(*Reprinted from Nature New Biology, Vol. 236, No. 64, pp. 93–94, March 22, 1972*)

# Spin Immunoassay Technique for Determination of Morphine

GENERAL techniques available for the assay of discrete chemical entities in biological fluids are inadequate. An ideal assay technique should provide rapid and accurate molecular recognition (specificity), high sensitivity, simplicity in execution, and adaptability to large classes of compounds. This report describes a general immunoassay method (referred to as "FRAT$^{tm}$", free radical assay technique[1]) which has all these advantages without the radiation hazards and manipulative disadvantages of other immunoassay techniques.

The technique involves the spin labelling of macromolecules as introduced by McConnell[2]. The electron spin resonance (ESR) spectra of nitroxide radicals reveal anisotropy both of the hyperfine coupling with the nitrogen and of the $g$ value. When nitroxides tumble in solution at rates that are slow relative to their hyperfine frequencies ($\sim$40 MHz) their ESR spectra appear as broad envelopes of lines due to the summing of different signals from molecules in all possible orientations relative to the magnetic field. Rapid tumbling causes averaging of the field positions and gives rise to three sharp lines due to isotropic nitrogen hyperfine interactions. A relevant example of this motional effect is the ESR spectrum of the dinitrophenyl nitroxide I. Because it is small the molecule tumbles rapidly in solution and gives rise to three sharp ESR lines. But when it is

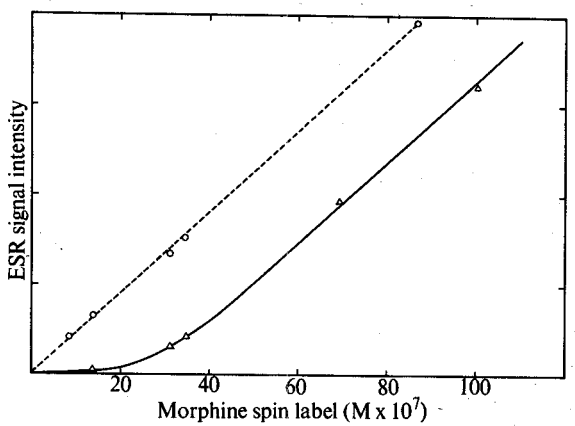
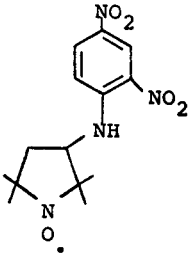
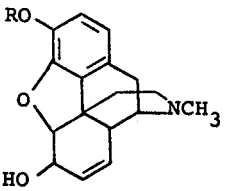



**Fig. 1** Signal intensity of low field ESR line as a function of morphine spin label concentration in the presence (——) and absence (– – –) of $\gamma$-globulin.

bound to large, slowly tumbling dinitrophenyl antibodies, a broadened ESR pattern characteristic of "immobilized" nitroxide radicals is observed[3].




I

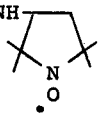



II

a R = H

b R = $CH_2CO$ BSA

c R = $CH_2CONH$




In our study rabbits were sensitized toward morphine IIa by injection with an emulsion of carboxymethylmorphine-BSA[4,5] conjugate IIb in complete Freund's adjuvant. The $\gamma$-globulin fraction of the serum was separated by ammonium sulphate precipitation and dialysis. Solutions with identical dilutions of $\gamma$-globulin were prepared with varying amounts of the spin labelled morphine analogue IIc. Fig. 1 shows plots of the intensity of the low field ESR lines of uncomplexed IIc against total IIc concentration with and without antibody. The horizontal displacement of the curve with antibody present corresponds, after correction for dilution, to a serum antibody binding site concentration of $2.4\times10^{-5}$ M. Because of the inhomogeneity of the antibody preparation, the association constant (based on equation (1)) varies with the ratio of spin label to antibody. The average association constant toward IIc determined where half the binding sites were occupied was $K=1.7\times10^{7}$ $M^{-1}$.

