Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 5, pp. 585–592, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00
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Digoxin-like immunoreactive substance: monoclonal and polyclonal RIA and FPIA compared

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Abstract: Digoxin-like immunoreactive substance (DLIS) was measured in 34 samples obtained from subjects not receiving digoxin: 10 uremic, 10 third trimester pregnancy, seven cord blood and seven normal. DLIS concentration was measured by four commercial polyclonal radioimmunoassay (RIA) systems: Clinical Assay (CA), Corning Immophase (CI), Diagnostic Products Double Antibody (DP), Kallestad Quantitope (KK), a monoclonal antibody (MA) assay and a Fluorescence Polarization Immunoassay (FPIA). In general, the cord blood samples were richer in DLIS. Digoxin immunoassays, MA and DP showed minimal interference by DLIS in all samples, whereas FPIA and CA exhibited the maximal cross-reactivity with DLIS. In cord blood samples, mean ±SD DLIS concentration ranged from 0.41 ± 0.13 (by CA) to 0.034 ± 0.02 ng ml^{-1} as measured by MA assay. In uremics, the mean DLIS concentration was below the detection limit of all RIA assays. The FPIA method showed a higher degree of cross-reactivity to DLIS, especially in the cord and pregnancy samples (0.42 ± 0.13 and 0.4 ± 0.14 ng ml^{-1}, respectively). DLIS in uremics was below the FPIA detection limit of 0.2 ng ml^{-1}. Overall, the degree of interference by DLIS in decreasing order was FPIA>CA>CI≥KQ>DP≥MA. The cord blood samples were re-analysed by FPIA (Digoxin-II assay) 4 months later, resulting in 2–4-fold higher DLIS concentrations for these samples. This appears to be due to the substitution of 5-sulphosalicylic acid as a protein precipitating reagent and this effect may have been accentuated by freeze–thaw cycles.

Keywords: *Digoxin-like immunoreactive substance; digoxin monoclonal antibody assay; radioimmunoassay; fluorescence polarization immunoassay.*

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This work has been presented in part at the 88th Annual Meeting of the American Society of Clinical Pharmacology of Therapeutics, Orlando, Florida, USA (March 1987).

Introduction

Digoxin is one of the most widely prescribed drugs in the United States. Because of a narrow therapeutic range (0.8–2.0 ng ml^{-1}), digoxin serum levels are frequently determined throughout therapy [1]. Over the past 15 years, the routine measurement of digoxin in body fluids has been accomplished almost exclusively through the use of various radioimmunoassay (RIA) and fluorescence polarization immunoassay (FPIA) procedures [2–8]. The accuracy and specificity of commercial digoxin *in vitro* diagnostic products have been the cause of great concern which is reflected by several recent publications [9–14]. This concern has been mainly because of (a) considerable interassay variability in serum digoxin concentration measurements in renal failure patients receiving digoxin [13]; (b) unexpected increases in serum digoxin levels in renal failure patients after digoxin is discontinued [15, 16]; and (c) false-positive serum digoxin concentrations in patients who are not receiving the drug [13, 15, 17]. In an effort to explain interassay variability, several compounds have been considered as cross-reactants. Digoxin metabolites cross-react in varying degrees with the antibodies used in most digoxin immunoassays. These metabolites may accumulate in renal failure patients and cause immunoassay measurements of digoxin to be inaccurate [12, 15, 16]. The presence of metabolites such as conjugates of digoxigenin monodigitoxoside (cardio-inactive metabolites of digoxin) may contribute to some of the variability in the serum digoxin concentration, but it does not explain why false-positive measurements occur in patients who have not been under digoxin therapy.

