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ISOLATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND QUANTITATION BY RADIOIMMUNOASSAY OF THERAPEUTIC CONCENTRATIONS OF DIGOXIN AND METABOLITES

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SUMMARY

A method combining high-performance liquid chromatography for separation of digoxin from three of its metabolites with subsequent quantitation of each compound by radioimmunoassay is described. The metabolites are shown to interfere with the radioimmunoassay procedure thus providing the need for separation prior to assay.

INTRODUCTION

Although radioimmunoassay (RIA) is by far the most commonly used method to quantitate therapeutic levels (0.5—2.5 ng/ml) of digoxin in plasma, antibodies used in this assay may not be specific for the parent drug. Stoll et al. [1] have shown that three digoxin metabolites: digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, and digoxigenin all cross-react with the digoxin "specific" antibody. Therefore pharmacokinetic studies based on the use of a nonspecific assay are open to question [2] especially if the studies were done in clinical settings, such as end-stage renal failure, where the accumulation of digoxin metabolites is possible.

It has been shown that the pharmacokinetics of digoxin are altered in human renal failure wherein the rate of digoxin elimination as well as the volume of distribution are reduced [2]. Since approximately 75% or more of a dose of digoxin is eliminated unchanged in the urine of a patient with normal renal function [3] the decrease in elimination in renal failure is to be expected. However, the surprising decrease in the volume of distribution from 10 l/kg in normal [4] to 5 l/kg in renal failure patients [2] is as yet unexplained and may well be an artifact of the RIA caused by the accumulation of digoxin metabolites which will react with the digoxin RIA antibody. Therefore, before a study of the pharmacokinetics of digoxin in renal failure could be undertaken

it was necessary to develop an analytical method specific for digoxin. This paper describes a high-performance liquid chromatographic (HPLC) procedure to separate digoxin from its metabolites with subsequent assay of the individual compounds by ^{125}I RIA.

EXPERIMENTAL

Materials

All solvents used in the extraction or chromatography were Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) "distilled in glass" grade. Water was distilled in an all-glass still. Digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, and digoxigenin were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind., U.S.A.). The RIA was performed using the Ab-TRAC (T.M.) Digoxin Solid Phase Radioimmunoassay Kit (^{125}I) manufactured by Becton Dickinson Immunodiagnostic (Orangeburg, N.Y., U.S.A.). Tritium-labelled digoxin was obtained from New England Nuclear (Boston, Mass., U.S.A.) and was purified before use by HPLC under the conditions described later. The scintillation solution used was Complete Counting Cocktail 3a70B manufactured by Research Products International (Elk Grove Village, Ill., U.S.A.). Tritium-labelled metabolites were prepared by hydrolysis of labelled digoxin with 1 *N* hydrochloric acid for 10 min at room temperature. The reaction mixture was then neutralized with sodium hydroxide and extracted into dichloromethane. The dichloromethane was evaporated to dryness under a gentle stream of nitrogen in a water bath at 37° and the residue redissolved in 0.2 ml of the chromatographic mobile phase. Separation of these hydrolysis products was accomplished by HPLC using the conditions described later. Half-minute elution fractions were collected and the radioactivity of an aliquot of each was measured by scintillation counting. Retention volumes of the tritium-labelled components were then compared to a chromatogram of the authentic (unlabelled) metabolites and the fractions from the hydrolysis products corresponding to the three metabolites were saved.

Plasma extraction

Approximately 5000 dpm of chromatographically pure [^{3}H]digoxin in 0.02 ml buffer was added to 1 ml of plasma in a 16 × 150 mm PTFE-lined screw-cap tube, vortexed, and allowed to stand for 5 min. The same amount of labelled digoxin was added to a scintillation vial for recovery monitoring.

The sample was shaken for 5 min with 12 ml of dichloromethane, centrifuged 15 min and the aqueous layer aspirated. The dichloromethane was then shaken with 1 ml of 0.1 *N* sodium hydroxide solution for 1 min, centrifuged for 15 min and the aqueous layer aspirated. After drying with about 0.5 g of anhydrous sodium sulfate, the dichloromethane was evaporated to dryness under a gentle stream of nitrogen at 37°. The residue was dissolved in 0.2 ml of HPLC mobile phase with vigorous vortexing.

