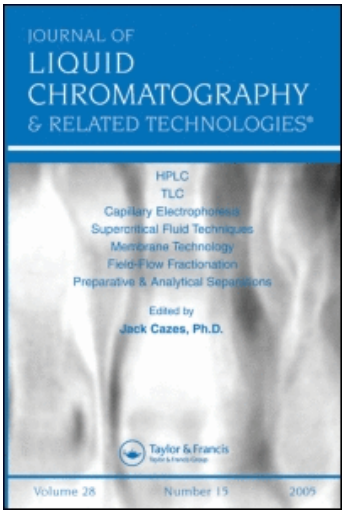

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HPLC/DAD Assay of Related Impurity Ethyl-4-oxopiperidine-1-carboxylate in Loratadine Through Derivatization with 2,4-Dinitrophenylhydrazine

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Abstract: Assay of ethyl-4-oxopiperidine-1-carboxylate (Impurity H) as related impurity in loratadine (active ingredient or in its pharmaceutical formulations) has been achieved through precolumn derivatization with 2,4-dinitrophenylhydrazine followed by HPLC/DAD. The derivatization reaction was found to be selective, fast, quantitative, and allows good sensitivity when monitored at 368 nm detection wavelength (absolute limit of quantitation is placed around 0.1 μg/mL, meaning 0.01% with respect to the amount of loratadine used in the test solution and 1/10 from the maximum allowed threshold for impurity H). Derivatization conditions and kinetics of the reaction are discussed. For convenience, the HPLC method used for separation of the derivatization product is identical to the method described in the European Pharmacopoeia 6.0, loratadine monograph, the test for related substances (other than impurity H). No interferences from the other compendial cited impurities of loratadine and from the derivatization reagent or related byproducts were observed. The assay was validated according to guidances in force. A linear response function was obtained over the concentration interval 0.1 to 8 μg/mL (0.01 to 0.8% impurity H in loratadine). The precision of the method against the derivatization product is characterized by a relative standard deviation of peak areas below 5%. The

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accuracy of the method is placed in the 95–105% interval (expressed as % bias from the theoretical concentration values). Stress studies were applied on loratadine to demonstrate that impurity H may represent a degradation byproduct. The method can be easily applied and may successfully replace the gas chromatographic/flame ionization detection (GC-FID) method designed for impurity H in the compendial EP monograph.

Keywords: 2,4-Dinitrophenylhydrazine, Ethyl-4-oxopiperidine-1-carboxylate, HPLC/DAD, Loratadine, Precolumn derivatization, Related impurities, Validation

INTRODUCTION

Loratadine, empirical formula $C_{22}H_{23}ClN_2O_2$ and chemical name ethyl 4-(8-chloro-5,6-dihydro-11H-benzo[5,6]cyclohepta-[1,2-b]pyridin-11-ylidene)-1-piperidine carboxylate, is a long acting tricycle non sedating antihistamine with selective peripheral histamine H_1-receptor antagonistic activity (second generation). It blocks peripheral effects of histamine release during allergic reactions, decreasing or preventing allergy symptoms. Human histamine skin wheal studies following single and repeated oral doses of loratadine have shown that the drug exhibits an antihistaminic effect beginning within 1 to 3 hours, reaching a maximum at 8 to 12 hours, and lasting in excess of 24 hours. Based on the its solubility determined under various pH conditions and its permeability through Caco-2 monolayers, loratadine was classified as a class II (high permeability, low solubility) drug according to the Biopharmaceutics Classification System (BCS).[1]

Synthesis of loratadine and other chemical class related compounds and their structure activity relationship has been already reviewed.[2] The profile of the related compounds in loratadine and the possibilities of assaying these compounds have been extensively studied.[3–7] According to European Pharmacopoeia, Ed. 6.0[8] determination of the related impurities of loratadine (impurities A, B, C, D, E, and F) is achieved by using a HPLC-DAD method, while determination of ethyl-4-oxopiperidine-1-carboxylate (namely impurity H) is achieved by the means of the GC/FID method (using isoamyl benzoate as internal standard). The lack of chromophores makes impurity H detectable with poor sensitivity in the UV domain, even at low wavelengths. As impurity B may be assayed through the compendial method without derivatization and due to the fact that its derivative with 2,4-DNPH is highly column retained under the RPLC separation mechanism, it seems rational to use the derivatization process only for assaying the impurity H. Consequently, 2,4-dinitrophenylhydrazine (2,4-DNPH, known as Brady's

reagent) has been considered as the derivatization agent for the carbonyl moiety.[9] The resulting derivatization products exhibit specific absorption in the 300–400 nm spectral range.

