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ANALYSIS OF DIGOXIN AT THERAPEUTIC CONCENTRATIONS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN DERIVATIZATION

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SUMMARY

A high-performance liquid chromatographic (HPLC) procedure has been developed for the analysis of digoxin in plasma at therapeutic concentrations. The assay method provides resolution of digoxin from its metabolites using a 15 cm × 4.6 mm HPLC column containing 3-μm octadecylsilane-bonded stationary phase. The effluent of the column is passed through a post-column reactor in which a fluorescent derivative is formed by the co-addition of hydrochloric acid and dehydroascorbic acid. Detection of the derivative is accomplished in a fluorometer with excitation at 336 nm and emission at 425 nm. The extraction efficiency for recovery of digoxin from plasma samples was 70% using chloroform—isopropanol (9:1) following a pre-wash with isooctane to remove endogenous substances. The calibration curve was linear (r = 0.9999) over the range 0.5—4 ng/ml digoxin in plasma using digitoxigenin as internal standard. The minimum detectable quantity of digoxin in plasma was 0.5 ng/ml at a signal-to-noise ratio of 4:1. Split-samples of digoxin control sera were assayed by the HPLC procedure and by the prescribed radioimmunoassay procedure. Excellent correlation was observed between the two methods (r = 0.999). No interference was noted when a selection of commonly co-prescribed drugs were evaluated for chromatographic co-elution or interference in detection with that of digoxin or the internal standard.

INTRODUCTION

The specific detection and the accurate quantitation of digoxin in biological fluids are problems encountered in drug plasma level monitoring. Because of the low therapeutic index of digoxin and the possibility of interference from metabolites and endogenous substances, a specific and sensitive analytical technique is essential. Although a host of biochemical methods have been

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reported for the measurement of digoxin in biological fluids, the principal methods currently employed in hospital laboratories are based on radioimmunoassay (RIA) procedures. While these methods are sensitive, the antibodies involved have frequently been reported to be non-specific and subject to cross-reactivity with some of the less cardioactive metabolites of digoxin [1] as well as with endogenous substances [2, 3].

Chromatographic methods, on the other hand, allow for the resolution of digoxin and hence provide freedom from interference from its metabolites, other drugs and plasma constituents. Chromatographic methods reported thus far include gas—liquid chromatography alone [4] or in combination with thin-layer chromatography [5] or mass spectrometry [6], thin-layer chromatography [7] and high-performance liquid chromatography (HPLC) [8–11]. Among the latter methods, two procedures [10, 11] have been developed which involve the collection of timed fractions of the column eluate and subsequent measurement by one of the RIA procedures. While the sensitivity of these HPLC—RIA methods was sufficient to monitor therapeutic plasma concentrations, the collection of timed column eluates may introduce methodological errors.

In the present study, an efficient extraction procedure was combined with a previously reported [12] HPLC assay method involving fluorogenic post-column derivatization to selectively determine digoxin at therapeutic plasma concentrations.

EXPERIMENTAL

Materials

Water, methanol, isopropanol, *n*-propanol and dichloromethane were of HPLC-grade quality and obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Glass-distilled quality isooctane was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Acetone and absolute ethanol were reagent-grade quality and were obtained from local suppliers.

The filter unit used in the extraction procedure consisted of a Nylon-66® membrane (0.45 μm, 13 mm diameter) and filter disk (Rainin Instruments, Woburn, MA, U.S.A.) housed in a Swinnex-13® Millipore filter holder (Millipore, Milford, MA, U.S.A.). This unit was used on a Luer-Lok® 5-ml Becton-Dickinson glass syringe (Becton-Dickinson, Mississauga, Canada). Digoxin, dihydrodigoxin, digoxigenin monodigitoxoside, digoxigenin bis-digitoxoside, digoxigenin and digitoxigenin were obtained from Boehringer (Mannheim, F.R.G.). Spironolactone, furosemide and quinidine were purchased from Sigma (St. Louis, MO, U.S.A.). Disopyramide was obtained from Roussel (London, U.K.) and procainamide and captopril were obtained from Squibb (Montreal, Canada). Propafenone was obtained from Knoll Pharmaceutical (Vaudreuil, Canada) and verapamil from G.D. Searle (Oakville, Canada). Trimethoprim-sulfamethoxazole was obtained from Hoffman-La Roche (Vaudreuil, Canada) and dipyridamole from Boehringer Ingelheim (Burlington, Canada).

