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Note

Improved method for assaying digoxin in serum using high-performance liquid chromatography–radioimmunoassay

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Although radioimmunoassay (RIA) is the most commonly used method to quantitate digoxin in the serum, most antibody populations cross-react with some of the major metabolites of digoxin, viz., digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, and dihydrodigoxin [1, 2]. This lack of specificity has led to the development of more specific assays for digoxin. These include gas chromatography—mass spectrometry (GC—MS) [3, 4], Sephadex LH-20 column chromatography followed by ultraviolet detection [5], and separation of digoxin and its metabolites by high-performance liquid chromatography (HPLC) with collection of fractions to be quantitated by RIA [6–8].

Most of the above assays have inherent disadvantages. Procedures involving GC—MS, although sensitive, are expensive and tedious. Some HPLC–RIA methods [6, 7, 9, 10] require [^{3}H]digoxin as an internal standard to monitor extraction (due to variability in extraction efficiency) or require [^{3}H]digoxin to be administered to patients.

We have developed an assay that has some advantages over currently available procedures. The extraction of digoxin from serum is over 90%, from serum as compared to previous assays where the extraction varied from 54 to 78% [7] or 70 to 80% [9]. The assay does not require [^{3}H]digoxin to be administered to the subjects or patients or to be used in the assay as an internal standard. Interference in the RIA procedure (in which [^{125}I]digoxin is used) due to gamma emission from [^{3}H]digoxin is also eliminated.

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Kuhlman et al. [11], Sternson and Shaffer [12] and Gault et al. [13] have shown that digoxin hydrolyses to digitoxosides, viz., digoxigenin bisdigitoxoside, digoxigenin monodigitoxoside and digoxigenin. There is a possibility that digoxin hydrolysis may occur in assays using reversed-phase HPLC [7, 8, 14–16], since the mobile phase in such systems is comprised largely of water. Our use of a normal-phase HPLC system (modification in part of that by Loo et al. [6]), where the mobile phase is comprised of organic solvents, eliminates the possibility of hydrolysis during the assay. Furthermore, after collection of eluate fractions, the solvent can be easily evaporated to dryness prior to reconstitution for RIA. Evaporation of aqueous solvents (in reversed-phase HPLC) is extremely cumbersome and further enhances the possibility of degradation.

Some studies [8, 14, 15] have reported partial or complete separation of dihydrodigoxin from digoxin. We have had little success in reproducing this separation. Due to its very low absorbance, nanogram quantities of dihydrodigoxin cannot be monitored using ultraviolet (UV) spectrophotometry. Hence, during the developmental stages of the assay we monitored retention times for dihydrodigoxin using spectrofluorometry [17].

EXPERIMENTAL

Materials

All solvents used for the extraction or chromatography were HPLC grade from Fisher Scientific (Pittsburgh, PA, U.S.A.). Digoxin was obtained from Sigma (St. Louis, MO, U.S.A.). Digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Dihydrodigoxin was a gift from H. Hull (Burroughs Wellcome, Research Triangle Park, NC, U.S.A.). RIA was performed using the digoxin RIA kit (Diagnostic Products, Los Angeles, CA, U.S.A.).

Extraction

Serum (1 ml) was extracted with 0.5 ml methylene chloride and 4 ml chloroform in a screw-capped 10-ml glass tube. The tube was agitated for 15 min on a rotary mixer and then centrifuged at 1000 *g* for 5 min. The organic layer was pipetted into a 12-ml conical centrifuge tube. The serum remains were re-extracted and centrifuged again using the procedure above. The combined organic extract was evaporated to dryness on a vortex evaporator. The residue was reconstituted in 170 μl of mobile phase and 100 μl were injected onto the HPLC column using a fixed-volume 100-μl loop injector.

Chromatography

The HPLC system was a Waters M6000A solvent delivery system. The column was a LiChrosorb SI-100 (Hewlett-Packard, Palo Alto, CA, U.S.A.), 5-μm particles, 20 cm × 4.6 mm I.D. The mobile phase was comprised of hexane–ethanol–methylene chloride (75:18:7). The flow-rate was monitored at 3 ml/min and column pressures ranged from 103 to 207 bar. The retention times for digoxigenin, digoxigenin monodigitoxoside, digoxigenin bis-

digitoxoside and digoxin varied slightly with each batch of mobile phase that was prepared but typically were 5.2, 7.2, 9.8 and 13.2 min, respectively. Retention times (during development of the assay) were monitored by injecting 2.5 μg of digoxin and its metabolites on the column and detected using a Gilson variable-wavelength UV spectrophotometer at 230 nm. Retention times (prior to assaying of each batch of samples) were determined by injecting a mixture containing 20 ng of digoxin and 20 ng of each of the metabolites on the column. Fractions (0.5 min) of the eluate were then collected over a period of 20 min. Each fraction was evaporated to dryness, reconstituted in 0.5 ml blank plasma and assayed by RIA to confirm that digoxin was indeed separated from its metabolites.