Hydrazine reagents are more often used for derivatization of the carbonyl moiety in environmental analysis.[10] Specifically, 2,4-dinitrophenylhydrazine may be used for derivatization through solid phase extraction (SPE) or solid phase micro extraction (SPME) sample preparation techniques.[11–15] The precolumn derivatization process with 2,4-DNPH is susceptible for online coupling to the LC instrumentation via a common autosampler.[16] Some problems related to oxidative degradation[17] of the reagent as well as the isomerization of the derivatization products have been highlighted.[18] The use of derivatization with 2,4-DNPH in bioanalysis has been reviewed.[19] A similar approach related to the determination of 2-[(dimethylamino) methyl]cyclohexanone as related impurity in tramadol has also been reported.[20]

A real challenge was trying to use the unmodified EP compendial HPLC method for related compounds of loratadine (other than impurity H), to easily integrate the sample preparation procedure within the method, resulting in a unique tool for controlling the quality of loratadine as active ingredient or in pharmaceutical formulations. Derivatization reaction was studied and the whole application was validated as per requirements of the ICH guidelines.[21,22] The proposed method represents a straightforward alternative to the compendial GC-FID one, without sacrificing any of the requirements related to sensitivity and selectivity.

EXPERIMENTAL

Reagents

All organic solvents used during experiments were HPLC gradient grade from Merck (Darmstadt, Germany). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Potassium dihydrogen phosphate, ortho-phosphoric acid 85%, and sodium hydroxide, all GR for analysis, were from the same producer, as well as the derivatization reagent 2,4 dinitrophenylhydrazine (2,4-DNPH), quality reagent Ph.Eur.

Loratadine impurity H (ethyl-4-oxopiperidine-1-carboxylate, Mw = 171.19, CAS No. 29976-53-2), loratadine CRS, loratadine for system suitability (containing loratadine, loratadine impurity A, and loratadine impurity E, respectively), loratadine impurity E CRS (ethyl 4-[(11RS)-8-chloro-6,11-dihydro-5 H-benzo[5,6] cyclohepta-[1,2-b] pyridin-11yl) dihydropyridine-1(2H)-carboxylate), and loratadine

impurity F CRS (ethyl 4-[(11RS)-8-chloro-11-fluoro-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]pyridine) were purchased from European Pharmacopoeia, Council of Europe (Strasburg, France), while loratadine impurity D (8-chloro-11(pyridin-4-ylidene)-6,11-6-dihydro-11-H-benzo [5,6] cyclohepta-[1,2-b])-1-piperidine carboxylate) and loratadine impurity G (8-chloro-11-(1-methylpiperidin-4-ylidene)-6,11-dihydro-5H-benzo[5,6] cyclohepta[1,2-b]pyridine) were working standards having a declared content higher than 98%.

Unless specified, acetonitrile with an addition of 0.2% *o*-phosphoric acid 85% was used as the sample solvent. Derivatization reagent stock solution having a concentration of 5000 μg/mL was used during experiments. Stock solutions of loratadine, loratadine for system suitability, and individual related impurities of loratadine (D, E, F, G, H) were made in the same sample solvent at 1000 μg/mL concentration levels.

Apparatus

An Agilent 1100 series liquid chromatograph (G1312A-binary pump, G1322A-degasser, G1313A-autosampler, G1316A-Peltier column thermostat, and G1315A-diode array detector) was used during the experiments.

