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Digitalis

Clinical Value of the Radioimmunoassay of the Digitalis Glycosides*

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THE common clinical problem of digitalis intoxication (3) has in the past several years spurred rapid progress in the quantitation of serum or plasma digitalis concentrations. Immunochemical methodology has played an important role in these advances, and in this presentation we will consider technical aspects as well as clinical application of cardiac glycoside radioimmunoassays.

Assay Techniques in Current Use

During the 1950's, it became possible to label cardiac glycosides with ^{14}C and tritium (^{3}H). Administration of these labeled cardiac glycosides to experimental animals and human volunteers yielded useful information on the pharmacokinetics of these drugs (15). In addition, development of radioisotope technology laid the ground work for most of the assay techniques to be discussed subsequently. More recently, Lukas and Peterson (31) developed a double isotope dilution derivative assay for digitoxin which has been applied to measurements of digitoxin in plasma, whole blood, urine, and stool. Although technically demanding, this procedure has yielded much useful information on the pharmacokinetics of digitoxin. Watson and Kalman (54) have developed a gas-liquid chromatographic method for assay of digoxin. This procedure is also technically demanding, but has high specificity and should prove to be useful in the detailed definition of metabolites of digoxin.

After Schatzmann's discovery that cardiac glycosides are potent inhibitors of cellular monovalent cation transport, Lowenstein and Corrill (29) developed a red blood cell ^{86}Rb uptake inhibition assay for digoxin and digitoxin. This assay approach has been further developed by Bertler and Redfors (5), and by Gjerdrum (21). Serum digoxin and digitoxin concentrations have also been measured by direct determination of the extent to which these agents inhibit Na+, K+-ATPase (4). Burnett and Conklin (9) have applied this approach to the measurement of digoxin as well as digitoxin.

The techniques currently in most common use for determination of cardiac glycoside concentrations in biological fluids are competitive protein binding assays, either of the radioimmunoassay (37, 48) or enzymatic displacement (8) type. Competitive binding of cardiac glycosides to Na+, K+-ATPase has been used by Brooker and Jelliffe (8) for the measurement of digoxin and digitoxin concentrations, and they have reported results very similar to those obtained by radioimmunoassay. All of these methods for determination of serum cardiac glycoside concentrations have been reviewed in detail recently (11, 51).

Our own experience has been based on the radioimmunoassay approach. Butler and Chen (12) immunized rabbits with conjugates of digoxin and serum albumin, and obtained antibodies which were shown to bind digoxin. Antiserums obtained in this way have been characterized in detail (49)

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as will be discussed subsequently. The exceptionally high affinity and specificity of selected antiserums for digoxin allows quantification of subnanogram amounts of digoxin or digitoxin by convenient methods which are applicable in the well equipped clinical chemistry laboratory.

As outlined in figure 1, an aliquot of serum or plasma containing unlabeled digoxin, without prior extraction, is mixed with an appropriate tracer quantity of digoxin in a suitable buffer volume. The amount of tracer is selected to lie near the midpoint of the range of unknown concentrations to be measured. This amount of tracer then determines the amount of antibody to be used, since optimal results are obtained when the percentage of total tracer bound by antibody is about 37% to 50% in the absence of any competing unlabeled ligand. The high specific activity of tritiated digoxin commercially available and the high affinity of selected digoxin specific antiserums result in a potential sensitivity of 0.1 ng/ml or less. After incubation during which equilibrium is established between tritiated and unlabeled digoxin and antibody binding sites, dextran coated charcoal is added to separate antibody bound from free tritiated digoxin. It is important to expose both known samples used to construct the standard curve and unknown samples to charcoal for similar lengths of time, since the equilibrium shown in figure 1 may be pulled toward the left as free digoxin is bound to charcoal. This problem is of little consequence when very high affinity antibodies such as those just described are used, but becomes an increasing problem with lower affinity antiserums as illustrated subsequently. After centrifugation to remove charcoal, antibody bound tritiated digoxin in the supernatant phase is decanted into liquid scintillation counting medium and counted in a liquid scintillation spectrometer equipped with a radium 226 external standard for quenching correction.

