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COMPARATIVE IMMUNO-REMOVAL: A NOVEL METHOD FOR ESTIMATING THE CONCENTRATION OF AN UNKNOWN IMMUNOREACTIVE COMPOUND

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We describe a method for estimating the molar concentration of an unknown immunoreactive compound. The amount of antibody required to bind half of a standard is compared to the amount required to bind half of an equal number of immunoreactive equivalents of the unknown. We demonstrate the utility of the method using a morphine antibody affinity resin and compounds structurally related to morphine.

Antibodies raised against biologically important compounds often recognize substances with structures related to the immunogen. When such a compound is detected by radioimmunoassay (RIA), the amount is expressed as immunoreactive equivalents of the RIA standard, but the absolute amount is unknown. The unknown compound may crossreact poorly and thus be present in higher concentration than the immunoreactive equivalency indicates, or it may have higher affinity than the standard and thus be present in lower concentration.

We present here a novel procedure for determining the immuno-crossreactivity of an unknown compound relative to a standard. The molar concentration of unknown can then be calculated. We illustrate with a morphine immunoaffinity resin and compounds related to morphine, since the method was useful to us in purifying and identifying opiates from bovine tissue (1). The method has general applicability under specified conditions.

Theory of Comparative Immuno-Removal

The comparative immuno-removal method is based on the fact that the ratio of the concentration of antibody required to bind one-half of

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Abbreviations: RIA, radioimmunoassay; HPLC, high-performance liquid chromatography.

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immunoreactive equivalents of a standard and an unknown is equal to the ratio of their equilibrium dissociation constants. The initial step in the method is to test dilutions of a solution of unknown by standard RIA. The IC_{50} is the dilution of unknown that reduces bound tracer by 50%. The procedure is identical to obtaining the immuno-crossreactivity of known ligands relative to a standard, the ratio of IC_{50} values being equivalent to the ratio of affinities. However, in this case, the concentration of unknown can not be expressed in molar units but only as immunoreactive equivalents of the standard.

Next, the standard and unknown are used at the same multiple of their IC_{50} (i.e., the same number of immuno-equivalents) and the concentration of antibody required to bind one-half of each ligand is determined. Binding to the antibody is monitored by RIA of the ligand left free in solution after removal of antibody. The use of an immuno-affinity resin facilitates the separation of unoccupied and ligand-bound antibody from free ligand. The antibody conjugated to resin is the same as that used in the RIA.

Let L be free ligand, L_T be total ligand, R be free antibody, R_T be total antibody, LR be ligand-antibody complex, K be ligand-antibody dissociation constant. At equilibrium,

$$K = (L)(R)/(LR) \qquad \text{Equation 1}$$

Let subscript s denote a standard, subscript x the unknown. The amount of antibody that binds one half of standard and unknown are R_{Ts} and R_{Tx} respectively.

At 50% binding of ligand in the immuno-removal step, L=LR, and Equation 1 becomes $K = R$.

Since $R = R_T - LR$, $K = R_T - L$.

Since $L = (0.5)(L_T)$,

$$K_s/K_x = [R_{Ts} - (0.5)(L_{Ts})]/[R_{Tx} - (0.5)(L_{Tx})].$$

In RIA, K is directly proportional to IC_{50} as can be seen from the Cheng-Prusoff equation (2) describing competition:

$$K = [(K^* + L^*) / K^*] IC_{50}$$

where L^* is the concentration of tracer in the RIA and K^* is the equilibrium dissociation constant for tracer antibody interaction.

In the immuno-removal step, standard and unknown are used at the same multiple of their IC_{50}, therefore both ligands are at the same constant (M) times their respective K values. Thus,

$$L_{Ts} = (M)(K_s), \quad L_{Tx} = (M)(K_x)$$

$$K_s/K_x = [R_{Ts} - (0.5)(M)(K_s)]/[R_{Tx} - (0.5)(M)(K_x)].$$

Therefore,

$$R_{Ts}/R_{Tx} = K_s/K_x = L_{Ts}/L_{Tx}.$$

Thus, the ratio of total antibody concentrations required to bind 50% of the unknown and standard gives the immuno-crossreactivity (K_s/K_x). Since the concentration of standard (L_{Ts}) is known, the molar concentration of unknown (L_{Tx}) can be calculated.