The chromatographic column, Inertsil® ODS-2, (Chrompack, Varian, Cat. No. 4363), 250 mm L × 4.6 mm i.d. × 5 μm particle size was used (prior to use, column validation indicated a reduced plate height of 2.3 for *o*-xylene at 0.8 mL/min flow rate). All the other operational parameters of the chromatographic method were set according to the test "Related substances" under loratadine monograph 01/2008:2124 from the European Pharmacopoeia 6.0., except the injection volume which was set to 10 μL. The column was operated at 40 ± 1°C. The mobile phase consisted of a mixture of 50 mM aqueous potassium dihydrogen phosphate (pH 2.8 ± 0.05)/methanol/acetonitrile = 35/30/40 (v/v/v), used in the isocratic elution mode at a flow rate of 1.5 mL/min. The mobile phase should be obtained by premixing the constituents (followed by filtration); online pump mixing of the components leads to phosphate precipitation and tubing blockage. Detection made by the diode array spectrometer was using 220 and 368 nm wavelengths with a spectral width of 4 nm (reference at 560 ± 10 nm) and a response time of 0.5 s.

RESULTS AND DISCUSSION

Obviously, the HPLC test for related substances in the EP monograph considers the general synthesis pathway of loratadine as described in

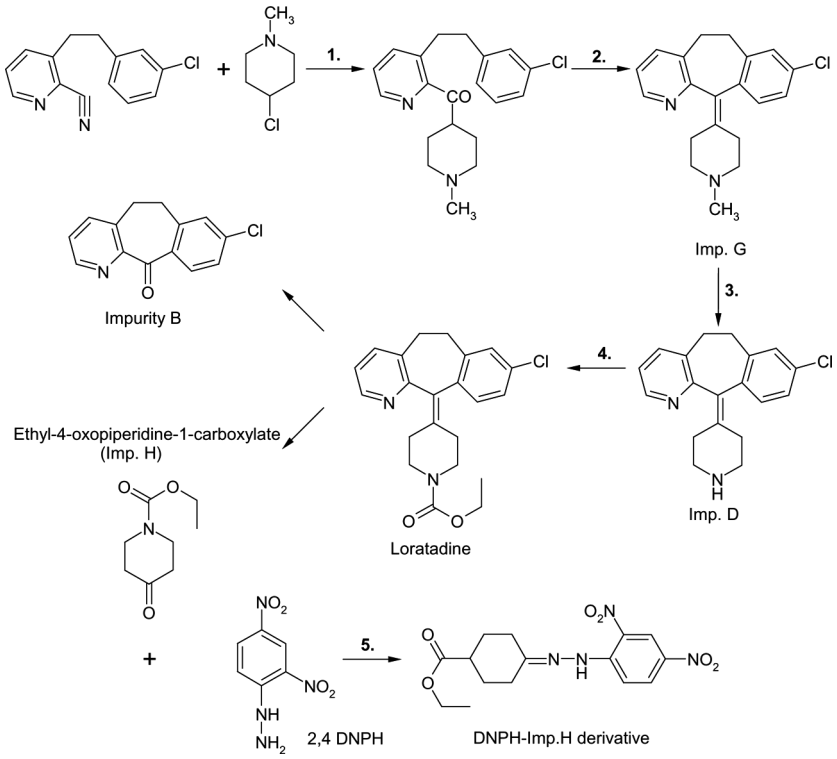

Figure 1. Synthesis pathways of loratadine; derivatization of impurity H with 2,4-DNPH.

Figure 1. Related impurities A, E, and F are byproducts from the synthesis dehydrocyclization stage 2 (the fluoro derivative assimilated with impurity F may be produced under action of boron trifluoride used in the main process). Related impurities D and G are synthesis intermediates from steps 2 and 3. Impurity C represents a related compound in the process starting material, 2-cyano-3-[2-(3-chlorophenyl) ethyl] pyridine. Impurities B and H are oxidative degradation products of loratadine, and consequently are important for evaluation of the active substance and stability of its pharmaceutical formulations. Both related compounds exhibit carbonyl moieties, and consequently are susceptible to 2, 4-DNPH derivatization. Unfortunately, impurity B was not commercially available as a reference product, and could not be submitted to the same rigorous derivatization study as impurity H. However, due to its specific UV absorption, impurity B may be assayed without derivatization through the compendial HPLC method. Its 2,4-DNPH derivative exhibits an increased retention and do not interfere with the target analyte.

