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SYNTHESIS, CHARACTERIZATION AND TOXICITY STUDY OF BEXAROTENE ESTERS

ABSTRACT

For awarding the educational and scientific degree ‚Doctor‘ in scientific specialty: 7.3 ‚Pharmaceutical Chemistry‘

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The dissertation work was discussed at a meeting of the departmental council of the Department of Pharmaceutical Chemistry at the Medical University - Varna and referred for defense before a Scientific Jury.

The dissertation covers 192 pages, 63 figures, 37 tables and 1 appendix with 5 figures. 213 titles were cited.

The experimental work on the dissertation work was carried out in the Department of Pharmaceutical Chemistry at the Medical University - Varna.

The public defense of the dissertation work will be held on 04.05.2023 at 1:00 p.m. on Webex platform at an open meeting of the Scientific Jury.

CONTENTS

List of commonly used abbreviations

RDH – retinoid dehydrogenase

RA – retinoic acid

RARs – retinoic acid receptors

ATRA – all-trans-retinoic acid

13cRA – 13-cis-retinoic acid

CTCL – cutaneous T-cell lymphoma

MDS – myelodysplastic syndrome

VPA – valproic acid

AML – acute myeloid leukemia

ALDH – aldehyde dehydrogenase

9cRA – 9-cis-retinoic acid

ADH – alcohol dehydrogenase

Treg – regulatory T cell

COVID-19 – coronavirus disease 2019

SARS-CoV-2 – severe acute respiratory syndrome - coronavirus 2

IFN – interferon

MS – multiple sclerosis

MeV – measles virus

HREs – hormonal response elements

PPAR – peroxisome proliferator-activated receptor

LXR – liver X receptor

DBD – DNA binding domain

LBD – ligand-binding domain

RXRs – retinoid X receptors

RAREs - retinoic acid response element

THR – thyroid hormone

ADR – adverse drug reaction

FDA – Food and drug administration

EGFR – epidermal growth factor receptor

PARP – poly-(ADP-ribose) polymerase

NF – nuclear factor

HPLC – high performance liquid chromatography

AD – Alzheimer's disease

PD – Parkinson's disease

CNS - central nervous system

ADME - absorption, distribution, metablozyme and excretion

I. INTRODUCTION

Retinoids are a class of chemical compounds derived from vitamin A. Both natural and synthetic retinoids have key roles in vision, embryonic development, regulation of cell proliferation and differentiation, homeostasis, and various aspects of metabolism.

Retinoids are used to treat a limited number of conditions, including skin conditions such as acne and psoriasis, and to treat certain types of cancer. Despite their good therapeutic effects, the application of retinoids is limited due to their toxicological profile. The reduction in adverse drug reactions with synthetic vitamin A derivatives has led to an increase in the use of retinoids as therapeutic agents and also to a better understanding of their mechanism of action.

Extensive studies on the mechanism of action of retinoids led to the characterization of nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). These receptors are members of the steroid/thyroid receptor superfamily and regulate the transcription of target genes upon ligand binding. The biological activity of nuclear hormone receptors, coupled with the ability to directly regulate their activity by small molecules, makes this superfamily a target for new drug discovery.

Bexarotene is a synthetic retinoid analogue with selectivity for all three retinoid X receptor isoforms and belongs to a group of compounds called rexinoids. These receptors play a role in the regulation of cell growth and differentiation through their ability to regulate transcription. Bexarotene’s selectivity for a particular isoform may provide therapeutic specificity and/or reduced toxicity. Bexarotene inhibits the growth of tumor cell lines of both hematopoietic and squamous cell origin. It also induces apoptosis in a number of tumor cell lines. It is approved for the treatment of cutaneous T-cell lymphoma (CTCL), both as an oral and as a dermal dosage form. Therapeutic effects have been observed in the treatment of breast and lung cancer. A number of studies have described the potential of Bexarotene in the treatment of neurological diseases such as Alzheimer's disease, Parkinson's disease and schizophrenia.

One approaches for optimizing drug profile is the application of the so-called ‚pro-drug design‘. The latter aims to improve the physico-chemical, pharmacokinetic, pharmacological, toxicological and even organoleptic properties of the molecule.

A pro-drug is a biologically inactive compound that is metabolized in the body to the active drug. A major trend in pro-drug development is the preparation of esters.

Esterases, which are present throughout the body, including the liver, blood, and other tissues, rapidly hydrolyze esters to release the active drug.

Pro-drugs can be classified as bioprecursors, classical pro-drugs, targeted drugs, mixed pro-drugs and co-drugs. The difference between them is the drug carrier.

Despite all the difficulties encountered in pro-drug design, pro-drug development is a faster and more feasible strategy than searching for an entirely new therapeutically active agent with suitable absorbtion, distribution, metabolism, excretion, toxicity (ADMET) properties.

A major problem is the prediction of bioconversion rates and pharmacological and/or toxicological effects of pro-drugs. The rate of hydrolysis and bioconversion can be affected by various factors such as age, health status and gender.

The structure of Bexarotene enables the application of the pro-drug design strategy. Its potential application in oncology and its ability to modulate a number of physiological effects provides the need for synthesis, characterization and investigation of new therapeutic applications of novel Bexarotene analogues.

II. AIMS AND TASKS

The aim of the present scientific study is to synthesize, structurally characterize and study a group of new, not described in the literature, Bexarotene ester derivatives and to prepare a toxicological profile of the retinoid analogs.

In connection with the implementation of the set goal, the following tasks are planned to be performed:

1. Development of a suitable methodology for the synthesis of new ester derivatives of the antineoplastic drug Bexarotene.
2. Development of a suitable method for monitoring the progress of the esterification reaction of Bexarotene.
3. Carrying out a qualitative characterization of the obtained Bexarotene derivatives by applying the following instrumental methods of analysis, including:
   - Melting temperature;
   - IR spectroscopy;
   - UV-VIS spectroscopy.
4. Development of HPLC method for establishing the purity of the newly synthesized derivatives.
5. Performance of a theoretical assessment of the toxicity of Bexarotene, its metabolites and the newly synthesized derivatives using *in silico* methods.
6. Determination of the overall toxicity of the newly synthesized Bexarotene derivatives by means of *in vivo* models.

III. MATERIALS AND METHODS

1. MATERIALS

Bexarotene (Free acid, Fluorochem); methyl alcohol (99.99 %, HPLC grade, Fisher Chemical); ethyl alcohol (≥ 99.8%, Analytical reagent grade, Fischer Chemical); 1-propanol (≥ 99% (GC), purum, Sigma-Aldrich); 1-butanol (≥ 99.5% (GC), Sigma-Aldrich); oxalyl chloride (98%, Sigma-Aldrich); thionyl chloride (99.5+%, Sigma-Aldrich); sulfuric acid (95-97%, Chem-Lab); aluminum powder, glacial acetic acid (≥ 99%, Fischer Chemical); ammonia (25%, Sigma-Aldrich); butanol (anhydrous, 99.8% Sigma-Aldrich); acetone (≥ 97%, Sigma-Aldrich); chloroform (anhydrous, ≥ 99%, Sigma-Aldrich); petroleum ether (80-100 ^{o}C, Fischer Chemical); ethyl acetate (≥ 99.8%, Fischer Chemical); hexane (99% HPLC, Lab-Scan); diethyl ether (99.5+%, Chem-Lab), formaldehyde (37%, Chem-Lab); water (HPLC grade, Fisher Chemical); Tween 20 (USP-NF, BP, Ph. Eur., CHIMEX Ltd – Dimitrovgrad), acetonitrile (≥ 99.9%, Sigma-Aldrich), dimethyl sulfoxide (99.9% for spectroscopy, Thermo Scientific), methanol (99.9% Extra Dry, Acros Organics).

An Ohaus Explorer Analytical balance with SmarText™ 2.0 software was used to weigh the quantities required for work.

During the synthesis, a magnetic stirrer Nahita Blue digital magnetic stirrer with heating, model 692/1 was used.

In the development of thin-layer chromatography, a DC-Fertigfolien ALUGRAM® SIL G/UV_{254} plate with a layer thickness of 0.20 mm, impregnated with a fluorescent indicator UV_{254}, was used.

For the visualization of the plates in the preparation of the thin-layer chromatography of the analyzed substances a UV light from a Nahita UV lamp was used.

The melting temperature measuring apparatus BUCHI Labortechnik AG M-565, Flawil/Switzerland was used to determine the melting temperature.

Ultrasonic bath UST1-100t, SIEL Ltd.

Infrared spectra in the interval 4000-500 cm^{-1} were taken on a Nicolet iS10 FT-IR spectrometer. Using ATR - a plug-in with a Smart iTR adapter.

UV-VIS spectra were recorded using a T60UV UV-VIS spectrophotometer in the wavelength range of 190 to 400 nm on UVWin Software 6.0.

A Thermo Scientific High Performance Liquid Chromatography (HPLC) apparatus model Spectra SYSTEM HPLC with manual injector, UV-VIS detector model Spectra SYSTEM UV2000 and Fluorescence detector model Spectra SYSTEM FL3000 were used for the development of the HPLC method. A Synergi 4u Hydro – RP 80A (250 x 4.00 mm) column protected with a Synergi 4u Hydro – RP 80A (250 x 4.00 mm) precolumn was used for the analysis. System control, data acquisition and analysis were performed using ChromQuest chromatographic data software, version 4.2.34. Analytical balance Sartorius A200S; ultrasonic bath UST1-100t, SIEL Ltd.

The OECD (Q)SAR Application Toolbox is a software application for evaluating the properties of chemical substances based on their molecular structure. The software assesses the hazard of various chemicals while reducing research time, cost and animal testing. A COMPAQ Presario SQ61 computer configuration with an AMD Turion™ II Dual-Core Mobile M500 2.20 GHz processor was used during the OECD (Q)SAR Application Toolbox analysis.

Molinspiration is a specialized chemical informatics software developed in Java. Molinspiration's tools are platform independent and can run on any PC, Mac, UNIX or LINUX machine. A COMPAQ Presario SQ61 computer configuration with an AMD Turion™ II Dual-Core Mobile M500 2.20 GHz processor was used during the Molinspiration analysis.

PreADME/Tox is a web-based application for predicting absorption, distribution, metabolism, excretion and toxicity and for building a library of drug-like molecules. A COMPAQ Presario SQ61 computer configuration with an AMD Turion™ II Dual-Core Mobile M500 2.20 GHz processor was used during the PreADME/Tox analysis.

During the *in vivo* general toxicity assay, 8-10 week old Wistar rats with an average weight of about 210 g were used. Diethyl ether (99.5+% Chem-Lab) was used to anesthetize the rats. Straight scissors for dissection 11.5 cm with a sharp tip were used to cut the sublingual vein, and straight scissors with a blunt tip 20 cm were used for dissection. Vacutainers 3 ml VACUSERA K2E K2EDTA were used for blood collection. The rats were humanely euthanized. Harvested organs were stored in sterile containers in formaldehyde (37%, Chem-Lab).

2. METHODS

2.1. General methodology for the synthesis of Bexarotene esters

To obtain the Bexarotene esters, the following synthesis methodology was followed:

0.03 g Bexarotene, pre-weighed to ±0.0001 g, is placed in a 100 ml one-neck round-bottom flask equipped with a reflux condenser. Alcohol is added to it in an amount sufficient to dissolve the Bexarotene. The mixing of the solution is carried out using a magnetic stirrer, with a stirring speed of 500 rpm and heating until the complete dissolution of Bexarotene and obtaining an absolutely clear homogeneous solution.

Oxalyl chloride (0.4 ml) was added slowly, dropwise, to the solution. After adding the entire amount of oxalyl chloride, the solution is subjected to continuous stirring and heating. The reaction conditions for conducting the synthesis and the alcohols used are presented in Table 1. At the end of the synthesis, after tempering, the solution remains clear and a precipitate separates in the flask. The resulting solution was dried on a Heidolph Hei-VAP Expert vacuum evaporator until the complete evaporation of the solvent.

Table 1. Reaction conditions and alcohols used for the preparation of Bexarotene esters.

Alcohol	***Temperature***	***Stirring speed***	***Reaction time***
Methanol	40 ºC	500 rpm	2 h
Ethanol	60 ºC	500 rpm	2 h
1-propanol	60 ºC	500 rpm	2 h
1-butanol	60 ºC	500 rpm	3 h

The drying of the samples is carried out under different conditions according to the alcohol used for the synthesis. The drying conditions of the samples are presented in Table 2.

Table 2. Conditions for evaporation of the solvent using a vacuum evaporator.

Alcohol	***Temperature***	***Stirring speed***	***Pressure***	***Time***
Methanol	50 ºC	100 rpm	194 mbar	15 min
Ethanol	50 ºC	100 rpm	95 mbar	15 min
1-propanol	50 ºC	100 rpm	25 mbar	20 min
1-butanol	50 ºC	100 rpm	16 mbar	20 min

2.2. Calculation of the yield obtained as a result of the synthesis

Theoretical yield refers to the amount of product that should be obtained after the chemical reaction is complete.

Practical yield is called the yield obtained after accounting for losses during synthesis. It gives information about what amount of product is obtained when applying precisely defined amounts of starting substances.

The ratio between the practical and the theoretical yield, expressed in percentages, is called the percentage yield. It is calculated using the following formula:

$$\%Y = \frac{PY}{TY} \text{x } 100 \text{ ,}$$

where:

%Y – percentage yield of the product obtained as a result of the synthesis;

PY – practical yield;

TY – theoretical yield.

2.3. Conduct TLC analysis to monitor the reaction process

Chromatography is a physical method for separating organic compounds based on the different distribution of components between two phases, one of which is stationary (stationary) and the other mobile (MF).

