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DETERMINATION OF TRITIATED DIGOXIN AND METABOLITES IN URINE BY LIQUID CHROMATOGRAPHY

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SUMMARY

A liquid chromatographic method for the determination of digoxin, digoxigenin, its mono- and bisdigitoxoside and dihydrodigoxin in urine is described. Doses of 100 μCi of [12α-^{3}H]digoxin and 0.5 mg (640 nmol) of digoxin were administered orally to eight healthy volunteers. The compounds were extracted from urine with methylene chloride containing 3% of heptafluorobutanol. After separation, fractions corresponding to digoxin and the metabolites were measured by liquid scintillation counting. Conjugates of the glycoside metabolites were determined indirectly after pre-treatment of the samples with β-glucuronidase—arylsulphatase. The detection limit was 0.1 nmol/l. Metabolites amounting to 0.5% of digoxin were assayed with a relative standard deviation of 5%.

The advantages of the method are a high recovery in the extraction step, short separation times and the possibility of separate assay of dihydrodigoxin.

INTRODUCTION

Digoxin is hydrolysed under acidic conditions to form digoxigenin and its mono- and bisdigitoxoside [1, 2]. In vivo, the corresponding dihydro compounds also appear [3—5], in addition to conjugates with glucuronic and sulphuric acids [6, 7]. The extent and clinical importance of digoxin metabolism have been discussed by several investigators [4, 5, 8—10], but our knowledge of the biotransformation remains incomplete. The methods available for the assay of digoxin and its metabolites are often tedious and time consuming. Watson et al. [11] used a gas chromatographic method with electron-capture detection. Liquid chromatographic separations using reversed-

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or normal-phase systems have been described [12—14] and applied to biological samples [9, 15—17].

In this study we aimed to combine a more efficient solvent extraction process than in previous methods and a high-performance liquid chromatographic system permitting short separation times. Moreover, the separate determination of dihydrodigoxin was desired. This method was applied to urine samples from volunteers given an oral dose of tritiated digoxin, the tritiated substance being used to obtain sufficient sensitivity.

EXPERIMENTAL

Materials

The liquid chromatograph consisted of a Model 71147 pump (LDC, Riviera Beach, FL, U.S.A.), an injection valve (Rheodyne, Berkeley, CA, U.S.A.) with a 100-μl loop, a stainless-steel separation column (150 × 4.5 mm I.D.) and a model 212 UV spectrophotometer (Cecil, Cambridge, Great Britain) with an 8-μl flow cell, operated at 220 nm. The liquid scintillation system was a Nuclear-Chicago Mark III (Searle, Des Plaines, IL, U.S.A.). Digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, digoxin and β-glucuronidase—arylsulphatase were obtained from Boehringer (Mannheim, G.F.R.). [12α-^{3}H]digoxin with a specific activity of 14 Ci/mmol was purchased from New England Nuclear Chem. (Dreieich, G.F.R.). Dihydrodigoxin and [12α-^{3}H]dihydrodigoxin were supplied by the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). 1H,1H-Heptafluorobutanol was obtained from Bristol Organics (Berkeley, Great Britain) and Insta-Gel from Packard Instruments (Downers Grove, IL, U.S.A.). Acetonitrile, *n*-heptane, methylene chloride, 1-pentanol, 2-propanol, 1-butanol and the buffer substances were of analytical-reagent grade and were obtained from E. Merck (Darmstadt, G.F.R.).

Extraction system

The distribution constants (K_D) between phosphate buffer of pH 6.5 (I = 0.1) and various organic phases were determined for digoxin and its hydrolysis products. Before and after extraction and equilibration with the organic phase the compounds were assayed in the aqueous phase by reversed-phase chromatography, UV detection and peak-height measurement. Tritiated dihydrodigoxin was used owing to its low UV absorbance. All extraction experiments were carried out in duplicate.

