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Physicochemical characterization of ezetimibe and its impurities

Katarzyna Filip [a,*], Krzysztof Bańkowski [a], Katarzyna Sidoryk [a], Joanna Zagrodzka [a], Marta Łaszcz [a], Kinga Trzcińska [a], Anna Szyprowska [a], Piotr Cmoch [a,b], Wioleta Maruszak [a]

[a] Pharmaceutical Research Institute, Rydygiera 8, 01-793 Warsaw, Poland
[b] Institute of Organic Chemistry Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw, Poland

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ABSTRACT

The physicochemical characterization of major degradation and process-related impurities associated with the synthesis of ezetimibe was performed. The possibility of forming the undesirable (*R,R,S*) stereoisomer of ezetimibe has been mentioned in literature (Vinod KK, Suhail A, Bhupendra T, Nitin G US 2010/0010212 A1, Ind-Swift Laboratories Limited WO 2008/096372), but no study of its structure determination has been published yet. This paper discusses the structure elucidation of the (*R,R,S*) stereoisomer as well as ezetimibe degradation product on the bases of NMR, IR and MS data. Other potential impurities of ezetimibe are also described. A selective and stability-indicating high-performance liquid chromatography method with dual UV detection was developed for the determination of chemical and stereochemical purity of ezetimibe. The characterization of particle size and shape for ezetimibe and its stereoisomer is also described.

1. Introduction

Ezetimibe, 1-(4-fluorophenyl)-3(*R*)-[3(*S*)-(4-fluorophenyl)-3-hydroxypropyl]-4(*S*)-(4-hydroxyphenyl)-2-azetidinone (Fig. 1), was discovered by a team of Schering-Plough research chemists as a result of extensive Structure Activity Relationship (SAR) studies and rational metabolic considerations [1,2]. Ezetimibe is a potent, metabolically stable cholesterol absorption inhibitor which strongly blocks the absorption of biliary and dietary cholesterol from the small intestine without affecting the absorption of fat-soluble vitamins, triglycerides or bile acids [3]. It may be used alone (marked as Zetia, Ezetrol or Ezedoc) or together with statins (e.g. ezetimibe/simvastatin) for the treatment of primary hypercholesterolemia, homozygous sitosterolemia, homozygous familial hypercholesterolemia (HoFH) and mixed hyperlipidemia [4,5].

The determination of a drug substance impurity profile, including potential degradation products and process-related impurities, is critical for the safety assessment of API and manufacturing process thereof. According to the guidelines issued by the International Conference on Harmonization (ICH) and European Pharmacopoeia it is mandatory to identify and characterize the impurities in a pharmaceutical product if present above the accepted limits of 0.1% [6,7].

As three asymmetric carbons in the ezetimibe molecule give rise to eight stereoisomers, the synthesis of the final product with the required stereochemistry is a significant challenge. Although different synthetic routes of ezetimibe and the intermediates thereof have been discussed in literature, the problem of its stereochemical purity is almost completely overlooked. This paper deals with the physicochemical characterization and structural elucidation of ezetimibe and process-related impurities found in the final product prepared according to the procedure described in Schering Co. patents [8,9] (Fig. 2). The stereochemical course of all steps of this process was studied. Each point was optimized and, as a result, the developed process allowed to obtain ezetimibe with high stereochemical and chemical purity. The final product (Fig. 1) and its main process-related as well as degradation impurities (isolated and/or synthesized) were characterized by infrared and nuclear magnetic resonance spectroscopy (IR, NMR), mass spectrometry (MS), high-performance liquid chromatography (HPLC) and differential scanning calorimetry (DSC) techniques. A selective and stability-indicating HPLC method for the determination of ezetimibe chemical and stereochemical purity was developed. The application of automated optical microscopy for the investigation of the final product samples led to the conclusion, that the morphology and size of precipitated ezetimibe are influenced by the manner of proceeding during the final crystallization.

* Corresponding author. Tel.: +48 22 456 38 93; fax: +48 22 456 38 38.
E-mail addresses: k.filip@ifarm.eu (K. Filip), k.bankowski@ifarm.eu (K. Bańkowski), k.sidoryk@ifarm.eu (K. Sidoryk), j.zagrodzka@ifarm.eu (J. Zagrodzka), m.laszcz@ifarm.eu (M. Łaszcz), k.trzcinska@ifarm.eu (K. Trzcińska), a.szyprowska@ifarm.eu (A. Szyprowska), p.cmoch@ifarm.eu (P. Cmoch), w.maruszak@ifarm.eu (W. Maruszak).

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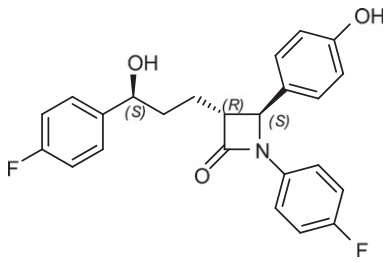


$C_{24}H_{21}F_2NO_3$

Mol. Wt.: 409.43

Fig. 1. Structure of ezetimibe.

2. Experimental

2.1. Materials and reagents

The samples of ezetimibe and its impurities were synthesized and/or isolated in Pharmaceutical Research Institute (PRI, Warsaw, Poland). The HPLC grade solvents (acetonitrile, ACN and methanol, MeOH) were purchased from POCH SA (Gliwice, Poland), trifluoroacetic acid (TFA) was purchased from Fluka & Riedel-de Haën (Seelze, Germany). Deionized water was prepared using MilliQ plus purification system (Millipore, Bradford, USA). Potassium bromide (FT-IR grade) and deuterated dimethylsulfoxide were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Chromatographic system

The chromatography was carried out using the Waters® Alliance HPLC systems consisting of Waters® e2695 Separations Module and Waters® 2489 UV/Vis or 2998 PDA detector. The chromatographic separation was performed using a Hypersil Gold C18 column (150 mm × 4.6 mm, 5 μm, Thermo Fisher Scientific). The mobile phase consisting of A (0.05% aqueous TFA + MeOH, 490:510, v/v) and B (ACN + mobile phase A, 300:100, v/v) with time gradient programme T (min)/B (%): 0/0, 18/0, 35/100, 36/0, 45/0 and the flow rate of 1.5 mL/min was used. The mobile phase solutions were filtered through a 0.45 μm filter. The injection volume was 20 μL and the detector wavelength was fixed at 210 nm (for the compound marked as EZ-2, Table 1) and 235 nm for other compounds. The column temperature was kept at 30 °C and the sample temperature at 10 °C. The analyzed samples were dissolved and diluted in the mixture of 0.05% aqueous TFA: MeOH (10:90, v/v); the concentration was about 2.0 mg/mL.

