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Review

Chromatography of cardiac glycosides

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CONTENTS

LIST OF ABBREVIATIONS

CLC	Column liquid chromatography
C.V.	Coefficient of variation
DG0	Digoxigenin
3-Epi-DG0	3-Epidigoxigenin
DG1	Digoxigenin monodigitoxoside

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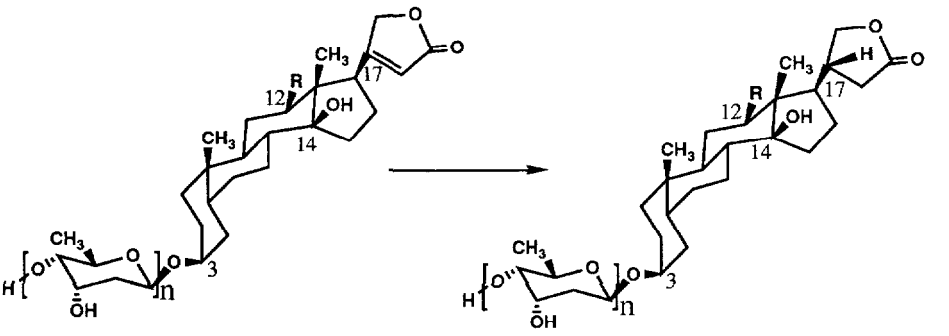
DG2	Digoxigenin bisdigitoxoside
DG3	Digoxin
DHDG0	Dihydrodigoxigenin
R-DHDG0	20*R*-Dihydrodigoxigenin
DHDG1	Dihydrodigoxigenin monodigitoxoside
R-DHDG1	20*R*-Dihydrodigoxigenin monodigitoxoside
DHDG2	Dihydrodigoxigenin bisdigitoxoside
R-DHDG2	20*R*-Dihydrodigoxigenin bisdigitoxoside
DHDG3	Dihydrodigoxin
R-DHDG3	20*R*-Dihydrodigoxin
S-DHDG3	20*S*-Dihydrodigoxin
DT0	Digitoxigenin
DT1	Digitoxigenin monodigitoxoside
DT2	Digitoxigenin bisdigitoxoside
DT3	Digitoxin
DHDT0	Dihydrodigitoxigenin
DHDT1	Dihydrodigitoxigenin monodigitoxoside
DHDT2	Dihydrodigitoxigenin bisdigitoxoside
DHDT3	Dihydrodigitoxin
FPIA	Fluorescence polarization immunoassay
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
HPLC	Hig-performance liquid chromatography
k'	Relative retention time
MS	Mass spectrometry
R_M	Lipophilicity factor based on retention factor (R_F)
RIA	Radioimmunoassay
TLC	Thin-layer chromatography
UV	Ultraviolet

1. INTRODUCTION

Cardiac glycosides have acquired an important place in current drug therapy since the introduction of the digitalis glycosides into medical practice by Withering in 1785. Of these 300 or more cardiotonic glycosides, which are naturally occurring steroids, digoxin (DG3) and digitoxin (DT3) are the most widely used compounds for the treatment of congestive heart failure and atrial arrhythmias. Among the latter DG3 remains the cardiac glycoside most extensively studied and actively used for therapy. Based on similarities in structure and its significantly greater clinical significance, relative to other cardiac glycosides, most of the ensuing discussions will use DG3 as the representative cardiac glycoside.

The sources of these digitalis glycosides are the plants *Digitalis lanata* and *D. purpurea*. These cardenolides consist of an aglycone or genin and three molecules



	Unsaturated lactone	Reduced lactone
R=OH	Digoxin series	
n=3	DG3 (Digoxin)	DHDG3 (Dihydrodigoxin)
n=2	DG2	DHDG2
n=1	DG1	DHDG1
n=0	DG0	DHDG0
R=H	Digitoxin series	
n=3	DT3 (Digitoxin)	DHDT3 (Dihydrodigitoxin)
n=2	DT2	DHDT2
n=1	DT1	DHDT1
n=0	DT0	DHDT0

Fig 1 Structures of digoxin, digitoxin and their corresponding metabolites

of the sugar digitoxose (Fig. 1). The genin is a steroid nucleus with an α–β unsaturated five-membered lactone ring at the C-17 position Two hydroxyl groups are also present at the C-3 and C-14 position. The sugar side-chain is attached to C-3 of the nucleus through the hydroxyl group. The only structural difference between DG3 and DT3 is the presence of an additional 12β-hydroxyl group in the former compound.

The clinical and biomedical significance of cardiac glycosides, particularly DG3 and DT3, arises from their uses in cardiac disease states ranging from congestive heart failure to cardiac arrhythmias which, if not adequately controlled, could result in mortality. Additionally DG3 has a generally accepted narrow therapeutic index of 0.5 to 3.0 ng/ml and a long half-life ranging from 20 tot 60 h. Following administration, the glycoside is metabolized to a variety of hydrolysis and reduction products. These factors lend additional complexity to initiating and maintaining adequate therapy, thereby requiring a clear understanding of the metabolism and pharmacokinetics of DG3 and making therapeutic drug monitoring a necessity.

