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DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–POST-COLUMN FLUOROGENIC ASSAY FOR DIGOXIN IN SERUM

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SUMMARY

A quantitative, sensitive and specific assay for digoxin was developed using a high-performance liquid chromatographic (HPLC) system with post-column (PC) fluorogenic derivatization Separation of digoxin from its metabolites was accomplished using a 15 cm×4 6 mm I D , 3-μm octadecylsilyl HPLC column and an optimum mobile phase of methanol–ethanol–isopropanol–dehydroascorbic acid (52 3 1 45, v/v) Concentrated hydrochloric acid, used as the PC derivatization reagent, was delivered by hexane displacement from a polyvinyl chloride pressure vessel Construction of the pressure vessel is described The mixture of HPLC effluent and PC reagent was passed into a 20-m knitted reactor (PTFE tubing) maintained at 79 0±0 2°C The resultant fluorophores were monitored by a fluorescence detector equipped with a 360-nm excitation filter and a 425-nm emission filter Specificity of this HPLC–PC assay for digoxin in the presence of its metabolites was demonstrated Also, numerous steroids evaluated did not produce fluorescence under these conditions An extraction procedure for evaluating digoxin in serum without interference from endogenous compounds was also developed Detector response to digoxin was linear from 0 5 to 3 3 ng extracted from serum

INTRODUCTION

Digoxin is the most commonly used digitalis glycoside for the treatment of congestive heart failure and certain disturbances of cardiac rhythm The low therapeutic index observed for digoxin and the clinical significance of digoxin therapy have necessitated the development of sensitive analytical methods for the quantitation of digoxin in biological samples. Digoxin may be analyzed by

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several methods including immunoassays, chromatographic procedures and various biological and chemical methods. The lack of specificity of the immunoassay methods for digoxin in the presence of digoxin metabolites [1–9], some co-administered drugs [10,11] and endogenous compounds such as the reported digoxin-like immunoreactive substances (DLIS) [12–16] has led to difficulties in interpretation of assay values. Attempts to compensate for this lack of specificity have included the use of chromatographic systems as elaborate sample handling methods prior to immunoassay [17–24].

The high-performance liquid chromatographic (HPLC) assay described here using post-column (PC) fluorogenic derivatization is a modification of the method initially reported by Kwong and McErlane [25]. While similar chromatographic procedures have been reported in the literature [26–28] they generally did not offer reliable delivery of PC reagents, nor were they evaluated for interference from potentially interfering compounds. An HPLC–PC assay for digoxin in serum offering sensitivity, specificity and dependable delivery of PC reagents is reported here.

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 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 a guard column and placed prior to the Spherisorb ODS II (3 μm), 15 cm×4.6 mm I.D 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 inline filter. The remaining HPLC system was comprised of a Model U6K injector (Waters Assoc , Milford, MA, U S.A.), a Waters Model 420 AC fluorescence detector with a modified quartz flow cell as previously described [25] (40 mm×1 mm I.D. quartz tubing) and an Altex CRIA Chromatopac data processor (Beckman Instrument).

A schematic diagram of the HPLC–PC fluorogenic system is shown in Fig 1 A Beckman Model 110 A single-piston metering system (Beckman Instrument) was used to pump hexane into the polyvinyl chloride (PVC) pressure vessel The reaction bath was maintained at 79 0 ± 0.2 °C using a Haake Model D1 constant-temperature circulator (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

The PVC pressure vessel consisted of two 5.1 cm diameter PVC socket weld flanges (Schedule 80 PVC), two 5.1 cm diameter blind flanges (Schedule 80 PVC) and 36.8 cm of 5.2 cm diameter PVC pipe (Schedule 80 PVC) (Scepter, Vancouver, Canada) Two 5.1 cm diameter full face 0.32 cm Hypalon gaskets (Custom Gaskets, Vancouver, Canada) were used between the pipe section flanges and the blind flanges. The ends of the PVC pipe section and the sockets

