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Method Development and Validation for Morphine in Chicken Plasma by RP-HPLC for Quantitative Analysis.

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ABSTRACT

The study focuses on the development and validation using reverse phase HPLC method for the quantitative estimation of morphine in chicken plasma, that is applicable to Forensic analysis in an accurate, precise and simple way. C18 column (250×4.6 mm, particle size 5 µm) with an isocratic method using mobile phase Methanol and 0.1% orthophosphoric acid in Milli-Q water (60:40 v/v) was used for the chromatographic separation. Morphine was extracted utilizing a liquid-liquid extraction method. The flow rate inside the column was kept at 1.0 ml/min and detected using a photodiode array detector at an appropriate wavelength of 240nm. The method was validated in accordance with ICH Q2(R2) guidelines, evaluating parameters including accuracy, precision, specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ). The method showed good linearity over concentration range of 1.66-8.33 µg/ml, with a correlation coefficient (r^2) of 0.9991. Precision studies indicated low %CV values, confirming reproducibility. Recovery studies demonstrate acceptable extraction efficiency. The developed method is simple, rapid, cost-effective, and suitable for routine quantitative analysis of morphine in forensic.

Keywords: Morphine, HPLC-UV; Forensic toxicology; Method Validation; Chicken Plasma; Forensic samples; Opioids analysis.

1. Introduction

Morphine is a powerful pain reliever that comes from the opium poppy plant. It's still one of the best ways to manage moderate to severe pain. Due to its importance for medical use and potential for misuse, it's crucial to have strong quality control and accurate measurement in forensic and pharmaceutals.so, the method used to analyse morphine need to be highly sensitive, precise and consistent.

There are various analytical techniques for quantification of Morphine including Capillary electrophoresis (CE), Gas Chromatography (GC), Gas Chromatography-Mass Spectroscopy (GC-MS), Isotopes Ratio Mass Spectrometry (IRMS), Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and reversed-phase high-performance liquid chromatography (RP-HPLC)[1,2].

Although GC-MS has been widely used in forensic toxicology, Morphine is unsuitable for direct GC analysis because of its highly polar, thermally labile, and non-volatile nature, requiring derivatization procedure that increase analytical complexity [3-5]. LC-MS/MS offers excellent sensitivity and selectivity for trace-level detection but involves expensive instrumentation, sophisticated maintenance, and highly trained personnel, limiting its accessibility in many laboratories [6]. IRMS is mainly restricted to specialized anti-doping and provenance studies due to its complex instrumentation and inability to provide routine quantitative analysis [7,8].

RP-HPLC is fully compatible with the physicochemical properties of morphine, eliminates derivatization requirements and allows reliable detection at 240 nm with simple sample preparation. Furthermore, HPLC systems are widely available, cost- effective and suitable for routine forensic and pharmaceutical analysis making RP-HPLC most suitable method for morphine quantification in biological matrices. The goal is to use this method for regular quality control checks. To make sure it works well, it tests for several important factors, including how well it measures the right amount, how accurate it is, how precise it is, and how specific it is to Morphine [9,6]

Despite the availability of several RP-HPLC methods for morphine quantification in human biological matrices, validated methods targeting animal plasma matrices for forensic purposes remain scarce. Chicken plasma represents a relevant and underexplored forensic matrix in the context of animal poisoning investigations and veterinary toxicology case work. To our knowledge, the present study describes the first fully validated RP-HPLC method for the quantitative determination of morphine specifically in chicken plasma, developed and validated in accordance with the latest ICH Q2(R2) (2023) guidelines. The method offers a simple, cost-effective, and derivatization-free approach suitable for routine forensic toxicology laboratories operating without access to high-end instrumentation such as LC-MS/MS.