2.3. Nuclear magnetic resonance (NMR) spectroscopy

All the NMR measurements were performed with a Varian-NMR-vnmrs600 spectrometer (at temperature 298 K) equipped with a PFG Auto XID ($^1H/^{15}N$—31P 5 mm) indirect probehead. Standard experimental conditions and standard Varian programs (ChemPack 4.1) were used. In order to assign the studied structures the following 1D and 2D experiments were employed: the ^1H selective NOESY, COSY, ^1H—13C gradient selected HSQC (*g*-HSQC) and HMBC (*g*-HMBC) optimized for $^1J(C—H) = 146$ Hz and $^nJ(C—H) = 8$ Hz, respectively. Additionally, the ^1H—15N gradient selected HMBC experiments optimized for $^nJ(N—H) = 5$ Hz were used to distinguish different types of nitrogen atom (amide/amine) in the investigated molecules.

The ^1H and ^{13}C NMR spectral data are given in relation to the TMS signal at 0.0 ppm. Nitromethane, whose signal is at 0.0 ppm, was used as an external standard for the ^{15}N NMR spectra. The concentration of all solutions used for the measurements was about 20–30 mg of compounds in the 0.6 cm^3 of solvent.

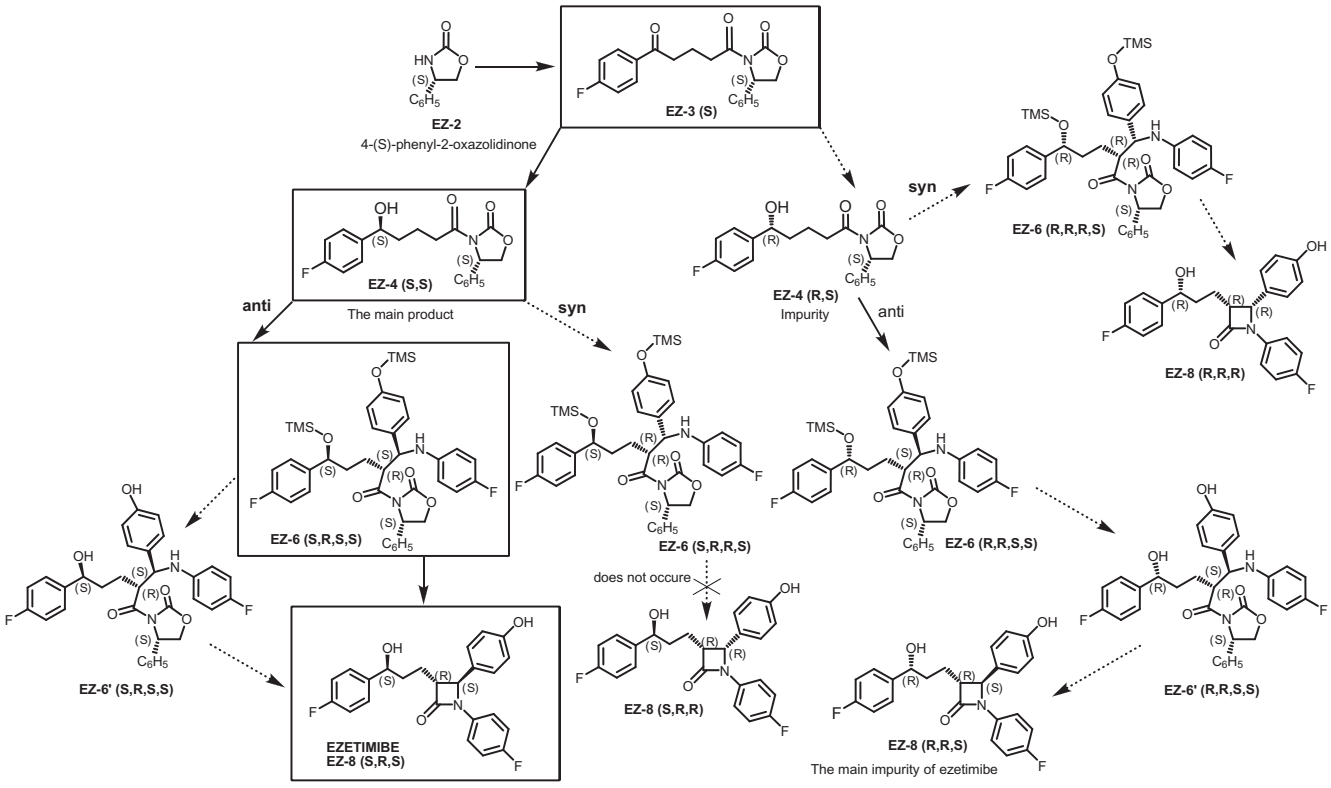
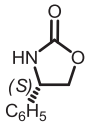
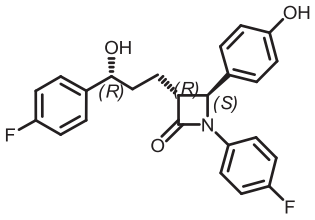
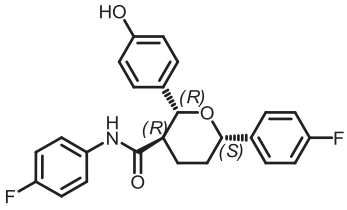
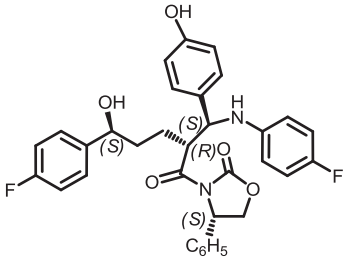
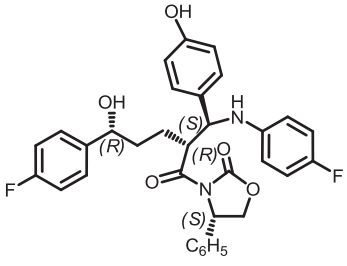


Fig. 2. The route of ezetimibe synthesis.

