DEVELOPMENT OF A HIGH - PERFORMANCE

LIQUID CHROMATOGRAPHIC ASSAY FOR DIGOXIN

IN PLASMA USING POST-COLUMN FLUOROGENIC DERIVATIZATION

by

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ABSTRACT

Digoxin is a cardiac glycoside obtained in purified form from the leaves of <u>Digitalis lanata</u>. The molecule is composed of a sugar portion and an aglycone (genin) portion. Chemically it is the drug of choice for the treatment of congestive heart failure and certain disturbances of cardiac rhythm.

The therapeutic significance of digoxin, and particularly the relatively narrow margin between a thérapeutic and toxic dose, has warranted the development of a specific and sensitive analytical method for their quantitation in biological samples. The principal method employed by most researchers and hospital laboratories for the assay of digoxin is based on the use of a radioimmunoassay (RIA) procedure. Present reports have shown that the digoxin metabolites, digoxigenin mono-digitoxoside, digoxigenin bis-digitoxoside and digoxigenin cross-react extensively in RIA. Dihydrodigoxin cross-reactivity depends on the RIA kit used. It was also shown that the RIA procedure gave false-positive results in specimens taken from non-digitalized renal patients. Such discrepancies raise questions regarding the "true" plasma digoxin concentrations measured by the current RIA method. Therefore, to ascertain the analytical reliability of available assays in measuring digoxin levels, this thesis reports the development of an HPLC separation of digoxin from its metabolites and some commonly co-administered drugs which, when coupled with post-column derivatization, will quantify

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the levels of intact digoxin in plasma.

Essentially this procedure is based on a novel method developed by Gfeller et al. (9). The post-column detection is based on the reaction of hydrochloric acid with the steroid portion of the cardiac glycosides. Fluorescence of the derivative is further enhanced by the addition of a hydrogen peroxide/ ascorbic acid mixture. The fluorogenic reactants are also employed in the method utilized in the United States Pharmacopeia and hence are considered to be reliable.

We have undertaken several modifications to the air segmentation post-column derivatization step developed by Gfeller's group (9). To increase the sensitivity of detection, a non-segmented post-column reactor is used. The glycosides are initially separated on a 15 cm octadecylsilane, 3u packing column, using a combination of methanol, ethanol, isopropanol and water as the mobile phase at a flow rate of 0.3 mL/min. A solution of dehydroascorbic acid and hydrochloric acid are added to the column effluent at 0.23 mL/min to form the fluorophore using a 10 m knitted reactor controlled at a temperature of 79° C. The glycosides react with HCl and dehydroascorbic acid to form the fluorophore which is monitored by a Waters fluorescence detector equipped with a 360nm excitation filter and a 425 nm emission filter.

The quantitation of digoxin in plasma is carried out by the incorporation of an internal standard, digitoxigenin. The method involves the extraction of the compound from plasma with methylene chloride containing 2% propanol. Endogenous substances, commonly

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co-administered drugs, and metabolites of digoxin do not interfere with the method. The total chromatographic time, including the post-column derivatization step, is about 40 minutes. The detection limit of the method is 0.5 ng/mL of digoxin in plasma. The average extraction recovery is 70% over the therapeutic concentration range. Detector response is linear from 0.5 ng/mL to 3.3 ng/mL of plasma.

Using HPLC separation and post-column detection with parallel quantitation by radioimmunoassay, we examined the plasma of digitalized patients. The mean HPLC / RIA ratios are 0.94 ± 0.3 (S.D.) (Acute Care Unit of the University Hospital) and 1.0 ± 0.34 (S.D.) (Vancouver General Hospital). These ratios are comparable to those found by specific HPLC-RIA results reported by other workers. The overall performance demonstrates that this system has the sensitivity, linearity and specificity desired for the determination of drug concentrations in plasma from digitalized patients.

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SYMBOLS AND ABBREVIATIONS

ACU	Health Science Acute Care Hospital
Al	Aluminum dift. for d
A-V	Atrio-ventricular
С	Centigrade
сс	cubic centimeter
CI	Chemical ionization
CM	centimeter
C.V.	coefficient of variation
DCM	dichloromethane
DLI	direct liquid introduction
DRP	digoxin reduction product
ECD	electron capture detector
ELISA	enzyme-linked immunosorbent
EMIT	enzyme multiplied immunoassay technique
ЕТОН	etha no l
g	gram
GC	gas chromatography
H ₂ 0 ₂	hydrogen peroxide
HCI	hydrochloric acid
HFBA	heptafluorobutyric acid
HFBI	heptafluorobutyrylimidazole
HPLC	High-performance liquid chromatography
HPLC-PC	High-performance liquid chromatography-post-column
ID	internal diameter
in or "	inches

INH	isonicotinylhydrazine
Intct	Intercept
Int. Std.	Internal standard
IPA	isopropyl alcohol
к+	potassium ion
LC-MS	liquid chromatography-mass spectrometry
m	meter
М	molar
MBTFA	N-methyl-bis-trifluoroacetamide
МеОН	methanol
mEq	milliequivalent
mg	milligram
MHPLC	micro high-performance liquid chromatography
min(s)	minute(s)
mL	milliliter
mM	millimolar
mm	millimeter
MS	mass spectrometry
mV	millivolts
n	# of samples
n	<pre># of pure solvent in the mixture (equation 2)</pre>
Na ⁺	sodium ion
ng	nanogram
nm	nanometer
OD	outer diameter
ODS	octadecylsilane
₽' or P'e	polarity
pg	picogram

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Pi	solvent polarity parameter of ith term
r	correlation coefficient
RFI	relative fluorescence intensity
RIA	radioimmunoassay
RP	reversed phase
RPM	revolution per minute
Rs	resolution
S.D.	standard deviation
t_{I} and t_{II}	retention time of dihydrodigoxin and digoxin
TLC	thin layer chromatography
u or um	micron
uL	microliter
USP	United States Pharmacopeia
UV	ultraviolet
۷	volts
VGH	Vancouver General Hospital
$w_I^{and} w_{II}^{I}$	bandwidth of the two bands at baseline
Σ	summation
Ø	volume fraction of component of solvent system

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INTRODUCTION

Digoxin is the most popular cardiac glycoside presently used for cardiac insufficiency. Although it is known to be a highly beneficial drug, it is also very potent and exhibits a narrow margin of therapeutic efficacy and safety. The determination of the optimal digoxin dosage in clinical practice is complex. For these reasons, plasma level and dosage schedules are frequently monitored and regulated. Problems with therapeutic efficacy continue to be reported in the literature (1) and many of these are ascribed to the analytical methodology employed.

Although a host of chemical, chromatographic and biological methods have been reported for measurement of digoxin in body fluids, the principal and most sensitive method employed in hospital laboratories is based on the use of radioimmunoassay procedures. Although these methods are sensitive, the antibodies involved have frequently been reported to be nonspecific and are subject to cross reactivity with some of the less cardioactive metabolites of digoxin, as well as with endogenous and exogenous substances with structural similarity to the aglycone portion of the digoxin molecule.

A rapid, specific assay for digoxin using physico-chemical measurement is therefore needed. High performance liquid chromatography (HPLC) combined with ultraviolet (UV) detection methods which are available (2,3) have been successful in facilitating the separation of digoxin from its metabolites, but the

applicability of this method to analysis in biological fluids has not been investigated due to the inherent lack of sensitivity of the UV detector and low absorptivity of digoxin. To achieve the necessary sensitivity required for therapeutic drug monitoring, numerous studies (4-8) have combined HPLC and radioimmunoassay (RIA) methods. Most of the methods described (4-6) either involved the administration of tritiated digoxin to the subjects or the use of tritiated internal standards prior to HPLC separation. These methods are often tedious and are not readily adaptable for routine monitoring of serum samples. The two most recent HPLC-RIA methods (7,8) offer some convenience for the clinical monitoring of digoxin plasma concentration in digitalized patients, however, timed collection of eluates employed in these methods is very critical and may introduce errors in the assay method. An efficient HPLC separation reported for digoxin and its metabolites coupled with highly sensitive post-column derivatization (9) offers a possibility to develop an extremely selective, sensitive and accurate method of analysis for digoxin.

The specific aims of the project were as follows: 1. To develop a highly selective and sensitive HPLC post-column derivatization procedure for the quantitation of therapeutic levels of digoxin without interference from its metabolites and other commonly co-administered drugs.

2. To develop suitable extraction procedures for effective recovery of digoxin from plasma.

3. To include other commonly co-prescribed drugs such as spironolactone, furosemide, quinidine etc. and to determine if these interfere with either the chromatographic process or detection system.

4. To test the precision and accuracy of the developed method using spiked samples in plasma obtained from the blood bank of the hospital and the Red Cross.

5. In collaboration with the hospital laboratories, to assay samples of plasma obtained from digoxin treated patients and to compare the results with those obtained by the hospital RIA method on the same sample.

1. LITERATURE SURVEY

Digitalis glycosides have been used for thousands of years to treat human diseases (10). The modern era of treatment with the glycosides begins with the work of William Withering in the eighteenth century. His papers introduced the successful use of digitalis for the treatment of patients suffering from dropsy (10). The progress in administration of digitalis glycosides can be seen today in the use of digoxin as the rational choice in the pharmacologic management of congestive heart failure and certain disturbances of cardiac rhythm.



FIG. 1 STRUCTURE OF DIGOXIN

1.1 Pharmacodynamic studies:

1.1.1 Chemistry

Digoxin (Fig. 1), a digitalis glycoside, consists of a genin and a sugar moiety bound by an oxygen atom. This glycoside can be isolated from the leaves of Digitalis lanata. The basic structure of

the aglycone or genin is a cyclopentaperhydrophenanthrene skeleton, which forms the base of all steroids. To the ring at C-17, is attached a five-membered beta-unsaturated lactone ring. The combination of the steroid nucleus and the lactone ring is known as the genin or aglycone. The sugar portion, a tridigitoxoside, is attached to the steroid nucleus at position 3; subsequent acid hydrolysis cleaves the digitoxoses and yields the corresponding aglycones. Hydroxyl substitutions are present at position 12 and 14. Carbons 10 and 13 of the genin moiety have a methyl group.

The following conditions must be fulfilled if the glycoside is to have cardioactivity (11):

 The steroid nucleus has a cis-cis configuration at C and D ring, which probably is involved in binding to the membrane-ATPase.
 This determines the specificity and extent of action.

2. An unsaturated lactone moiety ($\Delta^{\alpha,\beta}$ -structure) attached to the nucleus at C-17 is presumed to combine with the potassium (K⁺) center of the transport ATPase (12).

3. A sugar component is bound through an ether-type bond at C-3 of ring A of the nucleus. This auxillary sugar side-chain influences water solubility and pharmacokinetics of the molecule.

1.1.2 Effect of digoxin on myocardial function:

Each of the essential properties of cardiac muscle-contractility, conduction, refractoriness and automaticity-- are altered by digoxin. The principles of clinical use of digoxin are

based simply on two major effects of the drug.

1.1.2.1 Positive inotropic action:

It is increasingly recognized that the effectiveness of digoxin in the treatment of congestive heart failure is a consequence of its positive inotropic effects. A beneficial cycle is set up in which the increased force of myocardial contraction produces more efficient emptying of the heart chambers. The drug increases cardiac output, relieves the elevated ventricular pressure, pulmonary congestion and venous pressure, and the consequent reduction in the heart size further enhances its contractility. Diuresis is also brought about with relief of edema (13). The majority of evidence for the mechanism of this positive inotropic action to date suggests that this action is related to the ability to inhibit the Na-K ATPase in the myocardial sarcolemma (14). This action leads to an increase in intracellular sodium (Na^{+}) (15) which in turn results in an increase in intracellular calcium (16). This enhances excitation-contraction coupling in the muscle cells and hence augmentation of contractile force.

1.1.2.2 Cardiac electrophysiologic effects:

There is general agreement as to the clinical use of digoxin in the prevention, control and conversion of supraventricular tachycardias (17). In atrial fibrillation and flutter, glycosides slow the ventricular rates. This is achieved by the prolongation of the refractory period of the A-V node, which allows for fewer supraventricular impulses to reach the ventricles (17). In

paroxysmal supraventricular tachycardia, due to atrio-ventricular A-V node reentry, glycosides exert their vagally mediated effect to decrease the rate of impulse conduction as well as to prolong the refractory period in this structure (18,19). The cardiac electrophysiologic effects of digoxin are also believed to be due to the inhibition of the Na⁺pump which maintains intracellular electronegativity by actively transporting Na⁺out of the cell. An altered ratio of intracellular to extracellular Na⁺ and K⁺results in a reduction of the resting transmembrane potential (20), which leads to enhanced automaticity as well as reduced velocity of impulse conduction, especially in those areas such as the AV node where resting membrane potential is normally low (21).

1.1.3 Pharmacokinetics:

1.1.3.1 Absorption:

When administered by the oral route, digoxin is mainly absorbed at the level of the small intestine and to a lesser extent, in the distal small intestine and colon. Absorption shows considerable inter-individual variation, but averages 85% for the elixir and 60-80% for tablets (22). Numerous manuscripts (23-26) appearing in the last few years on digoxin bioavailability, have clearly documented important differences among the various preparations and have noted that the bioavailability and rate of absorption are strictly related to the rate of dissolution in the gut.

Postprandial administration and delayed gastric emptying decrease the rate of absorption and delay the time to peak serum

digoxin levels, but total quantity of drug absorbed is minimally altered. Drugs such as propantheline and metoclopramide, modify gastric motility and hence alter digoxin absorption (27). Other drugs may also interfere with digoxin absorption when administered concomittantly. These include kaolin pectate (28), cholestyramine (29) and neomycin (30). Disease states such as malabsorption syndrome also reduce the absorption of digoxin tablets (31).

1.1.3.2 Distribution:

The time course of the serum digoxin concentration indicates that the pharmacokinetics of digoxin should be described by a model containing at least two distinct compartments (32). After oral administration, peak serum concentration is reached at about 45 to 60 minutes. The next step is the distribution phase where the serum half-life ranges from 32 to 48 hours, which makes toxic reactions easier to manage if they occur (33). An equilibrium, or maximum peak concentration, occurs 4-6 hours after oral administration. For this reason, a correct evaluation of steady-state concentrations may be obtained only if sampling is performed at least 6-8 hours after drug intake.

Digoxin is 23-40% bound to serum protein (34-36). Decreased plasma protein binding of digoxin has been found in patients with uremia (37) but is insignificant for therapeutic effect. The glycoside is also bound to tissue proteins; high concentrations usually being found in the heart, kidney and liver (38-40). Quantitatively, however, the largest depot for digoxin is skeletal

muscle (41). Digoxin is poorly distributed to adipose tissue, therefore lean body weight is most relevant to dosing considerations (38-40).

The extensive distribution of digoxin in tissue is reflected by the large volume of distribution (Vd) (5.1-7.3 L/Kg) in healthy volunteers, but this drops to 2.6-4.3 L/Kg (32,42,43) in patients with renal failure. The Vd in neonates and infants is higher than that found in adults (in infants 2-81 days old, Vd is 9.9 L/Kg (44)). The basis for this difference seems to be due partially to an increased tissue binding of digoxin in the younger age group. Among other possible factors is a change with age in body composition --i.e., larger amount of total body water with a greater proportion in the extracellular water compartments and a reduced quantity of plasma protein available for drug protein binding.

1.1.3.3 Metabolism and elimination:

According to several reports, digoxin is mainly eliminated unchanged in urine, being subjected to limited metabolic degradation (45-47). From 67%-93% of a dose of digoxin is recovered in the urine, mainly as unchanged drug (48-50). However, mechanisms other than renal excretion of the unchanged compound may play a significant part in the disposition of digoxin in man in several instances (51-53).

Digoxin undergoes biotransformation by the liver. The chemical transformations that occur consist of saturation of the lactone ring to produce dihydrodigoxin or its aglycone, dihydrodigoxigenin, and

hydrolysis of the sugar moieties (54) (Fig. 2). Luchi and Gruber (55) observed that in patients requiring unusually high amounts of digoxin, digoxigenin and dihydrodigoxigenin accounted for more than 50% of the extractable excreted material, while the remainder was still in the unchanged form. Clark and Kalman (52) examined the distribution and excretion of metabolites in 50 patients and found that dihydrodigoxin was detectable in 50% of the patients and constituted between 1-48% of total glycoside. From 2% to 10% of the glycosides excreted in the urine were polar, water soluble metabolites.



Of the 100 patients receiving digoxin (56), seven subjects excreted more than 35% of digoxin in the urine as dihydrodigoxin and other dihydro derivatives. Approximately 10% of the drug was excreted in the urine as water-soluble metabolites. A mean of 15% was excreted in the stool, primarily as active as well as inactive metabolites.

In other studies, large amounts of the digoxin reduction

products or DRP were found in one subject (DRP greater than 35-40% of total urinary digoxin and its metabolites) (56,57,58). Since antibiotic therapy hinders formation of the DRP, they appear to be made exclusively by bacteria in the gastrointestinal tract (59). The generation, by intestinal bacteria, of large amounts of cardioinactive metabolites of digoxin with a reduced lactone ring (DRP) in some patients was associated with resistance to therapy and inappropriately high digoxin requirements (55).

