Research Article

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Evaluation of optimal urine screening and confirmation cut-off values for opiates, at a national reference laboratory

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Abstract

Objectives: To obtain optimal immunoassay screening and LC-MS/MS confirmation cut-offs for opiate group tests to reduce false positive (FP) and false negative (FN) rates.
Methods: A total of 126 urine samples, –50 opiate screening negative, 76 positive according to the threshold of 300 ng/mL by CEDIA method – were confirmed by a full-validated in-house LC-MS/MS method. Sensitivity, specificity, FP, and FN rates were determined at cut-off concentrations of both 300 and 2,000 ng/mL for morphine and codeine, and 10 ng/mL for heroin metabolite 6-mono-acetyl-morphine (6-MAM).
Results: All CEDIA opiate negative urine samples were negative for morphine, codeine and 6-MAM. Although sensitivity was 100% for each cut-off; specificity was 54.9% at CEDIA cut-off 300 ng/mL vs. LC-MS/MS cut-off 300 ng/mL and, 75% at CEDIA cut-off 2,000 ng/mL vs. LC-MS/MS cut-off 2,000 ng/mL. False positive rate was highest (45.1%) at CEDIA cut-off 300 ng/mL. At CEDIA cut-off 2,000 ng/mL vs. LC-MS/MS cut-off 300 ng/mL, specificity increased to 82.4% and FP rate decreased to 17.6%. All 6-MAM positive samples had CEDIA concentration ≥2,000 ng/mL.
Conclusions: 2,000 ng/mL for screening and 300 ng/mL for confirmation cut-offs are the most efficient thresholds for the lowest rate of FP opiate results.

Keywords: 6-MAM; codeine; confirmation; cut-off; immunoassay; LC-MS/MS; morphine; opiate; screening.

Introduction

Opiates are one of the five classes of abused drugs mandated for testing in Drug Testing Program of many European countries, the USA, and Turkey. To detect medical or nonmedical drug abuse and/or illicit drug use health care providers usually order urine drug testing. Opiate drug testing can be ordered for identifying heroin use, opioid group prescription drug monitoring to evaluate possible overdoses, to evaluate adherence to prescription medications, or for workplace testing [1–3].

Challenges in opiate screening interpretation depend on the metabolism of opioid group substances, their metabolites, and the cross-reactivity of drugs or substances which makes it hard to discriminate legal or illegal substance/drug use [4]. The cloned enzyme donor immunoassay (CEDIA) method used for opiate detection, with the application of the appropriate calibrator gives a positive result when morphine or codeine is present at greater concentrations than the defined threshold. All immunoassays and also CEDIA method may not be specific for the intended drug or drug class and may give cross-reaction with other prescription and nonprescription drugs. Studies performed by the manufacturer reported that more than 20 additional drugs and their metabolites that share structural similarities with the target compound cross-react with the antibodies used in immunoassays and can cause positive results when present at the tested concentrations [5, 6]. Verapamil, tramadol, venlafaxine, quetiapine, diphenhydramine, levofloxacine, ofloxacine, rifampin, dextrometorphan are some of these drugs interfere positively with opiate immunoassays [7]. False positive rates for drug abuse immunoassay

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tests change up to 34% [8]. For that reason, confirmatory tests with a more impressive method that have greater analytical specificity and lower detection limits, like Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis, are critical when a screening result is questioned or needs to be verified [9, 10]. Determination of "cut-off concentrations" for both screening and confirmatory tests distinguishes between positive and negative results. The results of drug or drug metabolite tests may be truly negative, or the drugs or their metabolites may be present at concentrations below the cut-off value used and thus be reported as negative or not detected [11]. Indeed, sampling time may be out of the detection period as detection period of opiate group drugs/metabolites in urine are approximately 2–5 days [6, 9].

As the intake of some codeine-containing drugs even at therapeutic amounts and ingestion of food products containing poppy seeds can give a positive opiate result with the 300 ng/mL screening cut-off value, the most appropriate thresholds have been extensively studied [12].

Countries usually use administrative cut-offs for drug abuse tests, which can change based on the aim of laboratory testing as clinical/forensic/workplace, the used laboratory technique (screening or confirmation), the used matrix, and established country laws [13, 14]. Substance Abuse and Mental Health Services Administration (SAMHSA) currently uses 2,000 ng/mL cut-off for opiate group "screening"; while in "confirmation" analysis 2,000 ng/mL for morphine, 2,000 ng/mL for codeine and 10 ng/mL for 6-monoacetylmorphine (6-MAM) – the main heroine metabolite in urine. However, some European countries still use 300 or 200 ng/mL screening cut-off values; and 300 ng/mL morphine, 300 ng/mL codeine and 10 ng/mL 6-MAM confirmation cut-off values [15, 16].

