

## DEVELOPMENT OF CHROMATOGRAPHIC METHOD FOR DETERMINATION OF DRUGS REDUCING CHOLESTEROL LEVEL – STATINS AND EZETIMIBE

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**Abstract:** The presented developed HPLC method and GC method may be used to separate and determine all analyzed 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) and ezetimibe using a single columns and a uniform methodology. In order to perform qualitative and quantitative tests of statins and ezetimibe the Symmetry C18 column 250 mm × 4.6 mm, 5 μm, the mobile phase: acetonitrile:water (70 : 30, v/v), adjusted to pH = 2.5 and a spectrophotometric detector for the HPLC method were used. For GC method column HP-1; 30 m × 0.25 mm × 0.25 μm and FID detector were selected. All results and statistical data obtained indicate good method sensitivity and precision. The RSD values are appropriate for both newly developed methods.

**Keywords:** hyperlipidemia, HPLC, GC, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, atorvastatin calcium, fluvastatin sodium, rosuvastatin calcium, lovastatin, simvastatin, ezetimibe

Hyperlipidemia (HLP) is a group of lipid metabolism disturbances of various pathological background and they are characteristic in that the blood levels of cholesterol, mainly low density cholesterol (LDL) and/or triglycerides, increase. Elevated levels of total cholesterol and LDL are tightly associated with an increased risk of ischemic heart disease and disturbances in the cerebral, coronary and peripheral circulation. The most important parameter of a lipid panel includes the LDL levels – the normal levels should be below 100 mg/dL, or even below 80 mg/dL according to the current standards of the European Society of Cardiology. Moreover, the cut-off value for the HDL levels has been elevated – the normal levels are those above 40 mg/dL for men and above 50 mg/dL for women, whereas a risk factor includes levels below 40 mg/dL. The third most important parameter includes the triglyceride levels – the normal levels are the ones that do not exceed 150 mg/dL.

First-choice medicinal products used in the treatment of hyperlipidemia, mainly in order to reduce the cholesterol levels, include 3-hydroxy-3-

methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors namely statins, and aryloxyalkylcarboxylic acids derivatives, namely fibrates (1-4).

Currently, a medicinal product belonging to a new group of agents affecting the lipid metabolism in the body has been introduced – and it is called ezetimibe. Ezetimibe selectively inhibits the absorption of exogenous cholesterol from a diet and present in the bile, as well as plant sterols in the intestines. It binds to the Niemann-Pick C1 like 1 protein (NPC1L1) in the epithelium of the intestinal mucous membrane. This protein plays a vital role in the absorption of cholesterol into the cells, therefore this agent reduces the cholesterol levels in the blood plasma. However, the mechanism of action of ezetimibe is different from the one observed for agents inhibiting the intestinal cholesterol absorption, ion-exchange resins and sitosterols (plant sterols) used so far in pharmacotherapy. Moreover, it has been observed that this new medicinal product does not affect the inhibition of gastrointestinal absorption in the case of triglycerides, fatty acids, bile acids or fat-soluble vitamins (A and D vitamins). Therefore,

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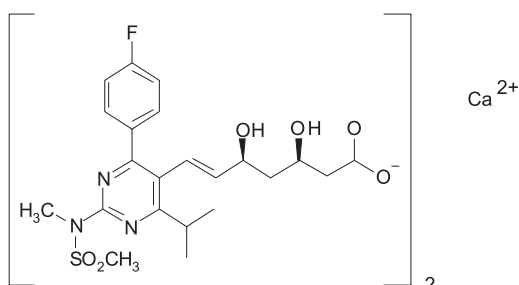
ezetimibe has been included in a new subgroup of medicinal products used in the treatment of elevated levels of total cholesterol and LDL fraction. It is used in the treatment of primary hypercholesterolemia (familial heterozygous and homozygous, and non-familial) and of familial homozygous sitosterolemia as a diet-supporting agent.

