SENSITIVE RADIOIMMUNOASSAY FOR DIGOXIN IN PLASMA AND URINE

J. G. Wagner[1], M. R. Hallmark, E. Sakmar and J. W. Ayres[2]

College of Pharmacy and Upjohn Center for Clinical Pharmacology
The University of Michigan
Ann Arbor, Michigan 48109

Received: 2-8-77

ABSTRACT

A reproducible and sensitive radioimmunoassay for digoxin in either serum, plasma or urine is described. Using 0.5 ml of serum or plasma, the assay sensitivity is 0.05 ng of digoxin/ml. The antiserum and tracer solutions employed are available in a kit sold in the United States. All other reagents were prepared in the laboratory. The assay allows measurement of digoxin in plasma or serum for 96 hours after single 0.5 mg doses of digoxin; this is necessary in human bioavailability studies to accurately estimate the total area under the digoxin concentration, time curve from zero to infinite time. In contrast, with the kit assay, employing 0.2 ml of plasma or serum, it has been reported that the 12 hr serum digoxin levels, after single 0.5 mg doses, are, in most subjects, below the sensitivity limit (about 0.5 ng/ml) of the assay.

INTRODUCTION

Attempts have been made to measure digoxin in human plasma or serum by gas-liquid chromatography (1-5). The method of Watson and Kalmen (5) involves seven steps and is far too complicated for routine use. A method based on digoxin inhibition of red cell ^{86}Rb uptake has been described (6-8) and less than 1 ng of digoxin may be detected by the method. A Na-K-ATPase displacement assay has been described (9); in our hands this assay was extremely variable and unreliable.

Butler and Chen (10) first reported on the preparation of digoxin-specific antibodies. Later, Smith and associates (11,12) characterized the antibodies and developed a radioimmunoassay (RIA) for digoxin in plasma or serum using ^{3}H-digoxin as the tracer. Subsequently, digoxin RIA kits became commercially-available. It was later shown that antibodies present in commercially-available antiserum were not specific for digoxin, but that digoxin, digoxigen-mono-digitoxoside and digoxigen-bis-digitoxoside all reacted with the antibody to about the same degree

---

[1] Send reprint requests to: Dr. John G. Wagner, Upjohn Center for Clinical Pharmacology, The University of Michigan, Ann Arbor, Michigan 48109

[2] Present address: School of Pharmacy, Oregon State University, Corvallis, Oregon 97331.

STEROIDS

(13,14). Digoxigenin also cross-reacts, but to a much lesser degree
(13,14). Dihydrodigoxin was also shown to be a common metabolite of
digoxin in man (15), and this metabolite was also shown to cross-react
with the antibody sold in commercially-available RIA kits (16,17).
Gault et al. (18) extracted digoxin and its metabolites from human urine
and separated some of them by column chromatography with Sephadex LH-20;
they reported that the highest percentage of metabolite found was 22%
in one patient, but that for 5 other patients peak percentages were 7
to 10%, and always occurred within the first day's urine collections.
They reported the possible presence of digoxin, digoxigen-mono-
digitoxoside, digoxigen-bis-digitoxoside, digoxigenin and dihydro deriva-
tives. Subsequently, Sugden et al. (19), using columns containing
diethylaminoethoxypropylated Sephadex LH-20, successfully separated
tritiated dihydrodigoxin from tritiated digoxin.

     Although the digoxin RIA lacks specificity for digoxin since it
also measures metabolites, the method has been extensively used in both
digoxin bioavailability studies and in therapeutic drug monitoring of
serum digoxin concentrations during therapy. The original method (11,12)
using ^{3}H-digoxin as the tracer, has been employed by most investigators,
but has been modified by others (14,20-25). An assay, employing
[125]I-labeled 3-0-succinyl digoxigenin tyrosine, as the labeled antigen,
has been reported (26) and kits, based on this method, have been used
clinically (27-29).

     The original RIA method (11,12) is sensitive to about 0.5 ng of
digoxin/ml of plasma or serum, and the kit procedure utilizes 0.2 ml of
plasma or serum. One modification, using 0.5 ml of plasma, was reported
to be sensitive to 0.2 ng/ml (25). The method of Stoll et al. (14),
from these laboratories, utilized 0.5 ml of plasma, and was reported to
have a sensitivity limit of 0.08 ng/ml. Recently, Wagner and Ayres (30)
showed that in human digoxin bioavailability studies it is necessary to
measure digoxin plasma or serum concentrations for 96 hr after single
0.5 mg doses of digoxin in order to obtain an accurate estimate of the
total area (0 to∞) under the concentration, time curves. Areas from
truncated curves were shown to yield erroneous estimates of bioavaila-
bility of digoxin. With the kit assay, employing ^{3}H-digoxin and 0.2 ml

of plasma or serum, the 12 hr serum or plasma levels after single 0.5 mg
doses are below the sensitivity limit of the assay. A sensitivity limit
of 0.2 ng/ml is still not sufficient, and a limit of 0.05 ng/ml is neces-
sary in human digoxin bioavailability studies.

Employing the readily-available tracer solution of ^{3}H-digoxin and
antiserum solution in a kit, which is commercially-available in the
United States, we have intensively studied each step in the digoxin RIA
procedure in order to determine optimum conditions. The resultant assay,
coupled with the new method of preparing calibration plots, gives good
reproducibility and a sensitivity limit of 0.05 ng/ml. It is shown that,
at a concentration of 0.1 ng/ml, the commonly-used logarithmic-logistic
(logit-log) type of calibration plot yields much larger standard devia-
tions for inversely-estimated concentrations, and, greater bias across
the whole curve (0-5 ng/ml) than the new calibration method which is
based on fitting normalized percent bound, concentration values to a
biexponential equation with a digital computer and a suitable nonlinear
estimation program.