Chromatographic systems

The separation columns were packed with LiChrosorb SI 60 (normal phase) or LiChrosorb RP-8 (reversed-phase), 5- or 7-μm particles (Merck). In the normal-phase system the eluent was *n*-heptane—1-pentanol—acetonitrile—water (64:26:9:1) and the flow-rate was 1.5 ml/min. The reversed-phase system used a mobile phase of phosphate buffer of pH 6.3 (I = 0.1)—2-propanol (83.5:16.5) and the flow-rate was 1.0 ml/min.

Studies with volunteers

The study on eight healthy volunteers was approved by the Ethical Committee, University Hospital, Linköping. Renal and hepatic functions, as judged from routine laboratory tests, were normal. The volunteers fasted for 8 h before and 2 h after the intake of 100 μCi of [12α-^{3}H]digoxin and 0.5 mg (640 nmol) of unlabelled digoxin given as rapidly dissolving tablets specially prepared by AB Hässle. Urine was collected in polythene bottles during the following intervals after drug intake: 0—4, 4—8, 8—12, 12—24, 24—48 and 48—72 h. Aliquots were kept at −18°C until taken for assay. The gastric pH was analysed using duplicate gastric aspirates taken immediately before drug intake.

Performance of analyses

Frozen urine samples were thawed and homogenized by shaking and 20-ml volumes were extracted for 15 min with 20 ml of methylene chloride containing 3% of heptafluorobutanol. After centrifugation at 1000 *g* for 10 min, 15 ml of the aqueous phase were transferred into a clean tube and re-extracted with 15 ml of new organic phase. Volumes of 10-ml of the organic phase from each extraction were combined and evaporated under nitrogen until about 0.5 ml remained, then 20 μl of 1-pentanol were added to prevent complete dryness and evaporation was continued until 20 μl remained. The residue was dissolved in 250 μl of mobile phase and 100 μl were injected on to the normal-phase chromatographic column. Fractions of the eluate corresponding to digoxin and its hydrolysis metabolites were collected in liquid scintillation vials and 10 ml of Insta-Gel were added before counting.

In order to determine dihydrodigoxin separately from digoxin, the same procedure was performed except that only one extraction was needed and phosphate buffer instead of 1-pentanol was added to prevent complete dryness. Reversed-phase chromatography was used for the separation process.

Conjugated metabolites were determined as the difference between an untreated sample and a sample incubated with β-glucuronidase—arylsulphatase at 37°C for 20—24 h. 25 μl of the enzyme were added per millilitre of sample, the pH of which was adjusted to 5.0 by the addition of buffer solution.

RESULTS AND DISCUSSION

Extraction

Different organic solvents and solvent mixtures were tested for the extraction of digoxin and its hydrolysis metabolites. Results from some of the systems are shown in Table I. Methylene chloride containing 1-butanol or heptafluorobutanol was found to be the most efficient extractant. Heptafluorobutanol is more volatile than the corresponding non-fluorinated alcohol, which facilitates evaporation. Repeated extraction with methylene chloride containing 3% of heptafluorobutanol according to the analytical procedure gave recoveries of 90—99% for the four components.

In order to determine approximately 0.5% of metabolites, it was necessary to concentrate the sample so that the volume injected (100 μl) corresponded to about 4 ml of urine. It was important that the organic phase was not

TABLE I

DISTRIBUTION CONSTANTS (K_D) FOR DIGOXIN AND HYDROLYSIS METABOLITES EXTRACTED FROM PHOSPHATE BUFFER (pH 6.5, I = 0.1)

Substance*	Log K_D		
	Methylene chloride	Methylene chloride—heptafluorobutanol (97:3)	Methylene chloride—1-butanol (90:10)
dg-g	0.23	0.57	0.56
dg-mono	0.00	0.34	0.52
dg-bis	0.43	0.86	1.05
dg	0.90	1.40	1.56

*dg = digoxin; dg-bis = digoxigenin bisdigitoxoside; dg-mono = digoxigenin monodigitoxoside; dg-g = digoxigenin.

completely evaporated as this resulted in hydrolysis of digoxin.

