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Targeted LC-MS Quantification of Eight Urinary Opioids using Validated 10-Fold Enrichment: Forensic and De-addiction Applications in Casework

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Limited access to liquid chromatography-tandem mass spectrometry (LC-MS/MS) can restrict targeted urinary opioid testing in some forensic laboratories. A fit-for-purpose single-quadrupole liquid chromatography-mass spectrometry (LC-MS) workflow was developed and validated for the quantification of urine morphine, codeine, 6-monoacetylmorphine (6-MAM), tramadol, 6-acetylcodeine (6-AC), heroin, fentanyl and methadone with 10-fold enrichment. The samples were subjected to passive hydrolysis, Oasis HLB solid-phase extraction (SPE) cleanup, enrichment, C18 separation, positive electrospray ionisation (ESI) full-scan acquisition and extracted-ion chromatogram (EIC)-based monitoring. This method was applied to 63 de-addiction and forensic casework specimens. The effective urine-equivalent limit of quantification (LOQ) was 50.0 ng/mL, corresponding to 0.5 µg/mL in the final enriched extract. Calibration was linear over 0.5-25.0 µg/mL with a correlation coefficient (R^2) of ≥ 0.990. Precision varied from 0.2% to 16.8%, bias from -17.3% to 18.0%, matrix effect (ME) from 85.8% to 109.1% and extraction recovery (RE) from 82.4% to 114.3%. Codeine and morphine were the most frequently quantified analytes, followed by 6-MAM and 6-AC. The method provides targeted urinary opioid quantification within a defined validation and LOQ-based reporting scope, without claiming equivalence to LC-MS/MS/multiple reaction monitoring (MRM) confirmation.

Keywords: Targeted urinary opioids, LC-MS, 10-Fold enrichment, De-addiction monitoring, Forensic toxicology.

INTRODUCTION

Opioid misuse has emerged as a major global public health and forensic concern, contributing substantially to drug related overdoses, morbidity and mortality owing to the increasing availability of psychoactive substances [1-5]. Accurate identification and quantification of opioids are therefore essential in forensic and clinical toxicology for cause-of-death investigations, impaired-driving assessments, workplace testing, violence-related cases and monitoring treatment compliance in drug de-addiction programs [6,7]. Among opioids, tramadol, fentanyl and methadone are of particular interest due to their widespread misuse and strong association with severe toxicity and fatal outcomes [8-16].

Fentanyl presents a unique analytical challenge due to its exceptionally high potency and its frequent use as an adulterant or substitute in illicit heroin preparations, significantly increasing the risk of accidental overdose [11,17-23]. Consequently, there is a growing need for highly sensitive and selective analytical methods capable of simultaneously detecting multiple opioids and heroin-related biomarkers at trace concentrations [18,23-28].

Urine remains the preferred biological matrix for routine toxicological analysis due to its non-invasive collection, extended detection window, suitability for high-throughput testing and capacity for long-term sample storage [23-25,29-32]. However, conventional immunoassay-based screening methods often suffer from cross-reactivity, variable cut-off values and

inadequate sensitivity toward synthetic and semi-synthetic opioids such as fentanyl and methadone, leading to potential false-positive and false-negative results [18,23,24,26-28,33]. To overcome these limitations, professional and workplace testing guidelines recommend chromatographic separation coupled with mass spectrometric confirmation for reliable identification of presumptive positive samples [7,24,32,34-38].

Among confirmatory techniques, liquid chromatography-mass spectrometry (LC-MS) has become the method of choice due to its high selectivity, sensitivity and suitability for polar and thermally labile opioids without requiring derivatization [4,37-41]. The interpretation of heroin use remains particularly challenging since heroin undergoes rapid metabolism following administration [42]. It is quickly converted to 6-monoacetylmorphine (6-MAM) and subsequently to morphine; notably, 6-MAM is considered a definitive biomarker of heroin consumption, whereas morphine may also arise from therapeutic morphine or codeine use [43,44]. Furthermore, the detection of 6-acetylcodeine (6-AC), a characteristic impurity of illicit heroin, alongside 6-MAM and other opioids, can provide additional forensic evidence supporting heroin exposure [45,46].

Confirmatory urine opioid testing often employs liquid chromatography-tandem mass spectrometry (LC-MS/MS) since multiple reaction monitoring (MRM) provides higher structural selectivity [28,47-55]. A direct head-to-head comparison with LC-MS/MS was not performed since LC-MS/MS instrumentation was unavailable during the course of this study. Therefore, the developed single-quadrupole LC-MS method is not intended to be presented as an equivalent or superior alternative to LC-MS/MS. Instead, this work focused on developing and validating a targeted, fit-for-purpose LC-MS workflow employing positive electrospray ionisation (ESI+), full-scan extracted-ion chromatogram (EIC) monitoring, passive chemical hydrolysis, Oasis HLB solid-phase extraction (SPE) cleanup, and a 10-fold sample enrichment step for the determination of eight pre-defined urinary opioids, namely morphine, codeine, 6-MAM, tramadol, methadone, 6-AC, fentanyl and heroin. The enrichment procedure improved analytical sensitivity, converting the validated extract limit of quantification (LOQ) of 0.5 μg/mL into an effective urine-equivalent reporting LOQ of 50.0 ng/mL, thereby enabling quantitative reporting within the validated LOQ-based analytical scope [54-58]. The applicability of the method was demonstrated through the analysis of 63 authentic urine samples collected from drug de-addiction treatment programs, rehabilitation compliance monitoring, and medicolegal case investigations.

EXPERIMENTAL

Methanol, ethyl acetate, isopropanol, acetone and acetonitrile (all LC-MS grade) were obtained from Thermo-Fisher Scientific (India). Type I ultrapure water (resistivity >18.2 MΩ/cm) was produced onsite using an XTRAPURE Plus Lab-Link water purification system (Mumbai, India). Acids and salts, such as ammonium formate, formic acid, glacial acetic acid and sodium acetate, were purchased from Sigma-Aldrich Ltd. (India). Analytical grade reagents, such as ammonia solution, sodium phosphate, sodium hydroxide, hydrochloric acid, sodium dihydrogen phosphate dihydrate, anhydrous sodium acetate and anhydrous sodium hydrogen phosphate, were provided by Rankem Chemicals (India). Oasis HLB SPE cartridges (60 mg, 3 mL) were purchased from Waters Corporation (Bengaluru, India) and Vadilal Chemical Ltd. (India) provided ultra-high purity nitrogen (grade 5.0). Cerilliant Corporation (Bangalore, India) provided certified reference materials for morphine, codeine, 6-MAM, tramadol, 6-AC, heroin, fentanyl, methadone and atropine. Since analyte specific stable isotope-labeled internal standards (SIL-IS) were temporarily unavailable, atropine was used as a replacement internal standard (IS). Although SIL-IS would provide stronger analyte-matched correction, atropine was consistently applied across calibrators, QC samples and case specimens and quantitative performance was evaluated through analyte-to-IS calibration, bias, precision, matrix effect (ME) and extraction recovery (RE) results. The equipment for sample preparation and storage consisted of a Waters 20-position SPE manifold (Massachusetts, USA), a Labman LMUC25D ultrasonic cleaner (India), a Neuation iTherm D150-4 dry heating block (India) and a MarkEn ultra-low temperature freezer with a temperature range of -20 to -86 ºC.

Preparation of calibration, quality control (QC) and IS solutions: Stock solutions of individual analytes were prepared in methanol at 1.0 mg/mL and then combined to prepare a mixed working solution containing 100 μg/mL of each analyte. An IS working solution was prepared in methanol at 100 μg/mL by dilution of a 1 mg/mL stock. Mixed calibration standards ranging from 0.5 to 25.0 μg/mL were prepared by serially diluting the mixed working solution with a 90:10 (v/v) diluent made of 10 mmol/L ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid. QC samples were prepared from the same mixed working solution and spiked into pooled urine to achieve target concentrations of 0.5, 1.0, 10.0 and 20.0 μg/mL in the final reconstituted extract, respectively. An IS was added to all calibrators, QC levels and case specimens to a final concentration of 10.0 μg/mL, after which all prepared samples were stored at -20 ºC when not in use. Routine quantification was performed with fresh working calibrators and QC samples, which were processed with analytical batches; the saved-calibration assessment described below was used only as a separate response-stability check.

Instrumentation: Chromatographic separation was performed using a Vanquish liquid chromatography system (Thermo-Fisher Scientific, USA) and an Acclaim C18 column (120 Å, 150 × 3.0 mm, 3 μm), which was maintained at 35 ºC. The autosampler was maintained at 8 ºC and the injection volume was set to 5 μL. Mobile phase A comprised 10 mmol/L ammonium formate with 0.1% formic acid, whereas mobile phase B comprised 0.1% formic acid-containing acetonitrile. The seal wash solvent consisted of 10% methanol in water and the needle wash solvent was a mixture of acetonitrile, isopropanol and acetone (45:45:10). Table-1 summarizes the LC flow rate and gradient program details. The total run time was 14.50 min, including column re-equilibration (12.25-14.50 min) and all gradient steps used a curve setting of 5.

Detection was performed using an ISQ EC mass spectrometer (Thermo-Fisher Scientific, USA) in ESI+ mode. The

TABLE-1
LC FLOW RATE AND GRADIENT PROGRAM USED FOR THE CHROMATOGRAPHIC SEPARATION OF THE TARGET ANALYTES

Time (min)	Flow (mL/min)	A (%)	B (%)	Curve
0.00	1.00	90	10	5
2.00	0.40	70	30	5
3.50	0.40	60	40	5
8.50	0.40	60	40	5
10.00	1.00	20	80	5
12.00	1.00	20	80	5
12.25*	1.00	90	10	5
14.50*	Stop			

*System re-equilibration period at initial mobile-phase conditions (12.25–14.50 min)

source settings consisted of a +3500 V spray voltage, 350 ºC vapourizer temperature and 325 ºC ion transfer tube temperature. The pressures of the sheath, auxiliary and sweep gases were 50, 7.5 and 1.5 psig, respectively. The data were acquired in full-scan mode over a mass-to-charge ratio (*m/z*) of 30-550 with a scan period of 1.0 s. The raw data were processed in Chromeleon 7 (v7.3.1.6535) using extracted ion chromatograms (EIC), which monitored the in-source fragment ions.