Chromatography

The HPLC system consisted of two Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pumps, a Model 660 solvent programmer, A Model U6-K injec-

tor and a Model 440 absorbance detector operating at 254 nm. Samples were chromatographed on a μBondapak C_{18} (Waters Assoc.) column (30 cm X 3.9 mm I.D.) with a 7-min gradient from 53% to 62% methanol in water. The fractions containing the digoxin and the metabolites were collected for further assay by ^{125}I RIA and scintillation counting and were evaporated to dryness in a rotary evaporator.

Radioimmunoassay

The dried down fractions from the chromatograph were reconstituted in 1.0 ml of the RIA kit buffer which had been diluted to 400 ml with distilled water containing 2% propylene glycol and 5% blank plasma. The propylene glycol and blank plasma are a modification of the RIA kit procedure. The plasma standards supplied with the kit were not used. Instead, standards were prepared in the modified buffer from weighed quantities of digoxin and the metabolites. The standards included a blank, 0.5, 1.0, 1.67, 2.5, 3.33, and 5.0 ng/ml. All samples and the standards were analyzed in duplicate. A blank plasma was also extracted and run through the procedure. The standard curve was plotted on logit—log paper to give the best straight line [5]. The abscissa was the log of the concentration and the ordinate was the logit of percent of ^{125}I trace level binding.

Recovery monitoring

The recovery of digoxin varied from 54% to 78% through the procedure prior to RIA. Therefore, an average recovery cannot be assumed and tritium-labelled digoxin was used to measure recovery through the procedure.

The amount of tritium-labelled digoxin added to the plasma sample as an internal standard was equal to about 5000 dpm in 0.02 ml. With the dilutions used this is about 0.1 ng and this added amount of digoxin must be taken into account when calculating the initial digoxin concentration of the sample. This correction is shown in the next section.

A 0.4-ml portion of the 1.0 ml reconstituted in buffer fraction from the liquid chromatograph was transferred to a scintillation vial and 10 ml of scintillation mixture added. This was counted for 20 min in a liquid scintillation counter along with the vials containing the total counts added at the beginning of the extraction. Added to the total counts vials was 0.38 ml of buffer to equalize any quenching by having the same volume of buffer in all samples. The calculation of the recovery was done as follows.

$$\text{Percent recovery} = \frac{\text{Counts in sample}/0.4}{\text{Total counts added}}$$

RESULTS AND DISCUSSION

Quantitation

RIA was performed on the samples taken off the liquid chromatograph with the standards prepared in buffer. Fig. 1 shows a typical standard curve for digoxin. The concentrations read off the standard curve were manipulated as

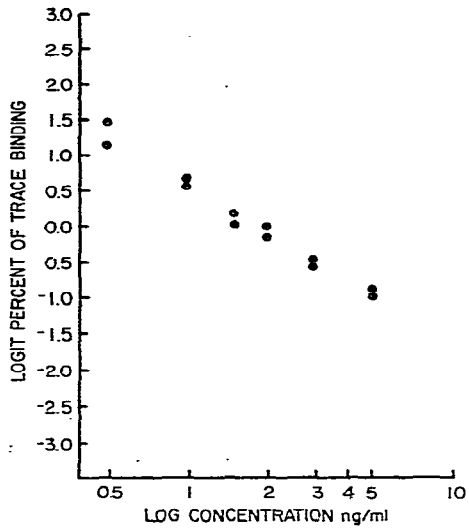

Fig. 1. Radioimmunoassay standard curve for digoxin.

follows to take into account the recovery loss, the amount of labelled compound added as recovery tracer and the blank reading.

$$C_i = \frac{\frac{C_f - C_b}{R} V_r - C_t V_t}{V_e}$$

where C_i = initial unknown concentration of the sample; C_f = final concentration from RIA; C_b = concentration of the blank extract; R = recovery of labelled tracer through procedure; V_r = volume used to reconstitute dried sample; C_t = concentration of labelled recovery tracer; V_t = volume of recovery tracer added; V_e = volume of sample extracted.

