A heterologous enzyme linked immunosorbant assay of morphine using penicillinase as label

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Abstract

A rapid, sensitive, specific and high through-put enzyme-linked immunosorbant assay (ELISA) method for determination of morphine in urine samples using penicillinase as label enzyme has been developed. No extraction or chromatography was included in this assay procedure. Immunoglobulin (Ig) purified poly-clonal anti-bodies against a C6-hemisuccinate derivative of morphine (M-C6-HS) conjugated to bovine serum albumin (BSA) was coated onto the wells of microtiter plate. A morphine-C3-hemisuccinate (M-C3-HS) was also prepared and the two derivatives were conjugated to penicillinase (M-C6-HS-P and M-C3-HS-P). The heterologous combination of antibody prepared against M-C6-HS-BSA and enzyme conjugate prepared for M-C3-HS-P showed better properties in term of sensitivity, reproducibility and slope of standard curve. The assay was sensitive from 20 pg/ml and detected up to 100 ng/ml of morphine in urine samples. The affinity of antibody in homologous assay was found to be 6.6×10^{10} l/mol and for heterologous assay was 3.2×10^{12} l/mol. The assay was completed within 4 h. The homologous assays performed under different conditions of coating, concentrations, duration, pH, etc. did not end up with a suitable standard curve. Hence it seems that the ability of morphine to displace the hapten enzyme conjugate dependds on the position of the enzyme coupled to the hapten molecule. This ELISA techniqu showed 100% correlation with immunochromatography (IC) and 90% percent correlation with latex agglutination inhibition (LAI) test in the results obtain with urine samples declared positive by authorities. ELISA also showed approximately 90% correlation with LAI-negative urine samples.

Keywords: *ELISA, Morphine, Penicillinase, Heterology*

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INTRODUCTION

Morphine (7, 8-didehydro-4, 5-epoxy-17-methyl-morphinan-3, 6-diol) is obtained from opium, derived from the milky exudate of the incised unripe seed capsules of the poppy plant, *Papaver somniferum*. This compound is a classical μ receptor and a potent narcotic analgesic with substantial potential for abuse and pose significant health problem for human society. There is a rising demand for drug testing which comes from authorities pressuring to curb the spread of substances of abuse and to provide greater protection to the members of society. According to the report of 1999 on drug abuse, 3 million people (around 5% of total population) have used one or the other type of drug of abuse in Iran. Currently the most frequently used biological sample for the identification of drug is urine analysis (Derks *et al*., 1985; Aoki *et al*., 1996, 1999; Stanley *et al*., 1991). Although use of other biological samples such as blood, saliva and even hair is being reported (Laurie *et al*., 1989; Edwards *et al*.,

1986; Kopecky *et al*., 1999; Gamaleya, 1993). But, urine analysis provides easy, stable, rapid and reliable results for drug of abuse. Two groups of test procedures have been reported. Chromatography, the old approach for drug analysis, although tedious, expensive and time consuming is still used widely as final confirmatory test and indisputable evidence (Stanski *et al*., 1982; Schanzel *et al*., 1999; Mule and Casella, 1988). This is because of consequences to lives, careers and reputations which follow reports of positive analytical findings. However, presently immunoassays are most commonly used screening test because their ease of performance, sensitivity and reasonable specificity (Catlin *et al*., 1992). Since the first radioimmunoassay (RIA) for morphine detection (Spector and Parker, 1970) the method have been changing and developing rapidly. Because of the problems encountered in using radioactive labels, other labels such as enzymes are reported for the immunoassay of morphine (Aoki *et al*., 1996, 1999; Stanley *et al*., 1991; Laurie *et al*., 1989). Penicillinase as label enzyme has been reported for a few small molecules (Jushi *et al*., 1983; Shrivastav *et al*., 1988; Pandey *et al*., 1990; Rassaie *et al*., 1992) and proteins (Shrivastav *et al*., 1988; Joshi *et al*., 1981; Joshi *et al*., 1978). This label was found to exhibit better properties in term of conjugation, end point detection and assay reproducibility (Sauer *et al*., 1989). In the present investigation, we have developed a simple, sensitive and high through-put ELISA for morphine measurement in urine samples, without any further treatment, using penicillinase as label enzyme. To best of our knowledge this is the first ELISA report for morphine detection using penicillinase as label.

