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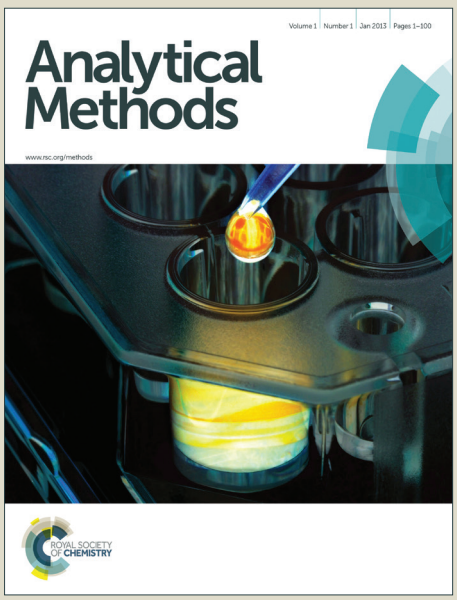

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Pharmacological and therapeutic properties of new derivatives of renin inhibitors and endothelin receptor antagonists, and the methods of their determination. - A review

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Abstract

According to the data of the World Health Organization (WHO), cardiovascular diseases are the most often diseases of the 21st century. They are the reason of about 17 millions of deaths each year, including 9.4 million of deaths caused by hypertensive disease or complications occurring during its course[1]. Therapeutic schemes in arterial hypertension include monotherapy or combination therapy involving administration of two or three drugs from various pharmacological groups, one of which should exhibit diuretic activity[2]. This review presents new information concerning analytical methods used in order to determine new substances of hypotensive activity belonging to two pharmacological groups, i.e., renin inhibitors and endothelin receptor antagonists.

1.Introduction

1.1 The role of renin-angiotensin-aldosterone system (RAA) in arterial hypertension.

RAA system plays a significant role in arterial hypertension pathogenesis (Fig. 1).

Fig. 1. Renin-angiotensin-aldosterone system

Angiotensin II which is formed in this system, affects cardiovascular system, and water-electrolyte balance. An activity of renin-angiotensin-aldosterone system is conditioned by the factors affecting renin secretion and depends on angiotensinogen regulating synthesis and metabolism – alpha-2 globulin formed in liver. Renin is a glycoprotein proteolytic enzyme, and its main source are the cells of renal juxtaglomerular apparatus, however the presence of renin-synthesizing gene was also noted inter alia within vascular wall and in central nervous system. Renin was discovered due to the works carried out by Swedish scientist Tiegerstadt[3]. In 1934, pathologists Harry Goldblatt elaborated the first animal model of arterial hypertension etiology[4]. The role of renin in the process of angiotensin I formation was described by two independent teams of Braun-Menendez et al.[5], and Page and Helmer.[6] Recognition of physiology and pathophysiology of RAA system resulted in the search for the substances affecting the changes in this system activity in terms of arterial hypertension therapy.

1.2. Drugs affecting RAA system activity

Basic substances of hypotensive activity affecting RAA system include angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARBs) and renin inhibitors. An activity of the drugs from the group of angiotensin converting enzyme inhibitors involves an inhibition of the reaction of angiotensin I conversion into angiotensin II. Enzyme called convertase is responsible for these compounds conversion. The first oral

drug from the ACEi group was captopril introduced to medicine in 1981. Further research within this group of drugs resulted in a release of several substances of various chemical structure and pharmacokinetic mechanism, which are commonly used in arterial hypertension therapy.

Blockers of angiotensin II receptor specifically combine with angiotensin I receptor (AT I). Blocking of AT I receptor causes an inhibition in biological function of angiotensin II, which is a peptide and its biological activity involves stimulation of aldosterone amount increase, i.e., mineralocorticoid hormone causing an increase in sodium ions and water absorption in kidneys. Absence of angiotensin II biological activity is also reflected in an inhibition of sodium ions resorption and potassium ions loss in renal tubules, which causes a reduction in circulating blood volume and cardiac load. An inhibition in angiotensin II activity results in a decreased peripheral tension, and thus reduced oxygen demand. The first substance blocking angiotensin activity was salarazin[7], which however was not used commonly as hypotensive drug due to short half-life and lack of possibility of its oral administration. The best recognized drug from ARBs group was losartan, which hypotensive activity was described by Timmermans et al.[8]. The search for other substances of similar activity resulted in an occurrence of the whole group of compounds so called sartans, which were included into the first-line medicaments in hypertensive disease therapy in the report of the World Health Organization and International Society of Arterial Hypertension in 1999.[9]

One of the new groups of drugs used in hypertension treatment are, so called, inhibitors of renin, which is formed in kidneys and then passes to blood and causes decomposition of angiotensinogen releasing decapeptide angiotensin I, which is then subject to decomposition in lungs, kidneys and other organs forming octapeptide angiotensinogen II. This octapeptide increases blood pressure, both directly by arteries stenosis, and indirectly by aldosterone release from adrenal gland; aldosterone is a hormone retaining sodium ions accompanied by an increase in extracellular fluid volume. Inhibitors of renin enzymatic activity cause decreased formation of angiotensin I and consequently proportional decrease in angiotensin II amounts. Such activity of renin inhibitors directly affects blood pressure decrease.[10]

Fig. 2. Endothelial system

Other group of substances introduced into hypertension therapy are the antagonists of endothelin receptor. Discovery of endothelins isolated from animal cells[11] as substances strongly affecting blood vessels, resulted in the search for the structures acting in an antagonistic manner towards them. Endothelins are the compounds of peptides nature composed of 21 amino acid residues, which are formed in the cells of blood vessels endothelium, brain, kidneys and endocrine glands. Their precursor is, so called, pre-proendothelin composed of 22 amino acid residues, which under an influence of intracellular endopeptidases is transformed to proendothelin composed of 38-39 amino acid residues, and then endothelin is formed as a result of endothelin-converting enzyme activity. Three isoforms of a structure of 21- amino acid peptides are known: endothelin 1 (ET-1), endothelin 2 (ET-2) and endothelin 3 (ET-3).[12] They are synthesized in different sites, ET-1 is formed inter alia in the cells of blood vesicles smooth muscles[13], nervous cells[14], ET-2 in kidneys[15], while the site of ET-3 synthesis and its role has been still not fully recognized. The factors stimulating endothelins formation include numerous substances, like adrenaline, angiotensin II, vasopressin and insulin. Endothelins bind with receptors which were divided into two groups: endothelin receptor type A (ET_A) and B (ET_B), and particular isoforms of endothelins are characterized by different affinity to specified receptor. It was confirmed in the research that receptor ET_A is characterized by a several-fold higher affinity to ET-1 and ET-2 than to ET-3, while receptor ET_B is characterized by equal affinity to all three kinds of endothelins. [16, 17, 18] The role of ET-1 has been described the best, and it involves binding mainly with A type receptor, which is observed in the cells of vessels smooth muscles and cardiac muscle, what constitutes 85% of all endothelin receptors. These receptors stimulation causes blood vessels constriction. B type receptor is mainly observed on the surface of endothelial cells and smooth muscles cells, and its activation results in transitory vasodilation as a result of an increased synthesis of nitric oxide and decreased intracellular concentration of calcium ions.[19]

2. Renin inhibitors

The new group of drugs being renin inhibitors include substances of various chemical composition but similar pharmacological activity. They all cause an inhibition in renin activity in renin-angiotensin-aldosterone system, causing in a consequence a decrease in blood pressure.

Fig. 3 Chemical structure of renin inhibitors

2.1. Aliskiren

(2S,4S,5S,7S)-5-amino-N-(2-carbamoyl-2,2-dimethylethyl)-4-hydroxy-7-{[4-metoxy-3-(3-metoxypropoxy)phenyl]methyl}-8-methyl-2-(propan-2-yl)nonanamide (Fig. 1) is the first oral, non-peptide direct renin inhibitor. Aliskiren is well soluble in water (solubility at pH 7.4 is > 350 $mg \cdot mL^{-1}$), and its molar mass is 551.8 $g \cdot mol^{-1}$. It is well absorbed after oral administration, maximum concentration in serum is observed after 1-3 h, its bioavailability after single 75 mg dose administration is 2.6%. Irrespective of the concentration, it binds with serum proteins in the range of 47-51%, half-life for aliskiren is from 23 to 70 h, and its metabolism occurs mainly in liver in small amount of about 20%.[20]

2.1.1. Separation methods

The separation methods were predominantly used for aliskiren determination. Lefèvrea and Gauronb[21] proposed HPLC method with fluorometric detection for aliskiren determination in blood serum and urine. The samples were extracted in an automated way from 400 μL of serum or urine with methyl alcohol–acetic acid mixture (99:1, v/v) on 100-mg Bond-Elut CN cartridges using the Gilson ASPEC system. Chromatographic separation was conducted on LiChrospher 100 RP8 5-μm particle size packed analytical column (25 × 0.4 cm I.D.). The mobile phase was acetonitrile-0.01 M potassium dihydrogen phosphate (65:35, v/v) mixture with a flow rate of 0.8 $mL \cdot min^{-1}$, fluorometric detection with excitation and emission wavelength of 280 and 330 nm, respectively, was used, an internal standard was (2S,4S,5S,7S)-N-butyl-5-amino-4-hydroxy-2,7-diisopropyl-8-[3-(3-methoxypropoxy)-4-methoxyphenyl]octanamide hydrochloride (CGP-56962A). Linearity range for the proposed method was 4.5-450 $ng \cdot mL^{-1}$ in serum, and 9.0–900 $ng \cdot mL^{-1}$ in urine, quantification limits were 4.5 $ng \cdot mL^{-1}$ and 9.0 $ng \cdot mL^{-1}$, respectively. HPLC method was used by F. Belalaet al.[22] The authors described a very sensitive method of aliskiren determination after derivatization with 1-naphthyl isocyanate. Caffeine solution in acetonitrile (50 $\mu g \cdot mL^{-1}$) was used as an internal standard. The research material was serum enriched with aliskiren addition. 0.5 mL of such prepared sample was subjected to extraction process using 1.5 mL acetonitrile and

centrifuged, supernatant after decantation was evaporated to dry form in nitrogen atmosphere at a temperature of 40°C. 0.5 ml acetonitrile was added to obtained dry mass and it was subjected to derivatization process which involved an addition of 0.225 mL of 0.5% 1-naphthyl isocyanate solution and 0.225 mL of 0.5% triethylamine solution to the sample, the whole was mixed and heated in a water bath for 35 min at a temperature of 50°C. After cooling to the room temperature, 0.5 mL of internal standard was added to such prepared post-reaction mixture. The course of derivatization reaction proposed by the authors is presented in Fig. 4.

Fig. 4. Derivatization reaction of aliskiren with 1-naphthyl isocyanate

Chromatography process was conducted on Shim-pack CLC-C18 column (250 × 4.6 mm i.d., 5 μm particle size), the mobile phase was acetonitrile-water-phosphoric acid mixture (45:55:0.01, v/v/v, pH 3.2), flow rate was 1 $mL \cdot min^{-1}$, UV detection with a wavelength of 230 nm was used. The linearity range for the method was from 5 $ng \cdot mL^{-1}$ to 400 $ng \cdot mL^{-1}$, detection limit was 0.5 $ng \cdot mL^{-1}$ and a limit of quantification was 1.0 $ng \cdot mL^{-1}$. Also HPLC-MS/MS method was used for aliskiren determination in blood serum[23], in which benazepril was applied as an internal standard. Samples preparation involved an addition of 500 mL phosphoric acid (2%, v/v) and 10 mL methanol benazepril solution to 100 μL serum. The whole was mixed, and then centrifuged at a temperature of 4^0C for 10 min. The extraction was performed using OasisW MCX solid-phase extraction (SPE) cartridges. SPE system was initially prepared by rinsing with ammonia solution in methanol (10%, v/v), and then methanol and 2% formic acid. Acidified serum samples were applied on SPE columns, and rinsed with 2 mL of 2% formic acid and 2 mL methanol. An analyte was eluted by three-times system rinsing with 0.5 mL and once with 0.3 mL of ammonia solution in methanol (10%, v/v). Eluent was evaporated to dry form in nitrogen atmosphere, and then dissolved in 100 μL of mobile phase, which was methanol-water-formic acid mixture (75:25:0.005, v/v/v). Such prepared samples were subjected to separation on XselectTM C18 CSH column, with mobile phase flow rate of 0.4 $mL \cdot min^{-1}$, total time of analysis was about 5 min, and the results obtained were characterized by a high precision and accuracy. The linearity range was 0.146–1200 $ng \cdot mL^{-1}$. Due to an application of small volume of the examined sample, the elaborated

method may be successfully used in monitoring of aliskiren level in pediatrics. Also the study on aliskiren determination in the saliva of healthy volunteers was conducted using LC-ESI-MS/MS.[24] Sample preparation for the study involved mixing of 100 µL of undiluted saliva with 10 µL of internal standard, which was benazepril hydrochloride solution (166 ng/ml) and 490 µL formic acid (2%, v/v). The sample was subject to extraction to solid phase (Oasis® MCX) using SPE columns which were suitably prepared by rinsing with 1 mL of a mixture of ammonia solution–methanol–acetonitrile (10:45:45, v/v/v), and then 1 mL of methanol and finally equilibrated with 2% formic acid. The samples were applied on such prepared SPE system and eluted with 1 mL of formic acid (2%, v/v) followed by 1 mL methanol–acetonitrile (50:50, v/v). Analyte was released by elution using 0.5 mL (three times) and one time 0.4 mL of a mixture of ammonia solution-methanol-acetonitrile (10:45:45, v/v/v). Eluent was evaporated at the temperature of 40°C. The sample was diluted using 100 µL mixture of acidified methanol-water (20:80, v/v). Chromatographic separation was carried out on a Xselect™ CSH C18 column (3.0 × 150 mm, 3.5 µm) protected by a corresponding Xselect™ CSH C18 guard column (3.0 × 20 mm, 3.5 µm). Time of analysis was 7.5 min with a mobile phase gradient of acidified methanol (A) and acidified water (B) (each 0.1% formic acid, v/v) to separate the compounds. Gradient was as follows: starting with 30% of A, after 0.2 min 50% A, at 6 min 80% A, at 6.3 min 100% A, decreased to 50% A at 7 min and staying at 50% for the rest of the run time. The elaborated procedure is characterized by a wide range of linearity, i.e. 0.586-1200 ng·mL^{-1}, low quantification limit, as well as good precision and accuracy. Based on the results obtained the authors concluded that the concentration of aliskiren in human saliva is measurable, but considerably lower than expected. The comparison of concentration in saliva and blood demonstrated considerable deviations, which may prove a complicated course of the process of drug distribution in human organism.

