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Group-Selective Immunoassay for the Detection of Morphine in Urine

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ABSTRACT

A new competitive inhibition immunoassay (group-selective immunoassay; GSI) has been developed to detect free morphine in urine with the Fab′ fragments of monoclonal antibodies (MAbs) ($1B_{12}F_9B_4$, IgG_1, κ, $K_{aff} = 9.66 \times 10^{10}M^{-1}$). At the first assay step, microtiter plates were coated with morphine-ovalbumin (M-6-S-OVA), in which free amino acids were protected by a glutaraldehyde cross-linking modification. The modification did not essentially influence the antibody-binding capacity of the immunosorbent. At the second assay step, anti-morphine MAbs' Fab′ fragments, in which free amino groups were biotinylated by *N*-hydrosuccinimide-biotin ester, were bound to chemically modified immunosorbent. The biotin residues were then detected by the streptavidin-peroxide conjugate. This method has a sensitivity of 3.50×10^{-15} mol/L using very little volume of sample, covering up to almost 1.20×10^{-11} mol/L of standard concentration of morphine with good reproducibility. Standard curve prepared in urine indicated a good correlation between the concentration of morphine and the value of OD ($y = 1/ax + b$; $r = 0.99939257$, $S = 0.01138127$). Coefficients of variation for this immunoassay were 1.41 ~ 6.61% within-a-day assay and 2.31 ~ 8.99% between days assay. The recoveries were 94 ~ 101.4% from negative urine and 95.2 ~ 107.5% from positive urine samples, respectively. This method has application as a specific screen for morphine in drug abusers, to study the metabolism of the drug in the body, or to screen the monoclonal antibodies (MAbs) against morphine.

INTRODUCTION

HEROIN, which is rapidly metabolized and excreted as free morphine and its glucuronides, is a dangerous drug of abuse and must be detected as part of any screening programme. Many methods have been used to study the pharmacokinetic properties of the drug[1,2] as well as to investigate the relationship between pain control and morphine concentration.[3] Unfortunately, most of the current techniques for measurement of morphine concentration have certain disadvantages, specifically in the detection of free morphine in various samples, so it is necessary for us to establish simple, sensitive, specific and high throughput assays for the qualification of morphine in urine and other biological fluids.

Here we describe a novel competitive inhibition immunoassay—improved group-selective immunoassay (GSI)[4] for detecting morphine in urine. The general principle of the method is based on chemical differences between the antigen and antibody molecules, created in our experiments by the protection of free amino groups in immobilized M-6-S-OVA conjugate by glutaraldehyde. This GSI method is more sensitive and has lower detection limit than conventional ELISA.

MATERIALS AND METHODS

Reagents

Morphine, Ovalbumin (OVA), *o*-Phenylenediamine(OPD), glutaraldehyde were purchased from Beijing Chemical Reagents Co. (China); bovine serum albumin (BSA) was purchased from Merk AG (Darmstadt, Germany), goat anti-mouse IgG-horseradish-peroxidase (IgG-HRP) was purchased from Jackson Immunoresearch, West Grove, PA. ELISA plates (96 wells) were purchased from Labsystems (Needham Heights, MA). Pepsin was purchased from Sigma (St. Louis, MO). All other reagents used in this study were of analytical grade and obtained from standard sources.

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Biotinylation of anti-morphine Fab′

Preparation of monoclonal antibodies against morphine. Morphine was conjugated with OVA (M-6-S-OVA) and BSA (M-6-S-OVA) as described by Akbarzadeh et al.[5] The conjugate was chromatographed on a Sephadex G-25 column, then stored at −20°C. The process of immunization and generation of MAbs was referred to Yang et al.[6]

Pepsin digestion of IgG to F(ab′)$_2$. 3–5 mg IgG in 0.5 mL was dialyzed against sodium acetate buffer, 0.1 mol/L, pH 4.2, containing NaCl, 0.1 mol/L, at 4°C. Pepsin (equal to 4% of IgG) was dissolved from porcine gastric mucosa in the dialyzed IgG solution. The mixture at 37°C was incubated for 12–16 h, and the pH of the digested IgG solution adjusted to 7.0 using Tris-HCl buffer, 2 mol/L, pH 8.0. The digested IgG solution was applied at pH 8.0 to a column (1.5 × 20 cm) of Sephadex G150 at a flow rate of 20 mL/h using sodium borate buffer, 0.1 mol/L, pH 8.0. The fraction volume is 1.0 mL.