In Turkey, drugs of abuse testing services for clinical, administrative, or forensic purposes are performed in medical laboratories in response to local demand [17–19]. Since 2015, the Turkish Ministry of Health has started new specific legislation on drug abuse testing in health institutions to guarantee quality and safety requirements in clinics and laboratories [20]. In these rules, mandatory five drugs to be screened, and their urine cut-off values were also defined [20]. For opiate group drugs agreed screening cut-off was 2,000 ng/mL; whereas it was 500 ng/mL for amphetamines, 300 ng/mL for benzodiazepines, 150 ng/mL for cocaine, and 15 ng/mL for marihuana. This cut-off brought discussion especially among psychiatrists and laboratory physicians. Clinicians claimed that they can miss too many true positive cases, even in presence of heroin and morphine in the urine matrix as they stay below the cut-off value.

In this study, our aim was to assign the optimal urine screening and confirmation cut-off values of opiate group abused drugs, by both evaluating 300 and 2,000 ng/mL screening and confirmation thresholds, at a national reference laboratory in Turkey.

Materials and methods

Subjects

After local ethical committee approval (08.12.2017/566) a limited dataset of consecutive patient results for drug abuse and toxicology screening test results were obtained from the laboratory data warehouse and used for analysis. All participants' rights were protected and written informed consent was obtained before the screening analysis.

This was a cross-sectional study hold during the period of three months -September and November 2015, in patients admitted to Kayseri Education and Research Hospital, in Turkey because of forensic or clinical toxicology aim of testing. A total of 126 urine samples, of those 50 opiate screening negative and 76 opiate screening positive according to the threshold concentration of 300 ng/mL by cloned enzyme donor immunoassay (CEDIA) method (Thermo Scientific™ Indiko™ Plus) were reanalyzed and confirmed by a full-validated in-house LC-MS/MS method, in an authorized national reference laboratory (Clinical Biochemistry Laboratory, Education and Research Hospital, Kayseri, Turkey) for quantitation and confirmation of drug abuse tests. Any drug screening positive instead of an opiate group or multiple drug positive samples even with the opiate group were excluded. Urine samples were stored at –80 °C between the screening and confirmation period. Freshly thawed urine samples were used for the LC-MS/MS method. Repeated freezing and thawing was avoided.

Material and chemicals

LC-MS/MS grade water, methanol, propanol, acetone, and acetonitrile were obtained from Sigma-Aldrich (Lyon, France); formic acid, ammonium acetate, sodium citrate, sodium acetate, potassium phosphate monobasic, and dibasic were purchased from Fluka-Analytical (Germany). The analytical standards (morphine, codeine, 6-MAM, morphine-d3, codeine-d3, 6-MAM-d3) were purchased from *Chiron* (Trondheim, *Norway*); IMCSzyme™ recombinant β-glucuronidase was obtained from Covachem LLC (Germany).

HPLC and MS/MS

LC-MS/MS analysis was performed on an ultra-high performance liquid chromatography (UHPLC) (Thermo Scientific Dionex Ultimate 3000) coupled with a triple-stage quadrupole mass spectrometer (TSQ Quantum Access MAX, Thermo Scientific). The instrument was operated with heated electrospray ionization (ESI), in positive ion and selected- reaction monitoring (SRM) mode with Thermo Scientific Accucore PFP (50 × 2.1 mm, 2.6 µm) analytical column and dual gradient pump (1,064 bar). Mobile phase A consisted 10 mM ammonium acetate and 0.1% formic acid in water, Mobile phase B consisted 10 mM ammonium acetate and 0.1% formic acid in methanol and

Mobile phase C consisted acetonitrile/isopropanol/acetone 9:9:2 (v/v/v). The column was kept at 40 °C and flow rate was 0.5 mL/min with a total run time of 12 min. HPLC and MS–MS conditions are given in Table1.

The opiate drug analytes, their corresponding internal standards, and the SRM transitions are shown in Table 2. Following optimization, at least two SRM transitions for each substance and each internal standard were monitored to provide sufficient identification of drugs and to calculate the ion ratio. Deuterated internal standards of analytes were used for quantitation. The quantifiers were used for all validation parameters. All data were analyzed by Thermo Scientific TraceFinder 2.1 software. Spectral confirmation was carried out with mzCloud mass spectral library.