2.1.2. Other methods

Except separation methods, also spectrophotometric and spectrofluorometric methods were used for aliskiren determination. Mai A. Ramadan and Muhammed B. Abuiriban[25] elaborated spectrophotometric method for aliskiren determination in pharmaceuticals using reaction of derivatization from o-phthalaldehyde and N-acetylcysteine in an alkaline buffer. The reaction involved 0.05% methanol solution of o-phthalaldehyde and 0.06% water solution of N-acetylcysteine. Powdered tablet mass was subject to the process of extraction with water within a time of 1 h. 2 mL of obtained solution was collected after filtering, 1.0 mL o-phthalaldehyde (0.05%), and 1.0 mL N-acetylcysteine (0.06%) and 1.0 mL borate buffer (0.2

M, pH 10.0) was added, the whole was mixed and left at a room temperature for 10 min. Then the sample was dissolved with distilled water and absorption spectrum was registered in the range of 200-500 nm. The reaction product was characterized by a spectrum of differentiated course with distinctly marked absorption maximum at λ=335 nm, which was used for quantitative analysis. Validation parameters were satisfactory, linearity range was from 10 $\mu g \cdot mL^{-1}$ to 200 $\mu g \cdot mL^{-1}$ with correlation coefficient of 0.9996, The limit of detection (LOD) and limit of quantification (LOQ) were 2.8 and 8.5 $\mu g \cdot mL^{-1}$, respectively. Other method of aliskiren derivatization was proposed by Zeynep Aydoğmuş et al.[26], who used dansyl chloride (DNS-Cl) in bicarbonate solution of pH 10.50 as a derivatization reagent, reaction was conducted for 20 min at 30°C in the dark. After that, the reaction product was extracted using 5 mL of dichloromethane. Organic layer was separated and dried over anhydrous sodium sulfate. The fluorescence of obtained product was measured at a wavelength of 501 nm after sample excitation with radiation of a wavelength of 378 nm. The course of derivatization reaction is presented in Fig. 5.

Fig. 5. Derivatization reaction of aliskiren with dansyl chloride

The validation parameters of elaborated spectrofluorometric method depended on the examined material. For the standard solutions they were as follows: linearity in the range of 100-700 $ng \cdot mL^{-1}$, correlation coefficient 0.9998, LOD 27.53 $ng \cdot mL^{-1}$, LOQ 91.76 $ng \cdot mL^{-1}$, for blood serum: 50.0-150 $ng \cdot mL^{-1}$, correlation coefficient 0.9986, LOD 4.91 $ng \cdot mL^{-1}$, LOQ 16.37 $ng \cdot mL^{-1}$. Similar method was used by the author in order to determine aliskiren concentration in pharmaceutical preparations and urine, and derivatization reagent was in that case fluorescamine[27], with procedure of samples preparation similar to described above. Fluorescence intensity was measured at a wavelength of 482 nm, after excitation with a wave of a length of 382 nm. The course of derivatization reaction is presented in Fig. 6.

Fig. 6. Derivatization reaction of aliskiren with fluorescamine

In this case, like in the method using dansyl chloride, the validation parameters were satisfactory and for the standard solutions they were as follows: linearity in the range of 140-1400 ng·mL^{-1}, correlation coefficient 0.9999, LOD 13.47 ng·mL^{-1}, LOQ 40.81 ng·mL^{-1}, for urine determination it was: 120-720 ng·mL^{-1}, correlation coefficient 0.9986, LOD 27.38 ng·mL^{-1}, LOQ 82.96 ng·mL^{-1}.

2.1.3. Determination of the presence of other substances

Combination treatment is preferred in arterial hypertension therapy, and it involves administration of a few substances of hypotensive activity. These may be combinations of active substances of various chemical structure and from different pharmacological groups. Therefore, there is a need to elaborate the methods of aliskiren determination in the presence of other substances of hypotensive activity. Such analysis include inter alia determination of aliskiren in the presence of diuretic which is hydrochlorothiazide. Ezzeldin et al.[28] proposed determination of these substances using HPLC and spectrophotometric methods. In case of HPLC method, methanol mixtures of examined substances were separated on Econosphere C-18 column, mobile phase was water-acetonitrile mixture (50:50 v/v), flow rate was 0.5 mL·min^{-1}, spectrophotometric detection at wavelength of 208 nm. Retention time was 2.13 min for aliskiren and 3.79 min for hydrochlorothiazide, linearity range in turn was 5-150 μg·mL^{-1} for aliskiren and 1-50 μg·mL^{-1} for hydrochlorothiazide. Other values of validation parameters prove high accuracy and precision of the elaborated method. Due to clear interference of the examined substances spectra, spectrophotometric method involved simultaneous equation method, where the authors were able to determine the concentrations of examined substances in their mixture using the values of absorbance amplitude at wavelengths of 277.48 nm for aliskiren and 267.48 nm for hydrochlorothiazide, as well as suitable mathematical equations. In case of spectrophotometric method application, the obtained linearity ranges were 5-150 μg·mL^{-1} for aliskiren and 1-41μg·mL^{-1} for hydrochlorothiazide.

Also RP-LC-PDA method was used in order to determine aliskiren and hydrochlorothiazide in pharmaceutical preparations and in the mixture.[29] Methanol extracts containing 150 μg·mL^{-1} aliskiren and 12.5 μg·mL^{-1} hydrochlorothiazide were prepared from grinded tablet mass, and they were exposed to ultrasounds activity for 7 min. The obtained solution was filtered using Whatman filter paper. Separation was carried out on chromatographic column Kromasil C18, 250 × 4.6 mm, 5 μm, mobile phase was acetonitrile-tetrahydrofuran-water mixture (71: 13: 16

v/v/v), flow rate was established on a level of 0.5 $mL \cdot min^{-1}$. Described conditions allowed to obtain good separation of examined substances with retention times of 3.7 min for aliskiren and 4.8 min for hydrochlorothiazide. Linearity was maintained in a wide range of concentrations, i.e. 1.2-480 $\mu g \cdot mL^{-1}$ and 0.1-40 $\mu g \cdot mL^{-1}$, respectively, with the values of correlation coefficients on a level of 0.9998 and 0.9994.

The studies on stability of the active substance are important in pharmaceutical analysis, since it significantly affects the quality of medicinal product. Sangoi et al. examined stability of aliskiren and hydrochlorothiazide in stress conditions using micellar electrokinetic chromatography (MEKC).[30] The studies on stability were conducted in various conditions: 0.2 M (3 h) and 2 M (5 h) hydrochloric acid – acidic hydrolysis; 0.2 M (3 h) and 2 M (5 h) NaOH - the alkaline hydrolysis; 60^0C; H_2O (60^0C) 48 h for hydrochlorothiazide and 30 h for aliskiren - neutral hydrolysis (thermal stress test); in 10% hydrogen peroxide - oxidative degradations; in UV-C (254 nm) light for 72 h for hydrochlorothiazide and 60 min for aliskiren - photodegradation. The samples for examinations were diluted with electrolyte composed of 47 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris), 47 mM sodium dodecyl sulfate (SDS) and 40 mL of ultrapure water. The pH was adjusted to 10.2 by adding 1M sodium hydroxide (NaOH) and the volume was made up to 50 mL with ultrapure water. MEKC method was performed on a fused-silica capillary (40 cm) at 28^0C. Applied voltage was 26 kV (positive polarity) and photodiode array (PDA) detector was set at 217 nm while ranitidine was used as an internal standard. In case of hydrochlorothiazide, such conditions allowed to obtain electropherogram proving this substance decomposition depending on examination conditions in the range from 2% for photodegradation process to 36% for neutral hydrolysis. The time of decomposition product retention which was 2.94 min was determined in all cases except photodegradation process, as well as the structure of formed product which was chlorothiazide. Examination of aliskiren stability in case of acidic and alkaline hydrolysis allowed to determine decomposition percentage which was 23% and 61%, as well as retention times which were 5.61 and 5.62 min, respectively. Oxidative degradation occurred in 46% and retention time of the product was 1.53 min. The highest decomposition of aliskiren on a level of 65% was obtained in photodegradation process, where three products of retention times of 1.53 min, 2.72 min and 5.62 min, were obtained; these products were not identified by the authors.

Hypertension therapy involves combination of renin inhibitors with calcium channel blocker or angiotensin II receptor antagonist. Amlodipine is one of the most commonly used calcium

channel blocker in this case. Spectrophotometric[31, 32], as well as chromatographic[33] method were used in an analysis of combined preparations for determination of aliskiren and amlodipine. Rameshbhai et al. examined aliskiren and amlodipine in tablets after active substance extraction using methanol. Absorption spectra were registered in the range of 200-400 nm with absorption maximum at 354.5 nm for amlodipine and 256.0 nm for aliskiren. Due to the interference of the spectra, the authors used absorbance correction method, which allowed an elimination of mutual effect of examined substances on the results of analysis. Examined linearity range was 20-120 $\mu g \cdot mL^{-1}$ for aliskiren and 10-60 $\mu g \cdot mL^{-1}$ for amlodipine, regression coefficients were 0.999 and 0.997, respectively. LOD and LOQ calculated using standard error of estimation were 3.68 $\mu g \cdot mL^{-1}$ and 11.16 $\mu g \cdot mL^{-1}$ for aliskiren, and 0.74 $\mu g \cdot mL^{-1}$ and 2.24 $\mu g \cdot mL^{-1}$ for amlodipine. Sai Sandeep Mannemalaa et al.[33] used HPLC method for an analysis of aliskiren and amlodipine in serum. Serum samples containing examined substances were mixed with hydrochlorothiazide solution, which played the role of an internal standard, and then they were subjected to the process of extraction with acetonitrile. Clear supernatant was evaporated and dry residue was dissolved in 250 μL of mobile phase, which was the mixture of di-potassium ortho-phosphate (25 mM, pH 4.2) and acetonitrile (60:40 v/v). 20 μL of such prepared sample was applied on Hibar C18 column (250 × 4.6 mm; Merck, Inc.), the flow rate of mobile phase was 0.8 $mL \cdot min^{-1}$, spectrophotometric detection with a wavelength of 232 nm was used in analysis. The separation allowed to obtain two well formed and separated peaks of the examined substances with retention times of 6.283 min for aliskiren, and 8.608 for amlodipine. Validation parameters determined by the authors for the elaborated method were satisfactory, which allowed to conclude that the method is suitable for examined substances analysis both in pharmaceutical preparations in order to control drug quality, and in biological material in pharmacokinetic examinations, as well as in drug level monitoring in patients treated with these substances.

Ion-Pair LC method[34] and capillary zone electrophoresis[35] were used in the analysis of three-component mixtures containing aliskiren, amlodipine and hydrochlorothiazide. In case of LC method, fragmented tablet mass was treated with 50 mL acetonitrile and subjected to ultrasounds activity for 15 min. Such obtained suspension was filtered, and the filtrate was diluted with acetonitrile in order to obtain suitable concentrations of the examined substances. Mobile phase was prepared by mixing acetonitrile with 25 mM octane sulphonic acid sodium salt monohydrate in water (60:40 v/v). Separation and determination of examined substances

was performed using Supelco, Discovery® HS (C18) column (250 × 4.6 mm, 5 μm) with UV detection λ = 232 nm. With mobile phase flow rate on a level of 1.2 mL· min^{-1}, the time of analysis was about 5 min, and peaks obtained as a result of separation well defined, with retention times of 2.25, 3.13 and 3.92 min for hydrochlorothiazide, aliskiren and amlodipine, respectively. Linearity ranges were 4-64 μg·mL^{-1} (hydrochlorothiazide), 32-320 μg·mL^{-1} (aliskiren) and 2-44 μg·mL^{-1} (amlodipine), regression coefficients were within the range 0.9993-0.9995. LOD and LOQ were estimated and found to be 0.855 and 2.951 μg·mL^{-1} for aliskiren, 0.061 and 0.202 μg· mL^{-1} for amlodipine, as well as 0.052 and 0.174 μg ·mL^{-1}, respectively, for hydrochlorothiazide.

In the year 2012, Chokshi et al.[36] and Kumaraswamy et al.[37] proposed concurrently the method of an examination of aliskiren and valsartan, which is a selective antagonist of AT_1 type angiotensin II receptor in pharmaceutical preparations, using RP-HPLC method. Similar mobile phase composed of methanol and potassium dihydrogenphosphate buffer and acetonitrile (adjusted to pH 3.0 with 1% orthophosphoric acid) was used in both cases, with flow rate on a level of 1 mL·min^{-1}. The difference was chromatographic method used for the separation, Palak V.Chokshi applied Hyper ODS2 Column C18, 250 × 4.6 mm (5 μm), and the separation in case of analysis described by Kumaraswama G. was conducted on Hiber@ Lichrosphere ® C18 column (250 × 4.6 mm, 5 μm particle size). The authors obtained satisfactorily separation of the examined substances, with retention times of 3.14 and 6.92min for aliskiren, and 6.5min and 7.94min for valsartan, respectively. Examined linearity ranges were the same in both cases, and they were 10-50 μg·mL^{-1}. Validation parameters of described analytical procedures were very similar and in both analyses fulfilled the requirements of the International Conference on Harmonization. In 2013, Goyani et al.[38] described pharmacokinetic examinations aimed at determination of distribution of aliskiren, valsartan and sitagliptin after oral administration to rats. The study included four groups of animals, the first one was administered aliskiren in a dose of 200 mg·kg^{-1} body weight, the second was given valsartan in a dose of 20 mg·kg^{-1}, and the third one sitagliptin in a dose of 15 mg·kg^{-1}, while the fourth one was treated with combination of all active substances in the examined doses. Blood was collected from all animals in suitable time intervals. Samples for analysis were subjected to liquid–liquid extraction process, in case of aliskiren, sitagliptin and irbesartan, which was an internal standard, the extraction was carried out using t-butyl methyl ether (TBME) in neutral environment. The same extraction reagent was used in case of

valsartan, but extraction was performed in acidic environment. The following parameters were examined in such obtained samples: the maximum plasma concentration (C_{max}) and the time to reach the maximum concentration (T_{max}). All analysis were performed using LC method with fluorometric detection. The mobile phase was the mixture of acetonitrile : 20 mM ammonium acetate buffer (35:65, v/v), and separation was conducted using Kromasil C_{18} column (250 × 4.6 mm, 5 μm), with flow rate of 1 $mL \cdot min^{-1}$. An application of fluorometric detection of a suitable sequence of excitation and emission waves length allowed to obtain the separation of the examined substances with following retention times: sitagliptin 3.69, valsartan 5.95 and aliskiren 8.91 min, linearity range was 25-2000 $ng \cdot mL^{-1}$ for aliskiren and sitagliptin and 50-4000 $ng \cdot mL^{-1}$ for valsartan. Pharmacokinetic parameters determined for the mixtures of substances demonstrated that the highest C_{max} value was characteristic for valsartan 1474.4 $ng \cdot mL^{-1}$, then sitagliptin 416.7 $ng \cdot mL^{-1}$ and aliskiren 328.4 $ng \cdot mL^{-1}$, while the second parameter T_{max} was 2, 1 and 0.5 h, respectively. The biological half-life time determined for particular substances was in the range of 1.4-3.7 h. The method described by the authors appeared to be useful both in concurrent determination of aliskiren, valsartan and sitagliptin, and also in pharmacokinetic examinations of these substances in rats blood serum.

