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ISOLATION AND QUANTITATION OF PICOMOLE QUANTITIES OF DIGOXIN, DIGITOXIN AND THEIR METABOLITES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

M. C. CASTLE

National Institute of General Medical Sciences and Laboratory of Chemical Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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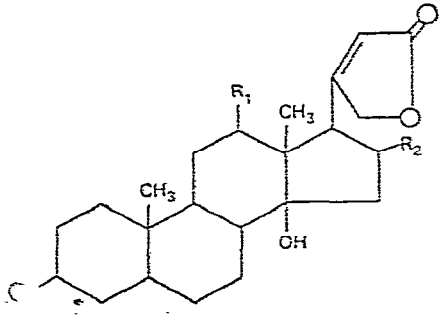
SUMMARY

The following high-pressure liquid chromatographic (HPLC) separations are described: (1) isocratic separation of digoxin and its metabolites, (2) isocratic separation of digitoxin and its metabolites, (3) gradient elution separation of digoxin, digitoxin and their metabolites, and (4) gradient elution separation of gitoxin from digoxin and its metabolites. These methods utilize a multi-wavelength UV detector set at 220 nm and a reversed-phase column with various mixtures of acetonitrile and water as the mobile phase. The feasibility of using these HPLC methods as qualitative and quantitative techniques for digitalis glycosides is discussed.

INTRODUCTION

Digoxin and digitoxin (Fig. 1) are steroid glycosides which are widely used in the treatment of congestive heart failure. Numerous studies have shown that these glycosides are metabolized by stepwise cleavage of the three digitoxose sugars attached at the C-3 atom of the steroid nucleus[1,2]. The metabolic products formed by this cleavage process are all cardioactive metabolites[3] and in some cases these



	R_1	R_2
DIGITOXIN	H	H
DIGOXIN	OH	H
GITOXIN	H	OH

Fig. 1. Structures of cardiac glycosides.

metabolites are more toxic than the parent compounds[4]. In addition to undergoing cleavage, digitoxin can also be hydroxylated at the C-12 position of the steroid nucleus to form digoxin[5,6]. Thus any investigation of the metabolic fate of digitoxin must consider a minimum of eight compounds: digitoxin and its three cleavage products and digoxin and its three cleavage products.

Numeous methods have been employed to separate digitoxin and its major metabolites. These methods include paper chromatography[7,8], column chromatography[9,10], thin-layer chromatography[11] and gas–liquid chromatography (GLC)[12,13]. All of these are less than ideal because they are time consuming and quantitation is difficult unless radioactive compounds are used. In recent years high-pressure liquid chromatography (HPLC) has emerged as a powerful analytical tool. HPLC has been employed successfully with numerous steroids and moderate success has been achieved in separating digitoxigenin and its glycosides[14].

The present study was undertaken to develop a rapid, reproducible method for separating and quantitating digoxin, digitoxin and their metabolites. A significant application of these HPLC techniques may involve the detection of impurities in clinical preparations of digoxin and digitoxin. Since gitoxin (Fig. 1) is a major contaminant of digoxin, a method to separate and quantitate these two drugs would also be useful. The use of HPLC to perform such an assay is also discussed in the present paper.

MATERIALS AND METHODS

Apparatus

The high-pressure liquid chromatograph used in these studies was a Waters Assoc. (Milford, Mass., U.S.A.) Model 202 complete with accessories for gradient elution chromatography. Samples were introduced on to the column through a Waters Assoc. U6K septumless injector. A Beckman Model 25 recording ultraviolet (UV) spectrophotometer was equipped with an 18-μl flow cell supplied by Waters Assoc. The column used throughout was a μBondapak C_{18} prepacked column (Waters Assoc.). This is a reversed-phase column (30 cm × 4 mm I.D.) containing 10-μm porous silica particles covalently linked with aliphatic hydrocarbon groups.

