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DIGOXIN AND METABOLITES IN URINE: A DERIVATIZATION—HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD CAPABLE OF QUANTITATING INDIVIDUAL EPIMERS OF DIHYDRODIGOXIN

HOWARD N. BOCKBRADER and RICHARD H. REUNING*

College of Pharmacy, The Ohio State University, Columbus, OH 43210 (U.S.A.)

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of digoxigenin, digoxigenin monodigitoxoside, digoxigenin bis-digitoxoside, digoxin, and dihydrodigoxin as the 3,5-dinitrobenzoyl esters. The method is applied to a 10 ml urine sample by adding digitoxigenin as internal standard, extracting with methylene chloride, derivatizing with 3,5-dinitrobenzoyl chloride in pyridine, chromatographing with a normal-phase system and detecting at 254 nm. Derivatized digoxigenin, digoxigenin mono- and bis-digitoxoside, and digoxin each yielded one symmetrical peak with the limit of sensitivity of the method being approximately 100 ng/ml. Analysis of a commercially obtained sample of dihydrodigoxin resulted in two well-separated, symmetrical peaks that represent the two epimers of derivatized dihydrodigoxin. Data indicate rapid and complete esterification of all primary and secondary alcohol moieties in the various molecules and the derivatives are shown to be stable in chloroform for at least four days. The procedure appears to be suitable for metabolic investigations and as a prototype for future analytical developments.

INTRODUCTION

The known metabolites [1—4] of digoxin (D3) shown in Scheme 1 include digoxigenin (D0), digoxigenin monodigitoxoside (D1), digoxigenin bis-digitoxoside (D2) and dihydrodigoxin (DHD3). The importance of metabolism as an elimination pathway for D3 is underscored by the results of Lukas [5], who used a specific double isotope dilution derivative method and found only 21—55% of an oral dose of digoxin excreted unchanged in urine and feces. Although D0, D1 and D2 usually account for less than 10% of overall urinary recovery, the percentage excreted as DHD3 has been found to vary widely. Dihydrodigoxigenin, the aglycone of DHD3 , was originally detected in the

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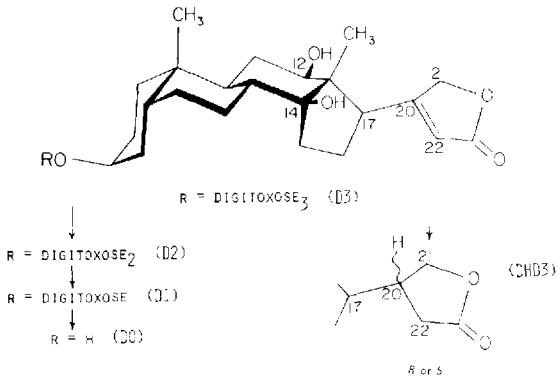

Scheme 1. Abbreviation scheme for digoxin and metabolites.

urine of a patient requiring unusually high doses of D3 [4]. This was followed by the discovery of DHD3 in human plasma [6]. The percentage of the total glycoside isolated as DHD3 has been found to vary widely between patients. Clark and Kalman [3] surveyed 50 patients and found a range of 1—47% of the total glycosides in the methylene chloride extract of urine present as DHD3. Peters and coworkers [7, 8], who investigated 100 patients receiving D3, reported a range of 2—52% of methylene chloride extractable drug plus metabolites present as reduced metabolites, with 53 subjects having over 10% and seven subjects having over 35% as reduced metabolites. This wide inter-subject variability in extent of formation of dihydro metabolites appears to be due to variability in the intestinal microbial flora that are responsible for forming the reduced metabolites [9, 10]. Lindenbaum et al. [10] estimate that about 10% of digoxin patients are substantial dihydro metabolite formers (i.e. $>$ 40% of urinary glycoside excretion attributed to dihydro metabolites).

The major analytical methods that have the selectivity and sensitivity for D3 and its metabolites are gas—liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). A GLC method [3, 6, 11, 12] has been developed which separates reduced metabolites from unreduced metabolites and is sufficiently sensitive for serum or urine samples. However, during the derivatization procedure D3 and DHD3 are converted to the corresponding derivatized aglycone. Recently, Heftmann and Hunter [13] reviewed the HPLC methods for steroids. Both reversed-phase [14—17] and normal-phase [14, 18—20] systems have been reported for digitalis compounds. Excellent specificity was achieved for D0, D1, D2, and D3; however, the molar absorptivities of D3 and its metabolites were inadequate for determinations in biological fluids. The lack of sensitivity has been overcome either by using tritiated digoxin [14, 17] or by collecting appropriate fractions and analyzing by radioimmunoassay [20—22]. The assay of D0, D1, D2, and D3 using derivatization with the ultraviolet chromophore 4-nitrobenzoyl chloride and normal phase HPLC has been reported [23, 24]. There has been no report of the application of this derivatization procedure to dihydro metabolites of digoxin or to determinations in biological samples.

