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Determination of tropicamide and its major impurity in raw material by the HPLC-DAD analysis and identification of this impurity using the off-line HPLC–FT-IR coupling

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ABSTRACT

This study presents TLC, HPLC-DAD and HPLC–FT-IR analyses concerning the detection, identification and determination of organic impurities of commercial tropicamide ((*R*,*S*)-*N*-ethyl-3-hydroxy-2-phenyl-*N*-(4-pyridylmethyl)propanamide) as a medical substance designed for the production of eye drops. In the examined samples from several random production batches, only one impurity (defined by Ph. Eur. 6th Ed.) was discovered in the amount sufficient for the quantitative analysis. On the basis of comparison of retention times, UV and IR spectra of the impurity and its synthesized standard, this impurity was identified as apotropicamide (*N*-ethyl-2-phenyl-*N*-(4-pyridylmethyl)prop-2-enamide). For the chemical identification of organic compounds occurring in the tropicamide samples, an off-line coupling of HPLC with FT-IR was used. The structure of a standard of apotropicamide was confirmed by ^{1}H NMR and ^{13}C NMR analysis. Validation of the HPLC-DAD method of determination both tropicamide and apotropicamide in the tropicamide medical substance was performed. This method is suitable for use in the quality control of tropicamide during its production.

1. Introduction

Tropicamide ((*R*,*S*)-*N*-ethyl-3-hydroxy-2-phenyl-*N*-(4-pyridylmethyl)propanamide) is a medical substance with parasympatholytic activity similar to atropine. However, it acts more rapidly (after ca. 15 min) and for a shorter time (1.5–3 h max.). It causes dilatation of the pupils, cycloplegia and increases intraocular pressure. For this reason, 0.5% and 1% aqueous solutions of tropicamide (Tropicamidum WZF POLFA S.A., Poland) are used in ophthalmology locally during medical examination of eyes and in short operations [1].

The European Pharmacopoeia (6th Ed.) [2] defines three possible organic impurities of tropicamide. The structures of tropicamide and these impurities are shown in Fig. 1. Impurities 1 and 2 are substrates in the synthesis of tropicamide. The impurity 3 arises as a result of the dehydration of tropicamide as a by-product in the final stage of the synthesis (Fig. 2). Due to its structural similarity to apoatropine, this compound has been called apotropicamide.

According to Ph. Eur (6th Ed.) the permissible level of organic impurities in tropicamide has to be determined using the TLC method and the limit test for the presence of (*R*,*S*)-tropic acid. Tropicamide is allowed to contain a single impurity in the amount of ≤0.5% and if there is more than one contaminant, the impurity occurring in the greatest quantity cannot exceed 0.2%. The maximum content of (*R*,*S*)-tropic acid must be below 0.05%. Over recent years the quality requirements for medical substances have been continuously increasing. In many pharmaceutical monographs the TLC test of substance purity has been replaced by the HPLC method.

According to the current recommendations of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) number Q3A(R2) [3] and Q3B(R2) [4], impurities of ≥0.1% should be identified. For their determination it is necessary to use standards of impurities or to estimate their content in solutions of various concentrations of the substance being tested.

The HPLC determination of tropicamide itself in pharmaceutical dosage forms has already been developed and validated [5–7]. However, until now nothing has been published on the concomitant HPLC separation and determination of both tropicamide and its impurities in a medical substance. In our study, samples of tropicamide from four production batches were examined using the HPLC method. They met the requirements of the Ph. Eur. (6th Ed.) monograph regarding the permitted level of organic impurities.

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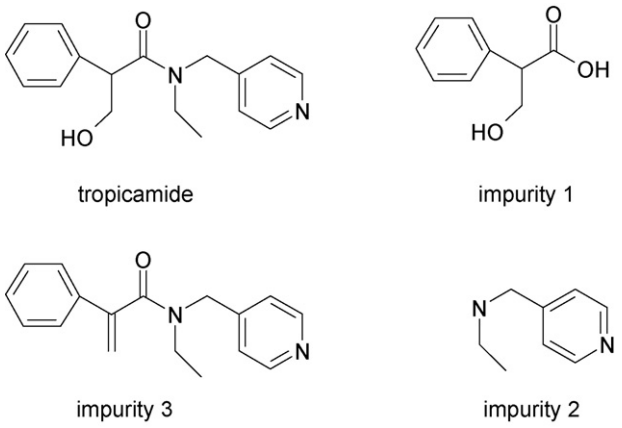

Fig. 1. (*R*,*S*)-*N*-ethyl-3-hydroxy-2-phenyl-*N*-(4-pyridylmethyl)propanamide (tropicamide) and its potential organic impurities by Ph. Eur.: impurity 1, (*R*,*S*)-3-hydroxy-2-phenylpropionic acid ((*R*,*S*)-tropic acid); impurity 2, 4-[(ethylamino) methyl]pyridine; impurity 3, *N*-ethyl-2-phenyl-*N*-(4-pyridylmethyl)prop-2-enamide (apotropicamide).

