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# DIGOXIN AND METABOLITES IN URINE AND FECES: A FLUORESCENCE DERIVATIZATION—HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE

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## SUMMARY

A high-performance liquid chromatography method is described for the determination of digoxin and its metabolites digoxigenin, digoxigenin monodigitoxoside, digoxigenin bis-digitoxoside and dihydrodigoxin (20*S* and 20*R*) excreted in urine and feces. The urine sample or fecal supernatant is extracted with methylene chloride in the presence of digitoxigenin or digitoxin as internal standard. Pre-column derivatization is achieved using 1-naphthoyl chloride with subsequent separation of the derivatized compounds on either a normal- or reversed-phase system with fluorescence detection. Recoveries for digoxin and all metabolites from fecal samples were in the range 60– 74%, which is comparable to that

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previously determined for urine samples. Standard curve data revealed linearity over a wide range of concentrations. Coefficients of variation for the analysis were less than 10% for all compounds over a range of 5—125 ng per ml urine and 10—250 ng per 200 mg feces. Peaks for digoxin and metabolites in urine and feces were obtained when human excreta were analyzed.

## INTRODUCTION

Digoxin (D3) is the drug of choice for treatment of congestive heart failure. Its metabolism has been the subject of numerous research investigations over many years. The structures of D3 and some of its known metabolites are shown in Fig. 1. D3 is metabolized by sequential cleavage of the digitoxose sugars attached at the 3-position. This yields digoxigenin bisdigitoxoside (D2), digoxigenin monodigitoxoside (D1) and digoxigenin (D0), all cardioactive metabolites. Metabolism also occurs by reduction of the 20,22-unsaturated lactone ring of D3, introducing an asymmetric center at the 20-position [1]. The two dihydrodigoxin (DHD3) epimers thus formed are 20*R*-DHD3 and 20*S*-DHD3. Since these two metabolites show considerably less cardioactivity than D3, it has been suggested that humans who form large amounts of these reduced compounds may require greater doses of D3 [2]. The reduced metabolite appears to be formed by gastrointestinal bacteria [2] and the predominant epimer in humans is 20*R*-DHD3 [3].

Methods of analysis have included radioimmunoassay (RIA) [4—11], gas chromatography [12—14] and high-performance liquid chromatography (HPLC) with RIA detection [15—18] or with ultraviolet detection [19, 20]. However, very few of these methods are suitable for quantitative determination of D3 and its metabolites in biological fluids. Most are lacking in either specificity for unchanged digoxin or the ability to quantitate metabolites separately, especially the epimers of DHD3. Many of the methods used to study human metabolism have utilized tritiated digoxin administration to humans and are not suitable for studies of D3 metabolism involving either a large number of patients or chronic administration of digoxin.

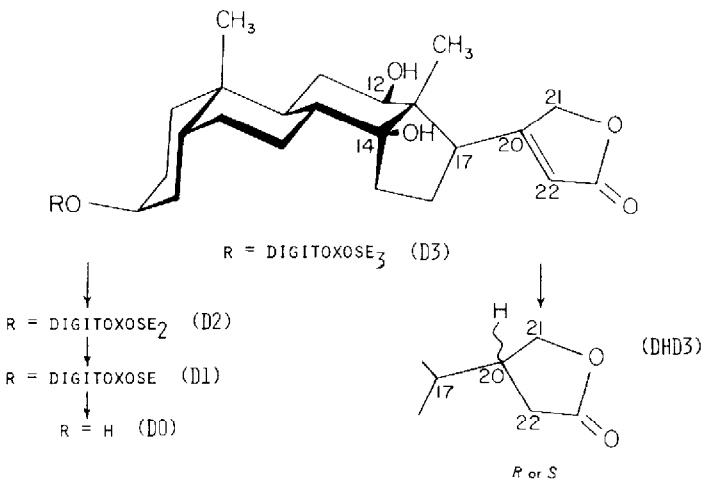


Fig. 1. Structural formulae of digoxin and metabolites.

