Quantification of Atropine in Leaves of *Atropa belladonna*: Development and validation of method by high-performance liquid chromatography (HPLC)

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The leaves of *Atropa belladonna* (L) are characterized by the presence of the alkaloid atropine, known for the antimuscarinic activity. They are used in therapy, mainly in homeopathic preparations. An HPLC method was developed and validated to quantify atropine in belladonna leaves. The samples were extracted with methanol, followed by acid-base extraction with 5% HCl and dichloromethane. Analysis by HPLC was performed on C18 column, in a linear gradient system using two mobile phases (water and acetonitrile), both acidified with trifluoroacetic acid. The determinations were performed using a reference standard and a diode array detector at 210 nm. The method was validated and proved to be specific/selective, comparing the UV profiles and the purity of the atropine peaks in reference and sample solutions and analyzing sample solutions with and without addition of standard, which produced an increase only of the area of peak of the sample, without changing the area of the adjacent peaks. The linearity (50 - 200 µg/mL) was provided by analysis of the analytical curves of atropine, with $r^2 = 0.9996$. LOD and LOQ were 3.75 and 11.4 µg/ml, respectively. The method was precise, reproducible and accurate, and presented recovery equal to 103.0%. The method was considered robust for the analyzed parameters. Four commercial samples were analyzed and the mean levels of atropine found ranged from 0.16 - 0.27 %. Thus, the method developed is effective for atropine quantification in *A. belladonna* leaves, because meets validation requirements of current legislation, besides presenting itself as a useful tool in the control of the quality of this plant material.

Keywords: *Atropa belladonna*; Atropine; HPLC method, Validation; Quality Control.

Introduction

Atropine is a tropane alkaloid that plays an important role in therapy because of its inhibitory action on acetylcholine. These metabolites are known as antimuscarinic or blocking substances of cholinergic muscarinic receptors. Although it can be performed by synthetic route, obtaining this metabolite is still performed from natural sources, mainly from plants of the Solanaceae family, such as *Atropa Belladonna* L., which present atropine as the major compound in its composition (1-4).

Scientific studies have been increasingly driven to develop analytical methodologies for the quantification of chemical markers in plant materials, resulting in quality materials that meet the control parameters recommended by the legislation due to the importance of raw materials of plant origin and plant medicines for the pharmaceutical industry. Plant medicines used as raw material in the production of herbal medicinal

products should be subjected to qualitative and quantitative analysis of the active ingredients and / or chemical markers, when known. The methodologies developed and used in official compendia (pharmacopoeias) must be validated and all validation steps (specificity/selectivity, linearity, limits of detection (LOD) and quantification (LOQ), precision, precision and robustness) must be proven (5-9). The official compendia describe the determination of total alkaloids in *A. belladonna* by titration method (10-12) and also by gas chromatography (13), but these methods employ large amounts of solvents, mainly in the extraction stage and are difficult to reproduce.

Developing analytical methods that are capable of identifying, separating or quantifying analytes in plant materials becomes a challenge, given the complexity of the matrices of these materials (14-16). Several authors describe methods to quantify tropane alkaloids in the Solanaceae Family's plant raw materials, with emphasis on *Datura*, *Atropa* and *Hyoscyamus* species. Among the

techniques used, High Performance Liquid Chromatography (HPLC) is the most widely developed for this purpose (17-24). Buffered mobile systems or having only one salt dissolved in their composition (K_2HPO_4 , KH_2PO_4 , for example) are best described for quantifying tropic type alkaloids (18-23). Ashtania and Sefidkonb (2011) (21) quantified total alkaloids (atropine and scopolamine) in *A. belladonna* leaf cultures using these systems. In 2005, Kursinszki and colleagues (24) developed a method, without using mobile phase systems with buffer solution, to quantify these alkaloids in cultivated roots of this species. Thus, a quantitative method to determine atropine in this plant drug becomes interesting considering the importance of guaranteeing the quality of this material for its use in the industry.

