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ORIGINAL

UHPLC Study on the Degradation Profiles of Olopatadine Hydrochloride in Eye Drops Subjected to Heat and Filtration Methods of Sterilisation

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Abstract A validated ultra-high performance liquid chromatography (UHPLC) method has been proposed, validated and used for the determination of olopatadine hydrochloride degradation products in olopatadine 1 mg mL^{-1} eye drops solution under the influence of two different sterilisation methods, heating and filtration, with good precision and accuracy. We found that the heat sterilization method yields a higher content of olopatadine hydrochloride degradation products in eye drops compared to unsterilized drug product or drug product sterilized by filtration, except for α-hydroxy olopatadine impurity, which remains stable with time and applied sterilization method. Contents of olopatadine related compound B shows a higher increase (from <0.005 to 0.044 %) when sterilised by heating than when subjected to aging and sterilization by filtration (increase up to 0.011 %). Similarly, total amount of all impurities is also increased from 0.13 to 0.49 % when the drug product is sterilised by heating instead of filtration (up to 0.39 %). Content of olopatadine related compound B and of all impurities is increased by aging, probably through thermal and oxidative degradation. Forced degradation studies were correlated with the sterilisation study and possible degradation pathways were identified. Olopatadine shows strong degradation under oxidative and moderate degradation under photolytic environment, with the olopatadine related compound B as the main degradation product. Sterilization of eye drops solution by filtration is recommended.

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Introduction

Olopatadine hydrochloride (Fig. 1) is chemically designated as {(11*Z*)-11-[3-(dimethylamino)propylidene]-6,11-dihydrodibenzo[*b,e*]oxepin-2-yl}acetic acid. It has an empirical formula $C_{21}H_{23}NO_3$ and a molecular weight of 337.412 g mol^{-1} [1]. It is the second-generation antihistamine used for allergic disorders such as ocular itching associated with allergic conjunctivitis [2]. Literature survey reveals that a number of different methods have been developed for the determination of olopatadine, including HPLC [3–8], HPTLC [9], LC–MS [10, 11] and spectrophotometry (UV–VIS) [12–14]. High-performance liquid chromatography (HPLC) has been used for the determination of olopatadine content and related compounds in biological fluids [11], in bulk drug form and pharmaceutical formulations, mainly in ophthalmic solutions [3–8, 15]. RP-HPLC is a fast, accurate and reliable method (retention time under 3 min), so it is especially applicable to drug products [3, 5, 6]. Different mobile phases such as methanol: 0.1 % formic acid (65:35) [3], 0.1 % orthophosphoric acid (pH 4.5) with triethylamine: acetonitrile (75:25) [15], methanol: phosphate buffer 60:40 [5], methanol: water (70:30) [6], phosphate buffer: methanol: triethylamine (55:45:0.1, pH 3.0) [7] or methanol: ammonium acetate buffer (80:20, pH 5.5) [8] were used in HPLC method. Detection wavelengths ranged from 244–246 nm [5, 6, 8] to 299–300 nm [3, 7] in HPLC and 206 [13]–220 nm [12] in UV spectrophotometry.

HPLC is considered a method of choice for the determination of olopatadine hydrochloride and its impurities

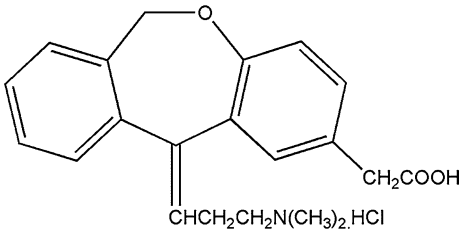


Fig. 1 Chemical structure of olopatadine hydrochloride. Figure taken from [3]

[3, 4, 16]. According to US Pharmacopoeia [16] and other sources [4], there are several known impurities of olopatadine hydrochloride, such as α-hydroxy olopatadine, olopatadine E-isomer, olopatadine related compound B and olopatadine related compound C. Degradation studies and analysis of olopatadine impurities are scarce, and only few such studies exist [4, 7, 13]. All forced degradation studies show that olopatadine strongly degrades in oxidizing environment and is subjected to acidic hydrolysis. Dey et al. [13] have shown that olopatadine undergoes degradation when exposed to heat (36 % of degraded drug product) and photolytic (23 %) conditions, but remains stable under alkaline conditions (4–6 %). In another study, Olopatadine HCl was susceptible to acidic, alkaline and oxidative conditions while it remained stable when exposed to direct sunlight and dry heat [7]. Dey et al. [13] did not try to detect impurities or identify degradation products, while Bhatt and Akhtar [7] found four unidentified degradation products. Mahajan et al. [4] found degradation of olopatadine under acidic, basic, and photolytic stress, while it was stable under oxidative and thermal stress conditions. They identified degradation products as olopatadine E-isomer, olopatadine ester E and Z.

Eye drops are sterile aqueous or oily solutions, emulsions or suspensions of one or more active substances intended for instillation into the eye [17]. In addition to its therapeutic effects, these solutions can be contaminated by various species of microorganisms which may cause serious eye infections of the cornea (keratitis) [18]. In order to avoid such a hazardous outcome and remove all pathogen microorganisms from the solution, sterilization is a crucial step in obtaining a safe eye drops product.

Products intended to be sterile should be sterilised in their final container by heating. For aqueous preparations, preferred procedure of sterilisation is by saturated steam under pressure, exposed to heating at a temperature of at least 121 °C for 15 min [19]. If sterilisation by heating is not possible due to formulation instability, an alternative method should be used, such as filtration [20], carried out under aseptic conditions. The sterilization procedure for aqueous products in the form of a decision tree is shown on Fig. 2. Solutions are filtered through a bacteria-retentive membrane with a nominal pore size of 0.22 µm or less or through any other type of filter known to have equivalent properties of bacteria retention [19].

Although sterilisation by heating is a preferred procedure, it may not be possible due to thermal instabilities of drug compounds, which can cause their decomposition and contamination with different impurities, some of them possibly toxic or hazardous. Thus, it is crucial to determine the stability of the drug product and the level of impurity contamination under the influence of sterilization by heating, and compare it to other non-heating methods such as filtration.

The purpose of this study was to determine the impact of heat sterilisation on the eye drops stability and to assist in the selection of the optimal sterilisation method. A validated ultra-high performance liquid chromatography (UHPLC) was proposed and validated for the determination of olopatadine hydrochloride degradation products because this method, with sub-2-µm column particles and mobile phases at high linear velocities, offers high resolution, sensitive and rapid analyses. Two samples of the eye drops solutions, one sterilized by heat and the other by filtration, were analysed for degradation profiles.

