Table I—Recovery of I and II from Plasma (n = 4)

Compound	Micrograms Added to 1 ml of Plasma	Mean Micrograms Recovered	Mean Percent Recovery	*SD* of Percent Recovery
I	1.00	0.996	99.60	1.11
	2.00	2.04	100.20	1.37
	Mean = 99.90 ± 1.25%			
II	1.00	0.47	47.39	0.01
	2.00	0.95	47.46	0.01
	Mean = 47.42 ± 1.64%			

Table II—GLC Estimation of I Added to Plasma

Added I, μg	n	Mean Peak Height Ratio I/II	*SD*	*RSD*
0.25	7	0.012	0.001	3.98
0.50	6	0.024	0.001	3.13
1.00	7	0.048	0.002	3.19
2.00	6	0.095	0.002	1.76
4.00	5	0.200	0.001	4.14
			Mean *RSD*	3.12
$y = mx$, where $m = 0.0492 \pm 0.0006$; $r^2 = 0.998$				

the advantage that the intact drug is measured. The technique is being used for investigating single- and multiple-dose pharmacokinetics.

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Binding of Codeine, Morphine, and Methadone to Human Serum Proteins

JOSEPH JUDIS

Abstract □ The binding properties of codeine, morphine (as representative opium alkaloids), and methadone (a synthetic pharmacologically similar compound) were studied with selected human serum proteins. The methodology involved equilibrium and dynamic dialysis using ^3H- and/or ^{14}C-labeled compounds. For estimation of the percent binding with equilibrium dialysis, concentrations of the ligand used were approximately therapeutic blood levels and another concentration 30–60 times higher. The percent binding to whole human serum ranged from about 20% for morphine to almost 60% for methadone. Of the human serum proteins investigated, the highest percent binding was found with albumin, except for methadone for which it was β-globulin III. The affinity for other serum proteins varied with the ligand. In studies with albumin using dynamic dialysis, the plots of nubar divided by free concentration *versus* nubar were similar for all three ligands studied and had positive slopes, unlike those reported for acidic compounds for which the slope is always negative. In studies of binding of one ligand in the presence of another, significant competition was demonstrated, suggesting that the same binding sites were involved.

Keyphrases □ Codeine—binding to human serum proteins □ Morphine—binding to human serum proteins □ Methadone—binding to human serum proteins □ Binding, protein—codeine, morphine, and methadone to human serum □ Protein binding—codeine, morphine, and methadone to human serum □ Analgesics, narcotic—codeine, morphine, and methadone, binding to human serum proteins □ Alkaloids, opium—codeine, morphine, and methadone, binding to human serum proteins

The binding of drugs to serum proteins is well established as a parameter in pharmacological and therapeutic activities of medicinal agents (1). Several reviews treated the methodology for studying protein binding and summarized the vast number of substances already investigated (2–5). Of the hundreds of compounds studied, the vast majority are acidic or nonpolar; few studies have

Table I—Binding of Methadone, Morphine, and Codeine to Various Human Serum Proteins[a]

Serum Protein Fraction	Concentration, mg/ml	Ligand, % Bound for Concentration Indicated: Methadone 9.3×10^{-8} Mole	Methadone 3.3×10^{-6} Mole	Morphine 5.5×10^{-8} Mole	Morphine 3.6×10^{-6} Mole	Codeine 5.6×10^{-8} Mole	Codeine 3.4×10^{-6} Mole
Albumin	40.0	31.16 (2.23)	21.84 (0.29)	23.24 (1.30)	16.56 (1.15)	14.26 (0.68)	28.50 (0.09)
α-Globulin IV-1	1.0	10.19 (1.28)	5.70 (0.51)	1.61 (0.07)	4.10 (0.98)	9.59 (1.46)	5.03 (0.21)
α-Globulin IV-4	5.0	12.53 (1.65)	4.58 (0.13)	6.42 (0.01)	7.56 (0.39)	12.34 (0.70)	17.05 (0.40)
β-Globulin III	7.0	37.75 (2.76)	10.90 (0.56)	7.39 (0.05)	3.16 (0.21)	7.98 (0.60)	3.68 (1.04)
γ-Globulin II	11.0	8.26 (0.72)	6.47 (0.40)	4.16 (0.20)	10.02 (2.72)	5.71 (0.72)	8.60 (1.16)
Human serum	—	59.78 (3.00)	39.54 (1.20)	24.02 (1.82)	20.08 (1.04)	29.01 (2.70)	22.35 (0.87)

[a] The number of moles of ligand added to each system is indicated as the concentration. Values in parentheses are standard deviations.