The presence of an endogenous and interfering digoxin-like immunoreactive substance (DLIS) in the digoxin-free sera of neonates and uremic patients [13, 17–19], pregnant women [20] and umbilical cord specimens [18, 21] has been reported, which further complicates digoxin monitoring in these groups of patients. Digoxin is also measured by FPIA (TDX System, Abbott Laboratories, Chicago, IL 60664, USA). Yatscoff *et al.* [22] concluded that in cord blood and uremic serum samples, the degree of interference of DLIS measured by FPIA procedure is generally less than that seen in commonly used RIAs for digoxin. The purpose of the present study was 3-fold: firstly, to compare the cross-reactivity of DLIS in four commercially available polyclonal immunoassay systems versus a monoclonal RIA method; secondly, to compare the results of these RIA systems with the performance of the FPIA method; and thirdly, to evaluate and compare the results obtained by the original FPIA method (TDX Dig-I) versus the more recently modified FPIA system (TDX Dig-II).

Experimental

Four [^{125}I]digoxin RIAs plus one FPIA method were used according to the manufacturer's specifications and without further modifications. In addition, digoxin monoclonal antibody assay (MA) (clone DI-22, lot 1, Miles Labs Elkhart, IN, USA) was also used according to an in-house procedure. For monoclonal antibody assay, standards were prepared in drug-free normal human serum; iodinated digoxin (lot 13,365 Corning Medical, Medfield, MA, USA) and dextran–charcoal as the separating agent were used. This was performed as a sequential assay. The four commercial RIAs were Clinical Assay Gamma Coat Solid Phase Digoxin Kit (CA), Lot 4108 (Travenol-Genetech Diagnostics, Cambridge, MA 02139, USA), Corning Immophase Digoxin Kit (CI), Lot 22595 (Corning Medical and Scientific, Medfield, MA 02052, USA), Diagnostic

Products Double Antibody Kit (DP), Lot 193 (Diagnostic Products Corp., Los Angeles, CA 90045, USA), and Kallestad ^{125}I-Digoxin Quantitope Kit (KK) Kallestad Inc. (Austin, TX 78701, USA). The logit-log method of data reduction was used for calculation of the data generated from all RIA systems. FPIA, Digoxin-I assay (D-I; TDX system, Abbott Laboratories, North Chicago, IL 60064, USA) was also used for analysis of all patient's samples. Subsequently, Digoxin-II assay (D-II; TDX system, Abbott Laboratories) was used for reanalysis of uremic, pregnancy and cord blood samples.

Two sets of quality control samples were analysed in duplicate with each run. The low, medium and high Lypochek Trilevel Controls, Lot 1000 (BioRad Laboratories, Richmond, CA 94804, USA) and the low and high level digoxin controls, Lot 02405 (Corning Medical Scientific, Medfield, MA, USA), were used as a mean of assay performance throughout the study. The normal sera were collected from seven healthy adult males. Twenty-seven samples from patients not receiving digoxin were analysed; 10 blood samples from uremic patients (chronic renal failure on dialysis, creatinine >10 mg dl^{-1}), 10 from women during the third trimester pregnancy, and seven cord blood samples. These samples were obtained by the clinical laboratory of Thomas Jefferson University and stored at −70°C until time of analysis. All samples assayed by the RIA and FPIA methods were quantitated in duplicates. To evaluate the lot-to-lot variability in measuring DLIS, all samples were re-analysed by RIA (KK) and FPIA (D-II) systems 4 months later using a new precipitating reagent. For the FPIA system, 5% trichloroacetic acid (TCA) was used as the precipitating agent in the first assay (D-I) and 3% 5-sulphosalicylic acid (5-SSA) in the second analysis (D-II). Because of quantitative assay problems, the protein precipitating reagent was switched from 5% TCA to 3% 5-SSA by the manufacturer during the time of our experiments.

