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# Specific and sensitive determination of digoxin and metabolites in human serum by high performance liquid chromatography with cyclodextrin solid-phase extraction and precolumn fluorescence derivatization

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**Abstract**

A precolumn fluorescence derivatization high performance liquid chromatographic method has been developed for the simultaneous determination of digoxin and its metabolites digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside digoxigenin, and dihydrodigoxin (20-*R* and 20-*S* epimers) in human serum. Digoxin and its metabolites were extracted from serum samples (containing digitoxin as internal standard) with a cyclodextrin solid-phase extraction (SPE) column. Fluorescent derivatives were formed by reaction of the analytes with 1-naphthoyl chloride in the presence of 4-dimethylaminopyridine under a nitrogen atmosphere in a glove box with controlled relative humidity (26% r.h. or less). The derivatives were isolated using cyclodextrin and C1 SPE columns sequentially, and determined by HPLC using silica column separation and fluorescence detection. Calibration curves were linear over the concentration range from 0.25 to 4.0 ng ml$^{-1}$. Recoveries of digoxin and its metabolites from serum ranged from 62 to 86%, and coefficients of variation from repetitive analyses ranged from 6.9 to 20.9% and from 5.8 to 12.2% at 0.5 ng ml$^{-1}$ and 2.0 ng ml$^{-1}$, respectively. This method has been shown capable of specifically determining digoxin and its major metabolites in serum, and has been successfully used in the determination of digoxin and its metabolites in serum samples collected from patients undergoing digoxin therapy. This method thus permits the investigation of digoxin metabolism and pharmacokinetics after the administration of commercial dosage forms.

*Keywords:* Cyclodextrin solid-phase; Digoxin; Digoxin metabolites; Fluorescence derivatization; HPLC; Human serum

## 1. Introduction

The cardiac glycoside digoxin ($D_3$) has been widely used in the treatment of congestive heart failure and certain cardiac arrhythmias. This drug is characterized by a narrow therapeutic range (typically 0.5–2 ng ml$^{-1}$ in serum), wide individual variability in dosage requirements, and complex metabolic pathways. Metabolism includes the hydrolysis of glycoside bonds to form cardioactive metabolites (i.e. digoxigenin bisdigitoxoside ($D_2$), digoxigenin monodigitoxoside ($D_1$), and digoxigenin ($D_0$)), reduction of the lactone ring to form a cardioinactive metabolite [(20-*R*]-dihydrodigoxin (*R*-$DHD_3$)), and conjugation reactions [1].

The most commonly used methods for the determination of digoxin in serum are immunoassays including radioimmunoassay and

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fluorescence polarization immunoassay. These methods are limited to varying degrees by non-specific interference from endogenous digoxin-like factors which are present in the serum of patients with certain diseases and interference from digoxin metabolites with varying degrees of cardioactivities [1–4]. More specific methods that combine the specificity of high performance liquid chromatography (HPLC) with the sensitivity of immunoassay [5–8] or radio-isotope detection [9,10] have the disadvantages of diverse cross-reactivities of metabolites with antibodies of different immunoassay methods [2,5,11] or the requirement of administering radiolabeled drug to patients.

An alternative approach combining the specificity of HPLC and the enhanced sensitivity of precolumn or postcolumn chemical derivatization has attracted more recent attention [12–18]. Precolumn derivatization to form UV absorbing derivatives has been shown to be adequate for the determination of digoxin and its metabolites in a standard mixture [12] and in urine samples [13]. The formation of fluorescent derivatives offers the potential for the determination of digoxin at serum concentrations less than 1 ng ml$^{-1}$ and has been used in the determination of digoxin and its metabolites in urine and feces [14,15]. Post-column derivatization, in which fluorescent degradation products of digoxin and its metabolites are formed in the presence of acid, was reported for the determination of digoxin from 0.5 to 4 ng ml$^{-1}$ in 3 ml plasma samples [16–18].

The lack of specific analytical methods has limited the investigation of digoxin metabolism and pharmacokinetics. Consequently, the metabolism of digoxin remains controversial [6,19–21] and little information has been provided to develop an understanding of the influence of various disease states on the metabolism [1].

This paper describes a precolumn fluorescence derivatization HPLC method for the simultaneous determination of digoxin and its major metabolites in serum. It is an extension of a previous report [14] which has been used for the determination of digoxin and its metabolites in urine and fecal samples [15]. The procedure involves the use of solid-phase extraction (SPE) on $\beta$-cyclodextrin columns to extract digoxin and its metabolites from serum samples, treatment of the extract with 1-naphthoyl chloride to form fluorescent derivatives, and HPLC to separate and measure the fluorescence of the resulting derivatives. This method maintains the specificity of HPLC while providing a detection limit comparable to that of immunoassay, and has been successfully used for the determination of digoxin and its metabolites in serum of patients on digoxin therapy.