Thin-layer chromatography (TLC) is a method with wide application in pharmaceutical practice, used both for the identification of substances and for their quantification. Purity tests can also be carried out, as well as to monitor the progress of chemical reactions. This chromatographic method has a number of advantages, such as high sensitivity, the ability to determine small amounts of components in complex mixtures, expressivity, immediate visual representation of the separated substances and, above all, simplicity of implementation.

A thin-layer chromatographic method was developed to monitor the reaction process of esterification of Bexarotene. In the preparation of the mobile phase, a solvent or a mixture of solvents such as ammonia, butanol, acetone, chloroform, hexane, ethyl acetate and petroleum ether are used in various combinations and ratios.

The disappearance of the chromatographic spot of the starting reagent Bexarotene from the reaction mixture, relative to the Bexarotene standard, is used to determine the end of the esterification process.

The analysis is carried out on UV-plates DC-Fertigfolien ALUGRAM SIL G/UV_{254} with a layer thickness of 0.20 mm impregnated with fluorescent indicator UV_{254}. Detection is performed with UV light on a Nahita UV lamp.

2.4. Characterization of the newly obtained analogues by means of instrumental methods

2.4.1. Characterization of the newly obtained analogues by means of melting point

The melting point of a substance is the temperature at which it changes from a solid to a liquid. At this temperature, the solid and liquid phases exist in equilibrium. The melting point of a substance is pressure dependent and is usually determined at a standard pressure such as 1 atmosphere or 100 kPa. A BUCHI Labortechnik AG M-565, Flawil/Switzerland melting point measuring apparatus was used to determine the melting point of Bexarotene and its newly synthesized esters.

2.4.2. Infrared spectroscopy

One of the main pharmacopoeial instrumental methods for determining the identity and characterization of pure substances and newly synthesized compounds is Infrared spectroscopy (IR spectroscopy or vibrational spectroscopy). It accounts for the interaction of infrared radiation with molecules through absorption, emission or reflection. For the qualitative identification of chemical substances or their functional groups, the analysis can be carried out in solid, liquid or gaseous form. To carry out analysis by means of infrared spectroscopy, a method of direct recording of spectra using an ATR attachment with a Smart iTR adapter is used.

Infrared spectra were taken in the interval 4000-500 cm^{-1} on a Nicolet iS10 FT-IR spectrometer.

2.4.3. UV-VIS spectroscopy

UV-VIS spectroscopy in the visible and ultraviolet regions is a type of electromagnetic spectroscopy that studies the interaction between light and matter. It is both a qualitative and quantitative method that measures the extent to which a chemical substance absorbs light. UV-VIS spectroscopy is based on the absorption of ultraviolet or visible light by chemical compounds, resulting in different spectra.

This spectral method has a number of advantages over other methods, namely: universality, sensitivity, selectivity, accuracy, ease of operation and sample preparation. Electronic spectra are observed in the visible (380 – 780 nm) and near UV (200 – 380 nm) region of the electromagnetic spectrum.

Qualitative characterization was performed by recording the spectra of Bexarotene and newly synthesized Bexarotene esters and determining their absorption maximum.

Spectra were recorded using a T60UV UV-VIS spectrophotometer in the wavelength range from 190 to 400 nm at 1 nm intervals on UVWin Software 6.0.

A comparative analysis was performed for the influence of the solvent on the absorption maximum.

A method for the quantitative determination of Bexarotene was developed and validated.

2.4.3.1. Preparation of a standart solution of Bexarotene for UV-VIS spectroscopic analysis

To prepare a Bexarotene standard solution, weigh 10.0 mg of a certified Bexarotene standard substance on an analytical balance with an accuracy of ±0.0001 g. In a 25 ml volumetric flask, place 10 ml of methanol before the addition of the standard substance. With the same solvent, the flask is made up to the mark. The resulting stock solution has a concentration of 0.4 mg/ml.

2.4.3.2. Preparation of working solutions of Bexarotene for quantitative UV-VIS spectroscopic analysis

From the obtained in item 2.4.3.1. Bexarotene standard solution, working standard solutions in the concentration range from 1.84 to 5.20 μg/ml are prepared by appropriate

dilutions. Five independent absorbance measurements were performed for each working solution.

2.4.3.3. Preparation of test samples of synthesis products

A 10.0 mg sample was weighed on an analytical balance with an accuracy of ±0.0001 g. 10 ml of methanol is added to it. The resulting solution was treated in an ultrasonic bath for 10 minutes and filtered through a Ministar® Syringe 28 mm membrane filter with a pore size of 5 μm. Add 10 ml of methanol to a 25 ml volumetric flask. The filtrate is quantitatively transferred to it. The volume of the flask is brought up to the mark with methanol. The resulting solution has a concentration of 0.4 mg/ml. By suitable dilutions of the stock solution, the test samples are prepared.

2.4.3.4. Validation of UV-VIS spectroscopic method

A validation procedure was conducted to prove the applicability of the method. Validation includes determination of the following analytical parameters:

- Linearity of the standard law;
- Precision;
- Accuracy;
- Limit of detection (LOD) and limit of quantification (LOQ).

The specified characteristics are in accordance with the requirements of ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology.

- *Linearity of the standard law*

Linearity is the interval of concentrations of the analyzed component in which the signal from the detector is linearly dependent on its concentration. To construct the standard curve, a basic standard solution of Bexarotene with a concentration of 0.4 mg/ml was prepared. Seven working solutions with concentrations of 1.84, 2.40, 2.96, 3.52, 4.08, 4.64 and 5.20 μg/ml were obtained from it by dilution. The absorbance of the obtained working solutions was measured five times. Linearity was assessed by the equation of the standard line and the correlation coefficient R^2.

- *Precision*

Precision is a parameter indicating the variation in the values of parallel measurements of the same sample obtained under certain conditions. A quantitative measure for assessing precision is the standard deviation (SD) calculated from the values of n number of measurements, as well as the relative standard deviation (RSD). Repeatability (consecutive five-fold analysis of three concentration levels, within the same day, under the same analytical conditions, by one operator) and intra-laboratory reproducibility (consecutive five-fold analysis of three concentration levels, on different days) were assessed.

- *Accuracy*

Accuracy is the degree of coincidence of the measurement result with the true value of the measured quantity. The assessment of accuracy is done by absolute or relative error, which show how much the average result deviates from the true value. The bias value is determined, as well as the relative deviation in percentages (b%), taking into account the real value of the standard substance of each of the analyzed samples. Absorbance was measured five times at the seven concentration levels.

- *Limit of quantification (LOQ)*

The limit of quantification is the smallest amount of analyte that the method can quantify with sufficient accuracy and precision. The limit of quantification is the concentration of the analyte giving a signal 10 times stronger than the background noise, i.e. signal-to-noise ratio = 10. Typically, the LOQ corresponds to the lowest concentration from the calibration graph.

- *Limit of detection (LOD)*

The limit of detection indicates the lowest amount of a substance in a sample that can be detected, but not quantified with an exact value. In many cases, the limit of detection is the analyte concentration giving a signal 3 times stronger than the background noise, i.e. the signal-to-noise ratio = 3 (or the standard deviation of the blank measurement).

2.4.4. Development of an HPLC method to establish the purity of the newly synthesized Bexarotene derivatives

Apparatus and chromatographic conditions:

The analysis was performed using a Thermo Scientific High Performance Liquid Chromatography (HPLC) apparatus model Spectra SYSTEM HPLC with manual injector, UV-VIS detector model Spectra SYSTEM UV2000 and Fluorescence detector model Spectra SYSTEM FL3000. A Synergi 4u Hydro – RP 80A (250 x 4.00 mm) column protected with a Synergi 4u Hydro – RP 80A (250 x 4.00 mm) precolumn was used for the analysis. System control, data acquisition and analysis were performed using ChromQuest chromatographic data software, version 4.2.34.

Conditions of the chromatographic system:

- Mobile phase - 60% methanol and 40% acetonitrile;
- Flow rate – 1.3 ml/min;
- UV detection – at 260 nm;
- Temperature of the chromatographic column – 40 °C;
- Temperature of manual injector – 25 °C;
- Sample volume for analysis – 20.0 µl.

The analysis was carried out in isocratic mode with a total duration of 15 minutes.

The basic standard solution of Bexarotene for performing the chromatographic analysis was prepared by dissolving an appropriate amount of the substance in an exact volume of 99.99% HPLC pure methanol.

2.5. *In silico* analysis for the prediction of the biological effect and theretical evaluation of the toxicity of Bexarotene, newly synthesized Bexarotene esters and their metabolites

he term *in silico* is derived from the computer component silicon, therefore *in silico* methods refer to methods or predictions using computational approaches. *In silico* methods can make rapid predictions for a large set of compounds with high throughput.

In silico pharmacology (also known as computational pharmacology) is a rapidly developing field. It develops software technologies to capture, analyze and integrate biological and medical data from many different sources. Specifically, it defines the use of this information

in creating computational models or simulations that can be used to make predictions, propose hypotheses, and ultimately provide discoveries or advances in medicine and therapy.

2.5.1. QSAR Toolbox software

The OECD (Organization for Economic Co-operation and Development) (Q)SAR ((Quantitative) Structure-Activity Relationships) Toolbox software (version 4.5) is designed for the risk assessment of chemical compounds. The risk assessment is carried out on the basis of the predicted metabolic changes of the chemical structure of the compounds. Adverse reactions or toxicity are always due to the parent compound. Often they are the result of its biotransformation to an active one.

Different biological species, organs and even different cells show different metabolic transformation.

Biotransformation of chemical substances often leads to the formation of biologically active metabolites. The determination of metabolites experimentally is a laborious, expensive and often not sufficiently informative process. This makes mathematical models in metabolic prediction very applicable.

The use of alternative methods to animal testing, such as the QSAR Toolbox, reduces the number of animal tests and at the same time reduces the time required for the study as well as the cost of the necessary animals.

The documented metabolic pathways in the QSAR Toolbox software database include the metabolism of over 200 organic compounds in mammals. This facilitates access to information on various classes of chemical compounds, with diverse functional groups including aliphatic hydrocarbons, halogenated hydrocarbons, alicyclic rings, furans, aromatic hydrocarbons and haloaromatic compounds, nitro derivatives, amines, etc. Toxicity resulting from metabolic activation can be predicted from data obtained from metabolic models.

- *In vivo rat metabolism simulator*

The current *in vivo* rat liver metabolic simulator (transformation table) represents electronically designed set of 671 structurally generalized, hierarchically arranged abiotic and enzymatic transformation reactions, which are characteristic for the metabolism for *in vivo* experimental systems such as rodent (mostly rat). The principal applicability of this simulator

is associated with the reproduction as well as the prediction of the metabolic activation reactions and pathways of xenobiotic chemicals, which may elicit *in vivo* genotoxicity effects.

- *Rat liver S9 metabolism simulator*

The current *in vitro* rat liver metabolic simulator (transformation table) represents electronically designed set of 551 structurally generalized, hierarchically arranged biotransformation reactions, which are characteristic for the metabolism for *in vitro* experimental systems such as rodent (mostly rat) liver microsomes and S9 fraction. The principal applicability of this simulator is associated with the reproduction as well as the prediction of the metabolic activation reactions and pathways of xenobiotic chemicals, which may elicit *in vitro* genotoxicity effects such as bacterial mutagenicity and chromosomal aberrations.

- *Skin metabolism simulator*

The Skin Metabolism Simulator mimics the metabolism of chemical compounds in the skin. It is hypothesized that enzymes in the skin may metabolize xenobiotics absorbed through it through reactions analogous to those identified in the liver. The simulator was developed as a simplified simulator of mammalian liver metabolism. The skin metabolism simulator contains a list of hierarchically ordered master transformations, which can be divided into two main types - rate-determining and non-rate-determining.

- *DNA binding by OASIS*

The profiler is based on Ames Mutagenicity model part of OASIS TIMES system. The profiler consists of 85 structural alerts responsible for interaction with DNA analyzed in Ames Mutagenicity model. The scope of the profiler is to investigate presence of alerts within target molecules which may interact with DNA.

- *Protein binding by OASIS*

The scope of the profiler is to investigate presence of alerts within target molecules responsible for interaction with proteins. The list of 112 structural alerts has been separated into 11 mechanistic domains. Each of the mechanistic domains has been separated into more than 2 mechanistic alerts. The profiling result outcome assigns a target to the corresponding structural alert, mechanistic alerts and domain.

2.5.2. Molinspiration software – Lipinski's rule of five

Molinspiration (https://www.molinspiration.com/cgi-bin/properties) offers a wide range of chemical informatics software tools supporting molecule manipulation and processing, including conversion of SMILES and SD files, molecule normalization, tautomer generation, molecule fragmentation, calculation of various molecular properties needed in QSAR, molecular modeling and drug design, high-quality imaging of molecules, molecular database tools supporting substructure and similarity search. The software also supports virtual screening, bioactivity prediction and data visualization.

Molinspiration software is used to calculate important molecular properties (miLogP, total polar surface area, molecular mass, number of hydrogen bond donors and acceptors, number of rotatable bonds) as well as to predict bioactivity for the most important drug targets (ligands for G-protein coupled receptors (GPCR), ion channel modulators, kinase inhibitors, nuclear receptor ligands, protease inhibitors, enzyme inhibitors).

Lipinski's rule is applied to determine drug-likeness. which is used to determine whether a chemical compound with pharmacological or biological activity has chemical and physical properties that would make it a likely orally active drug in humans. The rule describes molecular properties important to the pharmacokinetics of a drug in the human body, including their absorption, distribution, metabolism and excretion (ADME). However, the rule does not predict whether a compound is pharmacologically active.

Lipinski's rule states that an orally active drug has no more than one violation of the following criteria:

- No more than 5 hydrogen bond donors (the total number of nitrogen-hydrogen and oxygen-hydrogen bonds);
- No more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms);
- Molecular mass less than 500 daltons;
- Octanol-water partition coefficient (log P) not exceeding 5.

