4 in Table IV). With the parameter estimates, the computer-fitted excretion rate values (RB') and residual values listed in columns 4 and 5 of Table III were obtained. These values indicate that the interaction between iodoxamic acid and iopanoic acid in the liver can be described by the ligand exclusion model. In such a model, iopanoic acid acts as an inhibitor and competes with iodoxamic acid for binding to either of two identical sites within the liver, which presumably is the rate-limiting step in the liver's overall elimination of these radiographic contrast agents. However, because of the large standard error of the mean of the estimated values of K_m and K_i, the fit may be fortuitous. Further studies will be required to investigate this tentative conclusion.

Presumably, the experimental method and the data analysis used in the present investigation can be applied to the study of the interactions between other compounds (*e.g.*, indocyanine green, sulfobromophthalein, propranolol, lidocaine, and propoxyphene) that have high hepatic excretion. To apply this approach, one must have assay procedures for each compound independently and be able to measure the unbound concentrations in plasma water.

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Antibody Specificity Studies for Reserpine, Its Metabolites, and Synthetic Reserpine Congeners: Radioimmunoassay

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Abstract □ Progress in the development of radioimmunoassay techniques for reserpine and related compounds is reported. A conjugate of reserpine with human serum albumin was prepared, involving linkage at the indole nitrogen atom of reserpine. Injection of the purified conjugate into sheep elicited antibodies of high titer, which bound reserpine selectively. Tritiated reserpine was employed in the procedure, and dextran-coated charcoal was utilized to separate free and bound forms of the drug. Antibodies exhibited a selectivity for reserpine and did not cross-react significantly with major human metabolites. Cross-reactivity of antibodies with other reserpine derivatives (*i.e.*, syrosingopine, deserpidine, and rescinnamine) also was investigated. A stable tritiated or radioiodinated reserpine derivative of high specific activity is being sought to improve assay sensitivity for use in bioequivalence and bioavailability studies. In the absence of any extraction or concentration procedures, at least a 10-fold increase in immunoassay sensitivity would be required to follow reserpine levels in humans given normal doses of the drug. The methods show promise also for the assay of reserpine derivatives such as deserpidine, which exhibits cross-reactivity to reserpine antibodies.

Keyphrases □ Reserpine—antibody specificity studies, metabolites, synthetic congeners, radioimmunoassay □ Radioimmunoassay—analysis, reserpine, metabolites, synthetic congeners □ Antihypertensive agents—reserpine, radioimmunoassay, antibody specificity studies, metabolites, synthetic congeners

Levy *et al.* (1) reported the development of a sensitive and specific radioimmunoassay for the antihypertensive agent reserpine, with a sensitivity of 15 ng/ml in rat plasma and no interference from major metabolites. Application of the method in human bioavailability and bioequivalence studies, without preliminary extraction procedures, has been limited by sensitivity. This limitation results from the instability of tritiated reserpine of high specific activity. Experience in this laboratory with reserpine immunoassay development has been entirely analogous, but the method holds promise if a suitable tritiated or iodinated reserpine analog can be prepared.

Earlier reserpine assays, which were reviewed recently (2, 3), are useful for certain applications but involve time-consuming separations and extractions and have inadequate sensitivity and specificity for reserpine determinations in plasma or urine. A recent method (4) is reportedly sensitive to 200 pg/ml and, perhaps, to 50 pg/ml with less confidence. However, this procedure is indirect, involving extraction followed by TLC and a fluorometric densitometer scan. Recovery during extraction may be variable and must be established.

The purposes of this report are to compare specificities of antiserums produced in these laboratories during immunoassay development with the work of Levy *et al.* (1) and to discuss the potential utility of reserpine antiserums in the immunoassay of commercially available reserpine analogs such as deserpidine and rescinnamine.

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Table I—Data for Standard Radioimmunoassay Curve

Tube	Buffer[a], ml	Bovine Serum Albumin[b], ml	Reserpine Antiserum[c], ml	Reserpine (Cold)[d] Milliliters	Reserpine (Cold)[d] Nanograms	^{3}H-Reserpine[e], ml	Dextran-Coated Charcoal[f], ml
1	1.30	0.10	—	—	—	0.10	—
2	0.80	0.10	—	—	—	0.10	0.50
3	0.70	0.10	0.10	0	0	0.10	0.50
4	0.69	0.10	0.10	0.010	1.0	0.10	0.50
5	0.68	0.10	0.10	0.025	2.5	0.10	0.50
6	0.65	0.10	0.10	0.050	5.0	0.10	0.50
7	0.63	0.10	0.10	0.075	7.5	0.10	0.50
8	0.60	0.10	0.10	0.100	10.0	0.10	0.50
9	0.55	0.10	0.10	0.150	15.0	0.10	0.50
10	0.50	0.10	0.10	0.200	20.0	0.10	0.50