Methods and Experimental Setup

Instrumentation and Chromatograph

Waters Acquity H-class System (Waters Corporation, Milford, MA, USA) UPLC chromatograph was used in our analysis, equipped with quaternary solvent delivery pump (1,200 bar), Waters Acquity UPLC CSH C18 (100 × 2.1 mm, 1.7 µm) column, Waters Acquity UPLC H-class autosampler with flow through needle (FTN) injector, Waters Acquity UPLC PDA, 80-Hz detector with standard UPLC analytical flow cell (10 mm, 70 bar) and Empower software, version 3. Weighing for the analysis was performed on Mettler MX5 and XP205 (Mettler–Toledo International Inc., Columbus, OH, USA) electronic analytical balances. High-purity water for the analysis was prepared from Aqua Solutions water purification system. Sample solutions were sterilised by moist heat in Colussi U61-P/V autoclave (ICOS Impianti Group S.p.A––"Divisione Colussi", Cusano di Zoppola (PN), Italy) at 121 °C for 15 min, or by filtration using 0.2-µm, Supor® EKV (hydrophilic polyethersulfone) membrane filter (Pall Sciences, Pall India Pvt. Ltd., Mumbai, India).

Chemicals and Reagents

In-house standard of olopatadine hydrochloride was used. Acetonitrile of HPLC grade was obtained from JT Baker,

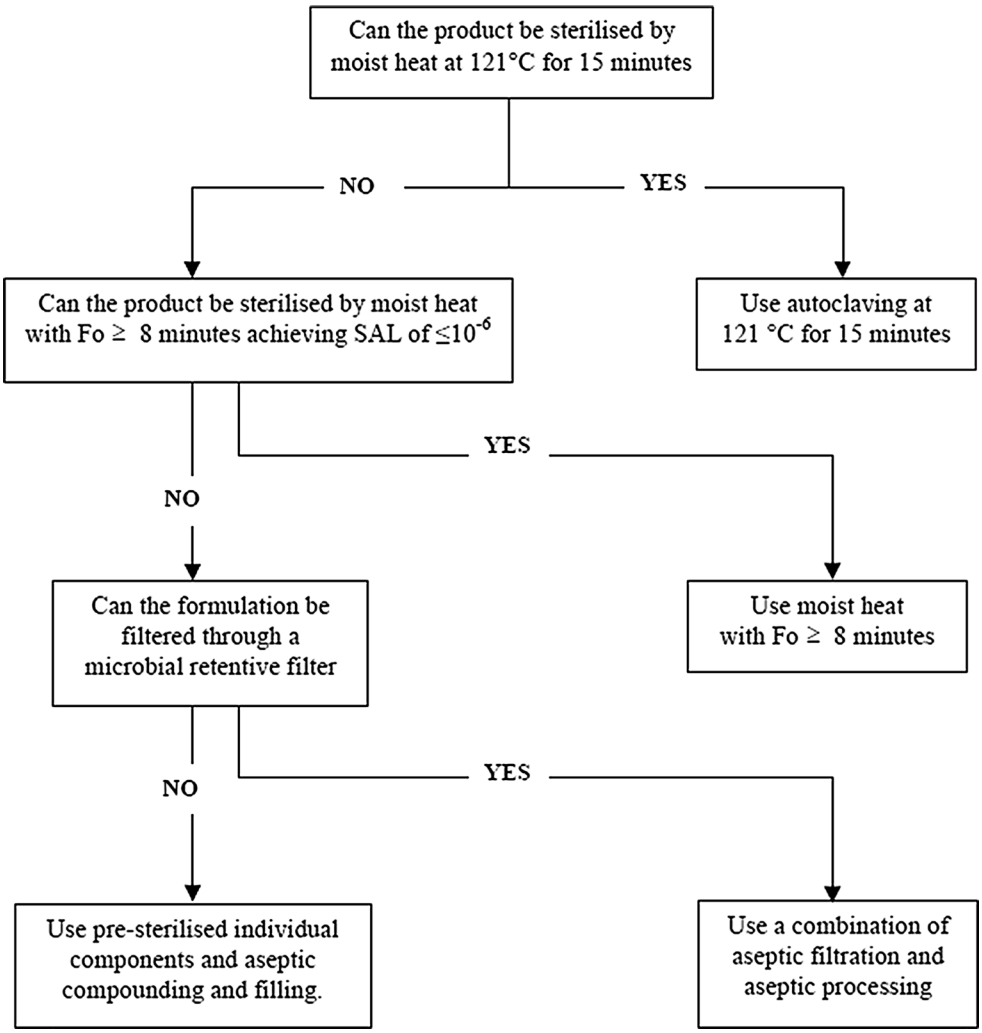


Fig. 2 Decision tree for sterilisation choices for aqueous products [20]

Germany. Sodium dihydrogen phosphate dihydrate and ortho phosphoric acid were purchased from Merck, Germany. Reference substances of α-hydroxy olopatadine, olopatadine E-isomer and olopatadine related compound C (USP impurity C) were purchased from Ragactives, Spain, and olopatadine related compound B (USP impurity B) from US Pharmacopoeia, USA.

Drug product

Eye drops product with a brand name OLOPAX (JGL dd, Rijeka, Croatia) consisting of 1 mg mL^{-1} of Olopatadine in the form of Olopatadine hydrochloride was used in this study. The unsterilized drug product samples were used in the study and then sterilized by filtration or by heating according to the described procedure. The sterilized drug product was analysed immediately or stored under appropriate storage conditions (see Table 1) for 1.5 and 3 months. The drug products were kept in white LDPE dropper bottles with tamper evident closure in accordance with the European Pharmacopoeia recommendations [17]. They were sterilised by ethylene oxide in order to minimize influence of the degradation of the packaging material. Excipients in the drug formulation include benzalkonium chloride, disodium phosphate dodecahydrate, sodium chloride, sodium hydroxide, hydrochloride acid and water.

Chromatographic Conditions

The chromatographic separation was achieved by using Waters Acquity CSH C18 column at the temperature of 25 °C. The mobile phase A consists of 6 mM phosphate buffer with pH adjusted to 2.4 with freshly diluted ortho phosphoric acid. The mobile phase B consists of acetonitrile: 0–16.19 min, B 10–75 %; 16.19–16.51 min, B 75–10 %; 16.51–17.81 min, B was held at 10 %. The flow rate of the mobile phase was kept at 0.343 mL min^{-1} and injection volume was 2.1 µL. Data were collected at 220 nm. High-purity water and acetonitrile in the ratio of 8:2 were

Table 1 Contents of impurities in Olopatadine eye drops of different ages sterilised by heating and by filtration

