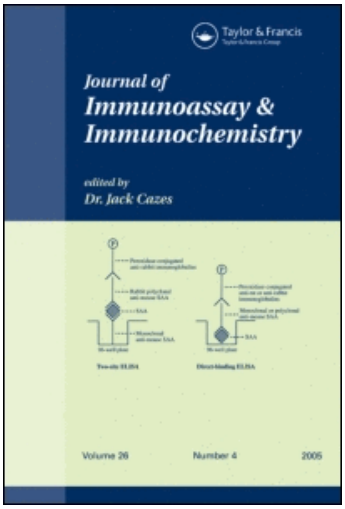

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DEVELOPMENT OF A SINGLE REAGENT POLARISATION FLUOROIMMUNOASSAY FOR THE DETECTION OF OPIATES IN URINE

The abbreviations used are: EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; TLC, thin layer chromatography; BSA, bovine serum albumin.

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ABSTRACT

The design, development, and optimisation of a simple polarisation fluoroimmunoassay to detect the opiate group of drugs, in urine, is described. Urine (10 µL) is added to 1.5 mL of a single reagent, prepared by mixing ovine anti-opiate serum with fluorescein-labelled morphine. After incubation for 30 min at 30°C, or 60 min at room temperature, fluorescence polarisation is measured. The assay can be used as a "stat" test or automated for large batch screening and detects the commonly abused opiates, heroin (through its metabolite morphine), codeine, and dihydrocodeine.

INTRODUCTION

Opiates are among the most commonly abused drugs and, because of the associated dangers, their detection is essential in any screening programme. Heroin, the most widely abused

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opiate, is rapidly and completely metabolised to morphine and excreted in the urine, primarily as the 3-glucuronide (1). Any assay for screening urines must, therefore, be able to detect morphine and its glucuronides.

Thin-layer chromatography (TLC) is commonly used for screening but requires a large volume of urine, is labour intensive, time consuming, and will not detect the major metabolite (morphine 3-glucuronide) unless a hydrolysis step is included. The need to screen increasingly large numbers of samples by simple techniques is being increasingly satisfied by commercial immunoassays such as Syva's (EMIT) enzymoimmunoassay system; Boehringer's haemagglutination inhibition test; Roche's Abuscreen; and more recently Abbott's TDx.

We describe the development of a simple immunoassay to detect most commonly abused opiates using fluorescence polarisation. The reagents could be adapted for use on the Abbott TDx but this instrument is complex and expensive. We have recently shown (2) that, provided the dissociation kinetics are relatively rapid, the assay can be simplified by equilibrating labelled analyte and antiserum to give a single reagent. Thus, the assay can be automated on simple inexpensive equipment. A small volume of urine is added to an aliquot of the single reagent followed by a short incubation and measurement of fluorescence polarisation.

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate isomer I, bovine serum albumin (BSA) (type A4503), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide, morphine-3-glucuronide, and gelatin were obtained from Sigma Chemical Co., Poole, Dorset, U.K.; chloroplatinic acid was from BDH, Poole, Dorset, U.K.; silica gel thin layer chromatography (TLC) plates for sample screening from Anachem, Luton, Bedfordshire, U.K.; and dihydrocodeine tartrate from MacFarlane Smith, Edinburgh, U.K. Morphine hydrochloride and codeine phosphate were obtained from the hospital pharmacy.

Assay tubes were 55 x 12 mm plastic disposable tubes (No. 55.484) from Sarstedt Ltd., Beumont Leys, Leicester, U.K.

Preparation of morphine 6-hemisuccinate

Morphine 6-hemisuccinate was prepared as described previously (3).

