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DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR DIGOXIN USING POST-COLUMN FLUOROGENIC DERIVATIZATION

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SUMMARY

An efficient high-performance liquid chromatographic (HPLC) separation for digoxin and its metabolites has been developed. Quantitation of digoxin at plasma levels was possible after the column effluent was passed through a fluorogenic post-column reactor. A study of the optimum post-column conditions was undertaken using a combination of ascorbic acid, hydrogen peroxide and hydrochloric acid, which was known to induce fluorescence in the digoxin molecule. Digoxin and its metabolites were separated on a 15 cm × 4.6 mm I.D., 3-μm reversed-phase (C_{18}) HPLC column using methanol—ethanol—isopropanol—water (52 : 3 : 1 : 45) as the mobile phase at a flow-rate of 0.3 ml/min. A solution of $1.1 \cdot 10^{-3}$ *M* hydrogen peroxide in a 0.1% ascorbic acid solution and concentrated hydrochloric acid were added into the post-column reactor through a peristaltic pump at a combined flow with a flow-rate of 0.23 ml/min. The mixture was passed into the 20-m reaction coil maintained at 79 ±1°C. The resulting digoxin fluorophore was monitored with a fluorescence detector. Detector responses were linear from 1.5 to 10 ng injected on-column. The overall performance demonstrated that this system has the sensitivity, linearity and stability desired in a digoxin plasma level determination. The total chromatographic time including the post-column derivatization step was about 40 min.

INTRODUCTION

Digoxin is the most widely used cardiac glycoside for the treatment of congestive heart failure and atrial fibrillation. However, digoxin exhibits a narrow therapeutic window and consequently toxic manifestations are frequently encountered during establishment of dosing regimens. For this reason, the dosages must be carefully adjusted and the levels in plasma or serum are routinely monitored to achieve optimum therapeutic effect while avoiding toxic episodes.

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Because the therapeutic concentrations are in the low nanogram range (0.5–3 ng/ml), highly sensitive assay techniques are required for measurement. Several methods have been developed for the quantitative determination of digoxin, free from interference of its metabolites. One such method [1] involves the use of thin-layer chromatographic (TLC) separation of drug and metabolite, followed by isolation, derivatization and analysis by gas chromatography (GC) using electron-capture detection. The minimum detectable quantity was 25 pg. However, the need for preparative TLC prior to the analysis made the procedure somewhat cumbersome. An analytical procedure using GC—mass spectrometry [2] has also been postulated for the identification and quantitation of dihydrodigoxigenin, a quantitatively variable metabolite of digoxin in humans. As such, the method was suitable for the aglycone portion of the molecule since the digitoxose sugar residues are unstable under the condition of the assay. A radiolabelled digoxin measurement by TLC has been reported [3] which was suitable for the intact drug and its known metabolites in plasma, saliva and urine after single-dose administration of labelled digoxin. While sensitive and selective, this method required the use of isotopic digoxin and would be inappropriate for routine measurements.

To date, the radioimmunoassay (RIA) procedure has offered the greatest sensitivity for digoxin measurement. However, the specificity of the method has frequently been questioned. Recently, several investigators have reported false positive results in non-digitalized [4] and uraemic patients [5] using commercial RIA kits. To overcome the lack of specificity of the RIA method, a number of investigators have included chromatographic separations prior to the measurement of digoxin. For example, Nelson et al. [6] combined a high-performance liquid chromatographic (HPLC) separation of digoxin followed by collection of the column eluate and subsequent RIA analysis. The relatively high coefficient of variation (21%) made this method somewhat less than appropriate for clinical measurements. As well, dihydrodigoxin, a metabolite that eluted near digoxin in the HPLC profile, may cross-react with many RIA kits [7]. Other HPLC—RIA methods have been reported by Loo et al. [8] and Morais et al. [9]; however, these also required the collection of timed eluate fractions with subsequent evaporation and RIA measurements.

The method described here has the necessary sensitivity and selectivity for digoxin plasma level monitoring and does not require the collection of eluates or subsequent evaporation and alternate assay. The assay technique involves a refinement of the method reported by Gfeller et al. [10] for the HPLC post-column fluorogenic determination of digoxin. This method was based on the fluorogenic reagents employed in the United States Pharmacopeia [11] for digoxin tablet content uniformity. The emphasis of the present study was placed on the optimization of reactor design, selectivity and sensitivity for the measurement of digoxin and its metabolites at expected plasma levels.

