A Sensitive, Rapid Radioimmunoassay for Morphine and Immunologically Related Substances in Urine and Serum

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A rapid, simple radioimmunoassay for morphine and immunologically related compounds is described and evaluated. The 2-h assay requires as little as 0.1 ml of urine or serum and provides a quantitative estimate of serum or urinary morphine. Individuals known not to be receiving heroin or other narcotics had apparent urinary morphine concentrations of less than 25 μg/liter and apparent serum concentrations of less than 10 μg/liter. Patients receiving as much as 100 mg of methadone gave results (expressed as morphine) of less than 25 or 10 μg/liter, respectively, for their serum and urine. Fifty-eight of 59 urines from known heroin users, sampled up to 48 h after self-administered heroin, contained more than 25 μg of morphine per liter; 32 of 37 sera obtained up to 12 h after heroin contained more than 10 μg/liter. Samples obtained from individuals receiving substances that may cross-react in the assay were tested and the results are discussed. We conclude that this radioimmunoassay may be suitable for routine screening of urine or serum for morphine.

Spector and Parker (1) have described a highly sensitive radioimmunoassay (RIA) for the detection of morphine and morphine-like compounds, in which use is made of rabbit antisera and [7,8-3H]dihydromorphine according to the Farr Technique (2). More recently, Van Vunakis et al. (3) described a radioimmunoassay for morphine that involves either rabbit or guinea pig antisera to morphine, morphine conjugate labeled with 125I and precipitation with specific antihuman serum. Other immunoassays are currently being used to identify and estimate morphine and morphine-like substances in urine and (or) serum (4–6, 11).

Radioimmunoassays in general, including the two mentioned above, are not generally applicable to large-scale testing of human biological fluids because of the time required for assay and the complexity of the techniques. We describe here a rapid, simple radioimmunoassay for morphine and morphine-like substances and demonstrate the applicability of the method to the detection of these substances in urine and serum specimens obtained from known chronic heroin addicts. The test system used is a modification of the assay originally described by Spector and Parker (1).

Materials and Methods

Components of the Assay System

*Tritiated dihydromorphine*: [7,8-3H]dihydromorphine with a specific activity of 2.5 Ci/g was prepared by the Chemical Research Department, Hoffmann-La Roche Inc.

*Antibody to morphine* was prepared in goats according to the method of Spector and Parker (1).

A saturated solution of (NH4)2SO4 was prepared in de-ionized water and used without neutralizing it.

*Phosphate buffered saline (pH 7.2)*: To 1 liter of de-ionized water was added 1.36 g of KH2PO4 and 8.55 g of NaCl (solution B). To 3 liters of de-ionized water was added 4.26 g of Na2HPO4 and 25.5 g of NaCl (solution A). At 25°C, solution B was added to solution A until the pH was 7.2.

*Normal goat serum* was obtained from healthy adult male and female goats, sterile filtered, and used without further treatment.

Preparation of Morphine Standards

*Normal human urine* was collected from healthy adults, allowed to stand at 4°C overnight, decanted from any precipitates that formed, and the decanted urine was filtered through a sterile 0.45-μm (av pore size) Millipore filter. We pooled and stored at 4°C each urine sample that by RIA did not appreciably differ in counts per minute (cpm) from those obtained with buffer.

*Human blood* was obtained by venipuncture from healthy adult volunteers, allowed to clot, and the serum was separated by centrifugation. The individual sera were also tested for acceptability as described for urine. Acceptable sera were pooled, sterile filtered through a 0.45-μm Millipore filter, and stored at −20°C until used.

*Morphine sulfate* (USP, Merck), prepared as a 20 μg/ml stock solution in HCl (10 mmol/liter), was stored at 4°C. The stock solution was then diluted in the pooled human urine or serum to give 0, 10, 25, and 50 ng of morphine per milliliter. Prepared standards were stored at 4°C.

Bray Scintillation Fluid

A modified Bray scintillation fluid was prepared, consisting of 240 g of recrystallized naphthalene (Eastman Kodak), 32 g of “Omifluor” (New England Nuclear Corp.), 400 ml of reagent-grade methanol, and 80 ml of reagent-grade ethylene glycol diluted to 4 liters with p-dioxane (“Spectrograde”; Matheson, Coleman, & Bell). Standard 20-ml screw-
capped scintillation vials (Packard Instruments) were filled with 12 ml each of the Bray scintillation fluid, capped securely, and stored in the dark at room temperature until used.