	Content (%)									
Age (month)	0				1.5			3		
Storage condition	25 °C/60 %				40 °C/75 %			40 °C/75 %		
Type of sterilisation	Before sterilisation	Heat	Filtration	Heat/ filtrate	Heat	Filtration	Heat/ filtrate	Heat	Filtration	Heat/ filtrate
α-Hydroxy olopatadine	0.040	0.037	0.040		0.041	0.038		0.037	0.033	
(*P* value)		0.71	0.99	0.73	0.75	0.64	0.46	0.61	0.23	0.53
Olopatadine E-isomer	0.028	0.037	0.029		0.037	0.031		0.037	0.030	
(*P* value)		0.16	0.98	0.16	0.17	0.53	0.31	0.16	0.79	0.20
Olopatadine related compound B	<0.005	**0.028**	0.005		**0.039**	0.009		**0.044**	0.011	
(*P* value)		**0.03**	0.90	**0.03**	**0.01**	0.34	**0.02**	**0.01**	0.22	**0.01**
Olopatadine related compound C	n.d.	n.d.	n.d.		<0.005	<0.005		0.015	0.009	
(*P* value)		–	–		0.39	0.45	0.88	0.06	0.16	0.26
Any other major impurity (RRT)	0.038 (1.44)	0.024 (1.43)	0.027 (1.44)		0.088 (2.11)	0.044 (0.30)		0.038 (0.36)	0.054 (0.36)	
Total impurities	0.130	0.169	0.131		**0.491**	**0.329**		**0.470**	**0.388**	
(*P* value)		0.12	0.89	0.12	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**

Impurities contents are given in relation to the olopatadine concentration in the eye drops sample solution (0.01 % corresponds to 0.02 μg mL^{-1}). *P* values between impurity contents of sterilized and non-sterilized fresh drug product are provided, together with the *P* values between impurity contents of heated and of filtered drug product of the same age (heat/filtrate). All $P < 0.05$ values are presented in bold, which show significant difference between impurity contents of compared drug products

n.d. not detected, *RRT* Relative retention time

used as a solvent for the preparation of sample, reference and system suitability solutions.

Preparation of Standard Reference Solutions

A standard reference solution containing 2 μg mL^{-1} of olopatadine was prepared from 22.2 mg of olopatadine hydrochloride salt, which corresponds to 20 mg of olopatadine. Olopatadine hydrochloride salt was dissolved in 100 mL volumetric flask with the solvent and 1 ml of the solution was diluted again in 100 mL volumetric flask with the solvent.

Placebo solution was prepared by dissolving 4.0 g of placebo in a 20 mL volumetric flask with the solvent.

Each impurity reference solution was prepared from 1.0 mg of reference impurity standard dissolved in a 50 mL volumetric flask with the solvent, and then diluted again in the ratio 1:20.

LOQ solution containing 0.02 μg mL^{-1} of olopatadine was prepared by diluting 1 mL of 2 μg mL^{-1} standard reference solution with the solvent in a 100 mL volumetric flask.

Preparation of Sample Solutions

Test solutions containing 200 μg mL^{-1} of olopatadine were prepared from Olopatadine 1 mg mL^{-1} eye drops solution. 4 g of eye drops solution (equivalent to 4 mg of olopatadine) was diluted with the water-acetonitrile solvent in a 20 ml volumetric flask. One sample solution was prepared for each type of sterilized drug product (by heating and by filtration).

Both sample and reference solutions were chromatographed, and the areas under the curve (AUC) of the peaks of each separated component, including degradation impurities of olopatadine, were determined. AUC values of sample solutions were compared to the values in reference solutions to obtain the concentrations of the impurities in the samples.

Method Validation

In order to study the olopatadine related substances in OLOPAX ophthalmic solution, we have developed and proposed a new UHPLC method. The UHPLC method was validated according to the ICH guidelines [21, 22] in order to assure reliable and repetitive results of the analysis for different parameters, including linearity, range, accuracy, precision, robustness, limit of quantization (LOQ), limit of detection (LOD) and selectivity. In validation procedures, we have also followed the recommendations from USP [16] (including chapters 1092 and 621), European Pharmacopoeia [17] and FDA [23], and from USP chapter 621 [24] and USP [16] on the requirements for the system suitability and RSD. The results of the validation procedure are shown in Table 2.

System Suitability

System suitability tests were carried out before and throughout every analysis to verify resolution, column efficiency, S/N ratio and repeatability of a chromatographic system. Tests included measuring the RSD of olopatadine peak responses in five injections (requirement: RSD <2.0 %), column efficiency (N), tailing factor (T) and the number of theoretical plates (req. $t > 4{,}000$) in olopatadine reference solution. LOQ solution was tested for S/N ratio (at least 10), while resolution between olopatadine and olopatadine related compound B was required to be more than 2.0.

Specificity

Specificity was tested on the solvent, placebo, sample and impurity solutions and on the sample solution spiked with each impurity. Olopatadine related compound B showed peak near the olopatadine component (RRT of 1.05), but it was well separated with resolution of 3.92. The peaks of olopatadine impurities are well-separated (resolution more than 1.5) from each other and from other related substances, placebo, solvent and olopatadine peaks.

Precision

Repeatability of measurement was tested on standard reference solution and RSD of 0.23 % was achieved for six injections.

The repeatability of the method was obtained from six different sample preparations spiked with known impurities isomer E and olopatadine related compound B. RSD of 0.47 % for isomer E and 0.37 % for olopatadine related compound B show that the method is precise. Intermediate precision has been tested by repeating the analysis of the sample spiked with known impurities by another analyst on the different instrument.

Accuracy

Recovery method was used to obtain accuracy by spiking the placebo in which known amounts of studied impurities and olopatadine were added to the placebo, corresponding to 0.025, 0.05, 0.1, 0.5, 1 and 2 % in relation to the label claim of olopatadine. Recovery was found to be between 99 and 104 % (100.8 % for 0.025 % impurity content, 104.2 % for 0.05 %, 101.0 % for 0.1 %, 100.9 % for 0.5 %, 99.5 % for 1 % and 99.5 % for 2 %), well inside the requirements

Table 2 Summary of validation parameters for estimation of impurities in Olopatadine 1 mg mL^{-1} eye drops

Parameter	Results	Acceptance criteria
Selectivity with forced degradation study	The method adequately separates all impurities and placebo peaks from each other and from the main peak of olopatadine hydrochloride	(a) No interference from solvent and placebo with the peaks of impurities and olopatadine (b) Olopatadine, impurities: $R_s > 1.5$; USP impurity B: $R_s > 2.0$
Precision:		
(a) Instrument precision	(a) RSD: 0.23 %	(a) RSD: <2.0 %
(b) Repeatability	(b) RSD: 0.47 % (isomer E); 0.37 % (USP impurity B)	(b) RSD: <2.0 %
(c) Intermediate precision	(c) RSD: 2.65 % (isomer E); 13.13 % (USP impurity B)	(c) RSD: <15.0 %
Accuracy	Recovery: 98.5–104.0 %	Recovery: 90–110 %
	Mean recovery: 101.0 %	
	RSD: 2.4 %	RSD < 5.0 %
Linearity and range	Impurity isomer E: 0.04–3.4 µg mL^{-1}; bias: 0.23 %; *r*: 0.997 Olopatadine related compound C: 0.04–3.4 µg mL^{-1}, bias: 0.54 %; *r*: 0.998 α-hydroxy olopatadine: 0.04–3.4 µg mL^{-1}, bias: 0.64 %; *r*: 0.999 Olopatadine related compound B: 0.02–4 µg mL^{-1}; bias: 0.25 %; *r*: 1.000	Bias: <5.0 % *r*: <0.99
Robustness:		
(a) Solution stability	(a) solutions are stable at room temperature for at least 72 h	Deviation of peak area from the initial value: <15.0 %
(b) Minor changes of method conditions	(b) the method is robust to small but deliberate variations in method parameters	S/N: >10 Olopatadine related compound B: $R_s > 2.0$
Limit of detection	Impurity isomer E: 0.02 µg mL^{-1} i.e. 0.01 % Other known and unknown impurities: 0.01 µg mL^{-1} i.e. 0.005 %	S/N: >3
Limit of quantification	Impurity isomer E: 0.04 µg mL^{-1} i.e. 0.02 % Other known and unknown impurities: 0.02 µg mL^{-1} i.e. 0.01 %	S/N: >10