Derivatization Reaction

Derivatization of impurity H with 2,4-DNPH is based on the process depicted in Figure 1, reaction 5. As it can be observed from Figure 2, labeling impurity H with 2,4-DNPH leads to a derivatization product, which does not interfere with loratadine, or any other related impurities. Monitoring the chromatogram at 368 nm adds selectivity to the separation process.

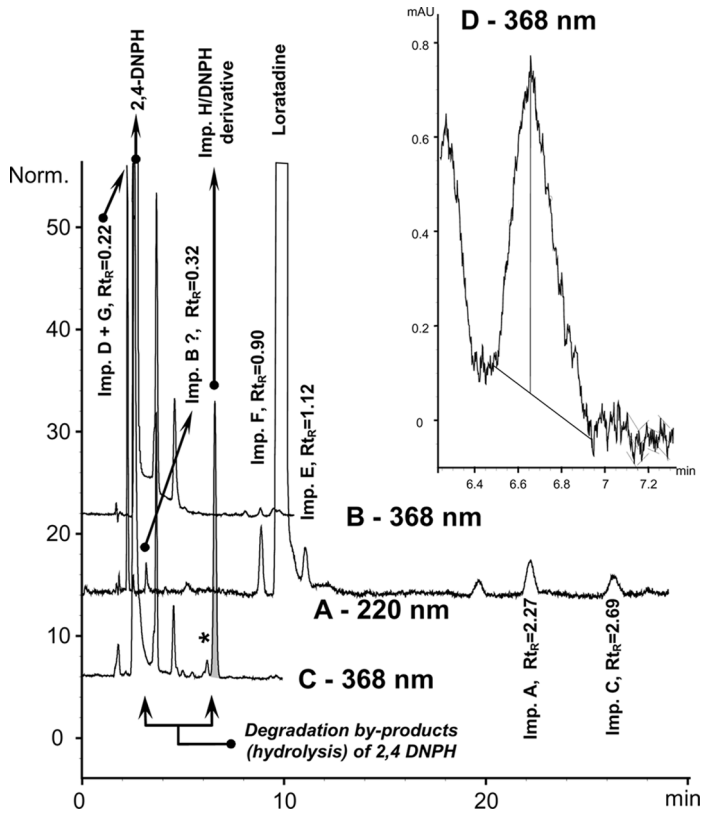

Figure 2. Typical chromatograms obtained on method application: A – loratadine and related compounds monitored at 220 nm, according to EP compendial method; B – derivatization reagent (2,4-DNPH), chromatogram monitored at 368 nm; C – chromatogram of a sample containing the derivatization product of impurity H with 2,4-DNPH, monitored at 368 nm; D – detail from a chromatogram corresponding to a sample having the concentration of impurity H at the LOQ level (0.1 μg/mL), monitored at 368 nm, used to evaluate the signal to noise ratio.

The derivatization reagent as excess and related byproducts (probably resulting from hydrolysis), although detected at 368 nm, are separated against the target compound.

The formation kinetics of the impurity H – 2,4-DNPH derivative has been investigated and the results are presented in Figure 3. It may be observed that after 20 min the derivatization reaction is quantitative. The derivatization product is stable in the reaction media, as the peak area integrated in the chromatogram of the solution injected 24 hours later is placed within the normal variation interval corresponding to a relative standard deviation (RSD%) of 2%.

The acidity of the reaction media is necessary to produce the condensation reaction, but no differences were observed through addition of phosphoric acid in the range 0.1–1% in the solvent (acetonitrile). The derivatization reaction carried out in the presence of a large excess of loratadine (concentration ranging from 1000 μg/mL to 5000 μg/mL) revealed no influences on the production yield and derivatization reaction time. The concentration of the derivatization reagent in the reaction media evaluated over 1000 to 5000 μg/mL interval is not influencing the process (large excess).