Preparation of morphine-6-hemisuccinate-BSA conjugate

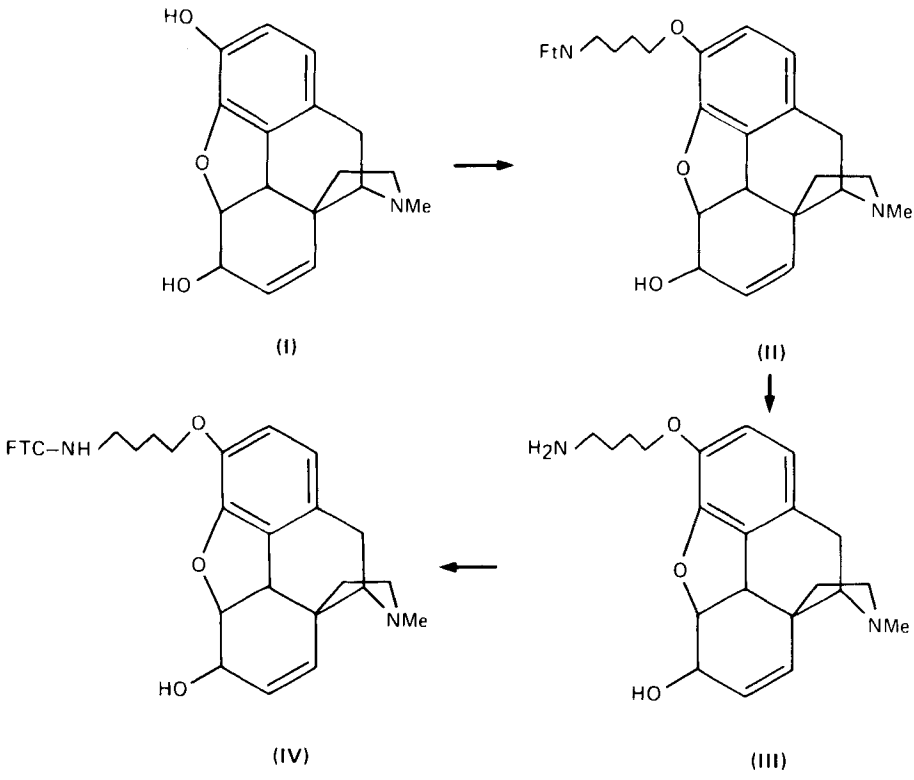
To a solution of morphine-6-hemisuccinate (12 mg) in pyridine/water (1:1 by volume) was added EDC (30 mg) and then, with stirring, a solution of BSA (30 mg) in water (1 mL). After 1 h at room temperature the solution was dialysed against three 1 L volumes of distilled water over three days. The retentate was lyophilised to yield 34.5 mg of immunogen.

Preparation of 3-O-(4-phthalimidobutyl)morphine (II) Figure 1)

To dry dioxane under nitrogen was added sodium hydride (75 mg, 60% in oil, 1.8 mmol) and morphine (500 mg, 1.7 mmol). The mixture was refluxed for 30 min, cooled to room temperature, and N-(4-bromobutyl)phthalimide (500 mg, 1.7 mmol) added. The reaction mixture was refluxed for 5 h, cooled, and water (50 mL) cautiously added. The aqueous solution was extracted with chloroform (3 x 50 mL), the combined extracts dried over anhydrous magnesium sulphate, filtered, and concentrated in vacuo. Flash chromatography of the dark residue, using the eluting solvent methanol/chloroform (15:75), gave the product as a light brown oil (318 mg, 40%) with R_f 0.6 upon TLC in the solvent system methanol/chloroform (1:4). The oil was refluxed with activated charcoal in methanol (20 mL) and ethyl acetate (20 mL), filtered, evaporated under reduced pressure, and crystallised (white crystals, mp 135-136 ^{o}C) from ethyl acetate.

Preparation of 3-O-(4-aminobutyl)morphine (III) (Figure 1)

To a solution of 3-O-(4-phthalimidobutyl)morphine (150 mg, 0.32 mmol) in methanol (2 mL) was added hydrazine hydrate (17 μL, 0.35 mmol). The mixture was refluxed for 3 h, cooled, and aqueous 1M hydrochloric acid (6 mL) added. The precipitated phthalhydrazide was removed by filtration, the filtrate extracted with ethyl acetate (2 x 3 mL), basified at 0 ^{o}C with



Ft = Phthalimide FTC = Fluoresceinthiocarbamyl

Figure 1. Synthesis of fluorescein-labelled morphine.

aqueous 2 M sodium hydroxide, and extracted with ethyl acetate (3 x 10 mL). The combined basic extracts were dried over anhydrous magnesium sulphate, filtered, and evaporated in vacuo to give the purified 3-O-(4-aminobutyl)morphine as a colourless oil (30 mg, 28%).