Due to non-availability of the apotropicamide standard (Fig. 1, impurity 3), the synthesis of this compound was devised and performed. The product's structure was confirmed by ^{1}H NMR and ^{13}C NMR analysis.

Conditions that allow effective separation of tropicamide from its potential organic impurities by the HPLC-DAD method were determined. The off-line HPLC–FT-IR coupling [8–14] was used to identify substances occurring on chromatograms obtained with the UV–vis detection. This method consists in physical solvents elimination in the process of nebulisation from eluate after chromatographic separation. Then, IR spectra of sample components from solid deposit are measured. This method is almost entirely free of the main problem, which occurs while measuring the IR spectra when the flow-through cell method is applied, where the interferences of solvent absorption bands with analytes exist.

However, this method also has its drawbacks and limits, since it is difficult to match the flow rate accurately so as to obtain good chromatographic separation and simultaneously achieve complete elimination of solvents. Quantitative losses of analytes in the deposition process, as well as distortion of quantitative data because of uneven thickness of the deposited sample also are a problem [15].

In recent years have appeared reports concerning application of chemometric methods in HPLC–IR analysis, as well as in the flow-through method and off-line HPLC–FT-IR [16–19]. They consist in developing chemometric algorithms which allow the elimination of the background eluent spectrum within the spectral analysis.

The content of apotropicamide in the studied production batches of tropicamide was determined.

Validation of the HPLC-DAD method was performed for both tropicamide and apotropicamide.

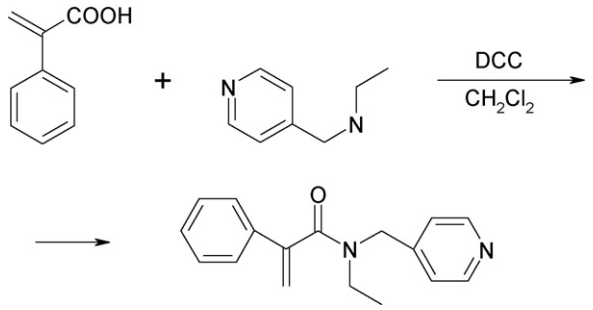

Fig. 3. Scheme of apotropicamide standard synthesis (DCC, dicyclohexylcarbodiimide).

2. Experimental

2.1. Chemicals and reagents

The tropicamide active substance (four batches produced for further industrial drug preparation in WZF POLFA S.A.) and the apotropicamide standard were synthesized in the Department of Drug Technology, Medical University of Warsaw. Tropicamide CRS from Promochem, (*R*,*S*)-tropic acid from Apollo Scientific LTD and 4-[(ethylamino)methyl]pyridine from Sigma–Aldrich Chemie GmbH were used. Water for chromatography was deionized by Milli-Q purification. Methanol of chromatographic grade and other solvents were all from Merck (Darmstadt, Germany).

2.2. Synthesis of the apotropicamide standard

2.2.1. Synthesis of *N*-ethyl-2-phenyl-*N*-(4-pyridylmethyl)prop-2-enamide (apotropicamide) (Fig. 3)

0.01 mole (1.48 g) of 2-phenylpropenic acid (atropic acid prepared by Baker and Eccles method [20]) and 0.01 mole (1.36 g) of 4-[(ethylamino)methyl]pyridine (Aldrich) were dissolved in 20 ml of anhydrous CH_2Cl_2 (POCH). This mixture was cooled down to −10 °C and then 0.011 mole (2.27 g) of dicyclohexylcarbodiimide—DCC (Aldrich) was added under stirring. The mixture was kept stirred at room temperature overnight. Precipitated dicyclohexylurea was then collected and washed with 50 ml of CH_2Cl_2. After evaporation of dichloromethane dark-yellow oil was obtained. The product was purified using column chromatography. Purification was carried out on Merck Kieselgel 60 (0.063–0.200) using the dichloromethane–methanol (POCH) solvent mixture 9:1 (v/v). After concentration of eluate it was analysed by TLC method described in Section 2.3.2. Structure confirmation is described below. The reaction yield was 1.8 g (67%).

2.2.2. Confirmation of the identity of synthesized apotropicamide

N-Ethyl-2-phenyl-*N*-(4-pyridylmethyl)prop-2-enamide is a thick, oily, yellow substance. Chemical formula: $C_{17}H_{18}N_2O$. Molecular mass: 266.35 (Table 1).

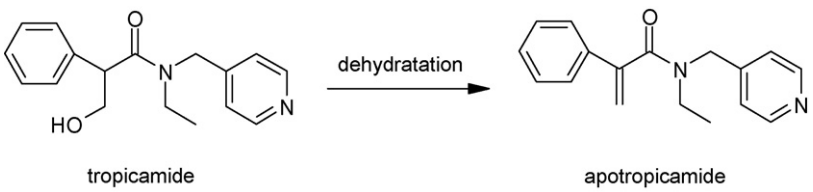

Fig. 2. Scheme of tropicamide dehydration to apotropicamide (major impurity).