The comparative immuno-removal method is based on the assumption that in both the RIA and immuno-removal steps, ligand binding to antibody follows the mass-law equations given above, which specify <u>free</u> ligand concentrations. Thus, by definition, immunoreactive equivalents of two compounds are the free concentrations that produce 50% reduction of tracer binding in the RIA. However, in most cases, only total concentrations are known; it is virtually impossible to measure free concentrations since this would require an independent analytical method with a sensitivity greater than RIA. When the free and total concentration of ligand differ significantly and immunoreactive equivalents of standard and unknown are based on total concentrations, the result will be an incorrect estimation of K_s/K_x and L_{Tx} in the immuno-removal step.

What are the conditions under which the comparative immuno-removal method gives an acceptable result when immuno-equivalents are based on the

total concentration of standard and the dilution of unknown that reduce the tracer binding by half in the RIA? To address this question we constructed a computer simulation based on the mass-law. We assumed that the tracer and the standard have the same affinity, that the tracer is used at a concentration 1/100 of its equilibrium dissociation constant, and that 30% of tracer is bound in the absence of standard or unknown. These conditions were chosen to be typical of RIA (3). Usually, high specific activity radioiodinated tracers are used at concentrations lower than their dissociation constants; and the tracer is the radiolabeled form of the standard and does not differ significantly in its affinity from the standard. We computed, for standard and comparison ligands of various affinities, the concentrations that reduce tracer binding by one-half. We then simulated the immunoremoval step using these computed total concentrations (L_T) of standard and comparison ligand.

The simulation of the immuno-removal step is based on the mass-law equation describing equilibrium:

$$LR = [(L)(R_T)]/[(L)+(K_L)]$$

Substituting $LR = L_T - L$, the following quadratic equation results.

$$L^2 - (L_T - K_L - R_T)(L) - (L_T)(K_L) = 0$$

For a given K_L and L_T, we computed by an iterative process the value of R_T that would result in L being one-half of L_T. We then computed the ratio of R_T values for a comparison ligand and a standard. The error ratio is a ratio in which the numerator is the ratio of the R_T values thus computed and the denominator is the true ratio of the dissociation constants.

The results of this simulation are shown in Figure 1. It can be seen that the comparative immuno-removal method works well for comparison ligands with dissociation constants greater than approximately one-tenth that of the standard. Serious error can occur for comparison high-affinity ligands with dissociation constants less than one tenth that of the standard. Additional simulations under different conditions of the RIA

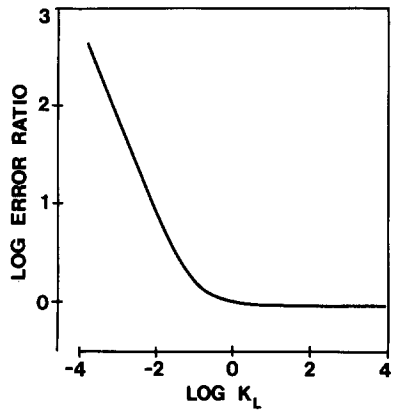

Fig. 1. Computation of error in the comparative immuno-removal method for ligands with various affinities. For details of simulation, see text. It was assumed that: K for standard equals 1; the tracer in the RIA step had the same affinity as the standard; tracer binding was 30% in the absence of competing ligand; and the concentration of tracer was 1/100 of its own dissociation constant. The error ratio compares the ratio of the dissociation constants for comparison ligand and standard determined by the immuno-removal method (i.e., from the R_T ratios) and the true ratio of their dissociation constants.

(i.e., higher or lower tracer binding or tracer concentration) gave qualitatively similar results, i.e., the affinity estimates for compounds with dissociation constants higher than the standard are essentially correct but affinity estimates for compounds with dissociation constants lower then the standard can only be regarded as minimal estimates.