Ezetimibe is used in monotherapy or more frequently in combination therapy with statins, if the

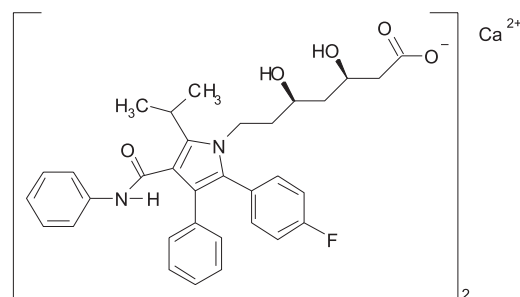
use of agents belonging to this group is contraindicated or poorly tolerated in high doses in these patients (5, 6).

Chemical formulas and names of medicinal products belonging to both therapeutic groups and used in combination therapy are presented in Figure 1.

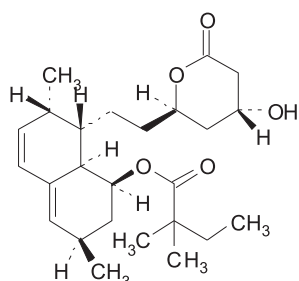
Available literature of recent years does not include any publications discussing the method to identify and determine ezetimibe in pharmaceutical



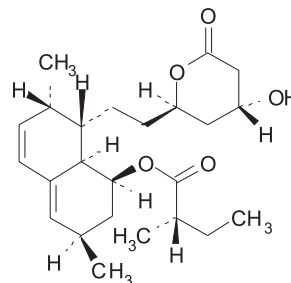
ROSUVASTATIN CALCIUM (C<sub>22</sub>H<sub>27</sub>FN<sub>3</sub>O<sub>6</sub>S) 2Ca  
bis[(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-  
[methyl(methylsulfonyl)amino]pyrimidin-5-yl](3R,5S)  
-3,5-dihydroxyhept-6-enoic acid] calcium salt



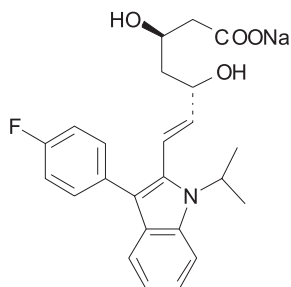
ATORVASTATIN CALCIUM (C<sub>22</sub>H<sub>27</sub>FN<sub>3</sub>O<sub>6</sub>S) 2Ca  
[R-(R\*,R\*)-2-(4-fluorophenyl)-â,â-dihydroxy-5-  
(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-  
1H-pyrrolo-1-heptanoate calcium



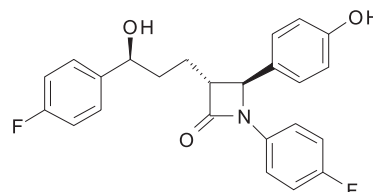
SIMVASTATIN (C<sub>25</sub>H<sub>38</sub>O<sub>5</sub>)  
(1S,3R,7S,8S,8aR)-8-[2-[(2R,4R)-4-hydroxy-  
6-oxotetrahydro-2H-pyran-2-yl]ethyl]-3,7-  
dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-  
1-yl 2,2-dimethylbutanoate



LOVASTATIN (C<sub>24</sub>H<sub>36</sub>O<sub>5</sub>)  
(1S,3R,7S,8S,8aR)-8-[2-[(2R,4R)-4-  
hydroxy-6-oxotetrahydro-2H-pyran-  
2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-  
hexahydronaphthalen-1-yl (2S)-2-  
methylbutanoate



FLUVASTATIN SODIUM (C<sub>24</sub>H<sub>25</sub>FNNaO<sub>4</sub>)  
[3R,5S,6E]-7-[3-(p-fluorophenyl)-1-(1-  
isopropyl)indol-2-yl]-3,5-dihydroxy-6-  
heptenoate sodium



EZETIMIBE (C<sub>24</sub>H<sub>21</sub>F<sub>2</sub>NO<sub>3</sub>)  
(3R,4S)-1-(4-fluorophenyl)-3-[(3S)-  
3-(4-fluorophenyl)-3-hydroxypropyl]-  
4-(4-hydroxyphenyl) azetidino-2-one

Figure 1. Chemical formulas and names of compounds belonging to statins and ezetimibe

products and biological materials. There are only few studies regarding clinical trials on the use of ezetimibe and statins in monotherapy and combination therapy (5, 6).

