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ASSAY OF DIGOXIN IN PLASMA BY GAS CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of digoxin in human plasma by gas chromatography with electron capture detection. The procedure involves addition of a labeled internal standard, [^{3}H]digoxin, extraction with methylene chloride and preliminary purification on a florisil column followed by thin-layer chromatography on silica gel. After formation of the heptafluorobutyrate derivative, the extract is rechromatographed on silica gel followed by gas–liquid chromatography. The method has been applied to the determination of known amounts of digoxin ranging from 5–100 ng per 10 ml plasma.

INTRODUCTION

A need has long existed for a reliable chemical method for the quantitation of digitalis cardenolides on a nanogram scale. Most of the published methods employ a colorimetric reaction[1–3] after thin-layer chromatography (TLC) or paper chromatography (PC). However, none of these reactions can be regarded as specific. More important, their use requires the presence of the cardenolides on a scale which exceeds levels normally found in plasma. Because of its high sensitivity and reproducibility, gas–liquid chromatography (GLC) has long appeared to workers as a possible solution to this problem. The first reported analysis of cardenolides by GLC was by JELLIFFE AND BLANKENHORN[4].

Subsequent papers dealt with GLC of silyl ethers of the cardenolides[5–7]. None of these papers dealt with the quantitation of digitalis glycosides with the exception of the paper by JELLIFFE[8], who first suggested the formation of halogenated derivatives and their detection by a gas chromatograph equipped with an electron capture detector. Although the successful preparation of trifluoroacetate derivatives of digitoxigenin and their separation by GLC was described, no follow-up on detection in biological systems was reported. In recent years the successful use of heptafluorobutyric anhydride as a derivatizing agent led us to reinvestigate the possibility of using this reagent for digoxin and to attempt its quantitation in amounts ordinarily present in plasma. We report here a method for the determination of digoxin in human plasma which employs gas chromatography with electron capture detection.

MATERIALS AND METHODS

Chemicals

Solvents were Mallinckrodt nanograde (Mallinckrodt Chemical Works, St. Louis, Mo.). Heptafluorobutyric anhydride (HFB) used for the formation of derivatives during the analyses was obtained from Peninsula Chemical Co., Gainesville, Fla. Heptafluorobutyric anhydride (double distilled) was obtained from Patton Chemical Co., San Jose, Calif. Heptafluorobutyrylimidazole was obtained from Pierce Chemical Co., Rockford, Ill. Digoxin was a gift from Burroughs Wellcome Laboratories, Tuckahoe, N.Y. Digoxigenin was purchased from Boehringer and Son, Mannheim, G.F.R. Digitoxigenin was purchased from Aldrich Chemical Co., Milwaukee, Wis.

Thin-layer chromatography

Plates (20 × 20 cm) coated with 0.3 mm Silica Gel 254 + 366 (without binder) (Brinkmann Institute, Westbury, N.Y.) were used for the first TLC step and were washed by allowing methanol to ascend to the upper edges. Precoated plates (Silicar 7GF, Mallinckrodt, St. Louis, Mo.) were used for the second TLC step and were purified by chromatography for 1 day with methanol and for 2 additional days with benzene. At the end of each day the solvent was changed. Fresh solvents were used for each assay. Samples were removed from the first TLC step by scraping, and transferred by suction into disposable Pasteur pipets, the narrow ends of which were packed with a small plug of silanized glass wool. In the second TLC step the silica gel was scraped onto weighing paper and transferred to a silanized glass tube. The nitrogen used to evaporate solvents was purified by passage through a filter containing molecular sieve 5A and Drierite.

Column chromatography

Florisil (80/100 mesh) was purchased from Applied Science Laboratories, Inc., Inglewood, Calif. Disposable 6-in. Pasteur pipets with tips packed with a plug of silanized glass wool served as columns. Florisil was dry packed. Each column was packed to a height of about 5 cm. The column was tapped gently to pack down the florisil. A small plug of silanized glass wool was inserted at the top of the column. The column was fitted into a vacuum bell jar through a rubber plug at the top. The side arm was connected to a water aspirator. The solvent flow rate through the column was about 3–5 ml/min. Prior to use, the column was washed with 10 ml of methanol–methylene chloride (1:100). During the wash time the solvent flow rate could be checked and adjusted if necessary. The wash solvents were collected in a 100-ml beaker placed directly under the column. Elution was carried out with 6 ml acetone which was collected in a cut-off 8 ml centrifuge tube held in position in a scintillator vial. The flow rate of 3–5 ml/min allowed the various steps including elution to be performed in a very short time (less than 10 min). Our experience has been that the flow rate is not critical. Solvents were applied using a Pasteur pipet fitted with a rubber bulb.