Table 1
Elemental analysis of the apotropicamide standard.

Chemical element	Calculated [%]	Found [%]
C	76.06	75.88
H	6.81	6.74
N	10.52	10.71
O	6.01	6.12

Table 2
Assignment of the ^{1}H NMR spectra of the apotropicamide standard. The chemical shifts are given in ppm from TMS, while the *J*-coupling constants are in Hz.

Group	Cis form	Trans form
$C5H_3$	0.977 t, *J* = 7.2	1.203 t, *J* = 7.2
$C4H_2$	3.243 q, *J* = 7.2	3.499 q, *J* = 7.2
$C6H_2$	4.7039 s	4.4245 s
C3H(a)	5.4288 s	5.3442 s
C3H(b)	5.7538 s	5.6567 s
C2′, C6′H, C3′, C5′H, C4′H, C3″, C5″H	7.023–7.472 m	
C2″H, C6″H	8.522–8.572 m	

s, singlet; t, triplet; q, quartet; m, multiplet.

Table 3
Assignment of the ^{13}C NMR spectra of the apotropicamide standard.

Group	Cis form [ppm]	Trans form [ppm]
C5	13.707	12.287
C4	43.331	41.335
C6	46.285	50.704
C3	114.267	114.336
C4′	122.889	121.941
C2′, C6′	125.949	125.703
C3″, C5″	128.929–129.953	
C3′, C5′	129.064	129.131
C1′	135.751	135.471
C2	145.177	144.931
C4″	146.840	146.345
C2″, C6″	150.164	150.199
C1	171.356	171.195

2.2.2.1. NMR analysis. The NMR spectra were acquired in $CDCl_3$ with the BRUKER Avance DMX 400WB spectrometer, using TMS as the internal standard. The proton and carbon-13 resonance frequencies were 400.1 and 100.6 MHz, respectively. The ^{1}H NMR and ^{13}C NMR spectra indicate that apotropicamide can adopt in solutions two structures with the cis–trans ratio of approximately 2:1 (Fig. 4, Tables 2 and 3).

2.2.2.2. IR analysis. The IR spectra were acquired with the PerkinElmer Spectrum 1000 spectrometer equipped with the Auto Image IR Microscope.

C═O	1640 cm^{-1}
C═C	1601, 1496 cm^{-1}
C–N	1414 cm^{-1}
═C–H	911 cm^{-1}
$C–C_{aromat.}$	787 cm^{-1}

The spectral analysis confirms the chemical structure of apotropicamide [21]:

For comparison, characteristic absorption bands for tropicamide were given [21]:

OH	3397 cm^{-1}
C═O	1638 cm^{-1}
C═C	1603 cm^{-1}
C–O(H)	1066 cm^{-1}
$C–H_{aromat.}$	790, 755 cm^{-1}

2.3. TLC

2.3.1. Instrumentation

Horizontal DS-Chamber for TLC (Chromdes, Licence: Med. Acad. Lublin, Poland), HPTLC pre-coated Plates 10 cm × 10 cm Silica Gel 60 F254 (Merck), UV lamp (254 nm) and a chamber with iodine were used.

2.3.2. Procedure

10 μl of a 2% solution of the studied tropicamide, as well as 10 μl of each of the standards: 1% solution of (*R*,*S*)-tropic acid and 0.1% solutions of tropicamide CRS, 4-[(ethylamino)methyl]pyridine and apotropicamide in a CH_3OH:H_2O mixture (1:1), were applied on two plates. One plate was developed in the mobile phase I: $CHCl_3$:CH_3OH:25% $NH_3·H_2O$ (9:1:0.3, v/v/v) [2], and the second in II: CH_2Cl_2:CH_3OH:25% $NH_3·H_2O$ (95:5:0.5, v/v/v) [21,22]. After drying at room temperature both plates were examined in UV light at 254 nm, and then inserted for 10 min into a chamber with iodine.

2.4. HPLC-DAD

2.4.1. Instruments

HPLC system (Dionex) consisted of P580 pump and UVD 340 S detector and HPLC column thermostat JetStream II Plus (WO Industrial Electronics), all controlled with Chromeleon software. Sample injection was made through Rheodyne injector valve with a 20 μl sample loop. Analytical column HyPURITY Advance, RP C18, 150 mm × 4.6 mm, 5 μm particle size (Hypersil) was used.

