duct formed after acid deamination of the amine) were unsuccessful.

If the antipodal potency ratios for the (+)- and (—)-esters (Ia) reflect receptor events (distribution factors have been shown to have only a minor influence on the potencies of the closely related methadone enantiomers) (Sullivan et al 1975), then the low values observed together with the reversal of receptor stereoselectivity relative to that shown towards methadone and many of its relatives (Casy 1973) provide further evidence of the conformational mobility of the methadone class of analgesic, a feature recently stressed by Henkel et al (1974, 1976).

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High sensitivity solid-phase exchange radioimmunoassay for morphine

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In recent studies (Fishman & Fishman 1974; Castaneda & Liao 1975) immobilized antibodies have been used as a probe in determining the quantitative and qualitative characteristics of steroid hormone binders which compete with the antibody for the same ligand. We felt that such methodology could also be applied to the study of the opiate receptor, and to that end we have raised and immobilized a morphine antibody. In the course of testing the immobilized system it was applied to the solid phase radioimmunoassay RIA of morphine and related opiates. A note by Steiner & Spratt (1978) prompts us to report the results obtained by us with our system insofar as RIA of morphine is concerned. In these applications the methodology we report exhibits several distinct advantages.

The first use of radioimmunoassay (RIA) for the assessment of a drug of abuse (morphine) concentration in body fluids was by Spector & Parker in 1970. Since then radioimmunoassays have been developed for methadone (Bartos et al 1977), barbituates (Spector & Flynn 1971), amphetamines (Cheng et al 1973), and other abused drugs (Castro & Malkus 1977). Other techniques which include haemoagglutination inhibition assays (Adler & Liu 1971), spin label assays (Leute et al 1972), and enzyme linked immunoassays (Rubenstein et al 1972) have also been developed for the measurement of morphine and its congeners.

The use of immobilized antibodies in other radioimmunoassays, has previously been reported (Catt et al 1968; Goldstein et al 1972; Castaneda & Liao 1975). One of the main advantages in using this technique is to eliminate the troublesome step of separating the complexed from the uncomplexed ligands since no precipitant or absorbant need be added after the initial binding of ligand.

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Antiserum preparation. Morphine-6-succinylbovine serum albumin (M-6HS-BSA) was prepared by modification of published procedures. Morphine (free base) was heated under reflux with succinic anhydride (Simon et al 1972) to give the morphine 6-hemisuccinate which after purification was conjugated with BSA using the mixed anhydride procedure described by Wainer et al (1972). The antigen which contained 9·2 mol ligand per mol was dissolved in phosphate buffered saline and emulsified with an equal volume of Freund's complete adjuvant to provide a concentration of 0·5 mg ml^{-1}. New Zealand white rabbits were injected with 1·6 ml of this suspension, 0·4 ml being injected into each footpad. Booster injections were administered three to five weeks later, and the rabbits were bled at seven day intervals. Aliquots of the serum obtained from the bleeding were stored and frozen. Hapten binding capacity of the antiserum samples at different dilutions was determined by incubation with [6-^{3}H]morphine (New England Nuclear) at 60 °C for 10 min and then overnight at 4 °C. The charcoal absorption technique (Odell et al 1975) was then used to separate complexed from free ligand. The charcoal was sedimented by centrifugation, and the supernatant was decanted and counted in a Packard liquid scintillation counter.

Immobilized antibody (IAb). Strips of water swollen unsintered polyvinylidene fluoride film (Roche Diagnostics, Nutley, N.J.), 0·6 × 3·0 cm were each submerged in 1·0 ml of antiserum solution (1 : 1000 dilution), and

the system was stored at ambient temperature for 16 h.

The antibody impregnated strips were washed several times with water, air-dried and stored at room temperature (20 °C). Check experiments conducted over three months showed that the activity of the immobilized antibody stored in water did not change significantly. This very firm binding of proteins to polyvinylidene fluoride is believed to comprise physical absorption aided by the entanglement of the protein in the strands of the polymer (Fishman & Fishman, Analytical Biochem., in press).

