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DEVELOPMENT AND VALIDATION OF A SIMPLE HPLC-UV METHOD FOR ANALYSIS OF HARMINE

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Abstract: In this study, a rapid, simple, and sensitive HPLC-UV method was developed and validated in accordance with ICH Q2 (R1) guideline for the quantitative analysis of harmine (HRM), a main β-carboline alkaloid found in the Peganum harmala (Uzerlik) plant. The method was established based on the UV spectral analysis, which indicated maximum absorbance for HRM at 243 nm. A mixture of methanol and ultrapure water containing 1% formic acid (50:50, v/v) was selected as the mobile phase. In the separation carried out under isocratic conditions, the retention time of HRM was determined as 5.5 min, and the entire analysis was completed in under 8 min. The method demonstrated excellent linearity within the concentration range of 1–36 µg/mL, with a correlation coefficient (R^2) of 0.99998. The accuracy and precision parameters of the method were found to be within acceptable limits, with relative error (RE) and relative standard deviation (RSD%) both below 2. The limits of detection and quantification (LOD and LOQ) were 0.269 µg/mL and 0.816 µg/mL, respectively. Also, it was determined that HRM standard solutions were stable for up to 7 days when stored at different temperatures (−20°C, 2–8°C, 25°C). In conclusion, this method offers a reliable alternative for the quantitative determination of HRM with high analytical quality, reproducibility, and short time. It can be widely used in routine laboratory practices for the monitoring of HRM in pure form and in various pharmaceutical formulations.

Keywords: Harmine, HPLC-UV method, Peganum harmala, Stability, Validation

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1. Introduction

Peganum harmala is a perennial hairless plant with an extensive geographical distribution in the northern hemisphere. It is prevalent in the tropical and subtropical regions of Asia, Africa, and America. This perennial woody plant, which typically grows between 30-70 centimeters in height, is abundant in Central Anatolia and naturally grows at altitudes as high as 1500 meters above sea level. The leaves of the plant are comprised of multiple parts; the flowers are solitary, large, and white. The fruit of the plant is a spherical capsule containing numerous seeds (Coode, 1967). The plant seeds are characterized by a strong odor and a slightly bitter taste. Active components of *Peganum harmala* are alkaloids, which are present in exceptionally high concentrations in the seeds and roots of the plant. The total alkaloid content of the plant is approximately 4-5% (Mahmoudian et al., 2002). Harman, harmine, harmol, harmalol and harmaline are the main β-carboline derivative alkaloids of *Peganum harmala*, and harmacillin, harmacilinin, harmalacidin, harmalanine, norharmine and isoharmine alkaloids have also been isolated in the studies about *Peganum harmala* (Ayoub et al., 1989; Chatterjee and Ganguly, 1968; Siddiqui, Khan, Faizi, and Siddiqui, 1987, 1988, 1989). Its alkaloids, most notably harmaline and harmine (HRM), have been demonstrated to exert various effects (Kara, 2011). Studies have shown that active alkaloids obtained from *Peganum harmala* seeds have different antifungal, antibacterial, antiparasitic and insecticidal effects. Many studies show that *Peganum harmala* has antioxidant and free radical scavenging effects (Akhtar et al., 2000; Astulla et al., 2008; Berrougui et al., 2006; Hamden et al., 2009; Hamden et al., 2008; Nenaah, 2010; Prashanth and John, 1999; Rharrabe, et al., 2007; Saadabadi, 2006). It has a long history of traditional use in treating diabetes in specific geographic regions (Bnouham et al., 2002). HRM is the most studied and examined alkaloid among these alkaloids (Pour and Moghadar, 2012). HRM ("7-methoxy-1-methyl-9H-pyrido[3,4-b]indole") was isolated for the first time in 1847 from the seeds of *Peganum harmala* (Figure 1). HRM, a principal alkaloid of *Peganum harmala*, has various pharmacological activities such as cytotoxic, antimicrobial, antitumor, antioxidant, antimutagenic, antigenotoxic, antifungal, antiplasmodial, and hallucinogenic properties (Patel et al., 2012). HRM reduces the effects of oxidative stress by scavenging free radicals through its antioxidant activity. Additionally, it

reduces the rate of vitamin E loss and prevents LDL oxidation due to this antioxidant activity (Berrougui et al., 2006). In recent years, HRM has demonstrated significant potential in the treatment of diabetes (Wang et al., 2015).