In determining the overall quality of the medicinal product, the main role is not only played by the research aimed at the possibility of active substance determination in pharmaceutical products in the presence of other active substances or excipients, but also the research allowing to determine an active substance in the presence of its metabolites, degradation products or impurities. In case of examination of aliskiren as substance of hypotensive activity newly introduced into therapy, attention should be paid to the publication concerning pharmacokinetic properties of this substance. Waldmeier et al.[39] widely described pharmacokinetics of aliskiren in healthy volunteers. The research material included plasma, urine, fat and feces. Suitably prepared samples were subjected to analysis using various methods in order to establish metabolic profile of aliskiren. Separation of metabolites was conducted using HPLC method with radiometric detection on LiChrospher 100-5 RP-18 ec column (5μm, 250 × 2 mm). Gradient elution using mobile phase solvent A (50 mM ammonium acetate adjusted to pH 6.0 with acetic acid) and solvent B (acetonitrile) was applied at a flow rate of 0.25 mL/min as follows: 0 to 35 min, 10 to 30% solvent B; 35 to 45 min, 30% solvent B; 45 to 50 min, 30 to 40% solvent B; 50 to 65 min, 40 to 90% solvent B; 65 to 70 min, 90% solvent B. In order to establish the chemical structure of the examined compounds, the authors applied LC-MS and ^{1}H NMR methods. Separation conditions

different than described above were used in case of LC-MS method application for determination of three metabolites, i.e., CC 8/3 Nucleodur C18 Pyramid column (5 μm, 250 × 4.6 mm i.d.; Macherey-Nagel), and mobile phase gradient was as follows: 0 to 10 min, 10 to 30% solvent B; 10 to 65 min, 30 to 90% solvent B; 65 to 70 min, 90% solvent B; 70 to 71 min, 90 to 10% solvent B; 71 to 80 min, 10% solvent B. Analysis of the results obtained allowed to establish the structure of nine compounds formed during metabolic processes of aliskiren in human organism. The authors also proposed the scheme of metabolic pathway of the examined compounds (Fig. 7.)

Fig. 7. Metabolism products of aliskiren

There were also the studies on an evaluation of aliskiren stability and possibility of its impurities determination in pharmaceutical preparations. M. Wrasse-Sangoi, M.S. Sangoi, P.R. Oliveira, L.T. Secretti, and C.M.B. Rolim elaborated and validated the method for aliskiren determination in the presence of the products of this substance degradation.[40] Aqueous extracts of the samples obtained by weighing and dissolution of specified amount of powdered tablet mass in water, were subjected to an activity of various factors. Stability of active substance was examined in 2 M hydrochloric acid - acidic hydrolysis, 2 M sodium hydroxide - basic hydrolysis (5h at ambient temperature), water was heated at 50°C for 96h - neutral condition, in 10% hydrogen peroxide, at ambient temperature for 30 h, protected from light - oxidative degradation, exposing the sample in quartz cuvette to 200 watt h/squaremeter of near ultraviolet light (UV-C) for 1h - photodegradation. Analysis involved liquid chromatography method with PDA detector. The separation was conducted on XBridge C18 column (150 × 4.6 mm i.d., with a particle size of 5-μm, 135 Å). Mobile phase was a mixture of acetonitrile-water (95:5, v/v) : phosphoric acid (25 mM, pH 3.0) (40:60, v/v). The results obtained by the authors proved a considerable decrease in peak area value during neutral hydrolysis, without additional peaks presence, which was explained by the lack of possibility of created product detection using PDA detector. Two additional peaks with retention times of 8.21 and 5.05 min occurred on a chromatograph as a result of acidic hydrolysis and in the process of oxidation, with concurrent decrease in the value of area of the peak derived from aliskiren. The highest percentage of separation was obtained in basic hydrolysis conditions (59.67%), and in photodegradation process (59.76%). In case of basic hydrolysis, three

additional peaks were obtained on chromatograph, and their Rf was 5.05, 6.15 and 7.87 min, respectively, while five additional peaks were noted in case of aliskiren photolysis process, with retention time in the range of 2.5-3.5 min.

An attempt to determine 3-amino-2,2-dimethylpropanamide as the product of aliskiren degradation was also undertaken.[41] Conditions of degradation process were as follows: acidic hydrolysis (0.1 M HCl at 90°C for 30 min), alkaline hydrolysis (0.1 M NaOH at 90°C for 30 min), oxidative hydrolysis (3% hydrogen peroxide at 90°C for 30 min), dry heated degradation (90°C for 30 min) and UV light degradation (254 nm for 24 h). Analysis was conducted using HILIC method (hydrophilic interaction liquid chromatography) with fluorometric detection. Separation of the examined substances was conducted on XBridge HILIC column (150 × 4.6 mm, 3.5 μm; Waters, USA) using mobile phase being a mixture of 10 mM K_2HPO_4 pH 7.2-acetonitrile (26:74; v/v) with isocratic elution. Detection of 3-mino-2,2-dimethylpropanamide in UV range is hampered due to low value of this substance absorption with a wavelengths of 205 nm. The reaction of post-column derivatization using o-phthaldialdehyde in thiols presence was applied in order to facilitate detection of 3-amino-2,2-dimethylpropanamidu formed during aliskiren degradation. This reaction allowed to obtain derivative of 1-alkyl-2-thioindoles characterized by high fluorescence value with waves length of 345 nm (excitation) and 450 nm (emission). It was observed as a result of the research conducted that the amount of 3-amino-2,2-dimethylpropanamide formed in the process of both acidic and alkaline hydrolysis is the highest and reaches the level of 0.27-1.07%. The method described is suitable for determination of aliskiren, except its degradation product, which is confirmed in validation parameters which are consistent with the values accepted by ICH for chromatographic methods.

Molleti et al.[42] presented the results of aliskiren determination in the presence of its impurities using reversed phase ultra performance liquid chromatography method (RP-UPLC). The authors analyzed impurities of active substance, which included four compounds:

Hydroxy impurity - (2S,4S,5S,7S)-N-(2-carbamoyl-2-methylpropyl)-5-amino-4-hydroxy-2,7-diisopropyl-8-hydroxy-8-[4-methoxy-3-(3-methoxypropoxy)phenyl]-octanamide hemi fumarate.

Acid impurity - (2S,4S,5S,7S)-N-(2-carbamoyl-2-methylpropyl)-5-amino-4-hydroxy-2,7-diisopropyl-8-hydroxy-8-[4-methoxy-3-(3-methoxypropoxy)phenyl]-octanoic acid.

Desmethoxy impurity - (2S,4S,5S,7S)-N-(2-carbamoyl-2-methylpropyl)-5-amino-4-hydroxy-2,7-diisopropyl-8-hydroxy-8-[3-(3-methoxypropoxy)phenyl]- octanamide hemi fumarate.

N-BOC impurity - tert-butyl(3S,5S,6S,8S)-8-(3-amino-2,2-dimethyl-3-oxopropyl carbamoyl)-6-hydroxy-3-4-methoxy-3-(3-methoxypropoxy)benzyl)-2,9-dimethyldecan-5-yl, carbamate.

The analysis was performed using Waters Acquity, BEH, C8 column (100 × 2.1 mm i.d.). The mobile phase consisted of 2.72 grams of potassium dihydrogen ortho-phosphate and 3.5 grams of 1-octane sulphonic acid adjusted to pH 2.0 as aqueous phase and acetonitrile as organic phase, with suitably changing gradient of particular components concentrations. The time of analysis with mobile phase flow rate of 0.5 $mL \cdot min^{-1}$ was about 6.5 min. Well formed and separated peaks were obtained on chromatogram, and they were derived both from aliskiren and the examined impurities with the following retention times: hydroxy impurity 3.2 min (LOD = 0.23 ppm and LOQ = 0.70 ppm), aliskiren 3.7 min, acid impurity 5.2 min (LOD = 0.36 ppm and LOQ = 1.10 ppm), desmethoxy impurity 5.6 min (LOD = 1.42 ppm and LOQ = 4.32 ppm), N-BOC impurity 6.1 min (LOD = 0.77 ppm and LOQ = 2.35 ppm). Also stress examination of aliskiren in various conditions was conducted in the analyses undertaken, the temperature during 24 h examination was 50^0C, and variable environment was created by 1 $mol \cdot L^{-1}$ HCl, 1 $mol \cdot L^{-1}$ NaOH, 10% H_2O_2 solution, and in order to examine the photostability, the sample was subject to irradiation at 1.2 million Lux for one hour. The determined percentage of substance degradation was in the range from 0.21% for photodegradation process to 89% in case of degradation in acidic environment. The method proposed by Srihari Molleti et al. may be applied both in an examination of active substance impurity, and in analysis of aliskiren stability.

Analytical examinations of medicinal substances which may substantially affect biological availability of the drug, its pharmacological activity and possible toxicity, should take into account the possibility of active substance occurrence in form of enantiomers. Aliskiren has four chiral centers, and may occur in a form of R- isomer. Ashok et al.[43] elaborated the method of aliskiren enantiomers separation. For this purpose, they used HPLC method with UV detection with wavelength of 228 nm, on Chiralpak-IC column (250×4.6mm) with 5-μm particles. Mobile phase was 0.1% n-butylamine in acetonitrile mixture (v/v). Both racemic mixture and aliskiren bulk sample spiked with (R)-isomer were separated in such conditions. Satisfactorily separation of the examined substances was obtained in both cases, with retention times of 12.47 and 12.57 min for (R)-isomer, and 15.78 and 15.59 min for aliskiren

depending on the kind of the examined sample. The range of linearity for (R)-isomer was from 0.6 to 4.5 μg·mL^{-1}. The method proposed by the authors for determination of (R) and (S) aliskiren enantiomers appeared to be precise and reliable, and thus it may be proposed as routinely used in an evaluation and control of drug quality.

2.2 Remikiren

Remikiren is (2S)-2-[(2R)-2-benzyl-3-(2-methylpropane-2-sulfonyl)propanamido]-N-[(2R,3S,4R)-1-cyclohexyl-4-cyclopropyl-3,4-dihydroxybutan-2-yl]-3-(1H-imidazol-4-yl) propanamide (Fig. 1), and its structure was elaborated in 1996. It is highly specific renin inhibitor, its in vivo activity on renin activity in serum and blood pressure was demonstrated in the study conducted by Kleinbloesema et al.[44] In order to determine the level of remikiren in blood serum, the authors used the method with fluorometric detection, using the reaction of active substance derivatization applying fluorenylmethyl chloroformate as derivatization reagent. Remikiren and internal standard (Ro 42-4661) were extracted from the serum using butyl acetate. Derivatization reaction was conducted for 1h after organic layer evaporation, and the sample was maintained at a room temperature. The separation was conducted on Nucleosil C_{18} 3 μm column, and a mobile phase was the mixture of 9% trifluoracetic acid 5 mmol·L^{-1} and 91% N-hexylmethylamine 0.35 g·L^{-1} in acetonitrile : 15 mmol·L^{-1} trifluoracetic acid (84.4:15.5; v/v) with pH 3.0. The following wavelengths were used in detection: 261 nm (excitation), 308 nm (emission), quantification limit was 1.3 ng·mL^{-1}. Similar method of remikiren determination was used in the research on remikiren pharmacokinetics in hypertensive patients.[45] As a reference method, the authors used HPLC method employing reversed phase chromatography on a C_{18} column and post-column irradiation using a BeamBoost® followed by coulometric oxidative electrochemical detection at +550 mV vs a H2/H' electrode, obtaining in this case quantification limit on a level of 0.2 ng·mL^{-1}. Other method for remikiren determination in blood serum was proposed by J. Leube and G. Fischer[46], and analysis was conducted using HPLC method with pre-column derivatization of the examined substance with 2,4-dinitrofluorobenzene and electrochemical detection, Ro-4661/000 compound was used as a standard. Derivatization process was performed automatically using borate buffer (0.2 M, pH 8.0) and 2,4-dinitrofluorobenzene. The separation was conducted on Novapak Cls column (150 × 3.9 mm I.D.) with acetic acid buffer (pH 7)-acetonitrile (100:85 v/v) mixture as mobile phase. These conditions allowed to obtain chromatographic separation with peaks of retention times of 11.4 min for remikiren and 17

min for internal standard. The range of linearity was 0.2-5 ng·mL^{-1} with quantification limit on a level of 0.3 ng·mL^{-1}. Statistical parameters of the elaborated method were satisfactory, and the procedure was proposed as useful in pharmacokinetic examinations of remikiren in hypertensive patients treated with this preparation.

The method of liquid chromatography with mass detection is used the most often in analysis of remikiren metabolic processes and in order to establish the structure of metabolism products.[47, 48, 49]. G. Hopfgartner and F. Vilbois used X-Terra MS C18 2.5 m analytical column, mobile phase composed of solvent A, which was 1 % acetic acid, and solvent B, which was the mixture of methanol-acetonitrile-acetic acid in the ratio 50:50:1 (v/v/v). The established gradient of concentrations at the start was 30% of solvent B concentration, which amount was automatically increased up to 70% during 10 min. The rate of mobile phase flow was 0.25 mL · min^{-1}. Based on an analysis of mass spectra, the authors managed to establish the probable structures of remikiren metabolites (Fig. 8).

Fig. 8. Metabolism products of remikiren

2.3. Enalkiren

Enalkiren is 3-amino-N-[(1S)-1-{[(1S)-1-{[(2S,3R,4S)-1-cyclohexyl-3,4-dihydroxy-6 methylheptan-2-yl]carbamoyl}-2-(1H-imidazol-5-yl)ethyl]carbamoyl}-2-(4methoxyphenyl) ethyl]-3-methylbutanamide (Fig. 1), and its activity involves an inhibition of the process of catalytic hydrolysis of angiotensinogen to angiotensin I, which causes in a consequence a decrease in the level of angiotensin II and prevents blood vessels constriction. The studies on an effect of enalkiren on this substance pharmacokinetics were performed in the 1990s on patients with congestive heart failure.[50] The authors used HPLC method for monitoring of drug level in patients blood serum. Serum samples containing enalkiren and internal standard (Abbot-64495) were subjected to the process of extraction using the mixture of ethyl acetate-hexane (7:3 v/v). After evaporation, the dry residue was dissolved in mobile phase which was composed of the mixture of methanol-acetonitrile-0.01 mol·L^{-1} tertamethyloammonium perchlorate in 0.1% fluoroacetic acid (pH=3). Such prepared samples were applied on C_{18} column (3 μm, 4.6 × 75 mm), the analysis involved spectrophotometric detection with

wavelength of λ = 205 nm. The linearity range in the examined conditions was 10-500 ng·mL^{-1}, correlation coefficient was 0.995, and quantification limit was 10 ng·mL^{-1}. Based on the results obtained, the authors determined pharmacological and pharmacodynamic model of enalkiren effect on blood pressure in the examined patients. HPLC method was also applied in the study on enalkiren effect on blood pressure decrease, the level of sodium elimination and renin-angiotensin-aldosterone system in monkeys with sodium deficiency.[51] In this case, blood serum samples were alkalized with Na_2CO_3 solution containing (H-β-ala-phe-his-cyclohexyl)ala(OH)-CHOH-isobutyl.2HOAC) as an internal standard. Then, the whole was extracted using in the first stage the mixture of ethyl acetate and hexane (7:3 v/v) and 0.1% trifluoroacetic acid in the second one. Aqueous phase was rinsed with 2 mL hexane and dosed on ODS2 3 /nmx5 cm chromatographic column (Regis, Morton Grove, Illinois). Solid phase and detection were the same like in the case of the study by Gupta et al. (acetonitrile, methanol, and 0.01 M tetramethyloammonium perchlorate (35:5:60) in 0.1% TFA, λ = 205nm.). In the study conducted the authors proved stable reduction of blood pressure during enalkiren administration. Observed hypotension was accompanied by an inhibition in serum renin activity and a decrease in angiotensin II level.