EXPERIMENTAL

Materials

Digoxin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, digoxigenin, dihydrodigoxin and digitoxigenin were obtained from commercial

sources (Boehringer Mannheim, Mannheim, F.R.G.) and were used without further purification. Spironolactone, furosemide (Sigma, St. Louis, MO, U.S.A.), disopyramide (Roussel, London, U.K.) and procainamide (Squibb, Montreal, Canada) were likewise used as received. Ascorbic acid (BDH, Toronto, Canada) and 30% hydrogen peroxide (American Scientific and Chemical Co., Portland, OR, U.S.A.) were used as received. Chromatographic solvents were HPLC grade (Fisher Scientific) and absolute ethanol was analytical grade.

Equipment

The HPLC system consisted of a Beckman Model 100A pump (Beckman Instruments, Fullerton, CA, U.S.A.), a Model U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and a direct-connect guard column (Milton-Roy, State College, PA, U.S.A.) packed with 37-μm, bonded octadecylsilane (ODS) phase (Whatman, Clifton, NJ, U.S.A.). Optimum separation was achieved using a Spherisorb ODS II, 3-μm, 15 cm × 4.6 mm I.D. column (Alltech Assoc., Deerfield, IL, U.S.A.).

The post-column reactor consisted of a multi-channel peristaltic pump (Manostat, New York, NY, U.S.A.) with acidflex tubing (Part No. 116-0538-09) and solvaflex tubing (Part No. 116-0533P04, orange–green collar), both obtained from Technicon Instruments (Tarrytown, NY, U.S.A.).

The 0.8 mm I.D. three-way tubing connectors, the 2 m × 0.8 mm I.D. mixing coil and the 0.3 mm I.D. reaction coils were polytetrafluoroethylene (PTFE) and were obtained from Omnifit (Cambridge, U.K.). A Model E51 constant-temperature circulator (Haake, West-Berlin, F.R.G.) was maintained at 79 ± 1°C.

A Model 420A filter fluorometer (Waters Assoc.) was equipped with a 360-nm excitation filter and 425-nm emission filter. The cell holder of the fluorometer was modified by replacing the stainless-steel inlet and outlet fittings with a single quartz tube (4 cm × 1 mm I.D.) which passed through the cell holder and was bent to exit out of the front of the detector. Back-pressure in the detector was generated by connecting a 1 m × 0.3 mm I.D. PTFE tube to the exit of the fluorometer to prevent bubble formation in the detector. A Chromatopac CR1A integrator (Shimadzu, Kyoto, Japan) was used for signal recording. A variable-wavelength ultraviolet detector, Model 155-10, with a 20-μl flow-cell (Beckman Instruments) was placed between the HPLC column and the post-column reactor tubing to determine band-broadening.

Preparation of standard solutions and reagents

Digoxin stock solution (1 mg per 100 ml) was prepared in absolute ethanol. For the calibration curve determination, aliquots equivalent to 2–10 ng were added to each of six PTFE-lined screw-capped centrifuge tubes, along with 2 ml of the internal standard, digitoxigenin (4 mg per 100 ml of absolute alcohol). Solutions of the metabolites of digoxin (digoxigenin, digoxigenin mono-digitoxoside, digoxigenin bisdigitoxoside, dihydrodigoxigenin and dihydro-digoxin) were prepared at a final concentration of 5 μg/ml in absolute ethanol and were added to the tubes containing digoxin (5 μg/ml) and digitoxigenin (50 μg/ml). These solutions were used for determination of the separation of digoxin and its metabolites.

The fluorogenic reagent, dehydroascorbic acid, was prepared at a concentration of $1.1 \cdot 10^{-3}$ *M* hydrogen peroxide in 0.1% ascorbic acid solution. Hydrochloric acid was used without dilution.

RESULTS AND DISCUSSION

Optimization of resolution: column selection

Initial studies were undertaken to optimize the HPLC resolution of digoxin and its metabolites. It is relevant to emphasize the importance of obtaining a short chromatographic time since as the bands widen with increasing retention time the later-eluting peaks exhibit a corresponding reduction in sensitivity of detection of the fluorophore formed in the post-column reactor. A diagram of the reactor system is shown in Fig. 1. In addition, it was necessary to achieve