Table 3 Peak resolution of olopatadine related compound B during 24 months

Period (months)	Resolution
0	3.9
3	3.5
6	3.7
12	2.9
18	2.9
24	2.6

(90–110 %), with the average value of 101.0 % and RSD of 2.4 %, less than the maximum acceptable value of 5 %.

Linearity and Range

Linearity was determined on olopatadine, impurity isomer E, α-hydroxy olopatadine, olopatadine related compound B and olopatadine related compound C with the data measured in the accuracy determination. Calibration curves were obtained by plotting the peak area versus the applied concentration. Linearity was validated and range determined to be between 0.04 and 3.4 μg mL^{-1} for impurity isomer E, α-hydroxy olopatadine and olopatadine related compound C and between 0.02 and 4.0 μg mL^{-1} for olopatadine related compound B. Calibration curve of impurity isomer E has regression line $y = 52842125.2x + 215.6$ with a bias of 0.23 % and correlation coefficient of 0.997, α-hydroxy olopatadine has $y = 39818799.3x + 440.6$, bias of 0.64 % and correlation coefficient of 0.9999, USP impurity B has $y = 56212581.5x + 267.1$, bias of 0.25 % and correlation coefficient of 1.000, while USP impurity C has $y = 48823619.5x - 520.9$, bias of 0.54 % and correlation coefficient of 0.998.

Solution Stability

Solution stability was tested on the standard reference solution, LOQ solution and the sample solution. Each solution was chromatographed at different time intervals during 72 h period (0, 4, 12, 24, 36, 48 and 72 h after the preparation of the solution) in order to find any differences in peak areas of olopatadine and impurities relative to the freshly prepared solution. Reference solution showed maximum deviation of 1.8 % in 12 h, 4.8 % in 24 h and 11.6 % in 72 h. LOQ solution showed deviation of up to 0.6 % in 24 h and up to 5.7 % in 72 h, while olopatadine and impurities contents in the sample solutions had maximum deviation of 0.3 % in 12 h, 2.3 % in 24 h and 2.8 % in 72 h. All measured deviations are less than the maximum acceptable value of 15 %, suggesting that the solutions are stable within 72 h.

Robustness

The robustness of the method was evaluated by deliberate variation of pH value (±0.2 pH), mobile phase flow (±0.01 ml/min), column temperature (±5 °C) and concentration of $NaH_2PO_4 \times 2H_2O$ in phosphate buffer (±3 mM). RSD of three injections was <0.3 %, resolution between olopatadine and olopatadine related compound B was more than 3.74, and S/N for LOQ reference solution was above 17.8.

Resolution of olopatadine related compound B was measured on the same column during validation and routine analysis for a period of 24 months. The results are shown in Table 3. It can be seen that the resolution never dropped below 2.6 which is above the minimal required resolution of 2.0.

LOD and LOQ

LOD and LOQ were determined by spiking the placebo solution with known concentrations of impurities. LOD was determined as a lowest concentration of impurity for which chromatographic response had S/N more than three. LOQ was determined in the same manner, but with S/N more than ten. LOD and LOQ were determined to be 0.02 and 0.04 μg mL^{-1} for impurity isomer E and 0.01 and 0.02 μg mL^{-1} for other impurities, accordingly.

Forced Degradation

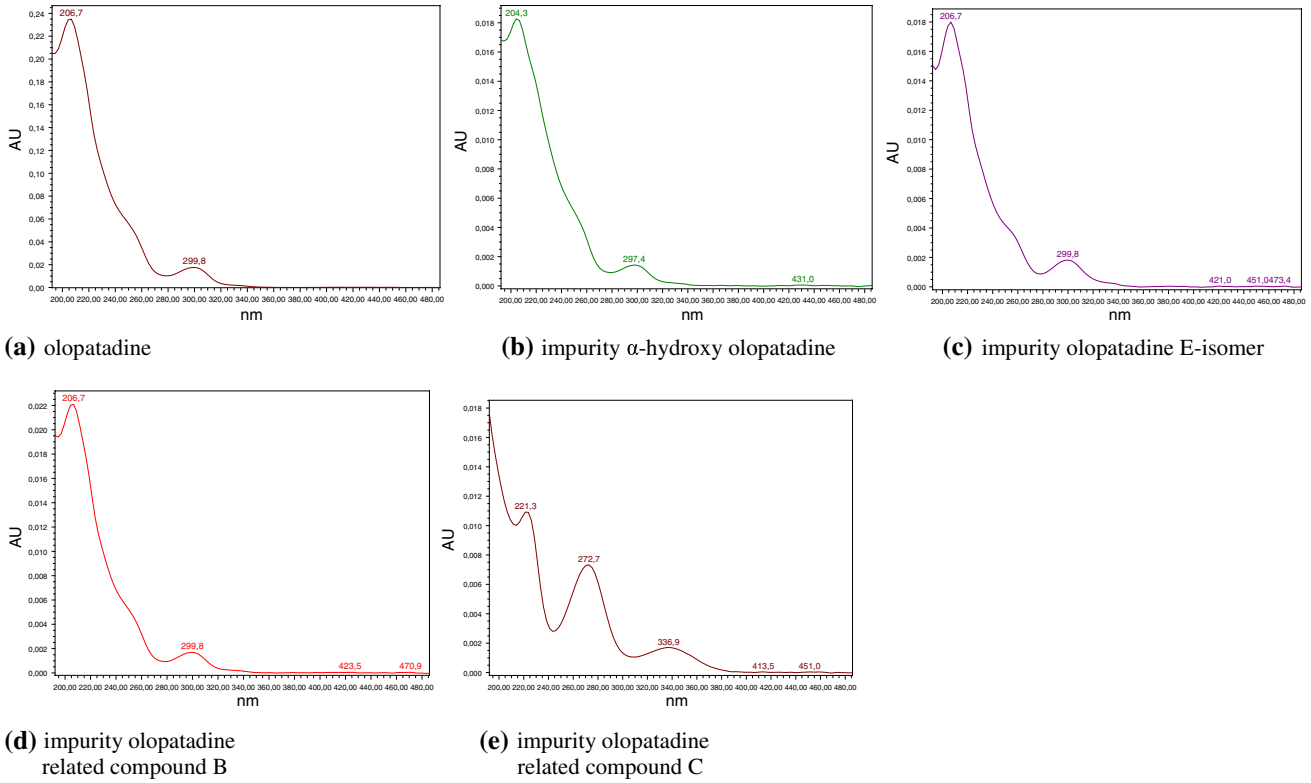
Forced degradation studies of both the drug sample and placebo were carried out under conditions of acid and alkali hydrolysis, dry heat, oxidation and UV photolysis. The following conditions were used:

