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SEPARATION OF DIGOXIN, DIGITOXIN AND THEIR POTENTIAL METABOLITES, IMPURITIES OR DEGRADATION PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and versatile series of high-performance liquid chromatographic systems are described for the resolution of digoxin, digitoxin and their potential metabolites or degradation products and impurities. These systems consist of isocratic, single-step gradient and linear gradient modes that provide resolution of the glycosides in 25, 17 and 14 min respectively. Digoxin, its mono- and bisdigitoxosides, digoxigenin and gitoxin, a potential impurity, may be isocratically separated in 11 min. The two semi-synthetic glycosides α- and β-acetyldigoxin are resolved and separated from digoxin and its metabolites in a chromatographic time of 23 min. Digitoxin and its metabolites or degradation products may be separated in as little as 9 min using an isocratic system.

The solvent systems employ varying proportions of methanol, water, isopropanol and dichloromethane and a conventional 5 μm bonded, octadecyl phase. Detection was accomplished using a variable wavelength detector set at 220 nm.

INTRODUCTION

Digoxin and digitoxin are cardiotonic secondary glycosides obtained from the leaves of *Digitalis lanata* or *Digitalis purpurea*. These purified extracts are the most commonly prescribed medication used in the treatment of congestive heart failure.

Both of the digitalis glycosides are metabolically converted to their respective bis- and monodigitoxosides and finally to the aglycone (genin) steroid by a stepwise elimination of the digitoxose sugars at carbon 3. In addition, the glycosides have been reported to give rise to dihydrodigoxin and dihydrodigitoxin[1–7] and it has also been noted that digitoxin can be converted to digoxin by β-hydroxylation in the liver[1,8,9].

In addition to metabolic cleavage of the digitoxose sugar residues, the acidic hydrolysis of digoxin and digitoxin to their bis-, mono- and aglycone fragments has been observed following *in vitro* tests[10–12]. The digitalis glycosides are also known to contain impurities such as gitoxin in digoxin and digitonin in digitoxin. The United States Pharmacopeia[13] specifies tests and limits to determine these, as well as other unspecified digitoxosides.

The assessment of plasma levels of drug and metabolites, and the detection of potential degradation products and impurities in drug formulations would therefore require methods that could resolve all of the above glycosides. In the past many investigators have used paper[14,15], thin-layer[16,17], gas–liquid[18,19] and column[20,21] chromatographic methods for the separation of digitalis glycosides. The wide variety of methods that have been reported up to 1974 have been reviewed by Page[22]. High-performance liquid chromatographic (HPLC) procedures have been used to separate various mixtures of cardiac glycosides[23,24]. Separation of digitoxin, digoxin and some of their metabolites by gradient elution in a single chromatogram has been reported by Castle[25] while separations of the high and low polarity groups of digitalis glyco-sides of the cardenolide series has been reported by Lindner and Frei[23]. HPLC has also been used by two groups of investigators to resolve digoxin and its hydrolysis products[26,27]. A recent paper by Fujii et al.[24] has reported a series of solvent systems using a micro-HPLC column for the resolution of various mixtures of digoxin, digi-toxin and their respective metabolites or degradation products, as well as lanatosides A and B. Unfortunately the methods employed to resolve the digitalis glycosides thus far have suffered from relatively long elution times, the need for gradient elution, or the need for specialized columns. In addition many methods have been suitable for only certain groups of the cardenolide series of digitalis glycosides. The need for a more robust HPLC method using conventional column technology that would be capable of the isocratic resolution of digoxin, digitoxin and their known metabolites and/or impurities in a single chromatogram is therefore evident. The development of such a method, as well as single-step gradient and linear solvent gradient HPLC modes, is the subject of the present paper. In addition, the isocratic resolution of the semi-synthetic digitalis glycosides, α- and β-acetyldigoxin from digoxin and its metab-olites is described.