Method

The HPLC post-column fluorogenic derivatization procedure has been

previously described [13]. Basically it consists of the post-column addition of hydrochloric acid and dehydroascorbic acid to the effluent of the HPLC column. The resultant fluorescent derivative of digoxin is detected in a fluorometer at an excitation wavelength of 360 nm and an emission wavelength of 425 nm.

Extraction procedure and calibration curve

Digoxin stock solutions were prepared in concentrations of 0.5, 2.5, 3.0, 6.0, 8.0 and 12.0 ng per 10 μl in methanol. The internal standard, digitoxigenin, was prepared at 80 ng per 10 μl of methanol. Six aliquots of 10 μl of each of digoxin stock solutions along with 10 μl of the internal standard solutions were placed in six PTFE-lined screw-capped centrifuge tubes each containing 3 ml of plasma. A blank sample of plasma without digoxin or digitoxigenin was also prepared. To effect precipitation of the proteins, 3 ml of acetone were added and the tubes were agitated on a vortex mixer (Vortex-Genie, Fisher Scientific) and subsequently centrifuged at 1000 *g* for 5 min. The supernatant was washed with 2 ml of isooctane by agitation on a vortex mixer and centrifuged at 1000 *g* for 5 min. The acetone—aqueous layer was removed and evaporated to a volume of approximately 3 ml under a gentle stream of nitrogen at 37°C. The remaining aqueous solution was extracted twice with 10 ml of dichloromethane containing 2% *n*-propanol by rotating the tubes on a Roto-Rak (Fisher Scientific) for 10 min. After centrifugation at 1000 *g* for 5 min the organic layer was removed and filtered through a Nylon-66 membrane housed in a Swinnex-13 filter holder. The resulting solution was evaporated to dryness under nitrogen at 37°C and reconstituted with 100 μl of water—methanol (50:50). The entire sample was injected onto the HPLC column.

Extraction efficiency

To determine the recovery of digoxin from plasma six aliquots of the digoxin stock solutions containing 0.5, 2.5, 3.0, 6.0, 8.0 and 12.0 ng of digoxin in methanol along with 80 ng of the internal standard, digitoxigenin were placed in each of six PTFE-lined screw-capped centrifuge tubes and these were brought to a volume of 100 μl with water—methanol (50:50). Each solution was injected onto the HPLC column.

Precision and sensitivity of assay

The assay precision was determined by preparing triplicate aliquots of plasma (3 ml) at each concentration of digoxin (0.5, 2.5, 3.0, 6.0, 8.0 and 12.0 ng) along with the internal standard (80 ng). The samples were extracted in the manner described above. Each extracted sample was injected once onto the HPLC column. The minimum detectable quantity was determined from these samples at a signal-to-noise ratio of 4:1. Additionally, day-to-day assay variability was determined by the preparation of triplicate plasma samples, each containing 2, 3 and 5 ng/ml digoxin along with 80 ng of the internal standard, digitoxigenin. These samples were prepared daily for three days and processed and analysed as described above.

Comparison of the HPLC procedure with the RIA method

Lypochek radioassay control sera (human), levels I, II and III (Environmental Chemical Specialties, Anaheim, CA, U.S.A.), were assayed by RIA according to the suppliers-recommended procedure (Nuclear Medical Labs., Dallas, TX, U.S.A.). The same samples were also extracted and assayed by the HPLC procedure described above. Triplicate samples were prepared of the three levels.

Determination of interference from other drugs

Solutions of the unionized forms of a number of drugs commonly co-prescribed were prepared. Thus, methanolic solutions of spironolactone, furosemide, disopyramide, captopril, dipyridamole, quinidine, verapamil, propafenone, procainamide and trimethoprim-sulfamethoxazole were prepared at concentrations of 3 mg/ml in methanol and 10 μl of each solution were injected onto the HPLC column.