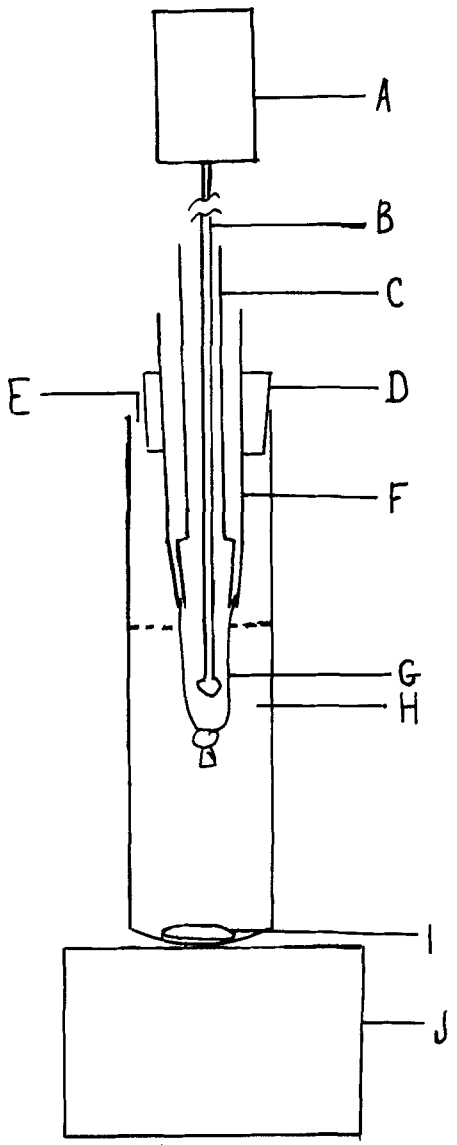

Figure 1—*Diagram of the apparatus used for dynamic equilibrium dialysis. Key: A, stirring motor; B, stirring rod; C, inner tube; D, rubber stopper; E, slot for withdrawing and adding buffer; F, middle tube; G, dialysis bag; H, 100 ml of buffer; I, stirring bar; and J, magnetic stirrer.*

concerned the serum protein binding properties of alkaloids and other basic organic compounds.

Only a handful of reports treated such widely used compounds as opium alkaloids or methadone. Morphine and methadone were shown to bind to serum proteins on the basis of decreased analgesic qualities resulting when the compounds were dissolved in serum prior to injection as compared to aqueous solutions (6). Several studies (7, 8) demonstrated the binding of morphine and methadone to human serum albumin and the binding of methadone to γ-globulin (9), but these studies involved nothing more quantitative than percent binding figures. In addition, the binding of morphine to α_1-globulin was demonstrated using electrophoresis (10), and the binding of papaverine to plasma proteins also was reported (11).

In view of the paucity of investigations of human serum protein binding of alkaloids, studies were conducted using codeine and morphine, as naturally occurring alkaloids, and methadone, as a synthetic organic base with pharmacological properties similar to the other two.

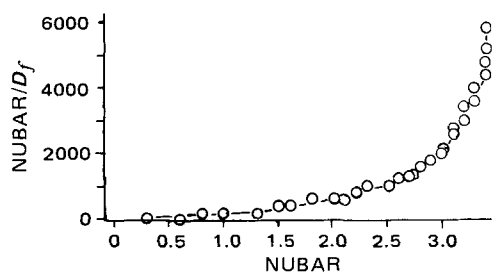

Figure 2—*Binding of codeine to human serum albumin determined by dynamic equilibrium dialysis. The plot is of nubar divided by free concentration* versus *nubar. A total of 3.368 × 10^{-5} mole of codeine was added at the beginning of the run, and the albumin concentration in the bag was 5.797 × 10^{-4}* M.