Further research added two more conditions: a polar surface area (PSA) of 140 A˚ and < 10 rotatable bonds, which correlate with drug permeability and flexibility, respectively.

2.5.3. PreADME/Tox software

Absorption, distribution, metabolism, excretion and toxicity (ADME/T) pharmacokinetic parameters of Bexarotene and the newly synthesized Bexarotene esters were evaluated using PreADME/T software (https://preadmet.qsarhub.com/adme/).

PreADME/Tox is a web-based application to predict absorption, distribution, metabolism, excretion and toxicity and to build a library of drug-like molecules using an *in silico* method.

Over 50% of drug candidates fail due to ADME/Tox deficiencies during development. This necessitates the use of computer methods to determine these metrics in order to reject compounds that are likely to fail.

2.6. Determination of general toxicity using *in vivo* models

A major approach to determining the overall toxicity of newly synthesized compounds with pharmacological potential is through pilot studies with experimental animals. They can give direction on the manifestation of a particular type of toxicity macroscopically and through biochemical studies. These studies are fundamental in determining the safety as well as the initial effective dose of the substances.

The present research is aimed at the 2nd phase of the preclinical stage in the creation of new drugs, namely pharmacological screening of the newly synthesized by our team ester derivatives of Bexarotene.

To determine the general toxicity of the newly synthesized esters of Bexarotene, the ethyl ester was used. The results obtained from the *in silico* analysis determine it as the least toxic and with the best physico-chemical characteristics.

2.6.1. Experimental animals

An *in vivo* model was applied to determine overall toxicity. Male sexually mature Wistar rats, aged 8-10 weeks, weighing 180 to 260 g, were used. The rodents are kept in an animal cage with a 12-hour day and night lighting cycle, with access to water and food *ad libitum*,

provided with optimal temperature, humidity and ventilation of the premises in accordance with the requirements for working with experimental animals (Regulation №. 20 of 01 November 2012 on the Minimum requirements for the protection and humane treatment of experimental animals and the requirements for the facilities for their use, breeding and/or delivery, in force from November 1, 2013, issued by the Ministry of Agriculture and Food, Official Gazette №. 87 of November 9, 2012) and in accordance with the rules of the Research Ethics Committee (REC) at the Medical University ‚Prof. Dr. Paraskev Stoyanov' - Varna. The scientific work was carried out with the permission of the Bulgarian Food Safety Agency (BFSA) №. 307/29.06.2022.

For the purposes of the study, 34 rats were used, divided into 5 groups:

- Controls - (n = 6);
- Bexarotene - 500 mg/m^2 (n = 6);
- Bexarotene - 750 mg/m^2 (n = 6);
- Ethyl ester of Bexarotene - 500 mg/m^2 (n = 8);
- Ethyl ester of Bexarotene - 750 mg/m^2 (n = 8).

2.6.2. Preparation of solutions

The control group was treated with a 25% aqueous solution of ethanol with a few drops of the suspending agent Tween 20. A total of 4 solutions were prepared - 2 of Bexarotene and 2 of ethyl ester of Bexarotene with a concentration of 16.19 mg/ml and 24.28 mg/ml in a volume of 34 ml. An average 1 ml were administered per rat at the above-mentioned doses by intragastric tube in the form of suspensions after sonication for 5 minutes.

The applied dose for the treatment of the rats was calculated according to the recommended initial single daily dose of Bexarotene - 300 mg/m^2 body surface (Brief characteristics of the product - Targretin 75 mg soft capsules).

The body surface of the rats was calculated according to Mosteller's formula [1]:

$$Body\ Surface = \sqrt{\frac{siz,cm\ x\ weight,kg}{3600}}\ , \text{m}^2$$

2.6.3. Experimental protocol

Following administration of Bexarotene and its ethyl ester, rats were observed for a period of 72 hours under normal conditions to assess the level of no effect and/or lethal dose. Rats were periodically monitored for behavioral changes and mortality.

After 72 hours, rats were anesthetized with diethyl ether and blood was collected for biochemical studies after transection of the sublingual vein into 5 ml VACUSERA CAT Serum GEL & Clot Activator vacutainers. At the end of the experiment, the rats were euthanized by breaking the neck, according to the requirements and organization of work in the Vivarium at the Medical University ‚Prof. Dr. Paraskev Stoyanov' – Varna.

In the medical laboratory ‚Lina' - Varna, the following laboratory tests were performed according to standard protocols: determination of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and C-reactive protein (CRP) to monitor the presence of liver toxicity.

When the rats were dissected, a macroscopic examination of all organs was performed, and the heart, liver, and kidney were taken for histological examination, which will be described in a subsequent scientific paper.

2.7. Statistical data processing

Statistical analysis was performed using built-in Excel functions to calculate standard deviation and ANOVA regression analysis. In the analysis, an acceptable level of confidence probability $p < 0.05$, divided into three ascending classes: $p < 0.05$, $p < 0.01$ (high significance) and $p < 0.001$ (very high significance) is accepted.

IV. RESULTS AND DISCUSSION

In order to achieve the aims of the present study, a synthesis of Bexarotene esters was carried out.

1. PREPARATION OF BEXAROTENE ESTERS

One of the methods for obtaining the more reactive acyl chloride, and subsequently an ester, is the reaction between a carboxylic acid and thionyl chloride ($SOCl_2$). This approach turned out to be inappropriate for the synthesis of ester derivatives of Bexarotene, since many by-products are obtained during the reaction, which greatly complicate the process of purification of the newly synthesized compound. In addition, thionyl chloride is on the List of Socially Hazardous Substances because it is reactive and corrosive. This necessitates the application of another approach for the synthesis of Bexarotene esters.

The preparation of acyl chlorides can also be done with oxalyl chloride ($(COCl)_2$). It is also used for oxidation of alcohols, formylation of arenes, preparation of oxalate diesters and others.

The production of acid chloride is an intermediate step in the synthesis of Bexarotene esters. The general scheme for the preparation of Bexarotene acid chloride is presented in Figure 1.

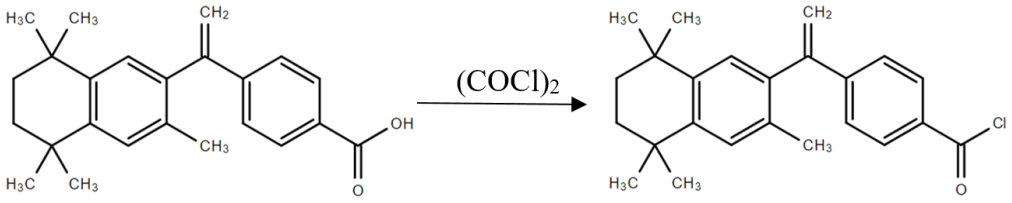


Figure 1. General scheme for the preparation of Bexarotene acid chloride.

In the direct interaction of oxalyl chloride and Bexarotene in an alcohol medium, an esterification process takes place, in which Bexarotene reacts with oxalyl chloride to form an acyl chloride and subsequently an ester. The *in situ* produced acyl chloride is extremely reactive and upon interaction with alcohol leads to the formation of the corresponding ester. The reaction scheme for the preparation of Bexarotene esters from Bexarotene acyl chloride is presented in Figure 2.

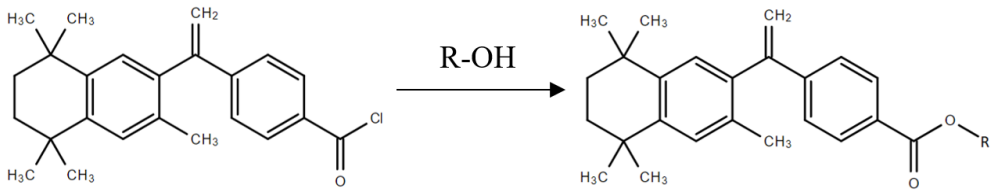
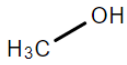
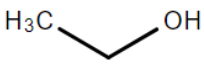
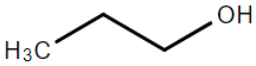
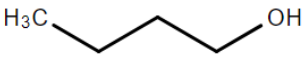


Figure 2. General scheme for the preparation of Bexarotene ester.

In the process of esterification, different alcohols can be involved. Simple aliphatic alcohols were used for the esterification. Due to the high reactivity of secondary and tertiary alcohols, only primary alcohols were used for the synthesis of Bexarotene esters. All of them are readily available, making them suitable reagents for the preparation of esters.

For the purposes of the present work, primary alcohols were used. Their structures and names are presented in Table 3.

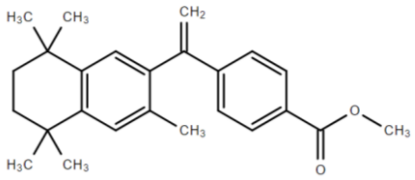
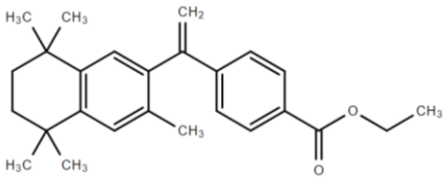
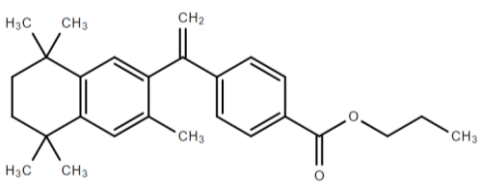
Table 3. Structure of alcohols used for the synthesis of Bexarotene esters.

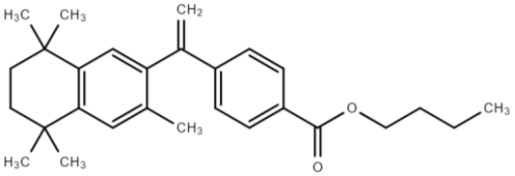
Bexarotene esters	**Alcohols used**	
	Structure	**Name**
E1		Methanol
E2		Ethanol
E3		1-propanol
E4		1-butanol

As a result of the described method of esterification and the alcohols used in the synthesis, 4 esters of Bexarotene were obtained. Their structural formulas, chemical names and physical characteristics are presented in Table 4.

The choice of alcohols for esterification was based on the reactivity of the alcohols and their ability to undergo an esterification reaction with Bexarotene. In future scientific work, the development of a method for the esterification of Bexarotene with more complex alcohols such as secondary, tertiary and aromatic is expected, with the aim to select alcohols that, after metabolism of the ester they release non-toxic products in the body.

Table 4. Structure, chemical name and physical characteristics of the obtained Bexarotene esters.

Compound	**Structure**	**Chemical name**	**Physical characteristics**
E1		Methyl 4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenil)ethenyl]benzoate	White crystalline substance, insoluble in water, soluble in methanol, ethanol, acetone and DMSO. Slightly soluble in hexane.
E2		Ethyl 4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenil)ethenyl]benzoate	White crystalline substance, insoluble in water, soluble in methanol, ethanol, acetone and DMSO. Slightly soluble in hexane.
E3		Propyl 4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-	White viscous substance, insoluble in

		naphthalenil)ethenyl]benzoate	water, soluble in methanol, ethanol, propanol and DMSO.
E4		Butyl 4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenil)ethenyl]benzoate	Yellow -brown substance with increased viscosity, sticky, insoluble in water, soluble in methanol, ethanol, butanol and DMSO.

In the course of the synthesis, some peculiarities are observed. During the synthesis of Bexarotene methyl ester, a white precipitate was observed to form after 1 hour of reaction time. For the other three esters, the solution remained clear until the end of the reaction time.

During drying on the vacuum evaporator, the formation of white precipitates was observed. The practical, theoretical and percentage yields of the newly synthesized Bexarotene esters are presented in Table 5.

Table 5. Molar mass, practical, theoretical and percent yield of the newly synthesized Bexarotene esters.

Bexarotene esters	***Molar mass, g/mol***	***Practical yield, g***	***Theoretical yield, g***	***Percentage yield, %***
E1	362.51072	0.222	0.31207	71.14
E2	376.54072	0.242	0.32415	74.66
E3	390.56592	0.216	0.33622	64.24
E4	404.59172	0.284	0.34830	81.54

2. CONDUCT TLC ANALYSIS TO MONITO THE REACTION PROCESS

During the Bexarotene esterification reaction, samples were taken for TLC analysis, which was performed at constant temperature and approximately uniform humidity, which may be factors in reproducibility.

To obtain spots with a correct shape and the absence of tails, solutions with concentrations falling in the range of 0.1 to 1.0 mg/ml were prepared.

The solvent or mixture of solvents present in the MF composition was used to dissolve the analytes.

For the good separation of substances from a given mixture, the method of applying the starting sample, the shape and size of the spots are of primary importance. The test solution was applied to the start using a ‚Hamilton' type syringe.

The thin-layer chromatographic analysis was carried out on a chromatographic plate DC-Fertigfolien ALUGRAM® SIL G/UV_{254} with a layer thickness of 0.20 mm, impregnated with a fluorescent indicator UV_{254}. Detection is performed with UV light on a Nahita UV lamp.

According to literature data, the mobile phases that are most often used in the analysis of Bexarotene and its analogues vary from two-component to four-component systems. Table 6 presents the composition of MFs used in various literary sources.

Table 6. Mobile phases used for TLC analysis according to literature sources.

MP	***A***			
Component	Ammonia	Butanol	Chloroform	Acetone
Ratio	1	4	3	3

MP	***B***		***C***		***D***	
Component	Hexane	Chloroform	Petroleum ether	Ethyl acetate	Hexane	Ethyl acetate
Ratio	2	8	1	6	4	1

The development of the thin-layer chromatographic method started with checking the affinity of solvents, as the only component of the MF, to the analyzed substances. With MF,

composed of hexane, spots applied to the starting line do not move - they stay on the starting line.