Standard curves can be plotted in several ways, including the two shown in figure 2. We have tended to use the reciprocal plot shown on the right because of its applicability to use with a computer program. The computer plots the best fit for rectilinear standard curves by least squares linear regression analysis, compares count rates for unknown samples, and prints out the concentration values for unknown samples.

The specificity of this assay system has been assessed by hapten inhibition experiments as shown in figure 3. None of the endogenous steroid compounds tested produced measurable displacement of ^{3}H-digoxin from antibody binding sites of this antiserum unless present in greater than 1000-fold molar excess.

This assay system can also be used for the

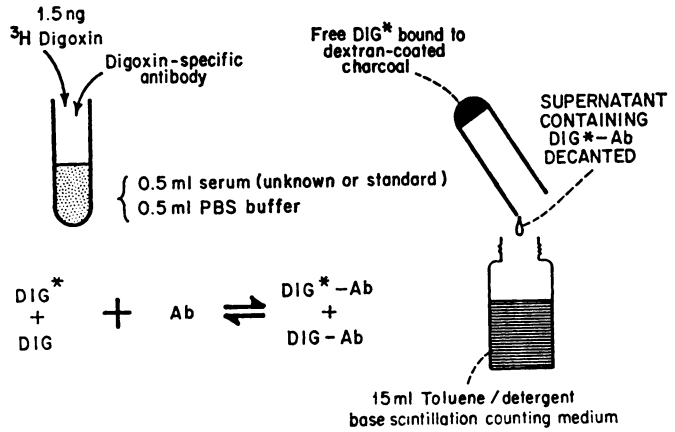

FIG. 1. Schematic representation of digoxin radioimmunoassay procedure. DIG, digoxin; DIG*, ^{3}H-digoxin; Ab, digoxin-specific antibody.

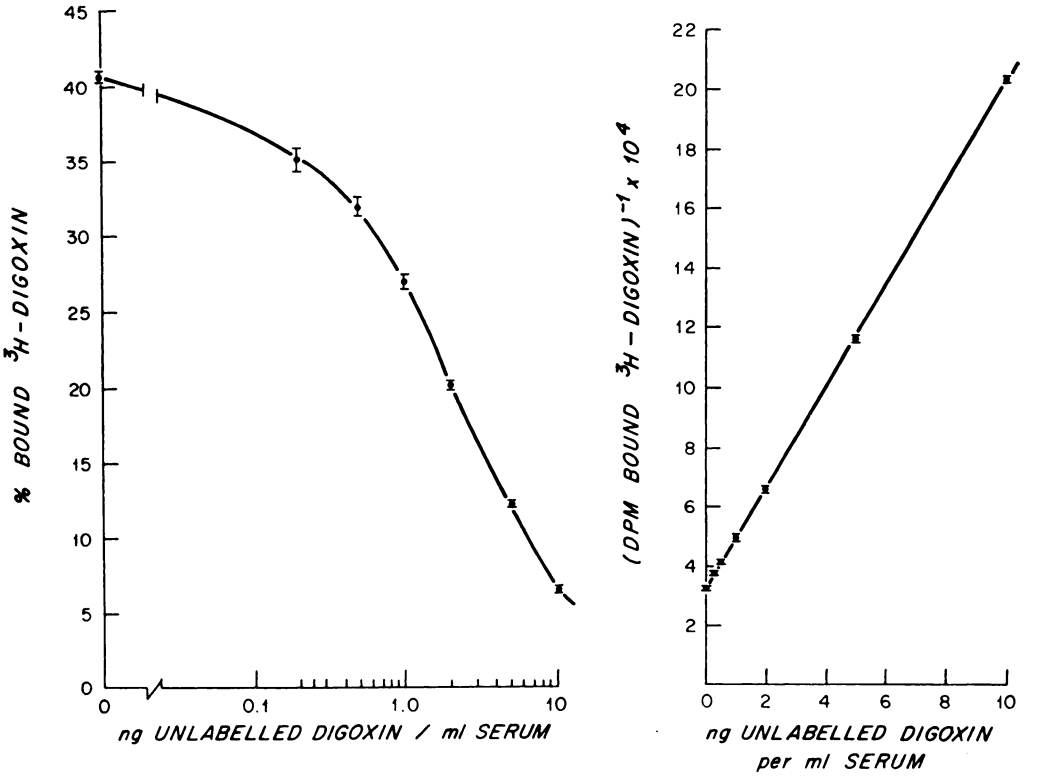

FIG. 2. Standard curves for digoxin radioimmunoassay. The same data plotted as percent of antibody bound ^{3}H-digoxin *versus* log unlabeled digoxin concentration on the left, and by a reciprocal plot on the right. The rectilinear plot obtained in the latter case facilitates computer data processing.