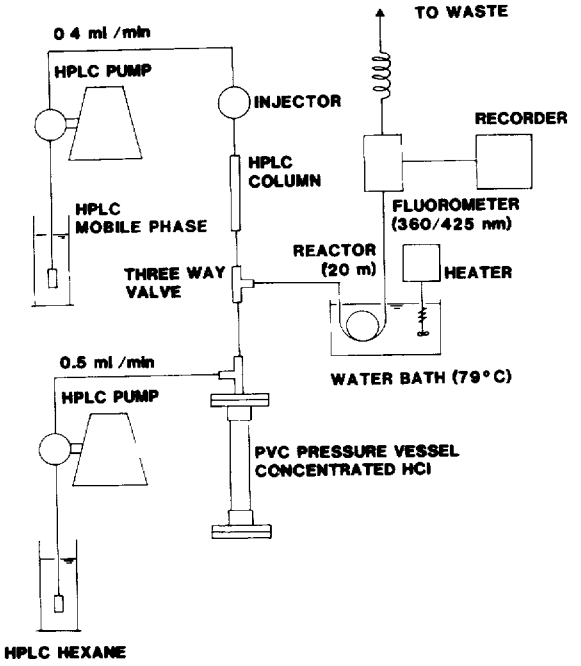

Fig 1 Schematic of HPLC-PC fluorogenic system

of the PVC flanges were joined using PVC glue (Scepter) Bolts (1 3 cm×5 cm National Coarse Thread, 316 stainless steel) (Indufast Fastners, Vancouver, Canada) were used to secure the socket and blind flanges together The top flange was drilled to accept 0.96 cm National Pipe Thread, and Swagelok fittings were used to provide an inlet for hexane and an exit for the concentrated hydrochloric acid. A diagram of the pressure vessel is shown in Fig. 2.

PTFE tubing (0 3 mm I D.) was used for the reactor and connection of the column and pressure vessel to the reactor. A three-way PTFE valve (Part No. 1102 Omnifit, Cambridge, U K.) was placed between the pressure vessel, column and reactor The PTFE tubing and connectors were obtained from Omnifit.

Materials

Digoxin, digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside, digoxigenin, dihydrodigoxin, dihydrodigoxigenin and digitoxigenin were purchased from Boehringer (Mannheim, F R.G) *R,S*-Mexiletine hydrochloride was obtained from Boehringer Ingelheim (Burlington, Canada) L-Ascorbic acid, hydrochloric acid and hydrogen peroxide (30%) were purchased from BDH (Toronto, Canada). Norethindrone, 17α-ethynyl estradiol, estrone, 6α-methyl-17α-hydroxyprogesterone acetate, estradiol, estrone-3-sulfate, testosterone, 19-nortestosterone, 17α-methyltestosterone, adrenosterone, 5α-androstane-3,17-