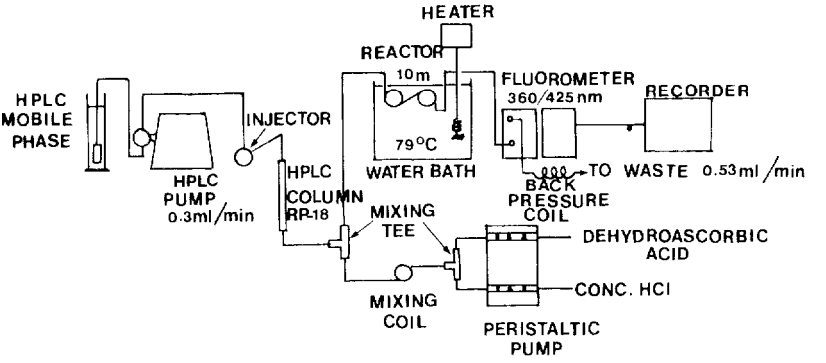

Fig. 1. Flow diagram of the fluorogenic reactor and HPLC system.

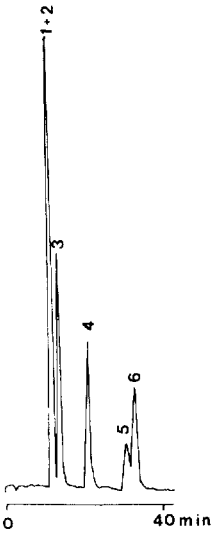

Fig. 2. Chromatogram showing optimal resolution obtained for digoxin and its metabolites. Chromatographic conditions: mobile phase, methanol—ethanol—isopropanol—water (52:3:1:45); HPLC flow-rate, 0.3 ml/min. Detection: fluorogenic reaction system as shown in Fig. 1. Peaks: 1 = dihydrodigoxigenin; 2 = digoxigenin; 3 = digoxigenin monodigitoxoside; 4 = digoxigenin bisdigitoxoside; 5 = dihydrodigoxin; 6 = digoxin.

resolution of dihydrodigoxin, a significant metabolite of digoxin in some patients, since this substance had not yet been effectively resolved according to literature reports. Loo et al. [8] had reported elution times for digoxin and dihydrodigoxin when these were introduced into an HPLC system. However, from an examination of peak widths and elution time in their manuscript, the separation would be inadequate for direct quantitation. Consequently, digoxin and its metabolites (digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, dihydrodigoxin and dihydrodigoxigenin) were examined using a number of partition columns and a variety of appropriate mobile phases. The goal in these trials was to develop maximum resolution of digoxin from its metabolites, notably dihydrodigoxin. It was not our purpose to resolve each of the metabolites from each other, since separation of digoxin, free from interference of its metabolites, was essential to its quantitation following post-column reaction. Effective separation of the earlier eluting metabolites has been previously reported [12]. However, dihydrodigoxin could not be included in this earlier work owing to its lack of an ultraviolet chromophore.

The chromatogram shown in Fig. 2 is representative of the maximum resolution (R_s=0.91) that could be obtained for the column types examined.

Optimization of resolution: mobile phase selection

The mobile phase found to provide maximum resolution of digoxin from dihydrodigoxin was determined from a systematic examination of a series of appropriate solvent combinations. The major requirement of the mobile phase was that it must be readily miscible with the aqueous post-column fluorogenic reagents and not influence the reaction. Wells et al. [13] studied the effect of methanol on the fluorescence of cardiac glycosides and found that methanol was an important component of the reaction medium. Similar observations

TABLE I

MOBILE PHASES, POLARITY AND RESOLUTION OBSERVED BETWEEN DIGOXIN AND DIHYDRODIGOXIN USING A 15 cm × 4.6 mm I.D., 3-μm RP-18 HPLC COLUMN

n	Mobile phase composition (%)					$P'e$*	R_s**
	Ethanol	Methanol	Water	Isopropanol	Dichloromethane		
1	–	70	30	–	–	663	0
2	–	40	47	9	4	727.3	0
3	5	55	40	–	–	710	0.3
4	5	50	45	–	–	735.5	0.65
5	4.95	54.46	39.60	0.99	–	706.81	0.298
6	4.95	49.50	44.55	0.99	–	732.0	0.752
7	2.97	58.42	37.62	0.99	–	698.3	0
8	2.97	54.46	41.58	0.99	–	718.5	0.808
9	2.97	51.49	44.55	0.99	–	733.6	0.909
10	2	55	42	1	–	721.4	0.516

*Polarity calculated from the following polarity values: ethanol, 4.3; methanol, 5.1; water, 10.2; isopropanol, 3.9; dichloromethane, 3.1.