Preparation of Test Reagent Tubes (10 × 75 mm Glass) for Use in the Assay

Each 10 × 75 mm glass reagent tube contained the following: 0.1 ml of undiluted normal goat serum, to stabilize the goat morphine antibody and to ensure that sufficient protein globulins were available to co-precipitate the antibody globulins after addition of (NH₄)₂SO₄; 0.1 ml of morphine goat antibody diluted in phosphate-buffered saline, to bind the desired amount of [³H]dihydromorphine; 0.02 ml of phosphate-buffered saline containing 2.92 ng of [7,8-³H]dihydromorphine with an activity of 7.3 nCi, or about 5500 to 6500 cpm; and 0.18 ml of phosphate-buffered saline (pH 7.2) containing sodium azide (1 mg/ml) to bring the total volume per tube to 0.4 ml. All tubes were filled and capped under aseptic conditions and stored at 4°C. Under these conditions the contents of the reagent tubes are stable for at least six months.

Test Procedure

0.1 milliliter of each morphine standard or specimen to be assayed was added to a reagent tube. The contents of the tubes were mixed with a vortex-type mixer and the tubes allowed to incubate for 1 h at 22°-26°C. Saturated (NH₄)₂SO₄ solution, 0.5 ml, was added to each tube, followed by vortex-type mixing. After 15 min of incubation at 22°-26°C to precipitate serum globulins, the tubes were centrifuged at 4000 × g for 15 min in a Sorvall SS-1 centrifuge with a fixed-angle rotor; 0.5 milliliter of clear supernatant fluid was withdrawn from each tube and added to a scintillation vial containing 12 ml of Bray’s solution. The contents of the vials were mixed on the vortex-mixer to fully disperse the sample and the vials were placed in the liquid scintillation spectrometer (Intertechique, Model SL-30) and allowed to equilibrate in the dark at 4°C for 30 min before counting. Each vial was counted for 2 or 4 min and cpm recorded. A standard curve was prepared, with cpm (unbound dihydromorphine) plotted on the Y-axis and ng of morphine per milliliter on the X-axis. The best line was then fitted through the points from 0 to 50 ng of the morphine standards per milliliter, diluted with either urine or serum.

The cpm for each unknown urine or serum was converted to nanograms of morphine equivalents per milliliter by use of the standard curve. The term morphine equivalents (ME) is used, because certain immunologically related substances will also give a positive reaction. Serum specimens having values greater than 50 ng ME per milliliter were not titrated further. If the unknown urine value was greater than 50 ng ME per milliliter, the urine was diluted 10- and 100-fold in saline and the test repeated. If the cpm value of the diluted urine was in the range of the standard curve, the concentration determined in nanograms per milliliter was then multiplied by the proper dilution factor to give total ME, in nanograms per milliliter. Urines having ME values higher than 5000 ng/ml were not titrated further. All urines and sera were assayed in duplicate or triplicate.

Results

Figure 1 depicts a typical standard curve derived from several determinations for a single set of urine standards tested against three batches of the same test reagents prepared at different times over a two-month period. A similar type of curve was obtained when the standards were prepared in serum. At higher morphine concentrations (100 or 200 ng/ml), the curve rapidly flattened, indicating that the antibody combining sites are saturated above the 50 ng/ml level.

To evaluate the sensitivity, specificity, and validity of the RIA, urines and (or) serum were obtained from the following populations:

(a) Individuals receiving no drugs, including alcohol, nicotine, and caffeine;
(b) Individuals who could have been using alcohol, nicotine, and (or) caffeine;
(c) Nonaddicts known to be receiving specific drugs; and
(d) Individuals known to be using heroin and (or) methadone.

The clinical results for these were as follows.

Establishing “Normal” Values

Urine. Because urines and sera obtained from individuals receiving no drugs could contain substances that cross-react with the antisera, it became critical to determine the range of values that one could expect in specimens obtained from individuals selected at random from a “normal” population. These data were accumulated over several months with specimens obtained from populations a and b described above.