With MF composed only of chloroform, the spots move slightly and a band is formed at the front.

With MF ethyl acetate the spots are washed out at the front, and with MF petroleum ether the spots are collected at the start.

An experiment was also carried out with MF methanol, due to the good solubility of Bexarotene and its esters in methanol. Spots with a large Rf value are obtained, and there's an entire area of the plate that fluoresces observed above the spots.

From the obtained results it is clear that esters have a different affinity compared to the studied monocomponent systems. This necessitates conducting an experiment with more than one component.

When performing the TLC analysis with MF A, the spots move sufficiently on the plate but are not well separated.

Analyzes were carried out with four-component systems with solvents used in the two-component (MF B, C and D), namely hexane, ethyl acetate, chloroform and petroleum ether in different ratios. The results obtained are unsatisfactory for a number of reasons: tails, front banding, irregularly shaped spots or poor spot separation are produced. Acetic acid was also added to this system to reduce tailing, but this did not significantly improve the results.

When performing the TLC analysis with MF B, the ester spots hardly moved from the start, and the Bexarotene spot moved slightly and formed a tail. In order to increase the polarity of the MF, 0.05 parts of methanol was added, which did not significantly change the result. With a gradient increase in the amount of methanol and chloroform, the spots begin to move and separate, but have an irregular shape resembling an inclined ellipse, the axes of which have a large difference in size. At all analyzed ratios of the components of MF B and methanol, a band is obtained at the front.

The use of MF composed of chloroform and methanol in a ratio of 10:1 was also conducted and then the gradient reduction of methanol was investigated. In these MFs, the Bexarotene spot is not detected and a band at the front is observed. On the other hand, the ester spots are present, but they are irregularly shaped and not well separated.

With MF C, the spots hardly move from the start, they have a very small Rf value, which is almost the same for all analyzed substances. With the same MF in a 1:1 ratio, the spots are also not well separated, necessitating the use of a different MF.

A MF composed of ethyl acetate and chloroform in a 1:1 ratio successfully separated the spots, no front band was obtained, but tails formed.

Performing the assay with MF D gave the best result. At a ratio of 4:1, as seen in Table 6, the spots of the test substances do not move significantly from the starting line. When adding a few drops of methanol to the MF to increase the solubility of the analytes, some of the problems described above reappear. A gradient decrease in hexane and increase in ethyl acetate was applied to improve separation. Also no methanol was used. With a ratio of hexane and ethyl acetate 1:1, we achieve good separation, a relatively correct shape of the spots of the substances under study, absence of tails and of a band on the front.

From the obtained experimental results, we can conclude that the best result is obtained with a 1:1 ratio of the components of the MF composed of hexane and ethyl acetate. The result of the TLC analysis is presented in Figure 3. The calculated Rf values are presented in Table 7.

Figure 3. Thin-layer chromatographic analysis of Bexarotene and the newly synthesized esters E1, E2, E3 and E4.

We can conclude that the MF composed of hexane and ethyl acetate in a ratio of 1:1 is suitable for following the esterification process of more complex esters of Bexarotene, due to the obtaining of spots with different Rf values.

Table 7. Rf values of Bexarotene and the newly synthesized Bexarotene esters.

Compound	***Bex***	***E1***	***E2***	***E3***	***E4***
Rf	0.75	0.55	0.64	0.66	0.72

3. STRUCTURAL CHARACTERIZATION OF THE OBTAINED DERIVATIVES BY INSTRUMENTAL METHODS

3.1. Characterization of derivatives by means of melting point

The melting point is the temperature at which a compound changes from a solid to a liquid aggregate state. This transition takes place slowly and in a certain temperature range.

The amount of heat required to melt a unit mass of a substance in crystalline form at a given pressure is called the specific heat of fusion. Its SI unit is J/kg.

Melting point is often used for qualitative identification of compounds and as an indicator of purity.

According to literature data, Bexarotene has a melting point of 230 – 231 ^{o}C. Melting point analysis of Bexarotene and the newly synthesized Bexarotene esters was performed. The obtained results are presented in Table 8.

Table 8. Melting point of Bexarotene and the newly synthesized esters.

Compound	***Bex***	***E1***	***E2***	***E3***	***E4***
Melting point	227 ^{o}C	154.6 ^{o}C	171.2 ^{o}C	-	-

From the obtained results, we can conclude that the synthesis of new ester derivatives of Bexarotene proceeds successfully, but not completely. Unmelted particles were observed at the determined melting point, possibly due to unreacted Bexarotene. For this reason, after taking into account the melting point of the esters, we continued heating to the melting point of Bexarotene. In all samples, after reaching 227 °C, complete melting of the particles and obtaining a homogeneous solution was observed.

The melting point of compound E3 and E4 was not determined due to inappropriate physical characteristics of the analytes described in Table 4 on page 29.

3.2. Infrared spectroscopy

In order to prove the structure of the newly obtained compounds, a comparative ATR-FTIR analysis of the spectra (presented in Figure 4 to 8) of the starting compound – Bexarotene and the newly synthesized esters of Bexarotene in the region 4000-500 cm^{-1} was carried out.

The ATR-FTIR spectrum of Bexarotene is presented in Figure 4.

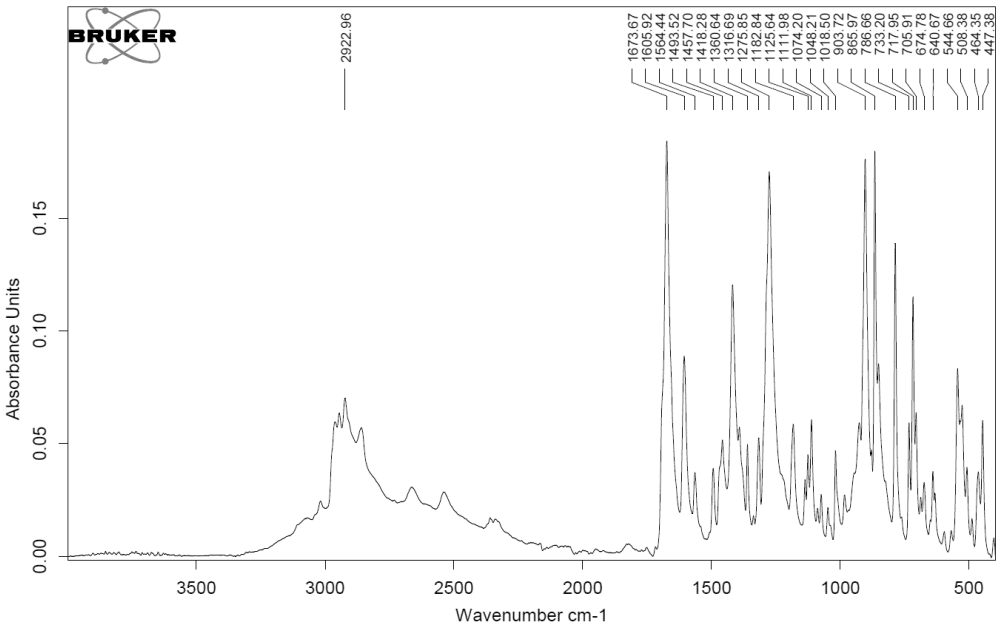


Figure 4. Data from conducted FT-IR spectral analysis of Bexarotene.

Analysis of the structure of Bexarotene shows the appearance of several bands due to the four methyl (-CH_3) and one methylene (=CH_2) group. The valence vibrations at 2872 cm^{-1} (sym.) and 2962 cm^{-1} (asym.) are characteristic of the methyl group, while the deformation vibrations are at 1380 cm^{-1} (sym.) and 1460 cm^{-1} (asym.). The methylene group has valence vibrations at 2853 cm^{-1} (sym.) and 2926 cm^{-1} (asym.), and deformation vibrations at 1470 cm^{-1} (scissor). When two methyl groups are at one C-atom a doublet appears at 1365 cm^{-1} and 1385 cm^{-1}.

The FT-IR spectrum shows bands between 1470 and 1600 cm^{-1}, characteristic of the skeletal vibrations of the carbon ring of the aromatic nucleus. A p-disubstituted benzene ring band between 855 and 790 cm^{-1} can also be detected.

The valence vibration of the C=O carbonyl group is one of the most intense. Its frequency is about 1720 cm^{-1}.

The performed FT-IR spectral analysis of the newly synthesized esters showed some structural changes. The spectra of the esters are presented in Figure 5, 6, 7 and 8.

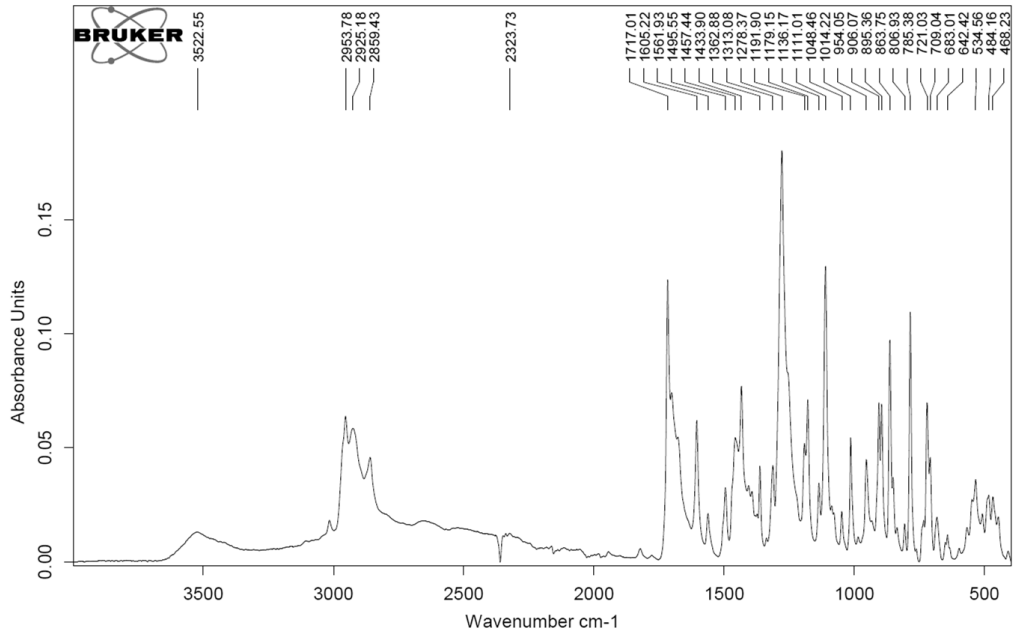


Figure 5. Data from conducted FT-IR spectral analysis of methyl ester of Bexarotene.

The peak at 1717 cm^{-1} is observed to be merged with two other peaks of lower intensity. This may be due to unreacted Bexarotene during esterification, as a peak at 1673 cm^{-1} is observed for Bexarotene.

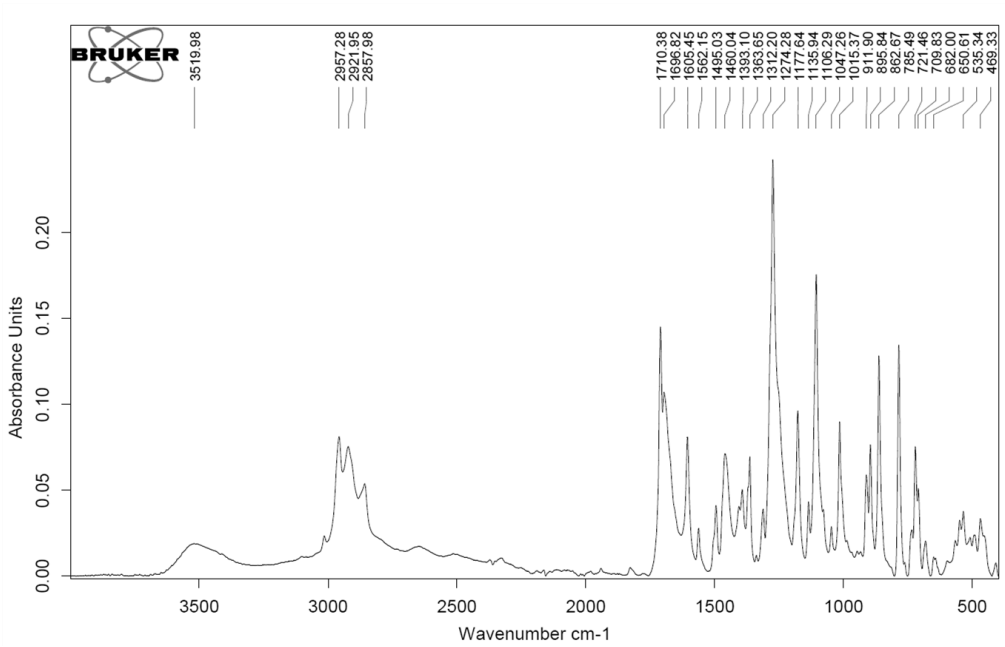


Figure 6. Data from conducted FT-IR spectral analysis of ethyl ester of Bexarotene.

