Fig. 1. Plots illustrating in vitro interference of DIGIBIND or DigiFab with 13 digoxin immunoassays.

Group A (*panels A1–A4*) includes methods with marked interference; group B (*panels B1–B4*) includes methods with moderate interference; and group C (*panels C1* and *C2*) includes methods with minimal interference. Within each group, *panels 1* or *3* represent assays performed on samples treated with DIGIBIND, *panels 2* or *4* represent assays performed on samples treated with DigiFab. Within groups A and B, *panels 1* or *2* represent assays in the absence of digoxin, *panels 3* or *4* represent assays in the presence of 40 μg/L digoxin. In group C (*panels C1* and *C2*), only data obtained in the presence of 40 μg/L digoxin are shown with ultrafiltrates (*UF*) for the TDx and AxSYM assays.

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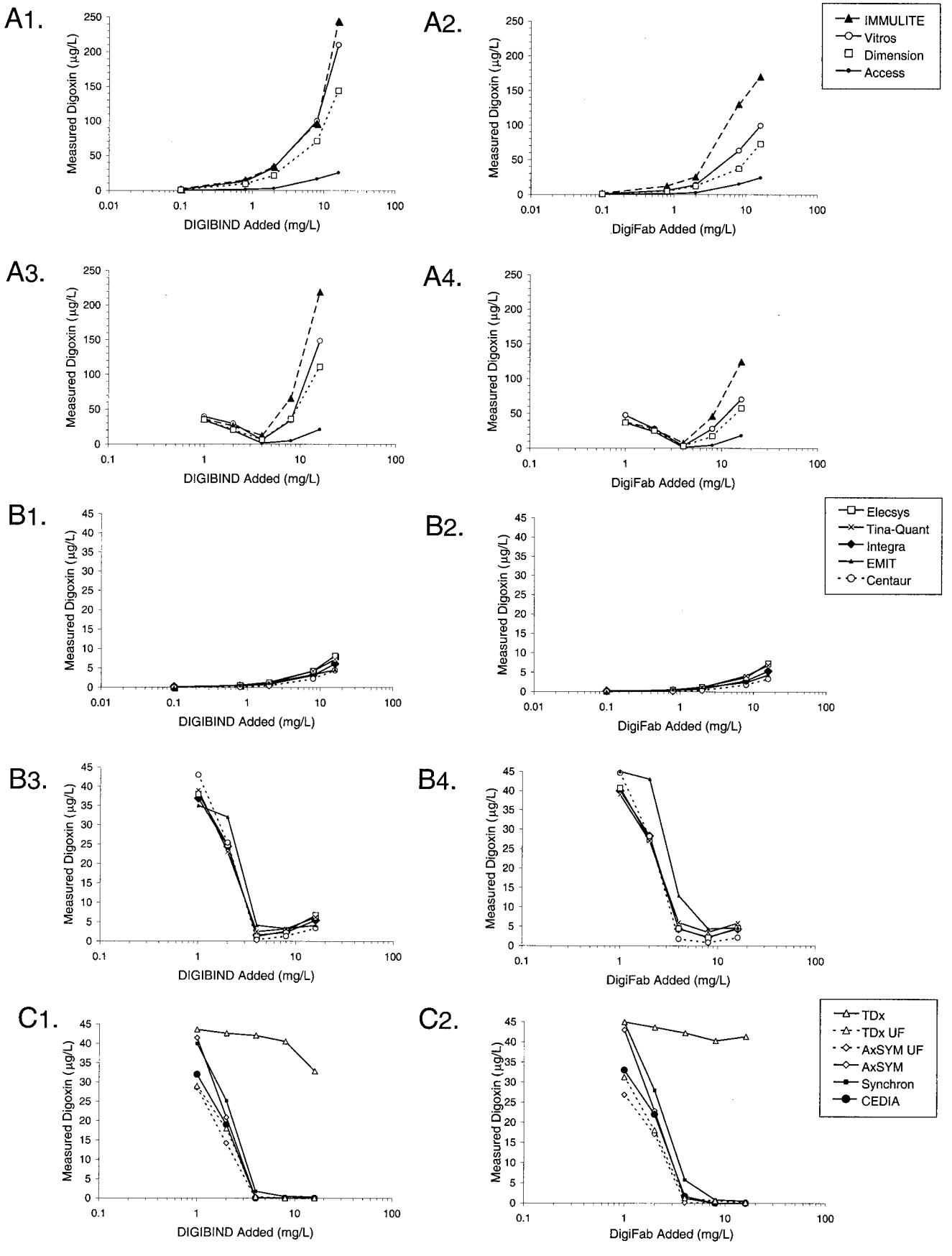
Digoxin is widely prescribed for the treatment of cardiac conditions *(1)*. Because of its narrow therapeutic range, digoxin-related toxicity resulting from acute or chronic overdose is common. Metabolites of digoxin as well as related compounds, including digitoxin, tanshinones, bufandienolide, and oleander, can contribute to or independently produce digoxin toxicity *(2, 3)*. Digoxin toxicity can be rapidly and safely reversed by administration of anti-digoxin immune fragments (Fab) such as DIGIBIND®, which has been available in the US since 1986. Therapeutic Fab products act by binding digoxin with high affinity (10^9–10^{10} L/mol), favoring movement of digoxin out of tissue and thus promoting elimination. Factors that impact dosing with Fab products include known or suspected digoxin load, patient weight and history, and renal function *(4–7)*.

