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STUDIES ON DIGITALIS

IV. A METHOD FOR THIN-LAYER CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF DIGITOXIN AND CARDIOACTIVE METABOLITES IN HUMAN BLOOD AND URINE

LIV STORSTEIN
Medical Department B, University Clinic, Rikshospitalet, Oslo (Norway)
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SUMMARY

A thin-layer chromatographic method for the separation of digitoxin and its cardioactive metabolites in one system is described. Pre-coated silica gel plates impregnated with 15% formamide solution in acetone were developed twice in the same direction (running distance 18 cm) with ethyl methyl ketone–xylene (50:50) as solvent. The system showed no border-zone effects, and the reproducibility was good. Samples (5 ml) of serum or urine were extracted with dichloromethane, the extracts were evaporated, the residues were dissolved in 70% ethanol, the ethanol solutions were washed twice with light petroleum and then evaporated, and the residues were dissolved in chloroform–methanol for application to the thin-layer plates. After development, the metabolites were scraped from the plates and analyzed by means of a modified rubidium-86 method. The recovery for the whole procedure was 59%, and the sensitivity of the method permitted the determination of down to 0.5 ng per spot. The method will facilitate the study of digitoxin metabolism in patients undergoing treatment with the drug.

INTRODUCTION

In order to study digitoxin metabolism in man, a method is required for the separation and determination of digitoxin and its cardioactive metabolites; some of the eight cardioactive substances have been separated, but not in a single system[1–10]. The aim of the work described here was to separate digitoxin and its seven cardioactive metabolites, in one thin-layer chromatography (TLC) system, by a method sufficiently sensitive to permit the determination of nanogram amounts of metabolites in samples from patients and to possess adequate accuracy.

MATERIALS AND METHODS

Thin-layer plates

A number of different types of plate were tried: silica gel according to Stahl

(Merck, Darmstadt, G.F.R.) and Kieselguhr G (Merck) spread on glass plates, pre-coated silica gel F_{254} TLC plates (Merck), pre-coated Kieselguhr F_{254} TLC plates (Merck), fast-running silica gel F_{254} (Merck) (silica gel–Kieselguhr) and Chromagram 6064 cellulose sheets (Eastman-Kodak, Rochester, N.Y., U.S.A.).

Solvent systems

The following solvent systems were tested: chloroform–pyridine; cyclohexane–acetone–acetic acid; ethyl methyl ketone–xylene; ethyl acetate–methanol–water; chloroform–methanol–water; ethyl methyl ketone–chloroform–formamide; and 1,2-dichloroethane–methanol–water[11].

Impregnation

Ascending-solvent impregnation with 10%, 15% or 20% formamide solution in acetone[12] was used for many systems. The plates were impregnated overnight in sealed glass jars, and the test substances were applied directly after removal of the plates from the jars.

Standard substances

The following standard substances were generously supplied by Boehringer (Mannheim, G.F.R.): digitoxin (DT-3), digitoxigenin bisdigitoxoside (DT-2), digitoxigenin monodigitoxoside (DT-1), digitoxigenin (DT-0), *epi*-digitoxigenin (*Epi*-DT-0), digoxin (DG-3), digoxigenin bisdigitoxoside (DG-2), digoxigenin monodigitoxoside (DG-1), digoxigenin (DG-0) and *epi*-digoxigenin (*Epi*-DG-0). The abbreviations shown in parentheses will be used in the text, DT signifying digitoxin and DG digoxin, and the arabic numbers giving the number of sugar molecules.

Detection reagent

Concentrated sulphuric acid in ethanol (1:4) was chosen for this purpose.

Extraction and purification procedure

Five millilitres of serum or urine from patients undergoing treatment with digitoxin were shaken with 15 ml of dichloromethane (Merck) for 10 min; after separation of the phases by centrifugation, 10 ml of the dichloromethane extract were evaporated to dryness at 50° on a water bath, then 3 ml of 70% ethanol were added to the residue and the solution was washed twice with 0.7-ml portions of light petroleum (b.p. 40–60°) (AnalaR; BDH, Poole, Great Britain). A portion (2.5 ml) of the ethanol extract was subsequently transferred to glass-stoppered conical tubes and evaporated on a water bath at 50°, in a stream of air. The dried extracts were dissolved in 25 μl of chloroform–methanol (50:50), and 15 μl of this solution were applied to the thin-layer plates. The standards were applied in 5-μg amounts at both edges of each plate, with 6 or 7 samples in between. The R_F values of the standards were the same whether the compounds were added to serum, urine or ethanol; a stock solution containing all the metabolites dissolved in pure ethanol was therefore used as standard.