The above necessity has resulted in the development of numerous analytical techniques for the quantition of DG3, clinically relevant cardioactive drugs and their corresponding metabolites, as subsequently discussed. However, the development of analytical techniques with adequate specifity in the presence of structurally similar metabolites and sensitivity in the clinically relevant nanogram range continues to present a formidable challenge to investigators in this area. Analytical procedures, reviewed here, which have been developed in the very recent years have made significant head-way in this very difficult area.

2 METABOLISM

Knowledge of the metabolism of the cardiac glycosides is essential in understanding the need for specific analytical methods for these drugs. DG3 and DT3 were previously thought to escape extensive metabolic degradation in the human body and were believed to be eliminated as unchanged drugs in the urine. However, it is now known that the metabolism of these drugs is significant in some patients [1–3]. Two major routes of metabolism of digoxin are recognized (Fig. 1) [1]. One route consists of cleavage of the digitoxose sugar moieties to form digoxigenin bisdigitoxoside (DG2), digoxigenin monodigitoxoside (DG1) and digoxigenin (DG0). DG0 is known to undergo further metabolism. However, these metabolites are incompletely characterized [4]. The bis- and monodigitoxosides of DG0 exhibit approximately the same cardioactivity as the tridigitoxoside, DG3, whereas DG0 and subsequent polar metabolites have been shown to be much less cardioactive [5,6].

The second route of metabolism of DG3 is the reduction of the double bond in the lactone ring to form dihydro compounds (dihydro reduction products), which show ten-fold lower positive inotropic effects than that of the parent glycoside [1,7]. Dihydrodigoxin (DHDG3), specifically the 20*R*-epimer of DHDG3, has been identified as a major metabolite of digoxin in humans [8]. This transformation of DG3 to DHDG3 is shown to take place within the gastrointestinal tract by the action of the enteric bacteria *Eubacterium lentum* [9,10], which not only reduces DG3 and DT3 to their respective dihydro products but further performs the reduction of their hydrolysis metabolites to their respective dihydro products as well [10,11]. Reduced (dihydro) metabolites appear to be a major route of metabolism for DG3 as evident from the reports indicating that these metabolites account for greater than 40% of total urinary glycosides in a significant minority of digoxin patients [12,13] and up to 66% of an enteric-coated formulation of DG3 [14].

The biotransformation of DT3 is equally complex, involving hydroxylation to form DG3, hydrolysis of the sugar side-chains of DT3 and DG3 and the reduction of the double bond in the lactone ring [3,15]. Evidence of conjugated and other metabolites have also been found, but the products are incompletely characterized [15].

This complex biotransformation of cardiac glycosides and the remarkable variation between subjects in the amounts of dihydro metabolites formed from DG3 underscores the importance of the need for specific assays to determine the relative levels of these drugs and their metabolites in order to set the correct dosage.

3 ANALYTICAL PROCEDURES

Although analytical methods for cardiac glycosides in the microgram range continue to be used by pharmacopeias and control laboratories, this review emphasizes those methods that permit quantitation in the nanogram range. Additionally, the vast majority of analytical methods published each year are devoted to DG3 and its metabolites, although analytical methods have occasionally been published for some of the other cardiac glycosides *e.g.* gitoxin [16] and DT3 and its metabolites [17–21]. Although a diverse range of analytical methods have been utilized for the quantitation of DG3 and its metabolites, it is the intent of this review to focus on the chromatographic methods for quantitating cardiac glycosides particularly emphasizing those advances that have been reported in recent years, details before 1985 being documented in earlier reviews [1,22]. Such currently employed analytical procedures for the quantitation of DG3 in plasma, serum or urine may be broadly classified into non-chromatographic and chromatographic methods for the purposes of this review.

3.1. Non-chromatographic methods

Several non-chromatographic methods exist for the quantitation of cardiac glycosides and are mentioned and briefly discussed below. These methods generally exploit physiologic/pharmacologic properties of cardiac glycosides or involve reaction with antibodies. The inherent non-specificity of the methods and lack of sensitivity for quantitation in the nanogram range constitute the principal reasons for their limited use. Additionally, some of the procedures are quite cumbersome Although non-specific, the non-chromatographic radioimmunoassay (RIA) procedure continues to be widely used in the clinical environment because of its relative ease of use and adequate sensitivity.