determination of serum deslanoside concentrations. Known amounts of deslanoside are used to construct the standard curve, and tritiated digoxin is used as tracer. Radioimmunoassay techniques have also been developed for digitoxin (37, 44), ouabain (45), and acetyl strophanthidin (42).

It seems appropriate at this time to review briefly a number of potential pitfalls which appear to have befallen some investigators who have employed radioimmunoassay techniques for the measurement of cardiac glycosides. Like any other techniques, these require a fundamental understanding of the molecular interactions involved and a number of recent publications and letters to editors suggest that this understanding is lacking in a number of instances. It goes without saying that meticulous attention to detail and continuous quality control are necessary to avoid supplying the clinician with erroneous results.

Assay results can be no more accurate than the preparation of standards for construction of the standard curve. It is, therefore, important to test crystalline digoxin (or other substance used as standard) for purity by thin layer chromatography or some other sensitive analytical technique. The material should then be dried to constant weight before careful gravimetric preparations of standards. Fresh standards should be made up every few weeks. Purity of tracer tritiated digoxin must be carefully monitored as well, since there is significant variation from lot to lot from commercial suppliers.

Most workers are aware that exogenous radioactivity in the serum of patients, such as that introduced in scanning procedures, can produce erroneous results. These isotopes are usually *gamma* emitters which are easy to differentiate from the low energy *beta* radiation emitted by tritiated tracer

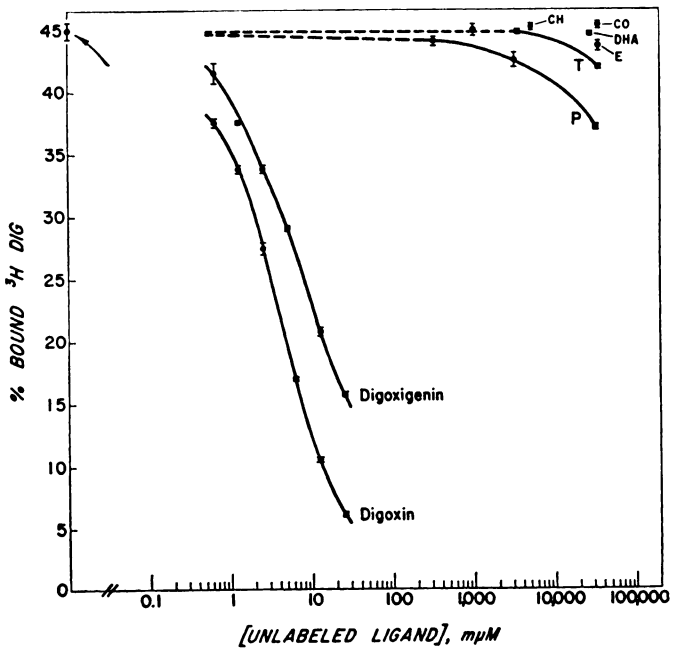

FIG. 3. Specificity of a selected rabbit digoxin-specific antiserum. ^{3}H-digoxin is displaced from antibody binding sites to the extent shown by unlabeled digoxin, digoxigenin, cholesterol (CH), cortisol (CO), dehydroepiandrosterone (DHA), 17β-estradiol (E), progesterone (P), and testosterone (T). The endogenous steroid compounds cause significant displacement only when present in concentrations greater than 1000-fold above those of digoxin.

compounds. It is convenient to keep one channel of the liquid scintillation spectrometer set to detect only events resulting from these higher energy *gamma* photons, so that one is immediately alerted if exogenous radioactivity is present in a sample. When found, the problem can be dealt with by extraction of the cardiac glycoside as in other procedures prior to measurement, or the method suggested by Butler (10) can be used to make the necessary correction.