2.MATERIALS AND METHODS

2.1 Reagents and Materials

Standard morphine, HPLC-grade methanol, Milli-Q water, orthophosphoric acid (OPA), sodium hydroxide, chloroform, isopropanol, volumetric flasks, micro-centrifuge (Eppendorf) tubes, and chicken blood (collected from a local abattoir) were used in this study. Chicken plasma was obtained by centrifugation of whole blood at 3000 rpm for 10 min and stored at −20 °C until use.

2.2 Chromatographic Conditions

Chromatographic separation was achieved using a C18 column (250 × 4.6 mm, particle size 5 µm) with an isocratic mobile phase consisting of methanol and 0.1% orthophosphoric acid in Milli-Q water (pH 2.5) in a ratio of 60:40% (v/v). The flow rate was maintained at 1.0 ml/min, with an injection volume of 10 µl and a run time of 7 min. Detection was performed using a photodiode array (PDA) detector at 240 nm. The optimized chromatographic conditions are summarized in Table 1.

2.3 Preparation of Mobile Phase

The mobile phase was prepared by mixing methanol and 0.1% orthophosphoric acid in Milli-Q water in the ratio of 60:40% (v/v). The mixture was filtered through a 0.45 µm membrane filter and degassed by sonication prior to use.

2.4 Preparation of Standard Stock Solution

A stock solution of morphine (1000 µg/ml) was prepared by dissolving 10 mg of morphine standard in 10 ml of mobile phase. The solution was sonicated and diluted with mobile phase as required.

2.5 Preparation of Working Solutions

Working standard solutions of morphine were prepared from the stock solution at concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 µg/ml in mobile phase by appropriate serial dilution.

2.6 Preparation of Calibration Standards in Plasma

Calibration standards were prepared by spiking 100 µl of blank chicken plasma with appropriate volumes of the working standard solution to achieve final concentrations of 1.66, 3.33, 5.00, 6.66, and 8.33 µg/ml. All spiked samples were processed using the liquid–liquid extraction procedure described below.

2.7 Liquid–Liquid Extraction (LLE) Procedure

One hundred microlitres (100 µl) of chicken plasma was transferred into an Eppendorf microtube containing the appropriate volume of morphine working standard. Thirty microlitres (30 µl) of 1 M sodium hydroxide was added and vortexed. Subsequently, 600 µl of a chloroform: isopropanol mixture (9:1, v/v) was added, vortexed for 3 min, and centrifuged at 15,000 rpm for 15 min at 4 °C. The organic (lower) layer was carefully separated and evaporated to dryness at room temperature or under a gentle stream of nitrogen at 40 °C. The dried residue was reconstituted in 600 µl of mobile phase and transferred to an HPLC vial for injection.

3. METHOD VALIDATION:

The validation procedure was confirmed to meet the specifications set by the ICH -Q2(R2) guidelines

3.1 System suitability:

This indicator's function is to determine the instrument's operating status and grant permission to move forward with the analysis of the next set of samples. In chromatographic techniques, system appropriateness tests are frequently used to confirm that the system has sufficient sensitivity, specificity, and reproducibility for the current analytical run and its use in a particular study. Samples representative of the batch was used at the start and end of each batch to determine the system's suitability. For morphine the %CV values for peak area and retention duration were calculated over

a period of six consecutive injections. Finding out if these %CV values fell within the range was the goal.

3.2 Specificity:

Specificity was evaluated by analyzing blank chicken plasma and morphine-spiked plasma at five different concentration levels. Chromatograms were examined for the presence of interfering peaks at the retention time of morphine

3.3 Calibration curve:

Five data points were used to create a calibration curve by precisely adding volumes of a working solution to a blank plasma sample. Morphine final concentrations of 1.66, 3.33, 5, 6.66 and 8.33 µg/ml were obtained using the previously indicated method. Plotting peak areas of morphine concentration produced the calibration curve. The data was interpreted using linear regression analysis. If the back-calculated concentrations of the standards were within a deviation range of ±15% (SD) from the nominal value, they were deemed acceptable.