Silica gel (100/120 mesh) could be substituted for florisil. The same washing and elution steps were applicable.

Digoxigenin heptafluorobutyrate

Digoxigenin (50 mg) was reacted with a mixture of 500 μl heptafluorobutyric anhydride (double distilled) and 2 ml benzene in a stoppered glass tube for 45 min at 80°. The excess reagents were then evaporated under nitrogen at 80° and the derivative crystallized from 70% aqueous methanol.

During the analysis, digoxigenin HFB was prepared by the direct action of heptafluorobutyric anhydride on digoxin. To form the HFB derivatives, the samples were transferred to silanized glass stoppered conical tubes (3 ml). Benzene (100 μl) and heptafluorobutyric anhydride (6 μl, from Peninsula Chemical Co.) were added for each plasma sample. The tubes were tightly stoppered and their tips placed to a depth of 2 cm into the holes of a heating block maintained at 90°. After 20 min excess reagent was evaporated under a stream of filtered nitrogen.

The derivative was characterized by mass spectrometer as the 3,12-diheptafluorobutyrate of digoxigenin (courtesy of Dr. WILLIAM HALPERN, Dept. of Genetics, Stanford University). The instrument used was a Finnigan 1015 Quadrapole mass spectrometer. Direct sample insertion was used.

Digitoxigenin heptafluorobutyrate

Digoxigenin (5 mg) was reacted with a mixture of 100 μl heptafluorobutyrylimidazole and 200 μl benzene in a stoppered conical tube at 90° for 1 h. The reaction was terminated by the addition of 1 ml water. Digitoxigenin HFB was extracted with 10 ml benzene. The solution was diluted to contain 0.72 ng/μl digitoxigenin HFB in benzene. No attempt was made to purify this derivative by crystallization. Its structure was not elucidated. The derivative was assumed to be the monoheptafluorobutyryl ester at C_3.

Both digoxigenin HFB and digitoxigenin HFB were stored in the cold when not being used. During a 2-month period no decomposition of these derivatives was observed.

[^{3}H]Digoxin

[12α-^{3}H]Digoxin was obtained from New England Nuclear, Boston, Mass. Radiopurity was checked by chromatographing aliquots of the diluted [^{3}H]digoxin (0.1 ng/μl) on silica gel in the system ethyl acetate–water–methanol (90:5:5) and determining the percentage of radioactivity in the digoxin area. Digoxin was chromatographed as reference in a separate lane and located by UV absorption at 2540 Å. Radioactive contaminants measured by this technique varied between 4 and 8% of the total radioactivity. When the level of contaminants was greater than 8%, a fresh dilution of [^{3}H]digoxin in benzene was prepared.

Tritium was counted in a Packard Tri-Carb liquid scintillation counter. To prevent absorption of trace amounts of [^{3}H]digoxin and the derivative to the walls of the counting vials, 1% ethanol was added to the scintillator solution. The scintillator fluid was made up by using 4 g Omnifluor (Packard Instrument Co.) per l of toluene. No correction was made for quenching. 10 ml of scintillator fluid was used. Counting time generally was 10 min. Counting efficiency for tritium was determined to be 28%.

Gas–liquid chromatography

The gas–liquid chromatograph was a Tracor MT 220Q with a ^{63}Ni (14.5 mCi)

electron capture detector. Silanized glass columns 4 ft. × 3.5 mm I.D. were packed with 3% OV-1 on Gas-Chrom Q (Applied Science Laboratories, Inc.) and operated at 240°. The carrier gas was 10% methane in argon at 100 ml/min. The detector was maintained at 350° and every 300 μsec a 55-V pulse of 10 μsec duration was applied. No purge gas was used. Under these conditions the minimum detectable level of digoxigenin HFB (a signal of greater than three times the background) was approximately 0.050 ng. The attenuation settings used for analysis were 1 × 256 and 1 × 128. Injections were made using a 10-μl Hamilton syringe (Hamilton Co., Whittier, Calif.). The detector response was displayed on a 1 mV chart recorder. Quantitation was made using peak heights.