In 2006, Kublin et al. have published a study on determination of a whole group of statins using gas chromatography with the FID detector (7). In 2010, a study of a method to determine all medicinal products belonging to statins using HPLC with the Symmetry C18 column and a spectrophotometric detector was published by the same authors (8). In 2012, Kublin et al. published an article presenting test methods for the most commonly used agents belonging to statins and fibrates using HPLC and GC (9).

In modern therapy of hypercholesterolemia simvastatin or atorvastatin at a dose between 10 mg and 40 mg daily and ezetimibe at a dose 10 mg daily are used most frequently. As a result, it is possible to reduce significantly statin doses that are used and to reduce the simvastatin dose of 80 mg, which is commonly used and poorly tolerated by patients, to approx. 10–20 mg with simultaneous use of ezetimibe at a dose of 10 mg. The medicinal product called Inegy, including simvastatin and ezetimibe at a dose

of 10 mg + 10 mg, has been already introduced to the American market. On the Polish market, this product has not been registered yet.

Due to lack of any reports regarding common methods for the identification and quantitative determination of agents belonging to statins and ezetimibe simultaneously, especially in medicinal products, it is justified to prepare methods to determine the contents of these compounds.

The study was assumed to prepare further simple, sensitive and unified quantification methods for the identification and quantitative determination that could be used to study a group of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (HMG-CoA), namely statins and ezetimibe in routine analyses, and to assess its analytical and economical aspects.

## EXPERIMENTAL

### Reference materials

Rosuvastatin calcium (MSN Laboratories Ltd.), atorvastatin calcium (Pharmathen), simvastatin (Aurobindo Pharma Ltd.), lovastatin (Teva), fluvastatin sodium (USP), ezetimibe (Merck).

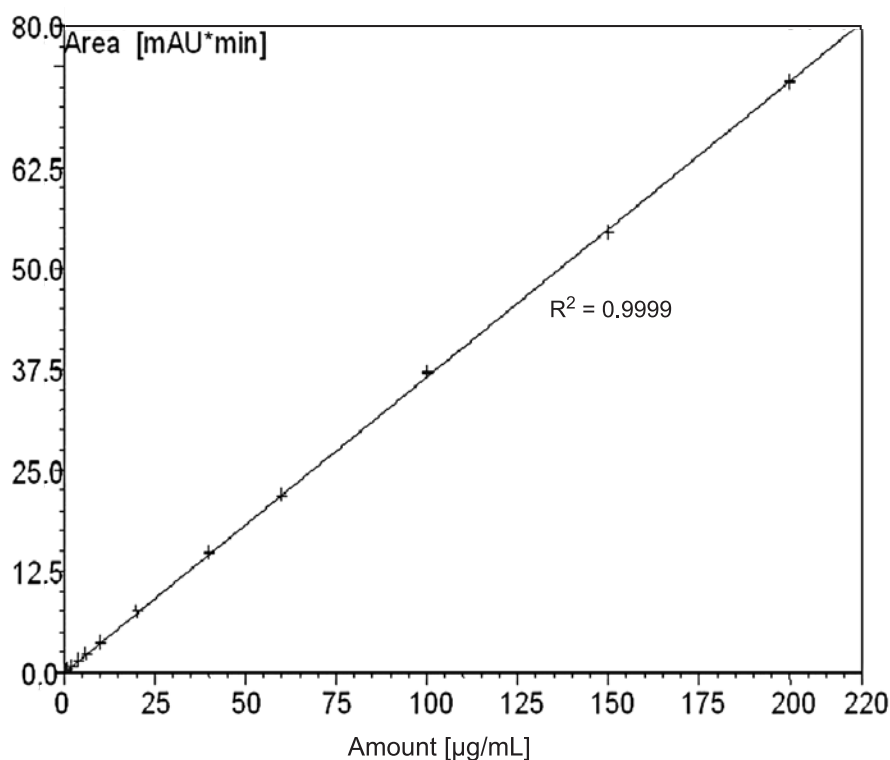


Figure 2. Regression curve for ezetimibe (HPLC)

### Medicinal products

Ezetrol – tablets 10 mg (MSD-SP Limited),  
Simorion – coated tablets 10 mg (Orion Pharma).