Reduction of the 20,22-unsaturated lactone ring of D3 introduces a center of asymmetry at the 20 position. Brown and Wright [25] reported the separa-

tion and isolation of the epimers of dihydrodigoxigenin (DHD0); however, they were unsuccessful in the separation and isolation of the epimers of DHD3. Watson et al. [6] reported that their GLC method did not separate DHD0 into two peaks. However, quantitation of the sum of the two DHD3 epimers in biological fluids has recently been achieved using radiolabeled drug and either DEAE-Sephadex column chromatography [26], HPLC [14] or a combination of column and thin-layer chromatographic procedures [27]. There have been no methods reported to date that are capable of separating the individual epimers.

This paper describes an HPLC method for the separation and quantitation of D3 and its metabolites and the application of the method to human urine samples. The most significant step in the methodology is the derivatization of D3 and its metabolites with 3,5-dinitrobenzoyl chloride, which facilitates separation and detection. The advantage of the method is the separation, detection, and quantitation of individual epimers of dihydro metabolites without use of radiolabeled drug.

EXPERIMENTAL

Materials

Hexane, methylene chloride, acetonitrile, pyridine, chloroform, and 2-propanol were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). D0, D1, D2, D3, DHD3, and digitoxigenin (DT0) were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The catalyst, 4-dimethylaminopyridine (Purum grade; Fluka, Buchs, Switzerland), was used as supplied, and the derivatizing agent, 3,5-dinitrobenzoyl chloride (DNBCl, Purum grade; Fluka), was recrystallized from petroleum ether and stored in a vacuum desiccator. All other chemicals and reagents were analytical grade or better.

Drug standards

Stock solutions containing D0, D1, D2, and D3 were prepared in 2-propanol at concentrations of 2.5, 5, 10, 12.5, 20, and 25 μg/ml. The internal standard, DT0, 20 μg/ml, was prepared in methylene chloride. The derivatizing agent (DNBCl) was prepared daily by dissolving in pyridine (85 mg/ml) with gentle warming.

Glassware

Glass culture tubes with PTFE-lined screw caps were used for the extraction and derivatization procedures (Corning, Corning, NY, U.S.A.). All glassware was soaked for 24 h in sulfuric acid—nitric acid (4:1), washed, and silanized for 2 min in a 1% solution of Dri Film (Pierce, Rockford, IL, U.S.A.) in toluene. After washing, the glassware was dried in an oven, and glassware for the derivatization procedure was stored in a vacuum desiccator.

Instrumentation and chromatographic conditions

A Model 5000 high-performance liquid chromatograph was equipped with a Model 960 ultraviolet (254 nm) detector (Tracor, Austin, TX, U.S.A.), and a

Rheodyne Model 7105 injection valve containing a 175 μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) Detector output was recorded on a Tracor Model T-11 recorder. A 100 μl aliquot of the derivatized sample was chromatographed at room temperature on a Partisil 10 column (25 cm × 4.6 mm I.D., 10 μm average particle size; Whatman, Clifton, NJ, U.S.A.) and was eluted isocratically with hexane—methylene chloride—acetonitrile (8:3:3 to 12:3:3). The mobile phase was pumped at approximately 1.8 ml/min and the percentage of hexane incorporated into the mobile phase was varied according to the degree of separation desired.

Extraction procedure

A 0.5 ml volume of the internal standard solution and 20 ml of methylene chloride were added to a 45 ml tube containing a 10 ml urine specimen. The tubes were tightly sealed, shaken for 15 min on a mechanical shaker (Eberbach, Ann Arbor, MI, U.S.A.), centrifuged for 20 min, and the aqueous phase was removed and discarded. After the addition of 15 ml of a 5% sodium bicarbonate solution to extract components that interfere with the derivatization procedure, the tubes were recapped, shaken, centrifuged, and the aqueous phase was removed and discarded as before. The organic phase was transferred to a 12 ml tube and gently evaporated to dryness at 50°C under a stream of nitrogen (N-evap®; Organomation Associates, Northborough, MA, U.S.A.). The tubes were tightly sealed and stored at room temperature prior to derivatization.