Experimental

Chemical and Standard

All solvents (HPLC and analytical grade) were purchased from Tedia (Fairfield, CA, USA). Chemical Standard of atropine ($\geq 99\%$) (Figure 1) was purchased from Sigma-Aldrich (São Paulo, Brazil). The other analytical grade reagents were purchased from F. Maia (Cotia, SP, Brazil) and Vetec (Duque de Caxias, RJ, Brazil).

Figure 1 Chemical structure of atropine.

Plant material and sample preparation

Four samples the *A. belladonna* leaves were commercially available and named Ab-01, Ab-02, Ab-03 and Ab-04. The sample Ab-01 was used to develop and validate the method. Approximately 0.25 g of the powdered drug (particle size - 180 μ m) was weighed accurately in 50 ml erlenmeyer flask and 15 ml of methyl alcohol was added. The flask was closed with plastic film and taken to the ultrasonic bath for 60 minutes (25, 26). The crude methanolic extract was filtered through filter paper into a 50 mL round bottom flask. The Erlenmeyer flask was washed with 2 x 1 mL methyl alcohol p.a. The solvent was removed on a rotary evaporator without exceeding 40 °C. The dried extract of the flask was transferred to a 250 mL separatory funnel using 25 mL of 5% hydrochloric acid (5% HCl) with ultrasonic aid to facilitate the solubilization of the extract in acid medium. The aqueous acid phase was extracted with 3 x 20 mL

dichloromethane using the first 20 mL to clear the residue from the 50 mL round bottom flask. The organic phases of the washes were discarded. The aqueous extract was basified with ammonium hydroxide (25% NH_4OH) to pH 10. The alkaloids were extracted with 5 x 20 mL of dichloromethane. The organic phases were combined and dried with 15 g of anhydrous sodium sulfate. Then it was filtered through filter paper into a porcelain crucible, the sodium sulfate from the filter paper was washed with 2 mL of dichloromethane. The solvent was evaporated to dryness in a water bath, without exceeding 40 °C. The dry extract of alkaloids was solubilized with methanol into a 5 mL volumetric flask and filtered through a 0.45 μ m membrane into a 2 mL vial.

Instrumentation and HPLC Conditions

The atropine analyses were performed using a Waters-Alliance 2695 equipped with a photodiode array detector (PDA) and an Empower software for data processing. Chromatographic separation was carried out in a Kromasil C18 column (150 x 4,6 mm i.d.; particle size, 5,0 μ m) guarded by a pre-column filter in-line Ultra (Phenomenex, USA). The mobile phase was composed of water (A) and acetonitrile (B), acidified with 0.01 e 0.08% trifluoroacetic acid (v/v), respectively, filtered through a 0.45 μ m porous nylon membrane and degassed in ultrasonic bath for 30 minutes. The gradient profile was adjusted as follows: 0.00 min. 5% eluent B, 15.00 minutes eluent 100% B. After isocratic elution was carried out up to 20.00 min eluent 100% B. Between the injections a 10 min equilibrium was performed using 5% B eluent. Sample were injected with auto-sampler in a volume of 10 μ L. Eluent system was pumped at a flow rate of 0.7 mL/min and detection wavelength was set to 210 nm.

Method Validation

System Suitability

Tests were performed to verify the adequacy of the chromatographic system for the analysis. During validation, peak area parameters, retention time and number of plates, referring to the peak atropine standard, were analyzed and evaluated by the relative standard deviation of the measurements on the different validation days.

Specificity/Selectivity

The data were obtained by comparing the ultraviolet profile and the purity of the atropine peak from the injection of the standard atropine solution (125 μ g/mL) and the sample solution and analyzing the areas of atropine in solution of the sample with and without addition of standard atropine.

Linearity and Limits of Detection and Quantification (LOD e LOQ)

Performed by injecting different dilutions of a stock solution of atropine standard (500 µg/mL). Three curves of the reference substance were constructed on different days with 7 points (injected in triplicate), corresponding to a concentration range of atropine between 50 and 200 µg/mL. The linearity of the method was verified from the measurement of the area of the peak referring to the chemical marker in the chromatogram. The regression coefficient was evaluated and the statistical treatment was performed using the least squares method and analysis of variance.