Validation

The validation procedure included method precision, bias, calibration linearity, the lower limit of quantitation (LLOQ), the limit of detection (LOD), carry-over, matrix effects, and external proficiency performance. For method validation results were evaluated based on pre-established criteria of 'Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology' in 2013 [21] and GTFC acceptance criteria [22] (Table 3). A quantifier ion and one qualifier ion were used with deuterated internal standards. Target ions at *m/z* 286.1, 152.1, and 165 for morphine and *m/z* 303.3, 165.1, and 183.1 for codeine and 328.1, 165.1, and 211 for 6-MAM were used. For calibration samples and validation studies, a 30 mL mixture of clean drug-free urine matrix samples of 3 healthy subjects was used. Concentration of the deuterium labeled internal standards in all samples was 200 ng/mL. Calibration curves were established in the concentration range of 0–2,000 ng/mL for morphine and codeine, and 0–1,000 ng/mL for 6-MAM with three injections at least at 9 different points. The concentrations of the calibrators were 1, 5, 10, 25, 50, 100, 200, 500, 1,000 and 2,000 (only for morphine and codeine) ng/mL. So calibrator reanalysis and injection precision were also checked. Bias and precision were calculated by five repeated infections of three different concentrations of metabolites. For determination of matrix effect five blank urine matrix extracts were spiked into five neat standard solutions at both high and low concentrations by postextraction addition method and ion suppression and/or enhancement were compared. Indeed, interference studies for other often abused screening mandatory drugs/metabolites (amphetamine, methamphetamine, MDMA, cocaine, tetrahidrocannabinol (THC), diazepam) were applied to check ion suppression and/or enhancement. Dilution integrity have been also studied for low specimen volume or excessively high concentrations above the established calibration range by repeating bias and precision studies at dilution ratios of 1:2, 1:10 and 1:50; which all met the acceptance criteria.

Table 1: HPLC gradient, mobile phase contents and MS–MS conditions.

HPLC flow gradient			
Time, min	**Mobile phase A, %**	**Mobile phase B, %**	**Mobile phase C, %**
0	0.5	5	0
0	0.5	5	0
0.5	0.5	40	0
3.6	0.5	95	0
6.6	0.5	100	0
7.8	0.5	0	100
8	0.5	5	0
12	0.5	5	0
MS/MS conditions			
Spray voltage, V			4,000
Vaporizer temperature, °C			300
Sheath gas pressure, arbitrary units			45
Auxillary gas pressure, arbitrary units			10
Capillary temperature, °C			300

Mobile phase A: 10 mM ammonium acetate and 0.1% formic acid in water. Mobile phase B: 10 mM ammonium acetate and 0.1% formic acid in methanol. Mobile phase C: acetonitrile/isopropanol/acetone 9:9:2 (v/v/v).

Sample preparation

For sample preparation after glucuronide hydrolysis dilution was applied. For each sample, a 500 µL aliquot of urine was spiked with 150 µL of internal standards solution and 10 µL of beta-glucuronidase enzyme in 340 µL potassium phosphate buffer (pH=5.0). After the incubation of samples at 60 °C for 1 h, samples were cooled, diluted with 1 mL deionized water and centrifuged. Then, 25 µL of sample was injected into the LC-MS/MS system. Codeine- d3, Morphine-d3 and 6-Acetylmorphine-d3 were used as internal standards.

Statistical analysis

For statistical analysis of the data analyse-it software (Analyse-it Software, Ltd., Leeds, UK) was used. Test sensitivity, specificity, FP and FN rates, likelihood ratio, and odds ratio values with Wilson 95% CI were presented for CEDIA and LC-MS/MS cut-offs –300 and 2,000 ng/mL.

Table 2: Opiate group drug analytes, their corresponding internal standards, and the SRM transitions of both analytes and corresponding internal standards.

Analyte	Precursor ion (*m/z*)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	Retention time, min	Corresponding internal standard	Precursor ion (*m/z*)	Qualifier ion (*m/z*)
Morphine	286.1	152.1	165	1.24	Morphine-d3	289.1	165.1
Codeine	300.2	165.1	215.3	2.24	Codeine-*N*-methyl d3	303.3	183.1
6-MAM	328.1	165.1	211	2.68	6-MAM-d3	331.2	165.1

Table 3: Summary of validation results.

Summary of validation results				
Parameter	**Opiates**			**Acceptance criteria**
	Morphine	**Codeine**	**6-MAM**	
Calibration, ppb	10–2,000	10–2,000	5–1,000 ppb	Calibration linear regression equation must be >0.99
Accuracy (bias), %[a]				Must not exceed ± 20%
1st concentration	−7.68	−3.12	−13.6	
2nd concentration	2.1	5.39	−0.76	
3rd concentration	0.42	−1.71	3.09	
Precision, %				
Within-run 1st concentration	4.77	2.57	13.20	Coefficiency of variance (CV)% must not exceed 20%
Within-run 2nd concentration	1.31	0.79	1.42	
Within-run 3rd concentration	1.58	1.49	1.01	
Between-run	1.74 to 7.00	0.82 to 2.03	1.73 to 5.0	
Analytical range, ppb				
LOB	2.61	−0.96	1.52	Lowest concentration that CV%<20% and S/N>3
LOD	11.06	11.94	2.13	Lowest concentration that CV%<20% and S/N>10
LLOQ	33.51	36.19	6.46	
Carry-over, %	4.71	2.2	No carry over	Carry-over after highest calibrator does not exceed 10% of LOD
Recovery, %	109.11	93.16	101.46	Recovery must not exceed 80–120%.
Processed sample stability, % (72 h)[b]	11.0	−3.5 to 11.76	−1.5 to 0.92	Bias must not exceed ± 20%
Matrix effect				
Relative recovery	81	87	114	Relative recovery must be 80–120%

