Detection of Morphine in Urine by Hemagglutination Inhibition, with Use of Lyophilized Reagents

Giulio Vanzetti, Mario Cassani, and Dante Valente

We describe a modified test of hemagglutination inhibition for the detection of morphine in urine, similar to the well-known test for pregnancy. The reaction takes place in test tubes or ampoules containing carefully matched amounts of lyophilized morphine antiserum and tanned human erythrocytes coated with morphine conjugated to rat serum albumin. The reagents are reconstituted by adding 100 µL of urine and 400 µL of water, and the result is read after 60 min. The detection limit, tested with the method of Gorodetzky (Clin Chem 19: 753, 1973), was about 200 ng of total morphine per milliliter of urine. For more than 2000 samples, results by our test agreed satisfactorily with those obtained by an accepted RIA method. The test is suitable for rapid screening in field work, monitoring subjects during detoxication, and use in nonspecialized laboratories. Confirmatory analysis is needed for quantitative measurements, forensic purposes, and discrimination between morphine and cross-reacting opiates.

Additional Keyphrases: abused drugs • opiates • screening • prototype kit

Many different methods are available for assay of illicit drugs in biological fluids; most of them require expensive equipment that often is lacking in nonspecialized laboratories. There is a need for simple methods suitable for untrained operators.

In 1971 Adler and Liu (1, 2) described a simple immunological test for morphine in urine. In that test, tanned sheep erythrocytes coated with rat serum albumin—morphine are agglutinated with a properly diluted morphine antiserum in microtiter plastic cups; if morphine is present in the urine added, it will bind the antiserum and inhibit agglutination. They developed similar hemagglutination-inhibition (HI) tests (3) for detection of other drugs of abuse, such as methadone, in urine.

These tests are inexpensive, sensitive, reasonably specific, and require no instrumentation. Even so, they are seldom used in the clinical laboratory. According to expert opinion and to our own experience, the quality control of the commercial reagents available so far has been variable (4, 5), which may in part explain the limited success of the test. Because well-documented information on the validity of the HI technique for morphine in urine has been scant (4, 6, 7), we thought it worthwhile to evaluate an updated, carefully standardized version of the HI test and compare results with an established RIA procedure.

At our request, a specialized manufacturer prepared the morphine antiserum and the sensitized erythrocytes, and lyophilized them in carefully matched amounts in glass tubes for individual assays. We applied this modified test to the detection of morphine in more than 2000 urine specimens, which we assayed simultaneously with the HI test and with a sensitive RIA procedure. We also evaluated the sensitivity of the HI test by the method of Gorodetzky (8, 9), with appropriate calibration standards.

Materials and Methods

Specimens. Urine specimens were obtained from several sources: hospitalized heroin addicts treated for or recovering from acute intoxication; outpatients taking part in a heroin detoxification program with methadone or clonidine; normal, nonaddicted subjects; and hospital patients not being treated with narcotics.

Reagents. The lyophilized reagents for the HI test—a bottom layer containing the buffered morphine antibody, and a top layer containing tanned human erythrocytes coated with rat serum albumin—were supplied in rubber-stoppered glass test tubes or in ampoules by Boehringer-Biochimia-Robin Research Laboratories, Monza, Italy.1

Morphine 3-glucuronide and 6-glucuronide were prepared by Boehringer-Biochimia-Robin; samples of codeine, dihydromorphine, ethylmorphine, hydromorphone, oxycodone, nalorphine, and naloxone were donated by Salars, Como, Italy. The purity of these products was verified by thin-layer chromatography.

The "Abuscreen" reagents for morphine RIA and a sample of dextromethorphan were supplied by Roche, Milano, Italy.

Calibration standards. To approximate the ester/free-drug ratio of urinary morphine, we prepared a stock solution containing morphine 3-glucuronide and morphine hydrochloride in distilled water in a 9/1 molar ratio, the final concentration of morphine being 1 g/L.

By diluting this stock solution with a pool of drug-free urine we prepared seven calibration standards containing 160, 170, 180, 190, 200, 210, and 220 ng of total morphine per milliliter. We used these to assess the sensitivity of the HI test (9).

To compare results of the HI test and the RIA, we tested urine specimens, usually in duplicate, after centrifugation for 10 min at 3000 rpm or filtration through ordinary filter paper.

For the HI test, we reconstituted the lyophilized reagents by adding 100 µL of urine and 400 µL of de-ionized water to each test tube, stirring manually, and incubating for at least 60 min at room temperature. Carefully avoid further stirring, which would disturb agglutination. Results, which did not change for at least 12 h, were rated positive if there was a distinct red ring at the bottom of the test tube, or negative if the ring was absent, atypical, or barely visible. Figure 1 shows typical results for negative and positive specimens.