### Reagents and equipment

High purity HPLC reagents: acetonitrile, methanol (Rathburn), orthophosphoric acid 85% (AppliChem), Dionex liquid chromatograph with a spectrophotometric detector. Agilent Technologies gas chromatograph type 6890N with FID detector.

### High performance liquid chromatography – HPLC

#### Qualitative analysis

##### Ezetimibe identification

The Symmetry C18 column, 250 × 4.6 mm, 5 μm and a spectrophotometric detector were used for the test (9, 10).

The following identification conditions were prepared: mobile phase: acetonitrile:water (70 : 30, v/v), adjusted to pH = 2.5 with 85% orthophosphoric acid; column temperature: 35°C; autosampler temperature: 15°C; mobile phase flow rate: 1.2 mL/min; injection volume: 10 μL; wavelength: 232 nm.

#### Preparation of calibration curve for ezetimibe

Standard solutions of ezetimibe in methanol at appropriate concentrations were prepared and a cal-

ibration curve for this compound was constructed (Fig. 2).

The linearity of the examined compound in the tested range of levels 0.010–199.92 μg/mL was observed.

The determination limit was 0.050 μg/mL, and the detection limit – 0.010 μg/mL.

#### Quantitative determination

##### Determination of ezetimibe

Under predetermined conditions, the selected ezetimibe levels were quantitatively determined in a medicinal product – Ezetrol tablets 10 mg.

A spectrophotometric detector and the wavelength of 232 nm were used during an analysis. Methanol was used as a solvent.

#### Preparation of standard and tested solutions

##### Standard solution of ezetimibe

Ca. 5 mg of a reference material - ezetimibe was weighed into a 100 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

##### Tested solutions

Ca. 50 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 5 mg of the active substance - ezetimibe) was weighed into a 100 mL measuring flask, approx. 50 mL of methanol was

Table 1. Retention times ( $R_t$ ) of tested compounds (HPLC).

COMPOUND NAME	RETENTION TIME [min]
ROSUVASTATIN CALCIUM	2.45
EZETIMIBE	3.01
ATORVASTATIN CALCIUM	3.41
FLUVASTATIN SODIUM	3.73
LOVASTATIN	7.65
SIMVASTATIN	9.96

Table 2. Retention time ( $R_t$ ) for tested compounds (GC).

COMPOUND	RETENTION TIME [min]
FLUVASTATIN SODIUM	2.26
ROSUVASTATIN CALCIUM	3.19
LOVASTATIN	4.14
SIMVASTATIN	4.41
ATORVASTATIN CALCIUM	5.51
EZETIMIBE	6.74

Table 3. Statistical assessment of results of determining the contents of ezetimibe in a medicinal product with HPLC and GC methods.

NAME OF COMPOUND / PRODUCT	NUMBER OF TESTS n	AVERAGE FROM ALL MEASUREMENTS X (%)	STANDARD DEVIATION S	CONFIDENCE INTERVAL $X \pm \Delta X$ Pu = 95% (w %)	RSD (%)
Ezetimibe tablets (Ezetrol) HPLC	10	99.04	0.02	99.04 ± 0.02	0.22
Ezetimibe tablets (Ezetrol) GC	10	100.21	0.04	100.21 ± 0.04	0.55

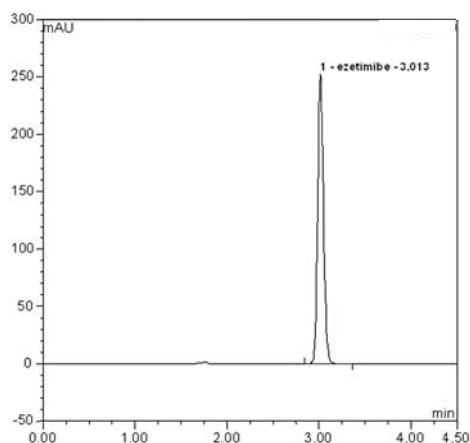


Figure 3. Chromatogram of the ezetimibe standard (HPLC)

