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Determination of Harmine and Harmaline in *Peganum harmala* Seeds by High-Performance Liquid Chromatography

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Abstract: A High-Performance Liquid Chromatography (HPLC) method was developed for determination of harmine, harmaline, harmol and harmalol in the extract of *Peganum harmala* seeds. The sample preparation was performed using liquid-liquid extraction. Chromatographic separation was achieved with a Tracer Excel 120 ODSA (150×4.6 mm) column, using a mixture of potassium phosphate buffer (10 mM, pH 7.0): acetonitrile (100:30; v/v) as mobile phase, in an isocratic mode at 1.5 mL min⁻¹. UV detection ($\lambda = 330$ nm) was used. The calibration curves were linear ($r^2 > 0.998$) in the concentration range of 0.5-20 $\mu\text{g mL}^{-1}$ for all analytes. The lower limit of quantification for all analytes was 0.5 $\mu\text{g mL}^{-1}$. The within and between day precisions in the measurement of QC samples at three tested concentrations were in the range of 0.6-10.2% for all analytes. The HPLC method is able to measure the harmala alkaloids in the plant extract. The method has suitable reproducibility, sensitivity and resolution for routine and accurate use with UV detection.

Key words: *Peganum harmala*, HPLC, harmine, harmaline, harmol, harmalol

INTRODUCTION

Harman alkaloids are naturally found in the plants such as *Peganum harmala* (Chatterjee and Ganguly, 1967; McKenzie *et al.*, 1975; Bais *et al.*, 2003), *Passiflora incarnate* (Halpern, 2004), *Banisteria caapi* (Bais *et al.*, 2003; Grella *et al.*, 2003) and *Tribulus terrestris* (Cheng and Mitchelson, 1997). These alkaloids proved to possess various pharmacological activities and neuromodulator properties. They stimulate Central Nervous System (CNS) of mammals (Frostholm *et al.*, 2000) and are mentioned as hallucinogen in the literature (Alen and Holmstedt, 1980; Kayir *et al.*, 2003). Harmaline, the major alkaloid in *Peganum harmala*, appears to act as a reversible monoamine oxidase inhibitor (MAOI) (Abdel-Fattah *et al.*, 1997) and in common with other β -carbolines bind to 5-HT receptors (Abdel-Fattah *et al.*, 1995; Helsey *et al.*, 1998).

Peganum harmala L. (Syrian rue), a wild-growing flowering plant belonging to Zygophyllaceae family,

grows in the semi-dried areas of the world (Rechinger, 1982). Different parts of the plant with local name as Esfand have been used in traditional medicine of Rouin region in Iran for the treatment of variety of human ailments (Siddiqui *et al.*, 1987). The seeds contain from 2.5 to 4% mixed harmala alkaloids (Wagner and Bladt, 1996) and are known to possess hypothermic (Abdel-Fattah *et al.*, 1995) and hallucinogenic properties (Lamchouri *et al.*, 1999; Grella *et al.*, 1998).

Although the separation and determination of β -carboline alkaloids such as harmaline and harmol have been investigated in several studies (Cepas *et al.*, 1996; Kartal *et al.*, 2003), the development of simple, efficient and sensitive analytical procedures are still important to researchers involved in the studies as diverse as pharmacology, toxicology and analytical chemistry (Picada *et al.*, 1997).

HPLC is generally applied for separation and quantification of harmala alkaloids with different types of

detection. Several methods described to the measurement of these alkaloids in plant tissue extract or biological samples. An HPLC method combined with fluorimetric detection and LC-Mass spectrometry was developed for determination of β -carbolines in foodstuffs (Adachi *et al.*, 1991). Solid-phase method for the extraction of harman and norharman alkaloids which described by Adachie group was together with using expensive fluorescent and mass detector, a facility that is not commonly available in every laboratory. The concentration of harman alkaloids in alcoholic beverage has been determined by HPLC (Bosin and Faull, 1988). However, they have not reported the sensitivity of their method. Also, fluorescence detection has been used by Bosin and Faull with a precolumn derivatization of alkaloids with acetic anhydride to facilitate the isolation and separation of β -carbolines (harman, norharman, harmol and norharmol). Efficient fractionation was reported with high-performance capillary electrophoresis method (Cheng and Mitchelson, 1997); but no quantitative data for assay of sensitivity of the method (limit of quantitation) has been mentioned. Chemiluminescence detection including derivatization with 2,4-dinitrophenyl oxalate has been used for the determination of β -carboline alkaloids (Cepas *et al.*, 1996). Although a high sensitivity of their method was reported, derivatization of the sample before detection caused too long sampling procedure and decreased reproducibility. Recently, a simple HPLC method for the analysis of β -carbolines was developed using UV detection (Kartal *et al.*, 2003). However, a very broad peak for harmaline and unacceptable resolution for harmalol and harmine were observed in the spiked chromatogram of four resolved analytes.

