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IDENTIFICATION OF SUBMICROGRAM AMOUNTS OF DIGOXIN, DIGITOXIN, AND THEIR METABOLIC PRODUCTS

ISOLATION BY CHROMATOGRAPHY AND PREPARATION OF DERIVATIVES FOR ASSAY BY ELECTRON CAPTURE DETECTOR

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SUMMARY

Digoxin, digitoxin and their principal metabolites were separated by paper chromatography and then by thin-layer chromatography. Appropriate spots were identified by their R_F values in comparison with known markers and then further characterized by gas–liquid chromatography after making volatile derivatives. The chromatographic procedures were demonstrated to have value for quantitative recovery and also for identification when combined with a gas chromatographic method reported earlier in this journal.

INTRODUCTION

Cardiac glycosides and their metabolites are present at low concentrations in biological fluids when these drugs are used in therapeutic doses. Previous attempts to study the metabolism of digoxin and digitoxin have usually required drugs labeled with radioactive isotopes[1–4]. Because of the undesirability of the use of labeled material in human subjects, it is appropriate to develop a chemical method to study the metabolism of these drugs at the subnanogram level.

Recently we described a chemical method for the determination of digoxin in plasma using a gas–liquid chromatograph equipped with electron capture[5]. This method involved the conversion of digoxin by heptafluorobutyric anhydride to form the highly fluorinated digoxigenin diheptafluorobutyrate (HFB). In the gas chromatograph an amount as low as 25 pg could be detected easily. The present paper describes the extension of the reaction with heptafluorobutyric anhydride to bis- and monodigoxigenin digitoxosides as well as digitoxin and its corresponding metabolic products. Since the reaction of heptafluorobutyric anhydride with digoxin and its metabolites* yielded digitoxigenin HFB, it was necessary to separate these glycosides prior to derivative formation. Therefore, in the first part of this report, we present an isolation and separation technique for digoxin, digitoxin and their metabolites. The separation technique was then applied to the analysis and purification of radiochemical digoxin and digitoxin from a commercial supplier.

* In this context we include digoxin, its bis- and monoglycosides and its aglycone.

MATERIALS AND METHODS

Thin-layer and paper chromatography

Solvents were obtained from either J. T. Baker or Mallinckrodt Chemical Co. Thin-layer plates were Mallinckrodt Chromar 7GF. Whatman filter paper Nos. 1 and 3 were used for paper chromatography. [^{3}H]Digoxin-12α (specific activity 5.26 mCi/mg) was obtained from New England Nuclear. [^{3}H]Digitoxin, also obtained from New England Nuclear, had a specific activity of 7.51 mCi/mg. Digoxin was generously supplied by Burroughs-Wellcome & Co., Inc. (U.S.A.). The digitoxin was obtained from Mann Research Laboratories, New York. The bis- and monoglycosides and the aglycones of digoxin and digitoxin were obtained from Boehringer Mannheim Corporation, New York.

Scintillation counting. All radioactivity measurements were made by liquid scintillation spectrometry. The scintillation fluid consisted of a mixture of 6 g 2,5-diphenyloxazole (PPO) and 0.5 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl POPOP) per liter toluene.

Isolation. The extraction of cardiac glycosides, digitoxin and digoxin, was carried out from an aqueous medium. Tissue to be analyzed was first homogenized in 4 vol. of water. For feces, the sample was lyophilized and the residue suspended in water. All samples were then treated in the same manner throughout the procedure.

The aqueous sample was extracted two times with 3 vol. of methylene chloride. If non-radioactive material was analyzed, [^{3}H]digoxin or [^{3}H]digitoxin was added so that quantitation of total recovery could be made. The per cent recovery of the metabolites was assumed to be the same as that of the parent drug. To remove materials in urine which interfere in the thin-layer chromatogram, the methylene chloride extract was washed twice with 1 *N* NaOH and then twice with water. The volume of methylene chloride was then reduced by evaporation under nitrogen at 50°. The residue was suspended in 30 ml or less of methylene chloride–methanol (99:1) and passed over a short (5 cm) silica gel column. The glycosides and their metabolites were then quantitatively eluted with acetone (about 7 ml), and the acetone evaporated under nitrogen.