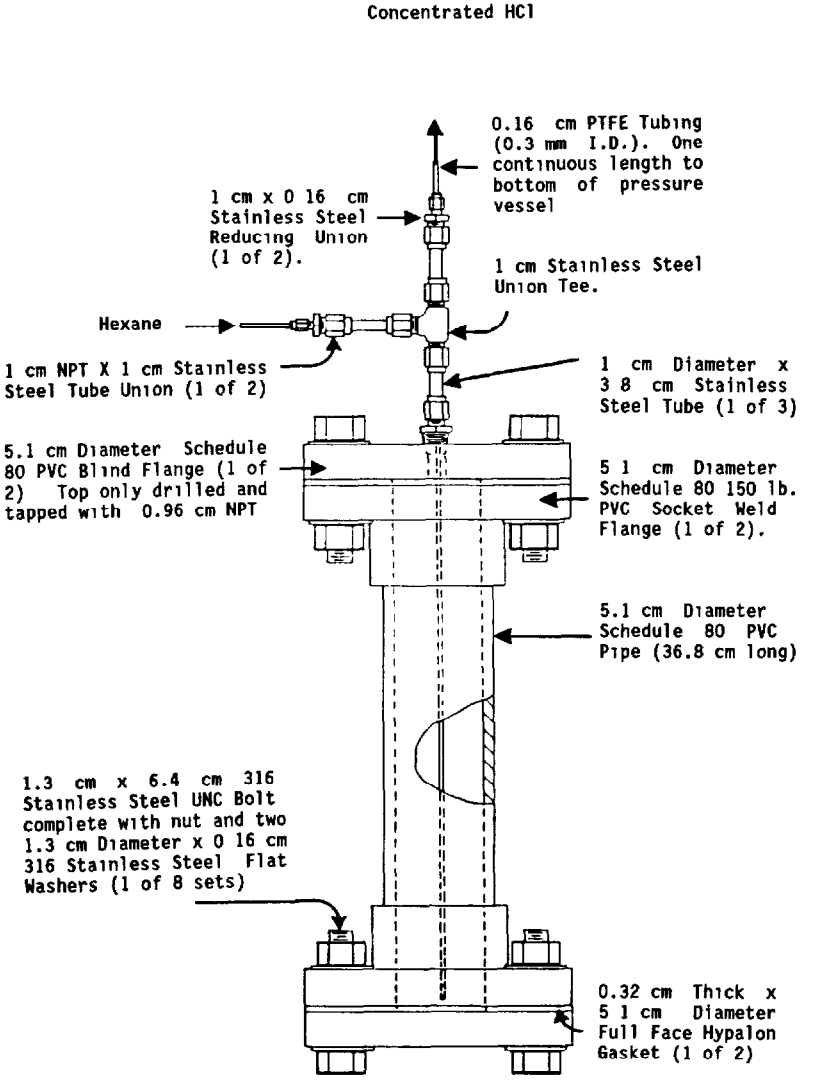

Fig 2 Diagram of the HPLC-PC pressure vessel with fittings

dione, Δ^4-androstene-3,17-dione, Δ^4-androstene-11β-ol-3,17-dione, deoxycorticosterone, 21-deoxycortisone, estriol, hydrocortisone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, Δ^5-pregnene-3β-20α-diol, 5-pregnen-3β-ol-20-one, cortisone, dehydroisoandrosterone, dehydroisoandrosterone-3-sulfate, 5β-pregnane-3α,20α-diol, progesterone and Reichstein's substance S were obtained from Sigma (St Louis, MO, U.S.A.). 17α-Estradiol and 17β-estradiol were samples from Ayerst Labs. (Montreal, Canada).

Reagent-grade absolute ethanol was purchased from Commercial Alcohols (Toronto, Canada). Reagent-grade acetone, methanol and *n*-propanol, glass-

distilled isooctane (2,2,4-trimethylpentane) and the remaining HPLC-grade solvents were purchased from BDH. Purified water was produced using the Milli-Q water purification system (Millipore, Milford, MA, U.S.A.).

Preparation of standard solutions and reagents

A stock solution of digoxin was prepared in ethanol (1 mg per 100 ml). The stock solution was diluted to give final concentrations of 1 5, 2.0, 3 0, 5.0, 7 0 and 10 ng per 10 μl. Calibration curve serum samples were spiked with 10 μl of the final digoxin solutions (1.5, 2, 3, 5, 7 and 10 ng per 3 ml of serum).

Digitoxigenin was used as internal standard for calibration curve samples and as external standard for the recovery study. A stock solution of digitoxigenin was prepared in ethanol (4 mg per 100 ml) and further diluted to give a final concentration of 80 ng per 10 μl. Serum samples were spiked with 20 μl of this as internal standard (160 ng of digitoxigenin).

Solutions of the metabolites of digoxin (digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, dihydrodigoxin and dihydrodigoxigenin), prepared in ethanol at a final concentration of 5 ng per 10 μl, were used for evaluating the separation of digoxin from its metabolites

The fluorogenic reagents dehydroascorbic acid and hydrogen peroxide diluted with phosphoric acid were prepared in the following manner: 250 mg of L-ascorbic acid were prepared in 500 ml of purified water and 1 ml of 30% hydrogen peroxide was prepared in 200 ml of purified water. Dehydroascorbic acid was prepared weekly by mixing dilute hydrogen peroxide (12.5 ml) and ascorbic acid solution (500 ml) and stirring for 2 h.

