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HPLC method for the analysis of harmol, harmalol, harmine and harmaline in the seeds of *Peganum harmala* L.

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Abstract

A simple and sensitive method for separation and determination of harmol, harmalol, harmine and harmaline has been developed and validated. Harmol, harmalol, harmine and harmaline were separated using a Metasil ODS column by isocratic elution with flow rate 1.5 ml/min. The mobile phase composition was Isopropyl alcohol–Acetonitrile–Water–Formic acid (100:100:300:0.3) (v/v/v/v) and pH adjusted 8.6 with triethylamine. Spectrophotometric detection was carried out at 330 nm. The linear range of detection for harmol, harmalol, harmine and harmaline were between 9.375–250, 30.750–246, 31.250–500 and 31.000–248 µg/ml, respectively. The method described was suitable for the determination of harmol, harmalol, harmine and harmaline in the seeds of *Peganum harmala* L.
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Keywords: Harmol; Harmalol; Harmine; Harmaline; High performance liquid chromatography; Validation

1. Introduction

Peganum harmala L. (Zygophyllaceae) is a herb native to dry area from Mediterranean east to northern India. The plant originated in Central Asia but now grows wild in Africa, Middle East, India, South America, Mexico, and Southern USA [1–3].

Seeds of *Peganum harmala*, a widespread species growing wild in Turkey and used as an antihea-moroidal, helmicide, and central nervous system (CNS) stimulating agent in folk medicine [4,5]. *Peganum harmala* has been used in Turkey for ritual purification for drift away the evil spirits by burning the seeds. It is known locally as 'üzerlik' and used for dyes in Turkish rugs, it was source of the richly coloured textile dye known as Turkish Red traditionally [4].

Seeds stimulant of the CNS, cause paralysis and are poisonous in strong doses [6]. Harmine and related alkaloids are serotonin antagonists, hallucinogens, CNS stimulants, and short-term monoamine oxidase (MAO) inhibitors. Small doses (25–50 mg) act as mild and therapeutic cerebral stimulant, sometimes producing drowsy or dreamy state for 1–2 h. Larger doses up to 750 mg may have hallucinogenic effects, the intensity of which varies widely with the individual [7].

These alkaloids have a wide spectrum of pharmacological actions, including MAO inhibition,

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binding to benzodiazepine receptors, convulsive or anticonvulsive actions, tremorogenesis, anxiolytic and behavioural effects, antioxidative action, and immunomodulatory effects. There were also some reports concerning the cardiovascular actions of harmala alkaloids [8].

Alkaloids in the seeds of *Peganum harmala* L. are derived from β-carboline (βCs) and tetrahydo-β-carboline (THβCs). These alkaloids that occur naturally in foods as a chemical condensation between indoleamines and aldehydes or α-keto acids [9]. This reaction may occur during food production, processing and storage. The occurrence of harman and norharman are known in well-cooked meat and fish at ng/g level. Minor amounts of them were reported in some alcoholic beverages and foodstuffs [9].

HPLC methods in combination with UV [10], chemiluminescence [11], fluorometry [12] have been used for selective and sensitive detection. Besides HPLC-MS [13], and GC-MS [9] are the techniques predominantly used for identification, separation and quantitation of βCs and THβCs. GC separation is greatly improved by chemical derivatization of βCs. Problems due to possible artefact formation during derivatization may also occur. Chemiluminescence detection was applied as an alternative to other spectroscopic techniques. But, HPLC combined with fluorescence and MS detection is often used because of its simplicity, good selectivity, and excellent sensitivity [2,9,13].

Proposed HPLC method with UV detection can successfully be used for the separation and quantitative determination of closely related βCs alkaloids in biological materials. Analytical data is presented to illustrate the usefulness of the method for the determination of *Peganum harmala* alkaloids. As a simple, sensitive, fast and easy method can be a good alternative to the current methods.