Biological specimens: For method development and validation, urine samples were collected from 10 healthy laboratory staff volunteers at least 2 weeks following any medication use to reduce potential interference. Specimens (10 mL each) were collected and screened for the presence of the target analytes. Negative specimens for the target analytes were pooled and centrifuged to remove particulate matter. The pooled and remaining individual samples were stored at -20 ºC until analysis.

The study was approved by the Institutional Ethics Committee of the National Forensic Sciences University (NFSU), Gandhinagar, India (Approval No. NFSU/SDSR/IEC/1054/2024) and was conducted in accordance with applicable ethical guidelines for research involving human biological specimens. A total of 63 anonymised urine samples collected between June and August 2025 were included in the study, comprising 24 specimens from individuals enrolled in a drug de-addiction program at Kanoria Hospital & Research Center (KHRC), Ahmedabad, India, 24 rehabilitation-compliance monitoring specimens from individuals with a history of illicit heroin use obtained from the Rwanda Forensic Institute (RFI), Kigali, Rwanda and 15 autopsy urine specimens from suspected opioid-related deaths. All samples were de-identified prior to analysis and no personal information was accessible to the investigators. The study utilized residual clinical and forensic specimens collected as part of routine treatment, rehabilitation monitoring or medicolegal investigations, with strict adherence to confidentiality and ethical requirements.

Statistical analysis: The results were categorized as not detected (ND), detected below the limit of quantification (< LOQ) or quantified at concentrations ≥ LOQ. Analytes were considered positive only when concentrations met or exceeded the LOQ. Positivity rates were expressed as n/N (%) with 95% confidence intervals, while quantified concentrations were summarized using the median, interquartile range (IQR), and range to minimize the influence of ND and < LOQ observations. Analyses of authentic casework samples were descriptive in nature due to differences in institutional, geographic, and casework backgrounds. Associations between morphine and codeine concentrations, as well as codeine distributions according to 6-AC status, were evaluated as exploratory trends among quantified samples. Bland-Altman analysis was applied exclusively to assess agreement between storage temperatures during method validation.

Passive chemical hydrolysis, SPE cleanup and extract enrichment: Urine samples (1 mL), including QC levels and case specimens, were spiked with 10 μL of an IS at 100 μg/mL, resulting in a final IS concentration of 10 μg/mL in the processed extracts. The samples were vortex, mixed with 0.5 mL of 0.1 mol/L sodium acetate buffer (pH 5.6) and incubated at 54 ºC for 6 h before extraction. The solution was then mixed with 0.1 mol/L sodium phosphate buffer (pH 6.0), added to the initial mixture in a 1:1 ratio and vortexed. The samples were cleaned using SPE with the Oasis HLB cartridges. First, the cartridges were conditioned with 1 mL of methanol, followed by 2 mL of a 2% NH_3 solution. The cartridges were then washed with 1.5 mL of 5% methanol solution and dried under vacuum for 5 min after sample loading. The analytes were then sequentially eluted with 1 mL of methanol and 1 mL of ethyl acetate. The combined eluate was dried to dryness under nitrogen at room temperature and reconstituted in 100 μL of a 90:10 mixtures of 10 mmol/L ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid. The reconstitution resulted in a 10-fold increase in urine concentration compared with the original urine. The reconstituted extract was transferred to a 300 μL glass micro-insert vial, capped and gently mixed before a 5 μL aliquot was injected for LC-MS analysis. The SPE steps were performed under a gravity flow, whereas the wash step was conducted using a 20-position SPE manifold under a controlled vacuum of 10 psi for 10 min.

To address the limited sensitivity of single-quadrupole LC-MS at concentrations below the lowest calibration level (0.5 μg/mL), a 10-fold enrichment approach was implemented to enable reliable quantification analysis of trace urinary analyte levels. This procedure was uniformly applied to all QC levels and casework samples. The results were back-calculated to express the original urine concentration by adjusting for the enrichment factor, which is 1/10th the concentration measured in the post-reconstituted extract, to account for the increased concentration caused by enrichment. This approach was chosen because extending the calibration curve below 0.5 μg/mL generated low-intensity EIC with matrix-related baseline noise, signal-to-noise ratios (S/N) < 10 and unacceptable precision and accuracy relative to the predefined acceptance criteria.

Validation design: Eight target opioids were validated in human urine according to standard forensic toxicology practices and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. Validation evaluated retention time (Rt) repeatability and ion ratios, linearity, precision, accuracy, analytical sensitivity, carryover, ME, RE, selectivity, sample

stability, co-elution effect and calibration stability [36,58,59]. Unless otherwise stated, post-enrichment extracts prepared from spiked pooled urine samples are used to report QC levels during validation.

The concentration-level design was parameter-specific: precision and accuracy were evaluated at low, middle and high QC levels, whereas ME/RE, stability and co-elution experiments used low and high QC levels to bracket the lower and upper concentration conditions of the validated LC-MS workflow.

Validation was interpreted within a targeted eight-analyte quantitative scope, with identification based on Rt, EIC response, qualifier/quantifier ion ratios where available, peak quality and absence of relevant interference.

Retention time (Rt) repeatability and ion-ratio criteria: Urine extracts at 0.5 μg/mL were analysed under fixed LC-MS conditions in full-scan mode with EIC processing. The repeatability of the Rt was assessed through 10 replicate injections (n = 10) within a single batch and was expressed as mean ± standard deviation (SD) and percent relative standard deviation (%RSD). Targeted identification was assessment using EIC ion ratios where available, with a tolerance of ± 20% and a Rt tolerance window of ± 2% relative to the calibrator. Under the tested conditions, tramadol was evaluated using quantifier-ion response, Rt agreement, chromatographic resolution, peak-quality assessment and absence of relevant interference, as a reliable qualifier ion could not be established under the applied single-quadrupole LC-MS conditions.

Linearity and analytical range: Five different calibration standard concentrations were prepared at 0.5, 1.0, 5.0, 10.0 and 25.0 μg/mL and each level was analysed five times (n = 5). The analyte-to-IS peak area ratio was used as analytical response. Linear regression with 1/x weighting was used to assess linearity. The analytical range was deemed acceptable when the correlation coefficient (R^2) was > 0.990 and the back-calculated concentrations for all calibration points fell within ± 20% of their nominal concentrations.

Precision and accuracy: The precision and accuracy of urine extracts were evaluated at three QC concentrations: 1.0, 10.0 and 20.0 μg/mL. Intra-day precision was evaluated using six replicates per level within a single batch (n = 6), whereas inter-day precision was assessed over three consecutive days (n = 18). The analyte-to-IS peak area ratio was used for quantification. Accuracy and precision were assessed against predefined performance criteria for the LC-MS method, which included a bias of no more than ± 20% and a %RSD of 20% or less.

Analytical sensitivity and carryover: The LOD and LOQ were evaluated by analyzing 10 individual blank urine samples and calculating the SD of the blank response at each analyte Rt and the calibration slope. The LOD and LOQ were calculated as follows: LOD = (3 × SD)/ slope and LOQ = (10 × SD)/slope. The estimates were empirically confirmed by analyzing five low-level processed urine extracts, ranging from 0.310 to 0.724 μg/mL. The urine-equivalent LOQ was verified by spiking blank urine at 50.0 ng/mL, then extracting and processing it through the full workflow, resulting in 0.5 μg/mL in the final enriched extract. LOQ acceptance required S/N ≥ 10, bias within ± 20%, %RSD ≤ 20%, Rt within ± 2% and ion-ratio agreement within ± 20% where qualifier ions were available. Carryover was assessed by injecting three pooled urine extracts immediately after the highest calibrator (25.0 μg/mL) and was considered absent when the response in the subsequent blank injections was ≤ 10% of the LOQ response.

ME, RE and selectivity: ME and RE were assessed at concentrations of 1.0 and 20.0 μg/mL in the extracts. Triplicate preparations (n = 3) were examined for three independent sets according to the Matuszewski approach, including (A) neat standards, (B) post-extraction spiked samples and (C) pre-extraction spiked pooled urine [60]. ME was calculated as (B/A) × 100 and RE as (C/B) × 100, with values of 80-120% considered acceptable. The selectivity of the method was evaluated by analyzing 10 blank urine samples that did not contain any analyte or IS and a pooled urine extract containing potential co-administered opioids (buprenorphine, hydrocodone, hydromorphone, meperidine, naloxone, naltrexone, oxycodone and oxymorphone). Based on the relevant ions and ion-ratio checks, no interfering peaks were observed at the Rt of the analytes or IS.

Stability and storage-temperature agreement (4 ºC *vs.* -20 ºC): The stability and storage-temperature agreement for the eight target analytes were assessed at concentrations of 1.0 μg/mL and 20.0 μg/mL in the extracts. Bench-top stability (at room temperature) was evaluated using triplicate aliquots (n = 3) at time point t_0 (reference time point), 5 h and 24 h. Stability was expressed as %Remaining = (mean at time t/mean at t_0) × 100 and precision was expressed as %RSD = (SD/mean) × 100, where acceptance was defined %Remaining being within ± 20% of t_0.

The long-term stability at 4 ºC and -20 ºC was evaluated by analyzing triplicate samples (n = 3) for each analyte, QC level, time point and storage condition on Days 0, 3, 7, 14 and 30, with results presented as %Remaining relative to day 0 (reference time point).