**Calculation based on the equation $R_s = (t_{II} - t_I)/0.5\ (W_I + W_{II})$ where t_I and t_{II} refer to the retention time of dihydrodigoxin and digoxin, and W_I and W_{II} to the basewidth of the two peaks, respectively.

were noted in the present studies, solvents such as acetonitrile and dioxane reduced fluorescence yield. The mobile phase reported by Desta et al. [12] was not suitable for the present post-column reaction because of quenching of fluorescence by dichloromethane and lowering of the boiling point of the mobile phase, thereby creating bubbles in the detector cells.

Solvent mixtures of three to four water-miscible solvents were combined in various proportions to achieve optimum resolution of digoxin and dihydrodigoxin. The solvent strength was calculated from the following equation [14]: $P'e = \Sigma_{i=1}^{n} \Phi_i P_i$, where $P'e$ is the total polarity, n is the number of pure solvents in the mixture, Φ_i is the volume fraction of the solvent and P_i is the solvent polarity parameter.

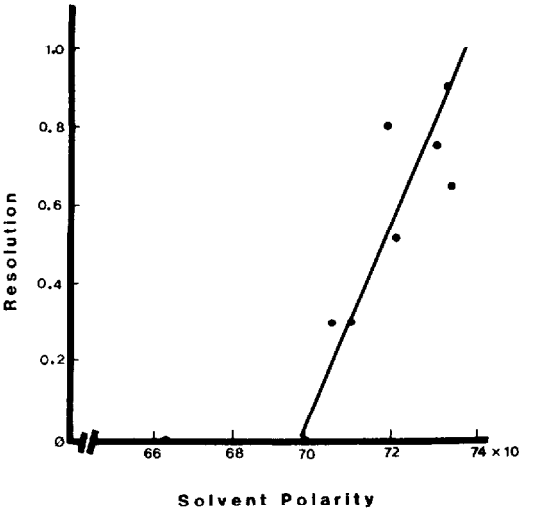

Fig. 3. Relationship between HPLC mobile phase polarity and resolution of dihydrodigoxin and digoxin.

Table I outlines the solvents investigated and their respective polarities and effect on resolution. A linear relationship was observed (Fig. 3) between solvent polarity and resolution of digoxin and dihydrodigoxin. The optimum solvent (n=9, Table I) was thus chosen for further studies. Increasing the polarity past this point led to unacceptably long retention times.

Optimization of fluorogenic reagent proportions

In order to achieve maximum sensitivity of detection of the fluorescent species formed in the post-column reactor, the concentrations of dehydroascorbic acid were altered while maintaining the quantity of hydrochloric acid constant, and then reversing the relationship. An optimal balance was found to be achieved with a dehydroascorbic acid/hydrochloric acid volume ratio of 0.1:0.5.

In addition, it was noted that the concentration of hydrogen peroxide in ascorbic acid (dehydroascorbic acid) affected the efficiency of fluorescence. In this study a very sharp maximum was observed (Fig. 4). Hence, this reagent was prepared daily to make certain that the optimal concentration of $1.1 \cdot 10^{-3}$ M hydrogen peroxide in ascorbic acid was maintained.

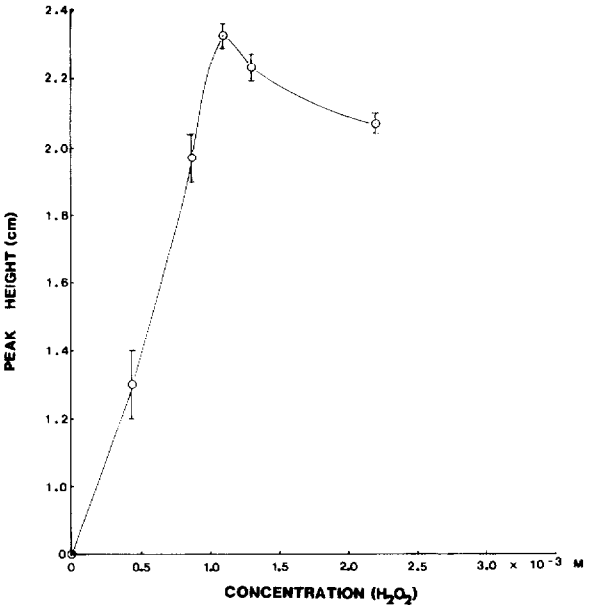

Fig. 4. Effect of hydrogen peroxide concentration in ascorbic acid on fluorescence yield of digoxin.