![Fig. 1. Composite standard curve for free morphine](image-url)
A total of 38 urine samples were obtained from 19 individuals who belonged to a group that did not indulge in alcohol, caffeine, nicotine, or drugs of any description. The maximum apparent morphine (morphine equivalents) in any urine was 2 ng/ml, and 33 of the 38 showed no detectable morphine equivalents. Because this population is not representative of a “normal” population, samples were obtained from 171 individuals (population b) who presented themselves to the clinic of a large industrial concern for yearly routine or pre-employment physical examinations or because of minor illness. The samples were identified only by number and we made no attempt to obtain a medical history or follow-up. Each sample was divided into two aliquots and 166 of the 171 were analyzed using two separate pools of antisera. As shown in Table 1, the distribution of quantitative results varied slightly, in part as a function of the antisera pool. With either pool, however, no more than 3% of the samples exceeded an ME of 25 ng/ml. The single sample with an ME greater than 50 ng ME/ml probably was submitted either by an heroin addict or by an individual who had recently received a substance that strongly cross-reacts. Samples obtained from an additional 30 individuals (population b) known not to be receiving drugs other than alcohol, caffeine and (or) nicotine were analyzed and with one exception were found to have an ME of less than 25 ng/ml of urine. On the basis of all the data accumulated with the “normal” population, we selected a sensitivity of 25 ng ME per milliliter as the concentration we would use to discriminate between a “positive” and “negative” sample.

Sera. Similar studies with sera obtained from 30 individuals (population b) established that no more than 3% of these samples contained greater than 10 ng ME per milliliter. Accordingly, we selected this sensitivity to differentiate between a “positive” and “negative” serum specimen.

Volunteers on Known Drugs

Since the RIA is designed as a diagnostic screening test for morphine and morphine-like substances, it is important to examine the clinical effect of other drugs on the test system. Spector and Parker (1), Spector (7) and others (3-6) have shown that substances similar in structure to the immunizing antigens will cross-react in vitro or in vivo to various extents. If RIA results are to be properly interpreted, the substances producing positive values in an individual’s urine or serum should be known.

Because most drugs are excreted in the urine both unchanged and as metabolites, it appeared that the most direct approach would be to examine the urine of subjects receiving drugs orally rather than to perform the tests on normal urine to which known amounts of drugs had been added. Accordingly, one to five volunteers per drug submitted one or more urine samples 4 to 12 h after ingesting the usual dose of one of the drugs listed in Table 2. The number of volunteers receiving each drug is small, but no urine contained more than 25 ng ME per milliliter, so it is unlikely that any of these drugs significantly cross-react.

Because dextromethorphan (widely used as an ingredient in cough syrups) is structurally related to morphine and thus might cross-react in the assay, we determined if urine obtained from volunteers receiving the maximal recommended dose of this substance would appear positive in the RIA. Four volunteers were given 30 mg of dextromethorphan contained in cough syrup (“Romilar CF”) every 6 h for four consecutive days, and urines and sera were collected before medication, 1 and 5 h after the first dose of the day, 6 h after each subsequent dose, and 24 h after the last dose. As shown in Table 3, all predemedication specimens had ME’s of 10 ng or less per milliliter. During the 4-day medication period, the four subjects produced a total of 75 urine specimens, 52 of which had ME’s of less than 25 ng/ml and 23 of which had ME’s of 25 to 46 ng/ml. Twelve hours after the last dose, the ME’s were all less than 25 ng/ml. Not shown are the serum results obtained during the same study; the ME’s for all were less than 10 ng/ml of serum.

Table 1. Apparent Concentrations of Morphine in Urines of Normal Individuals, Tested against Two Different Lots of Antisera

<table>
<thead>
<tr>
<th>ng ME’s/ml</th>
<th>Lot A</th>
<th>%</th>
<th>Lot B</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–15</td>
<td>162</td>
<td>97.6</td>
<td>153</td>
<td>89.5</td>
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<td>3</td>
<td>1.8</td>
<td>14</td>
<td>8.2</td>
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<tr>
<td>25–50</td>
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<td>0</td>
<td>3</td>
<td>1.8</td>
</tr>
<tr>
<td>&gt;50</td>
<td>1</td>
<td>0.6</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a Results expressed as morphine (“morphine equivalents”).

Table 2. Healthy Volunteers Submitted Urine Samples within 24 h of Receiving One of the Drugs Listed

<table>
<thead>
<tr>
<th>Drugs Listed</th>
<th>Penta</th>
<th>Ambobarbital</th>
<th>Secobarbital</th>
<th>Phenobarbital</th>
<th>Meprobamate</th>
<th>Diazepam (Valium)</th>
<th>Diphenhydramine (Benadryl)</th>
<th>Phenytoin (Dilantin)</th>
<th>Chlorpromazine (Thorazine)</th>
<th>Chlorprophenpyridamine (Chlortrimeton)</th>
<th>Amitriptyline (Elavil)</th>
<th>Oxymetazoline (Afrin)</th>
<th>Chlorpheniramine and phenylpropanolamine (Orade)</th>
<th>Dextropropoxyphene (Darvon)</th>
<th>Pentazocine (Talwin)</th>
<th>Diphenoxyalate (Lomotil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</table>

Each sample contained less than 25 ng morphine equivalents per ml.

