Optimization and Validation of High Performance Liquid Chromatography (HPLC) Method for Compendial Determination of Morphine in Seized Narcotic Evidences

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Abstract

Morphine is one of the most potent opioid analgesics, the World Health Organization (WHO) advises using it but long-term use can result in addiction and physical & psychological dependence. A high performance liquid chromatography (HPLC) method has been developed and validated as per International Conference on Harmonization ICH Q2 (R1) guidelines to determine morphine in distinct seized narcotic evidences. Very few studies are there on seized drug samples, mostly are on biological and pharmaceutical samples because of the complex nature of seized drug samples but its analysis is important to fight against drug trafficking and drug abuse. In this HPLC method, C18 column and photo diode array (PDA) detector at λ = 280 nm is used. Good level of stability and reproducibility is observed in the results with the particular composition of mobile phase used here. Linearity is reported in the concentration range of 0.5–15 µg/mL, and a remarkable correlation coefficient (R^2 = 0.9961) is determined which has not been reported earlier. The proposed method does not involve the use of expensive chemicals and shows better acquisition rate. Moreover, simple mobile phase preparation makes the entire procedure incredibly time-efficient making it the method of choice for routine morphine analysis.

Keywords

Morphine; opioid; addiction; seized; narcotic; evidences; high performance liquid chromatographic (HPLC).

1. Introduction

An opioid analgesic called morphine is used to relieve moderate to severe pain (fig. 1) (Khanna & Shukla, 1986; Osborne et al., 1990). The World Health Organization recommends it for the management of mild cancer-related pain. It is the preferred opioid for use in palliative and terminal care (Bosch et al., 2007). The commercial manufacture of significant alkaloids, including morphine, codeine, thebaine, narcotine and papaverine is derived from dried latex from the capsules of Papaver somniferum (Budvári-Bárány et al., 1997; Khanna & Shukla, 1986). Morphine is widely used to treat both short term and chronic cases of severe pain since it is a potent opioid analgesic. The morphinan-framed alkaloids, which include morphine, are found in the poppy plant. The drug has limited solubility in lipids but is soluble in water. Morphine-3-glucuronide (M3G) and morphine-6-glumronide (M6G) are the two primary metabolites of morphine in humans (Christrup, 1997).

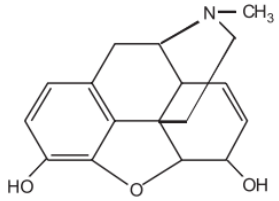


Fig. 1. Chemical Structure of Morphine

Thin-layer chromatography (TLC) which is one of the most used chromatographic techniques, is easy and cheap, and because of the possibility to manage extensive routines, it is commonly implemented for urine screening. Yet, despite the fact that derivatization techniques can enhance its effectiveness, it lacks some level of sensitivity and specificity (Marigo et al., 1986). And although, gas chromatography (GC) techniques have far greater inherent specificity and dependability, GC methods require rigorous and time-consuming sample preparation, including derivatization to diminish the high polarity of morphine and to enable electron-capture detection (Marigo et al., 1986).

Due to its inherent attributes of specificity, reliability, sensitivity, and, to some degree, reduced requirements for sample preparation, high-performance liquid chromatography (HPLC) is being implemented in a vastly increased number of laboratories (Budvári-Bárány et al., 1997; Krenn et al., 1998; Marigo et al., 1986). The preferred technique for analysing morphine and its metabolites simultaneously is HPLC (Bosch-Barrera et al., 2020; Chan, 2017). Based on each chemical component's distinct affinity for the adsorbent material found in the column or the mobile phase, which causes various components to move at different rates and separate, each chemical component is separated from the sample mixture using HPLC (Khanna & Shukla, 1986). It was once known as high-pressure liquid chromatography because it relies on high pressure pumps to facilitate quick separation (Sahu et al., 2018).

In the forensic science laboratory, innumerable narcotic drugs & psychotropic substances are received as evidences and the frequency of cases in which morphine is encountered is more. Usually, the laboratories adopt titration based conventional technologies, but we need to develop a robust technology for the routine analysis of morphine. Therefore, in the current study, an HPLC method has been developed and utilized to detect morphine in seized narcotic evidences.