Monitoring the free digoxin concentration after Fab administration may help ensure appropriate dosing, prevent deadly recrudescent toxicity, and determine when digoxin therapy should be resumed *(8–10)*. Monitoring free digoxin in serum is challenged by the positive interference that has been extensively described with DIGIBIND, which interferes with immunoassays by competing with assay capture antibodies. The degree of interference depends on incubation times, washing steps, and the affinity of capture antibody for bound vs free digoxin *(11–13)*. Consequently, monitoring of free digoxin in ultrafiltrates is a popular strategy for managing DIGIBIND-treated patients. Although ultrafiltration eliminates interference produced by large molecules, such as endogenous digoxin-like immunoreactive factors (DLIFs) and DIGIBIND, it does not eliminate interferences produced by small molecules known to interfere with digoxin immunoassays, such as spironolactone *(14)*. In addition, ultrafiltration methods are not standardized, may require matrix-specific calibration, add expense and manual manipulation, and lengthen turnaround time *(8, 15, 16)*.

DigiFab™ is a Fab preparation that was approved by the Food and Drug Administration in 2001 for treating potentially life-threatening digoxin toxicity or overdose. Fab is produced by immunization of sheep with digoxin (DIGIBIND) or digoxindicarboxymethylamine (DigiFab), followed by purification of the Fab from blood. The approximate molecular weights of DigiFab (46 000) and DIGIBIND (46 200) are similar, and a single vial of either DIGIBIND or DigiFab will bind ~0.5 mg of digoxin in vivo. As such, the clinical claims, dosing recommendations, and administration of DigiFab are identical to those of DIGIBIND. However, clinical studies have monitored DigiFab therapy by measuring digoxin in ultrafiltrates only *(17)*. The present study was designed to determine whether clinically relevant concentrations of DigiFab in serum interfere with 13 digoxin immunoassays and to compare results with DIGIBIND.

Single vials of DIGIBIND (38 mg; Glaxo Wellcome Inc.) and DigiFab (40 mg; Protherics, Inc.) were dissolved in 4 mL of type 1 water. DIGIBIND and DigiFab were added to pooled drug-free and DLIF-free serum to obtain final concentrations of 0.1, 0.8, 2, 8, and 16 mg/L. Increasing concentrations of each antidote (1, 2, 4, 8, and 16 mg/L) were also combined with 40 μg/L digoxin (Sigma-Aldrich) in pooled serum. Ultrafiltrates were prepared by use of Millipore Centrifree filters (30 kDa) and a fixed-angle centrifuge rotor (2000*g* for 30 min at ambient temperature). Prepared samples were aliquoted and stored frozen (−70 °C) until analyzed, a practice that should not affect the ability of DIGIBIND to bind digoxin *(18)*.

Digoxin was measured by 13 commercially available competitive immunoassays. Five homogeneous assays (Beckman-Coulter Synchron; Roche CEDIA, Integra, and TinaQuant; and Syva Emit 2000) and seven heterogeneous assays (Abbott AxSYM, Beckman-Coulter Access, Chiron ADVIA Centaur, Dade Behring Dimension RxL, DPC IMMULITE 2000, Ortho Vitros, and Roche Elecsys 2010) designed to quantify free digoxin in patient serum or plasma were evaluated. The CEDIA and TinaQuant as-


A1.
A2.
A3.
A4.
B1.
B2.
B3.
B4.
C1.
C2.
Measured Digoxin (µg/L)
DIGIBIND Added (mg/L)
DigiFab Added (mg/L)
IMMULITE
Vitros
Dimension
Access
Elecsys
Tina-Quant
Integra
EMIT
Centaur
TDx
TDx UF
AxSYM UF
AxSYM
Synchron
CEDIA

says were performed on the Hitachi 917 platform, and the Hitachi 717 platform was used to perform the Emit assay. The Abbott TDx/TDxFLx assay, which measures total digoxin in patient serum or plasma, was also used. Assays were calibrated and performed according to manufacturers' instructions, and three controls (Bio-Rad) were included with each run. Although most assays were performed with serum only, the TDx and AxSYM were performed with both serum and ultrafiltrate. Samples with values exceeding the reportable range of the assay were diluted and reassayed as recommended by the manufacturer. Plotted points represent a single result in most cases; points represent the average result when more than one result was obtained.