Optimization of post-column reaction temperature and time

A steady increase in reaction rate and sensitivity of detection was observed when the temperature of the post-column reactor bath was increased to 79°C. Above this temperature bubble formation in the detector cell occurred. Bubble formation could be suppressed at 79°C by connection of a 1 m × 0.3 mm I.D. PTFE coil at the outlet of the detector. The relative intensity of fluorescence at 79°C was approximately three times that seen at 59°C.

In addition to the reaction temperature, the reaction time was also studied for optimal efficiency. This study was most readily accomplished by varying the speed at which the peristaltic pump delivered the reagents, dehydroascorbic acid and hydrochloric acid. From the experimental data (Fig. 5) it was determined that a total flow-rate of 0.27 ml/min (HPLC pump plus peristaltic flow) was optimal for the reaction time. The HPLC pump flow-rate was maintained at 0.1 ml/min for the whole range of peristaltic pump speeds. In terms of actual reaction time, a total flow-rate of 0.27 ml/min required 7 min to traverse the 20 m × 0.3 mm coil and associated couplings. Reaction coil lengths of 10, 15, 20 and 25 m with 0.3 mm I.D. provided reaction times of 3, 5, 7 and 8.6 minutes, respectively, at these same flow-rates. The fluorescence intensity of digoxin for these four times are given in Fig. 6. The 20-m coil provided maximum sensitivity.

It has been established [15] that more dispersion of a peak occurs while traversing a straight reaction tube. This situation was suggested to improve dramatically when a coiled reaction tube is used. This phenomenon was deduced to be due to centrifugal forces acting on the flow pattern to produce secondary flows perpendicular to the main flow. As a result better mixing is achieved and band-broadening is reduced.

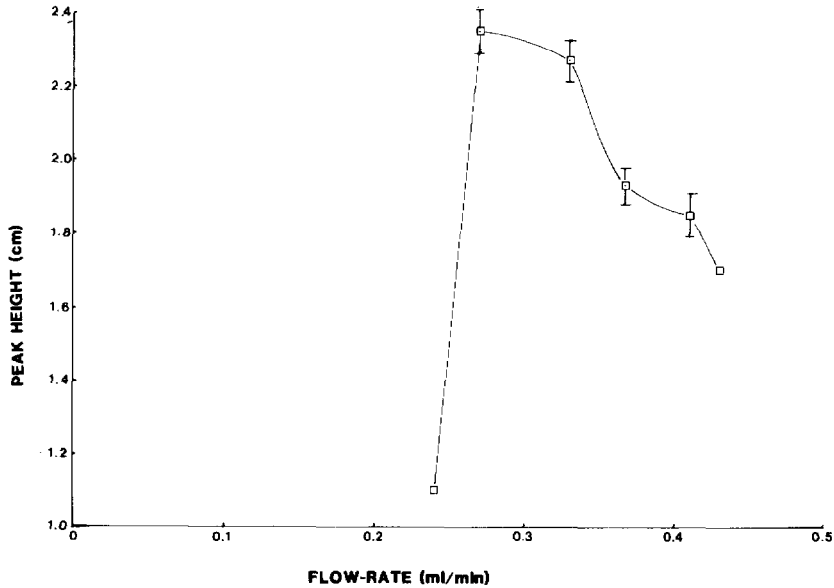

Fig. 5. Effect of post-column reactor flow-rate on fluorescence yield of digoxin.

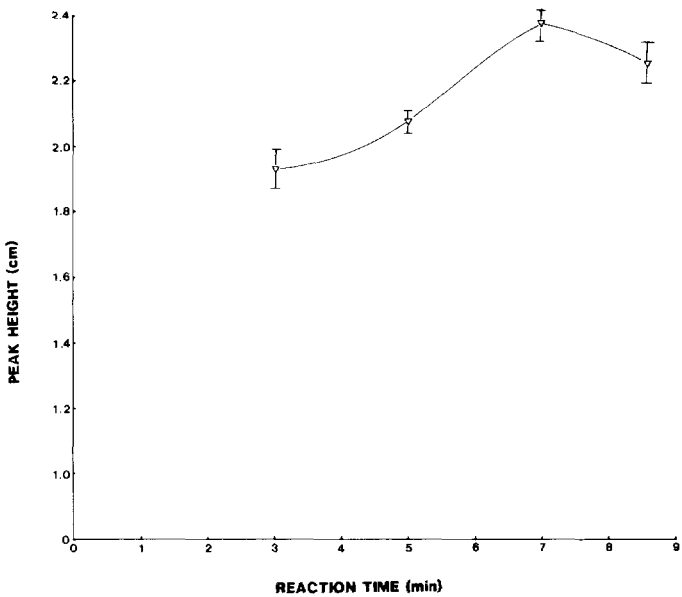

Fig. 6. Effect of reaction coil length on fluorescence yield of digoxin.