There are very few research articles on analysis of drugs in seized samples, most of the work has been carried out on biological and pharmaceutical samples (Cappelle et al., 2015; Jafari-Nodoushan et al., 2016; Mohseni et al., 2017; Rajaei et al., 2019). As seized drug samples are often limited in quantity, making it difficult to conduct multiple tests or replicate experiments. These are not pure substances but rather mixtures of multiple drugs or adulterants and analyzing such complex mixtures requires specialized techniques and expertise. Seized samples can be contaminated with other substances or impurities, which can interfere with the analysis and lead to inaccurate results. Moreover, some countries have legal restrictions on the possession and handling of controlled substances, which can make it furthermore difficult to conduct research and analysis on seized drug samples (Cole et al., 2011; Reuter, 1986).

But, analysis of seized drug samples is of major importance as the information obtained from these can also be used to track the source of the drugs and the organizations involved in their production and distribution. This, in turn, can aid in the investigation and prosecution of drug traffickers and can help law enforcement agencies to disrupt drug trafficking networks. It

helps in identifying new and emerging drugs, as well as variations in the potency and purity of existing drugs. This information can be used by public health officials to develop and implement effective drug prevention and treatment programs (Cole et al., 2011; Reuter, 1986).

2. Material and Methods

2.1. Reagents and chemicals

Morphine, codeine, thebaine and papaverine standard were procured from Government Opium and Alkaloid Factory (GOAF). Narcotic evidences were seized from the street. Acetonitrile, chloroform, triethylamine, sodium chloride, ammonia, isopropanol, anhydrous sodium sulfate and ultra pure water were obtained from Merck Chemicals (Darmstadt, Hesse, Germany). All the chemicals used were of HPLC analytical grade.

2.2. Liquid chromatography conditions

A Waters Aquity Arc High performance liquid chromatography-photodiode array detector (HPLC-PDA) was used for the proposed method's development and validation. The output signal was monitored and processed using Empower 3 software (Waters, USA).

2.3. Operating Conditions

The chromatographic column used in the present study was a C18, 5 micron particle size, 4.6×250mm (Waters, USA) and PDA detector at 280 nm was used. For mobile phase preparation, water was mixed with 0.1% triethylamine , and acetonitrile in the ratio of 45:55 (v/v). Analysis was carried out at flow rate of 2mL/min with the injection volume of 5-20uL.

2.4. Preparation of sample

Narcotic evidences were seized from the street. Firstly, 100mg of it was dispersed with 10ml of saturated aqueous sodium chloride at pH 10 (adjusted with dilute ammonia). Then, the mixture was extracted using three 20ml portions of chloroform/ isopropanol (3:1). And then organic layer was filtered through anhydrous sodium sulfate, or phase separation paper, and further, the organic solvent was evaporated to dryness. Finally, the residue was added in 10.0mL of mobile phase and used for examination.

2.5. Preparation of standard / stock solution

Accurately weighed 10mg of morphine standard and dissolved in 10.0mL of mobile phase to make 1000 PPM solution and then, serially diluted to make 15, 10, 5, 0.75, & 0.5 ug/ml (or PPM).

3. Results and Discussion

3.1. Validation of Morphine HPLC assay

3.1.1. System optimization

In order to achieve separation, numerous combinations of the C18 column with various solvent systems, including water with 0.1% TEA and acetonitrile, were investigated. The goal was to separate morphine in a chromatographically sound manner. When the morphine was

permitted to travel with acetonitrile and water containing 0.1% TEA, an improved retention profile was achieved. To maintain stable distribution of the analyte in the selected solvent during sample dissolution, the principle "the like dissolves the like" must be fulfilled. Because of its polar nature, morphine is soluble in all polar solvents, including water, methanol, and ethanol. Water is the most optimal solvent to be used to dissolve the target compound because it is the least expensive and has the strongest polarity of all these solvents (United Nations, 1998).

3.1.2. Selectivity & specificity

The method's selectivity was first evaluated against morphine while also being exposed to three other significant substances, codiene, thebaine, and papaverine. The test showed a distinct sequence of elution for all known substances, with morphine eluting a fair bit earlier than the other opiates (Fig. 2). The ability of an analytical method to quantify the analyte in the presence of other substances is known as selectivity. Sometimes, the words "specificity" and "selectivity" are used interchangeably. In general, the term "specific" denotes a method that yields a result for a single analyte only, whereas the term "selective" denotes a method that yields results for several chemical entities that may or may not be differentiated from one another (Chan, 2017). "Specificity is the ultimate of Selectivity," according to the International Union of Pure and Applied Chemistry (IUPAC). The IUPAC prefers to use the term selectivity and discourages the usage of specificity (Paithankar, 2013).

