Brazilian Journal of Analytical Chemistry
Pre-publication – Accepted after peer review
doi: 10.30744/brjac.2179-3425.AR-88-2025

ARTICLE

Comprehensive Extraction Optimization and LC–MS Validation for Detection of Illicit and Prescription Opioids in Human Urine

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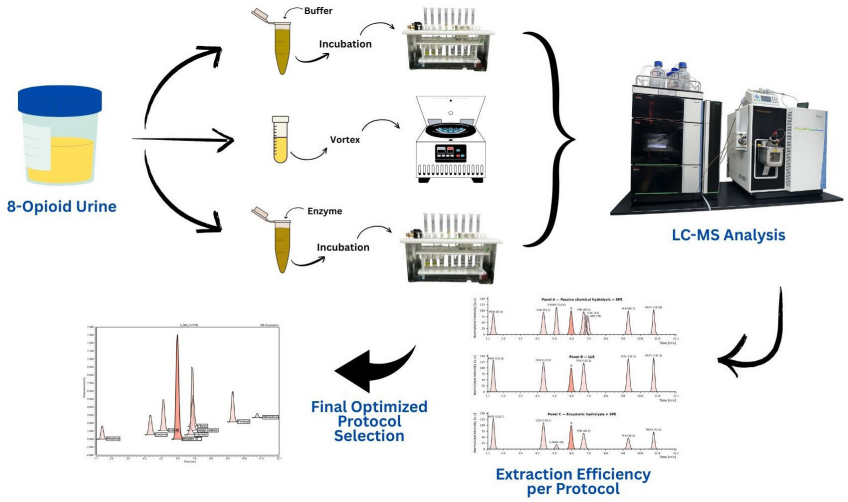

The misuse of opioids, including fentanyl, methadone, and heroin, has increased the demand for reliable and economical toxicological screening techniques. In resource-limited forensic settings, conventional approaches, such as enzymatic hydrolysis combined with tandem mass spectrometry (MS/MS), are often impractical. This study aimed to develop, optimize, and validate a reliable liquid chromatography–mass spectrometry (LC–MS) method for the quantification of eight opioids in urine using a single-quadrupole mass spectrometer. Three sample preparation strategies were comparatively evaluated: enzymatic hydrolysis followed by solid-phase extraction (SPE), liquid–liquid extraction (LLE), and passive chemical hydrolysis followed by SPE. Analyte stability (up to 96 h), carryover, matrix effect (ME), linearity, precision, and accuracy were assessed during method validation in accordance with the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The most consistent recovery (RE) was obtained with passive chemical hydrolysis followed by SPE at 54 °C for 6 h, with RE values ranging from 82.0 ± 2.1% to 114.4 ± 3.0%, whereas heroin showed a slightly lower RE (78.0 ± 3.4%) because of partial hydrolysis to 6-monoacetylmorphine and morphine. The method demonstrated excellent linearity, with the coefficient of determination (R^2) ranging from 0.991 to 0.998, good precision, expressed as relative standard deviation (%RSD) ≤ 16.8%, and acceptable bias (−13.0%

Cite: Ndikumana, E.; Pandey, A.; Dixit, A.; Soni, A.; Mahankar, N. Comprehensive Extraction Optimization and LC–MS Validation for Detection of Illicit and Prescription Opioids in Human Urine. *Braz. J. Anal. Chem.* (Forthcoming). http://dx.doi.org/10.30744/brjac.2179-3425.AR-88-2025

Submitted: September 3, 2025; **Revised:** October 21, 2025; December 6, 2025; December 25, 2025; January 16, 2026; January 22, 2026; **Accepted:** January 23, 2026; **Published online:** March 2026.

to 18.0%). All analytes exhibited ME values within 80–120%, indicating negligible ion suppression or enhancement. The applicability of the method to real-world samples was confirmed in urine casework: one sample tested positive for tramadol, whereas two were negative, supporting the method's forensic and clinical utility. Overall, the optimized single-quadrupole LC–MS method provides a reliable, cost-effective, and selective alternative for urinary opioid analysis, suitable for routine use in low-resource laboratories owing to its enzyme-free extraction and reliance on a single-quadrupole mass spectrometer.

Keywords: cost-effective analysis, method validation, single-quadrupole LC–MS, urine, opioid detection

INTRODUCTION

The International Narcotics Control Board (INCB) has reported a concerning increase in counterfeit opioid drugs, particularly in underdeveloped regions, where approximately 25–50% of dispensed prescription drugs may be fraudulent, thereby intensifying the risks associated with opioid use.[1,2] The cultural, economic, and criminal ramifications of the opioid crisis highlight the pressing need for robust analytical frameworks. Opioid-related deaths are rising sharply, placing a significant burden on healthcare systems and law enforcement worldwide. Heroin and fentanyl, along with their analogs, are primary drivers of this trend.[3-7] The spread of illicit synthetic opioids has increased drug trafficking and violent and organized crime, thereby worsening societal instability.[6,7] Given the widespread availability and increased potency of synthetic opioids, the forensic community has an ongoing need to improve toxicological methods that accurately identify and differentiate illicit opioids, prescription opioids, and their metabolites.[8-10]

In forensic investigations, identifying opioids in biological samples is crucial for evaluating drug use and intoxication, and for determining the cause of death, especially in cases involving overdoses, drug-facilitated crimes, and impaired-driving incidents.[11] The urinary marker 6-monoacetylmorphine provides definitive evidence of heroin use, distinguishing it from morphine use and ingestion of poppy seeds.[12-14] 6-acetylcodeine, an impurity in illicit heroin production, also serves as a forensic marker for heroin abuse.[14-16] Routine opioid screening in forensic and clinical toxicology is vital for assessing treatment adherence and identifying potential misuse, particularly for potent opioids such as fentanyl and methadone, which are frequently associated with fatal overdoses.[3,17]

Advances in analytical methods have revolutionized opioid detection and quantification, with liquid chromatography–mass spectrometry (LC–MS) becoming the preferred technique over conventional gas chromatography–mass spectrometry (GC–MS).[12,18] LC–MS offers increased sensitivity, selectivity, and flexibility for drug analysis in complex biological matrices, including urine and blood.[19,20] Urine-based assays typically employ β-glucuronidase hydrolysis with multi-hour incubations,[21-23] which can be inefficient and may cause loss of labile analytes.[14,24] Current analytical workflows often rely on tandem mass spectrometry (MS/MS) with multiple reaction monitoring (MRM) for detection, a highly effective yet resource-intensive approach that can limit accessibility in low-resource settings.[25,26] Rigorous validation is also required to ensure accuracy, precision, and specificity across matrices and conditions, as underscored by international analytical and forensic guidelines.[27-29]

These gaps motivated the development of a thoroughly validated, economically viable, and optimized single-quadrupole LC–MS method for the reliable quantification of opioids in forensic and clinical urine samples.

The physicochemical properties of the natural opioids (morphine and codeine), the semi-synthetic opioids (6-monoacetylmorphine, 6-acetylcodeine, and heroin), and the synthetic opioids (fentanyl, methadone, and tramadol) guided the development and validation of the LC–MS method. These opioids are weak bases ($pK_a \approx 8.7–9.3$), with lipophilicity ranging from that of morphine ($\log K_{ow} \approx 0.9$) to those of methadone and fentanyl ($\log K_{ow} \approx 4.0–5.0$), and molecular weights spanning 263–369 g mol^{-1}.[30-32] As shown in Figure 1, structural features that affect analytical behavior include free phenolic hydroxyls in morphine, codeine, 6-monoacetylmorphine, and tramadol (increasing polarity and causing earlier elution on C18); acetyl groups in heroin and 6-acetylcodeine (masking phenols, increasing lipophilicity, enabling retention, introducing

hydrolytically unstable esters); and the overall higher lipophilicity of fentanyl and methadone. All compounds contain basic nitrogen, which facilitates efficient ionization in positive electrospray ionization (ESI) mode. These properties determine the extraction recovery (RE) and ionization efficiency and thus inform the LC–MS method design and validation.[31-34]

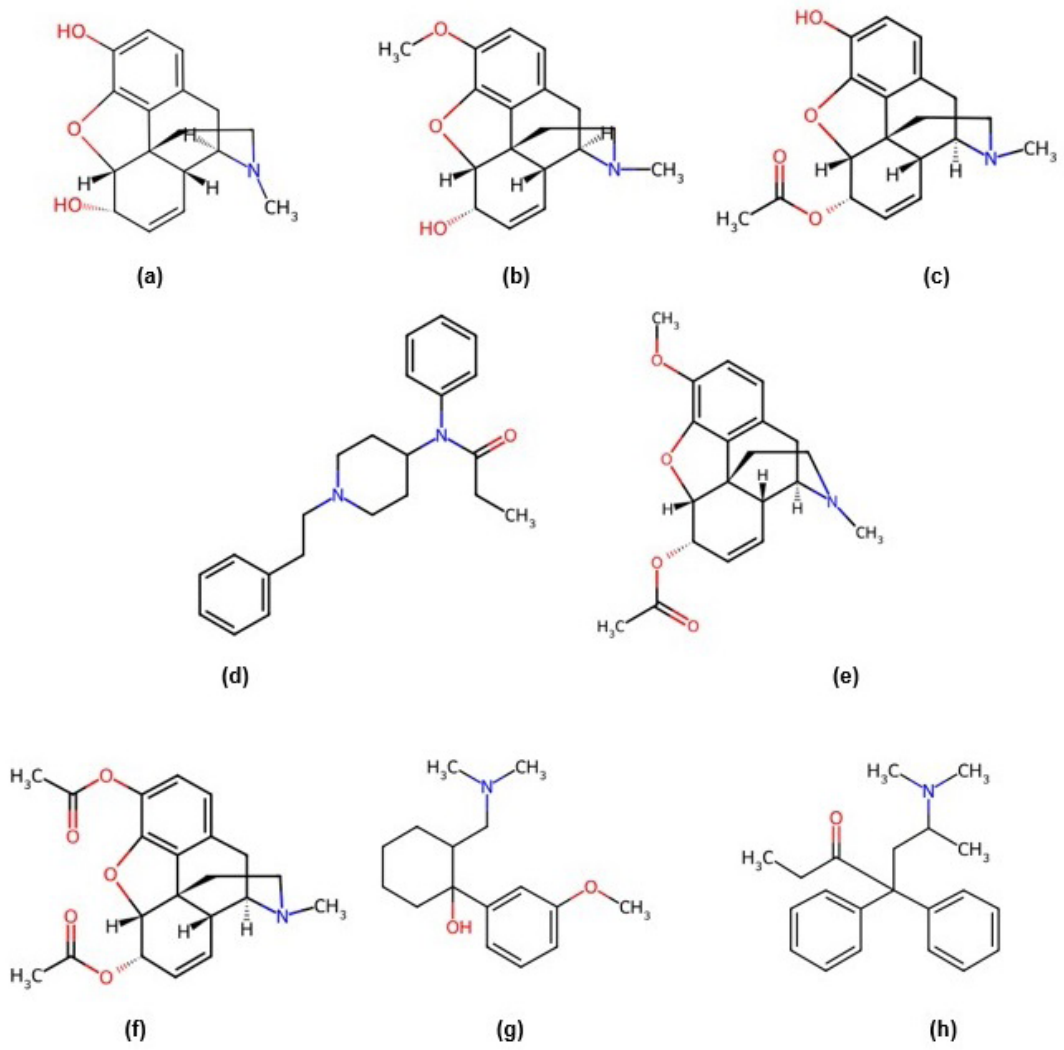

Figure 1. Chemical structures of the eight target opioids analyzed in this study: (a) morphine, (b) codeine, (c) 6-monoacetylmorphine, (d) fentanyl, (e) 6-acetylcodeine, (f) heroin, (g) tramadol, and (h) methadone.