Positive interference in the presence and absence of digoxin was observed with DIGIBIND and DigiFab (Fig. 1). Although interference was somewhat less with DigiFab (Fig. 1, panels A2, A4, B2, and B4) compared with DIGIBIND (Fig. 1, panels A1, A3, B1, and B3), these differences in magnitude are not likely to be clinically significant and may represent batch-specific differences *(18)*. The magnitude of the interference varied considerably with each method. As such, the methods were grouped as follows: group A (marked interference), IMMULITE, Vitros, Dimension, and Access methods; group B (moderate interference), Elecsys, TinaQuant, Integra, Emit, and Centaur methods; and group C (minimal interference), TDx, AxSYM, Synchron, and CEDIA methods. Interference in the absence of digoxin was observed at the lowest concentration of DIGIBIND tested (0.1 mg/L) with the IMMULITE (2.2 μg/L digoxin measured), Vitros (1.5 μg/L), and Dimension (0.9 μg/L) methods, whereas higher concentrations of the antidotes were required to observe interference in other group A and B methods. The interference in all group A and B methods was concentration-dependent and followed a linear relationship over the concentrations examined.

Interference was then evaluated in the presence of a digoxin concentration (40 μg/L) consistent with that observed clinically after a patient has been poisoned with digoxin *(8, 10, 17)*. Increasing concentrations of DIGIBIND or DigiFab reduced the amount of digoxin measured in all but the TDx method. The TDx method, the only method used here that incorporates a preanalytical protein precipitation step, provides results that are most consistent with total digoxin concentration in the presence of therapeutic Fab *(8, 10)*. Some inaccuracy was observed with the TDx method, as indicated by the results shown in Fig. 1, panels C1 and C2. The inaccuracy may be attributed to error introduced by manual dilution or by coprecipitation of digoxin with protein, a phenomenon suggested to occur with high protein concentrations *(12)*.

Data most consistent with free digoxin in the presence of Fab were obtained with ultrafiltrates (Fig. 1, panels C1 and C2). The discrepancies in results observed at 1 mg/L Fab (Fig. 1, panels C1 and C2) are curious. The predicted value of free digoxin at this concentration of Fab is 30 μg/L, which is similar to that seen with ultrafiltrates and with the CEDIA method. The reason that the AxSYM and Synchron methods modestly overestimated the digoxin concentration at this concentration of either DIGIBIND or DigiFab is unknown.

Equimolarity and consequent neutralization of 40 μg/L digoxin should be achieved with 4 mg/L of either Fab product. Complete neutralization of digoxin was observed with 4 or 8 mg/L Fab in the AxSYM, Synchron, and CEDIA methods (Fig. 1C), but was not observed with those assays in groups A and B (Fig. 1, panels A3, A4, B3, and B4). With Fab concentrations >4 mg/L, unbound Fab (antidote excess) would be expected. Accordingly, the

Table 1. Comparison of results from patients treated for digoxin toxicity with DIGIBIND.

			Measured digoxin,[b] μg/L				
Group	Assay	Reportable range,[a] μg/L	Pool 1 (DIGIBIND excess)	Pool 2 (Digoxin excess)	Pool 3 (Digoxin excess)	Pool 4 (Digoxin excess)	Pool 5 (~equimolar)
A	IMMULITE	0.2–8.0	77.4 (15 480%)	3.6 (206%)	7.9 (192%)	5.4 (180%)	5.7 (1146%)
	Vitros	0.4–4.0	58.0 (11 600%)	2.8 (159%)	6.0 (147%)	4.0 (133%)	3.8 (760%)
	Dimension	0.06–5.0	26.0 (5202%)	1.4 (82%)	2.9 (70%)	2.1 (71%)	1.8 (363%)
	Access	0.2–6.0	7.9 (1572%)	2.3 (132%)	4.9 (119%)	3.4 (112%)	1.8 (352%)
B	Elecsys	0.15–5.0	2.5 (504%)	3.0 (172%)	7.0 (172%)	4.3 (143%)	3.4 (680%)
	TinaQuant	0.15–7.5	3.4 (680%)	3.0 (170%)	6.9 (169%)	5.3 (177%)	4.4 (880%)
	Integra	0.13–5.0	2.5 (500%)	2.9 (165%)	6.0 (147%)	4.7 (157%)	3.2 (640%)
	Emit	0.2–5.0	3.9 (780%)	3.5 (199%)	4.4 (108%)	3.5 (117%)	4.4 (880%)
	Centaur	0.1–5.0	1.7 (330%)	2.7 (152%)	6.2 (152%)	5.0 (167%)	1.7 (344%)
C	TDx	0.5–5.0	23.7	7.5	12.9	8.4	33.2
	TDx UF[c]	0.5–5.0	<0.5	1.8	4.1	3.0	0.5
	AxSYM UF	0.3–4.0	<0.3	1.9	4.2	2.8	0.5
	AxSYM	0.3–4.0	<0.3 (100%)	1.8 (104%)	4.5 (110%)	4.1 (137%)	1.3 (264%)
	Synchron	0.2–5.0	<0.2 (100%)	3.0 (168%)	6.7 (164%)	4.1 (137%)	3.7 (730%)
	CEDIA	0.15–4.0	<0.15 (100%)	2.5 (142%)	5.0 (123%)	3.4 (113%)	1.3 (260%)

