

## Rapid latex agglutination inhibition reaction test for morphine in urine

Azim Akbarzadeh\*, Dariush Norouzian, Behrokh Farahmand and Davoud Nouri Inanlou

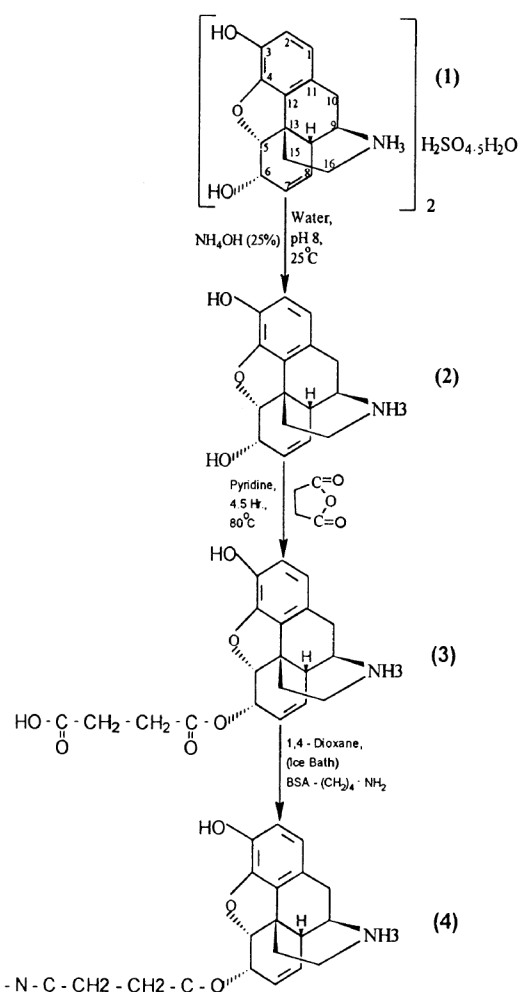
Department of Pilot Biotechnology, Pasteur Institute of Iran, Pasteur Avenue, Tehran 13164, Iran

A simple and rapid immunoassay of morphine based on latex agglutination inhibition reaction has been developed. Morphine-6-succinate is a morphine derivative, and its bovine serum albumin (BSA) conjugates were synthesized and characterized. The hapten density in the conjugate was determined spectroscopically to be 8.2 mol per mol of BSA. It was found that morphine-6-succinyl-BSA (M-6-S-BSA) exhibits favourable features in terms of immunogenicity and immunochemical specificity. A latex agglutination inhibition reaction test (LAIRT) using DEAE-cellulose purified rabbit IgG against M-6-S-BSA was found to give a sensitivity of 300 ng/ml of morphine. Various commonly used drugs and narcotics at concentration 0.1 mg/ml or less did not interfere with the test. Interference by normal urine was observed, but it could be eliminated by the inclusion of 0.78% normal rabbit serum. The sensitized latex was stable at 4°C for at least nine months. It was also stable to lyophilization and to at least four cycles of freezing and thawing. The total test time was 5 min. Comparison was made between LAIRT and enzyme-multiplied immunoassay (EMIT) on 100 urine samples collected from persons visiting the government narcotic clinic (Reference Laboratory, Tehran, Iran). While EMIT showed 84 positives and 16 negatives, LAIRT gave 68 positives and 32 negatives. The two tests showed no statistically significant difference ( $P < 0.05$ ).

MORPHINE is a central nervous system stimulant widely used by people in Iran. A recent study showed that drugs such as morphine were found in urine specimens of some persons visiting the government narcotic clinic (Reference Laboratory, Tehran, Iran). The abuse of morphine continues to be prevalent in some countries. Although there are few data about the abuse of morphine, we know that new users appear each day<sup>1</sup>. Such widespread drug abuse undoubtedly contributes to social problems. One way to deal with this drug problem is to have a rapid and economic means of identifying the users, so that appropriate measures can be taken. However, the commercially available enzyme-multiplied immunoassay (EMIT) costs about US\$ 6.00 per test locally, while the fluorescence polarization immunoassay (FPIA) uses expensive equipment and is not suitable for on-site test in developing

countries. A method for detection of morphine is hemagglutination-inhibition test which takes 3 h to be completed<sup>2</sup>. A latex agglutination inhibition tube test takes 2 h to be completed<sup>3</sup>. The abuscreen kit, produced by Roche Co., costs about US\$ 4.00 per test in Iran<sup>4</sup>. On the contrary, the latex agglutination inhibition reaction test (LAIRT) that was developed in our laboratory is specifically sensitive to morphine and costs about US\$ 0.5 per test and takes 5 min to be completed. Thus, it is highly desirable to have simple, rapid and economical test(s) to screen drug users for morphine. Here, the characteristics of LAIRT are reported<sup>5</sup>.