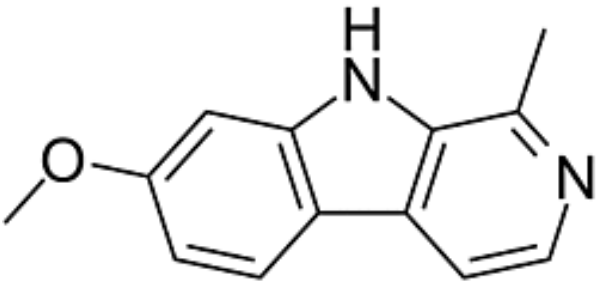

Figure 1. Chemical structure of HRM.

A popular analytical technique for separating, identifying, and quantifying the amounts of each component in a mixture is high-performance liquid chromatography (HPLC). It is recognized as one of the most precise analytical techniques for both quantitative and qualitative drug/active substance analysis (Ali, 2022; Al-Shami, Naseef, Moqadi, and Kanaze, 2024).

The objective of the current study was to develop and validate a rapid and easily executed analytical method for determining the HRM using HPLC.

2. Materials and Methods

2.1. Materials

HRM and methanol (HPLC grade) were purchased from Thermo Scientific (India) and Isolab (Germany), respectively. Also, Millipore ("Direct-Q® 3UV, USA") was used to obtain ultrapure water.

2.2. Methods

2.2.1. UV Wavelength Determination

To develop the analytical method, a standard solution of HRM was first prepared in methanol at a concentration of 10 µg/mL. Then, a UV spectrum was taken in the 200-400 nm wavelength range, and the wavelength at which it gave the maximum absorbance was determined.

2.2.2.Chromatographic System and Conditions

In the current study, an HPLC system ("Thermo Finnigan Surveyor, USA") with an autosampler and UV detector was used. Data was gathered and processed using the chromatographic software Chromoquest. Utilizing a mobile phase made of methanol and ultrapure water consisting of 1% formic acid (50:50, v/v), separation was accomplished on a C18 column (MZ Analysentechnik, Germany; 5µm, 4.6mm×250mm) while maintaining a constant column temperature of 25°C. The injecting volume of sample, the flow rate of mobile phase and UV detection wavelength were set as 10 µL, 1.0 mL/min, and 243 nm, respectively.

2.2.3. Preparation of Standard Solutions

Standard solutions were prepared at six different concentrations in the range 1-36 µg/mL, starting from the stock solution of HRM at a 50 µg/mL concentration in methanol, obtained by appropriately diluting it with methanol.

2.2.4. Validation of Analytical Method

Analytical method validation is performed to demonstrate that the method developed for quantification provides accurate, specific, and reproducible results. In our study, analytical method validation was performed according to ICH (Q2) R1 guideline, and linearity, accuracy, precision, specificity, limits of detection and quantification (LOD and LOQ), and stability parameters were examined (ICH Topic Q2 (R1), 2006) .

2.2.4.1. Linearity

For the linearity parameter, the standard solutions (n=6) were analyzed in the concentration range of 1–36 µg/mL. The calibration curve for HRM was obtained by plotting the concentration versus the corresponding peak area. Linear regression analysis was used to get the regression equation and coefficient of determination (R^2).

2.2.4.2. Accuracy

The accuracy parameter expresses the closeness of the values obtained from the analysis to the real values (Shabir, 2003). The accuracy parameter was studied as intra-day and inter-day. Freshly prepared standard solutions in six replicates (2, 16, and 32 µg/mL) were assayed for HRM on the same day and three consecutive days to determine intra- and inter-day accuracy (expressed as relative error; RE).