3.1.1 Immunoassays

These assays include RIA, enzyme immunoassay and fluorescence polarization immunoassay (FPIA) RIAs are based on the work of Yalow and Berson [23] on the detection of insulin. RIA since its development for DG3 has currently become the single most performed drug assay in the world. The assay normally involves incubation of the antigen and antibody for a set time, usually at equilibrium. The antibody-bound antigen is then separated from the free antigen and the radioactivity in one of the fractions is measured in order to determine the unknown concentration of the antigen from the measured count rates. ^{125}I-Labelled DG3 is used in virtually all digoxin RIA kits today.

The general capabilities and limitations of DG3 RIA have been summarized [24,25]. Considerable difficulty has been encountered due to the lack of specificity of [^{125}I-]DG3 kits. The antibody reacts with some of the DG3 metabolites [26] including DHDG3 [27] and also with certain unidentified endogenous substances called digoxin-like immunoreactive substances [1,2]. Antibodies against DHDG3 have been developed [28], although they have not been commercially marketed. In spite of its lack of specificity, ease of use and clinical applicability render RIA a useful and much utilized procedure for the quantitation of DG3 in the 0.5–3 ng/ml range. However, it has been the authors' experience with these assays that it is not unusual to find coefficients of variation (C.V.) of 20% at the low end of the range to 10% at concentrations of 3.0 ng/ml.

3.1.2. Determination of cardiac glycosides by ATPase inhibition

Following isolation of the Na^+, K^+-activated ATPase and the discovery that cardiac glycosides are able to inhibit ATPase activity by binding to the enzyme [29], this property has been utilized in the quantitation of cardiac glycosides. The method is based on the inhibition of ATP hydrolysis or the displacement of radiolabelled oubain from its binding site on the ATPase by other cardiac glycosides. Unlike RIA this procedure measures cardiac glycosides and those metabolites that possess an intrinsic ability to inhibit the ATPase enzyme and is therefore quite non-specific. Results obtained for urine samples by the two methods show differences, since polar and non-polar metabolites of cardiac glycosides occur in remarkably high concentrations in the urine [30,31]

3.1.3. Rubidium uptake by erythrocytes

This is an outdated method that is minimally used and is based on the inhibition by cardiac glycosides of the active uptake of potassium in to human erythrocytes [32,33]

3.1.4. Double isotope dilution derivative assay

This assay was originally used for the analysis of amino acids and steroids and has also been applied to the analysis of DG3 [34]. The sample is usually spiked with [^{3}H]DG3 for recovery calculations and is then extracted and converted to its acetate derivative with [^{14}C]acetic anhydride. The ^{14}C and ^{3}H content of the derivative, the specific activity of the ^{14}C derivative and the fraction of ^{3}H recovered are used in calculating the DG3 content. The several extractions and chromatographic clean-up procedures required and the poor precision of the assay at levels of 2 ng/ml detract from the applicability of this procedure.

3.2. Chromatographic principles and methods

Prior to discussing the advantages and disadvantages of recent chromatographic procedures listed in Table 1 it is pertinent to review and understand those

underlying principles that are relevant to the chromatography of cardiac glycosides. Application of the principles discussed below should also incorporate procedures that adequately validate each assay.

An entire review can be effortlessly devoted to addressing validation techniques for chromatographic procedures. However, the intent here is to emphasize the need for adequate validation procedures when quantitating DG3 and/or metabolites. With the exception of a few methods, validation results have been conspicuously absent in recently published chromatographic procedures for quantitating DG3. It is hoped that future chromatographic procedures for DG3 and/or metabolites will at least demonstrate acceptable limits of variability (*e.g.* C.V less than 10%), for intra- and inter-day reproducibility of the standard curves, and for recoveries of standards and quality control samples which are stored and assayed under conditions identical to those of the actual samples In the absence of adequate validation, the transfer of these published procedures to the clinical setting for day-to-day quantitation of samples still remains unassured.

3.2.1. Relationship between structure and chromatographic behavior

Although no correlation of chromatographic properties and molecular mass has been demonstrated for cardiac glycosides, it has been generally accepted that chromatography of cardenolides, when using high-performance liquid chromatography (HPLC), is dependent upon the properties of the stationary phase, the composition of the mobile phase or eluent, and the inherent structural properties of the glycone and functional groups of the glycone. In an excellent investigation of these relationships Davydov [35] demonstrated the relationship

$$\ln V_s = \ln V_{s\ gl} + \ln V_{s\ agl}$$

using HPLC procedures, where the retention volume (V_s) for the cardenolide is related to those for the glycone ($V_{s\ gl}$) and the aglycone ($V_{s\ agl}$). For cardenolides with similar aglycones the above relationship was shown to be largely influenced by the properties of the glycones. This relationship was demonstrated for cardiac glycosides ranging from DG3 and oubain to strophanthin and eryzimin. It has also been shown that the relative retention times of cardiac glycosides can be predicted with a high degree of reliability by taking into consideration their lipophilicity. The lipophilicity of a large number of cardenolides has been characterized based on R_M values obtained from thin layer chromatography (TLC) or HPLC [36]. The k' values in increasing order are DG0, DG1, DG2 and DG3. This order generally corresponds to their retention times on normal-phase chromatographic systems and to the reverse order on reversed-phase systems Such predictability can also be extended to derivatives of cardiac glycosides as evident from the retention times reported for the 1-naphthoyl chloride derivatives of DG3 and its metabolites [37].