Standard curves were prepared daily by adding 1 ml of the appropriate stock solution(s) to a 45 ml tube containing drug-free urine and internal standard, and extracting as described. Standard curves were analyzed by unweighted linear least-squares regression.

Derivatization procedure

A 200 μl volume of the derivatizing solution was added to the dried sample and the reaction was carried out for 10 min at room temperature with gentle shaking. The derivatized sample was carefully evaporated to dryness by removing the pyridine with a stream of nitrogen at 50°C. The excess derivatizing agent was hydrolyzed with 2 ml of a 5% sodium bicarbonate solution containing 2 mg/ml 4-dimethylaminopyridine. After shaking for 5 min, 1 ml of chloroform was added to solubilize the derivative and the tubes were rocked on an Aliquot Mixer (Ames, Elkhart, IN, USA). The aqueous layer was discarded and the organic phase was mixed for 2 min with 2 ml of a 5% sodium bicarbonate solution. The aqueous layer was discarded and 3 ml of a 0.05 *M* hydrochloric acid solution containing 5% sodium chloride was mixed with the organic phase for 2 min to remove any residual pyridine. After the organic layer was washed three more times with the acidic solution, the chloroform was ready for chromatographic analysis.

Extent of derivatization

Samples (1 mg) of D0, D1, D2, and D3 were carried through the derivatization procedure and the chloroform solution was evaporated to dryness at 50°C under a nitrogen stream and further dried at room temperature under high

vacuum for 1 h. A sample (200 μg) of DHD3 was derivatized and chromatographed as described except that two columns were used in series and the sample was subdivided into aliquots. The HPLC eluates corresponding to the derivatized *R* and *S* epimers [28] were collected and re-chromatographed several times. After solvent evaporation the samples were dried under vacuum at 80°C for 1 h. Each derivatized sample of D0, D1, D2, D3, *R*-DHD3 or *S*-DHD3 was dissolved in deuterated chloroform, filtered through cotton and analyzed on a Bruker HX-90 nuclear magnetic resonance (NMR) spectrometer.

Stability of derivatized compounds

Samples of DT0, D0, D1 (120 μg each), D2 (180 μg), and D3 (240 μg) were derivatized and a 175 μl aliquot of the final chloroform phase was injected onto the chromatographic column 0, 60, 120, 240, 510, 770, 1400, 2790, and 4260 min after completion of the derivatization procedure. The area of each of the chromatographic peaks was measured in triplicate using a planimeter.

Extraction efficiency

Samples of D0, D1, D2, D3, and DHD3 were added to drug-free urine and prepared according to the extraction and derivatization procedures except that the internal standard solution was added to the methylene chloride after extraction and just prior to evaporation. The extraction efficiency of each compound was calculated by comparing the peak height ratio of extracted samples to the peak height ratio of corresponding unextracted drug standards.

Precision

Drug-free urine specimens were supplemented with D0, D1, D2, and D3 at concentrations of 1 and 0.1 μg/ml and quantitated according to the extraction and derivatization procedures. Six samples at each concentration were analyzed for within-day assay variability and accuracy. In a similar manner, DHD3 was assayed at total (sum of both epimers) added concentrations of 2.5 μg/ml and 0.44 μg/ml (five replicates each).

Molar absorptivity and ratio of the two epimers of dihydrodigoxin

A 50 μg sample of DHD3 was derivatized and three 100 μl aliquots of the final chloroform phase were chromatographed separately. The peak area for each epimer was measured in triplicate using a planimeter. The peak having the larger area was *R*-DHD3 [28]. A ratio of the average area for the two peaks in each chromatogram was used to determine the ratio of *R*-DHD3 to *S*-DHD3.

The molar absorptivities of derivatized *R*-DHD3 and derivatized *S*-DHD3 were determined on a Cary 16 spectrophotometer (Cary Instruments, Monrovia, CA, U.S.A.) at concentrations of $5.58 \cdot 10^{-6}$ *M* and $5.01 \cdot 10^{-6}$ *M*, respectively. The derivatized epimers were purified as described in the previous subsection and weighed samples were dissolved in chloroform.

Application to human urine sample

The assay was used to determine digoxin and metabolites in urine from a 68-year-old female patient taking one 0.125 mg digoxin tablet daily. A column

4.6 mm × 25 cm packed with 5 μm LiChrosorb Si 60 (E. Merck, Darmstadt, F.R.G.) was used for improved efficiency together with a Model 110A pump (Altex, Berkeley, CA, U.S.A.). Other HPLC components were the same as described previously.