This study presents an innovative and cost-effective single-quadrupole LC–MS assay for the simultaneous analysis of eight opioids in urine. It minimizes the need for expensive MS/MS instrumentation and eliminates the need for β-glucuronidase hydrolysis, offering a practical solution for resource-limited laboratories. Using simple procedures, the method employs passive chemical hydrolysis followed by solid-phase extraction (SPE), and through systematic assessment of incubation times, a 6 h incubation at 54 °C was identified as optimal, yielding higher RE efficiency than shorter or longer durations.[21-23] Furthermore, the method avoids the routine use of stable isotope-labeled internal standards (SIL-IS), preserves labile opioids, simplifies sample preparation, and enables reliable, consistent, and legally defensible quantification in forensic and clinical contexts.

MATERIALS AND METHODS

Chemicals and equipment

LC–MS grade methanol (≥ 99.9%), ethyl acetate, and acetonitrile were obtained from Thermo Fisher Scientific (Mumbai, India). Ultrapure water (Type I; 18.2 MΩ·cm at 25 °C) was produced in-house using the XTRAPURE Plus LabLink water purification system (Mumbai, India). Sigma-Aldrich (Karnataka, India) supplied LC–MS grade acids and buffers, including ammonium formate (LiChropur, ≥ 99%), formic acid (98–100%), acetic acid (LiChropur, 100%), and sodium acetate. Additional analytical grade reagents, including ammonia solution (≥ 25%, EMSURE ISO), phosphate buffers, sodium chloride, magnesium sulfate, and sodium azide, were obtained from VWR Chemicals and RANKEM (Gurugram, India). Oasis HLB SPE cartridges (60 mg, 3 mL) were purchased from Waters Corporation (Bengaluru, India). β-glucuronidase (abalone-derived aqueous solution; ≥ 100,000 U mL^{-1}) was obtained from Sigma-Aldrich. Vadilal Chemical Ltd (Gujarat, India) supplied ultra-high purity nitrogen gas (grade 5.0). Cerilliant Corporation (Bangalore, India) provided certified reference standards for the target analytes (morphine, codeine, 6-monoacetylmorphine, tramadol, 6-acetylcodeine, heroin, fentanyl, and methadone). Because SIL-IS were temporarily unavailable, atropine was used as a substitute internal standard (IS). Equipment for sample preparation and storage included a Waters 20-position SPE manifold (Massachusetts, USA), a Labman LMUC25D ultrasonic cleaner (Tamil Nadu, India), a Neuation iTherm D150-4 dry heating block (Gujarat, India), and an ultra-low temperature freezer (MarkEn; −20 to −86 °C; Mumbai, India).

Biological specimen collection and handling

For method development and validation, urine samples were collected from ten healthy laboratory staff volunteers. The samples were screened for the target analytes. Negative specimens were pooled and centrifuged to eliminate particulates. Immediately after collection, sodium azide (0.1% w/v) was added to each urine sample as a preservative to limit microbial growth and endogenous enzymatic activity during storage and handling, thereby minimizing analyte degradation.[35,36] The pooled blank urine was stored at −20 °C until use in further method development and validation. To protect privacy, all demographic information was excluded, and donors provided samples at least two weeks after any medication use to prevent potential interference.

In addition, three urine specimens were obtained from Kanoria Hospital & Research Center, Ahmedabad, Gujarat, India, as part of a rehabilitation monitoring and de-addiction program, and were immediately analyzed using the validated assay. All procedures were approved by the Institutional Ethics Committee (Approval No. NFSU/SDSR/IEC/Certificate/1054/2024).

Preparation of calibration, quality control (QC), and IS solutions

Because of differences in solubility, 6-monoacetylmorphine, 6-acetylcodeine, and heroin were prepared in acetonitrile, whereas morphine, codeine, tramadol, fentanyl, and methadone were prepared in methanol; all stock solutions were prepared at 1.0 mg mL^{-1}. A mixed working solution was then prepared by combining the individual stocks to yield 100.0 µg mL^{-1} of each analyte. A working IS solution (100.0 µg mL^{-1}) was prepared in methanol by diluting the 1.0 mg mL^{-1} stock solution. Calibration standards (0.5–25.0 µg mL^{-1}) were obtained by serially diluting the mixed working solution in a 90:10 (v/v) diluent consisting of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid. QC levels at 0.5, 1.0, 10.0, and 20.0 µg mL^{-1} were prepared from the same mixed working solution by dilution to the target levels, followed by spiking into pooled blank urine. IS was added to all prepared samples (calibrators, QC samples, and case specimens) to a final concentration of 10.0 µg mL^{-1}. All study samples were stored at −20 °C when not in use.

LC–MS instrumentation and conditions

Chromatographic conditions

A C18 Acclaim 120 Å column (150 × 3 mm, 3 µm) was used for chromatographic separation on a Vanquish

liquid chromatography (LC) system (Thermo Fisher Scientific, USA). The column temperature was maintained at 35 °C, with a 5 µL injection volume and an autosampler temperature of 8 °C. The binary mobile phases consisted of 10 mM ammonium formate with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient was optimized to effectively separate analytes of differing polarity, starting at 90% A/10% B at 1.0 mL min^{-1} from 0.00 min, changing to 70% A/30% B at 0.4 mL min^{-1} at 2.00 min, and then to 60% A/40% B at 8.50 min. At 10.00 min, the flow rate was returned to 1.0 mL min^{-1} and the mobile phase was changed to 20% A/80% B. From 12.50 to 14.50 min, the system was re-equilibrated to the initial conditions (90% A/10% B). Consistent retention time (RT) and smooth solvent transition were achieved using a gradient curve value of 5.

Mass spectrometric conditions

A single-quadrupole ISQ EC mass spectrometer (Thermo Fisher Scientific, USA) was used for detection in positive ESI mode. The source operated at a vaporizer temperature of 350 °C and an ion transfer tube temperature of 325 °C, a spray voltage of +3500 V, and sheath, auxiliary, and sweep gas pressures of 50, 7.5, and 1.5 psig, respectively. The mass spectrometry (MS) data were acquired in full-scan mode over the mass-to-charge ratio (*m/z*) range of 30–550 with a scan time of 1 s. Data were then processed as extracted ion chromatograms (EIC). The analytes were identified by RT and two in-source fragment ions (quantifier and qualifier) for each analyte, with further confirmation based on the qualifier-to-quantifier ion ratio.

MS acquisition and ion selection

In the positive ESI mode, the analytes formed protonated molecules $[M+H]^+$. The data were acquired in the full-scan mode and processed as EIC. Specific ions were selected as quantifiers and qualifiers to provide structural information and strong signal intensity under in-source fragmentation conditions. The monitored ions (*m/z*; quantifier and qualifier) were: codeine (300.1 and 243.1); morphine (286.1 and 229.0); 6-monoacetylmorphine (328.1 and 211.1); 6-acetylcodeine (342.2 and 225.1); heroin (370.1 and 165.0); fentanyl (188.1 and 337.2); and methadone (265.1 and 105.1). A consistent qualifier ion was not established for tramadol under these conditions (Table II); therefore, identification relied on the quantifier ion together with RT and peak purity checks. Atropine, the IS, was monitored at *m/z* 290.1 and 124.1. All confirmation limits (RT repeatability and ion ratio tolerance) are detailed in the method development plan under the RT repeatability and ion ratio criteria section.

Optimization of extraction for analyte RE and matrix cleanup

We systematically evaluated three urine extraction methods to optimize analyte RE and matrix cleanup: liquid–liquid extraction (LLE), passive chemical hydrolysis followed by SPE, and enzymatic hydrolysis followed by SPE.

LLE

A 500 µL aliquot of pooled blank urine was spiked with a QC mixed standard containing the eight analytes and 5 µL of the IS (100.0 µg mL^{-1}). The sample was first basified with three drops of 5 N potassium hydroxide, then diluted with 250 µL of deionized water and mixed for 30 s. Salts were precipitated by adding 0.375 g of sodium chloride and 0.250 g of magnesium sulfate, and the mixture was vortexed for 5 min. Analytes were extracted with 1 mL of ethyl acetate, and the mixture was centrifuged at 3000 rpm for 15 min. The upper organic layer was collected and dried at ambient temperature under a gentle nitrogen stream. The residue was reconstituted in 50 µL of mobile phase, a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, centrifuged, filtered, and injected into the LC–MS system.

Passive chemical hydrolysis followed by SPE

A 500 µL aliquot of pooled blank urine was spiked with a QC mixed standard containing the eight analytes and 5 µL of the IS (100.0 µg mL^{-1}), then combined with 250 µL of 0.1 M acetate buffer (pH 5.6) and incubated

at 54 °C for 6 h. After cooling to room temperature, an equal volume (1:1) of 0.1 M phosphate buffer (pH 6.0) was added. The samples were processed using HLB cartridges preconditioned with 1 mL of methanol and 2 mL of 2% aqueous ammonia. After sample loading, the cartridges were washed with 1.5 mL of 5% methanol, dried under vacuum for 5 min, and sequentially eluted with 0.5 mL of methanol and 0.5 mL of ethyl acetate. The eluate was dried under nitrogen at room temperature, reconstituted in 50 µL of mobile phase consisting of a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, centrifuged, filtered, and injected into the LC–MS system.