The separation of digitoxin and its metabolites from digoxin and its metabolites was accomplished by a descending paper chromatographic system similar to that described by LAGE AND SPRATT[6]. The solvent for the paper chromatographic step was prepared by shaking formamide and chloroform (2:3) in a separatory funnel. This was allowed to stand overnight. The upper phase (formamide) was used to saturate the atmosphere by means of a paper lining (Whatman No. 1); the lower phase (chloroform) was the mobile phase. The chromatographic paper used for analysis, Whatman No. 3 (4 × 22 in.), was soaked 10–20 min in formamide–acetone (1:3) followed by 10–20 min air drying. The sample was then applied by means of 5 × 25 μl rinses with chloroform–methanol (1:1). The paper was placed in the chromatography jar and allowed to equilibrate for 1 h. Chloroform, saturated with formamide, was then added and the solvent front allowed to progress to 1–3 in. from the bottom of the paper. The paper was dried for 24 h at 70°.

When radioactive material was used, the radioactive areas along the paper were located by use of a Vanguard 800 autoscanner. When the compounds were unlabeled, they were located by reference to authentic compounds. For development of

reference compounds *m*-dinitrobenzene was used as described by WHITE AND OETH[7]. The areas corresponding to the reference compounds were then cut out, the compound eluted with methanol, and the solvent evaporated under nitrogen.

The eluted material was then transferred to thin-layer plates by 3 × 25 µl rinses of chloroform–methanol (1:1). Only one solvent system was used for the separation of digitoxin, bis- and monoglycosides of digitoxigenin, and digoxigenin. The plates were developed five times with isopropyl ether–methanol (9:1), a solvent system used by GRADE AND FORSTER[8]. To effect a complete separation of digoxin, bis- and monoglycosides of digoxigenin and digitoxigenin, it was necessary to utilize two solvent systems. First the plate was developed four times with diisopropyl ether–methanol (9:1). Then a single development with methyl ethyl ketone–chloroform (3:1) was used.

The areas corresponding to reference standards for digoxin, digitoxin and their metabolites were then removed by use of vacuum and a Pasteur pipette packed with glass wool. The individual compound was eluted with 3 ml of acetone, the acetone was evaporated to dryness, and the radioactivity was measured. Determination of radioactivity in the final sample served as a measure of the amount present.

Derivative formation and analysis by gas–liquid chromatography

Chemicals. Mallinckrodt nanograde (Mallinckrodt Chemical Works, St. Louis, Mo.) solvents were used throughout this study. Heptafluorobutyric anhydride was obtained from Peninsula Chemical Co., Gainesville, Fla.

Gas chromatography. The gas chromatograph was a Tracor MT220 (Tracor Inc., Austin, Texas) equipped with a ^{63}Ni (14.5 mCi) electron capture detector. The column was a 4 ft. × 2 mm I.D. glass U-tube. Packing was 3% OV-1 on Gas-Chrom Q (Applied Science, Inglewood, Calif.). Helium, at a flow rate of 50 ml/min, was the carrier gas. The column was maintained at 240°, and the detector at 350°. Before use, the column was conditioned with lecithin as previously described[5].

Preparation of HFB derivatives by heptafluorobutyric anhydride. HFB derivatives were prepared by the reaction with heptafluorobutyric anhydride. One microgram of each glycoside was reacted with 10 µl of heptafluorobutyric anhydride in 100 µl of benzene at 90° for 20 min. Excess reagent was removed under nitrogen and 1.0 ml of benzene added. The solution was then used directly for analysis by gas–liquid chromatography (GLC).