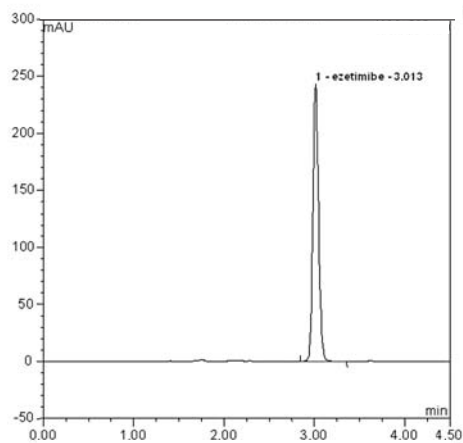


Figure 4. Chromatogram of the ezetimibe solution prepared from tablets (HPLC)

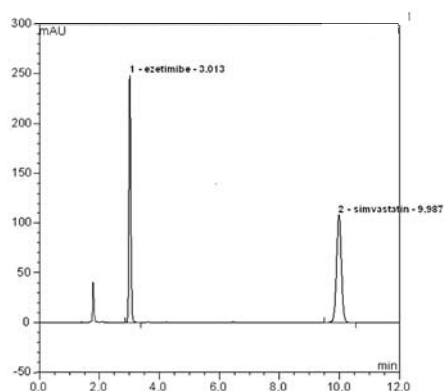


Figure 5. Chromatogram of the mixture of ezetimibe and simvastatin prepared from tablets at the wavelength of 232 nm (HPLC)

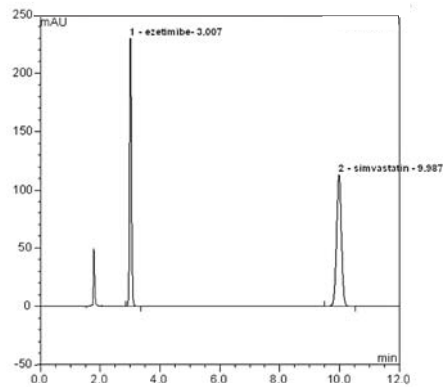


Figure 6. Chromatogram of the mixture of ezetimibe and simvastatin prepared from tablets at the wavelength of 238 nm (HPLC)

added and the solution was shaken mechanically for 30 min. Solutions were diluted to the volume, and filtered. The solutions obtained were used in tests. Table 3 presents results and a statistical assessment. Exemplary chromatograms are presented in Figures 3 and 4.

### Simultaneous quantitative determination of ezetimibe and simvastatin

#### Medicinal products: Ezetrol and Simorion

The determination was carried out at the following wavelengths: 232 nm for ezetimibe and 238 nm for simvastatin.

#### Preparation of standard solution of ezetimibe and simvastatin

Ca. 5 mg of a reference material (ezetimibe) and 5 mg of a reference material (simvastatin) were

transferred to a 100 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

#### Tested solutions

Approximately 50 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 5 mg of the active substance - ezetimibe) and 50 mg of a powdered tablet mass of Simorion (corresponding to approx. 5 mg of the active substance - simvastatin) were weighed into a 100 mL measuring flask. Approx. 50 mL of methanol was added and the solution was shaken mechanically for 30 min. Solutions were diluted with methanol and filtered. The solutions obtained were used in tests. Exemplary chromatograms are presented in Figures 5 and 6. Table 4 presents results and a statistical assessment.

### Gas chromatography (GC) method

#### Qualitative analysis

##### Ezetimibe identification

The following column was used: HP-1; 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m and the FID detector (8, 10). The identification conditions prepared: detector temperature: 320°C; injection chamber temperature: 300°C; column temperature: programme – initial temp. 190°C for 1 min; increment of 8°C/1 min to

the final temp. of 285°C/2 min; gas flow: 3.9 mL/min; injection volume: 1.0  $\mu$ L and split: 10 : 1.

