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Electrochemical detection of the 3,5-dinitrobenzoyl derivative of digoxin by high-performance liquid chromatography

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SUMMARY

Electrochemical detection of 3,5-dinitrobenzoyl derivatives of digoxin and its metabolites following high-performance liquid chromatography is reported. Partial resolution of derivatized digoxin and dihydrodigoxin was obtained using a Spherisorb ODS II analytical column Both single- and dual-electrode detection were investigated and a maximum sensitivity equivalent to 0.39 ng of digoxin was found with the dual-electrode method. This system has the necessary sensitivity and selectivity for development into a therapeutic monitoring assay method.

INTRODUCTION

Digitalis glycosides form one of the most beneficial group of drugs available to aid the failing heart. They are the drugs of choice for the treatment of congestive heart failure and certain disturbances in cardiac rhythm. Digoxin is clinically the most commonly used digitalis glycoside.

Metabolism of digoxin, by cleavage of the digitoxose residues, conjugation reactions and/or reduction of the unsaturated lactone ring, occurs mainly in the liver [1]. Digoxin is excreted largely by the kidneys via glomerular filtration and tubular secretion [1]. Some investigators have suggested that only a small proportion of digoxin is metabolized [2,3], while others have shown that 57–60% [4,5] is excreted as a metabolic product, largely dihydrodigoxin. The large variability in metabolism and excretion of digoxin and cardioactivity of

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digoxin metabolites [1] make it imperative that any reliable procedure for the measurement of digoxin also involves the resolution of the intact drug from its metabolites.

Digoxin may be analyzed by several methods including immunoassays and chromatographic techniques. The low therapeutic plasma levels for digoxin (0.5–2.0 ng/ml) [1] have prompted the development of extremely sensitive assay procedures. Immunoassays are used in the clinical laboratory due to their speed, precision, sensitivity and cost. One of the major problems with immunoassay methods for digoxin analysis is cross-reactivity of the anti-digoxin antibodies with digoxin metabolites, endogenous compounds such as 'digoxin-like immunoreactive substance(s)' and other drugs that may be co-administered with digoxin [6–8].

Various high-performance liquid chromatographic (HPLC) methods have been used for digoxin analyses [3,9–12]. The combination of HPLC with electrochemical detection (ED) has led to very sensitive assay methods for numerous drugs and endogenous compounds [13–15]. Although the utility of aromatic nitro derivatives for use with reductive HPLC–ED has been suggested [15], derivatized cardiac glycosides have not been detected electrochemically.

This report describes HPLC–ED of 3,5-dinitrobenzoyl derivatives of digoxin and its metabolites as well as partial resolution of derivatized digoxin and dihydrodigoxin.

EXPERIMENTAL

Apparatus

A Beckman Model 100 A dual-piston solvent metering system (Beckman Instrument, Fullerton, CA, U.S.A.) was used as the HPLC pump. The remaining HPLC system consisted of a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.) and an Altex C-RIA Chromatopac data processor (Beckman Instrument).

A fixed-wavelength (254 nm) ultraviolet detector (Beckman Model 153 detector, Beckman Instrument) was used to confirm the retention time of derivatized digoxin.

The Coulochem Model 5100A dual-electrode HPLC electrochemical detector, Model 5020 guard cell, Model 5010 dual-electrode analytical cell and Model 5012 screened wall jet cell with a gold electrode were purchased from ESA (Bedford, MA, U.S.A.). The guard cell electrode was placed before the injector and used to electrochemically clean the mobile phase before the introduction of samples. The analytical cell (Model 5010 or Model 5012) was connected directly after the analytical HPLC column as shown in Fig. 1.

A NewGuard holder equipped with a 1.5 cm×3.2 mm I.D. ODS cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) was used as guard column and placed prior to the Spherisorb ODS II (3 μm particle size) 15 cm×4.6 mm I.D.