Adsorption of digoxin to polythene and glass vials from aqueous solutions has been observed [18]. In this study, samples stored frozen for 6 months showed no loss of digoxin.

Chromatography

Normal- and reversed-phase chromatography were investigated. It was possible to achieve separations of digoxin and its hydrolysis products with both kinds of system. Well separated peaks were necessary in order to avoid overlapping of the radioactivity in the collection of the eluate fractions. A normal-phase system similar to that suggested by Lindner and Frei [13] was chosen for the analysis (Fig. 1), as short separation times and coinciding retentions for the dihydro compounds and the corresponding unsaturated compounds were obtained. For the separation of dihydrodigoxin from digoxin a reversed-phase system was used in which the dihydro compound was less retained than digoxin (Fig. 2). The k' and α values are given in Table II.

The order of elution of digoxin and its hydrolysis products was the same with both the normal- and the reversed-phase systems. Lindner and Frei [13] suggested that the retention mechanism was partition between polar solvent components enriched on the silica surface and the non-polar mobile phase. A reverse elution order would then be expected for hydrophobized silica with an aqueous mobile phase, but obviously other factors are involved in the retention [12, 16].

Recovery and relative standard deviation

The relative standard deviation was 2% on repeated analysis of urine samples containing 125 pmol (20 nCi) of [^{3}H] digoxin per millilitre. For the determination of hydrolysis products in urine, the relative standard deviation was 5% when measuring metabolites amounting to 0.5% of digoxin. The absolute yield from urine samples was in the range 85% (digoxigenin monodigitoxoside) to 99% (digoxin) in comparison with a standard solution injected directly on to the column.

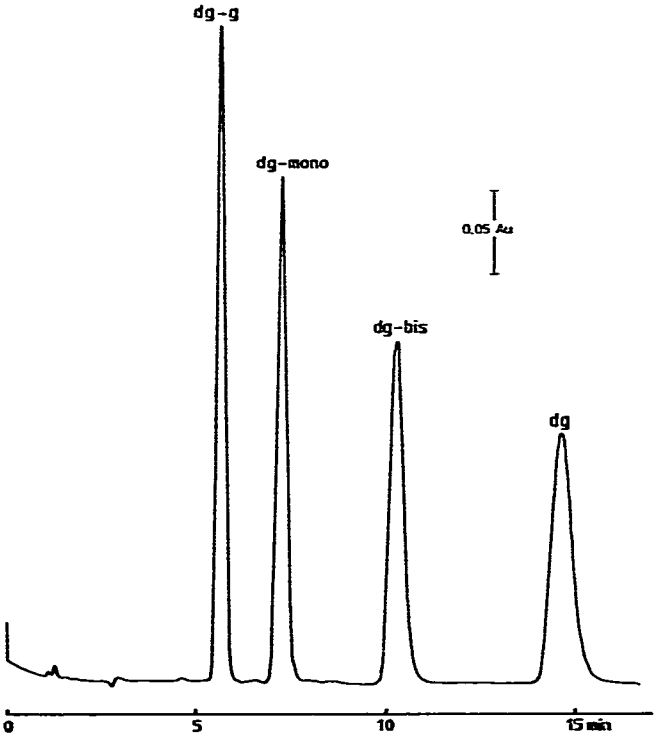

Fig. 1. Chromatogram obtained using a normal-phase system. Column packing: LiChrosorb SI 60 (5 μm). Mobile phase: *n*-heptane—1-pentanol—acetonitrile—water (64:26:9:1). Sample: 10 nmol of each substance in 100 μl of mobile phase. Detection 220 nm, a.u.f.s. 0.5. For abbreviations see Tables I and II.

In comparison, Nelson et al. [16] reported a recovery of 54—78% for digoxin, Loo et al. [15] an average of about 50% for digoxigenin and Gault et al. [17] a mean recovery of about 73%. The greater extraction with our method will increase the precision and the uniformity of recovery between samples from different subjects.