MATERIALS AND METHODS

Dimethyl formamide (DMF), gelatin, 1-ethyl 3- (3-dimethylaminopropyl) carbodiimide (EDC), bovine serum albumin (BSA), N-hydroxy succinimide ester (NHS), penicillinase (EC.3.5.2.6), penicillin V and structurally morphine-related molecules were obtained from Sigma Chemical Company, St Louis, Mo, USA. Immunochromatography rapid morphine detection (Sure Step) was obtained from Applied Biotech. Inc., USA. All other solvents and reagents were of analytical grade and purchased from E. Merck, Germany.

Preparation of antibody: A 6-hemisuccinate derivative of morphine was prepared following the method of Spector *et al*. (1973) with a minor modification. Morphine and succinic anhydride one gram each were dissolved in 20 ml of benzene and refluxed. After 30 min, succinic anhydride (1 g) was added and refluxed for 1 more hour. The solvent was removed by nitrogen gas flushing and the residue was dissolved in 10 ml of water (pH 2). The product was filtrated and treated with NaOH (2.5 N) till the pH of filtrate was adjusted to 9. The precipitate was removed by filtration, pH of solution was adjusted to 5 and left to stand at 4°C over night. The residue was collected and dried under vacuum over $CaCl_2$. The derivative (M-C6-HS) obtained was characterized by TLC (mobile phase, ammonium hydroxide, benzene, dioxan, ethanol 50:5:4:5) and infrared spectroscopy (peaks at 1700, 1369 and 1608 cm for carbonyl and carboxilic acid groups, respectively) and was conjugated to BSA following carbodiimide procedure. The derivative (M-6HS, 30 mg, 0.0744 mmol) and EDC (14.2, 0.0744 mmol) were dissolved in 2 ml of water and adjusted to pH 5.The product was incubated at room temperature while stirring for 1 h. BSA (52 mg, 0.00074 mmol) dissolved in water was added to the above solution drop by drop while stirring in a way that no precipitate was formed and allowed to stand at room temperature for 16 h. The final product was then dialyzed extensively, lyophilized and stored at 4°C. New Zealand rabbits were immunized following the low dose multi intradermal procedure of Vaitukaitis *et al*. (1991). Serum samples were prepared every month, characterized in terms of titer, specificity and affinity and stored until use.

Tracer preparation: Morphine 3-hemisuccinate (M-C3-HS) was prepared following the method of Wainer *et al*. (1992) with a minor modification as follow. Morphine (100 µg) and succinic anhydride (300 µg) were dissolved in 10 ml of pyridine and refluxed for 4 h. At the end of reaction time, pyridine was evaporated by nitrogen gas flushing. The residue was washed with hot ethanol × 5 times, recrystallized with 60% ethanol and dried in presence of $CaCl_2$. M-C3-HS obtained in this way was conjugated to penicillinase following an active ester method of Hosoda *et al*. (1979) briefly as follow. M-3HS (0.744 mmol) and NHS (0.744 µmol) were desolved 127 µl of DMF with constant stirring for 1 h at room temperature. To penicillinase (2 mg, 0.0744 µmol) dissolved in 500 µl of buffer (PBS, 0.1 mol, pH 8) was added 100 µl of pyri-

dine. The solution containing activated derivative was added to enzyme solution drop by drop for 20 min (no precipitate should form) and incubated at 4°C for 18 h. The final product which was morphine C3-hemisuccinat penicillinase (M-C3-HS-P) was chromatographed on a G-25 Sephadex pre-packed column and stored as reported by Shrivastav *et al*. (1988). Morphine-C6-HS was also conjugated to penicillinase following carbodiimide procedure as explained for immunogen preparation, chromatographed and stored (Shrivastav *et al*., 1988).