Table 1
Impurities of ezetimibe.

Structural formula	Chemical formula/MW	Systematic name
 EZ-2	$C_9H_9NO_2$ 163.17	4-(*S*)-phenyl-2-oxazolidinone
 EZ-8 (*R,R,S*)	$C_{24}H_{21}F_2NO_3$ 409.43	(3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*R*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-azetidin-2-one
 EZ-zanOH	$C_{24}H_{21}F_2NO_3$ 409.43	(2*R*,3*R*,6*S*)-*N*,6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2*H*-pyran-3-carboxamide
 EZ-6' (*S,R,S,S*)	$C_{33}H_{30}F_2N_2O_5$ 572.21	3-[[(2*R*,5*S*)-5-(4-fluorophenyl)-2-[(*S*)-[(4-fluorophenylamino)(4-hydroxyphenyl)methyl]-5-hydroxy-1-oxo-pentyl]]-4(*S*)-phenyl-2-oxazolidinone
 EZ-6' (*R,R,S,S*)	$C_{33}H_{30}F_2N_2O_5$ 572.21	3-[[(2*R*,5*R*)-5-(4-fluorophenyl)-2-[(*S*)-[(4-fluorophenylamino)(4-hydroxyphenyl)methyl]-5-hydroxy-1-oxo-pentyl]]-4(*S*)-phenyl-2-oxazolidinone

2.4. Infrared spectroscopy (IR)

The IR spectra of ezetimibe and isolated/synthesized impurities were recorded in the KBr (pellets) powder dispersion using a Nicolet Impact 410 spectrometer (Thermo Fisher Scientific).

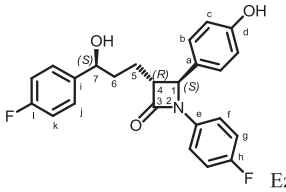
2.5. Differential scanning calorimetry (DSC)

The thermal analysis was carried out by the means of a differential scanning calorimeter DSC 822 with an IntraCooler (Mettler Toledo) in the nitrogen atmosphere. Accurately weighed samples (5–7 mg) were packed in aluminum pans with pierced lids. In the DSC experiments the samples EZ-8 (*S,R,S*) and EZ-8 (*R,R,S*) were heated from 25 to 200 °C, while EZ-zanOH was heated from 25 to 300 °C, with the scanning rate of 10 °C/min. The DSC instrument was calibrated using indium and zinc as standards.

2.6. Optical microscopy

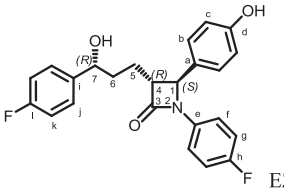
The measurements of particle shape and size distribution were done using an automated particle characterization system Morphology G3s (Malvern). The microscopic views were carried out

Table 2
NMR assignment for ezetimibe and its impurities.



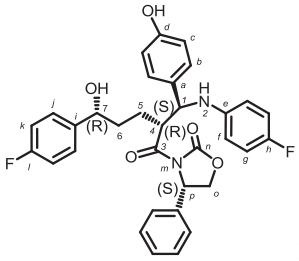
Ezetimibe, EZ-8 (*S,R,S*)

	^{1}H NMR	^{13}C/^{15}N NMR
1	4.81	59.58
a	–	127.88
b	7.18	127.52
c	6.76	115.71
d/(OH)	–/9.52	157.42
2	–	−224.8
e	–	133.98 [2.3 Hz]
f	7.22	118.22 [8.0 Hz]
g	7.13	115.79 [22.5 Hz]
h	–	157.05;158.96 [240.2 Hz]
3	–	167.33
4	3.09	59.40
5	1.73, 1.83	24.50
6	1.73	36.36
7/(OH)	4.50/5.28	71.05
i	–	142.14 [2.9 Hz]
j	7.32	127.50 [9.0 Hz]
k	7.11	114.64 [21.0 Hz]
l	–	160.06;161.98 [241.6 Hz]



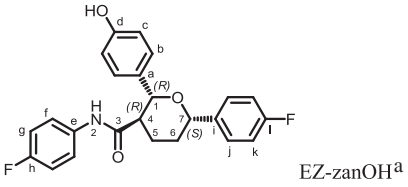
EZ-8 (*R,R,S*)

	^{1}H NMR	^{13}C/^{15}N NMR
1	4.79	59.78
a	–	128.83
b	7.18	127.55
c	6.76	115.68
d/(OH)	–/9.52	157.41
2	–	Not measured
e	–	133.99 [2.3 Hz]
f	7.22	118.21 [8.0 Hz]
g	7.13	115.78 [22.0 Hz]
h	–	157.04;158.95 [240.1 Hz]
3	–	167.29
4	3.08	59.52
5	1.73, 1.87	24.52
6	1.73	36.39
7/(OH)	4.56/5.28	71.11
i	–	142.08 [2.9 Hz]
j	7.34	127.56 [9.0 Hz]
k	7.11	114.64 [21.0 Hz]
l	–	160.06;161.99 [241.6 Hz]



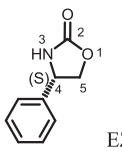
EZ-6' (*R,R,S,S*)

