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Pharmacokinetics of β-Methyldigoxin in Healthy Humans IV: Comparisons of Radioimmunoassays, Total Radioactivity, and Specific Assays of β-Methyldigoxin and Digoxin in Plasma

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Abstract □ A modified radioimmunoassay, using the displacement of the ^{125}I-digoxin derivative bound to antiserum, is presented. It permitted the monitoring of plasma for total glycosides up to 144 hr after oral and intravenous administrations of 0.3 and 0.6 mg of ^{3}H-β-methyldigoxin to healthy humans. In a specific plasma, the radioimmunoassay response of β-methyldigoxin was 86 ± 3% that of digoxin. Radioimmunoassay of plasma was highly correlated with liquid scintillation spectrometric analysis of total radioactivity, and plots of various studies showed intercepts not significantly different than zero. However, radioimmunoassay underestimated the radiolabeled plasma concentration by 12–38% and was dependent on the individual plasma. Since total radioactivity and radioimmunoassay can be expressed as a linear sum of the ^{3}H-digoxin and ^{3}H-β-methyldigoxin plasma concentrations, plots of ratios of total radioactivity to ^{3}H-digoxin concentration against ratios of ^{3}H-β-methyldigoxin to ^{3}H-digoxin plasma concentration were statistically evaluated to determine the specific activities of both glycosides in the two assays. The contributions of ^{3}H-β-methyldigoxin and its metabolite ^{3}H-digoxin were equivalent in liquid scintillation spectrometry, but the former ranged from 65 to 87% of the potency of the latter in the various radioimmunoassay studies. There was a significant difference in the estimated specific antigenicity of β-methyldigoxin at higher and lower plasma concentration ratios of β-methyldigoxin to digoxin, where the specific antigenicity was less at the higher ratios.

Keyphrases □ β-Methyldigoxin—oral and intravenous, pharmacokinetics, radioimmunoassay compared to radiochemical spectrometric assays, human plasma □ Pharmacokinetics—β-methyldigoxin, oral and intravenous, radioimmunoassay compared to radiochemical spectrometric assays, human plasma □ Radioimmunoassay—β-methyldigoxin, pharmacokinetic study after oral and intravenous administration, compared to radiochemical spectrometric assays, human plasma □ Cardiac glycosides—β-methyldigoxin, oral and intravenous, pharmacokinetics, radioimmunoassay compared to radiochemical spectrometric assays, human plasma

Radioimmunoassay has been applied to measure glycoside concentrations in biological fluids after administration of therapeutic dosages of digoxin and β-methyldigoxin (1–4). This radioimmunoassay of glycosides should be compared with other established methods, such as liquid scintillation spectrophotometry of labeled glycosides, to monitor total radioactivity or the specifically assigned radioactivity of separated parent drug and metabolites. Such comparisons should elucidate the specificity of such procedures.

Recent studies with radioimmuno- and ^{86}Rb-uptake assays investigated the mutual relationships between digoxin and its metabolites and quantified the fractional contributions of parent drug and metabolites (2, 5) to total activity. It was suggested that all cardioactive metabolites of digoxin and digitoxin also contribute to the total antigenicity and total uptake inhibition in the radioimmuno- and ^{86}Rb-uptake assays, respectively (2, 6). Potency differences were reported for different glycoside metabolites and derivatives in the radioimmunoassay (2). Equipotency can be assumed for the parent drug and metabolites when total radioactivity is monitored by liquid scintillation spectrophotometry.

This paper compares the radioimmunoassay and total and specific radioactivity methods used to monitor the glycoside ^{3}H-β-methyldigoxin (7, 8), which is about 50% metabolized, mainly to digoxin (7–9).

EXPERIMENTAL

Equipment—An automated-control γ-scintillation spectrometer[1] was used for determining the activity of the ^{125}I-digoxin derivative after addition to plasma in the radioimmunoassay procedure.

Materials and Methods—The ^{125}I-digoxin derivative, digoxin standard, buffer components, and antiserum used for the radioimmunoassay were obtained from the commercially available kit[2].