2.4.2. Chromatographic conditions

In the first part of the HPLC study, carried out using a diode array UV–vis detector (DAD), conditions for the separation of tropicamide and its potential organic impurities were determined. The volume of injection was 20 μl, the temperature 20 ± 0.02 °C and the flow rate of the mobile phase 0.6 ml/min. The appropriate chromatographic resolution was achieved in mobile phase consisting of methanol and water (30:70, v/v). The following solutions of substances in a mixture of methanol:water (50:50, v/v) were studied:

(a) tropicamide from the production batches (1 mg/ml);
(b) tropicamide CRS (0.1 mg/ml);
(c) apotropicamide (0.1 mg/ml);
(d) (*R*,*S*)-tropic acid (1 mg/ml);
(e) 4-[(ethylamino)methyl]pyridine (20 μg/ml);
(f) the mixture of solutions of all four standard substances b:c:d:e (1:0.5:2:2).

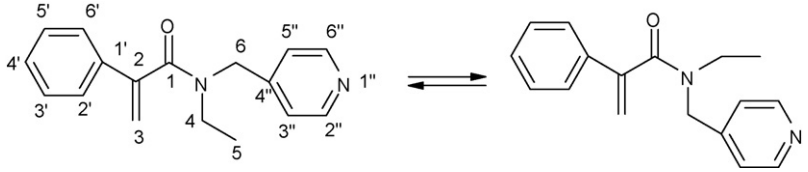

Fig. 4. Apotropicamide structural forms: cis (left) and trans (right).

With the aid of a diode array detector, UV spectra of the standard substances and the compounds appearing on chromatograms of the studied tropicamide samples were collected. In this way, the following wavelengths, close to the maximum absorbance, were selected: 225 nm (for (*R*,*S*)-tropic acid and 4-[(ethylamino)methyl]pyridine), 247 nm (for apotropicamide) and 257 nm (for tropicamide). Further detection was carried out at these wavelengths.

2.4.3. Determination of apotropicamide in the production batches

In the second part of the study, carried out using the HPLC-DAD method under previously optimized chromatographic conditions, the content of apotropicamide was determined in the studied production batches of tropicamide. Solutions of tropicamide of the 1 mg/ml concentration were analysed. The calibration curve (determined for the 247 nm wavelength) was plotted using the external standard method on the basis of the relationship of the peak areas to the following concentrations of apotropicamide standard solutions: 0.3, 0.6, 0.9, 3.0, 6.0, and 9.0 μg/ml.

2.4.4. Validation procedure

Subsequently, validation of the method was carried out for both apotropicamide and tropicamide. It included [23,24]:

1. Specificity—investigated by comparing chromatograms of the mobile phase, solutions of tropicamide CRS, apotropicamide standard, (*R*,*S*)-tropic acid, 4-[(ethylamino)methyl]pyridine and the mixture of solutions of these standard substances with the chromatogram of a solution of the studied tropicamide.
2. Linearity—the calibration curve was drawn using the external standard method for a range of concentrations from 0.3 to 9 μg/ml for apotropicamide and from 5 to 50 μg/ml for tropicamide. The mobile phase, which was used to prepare all solutions, was injected as a blank test.
3. Sensitivity—determined from the slope of the calibration curve.
4. Limit of detection—calculated on the basis of the standard deviation of the intercept (S_a) and slope (b) of the calibration curve according to the equation: $LOD = 3.3(S_a/b)$.
5. Limit of quantification—$LOQ = 3LOD$.
6. Precision (repeatability)—on the basis of seven determinations of the substance content in solutions in two concentrations: 0.9 and 6 μg/ml for apotropicamide, and 10 μg/ml and 30 μg/ml for tropicamide.
7. Accuracy (correctness)—investigated by determination of the substance in the following solutions: 0.30 μg/ml, 0.45 μg/ml, 0.60 μg/ml for apotropicamide, and 5 μg/ml, 20 μg/ml, 50 μg/ml for tropicamide. In the case of apotropicamide, solutions of 1% tropicamide were spiked with known amounts of apotropicamide. The true value was taken as the concentration of the substance (μg/ml) in the measured solution calculated on the basis of its weighed amount.

2.5. HPLC with off-line FT-IR

2.5.1. Instruments

HPLC system (see above) and LC-transform interface series 400 (Lab Connections) with germanium (IR transparent) disc covered underside with aluminium (IR reflective) coating were used. FT-IR spectrophotometer Spectrum 1000 with microscope Auto Image (PerkinElmer) was equipped with a turntable and the scanning control module (it has a variable speed motor drive to rotate the disc under the infrared microscope).

