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Comparison of Results for Quantitative Determination of Morphine by Radioimmunoassay, Enzyme Immunoassay, and Spectrofluorometry

In 1970, Spector and Parker [*1*] reported the production of antibodies to morphine as a 3-0-carboxymethoxymorphine-bovine serum albumin. They described a method for quantitative determination of morphine in serum by a competitive binding assay using the antimorphine antibody and radio-labeled morphine (radioimmunoassay) [*2*].

Subsequently, many new methods for morphine detection using immunological reagents have been developed. These techniques include enzyme immunoassay (EMIT®) [*3*], free radical assay (FRAT®) [*4*], and hemagglutination inhibition (HI) [*5*], in addition to radioimmunoassay (RIA).

The major contribution of the immunoassay tests to drug testing is their ability to detect drugs in urine hours or days after injection or ingestion of the drug. The greater sensitivity of the immunoassay tests compared to other methods for morphine detection (thin-layer chromatography, fluorometry, gas chromatography) has been well documented [*6–10*]. These studies have also investigated the incidence of false positives and false negatives, the selectivity of the immunological reagents, and the possible interfering drugs.

In this study we investigated the quantitative results (accuracy and precision) of the two immunological assays, RIA and EMIT®, as compared to two spectrofluorometric methods involving extraction. A variety of physiological samples encountered in morphine testing was studied. These samples included random urine from a methadone maintenance clinic and postmortem urine, blood, bile, brain, and lung tissue from heroin-induced or heroin-related deaths.

Materials and Methods

Samples

Random urine samples were collected from patients in a methadone treatment clinic for heroin addicts in Orange County, Calif. The samples were assigned a code number and split. Aliquots were assayed by RIA and EMIT® at the Beckman Instruments

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Radioimmunoassay Center and by fluorometry at the Orange County Coroner's Office Laboratory.

Coded aliquots of postmortem blood, urine, bile, brain, and lung tissue were sent to the immunoassay laboratory following analysis by fluorometry by the two participating forensic laboratories.

A check sample of morphine in whole blood was made up by a third party and sent as an unknown to the three participating laboratories as one of a continuing series of proficiency samples exchanged between toxicology laboratories in California. The sample was prepared by adding sodium secobarbital (Smith, Kline & French), diazepam (Roche), and morphine sulfate (Lilly) dissolved in small amounts of water and ethyl alcohol, to outdated blood bank blood with stirring and then stored under refrigeration. Concentrations of added drugs in the final solution were 10 μg/ml of secobarbital, 2.0 μg/ml of diazepam, and 1.15 μg/ml of morphine.

Radioimmunoassay

The reagents for radioimmunoassay of morphine, Abuscreen™ for morphine-^{3}H and Abuscreen™ for morphine-^{125}I, were obtained from Roche Diagnostics, Hoffmann-LaRoche, Inc., Nutley, N.J. 07110.

The tritiated kit was used without modification according to the manufacturer's suggestions for quantitative assay. The supernatant was counted on a Beckman Model LS-350 liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif. 92664).

The iodinated kit was used according to the manufacturer's suggestions for quantitative assay with two modifications: (1) a 30-min to 1-h incubation after addition of the antibody of the sample (in the sequential saturation procedure) and (2) an overnight incubation after adding the labeled morphine were added to the procedure. In June 1974, the manufacturer changed the instructions from a sequential saturation procedure to an equilibrium procedure. For the equilibrium procedure, the first incubation was omitted. The overnight incubation was retained, as it was found that the maximum binding was increased 20% by allowing a 16-h incubation, instead of the 10-min incubation found in the manufacturer's protocol. The supernatant was counted on a Beckman Biogamma counter (Beckman Instruments, Inc., Irvine, Calif. 92664).

The tritiated RIA and the iodinated RIA were compared by assaying duplicate urine samples. The values of duplicate pairs agreed well within the interassay repeatability in every case. Both random urine and postmortem urine samples were assayed.

In general, the tritiated radioimmunoassay gave a steeper standard curve (that is, a steeper slope of concentration plotted versus counts per minute) and, hence, better precision than the iodinated radioimmunoassay. Although the iodinated morphine radioimmunoassay required an overnight incubation, it was more convenient than the tritiated radioimmunoassay because many of the samples encountered in morphine testing caused quenching in liquid scintillation counting, which is avoided in gamma counting [*11*]. The results described in this paper for radioimmunoassay determination are those obtained with the ^{125}I-labeled radioimmunoassay for opiates.