Hoffmann and Halasz [16] proposed that geometrical deformation of a tube also leads to reduced band dispersion. To study the effect of reactor design, two different reactor coils were compared: one with an outer circular coil diameter of 50 cm and one with a diameter of 27 cm. The peak height, which reduces as band-broadening increases, was 42% greater for the smaller coil.

Studies by Uihlein and Schwab [17] and Deelder et al. [18] have shown

that knitted "fringes" (like those used for crochet work) can lead to further reductions in band-broadening. This type of reaction geometry was therefore also tested for effects on fluorogenic yield.

A 10 m × 0.3 mm knitted tube provided a base width of 2.2 min and an asymmetry factor of 2.5. On the other hand, the 20-m coiled reactor (coil diameter of 25 cm) had an asymmetry factor of 3. The fluorescence sensitivity of both reactors was identical. The shortened tube, however, allowed for increased HPLC pumping speeds and consequently shorter analysis time. Fine-tuning the combination of HPLC pump flow-rate and peristaltic flow-rate resulted in maximum sensitivity when the HPLC flow-rate was 0.3 ml/min and the total peristaltic flow-rate was 0.23 ml/min.

Band-broadening

In order to preserve the resolution obtained in the chromatographic column, band-broadening in the post-column reactor should be minimal. Therefore, a comparison between the different reactors was undertaken by inserting an ultraviolet detector (set at 214 nm) between the end of the HPLC column and the post-column reactor. Band-broadening, as determined from the difference in observed base widths between the ultraviolet detector and fluorescence detector, was found to be minimal with the knitted-reactor geometry.

Selectivity of assay

In order to be useful as a potential method for the determination of digoxin concentrations in the plasma of patients requiring this and other drugs for therapeutic effect, it was necessary to determine if commonly co-administered drugs would interfere with the measurement of digoxin. Accordingly, such

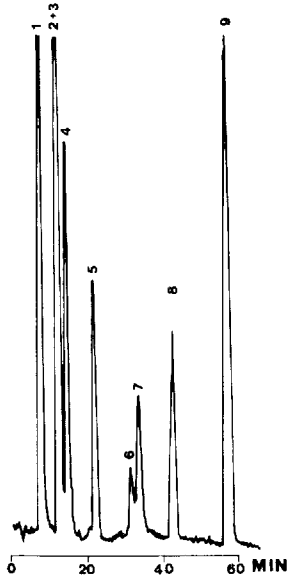

Fig. 7. Chromatogram of digoxin, its metabolites, spironolactone and furosemide. Chromatographic conditions as given in Fig. 2. Peaks: 1 = furosemide; 2 = dihydrodigoxigenin; 3 = digoxigenin; 4 = digoxigenin monodigitoxoside; 5 = digoxigenin bisdigitoxoside; 6 = dihydrodigoxin; 7 = digoxin; 8 = digitoxigenin; 9 = spironolactone.

drugs as furosemide, spironolactone, quinidine, procainamide, disopyramide, captopril, verapamil, dipyridamole and propafenone were evaluated for their elution time and fluorogenic reaction. As shown in Fig. 7, only furosemide and spironolactone exhibited fluorescence response at the excitation and emission wavelengths used. Also included in this chromatogram are the metabolites of digoxin at 5 ng of each and the chosen internal standard, digitoxigenin, at 50 ng.

Sensitivity, precision and linearity of assay

The injection of 10-μl samples of digoxin (0.1 μg/ml) provided detection limits of 1 ng at a signal-to-noise ratio of 6:1. The coefficient of variation for repeated injections of digoxin at the 3-ng level was 2% (n=10), indicating that the method was satisfactorily reproducible. The calibration curve, based on peak-height ratios of digoxin and internal standard (digitoxigenin), was linear over the range 1.5—10 ng per injection. Repeating the calibration curve daily for five days provided an overall coefficient of variation of 8%.

The HPLC system combined with fluorogenic post-column reaction has been shown to be an effective analytical procedure for the selective and sensitive determination of digoxin at therapeutic levels in the presence of its metabolites and other commonly co-administered drugs.

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