For the preparation of morphine base, a 10 g portion of morphine sulphate ((1), Figure 1) was dissolved in 160 ml of distilled water at room temperature. The pH of the solution was adjusted to 8.0 with NH<sub>4</sub>OH. The free morphine base ((2), Figure 1) was crystallized by precipitation at pH 8, filtered and dried at 60°C under vacuum<sup>1,6</sup>.



**Figure 1.** Preparation of morphine-6-hemisuccinate and conjugation to BSA. (1), Morphine-sulphate; (2) Morphine base; (3) M-6-S; (4), M-6-S-BSA.

\*For correspondence. (e-mail: Azimakbarzadeh@institute.pasteur.ac.ir)

Morphine-6-succinate (M-6-S) was prepared by heating morphine base with three equivalents of succinic anhydride in dry pyridine. This leads to the selective succinylation of the 6-hydroxyl group. Morphine free base (12 g) and succinic anhydride (7.5 g) in pyridine (150 ml) were placed in 1000 ml flask provided with a condenser and heated (water bath 80–85°C for 4.5 h). Pyridine was evaporated at reduced pressure, the residue was washed five times with hot ethanol, and re-crystallized twice from 60%. The yield of M-6-S was 80–85% of the theoretical yield<sup>5,7,8</sup> ((3), Figure 1).

For the separation of M-6-S from morphine by TLC the best solvent is ethanol/dioxane/benzene/NH<sub>4</sub>OH (8 : 1 : 10 : 1 by volume). Here the *R<sub>f</sub>* of morphine is 0.56 and that of M-6-S is 0.14. The product usually contained less than 2% morphine. The product was re-crystallized twice from 60% ethanol<sup>1,6-8</sup>.

Elemental analysis of M-6-S dried under reduced pressure at 60°C over P<sub>2</sub>O<sub>5</sub>: C, 62.3; H, 6.23; N, 3.7; calculated for C<sub>21</sub> H<sub>23</sub> NO<sub>6</sub>. H<sub>2</sub>O : C, 62; H, 6.2; N, 3.5 (refs 1 and 6).

M-6-S does not have a well-defined melting point, but decomposes above 200°C. It is readily soluble in water below pH 3 and above pH 8.5, but is much less soluble between these pH values (0.2 mg/ml at 2°C, and 0.5 mg/ml at 25°C), consistent with its zwitterionic structure. The compound is sparingly soluble in all organic solvents tested<sup>1,6</sup>.

Evidence that the morphine molecule was esterified with succinate, and that esterification occurred exclusively in position 6 is as follows: (i) Reaction of M-6-S with folin/phenol reagent demonstrated quantitative recovery of free phenolic group; (ii) When we tried to succinylate levorphenol, which lacks the 6-hydroxyl group, only unchanged levorphenol was recovered; (iii) Titration of M-6-S with NaOH showed the presence of one titratable group, with a pK of 4.2, typical of a carboxyl group; (iv) Acid hydrolysis of M-6-S yields morphine, as identified by TLC; (v) The UV spectrum of M-6-S is indistinguishable from that of morphine, in which the chromophore responsible for the peak at 285 nm is the phenolic ring; this is further evidence for the absence of reaction at this site; (vi) Portions of the IR spectra of M-6-S and morphine are shown in (1); the spectrum of M-6-S has lost the peak at 1100 cm<sup>-1</sup>, characteristic of a free secondary alcohol group; the succinyl group is indicated by a new C=O stretching peak at 1700 cm<sup>-1</sup> and by peaks characteristic of a COO-group at 1615 and 1380 cm<sup>-1</sup>; (vii) MS of M-6-S gave primary fragments at m/e 41 (100%), 44 (50%), 266 (17%), 384 (M-1) (5%) and 383 (M<sup>+</sup>) (2%). The peak at 385 agrees with the calculated molecular mass for M-6-S; the other peaks are consistent with a fragmentation scheme expected for M-6-S (refs 1 and 6).

In preliminary experiments, M-6-S was about as active in mice as morphine with respect to production of the Straub reaction and hyperactivity<sup>1,6</sup>.