2.2.4.3. Precision

The precision parameter expresses the degree of closeness of the values obtained from the analysis (Kandilli et al., 2018). The precision parameter was studied as intra-day and inter-day, and also injection repeatability was evaluated. Intra- and inter-day precision (expressed as percentage relative standard deviation-RSD%) was determined by assaying six replicates of freshly prepared standard solutions (2, 16 and 32 µg/mL) for HRM on the same day and three consecutive days, respectively.

2.2.4.4. Specificity

The ability of the method to separate the target compound's response from that of other sample components is known as specificity. The specificity of the method was assessed by analyzing the chromatograms to confirm the absence of discernible shoulders or interfering peaks. (Ugur Kaplan and Cetin, 2023). For the evaluation of the specificity parameter, methanol was analysed under the specified conditions, and it was determined whether it gave a peak in the same position as HRM.

2.2.4.5. LOD and LOQ

LOD is the lowest detectable but not always quantifiable analyte concentration in a sample.The minimum amount of an analyte that can be accurately and precisely measured in a sample is known as the limit of quantification, or LOQ. For HRM, LOD and LOQ were determined using the formulas "LOD=3.3*σ/S" and

"LOQ=10* σ/S", where S is the calibration curve's slope and σ is the intercept's standard deviation (Al-Shami et al., 2024).

2.2.4.6. Stability

The stability of the HRM was evaluated after 0, 1, 2, 3, and 7 days of storage at 25 °C or in a refrigerator (2-8 °C and -20 °C) in the standard solutions (2, 16 and 32 µg/mL) protected from light to reduce potential light effects. The stability parameter was examined by calculating the percentage recovery values (Ugur Kaplan and Cetin, 2023).

3. Results and Discussion

In the current study, a rapid, easily executed and sensitive HPLC-UV method for the determination of HRM was developed and validated. The chromatographic conditions were established to achieve a high performance of the analysis. In order to accomplish this, the UV spectrum of HRM (at 10 µg/mL concentration) was first taken, and the maximum wavelength was determined to be 243 nm (Figure 1). The obtained spectrum was found to be consistent with the literature (Yamagaki, Suzuki, and Tachibana, 2007). The literature has reported maximum wavelengths of 240-250 nm for HRM (Drugfuture, 2022; Mortazavi et al., 2021; Nafisi et al., 2012; Spectrabase, 2022; Robinson, 2019; Yamagaki et al., 2007).

According to the results of preliminary experiments, a mixture of methanol and ultrapure water consisting of 1% formic acid (50:50, v/v) was selected as the mobile phase. A C18 column (4.6mm×250mm, 5µm) carried out isocratic chromatographic separation at a flow rate of 1.0 mg/min with UV detection at 243 nm. In Figure 2, an HRM chromatogram was displayed under the chromatographic conditions mentioned above. The retention time of HRM was recorded to be 5.5 minutes. The total assay run time was less than 8 minutes, with a clear separation.

In our study, analytical method validation, including linearity, specificity, LOD, LOQ, accuracy, precision, and stability parameters, was carried out according to ICH (Q2) R1 guideline (ICH (Q2) R1, 2006).

The specificity parameter expresses the ability of the analytical method to measure the drug/active substance in the presence of excipients or components of matrix (Le, Phung, and Le, 2019). The chromatograms of the HRM standard solutions and methanol only were compared in order to determine the parameter (Kandilli et al., 2018). There was no peak visible in the area of the HRM peak in the methanol chromatogram. The well-resolved HRM peak demonstrated the high selectivity of the developed HPLC-UV method (Figure 2).

The linearity of an HPLC method is its ability to provide test results that are directly proportional to the drug/compound concentration in a sample over a given concentration range (Bhujbal, Rupenthal, and Agarwal, 2024). The calibration curve of HRM was obtained by plotting the peak areas (mAu) versus the concentrations of the active substances. The calibration curve equation was calculated as y=86504x-17881 for HRM using linear regression analysis (Figure 3). For HRM, a good linear relationship with a coefficient of determination (R^2) of 0.99998 was observed between its peak areas and concentrations over the concentration range (1-36 µg/mL). Furthermore, the LOD and LOQ values were determined as 0.269 µg/mL ve 0.816 µg/mL for HRM, respectively.