Table II—Binding of Methadone, Morphine, and Codeine to Various Human Serum Proteins[a] (Ranking in Order by Percent Binding)

	Methadone		Morphine		Codeine	
	9.3 × 10^{-8} Mole[b]	3.3 × 10^{-6} Mole	5.5 × 10^{-8} Mole	3.6 × 10^{-6} Mole	5.6 × 10^{-8} Mole	3.4 × 10^{-6} Mole
Rank 1	HS	HS	HS	HS	HS	ALB
Rank 2	B-III	ALB	ALB	ALB	ALB	HS
Rank 3	ALB	B-III	B-III	G-II	AG4	AG4
Rank 4	AG4	G-II	AG4	AG4	AG1	G-II
Rank 5	AG1	AG1	G-II	AG1	B-III	AG1
Rank 6	G-II	AG4	AG1	B-III	G-II	B-III

[a] Abbreviations for serum proteins are as follows: HS = human serum, ALB = human serum albumin, AG1 = α-globulin IV-1, AG4 = α-globulin IV-4, G-II = γ-globulin II, and B-III = β-globulin III. [b] Total concentration of ligand in cell.

EXPERIMENTAL

Materials—[^{14}C-*N*-Methyl]codeine hydrochloride[1], [^{14}C-*N*-methyl]morphine hydrochloride[1], and 1-^{3}H-*l*-methadone hydrobromide[2] had specific activities of 54, 57, and 135 mCi/mmole, respectively. Crystalline human serum albumin[3] and the other human serum proteins[3] were used as received. A liquid scintillation phosphor solution[4] was used in all radioactivity determinations. All other chemicals were reagent grade.

Methods—Equilibrium dialysis was employed for the estimation of the percent binding to specific proteins. However, to obtain more data efficiently for Scatchard-type plots, the dynamic equilibrium procedure was used. The procedure for the determination of protein binding using equilibrium dialysis was identical with that described previously (12, 13). All components of the system were dissolved in pH 7, 0.67 *M* phosphate

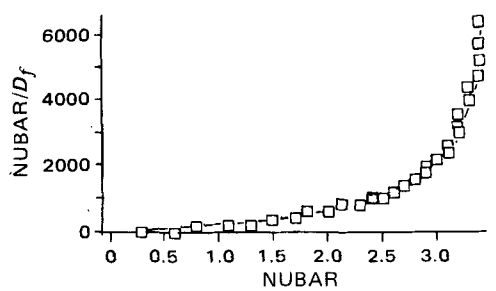

Figure 3—*Binding of morphine to human serum albumin determined by dynamic equilibrium dialysis. The plot is of nubar divided by free concentration* versus *nubar. A total of 3.527 × 10^{-5} mole of morphine was added at the beginning of the run, and the albumin concentration in the bag was 5.797 × 10^{-4}* M.

[1] Amersham/Searle Corp.
[2] New England Nuclear Corp.
[3] Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] Aquasol, New England Nuclear Corp.

Table III—Binding Parameters for Codeine, Morphine, and Methadone and Human Serum Albumin Calculated from Plots[a] of D_b/D_f versus D_b

Ligand	K_1	n_1	K_2	n_2	K_3	n_3
Codeine	280.6	0.209	4380	2.5	12,568	2.998
Morphine	388.3	0.047	3633	2.379	15,539	3.043
Methadone	487.75	0.23	2488	1.092	6,828	1.457

[a] D_b is concentration of bound ligand (molar), and D_f is concentration of free ligand (molar).

Table IV—Inhibition of Binding of ^{14}C-Codeine to Human Serum Albumin by Unlabeled Morphine

Absence of Unlabeled Morphine			Presence of Unlabeled Morphine[a]				
Total Moles of Codeine in System $\times 10^{-4}$	Beta	Nubar	Total Moles of Codeine in System $\times 10^{-4}$	Beta	Percent Decrease in Beta	Nubar	Percent Decrease in Nubar
0.22867	0.228	1.8005	0.22898	0.0978	57.15	0.7725	57.10
0.1627	0.5167	2.8986	0.16383	0.22196	57.04	1.2462	57.01
0.14232	0.6598	3.2378	0.142995	0.28154	57.33	1.3882	57.12
0.13146	0.7541	3.4185	0.131904	0.32268	57.21	1.4677	57.07

[a] A total of 1.75×10^{-5} mole of morphine added to the dialysis bag.