After about a week, the columns showed some evidence of absorption of the digoxigenin HFB derivative, resulting in a loss of linearity for subnanogram amounts of the derivative. Absorption of the derivative was compounded by the combination of the lactone ring and the underivatized hydroxyl group at C_{14}. The injection of microgram amounts of lecithin completely eliminated this problem. The lecithin was used as a solution in toluene at a concentration of about 1 μg/μl. Each morning two 3 μl injections of the lecithin solution conditioned the column to such an extent that an improvement of about 4× increase in peak height was obtained over untreated columns. The idea of using lecithin to condition columns in this manner was developed by WILLIAM LEACH, Syntex Corp., Palo Alto, Calif.

Assay procedure

As shown in Fig. 1, there are essentially seven steps for the assay: extraction, CC and TLC separation of digoxin from interfering materials, derivative formation, TLC separation of products from the reaction mixture, GLC, scintillation counting and quantitation. To correct for losses encountered in isolation of digoxin from plasma 7000 c.p.m. of [^{3}H]digoxin (1.1 ng) was added to the plasma prior to extraction and an equal amount was taken for liquid scintillation counting. 10 ml plasma was extracted once with 70 ml methylene chloride by shaking for 30 sec in a 250-ml separatory funnel. The methylene chloride was run into a 250-ml round bottom flask and evaporated to a volume of a few ml using a rotary type evaporator in a water bath at 50°. The extract was then transferred with three 5 ml aliquots of methanol–methylene chloride (1:100) to a florisil column which had just been washed with 10 ml methylene chloride. The column was then washed with 10 ml ethyl acetate. When the ethyl acetate had drained, digoxin was eluted with 6 ml acetone into a cut-off conical glass tube. The acetone was evaporated under nitrogen at 50°. The sides of the tube were then washed down with about 0.5 ml acetone and again the solvent evaporated. The residue was transferred using 3 × 25 μl methanol–methylene chloride (1:1) to a silica gel TLC plate. Four extracts were applied on a single 20 × 20 cm plate. TLC was then carried out in the solvent system benzene–methanol (5:1) to a height of 15 cm. Digoxin was located using the dye Orcein (Eastman Organic Chemicals, Rochester, N.Y.) as a marker. Orcein contained a number of dyes, the two most prominent ones chromatographed slightly ahead and just behind digoxin. The R_F value of digoxin in this system was 0.23. The dye spots are colored orange-red and can be located without difficulty in ordinary daylight. An area 1 × 1 cm was removed from the thin-layer plate by suction into a Pasteur pipet as described earlier, and eluted with 3 × 0.5 ml acetone into a 3-ml glass tube. The tube was centrifuged to

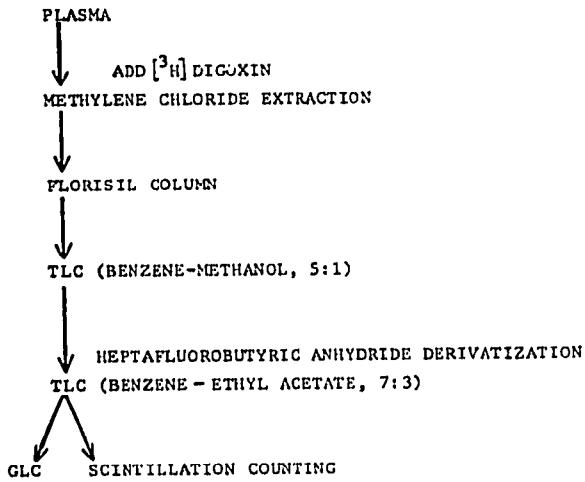

Fig. 1. Flow diagram for the isolation and derivatization of digoxin from plasma.

