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ORIGINAL ARTICLE

Determination of Heroin in Urine by Molecularly Imprinted Polymer-Based Solid-Phase Extraction and UV–Vis Spectrophotometry

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ABSTRACT

Background: Determination of heroin in urine is challenging because of matrix interference and heroin instability; therefore, this study aimed to develop a selective molecularly imprinted polymer-based solid-phase extraction method coupled with UV–Vis spectrophotometry for heroin determination in urine.

Methods: Heroin-imprinted polymers were synthesized by precipitation polymerization using benzoyl peroxide (BPO) as initiator, ethylene glycol dimethacrylate (EGDMA) as crosslinker, and either N-hydroxyethyl acrylamide (HEAA) or 3-(trimethoxysilyl)propyl methacrylate (TMSPMA) as functional monomers. Template removal was performed by Soxhlet extraction. Polymer formation and template removal were evaluated using FT-IR spectroscopy. MIP particles were packed into syringe SPE cartridges and applied to heroin-spiked urine samples (40–80 ppm). Heroin was quantified by UV–Vis spectrophotometry at 230 nm after MISPE clean-up and reconstitution in ethanol, using a global calibration curve (20–100 ppm).

Results: The UV–Vis calibration curve was linear across 20–100 ppm ($A = 0.0146C - 0.0607$; $R^2 = 0.9999$). In urine-matrix experiments ($n = 3$ replicates per level), recoveries ranged from 97.0% to 102.75%, with repeatability RSD = 1.24–3.83%. The global limit of detection was 0.010 ppm.

Conclusion: MISPE using heroin-imprinted polymers coupled with UV–Vis at 230 nm is a practical approach for determining heroin in spiked urine with acceptable within-run performance; however, expanded validation (selectivity and stability testing) is recommended for forensic-defensible application.

Key words: Heroin; Diacetylmorphine; Urine; Molecularly imprinted polymer (MIP); MISPE; UV–Vis spectrophotometry.

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INTRODUCTION

Papaver somniferum (opium poppy) is a member of the Papaveraceae family and a major natural source of pharmacologically active alkaloids. These compounds include isoquinoline derivatives such as papaverine, as well as phenanthrene derivatives such as morphine, codeine, and thebaine, which are important precursors for several clinically used and illicit opioids [1]. Because these alkaloids are widely encountered in medical and non-medical settings, their presence—or the presence of their metabolites—may be detected in biological specimens collected for clinical, occupational, or forensic purposes. This creates interpretive challenges in medicolegal toxicology, particularly when structurally related opioids and metabolites coexist in the same matrix [2].

Heroin (diacetylmorphine) is a semi-synthetic opioid produced by acetylation of morphine at the 3- and 6-hydroxyl groups (Figure 1), which increases lipophilicity and facilitates rapid central nervous system penetration compared with morphine [3]. After administration, heroin undergoes rapid enzymatic and non-enzymatic hydrolysis to 6-monoacetylmorphine (6-MAM) and subsequently to morphine, which is further metabolized mainly through glucuronidation to morphine-3-glucuronide and morphine-6-glucuronide [4, 5]. In urine, analytical determination is complicated by (i) heroin instability and potential degradation during collection, transport, and storage; (ii) the complexity of urine containing endogenous interferents; and (iii) interpretive ambiguity because morphine and related alkaloids may arise from sources other than heroin (e.g., codeine metabolism or opium exposure) if analytical selectivity is limited [6]. Consequently, robust heroin analysis depends not only on instrumental detection but also on appropriate pre-analytical handling, effective sample clean-up, and validation approaches that address matrix effects, selectivity, and analyte stability [7, 8].

Although chromatographic methods coupled to mass spectrometry are considered the gold standard for confirmatory testing, they may not be available in every laboratory due to cost and infrastructure requirements. In many settings, simpler analytical techniques may still be useful for screening or quantitative estimation when combined with selective extraction procedures and supported by adequate validation. This is particularly relevant for UV–Vis spectrophotometry, which is inexpensive and widely accessible but has inherently lower specificity in complex matrices; therefore, method reliability depends strongly on the selectivity of the extraction step and on validation measures that demonstrate acceptable performance [9].