Table V—Inhibition of Binding of ^{14}C-Morphine to Human Serum Albumin by Unlabeled Codeine

Absence of Unlabeled Codeine			Presence of Unlabeled Codeine[a]				
Total Moles of Morphine in System $\times 10^{-4}$	Beta	Nubar	Total Moles of Morphine in System $\times 10^{-4}$	Beta	Percent Decrease in Beta	Nubar	Percent Decrease in Nubar
0.28185	0.1111	1.0798	0.28099	0.07878	29.10	0.7633	29.31
0.20356	0.3237	2.2722	0.20372	0.22564	30.30	1.5851	30.24
0.15624	0.5555	2.9929	0.156634	0.38619	30.48	2.0859	30.31
0.13038	0.7533	3.3868	0.130796	0.52342	30.52	2.3607	30.30

[a] A total of 1.67×10^{-5} mole of codeine added to the dialysis bag.

buffer and incubated on a shaker in a 37° water bath for 24 hr, previously shown to be adequate for reaching equilibrium.

The dynamic equilibrium procedure used was a modification (14) of the Meyer and Guttman (15) procedure. The basic apparatus (Fig. 1) consisted of a collodion bag apparatus[5] modified by the attachment of dialysis tubing[6] (3.32 cm flat width), tied off at one end and held in the

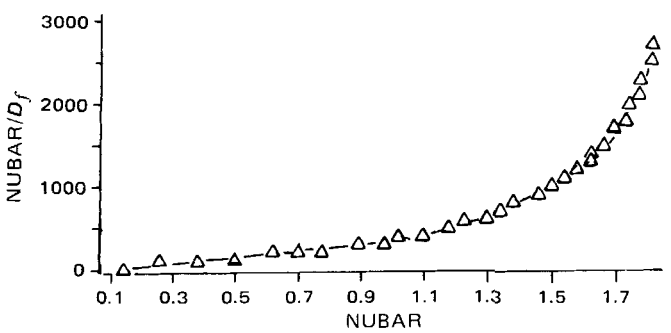

Figure 4—*Binding of methadone to human serum albumin determined by dynamic equilibrium dialysis. The plot is of nubar divided by free concentration* versus *nubar. A total of 2.589×10^{-5} mole of methadone was added at the beginning of the run, and the albumin concentration in the bag was 5.797×10^{-4}* M.

[5] No. 100/21, Schleicher and Schuell.
[6] Fisher Scientific Co.

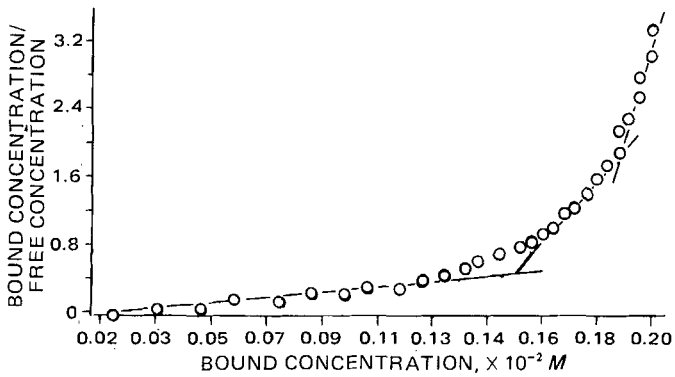

Figure 5—*Binding of codeine to human serum albumin determined by dynamic equilibrium dialysis. The plot is of bound concentration divided by free concentration* versus *bound concentration; amounts of albumin and codeine are the same as for Fig. 2.*

ground-glass joint at the end of the inner tubes of the collodion bag apparatus at the other end. The dialysis tubing was soaked overnight in phosphate buffer prior to use and cut to hold a volume of 5 ml.