Hydrogen peroxide diluted with phosphoric acid was prepared by dilution of 40 μl of 30% hydrogen peroxide of 5 ml with purified water and mixing this solution with concentrated phosphoric acid in a 1 1.ratio. The hydrogen peroxide diluted with phosphoric acid was prepared daily.

A 1-mg amount of each of the following steroids was weighed accurately, separately made up to 100 ml in methanol and the amounts indicated in parentheses were assayed by the HPLC–PC procedure. norethindrone (680 ng), 17α-ethynyl estradiol (18 ng), 17α-estradiol (96 ng), 17β-estradiol (37 ng), estrone (55 ng), 6α-methyl-17α-hydroxyprogesterone acetate (25 ng), estriol (55 ng), estrone-3-sulfate (30 ng), testosterone (133 ng), 19-nortestosterone (67 ng) and 17α-methyltestosterone (60 ng). For the following steroids, 1 mg of each was weighed accurately, made up to 10 ml in methanol, and approximately 500 ng of each were evaluated using the HPLC–PC assay: andrenosterone, 5α-androstane-3,17-dione, Δ^4-androstene-3,17-dione, Δ^4-androsten-11β-ol-3,17-dione, deoxycorticosterone, 21-deoxycortisone, estriol, hydrocortisone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, Δ^5-pregnene-3β,20α-diol, 5-pregnen-3β-ol-20-one, cortisone, dehydroisoandrosterone, dehydroisoandrosterone-3-sulfate, 5β-pregnene-3α,20α-diol, progesterone and Reichstein's substance S. A 1-mg amount of *R,S*-mexiletine hydrochloride was

weighed accurately, dissolved in 50% ethanol in purified water and taken to a final volume of 1 ml. This solution was then evaluated by the HPLC–PC fluorogenic assay.

Extraction and filtration equipment

A volac pipette controller for volumes of 1–20 ml (Scienceware, Pequannock, NJ, U.S.A) and Repipet dispenser (Labindustries, Berkeley, CA, U S.A.) were used for dispensing organic solvents during the extraction procedure

The filtration unit for filtering the solvent after extraction consisted of a Nylon 66 membrane (0.45 μm, 13 mm diameter) filter disc (Rainin, Woburn, MA, U.S A) in a Swinnex 13 Millipore filter holder (Millipore) attached to a 5-ml Luer-Lock Multifit B-D glass syringe (Becton Dickinson Canada, Mississauga, Canada).

A Vortex-Genie mixer (Fisher Scientific), a Labquake shaker (Labindustries, Berkeley, CA, U.S.A) and an IEC HN-SII centrifuge (Damon/IEC Division, Western Scientific, Vancouver, Canada) were used in the extraction of biological samples.

FP Vericel 47 mm, 0.45 μm membrane filters (Gelman Sciences, Ann Arbor, MI, U.S.A) were used with the Millipore all-glass filter apparatus (Millipore) for filtration of the mobile phase