Enzymatic hydrolysis followed by SPE

A 500 µL aliquot of pooled blank urine was spiked with a QC mixed standard containing the eight analytes and 5 µL of the IS (100.0 µg mL^{-1}). Then, 40 µL of β-glucuronidase and 250 µL of 0.1 M acetate buffer (pH 5.6) were added, and the mixture was incubated at 54 °C for 6 h. The sample was cooled to ambient temperature and mixed with an equal volume (1:1) of 0.1 M phosphate buffer (pH 6.0) after hydrolysis. SPE was performed on HLB cartridges preconditioned with 1 mL of methanol and 2 mL of 2% aqueous ammonia. After sample loading, the cartridges were washed with 1.5 mL of 5% methanol, dried under vacuum for 5 min, and sequentially eluted with 0.5 mL of methanol and 0.5 mL of ethyl acetate. The eluate was dried under nitrogen at room temperature, reconstituted in 50 µL of a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, centrifuged, filtered, and injected into the LC–MS system.

Unless otherwise specified, all QC levels are based on nominal concentrations in the final extracts, corresponding to a 10-fold enrichment relative to the original urine according to the relationship $C_{urine} = C_{extract} / 10$, where C_{urine} is the concentration in the original urine and $C_{extract}$ is the concentration in the final extract after 10-fold enrichment; this relationship was also applied to determine the back-calculated urine concentrations of analytes in casework specimens.

Assessment of analyte RE across three extraction methods

Three replicates (n = 3) of pooled blank urine samples were spiked and processed, resulting in a final extract concentration of 10.0 µg mL^{-1} per analyte. To determine analyte-specific extraction efficiency, samples were processed separately for each method (LLE, passive chemical hydrolysis followed by SPE, and enzymatic hydrolysis followed by SPE). The RE for each method was reported as the mean ± standard deviation (SD).

Method development plan

Evaluation of incubation time on analyte RE

Passive chemical hydrolysis followed by SPE was performed at 54 °C to assess the effect of incubation time on analyte RE and to maximize RE, as described in the Optimization of extraction for analyte RE and matrix cleanup section. A 500 µL aliquot of pooled blank urine was spiked with a mixed standard solution containing the eight analytes and the IS, then processed to achieve a nominal concentration of 10.0 µg mL^{-1} for each analyte and the IS in the final extract. Samples were incubated for 1, 6, 10, or 16 h before extraction. Three independent replicates were prepared at each time point (n = 3).

The extraction RE efficiency was calculated according to the Matuszewski protocol[37] by comparing the pre-extraction-spiked matrix with the post-extraction-spiked matrix, and the results were expressed as mean ± SD (%). The optimal incubation time was selected to preserve all analytes and provide consistent RE for subsequent validation.

RT repeatability and ion ratio criteria

The chromatographic precision and qualitative confirmation were evaluated under fixed LC–MS conditions, as described in the LC–MS instrumentation and conditions section, using the low QC (LQC) level (0.5 µg mL^{-1} in the final extract). The data were acquired in full-scan mode and processed as EIC. For RT repeatability,

the same QC was injected ten times (n = 10) within a single batch, and RT was recorded and reported as mean ± SD and relative standard deviation (%RSD) for each analyte. The designated quantifier and qualifier ions for each analyte were extracted as EIC, as described in the MS acquisition and ion selection section. The target ion ratio was determined and compared with those in other QC and unknown samples, with a tolerance of ± 20%. The RT acceptance criterion required that each analyte's RT in a sample be within ± 2% of the calibrator RT established from repeated injections. Tramadol confirmation under single-quadrupole in-source fragmentation, in the absence of a reliable qualifier ion, relied on the quantifier ion, the RT criterion, and chromatographic peak purity checks.

Validation and acceptance criteria

Eight opioids (morphine, codeine, 6-monoacetylmorphine, tramadol, 6-acetylcodeine, heroin, fentanyl, and methadone) were validated in human urine in accordance with standard practices in forensic toxicology and the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The validation assessed selectivity and specificity, the calibration model and linearity, the limits of detection (LOD) and quantification (LOQ), accuracy (bias, %), precision (repeatability and reproducibility), RE, matrix effect (ME), process efficiency (PE), carryover, and the stability of processed samples.[27,28]

Evaluation of linearity and analytical range

Calibration standards were prepared at five concentrations (0.5, 1.0, 5.0, 10.0, and 25.0 μg mL^{-1}). Five replicates were analyzed at each level. The analyte-to-IS peak area ratio was used as the instrument response, and data were processed in Chromeleon Console 7 (version 7.3.1.6535). The linearity was assessed using linear regression with 1/x weighting. The analytical range was established based on two criteria: (*i*) a coefficient of determination (R^2) ≥ 0.990 and (*ii*) back-calculated concentrations within ± 20% of the nominal values across all calibration levels.

Precision and accuracy: repeatability, reproducibility, and bias

Precision and accuracy were assessed in pooled blank urine samples at three QC levels, which corresponded to nominal concentrations of 1.0, 10.0, and 20.0 μg mL^{-1} in the final extracts. Intra-day (repeatability) was evaluated using six replicates for each level (n = 6) within a single batch, and inter-day (reproducibility) was evaluated over three consecutive days with 18 replicates (n = 18). The response of the instrument was expressed as the ratio of the analyte-to-IS peak area. The accuracy (bias, %) was calculated as [(observed mean − nominal value) / nominal value] × 100, and the precision (%RSD) was calculated as (SD / observed mean) × 100. The acceptance criteria required accuracy (bias, %) within ± 20% of the nominal value at each QC level and a precision (%RSD) ≤ 20% for both intra-day and inter-day measurements.

Analytical sensitivity and carryover assessment

The analytical sensitivity was evaluated by determining the LOD and LOQ. The background noise at each analyte's RT was measured in ten individual blank urine samples on the MS quantification channel. The blank signal variability and the calibration curve slope were used to calculate the LOD and LOQ. The LOD was defined as (3 × SD of blank urine signal) / slope of the calibration curve, and LOQ as (10 × SD of blank urine signal) / slope of the calibration curve. Practical evaluation was conducted by spiking pooled blank urine samples and processing them to yield concentrations of 0.310–0.724 μg mL^{-1} in the final extracts, after which five replicates (n = 5) were analyzed. The operational LOQ required a signal-to-noise ratio (S/N) ≥ 10, accuracy (bias, %) within ± 20%, and precision (%RSD) ≤ 20%. Given the 10-fold enrichment from urine to the final extract, the LOQ was also expressed as an effective urine concentration in the Analytical sensitivity and carryover subsection within the Results and discussion section. Qualitative confirmation required that the RT be within ± 2% of the calibrator value and that the qualifier-to-quantifier ion ratio be within ± 20% of the calibrator's ratio.

Three replicates of pooled blank urine extracts were analyzed immediately after injection of the highest calibrator (25.0 μg mL^{-1}) to assess carryover. For each blank extract, the peak area of the quantifier ion was measured at the specified RT for each analyte. Carryover was considered absent if the mean blank peak area was ≤ 10% of the LOQ response on the calibration curve.[38]

Assessment of ME, RE, PE, and selectivity

ME, RE, and PE were assessed using the Matuszewski protocol.[37] Two concentration levels in the final extract were assessed: 1.0 μg mL^{-1} at the LQC level and 20.0 μg mL^{-1} at the high QC (HQC) level. Three sample sets were prepared: Set 1 (A) comprised neat standards in 50 μL of mobile phase (90:10, v/v; 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid); Set 2 (B) comprised post-extraction-spiked samples prepared by adding analytes to extracted pooled blank urine; and Set 3 (C) comprised pre-extraction-spiked pooled blank urine processed using the optimized extraction protocol. Each set at each level was prepared in triplicate (n = 3). ME (%) was calculated as (B / A) × 100, RE (%) as (C / B) × 100, and PE (%) as (ME × RE) / 100. Alternatively, PE (%) was calculated as (C / A) × 100. Values within 80–120% were considered acceptable for ME, RE, and PE.

Selectivity was evaluated by analyzing ten individual blank urine samples.[39,40] Samples were extracted and injected without added analytes or IS to assess potential interferences at the RT of each analyte and at the IS RT by monitoring the relevant MS ions and target ion ratios. A pooled blank urine sample was also spiked with buprenorphine, hydrocodone, hydromorphone, meperidine, naloxone, naltrexone, oxycodone, and oxymorphone, then processed and analyzed. Interference was evaluated in the spiked pooled blank urine sample and blank donor samples. The acceptance criterion for selectivity was the absence of interfering peaks at each analyte's RT and at the IS RT.

Stability of processed urine samples

Stability was assessed using pooled blank urine samples at two QC levels (1.0 and 10.0 μg mL^{-1} in the final extracts). Three replicates (n = 3) were analyzed at each QC level immediately after extraction to establish the reference concentration at t = 0 h. The processed samples were stored at 4 °C and analyzed in triplicate at 24, 48, 72, and 96 h. Quantification was performed using new calibration curves prepared at each time point. Stability was expressed as percent change (% change), calculated as [(mean C_t − mean C_0) / mean C_0] × 100, where C_t is the mean concentration at each time point (24, 48, 72, and 96 h) and C_0 is the mean concentration at t = 0 h. The stability acceptance criterion was a % change within ± 20% of the initial concentration.

RESULTS AND DISCUSSION

Results of method development

Chromatographic separation

The developed LC–MS method demonstrated high chromatographic efficiency and selectivity, achieving baseline separation of all analytes, ranging from moderately polar species (e.g., morphine and codeine) to highly lipophilic species (e.g., methadone and fentanyl), within 11 min (Figure 2).

An Acclaim C18 column maintained at 35 °C provided baseline separation for all analytes, with sharp, symmetric peaks despite similarities in structure and retention behavior. A polarity-driven elution trend was observed: morphine (1.42 min) and codeine (4.35 min) eluted early owing to their hydrophilicity (log K_{ow} < 1.0), whereas more lipophilic opioids, including fentanyl (9.31 min) and methadone (10.79 min), were retained longer, consistent with their higher log K_{ow} values (Figure 1, Figure 2).

The method also resolved closely related analytes, including tramadol (6.70 min), 6-acetylcodeine (6.83 min), and heroin (6.95 min), demonstrating the ability of the method to separate structurally similar opioids. The separation between heroin and its hydrolytic metabolite, 6-monoacetylmorphine, was distinct, enabling precise identification and quantification in urine samples (Figure 2).