#### Preparation of a calibration curve for ezetimibe

Standard solutions of ezetimibe in methanol at appropriate concentrations were prepared and a calibration curve for this compound was drawn (Fig. 7).

The linearity in the tested range of levels 39.98–999.60  $\mu$ g/mL was observed. The determina-

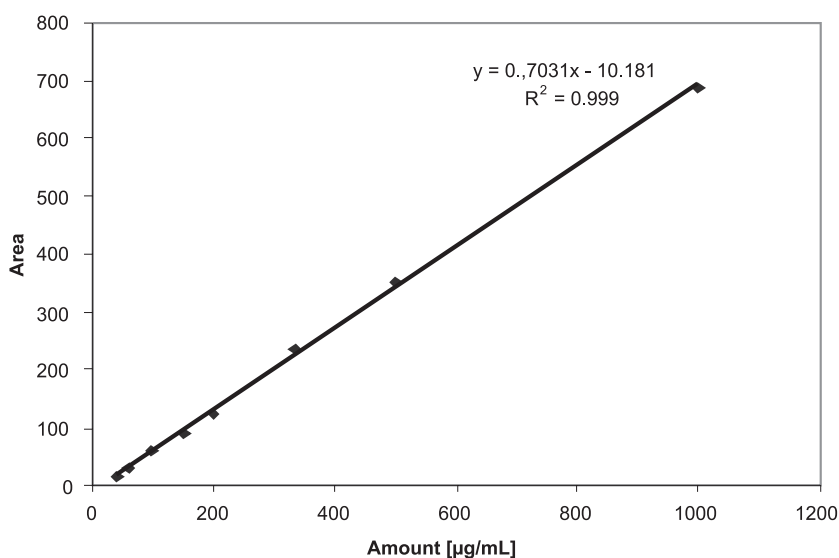


Figure 7. Regression curve for ezetimibe (GC)

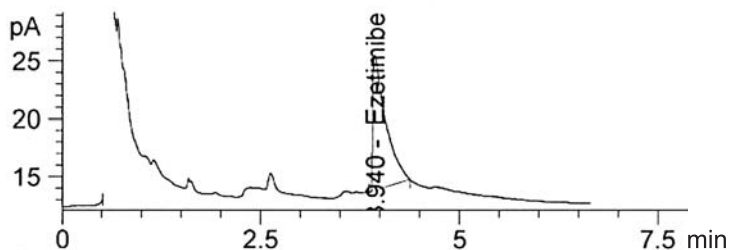


Figure 8. Chromatogram of ezetimibe standard (GC)

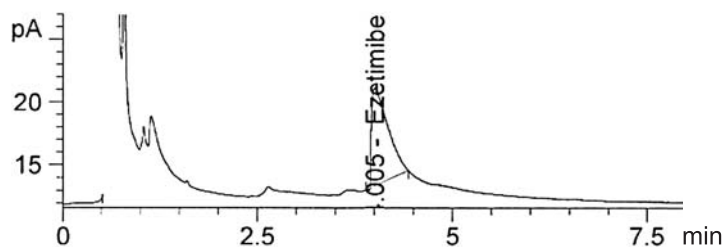


Figure 9. Chromatogram of the ezetimibe solution prepared from tablets (GC)

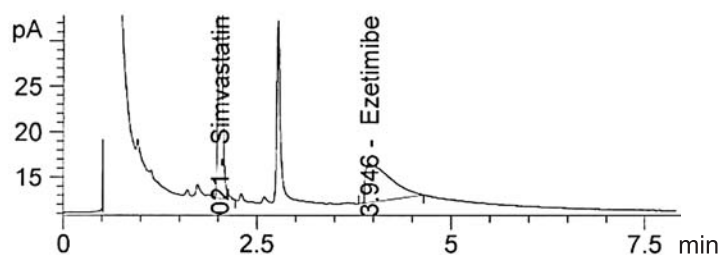


Figure 10. Chromatogram of the mixture of ezetimibe and simvastatin standards (GC)