The present work was preceded by development of a specific HPLC method for D3 and its metabolites which was previously reported [21]. This procedure involved derivatization of the glycosides with 3,5-dinitrobenzoyl chloride prior to HPLC with ultraviolet detection and had a lower detection limit of 50 ng/ml using a 10-ml urine sample. The excellent specificity of this approach with respect to known metabolites prompted consideration of fluorescent derivatives having potentially greater sensitivity. Described herein is a method by which D0, D1, D2, D3, 20*S*-DHD3 and 20*R*-DHD3 are extracted from urine or fecal samples, derivatized with 1-naphthoyl chloride (1-NC), and the derivatives determined by HPLC with fluorescence detection. Quantitation is accomplished by reference to standard curves using peak-height ratios with respect to either digitoxigenin (DT0) or digitoxin (DT3) as internal standard.

## EXPERIMENTAL

### *Materials*

Hexane, methylene chloride, acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) chloroform and 2-propanol (Mallinckrodt, Paris, KY, U.S.A.) were used as obtained. All solvents used for chromatography were of HPLC grade. The derivatizing reagent, 1-naphthoyl chloride, was used as supplied (Purum grade, Fluka, Buchs, Switzerland) and the catalyst, 4-dimethylaminopyridine (DMAP, Aldrich, Milwaukee, WI, U.S.A.), was repeatedly re-crystallized from hot benzene prior to use.

Drug standards, D0, D1, D2, D3, DHD3 (mixture of two epimers), DT0 and DT3, were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Stock solutions of all these standards were made up in 2-propanol with the exception of DT0 which was prepared in methylene chloride. Clindamycin (Upjohn, Barceloneta, Puerto Rico) was used as obtained.

### *Glassware*

Glass culture tubes with PTFE-lined screw caps were used for all procedures (Corning Glass, Corning, NY, U.S.A.). All glassware was soaked for 24 h in sulfuric acid–nitric acid (4:1), washed, siliconized in a 1% solution of SurfaSil® (Pierce, Rockford, IL, U.S.A.) in toluene, rinsed thoroughly and oven-dried before use.

### *Instrumentation and chromatographic conditions*

The isocratic HPLC system consisted of a Model 110A solvent metering pump (Beckman Instruments, Fullerton, CA, U.S.A.) and a Model FS970 fluorescence detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). Sample injections were made using either a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 175-$\mu$l loop, or a 3XL injector valve (Scientific Systems, State College, PA, U.S.A.) with a 20-$\mu$l external loop attached.

Three different columns were used: (1) 250 mm × 4.6 mm I.D., 5-$\mu$m LiChrosorb® SI60 (Jones Chromatography, Columbus, OH, U.S.A.), (2) 150 mm × 4.6 mm I.D., 3-$\mu$m Adsorbosphere® silica (Alltech Assoc., Deerfield, IL, U.S.A.), (3) 250 mm × 4.6 mm I.D., 5-$\mu$m Apex® ODS (Jones Chromato-

graphy). The column eluent was excited at 217 nm and fluorescence was monitored using a 340-nm emission cut-off filter, consistent with a maximum emission wavelength of 372 nm for the derivative of digoxin in hexane–methylene chloride–acetonitrile (5:1:1).

The mobile phase for all normal-phase analyses was hexane—methylene chloride—acetonitrile (5:1:1 to 6:1:1). For reversed-phase analyses, the mobile phase was 100% acetonitrile. Flow-rates were 1.8—2.0 ml/min and all chromatographic experiments were carried out at room temperature.

*Extraction*

A 1-ml volume of urine was pipetted into a test-tube containing 1 ml of DT0 (100 ng/ml) in methylene chloride and 1 ml of methylene chloride was added. When DT3 was used as the internal standard, 1 ml of a 100 ng/ml solution of DT3 in 2-propanol was pipetted into a test-tube and the solvent was evaporated before 1 ml of urine and 2 ml of methylene chloride were added. The amount of internal standard varied between experiments but the urine samples were always extracted with 2 ml of methylene chloride. The test-tube was shaken vigorously by hand four times (more shaking caused emulsion formation), centrifuged (1650 *g*), and the aqueous layer aspirated off and discarded. Two successive washes using 2 ml of a 5% aqueous sodium bicarbonate solution each time followed. The methylene chloride extract was transferred to a clean test-tube and evaporated under nitrogen at 50°C.