A combined 10-ml stock solution of digoxin, dihydrodigoxin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and digoxigenin was prepared at a concentration of 5 ng per 10 μl of each glycoside and digitoxigenin was added at a concentration of 80 ng per 10 μl. An aliquot of 10 μl of this solution was injected onto the HPLC column to determine the elution pattern of digoxin and its metabolites.

RESULTS AND DISCUSSION

A comparative study was undertaken to determine the most efficient extraction method for the recovery of digoxin from plasma. Preliminary experiments indicated that 80% of the known quantity of digoxin could be extracted from plasma with a solvent combination of chloroform–isopropanol (9:1). However, endogenous substances co-extracted in this system prevented reliable quantitation in the resulting chromatogram. It was noted that protein precipitation with agents such as trichloroacetic acid or hydrochloric acid led to

TABLE I

CALIBRATION CURVE DATA

Weight ratio*	Peak-height ratio**
0.022	0.210 ± 0.01
0.029	0.300 ± 0.02
0.044	0.450 ± 0.04
0.073	0.730 ± 0.07
0.102	1.02 ± 0.06
0.147	1.47 ± 0.10

Slope = 9.98
y-Intercept = 0.003
r = 0.9999

*Weight ratio calculated as digoxin weight/internal standard weight.
**Peak-height ratio calculated as digoxin peak height/internal standard peak height; values are the mean ± S.D. of triplicate analyses.

breakdown of the acid-labile glycoside, digoxin. Acetone, on the other hand, has been reported to be an efficient protein precipitant [14] and was found to provide a chromatographically cleaner extract. However, the appearance of an interfering peak in some plasma samples necessitated a pre-wash step with a neutral solvent. Hexane, heptane, benzene and isooctane effectively removed the interfering substance, however, isooctane provided a higher recovery of digoxin (70%).

Linearity of detection and assay precision were determined from the calibration curves. Triplicate samples of each of six concentrations of digoxin (0.5—4 ng/ml) containing the internal standard, digitoxigenin, were injected onto the HPLC column. The data given in Table I show the mean slope for the calibration curve. The minimum detectable quantity of digoxin was found to be 0.5 ng/ml at a signal-to-noise ratio of 4:1. Hence the sensitivity of the procedure is similar to that of the RIA methods. The chromatograms in Fig. 1 are representative of a typical 3-ml blank plasma extract, an extract to which had been added 3.0 ng/ml digoxin and 80 ng of the internal standard, and a plasma sample obtained from a patient receiving digoxin. The quantity of digoxin determined in this case was 1.8 ng/ml. Evaluations of plasma obtained from healthy volunteers, the Red Cross and a local hospital blood bank did not reveal any evidence of extraneous peaks in the chromatogram in the areas of elution of digoxin and the internal standard.

The inter-assay variability was determined by analysing triplicate samples of digoxin prepared in 3 ml of plasma at quantities of 2, 3 and 5 ng/ml, along with 80 ng of the internal standard. Each analytical method was repeated at one-day intervals. The coefficients of variation observed for these studies were 10% at the 2-ng level and 8% at the 3- and 5-ng levels (n = 9). The intra-assay variability was determined on one set of triplicate samples containing 2, 3 and 5 ng of digoxin. Analysis of these triplicate samples yielded coefficients of variation of 4% for the 2- and 3-ng levels and 5% for the 5-ng level (n = 9).

The specificity of the HPLC post-column procedure was evaluated by injection of several drugs frequently co-prescribed with digoxin. The selection of