LOD and LOQ were calculated from the data obtained in the linearity study and through Equations: $LOD = (3.3 \times s) / I$ and $LOQ = (10 \times s) / I$, at were, s = Mean standard deviation of the intercept of the three curves and I = Average slope of the three curves.

Precision

Repeatability (intra-days) was performed by analyzing six extracts from the sample, injected in triplicate in HPLC.

Intermediate precision (inter-days) was performed by analyzing, on three different days, three extracts were prepared and injected in triplicate on HPLC.

Interlaboratory reproducibility was performed in collaboration with the pharmacognosy laboratories of the Federal Universities of Paraná (UFPR) and of Pernambuco (UFPE). The sample and standard atropine were sent for analysis along with protocol describing the methodology. The analysis were performed in triplicate and the results found were compared and the DPR was calculated among the means of the determinations in the different laboratories. Atropine levels and the relative standard deviation (RSD) of the means were evaluated.

Accuracy

Standard atropine solution at concentrations of 80, 100 and 120% (relative to the extraction of plant material) was added at the final stage of the extraction process. The levels recovered from atropine and RSD of the means was evaluated.

Robustness

Performed by modifying previously established parameters of analysis. The atropine levels were calculated and the RSD and relation to the normal condition analysis was evaluated. The modified parameters were: mobile phase flow ($\pm 0,05$ mL/min); Wavelength (± 5 nm) and column supplier (Nano Separation Technologies - NST).

Analysis of Commercial Samples

Samples Ab-01, Ab-02, Ab-03 and Ab-04 were prepared as described in item 2.2 and analyzed in the developed and validated method.

Results

Method Development

In the development of the method, the standard and sample solutions produced the chromatograms shown in Figure 2, where it is possible to identify the atropine peak at the retention time of approximately 7.7 minutes.

Figure 2 Overlay chromatogram of the atropine sample and standard solutions.

Method Validation

System Suitability

The routine analysis of the atropine standard was achieved under the conditions of the method developed by HPLC. The parameter values and their relative standard deviation (RSD%) are shown in Table 1. In summary, the results for each parameter demonstrated that the system is suitable for atropine analysis.

Table 1 Results of system suitability for atropine quantification method in *A. belladonna* leaves.

Compound	Parameter	Mean	RSD (%)
Atropine	Retention Time (min)	7.691	0.8008
	Number of plates (N)	15231.2	3.523
	Peak Area (mV.s)	1971669	1.114

Specificity / Selectivity

The specificity/selectivity was evidenced with UV profiles of the atropine peak in similar reference and sample solutions, as well as the observation of the peak purity shown in Figure 3 (a and b). The selectivity was confirmed by the addition of a reference substance in the sample solution, which produced an increase only in the peak area of the sample, without changing the area of the adjacent peaks (Figure 3c). The areas measured before and after standard addition were 892459 and 1964117 for the atropine peak and there was no change in the other areas of the chromatogram. It was possible through the tests to observe that the method is specific and selective for analysis of the metabolite under study

Figure 3 (A) UV profile of the atropine sample solution and the standard; (B) Peak purity of atropine sample solution; (C) Overlay chromatogram of the atropine sample solution with and without addition of atropine standard.

Linearity, LOD and LOQ

Was determined of reference solutions with concentrations range 50-200 µg/mL, with regression equation $Y = 15490x + 18019$ and $r^2 = 0.9996$. LOD and LOQ were 3.75 and 11.3 µg/mL, respectively. The values obtained in the analysis of variance (ANOVA) show that there are significant differences between the different concentrations and respective areas (P -value < 0.05 and $F > F_{critic}$), with a confidence interval of 95% ($\alpha = 0.05$).

Precision

The method proved to be precise, with mean atropine levels of 0.267% and RSD equal to 2.78% for repeatability and intermediate steps. The results of Interlaboratory Reproducibility were also satisfactory, since the mean amount between laboratories was equal to 0.262% with RSD of 1.38%. Table 2 presents the results of this validation step.