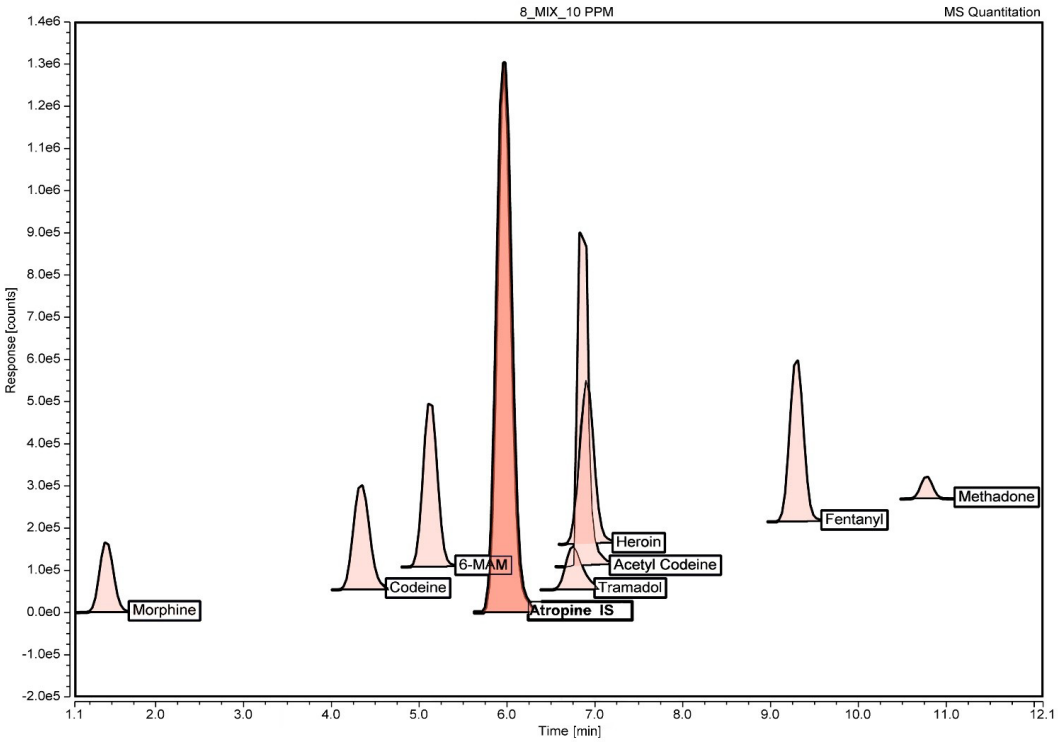

Figure 2. Representative LC–MS EIC showing the separation of eight target opioids and atropine IS at 10.0 μg mL^{-1}, prior to passive chemical hydrolysis followed by SPE. 6-monoacetylmorphine was labelled as 6-MAM.

Comparison of RE efficiency across three extraction methods

All analytes were successfully extracted with consistent RE and low variability using passive chemical hydrolysis followed by SPE. The RE (%) values (mean ± SD) were 87.5 ± 3.1% for morphine, 93.1 ± 2.7% for codeine, 114.4 ± 3.0% for 6-monoacetylmorphine, 95.5 ± 2.6% for tramadol, 82.0 ± 2.1% for 6-acetylcodeine, 78.0 ± 3.4% for heroin, 98.7 ± 3.1% for fentanyl, and 103.8 ± 1.9% for methadone. The chromatograms (Figure 3A) showed well-defined, intact peaks across the analyte panel, with %RSD values of approximately 2–3%, indicating good repeatability.

Enzymatic hydrolysis followed by SPE yielded variable results, with generally poor performance for ester-linked opioids. Heroin and 6-acetylcodeine were not detected, and 6-monoacetylmorphine was markedly low (19.0 ± 2.1%). Synthetic opioids, such as tramadol (66.6 ± 2.7%), fentanyl (46.6 ± 1.8%), and methadone (72.2 ± 2.2%), showed lower RE, whereas morphine (128.7 ± 2.0%) and codeine (110.1 ± 2.4%) showed elevated RE values (Figure 3C). This approach did not achieve comprehensive RE across the analyte panel, as indicated by the loss of ester-linked species in the chromatograms.

LLE favored lipophilic analytes and produced clear RE values exceeding 100% for several analytes, consistent with ESI ion enhancement. The observed RE values were 131.8 ± 2.5% for morphine, 122.9 ± 2.7% for codeine, 120.3 ± 2.6% for tramadol, 138.5 ± 2.8% for fentanyl, and 142.2 ± 1.9% for methadone. In contrast, heroin, 6-acetylcodeine, and 6-monoacetylmorphine were not detected, and their absence was confirmed in the chromatograms (Figure 3B).

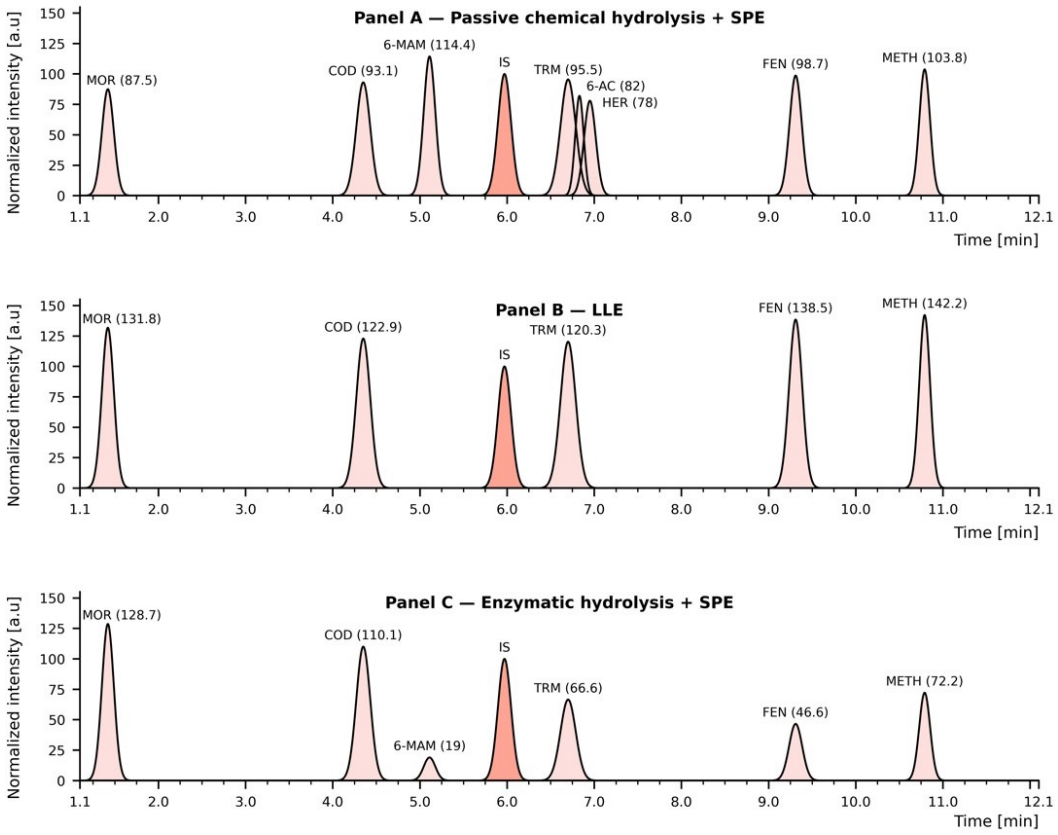

Figure 3. Chromatographic profiles of eight target opioids (10.0 µg mL^{-1}) obtained using three extraction approaches: (A) passive chemical hydrolysis followed by SPE, (B) LLE, and (C) enzymatic hydrolysis followed by SPE. Chromatograms are plotted as IS–normalized intensity. Values shown in parentheses above each analyte correspond to RE (%) for the respective method. Peak labels: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; IS, atropine; TRM, tramadol; 6-AC, 6-monoacetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Collectively, these data indicate that passive chemical hydrolysis followed by SPE provides the most comprehensive and well-balanced RE profile across all analytes. Enzymatic hydrolysis resulted in the loss of labile ester derivatives, lowering the RE for several targets. In contrast, LLE preferentially extracted lipophilic analytes but failed to recover ester-linked analytes and often produced clear RE values > 100%. Therefore, passive chemical hydrolysis followed by SPE was selected as the optimal extraction method for quantifying target urine opioids.

Effect of incubation time on RE

The RE values were inconsistent after 1 h at 54 °C. RE (%) for morphine (185.6 ± 3.2%), codeine (132.1 ± 3.4%), and 6-monoacetylmorphine (130.8 ± 2.9%) was high, whereas the RE (%) for tramadol (56.4 ± 3.5%), fentanyl (52.4 ± 2.1%), and methadone (49.3 ± 3.8%) was low. The RE of 6-acetylcodeine (98.9 ± 4.1%) was approximately 100%, whereas that of heroin (47.2 ± 3.3%) was markedly low, indicating an imbalance during early hydrolysis (Table I, Figure 4).

Table I. RE efficiency (%; mean ± SD) for analytes at 1, 6, 10, and 16 h of passive chemical hydrolysis followed by SPE at 54 °C (*n* = 3)

Analyte	1 h (%)	6 h (%)	10 h (%)	16 h (%)
Morphine	185.6 ± 3.2	112.0 ± 3.1	135.5 ± 3.0	145.5 ± 2.9
Codeine	132.1 ± 3.4	110.0 ± 2.7	120.2 ± 2.9	139.5 ± 3.0
6-monoacetylmorphine	130.8 ± 2.9	114.4 ± 3.0	90.1 ± 3.1	23.3 ± 3.1
Tramadol	56.4 ± 3.5	96.5 ± 2.6	82.2 ± 2.7	78.5 ± 2.7
6-acetylcodeine	98.9 ± 4.1	82.0 ± 2.1	55.4 ± 3.0	7.1 ± 3.4
Heroin	47.2 ± 3.3	78.0 ± 3.4	40.2 ± 3.1	n.d*
Fentanyl	52.4 ± 2.1	98.7 ± 2.8	90.6 ± 2.9	55.7 ± 3.0
Methadone	49.3 ± 3.8	103.8 ± 1.9	95.5 ± 2.7	55.6 ± 2.9

*n.d.: not detected.