MATERIALS AND METHODS

Antiserum 937 was raised against morphine conjugated to bovine serum albumin through an ethyleneamine linkage at position 3. Its properties in RIA, and the preparation of an immunoaffinity resin, have been described (1). For the immuno-removal step, 50 pmol of immunoreactive morphine equivalents (as determined by RIA) were placed in each microfuge tube. One ml of 150 mM sodium phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin, 0.1% Triton X-100, and varying amounts of immunoaffinity resin were added. After shaking overnight at 4°C, the tubes were centrifuged, and the supernatant was assayed by RIA. The amount of resin required to bind half of the immunoreactivity was determined by interpolation after conversion of the data to semi-logarithmic form (see Figure 2).

Morphine sulfate was from S.S. Penick Co. (Lyndhurst, NJ); codeine phosphate was from Burroughs Wellcome (Research Triangle Park, NC); thebaine was a gift from E. Brochmann-Hanssen. The purity of each compound was verified by reverse-phase HPLC.

RESULTS AND DISCUSSION

The results of immuno-removal of morphine, codeine and thebaine are shown in Fig. 2. Table 1 gives the resulting crossreactivity computations,

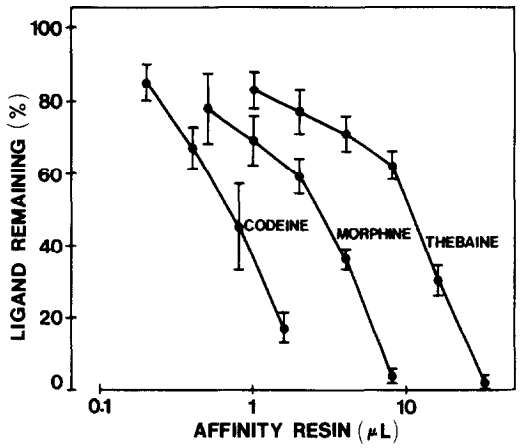

Fig. 2. Immuno-removal of morphine, codeine and thebaine by an antibody affinity resin. Titration was carried out as described under Materials and Methods. The molar amounts of each ligand are given in Table 1. The antibody resin was prepared as described. One ml of packed hydrated resin could completely bind 5 nmol of morphine. Three separate experiments were done; in each experiment the ligand remaining in solution was determined by RIA at each concentration of resin. Each point represents the mean and standard error of the mean for the three separate experiments.

the concentration estimates, and the comparisons with true values. The results of the immuno-removal method are -- within the limits of experimental error -- the same as the true values. Thus, the method of comparative immuno-removal could have been used to determine how many moles of codeine or thebaine were present, had these been unknown ligands.

The method is premised upon a homogeneous population of antibody binding sites, and therefore it should be most suitable with monoclonal antibodies.

Table 1. Estimation of the immuno-crossreactivity and concentration of codeine and thebaine using the comparative immuno-removal method

	morphine	codeine	thebaine
Actual Amount Added	50 pmol	21 pmol	190 pmol
Cross-reactivity by RIA*	100%	240%	27%
Amount of Resin for 50% Removal	2.6 μl	0.70 μl	11 μl
Estimated Cross-reactivity	100%	370%	24%
Estimated Amount Added	--	15 pmol	210 pmol

*From Goldstein et al. (1).

With polyclonal antibodies (as in our experiments), the result may be only approximate, since the antibody population primarily active in the RIA is not necessarily identical to that in the immuno-removal step. Moreover, as pointed out earlier, the method gives only minimal estimates of the affinity of unknown compounds with affinities much greater than the standard. Fortunately, it is uncommon for compounds to have greater affinity than the standard used in RIA; it can occur, however, as we have demonstrated for codeine. The minimal estimate of the affinity provided by the comparative immuno-removal method may be useful when it indicates that the unknown has higher affinity than the standard. Therefore, despite its limitation under some conditions, the method has general utility for estimating the concentration of an unknown immunoreactive compound.

The crossreactivity and molar estimates provided by the comparative immuno-removal method can be instructive when one is confronted with an unknown immunoreactive compound. Estimation of the actual amount of such a compound in an impure preparation is useful for determining the required scale of a purification effort. Knowledge of the crossreactivity can also prove that the unknown is a different compound from the standard if the crossreactivity is not 100%. Finally, if enough is known about the structural requirements for antibody recognition, a crossreactivity estimate for an unknown may provide structure information.

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