This report describes a simple and sensitive HPLC analysis of harmaline in the extract of *Peganum harmala* seeds. The method has been also applied to the measurement of harmine, harmol and harmalol as well.

MATERIALS AND METHODS

Standards and reagents: Harmaline, harmine, harmol and harmalol (analytical grades) were obtained from Sigma (St. Louis, MO, USA). All other chemicals and reagents including HPLC grade solvents were purchased from Merck (Darmstadt, Germany).

Plant material: The seeds of Syrian rue were collected from Abyaneh (Esfahan Province), Iran, in June 2003. Voucher specimens have been deposited in the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Apparatus and chromatographic conditions: The chromatographic separation was performed on a model 600 solvent delivery pump, a 600 E system controller, a 486 tunable absorbance detector (Waters, Millipore, Bedford, MA, USA) and a 746 data module Integrator. Samples were introduced by a rheodyne model 7725i injector fitted by a 20 μ L loop. The compounds were eluted with a mobile phase of potassium phosphate buffer (10 mM, pH 7.0): acetonitrile (100:30, v/v). The chromatography was performed isocratically on a Tracer Excel 120 ODSA (150 \times 4.6 mm i.d., 5 μ m, Teknokroma, Spain) at a flow rate of 1.5 mL min⁻¹ under room temperature (25°C). The eluents were monitored at 330 nm. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

Preparation of calibration standards: Starting from pooled stock solution of harmine, harmaline, harmol and harmalol (4 mM in methanol), standards were prepared using methanol as diluents. The calibration curve was performed with standards of the final concentrations of 0.5, 1, 5, 10, 15 and 20 μ g mL⁻¹ of each analyte, respectively.

Extraction and sample preparation: According to the literature (Kartal *et al.*, 2003), dried seeds of Syrian rue (1 g) were grinded and macerated three times with 25 mL methanol for 1 h. The extract was then filtered and evaporated in a rotary evaporator under vacuum at a temperature of 45°C. The residue was then treated with 25 mL hydrochloric acid solution (2% v/v), filtered and extracted three times with 10 mL petroleum ether to remove colorant impurities. The aqueous acid layer was then basified with ammonia to reach pH 10 and extracted three times with 25 mL chloroform. The organic solvent was then evaporated and yielded a total alkaloid extract of 0.17 g. The obtained residue was dissolved in 10 mL methanol and then filtered through a 0.45 μ m polypropylene filter. A 20 μ L aliquot was injected onto HPLC column.

RESULTS

Method development: At the beginning, the mobile phase described by Kartal *et al.* (2003) was used. The pH of this mobile phase was acidic and may lead to decreased column life. A mixture of methanol: acetonitrile: phosphate buffer (pH = 6.5) resulted in a chromatogram of low resolution between analytes. An increase in pH from 6.5 to 7.5 increased the resolution while the overall run time was also unacceptably increased. Finally a mixture of

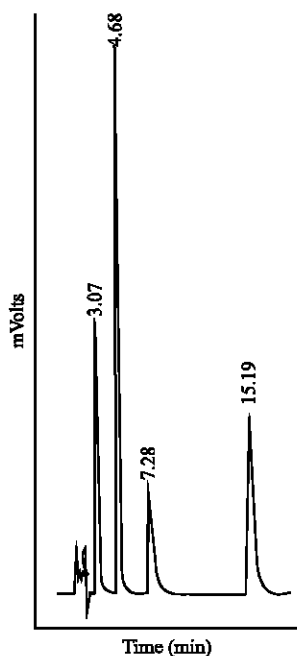


Fig. 1: The chromatogram of spiked methanolic sample of the harmol (3.07 min), harmalool (4.68 min), harmine (7.28 min) and harmaline (15.19 min)

potassium phosphate buffer (10 mM, pH 7.0): acetonitrile (100:30; v/v) provided suitable resolution in a shorter run time. Therefore, this mobile phase was used through the study. Flow rates between 0.8 and 1.8 mL min⁻¹ were studied. A flow rate of 1.5 mL min⁻¹ gave a reasonable separation time. Using a Tracer Excel 120 ODSA reversed-phase column, the retention times for harmaline, harmalol, harmine and harmol were observed to be 3.07, 4.68, 7.28 and 15.19 min, respectively (Fig. 1).

Linearity: The linearity of the method was studied in triplicate. The calibration curve (ranged from 0.5 to 20 µg mL⁻¹) was constructed by peak area of each analyte versus its concentration (µg mL⁻¹). The equation of the regression line, correlation coefficient (r²), Relative Standard Deviation (RSD) values of the slope and intercept for each compound are shown in Table 1. The method was specific as there was no interference with peaks of analyte in chromatograms.

