



Optimized Procedures for Simultaneous Quantitation of Low Concentration Levels of Morphine and Codeine in Urine Using Gas Chromatography–Mass Spectrometry

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Background: Detection techniques with high specificity, precision and accuracy are required for evidence of illicit drug intake. The study aim was to develop a low-concentration drug testing method for morphine and codeine in urine samples using gas chromatography-mass spectrometry (GC-MS) to establish a precise quantitative analytical method that improves upon limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy of currently available detection methods. **Methods:** Using a 300 ng/mL urine sample, solid phase extraction was performed using an automated solid phase method. All analyses were performed using a Hewlett-Packard (Palo Alto, CA) HP 6890 gas chromatograph interfaced to a HP 5973 mass selective detector (MSD) equipped with a DB-5MS column to acquire full-scan and SIM mass spectrometric data. Solid phase extraction was optimal at pH 9.0 in a 2 ml sample volume. An internal standard concentration of 100 ng/mL yielded optimal results. **Results:** Standard solution ranges (40-450 and 40-1500 ng/mL) significantly influenced LOQ. Calibration methods were not associated with LOD and LOQ for either MOR or COD. The intra- and inter-day precision values did not exceed 2% and were not different within groups. The accuracy of the examined method ranged from 97.8% to 103.3%. All parameters were validated in 33 clinical urine specimens. **Conclusions:** This developed method was successfully used for the determination of morphine and codeine in human urine for forensic identification. The examined protocol can be applied to simultaneous quantification of morphine and codeine at low concentration levels in urine.

Key words: Gas chromatography–mass spectrometry, urine specimen, internal standard method, limit of detection, limit of quantitation, morphine; codeine

INTRODUCTION

Morphine and codeine are naturally occurring alkaloids with high abuse potential. Morphine is a powerful narcotic analgesic and highly addictive. Codeine is a potent μ -opioid receptor agonist which is used for the treatment of adult cough. The presence of illicit and addictive drugs or their metabolites in urine is indicative of the previous intake.^{1,2} Urine analysis for morphine and codeine is used in forensic toxicology to determine drug abuse and diversion;³ however, testing for low-concentrations of morphine and codeine in urine can be

problematic during toxicology drug screens due to elevated threshold values for other available screening tools.

Concentrations of morphine and codeine can be determined simultaneously using micellar electrokinetic chromatography,⁴ disposable pipette extraction,⁵ high performance liquid chromatography (LC),⁶ LC–mass spectrometry (MS),⁷ and LC/triple quadrupole tandem MS.⁸⁻¹⁰ Gas chromatography–MS (GC-MS) has been widely used to

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detect the illicit drugs in blood and urine samples.^{11,12} Methods have been developed for the analysis of 6-acetylmorphine with morphine and codeine (components of heroin).^{10,13} Assays of morphine and codeine by GC-MS are capable of high sensitivity, specificity, and selectivity. GC-MS is considered to be superior to other analytical methods and can provide important diagnostic value to better determine drug use and diversion.

There are currently several recognized limitations and sensitivities for GC-MS that may impede the detection of low concentrations of morphine and codeine in urine.¹⁴⁻¹⁶ These include pH value in solid-phase extraction (SPE), different types and concentrations of internal standards (ISs), different ranges of standard curves, and variable methods of calibration. Other factors affecting the effectiveness of quantitative analysis of low-concentration drugs can also include sample pre-treatment, extraction recovery rate, chemical derivatization technique, contribution of ion exchange between the analyte and isotope IS drug, ionic strength of the analyte, and determination of the temperature increase program and calibration line method in GC-MS. The concentration of drugs in urine specimens may be less than threshold values commonly seen in GC-MS methodology, therefore eluding detection in high-risk patients. In order to avoid encountering false-negative results and variable sensitivity, enhanced tests/technology that provide improved clinical verification for the use of illicit and addictive drugs, including morphine and codeine, at low levels is needed.^{15,17}

The goal of this study was to address practical analytical needs by attempting to develop a low-concentration drug testing method for urine samples, and to establish a precise quantitative analytical method reflecting some of the aforementioned factors affecting quantitative performance, namely limit of detection (LOD) and limit of quantitation (LOQ), precision, and accuracy. When applied to the analysis of samples containing drugs at extremely low and extremely high concentrations, this method will enhance laboratory testing performance and quality management. Specifically, to generate improved quantitative effectiveness at low concentration levels, the following procedures were examined: (1) The extraction recovery of urine samples at different pH condition, (2) the effects of different types and concentrations of ISs, (3) the effects of different ranges of standard curve, and (4) the effects of different calibration methods. A linear calibration approach was adopted to elucidate the quantitative effectiveness of standard solutions based on selecting optimal procedures by comparing results of LOD and LOQ.