MATERIALS

Antiserum solution and tracer solution were pooled from Digoxin
Radioimmunoassay Kits [^{3}H][3]. Phosphate-saline buffer (pH 7.4 phosphate-
buffered saline solution) was prepared by dissolving 1.392 g of
dipotassium phosphate, 0.276 g of monosodium dihydrogen phosphate
monohydrate and 8.77 g of sodium chloride in sufficient water to make one
liter of solution. Charcoal suspension was prepared by suspending 125
mg of dextran[4] and 5 g of charcoal[5] in 100 ml of phosphate-saline buffer.
The liquid scintillation fluid was Unogel ® Emulsifier[6]. For the 30%
ethanol-water standard solutions of digoxin the digoxin was weighed on a
Cahn balance[7]. Pipetting was performed as follows: plasma and buffer
were pipetted with an Eppendorf Microliter Pipet[8]; 30% ethanol-water
standard solutions, antiserum solution and tracer solution were pipetted
with Lang-Levy Micro Pipets[9]; charcoal suspension was measured by syringe;

[3] Schwarz-Mann Catalog No. 0750-23, Division of Becton, Dickinson and
Company, Orangbury, New York.

[4] Dextran T-70, Pharmacia, Uppsala, Sweden.

[5] Norit A, Sigma Chemical Company, St. Louis, Missouri.

[6] Schwarz-Mann (address above).

[7] Cahn Division of Ventron Instruments Corp., Paramount, California.

[8] Brinkman Instruments Inc., Centiague Road, Westbury, New York.

[9] Arthur H. Thomas Company, Philadelphia, Pennsylvania.

10 µl aliquots of urine were measured by Eppendorf pipet; and Unogel®
was measured by L/I Repipet[10]. The scintillation counter was a Packard
Tri-Carb, Model 3320[11]. Centrifugation was performed in a Sorvall RC-3
General Purpose Automatic Refrigerated Centrifuge[12].

METHODS

I. Determination of Optimum Standards and Effect of Volume.

Calibration curves were prepared according to the method of Stoll
et al. (14) using known digoxin concentrations of 0.1, 0.5, 1, 2 and 5
ng of digoxin per ml of plasma. On one day analyst S obtained standard
curve data using 80 µl of kit (serum) standard and 420 µl of plasma.
In addition, standard curve data were obtained from 30% ethanol-water
standards where the volume and concentration of the standard were: 1 µl
for 0.1 ng/ml, 5 µl for 0.5 ng/ml, 10 µl for 1 ng/ml, 20 µl for 2 ng/ml
and 50 µl for 5 ng/ml, with 500 µl of plasma. On another day analyst S
obtained standard curve data using both kit standards and 30% ethanol-
water standards, with the total volume added being 50 µl in each case
with 500 µl of plasma.

II. Determination of the Kinetics of Association and Optimum Pre-Incubation Time.

There was no pre-incubation of unlabeled digoxin with antiserum in
the method of Stoll et al. (14). Phillips (22) claimed that pre-incuba-
tion increased both sensitivity and reproducibility of the digoxin RIA.
For each temperature (25°C and 37°C) 16 tubes were prepared, each
containing 500 µl of plasma, 500 µl of phosphate-saline buffer, 50 µl of
30% ethanol-water, 100 µl of antiserum solution and 100 µl of tracer
solution. The mixtures were vortexed. Then 500 µl of charcoal suspen-
sion (room temperature) was added to each of two tubes at 0, 5, 10, 15,
20, 30, 60 and 120 min. The charcoal contact time was 10 min for each.
Tubes were centrifuged at 2000 r.p.m. for 20 min. Each supernatant was
decanted into 15 ml of liquid scintillation fluid in a scintillation
vial and counted for 10 min. Hence, a series of duplicate B(0) values
were obtained for different reaction times. Plots of counts/min versus
time were made for each incubation temperature.

III. Determination of the Kinetics of Dissociation of the Digoxin-Antibody Complex as a Function of Temperature.

For each temperature (0°C, 25°C and 37°C) 14 tubes were prepared as
indicated under II above, except that the 50 µl of 30% ethanol-water
standard contained digoxin equivalent to 1 ng digoxin/ml plasma. After
the tracer solution was added, the tubes were incubated at the desired
temperature for 30 min. Then, 50 µl of a 30% ethanol-water solution, con-
taining the equivalent of 0.5 µg digoxin/ml, was added to each tube to
provide a 500-fold excess of digoxin. Two tubes were each incubated for
0, 5, 10, 20, 30, 60 or 120 min for each of the temperatures. After the
desired incubation time, 500 µl of charcoal suspension (room temperature)
was added, and the charcoal contact time was 10 min. Tubes were

---

[10] Labindustries, 1802 Second Street, Berkeley, California.

[11] Packard Instruments Co., Inc., Downers Grove, Illinois.

[12] Ivan Sorvall, Inc., Newtown, Connecticut.

centrifuged and the supernatants counted as indicated formerly under II above.

IV. Determination of Necessary Incubation Time After Tracer Solution
   Added.
   Sixteen tubes were prepared containing 500 µl of plasma, 500 µl of phosphate-saline buffer, 50 µl of ethanol-water standard (8 tubes equivalent to 0.1 ng digoxin/ml plasma and 8 tubes equivalent to 5 ng digoxin/ml plasma) and 100 µl of antiserum solution. All tubes were pre-incubated at 25°C for 60 min. Then, 100 µl of tracer solution was added to each tube. Two tubes for each of the two concentrations were incubated for 10, 20, 30 and 60 min at 25°C. Then, 500 µl of charcoal suspension (pre-cooled in an ice-bath) was added to each tube, and the charcoal contact time was 10 min under ice-bath conditions. Tubes were centrifuged and the supernatants counted as indicated under II above.

V. Determination of Optimum Charcoal Exposure Time Under Ice-Bath
   Conditions.
   Ten tubes were prepared, each containing 500 µl of plasma, 500 µl of phosphate-saline buffer, 50 µl of 30% ethanol-water standard equivalent to 1 ng digoxin/ml plasma and 100 µl of antiserum solution. Tubes were pre-incubated for 60 min at 25°C. Then, 100 µl of tracer solution was added to each tube, and the tubes were incubated for 60 min at 25°C. At the end of the incubation time, the tubes were placed in an ice-bath, and, after constant temperature had been attained, 500 µl of charcoal suspension (pre-cooled in an ice-bath) was added. Two tubes were used for each charcoal exposure time of 1, 5, 10, 30 and 60 min. At the end of the appropriate time, the tubes were centrifuged and the supernatants counted as indicated under II above.