A recent report (60) has expanded the determination of digoxin metabolism using tritiated digoxin in humans. Separation of the drug and its metabolites with subsequent RIA analysis of the collected fractions indicated that digoxin was excreted in urine as unchanged drug (55%), digoxigenin bis-digitoxoside (2%), digoxigenin mono-digitoxoside (0.8%), digoxigenin (0.25%) and dihydrodigoxin (0.3%).

Glomerular filtration is by far the major mechanism of excretion, although a small amount is subjected to both tubular secretion and reabsorption. The half-life of elimination is about 1.5 days and in renal failure this value may increase up to 5 days (22).

The variation in the reported amounts of the metabolites had led the investigators in the field to also suggest day-to-day fluctuations of the patients' digoxin levels (61); in addition the difficulty of separating toxic from nontoxic states in the usual setting (62) are not convincingly explained and required further

investigation of the analytical picture as compared to the clinical response.

1.2 Plasma digoxin levels:

Digoxin is given orally whenever possible; this is the most convenient and the safest route. The intravenous route is preferred only when minutes may mean the difference between life and death, as in patients with pulmonary edema from acute left ventricular failure. The intravenous route, however, is by far the most dangerous and its use may be rapidly fatal.

In several studies it has been demonstrated that very little of the variability in plasma digoxin concentration can be accounted for by consideration of factors such as dose, age, weight, etc. Thus, Wagner et al. (43) found that only 34% of the variability could be accounted for, by considering age, height, dose, body weight and renal function in 25 patients. It is clear that other variables need to be considered eg., patient compliance, variations in both absorption, metabolism, and perhaps the analytical procedure used.

Some workers have devised normograms or equations based on knowledge of the pharmacokinetic behavior of digoxin to determine the dose which will result in a given plasma digoxin concentration. These may, however, be unreliable and should be used with great circumspection and regard to the condition of the individual patient, rather than for the average values of pharmacokinetic variables of a population (63,64).

Digoxin plasma levels observed during chronic therapy with maintenance doses of 0.1-0.5 mg/day may range between 0.5 and 8.2 ng/mL (65,66). Therapeutic levels are considered to range between 1 and 2.5 ng/mL in normokalemic patients. Toxic symptoms such as gastric disturbances, headache, anorexia, loss of visual acuity, weakness, fatigue and mental confusion as well as cardiac symptoms are usually associated with levels greater than 3.0 ng/mL (67).

Serum digoxin levels are helpful in patient management since digoxin has been reported to have a low therapeutic index (1,68). The determination of the optimal dosage in clinical practice is difficult to quantitate. It is also often difficult to distinguish the endpoints of daily digoxin therapy both for evaluation of the desired therapeutic effect and for the possible sign of toxicity. Variations in absorption, distribution and renal and non-renal elimination of digoxin preclude accurate prediction of serum levels from a given dose. For these reasons, determination of serum digoxin levels can be valuable in titrating dosages. However, it is important to recognize that the serum digoxin level is only one of the criteria which a clinician should consider when making clinical decisions.

A patient's response to a drug as reflected by a serum level depends on a number of factors such as: age, acid-base balance, electrolyte balance, thyroid disease, renal impairment and the presence of other drugs. These may alter the sensitivity to digoxin and the level at which toxicity occurs. 1

Elderly patients are particularly prone to toxicity because of change in body composition. Lean body mass and creatinine clearance are reduced in elderly patients, thus allowing for a small volume of distribution and hence reduced excretion rate of the drug (69). A recent study revealed that the majority of clinically intoxicated geriatric patients had plasma levels within the therapeutic range. Because they might be unusually sensitive to the drug, the investigator concluded that the plasma digoxin level alone seems to be of limited value in screening elderly patients for intoxication and it has to be considered together with the entire clinical context (70).

Electrolyte imbalances, particularly those in hypokalemia are important sensitizing agents to the myocardium. Hence, digoxin intoxication may appear at normal serum glycoside levels in patients with hypokalemia. Thyroid disease may also affect the response to digoxin (71). The lower serum digoxin concentrations in hyperthyroid patients and higher serum concentrations in hypothyroid patients have been reported to be due to altered distribution volume (72-74), changes in renal excretion due to alterations in glomerular filtration rate (71), malabsorption (75,76), enhanced biliary excretion (75) and increased tissue uptake (77) of digoxin. Since a definite therapeutic range for digoxin is not known for patients with thyroid dysfunction (78), frequent monitoring of plasma levels may avoid the over-dosage in hypothyroid patients and obvious subtherapeutic dosing in hyperthyroid patients.

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The association of renal impairment, elevated serum digoxin

concentrations and evidence of toxicity is well documented. However, the relationship of the dysfunction and pharmacokinetic parameters that accompany renal impairment and alter digoxin disposition are not well delineated. Several studies on variables to predict serum concentrations based on renal function or creatinine clearance have not been encouraging. When adjustment is made for the altered distribution volume associated with renal impairment and indices of renal function are measured, a better predictability of serum digoxin concentration is observed (37,79,80). For this reason, the volume of distribution acts as an initial quide to the extent of the expected reduction in dose, and the dose subsequently can be readjusted according to the patient's clinical response using the plasma digoxin concentration as guide. It must be remembered, however, that the changes in volume of distribution in renal failure patients may also be the consequence of the insensitivity of assay methodology to true digoxin. In fact, Gibson and Nelson (81) found that digoxin metabolites in renal failure patients may result in the over-estimation of digoxin serum concentration by 6%-42% when the radioimmunoassay technique was utilized.

Certain agents have also been shown to modify the level of plasma digoxin. Digoxin disposition may be altered by changes in gastrointestinal absorption, tissue binding and body clearance by some drugs. For instance, renal clearance of digoxin has been shown to be diminished by spironolactone (39,83). However, spironolactone also interferes with serum drug immunoassay measurements by cross-reacting with the digoxin antibody. The nonspecificity of the

RIA kits for spironolactone or its metabolites may result in reporting of falsely elevated serum digoxin concentration (84,85).

The interaction of quinidine and digoxin, on the other hand, appears complex. Quinidine has been reported (150) to increase average digoxin concentrations from 0.9 ng/mL to 1.6 ng/mL. This interaction has since been confirmed by others, but its mechanism(s) is still not fully understood. It may be due to diminished distribution of digoxin to the tissue (151,152) as well as reduced renal clearance (151) resulting from inhibition of tubular secretion (153). The non-renal elimination of digoxin is also reduced by quinidine (152). The clinical significance of the interaction between digoxin and quinidine includes a greater risk of digoxin toxicity (86,87). Because of the inter-patient variation to quinidine's effect, frequent digoxin monitoring is advised.

Several other drugs which are co-administered with digoxin may also increase its serum concentrations. They include verapamil (154,155) (which increase the concentrations by 70-80%), nifedipine (+45%), amiodarone (+70%) (156) and disopyramide (15%) (157). How these interactions are produced is not clear. In one recent study (59), a five day course of erythromycin or tetracycline was found to raise the serum drug concentration by 43-116% in 3 volurteers who were known to reduce the drug to dihydrodigoxin. Though the mechanisms of these interactions with digoxin remain to be delineated, appropriate dosage adjustments can be made from the measurement of the plasma digoxin concentrations.

1.3.1 Chemical methods:

Direct measurement by ultraviolet spectroscopy is difficult for digoxin because the absorption maximum is at 217 nm (16,595 - molar extinction coefficient) (88). For quantitative analytical determination it must therefore be converted into more intensely absorbing derivatives.



Fig. 3 Chemical procedures for the photometric and fluorimetric determination of cardenolides

However, all chemical methods are liable to fail in the presence of large quantities of other substances and furthermore, because of their low sensitivity, they are seldom suitable for the estimation of cardioactive steroids in biologic samples. The chemical procedures for the photometric and fluorometric determination of cardenolides are summarized in Figure 3.

The reactions between the lactone side chain and the polynitroaromatic derivatives in alkaline solution - picric acid (89), 1,3-dinitrobenzene (90), 3,5-dinitrobenzoic acid (91) and tetranitrodiphenyl (92) - are based on the fact that C-C coupling of the unsaturated lactone ring with nitrated aromatic derivatives produces dye complexes which can be measured photometrically. The specificity of these reagents is low because chemical groups such as ketones also give an intense color.

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Both the Keller-Kiliani (93,94) and the Xanthydrol reactions (95) convert 2-desoxy sugars into characteristic colored derivatives. In this way all digitoxose-containing glycosides can be quantitatively determined. Owing to its high specificity for the sugar component of the glycoside, the reaction is scarcely influenced by the nature of the genin.

The reaction between digoxin and strong acids gives a lower limit of detection in the nanogram range using fluorescence spectroscopy. It was suggested by Jelliffe (96) that when digoxin is exposed to phosphoric acid, concentrated hydrochloric acid or trichloroacetic acid, the glycoside is first hydrolyzed to the genin and this will subsequently form the 14-anhydrogenin. The glycoside is further dehydrated to form the 14,16-dianhydrogenin.

The chemical methods so far described are nonspecific and glycosides accompanied by other substances or glycoside mixtures

have to be submitted to preliminary chromatographic separation or purification. Tschesche et al. (97) studied the sensitivity of various procedures. The test with polynitroaromatic derivatives is of much the same sensitivity as the Keller-Kiliani reactions, while the Baljet test is five times more sensitive, and the Xanthydrol reaction seven times more sensitive.

The fluorogenic reagent $(HC1/H_2O_2 \text{ or trichloroacetic})$ acid/chloramine or hydrochloric acid (HCl)), is suitable for direct use as a spray reagent for fluorometric detection of digoxin on chromatographic plates (96,98). The sensitivity limit is 10-20 ng per application. The first clinically applicable technique for chemical measurement of digoxin in urine was presented by Jelliffe (99). As digoxin is present in blood levels of approximately 0.5-2 ng/mL, direct determination by this technique is not feasible and the method could only be used after elaborate concentration of correspondingly large serum volumes.

1.3.2 Radioimmunoassay (RIA):

The original digoxin RIA method (100) employed ³H-digoxin. Immunologically this represented the ideal tracer due to its chemical identity with the unlabeled digoxin. However, due to low specific activity, long counting times were required. Since this time, many RIA kits using¹²⁵I labeled digoxin have become commercially available. Because of the speed and ease of performing
the RIA technique, combined with its sensitivity, the RIA method is the principal method employed in current references and hospital laboratories.

In the RIA assay, a small amount of the patient's serum is mixed in a buffer with radioactive digoxin (tracer) (Figure 4) and digoxin-specific antibody. The antibody binds the tracer in inverse proportion to the concentration of unlabeled digoxin present in the patient's serum. The tracer and the unlabeled digoxin are assumed to have equal affinity for the antibody binding sites. The unbound radioactive digoxin is then separated and the radioactivity of the bound tracer is counted in a scintillation instrument. Percent digoxin bound is converted to digoxin concentrations by reference to a standard curve prepared at the same time.



Figure 4: THE PRINCIPLE OF THE RADIOIMMUNOASSAY METHOD

Several studies have focused on the considerable discrepancies found in the values measured by the kits from different manufacturers (101-103). One study (38) showed a difference in accuracy between four digoxin RIA kits of as much as 30% at the proposed toxic concentration of 2ng/mL. The observed differences

might be due in part to variations in digoxin content of the standards supplied (102,105,106). Additionally, samples with subnormal serum albumin concentrations influence the accuracy of digoxin recovery (107,108). Recently, another study reported differences in digoxin concentrations in samples from patients with acute renal failure when the samples were measured by different immunoassays (109). Furthermore, another contribution to differences in the measurement by the use of RIA kits is poor specificity of the antibody. Cross-reactivity between a digoxin antibody and the primary metabolites, digoxigenin bis-digitoxoside and digoxigenin mono-digitoxoside was demonstrated by Stoll et al. (110). If these metabolites are eliminated in the urine it might be expected that. they could accumulate in the plasma of patients with renal failure, hence the apparent digoxin concentration as measured by standard I RIA would be higher than expected. The extent of cross reactivity of the digoxin antibody with dihydrodigoxin depends on the manufacturing procedure of the radioimmunoassay kit ranging from insignificant cross-reactivity (111,112) to as great as 30% (60,113-115).

Sensitivity of the RIA assay is generally between 0.2 and 0.4 ng/mL. A serum sample from a patient not receiving digoxin may be reported as containing 0.4 ng/mL of digoxin (84). The levels found in this group could not be explained and are probably caused by the discrepancies in the RIA antibodies.

Spironolactone, a potassium-sparing diuretic with a steroid-like structure is commonly co-administered with digoxin.

Interference by spironolactone with digoxin radioimmunoassay has been well documented, however, conflicting results regarding the interference were found by these workers (82,84). Silber et al. (82) suggested that neither spironolactone nor a metabolite, canrenone, should be used to determine assay interference, since unidentified metabolites, and not the parent compound or canrenone, are responsible for the falsely high values.

Anomalous serum digoxin concentrations using radioimmunoassay were also reported in patients with renal impairment (109,117,118). Graves et al. (118) suggested that a substance with digoxin-like immunoactivity is produced endogenously in the serum of digoxin-free patients with renal insufficiency. They also found inter-assay variability in this population and concluded that digoxin concentration measurements by current methods should be considered as questionable.

False-positive digoxin values were also found in newborn infants (119-121). Several commonly used commercial radioimmunoassays were found to measure some "apparent digoxin" values in a number of normal infants not receiving digoxin. Values falling well into the therapeutic range have been measured (120,121). These results cast doubts on the true, chemically useful serum concentrations as determined by the current RIA method.

An homogenous enzyme immunoassay (EMIT, or enzyme multiplied immunoassay technique) method has been employed for digoxin assay (122,123). The procedure shows sufficient sensitivity of 0.5 ng

digoxin /mL serum. The EMIT assay does not require separation steps, makes use of UV detection and may be readily automated. However, the incomplete inhibition of the G6-PD digoxin, upon binding, represents a problem, as do potential interferences with the enzymatic reaction by serum components. The cross-reactivity of the EMIT digoxin antibody with digoxin metabolites has been reported recently (124). These investigators found no cross-reactivity between dihydrodigoxin from two different lots, but extensive interference with the hydrolysis metabolites: digoxigenin, digoxigenin mono-digitoxoside and digoxigenin bis-digitoxoside. They suggested the EMIT method be used only for the accurate measurements of urinary digoxin.

Another immunoassay method utilizes heterogenous enzyme-linked immunosorbent or ELISA (125). The advantage of this procedure over EMIT lies in the complete differentiation of bound and free enzyme activity, however, the required separation step is a disadvantage of ELISA. Although the sensitivity is 0.3 ng/mL serum and intra-assay precision is good (8-14%), steroid-like structures such as spironolactone interfere with the assay method.

1.3.3 Biological assays:

The existence of a Na-K activated ATPase $(Mg^{+2}$ -dependent, Na-K activated ATP-phosphohydrolase) was discovered by Skou in 1957. Soon afterwards he also found that cardiac glycosides are able to inhibit ATPase activity by binding to the enzyme (126). Since some metabolites of digoxin are able to inhibit ATPase, this method lacks specificity. However, with respect to the minor extent to which the

metabolites often occur in plasma (127), the result obtained was said to be comparable to the RIA method. Other disadvantages of this assay are that the sensitivity strongly depends on the respective enzyme preparation, and that the preparation of the enzyme is a crucial step. Belz et al. (128) claimed that the discontinous displacement of ³H-Ouabain and the steep increase of unbound ³H-ouabain in the therapeutic range make it difficult to use the displacement assay for routine laboratory determination.

Rubidium-86 uptake by red blocd cells is dependent on the inhibition by cardiac glycoside of the 86 Rb flux across the human red cell in vitro. The dichloromethane extraction is a crucial step in the method since only cardiac glycosides and their metabolites are extracted, and not highly polar compounds such as glucuronides. If these substances are still cardioactive, and there are arguments that it could be so (129), the method would lead to a marked under-estimation of the cardioactive fraction in plasma. In addition, the performance of the complete 86 Rb analysis for plasma is much more time-consuming than RIA methods (130).

1.3.4 Chromatographic methods:

Watson and Kalman (131) developed a gas chromatographic (GC) assay for digoxin in human plasma based on the work by Jelliffe and Blankenhorn (132). This method involved the formation of the heptafluorobutyrate (HFBA) derivative of digoxin and detected by electron capture (ECD). However, they required 10 mL plasma and had to carry out elaborate processing in order to obtain the derivative

in a state suitable for injection. The derivatization step converts digoxin and its known metabolites to digoxigenin which would then not be distinguishable from each other. In a subsequent paper Watson et al. (133) combined a thin layer chromatographic (TLC) isolation of digoxin from its metabolites with subsequent detection of the HFBA derivative by GC-ECD. Unfortunately the method, although sensitive, involves a prior elaborate TLC preparative step.

Eichorst and Hinderling (134) developed a TLC method for the measurement of radiolabeled digoxin and its known apolar metabolites in plasma, urine and saliva after single-dose administration of labeled digoxin. However, this method is less than ideal for routine work because it is time consuming and requires administration of labeled digoxin.

Combined GLC-Mass spectrometry (GLC-MS) (54) has also been reported. This method, as the previous GLC method, would not be suitable for routine plasma sample assay because it is only applicable to the aglycone portion of the molecule.

Glycoside separations by high performance liquid chromatography (HPLC) are carried out on various types of column phases including silica gel (2, 135) ion exchange (136) and reversed-phase (RP) columns (eq. 135-138).

Reversed-phase columns are gaining wide acceptance in the separation of cardiac glycosides because of their almost universal applicability and their insensitivity towards polar contaminants. The separation is usually followed by UV detection at 220 nm and yields a lower limit of detection of 10 ng/injection (137).