TABLE 1

CHROMATOGRAPHIC METHODS FOR THE QUANTITATION OF DIGOXIN, DIGITOXIN AND THEIR METABOLITES

See also list of abbreviations.

Drug/metabolite quantitated	Method	Sample preparation	Column or layer	Mobile phase
DG3	HPLC–RIA	Bond-Elut or Sep-Pak column + methanol	C_8	Isopropanol–water (20:80)
DG3	HPLC–RIA	Acetonitrile denaturation	C_{18}	Ethanol–acetonitrile–water (3.3:25:71.7)
DG3,DG2,DG1, DG0,DHDG3	CLC–TLC–liquid scintillation	Chloroform or chloroform–methanol	Kieselguhr	Water–methanol–diethyl ether (1:1:1) and chloroform–methyl acetate (1:4)
DG3,DG2,DG1, DG0,DHDG3	HPLC–liquid scintillation	Methylene chloride–heptafluorobutanol	Silica or C_8	Heptane–1-pentanol–acetonitrile–water (64:26:9:1) Phosphate buffer–isopropanol (83.5:16.5)
DG3	HPLC–RIA	Chloroform–methylene chloride	Silica	Hexane–ethanol–methylene chloride (75:18:7)
DG3	CLC–RIA	Water–isopropanol followed by methanol	C_{18} (disposable column)	Water–isopropanol (15:85) followed by methanol
DT3,DT2,DT1, DT0	HPLC–RIA	Methylene chloride	C_{18}	Methanol–water (gradient)
DG3,DG2,DG1, DG0,DT3,DT2, DT1,DT0	HPLC–RIA	Clin-Elut column + ethyl acetate	C_{18}	Acetonitrile–methanol–water (20:20:60)
DG3,DG2,DG1, DG0,DHDG3	Pre-column derivatization with 1-naphthoyl chloride –HPLC	Methylene chloride	Silica or C_{18}	Hexane–methylene chloride–acetonitrile (6:1:1) Acetonitrile
DG3,DG2,DG1, DG0,DHDG3	Pre-column derivatization with 3,5-dinitrobenzoyl chloride–HPLC	Methylene chloride	Silica	Hexane–methylene chloride–acetonitrile (3:1:1)
DG3	Post-column derivatization with ascorbic acid and HCl–HPLC	Chloroform–isopropanol	C_{18}	Methanol–ethanol–isopropanol–water (52:3:1:45)

Internal standard	Detection limit	Range	Precision[a]	Specificity[b]	Reference
–	0 1 ng/ml	0 1–4 0 ng/ml	4–6%	Good	40
–	0.1 ng/ml	0 5–3 0 ng/ml	< 4% at 3 ng/ml	Fair	47
[^{3}H]DG3 and metabolites	0 1 ng/ml	0 1–4 0 ng/ml	< 5%	Excellent	46
[^{3}H]DG3 and metabolites	0 2ng/ml	–	< 5%	Good	43
–	0 5 ng/ml	0.5–4 0 ng/ml	11% at 0 5 ng/ml	Good	44
–	0 1 ng/ml	0 1–6.0 ng/ml	4 6–9 6%	Good	41
[^{3}H]DT3	0 3 μg/ml	0 5–20 μg/ml	3 0–5 0%	Good	18
[^{3}H]DT3 [^{3}H]DG3	10 ng/ml	10–100 ng/ml	–	Good	17
DT0 or DT3	5 ng/ml	5–250 ng/ml	< 10%	Excellent	37
DT0	100 ng/ml	100–1000 ng/ml	1 5–8 5%	Good	58
DT0	0 5 ng/ml	0 5–4 0 ng/ml	10% at 2 ng/ml	Fair	54,55

(Continued on p 224

TABLE 1 (*continued*)