- UV irradiation for 24 h with UV/VIS lamps turned on at 25 °C ± 2 °C/60 ± 5 % humidity
- Heat treatment at 80 °C for 6 days
- Acid hydrolysis (1 mol L^{-1} of phosphate acid) for 24 h
- Alkaline hydrolysis (1 mol L^{-1} sodium hydroxide solution) for 24 h
- Oxidative treatment (hydrogen peroxide 3 %) for 1 h

The chromatograms of treated samples were compared with untreated one and possible differences in olopatadine or impurities contents were determined.

Statistical Analysis

Statistical analysis was performed using the standard statistical procedure for comparison between the average content values of two samples. In all analyses, fresh and not-treated sample of eye drops solution was used as a benchmark



(a) olopatadine **(b)** impurity α-hydroxy olopatadine **(c)** impurity olopatadine E-isomer

(d) impurity olopatadine related compound B **(e)** impurity olopatadine related compound C

Fig. 3 Spectra of olopatadine and four known impurities

against which impurity contents of sterilized samples of different ages were compared. In forced degradation study, the untreated sample was used as a benchmark. We also compared impurity contents in samples of the same age treated by filtration and sterilization. Statistically significant difference between impurity contents in not-treated and sterilized samples was determined by calculating P value of Student t test. We used the criteria of $P < 0.05$, i.e. two samples do not have the same impurity content if their P value is below 0.05.

Results and Discussion

UHPLC Method for Determination of Impurities of Olopatadine in Ophthalmic Solution

The method proposed and used in our study was developed from the method for HPLC study of olopatadine recommended by USP [16]. First, it was optimized for HPLC and then transferred to UHPLC. UHPLC method was selected because this method, with sub-2-μm column particles and mobile phases at high linear velocities, offers high resolution, sensitive and rapid analyses. In order to achieve optimal resolution, peak symmetry, peak purity and number of theoretical plates, flow rates, injection volumes, sample concentrations, filter type and pore sizes were varied until satisfactory results were achieved.

USP method recommends two different methods for determination of olopatadine impurities: one for early eluting impurities (including α-hydroxy olopatadine, impurity isomer E and olopatadine related compound B) and another for late eluting impurities (including olopatadine related compound C). These two methods use different isocratic mixtures of phosphate buffer and acetonitrile for early (7:18) and late (1:1) eluting impurities. Resolution achieved using the method proposed in our study yielded better results compared to isocratic method proposed by USP in which unsatisfactory peak separations were achieved. Using octadecylsilyl silica gel for chromatography R as stationary phase and gradient elution of mobile phase in UHPLC method all impurities of interest (including USP late eluting impurities with olopatadine related compound C) are eluted in one run, contrary to the USP proposed method which requires different runs for early and late eluting impurities. This makes analysis of olopatadine impurities simpler and shorter (around 18 min for the proposed method and 35 min for both runs of the USP recommended method). The optimal separation was achieved by varying the gradients in the mixing of mobile phases.

The proposed method also differs from the USP method, as it does not use triethylamine in phosphate buffer, a

Table 4 Comparison of chromatographic conditions of the proposed UHPLC method and recommended USP method

	UHPLC method (this work)	USP recommended method for **early eluting impurities**	USP recommended method for l**ate eluting impurities**
Flow rate	0.343 ml/min	1 ml/min	1 ml/min
Injection volume	4.2 µl	30 µl	30 µl
Detector	UV 220 nm	UV 299 nm	UV 299 nm
Column	Octadecylsilyl silica gel for chromatography R (Acquity CSH C18 column is suitable), 2.1 x 100 mm; 1.7µm	Octylsilyl silica gel for chromatography R, 4.6 x 150mm; 5 µm	Octylsilyl silica gel for chromatography R, 4.6 x 150mm; 5 µm
Column temperature	25°C	25°C	25°C
Mobile phase	Mobile phase A 1g/l of sodium dihydrogen phosphate dihydrate R. Adjust with phosphoric acid, diluted R to a pH of 2.4 Mobile phase B Acetonitrile for chromatography R Time (min) / Mobile phase A (%) / Mobile phase B (%) 0 / 90 / 10 16.19 / 25 / 75 16.51 / 90 / 10 17.81 / 90 / 10	Buffer Dissolve 13.6 g of potassium dihydrogen phosphate R in 1 litre of water for chromatography R, add 1 ml of triethylamine R and mix. Adjust with phosphoric acid R to a pH of 3.0. Mobile phase Acetonitrile for chromatography R and buffer (7:18)	Buffer Dissolve 13.6 g of potassium dihydrogen phosphate R in 1 litre of water for chromatography R, add 1 ml of triethylamine R and mix. Adjust with phosphoric acid R to a pH of 3.0. Mobile phase Acetonitrile for chromatography R and buffer (1:1)
Run time:	17.81 min	at least 1.6 times the retention time of the major peak (~10 min).	at least 3 times the retention time of the olopatadine related impurity C peak (~25 min).

compound that modifies the column phase and cannot be easily rinsed. Consequently, the column can be used only for olopatadine analysis which is not preferred if large number of different analysis is performed on the same UHPLC column. The method was also optimized for wavelength detection in the range between 200 and 300 nm. Detection of impurities in UHPLC method is performed at 220 nm instead of USP prescribed 299 nm. Olopatadine and related impurities have one maximum absorption at 299 nm, but at lower wavelengths absorption is higher as can be seen from Fig. 3 where spectra of olopatadine and impurities α-hydroxy olopatadine, olopatadine E-isomer, olopatadine related compound B and olopatadine related compound C are presented. At 220 nm all impurities of interest have higher absorption and consequently higher signal to noise ratio than on 299 nm, which in turn makes lower concentration of impurities more visible on the chromatogram. The wavelength of 220 nm is chosen as the wavelength of compromise at which all impurities of interest are detected and the influence of the noise is minimal.

Comparison between chromatographic conditions of the proposed UHPLC method and USP recommended method for determination of olopatadine impurities are shown in Table 4.

Comparison Between Sterilisation by Heating and By Filtration

One of the goals of our work was to compare the effect of different sterilisation methods on degradation of olopatadine hydrochloride in Olopatadine eye drops product.