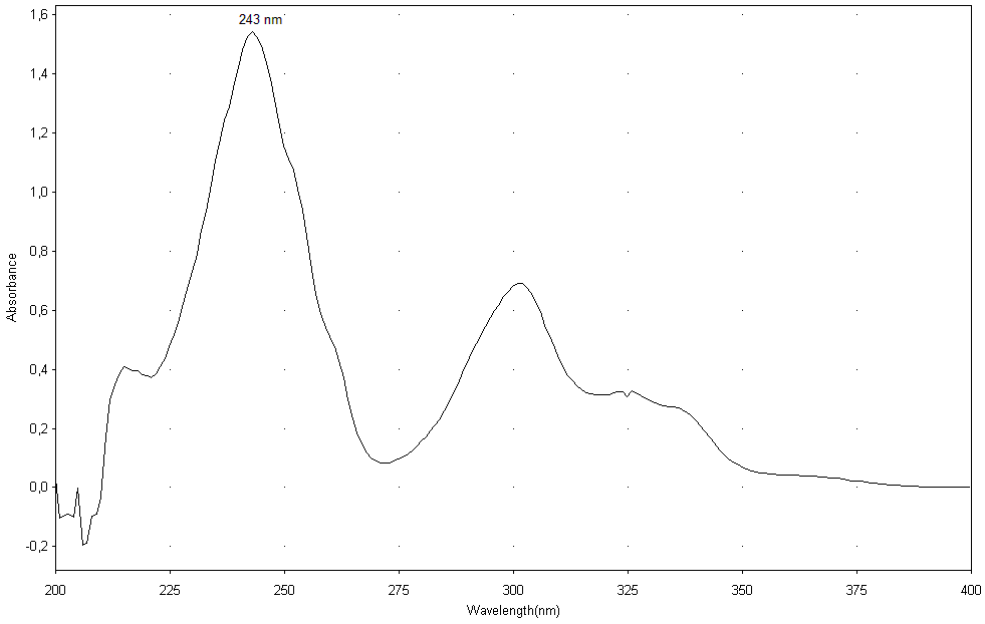

Figure 2. UV spectrum of HRM.