VI. Determination of the Minimum Amount of Liquid Scintillation Fluid
   Necessary.
   Eight tubes were prepared as under V above, except that there was no pre-incubation and the incubation time was 30 min at 25°C after tracer was added. The charcoal exposure time was 10 min. After centrifugation at 2000 r.p.m. for 20 min, the supernatants of each of two tubes were added to 8, 10, 12 or 15 ml c. liquid scintillation fluid, then counted for 10 min after visual observation.

VII. Quench correction.
   Fresh whole blood was collected and frozen in dry ice-acetone mixture, then thawed and frozen again. This provided hemolyzed blood from which the plasma was collected by centrifugation. Various mixtures were made of this red-colored plasma and plasma from non-hemolyzed blood. Two groups of duplicates of 10 different mixtures were made--one group corresponding to 110,000 DPM ^{3}H$_{2}$O/50 µl, and the other corresponding to 11,000 DPM ^{3}H$_{2}$O/50 µl. All vials also contained 50 µl of the pH 7.4 phosphate-buffered saline solution, 100 µl of antiserum and 50 µl of 30% ethanol-water (as used in the assay procedure) and 15 ml of liquid scintillation fluid. Instrument settings were: Red channel, 040-100 (A-B), 64% gain; Green channel, 040-1000 (C-D), 74% gain; and Blue channel, 300-1000 (E-F), 1.59% gain. The counts/min for each sample were divided by the DPM known to be present from standard ^{3}H$_{2}$O present, to obtain the % efficiency, then this value was plotted versus the

S **TEROIDS**

counts/min in the Blue channel obtained from AES counting.

VIII. Anticoagulant Study.

Fresh blood from a subject was drawn into Vacutainers containing sodium heparin, sodium citrate and disodium ethylenediaminetetraacetic acid (Na_2EDTA). The original Vacutainer containing sodium citrate contained this anticoagulant as an aqueous solution. To avoid dilution of the blood we evaporated off the water, leaving the dried sodium citrate. The blood samples were centrifuged and the plasma samples from the three types of tubes were spiked with 0, 0.05, 0.1, 0.5, 1, 2 and 5 ng of digoxin per ml of plasma, then all samples were assayed by the new procedure.

IX. Application of the Assay in Human Studies.

The assay method has been successfully used in two human digoxin bioavailability studies--one a four-treatment crossover study in 12 subjects with 14 sampling times, the other a three-treatment crossover study in 15 subjects with 15 sampling times. The "unknown" plasma samples from these two studies were assayed independently in the same laboratory by each of two analysts (designated "H" and "S"). All samples for one subject from all phases of one study were assayed on the same day by both analysts. Each day that "unknowns" were assayed, each analyst also prepared calibration data by using pooled pre-dose plasma (digoxin-free) of the same subjects that were used in the study. Also, a quality control sample, theoretically containing 0.9 ng of digoxin per ml of plasma, was prepared and aliquots were frozen; each day each analyst removed one of the vials from the freezer and assayed the sample along with the "unknowns". Before pooling the pre-dose plasmas for calibration purposes, each analyst assayed all pre-dose plasmas for a given study on the same day as well as obtaining calibration data that day.

X. Type of Calibration Plot.

The calibration data were plotted various ways as described in the literature (31-35) to see if a suitable linear relationship could be obtained without undue bias in any segment of the plot and with similar coefficients of variation for inversely-estimated concentrations at various known digoxin concentrations in the range desired, namely 0.05 to 5 ng/ml plasma. No satisfactory linear relationship could be found which satisfied the criteria. Results will be given for the logarithmic-logistic relationship (so-called "logit"-log plot) (31). For this type we plotted $\ln [\frac{1 - y}{y}]$ versus $\ln x$, according to equation 1. where $\ln$ stands for natural logarithm, $y = \frac{B(X)}{B(0)} \times 100$, x = digoxin concentration in ng of digoxin per ml of plasma, $B(X)$ is the % bound at digoxin concentration x and $B(0)$ is the % bound in the absence of digoxin. In such a plot the slope is positive, but the same magnitude as the slope of the often-used plot of $\ln [\frac{y}{1 - y}]$ versus $\ln x$ (31). The equation of the least squares line obtained may be represented as in equation 1, where $\ln Q$ is the intercept (corresponding to the value of $\ln [\frac{1 - y}{y}]$

when x = 1 and ln x = 0) and s is the slope of the line. Concentrations
were then inversely-estimated by rearranging equation 1 to equation 2
and programming an electronic calculator to solve equation 2.

$$\ln[\frac{1-y}{y}] = \ln Q + s \cdot \ln x \qquad \text{Eq.(1)}$$

$$x = e^{\left\{(\ln[\frac{1-y}{y}] - \ln Q) / s\right\}} \qquad \text{Eq.(2)}$$

The most successful calibration plot was obtained by fitting the
normalized % bound values, $\frac{B(X)}{B(0)}$ x 100, to a double exponential equation
of the type shown as equation 3, after Sullivan et al. (36), who fitted
% bound, [B(X)], x data in this manner. In equation 3, P(1), P(2), P(3)

$$\frac{B(X)}{B(0)} \times 100 = P(1) \cdot e^{-P(2) \cdot x} + P(3) \cdot e^{-P(4) \cdot x} \qquad \text{Eq.(3)}$$

and P(4) are the parameters estimated in the nonlinear least squares
fitting, performed with a digital computer and a suitable nonlinear
least squares program. Concentrations, x, were then inversely-estima-
ted from a known set of parameters and a known value of $\frac{B(X)}{B(0)}$ x 100 and
equation 3 using an iteration procedure with an electronic calculator.
In such a case x is incremented gradually and automatically, and the
final answer is obtained when the equation is satisfied with some
desired error. We used an error of 0.01 ng/ml of digoxin.