[1] Auto Gamma Counter, Packard Instruments Co., Downers Grove, Ill.
[2] ^{125}I-Digoxin derivative radioimmunoassay kit, Schwarz/Mann, Orangeburg, N.J.

Table I—Influence of Plasma Volume on Radioimmunoassay Efficiency[a]

Plasma Volume in 1 ml of Phosphate Buffer, μl	Replicate Measured Digoxin Concentrations by Radioimmunoassay, dpm/ml
50	1091, 1206
100	1183, 1218
200	1203, 1335
500	1162, 1248

[a] There was no statistically significant influence of the size of the assayed plasma on the radioimmunoassay ($F = 0.0052$; $df = 3, 4$; $p > 0.9$).

The seven volunteers received 0.3 and 0.6 mg of β-methyldigoxin orally and intravenously. Plasma glycoside concentrations were assayed by radioimmunoassay in five subjects (A, B, C, D, and G) (7, 8, 10) at the following times: 0, 1.5, 2, 2.5, 3.5, 7, 10, 15, 45, 60, and 90 min and 2, 3, 5, 7, 9, 11, 15, 20, 24, 36, 48, 60, 72, 84, 96, 120, and 144 hr after intravenous administration. Samples were assayed at the same times after oral administration except for 1.5, 2.5, and 3.5 min.

The methods and procedures for measurement of total plasma radioactivity and the radioactivity assignments to the separated drug and metabolite were described previously (7). The commercially available kit was modified in accordance with Stoll *et al.* (2) so that the wide glycoside concentration ranges found in plasma up to 144 hr after administration could be monitored by radioimmunoassay. The ^{125}I-digoxin derivative, rather than the ^{3}H-digoxin derivative, was used since the administered β-methyldigoxin was tritium labeled. This change alleviated the problem of quenching and led to increased sensitivity (11).

All plasma samples of a particular study were assayed at the same time. Individual calibration curves used the particular volunteer's blank plasma to which known concentrations of standard digoxin solutions were added.

Isotonic pH 7.4 phosphate buffer (1.0 ml) was added to 50–500 μl of plasma containing unknown concentrations of digoxin and/or β-methyldigoxin standard. Tracer, 10 μl, and 10 μl of antiserum were added to the mixtures. The mixtures were gently vortexed and incubated for 2 hr at 25° and then for an additional 16 hr at 4°. Equilibration was undoubtedly effected in the first 0.5 hr at room temperature but certainly was achieved by this procedure. The subsequent time under refrigeration was only to inhibit bacterial growth. Dextran-coated charcoal, 500 μl, was squirted into each mixture. Subsequently, at no more than 5 min, the samples were centrifuged at 1500 rpm for 10 min. The clear supernates were decanted and transferred to γ-counting tubes for measurement.

The influence of varied plasma volumes on the assay was studied since contradictions were reported (2, 12). Plasma blanks of 50, 100, 200, and 500 μl were spiked with digoxin standard so that the ultimate digoxin concentrations would be 2.0 ng/ml after dilution with phosphate buffer to the same 1.00-ml volume. Duplicate radioimmunoassays were performed (Table I), and no statistically significant difference was observed with decreasing fractions of plasma in the final solution.

Both the ^{3}H-digoxin and the ^{125}I-digoxin derivative radioimmunoassays were originally designed to measure unknown digoxin concentrations. However, these assays are not specific for digoxin but also respond to digoxin metabolites (2) and digoxin derivatives such as β-methyldigoxin (3, 4).

Since β-methyldigoxin is metabolized, mainly to digoxin (5, 9), the individual antigenic potencies of β-methyldigoxin and digoxin were evaluated in the radioimmunoassay over a significant concentration range. Plasma aliquots were spiked with either β-methyldigoxin or digoxin standards to give concentrations of 0.4, 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0 ng/ml. Controls with blank plasma were run at the same time.

RESULTS AND DISCUSSION

Typical semilogarithmic plots of total plasma glycoside concentrations as determined by total radioactivity as measured by liquid scintillation spectrophotometry and by "total antigenicity," *i.e.*, the total antigenic effect resulting from the sum of antigenic effective glycosides, as measured by radioimmunoassay are given against time for intravenous and oral administrations (Fig. 1) of β-methyldigoxin. Both methods of analysis permitted the measurement of plasma glycoside concentrations from 40.0 to 0.2 ng/ml.