The agreement was evaluated against a predefined ± 20 percentage point criterion and classified as good (within ± 10 percentage points), acceptable (within ± 20 percentage points) or poor (> ± 20 percentage points). The agreement between storage temperatures of 4 ºC and -20 ºC was assessed with a Bland–Altman analysis, using day-mean %Remaining values from days 3 to 30 (n = 4 paired time points; df = 3; two-sided t-critical, $t_{0.975,3} = 3.182$). Differences were defined as D = %Remaining at 4 ºC – %Remaining at -20 ºC with bias equal to the mean of D and limits of agreement (LoA) calculated as bias ± $t_{0.975,3}$ × SD(D).

Co-elution effect of 6-AC and heroin: The potential ionisation interactions arising from the close elution of 6-AC (6.83 min) and heroin (6.90 min) were assessed by urine extract co-injection at 1.0 and 20.0 μg/mL. Three sets of urine samples were prepared in triplicate (n = 3). The analyte pair was selected since both are acetylated opiates with similar functional groups, polarity and ionisation behaviour. Consequently, their proximity in elution may increase the chance of overlapping EIC responses (*e.g.*, acetyl-loss ions) and partner-driven ionisation effects in LC-MS. Set 1 consisted of 6-AC only to establish the reference mean peak areas and set 2 comprised heroin only at each level. Set 3 consisted of both analytes and was assessed with four concentration

combinations: L/L, H/H, H/L and L/H, where L and H denote the low and high concentration levels, respectively. The ionisation interaction was quantified as a percentage effect (%Effect) using %Effect = 100 × (Y/X–1), where Y is the analyte peak area in the mixed injection and X is the mean peak area for the corresponding single analyte injection at the same nominal concentration.

Saved calibration curve performance check: The day-1 calibration curve was electronically stored and reapplied to QC extracts at concentrations of 1.0, 10.0 and 20.0 µg/mL during six monthly checks [61,62]. At each QC level, triplicate QC extracts (n = 3) were analysed and back-calculated using the stored calibration curve at each monthly check. This assessment was conducted solely to assess response drift under the established method conditions and was not intended to replace the routine batch calibration or QC requirements. Acceptance was based on QC %bias within ± 20% and %RSD ≤ 20% at each validation check.

RESULTS AND DISCUSSION

Chromatography and MS performance: The LC-MS method demonstrated high chromatographic efficiency, resulting in the baseline separation of all eight target analytes on an Acclaim C18 column at 35 ºC within 11 min (Table-2), yielding sharp, symmetric peaks (Fig. 1). The Rt of compounds followed a polarity-driven trend, with early elution of morphine (1.42 min) and codeine (4.35 min), whereas more lipophilic substances such as fentanyl (9.31 min) and methadone (10.79 min), eluted later (Fig. 1). Importantly, closely eluted compounds including 6-AC (6.83 min) and heroin (6.90 min), were resolved at baseline (Fig. 1). A clear distinction was also established between heroin and 6-MAM, supporting targeted urine identification and quantification within the scope of the validated method (Fig. 1).

Analytes ionised in ESI+ were detected as protonated molecular ions ($[M+H]^+$). In this single-quadrupole LC-MS workflow, targeted identification employed protonated ions and in-source fragment ions, along with Rt agreement, chromatographic resolution, peak quality and qualifier/quantifier ion-ratio criteria. This approach does not provide the same structural selectivity as LC-MS/MS/MRM, where confirmation relies on precursor-to-product ion transitions. The monitored ions (*m/z*; quantifier and qualifier) comprised codeine (300.1 and 243.1), morphine (286.1 and 229.0), 6-MAM (328.1 and 211.1), tramadol (58.2; qualifier ion not established under method conditions), 6-AC (342.2 and 225.1), heroin (370.1 and 165.0), fentanyl (188.1 and 337.2), methadone (265.1 and 105.1) and atropine IS (290.1 and 124.1) (Table-2). The Rt repeatability at 0.5 µg/mL in the urine extract was acceptable, with %RSD values ranging from 0.000 to 0.563% across the panel (Table-2). Tramadol showed the highest variability in

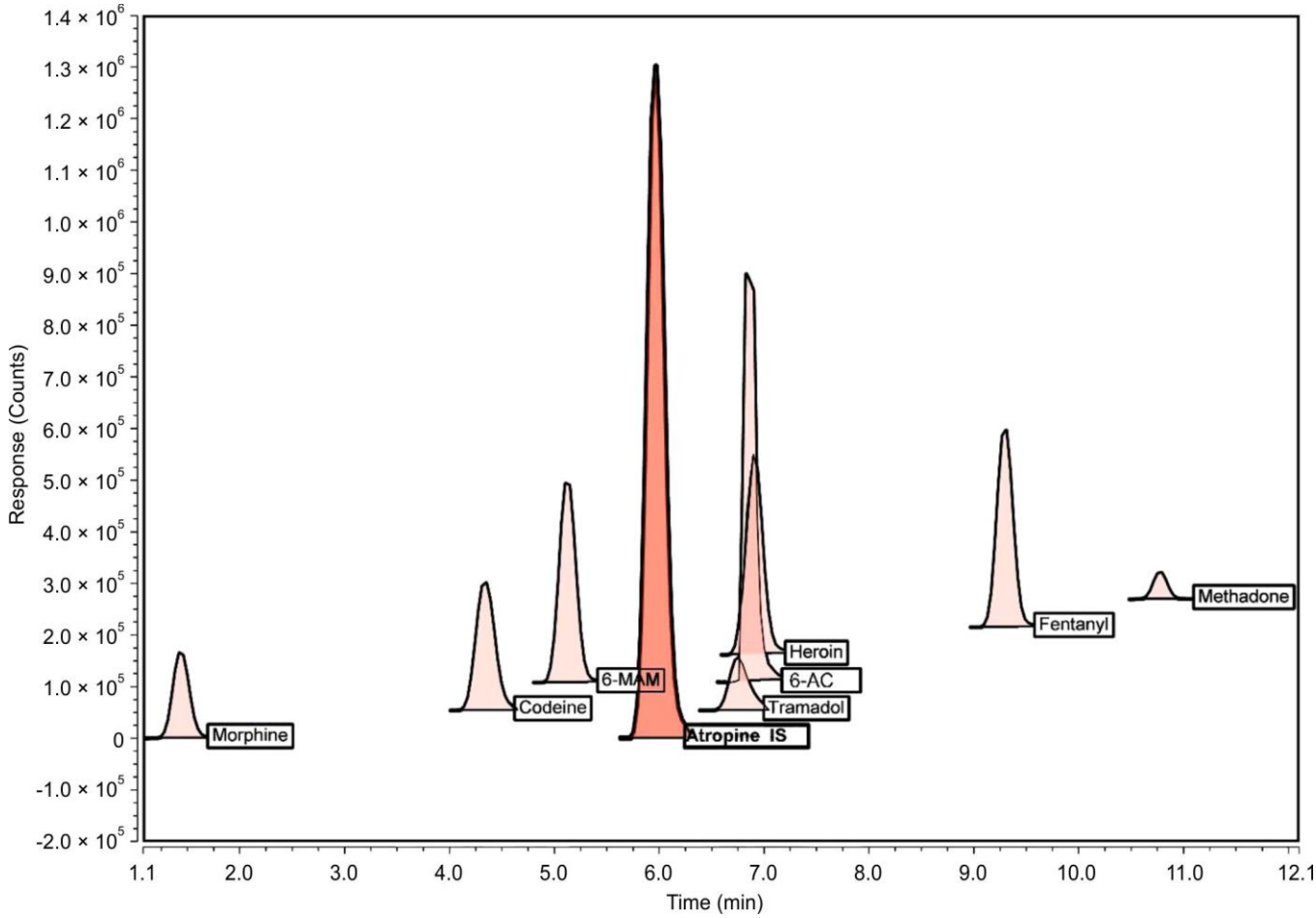

Fig. 1. LC-MS EIC showing chromatographic separation of the eight target analytes and IS (atropine) at 10.0 µg/mL in urine extracts

TABLE-2
ANALYTICAL PERFORMANCE AND VALIDATION CHARACTERISTICS OF THE LC-MS URINE ASSAY FOR TARGET OPIOIDS

Analyte	Rt (min)*		Calibration			
	Rt (± SD)	%RSD	Cal. range (µg/mL)	R^2	Range (%)†	Calibration equation
Morphine	1.42 (0.000)	0.000	0.5–25.0	0.991	94.2–116.9	y = 1.0012x + 0.4788
Codeine	4.35 (0.000)	0.000	0.5–25.0	0.995	81.2–109.5	y = 1.7815x + 0.7866
6-MAM	5.11 (0.015)	0.302	0.5–25.0	0.992	95.0–116.8	y = 2.2494x + 1.3040
Tramadol	6.70 (0.038)	0.563	0.5–25.0	0.994	85.3–109.7	y = 0.8128x + 0.2023
6-AC	6.83 (0.000)	0.000	0.5–25.0	0.995	80.0–109.3	y = 3.3292x + 2.1199
Heroin	6.90 (0.000)	0.000	0.5–25.0	0.996	81.2–110.4	y = 2.1559x + 1.9800
Fentanyl	9.31 (0.017)	0.181	0.5–25.0	0.998	86.2–105.5	y = 2.5831x + 0.0615
Methadone	10.79 (0.009)	0.088	0.5–25.0	0.997	89.2–106.3	y = 0.3007x + 0.0219
Atropine‡	5.97 (0.000)	0.000	NA	NA	NA	NA