[a]1st concentrations below cut-off values (25 ppb for morphine, and codeine; 5 ppb for 6-MAM), 2nd concentrations near cut-off values (200 ppb for morphine and codeine; 10 ppb for 6-MAM) and 3rd concentrations higher than the cut-off values (1,000 ppb for morphine and codeine; 100 ppb for 6-MAM). [b]Evaluation of length of time that metabolite in extracted samples stored at room temperature remains stable. LOB, Limit of Blank; LOD, Limit of Detection; LLOQ, Lower Limit of Quantification.

Sensitivity, specificity, and true and false positive and negative results of assays were calculated as described before [23].

Results

In this study, firstly we have validated a robust LC-MS/MS method for qualitative and quantitative analysis of opiates in urine. For opiate confirmation, opiate metabolites morphine, codeine and 6-MAM were quantified. Summary of validation results are given in Table 3. Linear calibration curves for all the analytes under investigation showed determination coefficients (r^2) equal or higher than 0.990 up to 2,000 ng/mL for morphine/codeine and up to 1,000 ng/mL for 6-MAM containing urine samples (Figure 1). The bias values at three different concentrations were within ±20% and at cut-off concentrations, it was 2.1% for morphine, 5.39% for codeine and −0.76% for 6-MAM, respectively. The intraday and inter-day precisions were within 20%. LLOQ of morphine, codeine and 6-MAM was found 33.51, 36.19, and 6.46 ppb, respectively. Zero calibrator containing urine matrix injected after the highest calibrator concentration did not present any detectable carryover. To determine matrix effect prepared and extracted 5 spiked matrix samples at both high and low concentrations using different blank matrices showed no significant ion suppression/enhancement occurred during chromatographic runs, as shown by relative recoveries between 80 and 120%. Processed samples were stable at room temperature for at least 72 h and bias did not exceed 20% for all metabolites. To check freeze-thaw stability, assays of real patient and QC samples were used. After any thawing cycle degradation differences were lower than 10%. Mid-term stability test, performed re-analyzing replicates of three real urine samples twice a month for 6 months, had similar results, with differences

always lower than 10%. Chromatograms obtained after the extraction of the spiked urine sample are shown in Figure 2.

Among the 126 urine samples, 50 were opiate negative (Group 0) and 76 were opiate positive with CEDIA according to 300 ng/mL cut-off, which is based on morphine and its cross-reactive substances.

In accordance with the screening test results, samples with an opiate concentration between 300 and 1,999 ng/mL and an opiate concentration of ≥2,000 ng/mL were confirmed by the LC-MS/MS method. Confirmation of 50 CEDIA opiate negative urine samples demonstrated that all samples were negative for morphine, codeine and 6-MAM, and quantitatively below the confirmation cut-offs 300, 300 and 10 ng/mL respectively (screening negative predictive value 100% at 300 ng/mL cut-off). Sensitivity, specificity, FP rate, FN rate and LR are presented in Table 4.

Based on the concordance between the results of CEDIA 300 ng/mL screening cut-off and morphine 300 ng/mL confirmation cut-off, sensitivity was 100%, specificity was 54.9%, FP rate was 45.1%. However, when the CEDIA cut-off was set at 2,000 ng/mL and morphine and codeine confirmation cut-off set at 300 ng/mL, sensitivity was again 100% and specificity was increased to 82.4% with an FP rate of 17.6%. At the both CEDIA and LC-MS/MS cut-off 2,000 ng/mL, sensitivity was 100% and specificity was increased to 75% with an FP rate of 25.0%. FN rate was 0% for CEDIA opiate screening at each cut-off (Table 4).

