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Full Length Research Paper

Simultaneous determination of atropine and scopolamine in different parts of *Hyoscyamus arachnoideus* Pojark plants by high-performance liquid chromatography (HPLC)

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A reverse phase high-performance liquid chromatography (HPLC) equipped with UV-PDA detector was used for the analysis of main tropane alkaloids, scopolamine and atropine, in the roots, aerial parts and seeds of *Hyoscyamus arachnoideus* Pojark plants collected from five different parts of Iran. Results showed that scopolamine was only the main alkaloid of two *H. arachnoideus* populations seeds, while atropine was the major alkaloid of almost all parts, especially roots. The highest and lowest content of tropane alkaloids was observed in populations from Zangane village and Ashtian Mountain, respectively. The alkaloid content of seeds was in general more than other parts tested. The linearity of the method was in the range of 4 to 400 µg/ml for atropine and 0.8 to 80 µg/ml for scopolamine. Limit of detection (LOD) and limit of quantification (LOQ) values were 5.15 and 17.4 µg/ml for atropine and 1.92 and 6.4 µg/ml for scopolamine.

Key words: *Hyoscyamus arachnoideus* Pojark, tropane alkaloids, validation, high-performance liquid chromatography (HPLC).

INTRODUCTION

Tropane alkaloids are natural compounds having in common, the 8-aza-bicyclo [3.2.1] octane structure. They are mainly occur in the Solanaceae, Erythroxylaceae, and Convolvulaceae families (Griffin and Lin, 2000; Humam et al., 2005). In the last few decades, more than 250 natural tropane alkaloids have been isolated from the different plant taxa and their biological properties have been the subject of many studies (Christen, 2000; Lounasmaa and Tamminen, 1993). Because of numerous pharmacological activities, tropane alkaloids are considered as an important class of natural products and some of which such as (-)-hyoscyamine, the more stable enantiomer of atropine, and scopolamine are widely used in therapeutics. Tropane alkaloids are competitive antagonists of the muscarinic acetylcholine receptor and classified as anticholinergic agents (Mateus et al., 1999). Because of the high cost of the industrial synthesis, tropane alkaloids are extracted from the plants of Solanaceae family and the investigation for new sources is still on going. So far, a number of analytical methods including gas chromatography (GC) (Majlat, 1982; Hartmann et al., 1986), gas chromatography-mass spectrometry (GC-MS) (Hashimoto and Yamada, 1983; Keiner et al., 2000), LCMS (Vepoorte and Niessen, 1984), high performance liquid chromatography (HPLC) (Fliniaux et al., 1993), thin layer chromatography (TLC), (Monforte et al., 1992) and capillary electrophoresis (CE)

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Table 1. Sources and geographical locations of *H. arachnoideus* populations tested.

Sample code	Locality	Altitude (m)
1	Malaier-Tore road, 5 Km before Zangane Village	2040
2	Malaier-Tore road, 5 Km before Zangane Village	2096 m
3	Ashtian road, 9 km before Khalajestan Village	2050 m
4	10^{th} km Hamadan to Malaier road	1700 m
5	Ashtian Mountain, Markazi province	1800 m

(Altria, 1998) have been used for determination of tropane alkaloids. More recently, analysis of tropane alkaloids and related compounds has been reviewed (Dräger, 2002). In the flora of Iran, the genus *Hyoscyamus* (Solanaceae) is represented by 19 herbaceous species, seven of which are endemic. One of these species is *Hyoscyamus arachnoideus* Pojark., a biennial herb 0.5 to 0.8 m in height with dense arachnose to tomentose trichomes. Worldwide, the plant is distributed in Iraq (Kurdistan) and some parts of Iran including west and center (Schonbeck-Temesy, 1972). To the best of our knowledge, there is no report on the alkaloid composition of *H. arachnoideus* different parts. Therefore, the aim of the present study was to determine main tropane alkaloids, scopolamine and atropine (Figure 1), in the roots, aerial parts and seeds of *H. arachnoideus* plants collected from different parts of Iran by reversed phase HPLC.

MATERIALS AND METHODS

Plant materials

Different populations of *H. arachnoideus* were collected after seed ripening stage from some parts of Markazi and Hamadan provinces according to geographical data presented in Table 1. Voucher specimens were deposited at the Herbarium of Natural Resources Faculty, University of Arak, Arak, Iran. After collection, plant materials were separated into roots, aerial parts and seeds and dried at room temperature for extraction of tropane alkaloids.