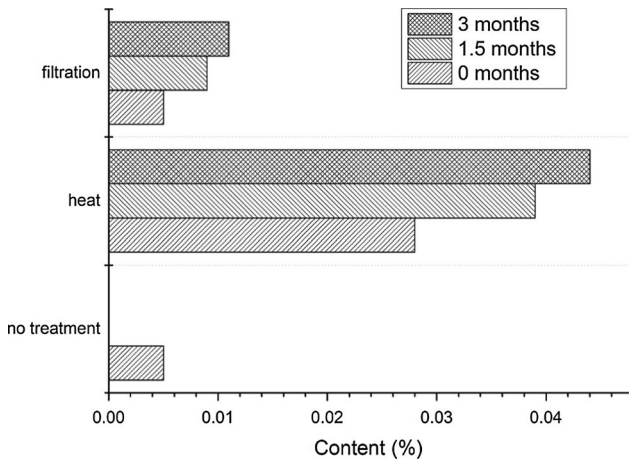


Fig. 4 Comparison of the content of olopatadine related compound B in eye drops solution for different ages of the drug product and for different types of sterilization (by heating and by filtration). Sterilization by heating significantly increases the content of olopatadine related compound B. In addition, its content increases by age of the drug product

Determination of impurities was performed before and after sterilisation of drug product and after it was stored at 40 °C/75 % RH for 1.5 and 3 months. Impurity results of drug products of different ages sterilised by heat and by filtration are presented in Table 1. *P* values of Student *t* test statistical analysis between content of each impurity in sterilized drug product of different age and content of fresh, not treated drug product are also shown. *P* values of comparison analysis between impurity content of drug products of

the same age sterilised by heating and by filtration are also given in a separate column. If P value is below 0.05, there is a statistically significant difference between impurity content of compared samples, and these cases are bolded in Table 1. The impurities contents are given in percentage, as the impurity concentration compared to the olopatadine concentration of 200 μg mL^{-1}, i.e. 0.01 % corresponds to 0.02 μg mL^{-1}. Known impurities, α-hydroxy olopatadine, olopatadine E-isomer, USP olopatadine related compound B and olopatadine related compound C were all found in tested eye drops solutions.

It can be seen how sterilisation method affects the increase of impurity content compared to unsterilized drug product. Olopatadine related compound B clearly shows statistically significant increase in sterilised product of all tested ages, regardless of the used sterilization procedure (heating or filtration). It also shows that sterilisation by heating increases its content when compared to the sample sterilised by filtration. It is evident that the impurity contents in the sample sterilised by heating is at least four times more than in the sample sterilised by filtration (Fig. 4). Also, the impurity content increases with age. Sterilisation by filtration does not increase the impurity contents in the fresh sample, and its increase in aged samples sterilized by filtration should be considered as a result of aging under the increased temperature, and not of filtration itself.

Drug product stored for 3 months and sterilised by heating also shows statistically marginal increase of olopatadine related compound C content and a possible increase of olopatadine related compound C if sterilized by heating and not by filtration. Olopatadine E-isomer also shows an increased content when sterilized by heating in drug products of all ages, including fresh drug product, although the *P* value is still above 0.05. According to our analysis, it is very unlikely that α-hydroxy olopatadine content is increased by sterilization (heating) or by age of the drug product.

Contents of all recorded impurities, shown in Fig. 5, also show statistically significant increase when sterilization by heating is used instead of filtration, especially in the case of aged drug products. Total content of impurities also significantly increases with the age of the drug product, which in turn shows that olopatadine degrades with time into its degradation products. It seems that the total impurity content decreases between 1.5 and 3 months, but the difference is too small to be considered statistically significant (*P* value of 0.16) and consequently total impurity contents in both aged samples should be considered similar.

Chromatogram of the drug sample sterilised by heating after 1.5 month of storage is shown in the Fig. 6. Peaks of all studied impurities are clearly visible. For comparison, chromatograms of the blank, placebo and reference

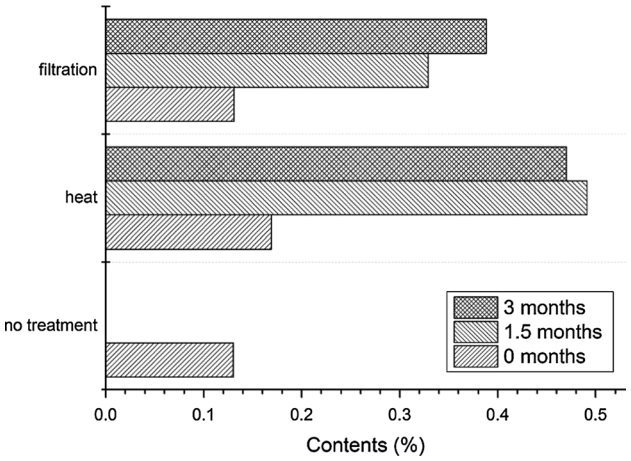


Fig. 5 Comparison of the content of all impurities in eye drops solution for different ages of the drug product and for different types of sterilization (by heating and by filtration). Sterilization by heating increases the total impurity content of aged drug product, as does aging of the drug product

solution containing olopatadine hydrochloride are shown in Fig. 7. It can be seen that no significant peaks are present at the retention times between 4 and 12 min where peaks of all four studied impurities are found, except the peak of olopatadine hydrochloride found at the retention time of around 6.2 min.

The contents of all studied individual and total impurities in the analysed ophthalmic solution are below the maximum acceptable values as required by both the USP and ICH guidelines [25]. Our in-house requirements are mostly based on USP requirements and comparison between these values and USP and ICH requirements are given in Table 5.

Degradation of Olopatadine

In order to identify the possible degradation pathways and provide a more comprehensive insight about the degradation of olopatadine, forced degradation studies were performed with the results given in Table 6. Content of olopatadine related compound B was increased under heating and photolytic conditions, while an extremely large increase was detected in oxidative environment. Olopatadine was substantially degraded under oxidative conditions (17 %), which clearly showed that olopatadine related compound B is a degradation product of olopatadine. Olopatadine is also moderately degraded under photolytic conditions, with increased contents of all impurities except olopatadine related compound C. Dry heating caused an increase in the contents of olopatadine related compounds B and C, similar with the results of heat sterilisation. An excess of olopatadine related compound C was found in oxidative environment. Figure 8 shows content of olopatadine