The immobilized antiserum was tested for RIA capability by two different procedures:

(1) Standard competition method: IAb strips are incubated at ambient temperature for a minimum of 12 h in 1·0 ml of [^{3}H]morphine solutions (0·01 M phosphate buffer, pH 7·4) with concentrations in the range of 10^{-12} to 10^{-9} M. The IAb strips are removed and aliquots (0·8 ml) of each of the morphine solutions before (input) and after incubation with IAb are mixed with 14 ml scintillant (Aquasol-2, New England Nuclear) and counted in a Packard liquid scintillation spectrometer. The amount of radiolabelled morphine bound to the antibody is calculated by subtracting from the input the amount of free [6-^{3}H]morphine present in the aliquot after incubation. Incremental additions of unlabelled morphine to the test solution progressively inhibit the binding of a standard amount of radiolabelled morphine, thereby producing a standard curve.

The effect of antiserum dilutions used in preparing IAb on the binding of morphine by IAb is shown in Fig. 1A. Dilutions of raw antiserum were made non-

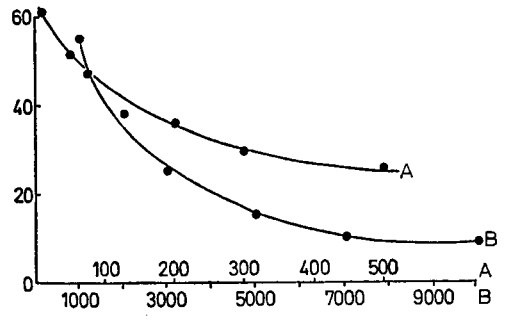

FIG. 1. A. Morphine binding by immobilized antiserum. Each polyvinylidene fluoride strip was treated with 1·0 ml of antiserum of given dilution. Each strip with immobilized antiserum was tested by incubation with 1·0 ml of [6-^{3}H]morphine containing 140 fmol of the labelled drug. Each point was determined in triplicate and average values are reported. Ordinate: % bound. Abscissae: (upper) pg morphine; (lower) antiserum dilution. B. Morphine standard curve using immobilized antibody. Each polyvinylidene fluoride strip was treated with 1·0 ml of antiserum of 1 : 1000 dilution.

sequentially from an initial 1 : 1000 dilution. The Fig. shows that approximately 70 fmol of morphine are bound, an amount sufficient to assay with sensitivity up to 500 pg of morphine. This contrasts with the report of Steiner & Spratt (1978) in which no appreciable binding of radiolabel to an unpurified immobilized antibody was obtained, and where purification of the antibody before immobilization was required to obtain effective binding.

Fig. 1B shows the morphine standard curve using IAb strips prepared from a 1 : 1000 dilution of the antiserum. Each point represents the average of a triplicate determination with the standard deviation being 3%. The sensitivity of the assay allowed for the detection of as little as 20 pg morphine. Although this technique produced satisfactory results, we wanted to avoid any possibility that on initial contact of IAb with morphine solution some loosely held antibody, a small fraction of the total, may be drawn into solution where it would compete for the drug with the immobilized antibody and thereby diminish the sensitivity of the assay. We therefore developed the alternate exchange procedure where this possibility is avoided.

(2) Exchange method: this procedure comprises two stages. IAb strips are first incubated with shaking at ambient temperature with 1·0 ml of [6-^{3}H]morphine solution for 2 h. The strips are then removed and placed in 1·0 ml solutions (phosphate buffer) containing morphine in concentrations ranging from 10^{-12} to 10^{-9} M.

An IAb strip preincubated in [6-^{3}H]-morphine and then placed in morphine-free solution serves as a blank. The exchange method is time sensitive and a few preliminary release experiments are made to obtain information on the incubation time needed to produce a curve of required sensitivity. In this particular assay the incubations are allowed to proceed for 45 min with shaking at ambient temperature after which time the strips are removed and aliquots (0·5 ml) of the solution are counted as described above.