Reduction of F(ab′)$_2$ to Fab′. F(ab′)$_2$ fraction against sodium acetate buffer, 0.1 mol/L, pH 5.0, was dialyzed at 4°C overnight. 2-mercaptoethylamine, 10 mmol/L, was added to the dialyzed solution at 37°C for 1.5 h, then iodoacetamide, 30mmol/L, was added at room temperature for 1 h. The above solution was added to a column of Sephadex G25 at a flow rate of 20 mL/h using sodium acetate buffer, 0.1 mol/L, pH 5.0.

Biotinylation of Fab′. Fab′ fraction against sodium phosphate buffer, pH 7.5, containing 1 g/L bovine serum albumin and 0.3 mol/L NaCl was dialyzed to a final volume of 0.5 mL, and then incubated with 25 μL of 42 mmol/L *N*-hydroxysuccinimidobiotin in dimethylsulfoxide, at 30°C, for 30 min. After incubation, the reaction mixture was incubated with 50 μL of 2 mol/L glycine-NaOH, pH 7.5, at 30°C, for 30 min, followed by the addition of 3.175 mL of 10 mmol/L sodium phosphate buffer, pH 7.0 containing 1 g/L bovine serum albumin, 0.3 mol/L NaCl, and 1 mmol/L $MgCl_2$.

Competitive inhibition enzyme-linked immunosorbent assay (ELISA)

Enzyme substrate solutions. The substrate solution containing *o*-phenylenediamine (OPD/H_2O_2) was prepared by dis-

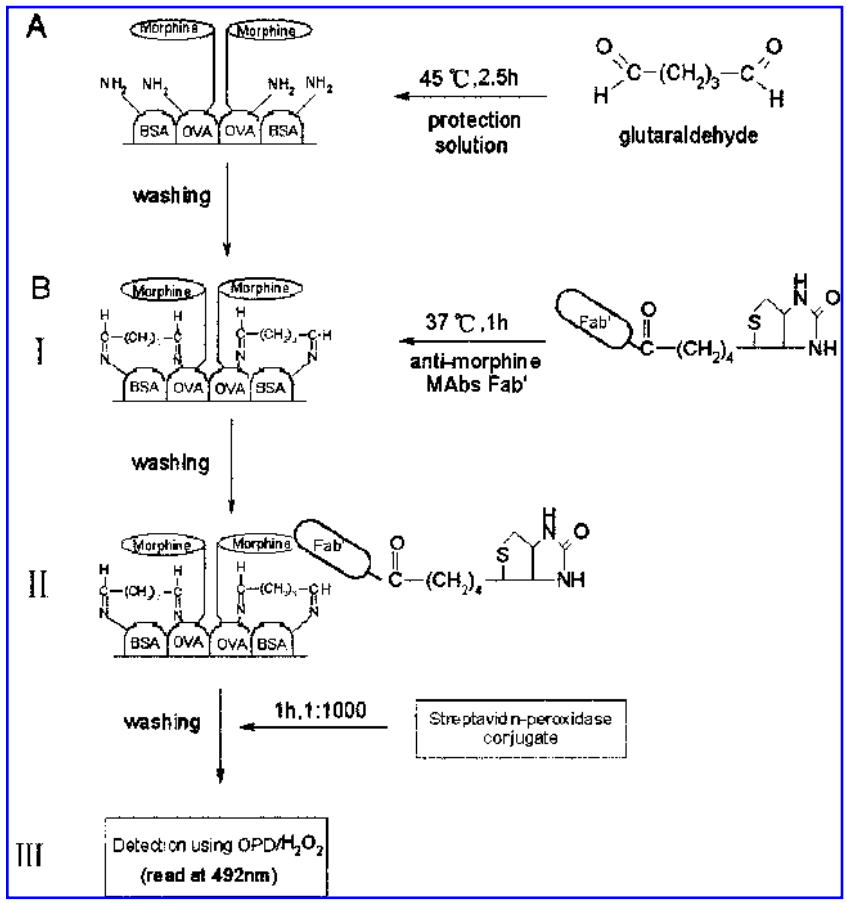

FIG. 1. Outline of group-selective immunoassay (GSI) using streptavidin-biotin system. (**A**) Preparation of chemically modified immunosorbent. Free amino groups on immobilized M-6-S-OVA conjugate are protected by glutaraldehyde. The microtiter plates with immobilized conjugate were treated with 2.5% glutaraldehyde solution in 0.01 M PBS, pH 8.0 for 2.5 h at 45°C. (**B**) Procedure of group-selective immunoassay (GSI). I: Incubation in GSI immunosorbent wells with biotinylated anti-morphine MAbs' Fab′ fragment dilutions in PBS containing 0.2% BSA for 1 h at 37°C. II: Streptavidin-HRP conjugates were added (incubation for 1 h at 37°C). III: Visualization of peroxidase activity with OPD and H_2O_2 at 492 nm.