2.4. Zanikiren

The next drug from the group of renin inhibitors introduced to arterial hypertension therapy is zanikiren, i.e. (2S)-2-benzyl-N-(1-((2S,3R,4S)-1-cyclohexyl-3,4-dihydroxy-6-methylheptan-2-ylamino)-1-oxo-3-(thiazol-4-yl)propan-2-yl)-3-(4-methylpiperazin-1-ylsulfonyl) propanamide (Fig. 1). The studies on the structure, effectiveness and bioavailability of zanikiren were conducted on various animal species like dogs, rats, monkeys and ferrets. The obtained results were widely presented in the papers of Rosenberg et al. [52, 53] and Kleinert et al. [54] In case of determination of the examined substance and its metabolites in serum, the authors used a direct method which was high-performance liquid chromatography [52, 54] or indirect methods involving renin inhibition assay [53] or plasma renin activity determination.[55] Two procedures were used in case of HPLC method application in the research. In dogs blood serum, the level of zanikiren and its two metabolites (des-methyl A-75247 and N-oxide A-80187) was determined using 40% aqueous solution of acetonitrile containing 0.1% solution

of trifluoroacetic acid as a mobile phase. Analysis was conducted using μBondpak C_{18} reverse-phase column and UV detection with a wavelength of 214 nm. Sensitivity of the method for each of the examined components was determined on a level of 100 ng·mL^{-1}, and recovery was 85±16% for zanikiren, 78±11% for metabolite A-75247 and 86±17% for A-80187. In order to determine zanikiren solubility in *in vitro* examination using HPLC method, the mixture of a following composition was used as a mobile phase: 1% aqueous perchloric acid-acetonitrile-methanol 49:38:13 (v/v/v), solid phase was 15-cm PRP column with spectrophotometric detection with wavelength of 214 nm.

3. Endothelin receptor antagonists (ERA)

The works on the substances of endothelin receptor antagonists character resulted in an isolation of cyclic pentapeptide from fermentation product of *Streptomyces misakiensis*, which was a natural analogue of ET-1 binding to receptor ET_A.[56] Further research were aimed at the change in peptide character of the substance, which in case of drugs is unprofitable due to the possibility of hydrolysis occurring with peptidases contribution, their decomposition after oral administration and inhibition of distribution by blood-brain barrier. Due to the character of activity, ERA were divided into two groups: selective blockers of receptor ET_A or ET_B, and non-selective blockers having an affinity both to receptor ET_A and ET_B. Selective inhibitors of endothelin receptor include: ambrisentan, atrasentan, darusentan, and sitaxsentan, while non-selective blockers include bosentan and tezosentan.

Fig. 9 Chemical structure of endothelin receptor antagonists

3.1 Bosentan

4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide was noted in the USA in 2001, and in Europe in 2002, and it was the first oral antagonist of endothelin receptors (ET_A/ET_B), registered as a drug of choice in pulmonary hypertension therapy. The studies preceding bosentan introduction on the market

required an elaboration of a range of analytical procedures allowing to determine its bioavailability, impurities level and presence of metabolites, or analysis of active substance in drug form.

3.1.1. Bioavailability and metabolites

The research on bioavailability and metabolism of bosentan proved that it is mainly metabolized in liver cells with a contribution of cytochrome P 450, and its bioavailability after oral administration ranges from 30 to 78%. Bosentan is metabolized to three products (Fig. 8), and its presence was noted analyzing various biological matrices. Lausecker et al.[57] observed the presence of bosentan metabolites in human serum and plasma, liver cells in dogs, their serum and bile, as well as in rats serum. Samples preparation involved their deproteinization using the mixture of acetonitrile-methanol (1:1 v/v), buffer solution pH 4 was added (Titrisol citrate /HCl) after supernatant centrifugation. Such prepared mixture was subject to liquid-liquid extraction process using 6 mL of n-chlorobutane–dichloromethane (8:2, v/v) mixture. Organic phase was evaporated and then dissolved in 1 mL acetonitrile-5 mM ammonium acetate–acetic acid (10:90:1, v/v/v). The separation was conducted using HPLC-MS-MS method with column-switching system, applying 25 × 4 mm Superspher RP-Select B trapping column (5 μm particle size, 60 Å pore size) and 150 × 2.1 mm Symmetry RP-18 analytical column (protected by a 10 × 2 mm Superspher RP-18 guard column). The mobile phases were the following mixtures: acetonitrile-methanol-5 mM ammonium acetate-acetic acid in the ratio 25:25:50:1 (v/v/v/v) - phase A, and phase B composed of the same components mixed in a ratio of 45:45:10:1 (v/v/v/v). The range of bosentan linearity in the applied analysis conditions was 1 - 10 000 $ng \cdot mL^{-1}$, while for its metabolites it was 2-2000 $ng \cdot mL^{-1}$. The upper limit of quantification (ULQ) for metabolites was 2000 $ng \cdot mL^{-1}$ since their level did not exceed 10% of primary bosentan content. The lower limit of quantification for bosentan was 1 $ng \cdot mL^{-1}$, while for its metabolites it was 2 $ng \cdot mL^{-1}$ which assured fulfillment of validation criteria for chromatographic methods. An application of column-switching system assured good separation of the examined substances with total time of analysis not exceeding 10 min, retention times were from 6 to 8.5 min. HPLC-MS-MS method was also used in the research on absorption, secretion and metabolism of bosentan in healthy men.[58] The study involved isotope labeled [^{14}C] bosentan in a form of oral suspension and solution for injection, and research material were serum, urine and feces. The method described by B. Lausecker was used in case of determination of bosentan and its metabolites

in serum, while HPLC method with radiometric detection was used for an analysis of feces and urine samples. Suitably prepared urine samples were separated on Superspher 100 column (250 × 4 mm; Merck, Darmstadt, Germany), and mobile phase was a mixture of 50 mM ammonium acetate buffer (pH 3.5), which contained 1.3% tetrahydrofuran, the second phase was acetonitrile in which tetrahydrofuran content was 3.0%. Feces samples dissolved in methanol were subjected to ultrasound activity for 20 min and centrifuged. Further sample preparation involved re-extraction to methanol, evaporation, dissolving of residue in the mixture of 50 mM ammonium acetate (pH 3) and methanol (1:1, v/v) and filtration. Such prepared feces samples were applied on HPLC column. It was observed based on analysis conducted that metabolite II was present in serum and feces, the presence of metabolite IV was noted in urine and feces, and it was not noted in serum samples. Metabolite II was represented in low amount in all examined matrices.

Fig. 10. Metabolism products of bosentan

3.1.2. Stability and impurities

Also the studies aimed at determination of bosentan stability and impurities presence were undertaken.[59, 60, 61] The authors examined stability of bosentan in stress conditions using acidic and alkaline hydrolyses, as well as analyzing photolytic and thermal decomposition. Jadhav et al. used RP-LC method with Zorbax SB-Phenyl column (250 × 4.6 mm, 5 μm) for separation of bosentan impurities, two mixtures constituted the mobile phase, i.e. solution A: (60% phosphate buffer, pH 2.5, and 40% methanol) and acetonitrile as solution B, UV detection at λ = 220 nm. Dissolved samples of the examined substance were subjected to an activity of concentrated hydrochloric acid for 2 h, 5 mol·L^{-1} sodium hydroxide 12 h, 6% hydrogen peroxide solution 24 h, at a temperature of 60^0C for 8 days, as well as light activity (according to ICH) for 9 days. Five various impurities of bosentan were taken into account in the research: Chloro impurity - impurity A, Hydroxy impurity - impurity B, Dimer impurity - impurity C, 4, 6-dichloro-5-(2-methoxyphenoxy)-2,2'-bipyrimidine - impurity D and 4-tert-butylbenzenesulfonamide - impurity E (Fig. 11.)

Fig. 11. Chemical structures of bosentan impurities

An application of suitable separation conditions allowed to obtain the chromatograph with well formed and separated peaks, which retention times were as follows: impurity A 16.119 min, impurity B 11.27 min., impurity C 26.65 min, impurity D 6.98 min, impurity E 4.85 min, retention time for bosentan was 12.25 min. The presence of impurities B and E was noted in the examined sample in case of acidic hydrolysis, while impurities A and B were observed in case of alkaline hydrolysis. The limit of detection (LOD) for all impurities was in the range from 0.025 to 0.068 $\mu g \cdot mL^{-1}$, while quantification limit was from 0.081 to 0.206 $\mu g \cdot mL^{-1}$.

3.1.3. Determination in pharmaceutical preparations

Annapurna et al.[61] conducted stress examination on two pharmaceutical preparations, Bosentas® and Lupibose®, containing bosentan as an active substance. The samples were prepared weighing tablet mass corresponding to bosentan content of 25 mg, the whole was transferred to 25 mL acetonitrile and subjected to ultrasounds activity for 30 min, the obtained solution was dissolved with mobile phase until the concentration of 100 μgmL^{-1} was obtained. Stress examinations involved determination of the content of bosentan and its degradation products in the samples subject to an activity of 1 $mol \cdot mL^{-1}$ HCl for 30 min at a temperature of 80^0C (acidic degradation), 1 $mol \cdot mL^{-1}$ NaOH for 30 min at a temperature of 80^0C (alkaline degradation), 3% H_2O_2 for 30 min in a thermostat maintained at 80ºC (oxidation degradation), UV light 365 nm for 4 hours (photolytic degradation), and temperature of 80^0C for 30 min. Analysis was conducted using HPLC-DAD method, on C18 column (250 × 4.6 mm, 5 μm), flow rate of mobile phase composed of the mixture of tetrabutylammonium hydrogen sulphate-acetonitrile (35:65 v/v) was 1.2 $mL \cdot min^{-1}$, detection at wavelength of 268 nm. Linearity of the elaborated method was in the range of 1.0-350.0 $\mu g \cdot mL^{-1}$ with correlation coefficient of $R^2 = 0.9999$, LOD and LOQ were 0.2684 $\mu g \cdot mL^{-1}$ and 0.8134 $\mu g \cdot mL^{-1}$, respectively. The study proved that the proposed method is suitable for successful determination of bosentan content in pharmaceutical preparations. The examined substance appeared to be stable, irrespective of an application of specified degradation conditions, its decomposition did not exceed 5%, and only in case of oxidation reaction an additional peak with retention time of 2.089 min was observed on the chromatogram.

The methods which in a fast, accurate and precise way allow the determination of the content of the active substances in various forms of drugs are significant in analytical studies concerning drug quality control. The main proposed methods in case of an analysis of bosentan containing pharmaceutical preparations include high-efficiency liquid chromatography and spectrophotometric method. R. Kalaichelvi and E. Jayachandran[62] proposed RP-HPLC method for bosentan determination in tablets. Fragmented tablet mass was extracted to methanol, and suitably diluted before applying on reverse phase Agilent XDB C18 chromatographic column (150 × 4.6 mm, i.d., 5 μm), phosphate buffer (pH-5) and acetonitrile in 45:55 (v/v) mixture was used as a mobile phase, and its flow rate was 1 $mL \cdot min^{-1}$, detection was conducted at a wavelength of 270 nm. The authors obtained satisfactory validation parameters in the proposed conditions, linearity was in the range of 25-150 $\mu g \cdot mL^{-1}$ (R = 0,.999), LOD and LOQ were 1.3344 $\mu g \cdot mL^{-1}$ and 4.043 $\mu g \cdot mL^{-1}$, respectively, and peak derived from the examined substance was well formed with retention time of 5.732 min. RP-HPLC method was also used in order to determine bosentan on C18 column.[63] The mobile phase in that case was the mixture of acetonitrile-10 mM ammonium acetate (pH 4.5) buffer (70:30, v/v), and detection was conducted at a wavelength of 265 nm. In this case, the linearity range was 5-70 $ng \cdot mL^{-1}$ with correlation coefficient on a level of 0.999, also the values of LOD and LOQ were lower (2.0 $ng \cdot mL^{-1}$ and 7.0 $ng \cdot mL^{-1}$), retention time during that analysis was 3.702 min. An application of Thermo Scientific C18 column and mobile phase composed of 20 mM ammonium bicarbonate (pH 5.0)-acetonitrile (70:30 v/v) mixture, with unchanged retention time allows to determine bosentan with comparable accuracy and precision, obtaining concurrently retention time reduction to 1.986 min.[64]

Determination of bosentan in pharmaceutical preparations is also possible using spectrophotometric method in UV range[65, 66] and thin-layer chromatography[67]. In case of an application of UV-VIS method, the authors obtained absorption spectrum with absorption maximum derived from bosentan localized in the range of 270-274 nm, depending on solvent used, in the described cases it was dichloromethane, mixture of methanol and octane 1-sulphonic acid (pH 3.5) (50:50 v/v), or methanol-water (60:40 v/v). In case of methanol and octane 1-sulphonic acid (pH 3.5) mixture application as a solvent, λ_{max} was 273 nm, the examined linearity range was 0.1-100 $\mu g \cdot mL^{-1}$ (r = 0.999), and the value of experimentally specified molar extinction coefficient $\varepsilon = 1.3293 \times 10^4$ $L \cdot mol^{-1} \cdot cm^{-1}$. Slightly different validation parameters were obtained using methanol-water (60:40 v/v) mixture as a solvent. The study was conducted at λ_{max} = 270 nm, examined linearity range was 10-90 $\mu g \cdot mL^{-1}$ (r^2 =

0.9993), while LOD and LOQ determined in these conditions were 1.892 μg·mL^{-1} and 6.872 μg·mL^{-1}, respectively. In case of thin-layer chromatography used for bosentan examination in pharmaceutical preparations, the authors used HPTLC silica gel 60 F_{254} plates as a solid phase, mobile phase optimum as regards analysis time and obtained peak symmetry was the mixture of methanol-ammonia (10:1 v/v). Detection of the obtained spots was conducted densitometrically at wavelength of 288 nm. Linearity level was from 10 to 80 ng/spot (r = 0.9991), and the coefficient of bosentan retention determined in these conditions was Rf = 0.76±0.02. Presented examples of analysis and validation parameters allow to conclude that both spectrophotometric method and HPTLC may be successfully used in analysis of pharmaceutical preparations containing bosentan, as alternative methods for more time-consuming and more expensive separation methods in the research on possible interactions of bosentan with other active substances administered for example in hypertension combination therapy, with drugs of anti-inflammatory activity or with hypoglycemic drugs. Elaboration of analytical methods allowing determination of a few active substances may be useful in concurrent monitoring of their level, or determination of possible interactions both in an aspect of their mutual effect on release, absorption, distribution, metabolism or secretion, as well as in analytical aspect involving an exclusion or determination of their mutual influence on analysis in body fluids. In case of an examination of bosentan interactions with a drug of anti-inflammatory activity, i.e. aceclofenac, Suganthi et al.[68] used RP-HPLC method. Optimum separation of the examined substances was obtained on Merk- LichroCART, C18 column (250 × 4 mm, 5 μm), mobile phase was the mixture of methanol-0.1% formic acid (pH 6.4) 70: 30 (v/v), flow rate was 1 mL·min^{-1}. Chromatogram with well separated, symmetric peaks was obtained in the presented conditions, and retention time for bosentan was 6.2 min, and 8.5 min for aceclofenac. The determined validation parameters were satisfactory and fulfilled the requirements of the ICH. Using the elaborated procedure and the method of equilibrium dialysis, the authors examined an effect of aceclofenac on bosentan binding with serum proteins. It was noted that bosentan binding with proteins decreased from 89.45% when the sample was free from aceclofenac, to 82.28% when aceclofenac content in examined sample was on a level of 30.0 μg·mL^{-1}. The results obtained allow to conclude that concurrent administration of bosentan with aceclofenac considerably deteriorates the process of bosentan absorption, the level of drug not bound with proteins increases considerably, which may result in liver damage. Other kind of interaction and possibility of concurrent determination of bosentan with glimepiride in human serum was described by Qiu et al.[69] The

study concerns the possibility of concurrent analysis of bosentan in the presence of glimepiride in biological matrix which is serum, and possibility of elaborated procedure application in pharmacological analysis. The separation of the examined substances involved an application of ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) with Acquity BEH C18 column (2.1 × 50 mm, 1.7 μm particle size). The mobile phase was the mixture of two solvents: solvent A: 0.1% formic acid in water and solvent B: acetonitrile. The concentrations gradients scheme was as follows: 35% B (0-0.5 min), 35-80% B (0.5-1.0 min), 80-35% B (1.0-1.5 min), with flow rate of 0.45 $mL \cdot min^{-1}$. Losartan solution was used as an internal standard. The samples were prepared adding 200 μL of internal standard solution at a concentration of 50 $ng \cdot mL^{-1}$ to 100 μL serum, the whole was mixed and centrifuged. determined validation parameters were satisfactory: linearity was 5-1000 $ng \cdot mL^{-1}$ for bosentan and 2.5-500 $ng \cdot mL^{-1}$ for glimepiride. Total time of analysis was only 1.5 min, and the obtained retention times were 0.57 min and 0.90 min, respectively. The authors emphasized that the described method is the first which allows concurrent determination of bosentan and glimepiride in serum, and may be used in clinical practice for examination of these substances pharmacokinetics in patients treated with them.