Reagents

The cardiac glycosides were purchased from Boehringer Mannheim (New York, N.Y., U.S.A.). Acetonitrile distilled in glass was obtained from Burdick and Jackson (Muskegon, Mich., U.S.A.). These highly purified solvents contain no preservatives and are thus ideal for use with UV detectors.

Procedure

Separate stock solutions of each cardiac glycoside or aglycone were carefully prepared by weighing the compound on a Cahn electrobalance and dissolving it in 95% ethanol. A 100-μl syringe was used to make all injections on to the column. Whenever possible, injection volumes of 50–75 μl were used to minimize errors in the injection procedure. Solvents were prepared immediately before use. Degassing of the solvent mixtures was not necessary even when gradient elutions were performed.

Operating conditions of the chromatograph varied somewhat depending upon

the separation to be performed. The specific conditions for each separation are presented with the chromatograms. Since the apparatus used in these studies utilizes a constant flow system, the operating pressure is a function of the solvent composition and of the flow-rate. The pressures in the chromatograms illustrated here were in the range of 2000–4000 p.s.i.

Each compound was chromatographed separately to determine purity and retention times. Peak heights and retention times for each compound were determined by averaging the values obtained from four separate chromatograms. These values were also used to determine the repeatability of the retention times and peak heights for each compound. Calibration curves (peak heights *versus* mass) were constructed using the averaged peak height values from four chromatograms.

RESULTS

Isocratic systems using mixtures of acetonitrile and water were developed for

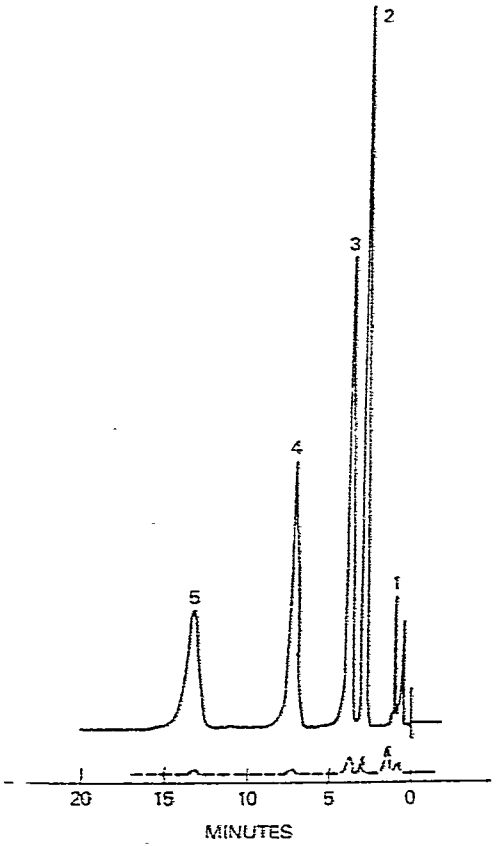


Fig. 2. Isocratic separation of digoxin and its metabolites by HPLC. Conditions: μBondapak C_{18} column; mobile phase, 25% acetonitrile in water, 3.0 ml/min; UV monitor at 220 nm, attenuation 0.1, chart speed, 0.2 in./min; temperature, 25°; 10 nmoles of each compound injected in 75 μl of ethanol. Lower chromatogram was carried out under identical conditions with the UV detector set at 254 nm. Peak identities: 1 = solvent peak; 2 = digoxigenin; 3 = digoxigenin monodigitoxoside; 4 = digoxigenin bisdigitoxoside; 5 = digoxin.