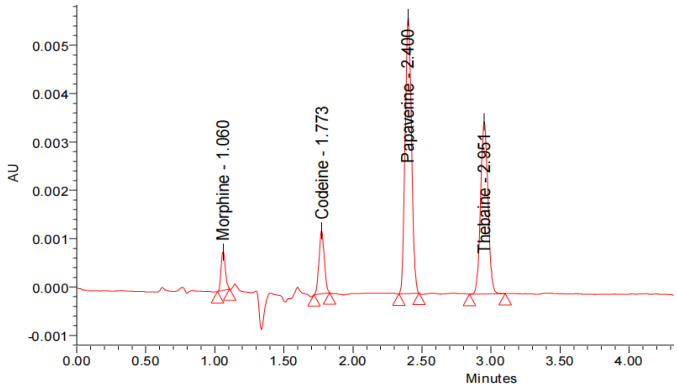
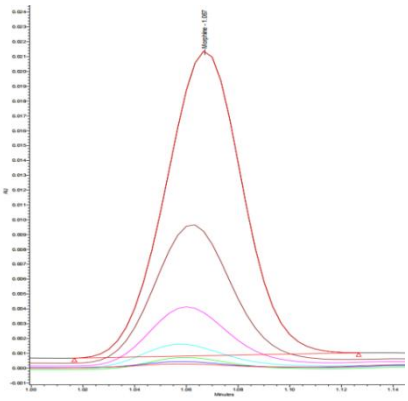


Fig. 2. HPLC Chromatogram of morphine in presence of other related interferents (Codeine, Thebaine & Papaverine) to access selectivity

3.1.3. Linearity

The potential of a method to produce test findings that are directly proportional to the sample concentration over a specified range is known as linearity. By diluting the standard stock (fig. 3) or separately weighing the sample components, the relationship can be apparent on the drug substance using the suggested methods. Visual evaluation of a plot of signals as a function of concentration or content of the analyte should be used to assess linearity (fig. 4). If a linear relationship exists, the test findings should be examined using the suitable statistical techniques, such as regression analysis. Regression line data can be used to calculate estimations of the degree of linearity (Table 1). It is typically stated as a variance around the regression line's slope. By using y = 2E+06x - 834.29 from the linear plot, R square value came out to be 0.9961. In some circumstances, the proper function of the analyte concentration should be used to depict the analytical responses. (Alquadeib, 2019; Chan, 2017; United Nations, 1998).



Regression Statistics	
Multiple R	0.998060156
R Square	0.996124075
Adjusted R Square	0.99534889
Standard Error	1057.021415
Observations	7

Fig. 3. Overlay of different serially diluted concentrations of Morphine ranging from 0.5 to 15 µg/ml.

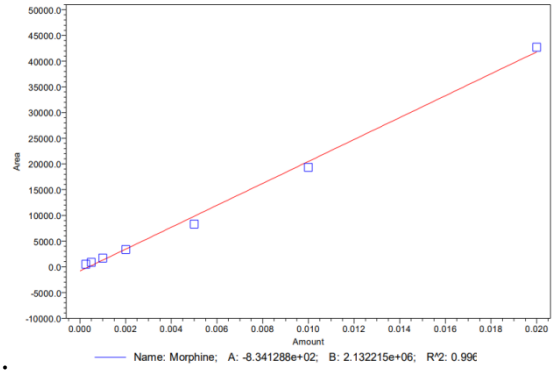


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Fig. 4. Standard Calibration curve of Morphine

3.1.4. Limit of Detection

The lowest concentration of analyte in the sample that can be detected but not absolutely quantified as an exact value is the limit of detection (LOD) of a specific analytical method (Alquadeib, 2019; Chan, 2017; Shabir, 1993; United Nations, 1998). Several methods can be used to determine the LOD. The signal to noise ratio is the foundation of the simplest strategy. By comparing measured signals from samples with known low analyte concentrations with those from blank samples, the signal to noise ratio may be calculated. The alternative method is based on the response's standard deviation and slope.

The LOD may be expressed as:

$LOD = 3.3\ \sigma / S$

Where, σ = the standard deviation of the response & S = the slope of the calibration curve

The slope is estimated from the calibration curve of the analyte and LOD is calculated to be 1.744 PPM.