Molecularly imprinted polymers (MIPs) are attractive sorbent materials because they can incorporate binding sites complementary in size, shape, and functional interactions to a target molecule [10, 11]. When integrated into solid-phase extraction formats, MIPs can enhance preconcentration and reduce matrix co-extraction, improving performance in complex biological samples [12, 13]. Therefore, the present study develops a syringe-format molecularly imprinted solid-phase extraction (MISPE) procedure using heroin-imprinted polymers as selective sorbents, coupled with UV–Vis determination at 230 nm, to extract and quantify heroin in urine.

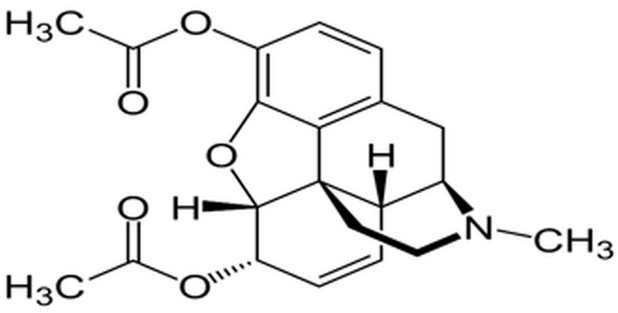

Figure 1. Chemical structure of heroin/diacetylmorphine

MATERIALS AND METHODS

Chemicals and reagents

Heroin reference material (diacetylmorphine) was supplied by the Medico-Legal Institute, Baghdad, Iraq. N-hydroxyethyl acrylamide (HEAA), 3-(trimethoxysilyl)propyl methacrylate (TMSPMA), and ethylene glycol dimethacrylate (EGDMA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol, acetonitrile, chloroform, glacial acetic acid, and benzoyl peroxide (BPO) were obtained from Merck (Darmstadt, Germany). Nitrogen gas (99.98%) was supplied by a local provider. All reagents were of analytical grade and used as received.

Administrative authorization and ethics

Controlled reference material: Handling and use of controlled reference materials were performed under institutional administrative authorization (College of Science, University of Baghdad; Letter No. 1300, dated 08/08/2023) with reference to the Iraqi Ministry of Health/Department of Forensic Medicine letter No. 18716, dated 17/07/2023.

Human urine specimens: De-identified human urine specimens were used for matrix (spiking) experiments. The study protocol was reviewed and approved by the Research Ethics Committee, College of Science, University of Baghdad.

Instrumentation

UV–Vis absorbance measurements were performed on a Shimadzu UV-1800 PC spectrophotometer at 230 nm using 1 cm quartz cuvettes. FT-IR spectra were recorded on a Shimadzu FTIR-8000 instrument over 400–4000 cm^{-1} using KBr pellets. Sonication was performed using an ultrasonic unit (Sonerx, W. Germany). ^1H NMR spectra (qualitative confirmation) were recorded in DMSO-d_6 at 499.69 MHz.

Preparation of molecularly imprinted polymers (MIP)

Molecularly imprinted polymers were prepared by precipitation polymerization using heroin as the template. The base formulation template:monomer:crosslinker molar ratio was 1:4:12 (mmol).

(i) **Template–monomer complexation:** Heroin (1.00 mmol; 0.369 g) was dissolved in 2.0 mL of porogen solvent (ethanol:acetonitrile, 1:9 v/v). Functional monomer was then added: either HEAA (4.00 mmol; 0.4605 g) or TMSPMA (4.00 mmol; 0.9934 g). The mixture was sonicated for 10 min to promote template–monomer interaction.

(ii) **Polymerization:** EGDMA (12.00 mmol; 2.3786 g) was added as crosslinker, followed by benzoyl peroxide (BPO) initiator (approximately 1% w/w of the total monomer + crosslinker; ~30 mg, ~0.12 mmol). The total volume was adjusted to 10.0 mL with ethanol:acetonitrile (1:9 v/v). The mixture was purged with nitrogen for 20 min, sealed, and polymerized in a water bath at 60°C for 10 h.

(iii) **Collection:** Polymer particles were collected by filtration or centrifugation, washed to remove residual reagents, and dried.

Non-imprinted polymer (NIP): A non-imprinted polymer can be prepared under identical conditions without adding heroin template to provide a selectivity control (imprinting factor). In the present work, method performance was evaluated using urine-matrix recovery and precision; MIP–NIP selectivity assessment is recommended as part of expanded validation.