Extraction procedure and standard curve samples

A modified serum extraction procedure previously described [29] was used. The isooctane wash (2 ml) was replaced by a 2-ml isooctane–dichloromethane (20 5) solvent wash to remove endogenous interference and reduce the amount of emulsion formed with the extraction solvent. For standard curve samples, 3 ml of blank serum were spiked with 10 μl of digoxin in ethanol (1.5–10 ng per 10 μl). For all standard curve and patient samples, 20 μl of internal standard solution (160 ng) were added to the serum After addition of 3 ml acetone, the sample was vortex-mixed for 20 s, centrifuged for 5 min at 1000 *g*, and the aqueous–acetone supernatant layer was transferred to a clean tube. This solution was then washed with 2 ml of isooctane–dichloromethane (20 5), vortexed for 60 s, centrifuged for 5 min at 1000 *g*, and the aqueous–acetone layer was partially dried under nitrogen using a 37°C water-bath for 20 min. The remaining aqueous layer was extracted twice with 10 ml of extraction solvent (dichloromethane–*n*-propanol, 98 2) and the combined organic phases were filtered (Nylon 66 membrane) and then dried under nitrogen in a 10-ml screw-capped PTFE-lined culture tube The residue was resuspended with 100 μl of methanol–water (50 50) in the same tube The entire sample was injected into the HPLC–PC system using a 100-μl Hamilton syringe (Hamilton, Reno, NV, U S.A).

Recovery and precision

The recovery of digoxin from serum using the final extraction procedure was evaluated. Serum samples (3 ml) to which 1.5, 3 and 10 ng of digoxin were added were extracted as described above. External standard solution (80 ng of digitoxigenin in ethanol) was added just prior to analysis. Recovery was calculated by comparing the peak-height ratio (digoxin to external standard) for extracted serum samples to that for unextracted samples of identical quantities

The precision of the extraction procedure was determined by repeated extraction of five serum samples (3 ml) spiked with 3 ng of digoxin and 160 ng of internal standard for four consecutive days.

RESULTS AND DISCUSSION

HPLC–PC assay development

Improvements to previously reported HPLC–PC fluorescence assay methods [25–27] are based on altering the method of delivering the PC reagents Concentrated hydrochloric acid was displaced from the pressure vessel by hexane which allowed for reliable delivery of the acid. The peristaltic pumps previously used for delivery of PC reagents [25–27] severely limited the choice of flow-rate and reactor size. These limits were effectively removed by using a second HPLC pump and hexane displacement of hydrochloric acid from a pressure vessel. PVC was the best material available for construction of the pressure vessel since it is easy to obtain, uses standard industrial pipe and fittings, is resistant to both concentrated hydrochloric acid and hexane and is light weight allowing for more convenient cleaning and filling with acid

The flow-rate of hydrochloric acid and the PC reactor length were optimized together since they both influence the time available for derivatization to occur. Changing the hexane flow-rates from 0.5 to 1 0 ml/min, with the 10-m reactor, gradually reduced the peak height (Table I) The shorter time spent in the reactor with the 1.0 ml/min flow-rate did not allow the development of maximum fluorescence. In order to increase the reaction time without reducing the hexane flow-rate below 0.5 ml/min, a 20-m reactor was used. With the 20-m reactor, the greatest sensitivity was obtained with a hexane flow-rate of 0.5 ml/min (direct injection of 0.585 ng digoxin with a signal-to-noise ratio of 4 1). Reduced baseline noise was also observed with the 20-m reactor

HPLC–PC fluorogenic assays reported for digoxin used concentrated hydrochloric acid derivatization and dehydroascorbic acid [25–27] or hydrogen peroxide solutions [28] for fluorescence enhancement. The HPLC–PC assay for digoxin reported by Reh and Jork [28] used a mobile phase of methanol–water–hydrogen peroxide diluted with phosphoric acid and PC addition of concentrated hydrochloric acid for digoxin derivatization. This allowed for separation of digoxin from digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, digoxigenin and lanatoside C [28]. Since it is desirable to resolve digoxin from

TABLE I

EFFECT OF HEXANE FLOW-RATE ON PEAK HEIGHT OF DIGOXIN USING THE HPLC-PC FLUORESCENCE ASSAY

Chromatographic conditions column, Spherisorb ODS II, 3-μm analytical column (15 cm×4 6 mm I D), mobile phase, methanol-ethanol-isopropanol-dehydroascorbic acid (52 3 1 45), HPLC flow-rate, 0 5 ml/min, post-column reactor, 10 m in 79 0±0 2°C water-bath, detection, fluorometer equipped with 360-nm excitation and 425-nm emission filters