Recovery during extraction and purification

Losses during the different steps of the procedure, and the total yield of the various cardioactive metabolites, were determined partly by addition of [^{3}H]DT-3

and partly by the modified rubidium-86 method[6]. Labelled DT-3 was used to determine the losses in the two washes with light petroleum, the tritium being counted in a Packard Tri-Carb scintillation counter. When using the rubidium-86 method for metabolites, samples were scraped from the plates, and 1 ml of physiological saline was added to each sample before extraction with 3 ml of dichloromethane; the extracts were evaporated to dryness and incubated with a red-cell suspension and a rubidium-86 solution as described in detail elsewhere[6]; DT-3 and its cardioactive metabolites inhibit the uptake of rubidium by the red cells. Standard curves for the metabolites were prepared for each run by adding known concentrations (5, 10, 20, 30, 40, 50 and 100 ng per ml) to 1-ml samples of physiological saline in duplicate.

RESULTS

Thin-layer chromatography

Table I shows some results obtained by using pre-coated silica gel F_{254} plates and three solvent systems. Pre-saturation of the tank with filter paper reduced the running distances and led to poorer discrimination between the standards than did chromatography without filter paper. Increasing the concentration of acetic acid from 1 to 10% in the cyclohexane–acetone–acetic acid system increased the running distances, whereas decreasing the amount of cyclohexane and increasing the amount of acetone led to poorer discrimination. Fig. 1 shows the best separation obtained with this system; DT-3 and DG-0 were not separated and separation of DG-3 from DG-2 was inadequate for our purpose.

Increasing the concentration of chloroform reduced R_F values when using chloroform–pyridine mixtures as solvents. Optimum separation within the DT and

TABLE I

TLC ON PRE-COATED SILICA GEL PLATES WITH THREE SOLVENT SYSTEMS

R_F values refer to standards dissolved in pure ethanol.

Solvent system and proportions	*Concentration of formamide solution in acetone used for impregnation (%)*	*R_F value*			
		DT-0	*DT-3*	*DG-0*	*DG-3*
Cyclohexane–acetone–acetic acid					
49:49:2	—	0.40*	0.23*	0.26*	0.16*
49:49:2	—	0.82	0.52	0.52	0.33
45:45:10	—	0.60*	0.45*	0.45*	0.34*
16:80:4	—	0.71	0.65	0.60	0.59
Chloroform–pyridine					
64:6	—	0.86	0.27	0.43	0.13
64:6	10	0.84	0.65	0.44	0.38
Ethyl methyl ketone–xylene–formamide					
50:50:0	10	0.55	0.28	0.27	0.12
50:50:4	10	0.47	0.20	0.20	0.09
50:50:4	15	0.52	0.29	0.21	0.10
50:50:4	20	0.54	0.33	0.19	0.09
70:30:0	10	0.72	0.59	0.50	0.36

* Development in tank lined with filter paper.