Drug/metabolite quantitated	Method	Sample preparation	Column or layer	Mobile phase
DG3	Post-column derivatization with HCl–HPLC	Methylene chloride–propanol	C_{18}	Methanol–ethanol–isopropanol–dehydro ascorbic acid (52 3 1 45)
DG0,DHDG0	Pre-column derivatization with 4-nitrobenzoyl chloride–HPLC	Extrelut column + methylene chloride	Silica	Hexane–methylene chloride–methanol (82 9·14 2:2 9)
DG3,DG2,DG1, DG0,DHDG3	Post-column derivatization with ascorbic acid and hydrogen peroxide–HPLC	–	C_{18}	Methanol–methylene chloride–water–isopropanol (41 3 50 6)
DG3	HPLC–Amperometry	On line clean-up with Lichrosorb DIOL column + phosphate buffer	C_{18}	Phosphate buffer
DG3	Post-column reaction with HCl–HPLC	Valve switching with anti-digoxin immunoadsorbtive cartridge	C_{18}	Phosphoric acid–hydrochloric acid–methanol (20 35:45)
DG3,DG2,DG1, DG0,DT3,DT2, DT1,DT0	Pre-column derivatization with 3,5-dinitrobenzoyl chloride–microbore HPLC	–	C_{18}	Acetonitrile–water (3 1)
DT3,DT2,DT1	TLC–RIA	Methylene chloride	C_{18}TLC and Si 250 TLC	Dioxane–methanol–water (2·5 3) Isopropyl ether–methanol (9 1)
β-Methyldigoxin, DG3,DG2,DG1, DG0	HPLC–FPIA	Chem-Elut column + methylene chloride	Silica	Heptane–isopropanol–methylene chloride–water (69 20 10·1)

[a] Precision for inter-day assays was not generally reported and should be taken into consideration

[b] Specificity ratings are subjective and are assigned based on separation of DG3 and DT3 from their corresponding metabolites

Internal standard	Detection limit	Range	Precision[a]	Specificity[b]	Reference
DT0	0 5 ng/ml	0 5–10 ng/ml	7 2% at 3 ng/ml	Fair	56
DT0	1 μg/ml	1–10 μg/ml	–	Fair	53
DT0	10 ng/ml	–	–	Fair	57
–	0 6 ng/ml	0 6–7 5 ng/ml	2 3% at 2 2 ng/ml	Unknown	50
–	0 3 ng/ml	0 5–4 0 ng/ml	7%	Unknown	61
Gitoxin	0.2 ng/ml	0 4–10 ng/ml	–	Good	19
[^{3}H]DT3 and metabolites	0 3 ng/ml	0 3–4 ng/ml	7 1% at 3 ng/ml	Fair	20
–	0 5 ng/ml	0 5–3 0 ng/ml	< 7%	Excellent	51

3.2.2. Exploiting radiolabelled cardiac glycosides

A large proportion of our early knowledge of the pharmacokinetics and biopharmaceutics of cardiac glycosides, specifically DG3, was from the use of radiolabelled DG3 [30,38]. Although major drawbacks including non-specificity and restrictions on the use of radioactive materials limit its current use, this method remains one of the most attractive methods for quantitation of cardiac glycosides

because of the high sensitivity provided. Additionally, the non-specificity of this procedure may be minimized by using some of the chromatographic techniques discussed herein prior to quantitation.

3.2.3. Sample preparation

Most of the currently used chromatographic procedures, for the quantitation of cardiac glycosides in the nanogram range vary in their sample extraction, chromatographic and detection procedures as indicated in Table 1. However, in most of the instances separate extraction procedures are not required for DG3 and its metabolites Most of the extraction procedures have utilized a direct extraction of the plasma or urine samples, occasionally following basification with sodium bicarbonate or a 5*M* sodium hydroxide solution. However, it is evident from Table 1 that a combination of methylene chloride and chloroform appears to be the most frequently used extraction method. However, for reasons not totally understood recoveries reported for DG3 and its metabolites have ranged from 54 to 78% [37,39], although recoveries greater than 90% have been reported for DT3 and metabolites [18]. More recent reports have indicated recoveries of 90% or greater for DG3 and metabolites using solid-phase extractions [40,41]. While these procedures show promise and provide relatively better ease of use and speed, it is the authors opinion and experience that further refinement of these techniques is needed before assaying a large number of samples on a regular basis, due to the need for frequent change of columns and inherent batch-to-batch variability of the solid-phase extraction columns. In the authors experience methylene chloride and chloroform serve as the best solvents for the extractions of DG3 and its metabolites [42]. It has also been shown that addition of a small amount of a relatively non-volatile component, *e.g* heptafluorobutanol or 1-butanol (to prevent complete evaporation and therefore minimize degradation), results in an appreciable increase in the distribution constant for the extraction of DG3 and metabolites from phosphate buffer [43]. In fact, a methylene chloride–chloroform mixture with a small amount of ethanol (to prevent adsorption of digoxin to glass) resulted in recoveries greater than 90% [42,44].

3.2.4. Thin-layer chromatography

These methods are currently deemed to be of value largely in the characterization of cardiac glycosides particularly in the microgram range. Procedures have been employed which utilize TLC with fluorescence detection [45], quantitation using RIA [20] or separation of DG3 and its metabolites using TLC followed by scintillation couting of the separated radioactive fractions [46]. The latter procedure [46] remains the only TLC procedure that demonstrated adequate specificity and sensitivity for the quantitation of digoxin and its known apolar metabolites However, more than one chromatographic system and [12α-^{3}H]DG3 and its correspondingly labelled metabolites are required for quantitation and recovery calculations. Therefore, to date, TLC remains a preparative tool in the quantitation of cardiac glycosides.