XI. Modification of the Plasma Assay for Urine.
      The assay for digoxin in urine was run essentially the same as for
plasma, except that 500 μl of the subject's pre-dose (zero hour) plasma
was used in place of the "unknown" plasma and the urine was introduced
as a 10 μl aliquot using an Eppendorf pipet. The validity of taking
such small aliquots was justified by repetitive pipettings of tracer
solution with the same pipet, addition of liquid scintillation fluid
and counting. For ten repetitive pipettings the coefficients of varia-
tion were 2.07% for the first counting and 2.64% for the second counting.
Then two vials were each counted 10 times, with the resultant coeffi-
cients of variation being 1.13% and 1.50%. Experiments were also done
to determine if the presence of plasma was necessary in the urine assay,
and, if so, how much. The normalized % bound values corresponding to
digoxin concentrations of 0.1, 0.5, 1, 2 and 5 ng/ml (total volume =
500 μl) were lower when buffer only was used, but not significantly
different for each concentration when 10, 50, 100, 150, 300 and 500 μl
of plasma were used. Hence, as little as 10 μl of plasma in a total
volume of 500 μl, has a maximal effect. Obviously then, changes in
albumin concentrations do not affect the assay, as has been reported by
Holtzman et al. (31), but challenged by Shaw (32). Why some minimum
amount of plasma is necessary is unknown.

Because of higher concentrations of digoxin in urine than in plasma, the concentrations used for the urine calibration curves were 5, 25, 50, 100 and 250 ng/ml--i.e. 50 times those used routinely in the plasma assay.

RESULTS AND DISCUSSION

I.[13] Determination of Optimum Standards and Effect of Volume.

Logarithmic-logistic plots were prepared (Methods section X, equation 1) from the data collected in this experiment. Using the natural logarithms of the variables the least squares lines were calcu-lated (see equation 1) and the slopes and intercepts of these lines are shown in Table 1.

Table 1. Parameters of Least Squares Lines of Logit-Log Plots and Coefficients of Variation from Inversely Estimated Concentrations for Experiment I.

Day	Type of Standards	Slope	Intercept	Correlation Coefficient	C.V. (%) from Inversely Estimated Concentrations
1	Kit	0.927	0.164	0.999	6.99
1	30% EtOH-H_2O (Volume not constant)	1.15	0.162	0.999	5.18
2	Kit	0.985	0.182	0.998	8.96
	30% EtOH-H_2O (Volume constant)	1.05	0.203	0.991	6.2

When labeled and unlabeled digoxin have the same affinities for antibody, Rodbard et al. (31) indicated that the theoretical slope of such a logit plot would be unity. The slopes on day 2, when volumes were held constant, do not differ from unity significantly. But, on day 1, when the volume was not held constant for the 30% ethanol-water stan-dards, the slope, 1.15, was significantly different from unity, indicat-ing a bias caused by non-uniform volumes added, probably caused by different amounts of ethanol, rather than a volume effect.

When digoxin concentrations were inversely estimated using equation 2, the coefficients of variation were lower on both days for the 30% ethanol-water standards than for the kit standards (Table 1). This result is a reflection that our own 30% ethanol-water standards were somewhat more accurate than the kit standards. On this basis we decided

[13] For ready reference, sections have same roman numerals as corresponding section in the Methods section.

to use our own standards rather than the kit standards.

II. Determination of the Kinetics of Association and Optimum
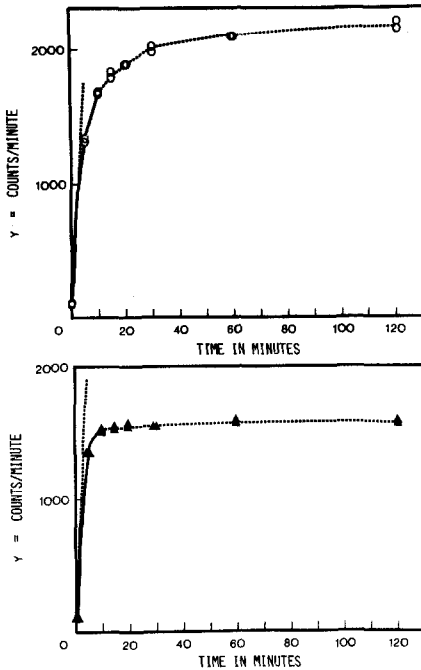   Pre-Incubation Time.

Fig. 1 - Kinetics of associa-
tion (see Methods and Results
II). Top curve (O), 25°C and
bottom curve (▲), 37°C.
Tangent lines represent initial
rates (see text).

Figure 1 presents the results of this experiment. The optimum pre-incubation time is that time when the counts/min are first in the asymptotic region of such a curve. This time allows as much as possible of the unlabeled digoxin to associate with the antibody before the labeled digoxin is added to fill up the sites unoccupied by the unlabeled drug. The results at 25°C indicated that a pre-incubation time of 60 min is about optimum, whereas at 37°C only about 15 min is necessary. However, because of rapid dissociation at 37°C (later Figure 2), the asymptote of the 37°C curve is appreciably lower than the asymptote of the 25°C curve. Hence, in order to obtain higher B(0) values, the pre-incubation at 25°C is more desirable. The first six points obtained at each temperature (solid lines in Figure 1) were fitted to the equation of a parabola (equation 4). The initial "reaction rate", dY/dt, could

S T E R O I D S

then be estimated by differentiating equation 4 with respect to time, t, yielding the result shown as equation 5. Initial rates, obtained by

$$Y = a_o + a_1 t + a_2 t^2 \qquad\qquad Eq.(4)$$

$$(dY/dt)_{t=o} = a_1 \qquad\qquad Eq.(5)$$

this method, from the data in Figure 1, were 331 and 364 counts/min^2 for 25°C and 37°C, respectively.

III. <u>Determination of the Kinetics of Dissociation of the Digoxin-Antibody Complex as a Function of Temperature.</u>
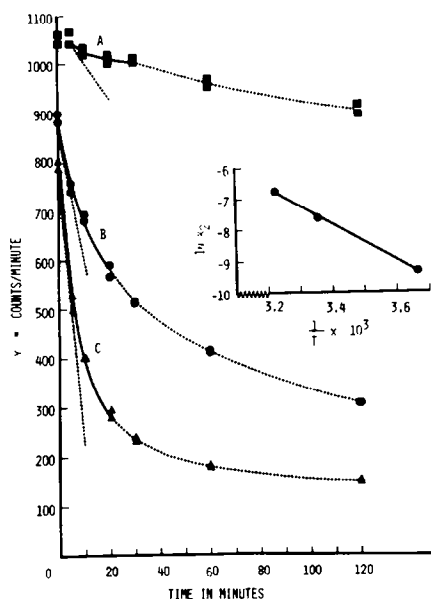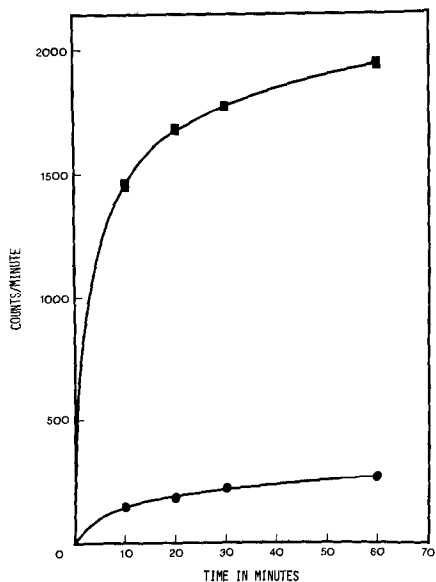