Chromatography

Fig. 2 shows a chromatogram of the separation of a mixture containing digoxin and the three metabolites. The sample contained 30 μg each of digoxin and the mono- and bisdigitoxoside and 10 μg of digoxigenin. The extinction coefficients of digoxin and the metabolites at the wavelength used (254 nm) prevent direct photometric detection in the therapeutic range (0.5—2.5 ng/ml). While other workers have demonstrated a 40-fold increase in sensitivity at 220 nm instead of 254 nm [6] one still would need at least a further 5—10-fold sensitivity improvement to measure digoxin and the metabolites directly. However, with the baseline separation achieved by this HPLC method the fractions containing each of the compounds can be collected and assayed by RIA which does have sufficient sensitivity.

Since pharmacological concentrations of digoxin and metabolites cannot be detected photometrically some other method must be used to determine the retention volumes containing these compounds. Chromatography with micro-

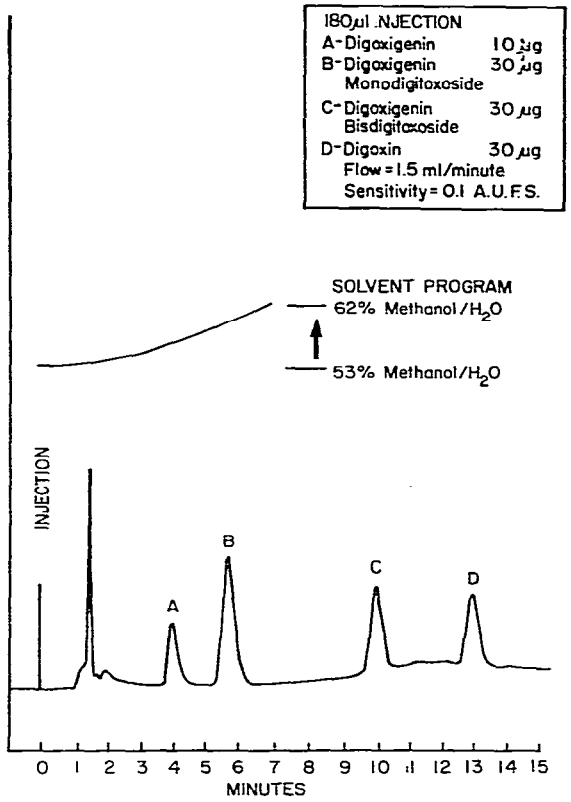

Fig. 2. Chromatogram of digoxigenin (A), digoxigenin monodigitoxoside (B), digoxigenin bisdigitoxoside (C), and digoxin (D). The chromatographic conditions are described in the text.

gram quantities as in Fig. 2 is helpful in initially determining the retention volumes with various solvent systems. However, this cannot be used prior to injecting extracts from plasma. We have found that after injection of microgram quantities of digoxin and metabolites for UV detection there was always a small but unavoidable carryover into subsequent injections which, although too small to be detectable photometrically, could easily interfere with the quantitation by RIA. This result is not unexpected since a 30-µg injection is ten thousand times higher than the nanogram levels normally achieved clinically and detectable by RIA thus carryover in the injection device at this level would not be surprising. Use of the tritium-labelled compounds works well to determine the retention volumes. The fractions can be collected coming off the column in scintillation vials, counted and retention volumes of the compounds determined.

Fig. 3 shows a typical chromatogram of a plasma extract. The retention volumes were determined in an earlier run. The peaks are endogenous material in the plasma.