2. Experimental

2.1. Chemicals

Harmol (Sigma; H-1000), harmalol (Sigma; H-0250), harmine (Sigma; H-8646) and harmaline (Sigma; H-2256) was obtained from Sigma Chemicals. Chromatographic grade-double distilled water, HPLC grade acetonitrile (Merck-100030), and isopropyl alcohol (Merck-101040), analytical grade formic acid (Merck-100264) were used.

2.2. Plant material

The plant material was collected from Van, Turkey on July 01, 2001. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Ankara University, Ankara, Turkey (AEF 21702).

2.3. Extraction of alkaloids

Two grams of dried and powdered seeds of *Peganum harmala* fruits was macerated four times with 50 ml methanol at 50 °C in a water bath for 1 h. The extracts were combined and evaporated to dryness. The residue was dissolved in 50 ml HCl (2%) then filtered. The filtrate was extracted two times with 20 ml petroleum ether. The aqueous acid layer was basified (pH 10) with NH_4OH and extracted four times with 50 ml chloroform. The chloroform layer was combined and evaporated to dryness, then the residues were dissolved in 25 ml methanol. The solution of alkaloid extract was passed through 0.45 μm filter and 10 μl extract was directly injected into the HPLC column. The results were obtained as a mean value of three separate injections.

2.4. Apparatus

The method development was performed with a LC system consisting of a Jasco model PU-980 pump and JASCO UV-975 UV/VIS detector. Samples were injected with a 7725 Rheodyne injector system with a 10 μl sample loop. The detector was set at 330 nm (0.02 a.u.f.s) and peak areas were integrated automatically by computer using BORWIN software programme.

Separation was carried out using a Metasil ODS column (5 μm, 150 × 4.6 mm I.D.; MetaChem Technologies Inc.). All the calculations concerning the quantitative analysis were performed with external standardisation by measurement of peak areas.

2.5. Stock and standard solutions

Harmol (12.50 mg), harmalol (12.30 mg), harmine (12.50 mg) and harmaline (12.40 mg) were accurately weighed into a 25 ml volumetric flask and dissolved in the mobile phase and filled up to volume with mobile phase.

2.6. Standard working solution

Standard working solutions were prepared individually in mobile phase for harmol, harmalol, harmine and harmaline. Aliquots from each working solution were combined and diluted with mobile phase to obtain a standard solution containing 250 μg/ml harmol, 246 μg/ml harmalol, 500 μg/ml harmine and 248 μg/ml harmaline.

3. Procedure

3.1. Chromatographic conditions

HPLC analysis was performed by isocratic elution with flow rate 1.5 ml/min. The mobile phase composition was Isopropyl alcohol–Acetonitrile–Water–Formic acid (100:100:300:0.3) (v/v/v/v) and pH adjusted 8.6 with triethylamine. All solvents were filtered through a 0.45 μm Milipore filter before use and degassed in an ultrasonic bath. Volumes of 10 μl each prepared solutions and samples were injected into the column. Quantification was effected by measuring at the 330 nm. The chromatographic run time was 10 min and the column void volume was 0.75 min.

Throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k'), the resolution (R), the selectivity (α) and peak asymmetry (T).

3.2. Calibration

Mixed standard solutions containing harmol (9.375–250 μg/ml), harmalol (30.750–246 μg/ml), harmine (31.250–500 μg/ml), harmaline (31.000–248 μg/ml) were prepared in the mobile phase.

Triplicate 10 μl injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph. The five concentrations of each compound were subjected to regression analysis to calculate calibration equation and correlation coefficients.

4. Results and discussion

4.1. Method development

The mobile phase was chosen after several trials with isopropyl alcohol, acetonitrile, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting Isopropyl alcohol–Acetonitrile–Water–Formic acid (100:100:300:0.3) (v/v/v/v) and pH adjusted 8.6 with triethylamine was selected to achieve maximum separation and sensitivity.

Flow rates between 0.5 and 1.5 ml/min were studied. A flow rate of 1.5 ml/min gave an optimal signal to noise with a reasonable separation time. Using reversed-phase ODS column, the retention times for harmol, harmalol, harmine and harmaline were observed to be 1.620, 2.971, 3.539 and 10.374 min, respectively. Total time of analysis was less than 12 min.

