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Note

Separation of digoxin from dihydrodigoxin and the other metabolites by high-performance liquid chromatography with post-column derivatization

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Dihydrodigoxin (Fig. 1) is formed by the reduction of the C-20–C-22 double bond of the lactone ring of the digoxin molecule and has been found to be the major dihydro metabolite of digoxin in man [1]. The production of this metabolite varies from patient to patient [1–5].

The cardiac activity of dihydrodigoxin has been reported to be about one tenth that of digoxin in laboratory animals [5–8]. The metabolic formation of a significant proportion of the relatively less active dihydrodigoxin from digoxin introduces a problem in digoxin therapy. An appropriate dosage regimen of digoxin would require a knowledge of the plasma digoxin level. This in turn will call for a sensitive analytical method that can specifically monitor digoxin levels in plasma or urine. Radioimmunoassay, although sufficiently sensitive for determining plasma levels of digoxin, lacks specificity, and therefore may measure both digoxin and its metabolites [9]; also digoxin radioimmunoassay has been reported to cross-react with dihydrodigoxin [10].

Radioimmunoassay and high-performance liquid chromatography (HPLC) have been combined for the determination of digoxin, digoxigenin and the mono- and bisdigitoxosides in biological fluids [11,12]. Various other methods, including column, paper and thin-layer chromatography, have been tried for the separation of digoxin and dihydrodigoxin and their derivatives [1,2,5,13–20], but satisfactory resolutions have not been obtained.

An HPLC method that completely separates digoxin from dihydrodigoxin and the other digoxin metabolites is described here. Dihydrodigoxin was detected fluorimetrically after post-column fluorigenic derivatization that employs the air-segmentation principle [21] with a previously unreported 100% fluid recovery

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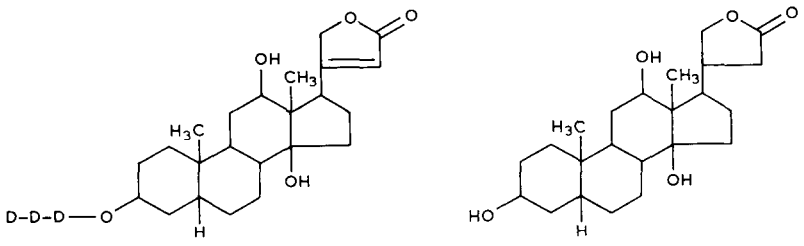

Fig. 1. Structural formulae of digoxin and dihydrodigoxin. Digoxin minus 3, 2 and 1 digitoxose sugar(s) results in digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside, respectively.

set-up. The HPLC separation was monitored with a dual-detector system in which the UV response was followed by the fluorescence detector, resulting in one continuous chromatogram with a total duration of about 36 min. With only UV detection, however, the HPLC separation takes less than 15 min.

EXPERIMENTAL

Apparatus

An Altex (Berkeley, CA, U.S.A.) high-performance liquid chromatograph equipped with a Model 110A pump, a 20-μl loop injection valve and a UV detector set at 254 nm (Altex Model 153) was employed. A 316 stainless-steel column (25 cm$\times$4.6 mm I.D.) pre-packed with Ultrasphere octadecylsilane (C_{18}) of particle size 5 μm was used. Recording was effected with an Omniscribe B-5000 dual-pen

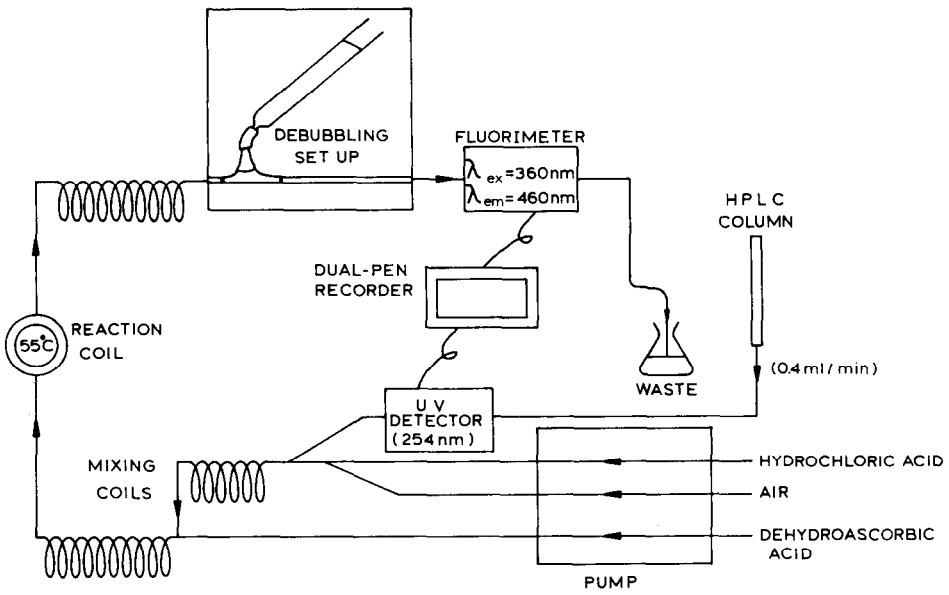

Fig. 2. Schematic diagram of the post-column fluorigenic derivatization system using the air segmentation principle with the 100% fluid recovery set-up.

recorder (Houston Instrument, Austin, TX, U.S.A.). The derivatized compounds were monitored with a Model 420 fluorescence detector (Waters Assoc., Milford, MA, U.S.A.).