When repeated analysis of urine samples to which 0.5 pmol (2 nCi) or [^{3}H]-dihydrodigoxin per millilitre had been added was performed, the relative standard deviation was 2% and the absolute yield was 99%.

Application

The method was applied to urine samples from eight subjects who had been given an oral dose of tritiated digoxin. The amounts of digoxin and metabolites (after deconjugation) excreted in the urine during 72 h averaged 42.2% (range 29.9—51.9%) of the dose (640 nmol). Digoxin constituted 90.3% (82.0—96.4), dihydrodigoxin 1.4% (0—6.2), digoxigenin bisdigitoxoside 3.6% (1.7—6.0), digoxigenin monodigitoxoside 2.2% (0.8—4.6) and digoxigenin 2.5% (0.5—6.0). These findings are within the ranges reported in other studies [9, 19].

The variation in metabolic pattern between different individuals is illustrated in Fig. 3, where the amounts of metabolites for two of the subjects (C and G)

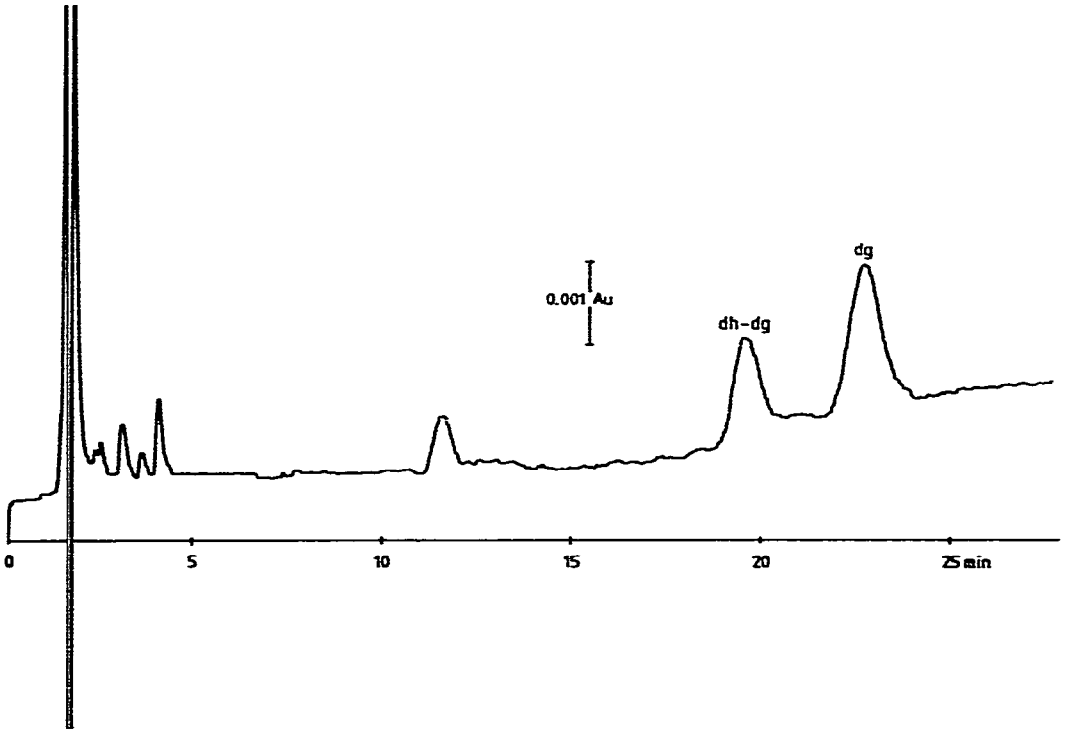

Fig. 2. Chromatogram obtained using a reversed-phase system. Column packing: LiChrosorb RP-8 (5 μm). Mobile phase: phosphate buffer (pH 6.3)—2-propanol (83.5:16.5). Sample: 100 nmol of dihydrodigoxin and 0.2 nmol of digoxin in 100 μl of mobile phase. Detection: 220 nm, a.u.f.s. 0.01. For abbreviations see Tables I and II.