The uptake of radiolabelled morphine by the IAb is calculated by subtracting the amount of labelled morphine remaining after incubation with the strip from the input of labelled morphine. The release of radiolabelled drug into solution is determined directly by counting a sample from each of the test solutions. The relation of unlabelled drug in solution to the amount of radiolabel released constitutes a calibration curve for the assay. The transfer of physically absorbed labelled morphine is less than 0·8% of drug in solution. This was determined by immersing the polymer strips free of antibody in labelled morphine solution for 1 h, draining them briefly along the walls of the tube, transferring the strips to a buffer solution and counting the buffer solution.

In Fig. 2 the calibration curve for the exchange method using IAb strips prepared as described in the methods section is presented. The slope of the curve is

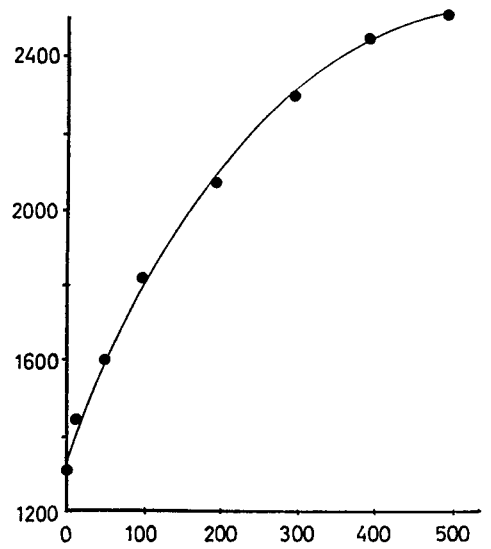

FIG. 2. Morphine calibration curve by the exchange method using immobilized antibody. 1/1000 dilution of Ab. Morphine 5353 counts min^{-1} were bound by the antibody. Incubation was carried out at room temperature for 45 min with agitation. Ordinate: counts min^{-1} released into solution. Abscissa: pg morphine.

much steeper than that of the standard curve in Fig. 1B providing for a greater sensitivity in the assay. A calibration curve of comparable sensitivity was obtained in the presence of 0·1 ml rat plasma or human urine indicating that the method is suitable to assay for morphine in body fluids. Apart from the possibility noted above, the reason for the greater sensitivity in the exchange procedure is due to it being a dynamic method in which measurements are not made at equilibrium. In this method the assay depends on the rate of exchange as a function of the concentration of unlabelled drug in solution. An important additional advantage of this method is the speed with which the assay can be carried out.

Table 1. Concentration necessary to inhibit [6-^{3}H]-morphine binding by 50%.

Compound	ID50 (pmol ml^{-1})	% cross reactivity
Morphine	0·438	100
Normorphine	6·68	6·5
Codeine	0·830	53
Levorphanol	0·952	46
Dextrorphan	>1000	<0·1

Each IAb strip was tested by incubation with 1·0 ml solution containing 0·14 pmol ml^{-1} [6-^{3}H]morphine, using the standard competition method.

The cross reactivity of several morphine related structures with the anti-serum is presented in Table 1. Normorphine (morphine without *N*-methyl) is fifteen fold less cross reactive than the parent compound. Codeine, the 3-methoxy derivative of morphine cross reacts extensively, a fact already established for antibodies raised with this antigen (Wainer et al 1973). The high stereospecificity of the antibody is apparent from the difference in binding of levorphanol and its enantiomer dextrorphan. The lack of cross reactivity of IAb with dextrorphan has already proved useful in preliminary studies of opiate receptor measurements.

The immobilized antibody technique reported here, therefore, exhibits a number of distinct advantages over other opiate measuring methods. Its simplicity and sensitivity makes it exceptionally useful in determining morphine concentrations in biological fluids and tissues, and a number of such studies are in progress.

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