Buffer was added through a slot cut in the rubber stopper at the top of the apparatus, and the contents of the bag were stirred with a motor-driven glass stirring rod. Incubation was at room temperature (24 ± 2°). The buffer was stirred with a polytef[7]-coated stirring bar driven by a magnetic stirrer. At the start of a run, protein and ligand solutions (total volume of 5 ml) were added to the bag and 100 ml of pH 7, 0.067 *M* phosphate buffer was added to the apparatus. At 30-min intervals for 3 hr, 50 ml of buffer was withdrawn with a syringe and replaced by 50 ml of fresh buffer.

Radioactivity was determined[8] in an aliquot of the withdrawn buffer. The amount of radioactivity added to the bag initially was determined from an aliquot of the stock solution of radioisotope and the counts per minute in the bag calculated for each sampling time. Free ligand counts per minute in the bag at each sampling time was calculated using the equation given by Meyer and Guttman (15) and the computer program developed by Dearden and Tomlinson (14). From the knowledge of the total and free concentrations of ligand, bound concentrations could be calculated; from the equation relating free concentrations and times of sampling, bound concentrations for intermediate time intervals could be calculated. Approximate estimates of binding parameters were calculated using the method of Sandberg *et al.* (16).

RESULTS AND DISCUSSION

Codeine, morphine, and methadone bound to the several human serum proteins to varying extents (Table I). The lower concentrations of the

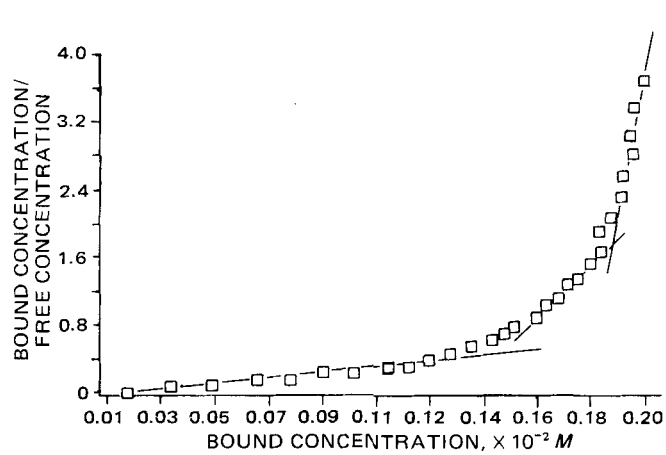

Figure 6—*Binding of morphine to human serum albumin determined by dynamic equilibrium dialysis. The plot is of bound concentration divided by free concentration* versus *bound concentration; amounts of albumin and morphine are the same as for Fig. 3.*

[7] Teflon, du Pont.
[8] Beckman model LS-133.

Table VI—Inhibition of Binding of ^{3}H-Methadone to Human Serum Albumin by Unlabeled Codeine

Absence of Unlabeled Codeine			Presence of Unlabeled Codeine[a]				
Total Moles of Methadone in System $\times 10^{-4}$	Beta	Nubar	Total Moles of Methadone in System $\times 10^{-4}$	Beta	Percent Decrease in Beta	Nubar	Percent Decrease in Nubar
0.25537	0.0868	0.76465	0.25722	0.04121	52.54	0.3655	52.20
0.20445	0.1859	1.3104	0.20475	0.09085	51.12	0.6414	51.05
0.14216	0.4035	1.9780	0.14196	0.19848	50.81	0.9716	50.88
0.11272	0.5900	2.2934	0.112692	0.28964	50.91	1.1255	50.92

[a] A total of 1.67×10^{-5} mole of codeine added to the dialysis bag.

Table VII—Inhibition of Binding of ^{3}H-Methadone to Human Serum Albumin by Unlabeled Morphine

Absence of Unlabeled Morphine			Presence of Unlabeled Morphine[a]				
Total Moles of Methadone in System $\times 10^{-4}$	Beta	Nubar	Total Moles of Methadone in System $\times 10^{-4}$	Beta	Percent Decrease in Beta	Nubar	Percent decrease in Nubar
0.26779	0.0684	0.63162	0.26536	0.02765	59.58	0.2530	59.95
0.20445	0.1859	1.3104	0.20312	0.07275	60.86	0.5096	61.11
0.15666	0.3374	1.8225	0.157693	0.12816	62.01	0.6969	61.76
0.11781	0.5511	2.2389	0.117747	0.21219	61.50	0.8616	61.52

[a] A total of 1.75×10^{-5} mole of morphine added to the dialysis bag.

ligands were approximately those indicated in the literature as therapeutic levels[9] (8, 17–20). When the concentrations were increased by a factor of 30–60, the percent binding did change, generally decreasing. When the ligands were arranged according to the percent binding in decreasing order, the greatest binding occurred with whole human serum with one exception (Table II) and with albumin next except for methadone at the lower concentration and codeine at the higher concentration. Otherwise, the order was different for the three ligands.