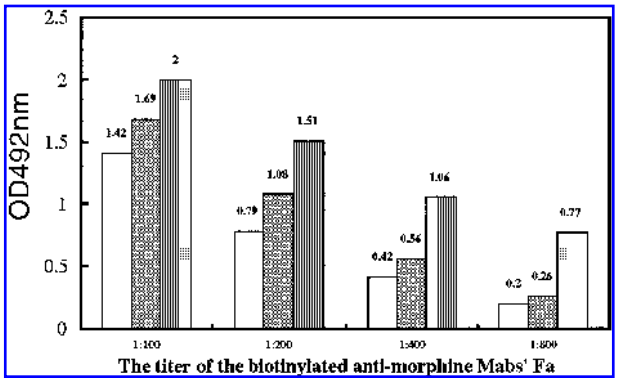

FIG. 2. Efficiency of free amino group protection in immobilized M-6-S-OVA conjugate after glutaraldehyde treatment. Open bars, ELISA immunosorbent (without glutardldehyde treatment); filled bars, GSI immunosorbent (with glutardldehyde and BSA treatment); hatched bars, GSI immunosorbent (with glutardldehyde but without BSA treatment).

solving 5 mg of OPD in 10 mL of 0.1 mol/L citrate/phosphate buffer, pH 5.0, and by adding 2 μL of 30% H_2O_2.

Preparation of immunosorbents for ELISA. Coating solution was prepared by dilution of the M-6-S-OVA conjugate in the coating buffer (0.05 mol/L Na-carbonate-bicarbonate buffer, pH 9.6) at 1 μg/mL. Then 100 μL of the coating solution was added to each well, and microtiter plates were left overnight at 4°C. The wells were washed three times and blocked by 1% BSA solution in 0.01 mol/L PBS (Na-phosphate buffer, pH 7.4) for 1 h at 37°C.

Preparation of immunosorbents for GSI. 200 μL of 2.5% glutaraldehyde solution in 0.01 mol/L PBS, pH 8.0 was added to each immunosorbent well prepared as described above. The wells were then incubated for 3 h at 45°C, washed, dried, and stored at 4°C.

Detection of free morphine in urine by GSI. The same volume of biotinylated anti-morphine MAbs' Fab′ fragments dilutions (with dilution of 1:100) and diluted samples were mixed and added to the wells. The plates were incubated for 1 h at 37°C, then washed and incubated again for 1 h with a solution of streptavidin-peroxidase conjugate in PBS (with dilution of 1:1000, containing 0.2% BSA). The plates were washed and peroxidase activity was measured by the reaction with *o*-phenylenediamine-H_2O_2.[7] Indirect competitive ELISA was carried out as previously described.[8]

TABLE 1. PRECISION OF WITHIN-A-DAY ASSAY[a]

Subjects	*Concentration of morphine (μg/mL of urine)*	*C.V. (%)*
Urine		
1	13.716 ± 0.193	1.41
2	0.307 ± 0.010	3.37
3	0.316 ± 0.011	3.58
4	0.928 ± 0.075	6.61
5	2.396 ± 0.363	3.03

[a]Each analysis was done in triplicate.

TABLE 2. PRECISION OF BETWEEN-DAYS ASSAY[a]

Subjects	*Concentration of morphine (μg/mL of urine)*	*C.V. (%)*
Urine		
1	14.600 ± 0.497	3.40
2	0.332 ± 0.021	6.32
3	0.307 ± 0.028	8.99
4	0.889 ± 0.058	6.54
5	2.354 ± 0.054	2.31

[a]Each analysis was done in triplicate.

RESULTS AND DISCUSSION

The most essential step of the proposed method is a complete protection of free amino groups in immobilized M-6-S-OVA conjugate without a decrease of the antigen-binding capacity. The best results were obtained after a treatment of immobilized M-6-S-OVA conjugate with glutaraldehyde interacting effectively with protein amino groups. A general reaction scheme is presented in Figure 1.

To optimize the conditions for free amino group blockage by glutaraldehyde, the following reaction parameters were tested: (1) protection solution with various glutaraldehyde concentrations (0.5–5%) in PBS of different molarities (0.01 ~ 0.1 mol/L); (2) NaCl content (0.1–1.0 mol/L); and (3) the pH (6.8–8.0). In all cases the protection was carried out at 45°C for 3 h. The results show that protection solution with 2.5% glutaraldehyde in 0.01 mol/L PBS (containing 0.15 mol/L NaCl, pH 8.0) was the best for free amino group protection in an immobilized M-6-S-OVA conjugate.

