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Analysis of atropine, its degradation products and related substances of natural origin by means of reversed-phase high-performance liquid chromatography

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Abstract

Chromatographic separation and quantification methods of tropa alkaloids were often described. In order to separate atropine from its degradation products ion-pair chromatography (IPC) has been frequently applied. Beside long equilibration times IPC often suffers from poor robustness. The aim of this study was to develop robust and simple HPLC methods for both stability testing of atropine solutions and limitation of related substances in atropine from plant material. Using a hydrophilic embedded RP18 column and a gradient elution gave baseline separation of all components.

Keywords: Stability studies; Pharmaceutical analysis; Atropine; Alkaloids

1. Introduction

Atropine is a parasympatholytic agent used for the treatment of e.g. spasm, bradycardia, surgery, or organophosphorus poisoning. It belongs to the group of tropa alkaloids and the skeleton consists of tropic acid and tropine. The racemic mixture of hyoscyamine can be isolated from plants of the solanaceae family, e.g. *Atropa belladonna* or *Hyoscyamus niger* [1]. Even though a huge number of synthesis pathways [2,3] have been described atropine for pharmaceutical purposes is always of natural origin. Thus, it can be accompanied by structural related substances occurring in the plants, i.e. norhyoscyamine and isomer, 6-hydroxyhyoscyamine, 7-hydroxyhyoscyamine, hyoscine (scopolamine), and littorine[1] [4]. Upon storage, degradation reactions can take place. Under slightly acidic conditions the ester hydrolysis leads to tropine and tropa acid, the latter eliminates water to give atropa acid. Under basic conditions atropine can directly eliminate a water molecule resulting in apoatropine (atropamine), which can hydrolyze to tropine and atropa acid on the one hand or form dimers to give belladonine on the other hand [3,5] (see Fig. 1). In any case, apoatropine has to be regarded as the main degradation product.

Atropine has a long standing tradition in clinical use. Hence, a lot of different analytical methods have been described with regard to the separation of the enantiomers (e.g. [6,7]) and related components in plants [8]. Since the stability of atropine is limited, a huge number of chromatographic methods on the separation of degradation products focusing on ion-pair high-performance liquid chromatography (IPC) have been reported [9–13]. Consequently, the newest edition of the European Pharmacopoeia [4] limits the related substances by means of the IPC using RP8 material as the stationary phase and gradient elution consisting of a mixture of an aqueous solution of phosphate buffer, sodium dodecyl sulphate and acetonitrile as mobile phase A and acetonitrile as mobile phase B. However, IPC methods especially in combination with gradient elution tend to be time-consuming due to long equilibration periods and they are often not very robust. Thus, the aim of this study was to develop a HPLC method without using ion-pair reagents. A hydrophilic embedded RP18 column seemed to be suitable to separate the

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[1] The European Pharmacopoeia (EP) monograph describes falsely isolittorine which has never been found in any plant.

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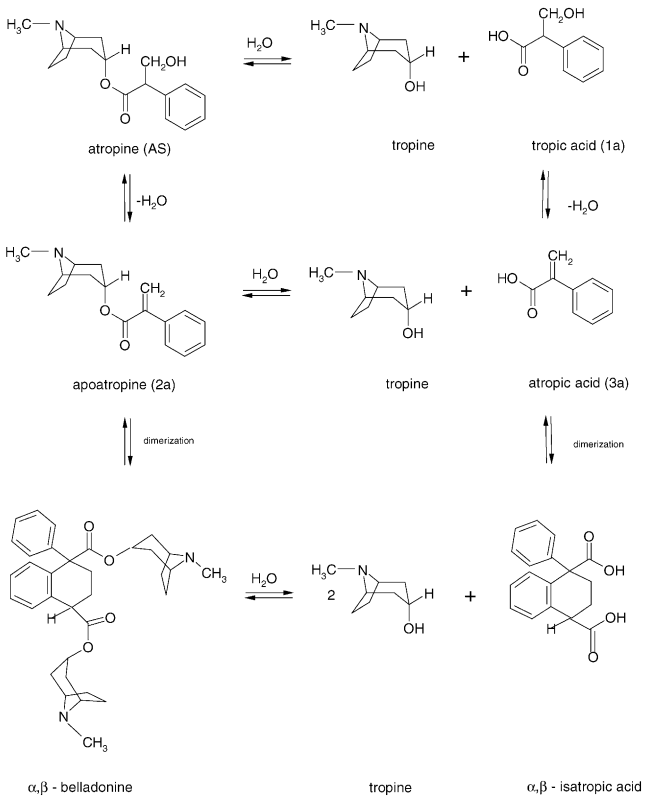

