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QUANTITATION OF DIGITOXIN AND THE BIS- AND MONODIGITOXOSIDES OF DIGITOXIGENIN IN SERUM

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SUMMARY

A specific assay is described for measuring the concentration of digitoxin and the bis- and monoglycosides of digitoxigenin in serum. The procedure includes: (1) addition of a tracer amount of tritium labeled parent compound to the serum in order to measure percentage recovery; (2) solvent extraction to separate polar and non-polar metabolites; (3) reversed-phase thin-layer chromatography of the non-polar fraction to separate digoxigenins from digitoxigenins; (4) thin-layer chromatography to isolate digitoxin, and the bis- and monoglycosides of digitoxigenin; and (5) use of an ^{125}I-radioimmunoassay to determine the concentration of the glycosides. Each of these three glycosides was administered intravenously to a normal subject, and the concentration of parent compound was measured in the serum at various times.

INTRODUCTION

In order to study the pharmacokinetics of digitoxin it is necessary to have a method which is sensitive enough for its quantitation at the concentrations found in serum after a single dose and specific enough to separate it from its many metabolites. Several methods have been described for the measurement of digitoxin in serum and urine. Assays based on Na^+, K^+-ATPase inhibition [1], red blood cell ^{86}Rb uptake [2], and competitive protein binding [3] have, in general, been replaced by radioimmunoassays (RIA) [4]. Although these methods are sufficiently sensitive to measure concentrations of digitoxin in biological fluids, they do not distinguish digitoxin from its metabolites.

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Therefore, a variety of chromatographic methods have been devised. These include a double isotope dilution derivative assay [5], gas—liquid chromatography (GLC) [6—8], high-performance liquid chromatography (HPLC) [9], column chromatography [10], and thin-layer chromatography (TLC) [11,12]. The double isotope dilution derivative assay and HPLC methods are generally not sensitive enough to directly measure the digitalis glycosides in serum, but HPLC has been combined with RIA for the quantitation of digoxin and its metabolites [13]. GLC requires three additional chromatographic steps and the preparation of derivatives and therefore is not suitable for the assay of large numbers of samples. Radiolabeled digitoxin has also been administered to man, and column or TLC techniques used to separate the parent drug from its metabolites [14,15]. However, the use of labeled material in human subjects is undesirable.

Pharmacokinetic studies which use non-specific methods to measure digitoxin are difficult to interpret due to the presence of metabolites in the serum. In some conditions, such as renal failure, patients have an increased proportion of metabolites, especially water-soluble metabolites [16,17]. Therefore, the pharmacokinetic parameters for digitoxin may be in error, and the percentage of digitoxin excreted may be overestimated when a non-specific assay is employed. Conflicting data obtained in azotemic subjects using non-specific assays for digitoxin have been summarized by Vöhringer and Rietbrock [18].

The method described here is sufficiently sensitive and specific to obtain pharmacokinetic information following the administration of a single dose of non-radioactive digitoxin or its metabolites, the bis- and monoglycosides of digitoxigenin. The method involves solvent extraction followed by TLC separation of the non-polar metabolites. The concentration of the parent compound is then measured using ^{125}I-RIA. The efficiency of the procedure is determined for each sample by addition of a tracer amount of the tritiated parent compound prior to extraction.

EXPERIMENTAL

Materials

The following unlabeled compounds were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.: digoxin (DG), digoxigenin bisdigitoxoside (bis-DG), digoxigenin monodigitoxoside (mono-DG), digoxigenin (genin-DG), digitoxin (DT), digitoxigenin bisdigitoxoside (bis-DT), digitoxigenin monodigitoxoside (mono-DT), digitoxigenin (genin-DT), and dihydrodigitoxin (DTH). [^{3}H]Digitoxin (13.8 Ci/mmol) and [^{3}H]digitoxigenin monodigitoxoside (34.8 Ci/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. [^{3}H]Digitoxigenin bisdigitoxoside (12.6 Ci/mmol) was obtained from Burroughs Wellcome, Research Triangle Park, NC, U.S.A. (through the courtesy of Dr. Ronald Cresswell). Each compound was purified by TLC prior to use. TLC plates (Si 250 TLC precoated, 250 μm) were obtained from J.T. Baker, Phillipsburg, NJ, U.S.A., and reversed-phase plates (KC_{18} precoated, 200 μm) from Whatman, Clifton, NJ, U.S.A.