Non-specific binding of GSI immunosorbent was tested in the presence or absence of BSA in washing solution. The results in Figure 2 demonstrate that, compared with conventional competitive ELISA (without glutaraldehyde treatment), GSI assay increases the OD value of the reaction about 30% and the background values for GSI are very low. With glutaraldehyde and no BSA treatment, the OD values are much higher, but the background values also increase correspondingly. It is clear that adding BSA can effectively decrease the non-specific binding caused by unreacted aldehyde groups.

TABLE 3. ANALYTICAL RECOVERY OF MORPHINE ADDED TO HUMAN URINE

Number of samples	*Concentration of morphine (pg/well)*		*Recovered (%)*
	Added	*Recovered*	
Urine			
N	25	23.5 ± 2.7	94.0
	50	50.7 ± 0.6	101.4
P_1	25	41.4 ± 1.4	104.4
	100	117.8 ± 8.8	102.5
P_2	25	39.3 ± 4.6	95.2
	100	123.0 ± 1.4	107.5

Each analysis was done in triplicate. N represents negative control; P_1 and P_2 represent positive samples 1 and 2, which contain 0.17 and 0.52 μg/mL of morphine, respectively. They are diluted 1000 times before use.

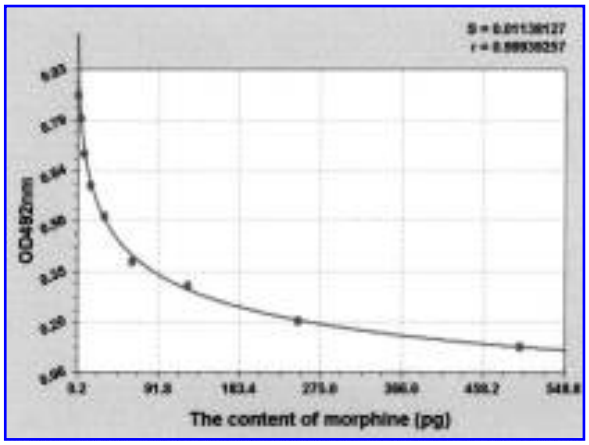

FIG. 3. The calibration curve of morphine in urine for MAbs.

We have investigated immunochemical properties of immobilized M-6-S-OVA conjugate by reaction with glutaraldehyde. It is evident that modification of amino groups will not change the antigenic properties of the immunosorbent, because morphine does not contain amino groups.[9] This method is especially suited for screening anti-morphine MAbs when only one kind of morphine-carrier protein conjugate is available.

The assay correctly identified all five abusers of morphine (heroin), as seen in Table 1 and Table 2, which show the results of five drug abusers with coefficients of variation of 1.41–6.61% within-a-day assay and 2.31–8.99% between days assay. Standard curve prepared in urine indicated a good correlation between the concentration of morphine and the value of OD ($y = 1/ax + b$; $r = 0.99939257$, $S = 0.01138127$) as shown in Fig. 3. It has a sensitivity of 3.50×10^{-15} mol/L using very little volume of samples, covering up to almost 1.20×10^{-11} mol/L of standard concentration of morphine with good reproducibility.

We also tested the recovery of morphine by adding a known amount of morphine to the negative and positive samples. Table 3 shows that the recoveries of morphine were 94–101.4% from negative urine and 95.2–107.5% from positive urine samples, respectively.

The assay with sensitivity enough to detect the free morphine in urine is also rapid compared to other analysis techniques: up to 48 tests in quadruplicate (on three plates with full standard curves) can be performed in 2 h.

ELISA type assays have the added advantage of using less expensive reagents than other assay methods; Microtiter plate readers are relatively inexpensive, and the colourmetric endpoint of the assay makes visual analysis of the plates entirely feasible. No scientific expertise is required to use this assay, and a positive (absence of color) or negative (presence of color) result can be roughly determined by a visual evaluation of the plate. This means that by following simple instructions non-scientific personnel can use this test outside the confines of a laboratory, which is ELISA's main advantage over existing morphine specific immunoassay techniques. This assay is intended as an initial screen for morphine abuse. It can be used in conjunction with, or in place of, any of the other commercially available tests. A positive result however, as in all screens of this nature, should always be backed up by a chromatographic procedure to confirm the presence of morphine.

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