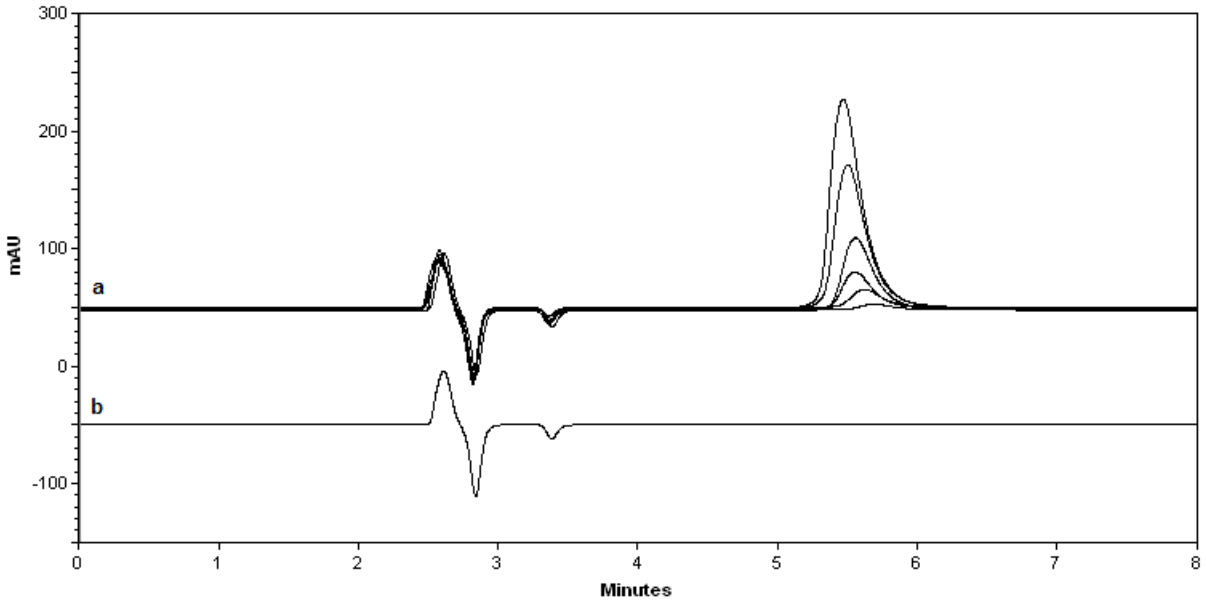

Figure 3. The chromatograms of the standard solutions of HRM (1- 36 µg/mL) (a); methanol (b).

The accuracy and precision (intra-day and inter-day) parameters were evaluated (at the concentrations of 2, 16, 32 µg/mL) in terms of RE and RSD%, respectively. The intra-day and inter-day RE values were found to be in the range of 0.326-1.545% and 0.514-1.164%, respectively. In addition, the intra- and inter-day RSD% values were determined to be between 0.475-0.951% and 0.271-0.604%, accordingly. For in vitro conditions, the obtained RE and RSD% should be <±2% and <2%, respectively (Mowafy, Alanazi, and El Maghraby, 2012). In our study, the values of RE and RSD% for HRM were found to be less than ±2% and 2%, respectively, for all injected concentrations. This indicates that the developed analytical method was accurate and precise. Also, the injection repeatability parameter was evaluated by calculating RSD% and was found to be in the range of 0.188-0.268% (Table 2).

Table 1. The results of accuracy and precision (intra-day and inter-day) of the method (Mean±SD; n=6)

	Concentration (µg/mL)	Found Concentration (µg/mL)	RE %	RSD (%)
INTRA-DAY	2	2.031 ± 0.010	1.545	0.475
	16	16.082 ± 0.132	0.515	0.818
	32	32.104 ± 0.305	0.326	0.951
INTER-DAY	2	2.023 ± 0.012	1.164	0.604
	16	16.082 ± 0.061	0.514	0.379
	32	32.201 ± 0.087	0.628	0.271

SD= standard deviation, RE= relative error, RSD= Relative standard deviation.

Table 2. The results of injection repeatability (Mean±SD; n=6)

Concentration (µg/mL)	Found Concentration (µg/mL)	RSD (%)
2	2.017 ± 0.005	0.268
16	16.060 ± 0.030	0.188
32	32.094 ± 0.198	0.198

SD= standard deviation, RSD= Relative standard deviation.

Table 3. The results of the stability study (Mean±SD; n=3)

Temperature	Time (Day)	Concentration (µg/mL)	Found Concentration (µg/mL)	Recovery (%)
25 °C	0	2	2.024 ± 0.006	100.000
		16	16.088 ± 0.113	100.000
		32	32.059 ± 0.070	100.000
	1	2	2.025 ± 0.008	100.049
		16	16.078 ± 0.130	99.938
		32	32.051 ± 0.140	99.976
	2	2	2.014 ± 0.005	99.481
		16	16.064± 0.068	99.853
		32	32.032 ± 0.134	99.916
	3	2	2.006 ± 0.005	99.102
		16	16.002 ± 0.029	99.465
		32	31.962 ± 0.108	99.697
	7	2	1.997 ± 0.007	98.669
		16	15.973 ± 0.061	99.289
		32	31.897± 0.111	99.496
2-8 °C	0	2	2.024 ± 0.006	100.000
		16	16.088 ± 0.113	100.000
		32	32.059 ± 0.070	100.000
	1	2	2.027 ± 0.015	100.122
		16	16.095 ± 0.113	100.048
		32	32.066 ± 0.153	100.024
	2	2	2.025 ± 0.006	100.052
		16	16.060 ± 0.079	99.825
		32	32.033 ± 0.043	99.920
	3	2	2.021 ± 0.011	99.841
		16	16.049 ± 0.038	99.758
		32	31.983 ± 0.020	99.762
	7	2	2.001 ± 0.007	98.844
		16	16.027 ± 0.065	99.620
		32	31.914 ± 0.023	99.549
-20 °C	0	2	2.024 ± 0.006	100.000
		16	16.088 ± 0.113	100.000
		32	32.059 ± 0.070	100.000
	1	2	2.033 ± 0.010	100.456
		16	16.096 ± 0.123	100.054
		32	32.088 ± 0.064	100.091
	2	2	2.029 ± 0.004	100.244
		16	16.093 ± 0.069	100.031
		32	32.043 ± 0.105	99.950
	3	2	2.031 ± 0.011	100.353
		16	16.055 ± 0.080	99.796
		32	32.014 ± 0.091	99.860
	7	2	2.023 ± 0.009	99.952
		16	16.035 ± 0.037	99.672
		32	31.989 ± 0.120	99.781