Fig. 1. Degradation pathways of atropine in an aqueous solution.

degradation products and additional by-products occurring in plants. On the one hand the method was developed for stability tests of solutions for eye drops (0.1 and 1% atropine) and injections (1% atropine). On the other hand a corresponding method was optimized to evaluate the related substances in atropine lots.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade except acetonitrile (HPLC grade). Atropine sulfate monohydrate and tropic acid were purchased from Fluka (Buchs, Switzerland), norhyoscyamine, 6-hydroxyhyoscyamine, 7-hydroxyhyoscyamine, hyoscine and littorine were provided by Boehringer Ingelheim (Ingelheim, Germany). Apoatropine was synthesized starting from atropine sulfate according to Hesse [14], and atropic acid from tropic acid according to Raper [15]. Benzalkonium chloride was purchased from Caelo & Loretz (Hilden, Germany), sodium chloride from Merck (Darmstadt, Germany) and Na_2Edetate dihydrate from Synopharm (Barsbüttel, Germany). Acetonitrile was purchased from Carl Roth (Karlsruhe, Germany), sodium dihydrogenphosphate dihydrate, and orthophosphoric acid 85% from Merck Eurolab (Bruchsal, Germany). All samples and buffers were prepared using ultrapure Milli-Q water (Millipore, Milford, MA, USA) and were filtered through a 0.2 μm membrane filter (Sartorius, Göttingen, Germany) prior to use.

2.2. Chromatographic conditions

2.2.1. Apparatus

The liquid chromatography was recorded on an Agilent System 1100 LC (Böblingen, Germany) consisting of a vacuum degasser, a binary pumping system forming a high pressure gradient by a static mixer (delay volume of 600–900 μL), an autosampler, a thermostatted column compartment, an UV-visible diode array detector and a LC 3D ChemStation equipped with HP Kayak XM600 and 3DSoftware (Version 8.04).

2.2.2. Stability studies

2.2.2.1. Standard solution I preparation. Solvent: a mixture of 80% 20 mM phosphate buffer, pH 2.5 (adjusted with conc. phosphoric acid) and 20% acetonitrile. Stock solution: 100.0 mg atropine sulfate monohydrate, 100.0 mg tropic acid, 100.0 mg apoatropine and 100.0 mg atropic acid were dissolved in 100.0 mL solvent, 10.0 mL of this solution was diluted to 100.0 mL with the solvent.

Sample solution preparation: atropine eye drop solution (0.1 and 1%): 0.1 g and 1.0 g atropine sulfate monohydrate, respectively, 10.0 g 0.1% benzalkonium solution (0.2 mg benzalkonium chloride, 1.0 g Na_2Edetate dihydrate, aqua ad iniectabilia ad 100.0 mL), 0.9 g NaCl and aqua ad iniectabilia ad 100.0 mL.

2.2.2.2. HPLC conditions. Separations were performed on a Thermo Hypersil Aquasil C_{18} analytical column (5 μm particle size, 125 mm × 4 mm i.d.) characterized by a hydrophilic endcapping (Thermo Hypersil-Keystone, Bellefonte, PA, USA).

Gradient elution was applied 20 mM phosphate buffer, pH 2.5 (adjusted with conc. phosphoric acid) and acetonitrile. Gradient was run with 20% acetonitrile from 0 to 3 min, 20–40% acetonitrile from 3 to 5 min and 40% acetonitrile up to 10 min, postrun with 20% acetonitrile for 2 min.