EXPERIMENTAL

Apparatus
        A Beckman High Performance Liquid Chromatograph (Model 322) equipped with dual pumps (Models 100A and 110A) and a Waters Associates injection loop (Model U6K) was used with a Hitachi variable-wavelength detector (Model 100-10) and a Shimadzu Chromatopac (Model C-RIA) electronic data system. The volume of the dynamically stirred mixing chamber and ancillary tubing was 200 μl. The column was a 25 × 0.46 cm Ultrasphere reversed phase (C_{18}) with 5 μm particle size, ob-tained from Beckman Instruments.

Materials
        Water, methanol, isopropanol and dichloromethane were HPLC quality (Fisher Scientific, Pittsburgh, PA, U.S.A.). All solvents used were of HPLC grade. The cardiac glycosides and aglycones (Table I) were obtained from Boehringer (Mannheim, G.F.R.) and were used without further purification.

Methods
        The glycosides were dissolved in the eluting solvent and injected into the chro-matograph immediately after preparation. Samples used for determination of chro-

TABLE I

STRUCTURES OF THE DIGITALIS GLYCOSIDES AND AGLYCONES INVESTIGATED
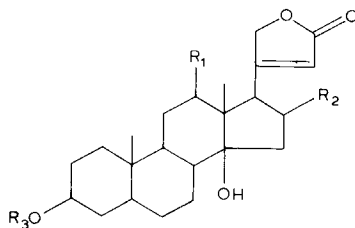
D = Digitoxose; Ac = acetyl.

Compound	R_1	R_2	R_3
Digitoxigenin	H	H	H
Digitoxigenin monodigitoxoside	H	H	D
Digitoxigenin bisdigitoxoside	H	H	D–D
Digitoxin	H	H	D–D–D
Gitoxin	H	OH	D–D–D
Digoxigenin	OH	H	H
Digoxigenin monodigitoxoside	OH	H	D
Digoxigenin bisdigitoxoside	OH	H	D–D
Digoxin	OH	H	D–D–D
α- and β-acetyldigoxin	OH	H	D–D–D | Ac

matographic characteristics were freshly prepared. Each data point on the calibration curve is an average of six determinations. The retention time of each compound was determined by separate injections of individual solutions of each sample. Solvent systems were prepared in sufficient quantities before use and degassing was not found to be necessary.

RESULTS AND DISCUSSION

The isocratic separation of digoxin, digitoxin and their metabolites or potential impurities is depicted in Fig. 1. The mobile phase consisted of water–methanol–isopropanol–dichloromethane (47:40:9:4). By a slight alteration in the composition of the mobile phase using the same eluents (43:35:15:7), the total elution time could be reduced from 25 to 13 min, but, the two early peaks due to digoxigenin and digoxigenin monodigitoxoside coalesced. However, the other seven glycosides still maintained baseline resolution. Gitoxin is a known impurity in digoxin formulations[13] and the compendial method specified for its detection is labour intensive. As shown in Fig. 1, this material may be resolved from digoxin in less than 11 min of chromatographic elution time. The chromatogram shown in Fig. 2 is representative of the fastest isocratic separation that could be achieved for the digitoxin series of potential metabolites or degradation products and was obtained with a solvent ratio of 44:34:15:7. In this case digitoxin, its mono- and bisdigitoxosides and digitoxigenin are completely resolved in 9 min.
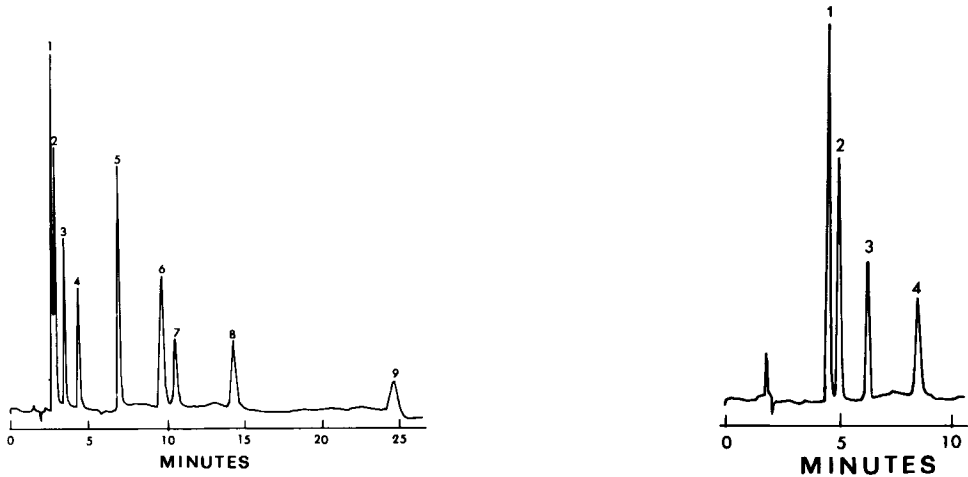
Fig. 1. Isocratic separation of digitalis glycosides. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = digitoxigenin; 6 = digitoxigenin monodigitoxoside; 7 = gitoxin; 8 = digitoxigenin bisdigitoxoside; 9 = digitoxin. Solvent system: water–methanol–isopropanol–dichloromethane (47:40:9:4); flow-rate 1.2 ml/min. 160 ng of each glycoside injected in 50 μl.