Analyte	Effective urine LOQ§ (ng/mL)	Mean ion ratio	Accuracy and precision				ME and RE	
			QC (urine extracts) (µg/mL)	%bias	%RSD intra-day	%RSD inter-day	Mean ME, % (%RSD)	Mean RE, % (%RSD)
Morphine	50	7.31	1	6.8	14.6	5.1	103.9 (6.8)	112.8 (8.2)
			10	15.0	0.5	1.6	–	–
			20	14.1	6.0	3.2	102.5 (1.0)	114.3 (1.3)
Codeine	50	8.75	1	7.1	2.9	2.2	103.1 (2.8)	111.0 (4.5)
			10	12.6	2.2	1.7	–	–
			20	13.4	3.2	1.6	102.0 (5.2)	109.0 (8.3)
6-MAM	50	3.85	1	11.9	2.2	4.0	101.1 (3.1)	113.8 (4.3)
			10	17.6	1.7	4.3	–	–
			20	12.5	6.5	2.2	100.8 (1.8)	114.0 (2.1)
Tramadol	50	ND	1	–11.0	5.3	3.4	108.2 (2.1)	91.5 (4.1)
			10	–2.0	2.9	5.3	–	–
			20	–6.1	0.3	1.3	106.4 (1.8)	96.8 (3.8)
6-AC	50	37.51	1	–13.0	13.3	8.2	89.7 (2.3)	87.1 (4.2)
			10	–10.9	2.5	3.9	–	–
			20	–6.3	1.8	2.7	98.6 (2.0)	88.7 (4.0)
Heroin	50	40.77	1	–17.3	10.6	7.8	85.8 (3.6)	82.4 (1.2)
			10	–15.9	8.3	6.7	–	–
			20	–15.4	0.2	5.6	89.2 (2.4)	85.8 (0.7)
Fentanyl	50	37.35	1	–6.0	7.0	4.1	96.9 (4.0)	89.6 (6.9)
			10	–4.4	5.5	2.1	–	–
			20	–3.7	3.9	3.9	98.5 (2.7)	98.8 (5.2)
Methadone	50	40.99	1	18.0	10.0	16.8	109.1 (1.9)	101.8 (3.3)
			10	3.8	4.5	8.1	–	–
			20	3.8	4.2	7.0	102.8 (1.0)	104.2 (1.6)
Atropine‡	NA	50.00	NA	NA	NA	NA	NA	NA

*Rt repeatability; †mean accuracy range; –, not applicable; ND, not detected; ‡IS; §derived after applying 10-fold enrichment factor; Rt repeatability and ion-ratio assessment at 0.5 µg/mL in urine extracts (n = 10). Accuracy and precision were evaluated at QC levels of 1, 10 and 20 µg/mL in urine extracts; intra-day precision was assessed with n = 6 and inter-day precision with n = 18.

Rt, whereas atropine IS displayed a stable Rt value of 5.97 ± 0.000 min (Table-2). Qualifier-to-quantifier ion ratios were within the ± 20% tolerance range only for analytes with reliable qualifier ions (Table-2).

Tramadol yielded weak, matrix-dependent in-source fragments and did not meet the criteria for a reliable qualifier ion. The tramadol findings were assessed using quantifier-ion response, Rt agreement, chromatographic resolution, peak-quality assessment and absence of interfering substances under the tested conditions. This limitation was retained as a specific method-performance boundary for tramadol. These criteria, together with the baseline chromatographic separation shown in Fig. 1, support targeted LC-MS reporting within the scope of the validated method but do not claim equivalence to LC-MS/MS/MRM confirmation.

Validation

Linearity: 1/x-weighted regression analysis revealed linear responses for all analytes, with R^2 ranging from 0.991 (morphine) to 0.998 (fentanyl) (Table-2). The calculated concentrations were within 20% of the predefined levels at each calibrator point, supporting the selected analytical range (Table-2). The ranges for the analyte-specific accuracy were 94.2-116.9% for morphine, 81.2-109.5% for codeine, 95.0-116.8% for 6-MAM, 80.0-109.3% for 6-AC, 81.2-110.4% for heroin, 86.2-105.5% for fentanyl, 89.2-106.3% for methadone and 85.3-109.7% for tramadol (Table-2). The calibration slopes fell between 0.3007 and 3.3292, whereas the *y*-intercepts varied from 0.0219 to 2.1199, consistent with the analyte-dependent detector responses (Fig. 2). These calibration data support routine quantitative application across the selected analytical

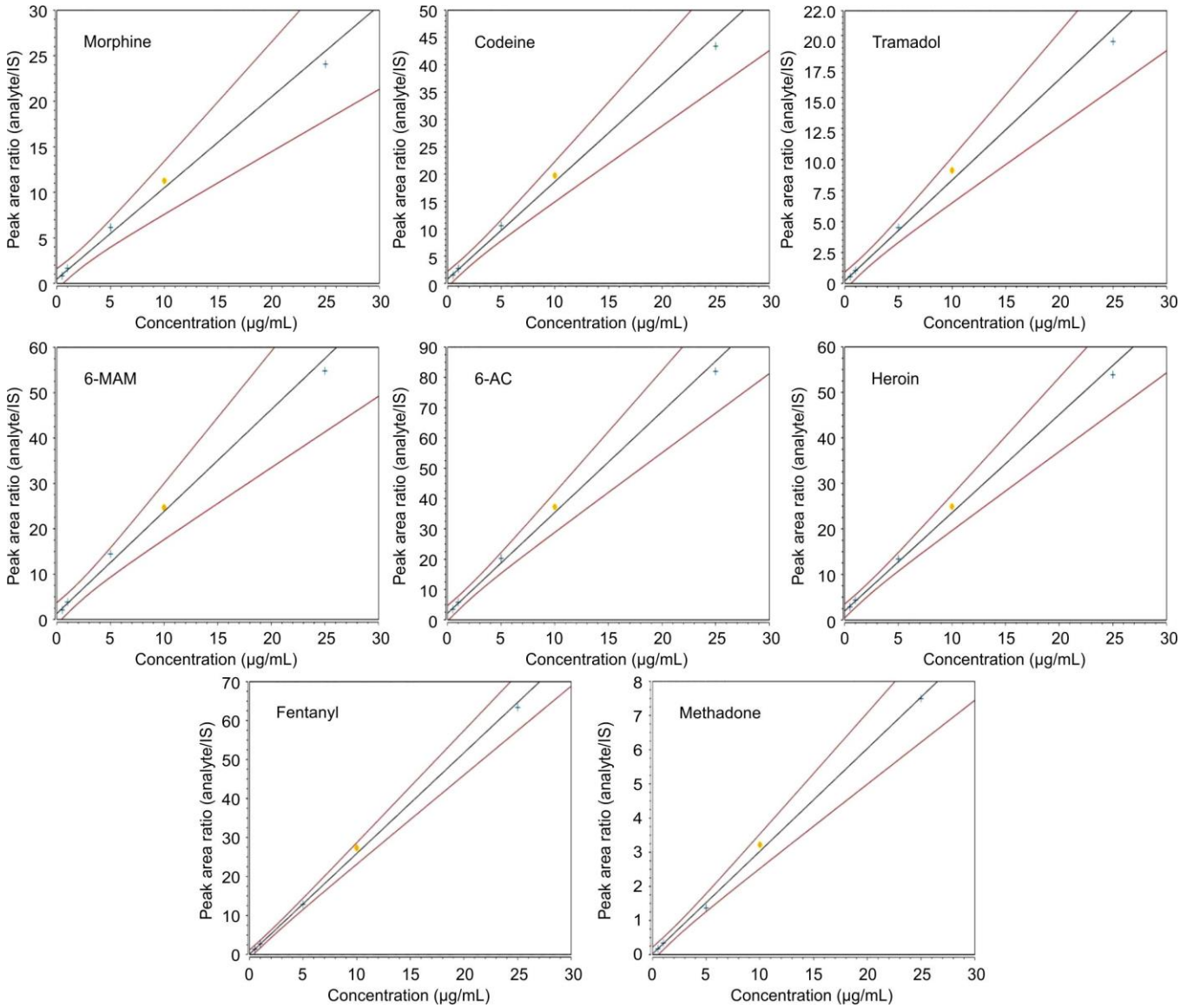

Fig. 2. Representative calibration curves for the eight target analytes, constructed from IS-normalised responses using 1/x-weighted linear regression over the validated concentration range of 0.5–25.0 µg/mL

range when 1/x-weighted regression is used. These calibration results were based on analyte-to-IS peak-area ratios and therefore reflect the atropine quantitative behaviour under validated conditions.

Precision and accuracy: Analyte responses were normalised to atropine levels to enable quantification. Across QC levels, the %bias ranged from -17.3% to 18.0%, while intra-day and inter-day %RSD ranged from 0.2% to 14.6% and 1.3% to 16.8%, respectively (Table-2). The results met the predefined performance criteria and support quantitative application within the scope of the validated targeted LC-MS reporting. The findings were interpreted together with the analyte-specific linearity, ME and RE results.

LOD, LOQ and carryover: The method provided an LOD of 0.3 µg/mL and an extract LOQ of 0.5 µg/mL within the validated calibration range. The original urine concentration was calculated as $C_{urine} = C_{extract}/10$, where C_{urine} is the analyte concentration in the original urine sample and $C_{extract}$ is the analyte concentration in the processed extract after reconstitution, as all QC and casework urine specimens were processed using the same 10-fold enrichment workflow. The original urine sample spiked with 50 ng/mL was equivalent to 0.5 µg/mL in the final enriched extract. A blank urine sample was spiked at the effective LOQ level before extraction and then processed according to the established workflow. The enriched extract showed detectable analyte responses, with no interfering blank peaks observed at the corresponding Rt and met the predefined criteria (Fig. 3). At concentrations below this reporting level, LC-MS/MS remains preferable due to the higher sensitivity.