3.4 Precision and accuracy:

Three duplicate samples with different concentrations—the Low-quality control (LQC: 1.66 µg/ml), Medium quality control (MQC: 5 µg/ml), and High-quality control (HQC: 8.33 µg/ml) samples—were examined as part of the intraday precision evaluation. The validation of the method's daily variation, with an emphasis on inter-day precision, was part of the evaluation of its reproducibility. Three sets of samples with different quantities—the LQC, MQC, and HQC samples—were examined as part of the analysis. Three separate times, the analysis was carried out. By calculating the coefficient of variation (%CV), a metric derived by dividing the SD by the mean and displaying the outcome as a percentage, the accuracy of intra- and inter-day assays was evaluated.

Six replicates were examined at three sample levels—LQC, MQC and HQC—in order to evaluate both intra-assay and inter-assay precision. While inter-assay accuracy was evaluated on three different days, intra-assay accuracy was evaluated on the same day. By calculating the percentage ratio between the determined concentration and the known concentration, the measurement accuracy was evaluated.

3.5 Recovery:

Analyte recovery was determined by comparing peak area of extracted QC samples (LQC, MQC and HQC) with peak areas of unextracted reference standards prepared at same concentration. Expressed as a percentage.

3.6 Stability

The assessment of the stability research was carried out as an essential element of the process of validating the approach. A stability test was done to assess the possible disintegration of morphine under different conditions.

i. Freeze-thaw stability:

A temperature of −20 °C was maintained for the storage of six replicates each for the LQC, MQC, and HQC samples. The samples were subsequently thawed completely at ambient temperature and promptly refrozen back to a temperature of −20 °C. The fore mentioned procedure was repeated twice, and thereafter, the samples were directly injected into RP-HPLC.

ii. Bench-top stability:

The primary aim of the bench-top stability experiment was to evaluate the stability of morphine in chicken plasma during 8 hours of exposure on the laboratory bench. The assessment was carried out using three distinct concentrations, specifically the LQC, MQC, and HQC Each concentration was reproduced six times.

iii. Short-term stability:

Samples were stored at room temperature for up to 24 hours before analysis.

iv. Long-term stability

The examination of stability within a certain setting. Long-term stability of morphine in chicken plasma was assessed by storing quality control samples (LQC, MQC, and HQC; n = 6 each) at −20°C for 30 days. This study entails the execution of studies aimed at evaluating the many physical, chemical, biological, bio pharmaceutical, and microbiological characteristics of a pharmaceutical compound. The investigations are conducted both during and after the projected shelf-life and storage duration of the samples. The storage conditions employed in these studies are indicative of the conditions anticipated in the target market.

4. RESULTS

4.1 Optimized method

The effects of various ACN, methanol, formic acid, and ortho-phosphoric acid compositions in the mobile phase on the resolution of morphine were investigated in a number of investigations. When using a mobile phase consisting of a (60:40%) mixture of methanol and 0.1% water, the best peak shape was observed. Morphine's peak reaction remained unchanged despite slight variations in its retention time due to this particular makeup. The chromatograms and ideal circumstances for blank