Standards and blood samples were diluted as required with normal serum (Hyland Laboratories, Division of Travenol Laboratories, Costa Mesa, Calif. 92626). Urine samples were diluted with drug-free normal urine provided in the Abuscreen™ kit.

Enzyme Immunoassay

The reagents for enzyme immunoassay of opiates were obtained from Syva Corp., Palo Alto, Calif. 94304, and used according to the manufacturer's instructions. Absorbance was read at 436 nm on a double-beam spectrophotometer using a heated sipper cell (Beckman Model 25, Beckman Instruments, Inc., Irvine, Calif. 92664).

Urine and bile samples were diluted with the buffer supplied with the EMIT® reagents, as needed to bring the drug concentration into the range of the standard curve.

Spectrofluorometry

Two spectrofluorometric methods were used. In Laboratory 1, 15 ml of blood, 15 g of brain, or 15 g of lung tissue were homogenized with 30 ml of saturated sodium bicarbonate solution. A 20-ml sample of urine or all the bile was hydrolyzed with 1/30 its volume of concentrated H_2SO_4 at 15 psi for 15 min, then saturated with $NaHCO_3$. The following procedure was used for extraction and washing of the sample. Extract with chloroform:isopropanol (4:1), extract the organic phase twice with 0.5*N* NaOH, wash the aqueous layer with chloroform, and discard the chloroform. Acidify the aqueous layer with 1 ml of concentrated HCl, wash with chloroform, and discard the chloroform. Saturate the aqueous layer with sodium bicarbonate and extract with 25 ml of chloroform:isopropanol (4:1). At this point, one tenth of each urine and bile sample was analyzed by fluorometry and the remainder set aside for analysis by other methods. Extract the organic phase with 1.5 ml of 0.2*N* H_2SO_4. Place 1.0 ml of the aqueous layer in a 12-ml centrifuge tube and add 2.0 ml of amino-methyl propanol (AMP) buffer. To prepare the AMP buffer, add 25 ml of 2-amino-2-methyl-1-propanol and 10 ml of concentrated HCl to 400 ml of water, allow to cool, and dilute to 500 ml. Mix the sample and buffer, pour into a cuvette, and place the cuvette in the fluorometer.

Fluorescence spectra and emission spectra of the solution are recorded following Reynold's [*12*] modification of the Goldbaum et al [*13*] procedure on a Perkin-Elmer Model MPF-2A fluorometer (Perkin-Elmer, Norwalk, Conn. 06856).

In the Laboratory 2 study, most of the samples used consisted of 5 ml of blood or 5 ml of urine and were not hydrolyzed. The sample pH was adjusted to 9.0 extracted with chloroform:isopropanol (4:1). The chloroform was washed with 0.02% borate buffer and then extracted with pH 4.6, 0.2*M* phosphate buffer. The aqueous layer was used for fluorometric determination using the Goldbaum et al [*13*] procedure on a Perkin-Elmer Model MPF-2A fluorometer.

Results

Immunoassay Precision and Recovery

The coefficient of variation of the calibration curve for the EMIT® assay was found to average 4.82% using ten duplicate determinations of four points on the curve. As shown in Table 1, an average of 0.45 μg/ml was recovered from a urine to which 0.50 μg/ml morphine (90% recovery) was added with a standard deviation (SD) of 0.057 μg/ml and a coefficient of variation (CV) of 12.7%. Repeatability was about ±10% on samples assayed again on different dates with different lots of reagents.

TABLE 1—*Precision and recovery for morphine.*

Radioimmunoassay (RIA)		
Standard Addition, ng/ml		25
Recovery, ng/ml		22.3
N = 20	47,789 ± 1479.8 SD cpm (3.0% CV)	
	22.3 ± 5.87 SD ng/ml (26.3% CV)	
Enzyme Immunoassay (EMIT®)		
Standard Addition, μg/ml		0.50
Recovery, μg/ml		0.45
N = 20	53.6 ± 3.7 SD EMIT® Units (6.9% CV)	
	0.45 ± 0.057 SD μg/ml (12.7% CV)	

The coefficient of variation of the calibration curve for the RIA assay was found to average 2.85% using ten duplicate determinations at four points of the curve. As shown in Table 1, an average of 22.3 ng/ml was recovered from a urine sample to which 25 ng/ml morphine (90% recovery) was added with a standard deviation of 5.87 ng/ml and a coefficient of variation of 26.3%. The values on samples assayed again on different dates with different lots of reagents differed by as much as ±50% of the average value over a year of study.