* In brackets: no. volunteers submitting samples.
In the survey of the additional 30 volunteers selected because they were not receiving any drugs (population b), one individual produced urines that occasionally had an ME >25 ng/ml. This individual had a dietary history that included a predilection for eating poppy seeds. Accordingly, to determine if poppy seeds contain cross-reacting substances, four volunteers each consumed about 15 g of poppy seeds in the form of a poppy-seed cake (baked 20 min at 100°C). During the subsequent 12 h of collection, the ME for urine obtained from three of the four volunteers was greater than 75 ng/ml; the ME for all samples from the fourth individual was less than 25 ng/ml. At present, we cannot explain these findings, because poppy seeds do not contain morphine. They do, however, contain narcotine and other as yet unidentified alkaloids (8), which could be responsible for the observed cross-reaction.

Heroin and (or) Methadone Users

Urine and (or) serum was obtained from a total of 110 patients applying for admission to a methadone clinic in Washington, D.C. The general nature of the clinic and population have been described by Du Pont (9). Criteria for inclusion in the study included: at least a year of daily heroin use; signs and symptoms of withdrawal when deprived of heroin; and the finding of needle marks adjacent to veins. Emphasis was placed on developing good rapport with the patient, to obtain a plausible estimate of the time elapsed between the last use of heroin and the collection of samples.

Many of the patients had received a small (10–20 mg) dose of methadone before submitting specimens. To eliminate the possibility that methadone or metabolites might cross-react in the assay, we obtained samples from an additional 22 patients who were selected from the regular clinic population on the basis of their long history of negative urines for morphine, as assessed by thin-layer chromatography. These patients were receiving orally 40 to 100 mg of methadone daily.

If sample volume was sufficient, the urine samples were analyzed by thin-layer chromatography in Dr. Bernard Davidow's laboratory at the New York City Bureau of Laboratories. The particular method used, which does not include an hydrolysis step, detects only free morphine (10). All of the specimens were processed in a double-blind design. Data plotted in Figure 2 show the concentration, in ME per milliliter, obtained by RIA and the results in terms of positive or negative by thin-layer chromatography for each urine sample as a function of time since admitted heroin use. With one exception, all the samples collected within 48 h of estimated heroin use contained more than 25 ng ME per milliliter, and a few samples were positive by RIA for as long as 78 h after heroin. Of the 71 total samples tested by RIA, 50 were also analyzed by thin-layer chromatography, and of these only 24 (or 48%) were positive for free morphine. Most of the urines positive by thin-layer chromatography had ME's of more than 1000 ng/ml by RIA and none of the samples tested by thin-layer chromatography were positive after 48 h. Considering the most important time interval, the initial 24 h since heroin, RIA failed to detect morphine equivalents in only 1 of 43 samples, while thin-layer chromatographic results were negative for 12 of 30 (40%) samples tested in the same interval.

Not shown in Figure 2 are the results for urine samples obtained from 30 patients who denied using heroin for at least one week; 22 of these 30 specimens were obtained from the reliable groups of patients who were receiving at least 40 mg of methadone daily. All 22 specimens had ME's of less than 25 ng/ml. In contrast, the other eight specimens were obtained from new applicants, and all eight were found to have ME's corresponding to 30 to 4500 ng/ml. Four of the eight had ME's of more than 200 ng/ml and were positive by thin-layer chromatography, so the patients submitting these samples probably supplied false information concerning the time since their last use of heroin. The other four, who had ME's of 30 to 200 ng/ml, might also have been untruthful, or it is even possible that these individuals excreted small amounts of morphine or metabo-
agglutination gave 0.0. three 6.

Results expressed as morphine ("morphine equivalents")

Discussion

The extreme sensitivity of the RIA is advantageous at least in two ways: (a) a negative result for urine is very strong evidence that clinically significant amounts of morphine or metabolites are not present, and, therefore, additional analytical procedures are not necessary; and (b) morphine can be detected for longer periods of time than with less sensitive methods. It is apparent, however, that certain drugs and substances may cause cross-reactions and that additional experience with the test may reveal other cross-reacting compounds. Therefore, to absolutely establish heroin usage, a nonserologic confirmatory test should be used.

In addition, the data clearly show that for the purpose of detecting recent heroin use, urine specimens are superior to serum.

Finally, from the data presented it is evident that the RIA described in this communication is a sensitive, simple test which can be carried out in less than 2 h, and thus would seem to be quite suitable for large-scale testing of urine or serum.

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References