Following the highest calibrator injection, the carryover was evaluated by injecting three pooled urine extracts. In subsequent injections, no detectable analyte peaks were observed at the target Rt and the mean blank response remained < 10% of the LOQ signal, thereby meeting the predefined carryover criterion. The absence of detectable carryover following high

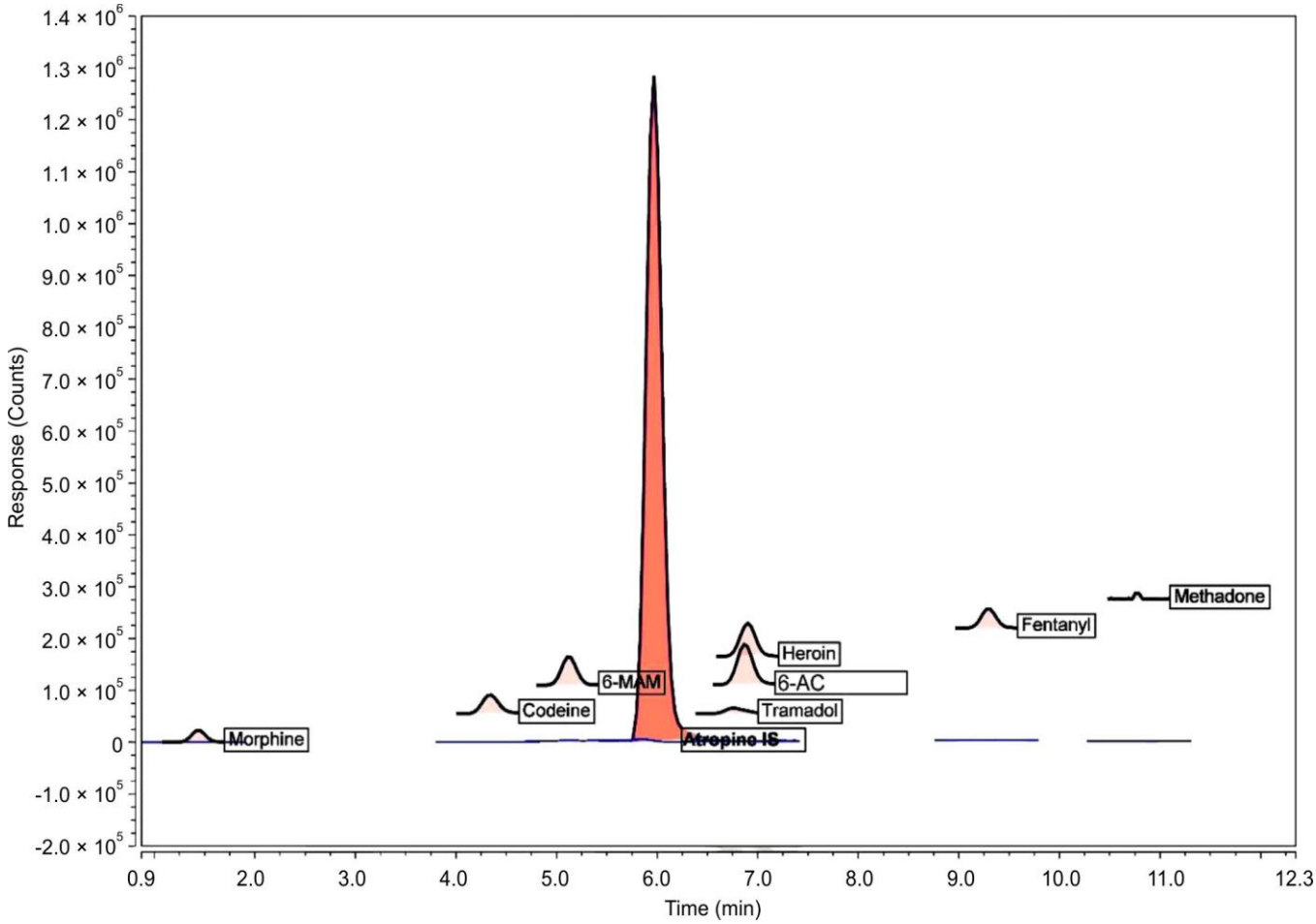

Fig. 3. Overlay of LC-MS EIC for a urine sample spiked at the effective LOQ level before extraction (50.0 ng/mL in original urine, equivalent to 0.5 µg/mL in the final enriched extract after 10-fold enrichment; light red) and a blank urine extract (blue), showing detectable analyte responses with no evident interfering peaks at the corresponding Rt under the established method conditions

concentration injection further supports the suitability of the method for sequential routine analysis.

ME, RE and selectivity: The presence of moderate ME in urine was well controlled at both concentrations. Across all target analytes, ME ranged from 85.8% to 109.1% at low QC (LQC) and from 89.2% to 106.4% at high QC (HQC), with a relatively low %RSD of 1.0-6.8% across the panel (Fig. 4b). Ionisation enhancement (> 100%) was observed for morphine, codeine, 6-MAM, tramadol and methadone, whereas 6-AC, heroin and fentanyl showed ion suppression (< 100%) (Fig. 4b). The RE of the extracted samples was consistently high and reliable, ranging from 82.4% to 113.8% at LQC and from 85.8% to 114.3% at HQC across the panel, with %RSD values of 0.7-8.3% (Fig. 4a). Atropine was used as a substitute rather than analyte-matched SIL-IS, thus ME and RE were evaluated analyte-by-analyte to assess whether recovery and ionisation differences remained controlled under the validated workflow.

No interfering peaks were observed at the Rt of any target analyte or IS in the blank and interference samples. The predefined identification criteria were met where qualifier ions were available, with ion ratios and Rt of analytes within accepted limits. These results support targeted selectivity under the tested LC-MS conditions, while acknowledging that this selectivity is not equivalent to LC-MS/MS/MRM-based structural confirmation.

Stability and inter-storage agreement (4 ºC *vs.* -20 ºC): All eight analytes at both low and high levels remained within the predefined long-term stability window of 80-120% mean %Remaining across storage Days 3-30 under both conditions (Fig. 5b-c). At 4 ºC, the mean %Remaining ranged from 82.6% to 109.3% at LQC and 83.8% to 110.8% at HQC (Fig. 5b). At -20 ºC, the mean %Remaining ranged from 84.2% to 106.4% at the LQC and 86.4% to 109.7% at HQC (Fig. 5c). The heaviest decline over time was seen for heroin, which almost reached the lower acceptance threshold by Day 30, whereas methadone showed a relatively small difference between storage conditions at the low level by Day 30 (Fig. 5b-c).

Bench-top (room temperature) stability met the predetermined acceptance limits (80-120% remaining) for all analytes at both the LQC and HQC (Fig. 5a). The mean %Remaining was close to 100%, with a range of 97.2-101.5% at LQC and 97.8-101.1% at HQC after 5 h and from 95.2-101.2% at LQC and 97.4-102.0% at HQC after 24 h (Fig. 5a). The highest decreases after 24 h were observed for the less stable analytes at the LQC level; however, all measured concentrations remained within the acceptable criteria, while %RSD

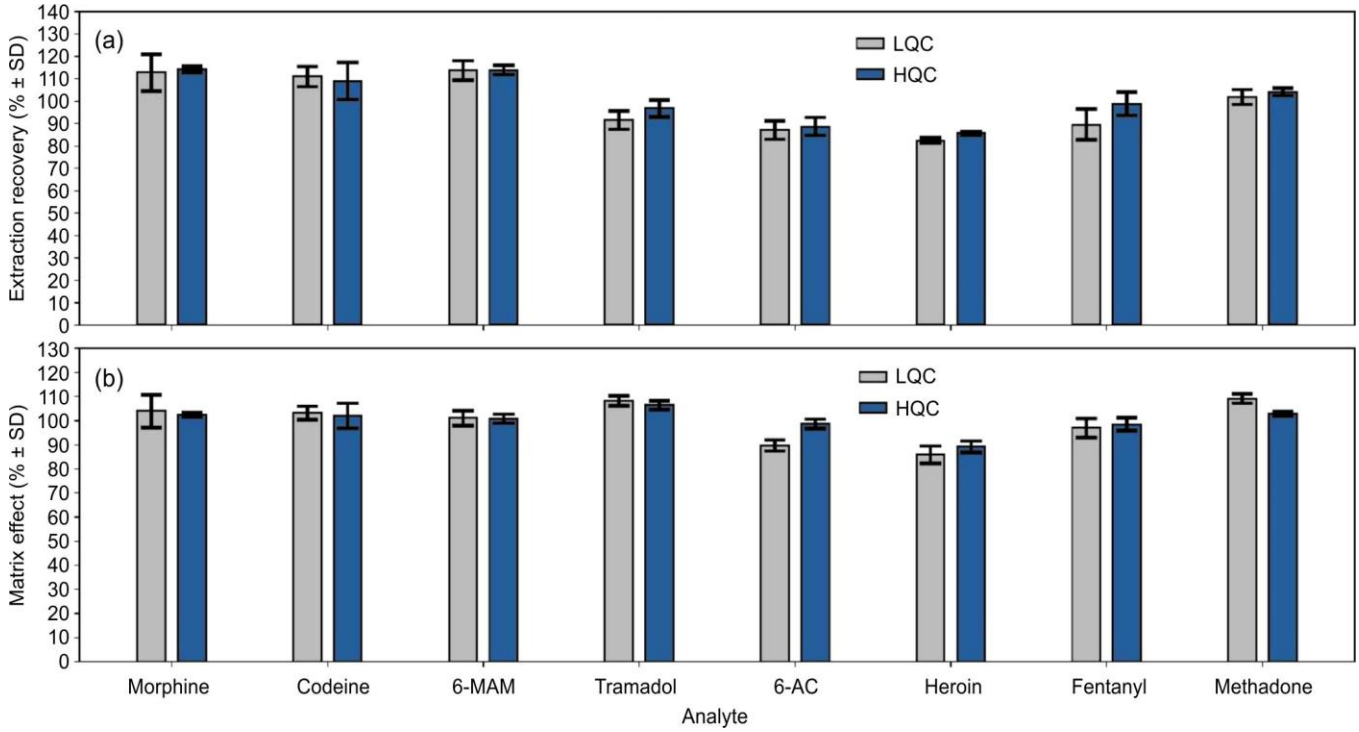

Fig. 4. RE (A) and ME (B) for target analytes at the LQC (1.0 µg/mL) and HQC (20.0 µg/mL) levels in urine extracts (mean % ± SD; n = 3)

values were generally ≤ 6% across all time points, confirming analyte stability at room temperature for at least 24 h (Fig. 5a).