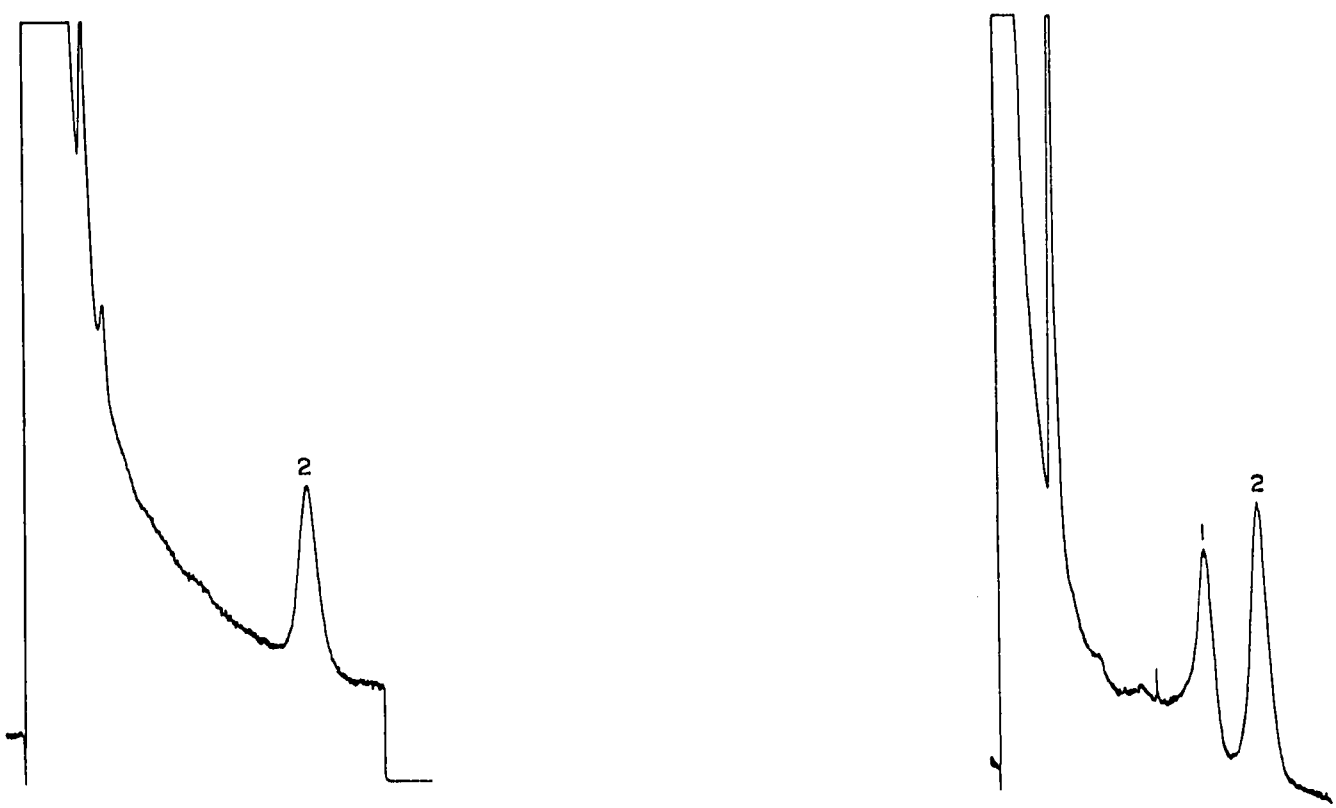

Fig. 2. The chromatogram on the left was obtained from 10 ml plasma carried through the procedure, $^1/_5$ the final sample was injected. Note the absence of any deflection at the t_r corresponding to digoxigenin HFB. The chromatogram on the right was obtained from 10 ml plasma to which 27.1 ng digoxin had been added. $^1/_{10}$ the final sample was injected. Peak 1 = 0.8 ng digoxigenin HFB. Peak 2 = 1.44 ng digitoxigenin HFB. The retention time of digoxigenin HFB was 9 min.

remove the small particles of silica gel not retained by the glass wool in the end of the pipet. The acetone was transferred to a 3-ml silanized glass tube and evaporated under nitrogen by placing the tips of the tubes into a sand bath at 50°. The HFB derivative was then formed as described and the excess reagents removed under nitrogen. The HFB derivative was then chromatographed on silica gel (5 × 20 cm) using benzene–ethyl acetate (70:30). Digoxigenin HFB was chromatographed as reference in a separate lane and was located by UV absorption at 2540 Å, and then covered with cellophane tape. To avoid contamination, the plate was channeled down the center. One plasma sample was run per plate. The solvent was allowed to run to a height of 10 cm. In this system the R_F value of the derivative was 0.35. The silica gel was scraped off onto weighing paper and transferred to a 3-ml silanized tube and 1 ml acetone added. The solution was mixed thoroughly on a Vortex shaker for about 10 sec and centrifuged 5 min at 5000 r.p.m. The acetone solution was transferred to a 3-ml silanized tube and evaporated as previously described. 12–50 μl benzene containing 0.72 ng/μl digitoxigenin HFB internal standard was added. 2 μl was then injected into the gas chromatograph. At the same time one-half the final sample was transferred into 10 ml scintillator fluid for counting.