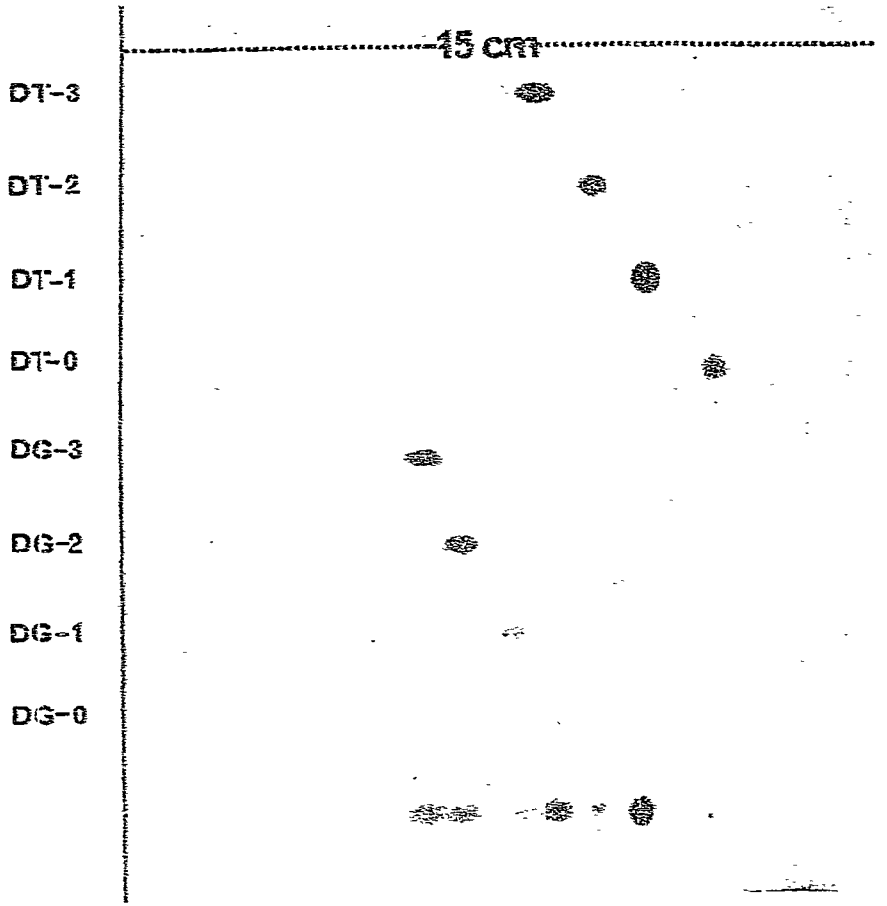

Fig. 1. TLC of DT-3 and related compounds on pre-coated silica gel plates (Merck); solvent system cyclohexane–acetone–acetic acid (49:49:2); front = 15 cm.

DG groups was achieved with the 64:6 system, but the two groups were not separated; DT-3 and DG-1, and DT-2 and DG-0, had identical R_F values. Impregnation of the plates with formamide–acetone changed the R_F values for metabolites containing sugar molecules, leading to separation between the DT and DG groups, but poor separation within the groups. The best results were obtained with formamide–acetone impregnated pre-coated silica gel plates (Merck) using ethyl methyl ketone–xylene as developing solvent. Increasing the concentration of the ketone increased the R_F values, but separation of the metabolites was poorer. Addition of formamide to the solvent slightly decreased the running distances. Impregnation of the plates with different concentrations of formamide (10, 15 and 20%) in acetone changed the separation both within and between groups. With 10% formamide solution in acetone, the DT and DG metabolites were not separated, whereas 20% formamide solution in acetone permitted separation of these metabolites. Impregnation with 15% formamide solution in acetone, and development with ethyl methyl ketone–xylene (50:50) using