	^{1}H NMR	^{13}C/^{15}N NMR
1	4.29	59.58
a	–	131.5
b	7.13	128.4
c	6.66	115.0
d/(OH)	–/9.29	156.4
2	5.96	−303.2
e	–	143.8 [1.2 Hz]
f	6.52	114.1 [7.6 Hz]
g	6.78	114.8 [22.0 Hz]
h	–	154.4 [231.7 Hz]
3	–	174.5
m	–	−226.6
n	–	154.0
o	4.07;4.74	70.0
p	5.54	57.5
Ph	–/7.29/7.08/7.14	139.1/125.6/128.3/127.5
4	4.30	48.0
5	1.16;1.60	26.6
6	1.35;1.48	36.2
7/(OH)	4.35/–	71.4
i	–	142.2 [2.7 Hz]
j	7.21	127.4 [7.4 Hz]
k	7.07	114.5 [21.1 Hz]
l	–	161.0 [241.6 Hz]



EZ-zanOH[a]

	^{1}H NMR	^{13}C/^{15}N NMR
1	4.64	80.76
a	–	131.2
b	7.21	128.2
c	6.68	114.7
d/(OH)	–/9.31	156.8
2	9.79	−247.3[b]
e	–	135.2 [2.3 Hz]
f	7.44	120.9 [8.0 Hz]
g	7.07	115.1 [22.2 Hz]
h	–	157.9 [240.4 Hz]
3	–	171.4
4	2.70	49.9
5	2.10, 2.13	28.2
6	1.55, 2.00	32.4
7/(OH)	4.63/–	78.1
i	–	139.1 [2.9 Hz]
j	7.43	127.7 [8.1 Hz]
k	7.16	114.8 [21.1 Hz]
l	–	161.3 [241.2 Hz]

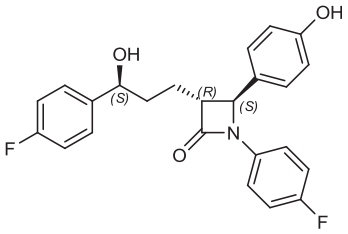
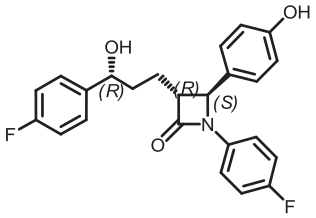
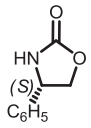
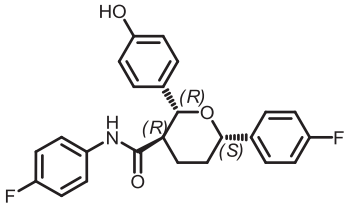
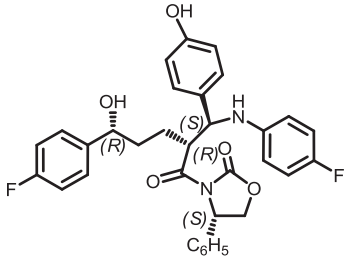


EZ-2

	^{1}H NMR	^{13}C/^{15}N NMR
2	–	159.0
3	8.20	-291.0
4	4.94 [6.52, 8.60 Hz] dd	55.2
5	4.00 [6.52, 8.50 Hz] dd	
	4.67 [8.50,8.60] pseudo t	71.5
Ph	7.34, 7.40	126.0, 128.0, 128.7, 141.0

[a] ^{1}H, ^{13}C NMR chemical shifts measurements taken on $CDCl_3$ solution containing two drops of DMSO-D_6 (better solubility of EZ-zanOH).
[b] Measurements of ^{15}N NMR chemical shift in DMSO-D_6 solution.

Table 3
IR bands for ezetimibe and impurities thereof.

Compound	Typical IR bands (cm^{-1})	Interpretation
 EZ-8 (*S,R,S*), Ezetimibe	3436, 3278	—O—H stretching vibrations
	3073–3015	—C_{Ar}—H stretching vibrations
	2927	Aliphatic —C—H stretching vibration
	1616–1597	Aromatic —C=C stretching vibrations
	1731	Amide —C=O stretching vibration
	1509	Amide —C—N stretching vibration
	1399	—C—H deformation vibration
	1224	—C_{Ar}—O and —C_{Ar}—F stretching vibrations
 EZ-8 (*R,R,S*)	3561, 3313	—O—H stretching vibrations
	3017	—C_{Ar}—H stretching vibration
	2928	Aliphatic —C—H stretching vibration
	1720	Amide —C=O stretching vibration
	1617, 1603	Aromatic —C=C stretching vibrations
	1511	Amide —C—N stretching vibration
	1396	—C—H deformation vibration
	1222, 1156	—C_{Ar}—O and —C_{Ar}—F stretching vibrations
 EZ-2	3246	—N—H stretching vibration
	3030–3013	—C_{Ar}—H stretching vibrations
	2927–2852	—C—H stretching vibration
	1744	Amide —C=O stretching vibration
	1488, 1457, 1401	—C—H deformation vibration
	1236	—N—H stretching vibration
	1097	—C—O stretching vibration
 EZ-zanOH	3315	—N—H and —O—H stretching vibrations
	3061	—C_{Ar}—H stretching vibration
	2975, 2930, 2909, 2848	—C—H stretching vibrations
	1654	—C=O stretching vibration
	1612, 1547, 1507	—N—H vibrations
	1212	—C—N stretching vibration
	1109	—C_{Ar}—O stretching vibration
	1086	—C_{Ar}—F stretching vibration
 EZ-6' (*R,R,S,S*)	3656, 3352, 3281	—N—H and —O—H stretching vibrations
	3031, 2946, 2876	Aliphatic and aromatic —C—H stretching vibrations
	1778, 1684	Amide —C=O stretching vibrations
	1604	Aromatic —C=C stretching vibrations
	1508	Amide —C—N stretching vibration
	1385, 1319	—C—H deformation vibration, —O—H deformation vibration and —C—O stretching vibration
	1210	—C_{Ar}—O and —C_{Ar}—F stretching vibrations

for the samples before and after the evaporation of the wet dispersion. The samples were dispersed in water with the addition of a surfactant. The measurements were done in diascopic light.