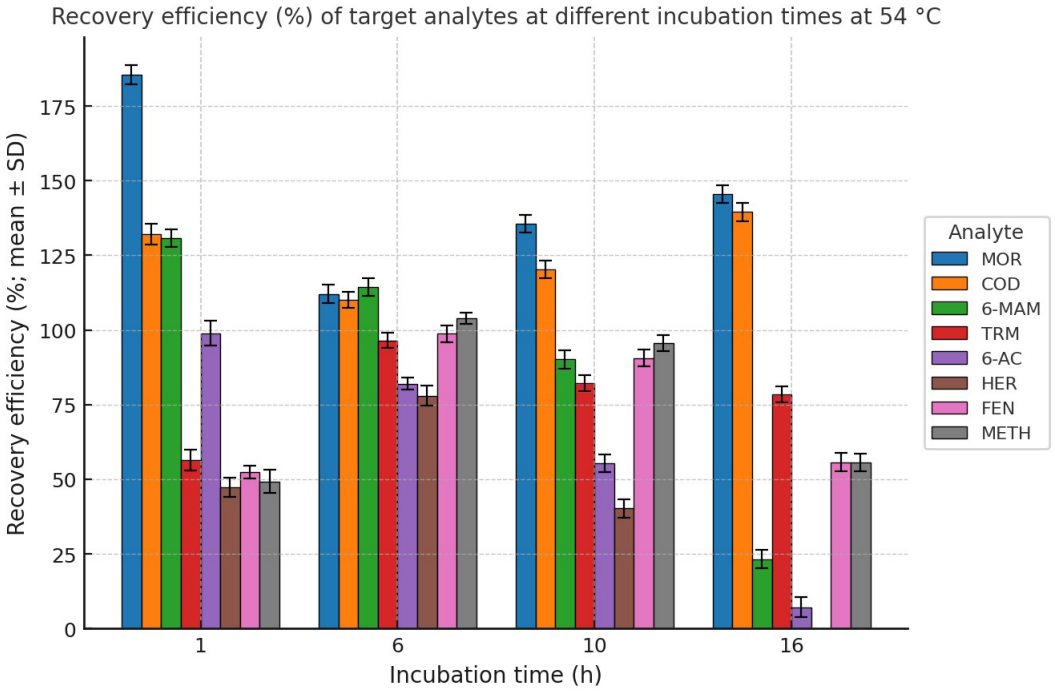

Figure 4. RE efficiency (%; mean ± SD) of target analytes at incubation times of 1, 6, 10, and 16 h during passive chemical hydrolysis at 54 °C followed by SPE (*n* = 3). Analytes are labeled as: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; TRM, tramadol; 6-AC, 6-acetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Extending the incubation period to 6 h yielded a consistent, panel-wide RE profile with low variability (≈ 80–115% across the panel). The RE values were 112.0 ± 3.1% for morphine, 110.0 ± 2.7% for codeine, 114.4 ± 3.0% for 6-monoacetylmorphine, 96.5 ± 2.6% for tramadol, 82.0 ± 2.1% for 6-acetylcodeine, 78.0 ± 3.4% for heroin, 98.7 ± 2.8% for fentanyl, and 103.8 ± 1.9% for methadone, confirming balanced hydrolysis and efficient extraction (Table I, Figure 4).

At 10 h, the RE values for ester-linked opioids decreased, whereas their deacetylated products increased: 6-monoacetylmorphine fell to 90.1 ± 3.1%, 6-acetylcodeine to 55.4 ± 3.0%, and heroin to 40.2 ± 3.1%; morphine increased to 135.5 ± 3.0% and codeine to 120.2 ± 2.9%. Tramadol (82.2 ± 2.7%), fentanyl (90.6 ± 2.9%), and methadone (95.5 ± 2.7%) remained within an intermediate RE range (Table I, Figure 4).

Degradation was evident by 16 h, as 6-monoacetylmorphine decreased to 23.3 ± 3.1%, 6-acetylcodeine to 7.1 ± 3.4%, and heroin was not detected. Further declines were observed for fentanyl (55.7 ± 3.0%) and methadone (55.6 ± 2.9%). Morphine and codeine continued to rise to 145.5 ± 2.9% and 139.5 ± 3.0%, respectively (Table I, Figure 4).

Collectively, these data indicate that 6 h at 54 °C provides the most well-balanced and reproducible RE across the panel, achieving RE values of approximately 80–115% with low variability (low SD values), whereas 1 h produces an unbalanced profile, and durations of 10–16 h lead to progressive loss of ester-linked opioids with reciprocal increases in morphine and codeine (Figure 4, Figure 5).

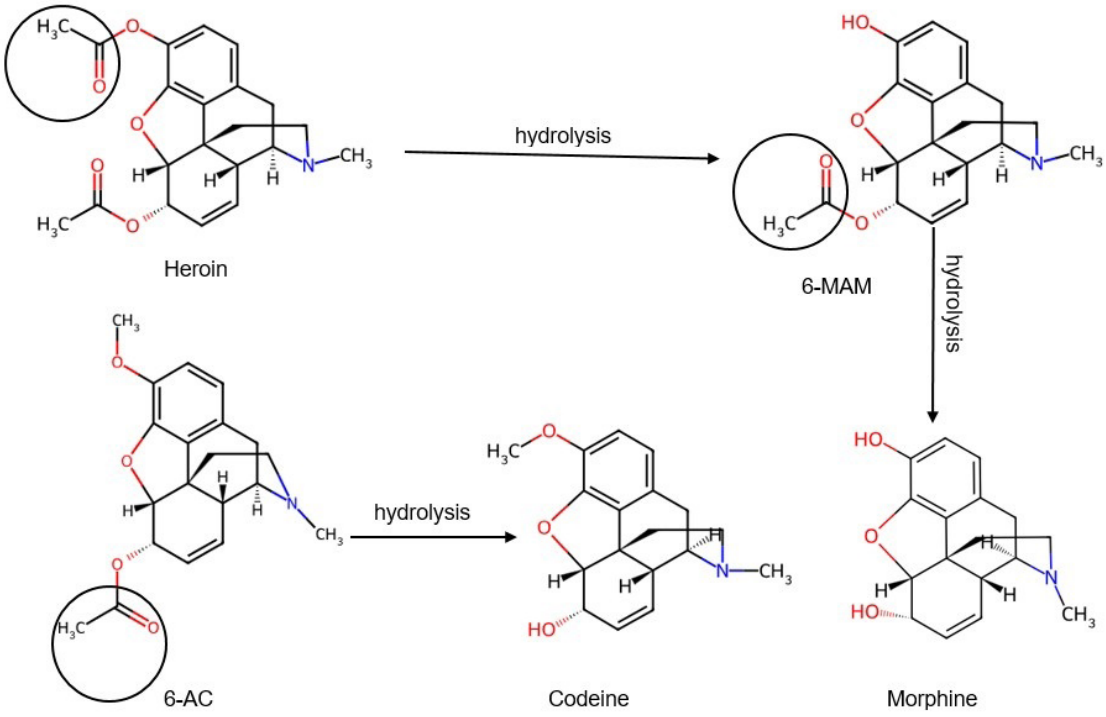

Figure 5. Hydrolytic instability of esterified opioids, illustrating ester cleavage and stepwise hydrolysis of heroin to 6-monoacetylmorphine (labeled as 6-MAM) and then to morphine, and of 6-acetylcodeine (labeled as 6-AC) to codeine.

RT repeatability and ion ratio performance

At the LQC level (0.5 μg mL^{-1}), RT was highly consistent across all analytes, with %RSD ranging from 0.000% to 0.563% (Table II). Tramadol showed the largest deviation (0.563%) but remained within acceptable limits; by comparison, fentanyl and methadone varied by 0.181% and 0.088%, respectively. The IS exhibited stable RT, supporting consistent normalization. Collectively, these observations demonstrate excellent chromatographic precision at the LQC level (Table II).

Table II. RT repeatability for analytes, including atropine as the IS, expressed as mean ± SD and %RSD, and MS transitions, with their *m/z* values and ion ratios, at the LQC level (0.5 µg mL^{-1}; *n* = 10)

Analyte	LQC (µg mL^{-1})	RT (min)		MS transitions		Ion ratio evaluation			
		Mean ± SD	%RSD	Quantifier (*m/z*)	Qualifier (*m/z*)	Ion ratio (%)	Range (±20%)	Lower limit (%)	Upper limit (%)
Morphine	0.5	1.42 ± 0.000	0.000	286.1	229.0	7.31	1.46	5.84	8.77
Codeine	0.5	4.35 ± 0.000	0.000	300.1	243.1	8.75	1.75	7.00	10.50
6-monoacetylmorphine	0.5	5.11 ± 0.015	0.302	328.1	211.1	3.85	0.77	3.08	4.62
Tramadol	0.5	6.70 ± 0.038	0.563	58.2	—	—	—	—	—
6-acetylcodeine	0.5	6.83 ± 0.000	0.000	342.2	225.1	37.51	7.50	30.01	45.01
Heroin	0.5	6.95 ± 0.000	0.000	370.1	165.0	40.77	8.15	32.62	48.30
Fentanyl	0.5	9.31 ± 0.017	0.181	188.1	337.2	37.35	7.47	29.88	44.82
Methadone	0.5	10.79 ± 0.009	0.088	265.1	105.1	40.99	8.20	32.79	49.19
Atropine	10.0	5.97 ± 0.000	0.000	290.1	124.1	50.00	10.00	40.00	60.00

— indicates values that were not applicable to tramadol.
Identification was confirmed using in-source fragments monitored by EIC, and ion ratios were calculated from the corresponding EIC peak areas.

The qualifier-to-quantifier ion ratios for analytes with a qualifier ion fell within the ± 20% tolerance range established from the calibrators (Table II), confirming specificity.[41] For example, morphine and methadone had an ion ratio of 7.31 (range, 5.84–8.77) and 40.99 (range, 32.79–49.19), respectively. In this study, the single-quadrupole LC–MS method produced only weak, matrix-dependent in-source fragments for tramadol, which did not meet the predefined criteria for a reliable qualifier ion because of insufficient S/N and lack of an interference-free response across urine matrices. Therefore, the identification of tramadol relied on three criteria: quantifier ion, RT within ± 2% of the calibrator value, and chromatographic peak purity checks. These criteria provide specificity without a qualifier ion. MRM qualifier transitions require tandem LC–MS. A qualifier ion was unavailable because this study used a single-quadrupole LC–MS system (Table II).