METHODS

Chemicals, reagents, and supplies

Morphine (M-005), Morphine-D3 (M-006), Morphine-D6 (M-086), Codeine (C-006), Codeine-D3 (C-007) and Codeine-D6 (C-041) of 1 mg/ml methanol solution with 99% purity were obtained from Cerilliant (Sigma-Aldrich). Reagents used for the derivation of the analytes and the ISs, Bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 10 g/L trimethylchlorosilane was purchased from Supelco (Bellefonte, PA, USA). Urine samples positive for illicit drugs and used for study analyses were obtained from the Division of Clinical Toxicology, Tri-Service General Hospital. Drug-free urine samples were provided by a member of the research group. All samples were kept at -20°C until analyzed.

Solid phase extraction

Using a urine sample (300 ng/mL), SPE was performed using the automated solid-phase method (Zymark Rapid Trace SPE Workstation). Manufacturer instructions were followed. Briefly, after extraction, the IS was added, derivatization and GC/MS analysis were then performed. The second set of samples of the same concentration were combined with methanol, followed by the addition of the IS. After blow-drying, derivatization and GC/MS analysis was performed. The calculation of extraction recovery rate was based on the strength ratio of identical ion pairs and was assessed by dividing the ratios for each concentration sample in the first set by the ratio of the corresponding concentration sample in the second set. A range of pH values was used to optimize extraction conditions.

Chemical derivatization

A screw-capped 10-ml glass extraction tube was used to collect 2 ml analyte, 100 μL IS (300 ng/mL), and 200 μL 12 N HCl solution. The extraction tube was then autoclaved (15 Psi, 120°C , 30 min). After cooling, a 1.5 ml phosphate buffer was added and mixed thoroughly. The sample was then adjusted to the indicated pH, followed by SPE. The organic was then transferred and evaporated under a stream of nitrogen. Once the samples were completely dried, 100 μL BSTFA and Ethyl acetate-mix (1:1) were added. The extraction tube was then capped and incubated at 100°C for 30 min. Samples were cooled to room temperature and 2 μL of derivatized samples were analyzed by a Hewlett-Packard (Palo Alto, CA, USA) HP 6890 gas chromatograph interfaced to an HP 5973 mass selective detector equipped with a DB-5MS column (30-M, 0.25-mm ID, 0.25- μm film thickness) to acquire full-scan and selected

ion monitoring (SIM) mass spectrometric data. SIM mode was applied to quantify analytes [Table 1].

Determination of ionization saturation concentration

In order to perform quantitative ion assessment and to determine a linear range, urine samples were diluted (from approximately 100 to 3000 ng/mL). The relationship between ion abundance and concentration of the ions selected was used in full-scan MS to assess the ionization saturation concentration of various ions in the analyte.

Preliminary selection of ions to be measured

After sample derivatization was performed, full-scan mass spectrometer data were employed to perform a preliminary selection of ionic fragments with little or no overlap between the isotope drug and analyte. Ions with high mass-to-charge ratio and high ionic strength were selected for ionic monitoring and analysis. Two to three ions for both the analyte and IS were selected, and samples were diluted from a concentration of 3000 ng/mL to 100 ng/mL, concentration against strength was recorded through SIM, and the linear regression coefficient was used to assess the selected stable ionic fragments.

Analytical effectiveness of different measurement methods

A comparison of the effect between one-point calibration, multi-point linear calibration, and polynomial calibration measurement methods on LOD and LOQ was performed. One-point calibration: (abundance of analyte ion/abundance of IS ion in analyte)/(ion abundance in threshold value standard solution/abundance of IS ion in threshold value standard solution) $\times$ concentration of threshold value standard solution = analyte concentration. Linear calibration: $y = ax + b$. Polynomial calibration: $y = a \times^3 + b \times^2 + cx + d$.