2.5.2. Procedure

After passing through the column and the diode array detector the sample was directed to the LC-transform interface, which was serially connected to the HPLC system. At the entry to the interface a stream of eluate passed through the splitter, in which (owing to a system of resistors) it was divided into a part directed to the outlet and a part directed further to the nebuliser capillary. The nebulisation process was carried out under the pressure of the inert gas—nitrogen, and at a raised temperature. Within a few milliseconds the mobile phase was vaporized at the capillary outlet and the dry remains of earlier separated analytes were deposited on the rotating germanium disc in the order, in which they exited from the chromatographic column. The germanium disc was rotating at a constant speed. The conditions of the entire process were experimentally adjusted as follows: mobile phase methanol: water (30:70 v/v), the flow rate of the mobile phase 0.3 ml/min, the division of the stream of eluate between the outlet and the nebuliser in the proportion of 80: 20, the pressure of nitrogen 20 psi, the temperature of nebulisation 122 °C, rotation of the Ge disc during the analysis at the speed of 10°/min with halting for the period of deposition of substances visible as peaks on the conventional chromatogram. The delay in time of deposition of analytes on the disc in relation to the retention times was ca. 1.5 min. After the deposition process had been finished, the disc was transferred to the IR spectrometer, placed under the microscope and the IR spectral analysis was carried out off-line (reflection–absorption technique). Measuring conditions of the IR spectra were as follows: spectral resolution – 4 cm^{-1}, number of averaged scans – 300, scan speed – 1 cm s^{-1}, size of the IR spot – 100 × 100 μm, interactive smooth – 10, the background recorded from the Ge disk in the same conditions.

3. Results and discussion

The aim of this study was the detection, identification and quantification of organic impurities of tropicamide in a raw material. Considering the ICH recommendations and the WHO report on the ensuring high quality of medical substances, samples of tropicamide from four production batches were submitted to thorough analysis. The TLC, HPLC-DAD and HPLC–FT-IR techniques were used.

N-Ethyl-2-phenyl-*N*-(4-pyridylmethyl)prop-2-enamide needed for this study as a standard, was synthesized by the method which so far has not been described for this compound. The product of this synthesis was subjected to the elemental analysis for carbon, hydrogen and nitrogen, as well as ^{1}H NMR, ^{13}C NMR and IR spectral analyses. These studies confirmed the identity of our compound with that reported in Ref. [21], as well as its occurrence in two forms: cis and trans at the ratio of approximately 2:1. The existence of these structures of apotropicamide is caused by hindered rotation around the C1–C7 bond. The analogical situation occurs in tropicamide, what was described by Petzold et al. [25].

All production batches of tropicamide fulfilled the requirements as to the level of organic impurities permitted by Ph. Eur. (6th Ed.). The limit test carried out in order to find (*R*,*S*)-tropic acid (in accordance with Ph. Eur.) gave a negative result, and more elaborate TLC analysis of 1%-solutions of tropicamide in two mobile phases (mobile phase I – according to Ph. Eur., mobile phase II – according to the literature [21,22]) has not detected any impurities. The studied samples gave only one spot on HPTLC plates, assigned to tropicamide CRS. The R_F values are presented in Table 4.

Next, the samples of tropicamide were analysed by HPLC-DAD. The diode array detector enabled us to measure UV spectra of the standards of tropicamide and its three potential impurities. Their comparison showed, that absorbance maxima of tropic acid are at 247, 252, 258, 264 nm (in accordance with Ref. [26]), and that their intensities are significantly different from those of other substances. The UV detector registered signals from (*R*,*S*)-tropic acid with greater sensitivity at lower wavelengths, for example

Table 4
The R_F values and retention times t_R of tropicamide and its possible organic impurities obtained in TLC and HPLC-DAD analyses, respectively.

Compound	R_F		t_R
	Mobile phase I ($CHCl_3$:CH_3OH:25% $NH_3 \cdot H_2O$ (9:1:0.3 v/v/v))	Mobile phase II (CH_2Cl_2:CH_3OH:25% $NH_3 \cdot H_2O$ (95:5:0.5 v/v/v))	Mobile phase III (CH_3OH:H_2O (30:70 v/v), 0.6 ml/min)
Tropicamide	0.49	0.81	9.3
(*R*,*S*)-Tropic acid (impurity 1)	0.05[a]	0.48[a]	7.4[a]
4-[(Ethylamino)methyl]pyridine (impurity 2)	0.54[a]	0.39[a]	2.2[a]
Apotropicamide (impurity 3)	0.69[a]	0.89[a]	14.5

[a] Measured only for pure substances.

Table 5
The content of apotropicamide in four production batches of tropicamide.

Tropicamide batch	Apotropicamide	
	[μg/ml]	[%]
3/01	0.460	0.005
8/01	0.337	0.003
2/02	0.249	0.002
1/03	0.572	0.006

at λ = 225 nm. This is in accordance with the literature [27–29]. Therefore, (*R*,*S*)-tropic acid is not visible in chromatograms registered at 247 nm. For this reason, for each sample the detection was made at 225 nm (significant absorbance of (*R*,*S*)-tropic acid) as well as at 247 nm (corresponding to the maximum absorbance of apotropicamide) and at 257 nm (close to the maxima from the rest of substances in the chromatogram).