Human fecal samples were diluted 5:1 (v/w) with clindamycin solution (5 μg/ml in double-distilled, deionized water) and homogenized by mechanically shaking for 15 min. This procedure was developed to arrest further metabolism of D3 by bacteria present in the feces. A 1-ml volume of a stock solution of DT0 (100 ng/ml in methylene chloride) or DT3 (100 ng/ml in 2-propanol) was pipetted into a test-tube, the solvent evaporated, and the tube weighed. Approximately 1 g of the fecal homogenate was poured into the test-tube; the test-tube and its contents were reweighed to determine the weight of fecal homogenate added. Distilled water (1 ml) was added, and the sample was vortexed for 30 s and shaken for 15 min. After centrifuging the sample for 1 h, the supernatant was poured off and extracted with 2 ml methylene chloride. The extract was washed twice with 5% sodium bicarbonate solution and evaporated under nitrogen at 50°C.

*Derivatization*

After the addition of 25 mg of DMAP and 10 μl of 1-NC to the dry extract, 100 μl of acetonitrile were added and the sample was capped and vortexed thoroughly. The test-tube was placed in a water bath at 50°C for 1 h.

*Preparation for chromatography*

The derivatized sample was centrifuged and evaporated at 50°C under nitrogen. Excess derivatizing agent was hydrolyzed by adding 2 ml of 5% sodium bicarbonate solution and shaking the sample mechanically for 5 min. To extract the derivative, 2 ml of chloroform were added and the test-tube was shaken by hand; the aqueous layer was aspirated off and discarded. Two washes with 5% sodium bicarbonate were carried out as before. Finally, three

washes, each using 3 ml of 0.05 *M* hydrochloric acid containing 5% sodium chloride, were performed and the chloroform was evaporated. The sample was dissolved in mobile phase before injection into the HPLC system.

*Standard curves*

Known amounts of D3 and metabolites were added to samples of digitalis-free urine or fecal homogenate followed by extraction, derivatization and chromatography as described above. The internal standard used for urinary studies was 25 ng DT3, while 100 ng DT3 were used for fecal studies. Standard curves were made by plotting peak-height ratios against the amount of glycoside added.

*Repeatability studies*

Repeatability of the quantitation method used for D3 and its metabolites in urine and feces was assessed by doing five analyses of the compounds added at a low concentration and five at a high concentration. The mean and the coefficient of variation (%) for the peak-height ratios were then calculated.

*Recovery from fecal samples*

An internal standard, 50 ng DT0, was added to each of eight 1-ml digitalis-free fecal homogenate samples. Four of the samples were supplemented with 250 ng of each of the glycosides (D0, D1, D2, D3, DHD3) and shaken for 10 min before centrifugation, extraction of the supernatant and further analysis. For the remaining samples the glycosides were added after extraction. Thus, the recovery of a particular compound was determined by dividing its peak-height ratio when it was added before extraction by its peak-height ratio when it was added after extraction.

## RESULTS AND DISCUSSION

The retention times for D3 and its metabolites for all three columns are listed in Table I. Although the reversed-phase system required the shortest total analysis time, the two epimers of DHD3 were not resolved. Thus, most of the developmental work was done using the 5-$\mu$m LiChrosorb SI60 column. Later experiments, using the short, 3-$\mu$m Adsorbosphere silica column, showed this column to be superior to the 5-$\mu$m column in terms of peak resolution, efficiency ($N > 60\,000$), sensitivity and total analysis time. It is noteworthy that a recent paper reported that 20*R*-DHD3 is the epimer formed in humans [3]. Thus, the reversed-phase system may also be useful for studies of D3 metabolism in humans.

Two cardiac glycosides were found to be appropriate internal standards for this HPLC method: DT0 and DT3. The latter is preferred since it is better resolved from the solvent front than DT0 and is, therefore, much less affected by interfering materials extracted from urine and feces which tend to elute with short retention times. In addition, the choice between two possible internal standards gives one flexibility in avoiding co-eluting peaks which may appear in biological samples.