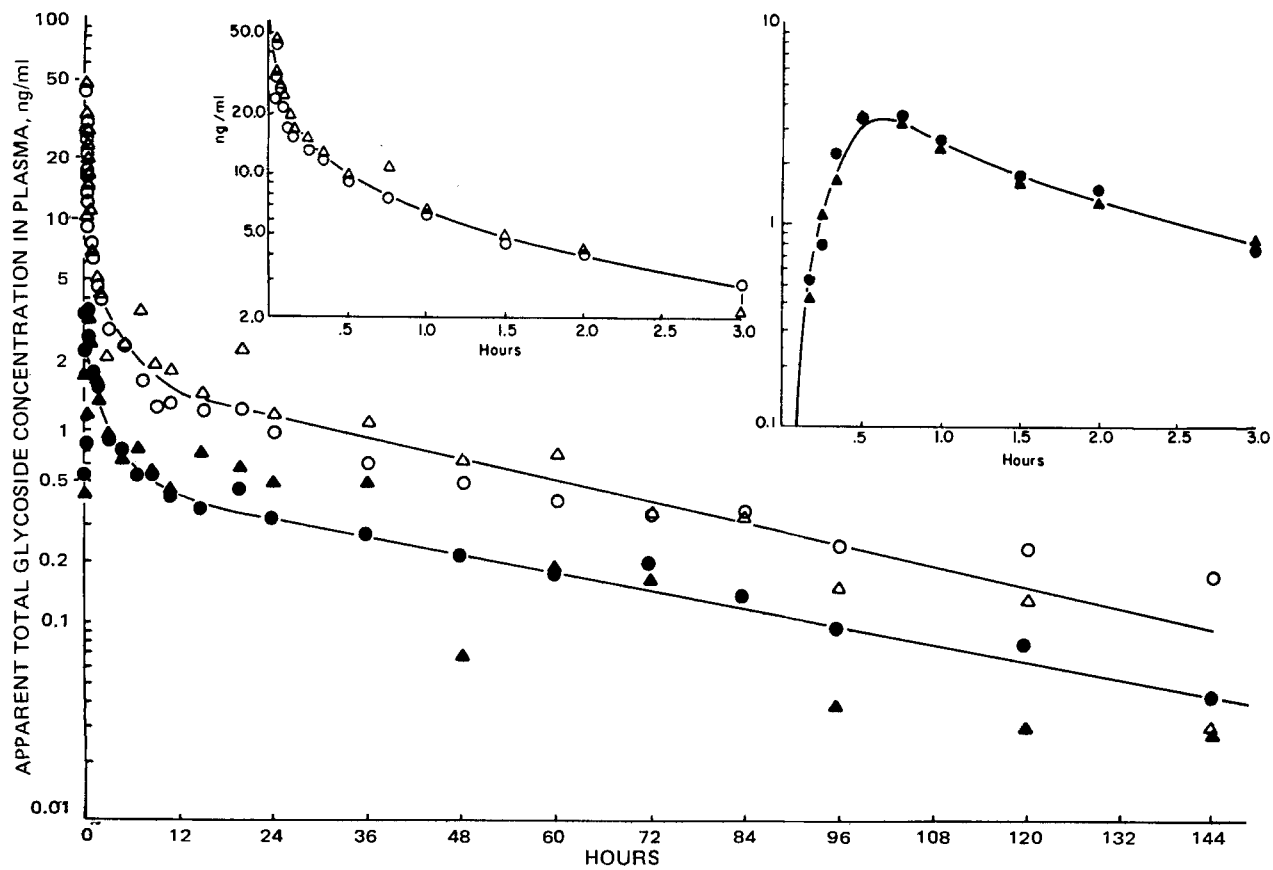

Figure 1—*Typical semilogarithmic plots of plasma concentrations of total glycosides against time by radioimmunoassay (▲, Subject B, 0.3-mg po dose; and △, Subject G, 0.6-mg iv dose) and liquid scintillation spectrometry (●, Subject B, 0.3-mg po dose; and ○, Subject G, 0.6-mg iv dose) after β-methyldigoxin administration. The insets expand the time axis over the initial period.*