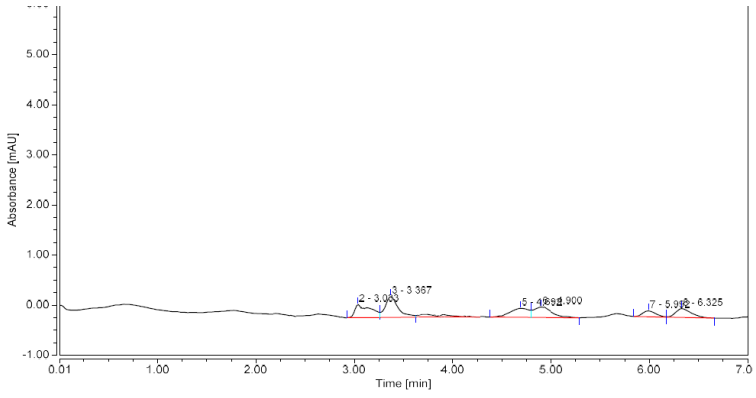


Figure 1. Blank (chicken plasma) chromatogram

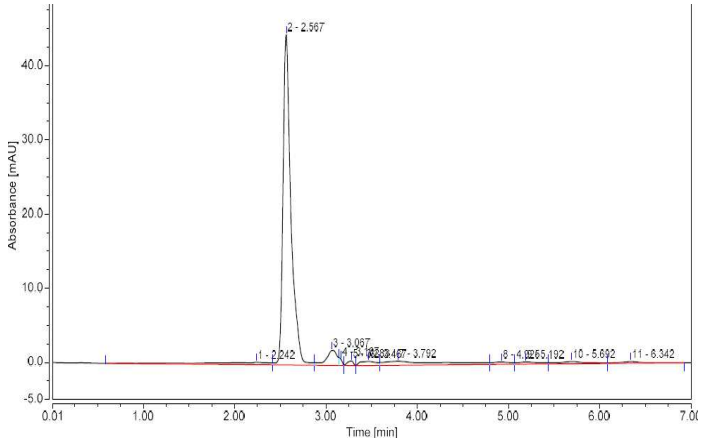


Figure 2. Chromatogram of morphine in chicken Plasma

Table 1. Summary of Optimized chromatographic conditions.

Parameters	Description
Equipment	HPLC - PDA
Column	C18 (250 × 4.6 mm particle size 5µm)
Mobile phase	Methanol: 0.1% ortho-phosphoric acid in water (pH: 2.5) in ratio of 60:40 v/v
Flow rate	1.00 ml/min
Injection volume	10 µl
Run time	7 min
Detection wavelength	240 nm
Retention time	2.56 min

4.2 Specificity

Five different concentration chicken plasma samples were used in the specificity evaluation. The chromatograms from chicken plasma samples are shown in Figures 1 and 2. No co-eluting peaks from naturally occurring compounds were observed. With a retention period of 2.56 minutes, the analyte morphine was eluted. However, the sample's execution time was optimized to seven minutes.

4.3 Linearity:

With an average correlation value (r2) of 0.9991, the standard curves showed linearity in the concentration range of 1.66–8.33 µg/ml for morphine. Quantification of the sample involves determining average peak area of the morphine concentration. The plotted data included average peak areas of plasma concentrations. The linearity data of morphine is given in Table 2, and the linearity curve of morphine is displayed in Figure 3.

Table 2. Linearity data of Morphine in Chicken Plasma

Sr. No.	Concentration (µg/ml)	Average Area ± SD	%RSD
1.	1.66	0.1323 ± 0.0017	1.27
2.	3.33	0.2373 ± 0.0036	1.54
3.	5.00	0.3528 ± 0.0047	1.35
4.	6.66	0.4647 ± 0.0083	1.78
5.	8.33	0.5606 ± 0.0064	1.14