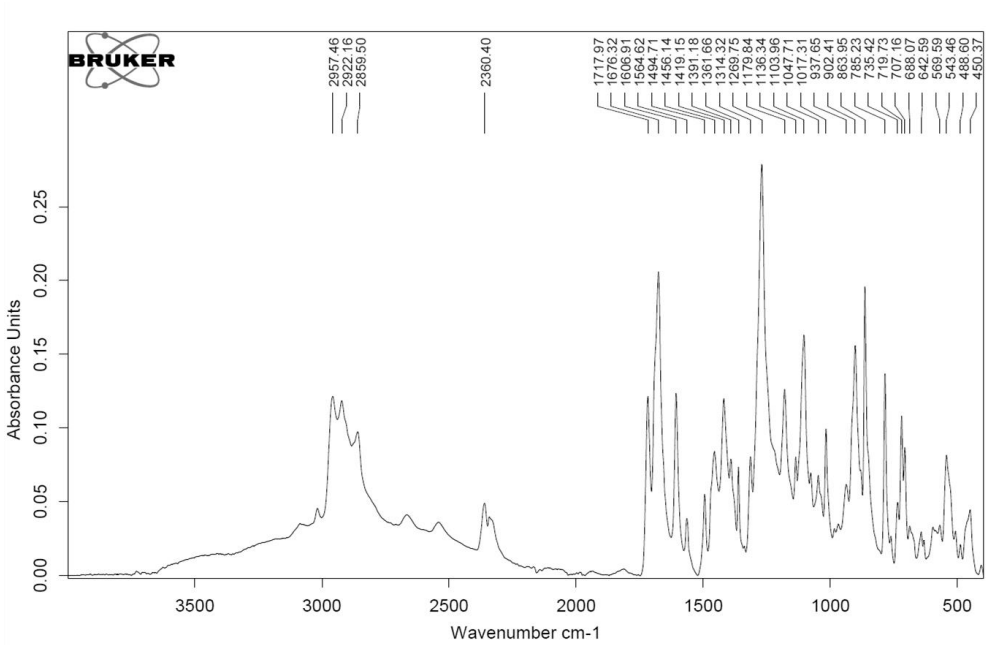


Figure 7. Data from conducted FT-IR spectral analysis of propyl ester of Bexarotene.

The presence of a peak at 1717 cm^{-1} and at 1676 cm^{-1} is due to incomplete esterification of Bexarotene, which appears as an impurity in the reaction mixture.

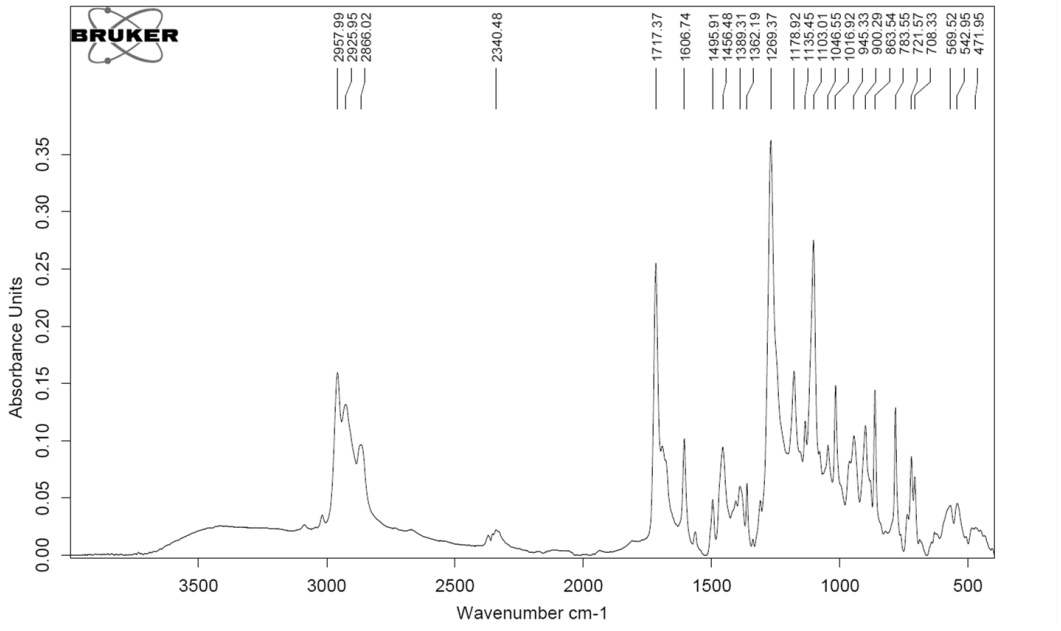


Figure 8. Data from conducted FT-IR spectral analysis of butyl ester of Bexarotene.

The spectra of the studied compounds are similar in all analyzed absorption regions, with several characteristic features being present, resulting from the presence of structural similarities common to Bexarotene and the newly obtained derivatives. The only structural difference between the newly synthesized esters is the length of the hydrocarbon chain in the ester moiety. Therefore, no significant changes were observed in the recorded spectra of the newly obtained Bexarotene analogues.

In the captured spectra in the region from 3000 cm^{-1} to 2800 cm^{-1} an extremely high similarity in absorption signals is observed. The resulting bands are close in location and intensity to the reported maxima. This is explained by the fact that some characteristic vibrations of the benzene nuclei, as well as symmetric and asymmetric vibrations of the C-H bond, are manifested in this region.

The most significant difference between the spectrum of Bexarotene and the newly synthesized esters is observed in the region from 1620 cm^{-1} to 1720 cm^{-1}, where the presence of a medium intensity band is reported in the spectrum of the esters. In the spectrum of Bexarotene, no similar band is reported. Its appearance clearly points to the structural changes in the carboxyl group and the esterification process that took place. The presence of an ester functional and the deformation oscillations characteristic of the C=O bond are reported in the region of the spectrum between 1730 cm^{-1} and 1715 cm^{-1}. The data obtained strongly correspond to the expected area of spectral banding characteristic of carboxylic compounds.

In the spectra of all analyzed newly synthesized esters of Bexarotene at a frequency of 1717 cm^{-1}, a band similar in intensity and position is reported. The occurrence of and can be attributed to the presence of a carbonyl group in the analyzed compounds.

Table 9. Location of spectral bands characteristic for the presence of a carbonyl group in the structure of the newly obtained Bexarotene esters, the interval from 1620 cm^{-1} to 1720 cm^{-1} was reported.

Compound	***Frequency, cm^{-1}***	
E1	1717.01	1605.22
E2	1710.38	1605.45
E3	1717.97	1606.91
E4	1717.37	1606.74

Additional confirmation of its presence in the spectrum is determined by the appearance of a band at the lower frequencies of the wave at about 1606 cm^{-1}. A similar band is observed

in the spectrum of all newly synthesized Bexarotene esters. The position of these bands in the spectra of the analyzed compounds is indicated in Table 9.

When analyzing the obtained spectrum of Bexarotene in the region 3000-2800 cm^{-1}, an intense band is observed at 2927 cm^{-1} and also at 2859 cm^{-1}.

Similar bands in the spectra of the newly obtained compounds are presented in Table 10.

Table 10. Location of spectral bands observed for the newly synthesized esters in the interval from 3000 cm^{-1} to 2800 cm^{-1}.

Compound	***Frequency, cm^{-1}***	
E1	2953.78	2859.43
E2	2957.28	2857.98
E3	2957.46	2859.50
E4	2957.99	2866.02

This gives us reason to assume that methyl residues are present in the molecule of the compounds. Their presence is confirmed by considering the bands present in the lower frequency region of the spectrum, in the interval from about 1365 cm^{-1} to 1450 cm^{-1}, corresponding to deformation C-H vibrations. Such a band is observed without shift in the spectra of all newly synthesized compounds. Its position is indicated in Table 11.

Table 11. Band position corresponding to strain C-H vibrations in the newly synthesized Bexarotene esters in the range of about 1365 cm^{-1} to 1450 cm^{-1}.

Compound	***Frequency, cm^{-1}***
E1	1362.88
E2	1363.65
E3	1361.66
E4	1362.19

A well-defined, highly intense band with a maximum at about 1275 cm^{-1} is reported in the spectra of all compounds analyzed. Its presence can be attributed to the presence of methyl groups. Since five $–CH_3$ groups are present in the structure of the compounds, this gives reason to assume that their corresponding vibrations will appear with greater intensity in the spectrum. The position of the bands attributed to the available $–CH_3$ groups is presented in Table 12.

Table 12. Position of the bands related to the methyl groups in the spectra of Bexarotene and the newly synthesized esters with a maximum at 1275 cm^{-1}.

Compound	*Frequency, cm^{-1}*
Bex	1275.85
E1	1278.37
E2	1274.28
E3	1269.75
E4	1269.37

Although minor, the shifts obtained can be attributed to the fact that the substitution in the side chain is at a considerable distance from the methyl groups in the Bexarotene backbone.

Weakly intense bands are observed in the interval from 2000 cm^{-1} to 1660 cm^{-1}, characteristic of benzene nuclei. In order to confirm their presence, an analysis was made of the bands in the spectrum interval between 1600 cm^{-1} and 1440 cm^{-1}. The most characteristic in this section is the band at about 1490 cm^{-1}. Its location in the spectra of the analyzed compounds is presented in Table 13.

Table 13. Spectral bands recorded in the interval 2000 cm^{-1} to 1660 cm^{-1}, characteristic of the presence of benzene nuclei.

Compound	*Frequency, cm^{-1}*
E1	1495.55
E2	1495.03
E3	1494.71
E4	1495.91

The type of substitution of the benzene nuclei was analyzed by reading the obtained spectrum bands in the interval about 2000 cm^{-1} and 1660 cm^{-1} as well as those between 900-650 cm^{-1}.

The infrared spectra of the four newly synthesized compounds are similar in the relative positions and intensities of the obtained peaks, which confirms the close structural relationship between them. This is mainly due to the presence of a Bexarotene backbone containing three cores and their associated methyl residues. Structurally, esters differ only in the length of the hydrocarbon chain in the ester part, which is not manifested by a characteristic band in the infrared spectrum of esters.

3.3. UV-VIS spectroscopy

3.3.1. Validation of the UV-VIS spectral method

The experimentally determined absorption maximum of Bexarotene (λ = 204 nm) was used to develop a quantification method.

Figure 9 shows the standard curve developed using a solution of the standard substance Bexarotene. The concentration range varies from 1.84 to 5.20 µg/ml.

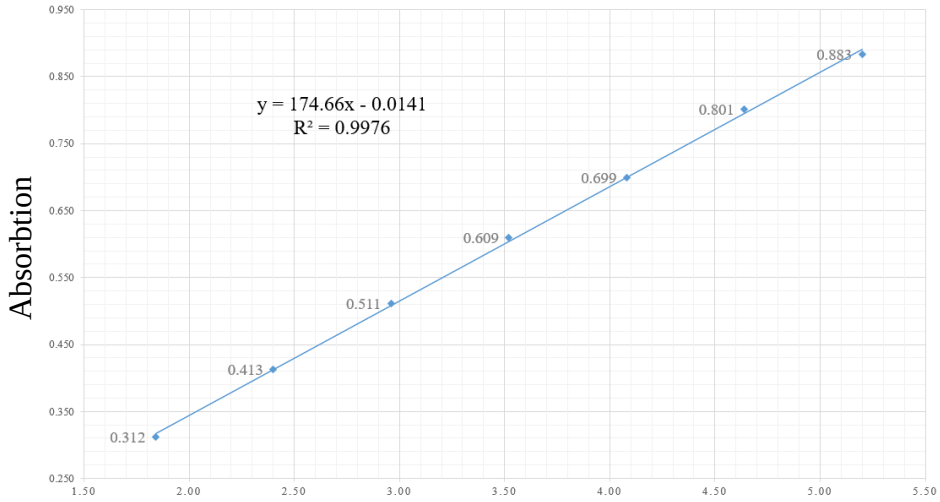


Figure 9. Standard line of Bexarotene standard solution in concentration range 1.84 – 5.20 µg/ml

The standard line was constructed to determine the linearity of the UV-VIS method.

Linearity

Linearity was assessed by the equation of the standard line and the correlation coefficient R^2.

The obtained results show that linearity (R^2 = 0.9976) was achieved in the target concentration range 1.84 – 5.20 µg/ml.

Precision

Data on the precision of the analysis are presented in Table 14.

Table 14. Evaluation of the repeatability and reproducibility of the UV-VIS spectral method.

Compound	*Concentration, µg/ml*	*Intraday analysis*		*Analysis within consecutive days*	
		SD	**RSD, %**	**SD**	**RSD, %**
Bexarotene	1.84	0.001483	0.496399	0.001924	0.642894
	3.52	0.002881	0.465123	0.001817	0.293757
	5.20	0.002280	0.258309	0.002864	0.324594

The calculated RSD values are acceptable and fall within the interval 0.25 – 0.64 %.

Accuracy

The absorbance at the seven concentration levels at the respective wavelength was measured five times. The obtained data are presented in Table 15.

Table 15. Evaluation of the accuracy of the UV-VIS spectral method.

Compound	*Concentration, µg/ml*	*bias*	*b, %*
Bexarotene	1.84	0.03	1.58
	2.40	0.05	1.89
	2.96	0.05	1.61
	3.52	0.06	1.84
	4.08	0.01	0.25
	4.64	0.04	0.82
	5.20	0.06	1.20

The calculated values of b% are permissible and fall within the interval 0.25 – 1.89 %.

Sensitivity was assessed by measuring the limit of detection (LOD) and the limit of quantification (LOQ).

Limit of quantification (LOQ)

The limit of quantification was calculated based on the signal-to-noise ratio ($S/N \geq 10$). Under the conditions of our experiment, the limit of quantification of Bexarotene was 0.5 µg/ml.

Limit of detection (LOD)

The detection limit indicates the lowest amount of a substance in a sample that can be detected. Under the conditions of our experiment, the limit of quantification of Bexarotene was 0.3 μg/ml.

The presented results show that the developed and validated UV-VIS spectrophotometric method for determining the concentration of Bexarotene is fast, simple and suitable for routine analyzes in daily laboratory practice. It is characterized by good linearity and high precision. Sample preparation and analysis time are relatively short, the cost of the method is relatively low.

3.3.2. Determination of absorbtion maximum of Bexarotene and newly synthesized esters of Bexarotene

UV-VIS spectroscopic analysis of Bexarotene and the newly synthesized Bexarotene esters was carried out in order to identify the spectral behavior and absorption properties of the studied compounds.