The agreement between refrigerated storage at 4 ºC and frozen storage at -20 ºC was evaluated through Bland-Altman analysis of day-matched mean %Remaining values on Days 3, 7, 14 and 30 as part of the validation storage-temperature experiment (Fig. 6). The bias values were small, ranging from -3.6 to +3.3 percentage points across analytes and concentration levels and the 95% LoA remained within the predefined ± 20 percentage point agreement threshold. This analysis was only used to evaluate the agreement of analytical storage under the tested conditions and was not related to casework interpretation or population-level inference.

The profiles at 4 ºC and -20 ºC were comparable over the assessed storage periods, with -20 ºC being the more conservative option for longer labile analytes storage. These findings support the storage-temperature agreement within the validation design, if the routine QC acceptance is met.

Co-elution effect (6-AC *vs.* heroin): Minimal co-elution effects were observed between 6-AC and heroin at all concentrations. All absolute %Effect values were ≤ 2.5%, indicating minimal suppression or enhancement of partner-related ions. At equivalent concentrations, only slight mutual suppression was observed: -1.3% for 6-AC and -1.4% for heroin under L/L conditions and -2.4% for 6-AC and -2.2% for heroin under H/H conditions, consistent with minor competition at higher total analyte load. A minor concentration-dependent pattern was observed in the asymmetric mixtures. High levels of heroin combined with low 6-AC produced a 2.5% decrease in 6-AC and a 1.2% increase in heroin, whereas low heroin combined with high 6-AC resulted in a 0.8% increase in 6-AC and a 2.1% decrease in heroin. These values are well below the predefined threshold of ± 20%, indicating that the close elution of 6-AC and heroin observed in Fig. 1 does not significantly affect their ionisation.

Accordingly, any matrix-related bias observed during validation is more likely attributable to matrix-related effects than to partner-driven interactions. These findings indicate that the chromatographic proximity of 6-AC and heroin is not the main cause of ionisation interference in this method.

Saved calibration curve performance check: The QC results from six monthly checks remained within the accepted limits, with %bias ranging from 0.8-13.1% at LQC, 3.4-9.7% at middle QC (MQC) and -6.0 to -0.2% at HQC and %RSD ranging from 2.9-13.6%, 4.8-13.5% and 4.9-12.1%, respectively. These findings indicate acceptable response stability under the tested conditions; however, they are not intended to justify routine six-month calibration reuse or replace batch-specific calibration and QC requirements in forensic casework. The validation results support the quantitative application of the method under the established conditions, if routine batch calibration, QC acceptance and the LOQ-based reporting criteria are maintained.

Forensic and de-addiction casework: A validated assay was applied to 63 urine casework samples *viz.* 24 from KHRC and 39 from RFI forensic cases, which comprised 24 samples for rehabilitation compliance-monitoring and 15 autopsy specimens (Table-3). Results were reported for the full cohort (N = 63) and stratified by specimen source to account for differences in context. The specimen-source groups differed by institution, geography and casework purpose, therefore, source stratified findings were descriptively interpreted and not used for direct population-level or geographical comparisons. Casework findings were interpreted as LOQ-based urinary analytical patterns rather than as definitive evidence of dose, route, timing, impairment, cause of death or individual exposure history.

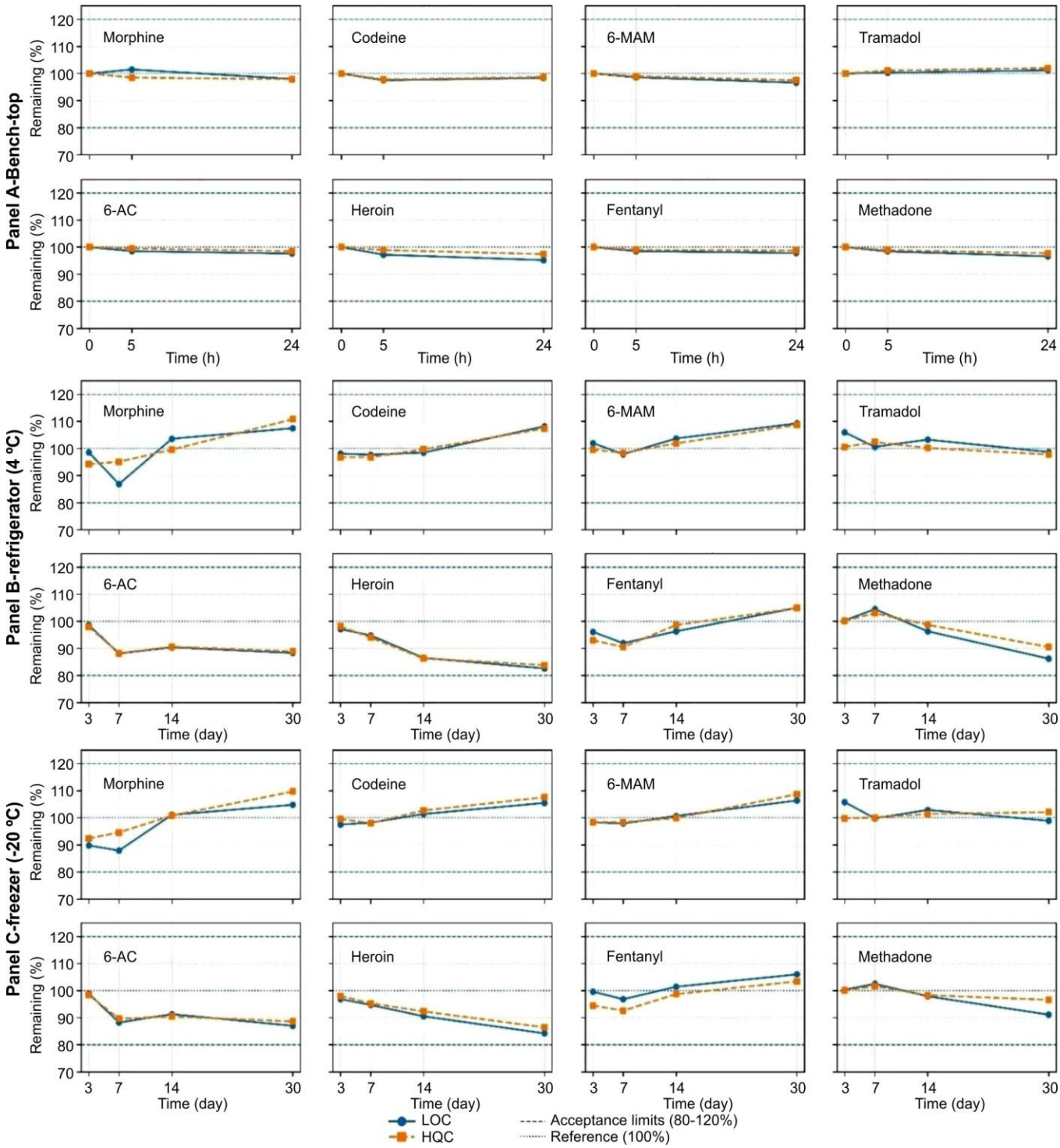

Fig. 5. Stability profiles of target analytes in urine extracts, expressed as %Remaining at LQC (1.0 µg/mL) and HQC (20.0 µg/mL) levels, under bench-top conditions at room temperature (A), refrigerated storage at 4 ºC (B) and frozen storage at –20 ºC (C)

Age and sex distributions and between-group comparisons: The full cohort comprised 88.9% males (56/63) and 11.1% females (7/63). The median age was 31 years, with a range of 17-63 years and an IQR of 23.5-40.5 years. The median age varied descriptively among the specimen-source groups: 32.5 years in the KHRC group, 22.0 years in the RFI-Rehabilitation group and 49.0 years in the RFI-Autopsy group. These demographic summaries highlight the diversity of the data set and were not interpreted as indicating population-level or geographical differences in opioid exposure.