Table II—Linear Regressions of Plasma Concentrations by Radioimmunoassay (RIA) on Concentration by Liquid Scintillation Spectrometry (LSC): RIA = mLSC + b

Subject	Dose, mg	Mode of Administration	Range, ng/ml	n[a]	$m \pm SE$	$b \pm SE$	r[b]	S_{RIA}[c]
A	0.6	Intravenous	0.10–20.0	29	0.858 ± 0.071	0.79 ± 0.51	0.919	1.97
A	0.6	Oral	0.16–6.2	23	0.731 ± 0.053	0.03 ± 0.14	0.949	0.48
B	0.6	Intravenous	0.10–32	29	0.618 ± 0.022	0.44 ± 0.24	0.983	1.05
B	0.3	Oral	0.03–3.6	24	0.893 ± 0.037	0.05 ± 0.05	0.982	0.18
C	0.6	Intravenous	0.20–88	27	0.850 ± 0.023	−1.11 ± 0.61	0.991	2.70
C	0.3	Oral	0.05–4.0	24	0.698 ± 0.081	0.27 ± 0.10	0.878	0.39
G	0.6	Intravenous	0.03–48	30	1.081 ± 0.017	0.34 ± 0.23	0.997	1.01
G	0.6	Oral	0.10–4.5	23	0.888 ± 0.043	0.12 ± 0.08	0.976	0.28

[a] Number of pairs. [b] Correlation coefficient. [c] Standard error of estimate of RIA by LSC.

Table III—Radioimmunoassays of Plasma β-Methyldigoxin in a System Calibrated against Digoxin

Concentration, ng/ml			Percent of Actual Concentrations	
Actual	Assayed			
0.40	0.40	0.30	100	75
1.00	0.80	0.85	80	85
2.00	1.60	2.10	80	105
3.00	2.40	2.75	80	71
5.00	4.00	4.70	80	94
10.00	>5.0	9.00	—	90
	Average ± SE		84 ± 4	87 ± 5
	Overall average			85.5 ± 3.2

Correlation of Plasma Concentrations of Glycosides by Radioimmunoassay and Liquid Scintillation Spectrophotometry of Total Radioactivity—Highly significant linear correlations existed between concentrations assayed by radioimmunoassay (RIA) and liquid scintillation spectrophotometry (LSC):

$$\text{RIA} = m\text{LSC} + b \qquad \text{(Eq. 1)}$$

The parameters of Eq. 1 and their variability are given in Table II for all studies conducted. Typical plots are given in Fig. 2.

The intercepts, b, in all cases were not significantly different from zero. The slopes, m, were significantly different among subjects and frequently within subjects, indicating that an individual plasma factor contributed to the variability in the radioimmunoassay response and that this plasma factor may vary within the individual.

In all cases except for Subject G, the slopes were significantly less than unity and indicated that the radioimmunoassay as established was not equivalent to the assay by liquid scintillation spectrophotometry. Radioimmunoassay alone would underestimate the radiolabeled material by 12–38% with Subject G excluded.

The radioimmunoassay was calibrated for digoxin by plotting the percent of added tracer bound to the antiserum against the added digoxin concentrations. Equivalent amounts of β-methyldigoxin were assayed at the same seven concentration levels between 0.40 and 10 ng/ml, and the experimental assay values were determined as digoxin-equivalent responses. β-Methyldigoxin was only 86% as responsive as digoxin in the plasma used for the radioimmunoassay (Table III).

Both of these compounds compete with ^{125}I-digoxin for binding sites. Since varying ratios of digoxin (DIG) and β-methyldigoxin (BMD) in plasma were assayed simultaneously, the presence of one could have modified the separate response of the other. Since:

$$\text{RIA} = m_R\text{BMD} + b_R\text{DIG} \qquad \text{(Eq. 2)}$$

it is possible that the factor m_R is not constant but varies as a function of the digoxin concentration.