The extraction efficiencies for D3 and its metabolites from urine using a

TABLE I

RETENTION TIMES OF 1-NAPHTHOYL CHLORIDE DERIVATIVES

System 1: Apex ODS, 100% acetonitrile. System 2: LiChrosorb SI60, hexane—methylene chloride—acetonitrile (6:1:1). System 3: Adsorbosphere Si, hexane—methylene chloride—acetonitrile (6:1:1).

| Glycoside derivatized | Retention time (min) | | |
|---|---|---|---|
| | System 1 | System 2 | System 3 |
| DT0 | 2.46 | — | — |
| D0 | 2.70 | 8.9 | 4.85 |
| D1 | 3.26 | 10.6 | 5.90 |
| D2 | 4.50 | 12.9 | 7.50 |
| DT3 | — | 14.3 | 8.10 |
| D3 | 6.24 | 15.5 | 9.40 |
| 20*S*-DHD3 | 6.80 | 16.4 | 10.0 |
| 20*R*-DHD3 | 6.80 | 18.2 | 10.9 |

TABLE II

RECOVERY OF DIGOXIN AND METABOLITES

The values in parentheses represent the coefficients of variation (%).

| Compound | Mean recovery ($n$ = 4) (%) | |
|---|---|---|
| | Urine* | Feces** |
| D0 | 46.0 (7.87) | 59.8 (12.1) |
| D1 | 36.0 (6.18) | 61.0 (14.1) |
| D2 | 61.2 (8.42) | 68.5 (10.0) |
| D3 | 73.2 (17.2) | 70.9 (11.5) |
| 20*S*-DHD3 | 71.9 (7.29) | 74.0 (12.8) |
| 20*R*-DHD3 | 76.0 (10.3) | 71.7 (14.5) |

*From ref. 21.

**Fecal samples contained 250 ng of each glycoside and peak-height ratios were measured with respect to 50 ng DT0.

method similar to the one described have been reported previously from this laboratory [21]. These data together with recoveries from feces are presented in Table II. The fecal recoveries represent compound added to the fecal homogenate whereas one metabolite, dihydrodigoxin, is probably formed in vivo inside the cell of *Eubacterium lentum* and may not be as readily extractable. However, low extractability does not appear to be a problem since dihydrodigoxin that is formed from digoxin by cultures of feces or cultures of *Eubacterium lentum* can be recovered by this extraction procedure to a degree equivalent to standards [22].

The standard curves for all the compounds (Table III) were linear over a wide range of concentrations for both urine and feces. Reproducibility of the method was found to be quite satisfactory for both urine and feces (Table IV). Coefficients of variation were well under 10% for drug and metabolites in both urine and fecal samples.

TABLE III

STANDARD CURVE EQUATIONS

PHR = Peak-height ratio with respect to internal standard. Independent variable in each equation is bracketed and is expressed in ng of glycoside per 1 ml of urine or 200 mg of feces. Numerical constants are the slope and intercept, respectively, for each compound. Column: 150 mm × 4.6 mm I.D., 3-μm Adsorbosphere silica. Mobile phase: hexane—methylene chloride—acetonitrile (6:1:1).

| Compound | Equation | Correlation coefficient |
|---|---|---|
| *Urine extracts (internal standard: 25 ng DT3)* | | |
| D0 | PHR = 0.0210 (D0) + 0.413 | 0.983 |
| D1 | PHR = 0.0116 (D1) + 0.0954 | 0.996 |
| D2 | PHR = 0.0275 (D2) + 0.117 | 0.995 |
| D3 | PHR = 0.0298 (D3) + 0.115 | 0.984 |
| 20*S*-DHD3 | PHR = 0.0157 (20*S*-DHD3) + 0.162 | 0.961 |
| 20*R*-DHD3 | PHR = 0.0318 (20*R*-DHD3) + 0.0118 | 0.993 |
| Range of sensitivity: 5—125 ng glycosides per 1 ml urine | | |
| *Fecal extracts (internal standard: 100 ng DT3)* | | |
| D0 | PHR = 0.00389 (D0) + 0.162 | 0.989 |
| D1 | PHR = 0.00351 (D1) + 0.0142 | 0.983 |
| D2 | PHR = 0.00587 (D2) + 0.0161 | 0.998 |
| D3 | PHR = 0.00429 (D3) + 0.0253 | 0.967 |
| 20*S*-DHD3 | PHR = 0.00509 (20*S*-DHD3) + 0.0718 | 0.997 |
| 20*R*-DHD3 | PHR = 0.00421 (20*R*-DHD3) + 0.132 | 0.965 |
| Range of sensitivity: 10—150 ng glycosides per 200 mg feces | | |