## 2. Experimental

### 2.1. Reagents and materials

Methylene chloride, chloroform, isopropanol, acetonitrile, and methanol were all HPLC grade. Water was HPLC grade except that used to rinse glassware. Hexane was UV grade and toluene was analytical reagent grade. The reagent used to prepare fluorescent ester derivatives, 1-naphthoyl chloride (1-NC; purum grade; Fluka, Ronkonkoma, NY), was distilled twice under vacuum, sealed in ampoules, and stored in a dessicator in the dark until used. The derivatization catalyst, 4-dimethylaminopyridine (DMPA; Aldrich Chemical Co. Inc., Milwaukee, WI) was purified by passing a solution of DMAP in acetonitrile (30%, w/v) through a filter funnel containing a layer of silica gel powder covered with a layer of activated charcoal. The filtrate was evaporated under vacuum and the dried residue was stored in a desiccator until used. The $\beta$-cyclodextrin SPE columns (Cyclobond® I, 1 ml reservoir) were from Astec (Whippany, NJ), and the C1 SPE columns (Bond Elut®, 1 ml reservoir) were from Analytichem International (Harbor City, CA).

### 2.2. Drug standards

Analytical standards of $D_0$, $D_1$, $D_2$, $D_3$ $DHD_3$ (a mixture containing approximately 75% [20-*R*]-$DHD_3$ and 25% [20-*S*]-$DHD_3$ [13]) and digitoxin ($DT_3$) were obtained from Burroughs Wellcome Co. (Research Triangle Park, NC). Stock solutions (10 μg ml$^{-1}$) of these standards in isospropanol were prepared. Working stock solutions (100 or 10 ng ml$^{-1}$) were made by diluting the stock solutions with methylene chloride. All solutions were stored in a refrigerator (−4°C) until used.

### 2.3. Glassware

Glass culture tubes with PTFE-lined screw caps (Corning Glass Co., Corning, NY) were used for all procedures. All glassware was immersed for at least 24 h in sulfuric acid–nitric acid (4:1, v/v), washed with water, treated with a 1% (v/v) solution of Surfasil® (Pierce Chemical Co., Rockford, IL) in toluene, rinsed thoroughly with double-distilled water, and dried in an oven before use.

### 2.4. Instrumentation and chromatographic conditions

The HPLC system consisted of a solvent delivery system (Kratos Spectroflow 400, Kratos Analytical Instruments, Ramsey, NJ or Varian model 2010, Varian Instrument Group, Walnut Creek, CA), a fluorescence detector (Kratos Spectroflow 980, Kratos Analytical Instruments) with a deuterium lamp, and a programmable integrator (Spectra-Physics, Piscataway, NJ). The excitation wavelength was set at 217 nm and fluorescence was monitored using a 340 nm emission filter. Sample injections were made using a 20 μl sample loop (model 7125, Rheodyne Inc., Berkeley, CA).

Separations were performed on a 150 mm × 4.6 mm i.d., 3 μm Spherisorb® silica column (Metachem Technologies Inc., Torrance, CA), protected by a disposable 15 mm × 3.2 mm i.d., 7 μm silica guard column (Applied Biosystems Inc., San Jose, CA). The mobile phase was hexane–methylene chloride–acetonitrile–methanol (36:6.3:5.4:0.2, v/v/v/v) at a flow rate of 1.6 ml $min^{-1}$. All chromatograpahic studies were carried out at room temperature.

### 2.5. Extraction of digoxin from serum samples

A 1.0 ml aliquot of serum was pipetted into a test tube containing 10.0 ng of digitoxin as the internal standard. The serum was diluted with a 1 ml of water and applied to a cyclodextrin SPE column which had been washed successively with 2 ml each of methanol, acetonitrile, isopropanol, and water immediately before use. After application of the diluted serum, the column was washed successively with 2 ml of water, 1 ml of 20% (v/v) methanol in phosphate buffer (pH 7.0; 7.5 mM monobasic potassium phosphate), 3 ml of water, and 1 ml of 10% (v/v) isopropanol in water. The column was dried for 5 min under vacuum, washed with ten 100-μl aliquots of methylene chloride, and dried for 5 min under vacuum. Digoxin and metabolites were eluted from the column with 1 ml of isopropanol, and the eluate was evaporated in a stream of filtered nitrogen at ambient temperature.