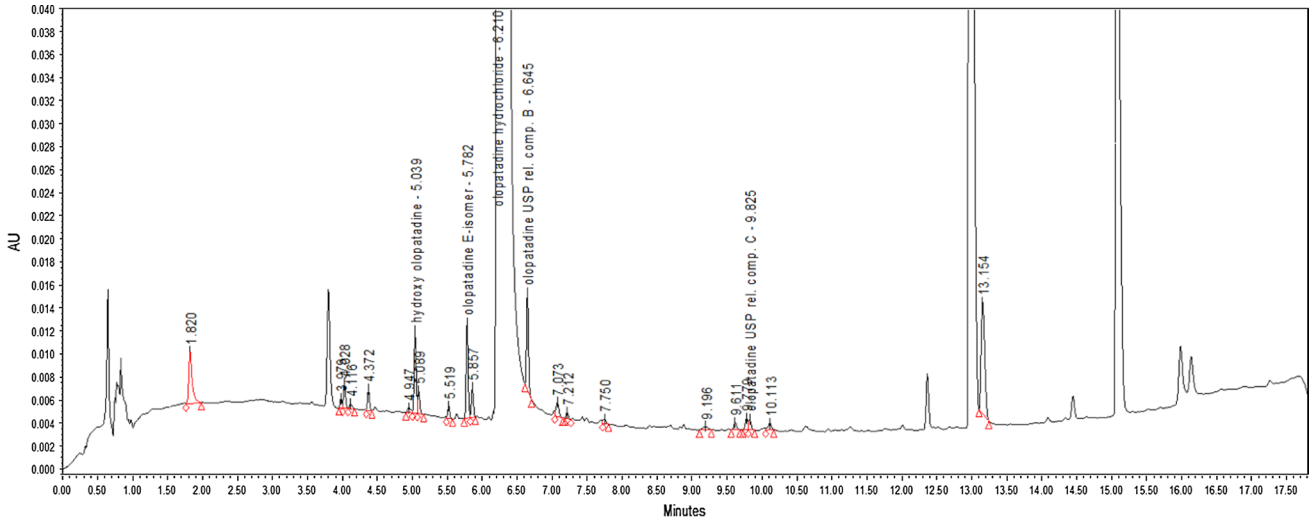


Fig. 6 Chromatogram of the sample sterilised by heating after 1.5 month of storage at 40 °C/75 % RH. More impurities are present on this chromatogram when compared to the chromatogram of the drug sample sterilised by filtration

impurity B and total impurity contents under different degradation conditions compared to the sterilization by heating and by filtration. An increase can be seen in dry heating, photolytic and especially oxidative environments, which shows that oxidation and thermal degradation are possibly the main degradation pathways of olopatadine.

Forced degradation studies fully support our findings that the sterilisation by heating considerably increases the content of olopatadine related compound B and of total impurities. Dry heating at 80 °C for 6 days significantly increases the contents of olopatadine related compound B and of total impurities, but do not increases the contents of other impurities. The same behaviour was found when the sample was sterilised by heating at 120 °C for 10 min. This strongly implies that the olopatadine related compound B probably originates from the thermal degradation of olopatadine. No other studied impurities increase under the heating environment, nor in the forced degradation study nor in sterilization by heating, implying that they are not formed by thermal degradation. Aged product shows an increase in the content of the total impurities, although sterilization by heating increases the content even more. Degradation under UV conditions increases the contents of all impurities, but this cannot explain the elevated total content of all impurities in the aged sample as they were stored in the dark conditions. On the other hand, oxidative environment increases total contents of impurities, contents of olopatadine related compound B and of olopatadine related compound C. Similar behaviour is found in the aged samples where contents of total impurities are increased even when the sample is sterilised by filtration. Contents of olopatadine related compound B and C show statistically marginal increase in the aged samples sterilised by filtration which is in accordance with the degradation studies if these two impurities are formed by degradation under the oxidative environment.

Degradation pathways can be further investigated through insight into the nature of impurities and the process of olopatadine synthesis. All studied impurities except olopatadine related compound B are by-products of olopatadine synthesis [26]. Olopatadine related compound C (Isoxepac) is the starting material of olopatadine synthesis. Its increase when the sample was subjected to heating or oxidation shows that a degradation pathway into the starting synthesis material is possible. Similarly, increase of α-hydroxy olopatadine and olopatadine E-isomer, intermediate products of olopatadine synthesis, under photolytic conditions shows similar degradation pathway. Olopatadine E-isomer was also found by Mahajana et al. [4] in a forced degradation study. Olopatadine isopropyl ester E and Z are formed in the second step of the synthesis by Wittig reaction from Isoxepac, followed by the hydrolysis and formation of olopatadine hydrochloride (Z/E: 4/1), source of olopatadine E-isomer [26]. α-hydroxy olopatadine is formed during the Wittig reaction in olopatadine synthesis. Degradation of olopatadine under heat treatment and in oxidative environment leads to the formation of olopatadine related compound B which is an N-oxide, possibly through the oxidation pathway. Amino and sodium oxides are reported as possible degradation products of olopatadine [27]. Among all excipients, disodium phosphate should act as an antioxidant synergist and stabilizer, preventing degradation of olopatadine.

Filtration should be used as a procedure of choice for sterilization of eye drops solution, but only if other