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Various attempts have been made to increase the detection sensitivity. For example, Natchmann et al. (2) proposed the separation and quantitation of digitalis glycosides by HPLC following pre-derivatization with 4-nitrobenzoyl chloride. UV detection at 254 nm permitted the detection of 5 ng/mL digoxin with 3:1 signal to noise ratio. Recently, Fujii et al. (3) described the separation and quantitation of 3,5-dinitrobenzoyl derivatives of cardiac glycosides and their metabolites for enhancing ultraviolet (UV) detectability by micro high-performance liquid chromatography (MHPLC). A detection wavelength of 230 nm was used to enhance the sensitivity and the limit of quantitative measurement of digoxin was less than 0.6 ng (signal to noise ratio 3/1). The application of this potential method in digitalized patients was not examined.

1.3.5 HPLC-RIA:

To overcome the problem of detector sensitivity Loo et al. (4) combined the resolving power of adsorption HPLC with the sensitivity of radioimmunoassay and applied the resulting technique (HPLC-RIA) to determine serum levels of digoxigenin in human subjects. This method, although sensitive, involves the administration of tritiated digoxin to the subjects, and therefore is not readily adaptable for routine monitoring of serum samples from digitalized patients. An even more ambitious HPLC-RIA method was employed by Nelson et al. (5) to quantify digoxin and its metabolites in plasma. The method employed "spiking" with ³H-digoxin for recovery monitoring, dichloromethane extraction, HPLC separation of the residue on a

reversed-phase column using a gradient solvent system, and quantitation of the collected fraction by RIA. Gault et al. (6) on the other hand, administered tritiated digoxin to volunteers, extracted radioactive digoxin and its metabolites from urine, separated them by RP-HPLC and used UV to detect the effluent prior to quantitating them by scintillation counting. The method described was able to detect three new extractable metabolites. Another HPLC-RIA also involving administration of tritiated digoxin but with higher extraction recovery was also reported (140). Because these methods involved the administration of tritiated digoxin they will therefore have the same disadvantage as the method by Loo (4).

Recently, Loo et al. (7) modified the method of Nelson et al. (5), by simplifying the extraction steps using a trace enrichment technique developed by Schauecker et al. (139). Another HPLC-RIA method (8) employed a combined internal standard and marker to follow the elution of digoxin and its metabolites. The latter method does not use labeled digoxin in the clinical samples until the usual quantitation by 3 H-RIA.

1.3.6 Post-column derivatization:

Post-column derivatization in HPLC offers a rather different approach to analysis than pre-chromatographic derivatization. Firstly, the compounds of interest are chromatographically separated before derivatization takes place. The advantages are that artifact formation is not critical and that the reaction does not have to go to completion or give well-defined derivatives-- provided that it is

reproducible.

The kinetics of the reaction are very important and determine the type of reaction detector to use. There are three different principles for the construction of such reactors: (i) tubular or capillary reactors, (ii) bed reactor and (iii) air segmentation streams (104).

The tubular reactors consist of a narrow tube usually 0.2mm-2.0mm ID through which the effluent-reagent mixture flows in the required reaction time. It is the simplest reactor and is recommended for fast reactions taking less than 30 sec.(116).

Packed bed reactors consist of a tube filled with granular inert material. This type of reactor is used with intermediate kinetics requiring actual reaction times in the reactor from one-half to several minutes (116). It can be looked at as a chromatographic column under non-retention conditions.

Segmented flow reactors are based on the segmentation of flowing liquid streams with air bubbles introduced into the stream at certain time intervals. The goal is to minimize band broadening. It requires a debubbling device between the reactor and detector. This approach is very efficient and has become of more interest for post-column derivatization with relatively slow reactions (more than 5 minutes).

In recent years, this analytical technique has attracted enhanced attention. One of the reasons for this attention is the current lack of suitable detectors for trace analysis in biological samples. The use of post-column derivatization can often improve the

desired detection sensitivity and the specificity of an HPLC method for specific trace analysis. Gfeller et al. (9) have reported the merits of post-column detection of cardiac glycosides with hydrochloric acid. The fluorogenic reaction was carried out in a modified Technicon Autoanalyzer system which employed the air segmentation principle and was based on the reaction of HCl with the steroid portion of the cardiac glycoside. Fluorescence of the derivatives was further enhanced by the addition of a hydrogen peroxide/ascorbic acid mixture. It has been found possible to detect amounts as small as 500 pg/2 ul injection of desacetyllanatoside C. However, Gfeller and his co-workers (9) did not report a minimum detectable quantity for digoxin.

2. EXPERIMENTAL:

2.1 Supplies

2.1.1 Chemicals

Digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, dihydrodigoxin, dihydrodigoxigenin and digitoxigenin were obtained from Boehringer (Mannheim, GFR) and were used without further purification. Spironolactone, furosemide and quinidine were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Disopyramide was purchased from Roussel (London, England) whilst procainamide HCl was obtained from Squibb (Montreal, Que., Canada). Other drugs such as Bactrim DS, Capoten, Colace, Isoptin, Persantine and Rythmonorm were obtained from local suppliers.

2.1.2 Reagents

All derivatization reagents for gas chromatography were purchased from Pierce Chemical Co., (Rockford, Il., USA). Dansyl hydrazine was obtained from Regis Chemical Co., (Morton Grove, Il., USA). Aluminum (Al) chloride, glycinamide, isonicotinylhydrazine (INH) and potassium ferricyanide were commercially available compounds and used as supplied. Hydrogen peroxide (H_2O_2) (30%) was purchased from American Scientific and Chemical (Portland, Or., USA) whilst hydrochloric acid (HCl) (37-38%) was obtained from Fisher Scientific Co.(Fair Lawn, N.J., USA). L-Ascorbic acid was BDH

laboratory reagent grade from BDH Chemicals (Toronto, Ont., Canada). Other common reagents were obtained from various suppliers.

2.1.3 Solvents

Water, methanol, isopropanol, n-propanol and dichloromethane were Fisher Scientific Co. HPLC quality (Fair Lawn, N.J., USA). "Glass distilled" quality 2,2,4-trimethylpentane (isooctane) was obtained from Burdick and Jackson Laboratories Inc., (Muskegon, Mi., USA). Acetone and absolute ethanol were reagent grade quality obtained from commercial sources and used as received.

2.1.4 Supplies for extractions

The filter unit consisted of a Nylon 66 membrane (0.45um, 13 mm diameter) filter disc (Rainin Instrument Co., Inc. Woburn, Ma., USA) housed in a Swinnex 13 Millipore filter holder (Millipore Corp. Belford Ma., USA). This unit was used on the end of a Luer-Lock 5cc Multifit B-D glass syringe (Becton Dickinson Canada, Mississauga, Ont., Canada).

The solid-phase cartridges Bond-Elut (Si, diol) were purchased from Analytichem International, Inc (Harbor City, Ca., USA).

2.1.5 Post-column derivatization supplies

2.1.5.1 Packed-bed reactor

The packed-bed reactor consisted of a 15 cm X 1.5 cm I.D. glass column (part # 6112, Omnifit Ltd., Cambridge, England) dry

packed with 20 um glass beads (a generous gift from Dow Corning Corp., Midland, Mi., USA).

2.1.5.2 Air-segmented reactor

The mixing and reaction spirals were essentially the same as described previously (9) except for the mixing tees and debubbler which were miniaturized using 1 mm I.D. quartz tubing. The mixing tees were constructed such that the reagent solutions entered at a 30° angle against the eluent stream. The debubbler was a miniaturized version of the Technicon C-5 debubbler.

2.1.5.3 Non-segmented reactor

The manifold was made from Solvaflex tubing (part # 116-0533P04, .015" I.D. orange/green collar, Technicon Instruments Corp., Tarrytown, N.Y., USA) for the delivery of dehydroascorbic acid and acidflex tubing (part # 116-0538-09 Technicon) for the delivery of HCl. A short stainless steel tube, inserted into the Śolvaflex tubing, served as a connection between tubings.

Polytetrafluoroethylene (PTFE) tubing of 0.3mm I.D. was used for the reactor and the connection between column and reactor to minimize sample band broadening. A 0.3mm I.D. PTFE tubing was also used as the restriction coil to provide increased resistance. A 0.8mm I.D. tubing was used for the mixing coil and connection between delivery tubings and the mixing coil.

A three-way PTFE connector (part # 1004 Omnifit Ltd., Cambridge, England) was likewise placed in the solvent delivery

lines from the reagents to the mixing coil. A 3-way PTFE valve (part # 1102 Omnifit Ltd., Cambridge, England) was placed between the column, the mixing coil and the reactor. Closing of the PTFE valve between the column, reactor and the mixing coil allowed for cleaning of the reactor system at the end of the day. All valves, connectors and tubings were manufactured by Omnifit Ltd. (Cambridge, England).

2.2 Equipment

2.2.1 High-performance liquid chromatographic (HPLC) system

The HPLC system consisted of a Beckman Model 100 A solvent metering system (Beckman Instrument, Inc., Fullerton, Ca., USA) equipped with a model U6K injector (Waters Assoc., Milford, Ma., USA), or a Rheodyne Model 7125 100 uL fixed loop injector (Rheodyne, Berkeley, Ca., USA), a Waters fluorescence detector model 420 AC with a homemade (1 mm ID X 40 mm length quartz tubing) flow cell (section 2.2.2) and an Altex CRIA Chromatopac Data Processor (Beckman Instrument, Inc.). A Schoeffel model FS-970 LC fluorometer (Acton, Ma., USA) was also used in this work for detector sensitivity assessment. The excitation and emission conditions were chosen as optimum for the determination of digoxin. A noise filter

constructed in the laboratory (Figure 5) was coupled between the detector and integrator.

To study band broadening, a variable wavelength ultraviolet (UV) detector (Beckman model 155 detector, Beckman Instrument Inc.) was placed between the post-column detection system and the analytical column. Band broadening of the post-column reactor was measured as the increase in peak width in minutes relative to the UV signal.

2.2.2 Flow cell

The Schoeffel detector titanium cell was manufactured to the exact specifications as the standard stainless steel cell for the FS 970 fluorometer.

The Waters detector flow cell block was modified to eliminate all stainless steel fittings. An all-quartz flow cell of 40 mm in length and 1 mm ID was fitted with acidflex tubing on both ends and this cell was held in place by epoxy glue on the cell block.

2.2.3 Post-column reaction system

A schematic diagram of the instrumental arrangement of the segmented-flow system is given in Figure 6. Air bubbles and the chemical reagents were introduced via a Technicon proportioning pump (Technicon Instruments Corp.). A Manostat Casette pump (Fisher



Figure 5. Schematic of the Noise Filter



Figure 6. Schematic diagram of the reaction detector unit for fluorescence detection⁹.

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Scientific Ltd.) with variable speed was used to supply the reagent solution for the packed bed reactor and non-segmented reactor. For all three reactors, the reaction bath was thermostated using a Haake model E51 constant temperature circulator supplied by Fisher Scientific Co. (Fair Lawn, N.J., USA).

Two nitrogen pressurized bottles (1 liter glass reservoir, Omnifit Ltd.) were also used to deliver the reagents (HCl and dehydroascorbic acid) to the reactor as an alternative to the peristaltic pump.

2.2.4 Extraction

A Vortex-Genie purchased from Fisher Scientific Co. was used to aid in the precipitation of proteins in plasma following addition of acetone, as well as to wash the supernatant with isooctane. A Fisher Roto-Rack model 343 was used in the extraction step and finally an IEC HN-SII Centrifuge, (Damon/IEC Division, Western Scientific, Vancouver, B.C., Canada) was used to separate the immiscible phases.

2.2.5 Gas chromatography

A Hewlett-Packard model 5880 A Series reporting gas chromatograph equipped with a Nickel-63 electron-capture detector (ECD) (Avondale, Pa., USA), and an on-column injector was used for GC analyses. The injection is made using a standard microliter

syringe with a fused silica needle (Hamilton Co., Reno, Nev., USA). A carbowax 20 M capillary column (20m X 0.2 mm ID) was used for the separation. The oven temperature was raised from 50° C to 250° C at a rate of 30° C/min while maintaining helium flow of 1 mL/min through the column. Initial temperature was maintained for 2 minutes before the temperature program. Other conditions were similar to the procedure by Watson et al. (133).

2.2.6 HPLC-Mass Spectrometry

An unmodified Hewlett-Packard model 1082 B liquid chromatograph was coupled to a model 5987 A GC/MS instrument equipped with direct liquid introduction (DLI) interface (Hewlett Packard Co., Palo Alto, Ca., USA) for LC/MS operation. The normal chemical ionization (CI) operating parameters of the mass spectometer for the LC/MS experiments were as follows:

electron voltage 230 V

emission current 300 uA

source temperature 250°C

ion source pressure 1.2×10^{-4} torr as measured at the CI GC/MS interface thermocouple

repeller 3 volt

electron multiplier 2200 volts

The HPLC eluent/CI reagent gas in this work was 75/25 (by volume) methanol:water at a flow rate of 0.8 mL/min.

2.3 Stationary phases

A 3 cm X 2.1 mm ID direct connect guard column (Mandel Scientific Co. Ltd., Rockwood , Ont., Canada) containing dry-packed 25-37 um CO:PELL ODS (octadecylsilane) pellicular media (Whatman Ltd., Clifton, N.J., USA) was placed prior to and in series with the analytical column.

The commercial columns used in these experiments were:

(a) Ultrasphere ODS (5u) 4.6mm X 25 cm (Beckman Instrument Inc.)

(b) Microsorb Short-One HPLC column C-18 (3u) 4.6mm X 10cm (Rainin Instrument Co., Inc.)

(c) Spherisorb ODS II (3u) 4.6mm X 15cm (Alltech Associates, Deerfield Il., USA)

Laboratory packed columns were as follows (on loan from Dr. Richard Wall, University of British Columbia, Faculty of Medicine, Dept. of Pathology, Vancouver, B.C., Canada):

- (a) Hypersil ODS (5u) 4.6mm X125mm
- (b) Lichrosorb RP 8 (10u) 4.6mm X 125mm
- (c) Hypersil ODS (3u) 4.6mm X 60 mm

2.4 Derivatization procedures

2.4.1 For Gas Chromatography and Electron-Capture detection

2.4.1.1 Reagent: N-Methyl-bis-trifluoroacetamide (MBTFA) (141):

About 0.5mg of digoxin or digoxigenin was reacted with 0.1 mL of MBTFA in dry pyridine for one hour at 65^{0} C. A volume of 2 uL of organic phase was injected.

2.4.1.2 Reagent: Tri-Sil Z (a mixture of trimethylsilylimidazole in dry pyridine 1.5 mEq/mL) (142):

A 1.0 mL sample was placed in a Reacti-vial (Pierce Chemical Co., Rockford, Il., USA) and was mixed with 1 mL of Tri-Sil Z. The mixture was shaken occasionally until the sample dissolved. This was then heated at 60° C for 10 minutes. The mixture was injected directly into the gas chromatograph.

2.4.1.3 Reagent: Heptafluorobutyrylimidazole (HFBI) (143):

A 20 uL aliquot of HFBI was added to 5 mg of digoxin. The mixture was capped and heated at 85^{0} C for 1 hour. This was then shaken for 2 minutes with 2 mL of toluene and 0.5 mL of distilled water. The toluene layer was removed and injected into the gas chromatograph.

2.4.2 In-vitro fluorometric assay method

Fluorescence spectra and intensity measurements were taken on an Aminco-Bowman Spectrophotometer (American Instrument Co., Inc. Silver Spring, Ma., USA) whose monochromators were calibrated

against the Xenon line emission spectrum and whose output was corrected for instrumental response by means of a quinine sulfate standard solution.

2.4.2.1 Sulfuric acid (145):

An aliquot of 0.1 mg of digoxin in 1 mL of chloroform was treated with 2 mL concentrated sulfuric acid (96%) and heated at 55° C for 7 minutes. The fluorescence intensity was measured at room temperature at 420 nm emission, 390nm excitation. The background intensity from sulfuric acid and chloroform was also measured, and subtracted from the intensity obtained for the drug solution.

2.4.2.2 Acetic anhydride/ acetyl chloride/ trifluoroacetic acid (146):

Solution A: An aliquot of 10 mL each of acetic anhydride and acetyl chloride were mixed in a glass-stoppered bottle and kept in the refrigerator.

Solution B (reagent): A 4 mL aliquot of solution A was pipetted into a 10 mL volumetric flask and the volume was made up to 10 mL with trifluoroacetic acid.

Procedure: An aliquot of 0.1 mg of digoxin in 1 mL chloroform was mixed with 2 mL of solution B. The solution was placed in a water bath at 55[°]C for 7 minutes and was allowed to stand at room temperature before reading against the reagent blank. Excitation and emission intensity were set at 345/435 nm respectively.

2.4.2.3 Dansyl hydrazine:

One mg of digitalis glycoside sample was dissolved in 1 mL of methanol to which a 2 fold molar excess (1.36 mg) of dansyl hydrazine and 2 drops of glacial acetic acid had been added. The solution was heated at 70° C for 15 minutes and was then evaporated to dryness under vacuum. The residue was dissolved in 0.5 mL of HPLC mobile phase.