### 2.6. Derivative formation

Fluorescent 1-naphthoate esters of digoxin and its metabolites were prepared by sequential addition of 50 μl of 10% (w/v) DMAP in acetonitrile and 50 μl of 4% (v/v) 1-NC in acetonitrile to the dried residue in each tube under a nitrogen atmosphere in a glove box. The relative humidity of the atmosphere in the glove box was maintained at 26% or less at 20°C by sweeping the glove box with a constant stream of dry nitrogen. The tubes were capped and the reaction mixtures thoroughly mixed. The tubes were then removed from the glove box and placed in a heating block (Techne Dri-Block Heaters) at 50°C for 1 h. The tubes were centrifuged briefly and the reaction mixture was evaporated under nitrogen. Excess reagent was removed by hydrolysis with 2 ml of 5% (w/v) sodium bicarbonate solution (pH 10.0). The mixtures were shaken for 1 min and 2 ml of chloroform were added to extract the glycoside derivatives. The tubes were shaken by hand, centrifuged, and the aqueous layer was aspirated and discarded. The chloroform solution was washed once wth 2 ml of 5% sodium bicarbonate solution and then twice with 2 ml of 0.05 N HCl; the chloroform solution was evaporated under nitrogen at room temperature.

### 2.7. Isolation of fluorescent derivatives

The residue remaining after derivative formation was dissolved in 200 μl of methylene chloride and applied to a cyclodextrin SPE column which had been rinsed successively with 2 ml each of methanol, acetonitrile, and methylene chloride immediately before use. After application, the column was rinsed with eight 100-μl aliquots of methylene chloride to elute interfering substances. The glycoside derivatives were then eluted with 1 ml of methanol and the methanol eluate was evaporated under dry nitrogen.

The dried residue was then dissolved in 250 μl of acetonitrile and applied to a C1 SPE column which had been rinsed successively

with 2 ml each of methanol and acetonitrile immediately before use. The tube was rinsed with another 250 µl aliquot of acetonitrile which was also applied to the column. Another 500 µl portion of acetonitrile was then applied to the column and the combined eluates were evaporated under dry nitrogen. The residue was dissolved in mobile phase before injection into the HPLC.

### 2.8. Calibration curves

Calibrators were prepared by adding known amounts of $D_3$, metabolites, and 10 ng of the internal standard ($DT_3$) to 1.0 ml aliquots of drug-free serum. Calibrators were prepared at concentrations of 0.25, 0.50, 1.0, 1.5, 2.0, and 4.0 ng ml$^{-1}$ for each of the analytes (i.e. $D_0$, $D_1$, $D_2$, $D_3$, and $DHD_3$). The concentration range for [20-*R*]-$DHD_3$ thus extended from 0.1875 to 3.0 ng ml$^{-1}$ since this epimer represented 75% of the total amount of $DHD_3$. Calibration curves were prepared by plotting peak height ratios against the known concentrations. The calibration equation and correlation coefficient for each analyte were obtained by unweighted least-squares linear regression analysis. A set of calibrators was prepared and analyzed with each set of test samples.

### 2.9. Determination of analyte concentrations

Concentrations of digoxin and its metabolites were determined by calculation of the peak height ratio and interpolation of the respective calibration curve. Concentrations of $DHD_3$ were based on measurement of the later eluting peak for the pair of peaks due to $DHD_3$ since this peak corresponded to the 20-*R* epimer which is the only epimer reported from human studies [25,26].

### 2.10. Precision and accuracy

Drug-free serum aliquots were supplemented with $D_3$ and its metabolites at concentrations of 0.5 and 2 ng ml$^{-1}$, and determined as described above. Five test samples at each concentration were analyzed for the determination of within-run assay variability. The mean values and coefficients of variation (CV%) of the observed concentrations were calculated.

### 2.11. Recovery

The relative recoveries of digoxin and its metabolites from serum by SPE extraction procedures were determined by adding $D_3$ and its metabolites to drug-free serum and analyzing the resulting test samples according to the extraction and derivatization procedures except that the internal standard was added to the dried residue just before the derivatization step. The relative recovery of each analyte was calculated by comparing the peak-height ratio of the analyte in the extracted test samples to those of corresponding unextracted standards.

### 2.12. Optimization of elution from cyclodextrin columns

Various solvents were investigated for their ability to elute the glycosides and their derivatives from cyclodextrin SPE columns. Recoveries of the glycosides or their derivatives were expressed relative to the recoveries observed with methanol as elution solvent.