TABLE II

CAPACITY FACTORS (k') AND SEPARATION FACTORS (α) FOR DIGOXIN AND METABOLITES

Substance*	Normal-phase LC		Reversed-phase LC	
	k'	α	k'	α
dg-g	3.15		1.81	
		1.32		1.55
dg-mono	4.15		2.81	
		1.50		2.37
dg-bis	6.23		6.66	
		1.47		2.02
dg	9.15		13.44	
		1.05		1.15
dh-dg	9.62		11.69	

*Abbreviations as in Table I; dh-dg = dihydrodigoxin.

are given. Both subjects had a gastric pH of 1.5 prior to drug intake and during 72 h 39.5 and 40.8% of the dose was recovered. The metabolites expected to be formed by intragastric hydrolysis, digoxigenin and its mono- and bis-digitoxoside, amounted to 3.0% for subject C and 11.8% for subject G. More than half of the amount of digoxigenin and its monodigitoxoside were found to

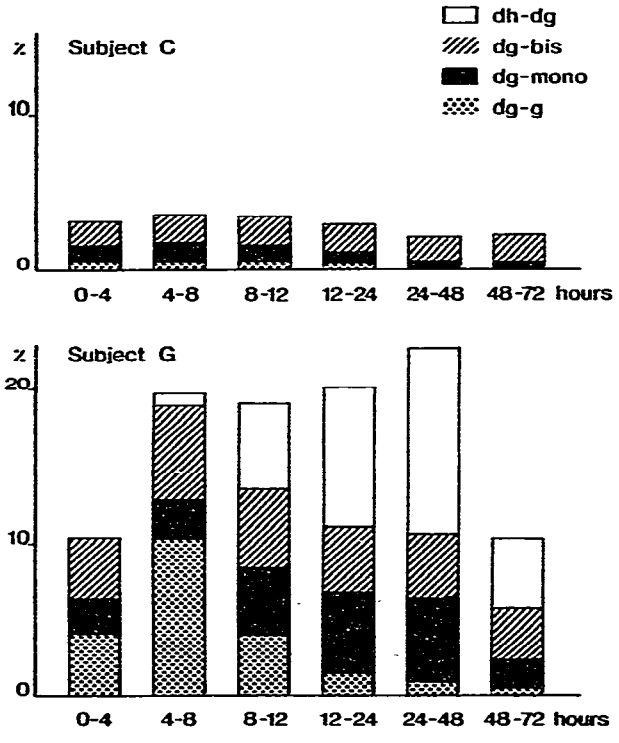

Fig. 3. Excretion of digoxin metabolites after deconjugation in two volunteers expressed as a percentage relative to the total recovery in each collection interval. For abbreviations see Tables I and II.

be present in the conjugated form. In relation to the rate of hydrolysis in vitro [2], the amounts of metabolites are low, which may reflect a rapid gastric emptying rate or a slower hydrolysis in vivo.

In subject G, 6.2% of the recovered dose was in the form of dihydrodigoxin. It has been suggested that this metabolite is formed by bacteria in the lower intestine, but this has not been proved [5]. As can be seen in Fig. 3, dihydrodigoxin is excreted later than the other metabolites, which does not seem to be consistent with the reported half-life of 1.2 h [9].

In conclusion, the proposed method for the determination of tritiated digoxin and its metabolites shows advantages over previous liquid chromatographic methods in terms of improved recovery in the solvent extraction procedure, short separation times and the possibility of the separate assay of the dihydro metabolite of digoxin. The use of ^{3}H-labelled digoxin and selective measurement of digoxin by liquid chromatography give increased accuracy and additional important information compared with conventional radioimmunoassay.

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