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Problems with the immunoassay of digoxin*

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Abstract: Factors that affect the standardization and reliability of the radioimmunoassay of digoxin are reviewed. Some new data are presented on standardization and suggestions are made for dealing with problems in the design and techniques of assays.

Keywords: *Immunoassay; radioimmunoassay; digoxin; plasma; interlaboratory variation; matrix interference.*

Introduction

Reliable methods for the determination of digitalis glycosides in biological fluids are a prerequisite for the rapidly expanding knowledge of the pharmacokinetics of these drugs. The usefulness of such measurements for therapeutic guidance especially of digoxin is also well documented [1–8] although doubts have been cast on their value [9, 10]. Since the therapeutic indices of the glycosides are so narrow, there is a need to determine serum drug concentrations with good accuracy and precision. So far the predominant technique for the assay of digitalis glycosides has been radioimmunoassay (RIA) which was introduced for digoxin by Smith *et al.* in 1969 [1]. RIA of digoxin has comparatively poor precision and is subject to non-specific interference and varying cross-reactivity to digoxin metabolites as well as problems with standardization [11].

In the last ten years the authors have studied problems in the standardization and reliability of the RIA of digoxin. These studies are reviewed and some new data on standardization are presented.

Experimental

The accuracy and precision of commercially available RIAs of digoxin were evaluated within the authors' laboratory and as an interlaboratory study. In addition, matrix influence and standardization were studied using different immunoassays for digoxin available as commercial kits. Brief descriptions of study designs and of immunoassay kits are given in Tables 1 and 2. Details of the experimental design and conditions of some of these studies have been described previously [11–16]. A randomized order of samples and standards was used in all RIA studies.

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Table 1
Specification of digoxin immunoassay kits used in different studies

Kit		Buffer	Separation Technique	Tracer Digoxin
SM^{3}H	Digoxin RIA Kit ^{3}H formerly from Schwarz/Mann now from Becton Dickinson (BD) Orangeburg, NY, USA	0.01 M Phosphate 0.15 M NaCl pH 7.4	Dextran-coated charcoal (0.25% dextran + 1% Norit A charcoal)	^{3}H-digoxin
SM^{125}I	Digoxin RIA Kit ^{125}I formerly from Schwarz/Mann now from BD, Orangeburg, NY, USA	0.01 M Phosphate 0.15 M NaCl pH 7.4	Dextran-coated charcoal (0.25% dextran + 1% Norit A charcoal)	3-*O*-succinyldigoxigenin-L-tyrosine (^{125}I)
CAG	Gammacoat ^{125}I Digoxin RIA Kit from Clinical Assays (CA), Cambridge, MA, USA	0.01 M Tris 0.14 M NaCl pH 7.4	Antibody-coated tubes	^{125}I-digoxin derivative or 3-*O*-succinyldigoxigenin-L-tyrosine (^{125}I)
NEN ^{125}I	New England Nuclear (NEN), North Billerica, MA, USA	0.02 M Phosphate + BSA 0.5% pH 7.4	Coated charcoal (0.5% BSA + 0.25% Norit A charcoal)	Digoxin–histamine-^{125}I-conjugate
Phabebas ^{125}I	Pharmacia Diagnostics AB, Uppsala, Sweden	Phosphate + 0.3% HSA, pH 7.4	Sephadex-coupled antibody	Digoxin ^{125}I-derivative
EMIT	SYVA, Palo Alto, California	0.055 M Tris HCl pH 8	Homogeneous	Digoxin-glucose-6-phosphate dehydrogenase
Farmos ^{125}I	Farmos Digoxin ^{125}I Radioimmunoassay Kit, Farmos Diagnostica. Turku, Finland	0.1 M Tris HCl pH 7.4 + 1% γ-globulins	PEG	^{125}I-digoxin derivative
Thorell	(J. I. Thorell and S. M. Larsson, 1978)	Barbitone + BSA 0.0025%, pH 8.6	Double antibody	Digoxin-BSA-^{125}I-derivative

PEG = polyethyleneglycol; BSA = bovine serum albumin; HSA = human serum albumin.