[a] Buffer was 0.15 *M* NaCl and 0.01 *M* sodium phosphate, pH 7.4. [b] Bovine serum albumin was 5 g of 2X crystalline bovine serum albumin (Armour)/100 ml. [c] Reserpine antiserum was diluted 1:50 with buffer; final dilution was 1:500 in tube. [d] Reserpine (cold) was 0.10 ng/μl in 10% ethanol in buffer. [e] ^{3}H-Reserpine was 0.10 ng/μl in 10% ethanol in buffer (10 ng/tube, ~5000 dpm). [f] Dextran-coated charcoal suspension contained 0.01 g of radioimmunoassay grade charcoal/ml and 0.25 mg of dextran (radioimmunoassay grade)/ml.

EXPERIMENTAL

Materials—The following were used: human serum albumin[1] (crystalline, B grade), bovine serum albumin[1] (crystalline, A grade), reserpine[2], Freund's complete adjuvant[3], formalin solution[4], dextran[5], charcoal[5], a commercial liquid scintillation fluid[6], deserpidine[7], rescinnamine[2], syrosingopine[8], methylreserpate[8], reserpic acid[8], and ^{3}H-reserpine[9]. Tritium counting was performed using a liquid scintillation spectrometer with automatic external standardization to enable computation of efficiency and disintegrations per minute of individual samples[10].

Reserpine Conjugation with Human Serum Albumin (Antigen Preparation)—To 5 μCi of ^{3}H-reserpine in 2 ml of ethanol was added 150 mg of reserpine. After solvent removal *in vacuo*, the solid was dissolved in 3.6 ml of acetic acid and added to 400 mg of human serum albumin in 4 ml of distilled water. To the resulting clear solution was added 2.4 ml of 38% formaldehyde, and the mixture was warmed at 50° under nitrogen for 1 hr. Solvents were removed *in vacuo* at room temperature (10 hr), and the residue was slurried in 10 ml of 5% sodium bicarbonate, collected by centrifugation, washed several times with 50% ethanol, and dried.

The solid product was thoroughly washed with chloroform–methanol (1:1) until no further reserpine appeared in the washings as determined by counting tracer radioactivity. Digestion of 2-mg samples of the 350 mg of conjugate obtained was carried out on a steam bath in a mixture of 10 drops of 30% hydrogen peroxide and 30 drops of 70% perchloric acid, and the digest was counted in a scintillation cocktail. Two separate determinations indicated 4.1 and 4.0 moles of reserpine/mole of human serum albumin (based on mol. wt. 70,000).

Immunization and Titer Determination—Two sheep were injected intramuscularly at several sites at monthly intervals with a solution of 3 mg of conjugate in 1.5 ml of physiological saline emulsified with 1.5 ml of Freund's complete adjuvant. Bleedings were made from the neck vein ~7–10 days after each booster injection. Serum, prepared by the usual procedures, was tested for reserpine-binding ability using 10 or 2 ng of ^{3}H-reserpine, according to the procedure described later.

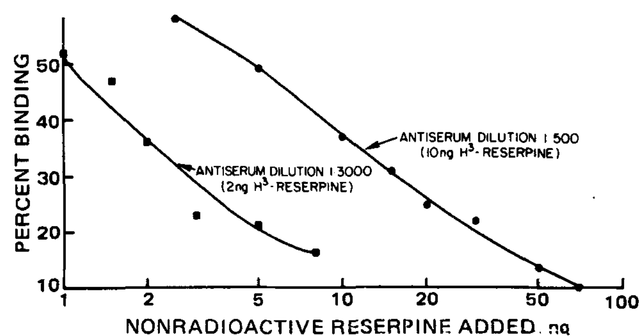


Figure 1—*Standard reserpine radioimmunoassay curve.*

Both sheep responded similarly to the conjugate, producing antiserum of usable titer (*i.e.*, 1:500–700 with 10 ng of ^{3}H-reserpine and 1:3000 with 2 ng of ^{3}H-reserpine) after 2–3 months. Bleedings taken subsequently up to 18 months maintained approximately equivalent titers and specificity. In experiments described later, serum from one sheep (LT 7-8-76) was employed.