After injection of 20 μL of the standard solution I and 20 μL of a 1:100 dilution of the sample solution (1%), respectively, the HPLC system was operated at a flow rate of 2.0 mL/min. The column temperature was set at 25 °C and the detection wavelength at 215 nm. The analyses are not valid in the case the resolution of atropine and tropic acid is less than two.

2.2.3. Related substances analysis

2.2.3.1. Standard solution II preparation. Stock solutions: 1.0 g atropine sulfate monohydrate, 2.0 mg 7-hydroxyhyoscyamine, 2.0 mg 6-hydroxyhyoscyamine,

2.0 mg hyoscine, 4.0 mg norhyoscyamine, 2.0 mg littorine, 2.0 mg tropic acid, 3.0 mg apoatropine and 2.0 mg atropic acid, respectively, were dissolved in 100.0 mL in the solvent. Then 10.0 mL of each solution was diluted to 100.0 mL with the solvent. The mixture of all components was prepared by using 10.0 mL of each stock solution and 10.0 mL of the solvent.

2.2.3.2. HPLC conditions. Separations were also performed on a Thermo Hypersil Aquasil C_{18} analytical column (see Section 2.2.2). Gradient elution was applied 20 mM phosphate buffer, pH 2.5 (adjusted with conc. phosphoric acid) and acetonitrile. Gradient was run with 25% acetonitrile from 0 to 4 min operating at a flow rate of 0.6 mL/min, 23% acetonitrile from 4 to 6 min at a flow rate of 1.0 mL/min and 45% acetonitrile up to 12 min at a flow rate of 1.0 mL/min, postrun with 25% acetonitrile for 2 min. The column temperature was set at 25 °C and the detector wavelength at 215 nm. Then 20 μL of the standard solution II was injected. The analyses are not valid in the case the resolution of 7- and 6-hydroxyhyoscyamine acid is less than 1.2.

3. Results and discussion

3.1. Stability studies and assay

Since the degradation produces both acidic components, i.e. tropic acid and atropic acid, and a basic compound, i.e. apoatropine, the simultaneous separation using conventional reversed phase chromatography is difficult. Depending on the pH of the mobile phase either component will not show retention. Therefore, ion-pair reagents are often added to the mobile phase; depending on pH cationic and anionic reagents, such as tetrabutylammonium sulfate and heptanesulfonic acid, were applied, respectively [5,9–13]. In order to avoid ion-pair reagents a RP18 column was used which is characterized by a hydrophilic endcapping. The polar groups of this material are capable of interacting with ionized and neutral polar moieties of the analytes and they allow to apply a highly aqueous mobile phase. Thus, in acidic media of pH 2 to 3 the acids will be neutral and the basic components will be positively charged. Taking the cut-off value of the phosphate buffer of 210 nm into consideration the detection wavelength was set at 215 nm. At this wavelength all compounds studied show a sufficient absorption and an overloading of the column can be avoided. Adding 20% acetonitrile gave a rather good separation of the atropine, tropic acid, atropic acid and apoatropine (see Fig. 2a). In order to shorten the analytical time and improve the sensitivity and peak shape of atropic acid and apoatropine a gradient, characterized by an increased amount of acetonitrile, was applied after the elution of tropic acid. However, atropic acid and apoatropine are eluted isocratically in order to guarantee robustness (see Fig. 2b).