3.1.5. *Limit of Quantitation*

The lowest amount of analyte in the sample that can be quantitatively measured with enough precision and accuracy is the Limit of Quantitation (LOQ) of a specific analytical method (Alquadeib, 2019; Chan, 2017; Shabir, 1993; United Nations, 1998). It is mostly impacted by the sensitivity of the detector and the accuracy of sample preparation. The same method used

to estimate the LOD can also be used to establish the LOQ. Based on the standard deviation of the response and the slope it is calculated by the formula:

$LOQ = 9.8 \sigma / S$

Where, σ = the standard deviation of the response & S = the slope of the calibration curve

The value of S and σ are estimated as for the LOD and LOQ is calculated to be 5.28 PPM.

3.1.6. Accuracy

The degree of agreement between the value regarded as a conventional true value or an accepted reference value and the value observed is expressed as an analytical method's accuracy (Alquadeib, 2019; Chan, 2017; United Nations, 1998). As practically no measurement method is perfect, it is impossible to exactly determine the true or actual value in any given measurement. Analyzing a sample with a known concentration will allow you to determine the accepted true value for accuracy assessment. The results of the accuracy experiments are often compared to those of a certified reference material with known purity or by assessing the recovery of the sample spiked with analyte of interest into the matrix of the sample (a placebo). If the sample's placebo is unavailable, the standard addition approach is applied. By using methods for impurity quantification, a sample with known amount of impurities is evaluated. Accuracy is accessed using five concentrations (15,10,5, 0.75 & 0.5 ug/ml) per three replicates each of the total analytical procedure. Overall accuracy is found to be 97.956 % (table 1).

Table 1 Accuracy determination for method validation

Injection	**Actual concentration (PPM)**	**Observed concentration (PPM)**	**X avg**	**Accuracy**	**Accuracy Avg**
1	15	15.3580	15.1066	98.34	98.03
2	15	15.3021		98.71	
3	15	14.6598		97.04	
1	10	10.4281	9.9692	95.40	96.93
2	10	9.9478		99.79	
3	10	9.5316		95.61	
1	5	4.9813	5.1438	96.84	97.88
2	5	5.1659		99.57	
3	5	5.2842		97.27	
1	0.75	0.7239	0.7433	97.39	98.26
2	0.75	0.7542		98.53	
3	0.75	0.7517		98.87	
1	0.5	0.5105	0.5160	98.93	98.68
2	0.5	0.5113		99.09	
3	0.5	0.5262		98.02	
				Overall	97.956

3.1.7. Precision

Precision is essential to assure that the instrument can produce a set of close-by results (Alquadeib, 2019; Chan, 2017; Shabir, 1993; United Nations, 1998). The percentage relative standard deviation (%RSD) for the morphine peak area obtained from repeated

injections was used to measure the method's precision. To evaluate intra-day precision, two consecutive injections of three aliquots at five different morphine concentration levels (15, 10, 5, 0.75 & 0.5 mg/mL) were made on the same day with an RSD ≤ 2.24% (table 2), the measure was deemed excellent, suggesting that the analyte was sufficiently consistent in the dissolving solvent and mobile phase utilised in this method. The aliquots were examined on two consecutive days for inter-day precision, and they displayed an RSD ≤ 2.79% (table 3).

Table 2 Intraday Precision of the method

Injection	**Actual concentration**	**Observed Concentration (Shift 1)**	**Observed Concentration (Shift 2)**	**Error %**	**Precision %**
1	15	15.3580	15.3021	0.373	99.627
1	10	10.4281	9.9478	4.803	95.197
1	5	4.9813	5.1659	3.692	96.308
1	0.75	0.7239	0.7542	4.04	95.96
1	0.5	0.5105	0.5113	0.16	99.84
				Mean	97.3864
				SD	2.18125
				RSD	2.23979

Table 3 Interday Precision of the method

Injection	**Actual concentration**	**Observed Concentration (Day 1)**	**Observed Concentration (Day 2)**	**Error %**	**Precision %**
1	15	14.6598	14.2838	2.506	97.493
1	10	10.4281	10.0212	4.069	95.931
1	5	4.9813	5.0354	1.082	98.918
1	0.75	0.7239	0.6635	8.053	91.946
1	0.5	0.5105	0.4845	5.2	94.8
				Mean	95.8176
				SD	2.66726
				RSD	2.78368

3.1.8. Population study

The developed method was successfully used to identify and quantify 10 different seized samples which were suspected to be having morphine. Fig. 5 shows the overlay of standard with seized sample of opium. And fig. 6 is showing an overlay of morphine peaks obtained from all the 10 seized samples.