[a] Reportable ranges were obtained from the manufacturers.
[b] Values in parentheses are the percentage of the TDx ultrafiltrate, where <0.5 = 0.5.
[c] UF, ultrafiltrate.

digoxin measured by group A and B methods increased linearly with Fab concentrations >4 mg/L. No increase in digoxin was observed with group C methods. The data presented in Fig. 1 suggest that positive interference from unbound Fab should be expected with all group A and B methods. As such, accurate interpretation of free digoxin concentrations in samples containing near-equimolar antidote or antidote excess requires evaluation of ultrafiltrate or use of an assay with which the immunotherapeutic agent does not interfere. The mechanism of Fab interference in specific digoxin immunoassays may be attributable to differences in the affinity and/or specificity of the capture antibodies used in each assay. It is of interest that the presence of a wash step (heterogeneous design) did not consistently prevent or minimize Fab-induced interference.

Because it is known that in vitro samples may not accurately represent samples collected in vivo *(13)*, samples obtained from patients treated with DIGIBIND for digoxin toxicity were also evaluated. Residual patient serum was collected and pooled according to human subject guidelines approved by the Institutional Review Board (University of Utah). The data in Table 1 are expressed as measured digoxin (μg/L) and as a percentage of the ultrafiltrate result from the TDx method, a method reported to be free of matrix bias and clinically demonstrated as suitable for monitoring DIGIBIND-treated patients *(19)*. Results for ultrafiltrates obtained with the TDx method agreed well with results obtained for both serum and ultrafiltrates in the AxSYM method. Under conditions of DIGIBIND excess (Table 1, pool 1), a pattern of positive interference was observed that corresponded well with the in vitro predictions derived from Fig. 1. Thus, total digoxin (TDx) was 23.7 μg/L, whereas free digoxin was undetectable when evaluated with group C methods and in an ultrafiltrate. The highest concentrations of apparent digoxin were observed with the group A methods in a pattern consistent with that seen in vitro. Likewise, moderate but clinically significant overestimations of digoxin were observed with group B methods.

Under conditions of digoxin excess (Table 1, pools 2–4) or in the presence of equimolar digoxin and DIGIBIND (Table 1, pool 5), the extent of neutralization varied considerably with the method. Furthermore, the pattern of results was not consistent with that observed in vitro. For example, both the highest and lowest results for pools 2–4 were observed with group A methods (IMMULITE and Dimension, respectively), methods for which marked interference was observed in vitro. For pool 5, digoxin results were overestimated by all methods compared with results obtained after ultrafiltration. Thus, results obtained with group A methods were 352-1146% of the results obtained for ultrafiltrates, results with group B methods were 344–880% of the results obtained for ultrafiltrates, and results with group C methods were 260–730% of the results obtained for ultrafiltrates. These data suggest that the degree of Fab-induced positive interference observed in vivo cannot be predicted solely from in vitro data, particularly when the molar ratio of Fab to digoxin is $\leq$ 1. A possible explanation for the difference in results between the in vitro and in vivo specimens is the contribution of digoxin metabolites and DLIFs to in vivo specimens. Such compounds would be extracted from tissue stores by Fab therapy and are likely to interfere with digoxin immunoassays, depending on the corresponding affinity of and reactivity with capture antibodies. Further research is required to fully characterize these interactions.

In conclusion, positive interference should be anticipated when samples from patients treated with either DIGIBIND or DigiFab are tested with any of the following digoxin immunoassays: Access, Synchron, Centaur, Dimension, IMMULITE, Vitros, Elecsys, CEDIA, Integra, TinaQuant, and Emit. Although minimal interference was observed with the AxSYM method, ultrafiltration remains the best strategy for accurate determination of free digoxin concentrations in the presence of therapeutic Fab products.

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