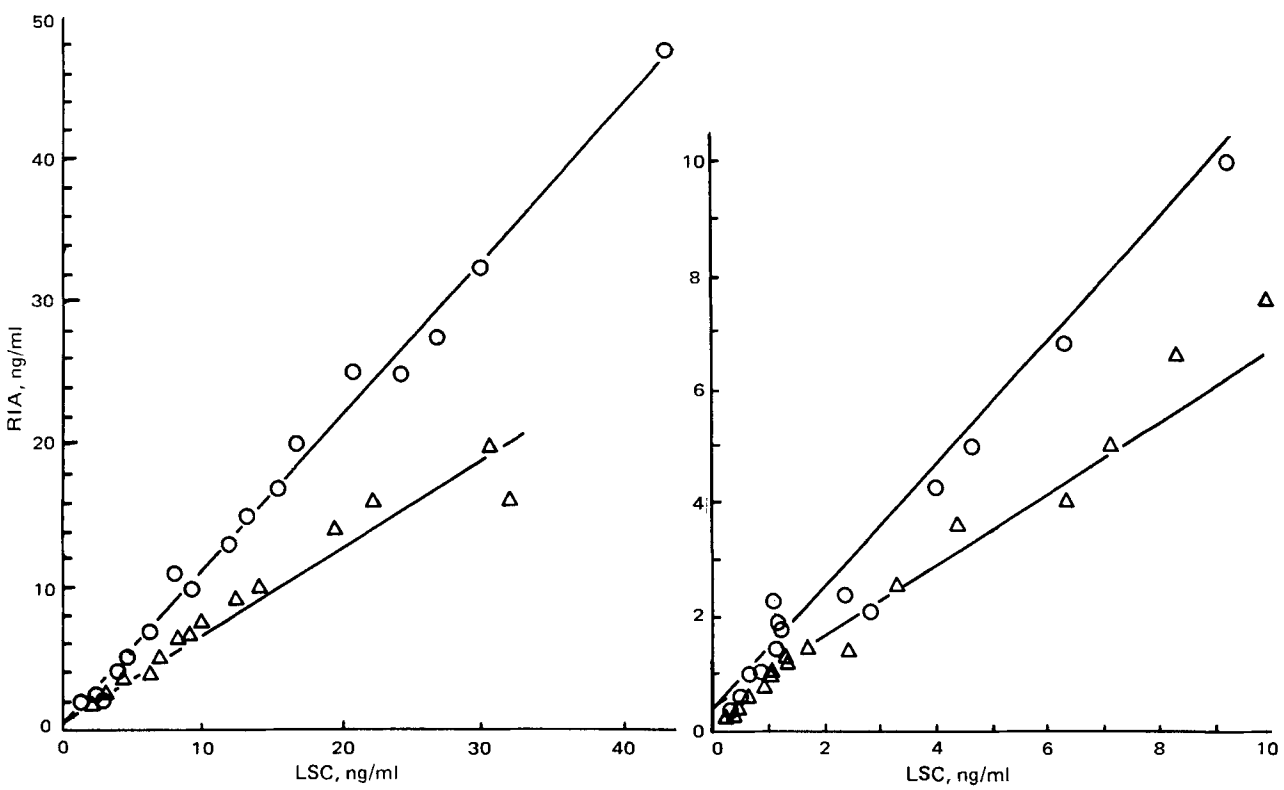

Figure 2—*Typical regressions of assays of total glycosides in plasma by radioimmunoassay (RIA) against assays by liquid scintillation spectrometry (LSC) for Subjects B (△) and G (○) given 0.6 mg iv of β-methyldigoxin. The lines drawn over the shorter range are the best fit over the longer range.*