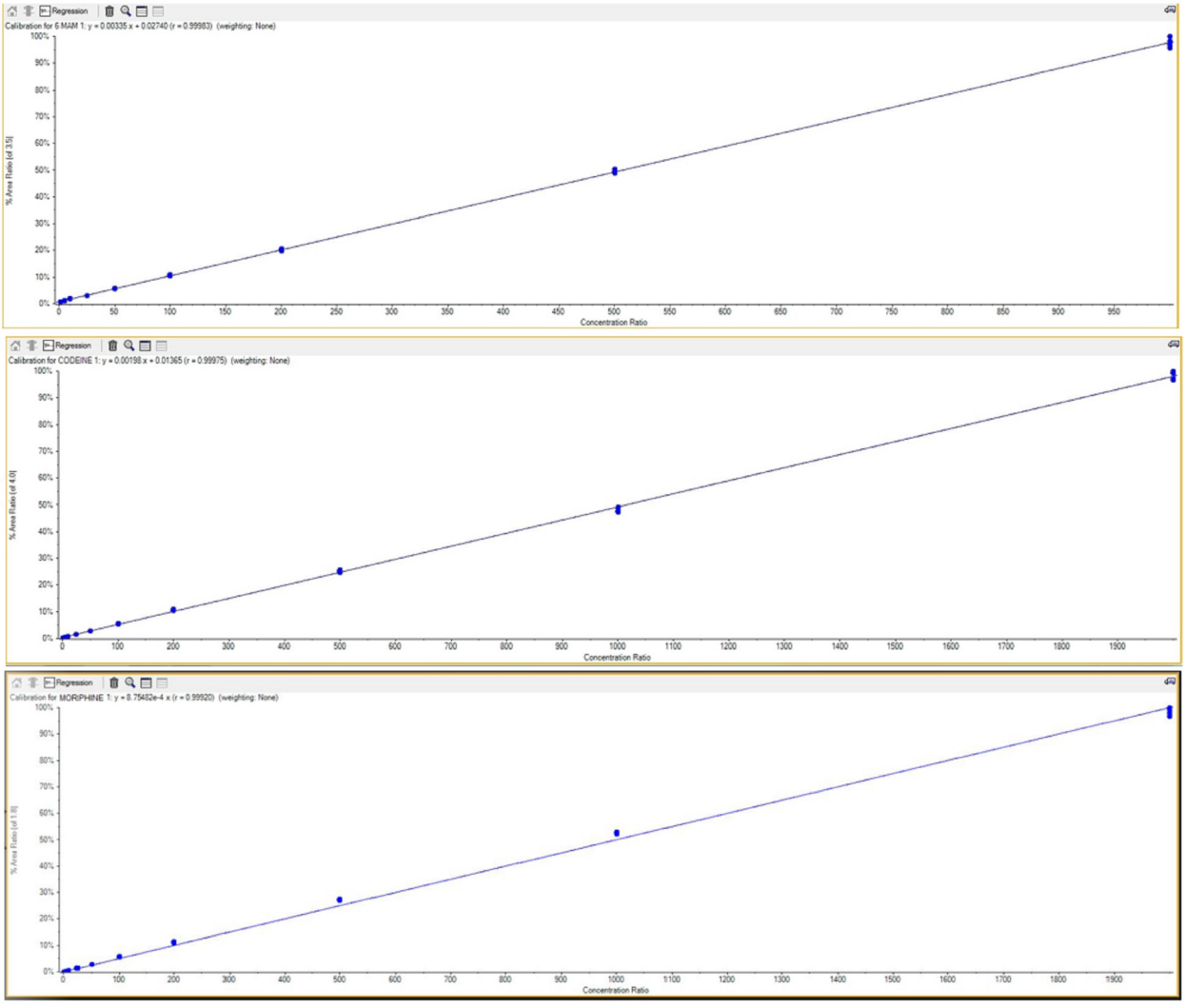

Figure 1: Calibration curves of 6-MAM (a), codeine and morphine.