Recoveries

The addition of 2% propylene glycol and 5% blank plasma to the RIA buffer

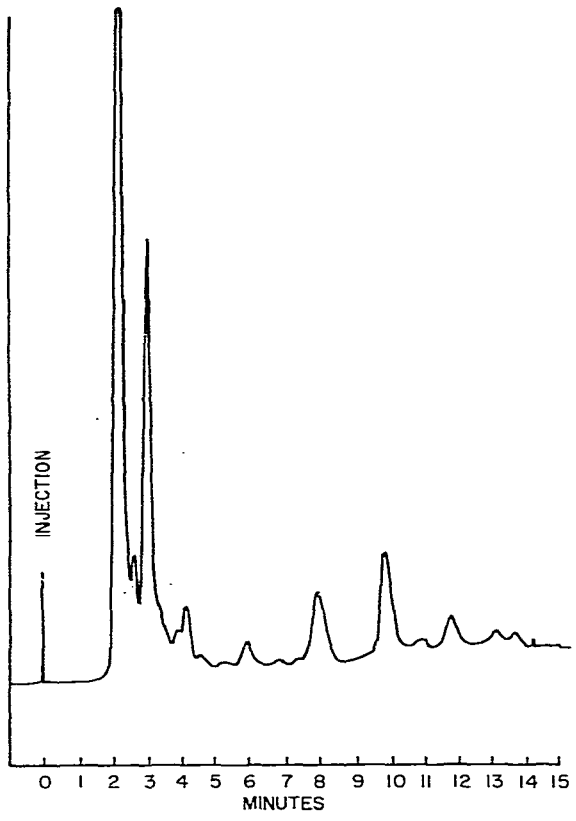

Fig. 3. Chromatogram of extracted plasma.

was necessary to obtain complete dissolution of the HPLC fractions after evaporating to dryness.

The ratio of the recoveries of the metabolites relative to digoxin (Table I) was determined as follows: Tritium-labelled digoxin and each of the three tritium-labelled metabolites were added to five plasma samples. The plasma samples were extracted and injected onto the liquid chromatograph. The eluted fractions containing the digoxin and the metabolites were collected and scintillation counted. The recovery of each compound was determined by the ratio of the amount of labelled compound added to the amount found in the eluted fraction.

Repeated digoxin assay on several days

Precision studies were carried out over several days by repeated analyses of plasma samples with added digoxin. The results are shown in Table II. Adversely affecting the precision of this assay is the necessity of adjusting the result read from the RIA standard curve for the losses in recovery as determined with the tritium-labelled tracer. For example, a sample with 2.0 ng/ml and 70% recovery should give an RIA value of 1.4 ng/ml. The error associated with the RIA result is about ± 0.1 ng/ml and the error in the tracer recovery result is ± 2%. Thus the calculated result could be between 1.8 and 2.2 ng/ml. As the

TABLE I

OVERALL RECOVERY (EXTRACTION AND CHROMATOGRAPHY) OF DIGOXIN AND METABOLITES

Compound	Trial No.	Recovery (%)	Metabolite/digoxin recovery ratio	Mean, S.D.
Digoxigenin	1	73.9	0.99	0.97, 0.03
	2	67.5	0.91	
	3	72.3	0.99	
	4	69.2	0.99	
	5	72.5	0.97	
Digoxigenin monodigitoxoside	1	68.1	0.91	0.89, 0.03
	2	64.8	0.87	
	3	62.2	0.85	
	4	62.5	0.89	
	5	68.5	0.91	
Digoxigenin bisdigitoxoside	1	70.9	0.95	0.97, 0.02
	2	71.0	0.95	
	3	72.3	0.99	
	4	70.4	1.00	
	5	73.4	0.98	
Digoxin	1	74.9		
	2	74.5		
	3	73.2		
	4	70.2		
	5	74.9		

TABLE II

PRECISION OF REPEATED DIGOXIN ASSAYS

Digoxin (ng/ml)	*n*	S.D. (ng/ml)	Coefficient of variation (%)
0.6	8	0.12	21.1
1.1	17	0.18	15.9
2.4	15	0.17	7.3
3.4	18	0.33	9.8
5.7	7	0.21	4.1

actual amount of digoxin or the recovery goes down the effect on the error increases. Analyses with lower than 50% recovery should be repeated. More than 1 ml of plasma can be extracted for samples with low levels of digoxin.

Assay of digoxin together with metabolites

Stoll et al. [1] found that digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside were capable of binding to the antibody of a commercially available kit to nearly the same degree as digoxin itself. Digoxigenin had somewhat less affinity.