$$AB + IIc \rightleftharpoons Ab\text{–}IIc \quad (1)$$

$$Ab\text{–}IIc + IIa \rightleftharpoons A\text{–}IIa + IIc \quad (2)$$

Admixture of aqueous morphine solutions with a solution of $2.4\times10^{-6}$ M in antibody binding sites and $2.7\times10^{-6}$ M in spin label IIc produced increases in the ESR signal of unbound spin label due to competition for antibody sites (equation (2)). Fig. 2 compares the observed effect of morphine concentration on signal intensity (curve *A*) against that calculated (curve *B*)

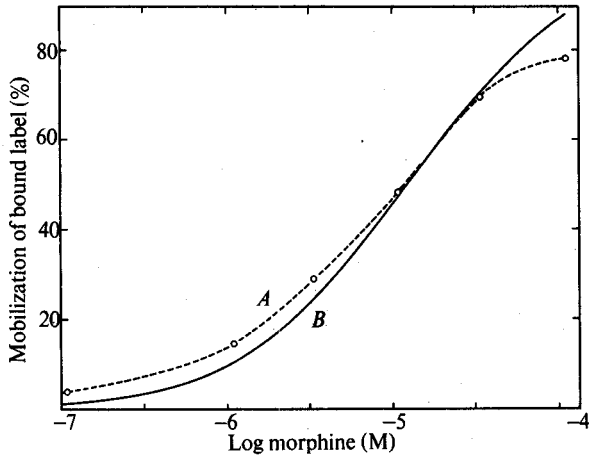



**Fig. 2** Percentage of unbound spin label as a function of morphine concentration. *A*, experimental; *B*, calculated.

with the assumption of a uniform association constant of $K_{morphine} = 3.1 \times 10^6\ M^{-1}$. The deviation from the calculated curve at high morphine concentration can be attributed partially to a small (~15%) antibody population with a very large preferential binding to the spin labelled morphine.

The data points in Fig. 2*A* were obtained allowing 25 min for equilibration at each concentration. Premixing of morphine and spin label before addition of antibody permitted more rapid equilibration. Reproducible data could also be obtained with 0.5 min equilibration without premixing although there were somewhat larger deviations from the calculated curve at high morphine concentrations. The latter conditions are ideal for rapid analysis of urine or saliva. A disposable glass capillary ESR cell can be used for this purpose, requiring only 20 $\mu$l. of test sample. The assay takes less than 1 min to complete.

The higher antibody affinity for IIc than for IIa implies that attachment to the phenolic oxygen of morphine may strengthen binding to the antibody. This is borne out by the observation of higher affinities for codeine (II, R–$CH_3$) and ethyl morphine ('Dionin') (II, R=$C_2H_5$). The morphine metabolite, morphine glucuronide, on the other hand, has somewhat weaker binding. Table 1 gives association constants for these and other compounds which demonstrate that only closely related morphine analogues can be detected even at the highest expected concentrations in body fluids. Of particular significance is that synthetic morphine substitutes such as methadone and propoxy-

phene ('Darvon') and unrelated drugs such as barbiturates and amphetamines are not recognized by the antibody.

**Table 1** Association Constants for Binding of Various Drugs by Morphine Antibodies

| | $K \times 10^{-6}$ ($M^{-1}$) |
|---|---|
| Morphine | 3.1 |
| Morphine[3]–O–glucuronide | 2.3 |
| Codeine | 29.0 |
| Hydrocodone | 19.0 |
| Ethyl morphine | 34.0 |
| Methadone | 0.00025 |
| Propoxyphene | 0.0021 |
| Amphetamine | 0.00044 |

This new technique provides a simple rapid test for heroin use by determination of the heroin metabolites, morphine and its glucuronide, in urine or saliva. The ability to distinguish morphine from methadone and unrelated commonly abused drugs suggests that the method should be well suited for use in heroin treatment programmes.

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Received August 23, 1971.

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Printed in Great Britain by Flarepath Printers Ltd., St. Albans, Herts.