3. Results and discussion

During the evaluation of the manufacturing process (Fig. 2) all compounds described in Table 1 were taken into account as potential impurities of the final ezetimibe. Careful examination of stereochemistry for the all steps of ezetimibe synthetic process has shown that under certain conditions the undesired isomer of intermediate EZ-4 can be formed and, as a result, up to four various stereoisomers of the ezetimibe precursor EZ-6 are possible (Fig. 2; the compounds from the proper route of synthesis are framed). The EZ-6 (*R,R,R,S*) was not isolated; the compound EZ-6 (*S,R,R,S*) was isolated but the following cyclization thereof was not observed. The intermediate EZ-6 (*S,R,S,S*) is commercially available and contains EZ-6 (*R,R,S,S*) as the main impurity. The products of deprotection of hydroxyl groups in EZ-6 (*S,R,S,S*) and EZ-6 (*R,R,S,S*) (marked here as EZ-6′ (*S,R,S,S*) and EZ-6′ (*R,R,S,S*), respectively) can also be observed as contaminants of EZ-6 (*S,R,S,S*). Both deprotection products were isolated. Unfortunately, EZ-6′ (*S,R,S,S*)

turned out to be unstable during storage and a full physicochemical characterization thereof was impossible. In the developed HPLC gradient method EZ-6′ (*S,R,S,S*) and EZ-6′ (*R,R,S,S*) were eluted from a chromatographic column at the same time. Therefore, only EZ-6′ (*R,R,S,S*), as the precursor of the main ezetimibe contaminant (isomer (*R,R,S*)), was considered as the potential impurity working standard. The forming of (*R,R,S*) isomer is very inconvenient because this impurity is difficult to remove from the final product. The following optimization of the manufacturing and purification process resulted in the obtaining of ezetimibe EZ-8 (*S,R,S*) batches entirely free of EZ-2 and EZ-6′ contaminants, whereas (*R,R,S*) isomer was found to be present in amounts below the accepted 0.15% (according to [7]). From among the impurities listed in Table 1, (*S*)-4-phenyl-2-oxazolidinone (EZ-2) is commonly known and commercially available and the structure of the degradation product formed under alkaline conditions (here marked as EZ-zanOH) has already been discussed [10,11]. However, no published report describing the complete structural characterization of the main process-related impurity of ezetimibe, (*R,R,S*) stereoisomer (marked here as EZ-8 (*R,R,S*)), has been found in literature. The working standards of the afore-mentioned compounds were synthesized or isolated and their structures were confirmed based on NMR, IR, and MS data. For ezetimibe (EZ-8 (*S,R,S*)) and its (*R,R,S*) stereoisomer the morphological analysis was done using automated optical microscopy. The thermal analysis of compounds: EZ-zanOH, EZ-8 (*R,R,S*) and ezetimibe is also described in this paper.

3.1. Structural confirmation of ezetimibe and its impurities by NMR and IR

The working standard sample (EZ-8 (*S,R,S*) 202/025/3) of ezetimibe, obtained by process depicted in Fig. 2, was used for structural elucidation. The assignment of $^{1}H/^{13}C$ NMR signals was established based on two-dimensional (^{1}H–1H NOESY, ^{1}H–1H COSY, ^{1}H–13C *g*-HSQC, ^{1}H–13C and ^{1}H–15N *g*-HMBC) NMR experiments (Table 2). A detailed analysis of the obtained results confirmed the structure of the investigated compound. Our assignment of all $^{1}H/^{13}C$ NMR signals is almost in accordance with the data previously obtained by Raman and co-workers [12], with one exception. In the paper [12] the signals of quaternary carbons of the phenyl rings containing fluorine atom should be assign inversely, what is obvious after careful analysis of the ^{1}H–13C *g*-HMBC correlation. Small differences in $^{1}H/^{13}C$ NMR chemical shifts between our data and the measurements described in [12] are probably due to different concentration of ezetimibe samples used for the NMR experiments. The ^{1}H and ^{13}C NMR experiments were also performed for the epimer EZ-8 (*R,R,S*) and, in comparison with those done for ezetimibe, small differences in chemical shifts (ca. 0.2 ppm) were observed especially for carbons C1 and C4. However, these differences are negligible and cannot be used in the process of discrimination of the spatial ezetimibe structure. For the unequivocal distinction of stereoisomers: EZ-8 (*S,R,S*) (ezetimibe) and EZ-8 (*R,R,S*) (impurity), additional studies (IR and optical rotation measurements, see Tables 3 and 4) were needed. Ezetimibe and (*R,R,S*) stereoisomer thereof have completely different IR spectra and the specific rotation of ezetimibe is opposite in direction to its (*R,R,S*) stereoisomer.

Table 4
The values of the optical rotation for ezetimibe and its impurities.

Compound	Sample number	$[\alpha]_D$ (20 °C, methanol) (°)
Ezetimibe, EZ-8 (*S,R,S*)	202/025/3	−27.5
	202/026/3	−27.5
	202/017/1	−26.6
EZ-8 (*R,R,S*)	202/042/1	+14.1
EZ-2	B-209/09	+56.2
EZ-zanOH	202/045/1	−122.8
EZ-6′ (*R,R,S,S*)	202/044/1	+18.3

The structures of ezetimibe impurities: EZ-2, EZ-zanOH, as well as, EZ-6′ (*R,R,S,S*) – the precursor of the (*R,R,S*) isomer of ezetimibe were confirmed based on two-dimensional (^{1}H–1H NOESY, ^{1}H–1H COSY, ^{1}H–13C *g*-HSQC, ^{1}H–13C and ^{1}H–15N *g*-HMBC) NMR experiments (Table 2).