Digoxin cannot be detected spectrophotometrically in the ng/ml range, but can be assayed by RIA. Hence, post injection, the eluate fraction corresponding to the retention time of digoxin was collected off the HPLC column. This fraction was evaporated to dryness. The residue was reconstituted in 0.5 ml of blank plasma and this was assayed for digoxin using the RIA kit mentioned before.

Radioimmunoassay

RIA was conducted on the HPLC eluate fractions. The fraction corresponding to digoxin (typically from 12 to 15 min) was collected in a 12-ml centrifuge tube and was evaporated to dryness on a vortex evaporator. The residue was reconstituted in 0.5 ml blank plasma and assayed by RIA. The RIA kit used is highly selective for digoxin in the presence of dihydrodigoxin [18]. Further, the specifications supplied with the kit indicate a cross-reactivity of only 1.4%. This selectivity for digoxin was verified as follows. A representative set of samples (n = 12) was obtained from pharmacokinetic studies conducted in normal beagle dogs administered 0.05 mg/kg digoxin as an intravenous infusion over a 5-min period. These samples were assayed as such and also after spiking them with dihydrodigoxin to result in a final dihydrodigoxin concentration of 4 ng/ml. The concentrations obtained were not significantly different, indicating that clinical levels of digoxin quantitated using the RIA kit used will not be significantly altered in the presence of dihydrodigoxin. The results are in agreement with those reported in a previous study [18].

Analyses

The standard curve and samples were done in duplicate. The concentrations used for the standard curve were 0.5, 1.0, 2.0 and 4.0 ng/ml digoxin. The standards (containing known concentrations of digoxin in serum) were extracted and assayed by HPLC—RIA to obtain a measured concentration. The standard curve was then obtained by regression of the known and measured concentrations. A set of controls (frozen serum samples spiked with 0.50 and 3.50 ng/ml digoxin) was run before and after each batch of samples to be assayed. The concentrations of the samples were obtained from the standard curve for that day.

RESULTS

Extraction

Various solvents were tried for optimizing extraction of digoxin from serum. Most of the systems exhibited non-linearity, low recoveries or non-reproducibility. A double extraction using 4 ml of chloroform and 0.5 ml of methylene chloride gave almost complete extraction which was reproducible. The regression line has a slope of 1.04 ± 0.11 and a mean value for the intercept not significantly different from zero (*hypothesis* H_0:mean = 0 was not rejected, $p = 0.6$). The correlation for the regression line was 0.99. The slope of the line indicates an extraction efficiency of around 100%. While the extraction procedure is tedious, it is accurate and reproducible and requires no internal standard. Furthermore the organic solvents used are easily evaporated.

Chromatography

Digoxin was separated from its metabolites (except dihydrodigoxin) on the column. A typical chromatogram showing separation of digoxin from its major

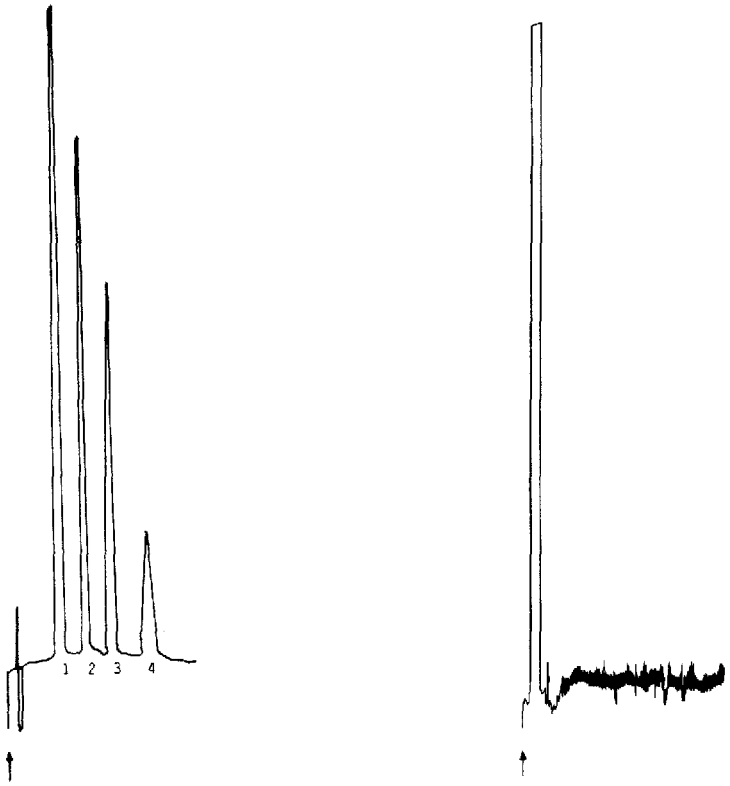

Fig. 1. Typical chromatogram of a standard mixture. The peaks and the retention time in minutes are as follows: 1 = digoxigenin (5.2 min); 2 = digoxigenin monodigitoxoside (7.2 min); 3 = digoxigenin bisdigitoxoside (10.0 min); 4 = digoxin (13.6 min). The peaks represent 2.5 μg of each compound injected on the column (0.1 a.u.f.s.).