Consequently, the sample preparation procedure for assaying impurity H in loratadine can be achieved as follows: 10 mg of the active ingredient

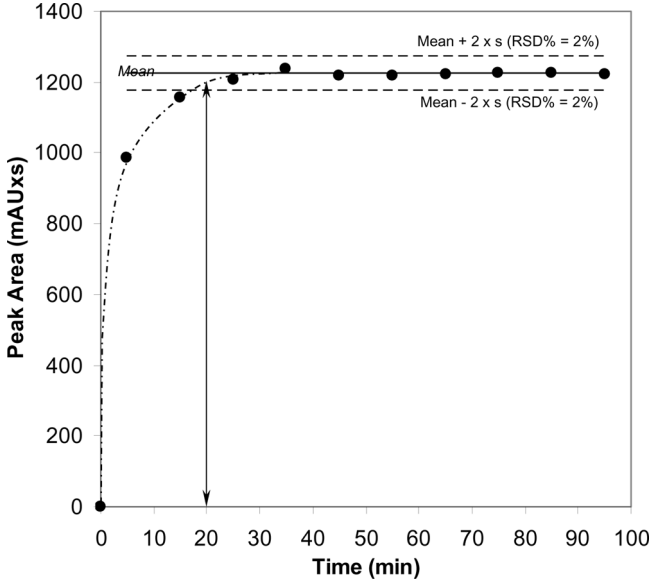

Figure 3. Kinetics of the derivatization reaction between impurity H and 2,4-DNPH.

(or a quantity of finished product corresponding to 10 mg of loratadine) are transferred in a 5 mL volumetric flask; 5 mL of a stock solution containing 5 mg/mL 2,4-DNPH in 0.2% phosphoric acid in acetonitrile is then added; the completion to mark is made with the same solvent (0.2% phosphoric acid in acetonitrile); ultrasonication is applied for 10 min., and the sample is left for completion of the derivatization reaction for the next 10 min.; filtration through 0.45 μm pore size membrane or centrifugation may be necessary; the solution is then transferred to the injection vial. The test solution is equivalent to 1000 μg/mL and to 2500 μg/mL 2,4-DNPH.

The assay of impurity H in loratadine (as active ingredient or in pharmaceutical formulations) is achieved through comparison to a standard solution containing 1 μg/mL impurity H (0.1% against loratadine in the test solution) and 2500 μg/mL 2,4-DNPH in acetonitrile with addition of 0.2% phosphoric acid. This solution may be also used as a system suitability test in the procedure (acceptance limits as follows are suggested: relative retention time of impurity H derivative versus 2,4-DNPH should be at least 2.5; efficiency of the peak corresponding to the target derivative should be at least 7000 theoretical plates; peak symmetry should be 1 ± 0.1; RSD% for the peak areas of the target derivative from three replicate injections should not be higher than 1%).

Method Validation

The selectivity of the method took into consideration the following aspects: potential interferences produced by the derivatization reagent and its related impurities; potential interferences produced by loratadine and its related impurities (other than impurity H); potential interferences brought by excipients used in the common pharmaceutical formulations (starch, lactose, silicon dioxide, cellulose, magnesium stearate, hydrogenated vegetal oils).

As illustrated in Figure 2, derivatization reagent (and related compounds) and loratadine (and related compounds) are not interfering with the derivatization product of impurity H with 2,4-DNPH. Detection made at 368 nm induces a supplementary selectivity, as loratadine and its related compounds (A–G) have no specific absorption at the given wavelength. No interference could be observed from excipients used in formulation (simultaneously considering the derivatization process and the chromatographic separation).

To obtain a better insight on the possibility of degradation of loratadine to impurity H, physical and chemical stress conditions have been applied to the active ingredient (alone or in presence of the excipients). Some of the physical factors have been applied on the active ingredient

in its solid form. The same physical factors and the chemical ones were applied on loratadine already brought in solution (concentration level of 10 mg/mL). Once the stress was ended, the solid material or the solutions were processed according to the sample preparation procedure (to obtain concentrations equivalent to 1000 μg/mL loratadine and 2500 μg/mL 2,4-DNPH). Quantitation of impurity H was made through comparison with the standard solution (0.1%). Results are given in Table 1.

Obviously, UV light exposure of loratadine in solution produces impurity H at a higher extent. The alkaline and oxidative conditions may induce formation of impurity H at a lower extent. The presence of the excipients matrix does not enhance or suppress degradation of loratadine through formation of impurity H.