The spectra of the analyzed compounds were taken in methanol solvent, against a pure methanol blank sample, and the corresponding graphical expressions are shown in Figure 10 to Figure 14. Sample solutions were prepared at a concentration that fell within the absorbance range between 0.500 and 1.00.

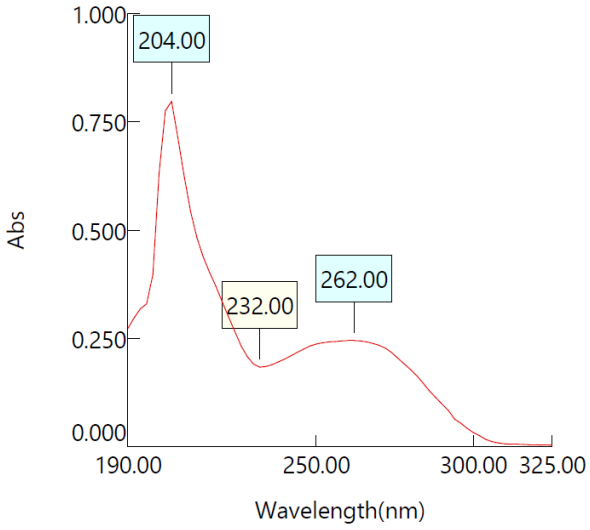


Figure 10. UV-VIS spectrum of Bexarotene in methanol.

The recorded spectrum of Bexarotene shows that it has two absorption maxima (peaks) at 204 nm and 262 nm. The measured absorbance is 0.798 and 0.245, respectively. An absorption minimum (valley) at 232 nm with an absorbance of 0.184 was also observed.

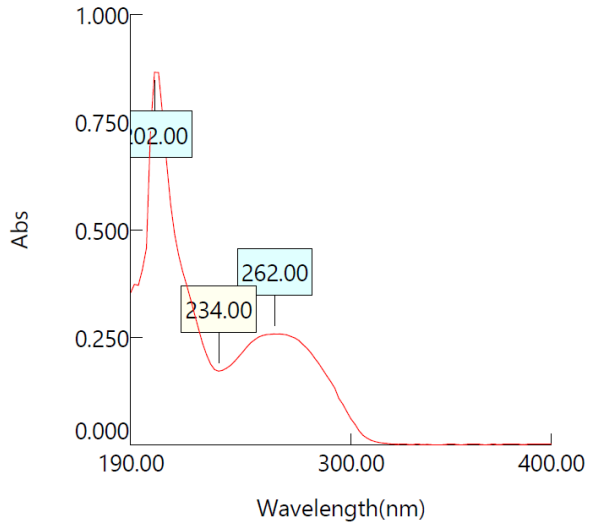


Figure 11. UV-VIS spectrum of methyl ester of Bexarotene (E1) in methanol.

Figure 11 shows the spectrum of methyl ester of Bexarotene (E1), which shows 2 absorption maxima (peaks) at a wavelength of 202 nm and 262 nm. The measured absorbance is 0.866 and 0.258, respectively. An absorption minimum (valley) at 234 nm with an absorbance of 0.172 was also observed.

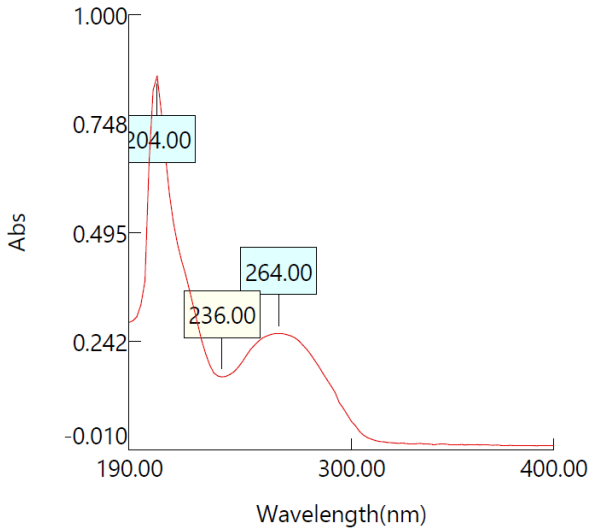


Figure 12. UV-VIS spectrum of ethyl ester of Bexarotene (E2) in methanol.

Figure 12 shows the spectrum of ethyl ester of Bexarotene (E2), which shows two absorption maxima (peaks) at wavelengths of 204 nm and 264 nm. The measured absorbance is 0.858 and 0.260, respectively. An absorption minimum (valley) at wavelength 236 nm with absorbance 0.159 is also observed.

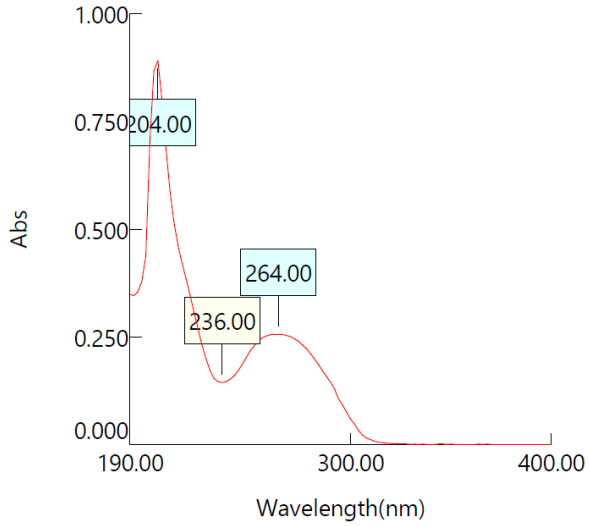


Figure 13. UV-VIS spectrum of propyl ester of Bexarotene (E3) in methanol.

Figure 13 shows the spectrum of propyl ester of Bexarotene (E3), which shows 2 absorption maxima (peaks) at wavelengths of 204 nm and 264 nm. The measured absorbance is 0.892 and 0.256, respectively. An absorption minimum (valley) at wavelength 236 nm with absorbance 0.144 is also observed.

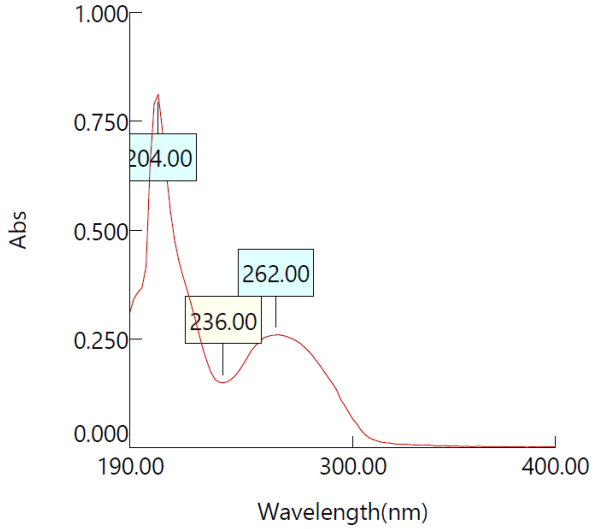


Figure 14. UV-VIS spectrum of butyl ester of Bexarotene (E4) in methanol.

Figure 14 shows the spectrum of butyl ester of Bexarotene (E4), which shows 2 absorption maxima (peaks) at a wavelength of 204 nm and 262 nm. The measured absorbance is 0.812 and 0.259, respectively. An absorption minimum (valley) at wavelength 236 nm with absorbance 0.149 is also observed.

3.3.3. Comparative analysis of the effect of solvents on absorbtion maxima

A comparative analysis was carried out when changing the solvents. Solvents with different cutoff values, presented in Table 16, were used.

Solvents dimethyl sulfoxide (DMSO) and acetone are not suitable for UV-VIS spectroscopic analysis of Bexarotene and its esters. Despite the good solubility of the analyzed substances in these solvents, their cutoff values are high, and below this value they absorb light.

Table 16. Solvents used and their cutoff values.

Solvent	***UV cutoff, nm***
Methanol	205
Hexane	195
Acetonitrile	190
Dimethyl Sulfoxide (DMSO)	268
Acetone	330

The spectra of the analyzed compounds were taken in solvent acetonitrile, against a pure acetonitrile blank sample. Figure 15 shows the spectrum of Bexarotene with acetonitrile solvent.

There are 2 absorption maxima (peaks) at wavelength 200 nm and 250 nm observed on the spectrum. The measured absorbance is 0.744 and 0.185, respectively. An absorption minimum (valley) at wavelength 232 nm with an absorbance of 0.145 is also observed.

Running the assay with the newly synthesized Bexarotene esters gave identical results. The hypsochromic (blue) shift of the absorption maximum is due to the used solvent, which has a smaller cutoff value. However, a plot similar to that presented in Figure 10 was observed. Analogous results were obtained with the solvent hexane versus the hexane blank sample.

Due to concerns about the occurrence of instantaneous hydrolysis processes due to the presence of water in the used solvents during the dissolution of the esters, an attempt was made

with absolutely dry methanol. The results obtained are similar to those with 99.99%, HPLC grade methanol.

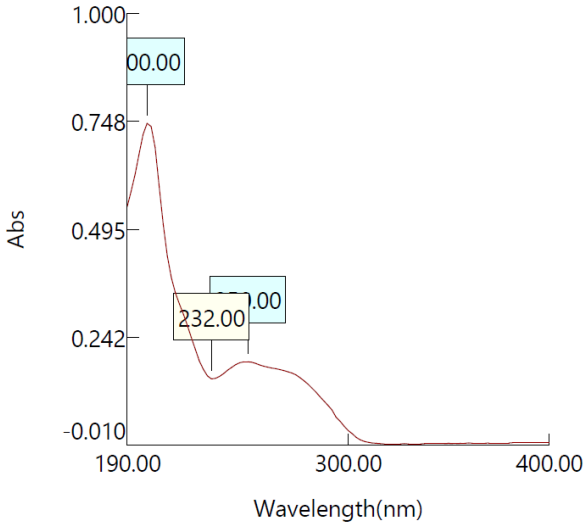


Figure 15. UV-VIS spectrum of Bexarotene with acetonitrile as solvent.

On the other hand, when determining the melting temperature, we found crystals of unreacted Bexarotene. This can degrade the spectra by giving bands that overlap those of the esters. Therefore, during the qualitative analysis of the esters, a fraction was collected from the liquid chromatograph at their respective retention time, thus confirming that the samples were pure. Since the HPLC samples were dissolved in methanol and acetonitrile in a ratio of 3:1, the UV-VIS spectral analysis was performed in the same solvents against a blank sample of methanol and acetonitrile (3:1).

Figure 16 shows the spectrum of methyl ester of Bexarotene with solvent methanol and acetonitrile (3:1).

There are 3 absorption maxima (peaks) at wavelengths of 202 nm, 258 nm and 320 nm observed on the spectrum. The measured absorbance was 0.662, 0.494 and 0.206, respectively. 4 absorption minima (valleys) at wavelengths of 196 nm, 232 nm, 300 nm and 358 nm are also observed. The measured absorbance was 0.372, 0.319, 0.143 and 0.060, respectively. Running the assay with the other Bexarotene esters gave identical results.

The absorption maxima obtained using methanol and acetonitrile as solvents were identical to those obtained using pure methanol as solvent, but two new peaks were also obtained.

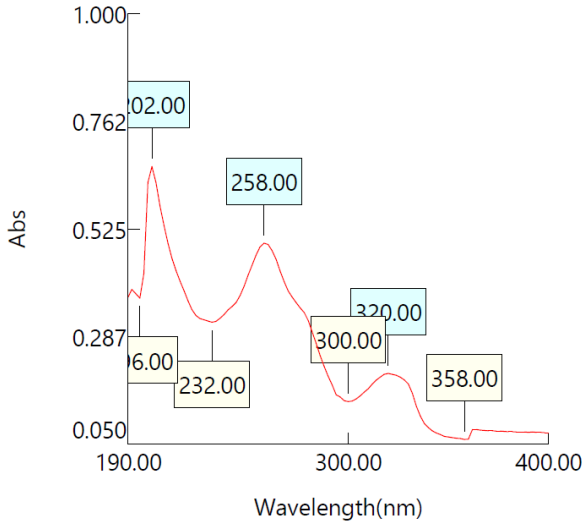


Figure 16. UV-VIS spectrum of Bexarotene with solvent methanol and acetonitrile (3:1).

From the comparative analysis, we can conclude that any solvent with a low cutoff value is suitable for conducting the analysis, but for the purpose of the scientific work, methanol was used because it is the safest.

The identical results obtained during the analysis are due to the close structure of the carboxylic acid present in the Bexarotene molecule and the ester group in the molecule of the newly synthesized esters of Bexarotene. The carboxyl functional group gives an absorption maximum at a wavelength of 210 nm and the ester at about 207 nm. This makes the method unsuitable for qualitative differentiation of the newly obtained Bexarotene esters by means of UV-VIS spectrophotometry.

The newly developed and validated method for the quantification of Bexarotene is not suitable for the determination of mixtures of Bexarotene and its esters.

3.4. HPLC method for establishing the purity of the newly synthesized derivatives

High-performance liquid chromatography (HPLC) is a specific form of column chromatography commonly used in analysis to separate, identify, and quantify components in complex mixtures.

HPLC uses a column that contains a stationary phase, a pump that moves the mobile phase through the column, and a detector that indicates the retention time of the individual analytes. The retention time varies depending on the interactions between the stationary phase,

the analyzed molecules and the solvent used. Based on the retention time, a conclusion can be made about the composition of the components in the analyzed mixture.

HPLC is a versatile, reproducible chromatographic technique for the evaluation of drug substances. It has a wide scope of application in various fields concerning the quantitative and qualitative evaluation of active substances.