SD: standard deviation.

In a study by Kartal et al. (2003), a combination of "isopropyl alcohol:acetonitrile:water:formic acid" (100:100:30:0.3; v/v/v/v) adjusted to pH 8.6 was used as a mobile phase for the determination of *Peganum harmala* alkaloids (harmine, harmaline, harmalol, and harmol). Metasil ODS column (5 mm; 150/4.6 mm I.D.) was employed for the determination of alkaloids, and UV wavelength and flow rate were selected as 330 nm and 1.5 mL. The retention time, LOD and LOQ values of harmine were reported as 3.530 min, 4.690 ug/mL and 15.630 ug/mL, respectively (Kartal, Altun, and Kurucu, 2003).

In a study, HPLC method was developed to determine HRM, harmaline, harmol and harmalol alkaloids in extracts obtained from *Peganum harmala* seeds. Potassium phosphate buffer (10 mM, pH 7.0) and acetonitrile mixture (100:30, v/v) were used as mobile phase, and the flow rate was 1.5 mL/min. In the analysis using 330 nm UV wavelength, the retention time for the HRM standard (prepared in methanol) was determined as approximately 7.3 min. The method was reported to be able to preserve harmala alkaloids in plant extracts and to be routine and accurate with adequate reproducibility, sensitivity and resolution. (Faramarzi, Venus, Amini, and Rouini, 2008).

In another study, chromatographic conditions were defined as "detection wavelength: 320 nm; flow rate: 1.0 mL/min, mobile phase: methanol: 0.01 mol/L ammonium sulfate solution: diethylamide (40:60:1; pH was adjusted to 3.8 ± 0.1 with phosphoric acid)" to determine the

amount of HRM loaded into micelles and the retention time of HRM was determined as approximately 8.2 min (Bei et al., 2013).

In our study, the stability of HRM in standard solutions at three different concentrations (2, 16, and 32 µg/mL) was evaluated by calculating the percentage difference from zero-time injections. After preparation of HRM standard solutions, they were stored at different temperatures (-20°C, 2-8°C and 25°C) and analyzed at 0, 1, 2, 3 and 7 days. It was determined that the recovery (%) values were 98.669%-100.456% at -20 °C, 2-8 °C and 25 °C. In order to be able to say that the active substance is stable under the mentioned conditions, the calculated recovery (%) values are required to be within the range of 95-105% (ICH Q1A (R2), 2003; Osel, Planinšek Parfant, Kristl, and Roškar, 2021; Shabir, 2003). HRM in the standard solutions were stable for 7 days at -20°C, 2-8°C and 25°C.

4. Conclusion

A simple, fast, robust, and economical HPLC-UV method was therefore developed in this study and then successfully validated for the analysis of HRM, one of the major β-carboline alkaloids of *Peganum harmala*. This method might be used for routine analysis of HRM in pure form and as well as various pharmaceutical formulations.

Author Contributions

The percentages of the author's contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	G.M.D	A.B.U.K.	M.G.	M.C.
C	25	25	25	25
D	25	25	25	25
S	-	25	50	25
DCP	40	40	10	10
DAI	10	30	30	30
L	25	25	25	25
W	25	25	25	25
CR	25	25	25	25
SR	20	25	30	25
PM	20	25	30	25
FA	20	25	30	25

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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