### 2.13. Optimization of reaction conditions

The effects of atmospheric moisture as reflected by the relative humidity, absolute and relative amounts of 1-NC and DMAP, and reaction volume were investigated to optimize the conditions for derivative formation. For determining the effect of atmospheric moisture, a mixture of digoxin and metabolites was derivatized at 26, 36, and 48% relative humidity (r.h.). Relative humidities of 26% and 36% were generated by passing a stream of dry filtered nitrogen through a glove box until the atmospheric moisture was reduced to 26% and 36% r.h., respectively. The relative humidity of 48% was the measured relative humidity in the laboratory at an ambient temperature of 20°C. Relative humidity was measured with a certified hygrometer (Fisher Scientific, Pittsburgh, PA).

### 2.14. Determination of digoxin in patient samples

Blood samples were collected from 86 hospitalized patients not undergoing digoxin treatment. Blood samples were collected into serum separator tubes (Autosep, Terumo Medical, Elkton, MD) and allowed to clot; serum samples were harvested and stored at −20°C until

Table 1
Recoveries [a] (%) of glycosides (prederivitization step) or glycoside derivatives (postderivatization step) from cyclodextrin SPE columns with different solvents

| Step | Eluent [b] | Recovery [a] (%) | | | | |
|---|---|---|---|---|---|---|
| | | $D_0$ | $D_1$ | $D_2$ | $D_3$ | $DHD_3$ |
| Prederivitization | $MeCl_2$ | ND | ND | ND | ND | ND |
| | AcCN | 24 | 25 | 37 | 36 | 39 |
| | IPA | 73 | 75 | 71 | 79 | 77 |
| | MeOH | 100 | 100 | 100 | 100 | 100 |
| Postderivitization | $MeCl_2$ | ND | ND | ND | ND | ND |
| | $MeCl_2$ | 54 | 56 | 52 | 38 | 39 |
| | AcCN | 56 | 48 | 47 | 46 | 52 |
| | EtOAc | 100 | 100 | 100 | 98 | 97 |
| | MeOH | 100 | 100 | 100 | 100 | 100 |

[a] Relative to those for methanol based on comparison of peak heights.
[b] The elution volume was 2 ml except where otherwise indicated.
[c] Ten aliquots of 100 μl each.
Key: $MeCl_2$, methylene chloride; IPA; isopropanol; MeOH; methanol; AcCN, acetonitrile; EtOAc, ethyl acetate; ND, none detected.

analyzed. Serum samples were stored for less than 2 months before assay. Digoxin concentrations were determined by the HPLC method and by an affinity-column-mediated, enzyme-linked immunoassay method (aca®, duPont Instruments, Wilmington, DE). Immunoassay determinations of digoxin concentrations were performed in the Clinical Chemistry Laboratory of the Ohio State University Hospitals (Columbus, OH) according to the manufacturer's instructions and quality control procedures. This method uses an antibody–enzyme complex to bind digoxin in serum. The free and bound forms of the enzyme–antibody complex are separated by an affinity column [22]. That fraction of the enzyme–antibody complex bound to digoxin is not retained on the column. The enzyme activity in the eluate is then measured spectrophotometrically and is proportional to the digoxin concentration.

Serum digoxin concentrations were also determined in blood samples collected from a patient receiving daily oral doses of 0.2 mg of digoxin as Lanoxicaps® (lot #6C2891, Burroughs Wellcome, Research Triangle Park, NC). Blood samples were collected immediately before dose administration on day 21 of digoxin therapy and at 2, 4, 6, 8, 10, 12, and 16 h after the dose. The blood samples were allowed to clot, and serum was harvested and stored at 20°C until analyzed.

## 3. Results and discussion

### 3.1. Optimization of extraction and derivatization conditions

The results of an investigation of elution strengths of various solvents for glycosides and their derivatives from cyclodextrin SPE columns are shown in Table 1. The elution solvent selected provided an optimal balance between high recovery of digoxin/metabolites and low recovery of interfering substances. For the extraction of digoxin and its metabolites from serum, the relative recoveries of glycosides were 70–80% for isopropanol compared to methanol. However, isopropanol eluted fewer interfering substances than methanol and was therefore chosen for the elution. For the isolation of derivatives, methanol was the strongest elution solvent among those tested, with fewer interfering substances eluted. Methylene chloride was found to be a good wash solvent for both prederivatization and postderivatization cleanup. In addition, multiple, small volume washes with methylene chloride were better than one, large volume wash in eluting interfering substances without significant elution of glycoside derivatives (Table 1).