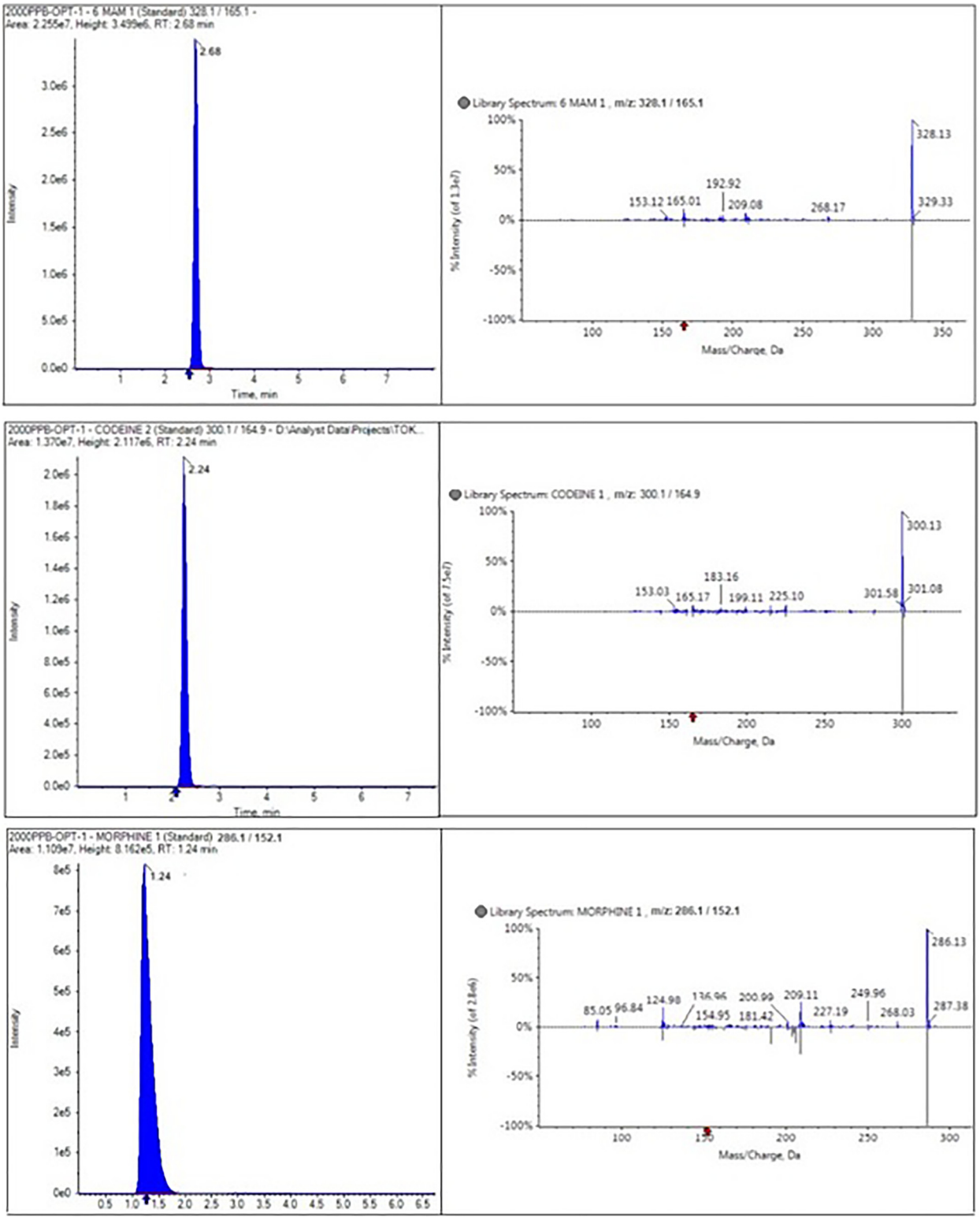

Figure 2: Chromatograms and MS/MS spectra of studied drugs 6-MAM, codeine, and morphine.

The opiate concentration of CEDIA positive 25 samples was between 300 and 2000 ng/mL (Group 1), 51 samples were above 2,000 ng/mL (Group 2). Confirmation of 25 CEDIA positive samples in Group 1 demonstrated that 24 samples (96.2%) were negative and had lower concentrations of morphine than the 300 ng/mL

Table 4: Sensitivity, specificity, false positive and false negative rates at different screening and confirmation cut-off values.

Opiate screening and confirmation (n=126)	Sensitivity (TP) (95% CI)	Specificity (TN) (95% CI)	False positive rate, %	False negative rate, %	Likelihood/odds ratio
CEDIA cut-off 300 ng/mL vs. LC-MS/MS cut-off 300 ng/mL	100 (90.1–100)	54.9 (44.7–64.8)	45.1	0	2.2
CEDIA cut-off 2,000 ng/mL vs. LC-MS/MS cut-off 300 ng/mL	100 (90.1–100)	82.4 (73.3–88.9)	17.6	0	5.7
CEDIA cut-off 2000 ng/mL vs. LC-MS/MS cut-off 2,000 ng/mL	100 (87.1–100)	75.0 (65.7–82.5)	25.0	0	4.0

False positive (FP)=screen positive and LC-MS/MS negative. False negative (FN)=screen negative and LC-MS/MS positive. Sensitivity=(TP)/(TP + FN). Specificity=(TN)/(TN + FP). Positive likelihood ratio (LR+)=TPR/FPR.

threshold; and all samples had 6-MAM below LLOQ (FP rate=96%) (Table 5). Confirmation of one sample showed a positive result with morphine=366 ng/mL concentration with the absence of codeine and 6-MAM.