The conventional binding curves, Scatchard plots (2, 3, 21) in which moles bound per mole of protein (nubar) divided by free ligand concentration (D_f) is plotted against nubar, are shown in Figs. 2–4. For most ligands studied previously (22–27), the Scatchard plot curve has a negative slope and is a straight line unless more than one type of binding site is involved. In the latter situation, a curve is found. The plots in Figs. 2–4 all have positive slopes and are curves, indicating more than one type of binding site.

Positive slopes have been interpreted to indicate possible cooperativity (28) and do not lend themselves to the analyses generally performed with typical Scatchard curves in terms of the calculation of binding parameters. However, to obtain approximate estimates of the binding parameters, the Sandberg *et al.* (16) method was used. This method involves plots of bound concentration of ligand divided by free concentration *versus* bound concentration (Figs. 5–7). The y-intercept is equal to nKP, the x-intercept is equal to n, and the slope is equal to K (n refers to the number of binding sites, K refers to the association constant, and P refers to the molar protein concentration). Each curve had three distinct segments, and each segment was treated as a separate curve for calculation (Table III).

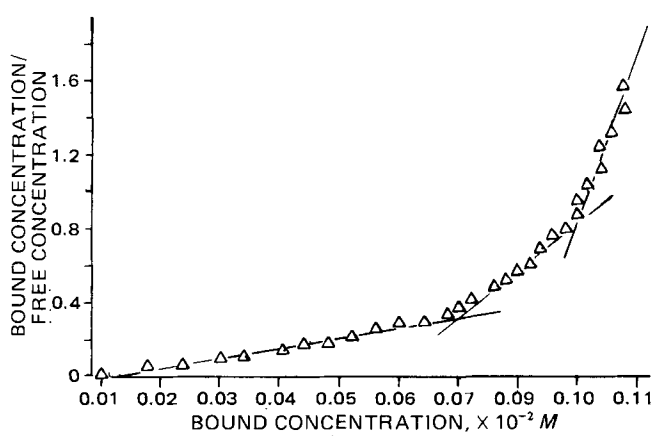

Figure 7—*Binding of methadone to human serum albumin determined by dynamic equilibrium dialysis. The plot is of bound concentration divided by free concentration* versus *bound concentration; amounts of albumin and methadone are the same as for Fig. 4.*

As an approach to determining whether the three ligands bind to the same sites on albumin, binding of one ligand was determined in the presence of another. Unlabeled morphine decreased significantly the binding of ^{14}C-codeine (Table IV), and codeine interfered with the binding of ^{14}C-morphine (Table V). Both codeine and morphine interfered with the binding of ^{3}H-methadone to human serum albumin (Tables VI and VII).

Codeine, morphine, and methadone apparently do bind significantly to human serum proteins, with all of the implications in terms of pharmacological action of drugs (1). Binding to albumin is greatest generally for the three ligands, which is characteristic of many molecules. The three compounds yielded binding plots (nubar divided by D_f *versus* nubar) that are clearly distinct from those of many anionic and nonpolar compounds. The curves for codeine, morphine, and methadone have positive slopes, and this feature may be characteristic of alkaloids and organic bases in general. The same types of plots were obtained in this laboratory in preliminary experiments with amphetamine, histamine, atropine, and epinephrine with human serum albumin as the protein. A curve with a positive slope (same type of plot) was found by Powis (29) in studies of the binding of tetracycline to albumin.

[9] Data on therapeutic serum levels for codeine were obtained from Carl W. Sigel, Burroughs Wellcome Co., Research Triangle Park, N.C.

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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.