The analytical precision of this method depends not only on the reproducibility of the various isolation steps but also on the reproducibility of the derivatization reaction. A notable feature of the present work was the

improved derivatization conditions compared to our previous method [14] which used a large molar excess of derivatization reagent under conditions of uncontrolled atmospheric moisture. In the present study, it was determined that the relative humidity was an important factor affecting the absolute yields of the 1-naphthoate esters of the glycosides. Fig. 1 shows chromatograms of a mixture of digoxin/metabolites derivatized at 26 and 36% relative humidities in a glove box. Reaction yields were greatly enhanced at lower relative humidity (26%, Fig. 1(a)) compared to those at higher humidity (36%, Fig. 1(b); or 48%, data not shown); this enhancement was more pronounced for those glycosides having three sugar moieties (i.e. $DT_3$, $D_3$ and $DHD_3$) compared to those having two or fewer sugar moieties (i.e. $D_0$, $D_1$, and $D_2$). This effect was attributed to the competition between water and the glycosides for the highly activated salt-like intermediate formed by the initial attack of DMAP on naphthoyl chloride. Reaction of water with the intermediate resulted in the hydrolysis of naphthoyl chloride rather than the formation of naphthoate esters of the glycosides [23,24].

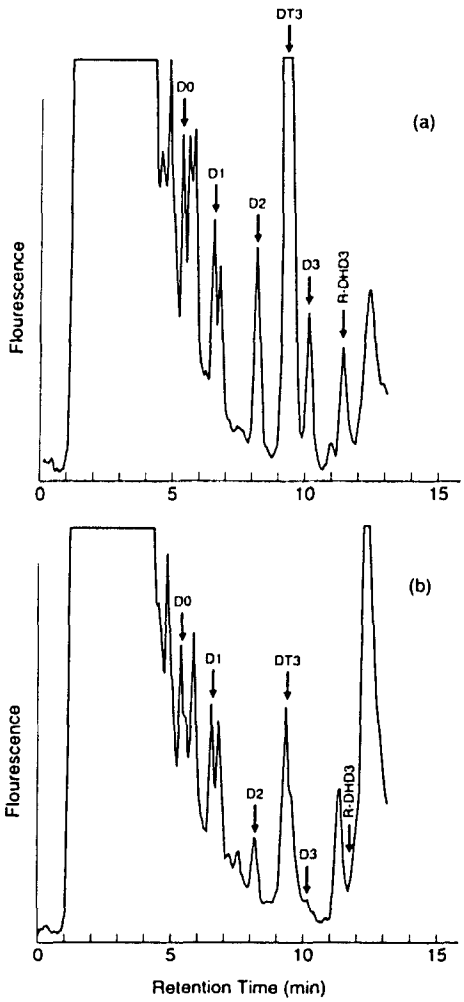


Fig. 1. Chromatograms of standard mixtures containing 1 ng each of $D_0$, $D_1$, $D_3$, $DHD_3$ and 10 ng of $DT_3$ as their naphthoate esters at (a) 26% r.h., and (b) 36% r.h. (Column 150 mm × 4.6 mm i.d., 3 μm Spherisorb® silica; mobile phsae, hexane–methylene chloride–acetonitrile–methanol (36:6.3:5.4:0.2, v v/v/v); flow rate, 1.6 ml $min^{-1}$, detector, Kratos fluorescence detector model 980; excitation at 217 nm emission filter, 340 nm.)

Increasing the amount of 1-NC from 2 to 10 μl did not improve the overall yields at 26% relative humidity, but did increase the yields at 48% relative humidity. However, more byproducts were generated and the reaction was less reproducible (i.e. variable yields of glycoside derivatives and interfering substances were observed) at 48% relative humidity. Our results indicated that the fluorescent yields were more reproducible and interfering substances less pronounced when the reactions were conducted under conditions of reduced relative humidity in a glove box. Therefore, all analytical studies were conducted in a glove box under conditions of reduced (i.e. relative humidity below 26%) relative humidity.

The extent of derivative formation was inferred from results of studies in which the amounts of 1-NC and DMAP were varied under controlled conditions of atmospheric moisture (26% rh or less) in a glove box. The peak heights of glycoside derivatives were not increased by increasing the amount of 1-NC from 2 μl (molar ratio, $1 \times 10^7$:1 for 1 ng of $D_3$) to 10 μl (molar ratio, $5 \times 10^7$:1) or of DMAP from 5 mg (molar ratio, $3.2 \times 10^7$:1 for 1 ng of $D_3$) to 25 mg (molar ratio, $1.6 \times 10^8$:1), and were reduced when less than 1 μl of 1-NC (molar ratio, $5 \times 10^6$:1) or 5 mg of DMAP (molar ratio, $3.2 \times 10^7$:1) are used.