Adequate quench correction is of particular importance. Samples from clinical sources often contain bile pigments or hemoglobin, among other materials which may quench significantly. Even when relatively small aliquots of serum or plasma are used, careful quench correction must be carried out either by addition of internal standards or by the use of a carefully calibrated external standard.

The most important and, one suspects, most common source of error in cardiac glycoside radioimmunoassay procedures is the use of inadequate antibody preparations. Many investigators have failed to recognize the importance of thorough characterization of antibodies prior to use in radioimmunoassay applications. The affinity constant of an antibody population is important as a determinant both of sensitivity (55) and of stability of antibody-hapten interaction. The importance of the latter can be illustrated by a comparison of two cardiac glycoside specific antiserums. It is possible to determine the rate constant for dissociation of the cardiac glycoside-antibody complex by observing the rate at which dextran coated charcoal sequesters labeled glycoside tracer molecules during the dissociation phase of the equilibrium state. Figure 4 shows the dissociation kinetics of a high affinity, high specificity digoxin-specific antiserum, designated antiserum 1,[1] which

[1] This antiserum was generously supplied by Dr. Vincent P. Butler, Jr.

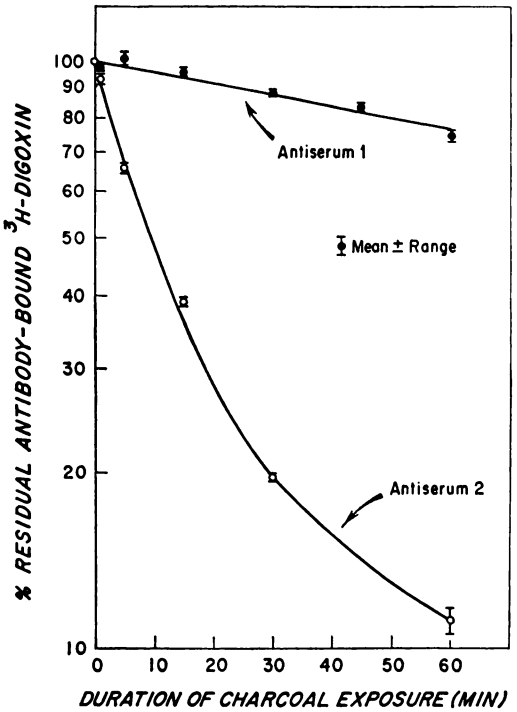

FIG. 4. Dissociation kinetics of ³H-digoxin-antibody complex. Percent of original binding is plotted as a function of duration of exposure to dextran coated charcoal. Antiserum 1, with an average intrinsic association constant of 1.7×10^{10} L/M shows slow dissociation of the complex, while antiserum 2 shows rapid dissociation leading to potential error in radioimmunoassay use.

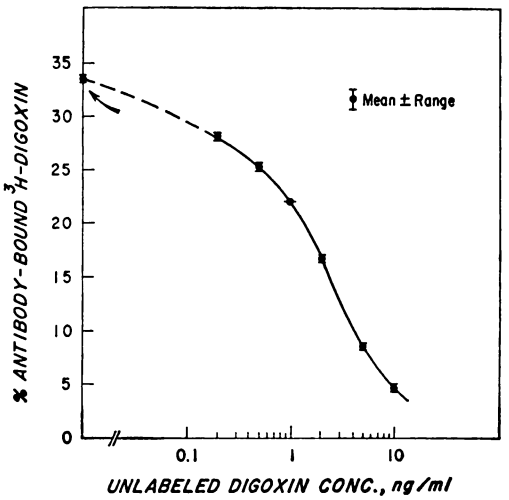

FIG. 5. Radioimmunoassay standard curve obtained with antiserum 2 (see fig. 4). Despite its unsatisfactory characteristics, this antiserum gives a curve which superficially appears adequate for radioimmunoassay use.

was used in the experiments illustrated in figures 2 and 3. Its digoxin complex dissociation kinetics are contrasted in figure 4 with another antiserum of inferior quality (antiserum 2) which has an average intrinsic association constant for digoxin more than an order of magnitude lower than that of antiserum 1. It is apparent that the complex formed by antiserum 2 tends to dissociate rapidly compared with that formed by antiserum 1. Thus, if charcoal is added sequentially to a series of standard and unknown samples, those in contact with charcoal for relatively longer times will show fewer counts in the supernatant phase. This will result in falsely high estimations of serum digoxin concentration in samples exposed for longer times than the standards. This is not a significant problem with antiserum 1, but an antiserum with the dissociation kinetics of antiserum 2 would require careful control of charcoal contact time.