The therapy using a few substances of hypotensive activity is a common pharmacological practice in pulmonary arterial hypertension (PAH) treatment. Therefore, it seems to be reasonable to elaborate analytical methods allowing to determine a few substances in the patients cured with combination therapy. Yokoyamaa et al.[70] presented the method of concurrent determination of bosentan, ambrisentan (endothelin receptor blockers), sildenafil and tadalafil (phosphodiesterase inhibitors) in serum of pediatric patients using LC-MS/MS method. Serum samples diluted with water containing internal standards of the examined substances (deuterated derivatives) were subjected to extraction process to the solid phase (Oasis WAX cartridges (Waters; 30 mg/1 mL; 30 μm), columns were rinsed with 2% solution of formic acid, and then they were eluted using methanol. Eluted samples were diluted using the mobile phase, which was the mixture of acetonitrile-5 mM ammonium acetate (45:55, v/v; pH adjusted to 5.0 with acetic acid). The separation was conducted on C18 column and an isocratic mobile phase running at a flow rate of 0.2 $mL \cdot min^{-1}$ for 5 min. The time of analysis in the elaborated conditions did not exceed 5 min, and determined retention times were as follows: 1.8 min for ambrisentan, 2.5 min for tadalafil, 3.3 min for sildenafil and 3.9 min for bosentan. Determined validation parameters were satisfactory, and the range of linearity for all examined substances was 2-1000 $ng \cdot mL^{-1}$. The method described was applied for

determination of the examined substances in children treated due to pulmonary hypertension, and was recommended by the authors for this kind of analysis, due to small amount of sample essential for analysis (total volume of blood needed for analysis does not exceed 2 mL).

3.2. Ambrisentan

(2S)-2-[(4,6-dimethylpyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenylpropanoic acid is a selective antagonist of endothelin receptor, recommended in pulmonary hypertension treatment (PAH), accepted in the therapy by World Health Organization. It was registered in oral form by the United States Food and Drug Administration in 2007. Chromatographic and spectrophotometric methods are predominant in analysis of ambrisentan both in pharmaceutical preparations and biological material.

Spectrophotometric methods were mainly used for ambrisentan determination in various forms of drugs. One of the proposed methods makes use of reaction between ambrisentan and solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in methanol (DDQ) or 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone in acetonitrile (CLA).[71] Tablet mass was subject to extraction process to 10 mL of methanol in case when DDQ was used as reagent, or 10 mL of acetonitrile with an application of CLA as reagent. After filtration of the samples, they were diluted with suitable solvent until examined substance concentration of 1 $mg \cdot mL^{-1}$ was obtained. Such prepared solutions were subjected to reaction with 2.0 mL of 0.1% DDQ or 2.0 mL of 0.5% CLA, at a room temperature for 15 min, in both cases the coloration was stable for 30 min. The samples were analyzed spectrophotometrically at λ_{max} = 560 nm for DDQ and λ_{max} = 520 nm, correlation coefficients for the applied reagents were R^2 = 0.9991 and R^2 = 0.9993, respectively. The range of linearity consistent to Beer's law was 5-50 $\mu g \cdot mL^{-1}$, and experimentally determined molar coefficient of absorption ε was $1.044 \cdot 10^5$ $L \cdot mole^{-1} \cdot cm^{-1}$ (DDQ) and $8.476 \cdot 10^4$ $L \cdot mole^{-1} \cdot cm^{-1}$ (CLA). The limits of detection and quantification for the method applied were 0.126 $\mu g \cdot mL^{-1}$, 0.383 $\mu g \cdot mL^{-1}$ and 0.152 $\mu g \cdot mL^{-1}$, 0.458 $\mu g \cdot mL^{-1}$, respectively.

Spectrophotometric determination of ambrisentan may also involve its reaction with methylene blue or safranin O in buffer environment, in which color complex extracting to chloroform is formed.[72] In this case, the authors extracted an active substance from tablet mass using methanol. After filtering, the filtrate was evaporated to dry form, and quantitatively transferred using a few portions of 0.1 M NaOH to measuring flask, and

completed with distilled water. Suitable amount of complex-forming reagent was added to such prepared samples, and it was 0.1% methylene blue solution, or 0.05 % safranin O solution. Formed complexes were subjected to the process of extraction to chloroform, organic phase was separated and the whole was completed with chloroform to suitable volume. Determined absorption spectra for particular reagents demonstrated absorption maximum at 525 nm for the complex with methylene blue, and 515 nm for the complex with safranin O. The course of reaction proposed by the authors is presented in Figs. 12 and 13.

Fig. 12. Proposed reaction of ambrisentan with methylene blue

Fig. 13. Proposed reaction of ambrisentan with safranin O

Examined range of linearity for that method was 1-15 $\mu g \cdot mL^{-1}$, with correlation coefficients of R^2 = 0.9991 and 0.9999, coefficients ε determined depending on reagents applied were $1.7911 \cdot 10^5$ $L \cdot mole^{-1} \cdot cm^{-1}$ for the complex with methylene blue, and $2.3272 \cdot 10^5$ $L \cdot mole^{-1} \cdot cm^{-1}$ for the complex with safranin O, with detection and qualification limits of 0.182 $\mu g \cdot mL^{-1}$ 0.175 $\mu g \cdot mL^{-1}$ and 0.551 $\mu g \cdot mL^{-1}$ 0.531 $\mu g \cdot mL^{-1}$, respectively.

Spectrophotometric method of ambrisentan determination in pharmaceutical preparations in the range of ultraviolet and visible light was also proposed by Adiki et al.[73] Analysis using the visible range was based on reaction of formation of the colorful product of the examined substance with 3-methyl-2-benzothiazolinone hydrazone in the presence of $FeCl_3$. The product formed as a result of reaction (Fig. 14) demonstrated absorption maximum at a wavelength of 625 nm. In this case, the range of linearity was from 10 $\mu g \cdot mL^{-1}$ to 30 $\mu g \cdot mL^{-1}$, and LOD and LOQ calculated based on straight equation were 2.7 and 8.3 $\mu g \cdot ml^{-1}$, with correlation coefficient of R^2 = 0.9986. In case of UV spectrophotometric method application for methanol ambrisentan solutions, analytical wavelength was 262.5 nm, and validation parameters for this kind of analysis were satisfactory and close to VIS spectrophotometric method.

Fig. 14. Proposed reaction of ambrisentan with 3-methyl-2-benzothiazolinone hydrazone

RP-HPLC method with UV detection was applied in the examination of ambrisentan stability and impurities, while LC-MS/MS method was used in order to determine the structures of degradation products.[74, 75] In the examination of impurities, Narayana et al. used SunFire C18 column, mobile phase was the mixture of potassium dihydrogen orthophosphate at pH adjusted to 2.5 with ortho-phosphoric acid in water and a mixture of acetonitrile-methanol using a simple linear gradient, UV detection with a wavelength of 225 nm was used. The samples were subjected to an activity of 0.5 mol·L^{-1} HCl 60^{0}C for 3 h, 0.5 mol·L^{-1} HCl 60^{0}C for 3 days, 3% H_2O_2 at room temperature for 3 days, in case of thermal and photolytic decomposition, the exposure on a given factor lasted 11 days. The presence of four impurities of ambrisentan was examined (Fig. 15).

Fig. 15. Chemical structures of ambrisentan impurities.

The retention times of peaks from particular impurities obtained in the examined conditions were as follows: 4.637 min Imp. 1, 12.566 min Imp. 2, 16.381 min Imp. 3 and 22.082 min Imp 4. Validation parameters of the method were consistent with the requirements of the ICH for chromatographic methods. Stress examinations of ambrisentan allowed to note the presence of decomposition products only in the case of alkaline hydrolysis (1 product of retention time of 2.304 min), and in case of acidic hydrolysis (3 products of retention times of 8.555 min, 16.338 min and 22.06 min). Due to the applied detection method, it was not possible to establish the structures of formed products. In case of thermo and photolysis, ambrisentan appeared to be stable substance which did not demonstrate any additional peaks on the chromatograph.

Establishing of the structures of formed degradation products was possible using LC-MS/MS method. Ramisetti and Kuntamukkala[75] conducted the separation using Agilent XDB C18 column (150 × 4.6 mm; 5 μm), mobile phase was composed of 10 mM ammonium acetate solution and acetonitrile. The process of ambrisentan degradation in this case was performed in acidic (0.5 M HCl, 60^{0}C, 3 h), alkaline (0.5 M NaOH, 60^{0}C, 8 h), neutral environment (H_2O, 60^{0}C, 8 h), and also ambrisentan susceptibility to oxidative (10% H_2O_2, 24 h), thermal (60^{0}C, 10 days) and photolytic stress was examined (UV light 254 nm, 10 days). The

degradation products (DP) of ambrisentan proposed by the authors determined based on LC-MS/MS analysis are presented in Fig. 16.

Fig. 16. Proposed degradation products of ambrisentan

The authors did not manage to establish the structure labeled as PD-4 (*m/z 319*), which was due to very low intensity of molecular ion peak.

The obtained results of stress examination for ambrisentan prove its lowest stability in acidic environment, in this case the presence of five degradation products was noted: DP-2; DP-3; DP-4; DP-5 and DP-6, while in case of oxidation stress the presence of degradation product DP-1 was observed. In other cases, ambrisentan appeared to be a stable substance and no products of its degradation were noted.

Like in other cases of the substances which may be observed as enantiomers, it seems to be reasonable to undertake the research on an elaboration of their determination method due to the possible differences in their pharmacological activity. Determination of ambrisentan enantiomers on chiral stationary phase was proposed by Michal Dous and Petr Gibala.[76] Ambrisentan samples (*S)* and (*R)* were prepared dissolving suitable amount of standard substance in the mixture of acetonitrile and water in ratio 4:6 (v/v). The authors verified in their study a usefulness of six columns with chiral phases. Optimum separation was obtained using Chiralcel OZ-3R column of a temperature of 35^0C, with mobile phase being a mixture of 20 mM sodium formate (pH 3.0)-acetonitrile (55:45; v/v), and flow rate of 1 ml·min^{-1}, and detection UV λ=263 nm. Well separated and formed peaks of *R* form of retention time on a level of 4.35 min, and *S* form which retention time was 4.93 min, were obtained in such conditions.

The combinations of ambrisentan with tadalafil are often used in a therapy of pulmonary hypertension.[77, 78] Therefore, Jayvadan K. Patel and Nilam K. Patel[79] proposed the method for concurrent determination of these substances in pharmaceutical preparations using reversed phase high-performance liquid chromatography (RP-HPLC). Optimum separation of the examined substances was obtained using Hypersil GOLD C18 column (150 x 4.6 mm; 5 μm) with mobile phase being a mixture of methanol-water-acetonitrile 40:40:20 (v/v/v). The

retention times in such conditions were as follows: 2.803 min for ambrisentan, and 7.127 min for tadalafil, the linearity ranges were 1-20 μg·mL^{-1} and 4-80 μg·mL^{-1}, respectively, with correlation coefficient of $R^2 = 0.997$.

3.3. Atrasentan

(2R,3R,4S)-4-(1,3-benzodioxol-5-yl)-1-[2-(dibutylamino)-2-oxoethyl]-2-(4-methoxyphenyl)pyrrolidine-3-carboxylic acid is an oral, selective blocker of A type endothelin receptor. It was proved in the research that its affinity to A type receptor is nearly 1000-fold higher compared to B type receptor. Unlike other substances from that pharmacological group, its effect on cells proliferation is used to a higher degree than its vasoconstrictive activity. Atrasentan was introduced on the market under commercial name of *Xinlay* by Abbot company, as preparation used in therapy of prostate cancer resistant on hormonal treatment. Therefore, atrasentan as an active substance will not be described in detail in this review, and some papers concerning an analysis of atrasentan[80], its metabolites and impurities[81,82] as well as pharmacokinetic examinations[83,84] may be found in the literature.

3.4. Sitaxentan

N-(4-chloro-3-methyl-1,2-oxazol-5-yl)-2-[2-(6-methyl-2H-1,3-benzodioxol-5-yl)acetyl]thiophene-3-sulfonamide is a selective blocker of A type endothelin receptors. Its affinity to this kind of receptor is about 6500-fold higher than to B type receptor.[85] It demonstrated good bioavailability in the research on animals after oral administration (50-60% in rats, 90-100% in dogs). However, the studies on its potential hepatotoxic activity resulted in a withdrawal of *Thelin* preparation by Pfizer company in 2010. The research on an evaluation of toxicity of sitaxentan and its pharmacokinetic preparations are still conducted.[86, 87] In 2013, Erve et al.[88] using LC-MS/MS method, examined and described an effect of sitaxentan on microsomes and hepatocytes of mice, rats, dogs and humans. Analysis of the metabolites was conducted on two kinds of columns: Poroshell 120 SB-C18 column (2.1 × 150 mm, 2.7 μm) and Zorbax Eclipse XDB-Phenyl column (4.6 × 250 mm, 5 μm). Mobile phase for column Poroshell was composed of 0.1% aqueous solution of formic acid and 0.1% solution of formic acid in acetonitrile. The total time of analysis maintaining suitable gradient of mobile phase concentrations was 20 min. In case of XDB-Phenyl column, the mobile phase was a mixture of 0.1% aqueous solution of formic acid and 0.1% solution of formic acid in methanol. The rate of mobile phase flow in these cases was 0.7 and 1 mL·min^{-1}, respectively.

The presence of three metabolites of sitaxentan was noted in the elaborated conditions (Fig. 17).

Fig. 17. Metabolism products of sitaxentan

Metabolite M1 was only noted in preparations originating from dogs, its retention time was 18.2 min, the presence of metabolite M2 with retention time of 17.1 min, and M3 which retention time was 5.4 min, was observed in all examined samples. The research proved that sitaxentan metabolism may lead to the products of potential hepatotoxic activity.