A detailed analysis and comparison of ^{15}N NMR spectra of all investigated compounds, as well as the mass spectral data, were crucial for the structure elucidation of EZ-zanOH degradation impurity. The result of a 2D ^{1}H-13C *g*-HMBC experiment was also

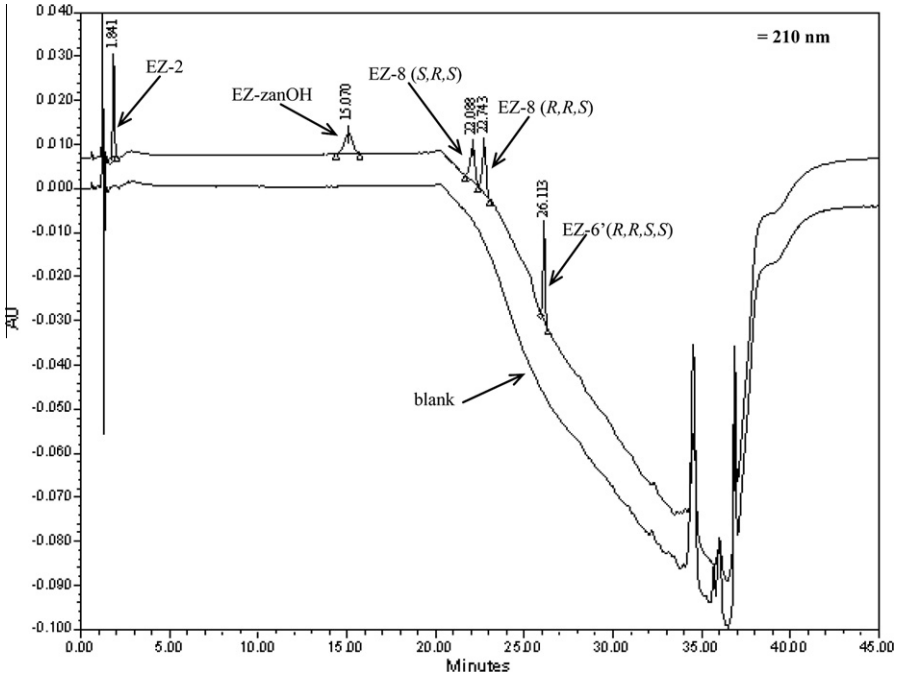


Fig. 3. Representative chromatogram generated to demonstrate the specificity and selectivity of the HPLC method (additionally, blank chromatogram is overlapped).

Table 5
The chromatographic parameters for the mixture of ezetimibe and its impurities at the level of 0.05%.

Compound	Retention time (min)	RRT	Resolution	Symmetry factor	s/n λ = 235 nm	s/n λ = 210 nm
EZ-2	1.87	0.08	–	1.20	0.59	55.05
EZ-zanOH	15.21	0.68	37.03	1.00	14.72	6.93
EZ-8 (*S,R,S*)	22.16	–	13.46	0.95	32.03	11.48
EZ-8 (*R,R,S*)	22.79	1.03	1.84	0.98	41.39	15.73
EZ-6′ (*R,R,S,S*)	26.12	1.18	13.89	0.96	28.24	23.18

helpful. The ^{15}N NMR spectral data obtained for ezetimibe revealed the signal at −224.8 ppm, corresponding to the amide nitrogen of β-lactam ring. In the ^{15}N NMR spectrum of EZ-6′ (*R,R,S,S*) the signal at −303 ppm was assigned as an amine nitrogen and the signal at −247 ppm in the spectrum of the degradation impurity EZ-zanOH corresponded to the amide nitrogen atom. The comparison of these ^{15}N NMR chemical shifts and the analysis of related chemical structures led to the conclusion that degradation product EZ-zanOH molecule possessed an amide group. This fact was confirmed by the careful examination of ^{1}H–13C *g*-HMBC spectrum of EZ-zanOH dissolved in $CDCl_3$ with a few drops of DMSO (for the solubility enhancement). In this 2D experiment the following correlations were observed: proton at 4.72 ppm with carbon at 78.33 ppm and proton at 4.59 ppm with carbon at 80.77 ppm. Additionally, the analysis of the ^{1}H selective NOESY experiments for protons at 4.72 ppm and 4.59 ppm suggested that both protons were in cis relationship, which indicated a fair proximity of both protons and thus formation of a six-member ring.

The —C═O stretching vibration of a lactam in ezetimibe and in the (*R,R,S*) stereoisomer thereof is at 1731 cm^{-1} and 1720 cm^{-1}, respectively. The IR spectral data of the impurity EZ-zanOH showed two strong peaks at 1654 cm^{-1} for —C═O stretching and 1547 cm^{-1} for —NH stretching, so it was concluded that an amide (—NH—C═O) group is present in the impurity molecule. The mass spectrum recorded for EZ-zanOH revealed the ion peak $[M + Na]^+$ at *m*/*z* 432.

Taking into account the above NMR, IR, MS spectral data as well as stress degradation and crystallographic studies described in literature [10,11], the structure of the isolated degradation product EZ-zanOH was proposed as (2*R*,3*R*,6*S*)-*N*,6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2*H*-pyran-3-carboxamide.

The analysis of the NMR and IR spectral data confirmed the structure of compounds EZ-2 and EZ-6′ (*R,R,S,S*). The characteristic IR band positions and NMR shifts for all studied compounds are tabulated (Tables 3 and 2).

3.2. Chromatography

The developed HPLC method allowed to determine the chemical as well as stereochemical purity of the final product (EZ-8 (*S,R,S*)). Because of the differences in the maximum absorption of the analyzed compounds (210 nm for EZ-2; 235 nm for ezetimibe, their (*R,R,S*) stereoisomer, and EZ-zanOH), HPLC studies were performed using two-channel detection. The analysis of the crude ezetimibe samples revealed the presence of three main impurities at retention times (RT) of 1.9 min, 22.8 min and 24.0 min, apart from the ezetimibe peak at RT of 22.1 min. Two compounds were identified as: stereoisomer of ezetimibe (RT 22.8 min, marked as EZ-8 (*R,R,S*)),