Template removal and particle preparation

Template removal was performed by Soxhlet extraction using ethanol/acetic acid (9:1 v/v) until heroin was no longer detectable in the wash solvent by UV–Vis at 230 nm. The particles were then washed with ethanol to remove residual acid, dried under vacuum, ground gently, and sieved to obtain approximately 125 μm particles for SPE packing.

Polymer formulation screening and selection

Different template:monomer:crosslinker combinations were prepared for each monomer system (Table 3) and initially screened based on particle formation and handling characteristics. Based on preliminary screening, the TMSPMA-based MIP (MIP1) and HEAA-based MIP (MIP2) were selected for urine-matrix recovery and precision studies.

SPE cartridge (syringe-column) preparation

SPE cartridges were prepared using 3 mL polypropylene syringes fitted with frits/plugs to retain the polymer particles. Polymer masses of 0.20 g or 0.40 g were packed to form a uniform sorbent bed. Cartridges were conditioned sequentially with 1.0 mL ethanol and 1.0 mL distilled water. Samples were passed through the cartridges using a peristaltic pump operated at a constant setting (~75 rpm), and the same flow conditions were maintained for all experiments.

Preparation of standards and calibration (global calibration)

A heroin stock solution was prepared in ethanol and diluted to working standards of 20, 40, 60, 80, and 100 ppm (ppm = $\mu g\ mL^{-1}$). Absorbance was measured at 230 nm to construct a global calibration curve (Figure 2). The calibration equation used for quantification was:

$$A = 0.0146C - 0.0607; \quad R^2 = 0.9999,$$

where A is absorbance and C is heroin concentration (ppm).

Urine specimens and sample preparation

De-identified urine specimens ($n = 6$) were used as blank matrix sources for spiking experiments. Each specimen (3.0 mL) was centrifuged at 5000 rpm for 10 min, and the supernatant was used for MISPE. For recovery and precision studies, the supernatant was spiked with heroin to final concentrations of 40–80 ppm, mixed thoroughly, and processed immediately.

MISPE procedure for urine samples

i. Condition the cartridge (Section 2.7).
ii. Load 3.0 mL of centrifuged urine supernatant (spiked sample).
iii. Wash the cartridge three times with 4.0 mL distilled water.
iv. Elute heroin with 2.0 mL acetonitrile:water (1:1 v/v) containing 4% (v/v) acetic acid into a clean vial.
v. Evaporate/dry the eluate at 50°C for 10 min.
vi. Reconstitute the residue in 1.0 mL ethanol and measure

absorbance at 230 nm.

Quantification and statistical calculations

Heroin concentrations were calculated from the global calibration equation (Section 2.8). Measurements were performed in triplicate (n = 3) at each concentration level.

$$\text{Recovery (\%)} = \left(\frac{C_{\text{found}}}{C_{\text{added}}}\right) \times 100$$

$$\text{Relative error (RE, \%)} = \left[\frac{(C_{\text{found}} - C_{\text{added}})}{C_{\text{added}}}\right] \times 100$$

Precision was expressed as the relative standard deviation (RSD, %) of triplicate measurements.

LOD determination (global): The limit of detection (LOD) was calculated once using LOD = $3.3\sigma/S$, where S is the slope of the global calibration curve and σ is the standard deviation of replicate blank responses (pooled blanks). Therefore, a single LOD value applies to all MIP types and masses. The global LOD reported in this study was 0.010 ppm ($\mu g\ mL^{-1}$).

RESULTS

Synthesis of heroin-imprinted polymers

Heroin-imprinted polymers (MIPs) were synthesized successfully by precipitation polymerization using either HEAA or TMSPMA as functional monomer and EGDMA as crosslinker. After polymerization, solid particles were collected, washed, and subjected to template removal. The dried particles were ground and sieved prior to packing into syringe-column SPE cartridges.

FT-IR confirmation of polymer formation and template removal

FT-IR spectra were recorded for heroin (template), unleached MIP (before template removal), and leached MIP (after template removal). The major bands are summarized in (Tables 1 and 2). After leaching, bands attributed to the heroin template—particularly aromatic features (e.g., Ar–H stretching and aromatic C=C stretching)—were absent or not observed, consistent with effective template removal. In contrast, characteristic ester bands of the polymer network (C=O and C–O vibrations associated with the EGDMA-based matrix and/or TMSPMA moieties) remained present after leaching, indicating preservation of the polymer backbone. Overall, the disappearance/reduction of template-associated aromatic bands after leaching supports the successful removal of heroin from the imprinted polymer cavities.