2.4.2.4 Hydrogen peroxide/ ascorbic acid/ hydrochloric acid (147):

The assay was done in accordance to the United States Pharmacopeial method for the digoxin tablet dissolution test. In this procedure 5 mg/mL digoxin solution was transferred to a glass-stoppered flask and 1.0 mL methanol was transferred to another flask to provide a blank. To this sample was added 1 mL of 2mg/mL ascorbic acid solution and the solution was then mixed. Concentrated HCl (5 mL) was added immediately to this mixture, followed with 1 mL of .012%v/v hydrogen peroxide solution. The reaction medium was stoppered and stored at room temperature for 2 hours before the fluorescence intensity was read.

2.5 Liquid chromatography and ultraviolet detection (138)

The glycosides were dissolved in the mobile phase and injected into the liquid chromatograph. The retention time of each compound was determined by separate injections of individual solutions of each glycoside. The mobile phase was prepared in sufficient quantities for daily use and degassing was not found to be necessary.

The mobile phase used in these experiments was composed of water/ methanol/ isopropanol/ dichloromethane (47:40:9:4). The flow rate of the HPLC was maintained at 1.2 mL/min.

2.6 Preparation of reagents

2.6.1 Reagents

2.6.1.1 Glycinamide/ Potassium Ferricyanide These reagents were prepared according to the method reported by Seki and Yamaguchi(148) where 0.5 g of glycinamide and 30 mg of potassium ferricyanide are dissolved in 0.3M borate buffer solution (pH 9.8).

2.6.1.2 Isonicotinylhydrazine (INH)- aluminum (Al) chloride solution

These reagents were prepared according to the protocol reported by Horikawa et al. (149). A 16 mM INH solution and an 80 mM aluminum chloride solution were prepared by dissolving the powders in methanol. The solutions were kept in an aluminum foil- wrapped container and stored in a dark place until required for use.

2.6.1.3 Dehydroascorbic acid

A stock solution of dehydroascorbic acid solution was prepared weekly and kept in a refrigerator until required for use.

Solution A: A solution containing 250 mg of ascorbic acid was made up to 500 mL with distilled water in a volumetric flask.

Solution B: A 1 mL aliquot of hydrogen peroxide (30%) was transferred to a 200 mL volumetric flask and made up to volume with distilled water.

The day prior to analysis, 100 mL of solution A and 2.5 mL of solution B were added dropwise to an Erlenmeyer flask, followed by stirring for 2 hours. This solution was kept in a refrigerator until needed.

2.6.2 Standard solutions

2.6.2.1 Digoxin standards

About 1 mg of digoxin reference standard was accurately weighed using an electrobalance model G (Cahn Instrument Co., Paramount, Ca., USA) into a 100 mL volumetric flask and made up to volume with methanol. Aliquots of this solution were diluted with methanol to yield working standards in the 1.5-10 ng/10 uL range.

2.6.2.2 Internal standard solutions

Standard solutions weighing approximately 4 mg/mL of digitoxigenin and 1 mg/mL each of 17- methyltestosterone, ethinylestradiol, prednisone, 17-estradiol, norethindrone, ouabain, cymarin, triamcinolone, diethylstilbestrol, mestranol and testosterone were individually prepared and injected into the liquid chromatograph to determine the retention times of each. An internal standard that elutes near the digoxin peak but which does not co-elute with any of the metabolites and endogenous material was considered to be ideal for this study. For this purpose

digitoxigenin, which eluted 10 minutes after digoxin, was used as the internal standard.

A stock solution of digitoxigenin was prepared in methanol at a concentration of 4 mg/100 mL. To each of the digoxin working standards were added 2 mL of this internal standard solution. These standards were made up to 10 mL with methanol. These working standards with the internal standard were used for the calibration curve. Another 2 mL of the internal standard solution was also transferred to a 10 mL volumetric flask and made up to volume with methanol. Plasma samples were spiked with 10 uL of this diluted internal standard.

2.6.2.3 Digoxin metabolites

One milligram of each of the metabolites of digoxin, namely digoxigenin, dihydrodigoxigenin, digoxigenin mono-digitoxoside, digoxigenin bis-digitoxoside and dihydrodigoxin, were accurately weighed and separately made up to 10 mL with methanol. An aliquot of 500 uL of each solution was transferred to a 10 mL volumetric flask and 10 uL of these were used for the specificity test of the HPLC post-column reaction.

2.6.2.4 Co-administered drugs:

Aliquots of 3 mg of furosemide, spironolactone and quinidine were weighed separately and dissolved in 10 mL methanol.

An aliquot of 3 mg of procainamide HCl was dissolved in water, basified to pH 8 with sodium hydroxide solution and extracted twice with 10 mL of dichloromethane. The combined organic phases were evaporated to dryness. The residue was dissolved in 10 mL of

methanol.

An aliquot of 1 mg of disopyramide (base) was made up to 10 mL with methanol.

Other drugs evaluated for fluorescent response using the post-column derivatization method included: Trimethoprim-sulfamethoxazole (Bactrim DS, Roche) Captopril (Capoten 100 mg tablets, Squibb) Docusate Sodium (Colace capsules, Bristol) Verapamil Hydrochloride (Isoptin Injectable 2.5 mg/mL, Searle) Dipyridamole (Persantine 75 mg tablets, Boehringer) Propafenone (Rythmonorm 300 mg tablets, Knoll).

Tablets and capsules were extracted in the manner described in the current Pharmacopoeiae. Extracts were usually evaporated and resuspended in the mobile phase and analyzed subsequently. The injectable preparation of verapamil was analyzed directly without further processing. Amounts of each drug analyzed were in microgram quantities, and hence were above plasma levels. The chromatogram of each drug examined was allowed to run for up to one hour to determine its elution profile.

In all such experiments, 10 uL of each drug solution in methanol was injected using a 25 uL syringe (Waters Associates 25 microliters, SGE Scientific glass engineering Ltd. Melbourne, Australia).

2.6.2.5 Mobile phase

The mobile phase was prepared by mixing the solvents and then degassing the composite by rapid stirring for 30 minutes. The mobile phases examined for this study are summarized on Table 3 of the Results and Discussion section.

2.7 HPLC post-column derivatization procedure

2.7.1 Column selection

Columns of different lengths and sizes of packing materials were examined individually by attaching columns enumerated in section 2.3 to the optimized non-segmented post-column derivatization system. Samples of digoxin mixed with its metabolites were injected and the resolutions of these were compared.

2.7.2 Optimized HPLC non-segmented post-column derivatization system

The HPLC post-column derivatization was carried out as shown in Figure 7.

The cardiac glycoside samples were injected via the Waters U6K universal injector. The glycosides were eluted from the 15cm, 3u ODS packed column equipped with an ODS guard column using an isocratic mobile phase composition; ethanol, methanol, isopropanol, water (3/52/1/45) at a flow rate of 0.3 mL/min. At the exit end of the column, the effluent was joined by the reagent line through a 0.8 mm ID PTFE tee. The post-column reagents (HCl and dehydroascorbic acid solution) were delivered by a peristaltic pump at a rate of 0.23 ± .01 mL/min and premixed in 2 m X 0.8 mm ID coil before joining the





effluent. The combined mixture was immediately passed into the 10 m knitted reactor maintained at 79 \pm 1⁰C. The glycosides, in the presence of HCl and dehydroascorbic acid, reacted to form the fluorophore which was monitored by a Waters fluorescence detector equipped with a 360 nm excitation filter and a 425 nm emission filter. The total flow rate of the system was found to be 0.53 mL/min. The flow rates in the HPIC and reagent line were measured by collecting the effluent in a graduated cylinder and timing with a stopwatch. Attached to the detector output was a homemade noise filter which was placed in series with the integrator. A back pressure coil of 1 m X 0.3 mm ID PTFE tubing was connectd to the fluorometer outlet to prevent bubble formation in the detector cell.

For constant proportioning of the peristaltic pump, the delivery tubes were stretched on the platen to avoid tube snaking and hence generating proportioning of equal pulsation. To maintain reproducible pumping rates for day-to-day operation, the acidflex tubing, which was found to stretch and flatten with time, was changed daily.

After each analysis day, the HPLC column was thoroughly rinsed with methanol and the post-column reactor was left dry. Baseline stability was obtained after the first hour of operation of the system.

2.7.3 Other alternative reagents employed for derivatization

In an attempt to eliminate the more volatile HCl, other

acidic reagents such as sulfuric acid (70%), perchloric acid or trichloroacetic acid were substituted for the concentrated HCl described above. All other post-column and HPLC parameters were maintained as before. Although several concentrations of sulfuric acid were tested (40, 60, 70 and 100%) 70% was chosen for its efficiency in derivatization and appropriate viscosity. The 100% sulfuric acid was too viscous to pump through the small internal bore PTFE reactor.

Procedures for the INH /Al and glycinamide / ferricyanide post-column derivatization reagents were mainly in accordance with the methods described in the literature (148,149). Since they were optimized for steroid determination, parameters such as the reagent flow rate and reaction temperature were modified to give the maximum response.

The optimized post-column procedure using these reagents were as follows: Digoxin solution was injected into the HPLC and eluted at a flow rate of 0.3 mL/min using the mobile phase (methanol/ ethanol/ isopropanol/ water 53/3/1/45). The effluent from the HPLC was mixed with the fluorescent reagents (INH/Al or glycinamide/ ferricyanide) at a flow rate of 0.2 mL/min and the mixed solution was heated in a PTFE coiled reactor (10 m X 0.3 mm) maintained in a water bath at 65° and 70° C respectively. Fluorescence was measured and the intensity of the peaks recorded. The excitation/ emission wavelength for INH/Al and glycinamide/

ferricyanide were 360/ 425nm and 334/ 395nm respectively. The derivatization using these two methods was found to be less efficient than the originally proposed method (procedure described in section 2.7.2).

2.7.4 Packed-bed reaction system

The glycosides were injected into the chromatograph and eluted using the isocratic mobile phase described in 2.7.2. The column effluent was joined by the reagents through a four-way PTFE tee. The mixing coil was omitted and the combined mixture was delivered directly into the packed bed reactor maintained at 79° C. All other parameters were similar to the non-segmented reaction detector. Although this reactor would seem to be ideal for the required reaction time proposed by Gfeller et al.(9), the instability of the beads for the packed-bed defeated the theory.

2.7.5 Air-segmented reaction system

A schematic diagram of the HPLC post-column derivatization used in this study is shown in Figure 6. The procedure used in this study was a modification of the system developed by Gfeller et al. (9). Digoxin standard solutions were injected onto the reversedphase column via the Waters Universal injector. The glycosides were eluted from the column using the mobile phase given in 2.7.2 at a flow rate of 0.4 mL/min. At the exit end of the column, 0.5 mL/min

of concentrated HCl, segmented with 0.5 cm air bubbles, was added to the column effluent through a 1 mm ID mixing tee described in 2.1.5.2. This segmented solution was passed into a 20-turn mixing coil after which .05 mL/min dehydroascorbic acid solution was added through a second 1 mm ID mixing tee. The combined mixture was then introduced into a 40-turn mixing coil. The fluorophore was formed in the 40-turn reaction coil maintained at 55° C in a water bath. The mixture was cooled to room temperature in a 30-turn glass coil, jacketed with running tap water. The mixture was introduced at a 90° angle against the air bubble stream. The combined, cooled mixture was debubbled before entering the fluorescence detector. The position of the debubbler was found to be significant for the efficient removal of air segments.

2.7.6 Optimization procedure for post-column derivatization

Before the post-column reaction conditions were studied, the HPLC mobile phase, octadecylsilane column, fluorescence detector and the reagent mixing tees were evaluated for maximum efficiency and detection sensitivity. Different HPLC mobile phases were examined to determine compatibility with the reaction medium, hence providing a maximum response. The 3u ODS column was selected as the optimum column packing as it gave satisfactory separation of digoxin and its metabolites. A Waters fluorescence detector with a 40 mm X 1

mm ID flow cell and a noise filter were satisfactory for detection of nanogram quantities of digoxin. As the source lamp output decreased with age, the detector response was kept constant by increasing the voltage on the photomultiplier tube. The mixing tees were of minimal internal diameter to minimize band spreading. The 1 m PTFE tubing connected at the exit end of the detector enabled the temperature of the reactor heating bath to be increased, thereby increasing the rate of post-column reaction.

2.7.6.1 Relative proportions of fluorogenic reagents

As it appeared that the HCl-dehydroascorbic acid fluorogenic reagents gave maximum sensitivity, the relative proportions of these were further examined to further increase detector response. The HPLC flow rate was initially maintained at 0.1 mL/min for subsequent experiments.

Internal diameters of 0.02, 0.015 and 0.010" for dehydroascorbic acid delivery tubings and 0.29, 0.53 and 0.63" ID acidflex tubings for HCl delivery were used to set the relative proportions of reagents for maximum fluorescence intensity studies. The other post-column reaction parameters described in 2.7.2 were held constant. In addition, concentrations of hydrogen peroxide in ascorbic acid and vice versa were also studied. The optimum concentration of ascorbic acid from .01 to 1% was initially determined by maintaining the concentration of hydrogen peroxide at

1.1 X 10^{-3} M. An optimal concentration of ascorbic acid was found to be 0.1%. Then hydrogen peroxide concentrations from 4.4 X 10^{-4} M to 2.2 X 10^{-3} M were also examined with the concentration of ascorbic acid at 0.1%. An optimum concentration of 1.1 X 10^{-3} M hydrogen peroxide was observed and used in the subsequent experiments. Further changes in the ratios of ascorbic acid / hydrogen peroxide did not increase fluorescence intensity of the derivative.

2.7.6.2 Reaction temperatures

Initial experiments were carried out using digoxin alone and an HPLC mobile phase of methanol. Temperatures of 45° , 50° , 55° , 57° , 59° and 64° C were used to determine the effect of temperature on the fluorescence intensity of digoxin. Response increased as the temperature increased. No data were obtained above 64° C (the boiling point of methanol). However, when the mobile phase described in 2.7.2 was used, the temperature could be further increased to 80° C, beyond this temperature the response remained constant. At about 80° C the reaction of the glycoside and the reagents was apparently complete. Therefore $79\pm1^{\circ}$ C was selected as the overall optimum reaction temperature.

2.7.6.3 Reaction kinetics

The effect of the flow rates of the reagents and flow rate

of the mobile phase on the reaction time, and consequently the detector response to the fluorophore formed, was investigated. This study was undertaken by varying the speed of the peristaltic pump which delivered the HCl and dehydroascorbic acid. Flow rates of 0.24, 0.27, 0.33, 0.37, 0.41 and 0.43 mL/min lead to average peak heights of 1.1, 2.35, 2.27, 1.93, 1.85 and 1.70 cm respectively.

Subsequent to this optimization, the length of the reaction coils was investigated. Coils of 10, 15, 20 and 25 m with 0.3 mm ID provided 3,5,7 and 8.6 minutes reaction times, respectively. In addition, different reactor geometries were also examined. They were: a 20 m length of 0.3 mm I.D. tubing with an outer coil diameter of 50 cm, a 20 m length, 0.3 mm I.D. tubing with an outer diameter of 2.7 cm ,a 10 m length coil of 0.3 mm I.D. tubing of 2.7 cm outer diameter and finally a 10 m knitted reactor. The knitted reactor was composed of fringes like those used for crochet work. The results of this investigation showed that the 10 m knitted reactor was found to give the least band broadening with comparable response to the 20 m coiled reactor.

As expected, the back pressure of the 10 m knitted reactor was less than the 20 m coil. As a result, the flow rate of the HPLC was increased further up to 0.45 mL/min. The response curve (Fig. 24 of the Results and Discussion section) showed an optimum flow rate of 0.3 mL/min for the HPLC mobile phase.
2.7.6.4 Detector wavelength

Excitation and emission maxima of the digoxin derivative were determined by alternately changing excitation and emission filters. An optimum excitation and emission wavelength of 360 and 425 nm respectively was found.

2.7.6.5 Control of baseline noise

Two nitrogen pressurized glass reagent reservoirs were used to generate pulseless flow of the reagents. The reservoir is of heavy-walled glass with three PTFE valves. The three individually controlled valves permit the application of gas under pressure to the bottle for delivery, venting or flushing of the reagent. One valve of the reservoir remained closed at all times during the operation, while the second valve was connected to a two-stage regulator attached to a nitrogen cylinder. The reagents from the two reservoirs were mixed via a three way tee. The flow of the reagents was regulated by altering the pressure of the nitrogen gas over the solution in the reservoir. Since the flow of dehydroascorbic acid had to be less than the flow of HCl, a restriction coil of varying length (3 to 7 m) was attached to the valve of the dehydroascorbic acid reservoir to add back pressure to that reservoir. A length of 5 m X 0.3 mm ID PTFE tubing was found to give sufficient back pressure and allow for an adequate amount of dehydroascorbic acid delivered relative to the delivery of HCl. Using this system, a nitrogen pressure of approximately 50 psi was sufficient to deliver 0.25

mL/min of reagents (HCl flow of .20 mL/min and dehydroascorbic acid flow of 0.05 mL/min) and the column flow rate was maintained at 0.15 mL/min. Higher HPLC column flow rates could not be achieved since these would necessitate higher flow rates of the fluorogenic reagents and the pressures necessary were above the maximum safe limit of the glass reservoirs. Flow of the reagents was easily halted by relieving the pressure in the reservoir via the unattached valve.

