Original Article

Comparison of ELISA and TLC Methods for the Morphine Detection in Urine of Drug Abusers

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ABSTRACT

Background: The current study was conducted to compare ELISA with thin layer chromatography (TLC) methods for diagnosis of morphine in the urine.

Methods: Positive urine samples for morphine confirmed by immunochromatographic strips were collected from the Imam Reza Toxicology Laboratory, Mashhad University of Medical Sciences, Mashhad, Iran in 2012 for the current study. Then, the collated urine samples (70) were analyzed by both ELISA and TLC methods.

Results: On analyzing samples by TLC, 57 out of 70 (81.4%) revealed morphine spot, whereas by ELISA method all samples were positive. The difference was statisticaly significant (*P*=0.0001). Both immunoassays had the same 100% positive results. The possible 18.6% false positive results might be due to drug interactions. TLC is more specific but time-consuming and less sensitive than ELISA is. However, TLC is an old method but more reliable than ELISA.

Conclusion: Contrary to the claim that commercially available ELISA kits have a high specificity for detection of morphine derivatives; it seems that false positive results may occur. It is thus recommended that all positive results obtained from ELISA be checked by a cheap widely available confirmation test of TLC or ideally by a quantitative technique such as GC-Mass spectroscopy, particularly for legal purposes.

Keywords: Confirmation Test, ELISA, Morphine, TLC, Urine.

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INTRODUCTION

Drug abuse is a critical problem throughout the world. Urine drug screens (UDS) are a frequent practice applied to detect common drugs of abuse. A few situations in which screening may be performed are including pre-employment, suspicion of drug abuse, random testing outlined in employment contract, military service, sports participation, legal/criminal, marriage, therapeutic drug monitoring and postmortem investigation [1].

UDS are generally done using immunoassay methods [2]. These assays for drugs of abuse are well-established [3-6]. They are planned to separate negative samples from the likely positive samples. Immunoassay UDS use specific antibodies against current drugs of abuse or their metabolites to detect them. The most commonly used UDS have been established based on immunoassay technique because it is cheap and rapid. Five different types of immunoassays are available: ELISA, enzyme-multiplied immunoassay (EMIT), fluorescence polarization immunoassay (FPIA), immunochromatography, and radioimmunoassay (RIA).

The most commonly drugs examined by a typical immunoassay are amphetamines, cannabinoid metabolites, cocaine metabolites, opiate metabolites, and phencyclidine (PCP). Expanded immunoassays are available to detect for tricyclic antidepressants, barbiturates, methadone, alcohol, and benzodiazepines. They may be useful when use of these substances is suspected [7].

A big difficulty with immunoassays is a false-positive result. Therefore, a confirmatory test with a high degree of specificity, such as, TLC, HPLC and GC-MS is needed to confirm a positive result made by immunoassay method. Confirmatory tests are more specific, accurate

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and reliable than immunoassay, but they are more expensive and time consuming.

TLC method is less expensive than HPLC and GC-MS in terms of capital equipment and other initial set-up costs. They are labor-intensive and generally require experience for accurate application. It is consisted of coating silica gel as a stationary phase on the surface of a glass or aluminum plate and the usage of special solvents (as mobile phase) for separation of spots. However, TLC is recommended as a confirmatory test for immunoassay screening results where experienced and trained staffs are available [8].

A screening test should be able to identify potential positive results with a high degree of reliability and sensitivity. These criteria are generally met by immunoassays. However, the antibodies used in immunoassays have relatively low specificities and may result in cross-reactivity [8]. But, it is recently observed that there are many brands of morphine ELISA kits by searching on the internet that their manufacturers claim to present their products with a high specificity for detection of drugs of abuse. This issue may seduce toxicologists to use the mentioned commercial kits without confirmatory tests.

Therefore, in this study, we aimed to evaluate the reliability of data obtained from a morphine ELISA kit versus a cheap available confirmatory analytical method named TLC.

MATERIALS AND METHODS

A total of 70 urine specimens from drug abusers of Mashhad which had positive results confirmed by membrane based immunochromatography assay (rapid strip test) for morphine derivatives in the Imam Reza Toxicology Laboratory, Mashhad University of Medical Sciences, Iran were collected during the summer (July to September 2012). All urine samples were daily collected and stored in refrigerator until analysis. The research project was approved by the Medical Research Ethics Committee of Mashhad University of Medical Sciences.

Elisa Procedure

The urine samples were centrifuged for 10 min with 2500g for eliminating of possible turbidity in the samples. Supernatants were used for the quantitative analysis. Urine matrix negative for morphine as negative calibrator and urine matrix containing 5, 25 and 100 ng/ml morphine as positive calibrators were used. Calibrators, controls and urine samples were diluted (1:10) with distilled water before assay.