RESULTS

The dinitrobenzoate derivatives of DT0, D0, D1, D2 and D3 each yielded a single, symmetrical, well-separated peak upon HPLC (Fig. 1A), whereas derivatized DHD3 yielded two symmetrical, well-separated peaks (Fig. 1B). Other research has shown that these two components of DHD3 are epimers having either the *R* (major) or *S* (minor) configuration at the C-20 position in the lactone moiety [28, 29]. Data on the capacity factor for each peak (DT0 = 4.6, D0 = 7.2, D1 = 9.2, D2 = 12.6, D3 = 16.9, *S*-DHD3 = 16.7, *R*-DHD3 = 21.0, using the 3:1:1 hexane—methylene chloride—acetonitrile mobile phase) together with the data in Fig. 1 indicate that the only incompletely resolved components are the derivatives of D3 and *S*-DHD3.

Reaction of DT0, D0, D1, D2, D3 and DHD3 with an excess of DNBCl in the presence of pyridine was carried out over times ranging from 3 to 60 min

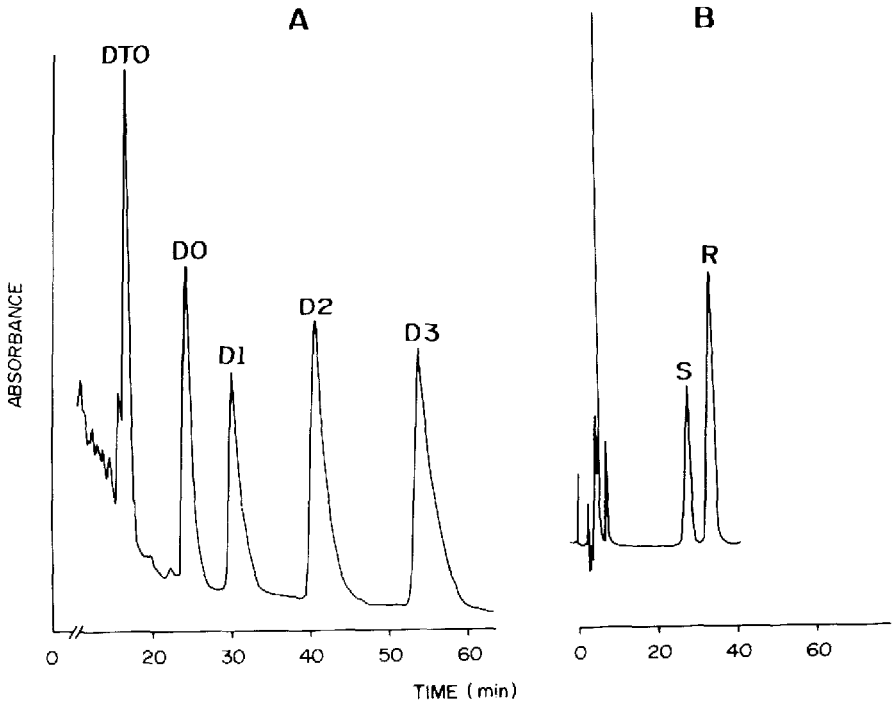

Fig. 1. (A) Representative chromatogram of 3,5-dinitrobenzoate derivatives of the internal standard digitoxigenin (DT0), digoxigenin (D0), digoxigenin monodigitoxoside (D1), digoxigenin bis-digitoxoside (D2), and digoxin (D3). Samples (1 μg/ml of each glycoside) were extracted from urine, derivatized and chromatographed using a mobile phase of hexane—methylene chloride—acetonitrile (3:1:1). (B) Representative chromatogram of 3,5-dinitrobenzoate derivative of dihydrodigoxin (DHD3). The two peaks represent the *S* (minor) and *R* (major) epimers [28, 29]. Samples were derivatized and chromatographed using a mobile phase of hexane—methylene chloride—acetonitrile (8:3:3).