2.7.6.6 & -Cyclodextrin addition

The effect of 9-cyclodextrin on the fluorescence intensity of the digoxin derivative was also examined. This was readily studied by introducing 9-cyclodextrin solution into the end of the reactor described in 2.7.1, via a three-way tee. The mixture was immediately passed into the fluorometer. Concentrations of 1.76 X 10^{-3} mM to 3 X 10^{-3} mM were studied. No change in sensitivity was noted. The wavelengths for excitation and emission were also considered; however, excitation/ emission wavelengths of 360/ 425nm were found to be ideal. In addition the reaction time of the digoxin derivative in the presence of 9-cyclodextrin was increased by inserting a 1 m coil between the tee and the detector. This final modification led to a decreased noise level; however, the sensitivity of detection was unchanged. While 9-cyclodextrin did reduce the baseline noise by a factor of 2, the expense of this reagent precluded its further use.

2.8 Reproducibility and linearity of the assay method

Day-to-day precision of the detection of digoxin standards was assessed by comparing the fluorescence response of digoxin at levels from 1.5 to 10 ng. A total of 10 analyses per day for 5 days were used in this study. Standard deviations of the peak height ratios were determined for each concentration and the mean of each concentration point was plotted against the weight ratios of digoxin/ internal standard. The coefficient of variation at the 3 ng level for a single day determination was 2%.

2.9 Plasma extraction procedure

2.9.1 Extraction procedure in water

2.9.1.1 Solvent-solvent extraction of digoxin in water

Water (5 mL) containing 1 ng/mL of digoxin was added to a screw capped tube. After the addition of an organic phase (see Table 8 of Results and Discussion section) the capped tube was placed on a rotating disk for 10 minutes at low speed to effect partition of digoxin into the organic phase. More rapid agitation led to emulsion formation. After centrifugation for 5 minutes at 2,500 rpm the lower layer was removed to a second tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 100 uL of the HPLC grade solvent used for chromatographic elution.

2.9.1.2 Solid phase cartridge extraction of digoxin in water

Cartridge activition step: In all cases of solid phase extractions, the cartridge was positioned in a Luer-lock needle mounted on a rubber stopper on a test tube equipped with a side arm connected to a vacuum line. Vacuum pressure was adjusted to 15 in. of mercury. Each cartridge was prewashed repeatedly with 5 mL methanol (3X) and then with water through opening and closing of the vacuum pressure line. This process activated the packing surface.

Extraction step: With the vacuum initially turned off, the "spiked" water containing known additions of digoxin was added. The sample was drawn through the column by vacuum and the drug was adsorbed on the column matrix. With the vacuum off, 10 mL of organic solvent (Table 9) was placed onto the column. The vacuum was turned on and the eluent was collected. The upper aqueous layer was discarded and the lower organic solvent was dried under a stream of nitrogen. The residue was reconstituted in 100 uL of the HPLC solvent used for chromatographic elution. The whole volume was injected into the chromatograph.

2.9.2 Extraction procedure in "spiked" plasma

2.9.2.1 Solvent-solvent extraction of digoxin in plasma

A volume of 3 mL of pooled human plasma, taken from the local blood bank, was pipetted into a 15 mL screw-capped tube. An

aliquot of 1 ng/mL of working digoxin standard was added and the mixture was mixed on a vortex mixer for 5 seconds. Then, 10 mL of the following organic solvents: dichloromethane, ethyl acetate and combination of either isobutanol, propanol ethanol or isopropanol and dichloromethane, as enumerated in Table 8 of the Results and Discussion section, were added and the mixture extracted for 10 minutes as described previously. After centrifugation for 5 minutes at 1000 rpm, the organic phase was transferred into a 10 mL screw-capped tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was reconstituted in 100 uL with the HPLC grade solvent used for chromatographic elution and the whole volume was injected into the chromatograph. Because of the presence of interfering peaks, only two of the solvents mentioned above are shown on Table 10.

2.9.2.2 Solid phase cartridge extraction of digoxin in plasma

To 3 mL of pooled plasma was added 1 ng/mL of digoxin in methanol. The plasma proteins were denatured by the addition of 3 mL of acetonitrile. After centrifugation for 5 minutes at 1000 rpm, the supernatant was passed through the cartridge by gravity with the vacuum in the test tube turned off. After 2 minutes, the vacuum was applied and the eluent discarded. The vacuum was then turned off and 10 mL of the following organic solvents: dichloromethane or dichloromethane/ isopropanol (Table 11) were added to the column and eluted through by gravity. After 1 minute of equilibration time, the

vacuum was turned on and the eluent was collected. This was then evaporated to dryness and the residue resuspended as before (2.9.1.2). The solid phase cartridge was activated as described in 2.9.1.2 " cartridge activation step".

Several modifications to the method described in Table 11 of the Results and Discussion section were also examined. The following three methods were also summarized in Table 12.

Method A involved the solvent/ solvent extraction of 3mL of plasma, containing 1 ng/mL of digoxin with 10 mL dichloromethane/ methanol (15:1). After shaking the tube for 10 minutes, the organic phase was transferred onto the activated cartridge. The cartridge was then washed with 2 mL of water and the vacuum was turned on to force the endogenous substances through the cartridge. With the vacuum off, 15 mL of dichloromethane were added onto the cartridge and the eluent was collected. The organic solvent was then evaporated and reconstituted as before (2.9.1.2).

Method B evaluated several bonded phases including diol, silica and ODS for their effectiveness in retaining digoxin from the plasma. Method B, similar to method A, also involved a pre-extraction step with two aliquots of 10 mL dichloromethane/ isopropanol (15:1). The organic phase was then transferred to a 10 mL test tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1 mL of solvent (such as water for reversed phase cartridges and chloroform for forward phase cartridges). This solvent was allowed to slowly pass through the extraction tube by gravity and the sample was eluted from the

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cartridge

using dichloromethane/ isopropanol (15:1) for the reversed phase cartridges and isopropanol for the silica cartridge. The vacuum was turned on after 1 minute and the eluent collected and processed as before (2.9.1.2).

Method C was designed to remove the interfering peaks present in the eluent found from the results of using method A and B. The procedure was similar to method B but with an additional solvent wash . After solvent-solvent extraction, the sample was resuspended in either water or chloroform depending on the type of cartridge used. The reconstituted sample was passed through the cartridge. The cartridge was flushed with either ethyl-ether/isopropanol, chloroform or ethyl acetate depending on the cartridge used. This washing step was found to remove the interfering endogenous substances. Digoxin was then eluted with solvent 4 which was either dichloromethane/ isopropanol or isopropanol (see Table 12).

2.9.3 Actual extraction procedure of digoxin in plasma Just prior to analysis, the frozen plasma samples were allowed to thaw at room temperature. In this procedure, from 1.5 to 10 ng/ 10 uL of digoxin solutions and 80 ng/ 10 uL internal standard solution was added to 3 mL of plasma. Initially this was deproteinized using 3 mL of acetone. The protein-free plasma was then washed with 2 mL of isooctane to remove any non-polar endogenous materials. The isooctane layer was then separated and the acetone layer was partially evaporated under a stream of nitrogen

for 20 minutes to a volume of approximately 3 mL. The remaining supernatant was then extracted with 2 aliquots of 10 mL of 2% n-propanol in dichloromethane. The combined organic phases were filtered and evaporated to dryness under nitrogen. The residue was resuspended in 100 uL of 50/50 methanol/ water. Plasma standards were prepared fresh for each assay run to establish linearity of the method. Injections of the resuspended residues into the liquid chromatograph were performed with the aid of a 1 mL syringe (Waters Assoc.). The schematic outline of the sample preparation is given in Figure 8. Plasma used in this study was obtained from The Red Cross Blood Bank and UBC Health Science Acute Care Hospital blood bank.

2.10 Recovery and precision of extraction

Recovery of digoxin from plasma following the method previously outlined (2.9.3) was checked by comparing the peak height of digoxin in plasma samples to those of the solutions of the drug in methanol of equivalent known concentration.

Repeated extractions of the same concentrations in plasma were also compared from day-to-day to determine their inter- and intra-assay variability.

2.11 Calibration curve and sensitivity of extraction

Concentrations of 0.5 to 3.3 ng/mL were prepared and analyzed. The relationship of peak height ratios to their respective concentrations was then determined.

The sensitivity of extraction was assessed by adding 0.2 ,



Figure 8. Extraction Scheme of Digoxin from Plasma

0.3 and 0.5 ng/mL of digoxin to plasma samples and extracting these as outlined in 2.9.3. A fluorescence response at the detector, with a signal to noise ratio of at least 2:1 was considered to be the minimum detection limit.

2.12 Specificity

Using drug-free plasma samples taken from the Red Cross Blood Bank (Vancouver, Canada) and UBC Health Science Acute Care Hospital (ACU) (Vancouver, Canada), the extraction procedure yielded blanks which were consistently free from interfering peaks in the retention area of digoxin (Fig.28 of Results and Discussion section).

Plasma pools, to which commonly co-administered drugs such as furosemide, quinidine, spironolactone, disopyramide, procainamide, persantine, propafenone, trimethoprim, sulfamethoxazole, captopril, docusate sodium and verapamil were tested for potential interference in the assay. Furosemide and spironolactone were among those drugs that were detected but did not elute in the retention area of digoxin. The other drugs did not show any peaks using this assay method. The parent drug and its respective metabolites were also well separated from digoxin in accordance with expected performance.

2.13 Quality control procedure

Lyphochek Radioassay control serum (human) levels I, II

and III supplied by Environmental Chemical Specialties (Anaheim, Ca., USA) were used as the quality control check for the correlation of the hospital RIA method and the HPLC post-column method developed. Level I lot (#19181), level II lot(#19182), and level III lot (#19183), were used in this study. Known assay values for level I were in the low range, level II were in the mid-range and level III were elevated. Each Lyphochek Radioassay control serum was reconstituted to 5 mL and extracted in the manner described in the plasma extraction procedure. The mean values determined for the HPLC post-column method were derived from replicate analyses. The UBC Health Science Acute Care Hospital RIA kits used to assay these samples were supplied by Nuclear Medical Labs. Inc. (Dallas, Tx., USA).

2.14 Analysis of cardiac patient plasma samples

Samples were obtained from UBC Health Science Acute Care Hospital and Vancouver General Hospital (VGH) (Vancouver, Canada). A portion of the plasma was analyzed by the hospital RIA method and the remaining plasma was stored in a refrigerator until required for HPLC analysis.

Water was sometimes added to the plasma sample to make up the volume to 3 mL to avoid changes in the partition coefficients in the extraction step. The supernatant was processed according to the procedure outlined above.

2.15 Calculations

Linearity of the assay procedure was established in the range 0.5 ng/mL to 3.3 ng/mL of plasma by analyzing spiked human plasma samples covering the range. A calibration curve, consisting of 6 different concentrations within the expected range of the samples to be analyzed was generated by least-square regression of the peak height ratios (drug/ internal standard) against the concentration ratios. The slope and intercept so obtained were used to calculate the concentrations of the patient samples.

The calculations of the correlation coefficient, slope and intercept of the standard curve of the assay method were dealt with similarly as above.

3. RESULTS AND DISCUSSION

3.1 Preliminary studies for the development of an analytical system for digoxin

3.1.1 On-column injection for capillary gas chromatography(GC)

The chemical instability of digoxin in the presence of HFBA has been shown to be the complicating factor in the analysis using GC-ECD (133). The close resemblance of digoxin to its hydrolysis products hampers the detection of degradation products or metabolites of digoxin. The extensive pre-purification step added to make the method specific, represents the major problem in this assay.

In the recent literature, on-column injection with capillary GC has been consistently proven to be useful when thermally labile compounds or high molecular weight compounds are to be analyzed (158). Because the sample is placed directly on the column without a flash vaporization step, those substances such as digoxin, which may degrade during a conventional syringe flash vaporization injection may be unaffected. In order to investigate the usefulness of on-column injection in GLC procedures, several experiments using mild reagents, such as Tri-Sil Z, MBTFA and HFBI were undertaken. The first two reagents did not lead to any peaks in the chromatogram that could be identified as the digoxin derivative.

HFBI, on the other hand, was described by the manufacturer as a mild reagent that does not release acid by-products that could lead to hydrolysis of labile compounds. Unfortunately, this reagent did lead to hydrolysis of digoxin to its aglycone and it was assumed to be due to the temperature required to effect derivatization (Figure 9). No other derivatizing reagent is commercially available that would offer any advantage in terms of sensitivity and specificity for the measurement of digoxin to those already tried.

3.1.2 Liquid chromatography- mass spectrometry

The development of combined high-performance liquid chromatography (HPLC) and mass spectrometry (MS) is arousing much interest as the technique shows a growing capability for handling non-volatile organic substances. Mass spectrometry is the most powerful technique available for structural characterization and is often the method of choice for the identification of compounds. By utilizing the high specificity of the mass spectrometer combined with the resolving power of the HPLC, the individual metabolites of digoxin could feasibly be assayed independently and therefore lead to accurate quantitation of digoxin itself.

A conventional reversed-phase octadecylsilane (ODS) 25 cm column was coupled to the IC/MS system using a spray interface. Several experiments were attempted to determine the chromatographic parameters for the digoxin standard samples. It appeared from these experiments that the concentrations of the samples and the amount of

Figure 9. Gas Chromatogram of HFB-Digoxin





water in the effluent affected the performance of the mass spectrometer. The mass spectra were found to vary with the concentrations introduced into the interface. Several mobile phases of different water composition were examined and each time a different fragmentation pattern was obtained.

The present LC-MS system equipped with a direct liquid introduction (DLI) interface requires solvent splitting. Only 10uL/min can be accepted by the mass spectrometer; therefore only 1% of the solute injected onto the conventional column can be introduced into the mass spectrometer at nominal flow rates of 1 mL/min. Consequently, the detection limit of this system is relatively unfavorable for digoxin therapeutic monitoring. The use of microbore packed columns which have become commercially available can be considered as an attractive and efficient solution to the problem because with such columns all the mobile phase can be continously delivered into the ion-source. However, the major disadvantage is that the entire LC system has to be converted to microbore capability to accomodate such columns. In addition, in order to use the LC-MS available for digoxin quantitation, modifications on the LC-MS interface would be needed. Therefore, the lack of reproducible ion spectra and the lack of a sufficiently efficient interface prevented further studies of this mode of analysis.

3.1.3 Liquid chromatography and UV detection

The assessment of plasma levels of digoxin and its metabolites would definitely require methods that could resolve all the metabolites from digoxin. We have reported (138) the usefulness of adapting a reversed-phase system to the HPLC analysis of this cardenolide as determined by spectrophotometric assay, based on the UV absorbance (220 nm) of the lactone ring. Unfortunately, these compounds exhibited relatively weak absorption maxima at this wavelength which was not sufficient for detection of nanogram amounts of digoxin in a biological matrix.

3.1.4 Fluorometric determination of digoxin

An initial attempt to develop a fluorescence assay for digoxin was accomplished by using a fluorotag, dansyl hydrazine. This tag would derivatize carbonyl functions such as that present in the lactone ring of digoxin to give a dansyl hydrazone derivative. However, sensitivity was poor and also yielded several derivatives as depicted by the chromatogram (Figure 10).

Analytical studies on cardiac glycosides so far have shown that specific reactions may only be expected from a reaction taking place somewhere in the steroid ring system. Methods based on such reactions were for the most part fluorometric. We therefore compared the fluorescence intensity generated by different existing fluorometric methods (Table 1). It is evident from the results that the United States Pharmacopeial (USP) method is the most sensitive of the procedures used. Consequently, attention was focused on this method, which depends on the action of hydrochloric acid in the

Figure 10. HPLC Chromatogram of Dansyl Hydrazone of Digoxin



Mobile phase: Methanol/ water (70:30); flow rate: 1.0 mL/min; fluorescence detection wavelength: excitation/emission 350/520 nm; sample size: 40ug/20uL

METHOD	WAVELENGTH (nm)	RFI n=3
Naik (a)	390/420	3.0 <u>+</u> 0.2
Jakovljevic (b)	345/435	0.8 ± .01
USP method (c)	375/420	7.1 ± 0.2

: .

(a) sulfuric acid (145)

(b) acetic anhydride, acetyl chloride and trifluoroacetic acid(146)

(c) ascorbic acid, hydrogen peroxide and concentrated hydrochloric acid (147).

presence of hydrogen peroxide and ascorbic acid in methanol. With this procedure, the excitation and emission spectra were determined for digoxin using an Aminco Bowman spectrofluorometer (Figure 11). The development and decay of digoxin fluorescence, using the USP method, from 1 to 7 hours were studied. It was found that by substituting water for methanol (as required in the USP) there was no change in the intensity of the fluorescence and that the fluorescence was stable throughout the period examined. This procedure was therefore adapted and modified for the post-column derivatization technique. Since water is preferred in any reversed-phase HPLC mobile phase for chromatographic resolution, this substitution was employed.

3.2 Optimization of the HPLC post-column derivatization method

3.2.1 Other reagents for derivatization

In order to minimize the deleterious effects of HCl fumes on the instrumentation, several attempts were made to substitute HCl with other less fuming acids such as sulfuric acid (70%), perchloric acid and trichloroacetic acid. Unfortunately, these acids produced less fluorescence intensity than did HCl (Table 2).