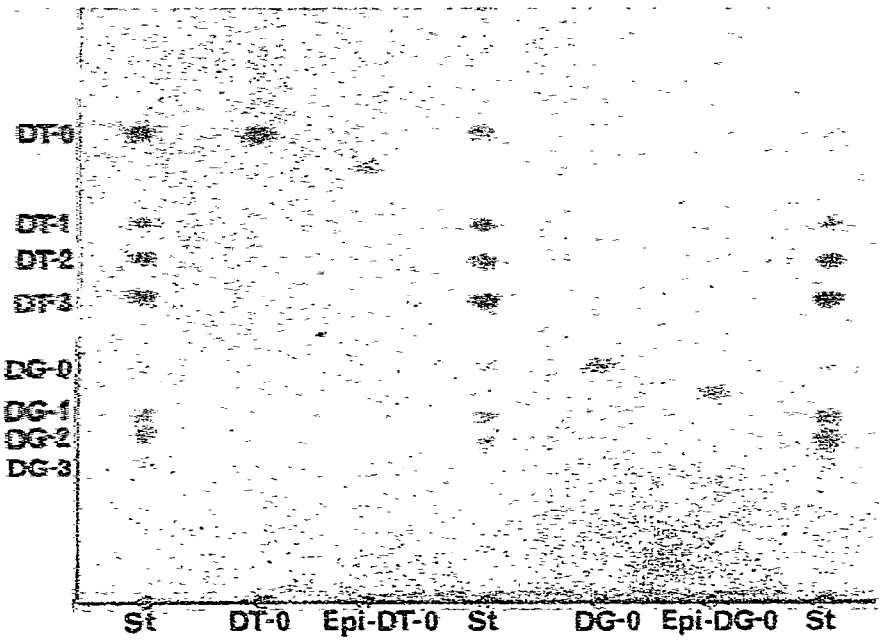

Fig. 2. TLC of DT-3 and related compounds on pre-coated silica gel plates (Merck) impregnated with 15% formamide solution in acetone; solvent system ethyl methyl ketone–xylene (50:50); front = 18 cm in each of two developments.

two runs in the same direction (running distance 18 cm) gave good separation of all eight metabolites (see Fig. 2), and these conditions were selected for further study. The plate was left at room temperature for 30 min before the second development.

Impregnation

The effect of formamide–acetone impregnation was to separate the hydroxylated metabolites (DG group) from the DT group and to give smaller and sharper spots as illustrated in Figs. 3a and 3b.

Thin-layer plates

The R_F values obtained with pre-coated plates showed better reproducibility than those obtained with laboratory-made silica gel or kieselguhr plates. On cellulose layers, the substances tailed off and were not separated. Pre-coated silica gel plates gave smaller and sharper spots than did fast-running plates (see Figs. 3b and 3c); impregnation had the same effect.

Detection reagent

Detection was satisfactory when 20% sulphuric acid solution in ethanol was used, with subsequent heating to 120°. The edges of the plates with the standards were cut off with a glass-cutter, then sprayed and heated; this was done because DT-3 and its cardioactive metabolites lose their biological activity when heated above 50°, and quantitation with the rubidium-86 method depends on this activity.