Confirmation of samples in Group 2 (opiate ≥2,000 ng/mL, n=51) demonstrated that 38 samples (75%) were positive for any opiate metabolite. Morphine concentration of 45 samples was >LLOQ (detected morphine LLOQ=33.51), codeine concentration of 25 samples was >LLOQ (detected codeine LLOQ=36.19), and 6-MAM concentration of 19 samples was >LLOQ (detected 6-MAM LLOQ=6.46) and the cut-off value (6-MAM cut-off=10 ng/mL). Thirty-five samples had a morphine concentration ≥300 ng/mL. Among the morphine negative 16 samples, three of them had codeine concentration ≥300 ng/mL and among these 16 samples, none of them had 6-MAM. In samples opiate screening concentration higher than 2,000 ng/mL FP rate was only 11% (Table 5).

Moreover, 6-MAM was positive in 18 samples, of which all were located in Group 2 (CEDIA opiate ≥2,000 ng/mL).

For discriminating morphine, codeine or heroin use and to identify the effect of only codeine presence on opiate screening concentrations, we investigated morphine/codeine ratio of samples. 51 samples had morphine/codeine ratio below 1, of which 44 were in opiate screening negative group. However 6 cases had "opiate screening concentration" above 2,000 ng/mL and, 1 case between 300 and 2,000 ng/mL. None of these 7 cases had 6-MAM>LLOQ.

Table 5: False positive and false negative numbers of opiate screening positive cases with opiate concentration between 300 and 2,000 ng/mL and opiate concentration higher than 2,000 ng/mL.

Opiate screening positive samples	n, %	False positive cases, n(%)	False negative cases, n(%)
Group 1 (Opiate concentration 300–2,000 ng/mL)	25 (31.9)	24 (96.0)	0 (%)
Group 2 (Opiate concentration ≥2,000 ng/mL)	51 (67.1)	11 (21.5)	0 (%)

Discussion

Drug test immunoassays differentiate negative from presumptive positive results. They do not identify what drug/metabolites are present and false-positive results cannot be distinguished from true positive results. To identify drugs/metabolites and for a true interpretation of test confirmation analysis needed with *LC-MS/MS* or gas chromatography-mass spectrometry (*GC-MS*) techniques.

In this study first, we developed a new, simple, validated opiate confirmation method. All parameters calculated for each analyte were adequate for the present study and acceptable for the established criteria of guidelines (Table 2).

Because of the interferences and high false-positive results Federal Workplace Drug-Testing Programs in the U.S. increased opiate screening and confirmation cut-off values to 2,000 ng/mL since 1998 [24] to reduce false-positive results sourced of poppy seed consumption and widely used medications such as codeine, ethylmorphine, dihydrocodeine, and oxycodone [24, 25]. By increasing the opiate cut-off to 2,000 ng/mL, the codeine and morphine confirmation rate reduced from 7.1 to 2.1%. The codeine-only confirmation rate lowered from 6.6 to 3.4%. It was concluded that by increasing opiate screening and confirmation cut-off values to 2,000 ng/mL more than 300% reduction was observed in the confirmed-positive rate of codeine and morphine. Indeed, a 47% reduction in codeine-only confirmations were observed in a urine drug-testing program where codeine was the major opiate used [25]. However, in some countries still, 300 or even 100 ng/mL opiate screening cut-offs are trend and available [4, 14, 15].

In this study, we investigated TP, TN, FP, and FN rates at proposed cut-off levels (300 ng/mL and 2,000 ng/mL) in Turkey for both screening and confirmation methods. Confirmation of 50 negative of 126 CEDIA opiate urine samples demonstrated that all samples were negative for morphine, codeine, and 6-MAM, demonstrating that CEDIA method was efficient in discriminating true negative results from positive results. Although sensitivity was 100% for CEDIA, specificity was 54.9% at CEDIA cut-off 300 ng/mL vs. LC-MS/MS cut-off 300 ng/mL, and 75% at CEDIA cut-off 2,000 ng/mL vs. LC-MS/MS cut-off 2,000 ng/mL. When CEDIA cut-off is increased to 2,000 ng/mL and compared with LC-MS/MS cut-off 300 ng/mL, specificity also increased to 82.4%, LR increased to 5.7, and FP rate decreased to 17.6%. FP rate was lowest at CEDIA cut-off 2,000 ng/mL vs. LC-MS/MS cut-off 300 ng/mL.

The high immunoassay FP rate in this study are in accordance with literature findings and opiate cut-off administration of SAMHSA [8]. Johnson-Davis et al. found false-positive rates as high as ~34% for opiates [8].