Hexane flow-rate (ml/min)	Mean peak height (n=4) (cm)
0 50	9 3
0 70	7 6
1 00	5 4

TABLE II

EFFECT OF VARYING THE AQUEOUS PORTION OF THE MOBILE PHASE ON PEAK HEIGHT USING THE HPLC-PC FLUORESCENCE ASSAY

Chromatographic conditions column, Spherisorb ODS II, 3-μm analytical column (15 cm×4 6 mm I D), mobile phase, methanol-ethanol-isopropanol-aqueous solution (52 3 1 45), HPLC flow-rate, 0 5 ml/min, hexane flow-rate, 0 5 ml/min, post-column reactor, 10 m in 79 0±0 2°C water-bath, detection, fluorometer equipped with 360-nm excitation and 425-nm emission filters

Aqueous portion of mobile phase	Peak height (cm)
Water–hydrogen peroxide diluted with phosphoric acid (30 15)	1 1
Water–hydrogen peroxide diluted with phosphoric acid (25 20)	1 9
Dehydroascorbic acid (45)	4 9
Dehydroascorbic acid–water (35 10)	4 4
Dehydroascorbic acid–hydrogen peroxide diluted with phosphoric acid (35 10)	3 5
Dehydroascorbic acid–hydrogen peroxide diluted with phosphoric acid (25 20)	3 9

dihydrodigoxin, the aqueous portion of a mobile phase previously reported to partially resolve digoxin from dihydrodigoxin [25] was replaced by the combination of hydrogen peroxide diluted with phosphoric acid and dehydroascorbic acid shown in Table II. Maximum sensitivity was obtained with a mobile phase consisting of 45 ml of dehydroascorbic acid, 52 ml of methanol, 3 ml of ethanol and 1 ml of isopropanol.

Maximum sensitivity

The maximum sensitivity obtained with the optimized HPLC–PC conditions given above using a 360-nm excitation filter and 425-nm emission filter combination was 0.5 ng digoxin per injection (directly) and 1 5 ng digoxin per 3 ml serum as shown in Fig 3

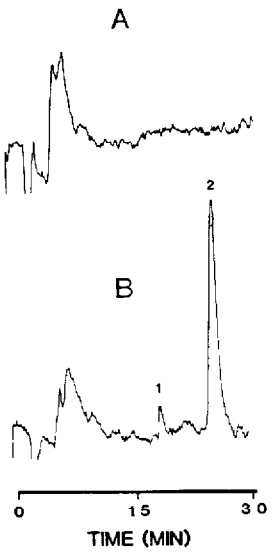

Fig 3 Chromatogram of (A) blank serum and (B) serum spiked with 1 5 ng of digoxin and internal standard Chromatographic conditions column, Spherisorb ODS II 3-μm analytical column (15 cm×4 6 mm I D), mobile phase, methanol–ethanol–isopropanol–dehydroascorbic acid (52 3 1 45), HPLC flow-rate, 0 4 ml/min, hexane flow-rate, 0 5 ml/min, post-column reactor, 20 m in 79 0 ± 0 2 °C water-bath, detection, fluorometer equipped with 360-nm excitation and 425-nm emission filters Peaks 1 = digoxin, 2 = internal standard (digitoxigenin)

For other HPLC–PC digoxin assay methods, the maximum sensitivity values reported were 0.1 ng digoxin per injection [28], 0 5 ng digoxin per injection [27] and 10 ng digoxin per injection [26]. The HPLC–PC assay described here is less sensitive than that described by Reh and Jork [28] but is still preferable for evaluating patient samples since partial resolution of digoxin from dihydrodigoxin was obtained. This HPLC–PC method has equal or better sensitivity than the other HPLC–PC methods reporting sensitivity to digoxin [25,27]. Removal of the problems associated with using a peristaltic pump for PC reagent delivery [25,27] make the HPLC–PC system developed here more appropriate for routine use.