Limit of detection and limit of quantification

The LOD and LOQ were determined based on the approach of "Standard Deviation (SD) of the Response and the Slope" whereby the LOD is expressed as $LOD = 3 s \sigma/m$, and the LOQ is expressed as $LOQ = 10 \sigma/m$. The σ is the SD of the response, and m is the slope of the calibration curve. The average LOD and LOQ were calculated. The empirical method for determining LOD and LOQ herein is performed by analyzing a series of standard solutions at a concentration of 40, 80, 150, 200, 250, 500, 1000, and 3000 ng/mL for six analytes. The LOD is defined as the lowest concentration at which the ion ratios meet within $\pm 20\%$ relative to those obtained for a calibrating standard assay. The LOQ is defined as the lowest concentration at which the ion ratios meet

Table 1: Selective results of qualitative and quantitative ion

Derivatization analytes	BSTFA Selective ions (m/z)		
MOR	429*	414	401
MOR-D3	432	417	
MOR-D6	435	420	
COD	371	372	343
COD-D3	374	346	135
COD-D6	377	349	

*Quantitative ions^o; MOR=Morphine; COD=Codeine; BSTFA=Bis (trimethylsilyl) trifluoroacetamide

acceptance criteria, and the assayed and target concentrations meet within $\pm 20\%$.

Precision and accuracy assessment

For intraday assessment, measurements of 5 urine samples in different batches with concentrations of 0, 150, 225, 375, and 450 ng/mL were taken during the same day (a total of seven tests were performed during the 1-day). For interday assessment, a new linear calibration line was established every day for 5 days based on measurements of different batches of urine samples with concentrations of 0, 150, 225, 375, 450 ng/mL. This test was performed using one-way analysis of variance.

The accuracy of the analytical method was assessed through two stages. After determining optimal conditions, the first stage consisted of establishing a calibration line based on samples with concentration points of 0, 150, 225, 375, 450 ng/mL prepared in-house. The accuracies of the measured concentrations were expressed in terms of percent degree of deviation.

Quantitative data analysis

The recovery rate was presented as mean $\pm$ SD for a given pH value in the MOR and chemical oxygen demand (COD) group, respectively. Differences between groups at each pH value were compared using an independent *t*-test. Statistical assessments were two-tailed and considered statistically significant at $P < 0.05$. Descriptive statistics and other analyses were performed using Microsoft Excel 2007.

RESULTS

Solid phase extraction

The SPE recovery rate directly determines LOD and LOQ. SPE conditions for two opiate drugs in urine samples were evaluated: sample pH and volume. Three urine samples with 300 ng/mL MOR and COD at a pH range of 4.0–12.0

were evaluated. The urine sample pH was adjusted with 11.8 N KOH and 9 N HCl to 4.0, 6.0, 8.0, 9.0, 10.0, 11.0, and 12.0. MOR recovery rates were good at pH values of 9.0 (72.02%) and 10.0 (71.67%), but the recovery rate was very poor at a pH of 4.0–8.0 and ≥ 11.0 (<50%). For COD, the recovery rate increased with increasing pH and was highest at pH 8.0, 9.0, and 10.0; a gradual decrease was observed at pH 11.0 and 12.0. Taken together, MOR and COD recovery rates were highest between pH 8.0 and 10.0 [Figure 1]. All urine samples in this study were extracted using a Varian extraction column, and pH 9.0 was used when performing the extraction.

Three urine samples at a volume of 1 mL, 2 mL, and 3 mL (plus 1.5 M phosphoric acid buffer solution) were evaluated. Two ion pair results were obtained following GC/MS analysis of each analyte and IS (MOR/MOR-D3 [m/z 429/432] and COD/COD-D3 [m/z 371/374]). The averages of the resulting three sets of ion-pair strength ratios were then obtained. The calculation of the extraction recovery rate was based on the ion-pair strength ratio of the analyte and IS, and was calculated by dividing the ratios of the samples that had undergone extraction by the ratios of the samples with corresponding concentrations that had not undergone extraction. With regard to the recovery rate for different sample quantities, in the case of both MOR and COD, the 2 mL and 3 mL samples had increased recovery rates compared with the 1 mL samples [Figure 2]. Based on these results, the experiments were performed at a pH of 9.0 in a 2 mL sample volume.