Standard Immunoassay Curve and Specificity Studies—Table I indicates the contents of the assay tubes utilized for the standard immunoassay curve. Bovine serum albumin (0.5%) was added to the buffer (physiological saline–phosphate buffer, pH 7.4) to minimize adsorption of ^{3}H-reserpine and cold reserpine onto vessel walls. Antiserum was freshly diluted from stock on the day of use. Following antiserum addition, varying quantities of the cold reserpine, metabolite, or other reserpine analog were added. Finally, 10 ng of ^{3}H-reserpine (~5000 dpm) was added to each tube.

Tubes were incubated at 37° for 2 hr and chilled in ice for 30 min, and 0.5 ml of dextran-coated charcoal suspension (Table I, footnote *f*) was added to desired tubes. After vortexing, suspensions were left at 0° for exactly 15 min and then centrifuged at 2° for 10 min. Exactly 1.0 ml of the supernate was removed carefully by automatic pipet and counted in 10 ml of scintillation cocktail in a counting vial. Percent binding figures were calculated. Nonspecific binding (~5%) was subtracted from the total percentage figures observed. The entire procedure was essentially identical to that described previously for methotrexate (5).

Typical standard curves obtained for two different antiserum dilutions are shown in Fig. 1. Curves apparently were reproducible in initial studies and were not shifted significantly by the addition of up to 0.1 ml (10% of the total tube contents) of human serum, plasma, cerebrospinal fluid, or urine to the assay medium. Curve fitting (linearization) was also carried out using logit transformation procedures (6), particularly for cross-reactivity (specificity) studies.

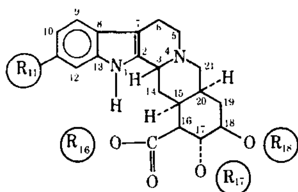
RESULTS AND DISCUSSION

Immunogen and Antiserum Preparation—By employing a modification of a literature procedure (7), reserpine was coupled through the indole nitrogen *via* formaldehyde treatment in acetic acid to lysine groupings of human serum albumin. The inclusion of tracer quantities of ^{3}H-reserpine enabled estimation of the conjugate reserpine content at 4 moles/mole of human serum albumin. This level compares closely to the "Mannich-type" conjugation carried out in dimethyl sulfoxide (using bovine serum albumin) by Levy *et al.* (1), which gave a hapten–carrier ratio of about 5. The coupling site at the indole nitrogen of reserpine appears to be ideal since major metabolic (hydrolytic) transformation sites, the trimethoxybenzoyl group at position 18 and the methyl ester at position 16 (Table II), remain unmasked as potential immunodeterminant groups, and the resultant antibodies should be able to distinguish reserpine from its major metabolic products reserpic acid, methylreserpate, and trimethoxybenzoic acid (8).

Each of two sheep responded similarly to initial antigen injections and monthly boosters. After 3 months, antiserum of usable titer (1:500–700 as determined using 10 ng of ^{3}H-reserpine and 1:3000 using 2 ng of ^{3}H-reserpine) resulted; these titers remained essentially constant over the 15 additional months during which the boosters were maintained. In

[1] Calbiochem Corp., La Jolla, Calif.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Difco Laboratories, Detroit, Mich.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] Radioimmunoassay grade materials, Schwarz/Mann, Orangeburg, N.Y.
[6] Scintaverse, Fisher Chemical Co., Raleigh, N.C.
[7] Gift of Abbott Laboratories, North Chicago, Ill.
[8] Gift of Ciba-Geigy Corp., Summit, N.J.
[9] New England Nuclear Corp., Boston, Mass. (specific activity 157 mCi/mmole, ^{3}H-trimethoxybenzoyl ring).
[10] Model 3255, Packard Instrument Co., Downers Grove, Ill.

Table II—Structures of Reserpine and Related Compounds



Compound	R_{11}	R_{16}	R_{17}	R_{18}
Reserpine	CH_3O	CH_3	CH_3	3′,4′,5′-Trimethoxybenzoyl
Deserpidine	H	CH_3	CH_3	3′,4′,5′-Trimethoxybenzoyl
Rescinnamine	CH_3O	CH_3	CH_3	3′,4′,5′-Trimethoxycinnamoyl
Syrosingopine	CH_3O	CH_3	CH_3	3′,5′-Dimethoxy-4′-ethylcarbonatobenzoyl
Methylreserpate	CH_3O	CH_3	CH_3	H
Reserpic acid	CH_3O	H	CH_3	H

comparison with these results, Levy *et al.* (1) reported a maximal titer of only 1:100 (1 ng of ^{3}H-reserpine) with their Mannich-type immunogen and eventually based their immunoassay on higher titer antiserum that originated from a "diazo-type" conjugate coupled in the indole benzene ring.