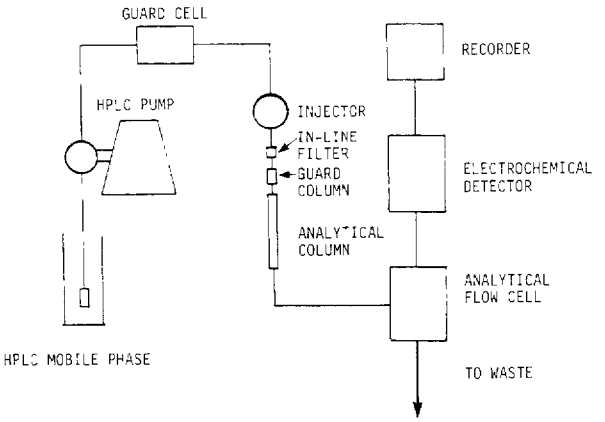

Fig. 1. Schematic of HPLC–ED system.

analytical column (Alltech Assoc., Deerfield, IL, U.S.A.). A direct-connect column prefilter (Alltech Assoc.) placed between the injector and guard column, was used as an in-line filter.

FP Vericel 45 mm diameter, 0.45 μm membrane filters (Gelman Sciences, Ann Arbor, MI, U.S.A.) were used with the Millipore all-glass filter apparatus (Millipore–Waters Assoc., Milford, MA, U.S.A.) for filtration of the mobile phase.

Materials

Digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, dihydrodigoxin and dihydrodigoxigenin were obtained from Boehringer (Mannheim, F.R.G.). Sodium bicarbonate, sodium hydroxide (Aristar grade), anhydrous sodium sulfate and hydrochloric acid were purchased from BDH (Toronto, Canada). Sodium acetate trihydrate (Gold label), glacial acetic acid (Gold label) and 3,5-dinitrobenzoyl chloride were purchased from Aldrich (Milwaukee, WI, U.S.A.). 4-Dimethylaminopyridine was obtained from Sigma (St. Louis, MO, U.S.A.).

Reagent-grade absolute ethanol was purchased from Commercial Alcohols (Toronto, Canada). HPLC-grade water produced with the Milli-Q water system (Millipore, Milford, MA, U.S.A.) was used throughout. Pyridine and the remaining HPLC-grade solvents were Omnisolv grade from BDH. Prior to use, the pyridine was distilled and stored over sodium hydroxide.

Methods

Digoxin (3 g) was derivatized in pyridine using 3,5-dinitrobenzoyl chloride as described by Fujii et al. [10]. The crude product was precipitated in cold dilute hydrochloric acid (1%, v/v, concentrated hydrochloric acid), reconstituted in ethyl acetate–hexane (1:1, v/v) and washed with 5% (w/v) sodium

bicarbonate solution containing 0.25% (w/v) 4-dimethylaminopyridine. After drying the organic phase with anhydrous sodium sulfate and evaporation in vacuo, the derivative was purified by recrystallization from methanol. A stock solution of the 3,5-dinitrobenzoyldigoxin derivative was prepared in methanol (1 mg per 100 ml) and further diluted to give a final concentration of 5 ng per 10 μl.

Derivatives of digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, dihydrodigoxin and dihydrodigoxigenin (3.0 mg each) were also individually prepared with 3,5-dinitrobenzoyl chloride, again using the procedure reported by Fujii et al. [10]. The cardiac glycosides were derivatized in pyridine which was subsequently evaporated under nitrogen. The residue was dissolved in HPLC-grade ethyl acetate, washed with 5% (w/v) sodium bicarbonate containing 0.25% (w/v) 4-dimethylaminopyridine and evaporated under nitrogen.

Sodium acetate buffer (0.1 *M*, pH 4.6) was prepared in the HPLC-grade water for use as an electrolyte with the electrochemical detector.

The HPLC mobile phases were prepared by mixing the individually measured solvents and filtering prior to use. The mobile phase was continuously degassed with helium.