Enzyme-linked immunosorbant assay

Checkerboard titration assay: Immunoglobulin fraction of anti-rabbit immunoglobulin prepared in goat was purified briefly as follow. Ammonium sulphate (33%) was added to serum samples of high titer and specificity. The precipitate was collected and dissolved in minimum quantity of PBS (pH 7.4) and dialyzed (PBS, pH 6.9) .The product was loaded onto a column of DEAE cellulose equilibrated with phosphate buffer pH 6.9, washed with the same buffer and high content protein fractions were collected, pooled and freeze dried.The resulting fine powder was collected and stored at 4°C. This product was coated onto the wells of microtiter plate (5 µg/well in PBS, 0.01 mmol, pH 7.2, here after called ELISA buffer). Coated wells were washed (with a 10 mmol PBS containing 0.05% Tween 20, here after called wash buffer), blocked with 10 mmol PBS containing 0.5% gelatin and 0.01% sodium azid here after called as blocking buffer, for 30 min at 37°C, washed and added with anti-serum obtained after each bleeding serially diluted in ELISA buffer (1:250, 1:500, 1:1000, 1:2000) for specific binding (SB) and normal rabbit serum (1:300) added to the corresponding wells and used as nonspecific binding (NSB) index, overnight at 37°C until dryness. At the end of incubation time wells were washed and the corresponding wells were added with different dilutions of tracer (M-C6-HS-P, 1:250, 1:500, 1:1000 and 1:2000) and (M-C3-HS-P, 1:200, 1:400, 1:800 and 1:1600), prepared in ELISA buffer incubated at 37°C for 2 h, washed and added with 100 µl of freshly prepared substrate solution. Rest of deprocedure was performed described earlier (Malakaneh *et al*., 1998).

Purification, titration and standard assay: Immunoglobulin fraction of high titer antibodies selected in above manner was purified following the method of Lewy and Sorb (1960). Different dilutions of antibody were coated onto the wells of microtiter plate and titrated with different dilutions of tracer both in homologous and heterologous combinations. Optimum titers were selected on these basis and the standard curves for different doses of morphine (0, 100, 1000, 10000 and 100000 pg/well) prepared in urine were constructed as described earlier (Rassaie *et al*., 1992).

Assay validity: Standard curve for best titer and combinations of antibody and antigen were selected on the basis of above experiments. Different doses of morphine standard were prepared in urine (normal individual indicated negative by RIA, IC and LAI). The standard curve was constructed and repeated for several times. The affinities were calculated as reported before. The slope of standard curve and a stock sample of morphine diluted serially (1:2, 1:4, 1:8) were drawn in parallel. The specificities were calculated for some of the structurally related molecules following the method of Abraham (1974). Urine samples reported to contain morphine (positive samples) and those reported to not contain morphine (negative samples) detected by LAI were obtained from Iranian Drug Control Department (IDCD). These samples were assayed using ELISA reported here and immunochromatography (IC) method in parallel.

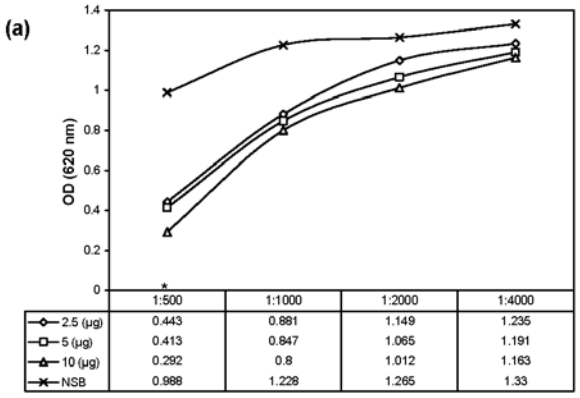
RESULTS

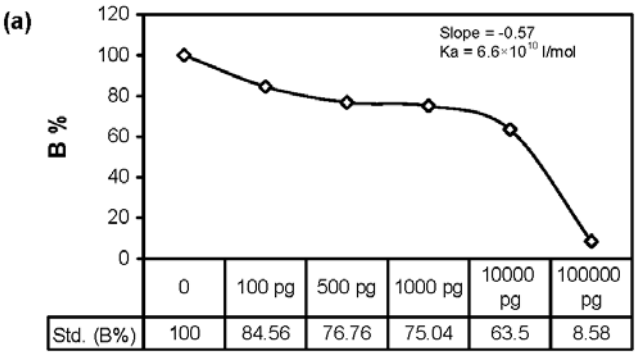
Table 1 presents the results for titration of antibodies at different times elapse after each blood collection. The data in this table indicated that the antibody and tracer

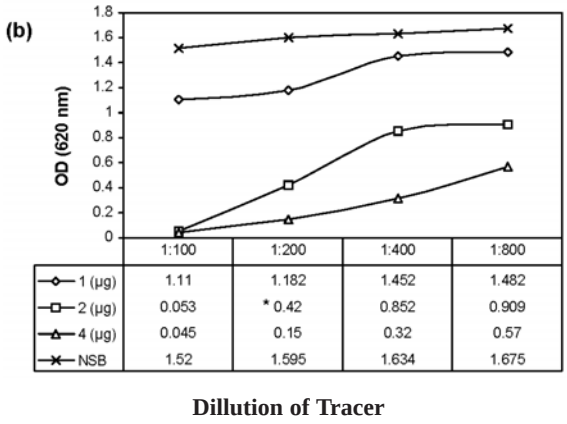
Table 1. Titer of antibody (M-C6-HS-BSA) and labeled antigen.