Extraction and chromatography of serum samples

A flow diagram of the analytical procedures is presented in Fig. 1. After collection, blood samples were centrifuged and the serum was stored frozen in glass vials at −20°C until assayed. A 2-ml aliquot of each serum sample was pipetted into a screw top centrifuge tube and ^{3}H-labeled parent compound (approximately 2×10^5 dpm) in 0.1 ml of phosphate-buffered saline [0.15 *M* sodium chloride, 0.01 *M* dipotassium hydrogen phosphate, 5% bovine serum albumin (Miles Laboratories, Kankakee, IL, U.S.A.) pH 7.4] was added. An aliquot of ^{3}H-labeled parent compound was also added to each of five scintillation vials containing 10 ml of scintillation fluid {5 g of 2,5-diphenyloxazole (PPO), 200 mg of 1,4-bis[2(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP), 100 g of naphthalene and dioxane to 1 l}. The serum was then extracted three times with 5-ml aliquots of dichloromethane (CH_2Cl_2). Each time, samples were mixed for 10 min on a Labquake® and then centrifuged to separate the water-soluble and dichloromethane-soluble fractions. The dichloromethane fractions were transferred with a Pasteur pipet to a centrifuge tube and the dichloromethane evaporated with nitrogen. The sides of each centrifuge tube were washed three times with 2 ml of dichloromethane and the solvent evaporated each time to concentrate the glycosides in the tip. The concentrate was dissolved in approximately 0.7 ml of chloroform–methanol (3:1) and spotted 2.5 cm from the bottom edge of a reversed-phase TLC plate. The

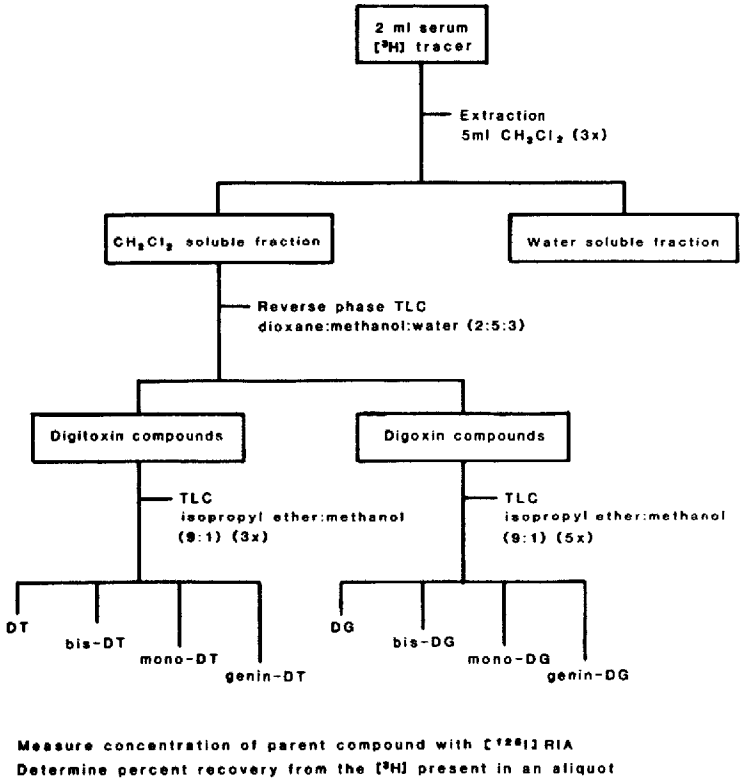

Fig. 1. Analytical procedures.

plates had been scored in 2.2 cm wide lanes separated by 0.7 cm lanes. Standard solutions of digoxin, digitoxin, and several of their metabolites (approximately 5 μg each) were spotted in either outside lane of the plates. The plates were then placed in a chromatography tank equilibrated with dioxane—methanol—distilled water (2:5:3) and developed until the solvent front had moved to a height of 19 cm. After drying, the lanes of the plate containing the standards were cut off with a glass cutter and sprayed with a freshly mixed chloramine-T—trichloroacetic acid reagent [3% chloramine-T (Eastman Organic Chemicals, Rochester, NY, U.S.A.)—25% ethanolic solution of trichloroacetic acid (1:4)] and heated in an oven for 8 min at 110° C. The digitalis compounds were visualized and marked under long-wavelength ultraviolet light. The area in each lane corresponding to the digitoxin compounds was identified based on the standards, and was scraped into one centrifuge tube. The silica gel was pulverized and the glycosides were eluted three times from the silica using 4 ml of ethanol. The pooled eluants were evaporated and the sides of the tubes washed down to concentrate them in the tip. This initial chromatographic step was necessary to separate digoxin and digoxin metabolites (which have been reported to be metabolites of digitoxin) from digitoxin and metabolites retaining the digitoxin steroid nucleus.