Table 2

Study	Assay method used	Design
Comparison of RB* and 4 kits for digoxin RIA [12]	RB, SM^3H, $SM^{125}I$, CAG, and NEN ^{125}I	45 samples from a standard plasma spiked with digoxin (2.6 nM) and 35 plasma samples from patients on digoxin therapy were assayed in duplicate by 4 commercial RIA kits. Seven assay runs with each kit and randomization of samples to runs and randomized placement of tubes within runs was practised
Inter- and intra-laboratory [13]	10 different methods, most frequently CAG, NEN ^{125}I and $SM^{125}I$	10 samples from a spiked normal plasma (digoxin 2.6 nM, SP) and a pooled plasma from patients on digoxin (PP) were sent openly on 10 different occasions to 27 laboratories in Sweden
Assay of standard material from four suppliers a = BD; b = Pharmacia; c = CA; d = NEN [11, 15]	Phadebas ^{125}I	Standards at levels 1, 2 and 4 μg/l were assayed in duplicate in a total of 8 assay runs together with standards prepared in normal human plasma spiked with WHO-digoxin
Comparison of standards in spiked normal human serum (WHO digoxin) and commercial standards	EMIT	Assay of commercial standards and standards in spiked normal human serum in duplicate, randomized, except for the first 6, which were in ascending order
Matrix influence from plasma samples from patients treated with spironolactone ($N = 7$), patients with uremia ($N = 11$) and patients with acute myocardial infarction (AMI, $N = 13$) [14]	SM^3H, CAG, $SM^{125}I$, NEN ^{125}I	Spiked samples from three categories of patients (2.5 nM) were randomly assigned to 6 assay groups each containing the same number of samples from each category. All samples were assayed in two different runs by each of the 4 methods. Standards and samples were assayed in duplicate and with the order of the single tubes in each run being randomized.
Matrix influence from the plasma of a patient with abnormal serum proteins [16]	NEN ^{125}I, Farmos ^{125}I, BD ^{125}I, Phadebas ^{125}I, Thorell	Assay in duplicate in two runs with each kit were performed using spiked and nonspiked patient's plasma together with spiked plasma standards; randomized placement of tubes within run.

* Inhibition of ^{86}Rb uptake into red blood cells according to Bertler *et al.*, 1972.

In an attempt to simplify the EMIT cad Digoxin Assay from SYVA (Paalo Alto, California) adapted to the Cobas BIO centrifugal analyzer (Roche Analytical Instruments Inc., Nutley, NJ 07110), attempts were made to omit pretreatment of standards and samples with the alkaline solution supplied. After this solution has been added, samples must be assayed within 5 min; otherwise a new portion of sample must be pipetted and treated with the solution, which is tedious and cumbersome. A comparison was made of the author's own standards in normal human serum [11] with those commercially supplied using the EMIT cad digoxin assay. Standards were assayed with and without alkaline pretreatment.

Results and Discussion

The relative standard deviation of the assay methods studied varied from 5–14%. The accuracy of the standard plasma varied by 30% and that of patients' samples by 40% [12].

The mean concentrations for the 27 laboratories obtained at the 10 assay rounds [13] together with ranges of round means and mean interassay standard deviation (ISD, nM) are given in Table 3. The performance of the best and worst laboratory is illustrated in Fig. 1. Table 4 gives ISDs and mean concentrations obtained for different laboratories with the most frequently used assay kits. It appears that the same kit gives an ISD varying as much as 0.10–0.43 nM when used by different laboratories. Results of studies on the effects of varying skill in handling the same assay method, as observed for the antiepileptic drug phenytoin [17], are in line with the present observations.

The precision of the digoxin immunoassay obtained in the authors' studies is in the same order of magnitude as that experienced by others [18–25]. A 95% confidence interval as wide as 1 nM is unacceptable for a drug with such a narrow therapeutic index as digoxin. To the authors' knowledge there is no recent evidence that the situation has improved. Rather, there is a tendency that more assay designs appear with new difficulties and other sources of error and apparently not much improvement of precision [26–30]. A number of different factors determine the precision obtainable in immunoassays [11, 18]. Of importance is high avidity of the antiserum, the structure of the

Table 3
Digoxin concentrations (C; nM) of SP and PP obtained at 27 Swedish laboratories at 10 assay rounds. The precision achieved by each laboratory is expressed as interassay standard deviation (ISD; nM). Significant differences between concentration means are shown: * = $P < 0.05$; ** = $P = < 0.01$; *** = $P < 0.001$. Significant differences ($P < 0.05$) in ISD between laboratories are given in absolute numbers out of the possible number of comparisons

	SP	PP
Mean C	2.59	2.46
S.D. of round means	0.03	0.03
S.D. of laboratory means	0.20	0.18
Range of round means	2.55–2.64	2.41–2.51
Range of laboratory means	2.15–2.85***	2.12–2.72***
Average S.D. of C within rounds	0.33	0.31
Average ISD	0.25	0.24
Range of ISD	0.07–0.61	0.05–0.43
Interlaboratory ISD differences	156/361	165/361