Preparation of 3-fluorescein-labelled morphine (IV) (Figure 1)

Thirty mg of the 3-O-(4-aminobutyl)morphine (90 μmol) was dissolved in methanol (4 mL), and fluorescein isothiocyanate (35 mg, 0.09 mmol) and triethylamine (50 μL) added. The reaction mixture was protected from light, and stirred at room temperature for 14 h. The solution was diluted with water (6 mL) and acidified with aqueous 1 M hydrochloric acid, to precipitate the crude product. This was filtered, dissolved in methanol (50 mL) and aliquots purified by preparative TLC with the developing solvent chloroform/methanol/acetic acid (50:50:1). 3-Fluorescein-labelled morphine (R_f 0.5) was eluted from the plates into methanol and its concentration in the eluates determined spectrophotometrically (4).

Preparation of antisera

Three mature ewes were immunised with the morphine-6-hemisuccinate-BSA immunogen following a previously described protocol (5).

Assay Buffer

All assay experiments were carried out in sodium borate buffer (100 mmol/L, pH 9.1) containing 1 g/L gelatin and 1 g/L sodium azide.

Assay standards

Morphine hydrochloride was used as primary assay standard throughout. A stock solution in assay buffer was prepared and then diluted in the same medium to give a range of standards from 1 to 64 mg/L of morphine.

Patients specimens

Urine specimens were obtained from the Department of Chemical Pathology, Hackney Hospital, London U.K., where they had been screened for opiates by TLC. All specimens were obtained from patients attending a drug dependency clinic and were stored, without preservative, at 4°C until assayed.

Polarisation fluorimetry

A modified LS-2 fluorimeter (Perkin-Elmer, Beaconsfield, Bucks) was used as described previously (6).

METHODS

TLC procedure

This was carried out as described previously (2) except that the developing solvent was ethyl acetate/methanol/ammonia (85:10:5) and after developing, the TLC plate was dried, sprayed with 5% sulphuric acid, heated for 1 min at 110 °C and, finally, sprayed with iodoplatinate. This was prepared by adding 12.5 mL of 40 g/L aqueous potassium iodide to 0.5 mL of

chloroplatinic acid (40% platinum) and the volume made up to 50 mL with distilled water. The procedure detects and identifies morphine, codeine, and dihydrocodeine but not glucuronides unless the sample is first hydrolysed. When required this was achieved by adding 1 mL of 6M HCl to 10 mL of urine and boiling for 30 min.

Boehringer drug test for opiates

Haemagglutination inhibition tests were carried out according to the manufacturer's instructions.

EMIT-d.a.u. (Svya) assays

These were carried out according to the manufacturer's instructions using a Gilford Stasar III spectrophotometer and the Syva Processor CP-5000 plus.

Single reagent polarisation fluoroimmunoassay (PFIA)

Fluorescein-labelled morphine at a concentration of 10 nmol/L was prepared in assay buffer. To this was added anti-opiate serum to give a dilution of 1/750. This working reagent was stored in the dark at 4°C until required, and brought to room temperature before use. To 1.5 mL of working reagent was added 10 µL of urine or standard. The solution was vortex mixed, incubated at room temperature for 1 h, and the fluorescence polarisation measured.

RESULTS

6-Morphine antisera

One sheep produced antiserum with excellent dissociation characteristics but a poor titre (1/120); another had a good titre (1/960) but poorer dissociation. The first bleeds from both sheep were pooled (1:1) and used for the single reagent assay development. Figure 2 shows the standard curve obtained at 20°C.

Specificity

Cross-reactivity studies were carried out (7). The results for the individual and mixed 6-morphine antisera in the single-reagent assay are shown in Table 1.