Therefore, sub-percent RT variability, together with compliant ion ratio checks at the LQC level, supports robust precision and reliable qualitative confirmation for single-quadrupole LC–MS and EIC-based analysis across the analyte panel (Table II).

Validation results

Linearity and range

Within the calibration range of 0.5–25.0 μg mL^{-1}, using five levels with 1/x weighting, all analytes demonstrated linear responses, with R^2 values ranging from 0.991 to 0.998 (Table III, Figure 6).[22,25,26,28,39] The back-calculated concentrations were within ± 20% of their nominal values across all levels, confirming a validated analytical range of 0.5–25.0 μg mL^{-1} for routine quantification (Table III, Figure 6). The accuracy across the curve was within the acceptance limits for each analyte: morphine (94.2–116.9%); codeine (81.2–109.5%); 6-monoacetylmorphine (95.0–116.8%); 6-acetylcodeine (80.0–109.3%); heroin (81.2–110.4%); fentanyl (86.2–105.5%); methadone (89.2–106.3%); and tramadol (85.3–109.7%) (Table III).

Table III. Calibration parameters and accuracy assessments for analytes determined by LC–MS (*n* = 5 per calibration level)

Analyte	Calibration range (μg mL^{-1})	Accuracy range*	R^2	Slope	Y-intercept	Calibration model
Morphine	0.5–25.0	94.2–116.9	0.991	1.0012	0.4788	Linear, 1/x weighting, Average
Codeine	0.5–25.0	81.2–109.5	0.995	1.7815	0.7866	Linear, 1/x weighting, Average
6-monoacetylmorphine	0.5–25.0	95.0–116.8	0.992	2.2494	1.3040	Linear, 1/x weighting, Average
6-acetylcodeine	0.5–25.0	80.0–109.3	0.995	3.3292	2.1199	Linear, 1/x weighting, Average
Heroin	0.5–25.0	81.2–110.4	0.996	2.1559	1.9800	Linear, 1/x weighting, Average
Fentanyl	0.5–25.0	86.2–105.5	0.998	2.5831	0.0615	Linear, 1/x weighting, Average
Methadone	0.5–25.0	89.2–106.3	0.997	0.3007	0.0219	Linear, 1/x weighting, Average
Tramadol	0.5–25.0	85.3–109.7	0.994	0.8128	0.2023	Linear, 1/x weighting, Average

* % of nominal value

The slopes reflected the relative detector response, with 6-acetylcodeine exhibiting the steepest slope (3.3292), followed by fentanyl (2.5831) and 6-monoacetylmorphine (2.2494), whereas methadone had the lowest slope (0.3007). Processing the data as analyte-to-IS peak area ratios in Chromeleon Console 7 (version 7.3.1.6535) confirmed the proportional signal response across the calibration range and supported the reliability of the calibration model for quantitative LC–MS measurements (Table III, Figure 6).

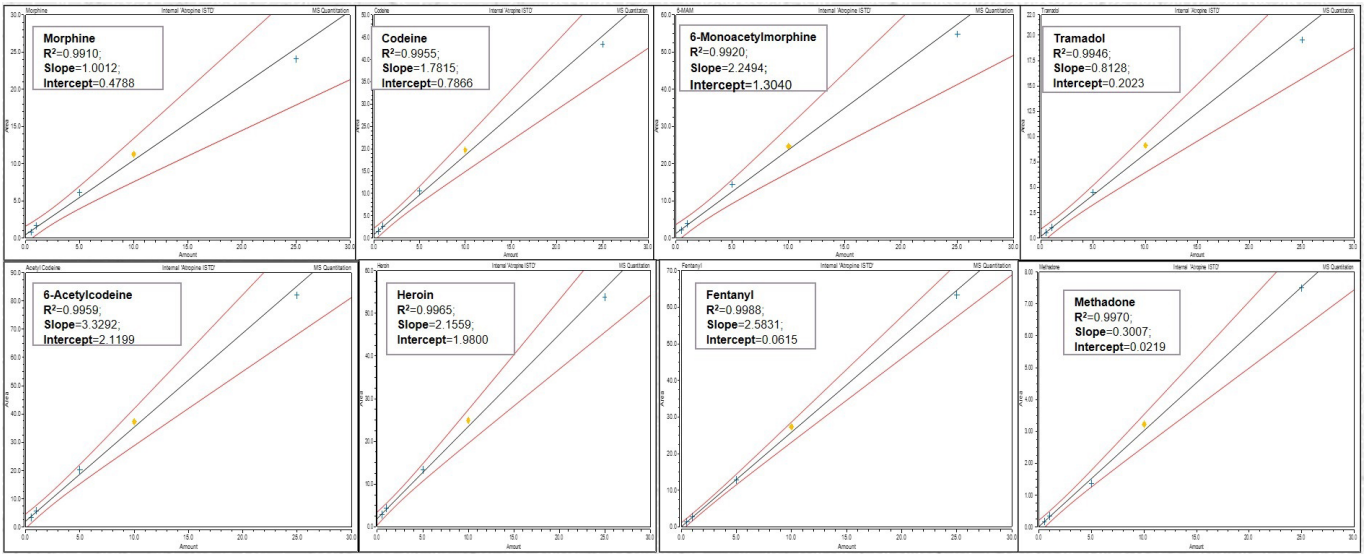

Figure 6. Calibration curves for analytes over the concentration range 0.5–25.0 µg mL^{-1} using linear regression with 1/x weighting. R^2 values were ≥ 0.991 for all analytes. Peak area ratios were plotted against nominal concentrations.

Precision and accuracy

The developed LC–MS method demonstrated high accuracy and precision at three QC levels (1.0, 10.0, and 20.0 µg mL^{-1}) for all analytes (Table IV).

Accuracy (bias, %) ranged from −13.0% for 6-acetylcodeine at the LQC level to 18.0% for methadone at the same level, and all values, excluding heroin, remained within the ± 20% acceptance limit.[27,28] Heroin showed a substantial negative bias (−24.0% to −16.9%), consistent with its known instability and partial hydrolysis during sample processing.

The only analyte that fell outside the accepted limits for accuracy was heroin (Table IV). Bias exceeded ± 20% at the LQC and middle QC (MQC) levels intra-day (−24.0% and −22.0%) and at the LQC level inter-day (−22.0%). The precision for heroin remained within the acceptable limits, indicating a systematic bias rather than random variability. This pattern is consistent with the reduced RE and ion suppression observed for heroin in the matrix study and is attributable to partial conversion to 6-monoacetylmorphine and morphine during processing. The bias decreased at higher concentrations (−17.0% intra-day; −18.0% inter-day), indicating a concentration-dependent effect.

The precision (%RSD) was excellent in both the intra-day (repeatability) and inter-day (reproducibility) assessments (Table IV). Intra-day %RSD ranged from 0.2% for heroin at the HQC level to 14.6% for morphine at the LQC level, whereas inter-day %RSD ranged from 1.3% for tramadol at the HQC level to 16.8% for methadone at the LQC level.

Table IV. Precision (%RSD) and accuracy (bias, %) for analytes at the LQC, MQC, and HQC levels (*n* = 6 intra-day; *n* = 18 inter-day)

Analyte	LQC/MQC/HQC (μg mL^{-1})	Intra-day (*n* = 6)			Inter-day (*n* = 18)		
		Mean (μg mL^{-1})	Bias (%)	Repeatability (%RSD)	Mean (μg mL^{-1})	Bias (%)	Reproducibility (%RSD)
Morphine	1.0/10.0/20.0	1.04/11.36/22.72	4.0/13.6/13.6	14.6/0.6/6.0	1.1/11.5/22.8	6.8/15.0/14.1	5.1/1.6/3.2
Codeine	1.0/10.0/20.0	1.09/10.52/22.04	9.0/5.2/10.2	2.9/2.2/3.2	1.1/11.3/22.7	7.1/12.6/13.4	2.2/1.7/1.6
6-monoacetylmorphine	1.0/10.0/20.0	1.11/11.34/22.14	11.0/13.4/10.7	2.2/1.7/6.5	1.1/11.8/22.5	11.9/17.6/12.5	4.0/4.3/2.2
Tramadol	1.0/10.0/20.0	0.92/9.65/19.25	−8.0/−3.5/−3.8	5.3/3.0/0.3	1.0/9.8/18.8	−11.0/−2.0/−6.1	3.4/5.3/1.3
6-acetylcodeine	1.0/10.0/20.0	0.88/8.87/18.56	−12.0/−11.3/−7.2	13.3/2.6/1.8	0.9/9.0/18.7	−13.0/−10.9/−6.3	8.2/3.9/2.7
Heroin	1.0/10.0/20.0	0.76/7.80/16.60	−24.0*/−22.0*/−17.0	10.6/8.3/0.2	0.8/7.9/16.4	−22.0*/−16.9/−18.0	7.8/6.7/5.6
Fentanyl	1.0/10.0/20.0	0.96/9.62/19.42	−4.0/−3.8/−2.9	7.0/5.5/3.9	1.0/9.6/19.3	−6.0/−4.4/−3.8	4.1/2.1/3.9
Methadone	1.0/10.0/20.0	1.02/10.32/20.56	2.0/3.2/2.8	10.0/4.5/4.2	1.2/10.4/20.4	18.0/3.8/1.8	16.8/8.2/7.0

Validation limits: accuracy (bias, %) within ± 20%; precision (%RSD) ≤ 20%.
*Values exceeding the accuracy limit were observed for heroin at the LQC and MQC levels (intra-day bias: −24.0% and −22.0%) and at the LQC level inter-day (−22.0%).
All precision values (%RSD) were within acceptable limits.

Precision improved as the concentration increased, with the MQC and HQC levels showing lower %RSD values than the LQC level. Codeine, tramadol, and fentanyl demonstrated stable performance, with %RSD consistently below 7.0% across all QC levels. At the LQC level, heroin and 6-acetylcodeine exhibited modest variability, but %RSD values remained within acceptable limits, supporting the robustness of the method.

Except for the noted heroin bias, all analytes met the bioanalytical method validation criteria, with no evidence of systematic bias or loss of precision across the tested concentration range (Table IV).