The optimal conditions allowing the separation of tropicamide from its potential organic impurities were determined. They have been described in Section 2.4.2. Retention times of all compounds are presented in Table 4. The chromatogram of the mixture containing standards of tropicamide and its impurities shows their good separation under the optimal conditions (Fig. 5 (1)). The representative chromatogram of tropicamide from one of the production batches shows for the examined concentration that only one of the possible impurities is present in the analysis (Fig. 5 (2)). The retention time of this impurity and its UV spectrum are identical to those of apotropicamide standard. In all studied samples, neither (*R*,*S*)-tropic acid (impurity 1), nor 4-[(ethylamino)methyl]pyridine (impurity 2) was detected. However, the presence of apotropicamide (impurity 3) was observed.

The established analytical method was used to determine apotropicamide in four production batches of tropicamide. The 1% solutions were analysed. The obtained results are presented in Table 5. They show that synthesized tropicamide is of very high quality (the content of apotropicamide was much below the Ph. Eur. stipulated limits, other impurities were not detected).

The proposed HPLC method was validated for both apotropicamide and tropicamide according to the ICH guidelines [24]. This method is specific and selective. No interferences were observed between any peaks from the compounds of our samples. In both cases, there is a straight-linear relationship between the peak area and the concentration of the compound (in the range of analysed concentrations). The calculated LOD and LOQ values for apotropicamide were 0.114 and 0.343 μg/ml, while for tropicamide: 0.511 and 1.533 μg/ml, respectively. The parameters related to the

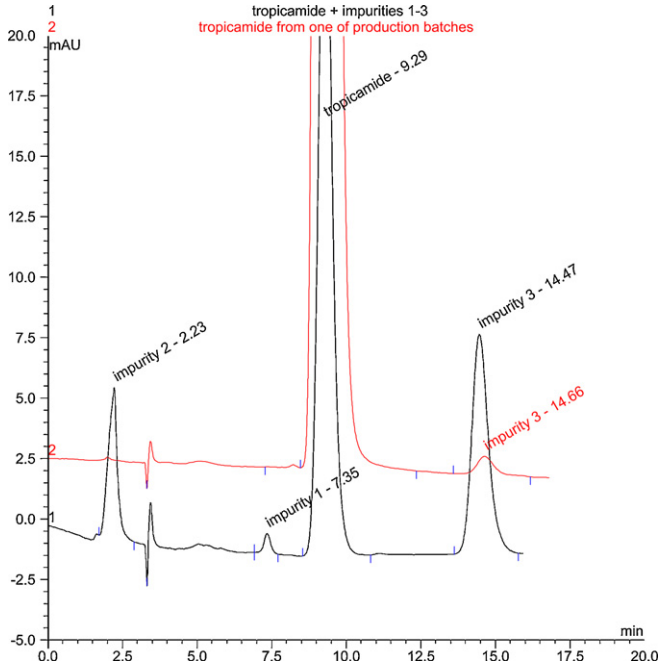

Fig. 5. (1) The chromatogram of the mixture containing standards of tropicamide and of its potential organic impurities. (2) The representative chromatogram of tropicamide from one of the production batches.

linearity, accuracy (correctness) and precision (repeatability) are presented in Tables 6–8.

The final step of this study was devoted to full identification of the impurity detected in the studied batches of tropicamide. Apart from the retention time and the UV spectrum identical to those of the standard, the IR spectrum of the impurity was compared with the IR spectrum of the synthesized apotropicamide standard. The off-line coupling of HPLC with FT-IR was used for this purpose (see Section 2.5.2). The IR spectra of substances visible on the conventional chromatogram of tropicamide were recorded from the dry remnants deposited on the germanium disc. They were compared with the IR spectra of tropicamide and apotropicamide standards measured from spots deposited on the germanium disc by applying their solutions with a syringe (1 μl of each solution, the 1 mg/ml concentrations). The lowest analytical tropicamide concentration which gave satisfactory IR spectra of its impurities was 1 mg/ml. The superimposed spectra of appropriate substances indicate their

Table 6
Linearity (the regression analysis data).

Compound	Range [μg/ml]	$y = bx + a$	r	S_a	t_a	S_b	t_b
Apotropicamide	0.3–9.0	$y = 1.3844x + 0.0859$	0.9998	0.0480	1.7888	0.0097	142.7653
Tropicamide	5.0–50.0	$y = 0.2537x + 0.0182$	0.9999	0.0393	0.4632	0.0014	180.8907

Table 7
Accuracy (correctness) of the assay.

Compound	Concentration [μg/ml]	Found-mean value [μg/ml]	Percentage recovery	S.D.	R.S.D.%
Apotropicamide	0.30	0.2593	86.4 ± 3.0	2.9698	3.44
	0.45	0.4170	92.7 ± 1.3	1.3655	1.47
	0.60	0.5512	91.9 ± 1.4	1.4910	1.62
Tropicamide	5	5.0323	100.65 ± 0.52	0.5459	0.54
	20	19.8744	99.37 ± 0.87	0.9159	0.92
	50	50.4597	100.92 ± 0.31	0.3251	0.32

Table 8
Precision (repeatability) of the assay.