Fig. 2. Isocratic separation of digitoxin series of glycosides. Sequence of elution: 1 = digitoxigenin; 2 = digitoxigenin monodigitoxoside; 3 = digitoxigenin bisdigitoxoside; 4 = digitoxin. Solvent system: water–methanol–isopropanol–dichloromethane (44:34:15:7); flow-rate 1.2 ml/min. 160 ng of each glycoside injected in 50 μl.

The glycosides, α- and β-acetyldigoxin are prepared by the enzyme hydrolysis of lanatoside C in *Digitalis lanata* leaf and previous attempts[28] to separate these two short acting semi-synthetic glycosides have not been fully successful. The chromatogram depicted in Fig. 3 shows that these two glycosides may be resolved in less than 24 min while maintaining complete separation of digoxin and its digitoxose residues.
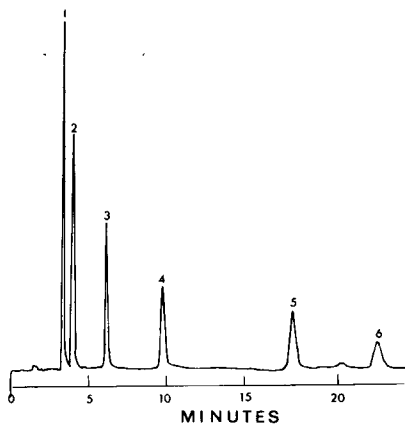

Fig. 3. Isocratic separation of α- and β-acetyldigoxin from the digoxin series of glycosides. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = α-acetyldigoxin; 6 = β-acetyldigoxin. Solvent system: water–methanol–isopropanol–dichloromethane (51:42:5:2); flow-rate 1.2 ml/min. 160 ng of each glycoside injected in 50 μl.