Calculation

A standard curve was established by chromatographing varying amounts of digoxigenin HFB from 0.30 ng to 1.6 ng with 1.44 ng digitoxigenin HFB. The peak heights and weights were determined and expressed as ratios. The peak height ratios were plotted on the ordinate *vs.* the weight ratios on the abscissa. From the chromatogram of a given plasma sample the heights of digoxigenin HFB and the internal standard were calculated and this ratio obtained. By referring this ratio to the standard curve the amount of digoxin present in the aliquot was obtained.

$$\text{ng digoxin (10 ml plasma)} = \frac{\text{c.p.m. [}^3\text{H]digoxin added}}{\text{c.p.m. }^3\text{H in final sample}} \times$$

$$\times \text{ ng digoxigenin HFB in final sample} - \text{ng [}^3\text{H]digoxin added}$$

Note: No correction was necessary from digoxigenin diheptafluorobutyrate to digoxin since the molecular weights are nearly the same (digoxin = 780, digoxigenin diheptafluorobutyrate = 782).

RESULTS

Digitoxigenin HFB was found to be an excellent internal standard for digoxigenin HFB from two viewpoints. Firstly it has the same chemical structure as digoxigenin with the exception of the C_{12} hydroxyl group, and secondly its retention time is longer than digoxigenin HFB and thus avoids the occasional contamination of the internal standard by interfering materials in the final extract.

The precision and accuracy of the method was checked by carrying out replicate determinations on known amounts of digoxin (27.1 ng). In addition, amounts of digoxin considered to be within the clinically-observed range (5–100 ng/10 ml of plasma) were added to plasma and the recovery determined. These results are summarized in Table I. In each instance the final result was corrected in terms of the

TABLE I

RECOVERY OF KNOWN AMOUNTS OF DIGOXIN ADDED TO 10 ML PLASMA

The amount added includes 1.1 ng [^{3}H]digoxin.

ng added	*ng found*
5.7	6.7
10.3	10.7
11.5	12.7
14.9	13.6
24.1	25.4
27.1	21.8[a]
27.1	27.7[a]
27.1	26.4[a]
27.1	28.0[a]
27.1	30.5[a]
27.1	27.9
47.7	43.2
73.3	69.7

[a] Mean value 27.1; standard deviation ± 3.0.

labeled internal standard ([^{3}H]digoxin) and a second internal standard (digitoxigenin HFB) added prior to gas chromatography.

The overall recovery as determined by tritium counting averaged 25%. Use of the internal standard [^{3}H]digoxin allowed the minimum number of steps (*i.e.*, extraction from plasma, elution from silica gel, and transferences) since the losses incurred would be accounted for in the radioactive counting procedure. This approach reduces the time of the analysis by a matter of hours.

Formation of the derivative digoxigenin HFB, from digoxin by the direct action of heptafluorobutyric anhydride, was found to be 80%. This was determined by GLC after reacting 20 ng digoxin with heptafluorobutyric anhydride (see MATERIALS AND METHODS). After removing the excess reagents under nitrogen, 20 μl digitoxigenin HFB (0.72 ng/μl) in benzene was added. 2 μl were then injected into the gas chromatograph and the weight calculated from the standard curve.

The minimum detectable quantity of pure standard digoxigenin HFB was about 0.050 ng. The minimum detectable concentration from plasma extract was about 0.100 ng. Since one-fifth the final sample could be injected into the gas chromatograph and the recovery for digoxin averaged 25%, $0.1 \times 5 \times 4 = 2$ ng sample represented the minimum detectable quantity.

DISCUSSION

The development of the electron capture detector has provided a means of measuring extremely small amounts of substances capable of interacting with thermal electrons. Since most steroids have little affinity for electrons they must first be converted to appropriate derivatives in order to be measured by electron capture. Digitalis glycosides share this property of steroids. Derivatives currently being used to confer electron-capturing properties in steroids include chloroacetates[9], heptafluorobutyrates[10], and pentafluorophenylhydrazones[11]. Of these derivatives heptafluorobutyric anhydride has been the most widely used. In the present method,

heptafluorobutyric anhydride was the derivative of choice. Other derivatives investigated included trifluoroacetic anhydride, monochlorodifluoroacetic anhydride, perfluorooctanoic anhydride, pentafluorobenzyl chloride, and hexadecafluoroanoyl chloride. Trifluoroacetic anhydride gave a single derivative with a sensitivity about one-fifth that of the heptafluorobutyric anhydride. Perfluorooctanoic anhydride gave three derivatives, presumably caused by splitting off of the OH at C_{14}, giving α and β anhydroderivatives. The remaining reagents yielded no derivatives or derivatives with such poor responses as to be of no further interest.