Results

The measured digoxin concentration in the quality control samples (lypochek trilevel controls and corning bilevel controls) were within the manufacturer specified range for all RIA kits. Figure 1 shows the individual values, for DLIS (reported as apparent digoxin concentration) as measured with four commercial digoxin RIA kits and the MA assay. The results of the analysis of these samples by FPIA (D-I) are included in Fig. 1. DP and MA exhibited the minimal interference by DLIS while CA demonstrated the highest interference by DLIS, especially for the cord blood samples. As shown in Fig. 1, cord blood samples seemed to be the richest in DLIS as detected by CI, CA, KK and FPIA (D-I) methods. In uremic patients, only one sample showed detectable DLIS by CI, DP and MA assays, while two sera exhibited apparent digoxin concentration above detection limit by CA and four sera were higher than 0.2 ng ml^{-1} by KK (see Fig. 1). The summary of results of cross-reactivity of DLIS with RIA and FPIA methods are listed in Table 1. For cord blood samples, re-analysis of the samples by TDX D-II assay was significantly higher than the initial values obtained by TDX D-I assay (1.2 ± 0.3 versus 0.42 ± 0.1 ng ml^{-1}, $P < 0.05$). As reflected in Table 1, FPIA showed higher DLIS concentration in most samples when compared with values obtained by the RIA methods. Overall, the degree of interference for DLIS in decreasing order was D-II>D-I>CA>CI≥KK>DP≥MA.

DLIS concentration in uremic, pregnancy and cord blood samples were measured by FPIA in two assays separated by a 4-month period. These data are shown in Fig. 2. The

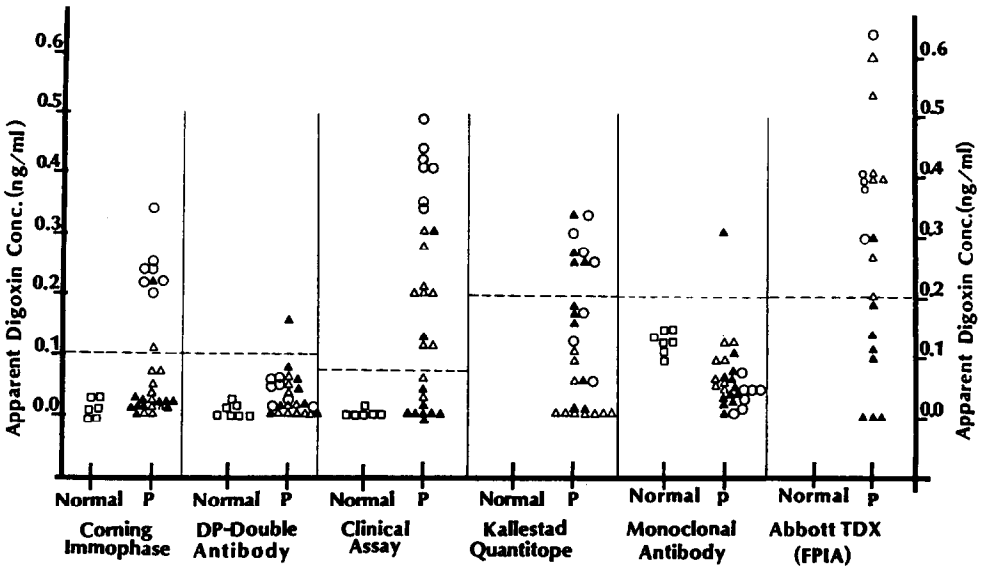

Figure 1
Apparent digoxin concentration (ng ml^{-1}) in serum of normal (□), uremic (▲), third trimester pregnancy (△) and cord blood from neonate (○) who did not receive digoxin previously. The logit-log method of data reduction was used, and each point represents the mean of duplicate measurements. ---, represents the detection limit for RIA and FPIA.