Assay validation

The standard deviation (SD) of the polarisation readings from 20 replicates of the zero-concentration standard (assay buffer) was calculated. The minimal detectable concentration at the 95% confidence level (8) was estimated as 0.35 mg/L of morphine or 0.25 mg/L of codeine. Normal urines contain endogenous fluorophores which, effectively, prevent attainment of these sensitivity levels. Therefore, thirty urines collected from members of staff were assayed and the mean and SD of the polarisation responses calculated. The mean response

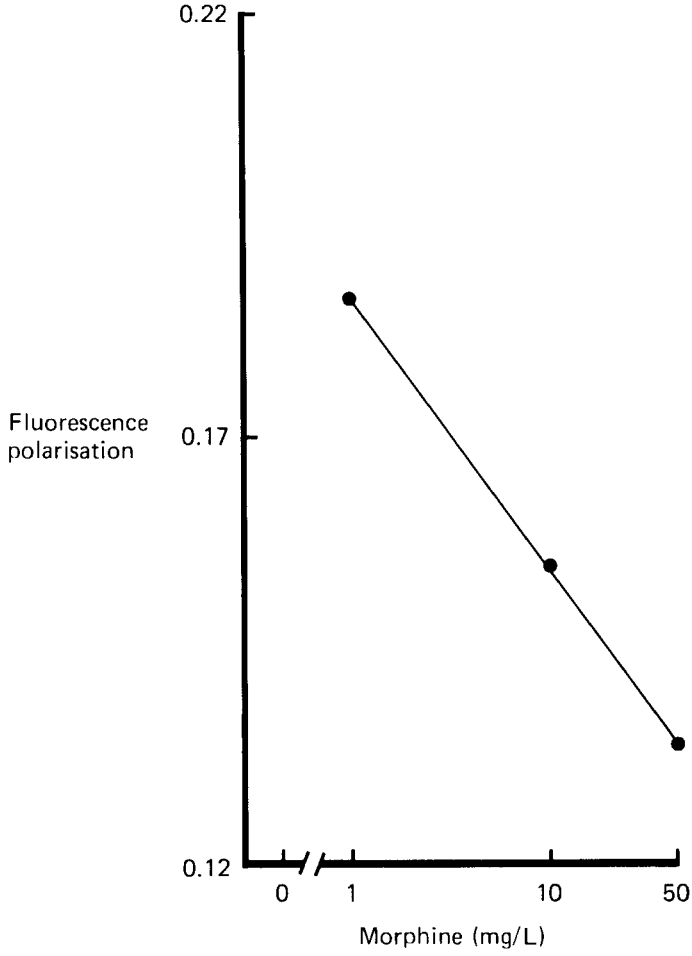


Figure 2. Standard curve, single reagent assay, room temperature.

Table 1

Percentage Cross Reactivity at 50% Displacement

6-Morphine Antiserum

	S 353-1	S 354-1	Mixed Sera
Morphine	100	100	100
Morphine-3-glucuronide	44	35	59
Codeine	379	160	234
Dihydrocodeine	167	80	151
Pethidine	1.0	1.1	1.1

Methadone, dipipanone, cocaine, amphetamine, secobarbital, and tetrahydrocannabinol all had less than 0.1 % cross-reactivity at the 1 g/L level.

corresponded to an apparent concentration of 0.15 mg/L of morphine or 0.13 mg/L codeine. The morphine concentration corresponding to the polarisation reading 3 SD from the mean was 0.7 mg/L (0.4 mg/L codeine) an indication of the variability of endogenous fluorophores. The cut-off level for an "opiate positive" result was set at 1 mg/L morphine equivalents to

enable a confident identification of a positive result and was also comparable to the detection capability of TLC (9).

Within-assay precision was assessed using three urines previously found to be positive for opiates. Thirty replicate assays of each was carried out and gave mean concentrations of 4.8 mg/L, 15.3 mg/L and 46 mg/L morphine equivalents with CVs of 9%, 8.1%, and 6.2% respectively. The same three urines were assayed on 10 separate days giving CVs of 12.0%, 9.7%, and 8.6% respectively for between-assay precision.

Morphine at concentrations of 5, 16, and 32 mg/L was added to a known normal urine. Recoveries of 94%, 93.7%, and 96.8% respectively were obtained. To a known positive urine with a concentration of opiates equivalent to 6 mg/L morphine was added 30 mg/L of morphine and to another with a concentration of 24 mg/L morphine equivalents was added 4 mg/L. Recoveries of 107% and 105% respectively were obtained.

Correlation with other screening techniques

All urines used for comparison had been screened for opiates by TLC (2) with a detection capability of 1-2 mg/L.