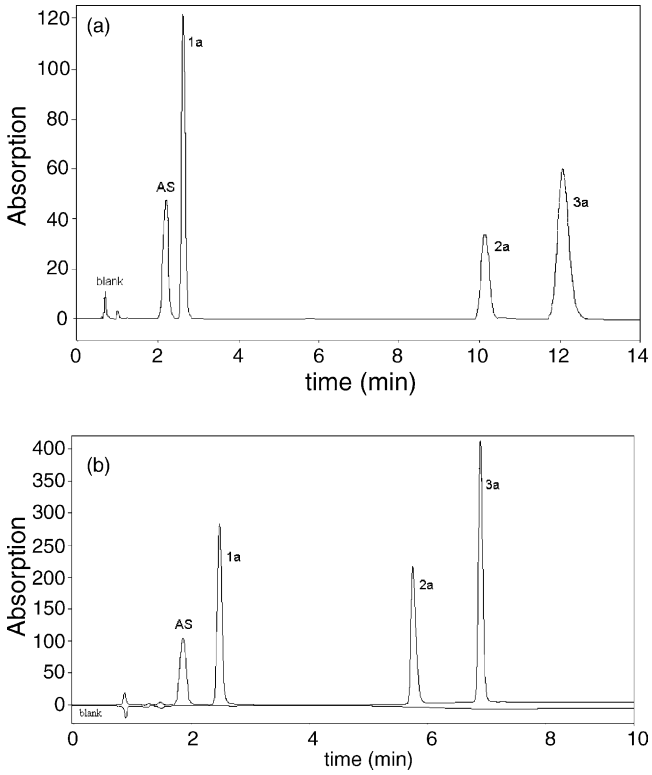

Fig. 2. Chromatograms of atropine and its degradation products applying (a) the isocratic method (80% 20 mM phosphate buffer, pH 2.5 and 20% acetonitrile) and (b) the gradient method starting isocratically with 80% 20 mM phosphate buffer, pH 2.5 and 20% acetonitrile for 3 min, increasing the acetonitrile percentage to 40% till 5 min and running again isocratically to 10 min.

The aforementioned method was also used for the atropine assay of solutions for eye drops and was validated in this respect. The linearity for a 0.1 and 1% atropine solution was studied at five equidistant concentration levels in a range from 8.0 to 12.0 mg atropine sulfate in 100.0 mL of the mobile phase. The calibration standards were measured six times randomly. Linear least-squares fitting method was used for computing the calibration line. Figures of merit of the calibration line can be found in Table 1.

3.2. Validation of the stability test method

The precision of the method used for both stability evaluation and assay was checked by the double measurement of six 1:100 dilutions of the 1% atropine eye drops and calculation of the content (see Table 1). The standard deviation was found to amount to 0.541, the relative standard deviation 0.0054 (0.54%) and the confidence interval ±0.433. Run-to-run precision of the peak area for the atropine peaks expressed as relative standard deviation (R.S.D.) of 10 replicate injections amounted to 0.09% which is acceptable. For checking the intralaboratory precision six 1:100 dilutions

Table 1
Figures of merit for linearity, precision and accuracy

Linearity	Range (mg/100 mL)	7.96–12.21	
	Intercept	17.42	
	Slope	85.84	
Precision of the method ($n = 6$)	Mean (mg/100 mL)	100.07	
	Standard deviation	0.541	
	R.S.D. (%)	0.54	
	Confidence interval	±0.43	
Precision of the system($n = 10$)	R.S.D. (%)	0.09	
Intralaboratory precision ($n = 6$)		Day 1	Day 2
	Standard deviation	0.541	0.452
	R.S.D. (%)	0.54	0.45
Accuracy ($n = 6$)		HPLC	Titration
	Mean (mg/100 mL)	100.07	99.7
	Standard deviation	0.541	0.270
	R.S.D. (%)	0.54	0.27
	Confidence interval	±0.433	±0.216

of the 1% atropine eye drops were measured at two different days, with two different column lots and two different mobile phase lots (see Table 1). The standard deviation of the second measurement was found to be 0.452 and the relative standard deviation 0.0045 (0.45%). The results of both measurements were compared by *F*-test and mean-*t*-test ($1-\alpha = 0.95$). The tests showed homogeneity of the variance and no significant difference between the mean values.

The accuracy was investigated comparing the results of the HPLC measurements for method precision with the titration method of the Ph. Eur 4.7 being a non-aqueous titration with perchloric acid/anhydrous acetic acid and a potentiometric detection (see Table 1). The standard deviation of the titration was found to be 0.27 and the relative standard deviation 0.27%. The results of the HPLC method and the titration method were compared by *F*-test and mean-*t*-test ($1 - \alpha = 0.95$). The tests showed homogeneity of the variance and no significant difference between the mean values. Taken together the HPLC method is able to provide accurate results.