Analytical sensitivity and carryover

The method achieved an LOQ of 0.5 μg mL^{-1} and an LOD of 0.3 μg mL^{-1} in pooled blank urine extracts. According to the relationship $C_{urine} = C_{extract} / 10$, the dried residues were reconstituted after extraction in 50 μL of mobile phase consisting of a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, to enrich the analyte concentration ten-fold relative to the original urine sample and allow accurate quantification within the calibration range (0.5–25.0 μg mL^{-1}) of the method. Therefore, an LOQ of 0.5 μg mL^{-1} in the final extract corresponds to an effective LOQ of 50 ng mL^{-1} in the original urine sample. The effective LOQ is compatible with commonly applied confirmatory urine cut-off concentrations in forensic and clinical toxicology, as recommended by the European Workplace Drug Testing Society (EWDTS) for several opioids, such as 300 ng mL^{-1} for morphine, 300 ng mL^{-1} for codeine, 100 ng mL^{-1} for tramadol, and 250 ng mL^{-1} for methadone.[42] However, analytes that require very low cut-off concentrations, such as 10 ng mL^{-1} for 6-monoacetylmorphine or sub-ng mL^{-1} for fentanyl, are still best suited to targeted tandem LC–MS methods with greater analytical sensitivity. At this LOQ, S/N, accuracy, and precision met the predetermined criteria; qualitative assessments of RT and ion ratio agreement were satisfactory, confirming sufficient sensitivity for routine quantification throughout the validation range.

Following injections of the highest calibrator (25.0 μg mL^{-1}), pooled blank urine extracts showed no analyte peaks at the target RT. The mean blank response was ≤ 10% of the LOQ signal, which met the predefined carryover criterion. These results indicate that the autosampler, injection pathway, and LC system did not retain quantifiable residues from high-concentration injections, minimizing the risk of false positives or biased low-level results and supporting the suitability of the method for high-throughput workflows. No carryover was observed.

ME, RE, PE, and selectivity

At the LQC level (1.0 μg mL^{-1}), ME ranged from 85.8% for heroin to 109.1% for methadone, falling within the predefined acceptance range of 80–120%.[43-45]

Table V. ME (%), RE (%), and PE (%) of analytes at the LQC and HQC levels in a urine matrix

Analyte	LQC and HQC (μg mL^{-1})	ME (%)	ME (%RSD)	RE (%)	RE (%RSD)	PE (%)	Ionization effect
Morphine	1.0	103.9	6.8	112.8	8.2	117.2	Enhancement
	20.0	102.5	1.0	114.3	1.3	118.2	Enhancement
Codeine	1.0	103.1	2.8	111.0	4.5	114.4	Enhancement
	20.0	102.0	5.2	109.0	8.3	111.2	Enhancement
6-monoacetylmorphine	1.0	101.1	3.1	113.8	4.3	115.0	Enhancement
	20.0	100.8	1.8	114.0	2.1	115.9	Enhancement

(continued on next page)

Table V-cont. ME (%), RE (%), and PE (%) of analytes at the LQC and HQC levels in a urine matrix

Analyte	LQC and HQC (μg mL^{-1})	ME (%)	ME (%RSD)	RE (%)	RE (%RSD)	PE (%)	Ionization effect
Tramadol	1.0	108.2	2.1	91.5	4.1	99.0	Enhancement
	20.0	106.4	1.8	96.8	3.8	102.9	Enhancement
6-acetylcodeine	1.0	89.7	2.3	87.1	4.2	78.1*	Suppression
	20.0	98.6	2.0	88.7	4.0	87.4	Suppression
Heroin	1.0	85.8	3.6	75.8*	1.2	65.0*	Suppression
	20.0	89.2	2.4	77.2*	0.7	68.8*	Suppression
Fentanyl	1.0	96.9	4.0	89.6	6.9	86.8	Suppression
	20.0	98.5	2.7	98.8	5.2	97.3	Suppression
Methadone	1.0	109.1	1.9	101.8	3.3	114.3	Enhancement
	20.0	102.8	1.0	104.2	1.6	107.1	Enhancement

ME (%) values > 100% indicate ion enhancement, whereas values < 100% indicate ion suppression. *Values outside the predefined acceptance range (80–120%) were observed for 6-acetylcodeine (PE = 78.1% at the LQC level) and heroin (PE = 65.0% at the LQC level and 68.8% at the HQC level; RE = 75.8% at the LQC level and 77.2% at the HQC level).

Ionization enhancement was observed for morphine, codeine, 6-monoacetylmorphine, tramadol, and methadone. Ion suppression was exhibited by heroin, fentanyl, and 6-acetylcodeine, most notably at the LQC level for heroin (85.8%) and 6-acetylcodeine (89.7%) (Table V; Figure 7a).

The RE values were generally high, ranging from 87.1% for 6-acetylcodeine at the LQC level to 114.3% for morphine at the HQC level (Table V; Figure 7b). Methadone, tramadol, and fentanyl were close to 100%, whereas heroin was lower (75.8–77.2%), consistent with hydrolysis to 6-monoacetylmorphine and morphine during preparation (Figure 5).

The PE values varied from 86.8% for fentanyl at the LQC level to 118.2% for morphine at the HQC level; 6-acetylcodeine (78.1% at the LQC level) and heroin (65.0% at the LQC level and 68.8% at the HQC level) were the exceptions (Table V; Figure 7b).[43,44] Morphine, 6-monoacetylmorphine, methadone, and codeine exceeded 110.0%, indicating ion enhancement, whereas tramadol and fentanyl were close to 100%, indicating minimal matrix loss (Table V, Figure 7a, c). The lower PE of heroin (65.0–68.8%) was associated with its chemical instability and ester cleavage susceptibility. The %RSD values for RE and ME were ≤ 8.3% for all analytes and QC levels (Table V).

The method meets the matrix performance validation standards and is suitable for high-throughput forensic toxicology applications. The reduced RE values for heroin and 6-acetylcodeine were consistent with their susceptibility to ester cleavage during processing, and the precision remained within acceptable limits across all QC levels.

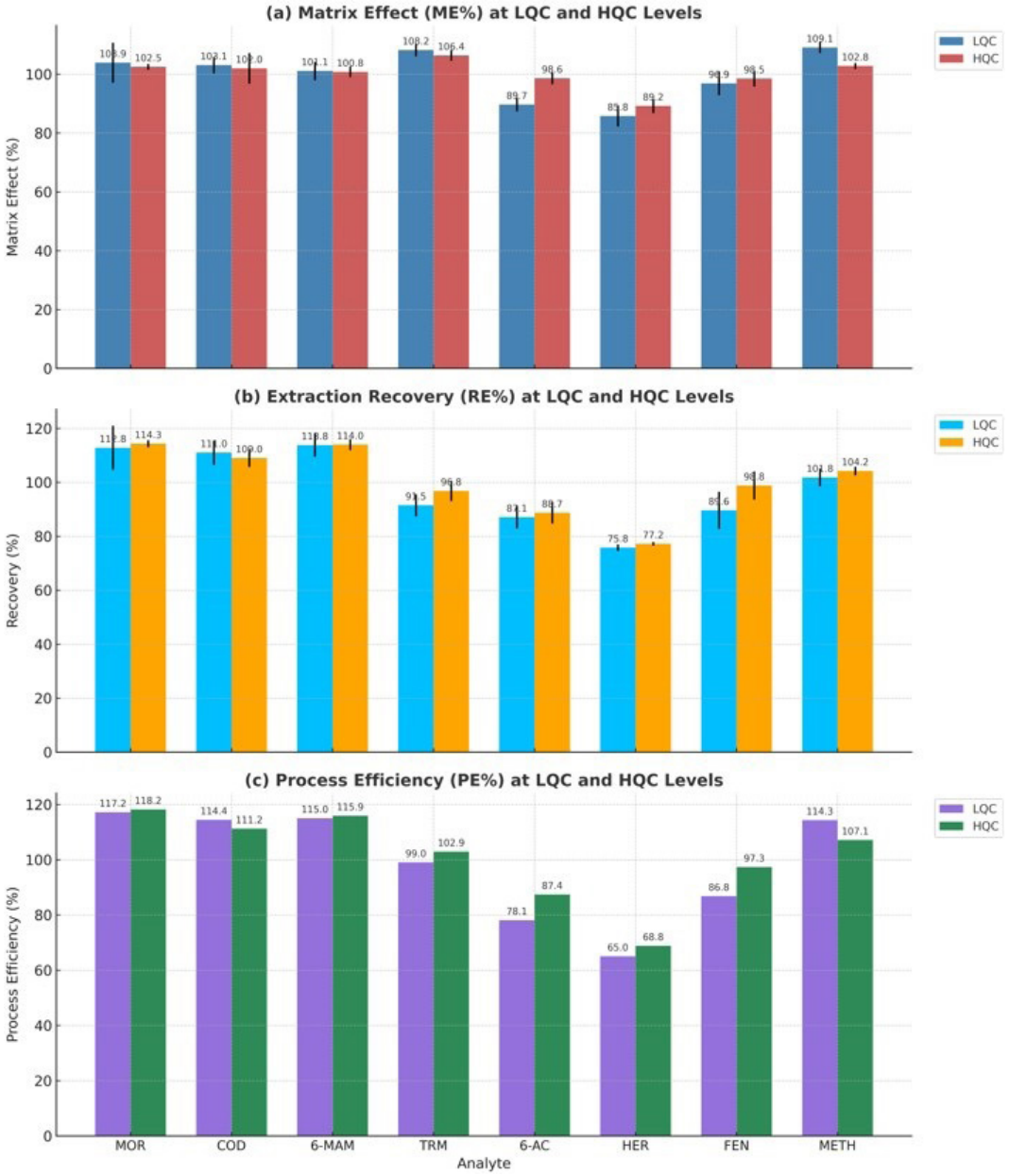

Figure 7. Panel (a) shows ME (%), panel (b) shows RE (%), and panel (c) shows PE (%) at the LQC (1.0 µg mL^{-1}) and HQC (20.0 µg mL^{-1}) levels. Bars in panels (a) and (b) represent mean ± SD (n = 3), whereas panel (c) shows PE (%) values at the same QC levels. Analytes are labeled as: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; TRM, tramadol; 6-AC, 6-acetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Values for heroin and 6-acetylcodeine that fell outside the 80–120% acceptance range were attributable to the PE calculation, defined as (ME × RE) / 100, and the hydrolytic instability of these esterified opioids (Figure 5). At the LQC level, 6-acetylcodeine exhibited mild ion suppression (89.7%) and reduced RE (87.1%), yielding a PE of 78.1%, which is consistent with the partial conversion to codeine (Table V, Figure 7c). Heroin showed the same trend at both QC levels, with PE values of 65.0% at the LQC level and 68.8% at the HQC level, driven by ion suppression (ME values of 85.8% and 89.2%, respectively) and reduced RE (75.8% and 77.2%, respectively). Low %RSD values indicate a systematic effect rather than a random

error. The results are consistent with the hydrolysis of heroin to 6-monoacetylmorphine and subsequently to morphine and the hydrolysis of 6-acetylcodeine to codeine (Figure 5). The other analytes were within the 80–120% acceptance range with low variability, reinforcing the overall method variability (Table V).