The RIA used was the Roche procedure (Abuscreen Reagents, morphine detection limit 40 ng/mL); results were quantified from a standard curve and expressed as concen-

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1 Since this paper was submitted, Boehringer-Biochimia-Robin has marketed these reagents, extensive work done in many laboratories has produced no false positives, and very few (<1%) false negatives.
The detection limit was further approximated by testing concentrations intermediate between those giving the last positive and the first negative result. For the Roche antiserum, the cross reactivity was estimated by RIA with the Abuscreen reagents.

RR*: relative reactivity = 100 divided by the compound’s molar equivalent of morphine.

Molar reactivity equivalent to 1.0 mol of morphine.
Table 2. Results Compared for 2265 Urine Specimens by HI and by RIA

<table>
<thead>
<tr>
<th>Concen by RIA, µg/L</th>
<th>Positive by HI</th>
<th>Negative by HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;200</td>
<td>1129 (99.8%)</td>
<td>2 (0.2%)</td>
</tr>
<tr>
<td>100–200</td>
<td>164 (81%)</td>
<td>39 (19%)</td>
</tr>
<tr>
<td>40–100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0–40</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
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Of about half of the specimens in this group classified as negative there was either a barely visible red ring or an atypical ring with irregular borders.

Table 3. Drugs Tested for Possible Interference with HI

<table>
<thead>
<tr>
<th>Concen, mmol/L</th>
<th>Phenylethylbarbituric acid</th>
<th>Diethylbarbituric acid</th>
<th>Phenytoin</th>
<th>Amphetamine</th>
<th>Dimethophrine</th>
<th>Promethazine</th>
<th>Trrazodone</th>
<th>Promazine</th>
<th>Chlorpromazine</th>
<th>Diazepam</th>
<th>Lorazepam</th>
<th>Fluorazepam</th>
<th>Nitrazepam</th>
<th>Flunitrazepam</th>
<th>Chlorndimethyl Diazepam</th>
<th>Metoclopramide</th>
<th>Amtriptilene</th>
<th>Clonidine</th>
<th>a-Methyl dopa</th>
<th>Acetaminophen</th>
<th>Chloroqine</th>
<th>Thioclochicoside</th>
<th>Tetracycline</th>
<th>Chloramphenicol</th>
<th>Acetylsalicylic acid</th>
<th>Gentisic acid</th>
<th>Theophylline</th>
<th>Ascorbic acid</th>
</tr>
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<tr>
<td>6.5</td>
<td>5.4</td>
<td>3.9</td>
<td>7.4</td>
<td>1.9</td>
<td>1.6</td>
<td>1.3</td>
<td>1.6</td>
<td>7.9</td>
<td>3.5</td>
<td>3.2</td>
<td>1.65</td>
<td>1.8</td>
<td>3.3</td>
<td>1.6</td>
<td>1.4</td>
<td>1.6</td>
<td>0.2</td>
<td>4.7</td>
<td>30.0</td>
<td>20.0</td>
<td>0.9</td>
<td>1.5</td>
<td>15.0</td>
<td>6.7</td>
<td>2.6</td>
<td>5.5</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

Concentrations tested were at least 25-fold the therapeutic concentration in plasma.

(b) Of the 203 specimens found to contain between 100 and 200 µg/L by RIA, 81% were positive by the HI test.
(c) All 931 specimens found to be negative or to contain <100 µg/L by RIA were negative by HI.

On the basis of the RIA results, the detection limit of our HI test system for urinary morphine was set at 200 µg/L, although most specimens containing 100 to 200 µg/L also gave positive results. This value is close to the detection limit estimated by the method of Gorodetsky: 99% = 215 (200–231, p = .95) µg/L. The difference may be ascribed to the different composition of our calibration standards, as compared with the average composition of urinary morphine metabolites, and to the different reactivity of the Roche and Boehringer antisera.

Interferences. Aside from cross-reacting opiates, we found no endogenous or exogenous compounds interfering with our modified HI test.

We tested the drugs listed in Table 3 for possible interference: the list includes several psychoactive drugs, some commonly used by drug addicts and others prescribed to them during rehabilitation. No interference was detected. We also assayed many pathological urine specimens from hospitalized patients not receiving narcotics; these included highly pigmented urine from icteric patients (30 specimens), urine with gross proteinuria (20 specimens), and hematuria (20 specimens). All gave negative results both with the HI test and with RIA. On the other hand, added morphine in a final concentration of 100 µg/L was easily detected, even in these specimens.

Suspended cells or particles may interfere, so centrifugation or filtration of urine is essential; its omission may lead to unreliable results. Bacterial contamination must be avoided; if the urine becomes strongly alkaline (pH 8), results may be falsely positive.