### 3.2. Chromatography

The retention times of the naphthoate esters of $D_0$, $D_1$, $D_2$, $DT_3$, $D_3$, [20-*S*] – $DHD_3$ and [20-*R*]-$DHD_3$ were between 4.5 and 11 min. Each derivative yielded a well-resolved peak, whereas the derivative of $DHD_3$ yielded two symmetric resolved peaks, which were previously shown to be due to the *R* and *S* epimers (in a mixture of 3.0 to 1) at the C-20 position in the lactone moiety of dihydrodigoxin [13,25,26]. It has also been shown that only the *R* epimer ([20-*R*]-$DHD_3$) is formed in humans

Table 2
Representative calibration curve equations for serum samples containing digoxin and its metabolites at concentrations ranging from 0.24 to 4.0 ng ml$^{-1}$ except for [20-*R*]-DHD$_3$, when they ranged from 0.1875 to 3.0 ng ml$^{-1}$

| Analyte | Intercept | Slope (ml ng$^{-1}$) | *r* |
|---|---|---|---|
| $D_0$ | −0.045 | 0.219 | 0.9992 |
| $D_1$ | −0.014 | 0.153 | 0.9993 |
| $D_2$ | −0.000 | 0.146 | 0.9997 |
| $D_3$ | −0.010 | 0.103 | 0.9997 |
| [20-*R*]-DHD$_3$ | −0.012 | 0.112 | 0.9960 |

PHR = a + *b* × [analyte] where PHR was the peak height ratio of the analyte to digitoxin, *a* was the intercept, *b* was the slope of the calibration curve, and [analyte] was the concentration of analyte in the serum expressed in nanograms per millimeter.

[25,26]. The mobile phase used in this study (hexane–methylene chloride–acetonitrile–methanol (36:6.3:5.4:0.2, v/v/v/v)) was slightly different from that previously reported [13] (i.e. hexane–methylene chloride–acetonitrile (6:1:1, v/v/v)). This mobile phase produced a better separation of glycoside derivatives from interfering peaks. In addition, the mobile phase was more stable at this composition, without phase separation upon standing. Adjustment of the ratio of the components of the mobile phase was occasionally needed to achieve better separation of dihydrodigoxin derivatives from interfering substances.

### 3.3. Linearity of calibration curves

The slopes, intercepts, and correlation coefficients for the calibration curves of digoxin and each of its metabolites are shown in Table 2. The calibration curves were linear ($r$ = 0.9960 or greater) over the concentration range from 0.25 to 4.0 ng ml$^{-1}$ in serum and the intercepts were not significantly different from zero. Chromatograms for digoxin and its metabolites extracted from serum at concentrations of 2.0 ng ml$^{-1}$ and 0.25 ng ml$^{-1}$, respectively are shown in Fig. 2.

### 3.3. Precision, accuracy, and recovery

The precision and accuracy of determination, and the recovery of digoxin and its metabolites at concentrations of 2.0 and 0.50 ng ml$^{-1}$ are summarized in Table 3. The precision and accuracy of this method were satisfactory, with coefficients of variation ranging from 6.9% to 20.9% at 0.5 ng ml$^{-1}$ and from 5.8% to 12.2% at 2.0 ng ml$^{-1}$. The observed concentrations were within ±20% of nominal values at 0.50 ng ml$^{-1}$ and within ±10% at 2.0 ng ml$^{-1}$. The recoveries of digoxin and metabolites from serum ranged from 62% to 86%. The recoveries of $D_3$ and $DHD_3$ were approximately equivalent to those of previous reports using liquid–liquid extraction for the determination of digoxin and its metabolites in urine and feces [13,14] whereas those of $D_0$, $D_1$ and $D_2$ were higher with the