The eluate corresponding to the digitoxin compounds area was transferred as described previously to silica gel TLC plates which had been dried overnight at 40° C. The plates were developed three times to the top of the plate with isopropyl ether—methanol (9:1) in a pre-equilibrated tank. They were dried between runs and standards were visualized as previously described. The area of each lane corresponding to the parent glycoside was scraped from the plate and eluted from the silica with three washings of 4 ml of ethanol. After evaporating the ethanol with nitrogen, the glycoside was dissolved in phosphate-buffered saline. A 0.1-ml aliquot was pipetted in duplicate from the test tube into a counting vial containing 10 ml of scintillation fluid and counted to 2% error. Quenching was corrected for using an external standard. The concentration of each compound was then determined with a digitoxin RIA. All serum samples were also assayed directly using the RIA.

The percentage recovery was calculated for each chromatographed sample by dividing dpm of ^{3}H recovered after chromatography by the total dpm added to each serum sample prior to extraction. Since ^{3}H tracer contributed to the total radioimmunoassayable parent compound after chromatography, the amount of digitalis glycoside present as ^{3}H-labeled compound was calculated from its specific activity and the dpm present and subtracted from the total assayable compound. RIA was used to calculate specific activity for each ^{3}H-labeled compound after each was chromatographed to greater than 97% purity. The concentration of non-radioactive material was then corrected for percentage recovery.

Digitoxin radioimmunoassay

The concentration of digitoxin and the bis- and monodigitoxosides of digitoxigenin was measured using ^{125}I-labeled digitoxin tracer (3-O-succinyl-digitoxigenin L-tyrosine) and antiserum from Becton-Dickinson, Orangeburg, NY, U.S.A. The assay procedure used was a modification of the one provided

with the Becton-Dickinson RIA kit. Digitoxin standards in the range of 3—60 pmol/ml were prepared in phosphate-buffered saline and run with each assay. Samples with concentrations above this range were diluted with phosphate-buffered saline. Duplicate 0.025-ml volumes of standard or sample were mixed with 1.0 ml of ^{125}I-labeled digitoxin tracer and then 0.1 ml of antibody was added. Each 10-ml vial of antibody was diluted with 3.5 ml of phosphate-buffered saline prior to use. After a 30-min incubation, 0.5 ml of a charcoal suspension [0.15 *M* sodium chloride, 3.6 m*M* sodium barbital, 3.6 m*M* sodium acetate, 0.03% Dextran T-70, and 1.25% Norit-A charcoal (J.T. Baker, Phillipsburg, NJ, U.S.A.)] was added. Following centrifugation at 1000 *g*, 1 ml of supernatant was pipetted into a tube for gamma counting. Samples were counted to a maximum error of 2%. Counts per min were plotted as a function of log of digitoxin concentration, and the concentration of digitoxin, and the bis- and monodigitoxosides of digitoxigenin was calculated from this standard curve. This was possible since the bis- and monodigitoxosides of digitoxigenin demonstrated an affinity for the digitoxin antibody indistinguishable from that of digitoxin.

Standard solutions of bis-DT, mono-DT, genin-DT, DTH, and DG were assayed in order to determine their binding affinities for the digitoxin antibody relative to that of digitoxin. Standard curves were prepared for each and compared to the digitoxin standard curve. The relative binding affinities were calculated from the concentration of each compound compared to digitoxin required to displace 50% of the labeled digitoxin bound in the absence of any unlabeled compound.

It was important that the composition of the sample and standard tubes be as similar as possible. Therefore, all serum samples from a subject following each drug administration were assayed at the same time. Since 0.025 ml of serum sample was required for each assay tube for the serum samples which were assayed directly, a 0.025-ml aliquot of serum from the same subject drawn prior to drug administration was added to each standard tube. If dilution of serum samples was necessary it was done with the same blank serum. The samples assayed after extraction and chromatography were reconstituted in the same phosphate-buffered saline used to prepare the standards, and, therefore, no adjustments were necessary.

Twenty standard samples of digitoxin at concentrations of 0, 3.3, 6.7, 13.1, 23.4 and 39.4 pmol/ml were prepared and assayed by the RIA to permit determination of the accuracy, precision, and sensitivity of the assay.