and was found to be complete within 5 min, as evidenced by constancy of peak heights subsequent to this time. Comparison of the integrated NMR for aromatic protons with that for C-18 and C-19 methyl protons yielded the following approximate number of esters per molecule of digitalis compound: D0, 2.1; D1, 3.2; D2, 3.9; D3, 5.1; *S*-DHD3, 5.4; *R*-DHD3, 5.3. Thus, these data indicate that all hydroxyl moieties except one are esterified on each molecule. This is consistent with the findings of Maerten and Haberland [30] who reported five-fold acetylation of digoxin with the tertiary C-14 hydroxyl remaining unesterified. Additional evidence for the five-fold extent of derivatization of the DHD3 epimers was obtained by comparing measured molar absorptivities (254 nm) of 51,560 mol^{-1} cm^{-1} for the derivatized *S* epimer and 51,030 mol^{-1} cm^{-1} for the derivatized *R* epimer with the molar absorptivity of about 10,000 mol^{-1} cm^{-1} at 254 nm for each 3,5-dinitrobenzoate group [31]. Derivatized DT0, D0, D1, D2, and D3 were found to be stable in chloroform for at least three days, as indicated by the constancy of HPLC peak area with respect to time reported for each compound in Table I.

TABLE I

STABILITY OF THE 3,5-DINITROBENZOATE DERIVATIVES OF DT0, D0, D1, D2, AND D3

Time (min)	Peak area as a percentage of the zero time value				
	DT0	D0	D1	D2	D3
0	100	100	100	100	100
60	91	96	96.5	99.4	97.8
120	99.1	101	101	104	102
240	95.5	98.8	99	104	103
510	111	106	112	109	106
770	105	105	112	104	103
1400	100	93.3	108	107	104
2790	103	98.4	109	106	104
4260	103	99.6	104	106	105

TABLE II

EFFICIENCY OF EXTRACTION OF DIGOXIN AND THREE METABOLITES FROM URINE

Values are the mean of 5 determinations, except for digoxin which is 3. Standard deviation in parentheses. The extraction procedure is described in the text.

Concentration (μg/ml)	Extraction efficiency (%)			
	D0	D1	D2	D3
2.5	51.7 (4.4)	38.9 (3.2)	63.3 (6.1)	71.0 (7.8)
1.0	44.5 (5.3)	40.0 (4.2)	65.0 (5.4)	70.9 (6.4)
0.50	46.4 (8.9)	36.6 (3.2)	60.0 (7.4)	71.0 (11.8)
0.25	47.7 (8.0)	33.5 (9.3)	64.2 (5.8)	75.5 (6.0)
0.10	39.5 (9.8)	31.0 (6.6)	53.5 (13.3)	77.8 (43.4)
Mean	46.0 (7.9)	36.0 (6.2)	61.2 (8.4)	73.2 (17.2)

TABLE III

EFFICIENCY OF EXTRACTION OF THE EPIMERS OF DIHYDRODIGOXIN FROM URINE

Values are the mean of 3 determinations; standard deviation in parentheses. The extraction procedure is described in the text.

S-DHD3		*R*-DHD3	
Concentration (μg/ml)	Extraction efficiency (%)	Concentration (μg/ml)	Extraction efficiency (%)
0.625	70.8 (8.8)	1.88	72.4 (4.2)
0.500	70.3 (5.8)	1.50	81.0 (13.3)
0.313	69.8 (6.6)	0.938	75.2 (17.5)
0.250	70.4 (4.1)	0.750	74.7 (4.5)
0.125	78.1 (11.4)	0.375	81.3 (9.1)
		0.188	71.3 (14.5)
Mean	71.9 (7.3)		76.0 (10.3)

Standard curves of peak height ratio (compound/internal standard) vs. concentration (μg/ml) for D3 and the various metabolites were linear and the *y*-intercept was not significantly different from zero ($P > 0.05$) except for D1. This was consistent with observations from analyses of drug-free urine samples in which the only appreciable chromatographic interference was at the retention time of D1. Slopes and intercepts, respectively, were as follows: D3, 0.560, −0.014; D2, 0.603, 0.002; D1, 0.447, 0.043; D0, 0.657, 0.069; *R*-DHD3, 0.615, −0.015; *S*-DHD3, 0.746, 0.019. Correlation coefficients were > 0.995 except for *R*-DHD3 which was 0.986. The extraction efficiencies for digoxin and metabolites are listed in Tables II and III. There does not appear to be any concentration dependence of the single methylene chloride extraction in the concentration ranges investigated. The extraction efficiencies for the DHD3 epimers and for D2 were comparable to that for D3 whereas the efficiencies of D1 and D0 were only about one-half that of D3. Reproducibility of the method at the low extreme of the assayable concentration range was excellent with a coefficient of variation of 5% or less for D3, D2, D1, D0 and *S*-DHD3 (Table IV). Accuracy for determination of known urinary standards was excellent (< 5% mean deviation from nominal value) in concentrations near 1 μg/ml for all six compounds and in concentrations near 0.1 μg/ml for *S*-DHD3 and *R*-DHD3 (Table IV). The mean deviation for D3 at 0.1 μg/ml was 17% (Table IV).