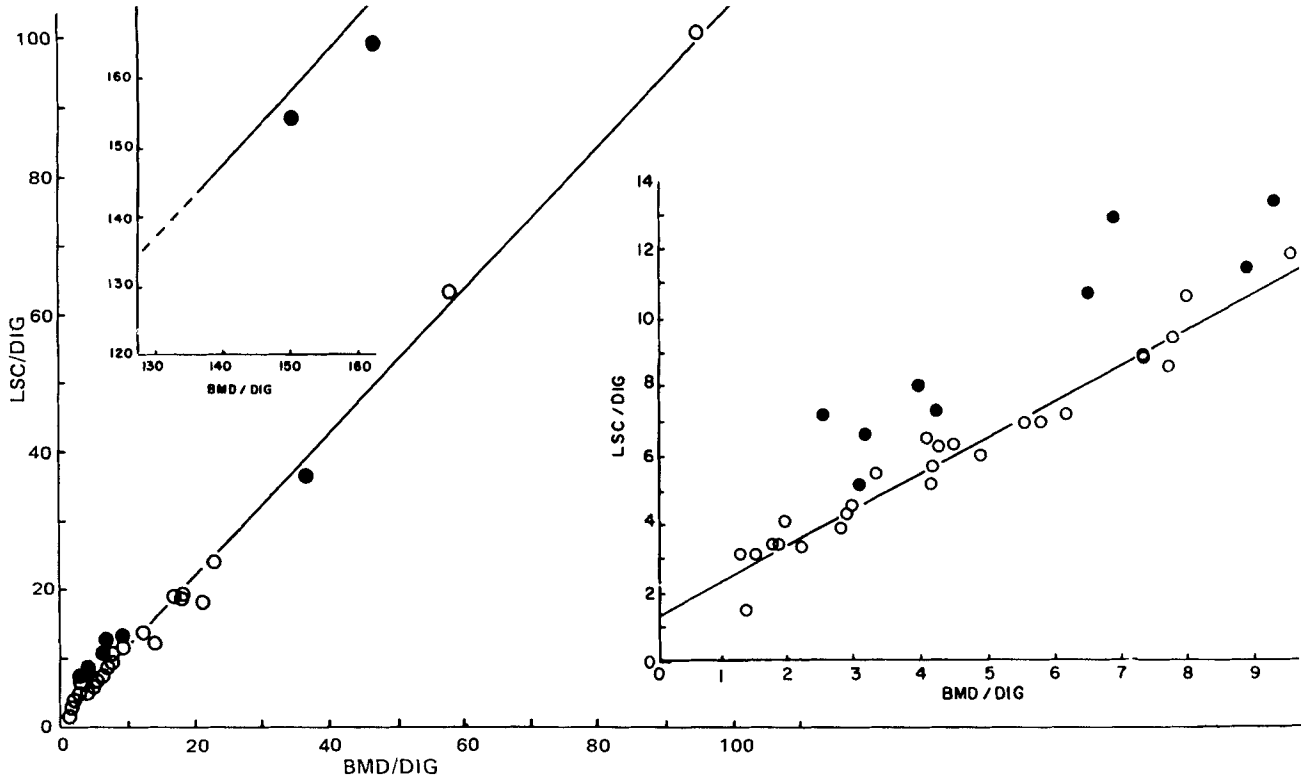

Figure 3—*Plots of the ratios of total glycoside plasma concentration by liquid scintillation spectrometry (LSC) to the plasma digoxin concentration (DIG) against the plasma concentration ratios of β-methyldigoxin (BMD) to digoxin (DIG) for the pooled available data from all subjects, oral and intravenous. The left inset shows the extrapolated regression line of best fit. The right inset is for the BMD/DIG range of 0–10 fitted separately. The dark symbols are for the data from Subject A, 0.6 mg po of β-methyldigoxin, which were excluded in fitting the shown regression line.*

It is also possible that the radiolabeled β-methyldigoxin metabolites or digoxin metabolites were monitored in the plasma by the liquid scintillation spectrometry but did not respond to the radioimmunoassay. However, studies on the TLC-separated β-methyldigoxin and digoxin from plasma did not show any significant amounts of other metabolites in plasma (7, 8).

Relative Potencies of β-Methyldigoxin and Digoxin in Several Plasma Assays after β-Methyldigoxin Administration—Total radioactivity (LSC) in a plasma aliquot can be expressed as the linear sum of the individual radioactivities of labeled β-methyldigoxin and digoxin:

$$\text{LSC} = m_L\text{BMD} + b_L\text{DIG} \qquad \text{(Eq. 3)}$$

where m_L and b_L reflect the relative potencies of β-methyldigoxin and digoxin, respectively, in the liquid scintillation spectrometric assay. The "total antigenicity" by the radioimmunoassay also can be expressed as a similar linear sum (Eq. 2) of the partial antigenicities.