Chemicals

Chloroform and methanol were purchased from Panreac (Spain), hyoscyamine and scopolamine as standards from Sigma (USA), ammonium solution 25% from Fluka (Switzerland), sulfuric acid 85 to 88%, anhydrous sodium sulfate 99% and potassium dihydrogen orthophosphoric acid 99% from Merck (Germany) and acetonitrile of HPLC grade from Caledon (Canada) chemical.

Instrumentation

Extractions were carried out using a power sonic 405 (Hwashin Technologies, Korea) ultra sonic chamber. A pH-meter, model CG-840 (Schott Gerate Gmbh, Germany) was employed to adjust pH in different stages. HPLC analyses were carried out on a C_{18} Lichrospher 100 column (5 µm, 250 x 4.6 mm) equipped with a K-1001 pump, K-2800 UV-PDA detector, and a 20 µl injection loop; all from Knauer (Germany). A 10 mm C_8 pre-column was coupled to the analytical column.

Extraction of alkaloids and chromatographic conditions

Plant materials were powdered and then sonicated for 10 min with 10 ml of chloroform-methanol-ammonium hydroxide (25%) (15:15:1) per 100 mg of sample. Extraction container was left at room temperature for 1 h then filtered and washed with 1 ml of chloroform twice. After solvent evaporation, 5 ml of chloroform and 2 ml of sulfuric acid (1 N) were added to dried sample and mixed thoroughly. Then chloroform fraction was removed and pH was adjusted to 10 by using NH_4OH. The alkaloids were extracted by chloroform (3 times, 1 to 2 ml). After addition of anhydrous Na_2SO_4, the extract was filtered and residue was washed with 1 to 2 ml of chloroform. Finally the extracted solvent was evaporated and the samples were dissolved in 0.5 ml methanol and kept at -8°C until analysis.

The samples were analyzed using a buffer containing 50 mM potassium dihydrogen orthophosphoric acid adjusted to pH 3.0 by orthophosphoric acid: Acetonitrile (80:20 v/v). The mobile phase was pumped at a constant flow rate of 1.4 ml min^{-1} and detection was carried out at a wavelength of 215 nm.

Preparation of calibration curves

Calibration curves were constructed by plotting peak areas versus concentration of atropine and scopolamine, and regression equations were calculated.

Method validation

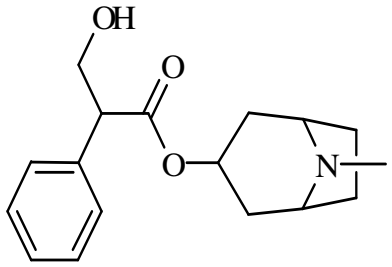
Method was validated in terms of sensitivity, linearity, precision, and recovery according to the guidelines of the International Committee of Harmonization (ICH) (1996).

RESULTS AND DISCUSSION

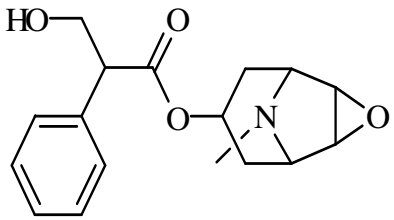
Results of HPLC analysis are presented in Table 2 that compares the values of atropine and scopolamine in different organs of *H. arachnoideus* plants collected from five parts of country. A good separation was achieved with resolution of 3.42. The retention time of atropine and scopolamine were 6.66 and 4.56, respectively (Figure 1). The linearity of the method was tested using the standard solutions of atropine and scopolamine. The calibration curve of atropine was linear in the range of 4 to 400 µg/ml, with correlation coefficient of 0.9997. The linearity

Table 2. Summary of validation parameters.