Since no effect of column lots, column temperature and mobile phase lots on the separation efficiency for peaks of atropine and tropic acid was observed (data not shown), the robustness of the method was checked with respect to more critical parameters such as variation of the flow rate (1.9; 2.0; 2.1 mL/min), detection wavelength (213; 215; 217 nm), variation of the composition of the mobile phase (content of acetonitrile: 18 volumes; 20 volumes; 22 volumes) and the pH of the 20 mM phosphate buffer (2.4; 2.5; 2.6) using the resolution of the peaks of a 0.01% atropine eye drop solution spiked with 0.01% tropic acid, and the peak area of atropine. Variations of the flow rate resulted in almost no variation of the resolution, but relatively large variations of the peak area indicating a limited robustness with respect to the flow rate. Since the wavelength of detection is in the heavily descending slope of the atropine absorption spectrum, a variation of the wavelength results in huge a variation of the peak areas. Hence, the method is not robust against variation of the detection wavelength. Variation of the pH value of mobile phase did not influence the resolution or the peak area. In contrast, variation of the content of acetonitrile in the isocratic part of the chromatogram diminishes the resolution in a way that a baseline separation between atropine and tropic acid is not longer guaranteed indicating that the method is not robust against the composition of the mobile phase. Taken together the method is not very robust against variations of the flow rate, the detection wavelength and the content of acetonitrile in the mobile phase. Hence, a system suitability test has to be performed before analysis. Since the separation of atropine and tropic acid is critical, the results of the analysis are only valid in the case the resolution of this pair is higher than two.

3.3. Analysis of related substances

The transparency statement of the new monograph atropine in Ph. Eur. 4.7 [4] consists of degradation products of atropine and related compounds of natural origin, namely apoatropine, noratropine, tropic acid, 6-hydroxyhyoscyamine, 7-hydroxyhyoscyamine, hyoscine and littorine (see Figs. 1 and 3).

In the next step of the study, it was checked whether the aforementioned method is capable of separating the natural components from atropine. Applying the gradient elution method described above all components are separated with exception of two pairs, 6-hydroxyhyoscyamine/hyoscine and littorine/tropic acid (see Fig. 4a). Thus, the method is a good starting point for further optimization. Increasing the amount of the acetonitrile, slightly changing the composition of the gradient and slowing down the flow rate to 0.6 mL/min in the beginning gave a sufficient baseline separation for a limit test of all components mentioned in the transparency statement of the new atropine monograph in Ph. Eur. 4.7. In addition,

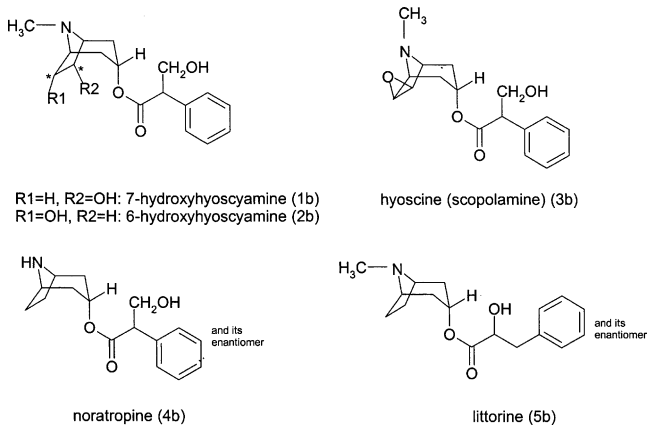

Fig. 3. Structural formulae of related substances of atropine, 7-hydroxy-hyoscyamine, 6-hydroxy-hyoscyamine, hyoscine, noratropine and littorine.

the natural compounds are well separated from the atropine peak which appears in a percentage of at least 99.0% in the drug substance (see Fig. 4b). The resolution values of all components were found to be higher than 1.2 (see Table 2).