The results with this RIA procedure were the same. Figs. 4—6, show the

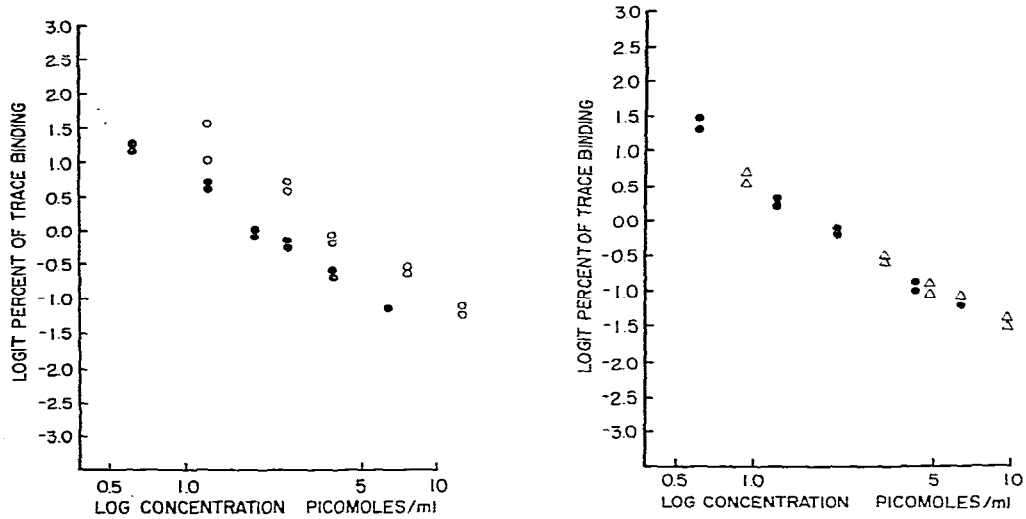

Fig. 4. Radioimmunoassay standard curve for digoxin (•) and digoxigenin (○) run simultaneously.

Fig. 5. Radioimmunoassay standard curves for digoxin (•) and digoxigenin monodigitoxoside (△) run simultaneously.

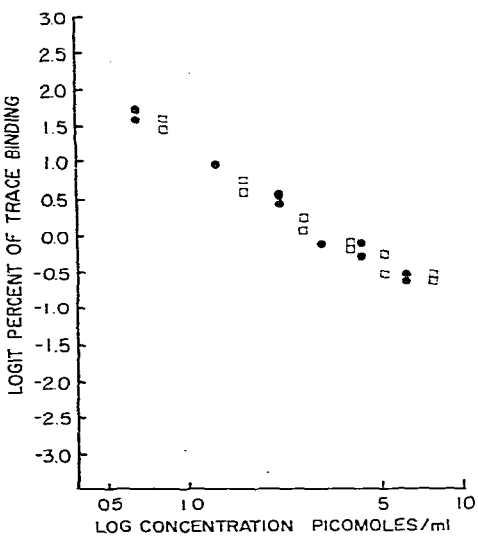

Fig. 6. Radioimmunoassay standard curves for digoxin (•) and digoxigenin bisdigitoxoside (□) run simultaneously.

molar RIA standard curves of digoxin compared with the three metabolites. The digoxin and metabolite standards in each case were assayed at the same time.

The effectiveness of the HPLC separation of digoxin from the three metabolites and their subsequent quantitation by RIA is shown in Table III. For each metabolite, three plasma samples (each in duplicate) were extracted, chromato-

TABLE III

ASSAY OF DIGOXIN TOGETHER WITH METABOLITES IN PLASMA SAMPLES

The concentrations of digoxin and metabolite in the third column were equal to those in the first and second columns, respectively.

Digoxin only (pmole)	Metabolite only (pmole)	Digoxin and metabolite (pmole)
1.17	2.97 (digoxigenin)	4.13
5.01	10.61 (Monodigitoxoside)	15.85
1.28	1.64 (Bisdigitoxoside)	3.29

graphed and assayed by RIA. The first two columns show the amounts found in the sample containing digoxin or metabolite only. The third column shows the sum of the number of pmoles of both digoxin and metabolite found in the sample containing both. This sum should be equal to the sum of the amounts in the first two columns, and in the three cases are nearly equal.

CONCLUSION

This study demonstrated that the presence of three of the metabolites of digoxin can interfere with the RIA of digoxin and that reversed-phase partition HPLC is suitable for separating the four compounds prior to the measurement of each by RIA.

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