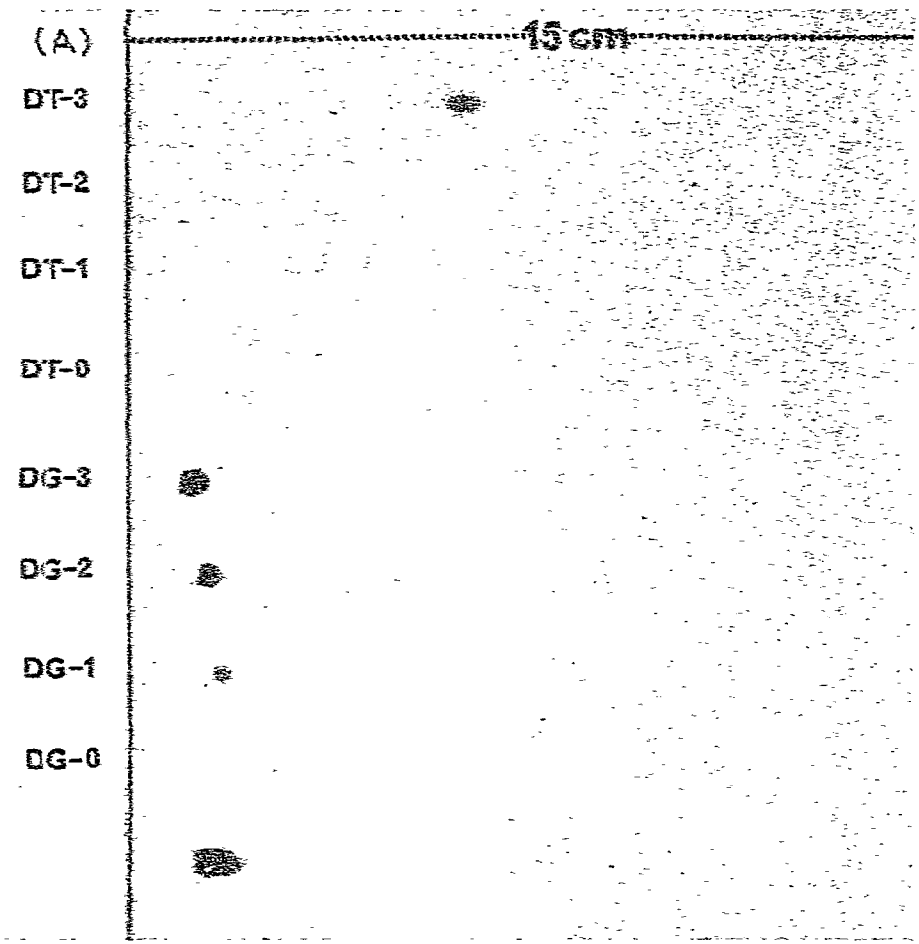

Fig. 3.

Epi-derivatives

The epimerized derivatives of DT-0 and DG-0 have R_F values between those of DT-0 and DT-1 and between those of DG-0 and DG-1, respectively (Fig. 2).

Reproducibility

The system that gave the best separation of all eight metabolites, pre-coated silica gel plates impregnated with 15% formamide solution in acetone and ethyl methyl ketone–xylene as solvent, gave good reproducibility, there being no border-zone effect (Fig. 2). The reproducibility was calculated for thirteen different plates where standards were added to serum, urine or ethanol; the following running distances were found (mean value, in cm, with SD in parentheses): DT-0, 12.29 (0.34); DT-1, 10.12 (0.46); DT-2, 9.12 (0.51); DT-3, 8.04 (0.56); DG-0, 5.81 (0.26); DG-1, 4.43 (0.18); DG-2, 3.74 (0.19); DG-3, 2.96 (0.25). When the value obtained for DT-3 was chosen as reference, the following R_x values were calculated: DT-0, 1.53 (0.08);

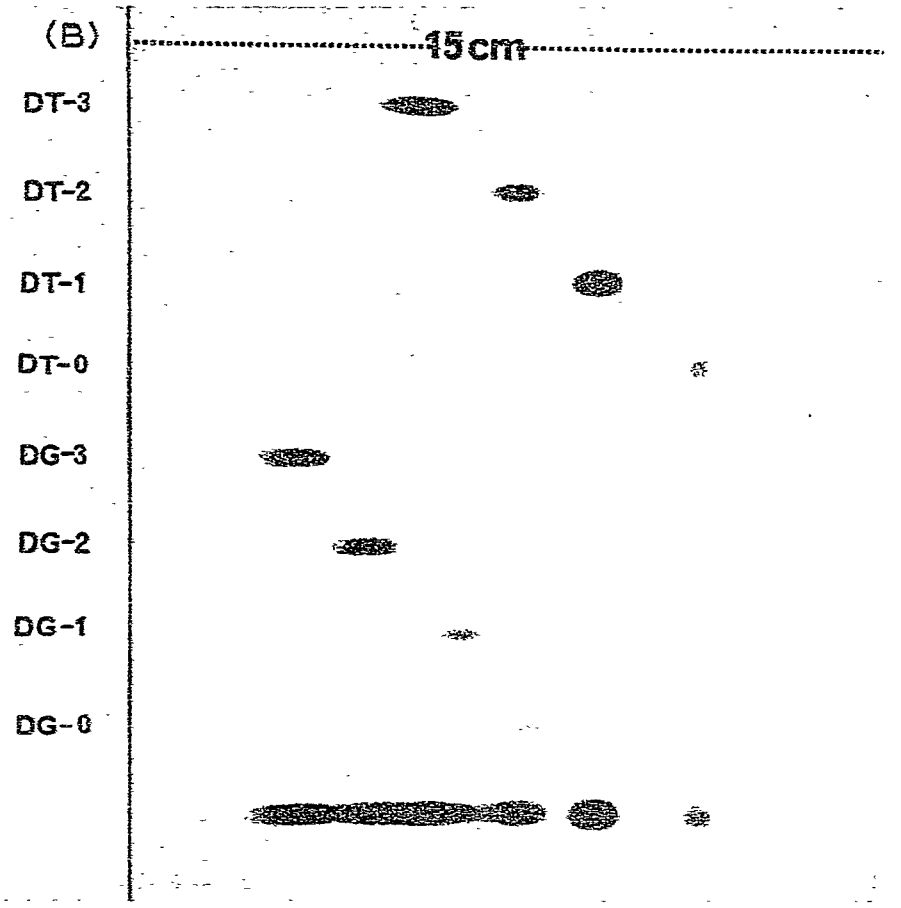

Fig. 3.