Human studies

The general utility and specificity of the method is illustrated by the following experiment. A normal subject, aged 23 years, was given a single intravenous dose of digitoxin after giving informed consent. After one month he was given the bis-DT and one month later the mono-DT. The dosing sequence was 1 mg of digitoxin (Crystodigin, Eli Lilly, Indianapolis, IN, U.S.A.), then 0.83 mg of bis-DT, and then 0.66 mg of mono-DT (i.e. 1308 nmol of each). Each intravenous dose was diluted to approximately 15 ml with sterile saline and injected intravenously over a 10-min period. Blood samples were drawn prior to injection and at approximately 5, 15, 30, 45 min and 1, 2, 4, 6, 8, 12 h and 2,

4, 6, 8, 12, 16, 24 and 28 days after injection. Exact sampling times were recorded.

RESULTS

While the initial reversed-phase chromatographic step separated the digitoxin from the digoxin classes of compounds, a second chromatographic step was necessary to separate individual compounds within the digitoxin class of compounds. The R_F values for both TLC systems for digoxin, digitoxin and the primary non-polar metabolites are given in Table I.

The mean (± S.D.) percentage recovery after extraction and chromatography was 58.3 ± 12.1% for digitoxin, 52.9 ± 10.8% for bis-DT, and 52.3 ± 3.8% for mono-DT. Approximately 5% of the ^{3}H-tracer remained in the serum after extraction. Therefore, losses occurred primarily during the TLC steps.

The digitoxin RIA standard curve was linear over the range 3—50 pmol/ml. All standard curves were prepared using digitoxin standards since there was little difference in the affinity (on a molar basis) of digitoxin, bis-DT, and mono-DT, for the digitoxin antibody. The relative binding affinities were 1.0, 0.94, and 0.96, respectively. Dihydrodigitoxin and digoxin were significantly less reactive with the digitoxin antibody than digitoxin, with relative binding affinities of 17.5 and 46.9, respectively.

The accuracy and precision of the ^{125}I-RIA were determined at five digitoxin concentrations (Table II). All but one of the mean values were within 2 pmol/ml of the calculated concentrations. The minimum detectable concentration was 1.6 pmol/ml.

The serum concentration versus time profiles for digitoxin, bis-DT and mono-DT following the administration of each separately as intravenous doses are presented in Fig. 2. The data obtained from the direct assay of the serum, and the data resulting from the extraction and chromatography are

TABLE I

TLC R_F VALUES FOR DIGITOXIN AND ITS METABOLITES

Compound	R_F × 100		
	TLC 1*	TLC 2**	
		3×***	5×***
Digitoxin	24	24	—
Bis-DT	30	41	—
Mono-DT	36	58	—
Genin-DT	43	79	—
Digoxin	54	—	14
Bis-DG	61	—	23
Mono-DG	69	—	40
Genin-DG	74	—	52

*TLC 1: reversed-phase system, dioxane—methanol—water (2:5:3).
**TLC 2: silica gel TLC, isopropyl ether—methanol (9:1).
***Plates were developed three or five times.

TABLE II

ACCURACY AND PRECISION OF [^{125}I] RIA FOR DIGITOXIN

Concentration (pmol/ml)		Mean deviation from theoretical value
Theoretical	RIA*	
3.34	3.77 ± 0.09 (6.4)	+ 0.4
6.67	7.99 ± 0.24 (5.2)	+ 1.3
13.07	12.43 ± 0.85 (6.8)	− 0.6
26.36	31.48 ± 2.22 (7.1)	+ 5.1
39.44	40.40 ± 1.95 (4.8)	+ 1.0

*Mean of 20 samples ± S.D. (coefficient of variation).

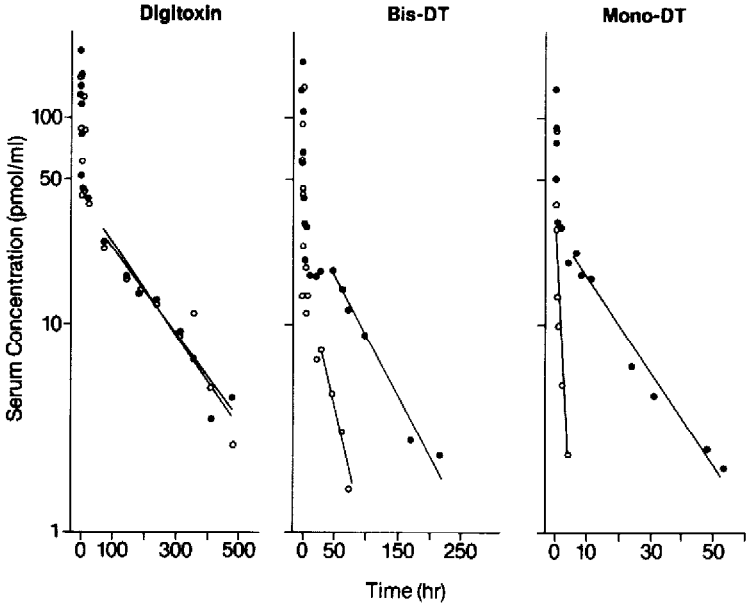

Fig. 2. Digitoxin, bis-DT and mono-DT serum concentrations as a function of time following the intravenous administration of equimolar doses of each. Concentrations measured directly in serum using RIA (•) and concentrations measured using a specific assay (○) are presented.