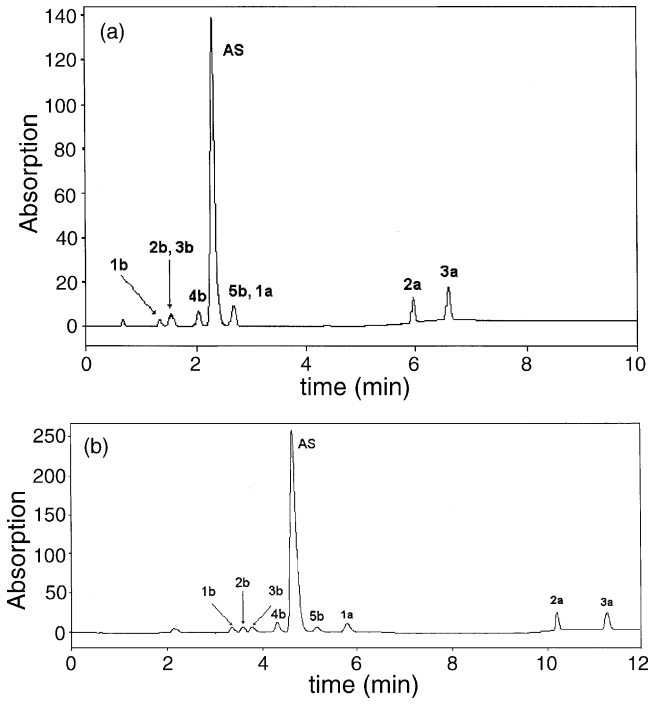

Fig. 4. Chromatograms of atropine related substances applying (a) the gradient method starting isocratically with 80% 20 mM phosphate buffer, pH 2.5 and 20% acetonitrile for 3 min, increasing the acetonitrile percentage to 40% till 5 min and running again isocratically to 10 min and (b) the gradient method starting with 25% acetonitrile from 0 to 4 min operating at a flow rate of 0.6 mL/min, 23% acetonitrile from 4 to 6 min at a flow rate of 1.0 mL/min and 45% acetonitrile up to 12 min at a flow rate of 1.0 mL/min, postrun with 25% acetonitrile for 2 min. 1b: 7-hydroxyhyoscyamine, 2b: 6-hydroxyhyoscyamine, 3b: hyoscine, 4b: noratropine, 5b: littorine, 1a: tropic acid, 2a: apoatropine, 3a: atropic acid, AS: atropine.

Table 2
Retention times, limit of detection of atropine and related substances and resolution factors of adjacent peaks in the order of increasing retention time

Substances	Retention time (min)	Resolution factor	Limit of detection (μg/mL)
Solvent	2.2	–	
7-Hydroxyhyoscyamine	3.4	5.72	0.03
6-Hydroxyhyoscyamine	3.6	1.26	0.03
Hyoscine	3.8	1.25	0.02
Noratropine	4.3	2.80	0.03
Atropine	4.7	1.72	–
Littorine	5.2	2.14	0.03
Tropic acid	5.8	2.52	0.02
Apoatropine	10.2	27.41	0.02
Atropic acid	11.2	8.12	0.02

3.3.1. LOD of the limit test method of related substances

The method was validated with respect to a limit test of all related substances of atropine sulfate. The selectivity was verified by spiking the atropine solution with the isolated or synthesized related substances. The limits of detection (LODs) of all related substances were estimated by means of the baseline noise method. The baseline noise was evaluated by recording the detector response before and after the peak. The LODs for each compound determined as signal-to-noise ratios (S/N) higher than three are shown in Table 2. The calculated limits of detection allow a limitation of all related substances to 0.1% in an atropine solution of 10 mg/100 mL.

Additionally, the robustness of the method was checked by measurements at two different days, with two different column lots and two different mobile phase lots. The tests showed no significant variation of the retention times and the resolution factors of the peaks (data not shown).

4. Conclusions

In this study two similar methods for the analysis of atropine sulfate without using a IPC reagent have been described. The first method is capable of precisely and accurately quantifying atropine sulfate in eye drops. The short retention time of atropine of about 2 min leads to short measurement periods and, thus, to low costs with regard to routine analysis. Additionally, this method allows a baseline separation of the main degradation products, i.e. tropic acid, atropic acid and apoatropine.

The second method is able to separate all related substances of atropine sulfate. The results of the study show that this method is a valuable technique for the determination of the degradation products of atropine sulfate and the related substances of natural origin in one run. Taking the LODs into consideration the method is appropriate to limit the related substances on a 0.1 percent level. When performing a suitability test as suggested on page 8, the method is as good as the method described in the current EP.

Acknowledgements

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