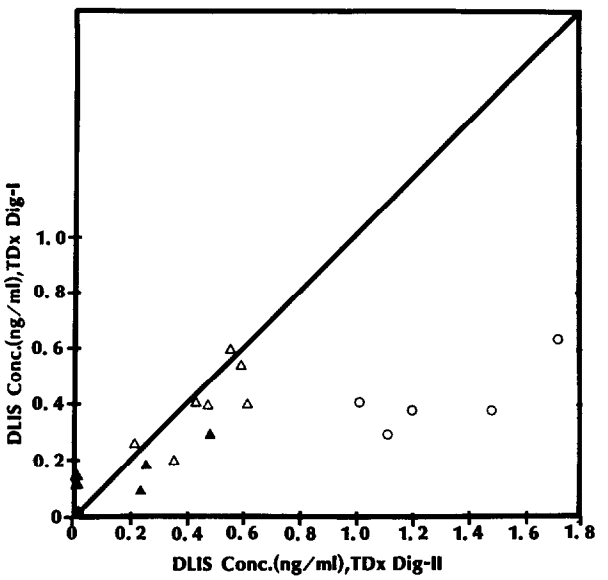

Figure 2
DLIS concentration (ng ml^{-1}) measured by FPIA in uremic samples (▲), third trimester pregnancy (△) and cord blood (○) obtained from patients who did not receive digoxin. There was a 4-month lag period between the first (TDX Dig-I) and second (TDX Dig-II) assays. ———, represents the line of identity, $y = x$.

Table 1
Digoxin-like immunoreactive substance concentration measured in normals, cord blood, uremics and third trimester pregnancy by both RIA and FPIA. Each sample was analysed in two replicate determinations. Data are presented as mean ±SD

	DLIS Concentration (ng ml^{-1})						
	RIA					FPIA (TDx)	
Sample	CI	DP	CA	KK	MA	D-I	D-II
Normals	0.007 ± 0.01	0.006 ± 0.01	0.007 ± 0.01	BDL	0.13 ± 0.01	BDL	BDL
(n = 7)	BDL	BDL	BDL		BDL		
Cord blood	0.24 ± 0.05	0.034 ± 0.03	0.41 ± 0.13	0.21 ± 0.09	0.03 ± 0.02	0.42 ± 0.13	1.2 ± 0.27*
(n = 7)	BDL	BDL			BDL		
Uremics	0.06 ± 0.07	0.08 ± 0.14	0.05 ± 0.1	0.17 ± 0.11	0.07 ± 0.08	0.11 ± 0.10	0.18 ± 0.21
(n = 10)	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Pregnancy	0.04 ± 0.03	0.02 ± 0.02	0.17 ± 0.09	BDL	0.07 ± 0.03	0.40 ± 0.14	0.49 ± 0.14
(n = 10)	BDL	BDL			BDL		

Abbreviations: MA, monoclonal antibody assay; DP, diagnostic products double antibody; KK, Kallestad quantitopes; CI, corning immophase; CA, clinical assay; D-I, digoxin-I assay; D-II, digoxin-II assay; BDL, the mean value was below assay detection limit.

*Mean was significantly different from D-I value ($P < 0.05$).

results of the second run (performed 4 months later by the FPIA, D-II method, showed a dramatic (2.5–4-fold) increase in DLIS concentration in the cord blood samples (see Table 1 and Fig. 2), while the repeat analysis of the same samples by RIA (KK) method revealed no change in the degree of interference by DLIS (data not shown).