Table 2 Results the precision step for atropine quantification method in *A. belladonna* leaves.

Precision						
Intra-days		Inter-days				
Replicate	% Atropine e	Replicate	% Atropine Day 1	% Atropine e Day 2	% Atropine e Day 3	
1	0.2651	1	0.2725	0.2681	0.2522	
2	0.2505	2	0.2760	0.2661	0.2527	
3	0.2803	3	0.2733	0.2638	0.2631	
4	0.2754	Mean	0.2739	0.2660	0.2560	
5	0.2685	RSD (%)	0.6666	0.8215	2.401	
6	0.2620					
Mean	0.2670	Mean: 0.2657		RSD (%): 2.776		
RSD (%)	3.922					
Reproducibility Interlaboratorial						
Replicate	% Atropine UFGRS	% Atropine UFPR	% Atropine UFPE			
1	0.2453	0.2602	0.2681			
2	0.2673	0.2595	0.2662			
3	0.2655	0.2606	0.2638			
Mean	0.2594	0.2601	0.2660			
RSD (%)	4.718	0.2000	0.8215			
Mean: 0.2618 %		RSD (%): 1.34 %				

Accuracy

The recovered levels of atropine standard are shown in table 3. The mean recovery of method was 103.0% with RSD of less than 5%, according to the official guidelines. In this way, developed method can be considered accurate.

Table 3 Results the recovery study to evaluate accuracy for atropine quantification method in *A. belladonna* leaves.

Solution	Standard Added (µg/mL)	Experimental Concentration (µg/mL)	Average Recovery (%)
SS*	-	56.02	-
ASS*	55.10	-	-
RS80*	44.08	100.7	101.4
RS100*	55.10	112.9	103.3
RS120*	66.12	125.0	104.4
Mean			103.0
RSD (%)			1.484

*SS – Sample Solution; ASS – Atropine Standard Solution; RS80,100,120 – Recoveries Solutions 80, 100, 120 %

Robustness

The method was robust, considering that no altered parameter produced a RSD result higher than 5% in relation to the mean atropine content of the analyzes under normal conditions. Table 4 shows the results obtained in the method validation step.

Table 4 Results of robustness step for atropine quantification method in *A. belladonna* leaves.

Variation	Atropine (%)	RSD (%) ^a
Mobile phase flow (0.65 mL/min)	0.2641	3.123
Mobile phase flow (0.75 mL/min)	0.2531	0.1125
Wavelength (λ = 205 nm)	0.2516	0.3123
Wavelength (λ = 215 nm)	0.2498	0.7992
Column supplier (NST)	0.2564	1.020

^aRSD calculated relative to the normal analysis condition

Analysis of Commercial Samples

Analysis of commercial samples revealed levels varying from 0.16 to 0.27% of atropine (Table 5), indicating marked variability between the different samples.

Table 5 Analysis result of atropine content in commercially obtained samples.

Replicate	Ab-01 (% atropine)	Ab-02 (% atropine)	Ab-03 (% atropine)	Ab-04 (% atropine)
1	0.2681	0.2051	0.1556	0.2769
2	0.2662	0.2100	0.1570	0.2705
3	0.2638	0.1931	0.1686	0.2702
Mean	0.2660	0.2027	0.1604	0.2726
RSD (%)	0.8215	4.2973	4.4572	1.3918

Conclusion

Considering the reports of determination of this type of secondary metabolite, using HPLC, in different plant materials, it was possible to obtain a method successfully developed for this purpose. The developed method was validated, showing precision, accuracy and robustness, with all the steps presenting RSD results below 5%.

A. belladonna is a plant material widely used in therapeutics and, since official quantification methods involve titration techniques or gas chromatography, the method developed in this work is advantageous because it uses a technique that quantifies in a simple, fast and efficient way of the active in the plant drug, can be applied to the quality control routines, of the studied plant drug, with confidence.

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