3.5. Darusentan

(2S)-2-(4,6-Dimethoxypyrimidin-2-yl)oxy-3-methoxy-3,3-di(phenyl)propanoic acid is a substance which is still a subject of clinical examinations. It was proved that it is selective antagonist of A type receptors, with an affinity 150-fold higher than towards B type receptors.[89] It may occur in two isomeric forms, and the research proved that satisfactorily pharmacological activity is only demonstrated for isomer (*S)*.[90] Thus, Gu et al. proposed the method of darusentan enantiomers determination in biological material using liquid chromatography method.[91] In this case, they used Chiralcel OD-RH column 150 × 4.6 mm, 5 μm (cellulose tris(3,5-dimethylphenylcarbamate)), mobile phase was the mixture of acetonitrile-water-formic acid (50:50:0.1,v/v/v). The retention times for particular enantiomers were in this case 10.03 min or 9.99 min for *R*-enantiomer, and 11.40 min and 11.44 min for *S*-enantiomer, depending on the matrix used (serum after an addition of suitable amount of examined substances; serum collected from rats after oral administration of darusentan racemate in amount of 3mg · kg^{-1}. Based on the examination of darusentan pharmacokinetics after oral administration of its racemic mixture, the authors concluded that in all the cases, irrespective of the dose applied, the concentration of *S* darusentan form in blood serum of rats is higher compared to *R* form, which may prove higher bioavailability of *S*-enantiomer.

4. Conclusion:

An effectiveness of arterial hypertension therapy is still unsatisfactory, and therefore there is a search for new drugs of hypotensive activity. The studies so far have resulted last years in an elaboration of new substances which may improve an effectiveness of various hypertension forms therapy. These compounds include renin inhibitors and endothelin receptor blockers. The breakthrough in the research in case of renin inhibitors, was an elaboration of aliskiren molecule, which was the first oral renin inhibitor. Further studies resulted in an elaboration of other compounds, which demonstrated pharmacological activity similar to aliskiren, and are concurrently characterized by higher bioavailability and specificity towards human renin. In case of endothelin receptor blockers, the breakthrough was a discovery of a substance of an activity blocking endothelin receptors which took place in 1990, and then its synthesis in 1991. The studies conducted resulted in obtaining of a range of substances of potential hypotensive activity. The drugs from these groups may become competitive towards angiotensin convertase blockers (ACE) due to the possibility of their administration in patients allergic to the drugs from ACE group. Administration of renin inhibitors and endothelin receptor blockers may be combined with other hypotensive drugs such as diuretics or angiotensin receptor blockers, which may lead to an increased hypotensive effect. Some part of discussed products is still on various stages of pharmacological and clinical examinations, and part has been already introduced to the therapy of hypertensive disease. Such wide development and introduction of new drugs to the therapy forces the researchers for an elaboration of new, precise, accurate analytical methods which may be applied both in the studies on bioavailability and in determination of metabolites and decomposition products.

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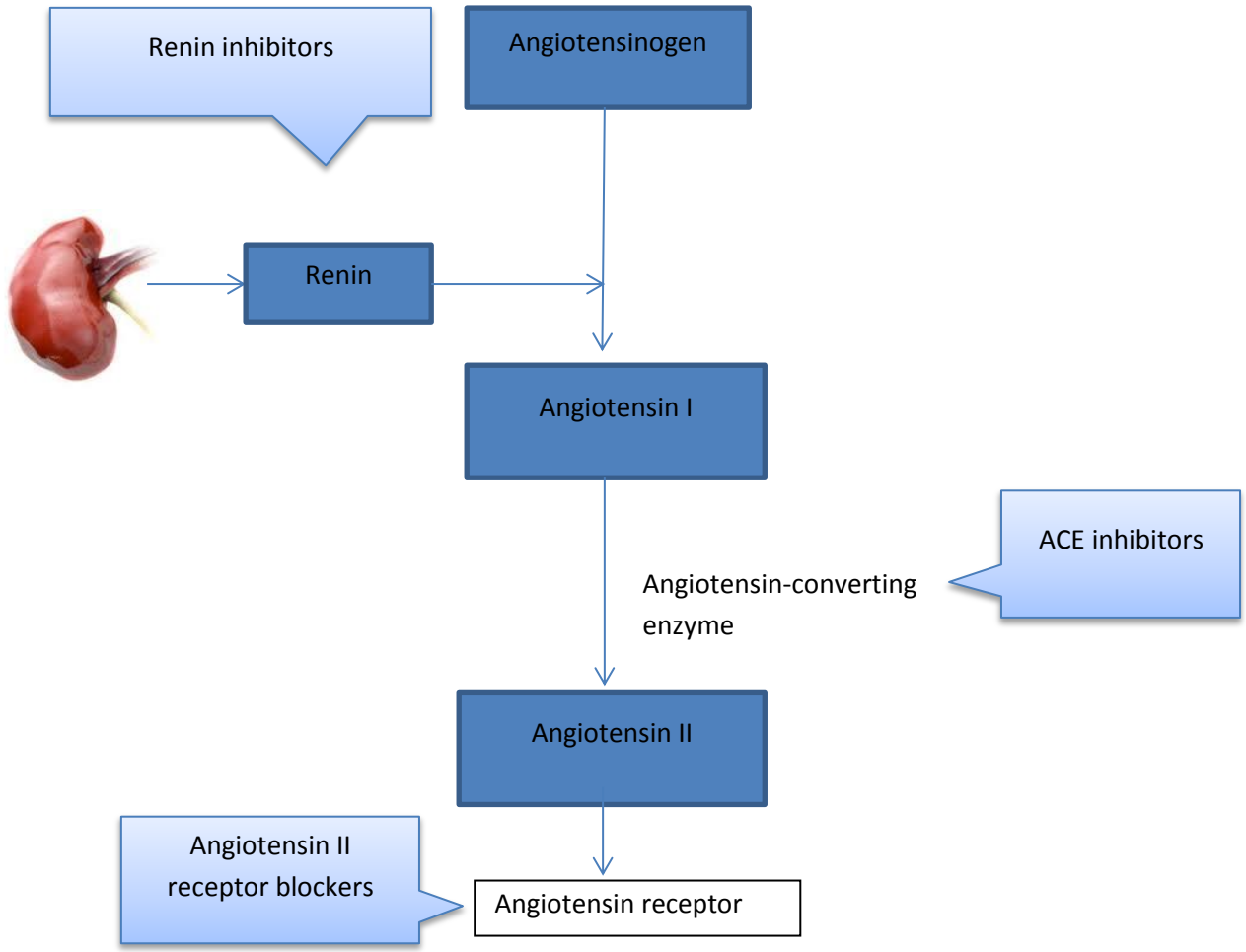