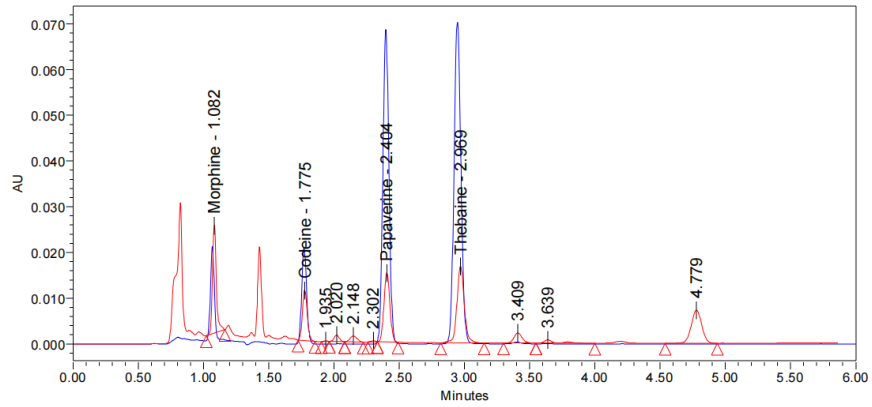

Fig. 5. HPLC Chromatogram overlay of 20 PPM standard and seized sample of unkown concentration

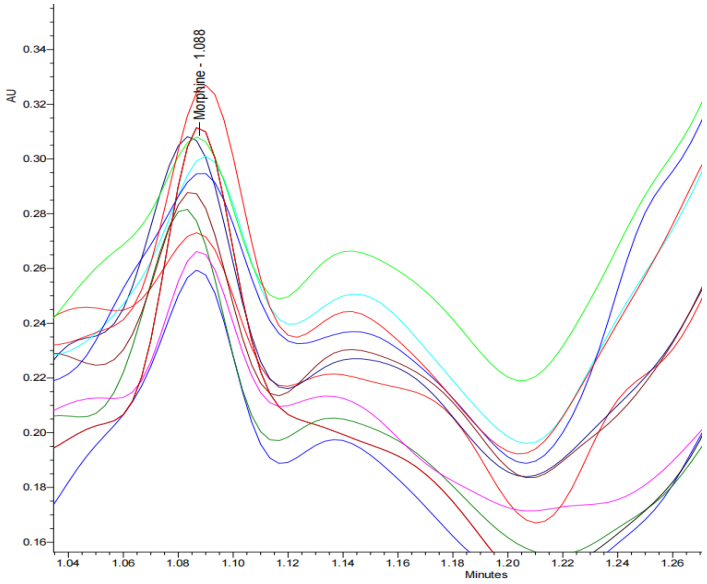

Fig. 6. HPLC Chromatogram showing Morphine presence in different seized samples.

4. Conclusion

The current study has been carried out for the detection of morphine in seized narcotic evidences. Very few studies are there on seized drug samples, mostly are on biological and pharmaceutical samples only because of the complex nature of seized drug samples and hardship in getting permission to work on such samples but it plays a vital role in law enforcement and public health efforts, providing important information that can help in the fight against drug trafficking and drug abuse. Although there isn't a single method that works well for all analytical requirements, methods can be chosen based on the necessary analytical figures of merit and practical variables. Here, an HPLC method has been developed on C18 column and a photo diode array (PDA) detector at λ = 280 nm is used. In order to create the mobile phase, water is mixed with acetonitrile and 0.1% triethylamine in a 45:55 (v/v) ratio, and the flow rate is held constant at 2.0 mL/min. With this composition of mobile phase, good stability and reproducibility is observed in the results. Linearity is reported in the concentration range of 0.5–15 µg/mL, and a remarkable correlation coefficient (R^2 = 0.9961) is determined which has not been reported earlier. The developed method is validated based on ICH guidelines. There are no system suitability parameters outside of the range. The

proposed method does not involve the use of expensive chemicals and shows better acquisition rate (runtime within 2 minutes). Moreover, the simple mobile phase preparation makes the entire procedure incredibly time-efficient making it the method of choice for routine analysis. Ultimately, the target analyte of interest i.e. morphine is detected and quantified in all the 10 distinct seized narcotic evidences with sufficient accuracy and precision.

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Conflict of interest

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

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