Linearity of the detector response at 368 nm toward the concentration of 2,4-DNPH derivative of impurity H has been studied over the interval 0.1 to 8 μg/mL (nine concentration levels: 0; 0.1; 0.25; 0.5; 1; 1.2; 2; 4; 8 μg/mL, respectively, three replicates per concentration level). Samples were prepared from impurity H reference standard substance in the presence of loratadine (1000 μg/mL) from a batch found to be impurity H free. The slope of the linear regression was 62.7 ± 0.2 and the intercept 3.3 ± 0.7 (mean $\pm$ s). The correlation coefficient was 0.99996, RSDs % of peak areas at each of the concentration levels ranged from 0.1 to 1.9%, and % biases of the back interpolated concentration values toward the known ones fall in the -1.5 to 5.2% interval. The limit of

Table 1. Assessment of loratadine degradation to its related impurity H under stress conditions

Active ingredient (state)	Stress agent	Duration (hours)	Produced impurity H (%)
solid	Temperature (60°C)	24	ND**
solid	UV radiation (254 nm, 16 W)	24	NQ***
solution	Temperature and acid (60°C + HCl 1 M)	24	NQ
solution	UV radiation (254 nm, 16 W)	24	0.2/0.18*
solution	Acid (HCl 37%)	24	ND
solution	Alkaline (NaOH 0.2 M)	24	0.03
solution	Oxidative (H_2O_2 3%)	24	0.03 / 0.02*
solution	Acid and oxidative ($HClO_4$ 70%)	24	ND

*result obtained in presence of the excipients matrix.
**not detectable.
***not quantifiable.

quantitation (LOQ) was evaluated according to the following statistical relationships:

$$LOQ = \frac{10 \times s_A - A}{B} \quad (1)$$

$$LOQ = \frac{2 \times t \times (s_A + c_m \times s_B)}{B + 2 \times t \times s_B} \quad (2)$$

where A and B are the intercept and the slope of the linear regression equation, s_A is the standard deviation of the intercept, s_B is the standard deviation of the slope, c_m is the mean of the concentration values used within the study, t is the student coefficient for $n-2$ degrees of freedom and a certitude level $P = 99\%$, n is the number of the concentration levels.[21–23]

Calculated LOQ values are in a good agreement (0.06 μg/mL from relationship 1 and 0.07 μg/mL from relationship 2). Analysis of the signal to noise ratio in the chromatograms corresponding to the lowest concentration level (0.1 μg/mL) used through calibration leads to a mean value of 10.2 (see Figure 2D). All results together are indicating that the LOQ of the method should be placed at the 0.1 μg/mL level, meaning 1/10 from the maximum admitted level of impurity H in loratadine.

As the compendial GC/FID method for assaying impurity H in loratadine imposes a signal to noise ratio of 10 for the chromatographic peak of the target compound representing the maximum allowed level (0.1% with respect to loratadine), it clearly results that the proposed derivatization based LC method is ten times more sensitive.

Precision (expressed as repeatability and intermediate reproducibility) was evaluated at the LOQ level (0.1 μg/mL), at 1 μg/mL (maximum accepted level of impurity H in loratadine active ingredient) and 2 μg/mL. Samples were produced by spiking loratadine (found free of impurity H) during application of the preparation procedure for test

Table 2. Results obtained during evaluation of precision carried out on the 2,4-DNPH derivative of loratadine impurity H

Concentration level (μg/mL)	Procedure	n	Mean experimental concentration (μg/mL)	RSD%	Mean % bias
0.1	repeatability	10	0.101	3.5	1
1.0		10	1.010	0.9	1
2.0		10	2.000	0.5	0
0.1	intermediate	6	0.102	1.8	2
1.0	reproducibility	6	1.010	2.5	1
2.0		6	2.020	2.3	1

Table 3. Results of the robustness study carried out on the assay of impurity H in loratadine through derivatization with 2,4-DNPH