<u>Figure 2</u>
Kinetics of dissociation of digoxin-antibody complex (see Methods and Results III). Curve A, ice-bath (0°C); curve B, 25°C; curve C, 37°C. Tangent lines represent initial rates (see text). Inset: Arrhenius plot for k$_2$.

Figure 2 presents the results of this experiment. The dissociation curves fall-off and approach asymptotic values. The rate of dissociation increases markedly with increase in temperature. The first eight points of each curve were fitted to the equation of a parabola (equation 4), and initial rates were estimated with equation 5. These "initial rates" were 5.709, 26.32 and 56.47 counts/min^2 for 0°C, 25°C and 37°C,

respectively. At 0°C there was an initial lag period of 5 min, after which there was a parabolic fall-off of counts/min from 5 to 30 min, with the rate being very much less than 25°C or 37°C. Thus, after incubation, tubes should be immersed in an ice-bath and charcoal treatment at 0°C is needed as reported by Samols et al. (37). The "initial rates" indicated above may be taken as a measure of $k_2 A^{\circ}$, where k_2 is the first order dissociation rate constant and A° is the initial amount of tracer-labeled digoxin in counts/min. The estimates of A° (a_0 of equation 1) were 1075, 881.6 and 784.2 counts/min for 0°C , 25°C and 37°C, respectively. From these values estimates of k_2 were made by dividing the appropriate $k_2 A^{\circ}$ value by the appropriate A° value, then multiplying by 60 to convert to sec^{-1}. The k_2 values obtained were 8.956×10^{-5}, 4.993×10^{-4} and 1.196×10^{-3} sec^{-1} for 0°C, 25°C and 37°C, respectively. Inset in Figure 1 is the Arrhenius plot based on these values of k_2. The calculated activation energy was 11.7 kcal/mol.

IV. Determination of Necessary Incubation Time After Tracer Solution Added.

Figure 3
Determination of necessary incubation time after tracer added (see Methods and Results IV). Top curve (■), 0.1 ng/ml; bottom curve, (●), 5 ng/ml.

Results of this experiment are shown in Figure 3. This experiment indicated that the time required for the labeled digoxin to occupy most of the sites unoccupied by the unlabeled digoxin was about 60 min, since the curves are approaching their asymptotic values at that time. Hence, an incubation time of 60 min at 25°C, the same as the pre-incubation time, is satisfactory.

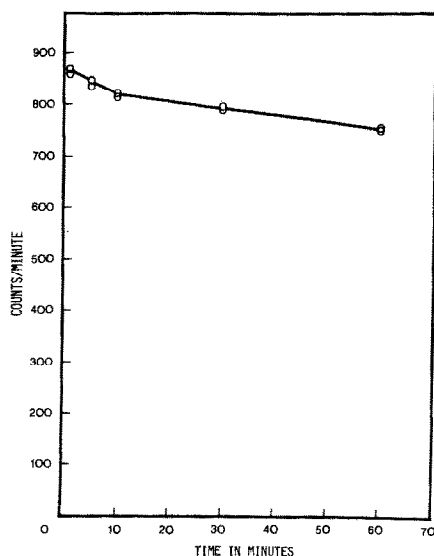V. <u>Determination of Optimum Charcoal Exposure Time Under Ice-Bath Conditions</u>.

Figure 4
Determination of optimum charcoal exposure time under ice-bath conditions (see Methods and Results V).

Results of this experiment are shown in Figure 4. There appeared to be an initial linear drop in counts/min for the first 10 min. These six points gave a least squares equation: counts/min = 8683 - 53.2 t ($r = -0.958$). At 10 min there was an abrupt change in slope, and, in the 10-60 min period the drop again appeared to be linear; the six points in this period gave the equation: counts/min = 8204 - 12.4 t ($r = -0.990$). We chose 10 min as the charcoal exposure time under ice-bath conditions, since any slight increase in exposure beyond 10 min

would not make a great difference in counts/min.

VI. Determination of the Minimum Amount of Liquid Scintillation Fluid Necessary.

The counts/min progressively increased from about 800 to 950 when from 8 to 15 ml of liquid scintillation fluid was used. When 8 ml were used the solution was definitely cloudy; when 10 or 12 ml were used the solutions were of intermediate clarity; when 15 ml were used, the solution was definitely clear. Hence, 15 ml of scintillation fluid was necessary.

Final assay procedure - To each tube is added the following in sequence: 500 μl of plasma, 500 μl of pH 7.4 phosphate-buffered saline solution and 50 μl of 30% ethanol-water; these are vortexed, then 100 μl of anti-serum solution is added and the mixture vortexed again. This mixture is pre-incubated for 60 min at 25°C. Then, 100 μl of tracer solution is added, the mixture is vortexed, then incubated for 60 min at 25°C. At the end of the incubation time, the tube is placed in an ice-bath, then 500 μl of charcoal suspension, pre-cooled in the ice bath, is added and the mixture is vortexed. After a 10 min charcoal time, the tube is centrifuged in a Sorval centrifuge (0-4°C) at 2000 r.p.m. for 20 min. The supernatant is decanted, being careful not to take any of the charcoal, into 15 ml of liquid scintillation fluid in a scintillation vial by shaking, then counted for 10 min in a scintillation counter.

In preparing tubes for the calibration data, digoxin-free plasma is used, and the same procedure is used for the B(0) values, but for the B(X) values the 50 μl of 30% ethanol water contains sufficient digoxin to provide the desired digoxin concentration.

With batch assays with a large number of tubes, the timer should be started when the charcoal suspension has been added to the last tube, so that the minimum charcoal contact time is 10 min.