TABLE IV

REPEATABILITY OF ANALYTICAL METHOD FOR DIGOXIN AND METABOLITES IN URINE AND FECAL SAMPLES

The values in parentheses represent the coefficients of variation (%). Concentrations refer to glycosides added.

| Glycoside | Mean peak-height ratio | | | |
|---|---|---|---|---|
| | From urine* | | From stool** | |
| | 25 ng/ml | 125 ng/ml | 10 ng/ml | 250 ng/ml |
| D0 | 0.345 (5.47) | 1.35 (5.35) | 0.215 (6.89) | 4.33 (4.36) |
| D1 | 0.426 (5.65) | 1.22 (2.60) | 1.29 (7.63) | 7.84 (5.42) |
| D2 | 0.437 (4.92) | 1.92 (4.68) | 0.205 (7.35) | 5.01 (6.56) |
| D3 | 0.309 (0.37) | 1.17 (4.59) | 0.165 (8.57) | 3.76 (4.44) |
| 20*R*-DHD3 | 0.421 (6.90) | 1.61 (4.30) | 0.217 (5.77) | 4.51 (4.81) |

*$n$ = 5; internal standard: 100 ng DT0; column: 250 mm × 4.6 mm I.D., 5-μm LiChrosorb SI60; mobile phase: hexane—methylene chloride—acetonitrile (5:1:1).
**$n$ = 6; internal standard: 50 ng DT0; column: 250 mm × 4.6 mm I.D., 5-μm LiChrosorb SI60; mobile phase: hexane—methylene chloride—acetonitrile (5:1:1).

The HPLC analysis can be used to study digoxin metabolism in humans. As an illustration, two human volunteers who were taking D3 chronically were studied by quantitatively collecting their urine and feces over a three-day

period. Representative chromatograms from the analysis of urine (Fig. 2) and fecal (Fig. 3) samples illustrate the effectiveness of this analytical method for identification of drug and metabolite peaks. Although not shown, chromatograms from digitalis-free urine and fecal samples from several humans, as well as the standard curves obtained in excreta from these subjects, indicate that interference from the biological material is minimal and that this method is capable of quantitating D3 and its metabolites at concentrations found in urine and feces. Further verification of peak identity can be achieved by re-chromatographing in a different HPLC system such as system 1 in Table I.