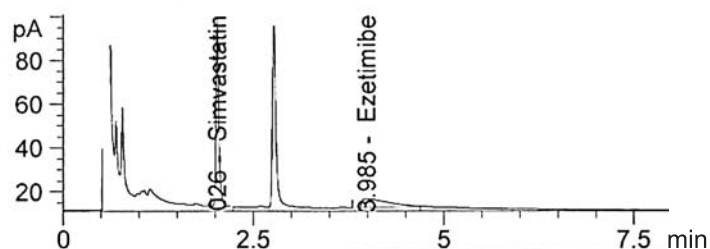


Figure 11. Chromatogram of ezetimibe and simvastatin solution prepared from tablets (GC)

Table 4. Statistical assessment of results of determining the contents of active substances in medicinal products with HPLC and GC methods parallelly.

NAME OF COMPOUND / PRODUCT		NUMBER OF TESTS n	AVERAGE FROM ALL MEASUREMENTS X (%)	STANDARD DEVIATION S	CONFIDENCE INTERVAL $X \pm \Delta X$ Pu = 95% (w %)	RSD (%)
HPLC	Ezetimibe tablets (Ezetrol)	9	99.06	0.05	$99.06 \pm 0.04$	0.46
	Simvastatin coated tablets (Simorion)	9	98.10	0.03	$98.10 \pm 0.02$	0.27
GC	Ezetimibe tablets (Ezetrol)	8	99.84	0.87	$99.84 \pm 0.73$	0.87
	Simvastatin coated tablets (Simorion)	8	95.48	0.26	$95.48 \pm 0.22$	0.28

tion limit was 39.984  $\mu\text{g/mL}$  and the detection limit – 19.992  $\mu\text{g/mL}$ .

#### Quantitative determination

Determination of ezetimibe with the GC method

Under predetermined conditions, the selected ezetimibe concentrations were quantitatively determined in a medicinal product – Ezetrol tablets 10 mg. The FID detector was used during analyses. Methanol was used as a solvent.

#### Preparation of standard and tested solutions

##### Standard solution of ezetimibe

Ca. 5 mg of a reference material was weighed into a 25 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

##### Tested solutions

Ca. 100 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 10 mg of the active substance - ezetimibe) was weighed into a 50

mL measuring flask, approx. 25 mL of methanol was added and the solution was shaken mechanically for 30 min. Solutions were diluted to volume, and filtered. The solutions obtained were used in tests.

Table 3 presents results and a statistical assessment. Exemplary chromatograms are presented in Figures 8 and 9.

### Parallel quantitative determination of ezetimibe and simvastatin

*Medicinal products: Ezetrol and Simorion*

The tests were performed under predetermined conditions used for the gas chromatography method.

*Standard solution of ezetimibe and simvastatin*

Ca. 5 mg of a reference material - ezetimibe and 5 mg of a reference material - simvastatin were weighed into a 25 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

*Tested solutions.*

Ca. 100 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 10 mg of the

active substance - ezetimibe) and 100 mg of a powdered tablet mass of Simorion (corresponding to approx. 10 mg of the active substance - simvastatin) were weighed into a 50 mL measuring flask, approx. 25 mL of methanol was added and the solution was shaken mechanically for 30 min. Solutions were diluted to volume with methanol and filtered. The solutions obtained were used in tests. Exemplary chromatograms are presented in Figures 10 and 11. Table 4 presents results and a statistical assessment.

### Identification of the mixture containing ezetimibe and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors - statins

The parallel test to identify HMG-CoA reductase inhibitors used in pharmacotherapy - statins and ezetimibe using the HPLC method and gas chromatography method was performed.

During the HPLC method a mixture of the following substances in methanol was used: atorvastatin calcium - 52.7 µg/mL, lovastatin - 48.8 µg/mL, simvastatin - 52.2 µg/mL, fluvastatin sodium - 50.5 µg/mL, rosuvastatin calcium - 51.5 µg/mL and ezetimibe - 51.9 µg/mL.