3.2.5. High-performance liquid chromatography

Since RIA methods for DG3 are not specific in the presence of its metabolites, investigators have turned towards the development of specific procedures using chromatographic methods as evident from some of the procedures listed in Table 1. However, detection using an ultraviolet (UV) monitor is not possible for concentrations of 20 ng/ml or lower for DG3 and some of its metabolites [17,18,44]. This is additionally complicated by the minimal UV absorbance of DHDG3. For these reasons a large number of the recently published methods have employed chromatographic separation of DG3 and its metabolites followed by collection of the eluate fractions and quantitation of the drug/metabolite in these fractions by RIA (or FPIA). These procedures are generally referred to as HPLC–RIA procedures.

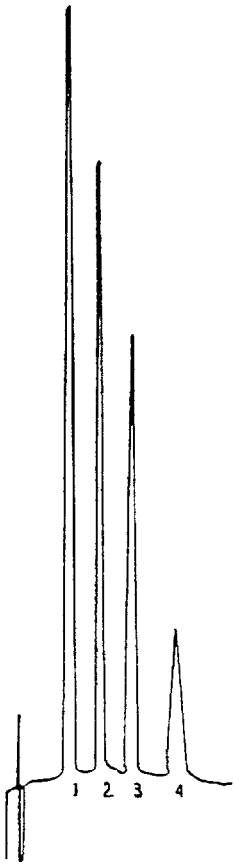


Fig 2. Typical separation of digoxin and its metabolites using a normal-phase column The peaks and the retention times are as follows 1 = DG0 (5 2 min), 2 = DG1 (7 2 min), 3 = DG2 (10.0 min), 4 = DG3 (13 6 min) The peaks represent 2 5 μg of each compound injected on the column (From ref 44 with permission.)

3.2.5.1. HPLC–RIA. These procedures [39,43,47–51] have generally improved the specificity and sensitivity of the quantitation of DG3 and its metabolites by separation of DG3 and its metabolites on column and subsequently applying the non-specific RIA procedure on the eluate fraction corresponding to DG3. Although these procedures have demonstrated column separation and specificity for DG3 in the presence of DG2, DG1 and DG0 (Figs. 2 and 3), the specificity of these methods for DG3 in the presence of DHDG3 remains questionable. Although separation of DG3 and DHDG3 has been reported in some of the recent publications, the confirmation of this separation remains uncertain, since detection of DHDG3 (which has extremely low UV absorbance) following separation on column was accomplished by UV detection. Consequently, quantitation of the collected fraction for DG3 by RIA is susceptible to inaccuracies since most RIA antibodies cross-react appreciably with DHDG3.

Since HPLC–RIA requires evaporation of the collected fractions prior to quantitation by RIA, use of reversed-phase systems for separation is strongly discouraged. Such systems result in the hydrolysis of the glycosidic side-chain due to the aqueous mobile phase during chromatography and evaporation. A normal-phase system with volatile solvents comprising the mobile phase was found to be ideal. The mobile phase of hexane–ethanol–methylene chloride (75:18:7) was found to give excellent results [44]. Also, addition of a modifier such as ethanol reduces the volatility of the mobile phase thus minimizing changes in retention times.

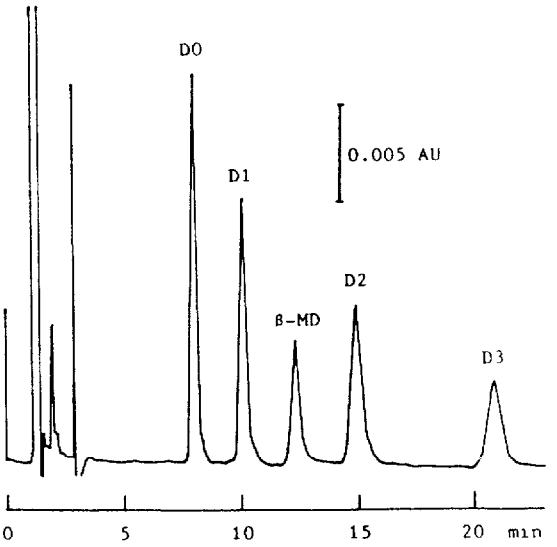


Fig 3 Chromatogram of β-methyldigoxin and its metabolites Chromatographic conditions HPLC column, 5-μm Wakopak Lichrosorb Si 60 (150 mm x 4 6 mm I.D), mobile phase, heptane–isopropanol–methylene chloride–water (69 20 10 1), flow-rate, 1,5 ml/min, detection, UV Peaks. D0 = DG0, D1 = DG1, β-MD = β-methyldigoxin, D2 = DG2, D3 = DG3 (From ref 51 with permission)