Other investigations of additional fluorogenic reagents were chosen from those that involved the functional groups present in the steroid ring structure of digoxin. Horikawa et al. (149)





Wavelength

TABLE 2. Effect of Different Acids on the Fluorescence Intensity of Digoxin Using Post-column Derivatization

Acid	Peak Height (cm)				
Hydrochloric acid	5.4				
Sulfuric Acid (70%) Perchloric Acid	1.1 3.0				
Trichloroacetic Acid	1.0				

developed a fluorometric method for the determination of ▲-3-ketosteroids in which the steroids react with isonicotinylhydrazine (INH) in a methanolic aluminum chloride solution to form hydrazones. These hydrazones fluoresce owing to complex formation with aluminum ions. Another method was that of Seki et al. (148) who used glycinamide/hexacyanoferrate in a weakly alkaline media of borate solution to detect urinary 17-hydroxyl-corticosteroids. Several parameters were changed to optimize the reactions but these proved to be unsuccessful since the derivative formed exhibited a signal less than 10% of the HCl induced fluorescent derivative.

3.2.2 Flow cell

Our progress had been hampered, in great measure, due to detector problems. Most of the detector flow cells available on the market are made of stainless steel, and a major drawback was encountered when it was realized that the rate at which hydrochloric acid was etching the cell surface of the Schoeffel 970 fluorescence detector, rendering it unserviceable in a few weeks. In order to minimize this etching and prevent damage to the detector, it was necessary to substitute the stainless steel flow cell. A titanium cell was finally suggested (159) and manufactured to the exact specifications as the standard stainless steel cell. When the titanium cell was installed and tested it was found to be more resistant to hydrochloric acid but still was etched. This etching, however slight, led to leakage of the HCl gas from the solution and

was suspected to damage the electronics of the instrument.

These problems with the Schoeffel detector required us to return to using the less sensitive Waters 420AC fluorometer. An all quartz flow cell of 40 mm in length and 1 mm ID, connected by acidflex tubing and held in place by epoxy glue, was used. Although the Waters fluorometer was not as sensitive as the Schoeffel instrument, the advantages of a non-corrosive cell were considered significant and it was therefore used in subsequent experiments.

3.2.3 Choice of chromatographic columns

An initial study to optimize the HPLC post-column fluorometric technique was focused on the development of a rapid method for the separation and accurate quantitation of digoxin. Dihydrodigoxin is quantitatively of major importance in some patients and therefore should be separated from digoxin if accurate measurements of digoxin are to be achieved. Thus far, the separation of dihydrodigoxin from digoxin has never been reported in the literature. As a consequence, in approaching this goal, we have included the dihydro-metabolites dihydrodigoxin and its aglycone, in the separation of digoxin.

It is relevant to emphasize the importance of obtaining a short chromatographic time. Since LC bands widen as retention time increases, later-eluting bands show a corresponding reduction in peak height and eventually disappear into the baseline.

Several reversed-phase columns were investigated as to their ability to separate digoxin and its metabolites using the

combination alcohol/water mobile phase. To compare the efficiency of these columns, it is necessary to have some quantitative measure of the relative resolution achieved (see Fig. 12 to 15). The resolution (Rs) of digoxin (I) and dihydrodigoxin (II) is defined as the distance between the two band centers, divided by the average band width (160):

 $Rs = \frac{(t_{II} - t_{I})}{(t_{2})(w_{I} + w_{II})} \qquad \text{equation 1}$

 $t_{\rm I}$ and $t_{\rm II}$ refer to the retention time of dihydrodigoxin and digoxin respectively. $w_{\rm I}$ and $w_{\rm II}$ refer to the bandwidth of the two bands.

The chromatograms in Figure 13 show that the separation and resolution could be optimized by choosing the appropriate column length and the size of the packing materials used. It was evident that the shorter columns reduce analysis time, albeit at the cost of resolution. Some of the selectivity can be restored, however, by using smaller size particles for packing the column. The 10 cm column showed an overall shorter chromatogram. A more dramatic difference between the 10 cm and 25 cm column is depicted in Figure 12. Additional separation selectivity was obtained by using a C-8 column (Figure 14) but at the expense of increased elution time. Finally, an acceptable total chromatographic time and resolution was achieved by using a 3u, 15cm length column (Figure 15).

Although the separation of dihydrodigoxin from digoxin was not completely resolved (Rs= .909), measurement of the quantity of digoxin would be accurate if peak height measurements were used.



Chromatographic conditions =

Mobile phase = Methanol - Water (70-30); flow rate = 0.1 mL/min; UV detection at 210 nm, for identification of the peaks see Figure 13

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Legends

- 1. dihydrodigoxigenin
- 2. digoxigenin
- 3. digoxigenin monodigitoxoside
- 4. digoxigenin bisdigitoxoside
- 5. dihydrodigoxin
- 6. digoxin

Chromatographic conditions =

Mobile phase = Methanol - ethanol - isopropanol - water (52-3-1-45); flow rate = 0.3 mL/min; post-column fluoresence detection using non-segmented reaction system as described in Figure 7.

Figure 14. Separation of Digoxin





Conditions and identification of peaks same as in Figure 13.

Digoxin and its Metabolites



3 um

Conditions and identification of peaks same as in Figure 13.

3.2.4 Effect of mobile phase

In the process of developing a specific assay, a change in separation selectivity was indicated. This was most readily achieved by a change of mobile phase selectivity. A systematic approach to selectivity optimization was undertaken to find the correct solvent strength of the mobile phase. Solvent mixtures of three or four water miscible organic modifiers were also used in fine-tuning solvent selectivity to give the best separation. Solvent strength can be accurately measured by polarity (P'). P' of a solvent mixture is the arithmetic average of the P' values of the pure solvents in the mixture, weighted according to the volume fraction of each solvent (161):

 $P' = \sum_{i=1}^{n} \emptyset_i P_i \quad \text{equation } 2$

n = the number of pure solvent in the mixture \emptyset = volume fraction of component of solvent system P_i^i = solvent polarity parameter

Table 3 shows the solvent strengths of the mobile phases used. Figure 16 illustrates the linear relationship of the resolution of digoxin and dihydrodigoxin with the polarities of the solvents.

The idea of combining liquid chromatography with post-column reactions for detection purposes has some drawbacks. The major limitation lies in the restriction placed on the choice of the chromatographic solvents since this will influence the reaction. Wells et al. (177) studied the effect of methanol on the fluorescence of cardiac glycosides and found that methanol is an

TABLE 3.	Mobile	Phase	Polarity	and	Resolution	of	Digoxin	from	Dihydrodi	igoxin
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n	MOBILE ETOH	PHASE MEOH	COMPOSITION WATER	(%) (a) IPA	DCM	P'e (b)	Rs (c)
1		70	30			663	0
2		40	47	9	4	727.3	?
3	5	55	40			710	0.3
4	5	50	45			735.5	0.65
5	4.95	54.46	39.60	0.99		706.81	0.298
6	4.95	49.50) 44.55	0.99		732.0	0.752
7	2.97	58.42	37.62	0.99		698.3	0
8	2.97	54.46	5 41.58	0.99		718.5	0.808
9	2.97	51.49	9 44.55	0.99		733.6	0.909
10	2	55	42	1		721.4	0.516

- (a) Polarity of solvents: ETOH (ethanol) 4.3, IPA (isopropanol) 3.9, MEOH (methanol) 5.1, DCM (dichloromethane) 3.1, WATER 10.2 (161)
- (b) Polarity calculated from ratio of solvents and using the polarity value (equation 2).
- (c) Resolution calculated using equation 1.





Solvent Polarity

important component of the reaction medium. Similar observations were found in the optimization of the mobile phase, that is, the combined alcohols (Table 3) were preferred as organic solvents for the post-column reaction, since other solvents such as acetonitrile and dioxane were found to decrease the fluorescence yield of digoxin. The mobile phase developed by Desta et al. (138) was not suitable for the present post-column reaction because of the quenching of the fluorescence by dichloromethane and the lowering of the boiling point of the mobile phase, therefore creating bubbles in the detector cell. The combination of the different proportions of alcohols in the mobile phase for this study was chosen to be #9 of Table 3.

3.2.5 Choice of reaction detector

The choice of a particular reaction detector type is influenced by the kinetics of the reaction since the primary aim is to preserve the original chromatographic resolution as much as possible. Band broadening is another crucial factor to consider in the construction of the proper reaction detector. On the basis of literature data, it could not be expected that, for a certain reaction time, a segmented system can compete with a tubular or a packed bed reactor. The three types of reactors were therefore compared.

3.2.5.1 Packed-bed reactor

A packed-bed reactor was examined in consideration to this

type of reactor can be favorable for reactions that take up to several minutes. The flow rates of the reagents and mobile phase were maintained at the rate optimized by Gfeller et al. (9). In order to minimize band broadening phenomena, good packing technique of the packed bed similar to the packing of HPLC column was used. Packings were initially washed with water then methanol to remove fines and organic impurities from the beads. Unfortunately, when the fluorogenic reagents (HCl and dehydroascorbic acid) were introduced into the bed, a yellowish discoloration was observed. Consequently, the back pressure inside the column increased preventing the reagents to flow through the bed. This was assumed to be due to the disintegration of the beads caused by the HCl solution. The instability of the glass beads under the reaction conditions precluded further experimentation with this type of reactor.

3.2.5.2 Air segmented reactor

Studies of the post-column reactor for the fluorogenic analysis were also undertaken using a continuous flow air segmentation system (Figure 6). Several modifications to the method developed by Gfeller et al. (9) were introduced. Miniaturization of the mixing tees, mixing coils, reaction spirals and debubbler reduced the band broadening and hence increased the sensitivity for detection of digoxin. The reagent delivery geometry was also altered to conform with the findings of Frei et al. (162,163) who noted that the construction of the mixing unit to be crucial, especially when eluent and reagent solutions are of widely differing densities. A

design, whereby the reagent solution enters at a 30° angle against the eluent stream, causes enough turbulence and good radial mixing to reduce band broadening by more than 30% as compared to addition at a 90° angle to the eluent flow. Therefore this was adapted as the mixing unit. It has also been noted in the literature that the Technicon (C5) debubbler itself contributes heavily to sample carry-over in the system (164). By reducing the internal volume of this C5 debubbler to a minimum, we anticipated a notable improvement in band broadening of the peaks. The least detectable amount achieved using this modified system was 5 ng/injection at signal/ noise= 3/1 (Figure 17). From the sensitivity observed and data derived after the band broadening study (see band broadening section 3.2.10) it appeared that the miniaturization only contributed to slight improvement in the performance of the system.

The beneficial effect of air segmentation in preventing carry-over has been emphasized extensively (165). However, there are technical drawbacks that are worth noting: (a) the streams have to be debubbled before they reach the flow cell; (b) the size of the air bubbles has to be controlled; hence, only glass coils are used for mixing to preserve the integrity of the bubbles(166); (c) a leak-free system is required for caustic reagents. The fluctuation in the solvent flowing pressure created problems for the acidflex tubing which held the mixing units and the reactor in series. It was observed that an increase in flowing pressure disconnected the acidflex tubing from the glass coils making it difficult to maintain the leak-free system. Therefore for the reasons enumerated, further
Figure 17. Low Level Detection of

Digoxin Using Air Segmentation System



Chromatographic conditions:

Column: 15 cm, 3 um ODS; mobile phase: methanol/ ethanol/isopropanol/ water (52:3:1:45); flow rate: 0.4 mL/min; post-column detection using air-segmented reaction system as described in Figure 6. experiments on this type of reactor were discontinued.

3.2.5.3 Tubular non-segmented reactor

Further studies on the post-column derivatization for the fluorogenic analysis of digoxin were undertaken using a non-segmented reactor as described in the experimental section. The elimination of the bubble segment in this case greatly increased the ease of maintaining the post-column reactor flow.

Several of the HPLC post-column derivatization parameters were investigated individually.

3.2.6 Relative proportions of reagents

By altering the concentrations of dehydroascorbic acid, while maintaining the quantity of HCl constant, and then reversing the relationship, an optimal balance was found (Table 4). A flow ratio of 0.1 dehydroascorbic acid to 0.5 HCl yielded an optimal fluorogenic efficiency for these two reagents.

In addition to this study, it was found that the concentration of hydrogen peroxide in ascorbic acid (dehydroascorbic acid) was affecting the efficiency of fluorescence. In this study (Figure 18) a very sharp maximum was observed. Hence, this reagent was prepared fresh daily to make certain that the optimum concentration was maintained $(1.1X10^{-3}M \text{ in a } 0.1\%w/v \text{ ascorbic acid solution})$.

I.D. DA Delivery Tubing (a)	Peak Height n=5
.02 "	1.95 <u>+</u> .05 cm
.015 .010	2.33 <u>+</u> .04 1.48 <u>+</u> .07

TABLE 4. Relative Proportions of Reagents

I.D. HCl Delivery Tubing (b)	Peak Height n=5
.29 "	1.80 <u>+</u> .07 cm
•53	2.35 <u>+</u> .06
.63	1.95 <u>+</u> .07

(a) dehydroascorbic acid tubing (Solvaflex) internal diameter where HCl tubing used was 0.53 "ID

(b) hydrochloric acid tubing (acidflex) internal diameter where DA tubing used was .015 "ID



3.2.7 Optimization of reaction temperature

Initial optimization of this parameter was studied with methanol as the mobile phase. In order to accelerate the reaction and simultaneously increase the fluorescence efficiency of the derivatization, the temperature of the bath was altered up to 64° C. Above $64^{\circ}C$, the methanol present in the reaction medium boiled and data could not be obtained. A reaction temperature of 59⁰C was therefore selected as the optimum reaction temperature when the mobile phase consisted of pure methanol (Figure 19). Following the development of the mobile phase for the separation of digoxin from its metabolites, it was found that the temperature of the reaction bath could be increased beyond the boiling point of methanol without bubble formation occurring in the detector cell. This increase in temperature considerably accelerated the reaction time in the post-column reactor. A restriction coil (1 m teflon tubing) connected at the exit end of the detector added back pressure to the system which facilitated still higher temperatures without boiling of the reaction medium, thereby further increasing the rate of the post-column reaction. Maximum peak height was obtained at a temperature of 79 \pm 1⁰C. Figure 20 shows the effect of the increased reaction temperature on the sensitivity of the post-column detection.

3.2.8 Reaction kinetics

In addition to the reaction temperature, the reaction time must also be optimized. This study was most readily accomplished by











HPLC post-column detection conditions same as in Figure 13. Amount of digoxin inejected : 1 ng.

of Detection

varying the speed of the peristaltic pump which delivers the HCl and dehydroascorbic acid. From the experimental data (see Figure 21) it was determined that the total flow rate of 0.27 mL/min (HPLC pump plus peristaltic pump flow) was optimal for the reaction time. The HPLC pump was operated at 0.1 mL/min for the whole range of the peristaltic pump speed. In terms of actual reaction time, a total flow of 0.27 mL/min requires 7 minutes to traverse a 0.3mm X 20m coil. Reaction kinetics were also studied by controlling the length of the capillary reactor. Reaction coils of 10, 15, 20 and 25m with 0.3mm internal diameter provided reaction times of 3, 5, 7 and 8.6 minutes respectively with combined 0.1 mL/min mobile phase and 0.17 mL/min total reagent flow rates. Response curves obtained for digoxin at the four reaction times are shown in Figure 22. The decrease in peak height response for the 25 m coil appeared to be due in part to peak dispersion in the reaction tube. The 20 m coil enabled maximum response with minimal peak broadening.

It has been established (165) that while traversing a straight reaction tube more dispersion is obtained. This situation was suggested to improve drastically when moving through a coiled reaction tube which was believed to create turbulent flow. This phenomenon was deduced to be due to centrifugal forces acting on the flow pattern; secondary flows perpendicular to the main flow direction are produced. As a result, better radial mixing and consequently, a reduction of band broadening occurs (see Figure 23).

With this knowledge in mind, two different reactors were studied which were wound to give an outer diameter of either 50 cm



Figure 21

100



101

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FIGURE 23. Secondary flow pattern in the cross section of a coiled tube!64

or 2.7 cm respectively. The peak height, which reduces as band broadening increases, was 42% greater for the narrow (2.7 cm) coil.

Hofmann and Halasz (167) proposed that geometrical deformation of a tube also leads to reduced band dispersion. In order to optimize the arrangement of the reaction tube, several types of reactor configurations were compared with respect to peak broadening.

The results obtained for the different reactor geometries examined are as follows:

(a) 20 m coils of 2.7 cm diameter post-column reactor had a basewidth of 3 minutes.

(b) 10 m coil of 2.7 cm diameter post-column reactor had a basewidth of 2.5 minutes.

Studies by Uihlein and Schwab (168) have shown that knitted ("fringes" like those used for crochet work) PTFE capillaries lead to further improvement with respect to peak broadening. This type of reactor geometry was therefore adapted to the fluorogenic system under investigation. A 10 m capillary tube was knitted into fringes and used as the reaction unit. A basewidth of 2.2 minutes was found. The asymmetric factor for the knitted configuration was 2.5 while the coiled reactor had an asymmetric factor of 3. The knitted capillary reactor had a slight advantage over the tightly coiled tube. This was similar to the observations made by Deelder et al. (169).

Initially, using the 2.7 cm diameter PTFE reactor (20 m) the flow rate of the HPLC pump was varied to optimize resolution

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and speed of analysis. A flow rate of 0.15 mL/min was found to give the best result without contributing excessive back pressure to the reagent delivery path. The decrease in back pressure of a 10 m reactor, as compared to the 20 m reactor, allowed us to increase the HPLC flow rate from 0.15 mL/min to 0.45 mL/min. Figure 24 shows the results from this study. The consequent increase in the flow rate of the HPLC pump shortened the analysis time by 15 minutes. A maximum flow rate of 0.3 mL/min from the HPLC pump with the peristaltic pump flow fine tuned at a flow rate of 0.23 mL/min was used in subsequent experiments.