VII. Quench Correction.

Linear regression analysis of the data from the two groups (110,000 DPM and 11,000 DPM ^{3}H$_2$O) indicated that there were no significant differences in either the slopes or intercepts of the % efficiency versus counts/min plots, indicating that the % efficiency relationship to quenching was not affected by the total radioactivity present in the

range studied. From all data the least squares regression line, forced
through the origin, was:

$$\% \text{ Efficiency} = (6.683 \times 10^{-5}) \text{ (AES)} \qquad \text{Eq.(6)}$$

Previously, in the same laboratory, Stoll et al. (14) obtained a slope
of 7.559×10^{-5} when hydrochloric acid, chloroform and water were used
rather than the mixtures of plasma from hemolyzed and non-hemolyzed
blood.

An electronic calculator program was written to calculate a % bound
value using the following equations, for the case where there is one
tube for the plasma sample, two tubes for the blank and two tubes for
the total count.

$$\% \text{ Bound} = \frac{S_c - B_c}{T_c - B_c} \times 100 \qquad \text{Eq.(7)}$$

$$S_c = \frac{S}{\frac{1}{100} [(AES)_S \times S1]} \qquad \text{Eq.(8)}$$

$$B_c = [\frac{B_1}{\frac{1}{100} [(AES)_{B_1} \times S1]} + \frac{B_2}{\frac{1}{100} [(AES)_{B_2} \times S1]}] / 2 \qquad \text{Eq.(9)}$$

$$T_c = [\frac{T_1}{\frac{1}{100} [(AES)_{T_1} \times S1]} + \frac{T_2}{\frac{1}{100} [(AES)_{T_2} \times S1]}] / 2 \qquad \text{Eq.(10)}$$

In equations 7 through 10, S_c, B_c and T_c are the corrected sample, blank
and total counts, respectively; S, B_1, B_2, T_1 and T_2 are counts/min for
sample, blank #1, blank #2, total count #1 and total count #2, respec-
tively; AES represents the counts obtained from the Blue channel for the
particular sample; and S1 represents the slope of the quench plot, name-
ly 6.683×10^{-5}.

VIII. and X. Anticoagulant Study.

Table 2 (next page) lists inversely-estimated concentrations
obtained from the biexponential fittings (equation 3) of the calibration
data in the anticoagulant study. All three anticoagulants may be used
with satisfactory results, as the data indicate. These data also show
the advantage of doing duplicate assays, since the mean of duplicates
usually has less bias than either individual value on the average.

Table 2

Inversely-Estimated Concentrations from Plasma Calibration Curve
Data (Biexponential Fits) in Anticoagulant Study

Known Digoxin Concentration (ng/ml)	Inversely-Estimated Concentrations of Digoxin (ng/ml)		
	Sodium Heparin	Dried Citrate	Na_2EDTA
0.05	0.05[a]	0.04	0.07
	0.05	0.06	0.04
	0.05[b]	0.05	0.055
0.10	0.10	0.07	0.10
	0.10	0.10	0.07
	0.10	0.085	0.085
0.50	0.41	0.53	0.52
	0.53	0.50	0.49
	0.47	0.515	0.505
1.0	1.13	0.98	0.95
	1.10	0.94	1.03
	1.115	0.96	0.99
2.0	1.91	2.12	2.02
	1.93	1.94	1.87
	1.92	2.03	1.90
5.0	----	4.95	5.28
	----	5.13	5.17
	----	5.04	5.225

[a]Duplicate assay values by same analyst on same day.
[b]Average of the duplicates.

Table 3

Data for Sensitivity Limit of Plasma Assay

Parameter	Normalized % Bound $= \dfrac{B(X)}{B(0)} \times 100$		
	Sodium Heparin	Dried Citrate	Na_2EDTA
Theoretical for $C = 0$	100.0	100.0	100.0
Computer-fitted for $C = 0$[a]	116.75	107.3	100.1
Observed average for $C = 0.05$	100.2	98.1	92.05
Computer-fitted for $C = 0.05$	100.0	99.2	92.9

[a]Value of P(1) + P(3) from fitted equation 3, where C is the digoxin concentration in ng/ml.

Comparing rows 2 and 3 in Table 3 for <u>each</u> anticoagulant separately indicates that with biexponential fitting (equation 3) the sensitivity of the assay is 0.05 ng/ml, since, for example, for sodium heparin 116.75 is significantly different from 100.2. In the range 0 to 0.05 ng/ml the apparent concentration can be negative or positive and is unreliable. Ability to estimate accurately 0.05 ng/ml is also supported by data in Table 2.

IX. and X. <u>Application of the Assay to Human Studies</u>.

Table 4

Summary of Results Obtained Independently by Two Analysts (H and S)
in Two Studies Using Pre-Dose Plasmas of Each Phase of the Studies

Study	No. of Samples per Subjects	Subject	Total Samples	Parameter	% Bound H	S
1	12	4	48	Mean	44.0	44.0
				Range	41.4-46.7	41.1-46.1
				C.V. (%)	3.34	2.90
2	15	3	45	Mean	45.8	43.1
				Range	44.0-48.0	40.8-45.8
				C.V. (%)	2.62	2.63

Table 4 summarizes the results obtained by the two independent analysts with the pre-dose plasma samples in two different human studies. The lower % bound value in each of the four groups in the table was higher than the corresponding B(0) value for the calibration data that day, indicating none of the pre-dose plasmas contained digoxin. The coefficients of variation in Table 4, namely 3.34, 2.90, 2.62 and 2.63, attest to the reproducibility of the assay.

Table 5 summarizes mean concentrations, coefficients of variation and bias values obtained from inversely-estimated concentrations for all the calibration data collected in one of the studies. Each mean in the table is derived from plasmas of 12 different subjects on 12 different days. The table also compares results achieved with the logarithmic-logistic type of calibration plot and the biexponential fits of the individual sets of calibration data. We ruled out the logarithmic-logistic plot since it produced very large standard deviations at a known digoxin concentration of 0.1 ng/ml, and for analyst S the data failed a linearity test (38) and produced considerable bias with a trend indicative of non-linearity.