3.4.1. Modification of a HPLC method for establishing the purity of newly Bexarotene esters

Methods for the analysis of Bexarotene based on different chromatographic conditions have been described in the literature. It has been reported that a system of acetonitrile and buffer (ammonium acetate and acetic xeline) can be used as the mobile phase; acetonitrile, water and glacial acetic acid; methanol and water (in a ratio of 9:1). Some authors consider the possibility of using the mobile phase of acetonitrile, water and tetrahydrofuran in a ratio of 70:20:10. In all described methods, UV detection was carried out in the 255-269 nm range.

The modification of the approaches already described in the literature for the analysis of Bexarotene consists in the selection of suitable chromatographic conditions, determination of a suitable wavelength for detection and selection of mobile phase. For the present analysis, a wavelength of 260 nm was chosen because it is reported to produce less noise, which facilitates the ability to quantify the presence of Bexarotene in a mixture.

In order to achieve a good separation of the substances under isocratic conditions, mixtures of solvents such as formic acid, water, acetonitrile, methanol and isopropanol in different combinations and ratios were tested as the mobile phase.

After conducting a number of studies, a system of methanol and acetonitrile in a ratio of 3:1 was selected as the most optimal mobile phase system, providing good separation, symmetrical peaks and high efficiency for the analysis.

3.4.2. HPLC analysis of Bexarotene and its analogues

The developed HPLC method allows the analysis of the composition of the compounds obtained as a result of the synthesis.

The peaks reported in each chromatogram should be attributed to the presence of certain components present in the composition of the individual samples. Since the elution time of each

compound is different, based on the obtained data, a conclusion can be drawn regarding the composition of the samples subjected to analysis.

For this purpose, chromatography of the standard substance Bexarotene was first performed, which plays the role of a witness (standard) in determining the component composition of the samples of the newly synthesized Bexarotene derivatives. A solution of Bexarotene with a concentration of 350.00 μg/ml in methanol was prepared. The resulting chromatogram is presented in Figure 17.

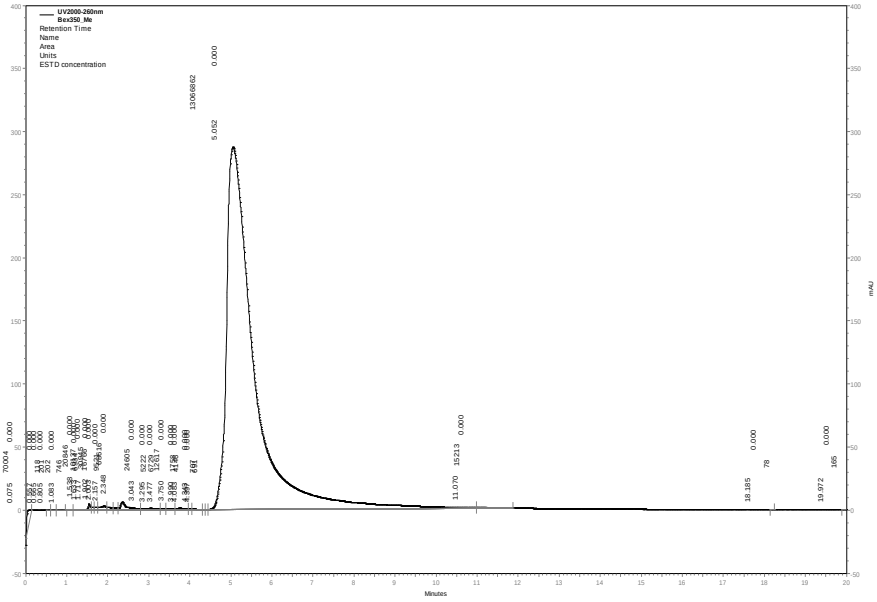


Figure 17. Chromatogram obtained from the analysis of a standard solution of Bexarotene with a concentration of 350.00 μg/ml

The resulting chromatogram shows the presence of one peak with a maximum at 5.052 min. The absence of any other peaks in the chromatogram gives us full reason to consider that the obtained peak refers to the retinoid Bexarotene and its retention time corresponds to 5.052 min.

The analysis of the methyl ester of Bexarotene (E1) was carried out according to the described chromatographic method, also used in the analysis of the standard substance Bexarotene. When conducting the analysis, a working solution with a concentration of 640.00 μg/ml was used.

In the resulting chromatogram, the presence of a distinct peak at a retention time of 3.482 min is reported. Thus, the observed peak can be attributed to methyl ester, characterized by a retention time of 3.482 min. The obtained data are presented in Figure 18.

The formation of a tail was observed in the chromatogram of Bexarotene, which could be corrected by acidifying the mobile phase. However, such a phenomenon was not observed in the chromatogram of the methyl ester of Bexarotene. The observed peak is high and with a narrow base, which indicates that the chromatographic system used is of sufficient efficiency.

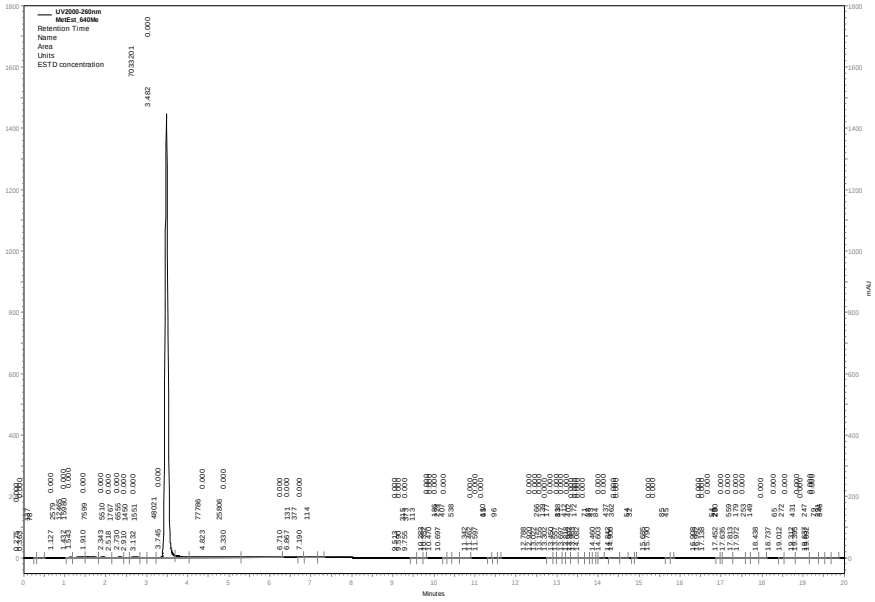


Figure 18. Chromatogram obtained in the analysis of methyl ester of Bexarotene (E1) with a concentration of 640.00 μg/ml

The analysis of the ethyl ester of Bexarotene (E2) was carried out according to the described chromatographic method used for the analysis of the standard substance Bexarotene. The concentration of the working solution used in the analysis is 510.00 μg/ml. The results of the analysis are presented in Figure 19.

Analysis of the chromatogram of the ethyl ester of Bexarotene shows the presence of three poorly resolved peaks. The peak with higher intensity in the resulting chromatogram should be attributed to the target synthesis product. The indicated peak was reported at a retention time corresponding to 3.752 min. A peak is also observed at about 4.278 min and at 4.578 min. It has not been determined what this peak corresponds to. Probably a by-product of the synthesis or unreacted Bexarotene.

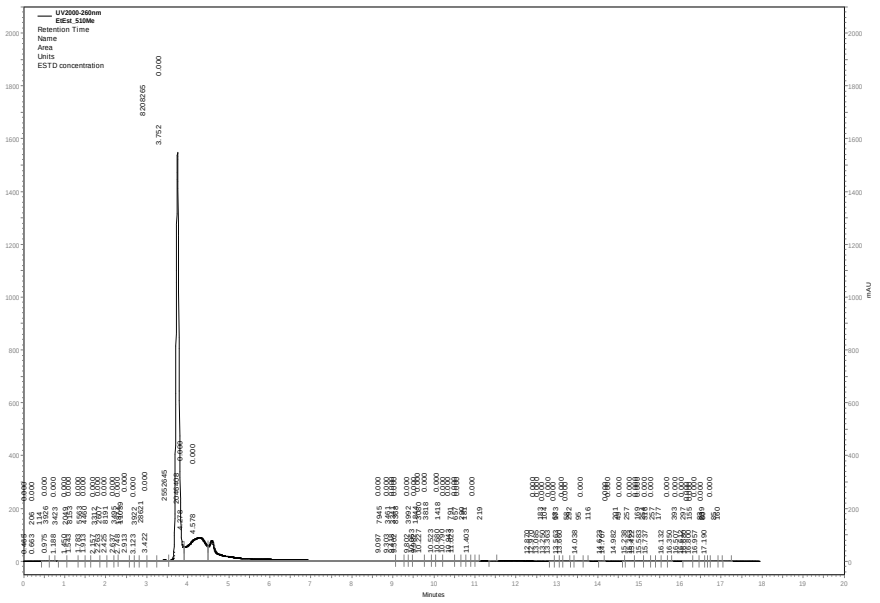


Figure 19. Chromatogram obtained in the analysis of ethyl ester of Bexarotene (E2) with a concentration of 510.00 µg/ml

The analysis of the propyl ester of Bexarotene (E3) was carried out according to the described chromatographic method used for the analysis of Bexarotene standard substance. The sample working solution concentration used was 800.00 µg/ml. The results of the analysis are presented in Figure 20.

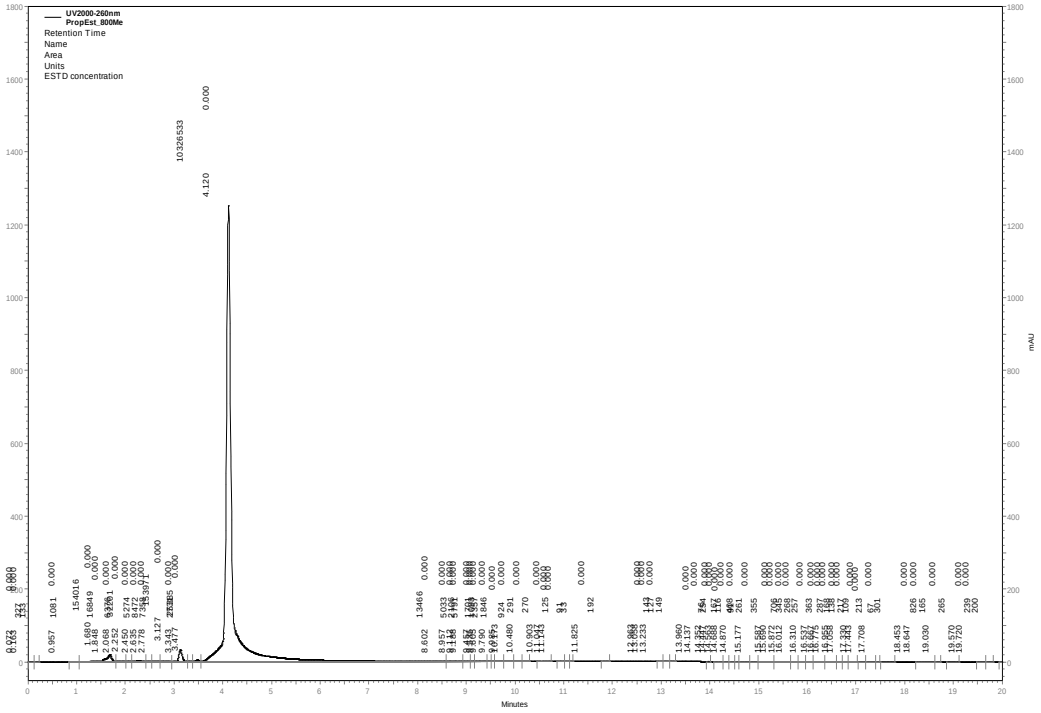


Figure 20. Chromatogram obtained in the analysis of Bexarotene propyl ester (E3) with a concentration of 800.00 µg/ml

When analyzing the chromatogram of the propyl ester of Bexarotene two peaks are reported. The peak with higher intensity in the resulting chromatogram should be attributed to the target synthesis product. The indicated peak was reported at a retention time corresponding to 4.12 min. A peak is also observed at about 3.127 min. It has not been determined what this peak corresponds to. Probably a byproduct of fusion.

The analysis of the butyl ester of Bexarotene (E4) was carried out according to the described chromatographic method used for the analysis of Bexarotene standard substance. Concentration of working solution of the sample is 790.00 μg/ml. The results of the analysis are presented in Figure 21.