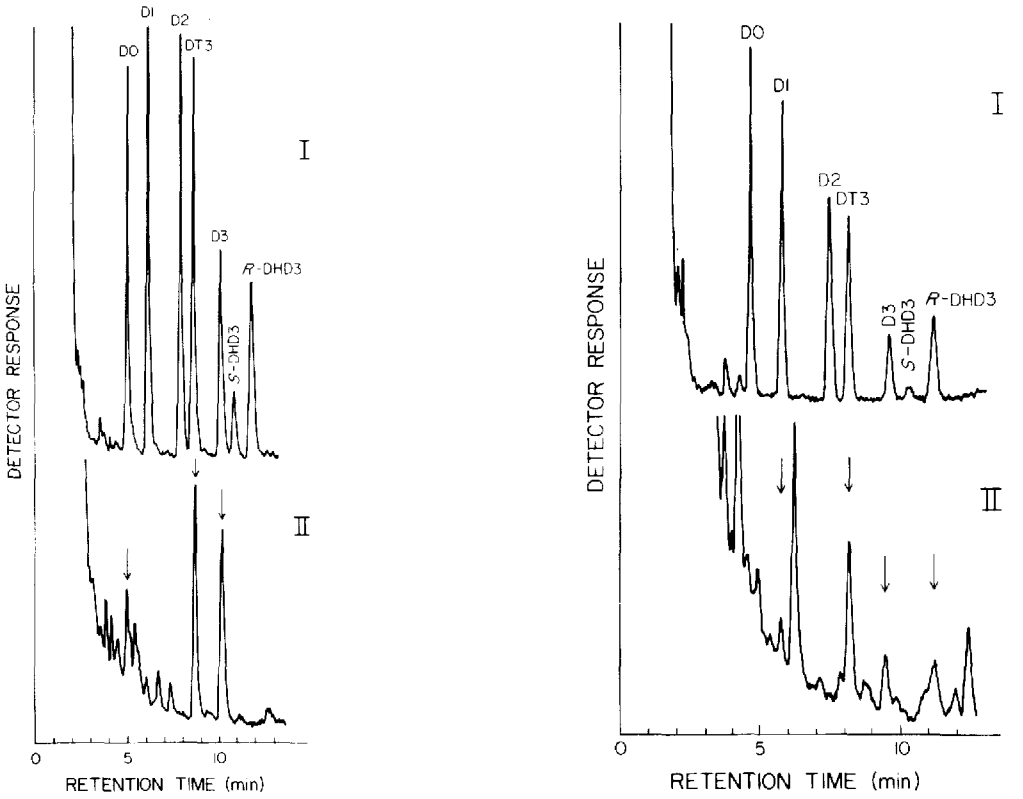


Fig. 2. HPLC profile of derivatized standards (I, equivalent of 2.0 ng of each compound on column; for DHD3 the 2.0 ng refers to the sum of *R*- and *S*-epimers) and an extracted and derivatized urine sample from a human subject receiving digoxin (II). Arrows indicate positions of identifiable peaks (*S*-DHD3 refers to 20*S*-DHD3 and *R*-DHD3 refers to 20*R*-DHD3). Assayed amounts were as follows: D3 = 51 ng/ml, D0 = trace. The samples were run on a 150 mm × 4.6 mm I.D., 3-μm Adsorbosphere SI column using a mobile phase of hexane—methylene chloride—acetonitrile (6:1:1) with 50 ng DT3 per ml urine as internal standard.

Fig. 3. HPLC profile of derivatized standards (I, 2.0 ng equivalent on column) and an extracted and derivatized fecal sample from a different human subject receiving digoxin (II). Arrows indicate positions of identifiable peaks (*S*-DHD3 refers to 20*S*-DHD3 and *R*-DHD3 refers to 20*R*-DHD3). Assayed amounts were as follows: D3 = 75 ng per 200 mg feces, D1 = 56 ng per 200 mg feces, *R*-DHD3 = 52 ng per 200 mg feces. Chromatographic conditions are identical to those for Fig. 2, with 100 ng DT3 per 200 mg feces as the internal standard.

Studies of stability under storage conditions were carried out on urine samples and fecal homogenates with D3 added. Measured peak-height ratios showed no statistical difference ($p > 0.05$, Jonckheere's test [23]) before or after 24 h of freezing. Similar studies showed that addition of clindamycin solution (5 $\mu$g/ml) had no effect on drug determination in feces over a 24-h period. Furthermore, addition of clindamycin at 5 $\mu$g/ml in a fecal homogenate was shown to prevent bacterial metabolism of D3 to 20*R*-DHD3 in a fecal sample which had displayed such conversion when incubated [2] in absence of the antibiotic.

In conclusion, the method for determination of D3 and its major extractable metabolites in urine and feces described in this paper is a powerful analytical tool which can be applied to metabolic studies of this drug. The ability to analyze fecal samples without administration of radioactive drug is a significant advantage of the method. Quantities as low as 5 ng of both drug and metabolites in 1 ml urine or 10 ng in 200 mg feces are detectable. Thus, it is possible to obtain an accurate profile of the extent of digoxin biotransformation to its extractable metabolites in humans.

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