A drawback of the normal-phase method is the necessity for regeneration of the silica column due to water adsorption and the necessity to monitor retention times of DG3 and its metabolites at frequent intervals to ensure that the appropriate eluent fractions are collected for subsequent quantitation using RIA. In this regard verification of the retention times by injection of radiolabelled standards is by far the best approach However, injection of a mixture containing 20 ng of DG3 and each of its metabolites followed by adequate washout of the column was found to be adequate in characterizing the retention times or any changes in the same [44]. Although the time required for such assays has been minimized by solid-phase extraction techniques described in recent procedures [40,41], HPLC–RIA remains a cumbersome process. Salient advantages of the assay include the improved specificity over RIA, including specificity in the presence of a DG3-like immunoreactive substance [52], and that automation of this procedure can be achieved to an appreciable extent. The principles of HPLC–RIA currently used in the quantitation of DG3 could potentially be applied to quantitation of some of its metabolites, *e.g.* DG2, DG1 and DG0 in the eluate fractions by virtue of the cross-reactivity of the RIA [42]. This has recently been demonstrated in an HPLC–RIA assay for DT3, DT2, DT1 and DT0 [18] HPLC–RIA therefore remains a viable analytical technique for the quantitation of DG3 and it metabolites (and other cardiac glycosides) with adequate sensitivity and specificity.

3.2 5.2. HPLC with derivatization. Since cardiac glycosides possess weakly absorbing UV chromophores, derivatization with strongly absorbing species have been used as a means to enhance their detectability. Both UV and fluorescence derivatization have been used for this purpose. During the last five years, several HPLC procedures employing either pre-column or post-column derivatization techniques have been reported [37,53–61]. The method developed by Jakobsen and Waldorff [53] utilized a (pre-column) derivatization technique with 4-nitrobenzoyl chloride prior to normal-phase separation with *n*-hexane–dichloromethane–methanol (82.9:14.2.2.9) as the mobile phase and UV detection at 258 nm. In this procedure, DG3 and DHDG3 were hydrolyzed to their respective genins by incubation with hydrochloric acid and extracted with an Extrelut column before derivatization. The approach was to obtain simple chromatograms with only two peaks, namely DG0 and dihydrodigoxigenin (DHDG0). The method did separate the two compounds well, but it lacked sensitivity. The idea of simplifying the analysis to determine the total reduced and non-reduced DG3 is interesting and could be useful with other more sensitive detection methods In this regard, the post-column derivatization method developed by Kwong and McErlane [54,55] for the analysis of DG3 and its metabolites is sensitive since the minimum detectable quantity of DG3 has been determined to be 0.5 ng/ml. This method involved solvent extraction of the compounds, separation on a C_{18} column using methanol–ethanol–isopropanol–water (52:3:1:45) as the mobile phase, followed by derivatization with ascorbic acid and detection using a fluorescence

monitor. The only major drawback of this method is the incomplete resolution of DG3 from DHDG3 (Fig. 4). A modified version of the method that has been recently reported [56] suffers the same problem as the above method. With a better separation procedure this method could become very useful for the study of DG3 metabolism.

The major goal of the method developed by Desta [57] for DG3 using a post-column derivatization technique with the fluorigenic species ascorbic acid was to overcome problems reported earlier in the separation of DHDG3 from DG3. Since this procedure involved post-column derivatization, the author was able to record chromatograms before and after derivatization with UV and fluorescence detectors, respectively. Since DHDG3 does not possess any chromophores, its separation could not be determined in the UV part of the method. However, in the fluorescence detection mode, they were well separated, although DG0 and DG1 were incompletely separated. The sensitivity and potential interference from biological matrices for this technique remain unknown, since the method has not been applied to biological samples.

A recently developed pre-column fluorescence derivatization–HPLC technique for the analysis of DG3 in urine and feces by Shepard *et al.* [37] not only

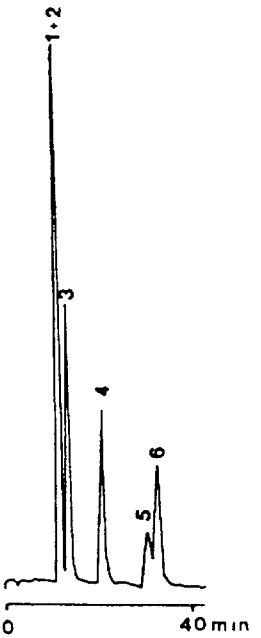


Fig. 4. Post-column fluorescence derivatization–HPLC profile of digoxin and its metabolites. Chromatographic conditions: derivatization, post-column with ascorbic acid; HPLC column, C_{18} (Spherisorb ODS II); mobile phase, methanol–ethanol–isopropanol–water (52:3:1:45); flow-rate, 0.3 ml/min; detection, fluorescence. Peaks: 1 = DHDG0, 2 = DG0, 3 = DG1, 4 = DG2, 5 = DHDG3, 6 = DG3. (From ref. 54 with permission.)