A solution of 0.2 g of M-6-S in 13 ml of 1,4-dioxane was vigorously stirred and cooled (ice bath) over a period of 20 min with a solution of 0.875 g of bovine serum albumin (BSA) in 19 ml of water, 0.7 ml of N NaOH, and 6.3 ml of dioxane. Subsequently, an additional 0.3 ml of N NaOH was added drop-wise, at which point the solution was somewhat turbid. After the reaction had proceeded for 30 min, further addition of 0.26 ml, of N NaOH resulted in a clear solution. Stirring and cooling were continued for a total of 4 h. The solution was dialysed for 18 h and brought to a pH of 4.6 with 0.5 ml of N HCl. The conjugate was precipitated and after storage in the cold for 6 h was collected by centrifugation. After being washed twice with water, it was redissolved in water by bringing the pH to 3.5 with dilute HCl. The clear solution was lyophilized, yielding 0.93 g of product, the yield of M-6-S-BSA was 86.5% of the theoretical yield ((4), Figure 1). The hapten density in the conjugate was determined spectroscopically to be 8.2 molecules per molecule of BSA (if we assume an *M<sub>r</sub>* of 67,000 for the protein)<sup>9,10</sup>.

Rabbits weighing about 2 kg each were immunized subcutaneously. The first immunization was made with 2.5 mg of the immunogen in sterile normal saline solution emulsified in complete Freund's adjuvant, while subsequent immunizations were done in incomplete Freund's adjuvant. Immunization was given over 7 weeks (once a week). The antiserum was absorbed with an equal volume of 2 mg/ml BSA and 2 mg/ml of M-6-S-BSA in 0.15 M phosphate-buffered saline (PBS), pH 7.2 for 30 min at room temperature. For the fractionation of rabbit antibody, the antisera from rabbits immunized with immunogen were first treated with ice-cold saturated ammonium sulphate to precipitate the immunoglobulin which was further purified by DEAE-cellulose ion exchange chromatography<sup>11-14</sup>.

The DEAE-cellulose purified immunoglobulin (IgG) was used in the sensitization of latex particle. About 10% suspension of polystyrene latex particle was first washed twice with glycine-buffered saline (GBS), pH 8.2. Coating of antigen to latex particle was accomplished as follows: 200 µl of IgG at a predetermined concentration in GBS was mixed with 10 µl of 10% latex suspension. The mixture was incubated in a waterbath at 37°C for 30 min and washed twice with GBS. After washing, the latex was suspended in GBS, pH 8.2 containing 0.5% normal rabbit serum and kept at 4°C until needed<sup>15</sup>.

The DEAE-cellulose purified IgG was diluted in GBS, pH 8.2 to 300, 150, 75 and 37.5 µg/ml and then allowed to bind to 10% latex suspension as described above. The optimal concentration of IgG was the one that detected the lowest amount of the M-6-S-BSA in the latex agglutination and also the lowest amount of morphine by LAIRT<sup>15</sup>.

The 0.5% sensitized latex suspension in GBS, pH 8.2 containing 0.5% normal rabbit serum (NRS) was stored

at 4°C and the stability of sensitized latex was checked, weekly and monthly. Some of these sensitized latex particles (the same lot) were tested for their ability to withstand cycles of freezing–thawing and lyophilization<sup>15</sup>.

The specificity of LAIRT developed for morphine detection was studied with the use of some commonly used drugs and narcotics. These drugs were individually dissolved in urine and tested for their interference on the LAIRT test; the highest drug concentration which did not affect the test was recorded.

Urine samples were collected in plastic bottles and were immediately cooled in ice. Upon arrival at the laboratory, they were stored at –20°C. Before use, the samples were centrifuged at 10,000 × *g* for 15 min at room temperature to obtain the clear supernatant<sup>15</sup>.

Urine samples were assayed in duplicates for morphine by EMIT. Samples which were positive for the drugs in this assay were subjected to an additional test using an morphine confirmation kit. Positive controls were urine samples included in the test kit and these were used to calibrate the assays. Urine samples obtained from ten laboratory personnel were used as negative control<sup>16</sup>.

Protein was determined by the method described by Lowry *et al.*<sup>17</sup>.

M-6-S, prepared as described here were white crystals obtained in 80% yield. Conjugation of M-6-S to BSA using 1,4-dioxane resulted in 86.5% recovery of the protein after dialysis. Respective molar extinction coefficients, as determined by optical densities at 280 nm were 0.325 for 1 mg/2 ml BSA, 1.1 for 1 mg/2 ml M-6-S and 0.375 for 1 mg/2 ml M-6-S-BSA. Based on the optical density of the conjugated product at these wavelengths (after solvent extraction to remove noncovalently bound ligand), 8.2 moles of M-6-S were coupled to each mole of BSA, since BSA contains a total of 59 lysine residues.

Effect of IgG concentration in the sensitization of latex particle was as follows. The optimal IgG concentration for coating latex particle was 300 µg/ml. The latex particle sensitized with anti-M-6-S-BSA could be agglutinated by the antigens (M-6-S-BSA) at 300 ng/ml. These concentrations of M-6-S-BSA were used in the LAIRT test in order to compete with morphine in the test system.