The apparatus for the post-column fluorigenic derivatization reaction is a modified version of that reported by Gfeller et al. [21] and is shown in Fig. 2. The reagents and air were pumped using a Technicon pump (Technicon Instruments, Chauncey, NY, U.S.A.).

Materials

Methanol, dichloromethane, isopropanol and water were of HPLC grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). Digoxin, dihydrodigoxin, digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside were purchased from Boehringer (Mannheim, F.R.G.). The reagents that were used for the post-column fluorigenic derivatization were ascorbic acid, hydrogen peroxide [both from the British Drug Houses (Canada), Toronto, Canada], concentrated hydrochloric acid (American Scientific and Chemical, Portland, OR, U.S.A.) and HPLC-grade water. Brij 35 (Atlas Chemical Industries Canada, Brantford, Canada) was used to reduce surface tension and minimize flow resistance.

Methods

The pump tubes (for hydrochloric acid, air and dehydroascorbic acid), the D_2 connectors, the mixing, reaction and cooling coils and the debubbler were similar to the set-up described by Gfeller et al. [21] except that (i) the D_2 connectors and debubbler were replaced by miniaturized ones (of 1 mm I.D.); (ii) the vertical exit of the debubbler was connected by means of Acidflex tubing to an overhanging glass tube; and (iii) the reaction chamber was maintained at 55°C. The HPLC eluate was passed through a UV detector, the reaction system (for post-column derivatization), the debubbler set-up and the fluorimeter before it was directed into the waste receptacle. The tubes that were used for fluid delivery were of the Acidflex type.

HPLC. Samples were dissolved in the eluent and the solution was injected into the chromatograph. The retention time of an individual compound was determined by separate injection of its solution. Solvent systems were prepared in sufficient volumes immediately before use, and degassing was found not to be necessary. The HPLC conditions are given in Fig. 3. The column back-pressure that was caused by the flow-rate at which the solvent system was pumped was below 266 bar.

100% Fluid recovery. The set-up for the 100% fluid recovery system consists of an 84 cm × 1.1 cm I.D. glass tube, tapering at its bottom to 1 mm I.D. where it is connected (by Acidflex tubing) to a debubbler (a modified version of the Technicon C_4 debubbler which in this instance has side-tubes of 1 mm I.D.). The glass tube is positioned as high as possible so that when it is three quarters full with the circulating fluid it exerts sufficient hydrostatic pressure on the surface of the liquid in the debubbler that the air-segmented fluid entering the debubbler will pass through the horizontal exit into the fluorescence detector, whereas the segments of air escape through the vertical exit as bubbles. This debubbling process is optimized so that 100% of the circulating air-segmented fluid will pass through