Equation 3 can be rearranged to:

$$\text{LSC/DIG} = m_L\text{BMD/DIG} + b_L \qquad \text{(Eq. 4)}$$

and typical plots of one ratio against the other are given in Fig. 2 and for the pooled data in Fig. 3. Statistical evaluations of the regressions for the available studies are given in Table IV. As anticipated, the parameters m_L and b_L were not significantly different from unity, and the contributions of digoxin and β-methyldigoxin to the total radioactivity were equivalent except for b_L for Subject A at the 0.6-mg po dose. The anomalous discrepancy in this intercept is obvious from the plotted solid symbols in Fig. 3, where the regression line was fitted to the pooled data with the data from this particular study excluded. There were no significant differences between the parameters for β-methyldigoxin (Table IV). Plots of LSC/BMD *versus* DIG/BMD gave similar information; the intercepts, m_L, were unity in all studies.

Similarly, Eq. 2 can be rearranged to:

$$\text{RIA/DIG} = m_R\text{BMD/DIG} + b_R \qquad \text{(Eq. 5)}$$

Table IV—Typical Linear Regressions of Ratios of Plasma Concentrations of Glycosides by Liquid Scintillation Spectrometry (LSC) and by Radioimmunoassay (RIA) to Plasma Digoxin Concentration (DIG) against the Ratios of Concentrations of β-Methyldigoxin (BMD) to Digoxin (DIG) in Human Plasma: LSC/DIG = m_LBMD/DIG + b_L; RIA/DIG = m_RBMD/DIG + b_R

Subject	Dose, mg	Mode of Administration	Range, BMD/DIG	n^a	$m_L \pm SE$	$b_L \pm SE$	$r_L{}^b$	$m_R{}^c \pm SE$	$b_R \pm SE$	$r_R{}^b$
A	0.6	Intravenous	1.4–23	11	0.930 ± 0.052	1.12 ± 0.73	0.986	0.870 ± 0.035 (0.80–0.90)	1.31 ± 0.49	0.994
A	0.6	Oral	5–165	9	0.999 ± 0.009	3.30 ± 0.71	1.000	0.871 ± 0.054 (0.60–0.90)	0.75 ± 3.0	0.989
B	0.6	Intravenous	1.9–95	11	1.050 ± 0.006	1.32 ± 0.20	1.000	0.706 ± 0.018 (0.70)	1.62 ± 0.62	0.997
C	0.3	Oral	2.8–21	7	1.013 ± 0.032	1.16 ± 0.30	0.998	0.649 ± 0.036 (0.67)	2.3 ± 0.34	0.993
Pooled	0.3, 0.6	Oral, intravenous	1.4–165	49	1.012 ± 0.010	1.87 ± 0.36	0.998	0.829 ± 0.017	1.04 ± 0.52	0.991
Pooled[d]	0.3, 0.6	Oral, intravenous	1.4–165	40	1.016 ± 0.010	1.39 ± 0.39	0.998	0.716 ± 0.014	1.76 ± 0.30	0.994
Pooled[d]	0.3, 0.6	Oral, intravenous	1.4–10	26	1.052 ± 0.046	1.23 ± 0.23	0.978	0.825 ± 0.082	0.981 ± 0.41	0.903

[a] Numbers of pairs. [b] Respective correlation coefficients. [c] Parenthetical values are estimates of m_R from the intercepts of plots of RIA/BMD *versus* DIG/BMD. When given as a range, the initial data were too scattered by this plotting method to conclude anything other than that the value lay within this range. Similar plots of LSC/BMD *versus* DIG/BMD were conclusive of intercepts, m_R, of unity in all cases. [d] Data of Subject A at the 0.6-mg po doses were excluded because of an anomalous intercept, b_L.