Standard Immunoassay Curve—With the components described in Table I and the conditions discussed under *Experimental,* typical standard immunoassay curves (Fig. 1) were obtained. Points were usually determined in triplicate, with a coefficient of variation of <8% in all cases when 10 ng of ^{3}H-reserpine was used. The position and shape of the curve were not influenced appreciably by the addition of up to 10% (0.1 ml) of human plasma, serum, or cerebrospinal fluid. With 2 ng of ^{3}H-reserpine, increased error was observed (Fig. 1).

Because of interest in obtaining radiolabeled material of higher specific activity (producing increased sensitivity), exhaustive work has not been carried out to determine optimum conditions for the assay with the available tritiated reserpine of low specific activity. The obvious deficiency of the system employing commercially available ^{3}H-reserpine is sensitivity insufficient to reach levels normally found (estimated by gross radioactivity measurements) in human subjects. These levels are 0.2–2.0 ng/ml according to one study after a single oral 0.25-mg dose (9). Levy *et al.* (1), using ^{3}H-reserpine of specific activity 1.7 Ci/mmole (of limited stability but ~10 times that available commercially), apparently encountered similar difficulties, with sensitivity limited to ~15 ng/ml of plasma, unless Tripp's extraction procedure was employed for primary reserpine concentration in the sample. In this laboratory, iodination procedures with iodine 125 have been unsuccessful, and similar difficulties were reported by Levy *et al.* (1).

Cross-Reactivity Studies (Specificity Determinations)—The major reserpine metabolites as well as several semisynthetic analogs were studied for their ability to displace ^{3}H-reserpine from antibody binding sites under the conditions outlined earlier. Metabolites were reserpic acid, methylreserpate, and 3,4,5-trimethoxybenzoic acid; commercially available synthetic analogs were rescinnamine, syrosingopine, and deserpidine (Table II). Results (Table III) are expressed as the ratio of the amount of compound to the amount of reserpine producing 50% binding inhibition (also expressed as percent cross-reactivity, derived from the reciprocal of the ratio in question). These figures were obtained *via* logit transformation linearization of sigmoid curves such as those shown in Fig. 1 (6).

The results in Table III indicate that major reserpine metabolites in humans, specifically reserpic acid, methylreserpate, and 3,4,5-trimethoxybenzoic acid, do not cross-react to any significant extent (<0.6%) with reserpine antibodies. Similar results were obtained by Levy *et al.* (1) using both their Mannich-type immunogen and a preferable "higher titer" antiserum involving coupling of bovine serum albumin through an azobenzoyl linkage adjacent to the 11-methoxy substituent of reserpine. Such results with all three conjugates are expected, because coupling sites are remote from the metabolism sites at the 16- and 18-positions; *i.e.,* these groups are left free as immunodeterminants in the hapten.

Table III—Cross-Reactivity of Reserpine Analogs and Metabolites in Reserpine Radioimmunoassay

Compound	Ratio of Amount of Compound to Amount of Reserpine Producing 50% Binding Inhibition: This Study	"Mannich" Immunogen (Ref. 1)	"Diazo-Coupled" Immunogen (Ref. 1)
Reserpine	1.0 (100)[a]	1.0 (100)	1.0 (100)
Reserpic acid	167 (0.6)	>2200 (<0.1)	>2000 (<0.1)
Methylreserpate	167 (0.6)	85 (1.2)	90 (1.1)
Serotonin	—	>2500 (<0.1)	>2500 (<0.1)
3,4,5-Trimethoxybenzoic acid	≫670 (<0.2)	>2300 (<0.1)	>2000 (<0.1)
Rescinnamine	38 (2.6)	5 (20)	130 (0.8)
Syrosingopine	≫670 (<0.2)	—	—
Deserpidine	1.33 (75)	—	—

[a] Figures in parentheses express percent cross-reactivity.

Of particular interest is the observation that deserpidine, which differs from reserpine only in the absence of an 11-methoxy grouping, was bound to antibody prepared in this work nearly as well as reserpine (75% cross-reactivity), indicating that the 11-position on the aromatic ring, which is rather close to the N-1 indole nitrogen atom, the point of coupling, is thus hindered as an immunodeterminant group in antibody formation. This observed cross-reactivity is of special interest as a potential basis for a deserpidine immunoassay. Little cross-reactivity was observed between antiserum and the two additional synthetic reserpine analogs, syrosingopine (0.2%) and rescinnamine (2.6%), although the latter was bound significantly to the Roche antibody ("Mannich immunogen," 20% cross-reactivity).

Such specificity studies, especially if combined with a stable tritiated or iodinated reserpine derivative (or one of the other drugs mentioned), could contribute valuable methodology for bioavailability, bioequivalence, and metabolism studies of reserpine or its analogs.

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