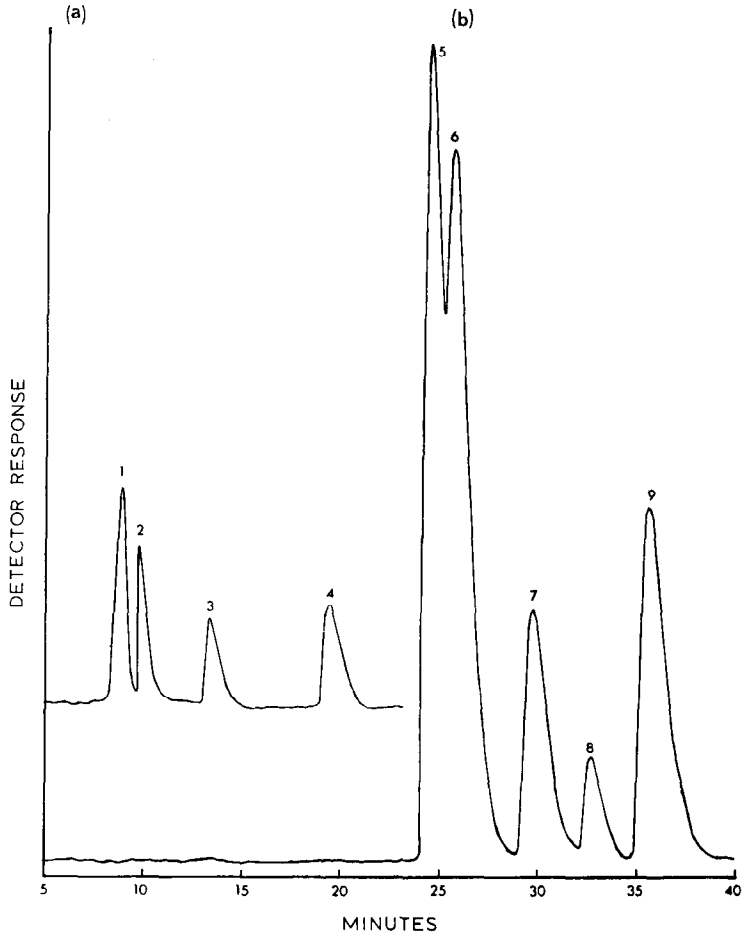

Fig. 3. Chromatogram showing the separation of a standard mixture of digoxin and the other digoxin metabolites as obtained by dual-detector monitoring. (a) Sequence of elution with UV detection: (1) digoxigenin; (2) digoxigenin monodigitoxoside; (3) digoxigenin bisdigitoxodise; (4) digoxin. (b) Sequence of elution with fluorescence detection: (5) digoxigenin; (6) digoxigenin monodigitoxoside; (7) digoxigenin bisdigitoxoside; (8) dihydrodigoxin; (9) digoxin. HPLC conditions: Ultrasphere-ODS column; solvent system, methanol–dichloromethane–water–isopropanol (41:3:50:6); flow-rate, 0.4 ml/min; UV detection at 254 nm, range, 0.02; fluorescence detection, $\lambda_{exc}=360$ nm, λ_{em}, cut-off = 460 nm, gain, 16; chart speed, 0.5 cm/min. The compounds were dissolved in the eluent.

the detector with the exclusion of the air segments by careful up and down adjustments of the position of the waste-containing flask, until the upward pressure (in the debubbler) exerted by the pump is equalized by the hydrostatic pressure of the fluid in the suspended glass tube. The fluid leaving the fluorescence detector had a flow-rate of ca. 1 ml/min and pumping the waste fluid out of the detector was found not to be necessary.

RESULTS AND DISCUSSION

The separation of digoxin (peaks 4 and 9) from dihydrodigoxin (peak 8), digoxigenin (peaks 1 and 5), digoxigenin monodigitoxoside (peaks 2 and 6) and

digoxigenin bisdigitoxoside (peaks 3 and 7) is shown in Fig. 3. The UV and fluorescence detector responses in Fig. 3a and b, respectively, were obtained with a dual-pen recorder after one sample injection. The initial portion of the chromatogram (Fig. 3a) shows the separation of digoxin from digoxigenin and its mono- and bisdigitoxosides after UV detection at 254 nm. Even though the sample also contains dihydrodigoxin, this compound does not have a peak in the UV-monitored chromatogram, as it does not have UV absorbance. After on-line post-column fluorigenic derivatization, however, all five compounds are detected by the fluorimeter after a total chromatographic and derivatization period of about 36 min. This is shown in Fig. 3b, where the separation of digoxigenin monodigitoxoside (6), digoxigenin bisdigitoxoside (7), dihydrodigoxin (8) and digoxin (9) is presented. It can be seen that digoxin (peaks 4 and 9) is completely isolated (baseline separation) from all of the metabolites. The resolution values obtained for the separation of digoxin and dihydrodigoxin (peaks 9 and 8, respectively, Fig. 3), after repeated injections of same sample were found to have a coefficient of variation of 1.8% ($n=10$). Similar calculations for triplicate samples on six consecutive days resulted in an overall coefficient of variation of 6.1%, indicating satisfactory reproducibility.

The presence of dihydrodigoxin in urine as a metabolite of digoxin in man has been reported [17]. Moreover, the possible appearance of the dihydro forms of digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside may not be ruled out [19]. It can be seen that dihydrodigoxin elutes faster than digoxin in the solvent system used in this study. Similarly, one may infer that the dihydro forms of digoxigenin and its mono- and bisdigitoxosides will elute faster than their respective unsaturated analogues. This will mean, therefore, that digoxin will elute last and still maintain its baseline separation. Hence, it appears that this HPLC system has the specificity that is desirable for the quantitation of digoxin in biological fluids by radioimmunoassay.

According to Fig. 3a, digoxin elutes at about 20 min at a flow-rate of 0.4 ml/min. The flow-rate had to be this low because of limitations imposed by the post-column derivatization set-up [21], which was necessary in order to detect dihydrodigoxin. However, for application of this HPLC method to the determination of digoxin in biological fluids, in combination with the radioimmunoassay, the reaction set-up would not be necessary, and so a shorter chromatographic time can be achieved. When the separation is monitored only by UV detection, it has been observed that digoxin elutes in less than 15 min at a solvent flow-rate of 1.2 ml/min.

The debubbling system that was developed for 100% fluid recovery prior to fluorimetric detection (Fig. 2) was found to be stable for long periods. Complete fluid recovery without the presence of any visible air bubbles could be maintained continuously for as long as 8 h. It should to be noted that the 100% fluid recovery system, by virtue of preventing possible sample loss, can be useful for collecting the relatively small amounts of separated dihydro metabolites of digoxin for subsequent quantitation using more sensitive methods. Using the set-up described,

the minimum detectable amount of digoxin (at a signal-to-noise ratio of 4:1) was 10 ng.

The specificity and short chromatographic time of this HPLC separation may make it suitable for the combined HPLC–radioimmunoassay determination of digoxin and its metabolites in biological fluids.

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