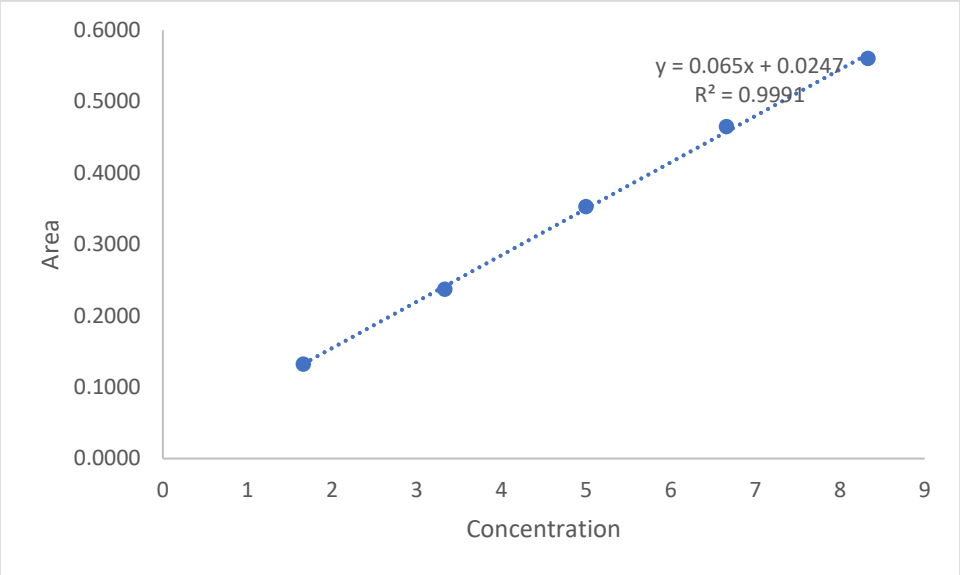


Figure 3. Linearity curve of morphine in chicken plasma

4.4 LOD and LOQ:

The determination of LOD and LOQ was conducted by independent investigations utilizing the calibration curve approach. The bioanalytical RP-HPLC method established the determination of the LOD and LOQ for Morphine in Table 3.

Table 3. LOD and LOQ data for Morphine

Name	LOD	LOQ
	Concentration (µg/ml)	**Concentration (µg/ml)**
Morphine	0.13	0.40

4.5 Precision and Accuracy

Three duplicates, each containing Morphine at 3 QC levels, were analyzed in order to assess precision and accuracy within a single experiment. Generally speaking, accuracy is defined as the state in which data falls between 85% - 115% of the true values. On the other hand, precision was evaluated by

calculating the %RSD, which has an acceptable range of ±15% except for the minimal measurable level. The accuracy level in LQC is expected to be between 80% and 120%, and the %RSD must be less than 2%. Precision and accuracy data were supplied.

Table 4. Accuracy and precision data of the Morphine in Chicken Plasma

	Intra-day precision (n=3)			Inter-day precision (n=6)		
QC SAMPLE	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Mean	0.1320	0.3574	0.5823	0.1338	0.3270	0.5826
SD	0.0013	0.0046	0.0060	0.0020	0.0039	0.0063
%CV	1.02	1.28	1.04	1.48	1.19	1.08
%mean accuracy	99.39	102.40	103.00	100.00	94.00	103.10

4.6 Recovery of morphine

Recovery of Morphine in LQC, MQC and HQC is 90%, 97.19% and 89.95% respectively. Recovery data is shown in Table 5.

Table 5. Recovery data of Morphine

	LQC		**MQC**		**HQC**	
Replicate number	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
1.	0.1309	0.1512	0.3560	0.3594	0.5681	0.6265
2.	0.1317	0.1455	0.3551	0.3651	0.5645	0.6320
3.	0.1334	0.1481	0.3463	0.3558	0.5539	0.6173
4.	0.1349	0.1461	0.3472	0.3689	0.5596	0.6351
5.	0.1327	0.1437	0.3569	0.3652	0.5649	0.6095
6.	0.1304	0.1411	0.3554	0.3634	0.5523	0.6188
Mean	0.1323	0.1470	0.3528	0.3630	0.5606	0.6232
SD	0.0017	0.0023	0.0047	0.0043	0.0064	0.0089
%RSD	1.27	1.59	1.35	1.17	1.14	1.42
%Mean recovery	90.00		97.19		89.95	
Overall %mean recovery	92.38					

4.7 Stability

i. Bench top stability

Percentage mean accuracy value of morphine in LQC, MQC and HQC were 101.21, 100.45 and 102.23%. Benchtop stability finding is presented in Table 6