Days after immunization	*M-C6-HS-P		*M-C3-HS-P	
	**Tracer	**Ab	**Tracer	**Ab
90	1:1000	1:4000	1:400	1:2000
120	1:1000	1:4000	1:400	1:2000
150	1:1000	1:4000	1:400	1:2000
180	1:1000	1:4000	1:400	1:1000

*enzyme conjugate.
**expressed in initial dilution.







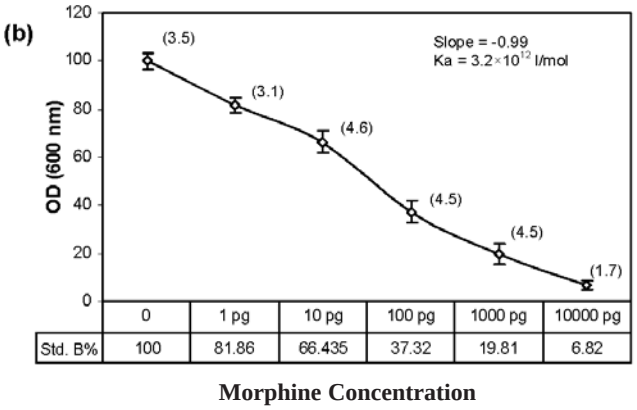


Figure 1. (a) Checkerboard titration assay of homologous system (anti-M-C6-HS-BSA and M-C6-HS-penicillinase); (b) Checkerboard titration assay of heterologous system (anti-M-C6-HS-BSA and M-C3-HS-penicillinase). *Dilution of antibody and tracer selected.

Figure 2. (a) Dose response curve for homologous system; (b) Dose response curve for heterologous system. Results of 10 experiments, values in parenthesis indicate CV and bars for SD.

Table 2. Specificity of anti-morphine antibody produced in this study.

SN	Molecule	% Cross-reaction
1	Ethyl morphine	820
2	Caffeine	>0.05
3	Salicylic acid	>0.05
4	Tebaen	11000
5	Ephedrine HCl	20
6	Codeine	75
7	Heroin HCl	151
8	Morphine	100

Heterologous system was used (anti-M-C6-HS-BSA and M-C3-HS-P).

titer were generally higher in case of homologous systems. A typical checkerboard titration assay for Ig purified antibody and two tracers are shown in figure 1 (a and b) where the difference in optical densities of each dilution curve is shown to be more in case of heterologous assay. A typical standard curve is shown in figure 2 (a and b) where a homologous (a) and a heterologous (b) combinations of antibody and enzyme conjugate were used. The heterologous assay was sensitive from 1 pg/well (10 pg/ml) and covered up to 10 ng/well. The slope of standard curve was calculated to be -0.99 for this system. However, the homologous standard curve was not sensitive (500 pg/well) and was not steep enough (slope = -0.57) to be used for further experiments. The affinities in term of binding of morphine to antibody as compared to morphine-enzyme bound to antibody was calculated to be 6.6×10^{10} l/mol for homologous combination and 3.2×10^{12} l/mol for heterologous assay. Table 2 presents results obtained for cross reactivity of antibody with morphine like compounds. The heterologous assay

system was used to calculate these results. Table 3 indicates our data obtained on percent of recoveries of morphine added to stripped urine samples (in three concentrations of low, medium and high) proved to not contain any reacting substances with anti-morphine antibodies. The results presented here indicate that the recovery is about 100% within the ranges under investigations. Table 4 represents our results for a number of urine samples provided by Drug Control Department (DCD), which were diagnosed (using a latex agglutination inhibition test, LAI) and reported to be positive. Out of 50 urine samples, five were found to be negative by ELISA developed in this experiments and immunochromatography (IC) test kits available in the market. These results indicated that five samples reported positive by LAI were actually negative by ELISA and IC, and one sample reported negative by LAI was actually positive by ELISA as well as IC.

In table 5 the results of 30 normal samples provided by the same agency and found to be negative by LAI were checked by ELISA and IC in which two samples were found to be actually positive for mor-

Table 3. Recoveries of morphine added to stripped urine.