Ten microlitres of the diluted calibrators or samples were added to the wells along with 100 µl of working enzyme conjugate (morphine derivative labeled with horseradish peroxidase) to compete on occupying the binding sites, then mixed gently and incubated for 30 min at room temperature. The liquid was poured out of the wells and the wells filled with 350 µl washing buffer and poured the liquid out again. This washing step was repeated four times. Then, 100 µl of substrate/chromogen were added to the wells and incubated for 30 min at room temperature**.** Attached enzyme conjugate converted the chromogen to a blue product and then 100 µ1 of the stop solution was added to the wells which lead to a color change from blue to yellow. Lastly, the absorbance was measured at 450 nm by a microplate ELISA reader within 30 min.

Tlc Procedure

For TLC assay, the possible morphine or its derivatives is needed to be extracted by a liquid-liquid extraction (LLE). To lower the urine sample pH in the range of 1-2, hydrochloric acid (HCl) drops were added to 50 mL of urine specimen and placed for 30 min in water bath for hydrolyzes. Next step was to add ammonia for neutralize the acidity to achieve a pH around 8.5-9. As the result, opioid compounds come in to the organic layer. Using 40 mL extraction solvents, urine samples were shaken in a separator funnel. After a while, the two-layer content was separated and the organic layer (lower layer) containing morphine was spilled into a beaker. After evaporated the organic solvent, the residues were come up at the bottom of the beaker.

TLC plates were activated by heating at 100º C for 20 min prior to use. The urine residues were reconstituted in methanol and then spotted on the plates along with morphine standard. The plates were marked by pencil one centimeter from the bottom to find out the spots were being put on this line. Afterwards, the plates were developed in mobile phase until the

level of eluent on the plate reaches up to 8 centimeters. The development solvent was added into the tank half an hour before the procedure. Compounds were separated by partition between the mobile and stationary phases. At the end, developed plates were dried and then examined by spraying Iodoplatinate solvent. The color spots were finally interpreted by comparison to the morphine standard spot.

Data were statistically analyzed using Fisher's Exact Test to determine significant differences in the data of the two methods. Statistical tests were conducted using INSTAT software (GraphPad, Inc., San Diego, CA). The two-sided *P* values of less than 0.05 were considered significant.

RESULTS

On analyzing samples by TLC, 57 out of 70 (81.4%) revealed morphine spot, whereas using ELISA method all the samples were positive. The difference was significant (*P* = 0.0001). There were not any changes between results obtained from rapid strip test and the ELISA. As a result, 13 cases out of 70 urine samples were false positive by ELISA technique.

DISCUSSION

Opioids are a group of drugs comprising both prescribed and illicit agents. Morphine and codeine are naturally occurring alkaloids from the opium poppy seed, *Papaver somniferum*. Opium is usually consumed by Iranian abusers [9, 10]. In our study, 13 out of 70 urine samples were false positive by ELISA technique. According to manufacturers' claims, a variety of non-related drugs to morphine derivatives had been checked over at different concentrations in urine and no cross-reactivity were found. Still, related compounds to morphine had been examined using the qualitative procedure and trivial cross reactivity was found [11]. According to our results and other studies, we think that some of the morphine ELISA kit brands have yet acquired less specificity and a high likelihood of false positives [12, 13]. The false positive results of ELISA may be due to drug interactions. Some of these drugs are used under physicians' orders and some of them are used intentionally. These substances can cause drug interaction in the results of rapid strip tests and ELISA and lead them to false positive or false negative. Agreement with this study, a similar study in Tabriz, Iran, showed that 25% of urine samples had false positive results by ELISA due to drug interaction [14].

The main finding of this study was the comparison of the results taken from ELISA techniques with TLC to distinguish the presence of opioids in abusers' urine samples. The limit of detection for strip test, ELISA and TLC was a concentration of 300, 5 and 300 ng/ml, respectively. This shows that ELISA method has a high degree of sensitivity in comparison to TLC. On the other hand, our results showed a low specificity for detection of morphine by ELISA. Finally, contrary to the claim that commercially available ELISA kits have a high specificity for detection of morphine derivatives, it seems that all positive results obtained from ELISA must be checked at least by a cheap widely available confirmation test such as TLC.

CONCLUSION

This study describes the importance of a cheap widely available urine drug confirmation test as TLC to find out any possible false positive result that may occur by immunoassay tests. It is thus recommended that all positive results obtained from immunochromatographic stripes or ELISA should be checked by a cheap widely available confirmation test of TLC or ideally by a quantitative technique such as GC-Mass spectroscopy, particularly for legal purposes.

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