Fig. 1. Renin-angiotensin-aldosterone system

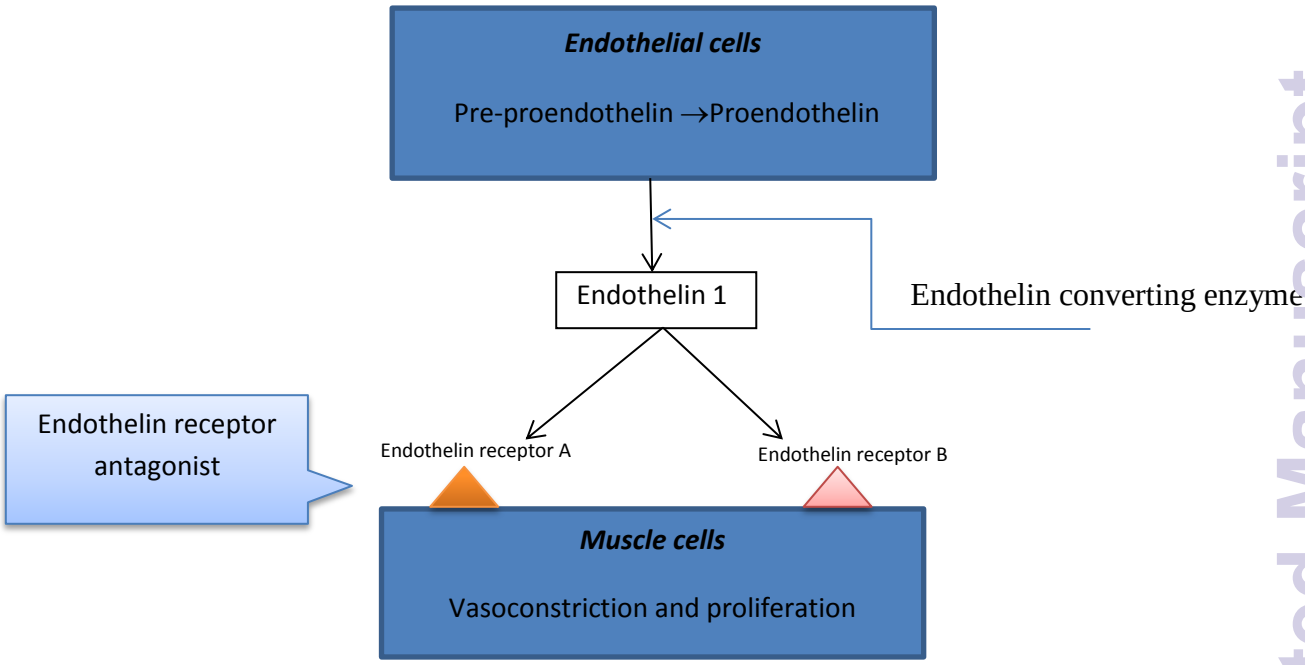

Fig. 2. Endothelial system

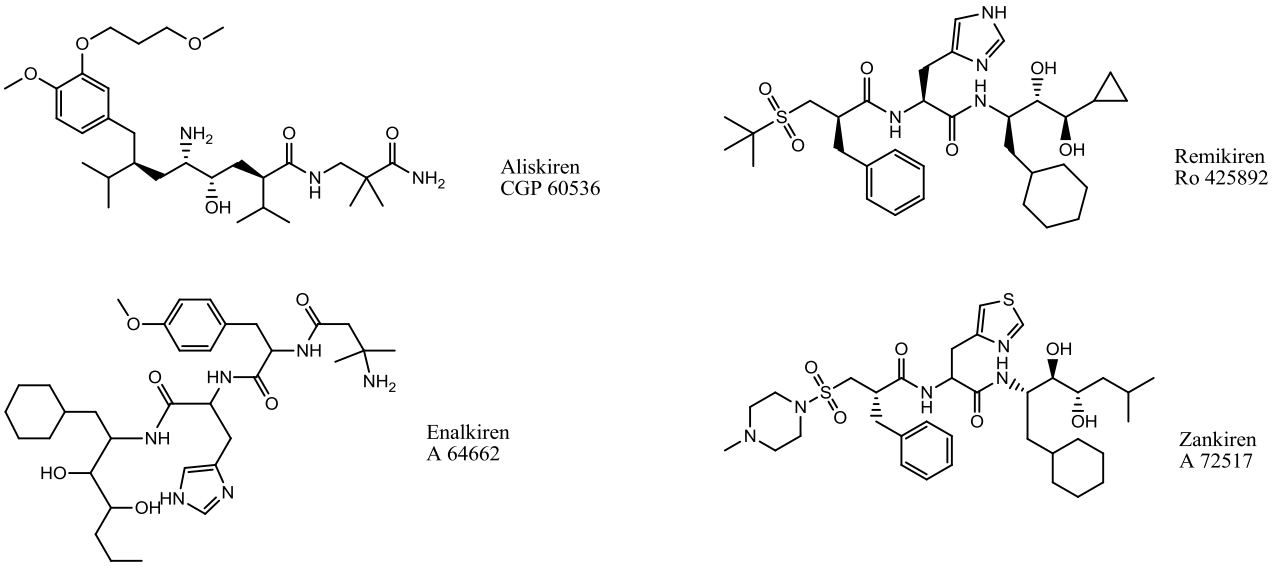

Fig. 3. Chemical structure of renin inhibitors

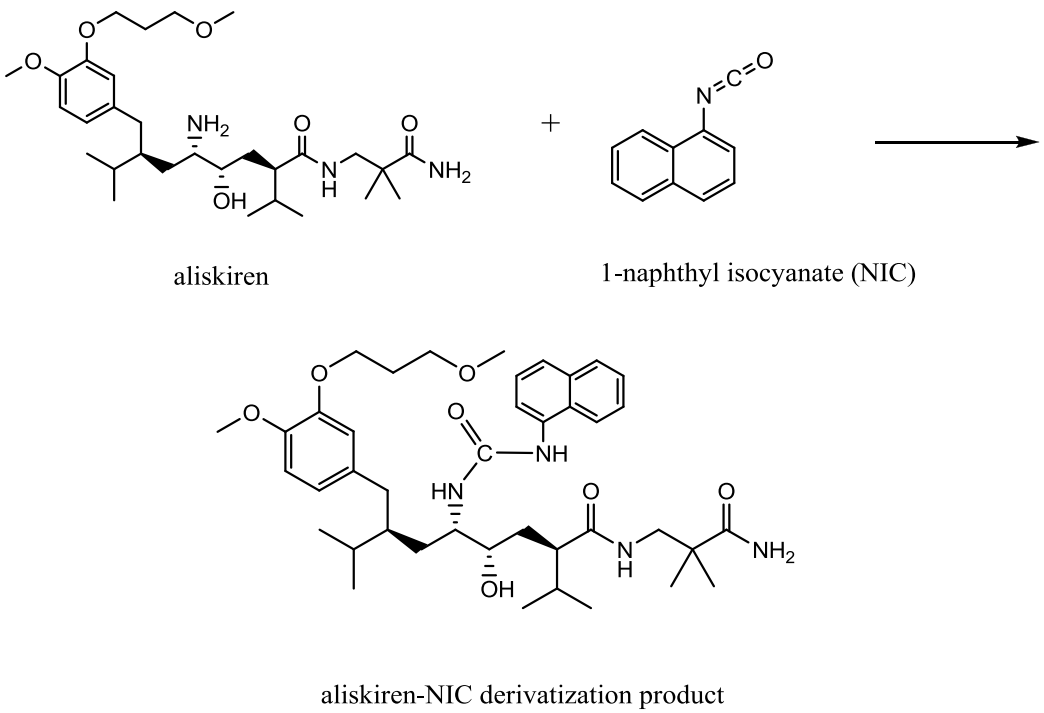

Fig. 4. Derivatization reaction of aliskiren with 1-naphthyl isocyanate

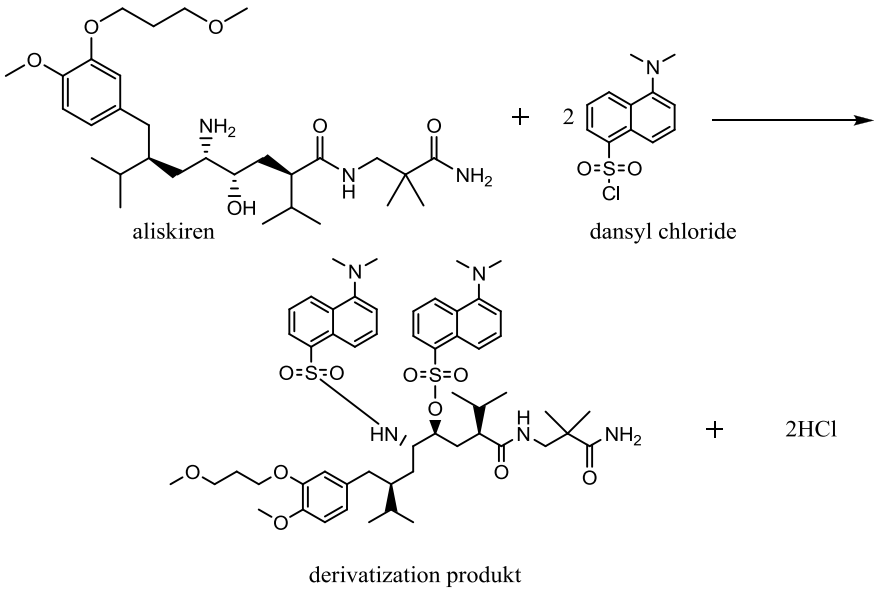

Fig. 5. Derivatization reaction of aliskiren with dansyl chloride

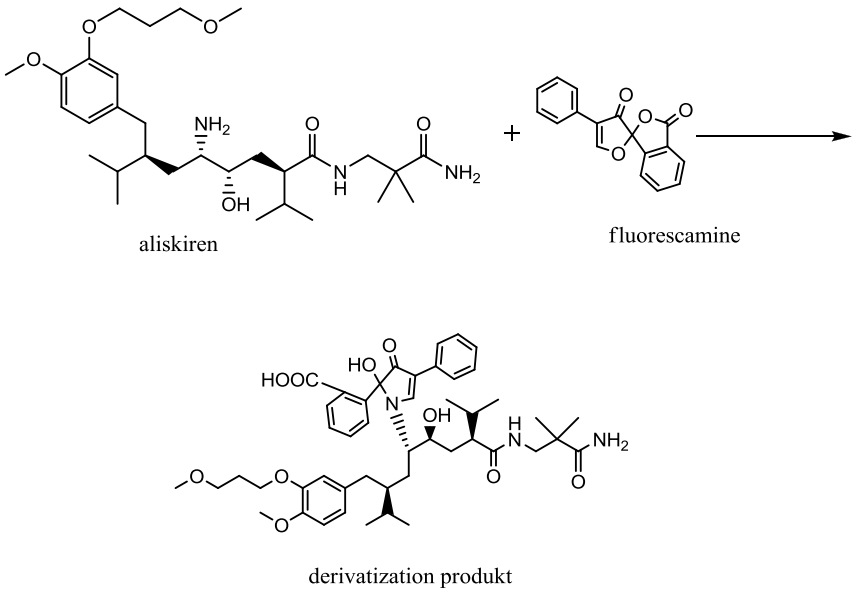

Fig. 6. Derivatization reaction of aliskiren with fluorescamine

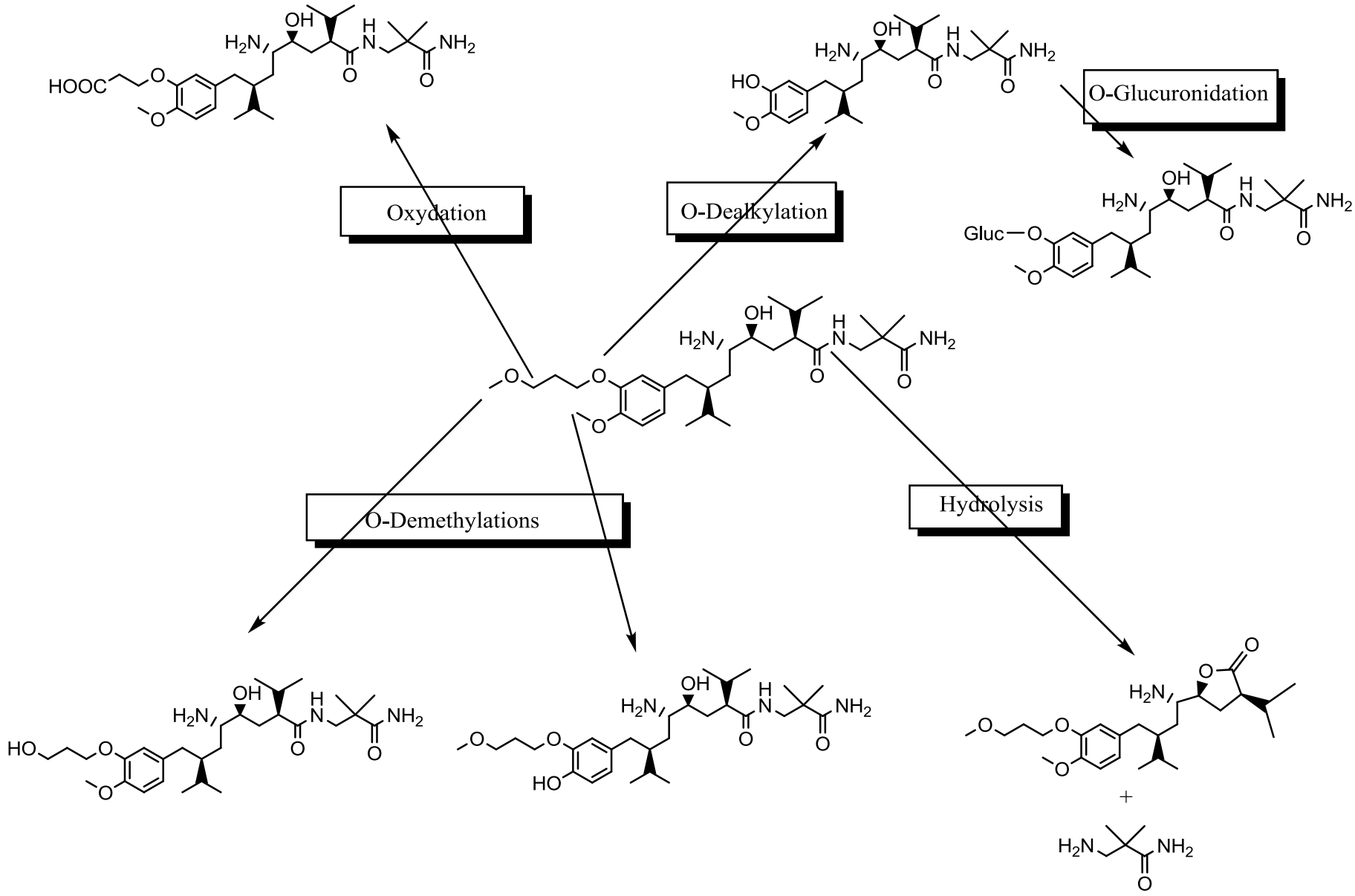

Fig. 7. Metabolism products of aliskiren

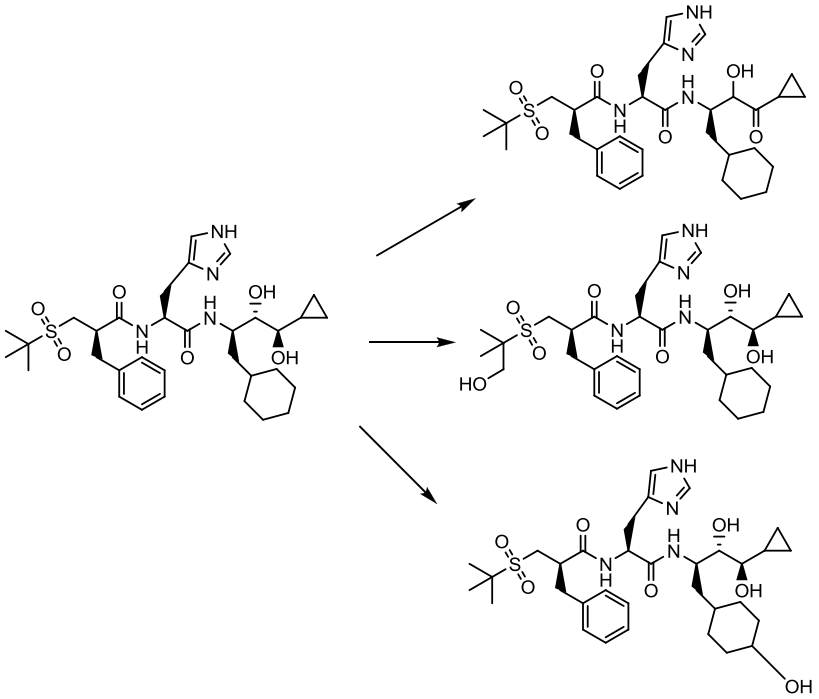

Fig. 8. Metabolism products of remikiren

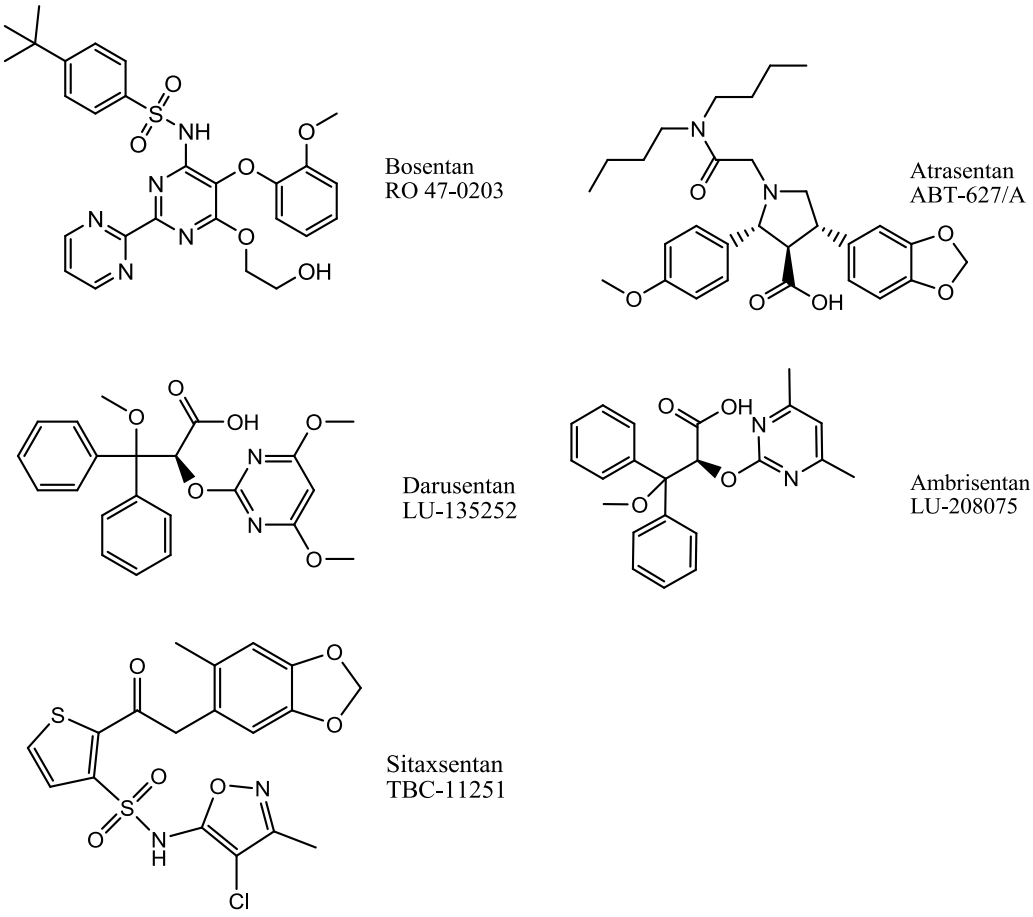

Fig. 9. Chemical structure of endothelin receptor antagonists