Discussion

In recent years, problems in measurement of serum digoxin by RIA methods [5, 23, 24], such as variable cross-reactivity of RIA systems towards known digoxin metabolites [25] have emerged. Additionally, comparison between several commercially available RIA, kits [19, 26, 27] have been reported. The finding of a DLIS in premature and full-term infants who were not receiving digoxin therapy [17, 19, 28] and the impact of DLIS on validity of digoxin measurements [29] has been the subject of several review papers. In the present study, the performance of four commercially available RIA kits with respect to interference by DLIS has been evaluated in samples from uremics, pregnant subjects and cord blood samples [30]. These results have been compared with data generated from an anti-digoxin MA assay (Fig. 1). The results clearly indicated that the highest degree of cross-reactivity of DLIS was observed in cord blood samples. Overall, the MA and DP double antibody systems showed no cross-reactivity with DLIS while CA exhibited the highest cross-reactivity with DLIS. This is consistent with the results reported by other investigators [13, 19–22]. A recent report by Witherspoon *et al.* [31] also confirmed that the DP double antibody RIA system did not detect DLIS in cord blood and in patients with chronic renal failure who were not receiving digoxin. That cord blood samples were richer in DLIS compared with uremic and pregnancy is in excellent agreement with the work of Hicks and Brett [28]. Since serum steroid concentrations are known to be increased in the newborn [32–34] and since digoxin is essentially a steroid derivative, the authors reported that these steroids may collectively represent the DLIS which interfere with most commercially available digoxin RIA systems [28].

In the second part of this study, the cross-reactivity with DLIS has been demonstrated by using the FPIA (D-I and D-II) for digoxin in two assays separated by 4 months. The data for cord blood samples revealed DLIS concentrations in the range of 0.0–0.6 and 0.0–1.7 ng ml^{-1} in the first (D-I) and second (D-II) assays, respectively (see Fig. 2). For cord blood samples, DLIS concentration measured by FIA was 2.5–4-fold higher in the second assay while the re-analysis of the same samples by the KK RIA method showed no change in the DLIS concentration. Generally, DLIS concentrations obtained by FPIA method were higher than those observed by various RIA systems, especially for the pregnancy and cord blood samples (see Table 1). This is in contrast to the results of Yatscoff *et al.* [22] who measured DLIS by RIA and FPIA methods in cord blood samples obtained from 30 neonates not receiving digoxin. These authors concluded that DLIS can be detected by the FPIA in the sera of neonates and uremic patients who are not under digoxin therapy, and the degree of interference is generally less than that seen in commonly used RIA for digoxin. At the present time, we do not have a clear explanation for this discrepancy between our data and the results obtained by other investigators. However, a 2.5–4-fold increase in DLIS, which was observed in the second assay of cord blood samples, may be explained by the change in the protein precipitating reagent by the manufacturer (change from 5% TCA employed in the D-I kit to 3% 5-SSA employed in the D-II kit). However, other factors such as lot-to-lot variability in

digoxin antibody specificity cannot be ruled out. This observation is in agreement with the recent note by Gault *et al.* [35] who reported higher digoxin concentration by FPIA D-II versus the D-I method in cord blood and sera from hepatic and renal failure patients who did not receive digoxin. Although the exact mechanism by which deproteinization with 3% 5-SSA may cause the elevated DLIS levels is not known, the release of additional DLIS from the protein binding site by 5-SSA versus TCA is a likely explanation. Until more specific assays are developed, DLIS will continue to complicate serum digoxin monitoring. Therefore, spurious digoxin levels are possible in patients with severe renal/hepatic dysfunction or uremia under digoxin therapy, unless the method of measurement has been characterized with respect to interference by DLIS.

In summary, we have characterized four commonly used RIA kits with respect to their degree of cross-reactivity with DLIS. These results were compared with those obtained from a digoxin MA assay. All methods showed some degree of cross-reactivity with DLIS with the exception of the DP double antibody system and the monoclonal assay. The sensitivity to DLIS varied among the assay systems. Measured DLIS levels were different according to the sample type. Cord blood was found to be richer in DLIS than others. Analysis of all samples by FPIA revealed a slightly higher degree of interference by DLIS compared with those obtained by the RIA procedures. Re-analysis of cord blood samples by FPIA using 5-SSA as a deproteinizing agent indicated an even higher apparent serum digoxin concentration when compared with TCA as a precipitant. This shows that FPIA is affected by the protein precipitating reagent used in this assay as well as by the length of time between the two analyses. This effect of 5-SSA may have been increased by repeated freeze–thaw cycles.

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[*Received for review 9 May 1988; revised manuscript received 31 October 1988*]