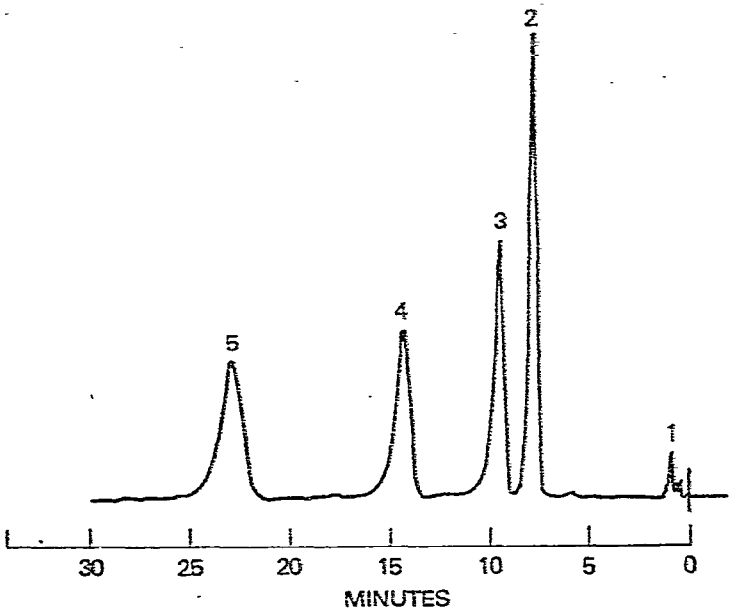


Fig. 3. Isocratic separation of digitoxin and its metabolites by HPLC. Conditions were identical to those in Fig. 2 except that the mobile phase was 33 % acetonitrile in water. Peak identities: 1 = solvent peak; 2 = digitoxigenin; 3 = digitoxigenin monodigitoxoside; 4 = digitoxigenin bisdigitoxoside; 5 = digitoxin.

the separation of digoxin, its bis- and monodigitoxosides, and digoxigenin (Fig. 2) and for the separation of digitoxin, its bis- and monodigitoxosides, and digitoxigenin (Fig. 3). In addition, a gradient elution system for the separation of all eight of these compounds is shown in Fig. 4. The isocratic systems are included here since many laboratories using HPLC may not have capabilities for generating gradients. With some HPLC systems (such as the Waters Assoc. apparatus used in these studies) gradients may be generated using an external gradient mixer and a single pump. However, with such an external system good repeatability of retention times and peak heights may be difficult to achieve.

In the systems described in Figs. 2 and 4, gitoxin co-chromatographs with digoxin bisdigitoxoside. Since gitoxin is often a major impurity of digoxin, a system to separate these compounds would also be useful. Again a gradient elution system was necessary as shown in Fig. 5.

The retention times for the various compounds are shown in Table I for both the isocratic and the gradient elution systems. The retention times in the first column were obtained with the isocratic systems illustrated in Figs. 2 and 3. The second column shows the retention times when all eight compounds were separated by gradient elution. The minimum detectable amount of each compound is also indicated in Table I. The minimum detectable amount is usually defined as the amount of a compound which will produce a peak twice the size of the deflections caused by the noise of the detector[15]. As before, the first column represents data obtained from isocratic systems (Figs. 2 and 3) and the second column illustrates results from a gradient