Formulation screening

Different template:monomer:crosslinker combinations were prepared for each monomer system (Table 3) and were initially screened based on particle formation and handling characteristics. Based on this screening, the TMSPMA-based MIP (MIP1) and HEAA-based MIP (MIP2) were selected for urine-matrix recovery and precision studies.

Calibration performance (UV–Vis)

A global UV–Vis calibration curve was constructed using heroin standards (20–100 ppm) prepared in ethanol and measured at 230 nm (Figure 2). The calibration equation was:

$$A = 0.0146C - 0.0607; \quad R^2 = 0.9999,$$

where A is absorbance and C is heroin concentration (ppm, $\mu g\ mL^{-1}$). The calibration curve was used for quantification of all extracted samples.

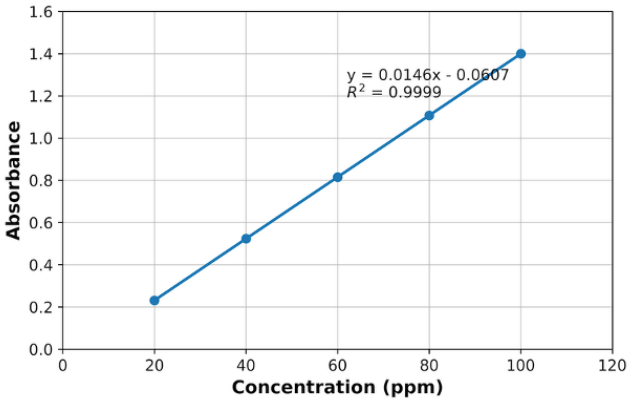

Figure 2. Calibration curve for heroin using 20–100 ppm standards.

Urine-matrix extraction performance (accuracy and precision)

Using the described MISPE conditions, heroin was quantified in spiked urine at 40–80 ppm. Across the tested MIP types (MIP1 and MIP2) and packing masses (0.20 g and 0.40 g), recoveries ranged from 97.0% to 102.75%, and repeatability expressed as RSD ranged from 1.24% to 3.83% (n = 3) (Table 4). These results demonstrate acceptable within-run precision and accuracy for spiked urine under the current experimental conditions.

