook Exp. Pharmacol.* 56/II:189 (1981).

Stewart J.T., Clark S.S. *J. Pharm. Sci.* 75:413 (1986).

Sun L., Spiehler V. *Clin. Chem.* 22:2029 (1976).

Svendsen C.N., Bird E.D. *Psychopharmacology* 90:316 (1986).

Takayanagi K., Jalife J. *Am. J. Physiol.* 250:H567 (1986).

Takiura K., Yuki H., Okamoto Y., Takai H., Honda S. *Chem. Pharm. Bull.* 22:2263 (1974).

Talbot R.G., Nimmo J., Julian D.G., Clark R.A., Nielson J.M.M., Prescott L.F. *Lancet* 11:399 (1973).

Tamm C. in "Proceedings of the First International Pharmacological Meeting", vol. 3, Wilbrandt W. (Editor), Pergamon Press, Oxford, England, p. 11 (1963).

Thomas R., Boutagy J., Gelbart A. *J. Pharm. Sci.* 63:1649 (1974a).

Thomas R., Boutagy J., Gelbart A. *J. Pharmacol. Exp. Ther.* 191:219 (1974b).

Thomas R., Brown L., Boutagy J., Gelbart A. *Circ. Res.* 46(Suppl. 1):1167 (1980).

Tilstone W.J., Semple P.F., Lawson D.H., Boyle J.A. *Clin. Pharmacol. Ther.* 22:389 (1977).

Tjaden U.R., Lankelma J., Poppe H., Muusze R.G. *J. Chromatogr.* 125:275 (1976).

Toseland P.A., Oldfield P.R., Murphy G.M., Lawson A.M. *Ther. Drug Monit.* 10:168 (1988).

Tucker A.R., Ng K.T. *Psychopharmacology* 81:86 (1983).

Tyler V.E., Brady L.R., Robbers J.E. in "Pharmacognosy", 7th edn., Lea and Febiger, Philadelphia, USA, p. 205 (1976).

USP 1985 "The United States Pharmacopeia", 20th edn., United States Pharmacopeial Convention Inc., Rockville, MD, USA, (1985).

Valdes R., Siegfried B. *Clin. Chem.* 29:1166 (1983a).

Valdes R., Graves S.W., Brown B.A., Landt M. *J. Pediatr.* 102:947 (1983b).

Valdes R., Brown B.A., Graves S.W. *Am. J. Clin. Pathol.* 82:210 (1984).

Valdes R., Graves S.W., Becker S.L. *J. Clin. Endocrinol. Metab.* 60:1135 (1985a).

Valdes R. *Clin. Chem.* 31:1525 (1985b).

Vasdev S., Longerich L., Johnson E., Brent D., Gault M.H. *Res. Commun. Chem. Pathol. Pharmacol.* 49:387 (1985).

Vemuri S., Sambamurthy K. *Indian J. Exp. Biol.* 18:669 (1980).

Venkatesh N., Al-Sarraf L., Kannan R., Singh B.N. *J. Pharm. Sci.* 74:1067 (1985).

Venkatesh N., Singh B.N., Al-Sarraf L., Kannan R. *J. Cardiovasc. Pharmacol.* 8:309 (1986).

Vinge E., Ekman R. *Ther. Drug Monit.* 10:8 (1988).

Volke J. *Bioelectrochem. Bioenerg.* 10:7 (1983).

Vorpahl T.E., Coe J.I. *J. Forensic Sci.* 23:329 (1978).

Wagner J.G. *Am. Heart J.* 88:133 (1974).

Wagner J.G., Dick M., Behrendt D.M., Lockwood G.F., Sakmar E., Hees P. *Clin. Pharmacol. Ther.* 33:577 (1983).

Waldorff S., Andersen J.D., Heebott-Nielsen N., Nielsen O.G., Moltke E., Sorensen U., Steiness E. *Clin. Pharmacol. Ther.* 24:162 (1978).

Wallick E.T., Dowd F., Allen J.C., Schwartz A. *J. Pharmacol. Exp. Ther.* 189:434 (1974).

Wang J., Bonakdar M., Morgan C. *Anal. Chem.* 58:1024 (1986).

- Wang J., Golden T., Tuzhi P. *Anal. Chem.* 59:740 (1987).
- Watson E., Tramell P., Kalman S.M. *J. Chromatogr.* 69:157 (1972).
- Weiler E.W., Lach H.J. *Clin. Chim. Acta* 104:337 (1980).
- Weiner C.P., Landas S., Persoon T.J. *Am. J. Obstet. Gynecol.* 157:368 (1987).
- Wellens H.J.J., Gorgels A.P., Braat S.J., Bar F.W., Vanagt E.J., Phaf B. *Am. Heart J.* 100:934 (1980).
- White P.C. *Analyst* 109:677 (1984).
- Wilkins M.R. *Trends Pharmacol. Sci.* 6:286 (1985).
- Withering W., reprinted in *Medical Classics* 2:295 (1937).
- Witherspoon L., Shuler S., Neely H., Sonnemaker R., Alyea K., Gilbert S. *Clin. Chem.* 33:420 (1987).
- Witty T.R., Remers W.A., Besch H.R. *J. Pharm. Sci.* 6:1248 (1975).
- Yang S., Hughes R.D., Williams R. *Hepatology* 8:93 (1988).
- Yannakou L., Diamandis E.P., Souvatzoglou A. *Ther. Drug Monit.* 9:461 (1987).
- Yatscoff R.W., Desjardins P.R.E., Dalton J.G. *Clin. Chem.* 30:588 (1984).
- Yoda A., Yoda S. *Mol. Pharmacol.* 10:494 (1974).
- Yusuf S., Wittes J., Bailey K., Furberg C. *Circulation* 73:14 (1986).
- Zaczek R., Coyle J.T. *J. Neural Transm.* 53:1 (1982).
- Zucker A.R., Lacina S.T., DasGupta D.S., Fozzard H.A., Mehlman D., Butler V.P., Haber E., Smith T.W. *Pediatrics* 70:468 (1982).