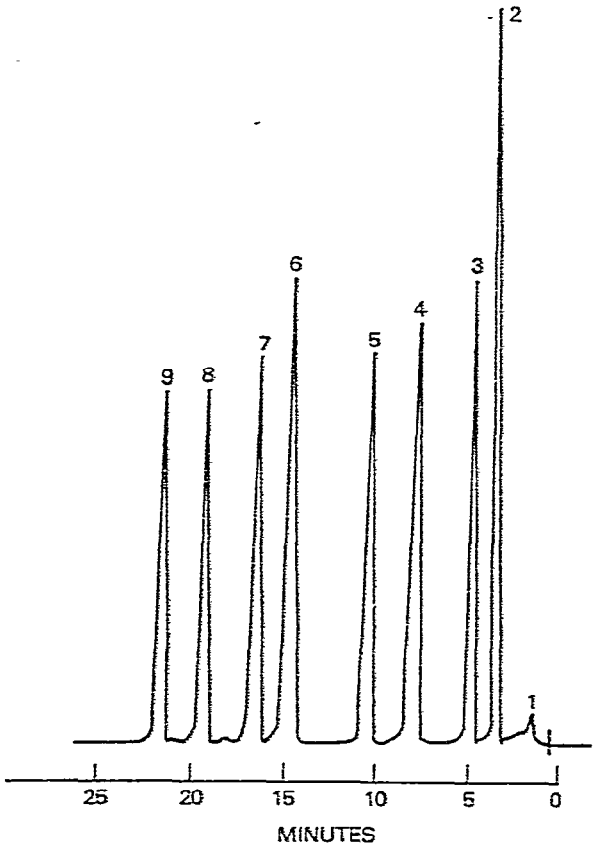


Fig. 4. Separation of digoxin, digitoxin and their metabolites by gradient elution chromatography. Conditions were identical to those in Fig. 2 with the following exceptions: the mobile phase consisted of a linear gradient of 25% acetonitrile in water to 40% acetonitrile in water at 5%/min; the flow-rate was 2.2 ml/min. Peak identities: 1 = solvent peak; 2 = digoxigenin; 3 = digoxigenin monodigitoxoside; 4 = digoxigenin bisdigitoxoside; 5 = digoxin; 6 = digitoxigenin; 7 = digitoxigenin monodigitoxoside; 8 = digitoxigenin bisdigitoxoside; 9 = digitoxin.

elution system (Fig. 4). These are the minimum amounts which can be detected with n attenuation of 0.01 a.u.f.s. At attenuations in the range of 0.01 to 0.1 a.u.f.s., a aseline drift of approximately 10–20% occurs when a gradient system is used.

The importance of using a detector which can record at 220 nm is illustrated i Fig. 2. These two chromatograms were obtained by making successive determi- ations with all conditions identical except the wavelength setting of the detector. s seen in the figure, the sensitivity is approximately 40 times greater for these eroids at 220 nm than at 254 nm.

Calibration curves for all eight compounds are shown in Figs. 6 and 7. Peak ights were measured for various amounts of each compound using the isocratic stems discussed previously (Figs. 2 and 3). The peak heights represented in Figs. 6 id 7 were obtained using a range of 0.5 to 4.0 nmoles of each compound and an tenuation of 0.1 a.u.f.s.