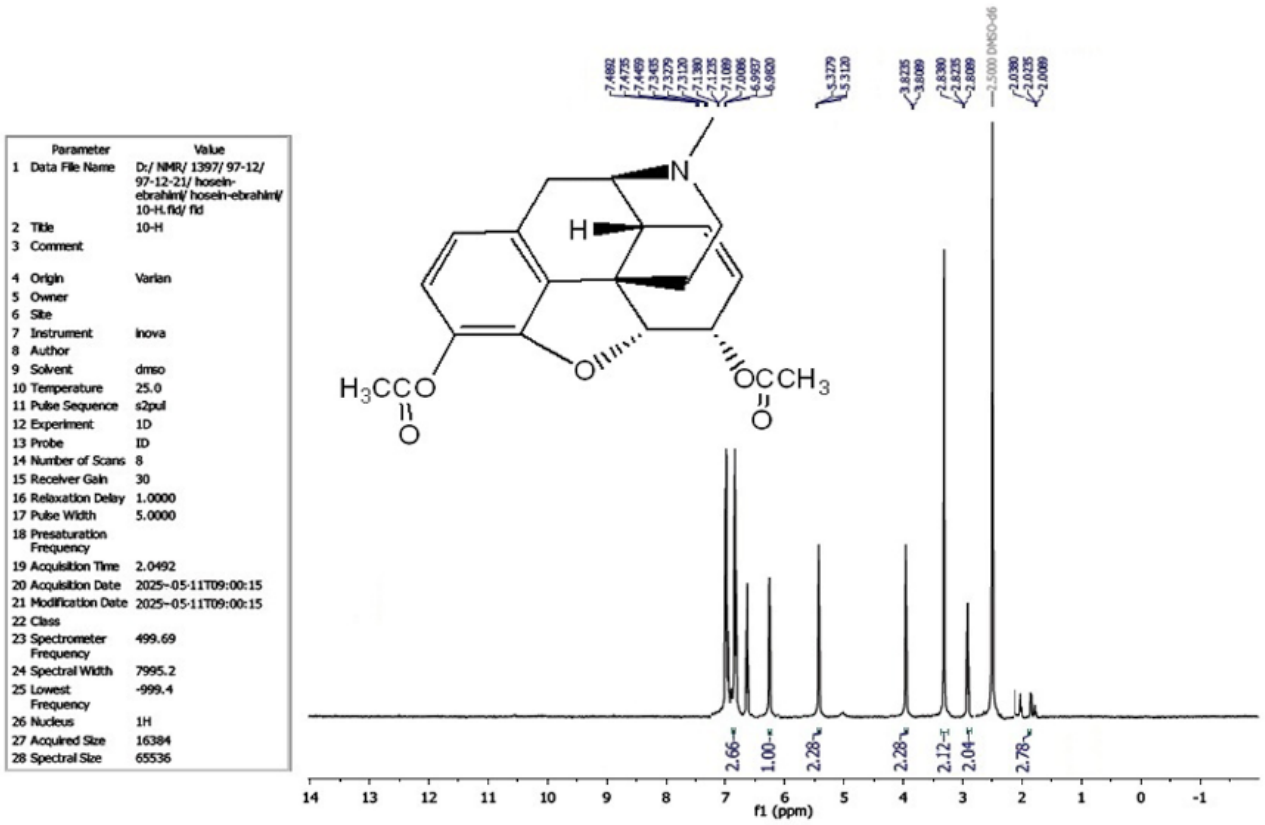

Figure 3. ^{1}H NMR spectrum of heroin extracted from MIP–SPE (DMSO-d_6, 499.69 MHz).

^{1}H NMR analysis (qualitative confirmation)

A ^{1}H NMR spectrum of the analyte recovered after MISPE (Figure 3) showed signals consistent with heroin (diacetylmorphine). This provides qualitative support for the identity of the extracted analyte following the MISPE procedure.

Table 1. Major FT-IR bands of heroin and TMSPMA-based MIP before and after template removal

No.	Assignment	Heroin (cm^{-1})	Heroin-MIP (TMSPMA), before leaching (cm^{-1})	Heroin-MIP (TMSPMA), after leaching (cm^{-1})
1	C=O (ester)	1735	1710	1710 (retained; polymer ester band)
2	C–H (aliphatic)	2954, 2862	2955, 2830	2940, 2820
3	Ar–H	3072	3060	Not observed
4	C=C (aromatic)	1596	1600	Not observed
5	C=C (aliphatic)	1612	1615	1620
6	C–O (ester)	1245	1255	1255 (retained; polymer ester band)
7	$C=CH_2$ (vinyl)*	—	1630	1615
8	Band in 1680–1665 cm^{-1} region*	—	1680	1665

* Bands primarily attributable to the polymer/monomer network rather than the heroin template.
Note: Ester bands (C=O ~1710 cm^{-1} and C–O ~1255 cm^{-1}) are expected to remain after leaching because the EGDMA/TMSPMA polymer backbone contains ester groups. Disappearance of aromatic/template-associated bands after leaching supports removal of the heroin template.

Table 2. Major FT-IR bands of heroin and HEAA-based MIP before and after template removal

No.	Assignment	Heroin (cm^{-1})	Heroin-MIP (HEAA), before leaching (cm^{-1})	Heroin-MIP (HEAA), after leaching (cm^{-1})
1	C=O (ester)	1735	1733	1733 (retained; polymer ester band)
2	C–H (aliphatic)	2954, 2862	2975, 2852	2966, 2844
3	Ar–H	3072	3045	Not observed
4	C=C (aromatic)	1596	1602	Not observed
5	C=C (aliphatic)	1612	1620	1615
6	C–O (ester)	1245	1238	1238 (retained; polymer ester C–O band)
7	$C=CH_2$ (vinyl)*	—	1610	1620
8	C=O (amide)*	—	1660	1675
9	N–H stretch*	—	3358	3455

* Bands primarily attributable to the polymer/monomer network rather than the heroin template.
Note: Ester bands (C=O ~1733 cm^{-1} and C–O ~1238 cm^{-1}) are attributable to the polymer network and are expected to remain after leaching. Disappearance of aromatic/template-associated bands after leaching supports removal of the heroin template.

