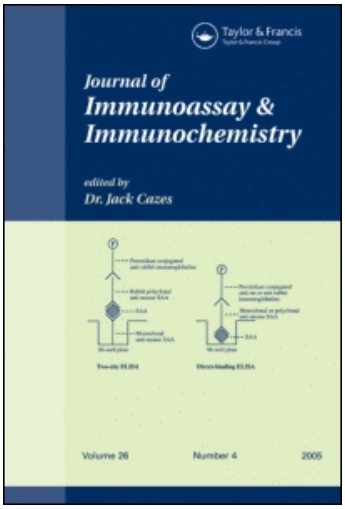

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A Simple Solid-Phase Radioimmunoassay for Morphine and Related Opiates Using A Mixture of Labelled Morphine and Antiserum Pre-Precipitated by Polyethylene Glycol

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A SIMPLE SOLID-PHASE RADIOIMMUNOASSAY FOR MORPHINE AND RELATED OPIATES USING A MIXTURE OF LABELLED MORPHINE AND ANTISERUM PRE-PRECIPITATED BY POLYETHYLENE GLYCOL

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ABSTRACT

A novel, solid-phase radioimmunoassay for morphine and related opiates is described in which radiolabelled morphine, antiserum and polyethylene glycol are premixed before being added to the samples or standards. The mixture can be stored at 2°C and used for several months until decay of the ^{125}I label leads to excessively long counting times. The method was developed using the Roche Abuscreen radioimmunoassay for morphine and has the particular advantages of simplicity (only 2 pipetting steps required instead of 5) and economy (800 assay tubes can be prepared from a 100-tube Abuscreen plus a 50% saving in operator time). The procedure can be applied to other assays providing the polyethylene glycol-precipitated antiserum is stable and that the antibody affinity for the labelled compound is not so high as to prevent displacement of the labelled compound from the antibody binding sites by unlabelled drug.

INTRODUCTION

A number of radioimmunoassay (RIA) methods have been developed for the analysis of morphine and closely-related opiates. Antisera raised against morphine conjugated to protein via the phenolic hydroxyl group (1-6) or via the alcoholic hydroxyl group (4, 6-10)

cross-react with a range of opiates and their metabolites, while conjugation via position 2 of the phenolic ring (11) or via the heterocyclic nitrogen atom (12-14) results in antisera capable of distinguishing between morphine and codeine. For forensic purposes, an RIA with a broad range of cross-reactivity is useful for determining frequently-used opiates or their synthetic analogues in biological fluids. Such an assay, the Roche Abuscreen for morphine, is commercially available and uses [^{125}I]morphine with an antiserum raised in goats against 3-O-carboxymethylmorphine conjugated to bovine serum albumin (15). However, Abuscreen kits are expensive (particularly in the U.K.) and the recommended protocol is laborious since it involves 5 pipetting steps. This paper describes a novel protocol that is convenient in practice, reduces the operator time by about 50% and enables 800 assay tubes to be prepared from a 100-tube Abuscreen kit. The protocol is not limited to the morphine Abuscreen but may be applied to various other assays.

MATERIALS AND METHODS

Abuscreen Radioimmunoassay Kits

These were purchased from Roche Products, Welwyn Garden City, Herts., UK.

Buffer

0.067M phosphate of pH 7.4 containing 0.1% sodium azide.

PEG Solution

23% w/v polyethylene glycol (PEG) of molecular weight 6000 in buffer.

Standard Solutions

Morphine sulphate is dissolved in buffer and diluted to give solutions containing 5, 10, 20, 40 and 80 ng/ml morphine base. The zero standard is buffer. The standard solutions are stored in silanized glass vials at $2^{o}C$. Other opiates and synthetic analogues are stored in aliquots of any convenient concentration at $-20^{o}C$ in silanized glass vials. For an assay, an aliquot is thawed and diluted with buffer to give the required range of standards.

Sample Preparation

Various dilutions of blood, urine and bile in buffer are assayed to ensure that at least one result lies on the standard curve. Suitable dilutions for blood are 1:2, 1:20 and 1:200. Higher dilutions are often required for urine and bile. The initial dilution of 1:2 merely conserves the sample.