The maximum absorption of harmol, harmalol, harmine and harmaline together were found to be at 330 nm and this wavelength was chosen for the analysis.

The chromatogram at 330 nm showed a complete resolution of all peaks (Fig. 1). Method was not susceptible to small variations of organic composition, pH and flow rates.

4.2. Linearity

Table 1 presents the equation of the regression line, correlation coefficient (r^2), relative standard deviation (RSD) values of the slope and intercept for each compound. Excellent linearity was obtained for compounds between peak areas and concentrations of 9.375–250 μg/ml with $r^2 = 0.9999$, 30.750–246 μg/ml with $r^2 = 0.9999$, 31.250–500 μg/ml with $r^2 = 0.9997$ and 31.000–

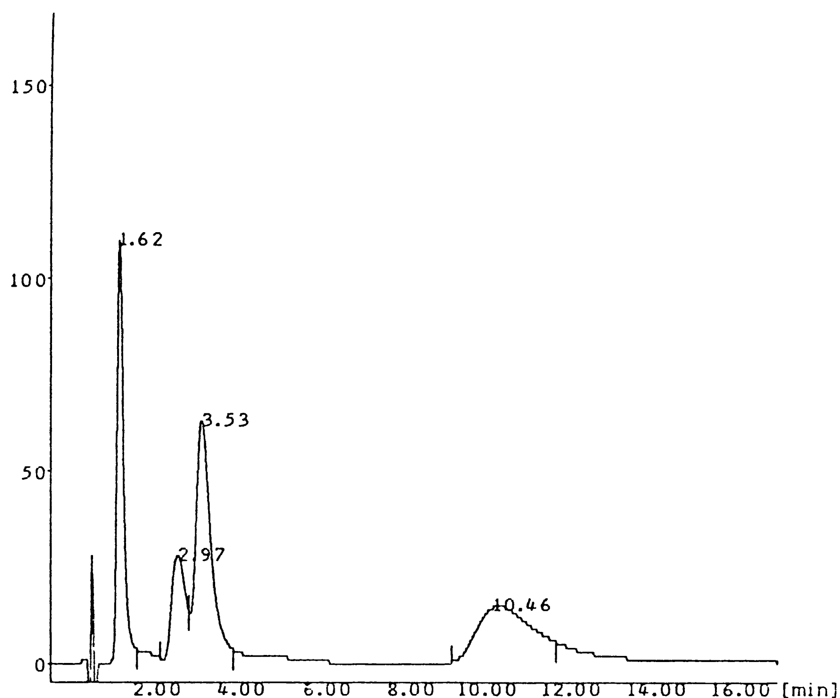

Fig. 1. Chromatogram of the mixture of harmol (1.62), harmalol (2.97), harmine (3.53) and harmaline (10.46) by developed HPLC method.

248 μg/ml with $r^2 = 0.9999$ for harmol, harmalol, harmine and harmaline, respectively.

4.3. Limits of detection and quantification

Limits of detection (LOD) were established at a signal-to-noise ratio (S/N) of 3. Limits of quantification (LOQ) were established at signal-to-noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by six injections of harmol, harmalol, harmine and harmaline at the LOD and LOQ concentrations. The LOD was calculated to be 3.125, 10.250, 4.690 and 10.330 μg/ml and the LOQ was calculated to be 9.375, 30.750, 15.630 and 31.000 μg/ml for harmol, harmalol, harmine and harmaline, respectively (Table 1).

4.4. Suitability of the method

The chromatographic parameters such as resolution, selectivity and peak asymmetry were satisfactory for these compounds (Table 2). The

Table 1
Linearity results, limit of detection (LOD) and limit of quantification (LOQ)

Compound	λ	Equation	r^2	Slope (RSD %)	Intercept (RSD %)	LOQ (μg/ml)	LOD (μg/ml)
Harmol	330	$Y = 9.929431X + 16.99812$	0.9999	0.383	2.390	9.375	3.125
Harmalol	330	$Y = 6.446235X - 43.9565$	0.9999	0.241	1.539	30.750	10.250
Harmine	330	$Y = 10.966X - 12.3125$	0.9997	0.297	11.486	15.630	4.690
Harmaline	330	$Y = 12.51171X + 14.57609$	0.9999	1.961	2.003	31.000	10.330

X = concentration (μg/ml); Y = area.