The repeatability of the peak heights and retention times for each compound

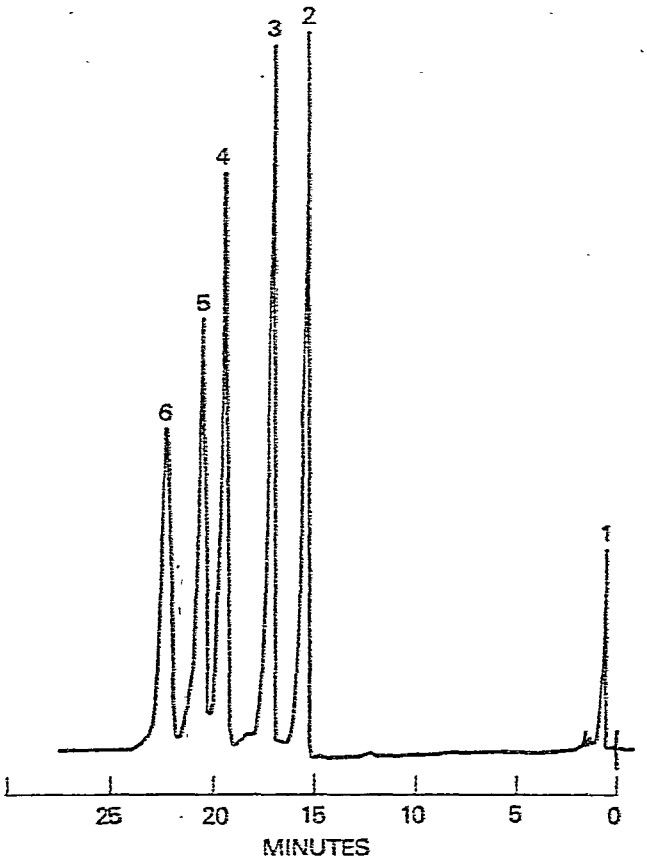


Fig. 5. Separation of digoxin and its metabolites from gitoxin by gradient elution chromatography. Conditions were the same as in Fig. 4 with the following exceptions: the mobile phase consisted of a linear gradient of 100% water to 30% acetonitrile in water at 6.67%/min. Peak identities: 1 = solvent peak; 2 = digoxigenin; 3 = digoxigenin monodigitoxoside; 4 = digoxigenin bisdigitoxoside; 5 = gitoxin; 6 = digoxin.

was also estimated. The relative standard error (standard error of the mean divided by the mean) was less than 1% in all cases.

DISCUSSION

Previous methods of separating and quantitating digoxin, digitoxin and their metabolites have involved time-consuming, often very complicated procedures. In the present investigation with HPLC, isolation of these compounds can be accomplished in less than 30 min. In addition, the data presented here also demonstrate that HPLC can be a valuable analytical tool for quantitating these compounds. When used in conjunction with a UV detector, as little as 4–20 ng of these compounds can be quantitated. The minimum detectable amounts of these compounds (Table I) are a function of the mode of separation (isocratic or gradient elution) as well as the molecular weight of the compound. In an isocratic system the compounds which elute

TABLE I

RETENTION TIMES AND MINIMUM DETECTABLE AMOUNTS OF DIGOXIN, DIGITOXIN AND THEIR METABOLITES BY HPLC

Compound	Retention times (min)		Minimum detectable amounts (ng)§	
	Isocratic	Gradient***	Isocratic	Gradient
Digoxigenin	2.8*	4.0	4	10
Digoxin monodigitoxoside	3.8	5.0	8	13
Digoxin bisdigitoxoside	7.1	8.0	16	10
Digoxin	13.2	10.5	39	14
Digitoxigenin	7.9**	14.8	9	4
Digitoxin monodigitoxoside	9.6	16.6	25	8
Digitoxin bisdigitoxoside	14.4	19.4	48	10
Digitoxin	23.0	21.5	76	11

* Conditions as shown in Fig. 2.
** Conditions as shown in Fig. 3.
*** Conditions as shown in Fig. 4.
§ Determined from peak heights which are at least twice the baseline noise at an attenuation of 0.01 a.u.f.s.

first have smaller elution volumes and the peaks are thus sharper than those which elute later (Figs. 2 and 3). The later eluting compounds with their larger elution volumes and broader peaks are more difficult to quantitate by the peak height method. In gradient elution chromatography this effect is less pronounced since elution volumes are fairly constant throughout the chromatogram (Fig. 4). The minimum

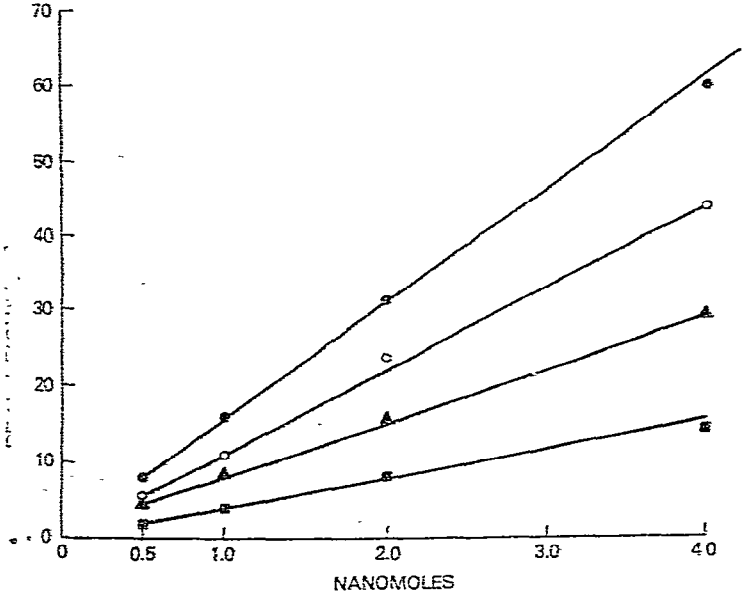


Fig. 6. Calibration curves for digoxin and its metabolites. ●, Digoxigenin; ○, digoxigenin monodigitoxoside; ▲, digoxigenin bisdigitoxoside; ■, digoxin.