The sensitivity of immunoassay methods and HPLC–radioimmunoassay (RIA) procedures for digoxin is considerably greater than that obtained for HPLC–PC methods of analysis. Immunoassay methods alone lack the desired specificity for digoxin, and chromatographic separation of digoxin from dihydrodigoxin was not described by most HPLC–RIA methods of digoxin analysis [17–20,22] The HPLC–RIA assay method that partially separates digoxin from dihydrodigoxin does not describe the resolution obtained [21,24] Therefore, the HPLC–PC assay described here is superior for digoxin analysis when adequate sample volumes (3 ml of serum) are available

TABLE III

RECOVERY OF DIGOXIN FROM SERUM SAMPLES

Concentration (ng per 3 ml of serum)	Recovery (%)	C V (%)	*n*
1 5	72 0	6 4	7
3 0	78 2	7 2	8
10 0	78 8	6 1	7

TABLE IV

PRECISION OF DIGOXIN ASSAY

Day	C V (%)
1	4 6
2	5 7
3	6 6
4	5 3
Mean	5 6

Recovery and precision

The recovery of digoxin was determined for blank serum spiked with 0.5, 3.0 and 10 ng of digoxin. Table III shows the percentage recovery for each concentration.

The precision of the HPLC–PC assay was determined using digoxin in ethanol rather than repeated injections from one extracted serum sample since the entire extracted serum sample was required for analysis. For the HPLC–PC assay the coefficient of variation (C.V.) for 1.5- and 10-ng injections of digoxin in ethanol was 4.7% ($n=4$) and 3.3% ($n=10$), respectively.

The precision of the extraction procedure was determined by repeated extraction of five blank serum samples (3 ml) spiked with 3 ng of digoxin and 20 μl of internal standard (160 ng) followed by HPLC–PC analysis and comparison of peak-height ratios The within-day C.V. is shown in Table IV. Analysis of variance for peak-height ratio means [$F_{(3,16)}=0\,184$ ($p=0.49$)] and for equality of variance [$F_{(3,16)}=0.22$ ($p=0\,88$)] verifies that there is no significant difference in day-to-day analysis

A greater inter-assay C.V. (8%) was reported for a similar extraction procedure [29]. This indicates that in comparison to this previous method the extraction procedure and HPLC–PC assay described here has a greater precision in relation to the mean values.

Calibration curve

Serum samples (3 ml) were spiked with 1–3.3 ng digoxin per ml and internal standard, extracted and analyzed using the HPLC–PC fluorogenic assay. The

peak-height and concentration ratios were then calculated using average values from four separate determinations. The correlation coefficient of 0.9876 and the y-intercept (0.0273) were not significantly different from zero (t-ratio = 1.23). The correlation coefficient observed here indicates that 97.5% of the total variability in the height and weight ratios are accounted for by mutual dependence of these ratios

Specificity

Digoxin metabolites. Using the final HPLC–PC system, digoxin is resolved from digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and dihydrodigoxigenin as shown in Fig 4. The resolution between digoxin and dihydrodigoxin ($r = 0.899$) was sufficient for peak-height quantitation. Since the HPLC–PC system separated digoxin from all of its metabolites, there was no interference from these metabolites in the analysis of digoxin.

Resolution of digoxin from dihydrodigoxin was not described for HPLC–RIA methods for digoxin analysis [17–20,22]. Along with the lack of specificity of the RIA used for digoxin in the presence of DLIS [30,31], interference from dihydrodigoxin may occur with these HPLC–RIA methods. Since the potential for cross-reactivity of dihydrodigoxin and DLIS exists with the RIA, the HPLC–PC method described in this report is better than the HPLC–RIA methods with respect to specificity for digoxin. The HPLC–RIA methods claiming partial separation of digoxin from dihydrodigoxin did not describe the resolution obtained by their chromatographic system [21,24].