Table 2
System performance parameters of harmol, harmalol, harmine and harmaline

Compound	t_r ($n = 9$, mean)	Area ($n = 9$, mean)	k'	R	α	T
Harmol	1.620 (1.043)[a]	1146.4 (3.279)[a]	1.160	5.066 (2.565)[a]	2.553 (1.317)[a]	1.062
Harmalol	2.971 (0.581)[a]	606 (4.436)[a]	2.962			2.625
				1.528 (4.503)[a]	1.255 (1.330)[a]	
Harmine	3.539 (1.629)[a]	1596.6 (2.662)[a]	3.718			1.754
				7.202(3.263)[a]	3.452 (1.338)[a]	
Harmaline	10.374 (1.367)[a]	1398.8 (4.790)[a]	12.831			2.778

RSD % = (standard deviation/mean) × 100; t_r = retention time.
[a] RSD % values are given in the parenthesis.

calculated resolution values between each peak-pair were not less than 1.50 and the selectivity were not less than 1.20.

k' were found to be 1.160, 2.962, 3.718 and 12.831 for harmol, harmalol, harmine and harmaline, respectively.

4.5. Precision

The precision of the method (within-day variations of replicate determinations) was checked by injecting nine times of harmol, harmalol, harmine and harmaline at the LOQ level. The precision of the method, expressed as the RSD % at the LOQ level were 1.680, 4.609, 2.711 and 3.078% for harmol, harmalol, harmine and harmaline (Table 3).

4.6. Accuracy

A standard working solution containing of the harmol, harmalol, harmine and harmaline, to give final concentrations, respectively 125, 100, 150, 112.5 μg/ml was prepared. The prepared mixture of standards was injected nine times as a test sample. From the respective area counts, the concentrations of the harmol, harmalol, harmine and harmaline were calculated using the detector responses. The accuracy, defined in terms of % deviation of the calculated concentrations from the actual concentrations are listed in Table 4. The results are obtained range of ±5%, the method is deemed to be accurate.

Table 3
Precision of the developed method at the LOQ level ($n = 9$)

Compound	λ	Peak area (mean)	RSD %
Harmol	330	102.00	1.680
Harmalol	330	151.83	4.609
Harmine	330	138.44	2.711
Harmaline	330	394.72	3.078

4.7. Semen Pegani extract

Quantitative determination of harmol, harmine and harmaline in the Van-Semen Pegani samples were carried out by RP-HPLC using external standard method (Fig. 2). The standard solutions of harmol, harmine and harmaline were added, respectively to Semen Pegani extracts and injected at each time. The area of peaks corresponding to standards were increased to prove the presence of these compounds (Table 5).

5. Conclusions

The developed method is suitable for the identification and quantification of harmol, harmalol, harmine and harmaline in biological materials. High percentage of recovery shows that the method can be successfully used on a routine basis.

The developed method was applied to the Semen Pegani sample extracts. The alkaloids harmol, harmine and harmaline in the extracts were well separated by HPLC. Harmol was the major alkaloid and its content (1.094%) was higher than harmaline (0.611%) and harmine (0.476%) in the samples (Table 5). Harmalol could not be

Table 4
Accuracy of the developed method ($n = 9$)

Compound	Spiked concentration (μg/ml)	Measured concentration (μg/ml) (mean ± SD)	RSD %	Deviation %
Harmol	125	118.92 ± 4.397	4.397	4.864
Harmalol	100	100.29 ± 3.876	3.864	0.290
Harmine	150	148.18 ± 2.926	1.975	1.213
Harmaline	112.5	112.50 ± 3.897	3.474	0.293

% Deviation = (spiked concentration − mean measured concentration) × 100/spiked concentration.