Reagent Mixture

One volume each of $[^{125}I]$morphine solution and antiserum from a morphine Abuscreen kit are mixed with 2 volumes of buffer in a plastic container and incubated at $2^{o}C$ for several hours. 16 volumes

of PEG solution are then added slowly with stirring and the mixture is stored at $2^{o}C$. Before use, the mixture is stirred to re-suspend the precipitate.

Assay Protocol

25µl of standard or sample and 500µl of reagent mixture are added to duplicate sets of plastic microcentrifuge tubes. A repeating dispenser can be used to add the reagent mixture since the precipitate remains in suspension for at least 30 minutes without further stirring. The tubes are stoppered, vortexed thoroughly and incubated for 18-24 hours at room temperature with continual mixing by inversion. After centrifugation (2 minutes, 12,000g), the supernatants are aspirated and the precipitates are counted in a γ-counter. Two extra tubes containing only 500µl of the reagent mixture are counted to measure the total radioactivity per tube. The results are plotted on any convenient co-ordinates.

RESULTS AND DISCUSSION

The addition of PEG to precipitate the bound fraction after incubation in RIA was first described by Desbuquois and Aurbach (16) and has since been widely used. It is more convenient than the ammonium sulphate precipitation normally used with the Roche Abuscreens because the precipitates produced by PEG are compact and waxy after centrifugation. The supernatants can therefore be

aspirated easily and virtually quantitatively, allowing the activity in the precipitated bound fractions to be counted. The separation was found to be 98% efficient and so it is not necessary to wash the precipitates before counting. Ammonium sulphate produces bulky precipitates that are easily disturbed and so aliquots of the supernatants have to be pipetted into separate tubes for counting or else the supernatants can be partially aspirated and the precipitates counted after one or more washes with 50% saturated ammonium sulphate. However, these are laborious procedures compared with the simple aspiration required after PEG precipitation.

The assay described here incorporates the advantages of PEG precipitation and includes a novel simplification of the usual assay protocol in that the [^{125}I]morphine, antiserum and PEG are added to the assay tubes as a mixture rather than sequentially. The result is effectively a solid-phase assay in which the morphine equilibrates between the aqueous phase and the precipitated antibodies. The particular advantages of the method are economy and simplicity; 800 assay tubes can be prepared without difficulty from a 100-tube Abuscreen and only 2 additions to each assay tube are required which reduces the operator time by about 50%. Once prepared, the reagent mixture is stable and can be used for several months until the activity of the [^{125}I]morphine has decayed to the point where excessively long counting times are required. This procedure, in which a labelled compound, antiserum and PEG are stored as a

mixture, does not appear to have been used previously, but a computer search of the literature at the time of writing revealed that a similar approach has been developed for thyroxine RIA (17) in which a freeze-dried preparation of antiserum and PEG is reconstituted and mixed with labelled thyroxine immediately before use.

The concentration of PEG is the assay tubes is 17.5%. This was found to be the minimum required for maximum precipitation of the bound fraction and is higher than the concentration used by Desbuquois and Aurbach (16). The precipitate settles on standing and so continual mixing is required during incubation. 18-24 hours are required for equilibrium to be attained. Should a result be required urgently, the assay can be carried out in a more conventional manner by incubating the sample or standards with $[^{125}I]$morphine and antiserum for 30-60 minutes before adding PEG. The same relative proportions of reagents are used as in the "mixture protocol".

The assay has been in use for several years and no problems have arisen. Blood and urine have virtually no effect on the standard curve and so samples require no pre-treatment other than dilution. Haemolysis, decomposition, sodium fluoride or phenyl mercuric nitrate preservatives and potassium oxalate anticoagulant do not affect the results. The inter-assay coefficient of variation was 7.7% (n = 15) when blood "spiked" with 80ng/ml of morphine was assayed by a number of operators.