The direct derivatization of digoxin using heptafluorobutyric anhydride resulted in a considerable simplification of the overall method as well as a saving of time. Initially, digoxigenin HFB was prepared from digoxin by hydrolyzing digoxin to digoxigenin which was then reacted with heptafluorobutyric anhydride to give the derivative. The procedure involved two separate steps, was time consuming and the yield of digoxigenin HFB from digoxin was about 60%.

A single step conversion was then speculated as arising from the combination of heptafluorobutyric anhydride and a relatively weak organic acid—the acid splitting the glycoside bond to liberate the genin which would then react with heptafluorobutyric anhydride to give the desired digoxigenin HFB. The experimental approach followed was to react 20 ng digoxin with heptafluorobutyric anhydride and to monitor the digoxigenin HFB peak with successive increments in acid concentration. The first set of reactions involving heptafluorobutyric anhydride quantitately cleaved the glycoside to give digoxigenin HFB. The reaction was then repeated using double distilled heptafluorobutyric anhydride to give the same percent conversion. The addition of 3% and 6% pentafluoropropanoic acid to heptafluorobutyric anhydride reduced the digoxigenin HFB peak considerably.

The direct derivatization of digoxin with heptafluorobutyric anhydride has great potential to the study of metabolic products of digoxin and digitoxin. At the moment we are investigating the derivatization of the known metabolics of digoxin and digitoxin using heptafluorobutyric anhydride. With the enhanced sensitivity of heptafluorobutyrate derivatives it should be possible to detect and characterize extremely small amounts of hitherto suspected metabolic products. Digitoxigenin heptafluorobutyrate was prepared with heptafluorobutyryl imidazole. This reagent was introduced by HORNING *et al.*[13], and has been used primarily for derivatizing amino groups. However, its use has also been extended to alcohols[14]. The byproduct, imidazole, is a weak base which confers basicity on the system as opposed to the anhydride which releases heptafluorobutyric acid. Such a reagent has distinct advantages when dealing with groups capable of being split off under acid conditions. Since all the glycosides and their aglycones possess such a group at the C_{14} position (the tertiary OH), the ability of this reagent to derivatize these compounds intact is significant. This is particularly important in dealing with unknown metabolites. We have derivatized both digoxigenin and digitoxigenin with substituted imidazole. We therefore propose the use of this reagent or the trifluoro analogue for the identification and derivatization of the aglycone metabolites of cardiac glycosides by GLC. The lower molecular weight obtained using trifluoroacetyl imidazole may be advantageous in identifying the molecular ion through mass spectroscopy.

Unexpectedly, digitoxigenin HFB was found to have a longer retention time than digoxigenin HFB on the OV-1 column. This longer retention time of digitoxigenin

heptafluorobutyrate on OV-1 was unexpected in view of the difference in molecular weight and polarity. The unpredictable migration of cardenolides in GLC has been previously noted[7]. The use of digitoxigenin heptafluorobutyrate as an internal standard improves greatly the precision of the method. In recent years the use of an internal standard in GLC with electron capture detectors has decreased. Note has been made of the inherent dangers in the use of an internal standard[12]. However, our experience has shown that the inclusion of an internal standard is an absolute necessity, particularly in dealing with the analyses of materials with short retention times (less than 10 min).

The method is reasonably simple and should present no problems, especially to workers experienced with GLC. At the moment batch procedures which will allow multiple analyses to be carried out are being investigated. The difficulties in establishing the method were largely overcome with the realization that the derivative could be quantitatively formed in a single step, and by the use of extremely clean TLC plates prior to GLC. The low level of digoxin detectable made it necessary to segregate the work area and the materials used from possible sources of contamination. The time for a single assay is less than 5 h. We plan to reduce this time by the use of liquid–liquid chromatography to replace the TLC steps.

Current methods for analyzing plasma digoxin include inhibition of Rb transport by the red blood cell[15], radioimmunoassay[16], and enzymatic isotope displacement[17]. The present method provides a viable alternative to the existing bioassay techniques for the quantitation of digoxin. The development of such a GC method for the determination of digoxin has obvious potential applicability to metabolic studies.

We are setting up a program to monitor plasma and urine levels of digoxin in patients to try to establish dosage regimens that will maintain desired chronotropic effects in atrial fibrillation and positive inotropic effects in cases of heart failure uncomplicated by arrhythmias.

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