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DT-1, 1.26 (0.04); DT-2, 1.14 (0.02); DG-0, 0.73 (0.03); DG-1, 0.55 (0.02); DG-2, 0.47 (0.02); DG-3, 0.37 (0.03).

Verification of separation

Two mixtures of standards were subjected to chromatography, the first containing DT-0, DT-2, DG-0 and DG-2, and the second containing DT-1, DT-3, DG-1 and DG-3; the mixtures were applied in spots containing 25 ng of each metabolite. In order to determine the extent of tailing between the spots, adsorbent was scraped from the plate between the spots and extracted. With very compact spots, the expected value would be zero, but with less sharply defined zones one would expect to find some of the activity from the metabolites above and below the spots. The following results (ng) were obtained with the rubidium-86 method as the mean of ten parallel determinations: DT-0, 0.9; DT-1, 0.0; DT-2, 0.1; DT-3, 0.6; DG-0, 0.3; DG-1, 0.1; DG-2, 1.2; DG-3, 0.0.

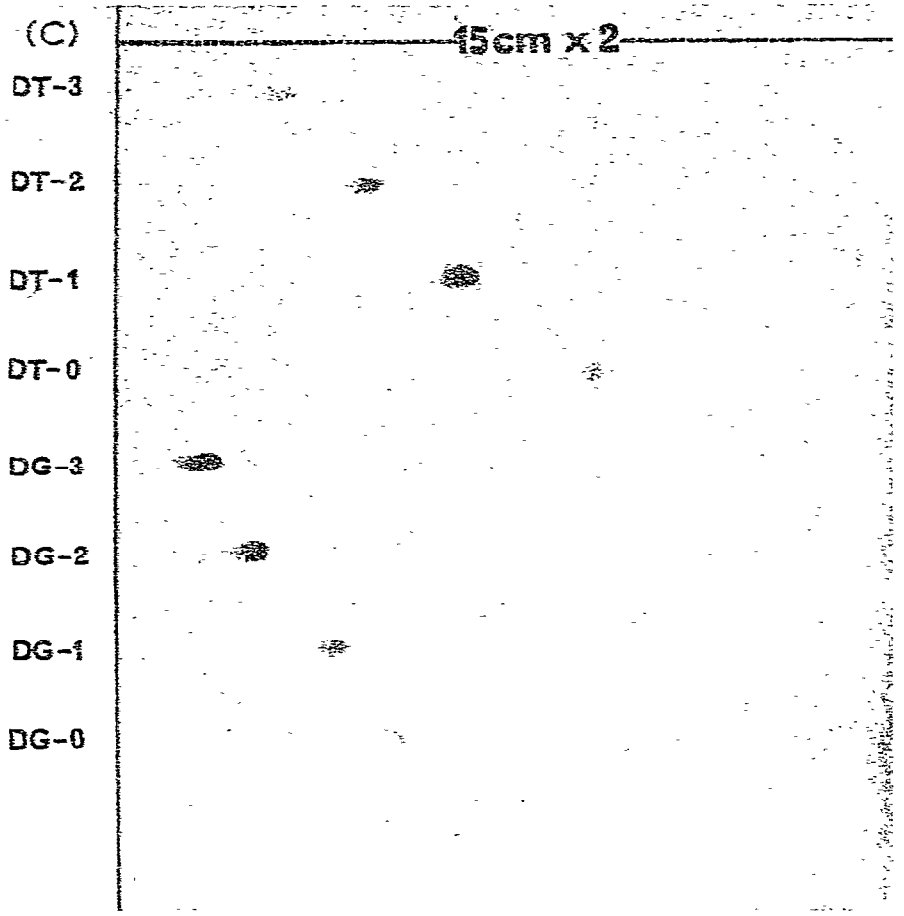

Fig. 3. TLC of DT-3 and related compounds on (a) pre-coated silica gel–Kieselguhr plates (Merck), fast-running, impregnated with 15% formamide solution in acetone; solvent system as in Fig. 2; front = 15 cm. (b) Conditions as in (a), but plate not impregnated. (c) Pre-coated silica gel plates (Merck); solvent system ethyl methyl ketone–xylene (70:30); front = 15 cm.