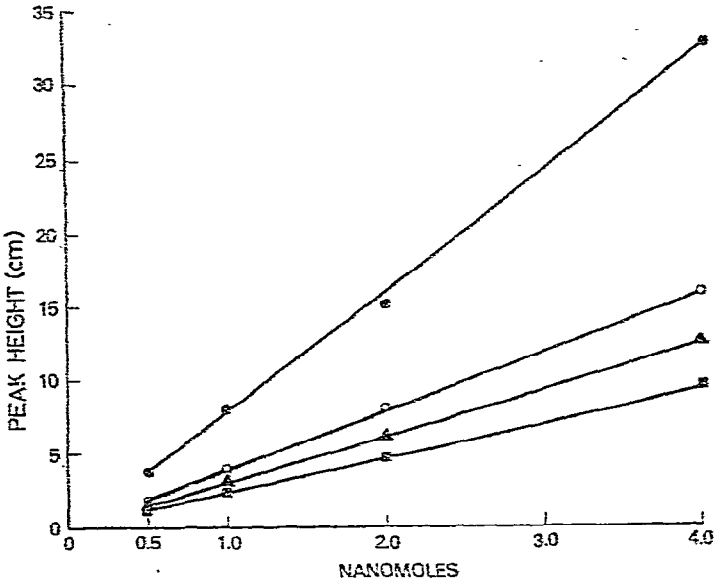


Fig. 7. Calibration curves for digitoxin and its metabolites. ●, Digitoxigenin; ○, digitoxigenin monodigitoxoside; ▲, digitoxigenin bisdigitoxoside; ■, digitoxin.

detectable amounts of these compounds are also related to the molecular weights. The UV absorbance of these steroids is derived from the unsaturation in the lactone ring. The presence of the digitoxose sugars at the C-3 atom of the steroid nucleus adds considerably to the molecular weight but has no effect on the UV absorbance. The aglycones thus have greater absorbance per ng (but not per nmole) than do the glycosides.

This method of analysis by HPLC should be applicable to the assay of clinical preparations of digitalis glycosides. Impurities could be identified and quantitated rapidly and efficiently. The application of this method to clinical and research situations is less apparent. The usual therapeutic range for serum digoxin concentrations is 0.7–1.5 ng/ml[16], which is beyond the sensitivity of this method of analysis. However, one of the great advantages of HPLC is that samples can be recovered in their original state. Thus each of the eight compounds in the present studies can be collected separately as they elute from the column. Once the compounds have been isolated they may then be subjected to quantitation by other more sensitive methods. For example, if radioactive compounds are used, the glycosides and their metabolites can be measured very accurately by scintillation counting. Another possibility would be to combine the HPLC system with the GLC technique developed by Watson *et al.*[11]. These workers claim a sensitivity of 25 pg using an electron capture detector. However, their method requires separation of digoxin, digitoxin and their metabolites by a series of thin-layer chromatographic techniques prior to the work-up for GLC. The HPLC method outlined in the present investigations would appear to markedly shorten and simplify this type of assay.

HPLC may also prove to be a valuable technique for the qualitative analysis of digitalis glycosides. The excellent resolution and repeatability of the peaks in the

present studies indicate that HPLC could be used as a rapid means of identifying unknown compounds suspected as being digitalis glycosides. The selectivity of the column appears to be such that this method of analysis could be extended to other cardiac glycosides as well.

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