FP rate was highest at CEDIA cut-off 300 ng/mL which may be caused by the consumption of poppy seeds, usage of unprescribed analgesics or cross-reactivity of some drugs or substances. Turkey is an important traditional major opium poppy producer country for medicinal and scientific purposes in the world [26]. Their seeds are usually used for food purposes. Individuals ingesting them may produce urine samples with morphine concentrations above the cut-off concentrations, as ingesting these foods can be falsely accused of illegal drug use [26, 27]. Determining the best discriminative cut-off levels for drug testing is significant, as healthcare providers try to determine whether a result is a true positive and to differentiate between someone who has been abusing morphine or heroin, or has consumed poppy seeds [28, 29].

In Turkey also unprescribed analgesic drug use is relatively in high rate, which of some combined with codeine to increase the analgesic power [30, 31]. The most commonly prescribed drugs that are represented in UDS surveys include opioid analgesics (codeine, hydrocodone, hydromorphone, morphine, oxycodone, and fentanyl) as well as amphetamine and several benzodiazepines [4]. Therefore, the high ingestion rate of opioid group drugs may be one of the reasons of high FP rates. Morphine/codeine ratio is used in forensic medicine to distinguish the consumption of codeine from abuse of morphine and other narcotics especially in investigating doping in sports. Codeine is found in combination preparations with acetaminophen, aspirin or ibuprofen in many over-the-counter drugs used for cold symptoms and analgesia. Morphine/Codeine ratio below 1 is considered as a sign of codeine only intake, whereas the ratio above 1 is considered as a sign of using morphine or heroin [32, 33].

In this study, 51 samples had morphine/codeine ratio below 1, of which 44 were in opiate screening negative group. However 6 cases had "opiate screening concentration" above 2,000 ng/mL and, 1 case between 300 and 2,000 ng/mL. None of these 7 cases had 6-MAM>LLOQ. To discriminate morphine, codeine or heroin consumption of these cases confirmation analysis is and interpretation according to ingestion time of drug, detection period etc. is necessary.

In the second part of the study we grouped CEDIA positive cases into two groups as urine opiate concentration between 300 and 2,000 ng/mL (Group 1), and opiate concentration above 2,000 ng/mL (Group 2). It was remarkable that in Group 1 only one sample among 25 samples was confirmed as positive; 96.0% of samples were negative. Specificity of 300 ng/mL cut-off was 3.8%, FP rate was 96% in Group 1 (opiate concentration between 300 and 2,000 ng/mL) according to 300 mg/dL morphine confirmation cut-off. In Group 2 FP rate decreased to 96%. The most remarkable finding was on 6-MAM in this part of the study. 6-MAM is a sensitive and specific unique metabolite that shows recent heroin intake. The presence of 6-MAM as an analytical target in confirmation analyses of blood or urine samples that screened positive for opiates is proof of recent use (2–8 h) of heroin [34]. In this study 6-MAM was positive in 18 samples; of which all were located in Group 2 (CEDIA opiate ≥2,000 ng/mL). This finding is in accordance with literature findings that, usually after heroin intake detected morphine concentration is higher than 2,000 ng/mL with its metabolite 6-MAM presence in urine above cut-off level (10 ng/mL) [35–38].

The limitation of using the higher opiate immunoassay cut-off is that, it can give much-reduced ability to detect opiates other than morphine or codeine that have lower cross-reactivity, as most of the opiate immunoassays predominantly use morphine as the assay calibrator and they best cross-react with morphine and codeine. So with the use of a high cut-off for opiate the synthetic or semi-synthetic-opiates can be missed. Some of the limitations in our study include a limited metabolite confirmation, which did not contain all of the synthetic or semi-synthetic-opiates. New studies including large series of cases and metabolites is needed. Another limitation was the insufficient prescription or food ingestion information of the patients, before the screening sampling; therefore, we were

unable to determine if drug use was illicit or legitimate. However therapeutic concentration follow up especially 100 ng/mL cut-off for morphine or codeine is used.

These results conclude that;

(1) Opiate screening cut-off 300 ng/mL has low specificity and a high rate of FP results.
(2) Most 6-MAM positive samples give results higher than 2,000 ng/mL with CEDIA screening.
(3) Immunoassay screening cut-off 2,000 ng/mL and confirmation cut-off 300 ng/mL are the most efficient thresholds for the lowest rate of FP results.

In conclusion, FP or FN results of drug abuse tests cause many social or economic problems in the population. For that reason, to minimize especially FP rate of immunoassay results and the number of confirmation tests needed are so crucial. For clinical care and pain-management monitoring, cut-offs may be based on lower concentrations for the urine opiate immunoassay test; however according to the results of this study "2,000 ng/mL" screening and "300 ng/mL" confirmation cut-offs are more appropriate for reporting of analyses intended for illicit substance use (e.g. heroin intake) and workplace drug testing.

Conflict of interest: None declared.

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