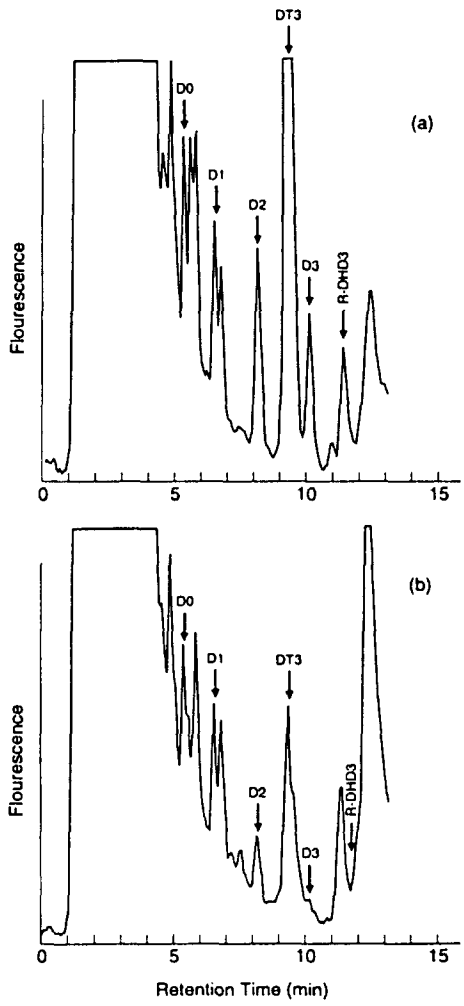


Fig. 2. Chromatograms of extracts of negative control serum samples to which (a) 2.0 ng ml$^{-1}$, or (b) 0.25 ng ml$^{-1}$ of $D_0$, $D_1$, $D_2$, $D_3$, $DHD_3$, and 10 ng of $DT_3$ (internal standard) were added. (Column, 150 mm × 4.6 mm i.d., 3 μm Spherisorb® silica; mobile phase, hexane–methylene chloride–acetonitrile–methanol (36:6.3:5.4:0.2, v/v/v/v); flow rate, 1.6 ml min$^{-1}$; detector, Kratos fluorescence detector model 980; excitation, 217 nm; emission cut-off, 340 nm.) The retention time of *S*-$DHD_3$ is not indicated in the figure because only *R*-$DHD_3$ is formed in humans.

Table 3
Relative recovery, precision, and accuracy for the determination of digoxin and the metabolites in serum

| Analyte | Recovery [a,c](%) | | Concentration observed [b,c] | |
|---|---|---|---|---|
| | 0.5 ng ml$^{-1}$ | 2.0 ng ml$^{-1}$ | 0.5 ng ml$^{-1}$ | 2.0 ng ml$^{-1}$ |
| $D_0$ | 76.7 ± 2.9 | 86.0 ± 3.3 | 0.5 (20.9) | 1.8 (12.0) |
| $D_1$ | 70.8 ± 7.6 | 84.2 ± 4.7 | 0.5 (17.9) | 1.8 (12.2) |
| $D_2$ | 79.5 ± 5.3 | 84.2 ± 4.9 | 0.6 (6.9) | 1.9 (9.3) |
| $D_3$ | 65.0 ± 9.1 | 69.1 ± 6.4 | 0.5 (13.4) | 1.8 (5.8) |
| [20-*R*]-$HD_3$ | 69.1 ± 10.6 | 61.6 ± 6.9 | 0.5 (14.6) | 1.8 (12.2) |

[a] Mean ± standard deviation.
[b] Mean (CV%).
[c] $n = 5$.

current method. The recoveries of digoxin and metabolites from serum by liquid–liquid extraction with methylene chloride increased as the number of sugar moieties increased, whereas for the present study, using cyclodextrin solid-phase extraction, recoveries increased with a decreasing number of sugar moieties. The $\beta$-cyclodextrin used for solid-phase extraction in this study has a torus-like structure with a hydrophilic external surface and a hydrophobic inside cavity [27]. The slightly lower recoveries of $D_3$ and $DHD_3$ compared to those of the sugar-hydrolyzed metabolites may be attributed to stronger interactions of the sugar hydroxy groups with cyclodextrin hydroxyl groups on the external surface resulting in less elution of these compounds from the column. This suggests that the hydroxy groups in the sugar moieties are responsible for retention, and suggests that polar interactions, possibly hydrogen bonding between cyclodextrin and the hydroxy groups on the sugar moieties, are the predominant factors that control the retention of these compounds.

### *3.4. Specificity and application to the determination of digoxin in patient serum samples*

Serum samples from hospitalized patients not undergoing digoxin treatment werre assayed for digoxin and its metabolites by the HPLC method described in this paper and by immunoassay. The patients were treated with the drugs listed in the appendix on the day of blood sample collection. The apparent digoxin concentration measured by HPLC was greater than 0.30 ng ml$^{-1}$ in only 1 of 86 (1.2%) patient serum samples and no metabolites were detected in any samples. The patient from whom this sample was collected was administered dobutamine, nystatin, aspirin, sucralfate, calcitriol, docusate sodium, vancomycin, cyclophosphamide, heparin, prednisone, acyclovir, and furosemide on the day of sample collection. On the other hand, the apparent digoxin concentrations measured by affinity-column-mediated, enzyme-linked immunoassay were greater than 0.30 ng ml$^{-1}$ in 24 of 86 (27.9%) serum samples collected from patients not receiving digoxin. The apparent serum digoxin concentrations in these samples ($n = 24$) ranged from 0.30 to 1.0 ng ml$^{-1}$ with a mean (±standard deviation (SD)) value of 0.41 ± 0.18 ng ml$^{-1}$. Furthermore, the apparent digoxin concentrations were greater than 0.30 ng ml$^{-1}$ in 9 of 11 (81.8%) serum samples in a subgroup of these samples collected from patients with hepatic failure; the apparent serum digoxin concentrations in these samples ($n = 9$) ranged from 0.30 to 1.0 ng ml$^{-1}$ with a mean (±SD) value of 0.52 ± 0.24 ng ml$^{-1}$. These results clearly demonstrate the greater specificity of the HPLC procedure, particularly when used to determine digoxin in samples collected from patients with hepatic failure.