both given to illustrate the differences between the specific and the non-specific assays. As can be seen from the figure there is little difference between the two assays when digitoxin was measured in the serum of the one subject studied. However, with bis-DT and mono-DT there appear to be significant concentrations of metabolite(s) which also react with the antibody. This would result in a considerable overestimate of the concentration of the parent compound if only total serum was assayed.

DISCUSSION

The method described was specifically designed to measure the pharmacokinetics of digitoxin and two of its metabolites, bis-DT and mono-DT, since there is interest in these latter two compounds as potential therapeutic agents. However, the method could also be applied to digitoxigenin as the digitoxin antibody readily reacts with this compound. The relative binding affinity for digitoxigenin was 1.16. This method in conjunction with a digoxin RIA could also be applied to digoxin, bis-DG, mono-DG and genin-DG. Since digitoxin is not a metabolite of digoxin the first chromatographic step would not be required. Five rather than three developments would be necessary to achieve adequate separation in the second chromatographic step (Table I). It would also be necessary to use a [^{3}H] digoxin tracer with high specific activity in order to minimize its contribution to the digoxin RIA.

The above assay was specific and sufficiently sensitive to allow measurement of non-radioactive parent drug for several half-lives. The use of a tritium tracer as an internal standard to permit corrections for recoveries to be made was essential due to the relatively low and quite variable recovery. This method also separates and allows identification of metabolites of the parent compound.

Digoxin and/or its metabolites have been reported to account for 7.5% of the daily excreted radioactivity following digitoxin administration, but to comprise only 2% of the metabolites in the serum of subjects on a maintenance dose of digitoxin [19,20]. Increased hydroxylation of digitoxin has been reported in patients with impaired renal function [17] but in another study this increase was not found [16]. If assay of the serum samples with a digoxin RIA indicates that digoxin and/or its metabolites are not present to a significant degree, the first chromatographic step may be omitted. The fact that there is minimal cross-reactivity between digoxin and the digitoxin antibody also increases one's confidence in omitting the first chromatographic step.

Dihydrodigitoxin has not been found to be a significant metabolite of digitoxin in man, but increased serum concentrations have been reported in azotemic patients [21]. Most TLC solvent systems, including this one, are unable to separate the dihydro compounds from their precursors. Since the Becton-Dickinson digitoxin ^{125}I-RIA antibody does not significantly cross-react with the dihydro compounds, it was assumed that concentrations of the dihydro compounds did not become sufficiently high so as to interfere. An RIA using an antibody which is specific for dihydrodigoxin but does not cross-react with digoxin has been described [22] and a similar assay may be developed to quantitate the dihydrodigitoxin compounds. A gas chromatographic—mass spectroscopic technique has also been reported for measuring dihydrodigitoxin in serum samples and could be used to quantitate these compounds after chromatography [21].

The method reported here is most useful for pharmacokinetic studies where it is necessary to separate the parent digitalis compound from its metabolites in serum. When digitoxin was administered to a subject, the half-life and area under the serum concentration—time curve calculated from data based on the specific assay were not very significantly different from those determined

by RIA of the total serum (7.3 vs. 4.9 days and 8.4 vs. 8.0 nmol h/ml). However, when bis-DT or mono-DT were administered their half-lives were much shorter and area considerably smaller when calculated from data based on the specific assay (23 vs. 56 h and 667 vs. 2417 pmol h/ml for bis-DT, and 1.6 vs. 18 h and 57 vs. 611 pmol h/mol for mono-DT). This suggests that there were significant levels of metabolite(s) that cross-react with the digitoxin RIA antibody. The water-soluble metabolites which were measured by RIA in the aqueous phase after the dichloromethane extraction were found to be present to a greater extent when bis-DT and mono-DT were given than with digitoxin. It appears that the fewer digitoxose sugars on the glycoside the shorter its half-life, and the more extensive the metabolism, especially the formation of water-soluble metabolites. These water-soluble metabolites are less active [23] and are present in the serum of subjects given mono-DT for many hours after mono-DT is no longer measurable.

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