Parameters	HPLC method	
	Atropine	Scopolamine
Linearity range (µg/ml)	4.0-400.0	0.8-80.0
Correlation efficient	0.9997	0.9991
LOD (µg/ml)	5.2	1.9
LOQ (µg/ml)	17.4	6.4
Recovery %	82.3	78.5
Intra-day precision (RSD %)	2.8-6.2	4.1-10.7
Inter-day precision (RSD %)	1.9-5.5	6.8-9.3



Atropine



Scopolamine

Figure 1. Chemical structures of atropine and scopolamine.

of scopolamine was from 0.8 to 80 µg/ml, with correlation coefficient of 0.9991. The calibration curves were represented by linear equations of Y=15292X+46705 and Y=9088.3X+6902.4 for atropine and scopolamine, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations LOD=3.3×N/B and LOQ=10×N/B where N is standard deviation of peak area (n=3), taken as measure of the noise and B is the slope of the corresponding calibration curve. The LOD and LOQ values were 1.92 and 6.40 ppm for scopolamine and 5.15 and 17.40 ppm for atropine, respectively. The intra-day and inter-day precision of the sample analysis were expressed in terms of relative standard deviation (RSD) percent with respect to peak area. The former was between 2.84 to 6.2% for atropine and 4.11 to 10.7% for scopolamine. Relatively higher values of RSD% for scopolamine are associated with its lower amounts in analyzed samples. The inter-day precision was calculated for atropine and scopolamine 3 times a week (Table 3). The accuracy of the method was evaluated by calculating the recovery of atropine and scopolamine by the standard addition method. The analyzed samples were spiked with extra concentration levels of 100 ppm from atropine and scopolamine and then mixtures were reanalyzed by the same method. The percent recovery was found to be 82.3% for atropine and 78.5% for scopolamine.

According to the results of present study, scopolamine was only the main alkaloid of two *H. arachnoideus* populations seeds. In contrast, atropine was detected as the principal alkaloid of almost all parts, especially roots. The highest and lowest content of tropane alkaloids was observed in plants of populations from Zangane village and Ashtian Mountain, respectively. On the other hand, the alkaloid content of seeds was in general more than other parts tested (Table 2). Differences among partitioning of tropane alkaloids in plants producing these compounds have been repeatedly reported. In consistent with our results, for example, it has been reported that in *Datura stramonium* the total amount of disappeared alkaloids in growing parts are equal to those found in the seeds (Demeyer and Dejaegere, 1997). However, Miraldi et al. (2001) found atropine as the main alkaloid of different plant parts at different stages of growth. These authors reported stems as the plant part with the highest content of tropane alkaloids (atropine and scopolamine). Chalabian and Majd (2004) also found the higher rate of hyoscyamine production than scopolamine in all of the samples collected from different phenological stages of *Hyoscyamus reticulatus*. According to literature, roots are primary sites of tropane alkaloids biosynthesis and subsequent modification of these compounds occurs in