3.2.9 Detector wavelength

Excitation and emission maxima of the derivatized digoxin were obtained by changing the excitation and emission filter combinations and are listed on Table 5. The emission maximum was found to be at 425 nm, whilst the excitation maximum occurred at 360 nm. Use of these filters gave the maximum sensitivity for the detection of digoxin.

3.2.10 Band broadening

In order to preserve the resolution of the column, the band broadening contribution of the reactor obviously should be as small as possible. Therefore, a comparison between the different reactors (Table 6) was made. An ultra-violet(UV) detector was inserted between the HPLC column and the post-column reactor system to measure relative band dispersion due to the reactor. Band



Optimization of HPLC flow rate

Figure24

HPLC flow rate (ml/min)

FILTERS nm (a)	PEAK HEIGHT (cm) n=3		
360/470	4.50 <u>+</u> .03		
360/425	6.25 <u>+</u> .04		
360/440	5.50 <u>+</u> .03		
360/450	. 0		
340/450	0		
360/460	1.50 +.02		
340/470	0		
340/440	0.50 <u>+</u> .01		
340/425	0.60 <u>+</u> .02		

TABLE 5. Filter Combinations for Optimum Detector Wavelength

(a) excitation/emission filters for Waters Fluorometer

TABLE 6. BAND BROADENING OF REACTION SYSTEM

DIMENSION	CONFIGURATION	TOTAL FLOW RATE (mL/min)	PEAK HEIGHT (cm)	PEAK WIDTH (min)	RESIDENCE TIME (min)
I. AIR SEGMENTATION: 11.3 m(L) X 9cm diameter 1 mm ID	Coil modified unmodified	0.53 0.53	3.0 2.75	2.5 2.8	15 15
<pre>II. NON SEGMENTED REACTOR: 20 m(L) X 2.7cm diameter 0.3 mm ID 10 m(L) X 2.7cm diameter 0.3 mm ID 10 m(L) X 0.3 mm ID</pre>	coil coil knitted	0.34 0.53 (a) 0.40 (b) 0.53 (a) 0.40 (b)	6.1 5.5 5.0 6.0 5.3	3.0 2.0 2.5 2.0 2.2	7 2 3 2 3
III. UV detection		0.30 (c)		1.8	-

(a) 0.3 mL/min HPLC flow rate
(b) 0.15 mL/min HPLC flow rate
(c) retention time for digoxin peak by UV detection: 30 minutes

broadening as determined from the peak width at base between UV and fluorescence detection of the different reactors investigated is shown in Table 6.

The data in Table 6 showed that the 10 m knitted configuration contributed the least to peak dispersion. This reactor was chosen as a compromise between the time needed for the reaction and the peak broadening due to the length of this coil, which might even destroy a separation already obtained in the column.

Since other components of the LC system may also contribute to peak dispersion, we examined the effect of using a Rheodyne loop injector and the Waters U6K Universal injector. It was found that the latter gave less dispersion with better reproducibility between injections. The design of this injection port has been reported to allow the introduction of the sample as a narrow band, thus reducing the occurrence of asymmetrical tailing peaks.

3.2.11 Baseline noise

Proper proportioning of the reagents is important since the ratio of the reagent and effluent may vary with time, leading to noisy and/or drifting baseline and ragged looking bands. A smooth, constant delivery of the reagent is essential, and this has been proposed to be most economically obtained with a nitrogen pressurized glass reagent reservoir (170). Unfortunately, the glass reservoirs available that are acid resistant are only rated to 25

psi, therefore making it difficult to propel the reagents efficiently through the 20 m reaction coil which has an estimated back pressure of 50 psi. In addition, the presence of HCl in the reagent makes it unsafe to operate. Therefore a peristaltic pump was, inspite of its shortcomings, used as the means of propelling the carrier streams. The main drawback of this type of pump is that the stream is never completely pulse-free. However, an improvement in the proportioning was achieved by frequent change of the acidflex pump tubes that delivered the HCl. The adjustment of the pressure of the acidflex tubing was also found to be important to achieve optimal stability of the flow of the reagents. These minor manipulations of the delivery of the reagents also improved the day-to-day reproducibility of the post-column derivatization method.

The mixing of effluent and reagents must occur before the completion of the reaction. Mixing in non-segmented streams is often slow. This problem has found to be alleviated by the use of a premixing PTFE coil before the addition of effluent and by the use of the knitted reactor (171). Poor mixing led to symptoms analogous to improper proportioning, mainly noisy baseline.

An alternate approach to suppress excessive baseline noise is the use of a noise filter. Thus, a noise filter (as described in the experimental section 2.2.1) was constructed and fitted between the detector and data system. The chromatograms in Figure 25 illustrate the difference obtained following incorporation of a noise filter.





A: WITHOUT NOISE FILTER B: WITH NOISE FILTER

3.2.12 Sensitivity

The incorporation of β -cyclodextrin, a fluorescence enhancer, into the manifold was initially proposed to increase the sensitivity of detection. Cyclodextrin, a monocyclic polymer is known to form an inclusion complex with digoxin and this has been found to increase the fluorescence intensity of a variety of organic compounds (172,173). Several modifications such as β -cyclodextrin concentration, detector wavelength and reaction times were considered. However, no apparent increase in fluorescence intensity was found when cyclodextrin was added into the reaction medium. This suggested that either there was no interaction of the β -cyclodextrin with digoxin under this time condition, or that the polymer was being degraded in the acidic environment as shown by Saenger (174).

3.2.13 Reproducibility and Calibration Curve

The coefficient of variation obtained for repeated injections of standards of digoxin at the 3 ng level was 2% (n=10), indicating that the present method is satisfactorily reproducible. A linear correlation between peak height ratios and concentration over the range of 1.5 ng to 10 ng per injection was observed (Table 7, Figure 26) which was adequate for current clinical needs. Good reproducibility was also obtained and assessed by repeating the calibration procedure on 5 different days. An overall average of 8% coefficient of variation was found.

Weight Ratio (a)	Peak Height Ratio (b)	<u>+</u> S.D.
.022	.210	.01
.029	.300	.02
.044	.450	.04
.073	.730	.07
.102	1.02	.06
.147	1.47	.10
	1	

TABLE 7. Data for the Standard Curve

(a) digoxin weight/internal standard weight

(b) digoxin peak height/internal standard peak height (mean of 5 days, repeated each day)



Weight Ratio

Figure 26

3.2.14 Choice of internal standard

An internal standard is a substance added to the sample at the earliest possible point in the analytical scheme to compensate for sample loss occurring during sample extraction, clean-up and final chromatographic analysis. The desirable characteristics of an internal standard are that it will co-extract with similar partition efficiency to that of the drug; that it has similar detection characteristics to that of the analyte; that it does not co-elute with the drug, metabolites or any biological endogenous substances and lastly, that it is readily available.

The choice of an internal standard in the present method was further complicated by the fact that it must, after passing through the post-column reactor, fluoresce at the chosen wavelengths (360/425 nm) similar to the drug being analyzed. In addition, the drug substance should not be a "co-administered" drug so that it would not be expected in the plasma of a significant number of patients. Within these limitations the following compounds were examined as possible internal standards: 17e'-methyl-testosterone (93 mins), ethinylestradiol (greater than 100 mins), prednisone (60 min), 17- estradiol (68 min), norethindrone (60 min), ouabain (18.4 min), cymarin (20 min), and digitoxigenin (40 min). Triamcinolone, diethylstilbestrol, mestranol and testosterone did not give any response.

Most of the steroids have long retention times and were not practical as internal standards. Cymarin and ouabain, on the other hand, were too polar and co-eluted with the metabolites.

Figure 27. Separation of Digoxin, its metabolites

and co-administered Drugs Using Post-Column

Detection



Chromatographic conditions:

Mobile phase: Methanolethanol- isopropanolwater (52/3/1/45); Flow rate: 0.3 mL/min; post-column fluorescence detection using nonsegmented reaction system as described in Figure 7.

Legends:

- 1. furosemide
- 2. dihydrodigoxigenin
- 3. digoxigenin
- 4. digoxigenin monodigitoxoside
- 5. digoxigenin bisdigitoxoside
- 6. dihydrodigoxin
- 7. digoxin
- 8. digitoxigenin (Int. Std.)
- 9. spironolactone

Digitoxigenin, however, fulfilled the criteria for an internal standard. Figure 27 depicts the chromatogram of the separation of digoxin, its metabolites and digitoxigenin and co-administered drugs eluted with the quaternary solvent system.

3.3 Application of the analytical procedure to plasma samples

3.3.1 Optimization of extraction method in spiked water

The efficiency of an extracting solvent depends primarily on the affinity of the solute for the extracting solvent as measured by partition coefficient, the phase ratio (volume of extracting solvent/ volume of sample), and the number of extraction steps. These studies can be divided into liquid-liquid extractions, solid-phase or cartridge extractions and combination of these two methods.

Initial experiments were carried out by extracting 1 ng/mL aliquots of digoxin in water (5 mL). The most appropriate methods in terms of recovery and time were ultimately tested with plasma aliquots containing the same quantity of digoxin.

A wide selection of pure solvents providing a wide range of solubility and selectivity properties was examined. Improved recovery of digoxin was observed by using a binary solvent mixture. In addition to this optimum adjustment in solvent polarity, recovery could be further increased by using larger volumes of solvent for the same sample quantity, or by using multiple sequential

TABLE 8.

SOLVENT-SOLVENT EXTRACTION OF DIGOXIN IN WATER_

Procedure:

5 mL water containing 1 ng/mL digoxin

Extract with organic solvent (see below)

Remove Organic solvent

Evaporate Organic solvent under nitrogen

Reconstitute with 100 uL HPLC mobile phase

Organic Solvents	Volume/ratio	Assay Recovery		
Dichloromethane (DCM)	15 mL	31%		
Ш .	2x 15.5mL	38%		
	3x 15 mL	47%		
11	4x 15 mL	69%		
Ethyl acetate	15 mL	36%		
DCM/isobutanol	15:2 (10 mL)	34%		
DCM/propanol	15:2 (10 mL)	41%		
DCM/ethanol	15:2 (10 mL)	31%		
DCM/methanol	15:2 (10 mL)	47%		
DCM/isopropanol	15:2 (10 mL)	42%		
DCM/isopropanol	15:1 (10 mL)	41%		
DCM/isopropanol	15:5 (10 ml)	42%		
DCM/isopropanol	2x 15:1 (10 mL)	75%		
Chloroform/methanol	2x 6:4 (10 mL)	55%		
Chloroform/isopropanol	2X 9:1 (10 mL)	80%		

extractions (see Table 8). There is a limit to the phase volume of the extracting solvent and the number of extractions performed before the method becomes tedious.

Results obtained from some of the organic solvents are presented in Table 8. The data indicated that the highest recovery of the drug could be expected in double extraction using either chloroform / isopropanol in a ratio of 9:1 or dichloromethane / isopropanol in a ratio of 15:1.

Solid-phase cartridge or bonded phase sorbents introduced for off-line sample preparation are widely used for trace enrichment and sample clean-up. Chemically they are similar to the column packings used in HPIC such as silica, diol $(C_2(OH)_2)$ or octadecylsilane bonded to silica, except for its particle size (usually 40 um). The cartridge is used to facilitate the sampling process and further increase the extraction yield. The sorbents are usually packaged in disposable polypropylene columns sandwiched between two polyethylene frits. The principle of the procedure involves the selective retaining of the compound of interest on the adsorbent as it first passes through the column and then subsequently the analyte is eluted with a specific solvent. Certain undesirable compounds which are adsorbed on the first pass can be selectively removed by washing with an intermediate solvent prior to final elution of the compound of interest (175).

The data given in Table 9 for the cartridge recovery of digoxin from water revealed an 81% yield when the elution solvent consisted of dichloromethane / isopropanol in a ratio of 15:1.

TABLE ĝ.

Solid Phase Cartridge Extraction of Digoxin in Water

Procedure:

Elute 5 mL of water containing Digoxin (lng/mL) through 2.8 mL cartridge

Extract cartridge with 10 mL Organic solvent (see below)

Evaporate Organic Solvent under nitrogen

Reconstitute Residue in HPLC mobile phase (methanol: Water. 50:50)

Cartridge		Organic Solvents	Ratio of Solvents	Recovery (%)
Octadecy1 RP-18	Silane	Chloroform/ Isopropanol	3:2	40
H	н	H H	6:1	43
H	11	Chloroform/Methanol	6:1	73
U II	н	Dichloromethane/Isopropanol	15:1	46
Silica		Chloroform/Isopropanol	3:2	65
н		н н	6:1	69
U U		Chloroform/Methanol	6:1	69
U II		н н	3:2	83
F1		Dichloromethane/Isopropanol	15:1	81
Diol	ć	11	15:1	81

Cartridges obtained from Analytichem International, Harbor City, CA., USA

Although this yield was similar to the previous solvent-solvent extraction, it was more efficient in that a smaller volume of organic solvent had to be evaporated.

3.3.2 Optimization of extraction method in plasma

The rate and extent of extraction may be different for a solute in a test system than in a practical sample. Partial association of drug substances with protein in plasma samples is one instance where the extraction efficiency may vary substantially from that obtained using water as a model system. Therefore, the subsequent step in the development of an extraction procedure is the isolation of the drug from the plasma sample. The solvent-solvent recovery experiments of digoxin from plasma are given in Table 10. Although only two solvents are listed in this Table they were representative of the results obtained for a number of solvents. In all cases, endogenous materials from the plasma were found to co-elute near digoxin and would therefore prevent its quantitation.

Since the polarity of digoxin was not clearly delineated, there was no consistency in the results from various bonded phases. Different solvents were evaluated to determine (a) how clean an extract was produced in terms of chromatographic background interferences and (b) how well digoxin was extracted. From the initial evaluation from water, dichloromethane / isopropanol showed the best yield with least chromatographic interference. However, the presence of an interfering peak in the plasma complicated the extraction procedure. Simple cartridge extraction was not enough to

TABLE 10.
SOLVENT-SOLVENT_EXTRACTION OF DIGUXIN FROM PLASMA
3mL plasma containing 1 ng/mL digoxin
Add 10 mL organic solvent (see below)
Tumble tube for 10 min to extract
Centrifuge at 1000 rpm for 5 min
Remove organic solvent
Evaporate organic solvent under nitrogen
Reconstitute with 100 uL HPLC mobile phase (methanol : water, 65:35)

Organic Solvents	Ratio of Organic solvents	Recovery (%)
Dichloromethane/Isopropanol Dichloromethane	15:1	_* _*

* Due to interference from plasma constituents, quantitation could not be done.

isolate the drug. Therefore, variation of the extraction methods using the 3 types of solid phases was investigated. They included; (a) precipitation of the protein in the plasma with a variety of organic solvents before its introduction through the cartridge, (b) prewashing of the adsorbed plasma sample to remove interfering endogenous compounds before its final elution from the cartridge, (c) a clean-up procedure by extraction with organic solvent before its passage through the cartridge (Table 11, 12).

The overall results from these extractions indicated that digoxin was either not readily removed from the cartridge or that digoxin in the presence of a small amount of water from the plasma, when combined with alcohol did not allow the drug to be adsorbed on the cartridge. The combination of solvent extraction and cartridge adsorption(method C Table 11) initially showed some promise. However, method C which combined a clean-up step and a cartridge extraction procedure was proven to be unreliable when plasma from a different source was used. In all cases, the extractions were found to become tedious and endogenous materials from plasma were found to interfere with the digoxin peak; hence, this method was abandoned.

It is obvious that more clean-up procedures were required prior to the LC analysis of the final sample extract. Since proteins are known to interfere with many analytical techniques, a deproteinizing step was introduced into the scheme. Reagents used to effect deproteinization were acetonitrile, trichloroacetic acid, dilute hydrochloric acid, methanol, acetone and a combination of zinc sulfate and barium hydroxide. The effect that each

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TABLE 11. SOLID PHASE CARTRIDGE EXTRACTION OF DIGOXIN IN PLASMA

Procedure:

3 mL plasma containing Digoxin (1 ng/mL) Precipitate plasma proteins with 3 mL acetonitrile Centrifuge at 1000 rpm for 5 min Pass supernatant through cartridge Discard first eluent from cartridge Elute cartridge with organic solvent Evaporate organic solvent under nitrogen Reconstitute residue in HPLC mobile phase

Cartridge	Organic Solvents (10 mL)	Ratio of solvents	Recovery (%)
Diol	Dichloromethane	1	0
Diol	Dichloromethane/isopropanol	15:1	0
Silica	n n'	15:1	0

TABLE 12

SOLVENT-SOLVENT EXTRACTION OF DIGOXIN COMBINED WITH SOLID PHASE CARTRIDGE ISOLATION

Method A

Method B

Method C

3 mL plasma containing 1 ng/mL digoxin

Extract with 10 mL Organic solvent 1 (see next page)

Pass organic solvent through cartridge

Wash cartridge with 2 mL water

Elute with organic solvent (15 mL)

Evaporate organic solvent

Reconstitute with 100 uL HPLC mobile phase 3 mL plasma containing 1 ng/mL digoxin

Extract with 2x10 mL solvent 1 (see next page)

Evaporate organic solvent 1 under nitrogen

Redissolve digoxin in solvent 2

Pass solvent 2 through cartridge

Elute cartridge with organic solvent 3

Evaporate organic solvent 3

Reconstitute with 100 uL HPLC mobile phase 3mL plasma containing 1 ng/mL digoxin

Extract with 2x10 mL solvent 1 (see next page)

Evaporate organic solvent under nitrogen

Redissolve in solvent 2

Pass solvent 2 through cartridge

Wash cartridge with solvent 3

Elute cartridge with solvent 4

Evaporate organic solvent under nitrogen

Reconstitute with 100 uL HPLC mobile phase

..... continued.....