Inter and intra-day assay precision and accuracy: The accuracy and precision were determined by preparing three replicate samples of each compound at concentrations of 0.5, 1 and 10 µg mL⁻¹. The results of the assay showed a mean RSD for within-day as 2.0, 2.8, 3.5 and 1.1% for harmalol, harmaline, harmol and harmine, respectively. The respective RSDs for between-day



Fig. 2: The chromatogram of *Peganum harmala* seeds extract: harmine (7.52 min) and harmaline (15.87 min)

Table 1: Linearity results of HPLC analyses of four harman alkaloids

Compounds	Equation	r ²	Slope (RSD%)	Intercept (RSD%)
Harmaline	y = 53675x-10091	0.9983	1.3	2.7
Harmalol	y = 51808x + 54966	0.9998	1.3	9.5
Harmine	y = 93485x+14601	0.9995	1.2	2.6
Harmol	y = 100518x-1775	0.9990	1.3	6.3

Table 2: Accuracy and precision of the method

Compounds	Conc. (µg mL ⁻¹)	Within-day variability		Between-day variability	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Harmalol	0.5	3.3	84.8	-	-
	1.0	1.9	91.5	-	-
	10.0	0.9	94.6	-	-
Harmaline	0.5	3.0	86.0	0.9	80.0
	1.0	4.6	88.0	2.5	83.0
	10.0	0.7	98.8	1.6	103.7
Harmol	0.5	3.6	112.0	-	-
	1.0	6.1	94.6	-	-
	10.0	0.9	95.9	-	-
Harmine	0.5	1.8	119.0	10.2	114.0
	1.0	0.6	97.0	2.6	119.0
	10.0	0.8	96.8	1.1	116.0

reproducibility in five different days were 1.5 and 4.6% for harmaline and harmine, respectively (Table 2).

Limits of detection and quantification: Limits of detection (LOD) were established at a signal-to-noise ratio (S/N) of 5 resulting in concentrations of 0.01, 0.05, 0.05 and 0.1 µg mL⁻¹ for harmine, harmaline, harmalol and harmol, respectively. The limit of quantification of the method defined as the minimum concentration that could be measured with a RSD < 15% was found to be 0.5 µg mL⁻¹ for all of four compounds.

HPLC chromatogram for *Peganum harmala* seeds extract: Figure 2 represents the HPLC chromatogram of harmine and harmaline in the extract of seeds of Syrian

rue. The standard solutions of harmine and harmaline were injected as well. Harmine was the major alkaloid and its content (0.465 g/100 dried seed) was higher than harmaline (0.355 g/100 dried seed). Harmol and harmalol did not detect in the sample.

DISCUSSION

HPLC is generally applied for separation and quantification of harmala alkaloids with different types of detection. Several methods described to the measurement of these alkaloids in plant tissue extract or biological samples. An HPLC method combined with fluorimetric detection and LC-Mass spectrometry was developed for determination of β -carbolines in foodstuffs (Adachi *et al.*, 1991). Solid-phase method for the extraction of harman and norharman alkaloids which described by Adachie group was together with using expensive fluorescent and mass detector, a facility that is not commonly available in every laboratory. The concentration of harman alkaloids in alcoholic beverage has been determined by HPLC (Bosin and Faull, 1988). However, they have not reported the sensitivity of their method. Also, fluorescence detection has been used by Bosin and Faull with a precolumn derivatization of alkaloids with acetic anhydride to facilitate the isolation and separation of β -carbolines (Harman, norharman, harmol and norharmol). Efficient fractionation was reported with high-performance capillary electrophoresis method (Cheng and Mitchelson, 1997); but no quantitative data for assay of sensitivity of the method (limit of quantitation) has been mentioned. Chemiluminescence detection including derivatization with 2,4-dinitrophenyl oxalate has been used for the determination of β -carboline alkaloids (Cepas *et al.*, 1996). Although a high sensitivity of their method was reported, derivatization of the sample before detection caused too long sampling procedure and decreased reproducibility. Analysis of harmol and its conjugated metabolites after enzyme hydrolysis in biological fluid has been also reported (Ching *et al.*, 1986). A high flow rate (3 mL min^{-1}), low retention time of harmol and separation of only one harman alkaloid (harmol) made their method usefulness. Recently, a simple HPLC method for the analysis of β -carbolines was developed using UV detection (Kartal *et al.*, 2003). However, a very broad peak for harmaline and unacceptable resolution for harmalol and harmine were observed in the spiked chromatogram of four resolved analytes.

CONCLUSION

The developed method is superior to the method of Kartal *et al.* (2003) in a) a more than two times higher k' (capacity factor) for first peak in chromatogram (harmalol)

(a minimum k' of 1.5 is recommended) as the k' reported for their first peak (harmol) is 1.16, b) a complete baseline resolution between second and third peaks (harmol and harmaline in our study with $\alpha \approx 2$ vs harmalol and harmine in their study), c) a much sharper peak for harmaline and finally d) more than three times lower limit of quantitation of analytes.

In conclusion, the present HPLC method is able to measure the harmala alkaloids in the plant extract. The method has suitable reproducibility, sensitivity and resolution for routine and accurate use with UV detection.

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