Internal standard type and concentration

ISs may cause severe cross-contamination of low-concentration analytes, and different amounts of

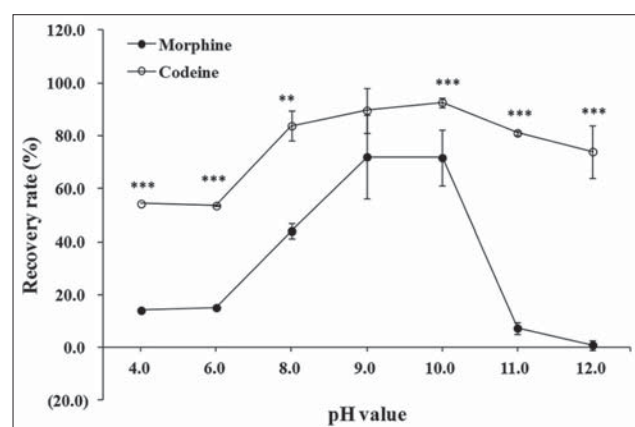


Figure 1: Comparison of recovery rate between morphine and codeine at different pH values. Data were presented as mean $\pm$ standard deviation for a given pH value. Differences between morphine and codeine were compared using an independent *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, indicated a significant difference between groups

the IS can cause differences in the characteristics of the calibration line. Three solutions (2 mL) with concentrations of 40–3000 ng/mL were examined with 100, 200, and 300 ng/mL IS added to determine their impact on LOD and LOQ. MOD-D3 and MOD-D6 were applied for MOR, while COD-D3 and COD-D6 were applied for COD. MOR-D3 and MOR-D6 were used as ISs for MOR, and COD-D3 and COD-D6 were used as an IS for COD. Using the BSTFA derivatization method, MOR-D6 was significantly better than MOR-D3 [Table 2], although this had no significant influence on LOD. These results indicate that the greater the amount of IS, the larger the LOD. However, different ISs may yield different LOD values even when the amount added is the same. Taking MOR-D3 and MOR-D6 as examples, MOR-D6 has relatively little interaction with analyte ions, and the LOD was significantly lower. Using the multi-point linear measurement method, 100, 200, and 300 ng/mL amounts of IS were used to determine LOQ [Table 2].

Ranges of standard curve

Three sets of standard solutions with respective concentration ranges of 40–450, 40–1500, and 40–3000 ng/mL were prepared, and the effect of the concentration ranges on

Table 2: Effects of different types and concentrations of internal standards for limit of detection and limit of quantitation

Analytes	ISs	ISs additive volume (ng/mL)					
		100		200		300	
		LOD	LOQ	LOD	LOQ	LOD	LOQ
MOR	MOR-D3	80	60	80	60	100	60
	MOR-D6	60	60	60	60	80	60
COD	COD-D3	60	60	60	60	80	60
	COD-D6	60	60	60	60	60	60

Unit=ng/mL; ISs=Internal standards; LOD=Limit of detection; LOQ=Limit of quantitation; MOR=Morphine; COD=Codeine

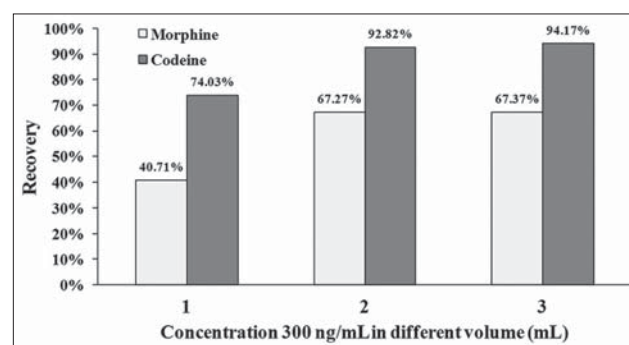


Figure 2: Recovery (%) for morphine and codeine at a concentration of 300 ng/mL in different volumes ($n = 4$ per group)

LOD and LOQ were determined [Table 3]. The LOD values for the two analytes were 60 ng/mL suggesting different standard solution concentration ranges have no direct influence on LOD. The MOR and COD LOQ values were 40 ng/mL for the three standard solution concentration ranges, except for the 40–3000 ng/mL concentration range, which was 120 ng/mL. These results indicate that higher standard solution ranges may have a significant influence on LOQ, but the 40–450 and 40–1500 ng/mL concentration ranges may be best applied in low-concentration testing.

Calibration methods

We had further compared the effects of different calibration methods, including one-point, multi-point linear, and polynomial methods for LOD and LOQ. For each calibration method, the LOD and LOQ for MOR and COD were 60 ng/mL and 40 ng/mL, respectively [Table 4]. These data suggest calibration methods are not critical in determining LOD and LOQ.