Table 6. Bench top stability data of Morphine

Concentration (µg/ml)	Replicate number n=6						Mean ± SD	%RSD	%Mean accuracy
	1.	2.	3.	4.	5.	6.			
LQC	0.1345	0.1315	0.1358	0.1301	0.1371	0.1343	0.1339 ± 0.0026	1.96	101.21
MQC	0.3491	0.3506	0.3624	0.3581	0.3585	0.3474	0.3544 ± 0.0061	1.72	100.45
HQC	0.5655	0.5742	0.5845	0.5721	0.5648	0.5775	0.5731 ± 0.0075	1.30	102.23

ii. Freeze-thaw at – 20 °C

The values of % mean accuracy of morphine found is 100.53, 100.48% and 101.59% respectively. Freeze thaw stability findings were presented in Table 7

Table 7. Freeze thaw stability data of Morphine

Concentration (µg/ml)	Replicate number n=6						Mean ± SD	%RSD	%Mean accuracy
	1.	2.	3.	4.	5.	6.			
LQC	0.1352	0.1365	0.1307	0.1298	0.1322	0.1334	0.1330 ± 0.0026	1.94	100.53
MQC	0.3467	0.3546	0.3648	0.3581	0.3538	0.3491	0.3545 ± 0.0065	1.83	100.48
HQC	0.5548	0.5623	0.5794	0.5699	0.5782	0.5721	0.5695 ± 0.0095	1.66	101.59

iii. Short term stability

The values of percentage of mean accuracy of morphine found is 100.30%, 100.17% and 102.30% respectively. Short term stability results were shown in table 8.

Table 8. Short term stability data of Morphine

Concentration (µg/ml)	Replicate number n=6						Mean ± SD	%RSD	%Mean accuracy
	1.	2.	3.	4.	5.	6.			
LQC	0.1362	0.1342	0.1329	0.1332	0.1302	0.1292	0.1327 ± 0.0026	1.94	100.30
MQC	0.3487	0.3442	0.3563	0.3572	0.3634	0.3503	0.3534 ± 0.0069	1.96	100.17
HQC	0.5775	0.5731	0.5831	0.5742	0.5656	0.5675	0.5735 ± 0.0064	1.12	102.30

iv. Long term stability

The values of percentage of mean accuracy of morphine found is 100.08%, 99.80% and 101.18% respectively. Long term stability results were shown in table 9.

Table 9 long term stability data of Morphine

Concentration (µg/ml)	Replicate number n=6						Mean ± SD	%RSD	%mean accuracy

	1.	2.	3.	4.	5.	6.			
LQC	0.1352	0.1283	0.1328	0.1318	0.1351	0.1313	0.1324 ± 0.0026	1.96	100.08
MQC	0.3610	0.3532	0.3483	0.3536	0.3513	0.3454	0.3521 ± 0.0053	1.52	99.80
HQC	0.5589	0.5774	0.5663	0.5703	0.5687	0.5613	0.5672± 0.0066	1.17	101.18

5. DISCUSSION

The developed RP-HPLC method was optimized for Morphine in chicken plasma. Analytes were separated using Methanol and water containing 0.1% OPA pH 2.5 (60:40% v/v) at 1 ml/minute flow rate on C18 Column (250 mm × 4.6 mm: particle size 5 µ). The method was also validated following United states food and drug administration (ICH Q2(R2)) guidelines. The retention time of Morphine is 2.56. In the Chicken plasma matrix, liquid–liquid extraction yielded clean samples with a recovery from ranges 85 - 115%. The method was linear over the ranges of 1.66–8.33 µg/ml, with r^2 of 0.9991. Therefore, the proposed method could be considered valid for routine drug analysis in biological matrices.

6. Conclusion

The suggested RP-HPLC method for the determination of morphine in chicken plasma is rapid, sensitive, and repeatable, with linear dynamic ranges of 1.66 – 8.33 µg/ml, respectively. It has been validated and adheres to the ICH Q2(R2) standards, demonstrating a high level of accuracy and precision following the guidelines.

Declarations

Funding

No funding was received for conducting this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

Akshay Kumar Gour: Conceptualization, Methodology, Investigation, Formal analysis, Writing original draft. Prajesh Prajapati: Supervision, Writing- review and editing. All authors read and approved the final manuscript.

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