As shown in figure 5, despite these rapid dissociation kinetics, antiserum 2 gives a radioimmunoassay standard curve which appears satisfactory for analytical use on superficial examination. Meade and Kleist (34) have reported that this problem of relatively rapid antibody-hapten dissociation kinetics exists with the components supplied in a commercially available digoxin radioimmunoassay kit. They have suggested an alternative means of separation of antibody bound and free digoxin which tends to circumvent this problem. This may be a reasonable alternative when using antibodies with rapid dissociation kinetics, provided adequate specificity can be maintained.

The specificity problem is also well illustrated by a comparison of the above two antiserums. Antiserum 1, as previously noted, shows high specificity for the homologous hapten digoxin (see fig. 3). In contrast, figure 6 shows a substantially lower degree of specificity for antiserum 2. It seems likely

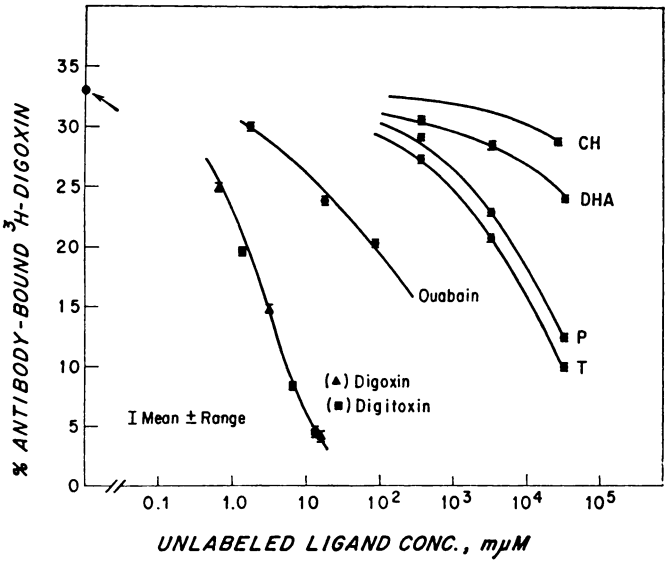

FIG. 6. Specificity of antiserum 2. In contrast to antiserum 1 (fig. 3), relatively poor specificity is reflected by displacement of ^{3}H-digoxin from antibody binding sites, leading to potentially erroneous results if used for radioimmunoassay purposes. Arrow on vertical axis denotes binding in absence of competing ligand. CH, cholesterol; DHA, dehydroepiandrosterone; P, progesterone; T, testosterone.

that use of inferior antiserums of this type explains the difficulties reported by some investigators with radioimmunoassay specificity. Unfortunately, commercial suppliers of radioimmunoassay materials have in general not supplied the purchaser with adequate data characterizing the antibodies supplied. The responsibility for quality control therefore rests with the user. Minimum documentation of the suitability of a given antibody for radioimmunoassay use would require hapten inhibition studies to define specificity, and charcoal contact time studies to define the range of contact times which could be used without introducing an unacceptable degree of error.

Finally, it is important to recognize that specificity of cardiac glycoside radioimmunoassay systems generally tends to increase with increasing duration of the incubation step (47). This should also be systematically studied in the process of setting up a radioimmunoassay procedure.

Rationale

Several lines of evidence have suggested that serum digitalis glycoside concentrations might be usefully related to pharmacological or clinical effects. It has long been apparent that digitalis toxic rhythm disturbances are dose-related phenomena, as are most of the extracardiac manifestations of toxicity (46). A number of studies are now available demonstrating that serum digitalis concentrations rise with increasing dosage (51). Thus, a correlation between serum digitalis concentration and clinical state would be expected, at least on a statistical basis. Further, the studies of Doherty and Perkins (16) have demonstrated a relatively constant ratio of serum to myocardial digoxin concentration in animals after the attainment of serum-tissue equilibrium. A relatively constant serum to myocardial digoxin concentration ratio was also observed in man (17). It is apparent, however, that total myocardial digoxin content includes non-specific drug binding as well as binding to specific receptors (33), so that total myocardial digoxin concentration cannot necessarily be expected to bear a one-to-one relationship to effect.