Proof of applicability

The percent coefficient of variation values calculated for intra- and inter-day precision of MOR and COD did not exceed 2% and were not significantly different ($P > 0.05$); hence, the examined method was considered precise for MOR and COD [Figure 3a and b]. The accuracies of the measured concentrations were expressed in terms of the percentage degree of deviation. The accuracies ranged from 99.82% to 101.33% for MOR and 99.11% to 102.84% for COD [Figure 3c]. Taken together, using the linear

measurement method, the accuracy of this analytical method ranged from + 1% to – 2%.

Four urine spikes of amphetamine drugs (amphetamine, methamphetamine, MDA, MDMA) were used to further examine the examined methodology [Table 5]. Samples B1, B2, and B3 were blind quality control samples that yielded results uniformly within $\pm 20\%$. Validation was carried out using 33 clinical urine specimens, [Samples P1 through P33 in Table 5]. The differences were uniformly $>\pm 20\%$ regardless of whether the concentration was ≥ 1000 ng/mL.

Table 3: Effects of different ranges of standard curve for limit of detection and limit of quantitation

Analytes	Standard solutions concentration range (ng/mL)					
	40-450		40-1500		40-3000	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
MOR	60	40	60	40	60	120
COD	60	40	60	40	60	40

Unit=ng/mL°; LOD=Limit of detection; LOQ=Limit of quantitation; MOR=Morphine; COD=Codeine

Table 4: Effects of different calibration methods for limit of detection and limit of quantitation

Analytes	Calibration methods					
	One-point		Multi-point linear		Polynomial correction	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
MOR	60	40	60	40	60	40
COD	60	40	60	40	60	40

Unit=ng/mL°; LOD=Limit of detection; LOQ=Limit of quantitation; MOR=Morphine; COD=Codeine

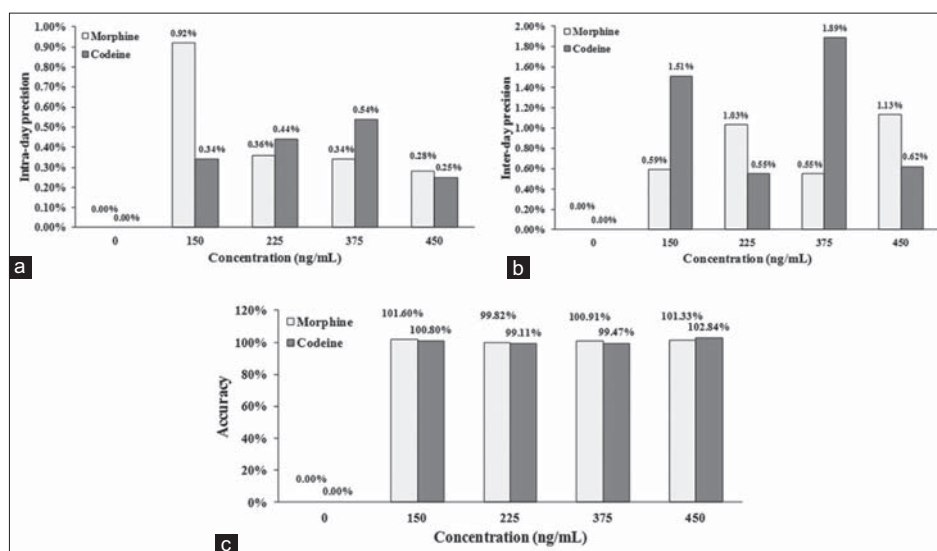


Figure 3: Intraday precision ($n = 7$) (a), interday precision ($n = 5$) (b), and accuracy ($n = 5$) (c) for morphine and codeine. Values represent coefficient of variation (%)

Table 5: The concentration of opium drug in urine specimen

Cases	Analytes					
	MOR			COD		
	TSGH	This study	Reproducibility	TSGH	This study	Reproducibility
B1	229	225	-1.75	229	225	-1.75
B2	388	381	-1.80	387	386	-0.26
B3	0	0	0.00	0	0	0.00
P1	0	0	0.00	550	558	1.45
P2	0	0	0.00	416	423	1.68
P3	0	0	0.00	3128	3606	13.26
P4	0	0	0.00	1665	1650	-0.90
P5	1448	1636	12.98	0	0	0.00
P6	1115	1219	9.33	0	0	0.00