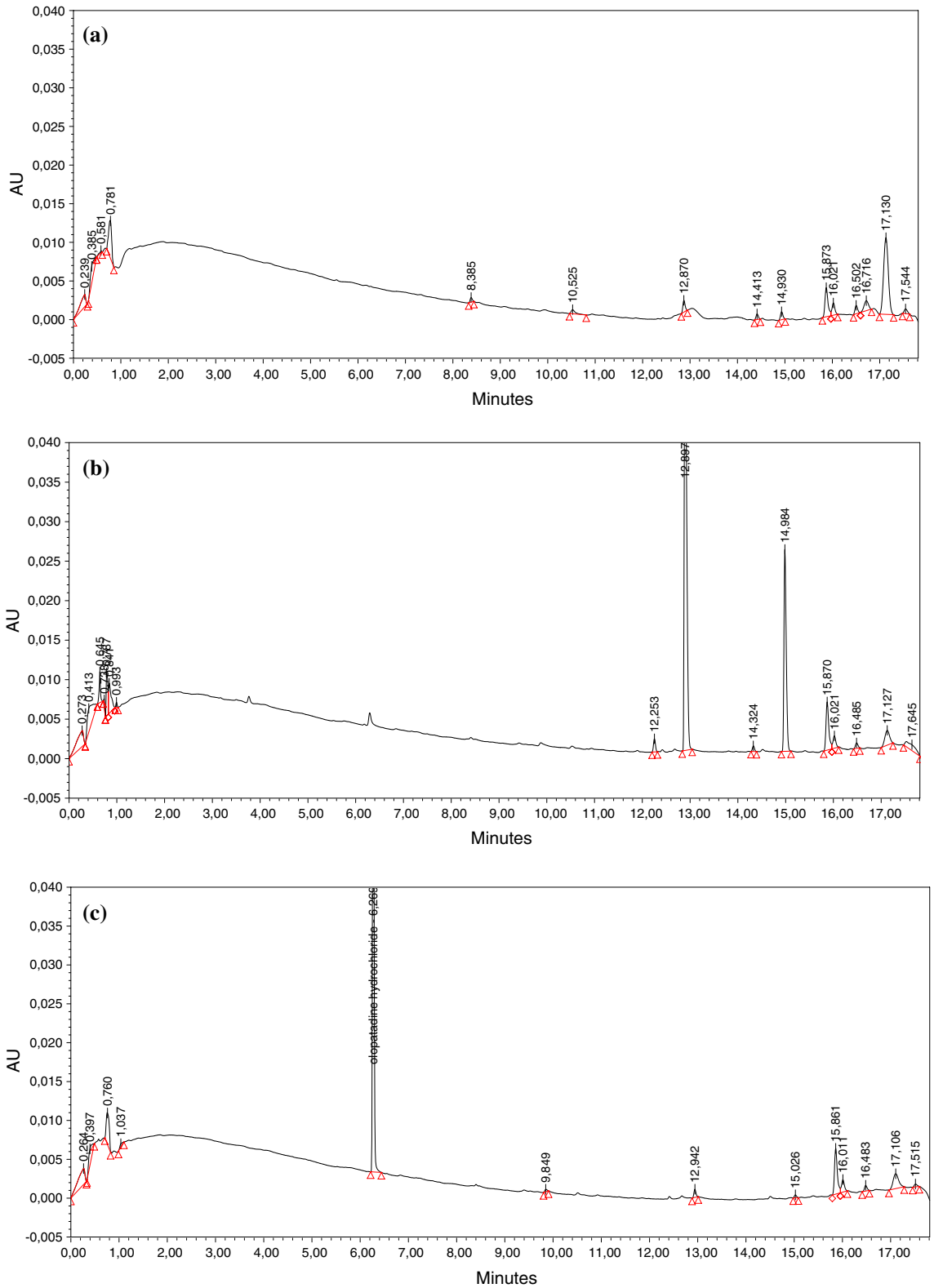


Fig. 7 Chromatograms of the blank (**a**), placebo (**b**) and standard reference solution (**c**). Blank and placebo solutions show no significant peaks at retention times between 4 and 12 min where the peaks of the studied impurities are found (compare to Fig. 6). Standard reference solution show only the peak of olopatadine hydrochloride and no impurity peaks

Table 5 Comparison of in-house, USP [16] and ICH [25] requirements for maximal content of impurities of olopatadine in the drug product

Impurity	Impurity requirement (%)			
	In-house		USP	ICH
	Release	Shelf life		
Olopatadine related compound B	0.5	2.0	2.0	1.0
Olopatadine E-isomer	0.5	0.5	0.5	1.0
α-Hydroxy olopatadine	0.5	0.5	0.5	1.0
Olopatadine related compound C	0.5	1.0	1.0	1.0
Any other impurity	0.5	0.5	0.5	1.0
Total impurities	1.0	3.0	3.0	/

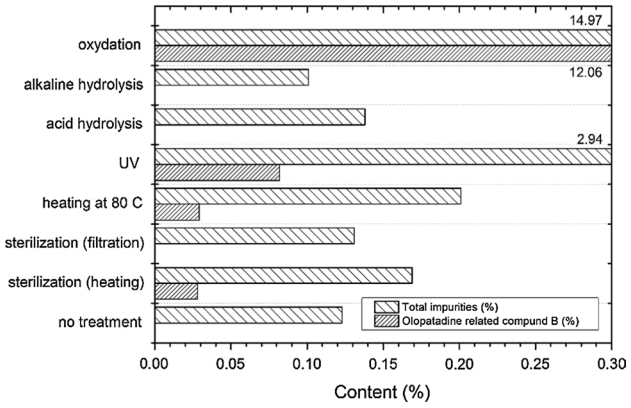


Fig. 8 Comparison of the content of all impurities and olopatadine related compound B in eye drops solution in different degradation environments and for different types of sterilization. Sterilization by heating, dry heating, photolysis and oxidation increase the contents of all impurities and of olopatadine impurity B

requirements, such as microbiological content and safety, are met. Sufficient sterilization and bacteria removal by filtration can be achieved if appropriate membrane filters are used [28].

Conclusion

High resolution and sensitivity of the proposed UHPLC method enabled us to determine with fair certainty the low amounts of impurities in olopatadine eye drops solution. The influence of the different sterilization procedures, heating and filtration, on the impurities content of the drug product has been successfully determined. The results were correlated with the degradation study and possible degradation pathways and conditions were established.

We can summarize our findings in the following way:

1. Sterilization by heating inevitably increases the content of olopatadine related compound B compared to the sterilization by filtration.
2. Olopatadine degrades strongly in oxidative environment, with olopatadine related compound B as its main degradation product.
3. Olopatadine related compound C shows an increase if an aged drug product is sterilized by heating compared to sterilization by filtration.

Table 6 Contents of impurities in olopatadine eye drops under forced degradation conditions

Forced degradation treatment	Untreated sample	Dry heat treatment at 80 °C for 6 days	UV	UV control	Acid hydrolysis	Alkaline hydrolysis	Oxidation
α-Hydroxy olopatadine	0.046	0.038 ($P = 0.19$)	**0.260 (0.009)**	0.043 (0.47)	0.043 (0.47)	0.042 (0.43)	0.040 (0.24)
Olopatadine E-isomer	0.034	0.039 (0.28)	**0.311 (0.008)**	0.033 (0.93)	0.035 (0.68)	0.041 (0.19)	0.025 (0.17)
Olopatadine related compound B	n.d.	**0.029 (0.02)**	**0.082 (0.005)**	n.d.	n.d.	n.d.	**12.058 (0.0006)**
Olopatadine related compound C	n.d.	0.009 (0.15)	n.d.	n.d.	n.d.	n.d.	**0.33 (0.008)**
Any other major impurity	0.043	0.059	1.347	0.05	0.043	NA	1.68
(RRT)	(1.39)	(2.11)	(0.64)	(1.41)	(1.41)		(0.98)
Total impurities	0.123	**0.201 (0.05)**	**2.94 (0.008)**	0.136 (0.45)	0.138 (0.45)	0.101 (0.26)	**14.97 (0.0007)**
Olopatadine (% degraded)		1.0 %	**5.7 %**	1.0 %	0.7 %	1.1 %	**16.7 %**

Impurities contents are given relatively to the olopatadine concentration in the eye drops sample solution (0.01 % corresponds to 0.02 μg mL^{-1}). Statistical P values are calculated relative to the untreated sample and given in parenthesis. All results with $P < 0.05$ are bolded and considered statistically different from the corresponding result of the untreated sample

4. Total amount of all impurities is increased when the drug product is sterilised by heating compared to sterilization by filtration, regardless of its age.
5. As expected, total amount of impurities increases with the age of the drug product.

We can conclude that the olopatadine related compound B in analysed samples is formed by thermal degradation in the presence of oxygen, while the total impurity content is increased by aging as the result of the similar conditions (but much lower temperature) during prolonged period.

Heat sterilization method gives a higher content of olopatadine hydrochloride degradation products in eye drops than sterilization by filtration. We can recommend the use of filtration procedure for sterilization of the tested eye drops if other requirements, such as microbiological safety, can be met through this procedure. Because the method separates the olopatadine hydrochloride from its degradation products, it can be also used as a stability-indicating assay.

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