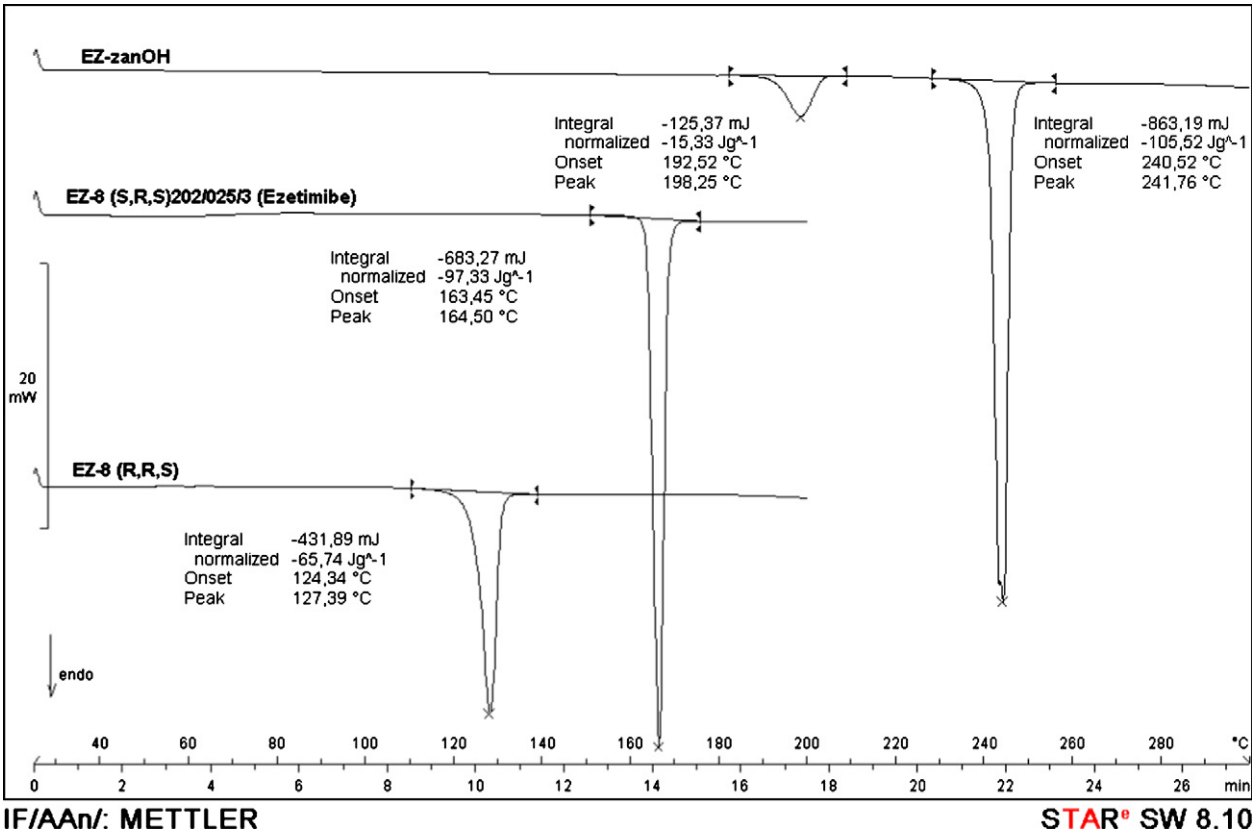


Fig. 4. The DSC curve of ezetimibe, EZ-8 (*S,R,S*), its (*R,R,S*) stereoisomer and degradation product formed under alkali condition (EZ-zanOH).

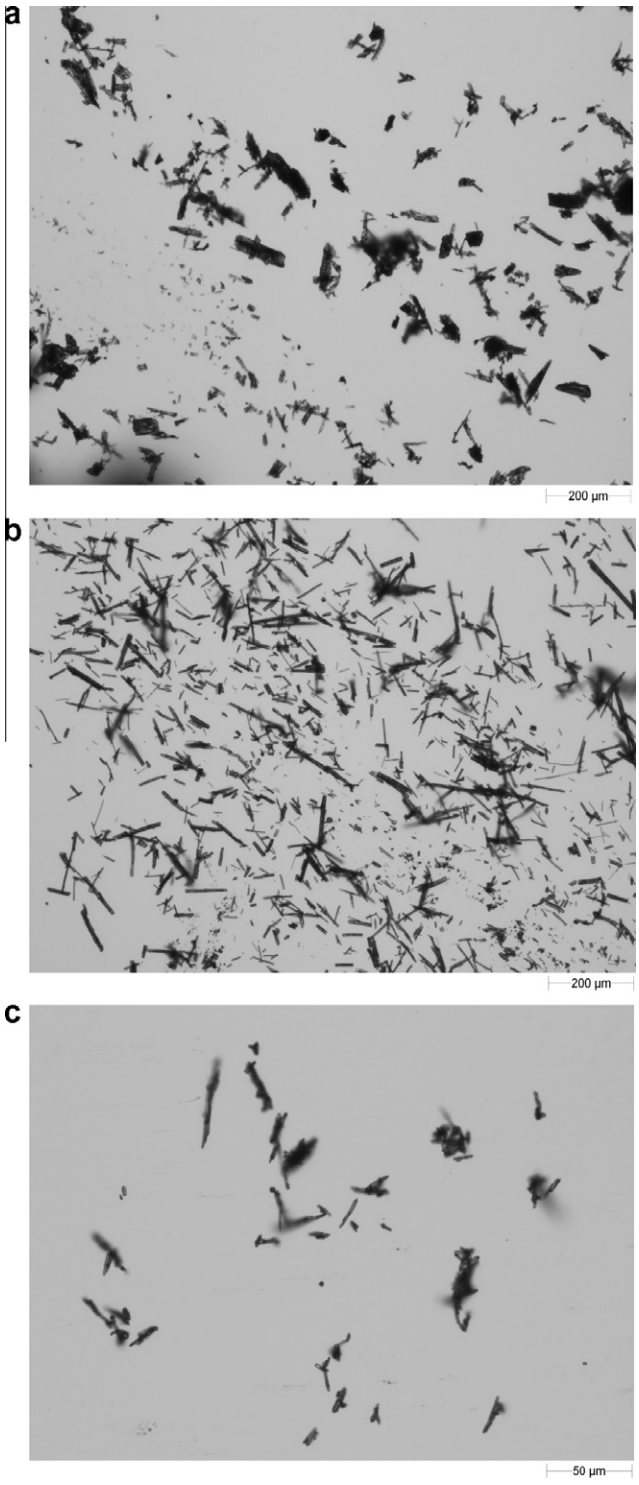


Fig. 5. The representative crystal pictures of ezetimibe (a. Sample No. EZ-8 202/025/3, b. Sample No. 202/017/1) and (*R,R,S*) isomer thereof (c. Sample No. EZ-8 (*R,R,S*) 202/042/1), all before dispersion.