Table 3. Polymerization recipes (template:monomer:crosslinker) used for heroin MIPs (prepared at 60 °C)

(A) TMSPMA-based MIPs

Formulation	Heroin (mmol)	TMSPMA (mmol)	EGDMA (mmol)	Solvent (10 mL)	Observed appearance
MIP1	1.00	4.00	12.00	EtOH	White
MIP2	1.00	5.00	10.00	EtOH	White/yellow
MIP3	1.00	3.00	14.00	EtOH	Pale yellow
MIP4	1.00	2.00	13.00	EtOH	White

(B) HEAA-based MIPs

Formulation	Heroin (mmol)	HEAA (mmol)	EGDMA (mmol)	Solvent (10 mL)	Observed appearance
MIP1	1.00	4.00	12.00	EtOH	White
MIP2	1.00	2.00	10.00	EtOH	Yellow
MIP3	1.00	3.00	12.00	EtOH	Yellow
MIP4	1.00	5.00	14.00	EtOH	White

Table 4. Accuracy and precision of heroin determination in spiked urine after MISPE/UV–Vis (230 nm).

MIP mass (g)	MIP type	Added (ppm)	Found (ppm)	LOD (ppm)	Recovery (%)	RSD (%)	RE (%)
0.20	MIP1 (TMSPMA)	40	38.8	0.010	97.000	2.943	−3.000
0.20	MIP1 (TMSPMA)	60	58.9	0.010	98.167	2.183	−1.833
0.20	MIP1 (TMSPMA)	80	78.3	0.010	97.875	3.281	−2.125
0.20	MIP2 (HEAA)	40	41.1	0.010	102.750	1.240	2.750
0.20	MIP2 (HEAA)	60	59.2	0.010	98.667	1.587	−1.333
0.20	MIP2 (HEAA)	80	81.5	0.010	101.875	1.860	1.875
0.40	MIP1 (TMSPMA)	40	39.5	0.010	98.750	3.826	−1.250
0.40	MIP1 (TMSPMA)	60	59.7	0.010	99.500	2.732	−0.500
0.40	MIP1 (TMSPMA)	80	81.3	0.010	101.625	2.632	1.625
0.40	MIP2 (HEAA)	40	40.9	0.010	102.250	2.528	2.250
0.40	MIP2 (HEAA)	60	58.9	0.010	98.167	3.814	−1.833
0.40	MIP2 (HEAA)	80	80.7	0.010	100.875	3.742	0.875

All measurements were performed in triplicate (n = 3) at each concentration level. Recovery (%) was calculated as $(C_{found}/C_{added}) \times 100$. Relative error (RE, %) was calculated as $[(C_{found} - C_{added})/C_{added}] \times 100$.
LOD (ppm) denotes the global limit of detection calculated once from the global calibration curve as LOD = $3.3\sigma/S$, where S is the slope of the calibration curve and σ is the standard deviation of replicate blank responses; therefore, the same LOD value applies to all MIP types and masses (global LOD = 0.010 ppm).

DISCUSSION

Heroin (diacetylmorphine) remains an important target in clinical and forensic toxicology because of its public health impact and frequent involvement in medico-legal investigations [14]. Urine is widely used for screening and retrospective assessment; however, analytical determination is challenging because urine is chemically complex and variable among individuals, and because heroin is intrinsically unstable, undergoing rapid hydrolysis to 6-monoacetylmorphine (6-MAM) and morphine. These processes occur in vivo and may continue during sample handling, transport, and storage, altering the detectable profile and complicating interpretation if pre-analytical conditions are not controlled or documented [15–17]. Therefore, reliable urine testing requires selective sample preparation and transparent validation of accuracy, precision, and stability-related uncertainty in the urine matrix [17].

Within this context, the present study demonstrates that heroin-imprinted polymers synthesized using either TMSPMA or HEAA as functional monomers can be applied as selective sorbents in a simple syringe-column MISPE workflow coupled with UV–Vis spectrophotometry at 230 nm. FT-IR comparisons obtained before and after template removal support effective leaching of the template with preservation of characteristic polymer-network bands, which is consistent with creation of imprint-derived binding sites. These observations agree with established MISPE concepts, where removal of the template generates cavities capable of preferential rebinding of the target molecule and can improve clean-up and enrichment compared with non-selective extraction approaches [18]. The feasibility of packing the particles into a syringe format also supports practical utility as a low-cost and rapid clean-up step in laboratories with limited access to advanced instrumentation.