A first trial compared the PFIA against TLC results. Of 168 specimens tested 118 were positive by PFIA and 108 positive by TLC (some specimens were also positive for other opiates). Morphine is mainly excreted as the 3-glucuronide (1) with which the PFIA antiserum cross-reacts. Any urine with an opiate

concentration (including metabolites) less than 10 mg/L will contain very little morphine and may not be detected by TLC without hydrolysis. When the 10 samples that were positive by PFIA but negative by TLC were hydrolysed by boiling with HCL all 10 were then found to be positive for morphine by TLC.

A second trial included other immunoassays available at the time. A further 90 specimens were screened by TLC, PFIA, EMIT-d.a.u., and haemagglutination inibition. Of these urines 28 were negative and 54 were positive by all four procedures (although nine of these were also negative by TLC until hydrolysed with HCl). A further 3 specimens were positive by all the immunoassays but negative by TLC even after hydrolysis. This does not imply that all the unconfirmed TLC positives were false but is due to the greater sensitivity of the immunoassays to opiates and their metabolites (9). If the total concentration of opiate and metabolites is close to the 1 mg/L cut-off of the PFIA then TLC may not detect it even after hydrolysis. Positive EMIT results were obtained for 3 specimens that were negative by all other methods and could be considered as false positives by EMIT. Finally 2 specimens that were weakly positive by PFIA (between 1 and 1.3 mg/L morphine equivalents) gave negative EMIT and TLC results; one was negative and one weakly positive by the haemagglutination inhibition test. This is probably a reflection of the greater sensitivity of immunoassays and variability of cross reaction between different antisera.

DISCUSSION

Assays designed for the initial screening for drug abuse should be simple, suitable for automation, and optimised to determine the presence, or absence, of a range of related drugs rather than being specific for a single drug. Combination reagents have been reported which satisfy this requirement (10,11,12). The alternative approach is to develop broad spectrum antisera which will detect a range of related drugs.

As the assay design was based on fluorescence polarisation it could be adapted for use on the TDx but the instrumentation is complex and expensive. By modifying the reagents to a single-reagent approach it could be used with less expensive instruments such as the Perkin-Elmer LS-2. This instrument and assay together provide a simple system both for stat assays and for large scale screening programmes. Furthermore, full automation of the assay could be achieved by using the Perkin-Elmer PF1-20. Once a positive sample has been detected, identification of the opiate can be carried out by TLC, GLC, or specific immunoassays (13).

It has been known for some time that antisera to morphine prepared through the 3-position are relatively "blind" to structural changes occurring at the 6-position, and vice-versa (14). Therefore an antiserum to morphine derivatised through the 6-position was prepared. When used with tracer prepared through the 3-position, the dissociation characteristics enabled

development of a single-reagent assay, equilibrium being reached after 1 h at room temperature or 30 min at 30°C. Thus, a single reagent assay could be used with the advantage of simplicity and ease of automation. Such incubation periods are only necessary for quantitation or accurate detection of very low levels close to the cut-off point. If only a qualitative result is required then a few minutes incubation is sufficient. Indeed, if a rapid result is essential, as in suspected overdose, the high levels of morphine will displace tracer beyond the cut off point within minutes of sample addition. However, if short incubation periods are used, so that equilibrium is not reached, then poorer precision would be expected.

By using a broad spectrum antiserum several opiates could be detected. Cross-reactivity studies suggested that the assay would be more sensitive to codeine and experiments showed that after a single 10 mg dose of codeine a positive result could be obtained for up to 36 hours (13). The disadvantage of using a broad spectrum antiserum is that it is likely to cross-react with "innocent" materials. Indeed, like other commercially available opiate antisera, this assay will give a positive result from poppy seeds (9,13).

Once the sample has been added to the reagent and equlibrium reached the polarisation reading is stable for several weeks provided it is not exposed to strong light. Thus polarisation readings can be taken at any convenient time;

indeed readings need not be carried out on site but can be sent to a central laboratory for processing. In our experience single reagents have proved extremely stable at 4 °C or at room temperature. An amphetamine reagent has remained stable for two years at room temperature provided it is stored in a brown bottle.

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