The following predetermined assay conditions were used: Symmetry C18, 250 × 4.6 mm, 5 µm column; mobile phase: acetonitrile : water (70 : 30, v/v), adjusted to pH = 2.5 with 85% orthophosphoric acid; column temperature: 35°C; autosampler temperature: 15°C; mobile phase flow: 1.2 mL/min; injection volume: 10 µL; wavelength: 232 nm.

All tested compounds were separated, and retention times obtained are presented in Table 1. The separation factor between two adjacent peaks of atorvastatin and fluvastatin (peaks 3 and 4 in Fig. 10) is 2.44. The chromatogram is presented in Figure 12.

During the gas chromatography (GC) method a mixture of following substances in methanol solutions was used: atorvastatin calcium - 52.7 µg/mL, lovastatin - 48.8 µg/mL, simvastatin - 55.2 µg/mL, fluvastatin sodium - 101.0 µg/mL, rosuvastatin calcium - 103.0 µg/mL and ezetimibe - 266.3 µg/mL

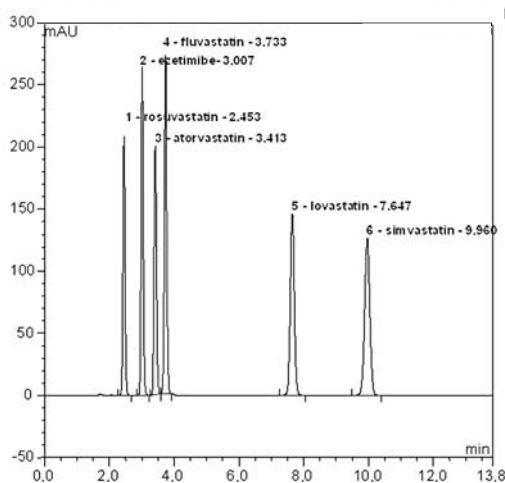


Figure 12. Chromatogram of the statin mixture and ezetimibe standards (HPLC)

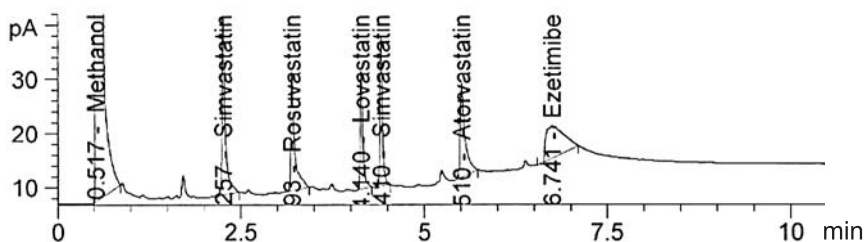


Figure 13. Chromatogram of the statin mixture and ezetimibe standards (GC)



The following identification conditions were developed: HP- 1 column; 30 m × 0.25 mm × 0.25 μm; detector temperature: 320°C; injection chamber temperature: 300°C; column temperature: programme – initial temp. 240°C for 1 min; increment of 10°C/1 min to the final temp. of 295°C, 2 min; gas flow: 3.9 mL/min; injection volume: 1.0 μL; split: 10 : 1.

All tested compounds were separated, and retention times obtained are presented in Table 2. The separation factor between two adjacent peaks of lovastatin and simvastatin (peaks 4 and 5 in Fig. 13) is 2.08. The chromatogram is presented in Figure 13.

### DISCUSSION OF RESULTS

With regard to the fact that treatment of hypercholesterolemia often involves combination therapy including medicinal products belonging to statins – simvastatin the most frequently and ezetimibe, this paper was aimed to perform a quantitative analysis using HPLC and GC methods of compounds that are the most frequent ingredients of medicinal products. Results and statistical data obtained presented in Table 3 and 4 indicate good method sensitivity and precision. The RSD values presented in Table 3 and 4 are appropriate for newly developed methods to determine ezetimibe alone, as well as ezetimibe and simvastatin simultaneously.

### CONCLUSIONS

HPLC and GC methods developed and presented herein may be used for simultaneous separation and determination of all tested (HMG-CoA)

reductase inhibitors – statins, and a newly introduced compound – ezetimibe, using one column and similar methods.

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