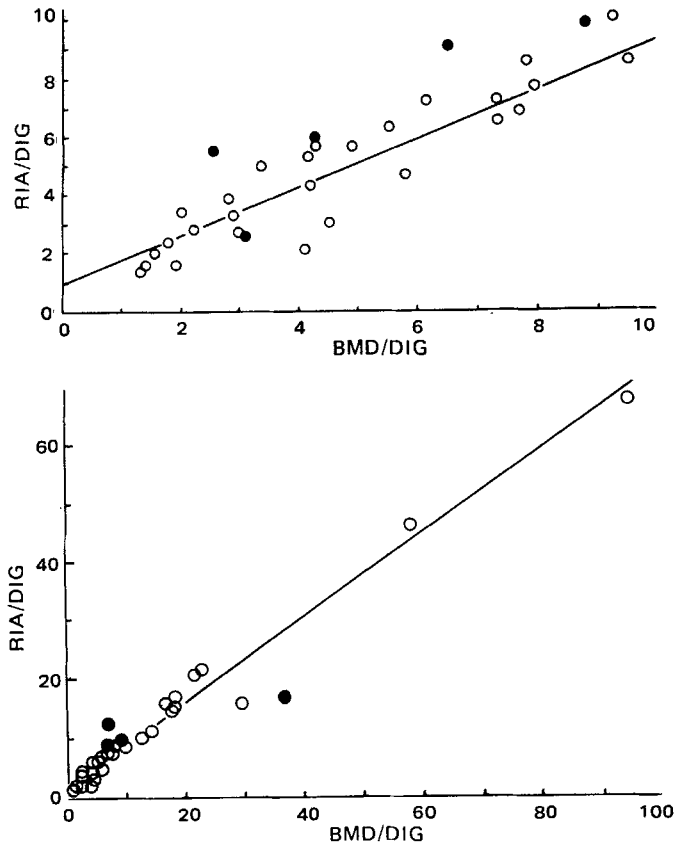

Figure 4—*Plots of the ratios of total glycoside plasma concentration by radioimmunoassay (RIA) to the plasma digoxin concentration (DIG) against the plasma concentration ratios of β-methyldigoxin (BMD) to digoxin (DIG) for the pooled available data from all subjects, oral and intravenous. The dark symbols are for the data from Subject A, 0.6 mg po of β-methyldigoxin, which were excluded in fitting the shown regression lines. Both ranges were fitted separately.*

and plots of the pooled data over several BMD/DIG ranges are given in Fig. 4. The regression lines were fitted for the particular ranges given where the anomalous data of Subject A at the 0.6-mg po dose were excluded. The statistical evaluations of the regressions for the pooled data over several ranges and for the available specific studies are given in Table IV. There were no significant differences among the various intercepts, b_R, which were not significantly different from unity. This result was a measure of the individual "antigenicity" of digoxin in the assay and was anticipated since the radioimmunoassay was calibrated for digoxin. However, individual m_R values, measures of antigenic potency of β-methyldigoxin in the assay, were significantly different for the separate studies. This finding was consistent with the previously discussed results for the regressions of RIA *versus* LSC (Table II).

The m_R values were significantly less than unity for the separate studies and for the pooled data of all available studies, consistent with the fact that the specific antigenicity of β-methyldigoxin was less than that of digoxin (Table III). The separate regressions for different ranges of β-methyldigoxin to digoxin ratios, BMD/DIG, showed significantly different slopes when the anomalous oral study for Subject A was excluded. The slope, 0.72 ± 0.014, for the longer range (1.4–165) or higher relative β-methyldigoxin concentrations was significantly less than the m_R = 0.03 ± 0.08 for the shorter range (1.4–10), indicative of higher specific potency for β-methyldigoxin in the presence of relatively large amounts of digoxin. This finding tends to confirm the previously stated possibility that the presence of one glycoside may modify the radioimmunoassay response of the other. Similar values for m_R were obtained (Table III) from the intercepts of RIA/BMD plots against DIG/BMD.

In summary, it can be stated that an *a priori* assumption of radioimmunoassay equivalency of glycosides with their derivatives and their cardioactive metabolites is unwarranted. The fundamental basis of a radioimmunoassay demands a competition for binding sites, and minor functional changes in a molecule can affect the binding constants. An assumption that the specific potency of an agonist in the presence of others is not modified by changes in their relative concentration is also unwarranted. These factors are further perturbed by interactions peculiar to an individual plasma.

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