Positivity rates and concentration summaries: Fentanyl and methadone were relatively rare, occurring in 3.2% (2/63; 95% CI, 0.9-10.9%) and 6.3% (4/63; 95% CI, 2.5-15.2%) of cases, respectively (Table-3). Heroin-related markers were detected in a considerable portion of the cohort, with 6-MAM detected in 30.2% of cases (19/63; 95% CI, 20.2-42.4%) and

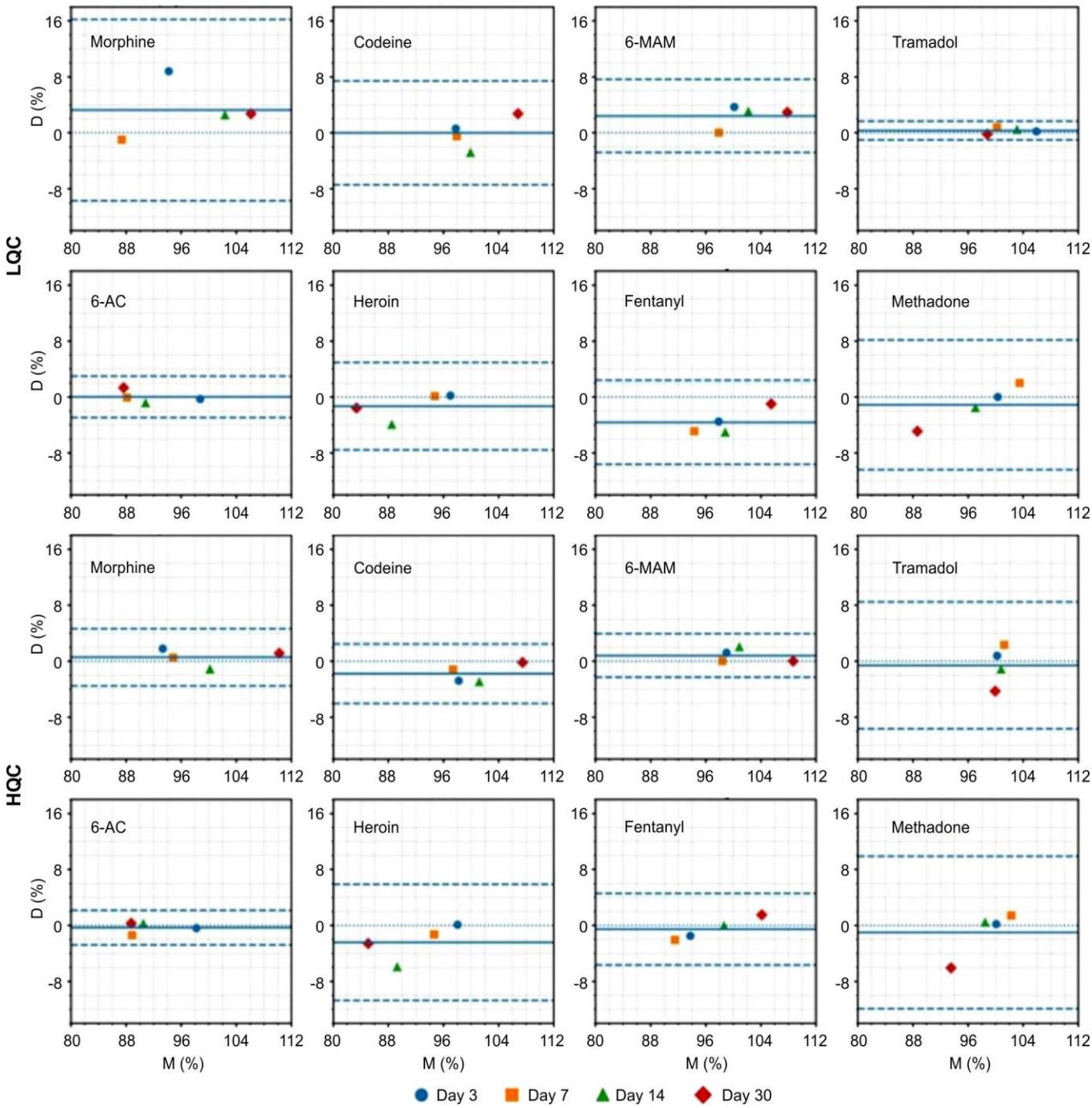

Fig. 6. Bland–Altman plots used in the validation storage-temperature agreement assessment, comparing %Remaining at 4 ºC and –20 ºC for LQC and HQC levels of the target analytes. For each time point (Days 3, 7, 14 and 30), M = [%Remaining at 4 ºC + %Remaining at –20 ºC]/2 and D = [%Remaining at 4 ºC – %Remaining at –20 ºC]. The solid line indicates the mean bias and the dashed lines indicate the t-distribution-based 95% LoA

6-AC quantified in 22.2% of cases (14/63; 95% CI, 13.7-33.9%) (Table-3), whereas tramadol was detected in 11.1% (7/63; 95% CI, 5.5-21.2%) of cases (Table-3). Heroin in its intact form was infrequently quantified, occurring at a rate of 1.6% (1/63; 95% CI, 0.3-8.5%) (Table-3).

Descriptive positivity patterns were observed after specimen source stratification (Table-3). These quantified positivity rates and their 95% CI are also illustrated in a forest plot (Fig. 7). Quantified detections were infrequent in the KHRC subgroup: morphine was detected in 4.2% of cases (1 out of 24 cases; 95% CI, 0.7-20.2%) and, codeine and tramadol each in 8.3% of cases (2 out of 24 cases; 95% CI 2.3-25.8%). No quantifiable results (0 of 24) were observed for 6-MAM, 6-AC, heroin, fentanyl or methadone (Table-3).

The RFI-Rehabilitation subgroup showed higher observed quantified positivity for both major opioids and heroin markers: morphine and codeine were detected in 100.0% (24 of 24 cases, 95% CI 86.2-100.0%); 6-MAM in 79.2% (19 of

TABLE-3
QUANTIFIED POSITIVITY RATES OF TARGET OPIOIDS IN URINE CASEWORK SPECIMENS BY SPECIMEN-SOURCE GROUP AND IN THE FULL COHORT

Analyte	KHRC (n = 24) n/N (%; 95% CI)	RFI-Rehabilitation (n = 24) n/N (%; 95% CI)	RFI-Autopsy (n = 15) n/N (%; 95% CI)	Full cohort (N = 63) n/N (%; 95% CI)
Morphine	1/24 (4.2%; 0.7–20.2%)	24/24 (100.0%; 86.2–100.0%)	9/15 (60.0%; 35.7–80.2%)	34/63 (54.0%; 41.8–65.7%)
Codeine	2/24 (8.3%; 2.3–25.8%)	24/24 (100.0%; 86.2–100.0%)	9/15 (60.0%; 35.7–80.2%)	35/63 (55.6%; 43.3–67.2%)
Tramadol	2/24 (8.3%; 2.3–25.8%)	0/24 (0.0%; 0.0–13.8%)	5/15 (33.3%; 15.2–58.3%)	7/63 (11.1%; 5.5–21.2%)
6-MAM	0/24 (0.0%; 0.0–13.8%)	19/24 (79.2%; 59.5–90.8%)	0/15 (0.0%; 0.0–20.4%)	19/63 (30.2%; 20.2–42.4%)
6-AC	0/24 (0.0%; 0.0–13.8%)	13/24 (54.2%; 35.1–72.1%)	1/15 (6.7%; 1.2–29.8%)	14/63 (22.2%; 13.7–33.9%)
Heroin	0/24 (0.0%; 0.0–13.8%)	1/24 (4.2%; 0.7–20.2%)	0/15 (0.0%; 0.0–20.4%)	1/63 (1.6%; 0.3–8.5%)
Fentanyl	0/24 (0.0%; 0.0–13.8%)	0/24 (0.0%; 0.0–13.8%)	2/15 (13.3%; 3.7–37.9%)	2/63 (3.2%; 0.9–10.9%)
Methadone	0/24 (0.0%; 0.0–13.8%)	4/24 (16.7%; 6.7–35.9%)	0/15 (0.0%; 0.0–20.4%)	4/63 (6.3%; 2.5–15.2%)

Data are presented as *n/N* (%) with Wilson 95% CI. Analyte was considered positive only when quantified at or above the validated LOQ. Results reported as ND/<LOQ were treated as negative for prevalence estimates.

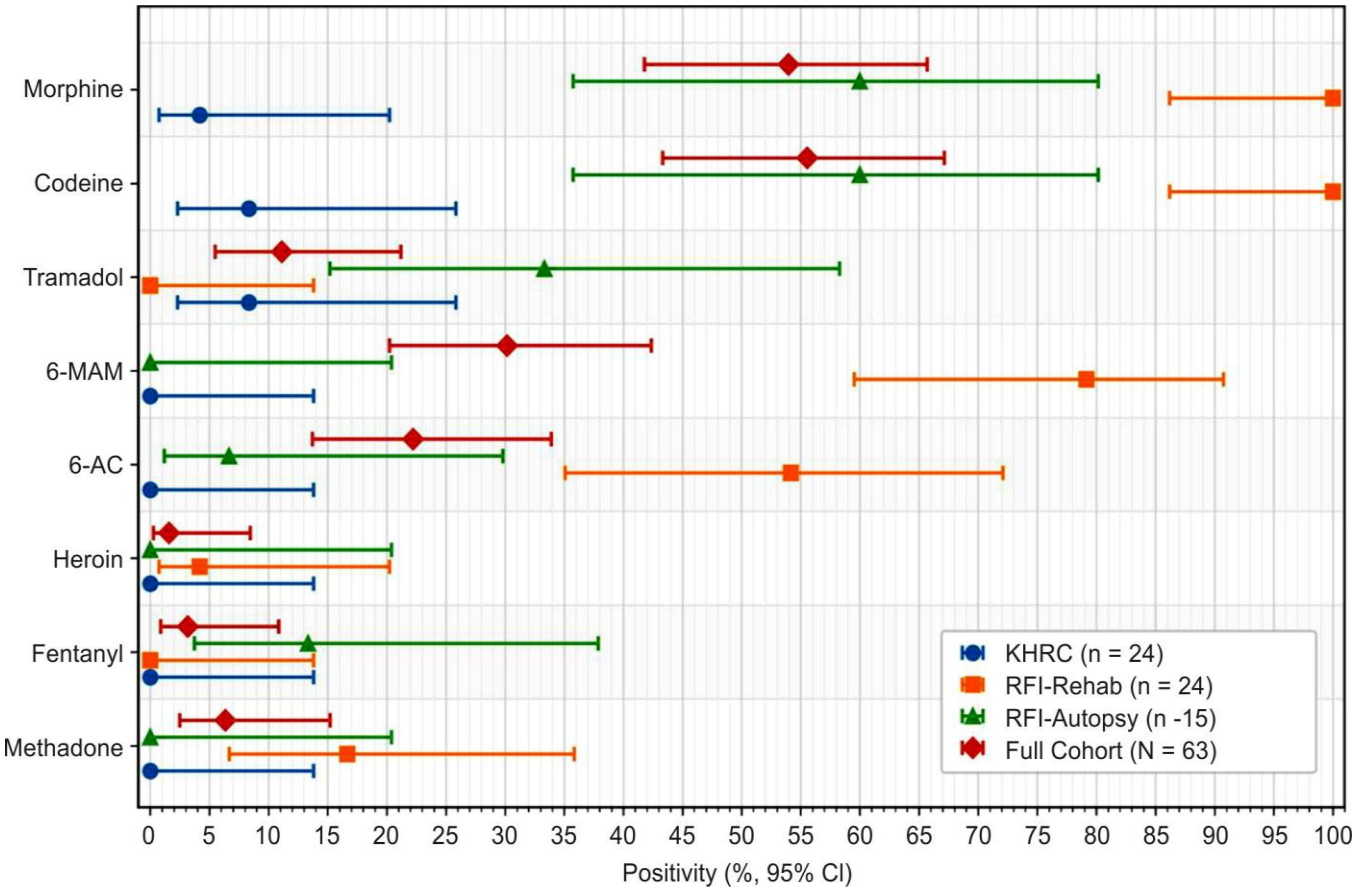

Fig. 7. Forest plot of quantified positivity rates with 95% CI for target opioids by specimen source group and in the full cohort. KHRC (n = 24); RFI-Rehabilitation compliance-monitoring group (n = 24); RFI-Autopsy group (n = 15); full cohort (*N* = 63)