Further rationale for the clinical use of serum cardiac glycoside concentration meas-

urements is found in the literature implicating Na^+, K^+-ATPase in the mechanism of cardiac action of digitalis (1, 7, 27, 40, 52). Evidence is available from studies of the squid giant axon and the red blood cell that this plasma membrane bound enzyme system is inhibited by cardenolides only when the latter are present at the external cell surface. Caldwell and Keynes (13) observed that sodium flux was inhibited only when ouabain was present at the outer surface of the squid giant axon. Analogous results were obtained by Hoffman (25) in experiments with human red blood cells. Thus, the digitalis sensitive site of Na^+, K^+-ATPase apparently lies in close proximity to the plasma compartment where it would be expected to be responsive to plasma cardiac glycoside concentrations. Although a reasonably convincing case can be made for Na^+, K^+-ATPase inhibition as an important mechanism of toxic electrophysiological digitalis effects, it should be noted that the mechanism of inotropic effect is less certain (28).

Finally, recent animal experiments have shown a close relationship between serum digoxin concentration and the electrophysiological effects of this drug on the heart. Serum digoxin concentration has been shown to be closely related to drug induced changes in cardiac automaticity in experiments in dogs (2). This relationship was apparent when tested by provocation of repetitive ventricular responses by low energy endocardial stimulation, or by digitalis tolerance testing with infusion of the rapidly acting cardenolide acetyl strophanthidin.

Clinical Experience

Studies from a number of laboratories, using several techniques, reflect substantial agreement concerning serum or plasma digoxin and digitoxin levels in patients receiving usual doses of these drugs. Table 1 shows mean serum digoxin concentrations in a number of groups of patients independently studied. Although not shown in this table, it is of note that larger doses of digoxin were associated with higher serum concentrations of the drug in all studies in which this relationship was examined (51). Not surprisingly, impaired renal function has also been uniformly associated with higher serum digoxin concentrations at any given dose level (50). Some of the variation in mean serum levels is attributable to differing times at which serum was obtained for digoxin concentration determination. It is reassuring to note that the mean values for non-toxic patients tend to cluster closely

TABLE 1

Serum or plasma digoxin concentrations: non-toxic and toxic patients

Authors (Ref.)	Method	Mean Conc. Non-toxic	Mean Conc. Toxic	Statistical Significance
Beller *et al.* (3)	Radioimmunoassay	1.0	2.3	Yes
Bertler and Redfors (6)	^{86}Rb uptake	0.9	2.4	Yes
Brooker and Jelliffe (8)	Enzymatic displacement	1.4	3.1	Yes
Burnett and Conklin (9)	ATPase inhibition	1.2	5.7	Yes
Chamberlain *et al.* (14)	Radioimmunoassay	1.4	3.1	Yes
Evered and Chapman (18)	Radioimmunoassay	1.38	3.36	Yes
Fogelman *et al.* (20)	Radioimmunoassay	1.4	1.7	No
Grahame-Smith and Everest (22)	^{86}Rb uptake	2.4	5.7	Yes
Hoeschen and Proveda (23)	Radioimmunoassay	0.8–1.3	2.8	Yes
Johnston *et al.* (26)	Radioimmunoassay	1.0	3.15	Yes
Morrison *et al.* (36)	Radioimmunoassay	0.76	3.35	Not stated
Oliver *et al.* (38)	Radioimmunoassay	1.6	3.0	Yes
Smith *et al.* (48)	Radioimmunoassay	1.3	3.3	Yes
Smith and Haber (50)	Radioimmunoassay	1.4	3.7	Yes

around the mean steady state blood level of 1.4 ± 0.3 (S.D.) ng/ml found by Marcus *et al.* (32) in a study of normal volunteers receiving 0.5 mg of tritiated digoxin per day.