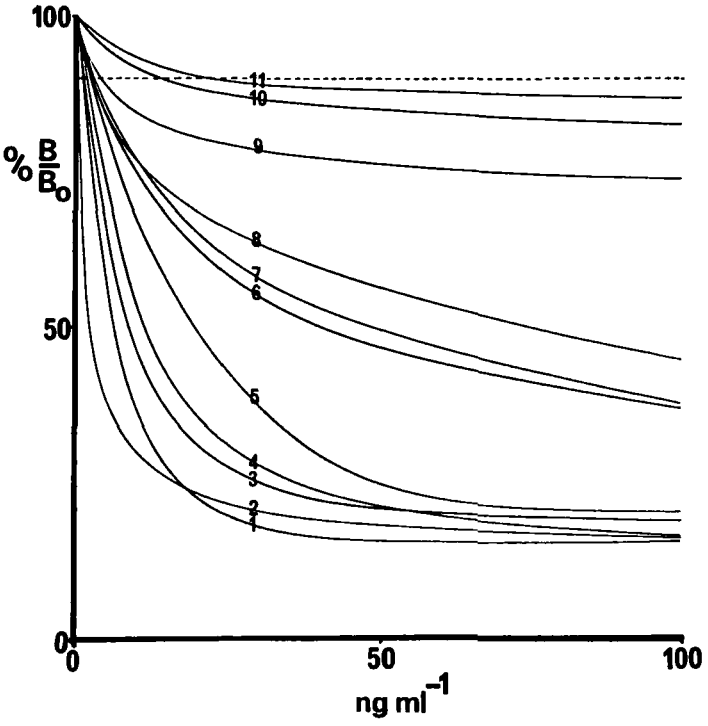

FIGURE 1. Standard curves of various morphinans. B = bound activity; B_0 = activity bound in absence of unlabelled drug.
1. Codeine. 2. Dihydrocodeine. 3. Morphine and morphine-3-glucuronide (as weight of morphine base). 4. Hydrocodone. 5. Morpholinylethylmorphine. 6. Dihydromorphine. 7. Methyldesorphine and hydromorphone. 8. Levomethorphan. 9. Codeine-6-glucuronide (as weight of codeine base). 10. Normorphine and levallorphan. 11. Oxycodone.
No cross-reaction with etorphine, morphine-N-oxide, naloxone.

Standard curves of a number of morphinans are shown in Fig. 1. The pattern of cross-reactivity shows no unexpected features and is determined primarily by the extent to which the 6-OH and N-methyl groups of the morphine molecule are modified. The increased cross-reactivity of codeine and dihydrocodeine compared with morphine

can be accounted for by the closer resemblance of codeine and dihydrocodeine to the conjugate used to raise the antiserum.

Blank blood samples at the initial dilution of 1:2 give a mean assay response of 98.3% B/B_0 with a standard deviation of 2.7% B/B_0. The horizontal line in Fig. 1 at 90% B/B_0 corresponds to the mean blank response plus three standard deviations and defines approximate "cut-off" values of less than 5ng/ml for the majority of the compounds examined after correction for the initial 1:2 dilution factor.

In theory, the principle of pre-mixing the labelled compound, antiserum and PEG before use in RIA should be broadly applicable but, in practice, not all assays give satisfactory results. For example, while the cocaine metabolite Abuscreen and a specific diazepam assay (18) both function as well as the morphine Abuscreen when adapted to a "mixure protocol", the barbiturate and phenyclidine Abuscreens failed to give standard curves and the methaqualone Abuscreen gave standard curves that became progressively shallower over a period of three weeks. The failure of the barbiturate and phencyclidine Abuscreens to give standard curves was due to lack of displacement of bound, labelled drug by unlabelled drug, presumably because the antisera had a higher affinity for the ^{125}I-labelled drugs than for the unlabelled drugs. The change in the methaqualone standard curves is less easily accounted for but may be

due to gradual deterioration of the precipitated antibodies in the reagent mixture. However, PEG can be used with the barbiturate, methaqualone, phencyclidine and also the amphetamine Abuscreens if the PEG precipitation is carried out after the incubation.

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