detected in our samples. It is possible that harmol could have been produced artifactually by aerial oxidation of harmalol during extraction [14]. Total alkaloid content of Semen Pegani varied between 2 and 5% according to literature [2,15,16]. Alkaloid composition in plants can change due to various factors such as soil, climate, collecting time and others. Also, the ratio of alkaloids in the plant

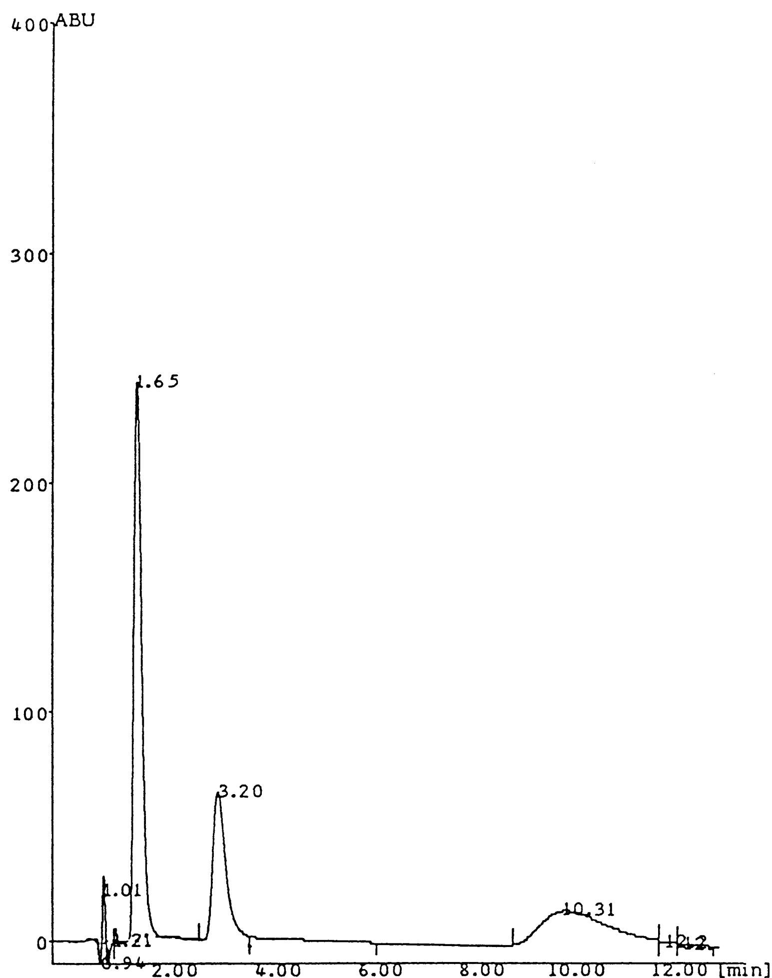

Fig. 2. Chromatogram of the Semen Pegani extract harmol (1.65), harmine (3.20), harmaline (10.31).

Table 5
Contents of alkaloids in Semen Pegani

Compound	Area ($n = 3$; mean)	Concentration (μg/ml) ($n = 3$, mean)	Alkaloid % ($n = 3$, mean) mean ± SD
Harmol	8705 (0.463)[a]	874.975 (0.464)[a]	1.094 ± 0.0049 (0.453)[a]
Harmine	4163.5 (1.087)[a]	380.796 (1.084)[a]	0.476 ± 0.0056 (1.188)[a]
Harmaline	6132.25 (2.496)[a]	488.994 (2.513)[a]	0.611 ± 0.0155 (2.546)[a]

RSD % = (standard deviation/mean) × 100; SD = standard deviation.
[a] RSD % values are given in the parenthesis.

need not necessarily be the same at all stages of growth [17]. Literature survey showed that there is not any report on alkaloid composition of Turkish Semen Pegani samples for comparison. This is first HPLC report on Turkish Semen Pegani alkaloids. The proposed method is simple, sensitive, rapid, specific and could be applied for quality monitoring of harmol, harmalol, harmine and harmaline in biological materials.

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