#	Operational parameter	Set value	Variation interval		RSD% for t_R	R_s (Target compound vs. compound*)	Assay % bias	Conclusion
			Min.	Max.				
1	Column temperature	40°C	39°C	41°C	≤2	≥1.5	±5	C
			35°C	45°C	>2	≥1.3	±7.5	
2	Ionic strength of the	50 mM	45 mM	55 mM	≤2	≥1.5	±5	NC
	inorganic buffer in the mobile phase		30 mM	80 mM	>2	≥1.4	±5	
3	% of the aqueous	35%	34%	36%	≤2	≥1.5	±5	NC
	component in the mobile phase		30%	40%	>2	≥1.5	±5	
4	pH of the aqueous	2.8	2.6	3.0	≤2	≥1.5	±5	NC
	component of the mobile phase		2.4	3.2	≤2	≥1.5	±5	
5	Flow rate	1.5 mL/min	1.4 mL/min	1.6 mL/min	>2	≥1.5	±5	NC
6	Column batch	Batch 6KS11022	Batch 7BS11082	Batch 7BS11080	≤2	≥1.5	±5	NC

C – critical parameter; NC – non-critical parameter.

solution. For repeatability, ten individual samples were processed at each of the concentration levels. For intermediate reproducibility, one sample per concentration level were prepared by different analysts and assayed on the same HPLC system in six experimental sessions, delayed by at least 24 hours one from another. Results are presented in Table 2.

RSD% of the absolute retention time values of the derivatization product was 0.4% (n = 30, repeatability) and 1.1% (n = 18, intermediate reproducibility). No evident trends in the variation of the considered parameters were observed.

Accuracy was addressed on samples containing impurity H at the same concentration levels as for precision (0.1; 1; 2 μg/mL). Spiking was made in the presence of loratadine and excipients used in formulation. Three separate samples were made at each concentration level, and each of the samples was injected in triplicate. Interpolation was made in the equation obtained under the linearity study. RSD% within a concentration level ranged from 0.6 to 2.5%, while recoveries were placed in the 96 to 104% interval.

Robustness study was mainly oriented to evaluate the influence of the operational parameters on the retention of the target compound, as its separation against a minor component from the 2,4-DNPH pattern (noted with * in Figure 2) seems critical. Incomplete separation may produce errors on integration, implicitly affecting the quantitative results. An overview on the results issued from the robustness procedure is given in Table 3.

The method is robust towards the operational parameters of the chromatographic separation. Column temperature is somehow critical, but only if excessive heating is produced. To obtain highly reproducible results in terms of retention it is advisable to keep column thermostat compartment close. Due to the column increased length, for thermostats with dual Peltier elements, it is recommended that both are heated at the required temperature.

CONCLUSIONS

Ethyl-4-oxopiperidine-1-carboxylate (Impurity H) has been assayed in loratadine (as active ingredient or in pharmaceutical formulations) using a sample preparation method based on derivatization with 2,4-dinitrophenyl hydrazine. The separation was achived by using the compendial liquid chromatography method for related substances (other than impurity H) from the European Pharmacopoeia, loratadine monograph 01/2008:2124. The proposed alternative succesfully replaces the GC/FID method dedicated to the assay of impurity H in loratadine, from the above mentioned pharmacopoeial monograph.

Derivatization allows monitoring of the impurity H derivative at 368 nm, inducing enhanced selectivity of the spectrometric detection process. Derivatization reaction is selective, relatively fast, and quantitative. Impurity B of loratadine may represent a substrate for the same derivatization reaction. However, impurity B may be accurately detected without derivatization through the compendial method. The derivatization compound corresponding to impurity B does not interfere with the derivatization product of impurity H.

Compared to the official GC/FID method, the assay of the 2,4-DNPH derivative of impurity H exhibits an increased sensitivity (10 times higher), as the determined LOQ is placed at the 0.1 μg/mL level (meaning 0.01% impurity H in loratadine). Method precision and accuracy are within 5% interval (expressed as RSD% and % biases, respectively). The method was found robust against its operational parameters. It has been demonstrated that UV irradiation, as well as the oxidative and the alkaline stress of loratadine in solution degrade the active ingredient leading to formation of impurity H.

The assay of impurity H may be readily integrated within the compendial LC method, requiring only preparation of two additional specific tests and standard solutions. The standard solution may be also used as a system suitability test (acceptance limits were also suggested). The derivatization procedure may be easely integrated within the official monograph, successfully replacing the GC alternative. It is cost effective and enhances on the overall efficiency of the analytical charactarization process of loratadine.

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