Table 5

Summary of Mean, Coefficient of Variation and Bias of Inversely-
Estimated Digoxin Concentrations[a]

Calibration Method	Analyst	Known	Mean Estimated	C.V.(%)	Bias[b] ng/ml	%
Logarithmic-logistic	H	0.1	0.100	29.8	0	0
with pooled data[c]		0.5	0.516	7.00	0.016	3.2
		1.0	1.036	7.54	0.036	3.6
		2.0	2.00	5.38	0	0
		5.0	4.85	5.16	-0.15	-3.0
Logarithmic-logistic	S	0.1	0.095	24.6	-0.005	-5.0
with pooled data[d]		0.5	0.546	8.54	0.046	9.2
		1.0	1.09	5.50	0.09	9.0
		2.0	1.97	5.35	-0.03	-1.5
		5.0	4.66	5.44	-0.34	-6.8
Individual subject	H	0.1	0.095	7.10	-0.005	-5.0
biexponential fits		0.5	0.498	4.18	-0.002	-0.4
		1.0	0.988	4.08	-0.013	-1.3
		2.0	2.10	4.48	0.10	5.0
		5.0	4.82	8.78	-0.18	-3.6
Individual subject	S	0.1	0.0967	5.09	-0.0033	-3.3
biexponential fits		0.5	0.500	2.26	0	0
		1.0	0.994	3.60	-0.006	-0.6
		2.0	2.04	3.53	0.04	2.0
		5.0	4.94	3.14	-0.06	-1.2

[a]Both analysts spiked digoxin-free pre-dose plasma of twelve subjects on
each of twelve different days. The normalized % bound values were used
to prepare both types of calibration plots.

[b]The bias expressed in ng/ml is the difference between the mean estimated
concentration and the known digoxin concentration. This value expressed
as a percentage of the known concentration is the % bias.

[c]These data passed the linearity test (38).

[d]These data failed the linearity test (38).

Table 6 summarizes results obtained with the quality control samples
in one of the human studies. Results obtained with individual subject
biexponential fits, biexponential fits of pooled data and logarithmic-
logistic plots of pooled data are compared. Results show the advantage
of performing two independent assays on each sample, since (although not
shown) the average of the two assays on any given day is usually closer
to the "true" value than either value alone. Also, as seen in Table 6,
the overall average, 0.90 ng/ml, obtained with the recommended calibra-
tion method (i.e. individual subject biexponential fits) is the actual

concentration. It should be noted that the coefficients of variation
obtained from the quality control samples, namely 6.23 and 8.18%, by the
recommended method, are somewhat higher than most of those obtained from
inversely-estimated concentrations from calibration curve data (Table 5).
Results obtained in the second human study are not reported, but were
very similar.

Table 6

Results Obtained with Quality Control Sample of Spiked Plasma
Assayed Each Day a Calibration Curve Was Prepared Independently
by Both Analysts (H and S)

| Parameter | Concentration of Digoxin (ng/ml) | | | | | |
| | Individual Subject Biexponential Fits | | Biexponential Fits of Pooled Data | | Logit-Log with Pooled Data | |
	H	S	H	S	H	S
Mean	0.94	0.85	0.93	0.84	0.99	0.89
Range	0.88-1.00	0.71-0.93	0.86-1.00	0.64-0.94	0.92-1.07	0.84-0.96
C.V. (%)	6.23	8.18	4.25	8.65	4.66	9.14
Pooled data						
Mean	0.90		0.89		0.94	
C.V. (%)	8.57		8.17		8.63	

XI. Modification of the Plasma Assay for Urine

From inversely-estimated concentrations of both analysts on three
separate days (i.e. 6 values per concentration), the coefficients of
variation were 5.77, 1.97, 1.68, 3.04 and 3.44% for known urine digoxin
concentrations of 5, 25, 50, 100 and 250 ng/ml, respectively. From six
quality control samples, assayed on the same three days, an overall
coefficient of variation of 6.19% was obtained, which is very comparable
to those obtained in the plasma assay.

GENERAL DISCUSSION

Various measures of precision of radioimmunoassays have been dis-
cussed in the literature. Among these are: (a) the coefficients of
variation calculated from % bound values; (b) the coefficients of varia-
tion calculated from normalized % bound values; (c) the coefficients of
variation calculated from the transformation on the ordinate of the cali-
bration plot, which is used to linearize the data; (d) the coefficients
of variation calculated from inversely-estimated concentrations; and (e)
the coefficients of variation calculated from assays of quality control
samples. These were discussed by Tembo et al. (39); it was shown that

the relative magnitudes of such coefficients of variation are usually
(e) > (d) > (c) > (a) > (b), hence great caution must be excercised in
comparing different authors' coefficients of variation for similar types
of assays. The most valid indicators of the precision of any assay are
(d) and (e) above. Also, Cekan (40) pointed out that: "Precision con-
nected with the determination of a standard curve is closely related to
the sensitivity of an assay. One can generally say that the higher the
precision, the higher the ability to distinguish a dose from zero or one
dose from another...The sensitivity, in the sense of the distiguishing
power of one dose from another, is also clearly dependent on precision."

     From quality control samples of sera Fraser et al. (41) reported
standard deviations of 0.24 and 0.20 ng/ml for mean digoxin concentra-
tions of 3.62 and 1.65 ng/ml, corresponding to coefficients of variation
of 6.6 and 12%, respectively. Hence, the value of 8.57%, reported in
Table 6 after application of the new assay, agrees well with their
values. Halkin et al. (42) reported a coefficient of variation of 8.9%
for a quality control urine sample containing 1.88 ng of digoxin/ml.
Our value of 6.19% for the quality control urine sample compares
favorably.

     The new assay developed differs from that recommended in the kit
instructions in several ways: (a) We use our own 30% ethanol-water stan-
dard solutions of digoxin, rather than the kit serum standards. (b) We
prepare our own buffer and charcoal suspension, rather than using those
in the kit. (c) We use 0.5 ml of plasma or serum, rather than the 0.2
ml recommended in the kit. (d) For the standard curve we use digoxin
concentrations of 0.1, 0.5, 1, 2 and 5 ng/ml (and sometimes 0.05 ng/ml),
rather than those recommended in the kit of 0.5, 1, 1.5, 2, 3 and 5
ng/ml. (e) We pre-incubate the unlabeled digoxin with the antiserum for
1 hr at 25°C, whereas the kit method has no pre-incubation step. (f)
We incubate for 1 hr after the ^{3}H-digoxin is added, whereas in the kit
method a 30 min incubation time is recommended. (g) We do the charcoal
treatment under ice-bath conditions, whereas the kit method recommends
room temperature conditions. (h) We use 15 ml of liquid scintillation
fluid, whereas the kit recommends 10 ml. Since the new assay covers the
range 0.05 - 5 ng/ml plasma or serum, whereas the kit assay covers the

range 0.5 - 5 ng/ml, we saw little reason to compare results in the common range of 0.5 - 5 ng/ml. The advantage of the new assay is accuracy and reproducibility in the range 0.05 - 0.5 ng/ml, which is not covered by the kit assay.