Recovery

The recovery during the complete extraction, purification and chromatography procedure was tested by adding known amounts of standards (50, 100, and 250 ng) to 5 ml of serum or urine and determining each metabolite by the rubidium-86 method. Recovery from urine was slightly higher than that from serum owing to the fact that extraction of DT-3 from urine with dichloromethane was complete, whereas the extent of extraction from serum was only 93.5% (ref. 14). There were no systematic differences in recovery between the various metabolites, and recovery was the same for the different concentrations studied. When 250 ng per sample was added, the mean yield for all the metabolites was 49.5 ng; complete recovery according to standardization of the method by taking aliquots from the different solvent phases should have yielded 84 ng, so that the actual recovery was 59%. Ten samples of the eight substances in a mixture were applied directly to thin-layer plates. After separation, the metabolites

were scraped from the plates and subsequently determined by the rubidium-86 method. This part of the procedure accounted for 36.5% of the reduction in recovery. The loss during the washes with light petroleum was small (0.4% of the added [^{3}H]-DT-3 during the two washes).

DISCUSSION

A variety of solvent systems for the paper chromatography and TLC of cardiac glycosides has been described in the literature[11]. Separation of DT-3 from its cardioactive metabolites in a single system has hitherto not been described, although separation of the substances in two systems presents no difficulties. Impregnated systems could be used to separate the DT from the DG group, and subsequent separation within the groups could be achieved with unimpregnated systems. In order to attain the highest possible accuracy, the search for a system that allowed separation of all eight substances was undertaken. The system described here gave very good reproducibility and no border-zone effect (this was important, because the small amounts of metabolites in samples from patients could not be located by using any detection reagent). The zones had to be scraped from the plates according to the location of standards (which were cut off in strips by a glass-cutter and heated to 120°). The extraction and purification procedure was standardized as far as possible to increase the accuracy by taking equal aliquots from various solvent phases. With the accuracy obtained (see Table II), ten parallel determinations on samples from patients should give reliable results. With DT-3 levels in serum or urine between 10 and 20 ng/ml, a 50-ml would be adequate for metabolite analysis.

TABLE II

STANDARD DEVIATION OF PARALLEL SEPARATIONS OF SERUM SAMPLES AT DIFFERENT DRUG CONCENTRATIONS

Number of samples	*Concentration range (ng/ml)*	*Mean concentration (ng/ml)*	*Standard deviation (ng/ml)*	*Coefficient of variation (%)*
9	0– 5	3.23	1.39	0.425
10	5–10	7.50	1.33	0.177
10	10–20	13.35	4.95	0.370
10	20–30	25.65	4.59	0.179
10	30–40	35.60	9.36	0.262

Since development of the proposed method, another method for the separation of DT-3, DG-3 and their active metabolites has been published. Watson and Kalman[15] combined paper-chromatographic separation of the DT and DG groups with TLC for separation within the groups (four or five developments in the same direction were necessary for satisfactory separation). Heptafluorobutyryl derivatives of the different metabolites were prepared and quantitatively determined by gas–liquid chromatography[16]. The extraction and purification procedure involved two extractions, four washes and passage over a silica gel column with subsequent elution before paper chromatography. The accuracy and total recovery of the method was not stated, but the sensitivity was very good, allowing measurement of down to 25 pg.

When comparing the methods, three factors are of prime importance: the sensitivity, the accuracy and the time needed for the whole procedure. The present method is sensitive, fairly accurate and has relatively few steps; the method of Watson and co-workers has better sensitivity, but it is more time-consuming and the accuracy was not stated.

The present work clearly demonstrates the problems involved in the separation of DT-3 from its cardioactive metabolites (see Figs. 1 and 3). It is necessary to run all the metabolites as standards to show that they are separated. In some earlier studies of DT-3 metabolism, all the standard substances have not been available to the investigator: interpretation of results may thus be very difficult.

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