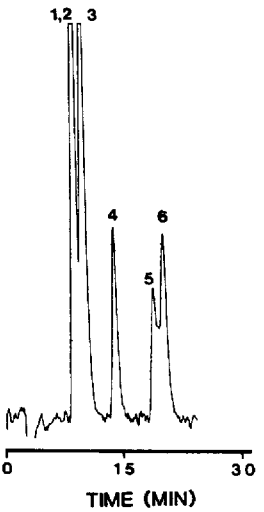

Fig 4 Chromatogram of digoxin and its metabolites by HPLC–PC Chromatographic conditions same as for Fig 3 Peaks 1 = digoxigenin, 2 = dihydrodigoxigenin, 3 = digoxigenin monodigitoxoside, 4 = digoxigenin bisdigitoxoside, 5 = dihydrodigoxin, 6 = digoxin

The HPLC assays using PC fluorogenic derivatization for digoxin reported by Gfeller et al. [26] and Reh and Jork [28] did not describe separation of digoxin from dihydrodigoxin. Baseline resolution of digoxin from dihydrodigoxin has been reported [27] but the sensitivity of this assay for digoxin was not adequate for therapeutic monitoring. The resolution of digoxin from dihydrodigoxin obtained with the HPLC–PC assay described here was slightly less than previously reported ($r=0.91$) [25]. Although the sensitivity of this assay was sufficient for evaluating patient samples [25,29], long chromatography time (40 min) and unreliable delivery of PC reagents made it less suitable for routine clinical use

Steroids Numerous steroids have been reported to cross-react with digoxin antisera [32–34]. Since cross-reactivity with digoxin antisera was reported, the specificity of immunoassay methods using these antibodies is questionable. Matthewson [34] reports that the following steroids eluted near the DLIS fractions using reversed-phase HPLC with RIA detection: dehydroepiandrosterone-3-sulfate, cortisone, cortisol, deoxycortisone, Δ^4-androstene-3,17-dione, progesterone and glycochenodeoxycholic acid. Samples of endogenous and synthetic steroids, including five of the steroids reported by Matthewson [34] to cross-react with the digoxin antisera, were evaluated to ensure that they did not interfere with the HPLC–PC assay procedure. No fluorescent peaks were observed after direct injection of these steroids in methanol. The steroids evaluated either do not elute from the HPLC system or do not produce a fluorescent product under these conditions and, therefore, would not interfere with the HPLC–PC analysis of digoxin.

Co-administered drugs It is imperative that drugs which may be co-administered with digoxin be evaluated for interference with the analytical method. Using a similar HPLC–PC assay for digoxin the following drugs were previously evaluated [25]: spironolactone, furosemide, disopyramide, captopril, dipyridamol, quinidine, verapamil, propafenone, procainamide and trimethoprim sulfamethoxazole. Only furosemide and spironolactone yielded a fluorescent response and under the conditions used they were chromatographically separated from digoxin [29]. The newer antiarrhythmic agent, mexiletine, was not previously evaluated. Therefore, mexiletine was assayed using the HPLC–PC assay developed here to determine if any fluorescence was produced under these conditions. No fluorescence was found on injection of 7 5 μg of mexiletine which would be the maximum expected in 3 ml of serum from patients within the therapeutic range for this drug [35].

CONCLUSIONS

An HPLC–PC fluorogenic assay using concentrated hydrochloric acid and dehydroascorbic acid derivatization was developed. Hexane, delivered by a second HPLC pump, was used to displace the concentrated hydrochloric acid from

a pressure vessel allowing for reliable and relatively pulse-free flow of acid into the PC reactor. Dehydroascorbic acid was added to the aqueous portion of the HPLC mobile phase. The chromatographic column separated digoxin from its metabolites prior to derivatization (Fig. 4) allowing for quantitation of digoxin in digitalized patient samples where metabolites may be present.

Numerous steroids have been reported to cross-react with digoxin antisera [32–34]. Steroid samples evaluated by the HPLC–PC assay in this study either do not elute from the HPLC system or do not produce a fluorescent product under these conditions. This indicates that the HPLC–PC assay developed here would be able to quantitate digoxin in the presence of the steroids tested.

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