(*S*)-4-phenyl-2-oxazolidinone (RT 1.9 min, marked as EZ-2) and confirmed by the co-injection of authentic samples. The identification of the third peak at RT 24.0 min proved to be unsuccessful, but the optimization of the final product purification process allowed to eliminate this impurity almost completely. The specificity and selectivity of the HPLC method was determined by analysing the spiked ezetimibe sample, containing impurities at the level of 0.15% into 100% EZ-8 (*S,R,S*). The representative chromatogram generated to demonstrate the specificity and selectivity of the HPLC method is shown in Fig. 3. The resolution between ezetimibe and impurities peaks meets the established criterion (>1.5). The expected detection limit was estimated by the analysis of the mixture solution of ezetimibe and impurities at the reporting level (0.05% according to [7], Table 5). The stability-indicating status of the developed HPLC method was demonstrated based on the degradation studies of ezetimibe. The stress studies conducted under alkaline conditions showed that ezetimibe decomposed to almost only one product (RT 15.2 min), marked as EZ-zanOH. As described above, the structure of this degradation product was proposed as (2*R*,3*R*,6*S*)-*N*,6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2*H*-pyran-3-carboxamide and was confirmed with the help of the NMR, IR and MS analyses of the isolated sample. The degradation studies of ezetimibe described in [10] demonstrate the formation of (2*R*,3*R*,6*S*)-*N*,6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2*H*-pyran-3-carboxamide under various stress conditions.

Table 6
The numerical values characterizing the obtained distributions for ezetimibe and its isomer; d [0.1], d[0.5], d[0.9] – volume fraction of distribution below 10%, 50% and 90%, respectively.

	EZ-8 202/025/3	EZ-8 (RRS) 202/042/1
Elongation, d [n,0.9]	0.674	0.698
Length d [n,0.9], µm	46.6	18.7
Width d [n,0.9], µm	20.8	7.4
Particle size distribution		
d [v,0.1], µm	16.3	6.2
d [v,0.5], µm	32.9	9.9
d [v,0.9], µm	64.9	16.4
D [4,3], µm	38.4	10.9

3.3. Thermal analysis

The thermal analysis was performed for ezetimibe, its stereoisomer and for the degradation impurity. The DSC curve of ezetimibe, EZ-8 (*S,R,S*), is shown in Fig. 4 with the calculated enthalpy and the melting point (onset). The DSC curve of EZ-8 (*S,R,S*) was characterized by an endothermic peak. The endothermic peak was a result of melting the substance at 163.45 °C with $\Delta H = 97.33\ J\ g^{-1}$. For the ezetimibe (*R,R,S*) isomer, the DSC curve (Fig. 4) showed an endothermic peak, at 124.34 °C with $\Delta H = 65.74\ J\ g^{-1}$, resulting from the substance melting. The DSC curve of the compound EZ-zanOH (Fig. 4) was characterized by two endothermic peaks. The first peak came from the melting of an unknown impurity at 192.52 °C with $\Delta H = 15.33\ J\ g^{-1}$. The second peak was due to the melting of the substance at 240.52 °C with $\Delta H = 105.52\ J\ g^{-1}$.

3.4. Particle size and shape measurements

The determination and comparison of particle size and shape distribution for three ezetimibe samples (202/025/3, 202/026/3 and 202/017/1) as well as the morphological analysis of the ezetimibe stereoisomer (EZ-8 (*R,R,S*)) were performed using automated optical microscopy. The particles observed in the ezetimibe samples 202/025/3 and 202/026/3 were similar, while the crystals of the third batch (202/017/1) had a completely different shape. The particles of 202/017/1 were needle-shaped, whereas in the case of 202/025/3 and 202/026/3 the crystals showed rather a columnar shape. The examples of view for the tested ezetimibe samples are shown in Fig. 5a and b.

The measurements done for ezetimibe working standard (202/025/3) and its isomer (EZ-8 (*R,R,S*) 202/042/1) pointed out the similarity between particles in the range of the elongation parameter (on a scale from 0 to 1, where 1 corresponds to acicular shape).

However, the particles were characterized by a completely different size. The particles of ezetimibe 202/025/3 showed rather a columnar shape, while the particles of EZ-8 (*R*,*R*,*S*) isomer (202/042/1) were much smaller (taking into account both width and length) and rather acicular. The numerical values characterizing the obtained distributions are presented in Table 6. The representative crystal pictures of isomer EZ-8 (*R*,*R*,*S*) and ezetimibe (EZ-8 (*S*,*R*,*S*)) before dispersion are presented in Fig. 5.

4. Conclusion

The performed studies shed light on the stereochemistry of the processes taking place during the ezetimibe synthesis carried out according to Fig. 2. It was proved that the proper stereochemical purity of the intermediate EZ-6 is a key feature in the obtaining of optically pure ezetimibe. Structure elucidation of the main process-related impurity (the (*R*,*R*,*S*) isomer) as well as the isolated degradation product was discussed and physicochemical characterization of these compounds was presented. The developed high-performance liquid chromatography method proved to be selective and stability-indicating and allow to determine chemical and stereochemical purity of ezetimibe.

The determination of the impurity profile and elucidation of the structures of the main contaminants is very important to comply with the regulatory norms as well as for assessing the quality of ezetimibe as an API. The presented studies can be also helpful in preparing pharmacopoeial monograph of this substance.

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