RESULTS AND DISCUSSION

Separation by paper and by thin-layer chromatography

The result of the paper chromatographic (PC) separation is shown in Table I. As indicated by the R_F values, the bis-metabolites of both digoxin and digitoxin do not separate from their aglycones. Because this separation is not complete, and because of the uniformly low R_F values for the digoxin class, the separation on paper is utilized to separate the digoxin and digitoxin classes. In studies on the metabolism of digoxin, this chromatographic step is not necessary.

The result of the separation by thin-layer chromatography (TLC) is shown in Table II. As indicated by the R_F values of the metabolites, the set of developments with isopropyl ether–methanol (9:1) effects a complete separation of digitoxin and

its metabolites, whereas a development in methyl ethyl ketone–chloroform (3:1) is necessary for an adequate separation of the digoxin class.

The recovery of radioactive digoxin during the procedure was studied in the following way. [^{3}H]Digoxin was added to a rat liver homogenate. The analysis, as previously described, was carried out, the [^{3}H]digoxin was isolated, and the radioactivity was measured. The overall recovery of [^{3}H]digoxin averaged 75 %.

The purity of [^{3}H]digoxin and [^{3}H]digitoxin from a commercial supplier was determined. Table III presents the results. In the case of digitoxin, contamination due to compounds that co-chromatograph with the available metabolites exceeds 50 %. The major contaminants chromatograph with digoxigenin, digitoxigenin bisglycoside, and digoxigenin monoglycoside. In the case of [^{3}H]digoxin, 94 % of the total radioactivity chromatographed with authentic digoxin. The principal contaminant co-chromatographed with the digoxigenin bisglycoside.

Analysis by gas chromatography

The cardiac glycosides, digoxin and digitoxin, and their bis- and monodigitoxosides were derivatized directly to the corresponding genin HFB using heptafluoro-

TABLE I

PC R_F VALUES OF DIGOXIN, DIGITOXIN AND THEIR METABOLITES

Paper: Whatman No. 1; solvent: chloroform saturated with formamide.

Compound	*R_F value*
Digoxin	0.50
Digoxin bisglycoside	0.41
Digoxin monoglycoside	0.35
Digoxigenin	0.41
Digitoxin	0.94
Digitoxin bisglycoside	0.86
Digitoxin monoglycoside	0.80
Digitoxigenin	0.87

TABLE II

TLC MIGRATION VALUES (cm) OF DIGOXIN, DIGITOXIN AND THEIR METABOLITES

Thin layer: silica gel; solvents: (a) isopropyl ether–methanol (9:1) (five developments), (b) methyl ethyl ketone–chloroform (3:1); the solvent front was 15 cm.

Compound	*Solvent a*					*Solvent b*
	1	*2*	*3*	*4*	*5*	
Digoxin	0.3	0.7	0.9	1.1	1.3	3.9
Digoxin bisglycoside	0.5	1.2	1.5	1.8	2.0	5.0
Digoxin monoglycoside	1.1	2.0	2.6	3.1	3.4	6.6
Digoxigenin	1.3	2.5	3.2	3.8	4.1	7.8
Digitoxin	1.2	2.3	2.6	3.2	3.6	—
Digitoxin bisglycoside	2.1	3.5	4.4	5.0	5.5	—
Digitoxin monoglycoside	3.1	5.0	6.3	7.3	8.0	—
Digitoxigenin	4.3	6.9	9.3	9.5	10.4	—

butyric anhydride: digoxin, bis- and monodigitoxoside digoxigenin giving digoxigenin HFB, and digitoxin, bis- and monodigitoxoside digitoxigenin giving digitoxigenin HFB. The formation of the genin HFB in each case was deduced from the identity of the GLC retention times of the respective derivatives. The yield of digoxigenin hepta-

TABLE III

IMPURITIES ASSOCIATED WITH COMMERCIAL [^{3}H]DIGOXIN AND [^{3}H]DIGITOXIN

Thin layer: silica gel; solvents: (a) isopropyl ether–methanol (9:1), (b) methyl ethyl ketone–chloroform (3:1); the solvent front was 15 cm.