Comparison of Results: Urine Samples

Since different laboratories and different analysts were involved in the comparison, as well as different methods, the criterion for agreement was based on interassay repeatability, rather than on the intra-assay standard deviations. The values were considered in agreement if the values for morphine by one method plus or minus the repeatability of that method overlapped the value by the second method plus or minus the repeatability of the second method.

Comparing the enzyme immunoassay to the radioimmunoassay on 51 urine samples, the values agreed in 41 cases and did not agree in 10 cases. Six of the disagreeing cases involved codeine. The presence of codeine caused false high values in both immunoassays, with the enzyme immunoassay showing much higher values. Where we have quantitative data on the codeine concentrations, the immunoassay results were not the simple weighted sum of the morphine and codeine concentrations, but were much higher. The presence of naloxone in another sample gave a positive, but unequal, response to the immunoassays. The cause for disagreement in the remaining three samples could not be explained at this time.

There was no difference in analytical characteristics between random and postmortem urine samples.

Comparing the immunoassay results to the spectrofluorometric results on the 38 urine samples from Laboratory 1, there was agreement in only about one half of the cases (21 agreed, 17 disagreed). In 8 cases the immunoassays gave positive values not found by fluorometry. Positive values found were in agreement in 13 additional cases and disagreed in 9 cases. Disagreement was apparently due to the presence of codeine in 7 of these disagreeing cases, as determined by independent tests.

The 15 urine samples from Laboratory 2 were not hydrolyzed; therefore, only free morphine was determined. As expected, the immunoassay values for total morphine were much greater than the values for free morphine by spectrofluorometry. In 8 cases the results using fluorometry (Laboratory 2) were much lower than the immunoassay results, or were reported as "not detected" where immunoassays were able to detect morphine or other opiates present. In three cases codeine was present, causing false high values for the immunoassays. Case history revealed that in an additional case, naloxone was present, causing a false positive immunoassay result. In 3 cases the values were the same for the immunoassays and the fluorometry assay.

Table 2 shows some of the typical values obtained by these methods on random urine samples. The values in Line 7 were typical of the false high values obtained when codeine was present. The lower values found by fluorometry in urine samples were not unexpected because previous studies [*12-14*] have recognized the presence of interfering substances in urine samples which quench fluorescence.

Comparison of Results: Blood Samples

The enzyme immunoassay was not applicable to postmortem blood samples because of the intense color due to hemolysis. The hemolysis in postmortem blood and tissue samples also made use of the tritiated morphine radioimmunoassay difficult. Although

TABLE 2—*Typical results of methadone clinic random urine samples.*

Sample	EMIT®, μg/ml	RIA, μg/ml	Fluorometry, μg/ml
1	3.4	3.50	0.8
2	ND	ND	ND
3	7.8	8.8	6.4
4	4.0	3.0	0.5
5	1.3	0.4	ND
6	9.2	10.0	9.6
7	250.0	244.0	1.9
8	3.0	0.80	ND
9	3.8	5.2	ND
10	1.0	1.55	ND

ND = not detectable.

the liquid scintillation count rate can be corrected for quench error [11], the low counting efficiencies increase the uncertainty of the count rates obtained and require longer counting times.

Comparing the radioimmunoassay results to the amphoteric extraction fluorometry results using 20 ml of blood (Laboratory 1) for 26 cases, 20 results agreed, 4 results disagreed, and in 2 cases the radioimmunoassay detected morphine or opiates which the fluorometric method did not detect. In one of the disagreeing cases, codeine was known to be present and accounts for the false high value found by the radioimmunoassay method.

Comparing the radioimmunoassay results to the base extraction fluorometry results (Laboratory 2) for 34 cases, in 7 cases the results agreed, in 13 cases the radioimmunoassay detected morphine or opiates which were not detected by the fluorometry method, in 6 cases traces found by emission spectra and not confirmed by the excitation spectra in fluorometry (as used by this laboratory) were confirmed by radioimmunoassay, and in 8 cases the values obtained did not agree. In 2 of the disagreeing cases, codeine was known to be present, which resulted in false high values by radioimmunoassay. In a third case in which the radioimmunoassay gave a positive result and the fluorometry detected no morphine, the deceased had received naloxone, a narcotic antagonist. These cases are discussed below.