Discussion

HI tests have been remarkably successful in the assay of urinary constituents, e.g., choriongonadotropin in a popular test for pregnancy (11-14). We believed that a similar test with stable, conveniently lyophilized reagents for morphine in urine might prove useful in the clinical laboratory, but the procedure needed validation by comparison with an established quantitative method of sufficient sensitivity.

To our knowledge, the only paper dealing with the assay of drugs subject to abuse in which the HI test for morphine in urine was evaluated extensively by comparison with other analytical procedures in current use was published in 1974 (4).

In that investigation, which involved assaying 422 urine specimens, the HI test and other sensitive immunoassays (with detection limits of either 500 or 30 µg/L, for two sensitivity levels) were compared with a thin-layer chromatographic procedure of limited sensitivity (lowest detectable concentration, 1–5 mg/L).

Thus the immunoassays (including HI) gave a large number of "false positives"; but the authors themselves noted that most were probably true positives of low concentration not detectable by thin-layer chromatography.

To avoid that risk, we used as a comparison method a sensitive RIA test with Abuscreen reagents, whose detection limit was much lower than that of our HI test.

We also modified the HI technique to optimize reaction conditions. The main characteristics of our HI test as compared with the original procedure of Adler and Liu, are:

- in our test, lyophilization in the presence of phosphate buffer and EDTA ensures reagent stability and consistency of results;
- rigorous protocols were enacted for the preparation and control of all reagents;
- instead of rabbit, we use human erythrocytes, which settle more rapidly;
- we use a fivefold reaction volume with a higher concentration of erythrocytes and glass test tubes or ampoules instead of Microtiter plastic cups; this ensures easy and clearcut final reading; and
- compared with the current antisera raised with bovine serum albumin—carboxymethylmorphine as immunogen, the Boehringer antisera raised in rabbits with rat serum albumin—morphine glucuronide have a different cross-reaction profile; results obtained with selected antisera of this kind have been quite consistent.

Our variant is less sensitive to urinary morphine than is the original test of Adler and Liu. Our detection limit is 200 µg of total morphine per liter or 50 µg of nonesterified morphine per liter, as opposed to 20 µg of nonesterified morphine per liter in the original test (2). We tried to lower our detection limit but found that the test became less reliable. The sensitivity of the modified test, however, still
exceeds that of other nonradioactive, commercially available tests (e.g., 500 μg/L for enzyme immunoassay), and is adequate for most purposes.

The assessment of sensitivity by the method of Gorodetzky deserves comment. As a rule, the sensitivity of the HI system for urinary morphine has been estimated by testing free morphine solutions; but only a minor proportion of morphine, about 10%, is present in urine in the free form. Most is excreted as the 3-glucuronide, with smaller amounts of the sulfate and 6-glucuronide conjugates (15). On the other hand (Table 2), the Boehringer antisera have much less affinity for the esters than for nonesterified morphine; the amount of 3-glucuronide needed for a given hemagglutination-inhibition activity is about five or six times greater, and that of the 6-glucuronide is about 20 times greater, than that of the free drug.

To compensate, at least partly, for the different reactivities of nonesterified morphine and its esters, and to approximate the ester/free drug proportion found in human urine, we used for the calibrating solutions a 9:1 mixture of morphine 3-glucuronide and morphine hydrochloride (molar equivalents). We found a value of about 215 μg/L for 99% of runs, representing "sensitivity" in Gorodetzky’s definition, in reasonably good agreement with sensitivity as assessed by comparison with RIA results.

Interestingly, a modest 37% increase in the morphine content of the calibrating solutions (from 160 to 220 μg/L) was enough to swing results from 100% negative to 100% positive; the concentration range in which replicate tests may give discordant results is narrow. For a qualitative test with subjective reading, this seems an argument in favor of consistency.

Variations in the quality of commercial reagents prepared by different manufacturers may explain the heretofore limited success of the HI test for morphine. Our findings, at any rate, show that with rigorous control and standardization of reagents the HI test yields results in excellent agreement with those of a generally accepted RIA method.

The HI test yields only qualitative information—an all-or-none response—although it could give semiquantitative data with stepwise dilutions of urine. For truly quantitative results, other immunoassays are recommended. Like other immunoassays, the HI test is not strictly specific for morphine, because the antisera cross react with other opiates. For discrimination, chromatography is in order.

Despite these limitations, the HI test has the undeniable merit of being technically simple and not requiring complex equipment. We conclude that through the use of well-matched, lyophilized reagents, and careful standardization and control of each reagent lot with positive and negative urine specimens, the HI test for morphine gives definite and reliable results. The HI technique may therefore prove useful in many circumstances, as in field work for rapid screening of addicts, monitoring of subjects during detoxication, emergency work, and use in nonspecialized laboratories.

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References