Compound	Concentration [μg/ml]	Found-mean value [μg/ml]	S.D.	R.S.D.%	Confidence interval
Apotropicamide	0.9	0.8456	0.0120	1.42	0.846 ± 0.015
	6	6.0140	0.0499	0.83	6.014 ± 0.046
Tropicamide	10	10.1774	0.0334	0.33	10.177 ± 0.035
	30	29.6031	0.0792	0.27	29.603 ± 0.098

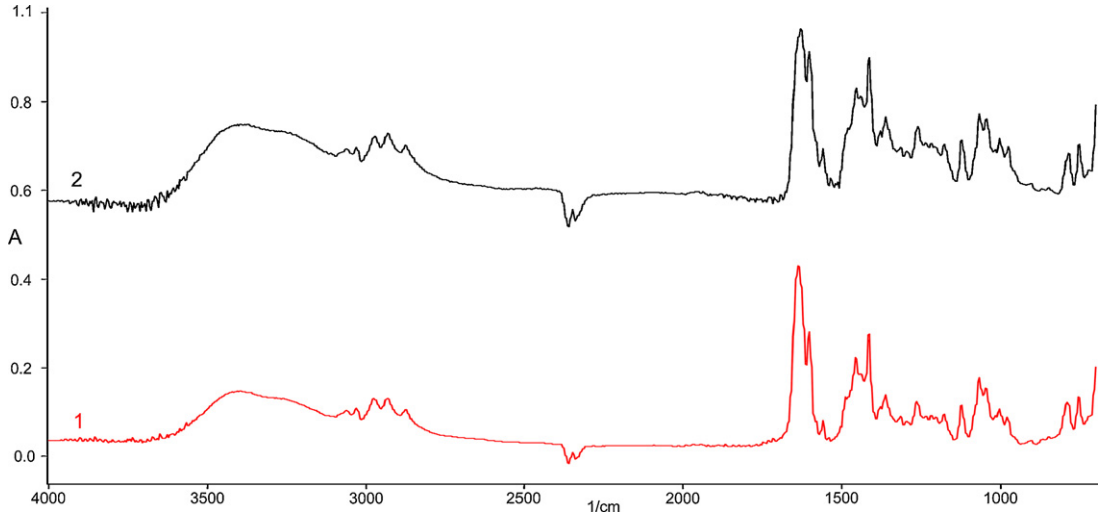

Fig. 6. Comparison of the IR spectra of tropicamide standard (1) and tropicamide (2) from one of the production batches.

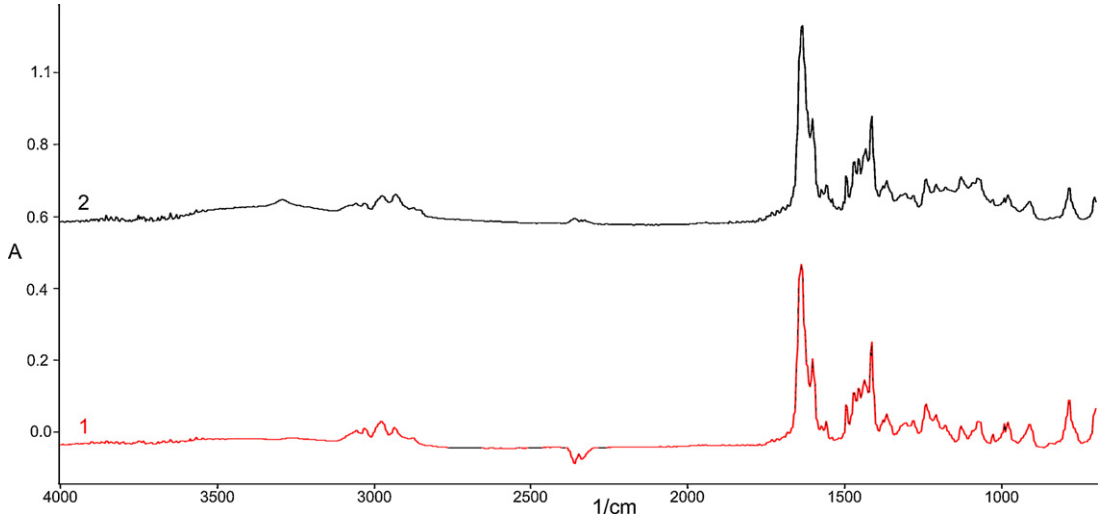

Fig. 7. Comparison of the IR spectra of apotropicamide standard (1) and apotropicamide (2) present in tropicamide from one of the production batches.

identity. They are shown in Figs. 6 and 7. The identification of compounds does not raise any doubts. Small differences between the bands intensity in the 1475 and 1700 cm^{-1} regions for tropicamide and its standard, and in 1000–1300 cm^{-1} region for apotropicamide and its standard can be a result of the imperfection of the sample deposited on Ge disk. This problem is described by István et al. [19].