SN	Concentration added (pg/ml)	Concentration recovered (pg/ml)	Recovery (%)
1	Low 50	48±0.6	98
2	Medium 500	510±15	102
3	High 5000	5095±50	103

Heterologous system was used (anti-M-C6-HS-BSA and M-C3-HS-P)

Table 4. Assay of urine samples reported to be positive by IDCD.

SN	OD	ELISA	IC	LAI	SN	OD	ELISA	IC	LAI
1	0.14	-	-	+	26	1.37	+	+	-
2	1.26	+	+	+	27	1.58	+	+	-
3	1.57	+	+	+	28	1.40	+	+	+
4	1.68	+	+	+	29	1.93	+	+	+
5	0.18	-	-	+	30	2.15	+	+	+
6	2.00	+	+	+	31	1.72	+	+	+
7	1.80	+	+	+	32	2.07	+	+	+
8	1.50	+	+	+	33	1.43	+	+	+
9	1.80	+	+	+	34	2.39	+	+	+
10	1.98	+	+	+	35	1.82	+	+	+
11	1.48	+	+	+	36	2.54	+	+	+
12	1.90	+	+	+	37	2.04	+	+	+
13	1.69	+	+	+	38	2.50	+	+	+
14	2.11	+	+	+	39	2.05	+	+	+
15	1.37	+	+	+	40	1.38	+	+	+
16	1.47	+	+	+	41	0.10	-	-	+
17	1.67	+	+	+	42	1.40	+	+	+
18	1.54	+	+	+	43	1.74	+	+	+
19	1.65	+	+	+	44	1.50	+	+	+
20	2.20	+	+	+	45	1.41	+	+	+
21	0.15	-	-	+	46	1.59	+	+	+
22	1.63	+	+	+	47	2.00	+	+	+
23	1.59	+	+	+	48	1.81	+	+	+
24	1.20	-	-	+	49	1.42	+	+	+
25	1.72	+	+	+	50	1.53	+	+	+

*S 0=0.20, S 10 pg=0.35, S 100 pg=0.47, S 1 ng=1.01, S 10 ng=1.32, S 100 ng=1.47
*Optical densities obtained for standard doses of morphine
OD=Optical densities in ELISA

Table 5. Assay of negative urine samples provided by IDCD.

SN	OD	ELISA	IC	LAI	SN	OD	ELISA	IC	LAI
1	0.06	-	-	-	16	0.12	-	-	-
2	0.10	-	-	-	17	0.13	-	-	-
3	0.08	-	-	-	18	1.23	+	-	-
4	0.09	-	-	-	19	0.14	-	-	-
5	1.50	+	+	-	20	0.03	-	-	-
6	1.49	+	+	-	21	0.12	-	-	-
7	0.10	-	-	-	22	0.15	-	-	-
8	0.06	-	-	-	23	0.12	-	-	-
9	0.04	-	-	-	24	0.11	-	-	-
10	0.07	-	-	-	25	0.15	-	-	-
11	0.10	-	-	-	26	0.07	-	-	-
12	0.12	-	-	-	27	0.37	+,-	-	-
13	0.20	-	-	-	28	0.11	-	-	-
14	0.16	-	-	-	29	0.12	-	-	-
15	0.18	-	-	-	30	0.15	-	-	-

*S 0=0.20, S 10 pg=0.35, S 100 pg=0.47, S 1 ng=1.01, S 10 ng=1.32, S 100 ng=1.41
*Optical densities obtained for standard doses of morphine
OD=Optical densities in ELISA

phine test by ELISA and IC. However, these two samples were confirmed to be positive when tested by HPLC. One sample having a weak positive indication in ELISA (0.37 OD) equivalent to around 2 ng morphine/ml was found to be negative by IC and LAI and found to be positive by HPLC. The result of parallelism test is shown in figure 3, where the slope of the standard curve was calculated to be 0.96 and that of sample dilution 0.99. Finally samples containing different amount of morphine were pooled and measured in three ranges of concentrations viz, low, medium and high, the results of which are shown in table 6. These were assayed in five different occasions each time in 4 replicates. Percent coefficient of variation (CV%) for inter- and intra-assays were found to be 0.79-13 and 1.8-3.14, respectively.