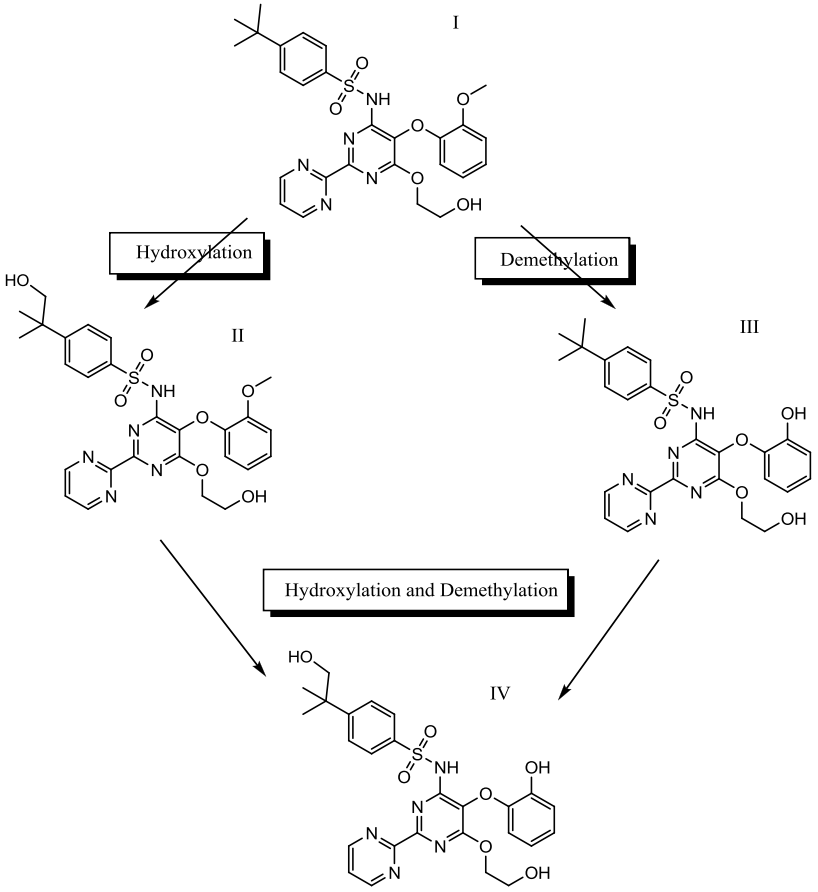

Fig. 10. Metabolism products of bosentan

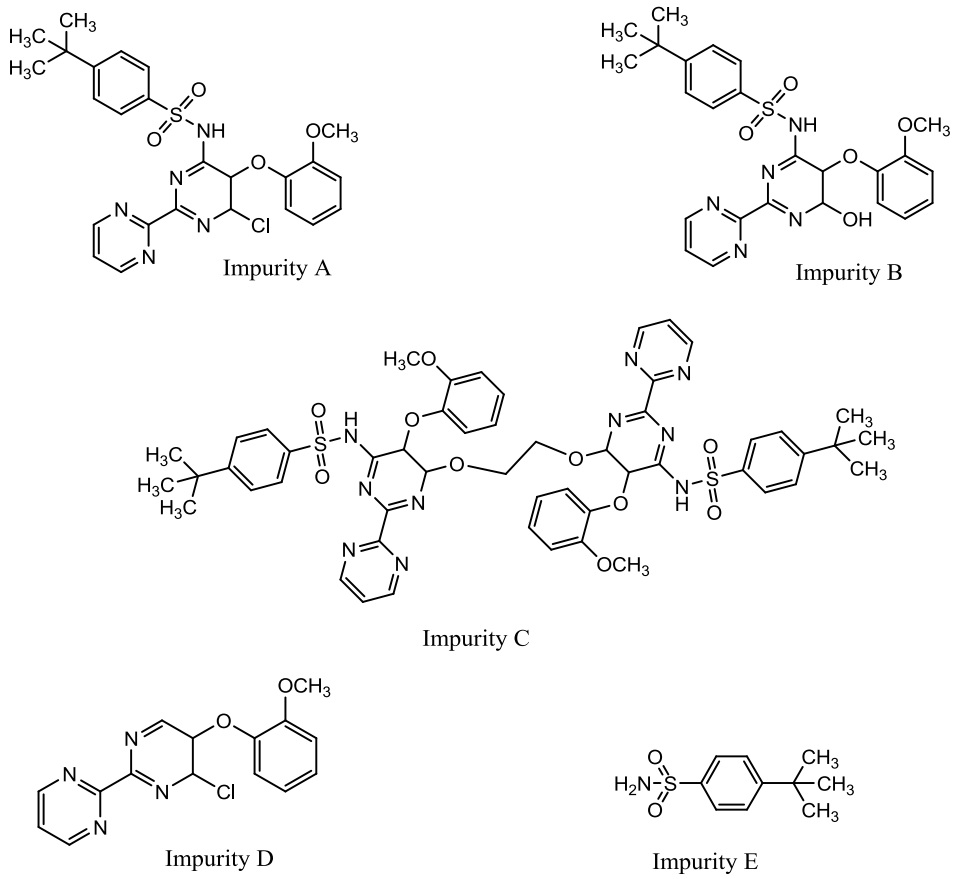

Fig. 11. Chemical structures of bosentan impurities

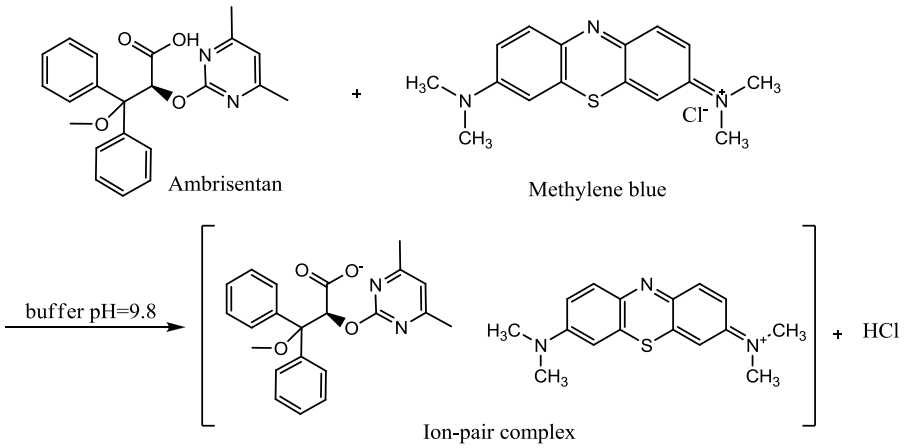

Fig. 12. Proposed reaction of ambrisentan with methylene blue

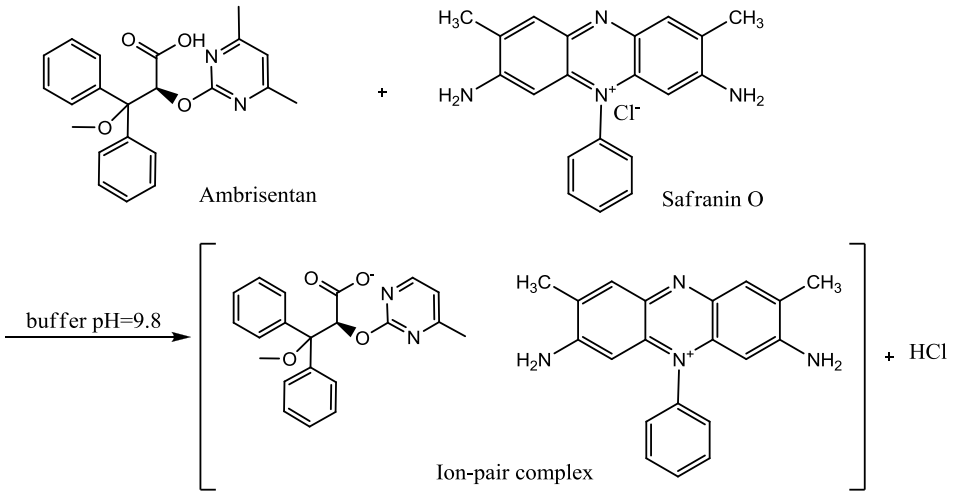

Fig. 13. Proposed reaction of ambrisentan with safranin O

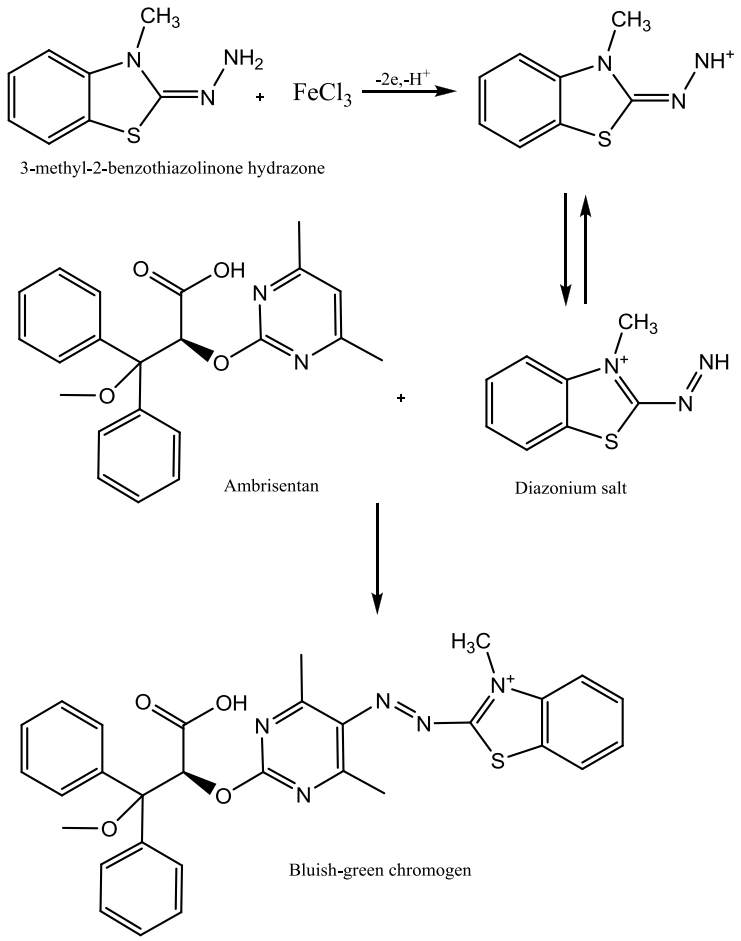

Fig. 14. Proposed reaction of ambrisentan with 3-methyl-2-benzothiazolinone hydrazone

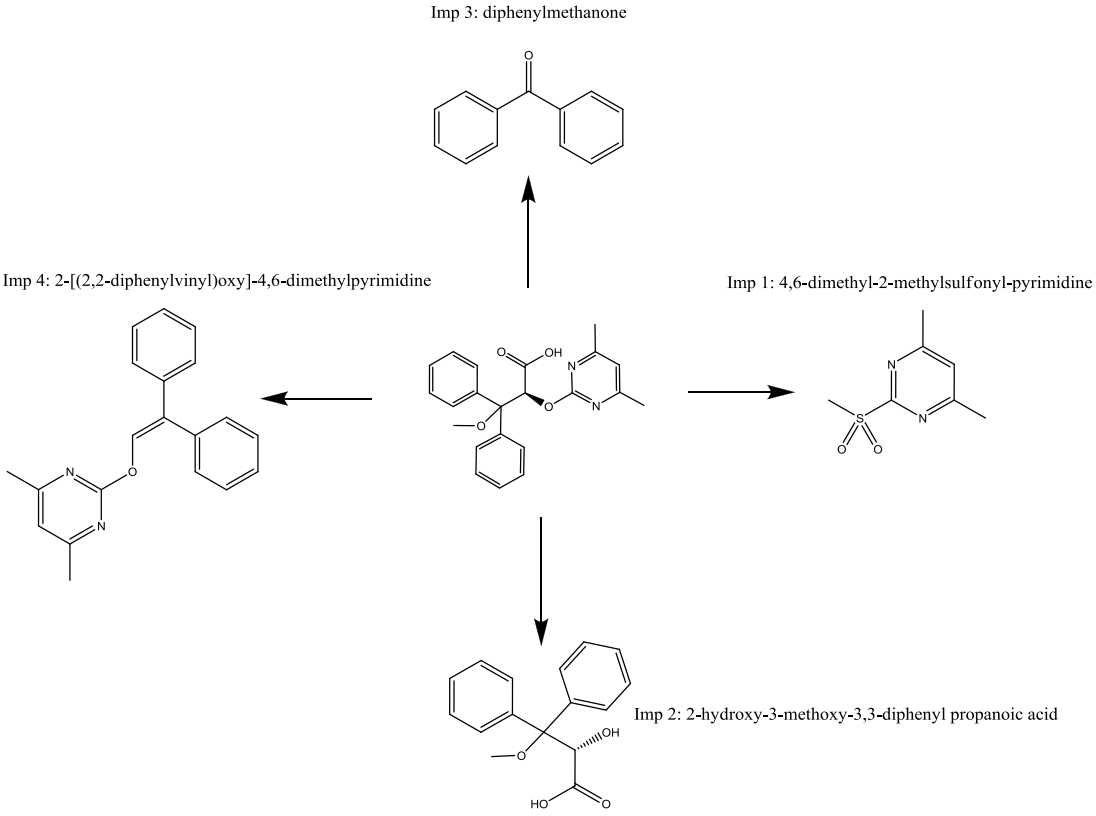

Fig. 15. Chemical structures of ambrisentan impurities.

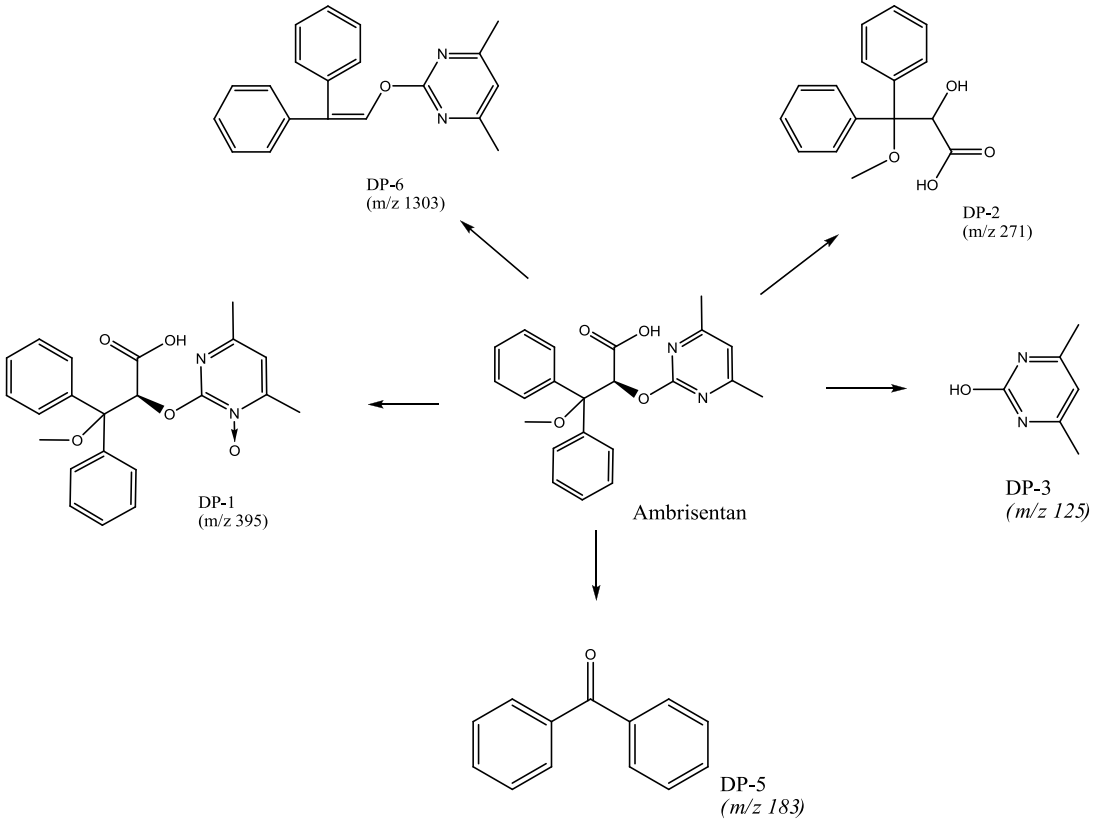

Fig. 16. Proposed degradation products of ambrisentan

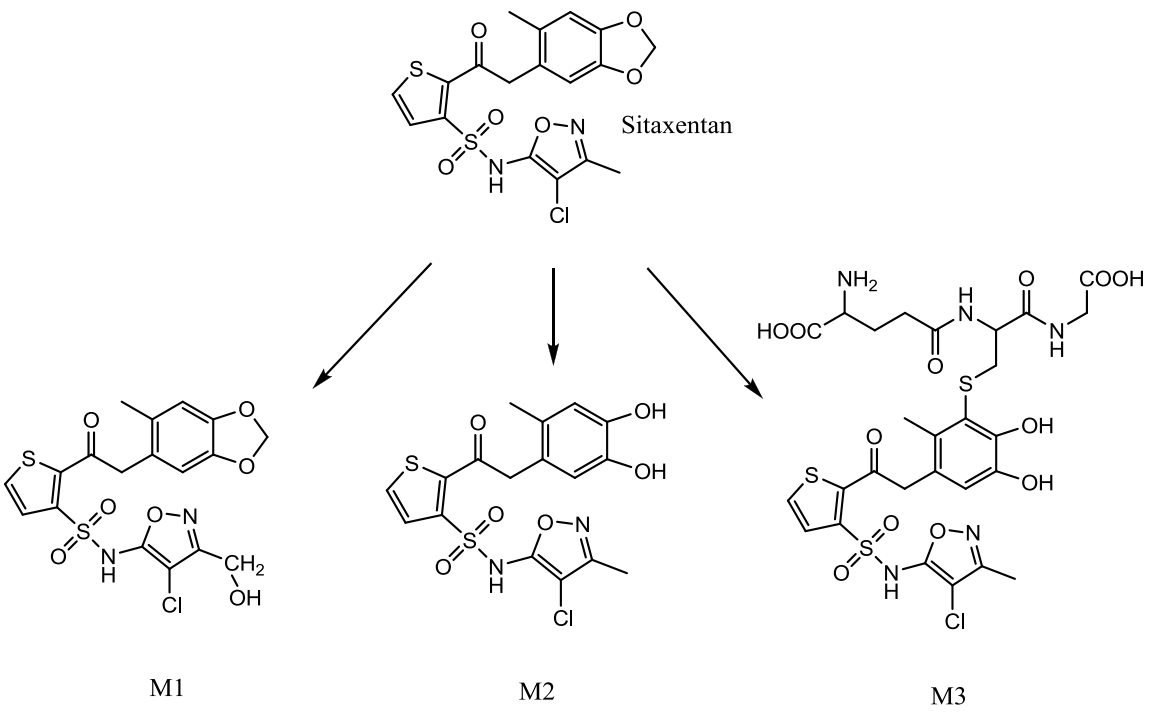

Fig. 17. Metabolism products of sitaxentan