Studies of the relationship between serum digoxin concentration and occurrence of digitalis intoxication are also summarized in table 1. Despite the multiple variables known to influence cardiac response to digitalis glycosides, it can be seen that significantly higher mean serum digoxin concentrations were observed in toxic patients compared with non-toxic patients in nearly all studies published to date; the study of Fogelman *et al.* (20) was the only exception. This summary includes a total of more than 1000 patients studied. In general, the mean digoxin concentrations observed in patients with toxic manifestations are about 2-fold higher than those of patients without toxicity. Despite the significantly different mean levels, however, overlap has been observed in most series, and it must be emphasized that no arbitrary level can be chosen which clearly differentiates toxic from non-toxic serum digoxin concentrations. Serum concentration data are most useful when interpreted together with all other relevant variables in the clinical context. A number of the most important of these variables are listed in table 2. There is some question whether age *per se* deserves a place on this list beyond the extent to which it correlates with renal function (19).

In our experience, ischemic heart disease has been particularly prevalent among patients with cardiac toxicity occurring at relatively low serum digoxin or digitoxin concentrations (46). This is undoubtedly due to, at least in part, the electrophysiological abnormalities accompanying focal ischemia, which are in many respects similar to the electrophysiological abnormalities induced by digitalis excess (24, 43). Clinical evidence supports the concept that less digitalis is required to precipitate overt rhythm disturbances in the diseased heart compared with the normal heart (53). On the other hand, one sees occasional patients who require serum digoxin concentrations of 3 ng/ml or greater to maintain therapeutically appropriate ventricular responses to supraventricular tachyarrhythmias such as atrial flutter or atrial fibrillation.

TABLE 2

Factors affecting individual sensitivity to cardiac glycosides

Serum electrolytes (K^+, Mg^{++}, Ca^{++})
Adequacy of tissue oxygenation
Acid-base balance
Age (?)
Renal function
Thyroid status
Autonomic nervous system tone
Other drugs concurrently received
Type and severity of underlying heart disease

Similar considerations apply to the evaluation of serum digitoxin concentrations. The results of a number of studies of serum or plasma digitoxin concentrations are summarized in table 3. It is apparent that mean serum digitoxin concentrations in non-toxic patients are about 10-fold higher than those of digoxin, presumably due to the substantially greater serum protein binding of digitoxin (30). As in the case of digoxin, mean concentrations in toxic patients have been found to be significantly higher in all studies in which statistical evaluation was done. Again, one cannot arbitrarily assign a diagnosis of digitalis intoxication to a patient on the basis of a higher than average serum digitoxin concentration due to the multiple factors which influence cardiac response to these drugs. Patients on usual maintenance doses of digitalis leaf usually have serum digitoxin concentrations comparable to those of patients on usual maintenance digitoxin doses, when measured by radioimmunoassay (44) or by enzymatic displacement (8).

Space and time do not allow a detailed review of the role of digitalis assay techniques in studies in the clinical pharmacology of digitalis glycosides. These have been summarized recently (51), and there is reason to anticipate that one of the most important results of wide-spread availability of cardiac

TABLE 3

Serum or plasma digitoxin concentrations: non-toxic and toxic patients

Authors (Ref.)	Method	Mean Conc. Non-toxic	Mean Conc. Toxic	Statistical Significance
Beller *et al.* (3)	Radioimmunoassay	20	34	Yes
Bentley *et al.* (4)	ATPase inhibition	23	59	Yes
Brooker and Jelliffe (8)	Enzymatic displacement	31.8	48.8	Not stated
Lukas and Peterson (31)	Double isotope dilution derivative	20	43–67	Not stated
Morrison and Killip (35)	Radioimmunoassay	25 (0.1 mg/day) 44 (0.2 mg/day)	53	Yes
Rasmussen *et al.* (39)	^{86}Rb uptake	16.6	48.7	Not stated
Ritzmann *et al.* (41)	^{86}Rb uptake	19	39–51	Not stated
Smith (44)	Radioimmunoassay	17	34	Yes

glycosides measurement techniques will be increased information on the pharmacology of these drugs in a wide variety of clinical, circumstances.

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