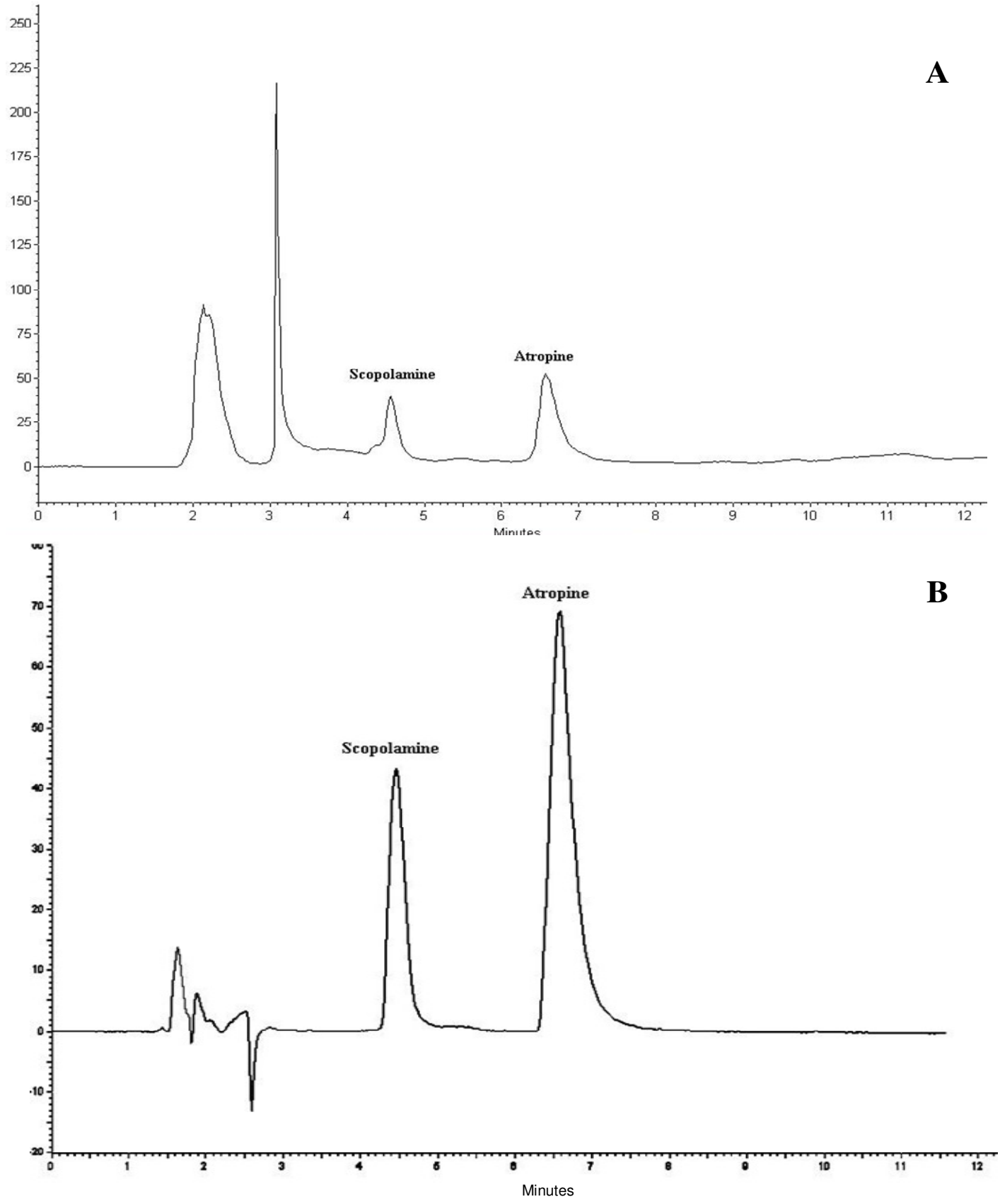


Figure 2. HPLC chromatogram of a sample (A) and standard (B).

Table 3. Atropine and scopolamine composition of different populations of *H. arachnoideus*.

Sample code		Amount (mg/Kg) DW		Atropine/Scopolamine
		Scopolamine	Atropine	
Roots	1	44.6 ± 3.0	180.0 ± 1.5	4.0
	2	4.8 ± 0.3	195.0 ± 1.5	40.0
	3	15.7 ± 0.9	255.1 ± 1.1	16.0
	4	4.6 ± 0.4	395.1 ± 1.8	85.8
	5	12.6 ± 0.3	275.6 ± 1.2	21.0
Aerial parts	1	86.3 ± 1.0	120.0 ± 1.2	1.4
	2	21.2 ± 0.6	190.0 ± 3.0	9.0
	3	34.0 ± 0.4	310.1 ± 3.0	9.0
	4	42.6 ± 1.2	50.1 ± 1.1	1.1
	5	48.8 ± 0.5	125.1 ± 1.2	2.6
Seeds	1	431.0 ± 3.0	448.0 ± 4.0	1.0
	2	829.0 ± 4.3	477.0 ± 4.1	0.6
	3	52.3 ± 0.8	90.0 ± 0.9	1.7
	4	81.2 ± 1.0	29.2 ± 0.4	0.4
	5	50.8 ± 0.6	65.0 ± 0.7	1.3

the aerial parts (Dräger, 2002; Miraldi et al., 2001). In this study, the atropine to scopolamine ratio was high in roots but it descends in aerial parts and seeds near to, or even beneath 1. This phenomenon can be attributed to the transformation of atropine to scopolamine through its transfer to aerial parts. Results of present study also showed the different ability of populations tested in the production of tropane alkaloids. Populations from Zangane village were identified as the most capable populations in the accumulation of these medicinally important compounds. Genetic factors, climatic conditions and/or interaction between them are possible reasons for the variability observed (Hadian et al., 2010; Loziene and Venskutonis, 2005). In conclusion, our findings confirmed and extended results of previous studies on the different patterns of tropane alkaloids accumulation in the organs of producing plants (Chalabian and Majd, 2004; Demeyer and Dejaegere, 1997; Miraldi et al., 2001). In all cases, with some exceptions, atropine was the predominant alkaloid of different plant parts and the ratio of atropine to scopolamine was decreased from roots to seeds.

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