Overall, the HPLC method is adequately sensitive, precise and accurate for it to be used as a routine method for determining apotropicamide in tropicamide. It can replace the approximate determination of the apotropicamide concentration in the tropicamide material carried out by the TLC method still recommended by Eur. Ph.

4. Conclusions

The proposed HPLC method for determination of apotropicamide in the raw tropicamide material is adequately sensitive, precise and accurate. For this reason, it can replace the approximate TLC analysis, still recommended by Ph. Eur, and can be applied in the quality control of tropicamide.

Our results show that tropicamide analysed in this study is of very high quality. All production batches fulfilled the purity requirements imposed by Ph. Eur. The good performance of the HPLC method allowed us to determine the content of apotropicamide (impurity 3) at the level about 100-fold lower than that permitted by Ph. Eur.

A very significant element in this study was the use of the off-line coupling of HPLC with FT-IR for the identification of the principal tropicamide impurity, which is apotropicamide. In spite of the presence of small amounts of apotropicamide in the raw material, we managed to acquire the infrared spectrum of this compound and to compare it with that of the apotropicamide standard. This is one of scarce examples of successful HPLC–FT-IR applications to the pharmaceutical analysis.

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References

[1] W. Kostowski, Pharmacology, PZWL, Warsaw 2001.
[2] The European Pharmacopoeia, 6th edition with Supplements.
[3] Int. Conf. on Harmonization (ICH), Impurities in New Drugs Substances, ICH-Q3A(R2), 2006.
[4] Int. Conf. on Harmonization (ICH), Impurities in New Drug Products, ICH-Q3B(R2), 2006.
[5] J. Hermansson, J. Chromatogr. 298 (1984) 67–78.
[6] M.J. Galmier, A.M. Frasey, S. Meski, E. Beyssac, J. Petit, J.M. Aiache, C. Lartigue, Biomed. Chromatogr. 14 (2000) 202–204.
[7] M. Amanlou, G. Asmardi, P.C. Andalibi, N. Javadi, F. Khodadady, Z.B. Omarny, J. Chromatogr. A 1088 (2005) 136–139.
[8] G.W. Somsen, C. Gooijer, N.H. Velthorst, U.A.Th. Brinkman, J. Chromatogr. A 811 (1998) 1–34.
[9] G.W. Somsen, C. Gooijer, N.H. Velthorst, U.A.Th. Brinkman, T. Visser, J. Chromatogr. A 756 (1996) 145–157.
[10] M. Ludlow, D. Louden, A. Handley, S. Taylor, B. Wright, I.D. Wilson, Anal. Commun. 36 (1999) 85–88.
[11] M. Ludlow, D. Louden, A. Handley, S. Taylor, B. Wright, I.D. Wilson, J. Chromatogr. A 857 (1999) 89–96.
[12] R. Vonach, B. Lendl, R. Kellner, Anal. Chem. 69 (1997) 4286–4290.
[13] S. Bourne, An. Lab. 30 (1998).
[14] I.D. Wilson, J. Chromatogr. A 892 (2000) 315–327.
[15] Y. Li, P.R. Brown, J. Chromatogr. Sci. 41 (2003) 96.
[16] F. Cuesta Sánchez, B.G.M. Vandeginste, T.M. Hancewicz, D.L. Massart, Anal. Chem. 69 (1997) 1477.
[17] R.J. Dijkstra, H.F.M. Boelens, J.A. Westerhuis, F. Ariele, U.A.Th. Brinkman, C. Gooijer, Anal. Chim. Acta 519 (2004) 129.
[18] H.F.M. Boelens, R.J. Dijkstra, P.H.C. Eilers, F. Fitzpatrick, J.A. Westerhuis, J. Chromatogr. A 1057 (2004) 21.
[19] K. István, R. Rajkó, G. Keresztury, J. Chromatogr. A 1104 (2006) 154–163.
[20] J.W. Baker, A. Eccles, J. Chem. Soc. 130 (1927).
[21] U. Timm, B. Göber, H. Döhnert, S. Pfeifer, Pharmazie 32 (1977) 331–334.
[22] R. Pohloudek-Fabini, E. Martin, V. Gallasch, Pharmazie 37 (1982) 184–187.
[23] N.A. Epshtein, Pharm. Chem. J. 38 (2004) 4.
[24] Int. Conf. on Harmonization (ICH), Validation of Analytical Procedures, ICH-Q2(R1), 2005.
[25] R. Petzold, U. Timm, B. Göber, Pharmazie 33 (1978) 651–654.
[26] H. Göber, et al., Pharmazie 31 (1976) 550–553.
[27] P.J. Simms, R.W. Towne, C.S. Gross, R.E. Miller, J. Pharm. Biomed. Anal. 17 (1998) 841–849.
[28] B. Dräger, J. Chromatogr. A 978 (2002) 1–35.
[29] P.K. Harrison, J.H. Tattersall, Naunyn-Schmiedeberg's Arch. Pharmacol. 373 (2006) 230–236.