Fig. 2. Typical chromatogram obtained on injecting an extracted serum sample obtained from a beagle dog administered 0.05 mg/kg digoxin (0.01 a.u.f.s.).

metabolites is shown in Fig. 1. A typical chromatogram obtained on injecting an extracted serum sample (obtained from pharmacokinetic studies in dogs) is shown in Fig. 2. It is evident that biological samples of digoxin cannot be quantitated using UV spectrophotometry.

Linearity

The typical standard curve was linear. The slope of the standard curve had a mean ± standard deviation of 0.96 ± 0.09 and for the intercept was −0.03 ± 0.06 (n = 5). The correlation coefficient was 0.99 or greater for each standard curve. The low standard deviations for the slope and the intercept indicate that the standard curve remained essentially constant over a eight-month period. It is also evident that storage of spiked frozen serum had a negligible effect on the standard curve.

Recovery

The precision of the method is estimated from the standard deviations for the repeated measurements made on two sets of controls. This was achieved by running a set of controls at the start and completion of each batch of samples. The recoveries for these repeated measurements are given in Table I. The recovery at a spiked concentration of 0.50 ng/ml digoxin is 100.17 ± 10.97% and for a spiked concentration of 3.50 ng/ml is 99.91 ± 8.97%. The overall

TABLE I

RECOVERY OF CONTROLS BY HPLC—RIA

Day	Spiked concentration: 0.5 ng/ml		Spiked concentration: 3.5 ng/ml	
	Measured concentration (ng/ml)	Recovery (%)	Measured concentration (ng/ml)	Recovery (%)
1	0.44	88.00	3.22	92.00
	0.39	78.00	3.25	92.86
	0.43	86.00	3.31	94.57
	–*		3.72	106.28
2	0.55	110.00	3.05	87.14
	0.55	110.00	3.30	94.28
	0.52	104.00	3.74	106.86
	0.53	106.00	3.73	106.57
3	0.54	108.00	4.05	115.71
	0.54	108.00	3.92	112.00
4	0.48	96.00	3.25	92.86
	0.55	110.00	3.54	101.14
	0.49	98.00	3.38	96.57
Mean	0.50	100.17	3.50	99.91
S.D.	0.05	10.97	0.31	8.79

*Lost sample due to spillage.

day-to-day precision is indicated by the low standard deviations associated with the recovery of the controls. The mean recoveries of 100.17 and 99.91% at 0.50 and 3.50 ng/ml is indicative of the accuracy of the assay. The within-day precision and recovery calculated for each of the days was found to be less than 10% for the controls.

The accuracy and precision of the standard curve is evident from the low standard deviations for the slope and the intercept and high correlation coefficients.

Application

Serum samples obtained from normal beagle dogs administered digoxin for ongoing pharmacokinetic studies were assayed using both HPLC—RIA and direct RIA. Over 40 samples were assayed by both methods. A linear relationship was observed between the values obtained by both methods, with a slope of 1.02 and an intercept not significantly different from zero (p = 0.78). Values obtained by using direct RIA were plotted on the abscissa. (Fig. 3). The results are similar to those of Gibson and Nelson [19, 20] in patients. They reported that the values obtained by direct RIA and those obtained by HPLC—RIA were essentially identical in patients with glomerular filtration rates greater than 40 ml/min. They observed that the differences in the values may be significant in patients with renal failure presumably due to accumulation of cross-reacting metabolites.

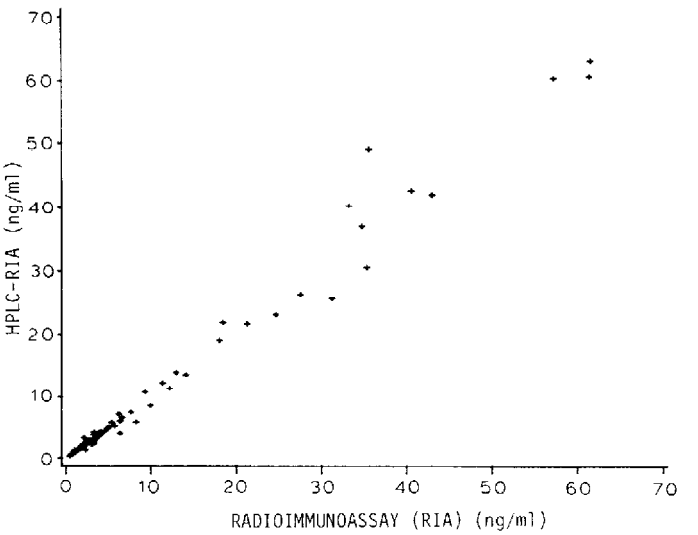

Fig. 3. Comparison of values obtained on assaying serum samples (obtained from dogs administered digoxin) by HPLC—RIA and that by direct RIA. The regression line is described by HPLC—RIA = 1.02 RIA − 0.09 (r^2 = 0.99).

CONCLUSION

We have described an accurate and precise method for assaying digoxin in serum. The assay possesses substantial improvements over existing assays. The specificity of the assay could be of clinical value in patients with renal failure

and cumulation of metabolites of digoxin. This assay will also be useful in pharmacodynamic studies in dogs to assess the role of cardioactive metabolites.

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