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Method of Extraction	Cartridge	ORGANIC So Solvent 1	OLVENT RATIOS A Solvent 2	ND VOLUME (a) Solvent 3	Solvent 4	Recovery (%) (b)
A	DIOL	DCM/MeOH (15:1) 10 mL			-	N.D.
Β.	DIOL	DCM/IPA (15:1) 2x10 mL	water 1 mL	DCM/IPA (15:1) 5 mL		N.D.
В	SILICA	п	CHL 1 mL	IPA 5 mL		O
В	C-18	11	water 1 mL	DCM/IPA (15:1)		N.D.
C	DIOL	11	n	ET20/IPA (20:1) 3 mL	DCM/IPA (15:1) 5 mL	0
С	SILICA	н	CHL 1 mL	CHL 1 mL	IPA 5 mL	0
C	DIOL	n	CHL 3 mL	ETOAc 3 mL	IPA 3x10 mL	75%

(a) Solvent abbreviations :

DCM = dichloromethane IPA = isopropanol CHL = chloroform

ETOAc = ethyl acetate MeOH = methanol ET20 = diethyl ether

(b) N.D. = none detected due to interference from plasma.

deproteinizing reagent could have on the assay of a drug must be carefully considered. For example, digoxin, an acid-labile drug would not withstand deproteinization with trichloroacetic acid and hydrochloric acid. Organic solvents, on the other hand, deproteinize by destroying quaternary and tertiary structures of the proteins and are thus unlikely to hydrolyze digoxin. Although acetonitrile was reported to precipitate 99.9% of proteins, the precipitate formed was found to trap some of the solvents therefore decreasing the extraction efficiency. Methanol, on the other hand, was found to be more difficult to evaporate. Among these deproteinizing agents, acetone was found to be the most convenient to use because it has been reported to precipitate 99.1% of the protein (176) in plasma, and it is water miscible and quite volatile. By evaporating acetone from the plasma sample, subsequent extractions could be efficiently performed with appropriate organic solvents.

The presence of an interfering peak in some of the pooled plasma blanks, even after protein precipitation, prompted a search for an intermediate solvent to eliminate this endogenous interfering material. A variety of non-polar solvents were therefore examined. These were: hexane, heptane, benzene and isooctane. The most efficient solvent was found to be isooctane (Table 13) at a volume of 2mL isooctane to 3mL of plasma.

Although liquid extraction is simple and does not require complex equipment, it is not entirely free of practical problems. Extraction procedures using dichloromethane usually result in an
TABLE 13. Recovery of Digoxin Using Different Non-Polar Solvent Washes

Non-Polar Solvents	Recovery n=2
hexane	55%
heptane	65%
benzene	40%
iso-octane	70%

emulsion that is not readily separated. More persistent emulsions were formed in some of the plasma samples investigated and these could not be disrupted by high-speed centrifugation. As a consequence, the organic phase was filtered through a Nylon 66 membrane filter. This step also served to protect the column frit from clogging due to debris contained in the extractants.

Finally, to optimize the extraction procedure with respect to recovery and specificity, the proportion and polarity of the alcohol in the extractant were re-examined. Quantities of 2, 6, 10, 20% isopropanol and 2% n-propanol in dichloromethane were used in this study. It was found that by increasing the amount of isopropanol in dichloromethane, the area of the interfering peak also increased proportionately. By using n-propanol instead of isopropanol, a cleaner chromatogram could be obtained. Overall 2% n-propanol in dichloromethane was found to be more selective as an extraction solvent than 2% isopropanol in dichloromethane.

In an attempt to facilitate the extraction procedure and further increase the extraction efficiency, the solid phase extraction was re-examined. This involved the deproteinization of the "spiked" plasma sample with acetone and delipidation with isooctane with subsequent evaporation of the acetone. The remaining supernatant was applied on the diol Bond Elut column and extracted with 10 mL 2% propanol in dichloromethane. Although the extraction step was simplified, the recovery using the disposable mini-columns was found to be 30% less efficient than the manual extraction method.

The final extraction procedure used was based on the scheme described in Figure 8. A typical HPLC elution pattern of digoxin standard and the internal standard, digitoxigenin, extracted from plasma is shown in Figure 28. There were trace amounts of endogenous fluorescent contaminants which eluted before digoxin in the plasma sample (Figure 28 blank).

3.3.3 Recovery and precision of the extraction method

After an orderly logical progression through the development of the extraction procedure had been examined, an evaluation of this procedure is always required. This involved the determination of its efficiency and accuracy. Percentage recoveries were determined using blank plasma samples spiked separately with digoxin at 2, 3, 5 ng/mL plasma levels. The results are shown in Table 14. "Spiked" plasma samples covering these same concentrations were also analyzed in triplicate on 3 different days to give the inter-assay coefficient of variation. An average recovery of 70% was found for these samples.

3.3.4 Calibration curve and method sensitivity

In a linearity study, plasma samples at concentrations of 0.5 to 3.3 ng/mL were prepared, extracted and analyzed. Calibration samples were prepared from plasma samples taken from the blood bank of the Health Sciences Hospital, Acute Care Unit, UBC, Vanc., Canada. The data obtained are summarized in Table 15. Linear correlations between peak height ratios and weight ratios over the

Blank and Spiked Plasma



30

0

Plasma containing 3 ng of digoxin (1) and 80 ng of digitoxigenin (2) (internal standard). Plasma extracted according to the extraction procedure on Figure 8.

60 min

TABLE 14. Reproducibility and Recovery Data

		C.V.	. 8
Concentration Added (ng/ 3mL)	% Recovery	Intra-assay n=3	Inter-assay n=3
2 3	71 69	4 4	10 8
5	69	5	8

TJ •	Data	TOL	riasilia	Stanuaru	curve	

TADE 15

Weight Ratio (a)	Peak Height Ratio (b)	C.V.8
.022	.277 <u>+</u> .025 cm	9'
.029	.358 <u>+</u> .036	10
.044	.504 <u>+</u> .040	-8
.073	.762 + .060	8
.103	1.16 + .098	8
.147	1.54 + .168	11

(a) digoxin weight/internal standard weight

(b) digoxin peak height/internal standard peak height (mean of 5 days)







CALIBRATION CURVE OF DIGOXIN IN PLASMA SAMPLES

concentration range were found. The coefficient of correlation calculated showed acceptable variation (Figure 29). The method's limit of detection was found to be 0.5 ng/mL with a signal to noise ratio of 4 to 1. Therefore, the sensitivity of the method described is appreciably the same as that observed in the RIA method.

3.3.5 Specificity

Patients undergoing therapy with digoxin may also be treated with a variety of other drugs depending on their overall state of health. Diuretics such as spironolactone and furosemide are frequently used in cardiac patients to relieve the strain on the heart. On some occasions antiarrhythmic agents such as quinidine, verapamil or propafenone may be co-administered to aid in regulating both the beat strength and rhythmicity of the heart beat. If present, these agents could interfere chromatographically with the digoxin peak. This interference problem could easily be circumvented by determining their potential for co-elution with digoxin. Drugs in this study included furosemide, spironolactone, quinidine, procainamide, disopyramide, dipyridamole, verapamil, propafenone, captopril and other common drugs such as dioctyl sodium sulfosuccinate, trimethoprim and sulfamethoxazole. Only furosemide and spironolactone gave some response near the eluting peaks of interest (Figure 27). Included in this chromatogram were the metabolites of digoxin present in 5 ng amounts each. Although dihydrodigoxin was incompletely resolved from digoxin, the interference was minor since the fluorescent intensity of this

metabolite was approximately one-half of the digoxin response. Figure 27 shows the separation of digoxin from its metabolites and co-administered drugs, indicating that the present method is satisfactorily specific.

The final test for the specificity of this method involved the extraction of a series of plasma samples obtained from the Red Cross, the blood bank of the Health Science hospital and samples from the staff and graduate students in the Faculty of Pharmaceutical Sciences . It is considered that a method that is applicable to the pooled plasma of different sources is indeed a robust method. The overall result of this experiment showed no interference in the retention area of interest of digoxin.

3.3.6 Quality Control Procedure

To assess the accuracy and precision of this method, a Lyphochek Radioassay control serum was used. This offers a tri-level range of digoxin concentrations for intra-laboratory quality control. Assay values for Level I are in the low range, Level II is mid-range and Level III is elevated. The assay values obtained were subsequently compared to those determined by the Health Science hospital RIA method on the same sample (Figure 30). The results obtained during the analysis of the same sample by the two independent detection techniques are summarized in Table 16. As shown in Figure 30, in levels I to III, a good correlation was found between the amounts of digoxin determined by HPLC post-column procedure and the RIA method.

HPLC-PC and RIA Method on

Radioassay Control Serum



RIA Method

LEVEL	HPLC POST-COLUMN n=2	RIA (a)
I	0.598	0.55
II	2.07	2.25
III	3.09	3.24

TABLE 16. Data for Quality Control Procedure

(a) Nuclear Medical Labs (ng/mL)

3.4 Determination of digoxin in patients' plasma by HPLC post-column derivatization

Direct radioimmunoassay (RIA) of digoxin in plasma is sufficiently sensitive, rapid and inexpensive but lacks the desired selectivity (110). Apparently, high cross-reactivity values have been reported between digoxin antibodies and digoxin metabolites (110). In addition, the antibodies used have been shown to cross-react with several co-administered drugs (82, 84) as well as a "digoxin-like substance" (118) present in plasma. This interference must be eliminated, to avoid the over-estimation of digoxin, especially because this drug has a low therapeutic index. In view of the occurrence of false-positive digoxin values obtained during RIA assays, this method should be complemented by an independent chemical method with a very high degree of specificity. The method presented here allows for the detection of digoxin at levels at least equal to the useful detection limit established for the RIA procedure.

In assessing the accuracy of the HPLC post-column method, patient samples containing digoxin, ranging from sub-therapeutic to toxic concentrations were processed by the HPLC post-column derivatization method. Results obtained were compared to the RIA method. A total of 42 samples (24 from Health Science Acute Care Hospital, 18 from Vancouver General Hospital), collected during digitalization, were extracted and analyzed for digoxin.

Patients RIA HPLC-PC DETECTION 1 0.7 0.707 1.010 2 1.3 0.896 0.689 3 0.6 1.170 1.950 4 1.3 0.943 0.725 5 1.2 1.190 0.990 6 1.4 0.830 0.590 7 0.9 0.570 0.630 8 0.5 0.500 1.000 9 1.0 1.070 1.070 10 1.2 1.050 0.875 11 1.1 1.360 1.240 12 1.3 1.610 1.240 13 1.3 1.610 1.240 13 1.3 1.610 1.240 14 0.8 0.830 1.040 15 1.2 1.370 1.140 16 1.4 1.120 0.800	(Acute Care Hospital)	Digoxin Concentration ng/mL		HPLC/RIA RATIO
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	Patients	RIA	HPLC-PC DETECTION	
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$ \begin{array}{c} 1\\ 2\\ 3\\ 4\\ 5\\ 6\\ 7\\ 8\\ 9\\ 10\\ 11\\ 12\\ 13\\ 14\\ 15\\ 16\\ 17\\ 18\\ 19\\ 20\\ 21\\ 22\\ 23\\ 24\\ \end{array} $	$\begin{array}{c} 0.7\\ 1.3\\ 0.6\\ 1.3\\ 1.2\\ 1.4\\ 0.9\\ 0.5\\ 1.0\\ 1.2\\ 1.1\\ 1.3\\ 1.3\\ 0.8\\ 1.2\\ 1.4\\ 2.0\\ 2.7\\ 1.4\\ 1.2\\ 1.8\\ 2.1\\ 0.9\\ 1.1 \end{array}$	0.707 0.896 1.170 0.943 1.190 0.830 0.570 0.500 1.070 1.050 1.360 1.610 1.530 0.830 1.370 1.120 2.010 1.800 0.769 1.260 1.730 1.550 0.560 0.850	$\begin{array}{c} 1.010\\ 0.689\\ 1.950\\ 0.725\\ 0.990\\ 0.590\\ 0.630\\ 1.000\\ 1.070\\ 0.875\\ 1.240\\ 1.240\\ 1.240\\ 1.240\\ 1.180\\ 1.040\\ 1.140\\ 0.800\\ 1.010\\ 0.670\\ 0.550\\ 1.050\\ 0.960\\ 0.740\\ 0.620\\ 0.770\end{array}$

TABLE 17. Digoxin in Plasma of Digitalized Patients

mean + S.D. (0.94 +0.30)

.....continued.....

VGH PATIENTS	Digoxiı	n Concentration ng/mL	HPLC/RIA RATIO
	RIA	HPLC-PC DETECTION	
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18	0 0.3 2.3 2.0 2.5 1.7 1.3 0.8 1.2 1.3 0.7 1.3 0.6 1.1 0.8 0.9 2.0 0.5	$\begin{array}{c} 0\\ 0.300\\ 1.720\\ 1.070\\ 2.220\\ 0.876\\ 1.970\\ 1.400\\ 0.825\\ 1.140\\ 0.780\\ 1.120\\ 0.860\\ 1.400\\ 0.91\\ 0.850\\ 1.520\\ 0.500\end{array}$	0 1.0 0.75 0.535 0.888 0.515 1.520 1.750 0.688 0.877 1.110 0.862 1.430 1.270 1.140 0.940 0.760 1.000

mean + S.D. (1.0 + 0.34)

The results obtained during the analysis of the same samples by the two independent methods are summarized in Table 17. Average ratios of 0.94 ± 0.3 (ACU) and 1.0 ± 0.34 (VGH) were obtained. A range of 0.52 to 1.95 was found for these samples. This is comparable with those results observed by Loo et al. (7) (0.84±0.13) and the non-dialyzed patients observed by Gibson and Nelson (81) (1.06 ± 0.09) . From Table 17, ten of the samples showed a ratio of greater than one. Of the 6 patients from the Vancouver General Hospital whose medication profiles were followed, two patients who showed higher HPLC/RIA ratio (#13,14) were also on quinidine. The validity and significance of this finding requires further investigation. As shown in Figure 31, for samples with levels of 0.3to 2.7 ng/mL, correlations of 0.78 (VGH) and .79 (ACU) were found between the amounts of digoxin determined by the two methods. A y-intercept of 0.334 with a T-test significance of less than 5% was found, suggesting that this intercept was not significantly different from zero. The regression line in Figure 31 showed a consistent and appreciable bias between the two methods, with values being higher for the RIA procedure than for the HPLC post-column method. The quality criteria (reproducibility in section 3.3.3, linearity in section 3.3.4, specificity in section 3.3.5 and comparison with standard sera with the RIA method in section 3.3.6) showed that the HPLC post-column method is accurate in the determination of "true" digoxin concentrations. Although the results from Table 17 are comparable to the HPLC-RIA methods (7, 81), the method described here is more advantageous in that it allows for

Figure 31. Correlation Between Plasma Digoxin Levels



unattended direct quantitation. The HPLC-RIA methods on the other hand, required a collection step after the chromatographic procedure. This step is very critical and may introduce error in the assay method.

The reasons for the low correlation between the two methods may be due to several contributing factors. For example, it has been reported that the digoxin metabolites cross-react extensively in the RIA procedure (110). Dihydrodigoxin has also been shown to cross-react with the antibody (114). Recently, it was also reported that substantial differences in digoxin concentrations were found to exist when different immunoassay methods were used (118). Such differences between these immunoassays raise the question about the determination of true, clinically useful plasma digoxin concentrations by the current RIA method. The unavailability of a homogenous study population may have impaired the proper evaluation of the correlations between the two methods. Finally, while the RIA kits may be measuring an unknown endogenous substance in the plasma, the HPLC method described here has been shown to be specific and accurate (as depicted in the quality control procedure section 3.3.6) for the measurements of the true plasma digoxin concentrations.

4. SUMMARY AND CONCLUSIONS

The potential of the proposed HPLC post-column method for the fluorescent detection of digoxin has been demonstrated. The method involves the separation of digoxin from its metabolites and post-column derivatization. The production of the fluorophore is based on the reaction of hydrochloric acid with the steroid portion of the cardiac glycoside. Fluorescence of the derivative is further enhanced by the addition of a hydrogen peroxide/ ascorbic acid mixture. The reactor described is simple, easy to build and versatile. The method has the resolution and sensitivity desired for the analysis of digoxin in plasma collected from patients who were undergoing digitalization. The relatively low correlation between the HPLC and the RIA methods supports the idea that the RIA procedure fails to provide accurate measurement of digoxin levels in plasma. This is further supported by the knowledge that the RIA procedure measures drug and metabolite concentrations with equal reactivity. Hence, the HPLC procedure presented here provides for accurate determination of digoxin levels in plasma, free of interference from digoxin metabolites and many commonly co-administered drugs.

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