Since the molar absorptivities for the two derivatized epimers of DHD3 were essentially the same, the relative areas under the chromatographic peaks for the two epimers can be used to determine the epimeric composition of the commercially supplied DHD3, assuming that any on-column loss is the same for both epimers. The ratio found was 3.0 to 1 (*R*/*S*).

Currently this method is being utilized to detect digoxin and dihydrodigoxin in urine from patients. Results from a patient taking oral digoxin are shown in Fig. 2. Chromatographic evidence for digoxin and the *R* epimer of dihydrodigoxin was obtained.

TABLE IV

ACCURACY AND REPRODUCIBILITY OF ASSAY FOR DIGOXIN METABOLITES IN URINE

Compound	Concentration of prepared standards (μg/ml)	Measured concentration (mean of 5 or 6 determinations)	C.V.* (%)
D3	1.0	0.954	1.2
	0.1	0.117	1.7
D2	1.0	1.021	2.4
	0.1	0.124	3.5
D1	1.0	0.985	3.8
	0.1	0.188	4.9
D0	1.0	0.981	2.9
	0.1	0.156	3.1
S-DHD3	0.625	0.620	2.6
	0.0625	0.0606	4.2
R-DHD3	1.875	1.833	4.6
	0.375	0.382	8.5

*Coefficient of variation.

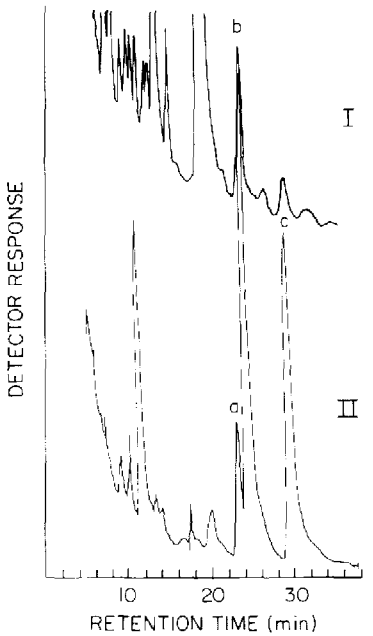

Fig. 2. Chromatogram (HPLC) of derivatized standards (II) and of an extracted and derivatized urine sample (I) from a patient receiving digoxin. Peaks identified as a, b, and c correspond to derivatized *S*-dihydrodigoxin, derivatized digoxin and derivatized *R*-dihydrodigoxin, respectively. For the standards, 10 μg of digoxin and of the dihydrodigoxin epimeric mixture were added to 10 ml of blank urine and assayed (II). The mobile phase consisted of hexane—methylene chloride—acetonitrile (3:1:1).

DISCUSSION

The importance of this analytical procedure lies more in its application to metabolic studies and to future analytical method development for digitalis compounds than in its application to routine determinations of urinary digoxin and metabolites. The procedure described has sufficient sensitivity to quantitate digoxin and the major metabolite, DHD3, in urine. Results of others [26] indicate there would be insufficient sensitivity with this procedure for determining urinary concentrations of the very minor metabolites D2, D1 and D0. More importantly, however, this is the first analytical procedure that has reported separation of the individual epimers of dihydrodigoxin. Use of a column with improved efficiency in studies in patients has also permitted separation of D3 and the minor epimer of DHD3 (Fig. 2), which was not possible under the conditions used for analytical development. Thus, this methodology provides the first opportunity to separate, isolate and identify the individual epimers of DHD3 and to determine which is/are formed in animals and man [28, 29].

The second important aspect of this method is that it points out a potential direction for development of more sensitive methods that would still retain specificity for the individual epimers of DHD3 as well as the other metabolites of digoxin. Two techniques that potentially have sufficient sensitivity for serum concentrations (subnanogram/ml range) are acylation with either fluorescent moieties or with radiolabeled derivatizing agents. If the excellent specificity shown in this investigation with the dinitrobenzoyl derivative (Fig. 1A and B) can also be achieved with derivatives having enhanced detectability, then this direction of research may yield a specific method for serum digoxin and its metabolites. The rapidity and completeness of dinitrobenzoyl derivatization, stability of the derivatives (Table I), linearity of standard curves, and reproducibility of the method (Table IV) all indicate that this type of derivatization is a promising avenue for further research.

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