Selectivity was assessed using ten individual blank urine samples and a pooled blank urine sample spiked with additional non-target opioids (as described in the methods). Neither the individual blanks nor the spiked pooled sample showed interfering peaks at the RT of any target analyte or the IS. The ion ratios and RT values remained within ± 20% and ± 2% of the calibration values, respectively. These data confirm adequate selectivity and specificity under the tested conditions.

Stability of processed urine samples

The stability of the processed samples at 4 °C was assessed for each analyte at two QC levels (1.0 and 10.0 μg mL^{-1}) over 96 h, with results expressed as % change from the initial time point at t = 0 h. Across QC levels and time points, all analytes remained within the ± 20% acceptance limit (Table VI, Figure 8).

Table VI. Time-dependent stability of analytes in processed urine samples stored at 4 °C for 0–96 h, expressed as % change, with %RSD values calculated for triplicate measurements (*n* = 3)

Analyte	QC levels (μg mL^{-1})	*Mean Concentration at t = 0 h	% change from t = 0 h				%RSD at 96 h
			24 h	48 h	72 h	96 h	
Morphine	1.0	1.14	−1.4	−2.8	−5.8	−18.3	4.0
	10.0	11.32	−1.0	−2.2	−4.9	−15.6	3.5
Codeine	1.0	1.16	−11.0	−14.6	−16.5	−18.2	3.9
	10.0	10.81	−5.0	−7.7	−15.2	−16.2	3.5
6-monoacetylmorphine	1.0	1.13	−2.8	−8.4	−7.8	−14.1	3.9
	10.0	11.50	−1.3	−4.9	−9.7	−10.8	4.2
Tramadol	1.0	0.92	−2.1	−7.7	−10.1	−17.8	3.5
	10.0	9.60	−7.2	−6.3	−9.1	−15.4	2.4
6-acetylcodeine	1.0	0.85	−9.1	−12.0	−11.9	−19.5	4.8
	10.0	8.84	−6.5	−8.6	−14.2	−17.0	3.1
Heroin	1.0	0.76	−5.0	−8.3	−13.3	−18.1	3.5
	10.0	7.63	−0.7	−2.5	−4.6	−15.7	3.0
Fentanyl	1.0	0.90	−3.2	−6.5	−15.2	−18.4	2.7
	10.0	9.80	−3.8	−6.0	−10.5	−13.2	2.3
Methadone	1.0	1.02	−5.6	−14.6	−16.0	−19.8	4.8
	10.0	10.25	−1.9	−9.4	−15.1	−15.7	3.3

*Reference concentration at t = 0 h.
Analytes were considered stable when the % change was within ± 20%; values outside this range indicated instability.

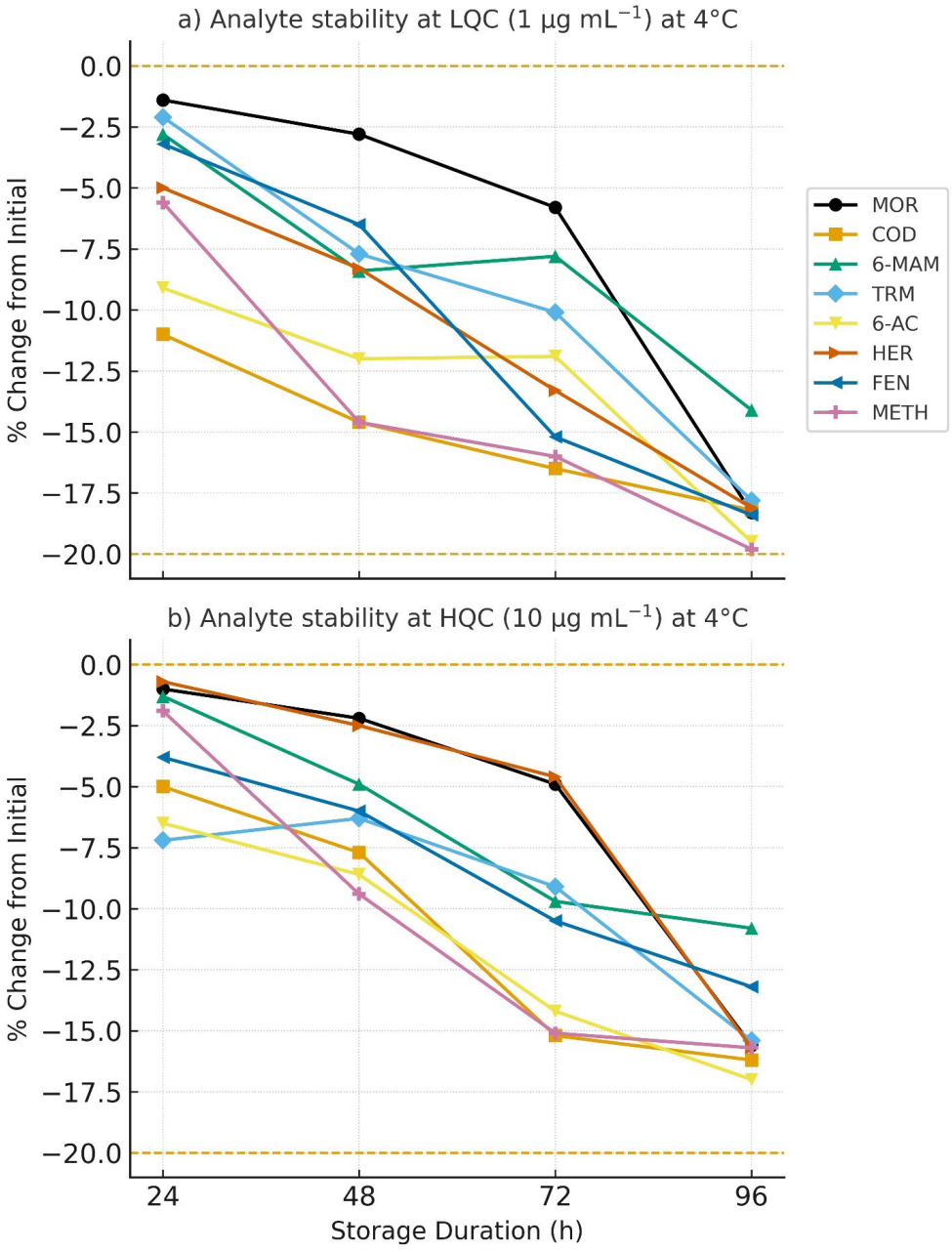

Figure 8. Stability profile of the analytes in processed urine samples stored at 4 °C for up to 96 h: panel (a) LQC level (1.0 µg mL^{-1}); panel (b) HQC level (10.0 µg mL^{-1}). Analytes are labeled as: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; TRM, tramadol; 6-AC, 6-acetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Concentrations generally decreased with storage duration, and this effect was more noticeable at the LQC level (Table VI, Figure 8a). At the LQC level after 96 h, the largest decreases were observed for methadone (−19.8%), 6-acetylcodeine (−19.5%), fentanyl (−18.4%), and both codeine and morphine (≈ −18.0% each) (Table VI, Figure 8a). At the HQC level, the greatest decline was −17.0% for 6-acetylcodeine, with smaller variations for the other analytes (Table VI, Figure 8b). Heroin and 6-monoacetylmorphine exhibited moderate time-dependent losses consistent with partial hydrolysis in an aqueous medium (Table VI, Figure 8).

High precision was maintained throughout the storage period, with %RSD ≤ 4.8% at 96 h, confirming reproducible quantification (Table VI). Collectively, these findings indicate that processed urine extracts remain stable at 4 °C for up to 96 h, although some analytes at the LQC level approached the acceptance threshold near the end of the storage period (Table VI, Figure 8).

METHOD APPLICABILITY

Urine specimens from three anonymized cases were collected in August 2025 at Kanoria Hospital & Research Center. Immediately after collection, the specimens were analyzed using the validated assay as part of a rehabilitation monitoring and de-addiction program. Each sample was analyzed in duplicate alongside calibrators, three QC levels, and three blanks. QC performance was considered acceptable when the measured concentrations were within ± 20% of the nominal values. Applying the relationship $C_{urine} = C_{extract} / 10$, one of the three specimens tested positive for tramadol, whereas the other two tested negative for the target opioids. Table VII summarizes the results.

The limited number of urine case specimens constrained the sample size available for analysis. These findings are preliminary but indicate the applicability of the proposed method in real-world contexts. Further large-scale case studies should be conducted to confirm and extend the findings of this study.

Table VII. Results for authentic urine samples analyzed using the validated single-quadrupole LC–MS method

Sample ID	Morphine	Codeine	6-monoacetylmorphine	Tramadol	6-acetylcodeine	Heroin	Fentanyl	Methadone
S1	—	—	—	—	—	—	—	—
S2	—	—	—	0.732	—	—	—	—
S3	—	—	—	—	—	—	—	—

— not detected; units: μg mL^{-1}.

CONCLUSIONS

This study developed and validated a cost-effective, sensitive single-quadrupole LC–MS assay for the simultaneous determination of eight opioids in human urine. The method used passive chemical hydrolysis optimized at 54 °C for 6 h and achieved high RE for most analytes, ranging from 82.0 ± 2.1% for 6-acetylcodeine to 114.4 ± 3.0% for 6-monoacetylmorphine, with slightly lower RE for heroin (78.0 ± 3.4%) because of its instability during sample processing. Passive chemical hydrolysis followed by SPE outperformed both enzymatic hydrolysis followed by SPE and LLE, and the validation met the following predefined criteria: $R^2 \geq 0.991$, precision (%RSD) ≤ 16.8% for both intra-day and inter-day measurements, accuracy (bias, %) within ± 20%, acceptable ME, processed sample stability, and carryover. The applicability of the method was supported by casework: one of three case specimens tested positive for tramadol. Overall, the method provides a reliable, accessible option for clinical and forensic laboratories, particularly in resource-limited settings where access to MS/MS instrumentation and enzymatic hydrolysis is constrained.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The research did not obtain any specific funding from public, commercial, or nonprofit agencies.

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