RESULTS AND DISCUSSION

HPLC–ultraviolet detection

The 3,5-dinitro derivatives of digoxin and its metabolites (3-mg quantities of each) were evaluated using HPLC with ultraviolet detection at 254 nm with a mobile phase consisting of acetonitrile–acetate buffer (20:7, v/v). The identity of the digoxin derivative was established by comparison of the melting point of its derivative with that reported (m.p. 199–204 °C; lit. [10], 203–205 °C)

TABLE I

HPLC RETENTION TIMES OF 3,5-DINITROBENZOYL DERIVATIVES

Chromatographic conditions: HPLC column, Spherisorb ODS II 3 μm (15 cm × 1 6 mm I.D.); flow-rate, 1.0 ml/min; mobile phase, acetonitrile–water (20 7, v/v); ultraviolet detection at 254 nm.

Glycoside	Retention time (min)
Dihydrodigoxigenin	5.63
Digoxigenin	5.64
Digoxigenin monodigitoxoside	9.63
Digoxigenin bisdigitoxoside	16 90
Dihydrodigoxin	35.81
Digoxin	37.23

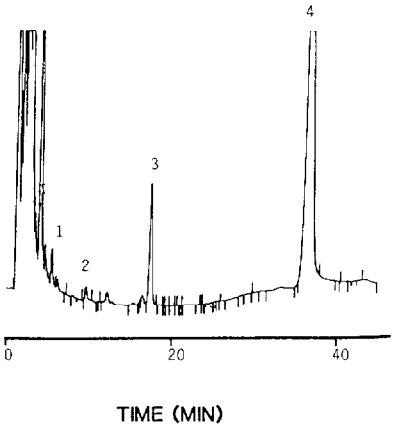

Fig. 2 Chromatogram of 3,5-dinitrobenzoyl derivatives of digoxin with ultraviolet detection. Chromatographic conditions: HPLC column, Spherisorb ODS II 3 μm (15 cm $\times$ 1 6 mm I.D.); flow-rate, 1.0 ml/min; mobile phase, acetonitrile–water (20 7, v/v); ultraviolet detection at 254 nm; range, 0.005 a.u.f.s. Peaks: 3,5-dinitrobenzoyl derivatives of (1) dihydrodigoxigenin and/or digoxigenin, (2) digoxigenin monodigitoxoside, (3) digoxigenin bisdigitoxoside and (4) digoxin.

along with determination of relative retention times observed for digoxin and its metabolites with those noted by Fujii et al. [10]. The retention times observed in the present study are shown in Table 1. When digoxin was evaluated for degradation using this derivatization procedure, small amounts of the metabolites dihydrodigoxigenin and/or digoxigenin, digoxigenin monodigitoxoside and digoxin bisdigitoxoside were observed in the chromatogram (Fig. 2). Drug-free samples derivatized simultaneously indicated that the reagents were not responsible for the peaks observed with the digoxin sample. There was no evidence of the presence of the metabolites in the original digoxin sample when evaluated by a previous HPLC procedure [11].

HPLC-ED

Single-electrode detection. In order to determine the detection sensitivity limits attainable for the dinitrobenzoyl derivative of digoxin, the recrystallized derivative was evaluated using a mobile phase of acetonitrile–acetate buffer (20:7, v/v) with electrochemical reduction using one glassy carbon electrode of the Model 5012 screened wall jet cell at potentials from 0 to -0.85 V. The results indicated that a potential of -0.80 V gave the greatest response. A second electrode consisting of a gold wall jet cell was also evaluated but did not provide the sensitivity found with the carbon electrode.

One of the difficulties found by many investigators using HPLC for the analysis of digoxin is the close elution of digoxin and dihydrodigoxin. The limits of detection and resolution of derivatized digoxin from derivatized dihydrodigoxin using a variety of mobile phases are shown in Table II. The limit of

TABLE II

SENSITIVITY AND RESOLUTION OF DERIVATIZED DIGOXIN AND DIHYDRODIGOXIN USING HPLC–ED

Chromatographic conditions: HPLC column, Spherisorb ODS II 3 μm (15 cm×4.6 mm I.D.); flow-rate, 1.0 ml/min, Model 5020 pre-injector guard cell electrode, −0.85 V pre-injector; detection, carbon electrode −0.80 V. A = HPLC-grade methanol; B = ethanol; C = HPLC-grade acetonitrile; D = HPLC-grade isopropanol; E = 0.1 *M* acetate buffer at pH 4.6.