The effect of urine in the LAIRT test was studied. The experiment was carried out using urine from 20 women undergoing pregnancy test at Emmam Khomeini Hospital and 20 urine samples from opiate patients visiting the government narcotic clinic (Reference Laboratory, Tehran, Iran), and who had been shown to be positive to morphine by a Syva EMIT morphine test. In the LAIRT test, there were some non-specific agglutinations when certain urine samples were mixed with the antibody-sensitized latex particle in the diluent in the absence of M-6-S-BSA. These non-specific agglutinations could be eliminated by adding NRS at a final concentration of 0.78%.

For the determination of morphine by LAIRT, the latex particles were sensitized with DEAE-cellulose purified anti-M-6-S-BSA IgG. Anti-M-6-S-BSA IgG-sensitized latex particle reacted with morphine. The sensitivity of LAIRT test in the determination of morphine using anti-M-6-S-BSA IgG-sensitized latex was 300 ng/ml, while the detection limit for M-6-S was 250 ng/ml.

Various commonly used drugs studied did not affect the LAIRT test. Acetaminophen, caffeine and phenobarbital sodium did not inhibit the test at less than 0.1 mg/ml.

The IgG-sensitized latex particles stored as described earlier were stable at 4°C for at least nine months without any change in their sensitivity of morphine detection. The particles were also stable to four cycles of freezing–thawing and to lyophilization.

Urine specimens from a total of 100 patients visiting the government narcotic clinic (Reference Laboratory, Tehran, Iran) were collected. Most urine specimens were cloudy in appearance and precipitated heavily upon cooling in ice. The average pH was 6.45 ± 1.2. The samples were assayed for morphine using EMIT morphine assay and LAIRT. The results of the tests are shown in Table 1. The EMIT assay showed 84 positive cases for morphine, while 16 cases were negative. In LAIRT however, 68 urine samples were positive for morphine, while 32 cases were negative. The results of the two assays were not significantly different ( $P < 0.05$ ) by the  $\chi^2$  McNemar test<sup>18,19</sup>.

The problem of morphine abuse in various developing countries, especially in Iran, is extremely serious. It was shown that 2% of the population in Iran uses morphine. This figure has been repeatedly confirmed by the Iran

**Table 1.** Result of morphine detection in urine samples from the Reference Laboratory using LAIRT and EMIT morphine assay

| LAIRT | EMIT |    | Total |
|-------|------|----|-------|
|       | –    | +  |       |
| +     | 7    | 61 | 68    |
| –     | 9    | 23 | 32    |
| Total | 16   | 84 | 100   |

Office of the Narcotics Control Board. In developing countries, drug abuse is harmful for the country's progress. One way to discourage drug abuse is to identify the users, so that appropriate measures can be taken. For this purpose, various commercial immunoassays of morphine are available. These tests, however, are very expensive, require expensive equipment/expertise or use unstable reagents. The total test time required is also important. The test time should be reasonably short so as to get quick results. This should be possible with careful design of the immunogen. It is shown in the present study that subtle structural changes in the immunogen could affect the specificity of the antibody. Thus antibodies raised against [M-6-S-BSA] react well with morphine. Moreover, the antibody raised against an immunogen with morphine via the 6th-position of the phenyl ring, is exquisitely specific for morphine. In the present study, the haptenic group is M-6-S and the antibody is directed against morphine with a succinyl group. The detection limits of morphine are 300 ng/ml. It is interesting to note that while M-6-S-BSA induced barely detectable specific antibody in rabbits, antisera with high titers were elicited against M-6-S-SBA. This is most likely due to the presence of a 4-atom 'spacer' between the haptenic group and the carrier protein in the immunogen. It has been shown that a good immunogen must attain a certain minimum ligand density, probably about 15 for BSA. An attempt was therefore made to determine the ligand density in M-6-S-BSA by spectroscopic procedures, the ligand density was found to be 8.2 mole of morphine per mole of BSA. The results of EMIT and LAIRT on the urine samples although not statistically different, show fewer positive cases by LAIRT. This is probably because of the lower sensitivity of LAIRT compared to EMIT. There were seven cases in which LAIRT showed positive results, while EMIT showed negative results. These cases might be false positives of LAIRT due to interfering substances in the urine. False negatives by EMIT could not be ruled out. In spite of the slightly lower sensitivity towards morphine by LAIRT, the test has several advantages. It is economical (approx. US\$ 0.50 per test at

Pasteur Institute, Tehran). It does not require expensive equipment or expertise. The reagents are quite stable to the usually not so optimal transportation and storage conditions available in tropical developing countries and the test time is reasonably short (about 5 min). LAIRT should be useful in the screening of morphine abuse in Iran and other countries.

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