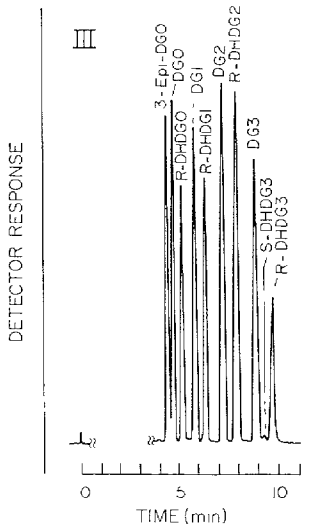


Fig 5 Pre-column fluorescence derivatization–HPLC profile of DG3, DHDG3, their hydrolysis products and 3-epidigoxigenin Chromatographic conditions derivatization, pre-column with 1-naphthoyl chloride. column, silica (Adsorbosphere), mobile phase, hexane–methylene chloride–acetonitrile (27 5 5), flow-rate, 1.2 ml/min, detection, fluorescence (A Chadrasekaran, L W Robertson and R H Reuning, unpublished results)

separated DHDG3 from DG3 and other metabolites, but also separated the individual epimers of DHDG3. The column, mobile phase and extraction conditions were similar to the ones used in a previously reported HPLC–UV method [37,58], which was based on a pre-column derivatization technique with 3,5-dinitrobenzoyl chloride. Both of these methods separated the individual epimers of DHDG3 from DG3 as well as other metabolites. Also, the fluorescence derivatization method is sensitive (5 ng/ml for urine, 10 ng/200 mg for feces). Recently this method was found to be capable of even separating DG2, DG1 and DG0 from their respective dihydro metabolites (Fig. 5) [59]. An important feature of this method is its ability to analyze fecal samples. This is significant, considering the fact that the dihydro metabolites are formed in the lower gastrointestinal tract by enteric bacteria. This method has already been successfully applied to study the metabolism of DG3 in humans [59,60]. Extension of this technique to plasma samples looks promising [60].

Microbore HPLC separation following derivatization also seems to offer the much needed sensitivity for cardiac glycosides, as demonstrated by the method developed by Fuji *et al* [19]. This method involved pre-column derivatization with 3,5-dinitrobenzoyl chloride, separation of the derivatives on a reversed-phase column with a mobile phase of acetonitrile–methanol–water (3:1:1) at a flow-rate of 8 μl/min and UV detection at 230 nm. Interference due to dihydro metabolites and other endogenous materials is not known, since this method has not been applied to biological samples.

In a recently reported HPLC–fluorescence reaction detection method for DG3, Reh [61] has used an on-line immunoadsorptive clean-up procedure to minimize the assay time by eliminating the extraction step. In this method, a polyclonal antibody against DG3 covalently linked to Spherosil with valve-switching was used. The sensitivity of this method is good, as the detection limit has been determined to be 300 pg/ml. Again, the specificity is not known, since metabolites, especially the dihydro metabolites, have not been tested for interference. It is also possible that the column may not be stable for repeated sample analysis.

It is clear from the above discussions that fluorescence derivatization–HPLC offers the sensitivity required for detecting DG3 and its metabolites. Further advances in this field are expected to arise by combining microbore HPLC with fluorescence derivatization.

3.2.6. Gas chromatography–mass spectrometry

Few gas chromatographic–mass spectrometric (GC–MS) methods have been developed for the analysis of cardiac glycosides, due to the low volatility of these compounds. Watson and co-workers [62–64] used a chemical derivatization technique with heptafluorobutyric anhydride as a means to overcome the volatility problem. The drawback of this technique is its inability to distinguish the glycosides from their aglycones, since the glycosides are hydrolyzed to their genins during the derivatization reaction [63]. Although molecular ions are not obtained for cardiac glycosides via electron ionization, Greenwood and co-workers [65,66] successfully developed a method to measure DG3 and DHDG3 by using their characteristic fragment ions. Recently Shomo *et al.* [21] have used laser desorption technique to characterize DG3, DT3 and their metabolites. Since this technique is sensitive and produces pseudomolecular ions as the most intense ions, it offers an excellent means for measuring these drugs and their metabolites.

4 SUMMARY

Most of the recently reported methods for the quantitation of cardiac glycosides have been for digoxin and its metabolites. Recent procedures using high-performance liquid chromatography–radioimmunoassay (HPLC–RIA) and HPLC following derivatization show appreciable improvements in accuracy and specificity for quantitating digoxin in the low nanogram range. Gas chromatographic procedures have been explored to a very limited extent and further advances in the quantitation of cardiac glycosides are anticipated to arise from the use of laser desorption–Fourier transform mass spectrometry. However, currently, HPLC with derivatization and HPLC–RIA techniques remain the techniques of choice for quantitation of digoxin and/or its metabolites based on considerations of ease of use, sensitivity, specificity, accuracy and reproducibility.

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