Area isolated	[^3H]*Digitoxin* (*solvent a*)		[^3H]*Digoxin* (*solvent a*; *solvent b*)	
	d.p.m.	%	*d.p.m.*	%
Digoxin	422,980	0.4	354,000,000	94.0
Digoxin bisglycoside	250,670	0.3	12,000,000	3.2
Digoxin monoglycoside	9,630,280	9.6	5,400,000	1.4
Digoxigenin	29,700,000	29.7	3,650,000	1.0
Digitoxin	45,000,000	45.0	—	—
Digitoxin bisglycoside	14,400,000	14.4	—	—
Digitoxin monoglycoside	328,610	0.3	—	—
Digitoxigenin	76,660	0.1	—	—

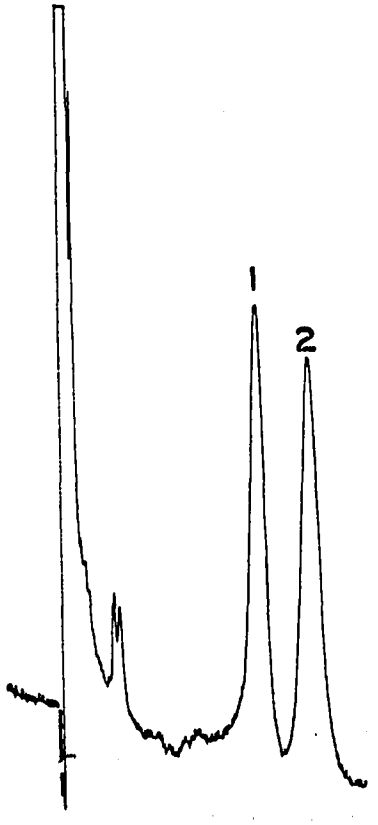

Fig. 1. Gas chromatogram obtained from digoxigenin HFB and digitoxigenin HFB. Peak 1 = 0.5 ng digoxigenin HFB; peak 2 = 1.0 ng digitoxigenin HFB. The retention time for digoxigenin HFB was 9 min, and that for digitoxigenin HFB 11.3 min.

fluorobutyrate was about 80 % and of digitoxigenin heptafluorobutyrate about 70 %. Two small peaks of shorter retention times were assumed to be the anhydro derivatives formed by the elimination of the C-14 hydroxyl group. As reported earlier[5] digitoxigenin HFB had a longer retention time on 3 % OV-1 than digoxigenin HFB. This order of appearance was unexpected in view of the differences in polarity and molecular weight. The gas–liquid chromatogram of a mixture of digoxigenin HFB and digitoxigenin HFB is shown in Fig. 1. The lower limit of detection (defined by a peak height at least five times the base line) for both digoxigenin HFB and digitoxigenin HFB was about 25 pg.

An accurate and relatively rapid method has been presented for the isolation of the metabolic products of digoxin and digitoxin. This method, utilizing one solvent extraction followed by PC and TLC, offers advantages over existing isolation and separation techniques. Many studies of the distribution and metabolism of digoxin and digitoxin have employed methods of multiple solvent extractions, followed by alumina oxide chromatography and/or PC or TLC[9–15]. The necessity of multiple solvent extractions and/or rigid combustion techniques dictates that such procedures be extremely costly in time, thereby reducing the utility of the method. The use of multiple extractions presents distinct technical problems when dealing with many samples. The use of alumina oxide chromatography, as a clean-up and separation technique for digoxin, digitoxin and their metabolites, is almost universal in the study of the metabolism of cardiac glycosides. Alumina oxide chromatography contributes to molecular rearrangements of a considerable number of steroids[16]. For this reason, alumina oxide chromatography was avoided.

There are several PC methods for cardenolides in the literature[17–20]. These were found to be unacceptable for quantitative separation. Several TLC techniques were also tried[8,21,22]. These proved to be qualitatively but not quantitatively useful.

Studies on the metabolism in man of the cardiac glycosides are currently in progress using the various chromatographic steps as described in this paper.

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