TSGH=Results provided by the clinical toxicology laboratory at Tri Service General Hospital (derivative agents: BSTFA); This study=Results acquired from this study; BSTFA=N,O-bis (trimethylsilyl) trifluoroacetamide; MOR=Morphine; COD=Codeine

DISCUSSION

The currently examined GC-MS method demonstrated a precise and accurate low concentration drug testing method for morphine and codeine in urine. Briefly, we verified the optimized procedures for simultaneous quantitation of low concentrations of MOR and COD. The data showed the best recovery of SPE for a urine specimen was obtained at pH 9.0. In the concentration range of 40–1500 ng/mL linear calibration curves, with 100 ng/mL IS, 50 μ L BSTFA derivatization and 50 μ L ethyl acetate had presented as a validated method with LOD and LOQ at 60 ng/mL and 40 ng/mL, respectively. The intra- and inter-day precision values did not exceed 2% and were not different within groups. The accuracy of the examined method ranged from 99.11% to 103.3%. All parameters were validated in 33 clinical urine specimens containing four types of amphetamine drugs.

The current results are consistent with previous reports where GC-MS had been used to evaluate low concentrations of MOR and COD in urine. Zhang *et al.* reported a stable, selective, and sensitive GC-MS methodology for the simultaneous determination of MOR and COD in human urine.¹¹ Specifically, a reported intraday precision of 12% or less, and an interday precision of 13% or less. This compares with the currently reported intraday and interday precision of 2% or less. In addition, the accuracy of the methodology reported by Zhang *et al.* ranged from 87.2% to 99.7%, whereas the current study demonstrated an accuracy of 99.82% to 101.60% for MOR and 99.11% to 102.84% for COD. Differentiating factors in the current study to previous reports

are the findings clarifying the impact of the concentration of an IS, optimal standard solution ranges, as well as the minimal impact of calibration methodology on LOD or LOQ.

Considering LOQ is the minimum concentration point obtained by means of multi-point linear measurement using the foregoing standard solution within 20% of the theoretical concentration. Because of this, parameters influencing LOD will directly determine the LOQ results. In the case of opiate drugs, experimental results have shown that the addition of 100 ng/mL IS will enable the effective implementation of chemical derivatization. Apart from the addition of 50 μ L BSTFA as a derivatization agent, 50 μ L ethyl acetate solution was also added to increase the cosolvency of the derivatization agent and facilitating reaction of the derivatization agent with the analyte. The use of MOR-D6 and COD-D6 as ISs is optimal.

Recent reports, using different GC-MS methodology, have demonstrated varying levels of accuracy and precision in the analysis of urine for opiates, including MOD and COD.^{18,19} These reports also begin to address the need for optimized derivatization approaches for multiple opioids in urine.¹⁹ The currently described methodology represents a standardized procedure for the detection of low concentration illicit opiates, with results clearly identifying improved precision and accuracy. Furthermore, calling additional attention to the benefits of an appropriate IS for use in GC-MS analyses.²⁰ Taken together, the use of a standardized and routine methodology provides control of the entire analysis which should positively impact screening results.²¹

Based on the results of the current study, the use of GC-MS for the evaluation of morphine and codeine at low concentrations is recommended. To generate the better quantitative effectiveness at low concentration levels, the following variables are recommended to be included in any standard operating procedure development: (1) the extraction recovery of urine samples at different pH condition, (2) the effects of different types and concentrations of ISs, (3) the effects of different ranges of standard curve, and (4) the effects of different calibration methods. The linear calibration approach was adopted to elucidate the quantitative effectiveness of standard solutions based on selecting optimal procedures by comparing results of LOD and LOQ.

The current study contained several limiting factors that should be recognized: (1) It is not clear if the recommended “optimal” methodology and GC-MS parameters identified are universal; among the examined variables is the make and model of GC-MS; (2) For those countries where individuals addicted to illicit drugs tend to use multiple substances, including opioids and cocaine, this study may not be applicable.

CONCLUSIONS

The aim of the current study was to develop a low-concentration drug testing method for morphine and codeine in urine samples using GC-MS to establish a precise quantitative analytical method that improves on LOD, LOQ, precision, and accuracy of currently available detection methods. The currently developed methodology was validated by 33 clinical specimens for the determination of morphine and codeine in human urine for forensic identification. The examined protocol can be applied to the simultaneous quantification of morphine and codeine at low concentration levels in urine.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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