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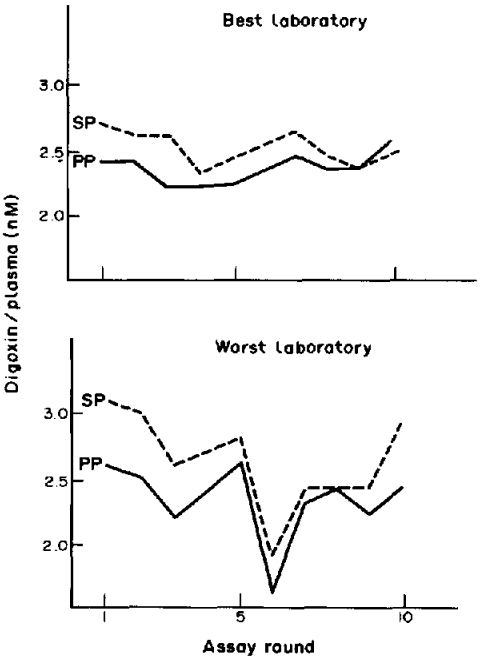

Figure 1
A comparison of assay results of a standard pool (SP spiked with digoxin 2.6 nM/l) and a pooled patient plasma (PP) obtained by the best (above) and the worst (below) laboratory taking part in an interlaboratory study of digoxin assays performed in Sweden in 1977.

Table 4
Digoxin assays of SP and PP performed with the three most frequently used RIA methods (N refers to the number of laboratories). The concentrations (C; nM) are given as mean ±S.D. The precision obtained with the RIAs is expressed as average interassay standard deviation (ISD; nM). Ranges are given within brackets. Significant differences in mean C within groups of laboratories using the same RIA are shown; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Significant differences ($P < 0.05$) in ISD within such groups are given in absolute numbers out of the possible number of comparisons

RIA	C	ISD
CAG	SP 2.62 ± 0.15 (2.48–2.81)	0.31 (0.14–0.43) 13/21
$N = 7$	PP 2.42 ± 0.16 (2.14–2.59)**	0.25 (0.10–0.43) 7/21
NEN ^{125}I	SP 2.70 ± 0.10 (2.59–2.85)	0.25 (0.12–0.40) 8/21
$N = 7$	PP 2.57 ± 0.11 (2.40–2.72)	0.24 (0.10–0.38) 10/21
SM^{125}I	SP 2.32 ± 0.15 (2.15–2.51)**	0.19 (0.15–0.23) 0/6
$N = 4$	PP 2.66 ± 0.16 (2.12–2.50)**	0.22 (0.17–0.27) 0/6

Reproduced from *Acta Pharmacologica et Toxicologica* **45** 66–72 (1979), see ref. [13].

labelled ligand, incubation time, design of the reaction conditions and the separation procedure. Among the factors of importance for the accuracy of an immunoassay are the method of standardization, the cross-reactivity of metabolites and of structurally similar compounds, and non-specific interference.

The experience of the present authors of matrix interference is shown in Table 5 [14]. Two of the four assay methods studied showed a significant influence from spironolactone metabolites, another was affected by uraemic plasma and a third by plasma from patients with acute myocardial infarction. Others have reported similar experiences [31,

Table 5
Precision of digoxin radioimmunoassays and matrix effects

Mean digoxin concentration measured after adding 2.5 $nmol^{-1}$

Assay method	Spironolactone	Plasma category Uremia	AMI	Differences between categories
SM^3H	2.46	2.32	2.39	232 ≠2.46*
$SM^{125}I$	2.62	2.57	2.62	NS
CAG	2.68	2.46	2.46	2.46*≠2.68*
$NEN^{125}I$	2.77	2.64	2.56	NS

Significant differences from 2.50 nM are marked by underlining, and between RIA methods by asterisks: ——; or * = $P < 0.05$; ═══; or ** = $P < 0.01$. NS = not significant. Differing RIA methods are connected with lines. [a]Refers to both uraemia and AMI.
Reproduced from *Clinical Biochemistry* **14** 67–71 (1981), see ref. [14].

Table 6
Results of radioimmunoassays of plasma from a patient not taking digoxin

Radioimmunoassay used	Concentrations in sample from patient: without added digoxin nM	with added digoxin* nM	Normal plasma: with digoxin† nM
$NEN^{125}I$	4.4	5.0	2.0
$Farmos^{125}I$	3.1	3.9	2.1
$BD^{125}I$	1.4	1.5	2.0
$Phadebas^{125}I$	0.6	0.7	2.0
Thorell	0.25	0.6	1.9

*1.5 nM, †2 nM.
Reproduced from *N. Engl. J. Med.* **310** 725 (1984), see ref. [16].