24 cases, 95% CI 59.5-90.8%); and 6-AC in 54.2% (13 of 24 cases, 95% CI 35.1-72.1%) indicating a higher frequency of heroin-related marker detection in specimens from this monitored subgroup (Table-3).

In the RFI-Autopsy subgroup, morphine and codeine were found in 60.0% (9 out of 15 cases; 95% CI, 35.7-80.2%), whereas tramadol was present in 33.3% (5 out of 15 cases; 95% CI, 15.2-58.3%). Heroin markers were infrequent, with 6-MAM not being quantified (0 of 15) in any specimens; the upper 95% CI was 20.4% and 6-AC was quantified in only 6.7% of the samples (1 of 15; 95% CI, 1.2-29.8%) (Table-3). Patterns of heroin-marker positivity varied across specimen-source groups (Fig. 7).

Autopsy urine specimens with a lower frequency of quantified heroin-related markers were interpreted with caution. Low or non-quantified 6-MAM, 6-AC, or heroin levels may result from marker instability, delayed sampling and post-collection handling, whereas morphine may remain measureable. These explanations should be viewed as possible analytical and pharmacokinetic considerations rather than confirmed causes, due to the lack of available case histories, intake times, clinical information and investigative context.

Concentration distribution and ratios: Concentration summaries were calculated only from results that were at or above the validated LOQ. The median morphine concentration was 1241.45 ng/mL, with an IQR of 465.93-1435.60 ng/mL and a total range of 67.30-2662.90 ng/mL. The median codeine concentration was 474.80 ng/mL, with an IQR of 163.75-721.33 ng/mL and a total range of 70.80-951.00 ng/mL. Heroin-related markers were quantified at lower concentrations: 6-MAM showed a median of 116.80 ng/mL, with an IQR of 100.45-140.30 ng/mL and a range of 68.30-183.00 ng/mL, whereas 6-AC showed a median of 78.65 ng/mL, with an IQR of 72.56-92.48 ng/mL and a range of 59.72-149.20 ng/mL.

The lower concentrations and frequency of acetylated heroin-related markers were interpreted as descriptive urinary analytical patterns within a validated LOQ-based framework rather than as evidence of differences in dose, timing, route, exact source, or geographic drug-use patterns (Table-3).

The variability in positivity rates and concentrations within the combined KHRC and RFI cohort may reflect differences in the specimen-source context, urine dilution, analyte stability and unrecorded case-level factors. Due to these variables were not available for individual specimens, the observed patterns were treated only as descriptive analytical findings rather than as verified determinants of exposure or population-level comparisons.

Tramadol was quantified in seven cases within the targeted LC-MS reporting scope, with a median concentration of 381.00 ng/mL and a range of 56.00-732.00 ng/mL. Methadone was quantified in four cases, with a median concentration of 623.10 ng/mL and a range of 316.30-951.00 ng/mL. Fentanyl was quantified at 52.30 and 65.10 ng/mL in two specimens, whereas heroin was quantified at 63.10 ng/mL in one specimen. Individual concentrations were considered more informative than broad statistical summaries.

Analyte ratios were used solely as exploratory pattern-supporting descriptors and were calculated only when both analytes in a pair were quantified at or above the validated LOQ. The codeine-to-morphine ratio was calculated in 34 specimens, with a median of 0.399, an IQR of 0.275-0.579 and a range of 0.155-9.346. The ratio of 6-MAM to morphine was calculated in 19 specimens, with a median of 0.079, an IQR of 0.069-0.093 and a range of 0.039-0.125. The 6-AC-to-codeine ratio was calculated in 14 specimens, with a median of 0.109, an IQR of 0.092-0.135 and a range of 0.072-0.207. These ratios helped describe co-quantified urinary analyte patterns but were not interpreted as case or population-level evidence.

Morphine-codeine association: Morphine and codeine concentrations were descriptively visualised in the 34 specimens where both analytes were quantified at or above the validated LOQ (Fig. 8). The scatter plot was used solely to show the exploratory covariation between the measured urinary concentrations under the analytical conditions. This association was not treated as hypothesis-driven evidence and does not establish case-level exposure history, metabolic direction, causality, or geographical difference.

Codeine distributions according to 6-AC status: Codeine concentrations were descriptively visualised using a box-and-whisker plot, with overlaid individual data points for the 35 specimens in which codeine was quantified (Fig. 9). The 6-AC-positive group included 14 cases and showed a higher median codeine concentration than the 6-AC-negative group, which included 21 cases. The median codeine concentration was 763.90 ng/mL in the 6-AC positive group and 193.30 ng/mL in the 6-AC negative group (Fig. 9). This comparison was performed only as an exploratory pattern check and should not be interpreted as proof of the absence of a source, dose, timing or geographical differences. The concentration data alone cannot rule out other possible sources of codeine exposure.

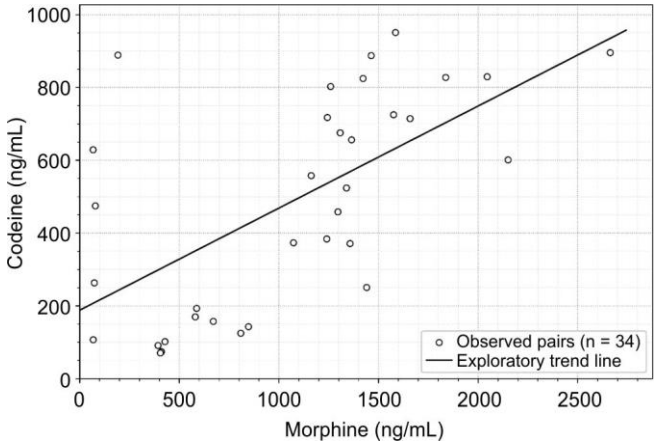

Fig. 8. Scatter plot of urine morphine and codeine concentrations in co-quantified specimens (n = 34 pairs), with an overlaid exploratory trend line to visualize covariation

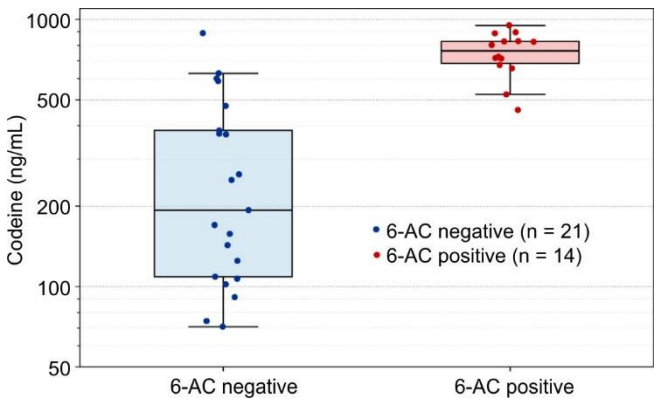

Fig. 9. Descriptive box-and-whisker plot of codeine concentrations in quantified specimens stratified by 6-AC status, with overlaid individual data points. The boxes represent the median and IQR and whiskers extend to the most extreme values within 1.5 × IQR of the quartiles according to Tukey's rule. Values beyond the whiskers are presented as individual observations

This study presents a targeted, fit-for-purpose single-quadrupole LC-MS workflow for eight urinary opioids by integrating SPE cleanup, 10-fold enrichment, C18 chromatographic separation, full-scan EIC monitoring, validation and LOQ-based reporting. This method is not proposed as equivalent or superior to LC-MS/MS/MRM confirmation. The contribution of this study lies in its combined sample preparation, enrichment, validation and authentic casework application within a defined eight-analyte reporting scope. Atropine-

normalised quantification and tramadol reporting were performed within the validated method-performance boundaries. Casework findings should be interpreted conservatively as descriptive urinary analytical patterns from heterogeneous source groups, not as population-level geographic comparisons or definitive evidence of dose, timing, route, impairment, cause of death or individual exposure history.

Conclusion

A 10-fold enrichment, single-quadrupole ESI+ LC-MS method was developed for the targeted quantification of eight urinary opioids, including heroin-related markers, within a defined validation and reporting scope. Validation supported quantitative analysis under established method conditions with acceptable calibration, precision, matrix effect (ME), extraction recovery (RE), stability, carryover and LOQ-based reporting performance. The workflow was useful for describing urinary opioid patterns in 63 authentic urine specimens from de-addiction and forensic casework. The method is not proposed to be equivalent to or a substitute for LC-MS/MS/ MRM confirmation, but rather provides value through its integrated approach to sample preparation, enrichment, chromatographic separation, validation and authentic urine application for the selected eight-analyte panel.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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