REFERENCES

1. Jelliffe, R. W. and Blankenhorn, D. H., J. Chromatogr. 12, 268-270 (1963).
2. Wilson, W. E., Johnson, S. A., Perkins, W. H. and Ripley, J. E., Anal. Chem. 39, 40-44 (1967).
3. Maume, B., Wilson, W. E. and Horning, E. C., Anal. Letters 1, 401-415 (1968).
4. Wilson, W. E. and Ripley, J. E., Anal. Chem. 41, 810-815 (1969).
5. Watson, E. and Kalman, S. M., J. Chromatogr. 56, 209-218 (1971).
6. Lowenstein, J. M., Circulation 31, 228-233 (1965).
7. Lowenstein, J. M. and Corrill, E. M., J. Lab. Clin. Med. 67, 1048-1052 (1966).
8. Gjerdrum, K. Acta Med. Scand. 187, 371-379 (1970).
9. Marcus, F. I., Ryan, J. H. and Stafford, M. G., J. Lab. Clin. Med. 85, 610-620 (1975).
10. Butler, V. P.,Jr. and Chen, J. P., Proc. Natl. Acad. Sci. 57, 71-78 (1967).
11. Smith, T. W., Butler, V. P.,Jr. and Haber, E., Biochemistry 9, 331-337 (1970).
12. Smith, T. W., Butler, V. P., Jr. and Haber, E., N. Engl. J. Med. 281, 1212-1216 (1969).
13. Larbig, D. and Kochsiek, K., Dtsch. Med. Wochenschr. 97, 1310-1312 (1972).
14. Stoll, R. G., Christensen, M. S., Sakmar, E. and Wagner, J. G., Res. Com. Chem. Pathol. Pharmacol. 4, 503-510 (1972).
15. Clark, D. R. and Kalmar, S. M., Drug Metab. Dispos. 2, 148-150 (1974).
16. Greenwood, H., Snedden, W., Hayward, R. P. and Landon, J., Clin. Chim. Acta 62, 213-224 (1975).
17. Kramer, W. G., Bathala, M. S. and Reuning, R. H., Res. Com. Chem. Path. Pharmacol. 14, 83-88 (1976).
18. Gault, M. H., Ahmed, M., Symes, A. L. and Vance, J., Clin. Biochem. 9, 46-52 (1976).
19. Sugden, D., Ahmed, M. and Gault, M. H., J. Chromatogr. 121, 401-404 1976).
20. Barrett, M. J. and Cohen, P. S., Clin. Chem. 18, 1339-1342 (1972).
21. Meade, R. C. and Kleist, T. J., J. Lab. Clin. Med. 80, 748-754 (1972).
22. Phillips, A. P., Clin. Chim. Acta 44, 333-340 (1973).
23. Zeegers, J. J. W., Maas, A. H. J., Willebrands, A. F., Kruyswijk, H. H. and Jambroes, G., Clin. Chim. Acta 44, 109-117 (1973).
24. Beach, H. R. and Watanabe, A. M., Clin. Chem. 21, 1315-1329 (1975).
25. Marcus, F. I., Dickerson, J., Pippin, S., Stafford, M. and Bressler, R., Clin. Pharmacol. Ther. 20, 253-259 (1976).
26. Lader, S. R., Court, G., Johnson, B. F. and Hurn, B. A. L., Scand. J. Clin. Lab. Invest. 29, 14.10 (1972), Suppl. 126.
27. Weintraub, M., Au, W. Y. W. and Lasagna, L., J. Am. Med. Assoc. 224, 481-485 (1973).

28. Park, H. M., Chen, I. W., Manitasas, G. T., Lowey, A. and Saenger, E. L., J. Nucl. Med. 14, 531-533 (1973).

29. Calesnick, B. and Dinan, A., Clin. Chem. 22, 903-905 (1976).

30. Wagner, J. G. and Ayres, J. W., Bioavailability assessment: Methods to estimate total area (AUC→∞) and total amount excreted (A_e^{∞}) and importance of blood and urine sampling schemes with application to digoxin, J. Pharmacok. Biopharm., submitted January 12, 1977.

31. Holtzman, J., Shafer, R. and Erickson, R., Clin. Chem. 20, 1194-1198 (1974).

32. Shaw, W., Clin. Chem. 21, 636 only (1975).

33. Rodbard, D., Bridson, W. and Rayford, P. L., J. Lab. Clin. Med. 74, 770-781 (1969).

34. Harding, B. R., Thompson, R. and Curtis, A. R., J. Clin. Pathol. 26, 973-976 (1973).

35. Rodbard, D., Clin. Chem. 20, 1255-1270 (1974).

36. Sullivan, T. J., Stoll, R. G., Sakmar, E., Blair, D. C. and Wagner, J. G., J. Pharmacok. Biopharm. 2, 29-41 (1974).

37. Samols, E., Lewis, M. and Bell, D., S. Med. J. 67, 1392 only (1974).

38. Documenta Geigy, Scientific Tables, Sixth Edition, 1962, pp. 173-178.

39. Tembo, A. V., Schork, M. A. and Wagner, J. G., Steroids 28, 387-403 (1976).

40. Cekan, Z., J. Steroid Biochem. 6, 271-275 (1975).

41. Fraser, E. J., Leach, R. H., Poston, J. W., Bold, A. M. Culank, C. S. and Lepedo, A. B., J. Pharm. Pharmac. 25, 968-973 (1973).

42. Halkin, H., Sheiner, L. B., Peck, C. C. and Melmon, K. L., Clin. Pharmacok. Ther. 17, 385-394 (1975).