Analytically, the method showed excellent linearity over the studied range (20–100 ppm) with the regression equation $A = 0.0146C - 0.0607$ and $R^2 = 0.9999$ (Fig. 2). It should be emphasized that this calibration represents a global UV–Vis calibration (standards prepared and measured in ethanol), which was then used to quantify urine extracts after MISPE clean-up and reconstitution in ethanol. In urine-matrix (spiked) experiments, recoveries of 97.0–102.75% with repeatability up to 3.83% RSD (n = 3) indicate acceptable within-run performance under controlled spiking conditions. These results suggest that the selected MISPE conditions can reduce matrix interference sufficiently to enable UV–Vis quantification over the examined concentration range. The generally low RSD values further indicate that extraction and measurement were reproducible at the within-run level. Collectively, these findings are consistent with reports describing MIP-based sorbents as effective tools for sample clean-up and preconcentration in complex matrices [19, 20]. In the present study, a single global LOD of 0.010 ppm was reported based on the global calibration curve, which provides a transparent sensitivity estimate applicable to all tested MIP types and masses. Despite these strengths, interpretation of heroin in urine must be approached cautiously because diacetylmorphine may hydrolyze during collection and storage, narrowing the time window for detecting the parent drug and shifting the

measured profile toward metabolites [17]. In forensic practice, 6-MAM is widely considered a specific marker of heroin exposure, whereas morphine is less specific because it may also arise from codeine metabolism or therapeutic morphine administration. Accordingly, studies reporting urine determination of heroin should document specimen handling conditions that influence hydrolysis (e.g., time-to-analysis, storage temperature, and pH control, and the use of preservatives where applicable) and should clarify whether the workflow is intended for screening/estimation or confirmatory interpretation of heroin exposure [21, 22]. In the present work, performance was assessed using spiked urine under controlled conditions, and broader stability-controlled evaluation would further strengthen interpretive confidence.

A key analytical limitation is that UV–Vis spectrophotometry lacks structural specificity compared with chromatographic confirmation, and urine contains endogenous chromophores and variable salts/proteins that can contribute to non-specific absorbance. Consequently, the reliability of an SPE–UV approach depends strongly on demonstrating that MISPE sufficiently suppresses co-extracted interferences. In the present study, MIP performance was evaluated using urine-matrix recovery and precision; however, selectivity was not quantified using a non-imprinted polymer (NIP) control (imprinting factor). Publication-strength validation commonly includes direct comparison with an NIP, calculation of selectivity or imprinting factors (MIP/NIP), and interference testing with structurally related opioids and metabolites (morphine, codeine, and ideally 6-MAM) to support the claim that the measured UV–Vis signal is attributable to the target analyte rather than co-extracted species [23]. Including these elements would strengthen the method's defensibility, particularly for forensic interpretation.

Finally, to prepare the method for routine or forensic implementation, expanded validation in accordance with toxicology standards is recommended. This includes assessing matrix effects across multiple urine sources, evaluation of inter-day precision (and between-analyst precision where feasible), and stability studies relevant to heroin hydrolysis during realistic handling and storage [17]. Addressing these points would strengthen confidence in applying MISPE–UV workflows for complex biological matrices and clarify the appropriate intended use.

CONCLUSION

MISPE using heroin-imprinted polymers coupled with UV–Vis at 230 nm provides a practical approach for determining heroin in spiked urine with acceptable within-run performance (recoveries 97.0–102.75%, RSD up to 3.83%, and a global LOD of 0.010 ppm). Because heroin is unstable in urine and UV–Vis lacks structural specificity, expanded validation—particularly selectivity testing with an NIP control and interference assessment, as well as stability and inter-day precision studies—is recommended before forensic-defensible routine application.

ETHICAL DECLARATIONS

Ethics Approval and Consent to Participate

Handling and use of the controlled reference material were conducted under administrative authorization from the College of Science, University of Baghdad (Letter No. 1300, dated 08/08/2023), with reference to the Iraqi Ministry of Health/Department of Forensic Medicine letter No. 18716, dated 17/07/2023. De-identified human urine specimens were used for spiking experiments. The study protocol was reviewed and approved by the Research Ethics Committee, College of Science, University of Baghdad.

Consent for Publication

None.

Availability of Data and Material

The datasets are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest.

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Use of Generative Artificial Intelligence

The authors declare that ChatGPT, a generative AI tool developed by OpenAI, was used solely to enhance clarity and grammatical accuracy during the final editing phase. It was not used for content generation, data analysis, or interpretation.

Authors' Contributions

All authors contributed equally to the design and conception of the study. All authors reviewed the manuscript and approved the final manuscript.

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