Serum digoxin concentrations were also determined in samples collected from a patient receiving daily oral digoxin doses as Lanoxicaps® (0.2 mg; Burroughs Wellcome Company, Research Triangle Park, NC) and are shown in Fig. 3. Serum concentrations increased from 0.70 ng ml$^{-1}$ at the beginning of the dosing interval on the 21st day of treatment to a maximum serum concentration of 1.6 ng ml$^{-1}$ 4 h after the dose, and declined to 0.86 ng ml$^{-1}$ 16 h after the dose. No digoxin metabolites

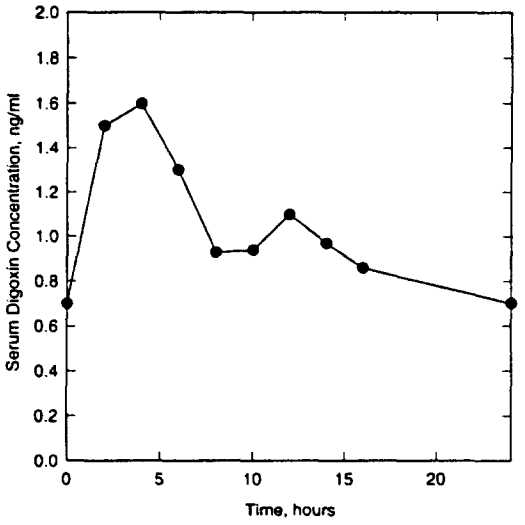


Fig. 3. Serum digoxin concentrations at day 21 in a patient receiving daily oral doses of 0.2 mg of digoxin as Lanoxicaps®.

were detected in the serum samples from this patient.

In conclusion, a sensitive and specific analytical method has been developed for the simultaneous determination of digoxin and its major metabolites in serum. Concentrations as low as 0.25 ng of both digoxin and its metabolites in 1.0 ml of serum were determined with acceptable precision and accuracy. The sensitivity was comparable to that of the immunoassay, but the specificity was greater, as is indicated by the results of an analysis of serum samples from patients not treated with digoxin. This is the first specific analytical procedure that has reported the separation and determination of digoxin and its metabolites at serum concentration less than 0.5 ng $ml^{-1}$ by HPLC without using radioactivity or immunoassay detection. This method therefore offers the advantage of unambiguous investigation of digoxin metabolism and pharmacokinetics and of the influence of the disease state on digoxin metabolism after the administration of commercial dosage forms. It also permits the assessment of the specificity of current immunoassay methods for serum digoxin.

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## Appendix: list of drugs administered to 86 hospitalized patients who were not receiving digoxin treatment

Acetaminophen
Acetazolamide
Acyclovir
Albuterol
Allopurinol
Amiodarone
Amitriptyline
Amoxicillin
Ampicillin
Aspirin
Atenolol
Atropine
Azathioprine
Bumetanide
Calcitriol
Captopril
Carbamazepine
Cefazolin
Cefoperazone
Ceftazidime
Ceftizoxime
Cephalexin
Chlordiazepoxide
Ciprofloxacin
Clavulanic acid
Clindamycin
Clonidine
Clotrimazole
Codeine
Conjugated estrogens
Cytoxan
Diazepam
Diphenhydramine
Dipyridamole
Dobutamine
Docusate sodium
Dopamine
Enalapril
Erythromycin
Famotidine
Fluconazole
Furosemide
Gemfibrozil
Gentamicin
Glyburide
Heparin
Hydralazine
Hydrochlorothiazide
Ibuprofen
Ipratropium bromide
Isosorbide dinitrate
Isradipine
Labetalol
Lidocaine
Lorazepam
Lovastatin
Medroxyprogesterone acetate
Meperidine
Metoclopramide
Metolazone
Metoprolol
Midazolam
Minoxidil
Morphine
Nicotine
Nifedipine
Nitroglycerin
Norepinephrine
Nystatin
Oxybutynin
Oxycodone
Pentoxyphylline
Phenytoin
Piroxicam
Prednisone
Procainamide
Procaine
Promethazine
Propoxyphene
Ranitidine
Sotalol
Spironolactone
Sulbactam
Sulfamethoxazole
Sulfisoxazole
Temazepam
Tetracycline
Timolol
Tobramycin
Triamcinolone acetonide
Triamterene
Trimethoprim
Vancomycin
Verapamil
Warfarin

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