Results of two blood samples from Laboratory 2 in which different values were obtained by radioimmunoassay were rechecked by both Laboratory 1 and Laboratory 2 each using the amphoteric extraction with fluorometry. The two fluorometry values were in agreement with each other and with the value found previously. This suggests that the differences observed are truly due to the nature of the methodology, rather than the laboratory technique. These cases are discussed below.

Comparison of Results: Bile

Bile samples were diluted with buffer supplied with the EMIT® reagents for the enzyme immunoassay and with negative control serum for the radioimmunoassay. Of the eight samples assayed, the radioimmunoassay and the enzyme immunoassay values agreed in five cases, disagreed in two, and in one case the sample color caused such high background absorbance that EMIT® could not be used. Of the eight samples, immunoassay and fluorometry values agreed in six cases and disagreed in two.

Comparison of Results: Brain and Lung Tissues

While only six tissue samples were compared, the results were in satisfactory agreement in all but one case. This is encouraging since different tissue sections from each organ were used by the different laboratories. The values obtained are shown in Table 3.

TABLE 3—*Results of tissue samples.*

Tissue	Radioimmunoassay, μg/g	Fluorometry, μg/g
Brain	0.054	0.05
	0.059	0.10
	0.025	0.02
Lung	0.356	0.21
	0.216	0.14
	0.108	0.01

Results of Interlaboratory Check Sample

The check sample made up by a third party and sent as an unknown to the three participating laboratories contained 1.15 μg/ml morphine as the free base, in whole blood. Radioimmunoassay gave an average value of 1.20 μg/ml with a range from 0.98 μg/ml to 1.40 μg/ml for five determinations on five different days. Laboratory 1, using the amphoteric extraction and fluorometry, found 0.94 μg/ml. Laboratory 2, using a total base extraction and fluorometry, found 1.17 μg/ml.

The good agreement of the values by the different laboratories and the different methods on this spiked sample show that all the participating laboratories in the study were in good control of their methods and gave accurate answers.

Case Histories

Case 1—A 23-year-old male hanged himself in county jail during heroin withdrawal. The deceased had received Narcan® (naloxone HCl—a narcotic antagonist), bicarbonate, and epinephrine. No morphine was found in the lung, urine, or blood by fluorometry. A level of 30 μg/ml was found in the bile by fluorometry. However, the immunoassay tests gave opiate concentrations of 0.39 μg/ml (enzyme immunoassay) and 0.14 μg/ml (radioimmunoassay) in the urine and 0.011 μg/ml (radioimmunoassay) in the blood.

Naloxone (Narcan® injection) was diluted with serum and tested for cross-reactivity in the radioimmunoassay. The relative reactivity compared to morphine (reported as 1.00) was 0.001, in vitro. This low cross-reactivity would lead one to expect that naloxone would not interfere in the radioimmunoassay for morphine. However, the above radioimmunoassay results, which were confirmed by an independent laboratory, suggest that naloxone and its metabolites cross-react with the immunoassay antibody in vivo in physiologic urine and blood samples.

Case 2—A 19-year-old male was found with a syringe and other paraphernalia adjacent to the body. There was a strong suspicion that this was a suicide using heroin. Brain, lung, and bile were all positive for morphine by fluorometry. Morphine levels in the blood were 0.07 μg/ml (fluorometry) and 0.06 μg/ml (radioimmunoassay). Morphine levels in the urine were 3.3 μg/ml (enzyme immunoassay), 2.4 μg/ml (radioimmunoassay), and 2.4 μg/ml (fluorometry). Values by all methods were in agreement.

Case 3—A 19-year-old male was found in his residence in full rigor. The codeine concentration in the blood was 0.90 μg/ml and the morphine concentration was 0.89 μg/ml by fluorometry. Radioimmunoassay gave 18.4 μg/ml of total opiates. In the urine, 33.0

μg/ml of codeine and 1.37 μg/ml of morphine were found by fluorometry. Enzyme immunoassay gave a level of 1150 μg/ml and radioimmunoassay gave a concentration of 242 μg/ml of opiates.

This case illustrates the false high values found with immunoassays when codeine is present. These values are greater than the sum of the antibody response to morphine and codeine.