Mobile phase composition					Resolution of digoxin and dihydrodigoxin	Limit of detection (derivatized digoxin) (ng)
A	B	C	D	E		
40	3	40	3	15	None	
40	3	40	3	18.4	0.7	8.8
40	3	50	3	20	0.6	
40	3	60	3	22	0.5	2.8
40	3	60	2	22	0.75	2.8
40	3	60	2	22	0.70	2.2

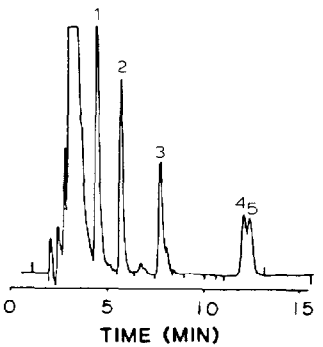

Fig. 3. Chromatogram of digoxin and its metabolites as their 3,5-dinitrobenzoyl derivatives using electrochemical detection. Chromatographic conditions: HPLC column, Spherisorb ODS II 3 μm (15 cm×4.6 mm I.D.); flow-rate, 1.0 ml/min; mobile phase, methanol–ethanol–acetonitrile–isopropanol–acetate buffer (0.1 *M*, pH 4.6) (40 3 60:2 22, v/v); Model 5020 pre-injector guard cell electrode, −0.85 V; detection, carbon electrode −0.80 V. Peaks: 3,5-dinitrobenzoyl derivatives of (1) digoxigenin, (2) digoxigenin monodigitoxoside, (3) digoxigenin bisdigitoxoside, (4) dihydrodigoxin and (5) digoxin.

detection was found to be 2.2 ng of derivatized digoxin (representing 0.98 ng of digoxin) using the recrystallized sample and a mobile phase consisting of methanol–ethanol–acetonitrile–isopropanol–buffer (40:3:60:2:22, v/v). The chromatogram shown in Fig. 3 is representative of the resolution of derivatized digoxin and its metabolites using this mobile phase with electrochemical detection.

Dual-electrode detection. Using a Model 5010 flow cell with two glassy car-

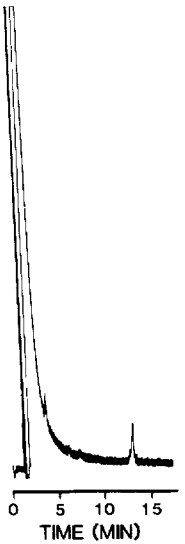

Fig. 4. Chromatogram of 3,5-dinitrobenzoyldigoxin (0.89 ng) using dual-electrode detection. Chromatographic conditions: HPLC column, Spherisorb ODS II 3 μm (15 cm$\times$4.6 mm I.D.); flow-rate, 1.0 ml/min; mobile phase, methanol–ethanol–acetonitrile–isopropanol–acetate buffer (0.1 *M*, pH 4.6) (40:3 60 2:22, v/v); Model 5020 pre-injector guard cell electrode, −0 80 V; detection, first electrode −0.80 V, second electrode +0.80 V; recording second electrode Digoxin elutes at 13 min.

bon carbon electrodes, 3,5-dinitrobenzoyldigoxin was reduced at the first electrode (−0.8 V) and the reduced product was then oxidized at the second electrode (+0.8 V). Recording from the oxidative electrode gave a maximum sensitivity of 0.88 ng (signal-to-noise ratio=5) of the recrystallized sample of derivatized digoxin (equivalent to 0.39 ng of digoxin) as shown in Fig. 4. This procedure decreased the baseline noise seen when recording from the reductive electrode and improved the time for equilibration of the system.

The dual-electrode procedure described provided adequate sensitivity to that required for the determination of therapeutic levels of digoxin in 1 ml of plasma.

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