Although isocratic solvent systems are generally preferable to linear gradient or step gradient modes as the isocratic mode does not require additional pumps, solvent switching valves or column re-equilibration, the digitalis glycosides were subjected to these techniques to determine if there was any gross benefits in terms of overall resolution or time. Fig. 4 is representative of a single-step gradient that was generated by using two HPLC pumps, although the use of a solvent switching valve would also serve the same purpose. One pump delivered a solvent mixture consisting of water–methanol–isopropanol–dichloromethane (49:41:7:3) for 5 min. At this point the first pump was stopped and the second pump began delivery of a solvent mixture containing the same solvents in a ratio of 41:34:17:8. The total elution time for digitoxin was 17 min but column re-equilibration was found to require 15 min before an identical chromatogram could be generated. A linear gradient starting with water–methanol–isopropanol–dichloromethane (49:41:7:3) that was altered to (38:32:20:10) starting from time 2.5–3 min provided the shortest total elution (Fig. 5) of the eight digitalis glycosides (14 min). Although the single-step gradient and linear gradient modes reported here lead to considerably faster elution times than the 21.5 min reported for an earlier gradient solvent system[29], it is considered that little benefit could be gained over the isocratic solvent mode depicted in Fig. 1 since the gradient modes require column re-equilibration and more sophisticated instrumentation. Quantities of each of the glycosides were increased to accommodate the shift in baseline observed due to the gradient profile.
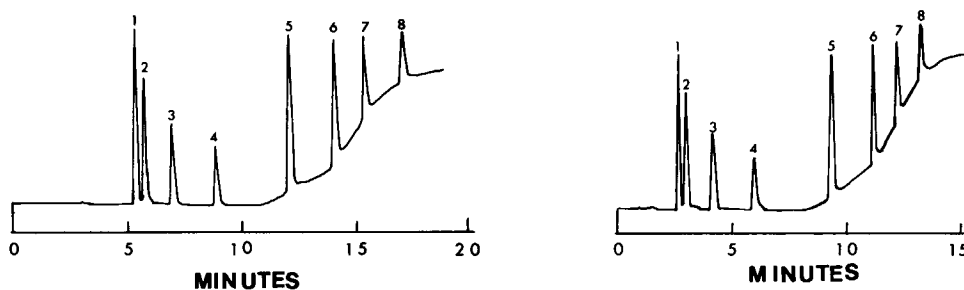

Fig. 4. Single-step gradient separation of digitalis glycosides. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = digitoxigenin; 6 = digitoxigenin monodigitoxoside; 7 = digitoxigenin bisdigitoxoside; 8 = digitoxin. Solvent system at time 0: water–methanol–isopropanol–dichloromethane (49:41:7:3). Solvent ratio at 5 min changed to 41:34:17:8. Flow-rate: 1.2 ml/min throughout. 16 μg of each glycoside injected in 50 μl.

Fig. 5. Linear gradient elution of digitalis glycosides. Refer to Fig. 4 for identification of glycosides. Solvent system at time 0: water–methanol–isopropanol–dichloromethane (49:41:7:3). Solvent was linearly changed from time 2.5–3.0 min to water–methanol–isopropanol–dichloromethane (38:32:20:10) and was maintained at these proportions until completion of the chromatographic elution. Flow-rate: 1.2 ml/min. 16 μg of each glycoside injected in 50 μl.

The retention times of each of the digitalis glycosides were found to be reproducible with a relative standard deviation of 0.5% ($n = 6$ for each glycoside). A summary of the retention times of the digitalis glycosides evaluated is given in Table II. The capacity factor (k') value for digoxigenin in Fig. 1 is 1.46. This value is below the optimum value of 2 for chromatographic methods but within the minimum value of 1 accepted by most researchers in the field[30].

TABLE II

TOTAL RETENTION TIMES* OF DIGITALIS GLYCOSIDES UNDER THE CONDITIONS SPECIFIED IN THE CORRESPONDING FIGURES**

Compound	Fig. 1	Fig. 2	Fig. 3	Fig. 4	Fig. 5
Digoxigenin	2.8		3.4	5.5	2.8
Digoxigenin monodigotoxoside	2.9		4.0	5.9	3.2
Digoxigenin bisdigitoxoside	3.6		6.1	7.1	4.4
Digoxin	4.5		9.8	9.0	6.3
Gitoxin	10.6				
Digitoxigenin	7.0	4.5		12.2	9.6
Digitoxigenin monodigitoxoside	9.8	5.0		14.9	11.4
Digitoxigenin bisdigitoxoside	14.4	6.3		15.5	12.4
Digitoxin	24.5	8.5		17.2	13.6
α-Acetyldigoxin			18.0		
β-Acetyldigoxin			23.4		

* Time to the nearest tenth of a minute.
** Times are the mean of six determinations.

The HPLC systems reported in this paper provide good isocratic and solvent program modes for the resolution of the major therapeutic glycosides, digoxin and digitoxin as well as their potential impurities or degradation products. By only subtle changes in the relative composition of four solvents, the glycoside or series of glycosides can be eluted in convenient chromatographic times. In addition, the first reported complete HPLC resolution of α- and β-acetyldigoxin was readily accomplished by the isocratic solvent mode.

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