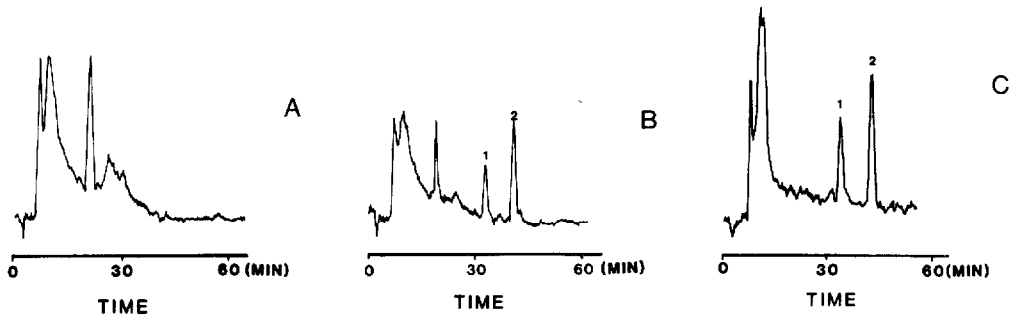

Fig. 1. Typical chromatograms of blank plasma (A), spiked plasma containing 3 ng of digoxin and 80 ng of digitoxigenin in a 3-ml plasma aliquot (B) and a sample obtained from a patient receiving digoxin therapeutically (quantity determined was 5 4 ng/ml in a 3-ml plasma aliquot, or 1.8 ng/ml) (C). Chromatographic conditions: mobile phase, methanol—ethanol—isopropanol—water (52:3:1:45); flow-rate, 0.3 ml/min; post-column fluorescence detection using non-segmented reaction system. Peaks: 1 = digoxin; 2 = digitoxigenin, internal standard.

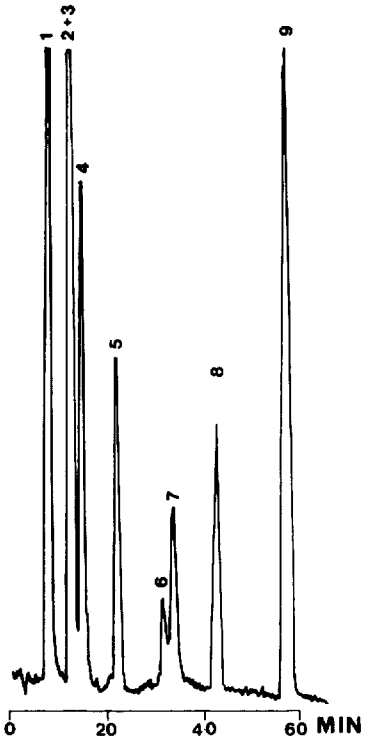

Fig. 2. Chromatogram of digoxin, its metabolites and co-administered drugs using post-column detection. Chromatographic conditions: same as Fig. 1. Peaks: 1 = furosemide, 2 = dihydrodigoxigenin; 3 = digoxigenin; 4 = digoxigenin monodigitoxoside; 5 = digoxigenin bisdigitoxoside; 6 = dihydrodigoxin; 7 = digoxin; 8 = digitoxigenin, internal standard, 9 = spironolactone.

these agents was based on a local survey of cardiologists and is therefore considered to be representative. Accordingly, aliquots of methanolic solutions of furosemide, spironolactone, quinidine, procainamide, disopyramide, dipyridamole, verapamil, propafenone, captopril and trimethoprim-sulfamethoxazole were injected onto the HPLC column. The quantities of each drug injected were above therapeutic levels in order to ascertain that co-elution with digoxin or the internal standard would not be a problem. In this experiment, only furosemide and spironolactone yielded a response under the conditions employed for the post-column fluorogenic reaction as shown in Fig. 2. Also included in this chromatogram are the reported metabolites of digoxin: digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and dihydrodigoxin. Although the resolution of the latter metabolite from digoxin was incomplete, interference of dihydrodigoxin with peak-height measurement of digoxin was minor. In addition, the fluorogenic response of dihydrodigoxin is approximately one half of that of digoxin, hence only minimal quantities would be observed in therapeutic drug monitoring.

In order to compare the HPLC post-column assay for digoxin with an established procedure, a RIA method was employed. Control sera were obtained that offered three-level ranges of digoxin in lyophilized plasma samples. Such samples are frequently used for intra-laboratory quality control

in clinical laboratories. When triplicate samples at each concentration were analysed by the HPLC method and by the prescribed RIA method, excellent correlation between the two techniques was observed (slope = 0.92, intercept 0.07, $r = 0.999$).

In summary, the HPLC post-column procedure developed has been shown to be sufficiently sensitive to assay digoxin at therapeutic levels and unlike the RIA procedure is not susceptible to interference from the metabolites of digoxin.

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