32]. In another study [16] results with plasma from a patient with an abnormal protein pattern, who had not taken digoxin for 2 months were dependent on which assay method was used (Table 6). Results with two methods gave an impression of overdose, whereas results with a third method gave a result within the therapeutic range. Only two of the methods gave results in the region of the detection limit of the assays.

The influence of the matrix is sometimes difficult to foresee and explain fully but some experiences in the course of the studies are worthy of notice. An assay that is better optimized in respect of amounts of reagents (assay design) and time for equilibration gives better precision and is less prone to suffer from matrix influences. It is also important to have antisera with a sufficiently high affinity constant (at least in the order of 1.10^{-10}l mol^{-1}). Separation procedures based on adsorption give different kinds of matrix influences and should be abandoned especially since charcoal is also difficult to define [33]. Assay systems should be characterized for cross-reactivity to spironolactone metabolites as well as to hydrolysis metabolites of digoxin and to dihydrodigoxin. There is insufficient information of this nature from commercial suppliers.

Results of standards from four commercial sources are shown in Table 7 [11, 15]. A variation up to 30% was found. These deviations are not easy to understand but might be explained by the degree of purity of the digoxin substance and also its dissolution and dilution as well as the matrix used (plasma or serum) [34]. A variation of standards by as much as 30% between different commercial sources is unacceptable for standardization. From other reports a variation of up to 50% between different commercial sources can be expected [35]. Evidence that the matrix is of great importance in standardization has also been reported by others [36–38]. Such deviations are unacceptable in view of the therapeutic range [3].

In an attempt to simplify and speed up the EMIT cad digoxin assay, attempts were made to omit the alkaline pretreatment procedure in the kit since samples have very low stability after addition of this reagent and cannot be reassayed as such after treatment. It was found that standards differed by up to 50% (Fig. 2) when not treated whereas after treatment results were comparable. It appears that factors in the matrix have importance for the outcome of the assay results.

How then should problems in the immunoassay of digoxin be dealt with? First, the design of the assays should be optimized and only antisera with sufficiently high affinity

Table 7
Mean digoxin concentrations measured by RIA without extraction (Phadebas) in commercial standard preparations

Specified concentration (nM)	Kit source	Concentration measured ($\bar{x} \pm SD$, $N = 8$)
1.3	SM	1.25 ± 0.09
	CAG	1.22 ± 0.13
	Phadebas	1.20 ± 0.06
	NEN	0.99 ± 0.13
2.6	Phadebas	2.52 ± 0.12
	SM	2.44 ± 0.16
	CAG	2.34 ± 0.13
	NEN	1.98 ± 0.09
5.1	SM	4.08 ± 0.24
	Phadebas	4.06 ± 0.19
	CAG	3.96 ± 0.22
	NEN	3.65 ± 0.14

Significant deviations from the specified concentrations are marked by underlining and between RIA methods by asterisks connecting the differing preparations: ——; or $^{*}P < 0.05$; ═══; or $^{**}P < 0.01$; ═══ or $^{***}P < 0.001$. N refers to number of assay runs.

Reproduced from *Acta Pharmacologia et Toxicologia* **59**, Suppl. IV (1986), see reference [11].

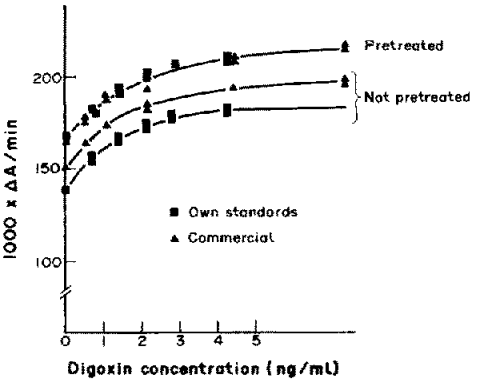

Figure 2
A plot of absorbance difference Δ A per min ($1000 \times \Delta A\ min^{-1}$), a measure of enzyme activity, versus digoxin concentration for own standards in spiked normal serum as compared with commercial standards from SYVA in the EMIT cad digoxin assay system.

constants should be used. Further, the assays will gain from better separation procedures than that using dextran-coated charcoal. Manufacturers must also solve the problems of standardization. This includes making proper solutions, adequate dilutions and the use of a common well-defined matrix. Kits should be characterized for cross-reactivity to metabolites of digoxin and structurally similar interfering compounds like spironolactone metabolites and the digitalis-like immunoreactive substances (DLIS). In the meantime a reference method would be of value for samples where problems may be encountered. It should be a responsibility of the laboratory performing digoxin assays to take part in quality control programmes supervised by laboratories with good knowledge of the field.

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