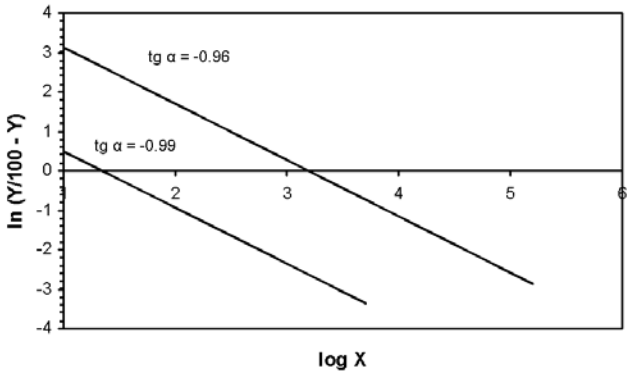


Figure 3. Test of parallelism for standard and sample dilution curves linearized.

DISCUSSION

In the detection of drugs of abuse a fast, reliable, specific and easy handling screening method is usually appreciated and preferred. The main task of these assays would be to detect the small number of positive samples from a large group of suspected samples. However, due to implications involved in the report of positive samples specially when a sever punishment is involved it is essential to perform a confirmatory test such as chromatography (TLC, HPLC or even liquid or gas chromatography-mass spectrometry). But these procedures are tedious, time consuming and expensive to perform in routine diagnostic laboratories. Currently immunoassays are the method of choice for fast and accurate detection of various drugs in different biological fluids. Indeed ELISA tests are used frequently for morphine detection in different biological samples (Aoki *et al*., 1996, 1999; Stanley *et al*., 1991; Laurie 1989). In this study we have developed a simple (detection without reader requirement), rapid (4 h incubation time), high through-put (80 samples within

Table 6. Inter- and intra-assay variations of ELISA results.

SN	N	Low (ng/ml) Mean±SD	CV%	Medium (ng/ml) Mean±SD	CV%	High (ng/ml) Mean±SD	CV%
Intra-assay							
1	4	0.57±0.039	6.8	1.71±0.051	3	2.01±0.016	0.79
2	4	0.59±0.042	7.1	1.74±0.047	2.7	2.04±0.12	6
3	4	0.56±0.043	7.7	1.77±0.046	2.6	2.15±0.09	4.4
4	4	0.56±0.074	13	1.68±0.033	2	1.99±0.05	2.6
5	4	0.57±0.052	9	1.72±0.022	1.3	2.20±0.02	0.9
Inter-assay							
4 × 5		0.57±0.01	2.1	1.7±0.03	1.8	2.0±0.07	3.1

N=number of replicates for each concentration was performed
4×5=Each concentrations were repeated five times in different days

the incubation time for each plate) and sensitive (1 pg/well or 20 pg/ml) for the detecting morphine in minor amount of urine sample (50 µl). The standard curve extended from 20 pg/ml to 200 ng/ml showing a good sensitivity in lower concentrations and saturation up to 100 ng of sample. However, this property of the assay seems to be dependent on the quality of both antibody and enzyme label. Therefore, it was observed that heterologous system worked better in term of sensitivity, specificity and other assay validation parameters. We observed and reported similar effect in case of testosterone enzyme immunoassay (Rassaie *et al*., 1992). This type of observations was first reported by Van weeman and Schuurs (1973) and further was confirmed by number of other workers (Chappy *et al*., 1992; Arakawa *et al*., 1979; Hosoda *et al*., 1981). However, the ELISA method reported by Aoki *et al*. (1996) used a C3 derivative to produce both immunogen and enzyme label with alkaline phosphatase. In this assay, a sensitivity of 6 pg/well ranging up to 600 pg/well with a cross-reaction of 1321% with codeine was reported. Laurie *et al*. (1989) reported a nanogram sensitive assay avoiding the labeling of antigen and used a second antibody label. Although this would have prevented the affinity problem but it has greatly affected the sensitivity. In our assay changing the position of enzyme conjugation we were able to obtain a sensitive assay with a reasonable specificity (codeine cross reaction 75%). Inter- and intra-assay coefficients of variation (CVs) for tests performed in replicates and different occasions were found to be within the acceptable limits of standardization of method. Using this assay system a correlation of almost 100% with IC was observed for normal and positive samples. However, number of false positive and false negative detection was observed when using LAI. It should be further enphesized that the optical density used for the end point detection in our experiments lies around 650 nm, the results for positive and negative samples can be well elaborated with naked eye, which could be considered as a major advantage for a simple test for mass screening. In summery, it is suggest that the use of ELISA with penicillinase as label enzyme is a convenient method for accurate, sensitive and large sample screening of morphine in urine.

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