Case 4—A 29-year-old male died within three hours after purchase of heroin. Powder near the body assayed as 4.6% heroin, 21.0% procaine. Lung and bile were positive for morphine by fluorometry and brain tissue gave 0.9 μg/g of morphine. Morphine concentrations in blood were 0.01 μg/ml (fluorometry) and 0.06 μg/ml (radioimmunoassay). No morphine was detected in the urine by fluorometry or by enzyme immunoassay, but 0.050 μg/ml was detected by radioimmunoassay.

Case 5—Witnesses reported that the deceased, a 23-year-old male, collapsed and died in less than one hour after the injection of heroin. Brain and lung were positive for morphine and bile gave 3.2 μg/ml by fluorometry. Blood levels were 0.02 μg/ml (fluorometry) and 0.060 μg/ml (radioimmunoassay). Morphine was not detected in the urine by fluorometry or by enzyme immunoassay, but 0.100 μg/ml opiates were found by radioimmunoassay. A blood alcohol concentration of 80 mg/dl was also found.

Case 6—A 21-year-old female died within three hours after heroin injection. In the blood, 0.01 μg/ml of morphine was found by fluorometry and 0.533 μg/ml by radioimmunoassay; in the brain, 0.05 μg/g (fluorometry) and 0.545 μg/g (radioimmunoassay); and in the lungs, 0.21 μg/g (fluorometry) and 0.36 μg/g (radioimmunoassay). Morphine was also found in the bile. No other drugs were found in the blood. Results by radioimmunoassay were much higher than results by fluorometry, except in the lung.

Conclusion

The agreement of the participating laboratories on the spiked check sample and other proficiency samples suggests that the differences described in this paper are due to the different behavior of spectrofluorometry and immunoassay procedures when complex physiological samples containing unknown metabolites are analyzed.

The two immunoassays, although using very different detection methods (absorbance versus nuclear counting) appear to detect the same morphine species. False high values were obtained with the immunoassays in cases in which codeine was present due to cross-reactivity of the codeine with the morphine antibody. In addition, naloxone was found to interfere by giving false positive values in urine samples by the immunoassays, even though we found that the relative cross-reactivity of naloxone in vitro was only 0.001 (relative to morphine as 1.00). High values by the immunoassays in many cases may have been caused by other common drugs listed by the reagent manufacturer [*15*] as cross-reacting with the antimorphine antibodies, such as meperidine (0.002), dextromethorphan (0.001), chlorpromazine (0.001), oxycodone (0.002), and hydrocodone (0.002).

The immunoassay values were often higher than the fluorometry values. This may have been because the immunoassays detect total morphine, that is, both free morphine and morphine glucuronide. Since the biological fluids and tissue homogenates are assayed directly by the immunoassays, they do not reflect the possible loss due to extraction efficiency, which lowers the fluorometry values.

Extraction procedures do not achieve 100% extraction efficiency due to partition coefficients, trapping of the morphine due to clotting of the sample, absorption, and existence of the morphine as the water-soluble glucuronide. The immunoassays are applied directly to unextracted biological fluid or tissue homogenate and react with all opiate-condensed ring structures, regardless of their conjugation with glucuronic acid or other metabolic alteration in oxidation state.

Quenching interferences in the fluorometric assay, which are not well understood, may also have been responsible for the lower values by fluorometry.

The optical methods—spectrofluorometry, tritiated radioimmunoassay using liquid scintillation counting, and the enzyme immunoassay using absorbance—are all subject to optical interferences due to quenching of various types. On the other hand, the iodinated radioimmunoassay was not affected by optically absorbing sample components, did not require extraction, and was easiest to use.

In conclusion, the immunoassay techniques for morphine provide a sensitive test for the detection of morphine concentrations which is comparable to and complementary to the fluorometric methods.

Summary

The quantitative results (accuracy and precision) for determination of opiates by radioimmunoassay (RIA), enzyme immunoassay (EMIT®), and spectrofluorometry on split samples are compared. A variety of physiological samples were studied, including random urine from a methadone maintenance clinic and postmortem urine, blood, bile, brain, and lung tissue from heroin-induced or heroin-related deaths. The opiate concentrations detected by the two immunoassay methods were in good agreement with each other in the absence of interfering substances which are believed to react with the antimorphine antibodies. The immunoassay results were in agreement within the relative standard deviation with the fluorometry results in 55% of the urine samples and 80% of the blood samples.

The immunological methods are superior to fluorometry for quantitation of morphine in urine samples due to quenching interferences in fluorometry from urine. They were comparable to fluorometry for quantitation of morphine in blood samples.

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