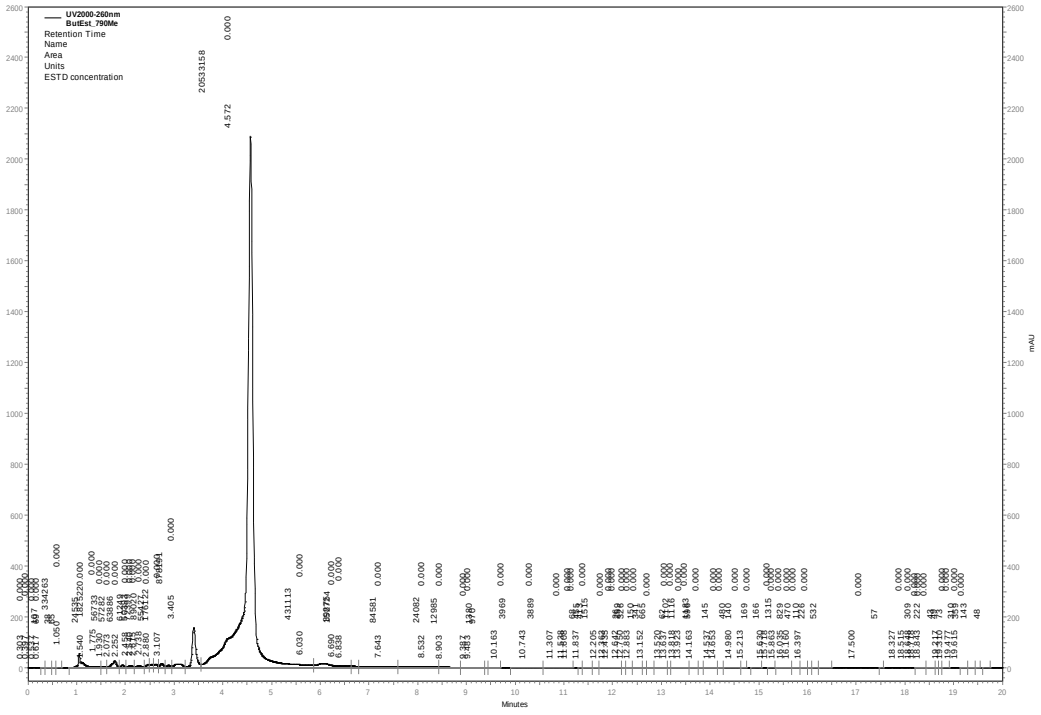


Figure 21. Chromatogram obtained in the analysis of Bexarotene butyl ester (E4) with a concentration of 790.00 μg/ml

The chromatographic data obtained show the presence of a high intensity peak at a retention time of 4.572 min. The peak with higher intensity in the resulting chromatogram should be attributed to the target synthesis product.

Another peak with a retention time of 3.405 min and a shoulder of the high intensity peak was also observed in the chromatogram, which were probably due to impurities from the synthesis of the butyl ester of Bexarotene.

From the presented chromatograms, we can conclude that the retention times of all analyzed substances have close values, but nevertheless they can be differentiated as different compounds. The similar retention times are due to their related structures.

4. *IN SILICO* METHODS FOR PREDICTING BIOLOGICAL ACTIVITY OF BEXAROTENE ESTERS

4.1. Prediction of potential metabolic activity of Bexarotene newly synthesized Bexarotene esters and their metabolites using QSAR Toolbox

A study of the potential metabolic activity of the structure of Bexarotene and the newly synthesized esters was carried out using metabolic models.

The application of QSAR models allows to identify and characterize the physicochemical properties of the structure of Bexarotene and its esters, as well as some of the properties affecting the biological activity of the preparation.

For a complete study on the activity of Bexarotene esters, it is important to evaluate not only the starting compound, but also the experimental and theoretical prediction of its possibilities for biological activation, as a result of which the corresponding metabolites are formed. They, in turn, can show stronger biological activity, as well as lead to a change in the safety spectrum.

The characterization of the obtained Bexarotene derivatives includes the determination of their ability to form active metabolites, as well as their potential interaction with DNA and proteins. The information obtained is essential for the study of the biological potential of the newly synthesized compounds and their safety profile.

For the purpose of the scientific work, a study of the potential metabolic activity of the structure of Bexarotene was carried out by means of the application of a mathematical model.

This model enabled us to identify and determine the physicochemical properties of Bexarotene and its esters, as well as some of the properties influencing the biological activity of the molecules.

The results obtained for the binding of Bexarotene and its esters to DNA and proteins are presented in Table 17.

Table 17. Binding of parent compounds to DNA and proteins.

Compound	*Bexarotene*	*E1*	*E2*	*E3*	*E4*
DNA binding	-	-	-	-	-
Protein binding	-	-	-	-	-

From the *in silico* analysis, it can be seen that neither Bexarotene nor its newly synthesized esters bind to DNA and proteins, therefore they do not cause mutagenicity and genotoxicity. It follows that the esters have a safety profile identical to that of Bexarotene.

4.1.1. *In vivo* rat metabolism simulator

The set of molecular transformations in the liver metabolism simulator consists in part of 30-40 abiotic (non-enzymatic) and 630-640 enzymatic reactions. Abiotic reactions are believed to occur at a very high rate and these reactions are given a higher priority. This subset of reactions also includes transformations of highly reactive functional groups and intermediates, such as tautomerization, arene epoxide rearrangement to phenols, etc.

The simulator also contains 520-530 phase I enzymatic transformations, such as aliphatic C-oxidation, aromatic C-hydroxylation, oxidative N- and O-dealkylation, epoxidation, ester and amide hydrolysis, reduction of carbonyl groups, reduction of nitro- and azo- groups, N-hydroxylation, oxidative deamination, beta-oxidation, ring cleavage, hydrolytic cleavage, introduction of aromatic fragments, decarboxylation, dehalogenation, etc. The model includes 100-110 phase II enzymatic transformations, such as glucuronidation, sulfation, glutathione conjugation, N-acetylation, etc., which, in contrast to *in vitro* systems, are thought to occur with high priority *in vivo*. By means of QSAR Toolbox, only the metabolites (products) obtained from phase I reactions are visualized.

The potential metabolic activity of the Bexarotene structure was investigated using metabolic models.

Applying a mathematical prediction model allowed us to identify and determine the physicochemical properties of the Bexarotene structure, as well as any of the properties influencing the biological activity of the drug.

As a result of the mathematical prediction performed using the *in vivo* rat metabolism simulator of Bexarotene the obtained metabolites are presented in Table 18. The metabolism of Bexarotene esters E1, E2, E3 and E4 are presented in Tables 19, 20, 21 and 22, respectively.

Table 18. Numbers and structure of predicted metabolites of Bexarotene obtained after *in vivo* rat metabolism simulator.

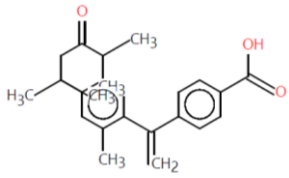
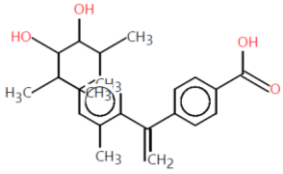
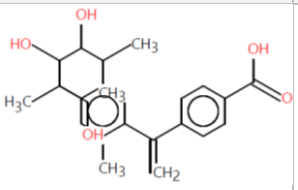
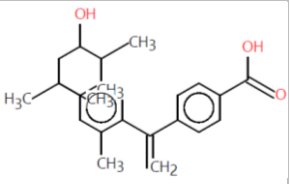
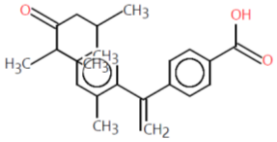
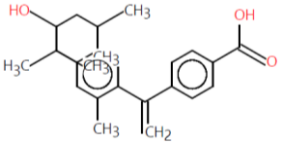
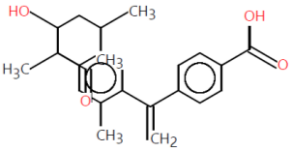
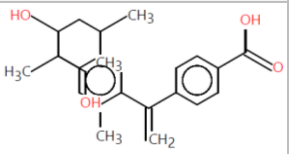
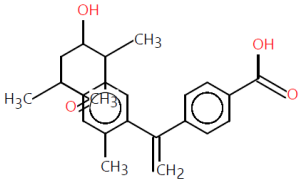
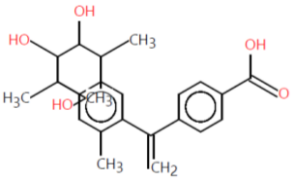
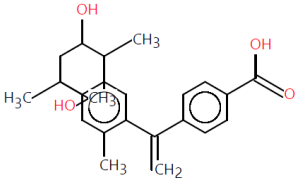
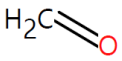
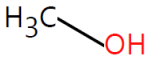
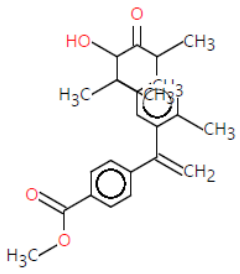
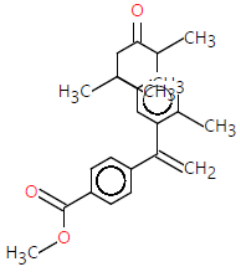
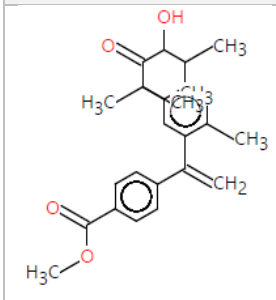
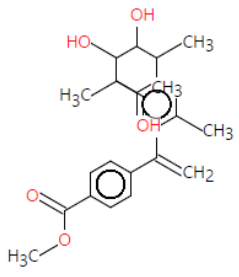
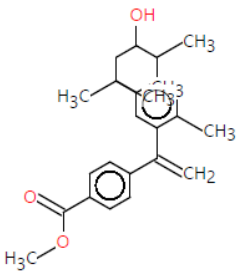
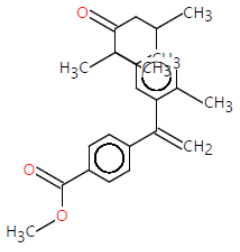
1	2	3	4
			
5	**6**	**7**	**8**
			
9	**10**	**11**	
			

Table 19. Numbers and structure of predicted metabolites of compound E1 obtained after *in vivo* rat metabolism simulator.

1	2	3	4
			
5	**6**	**7**	**8**
			
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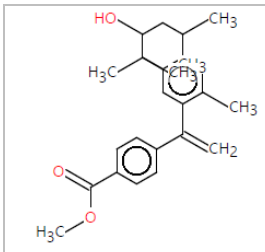	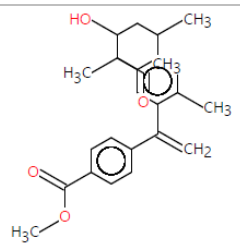	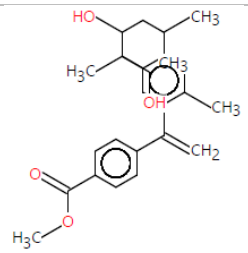	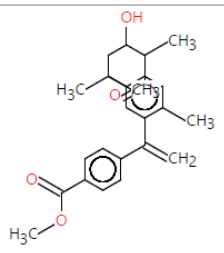
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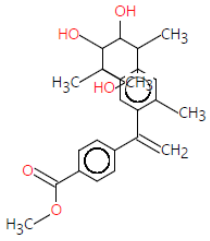	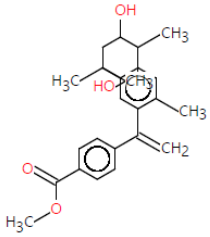	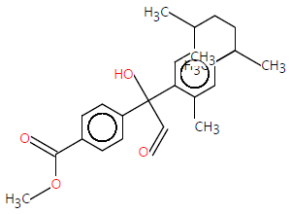	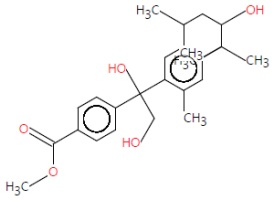
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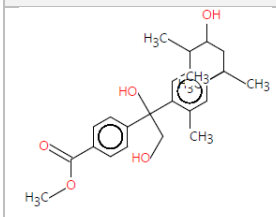	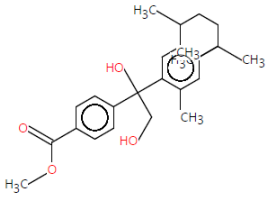	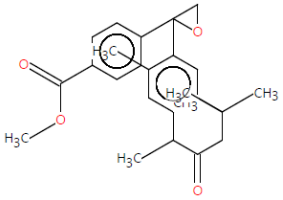	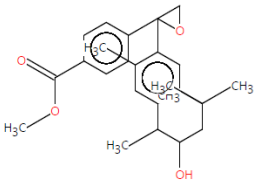
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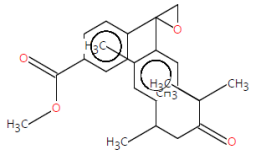
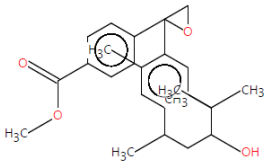
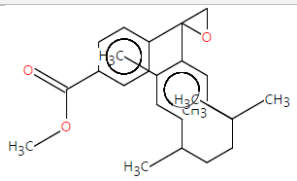
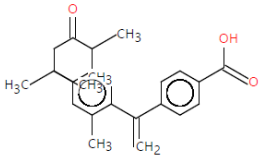
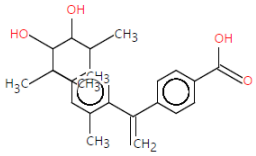
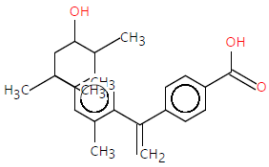
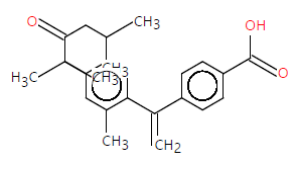
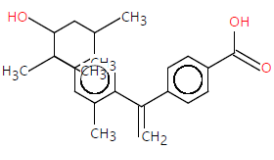
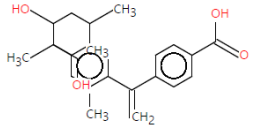
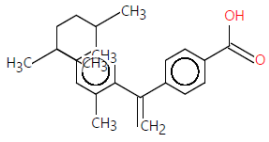
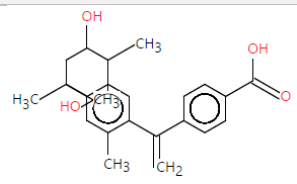
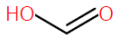
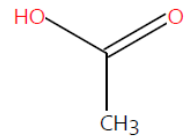
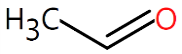
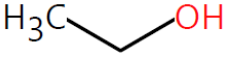
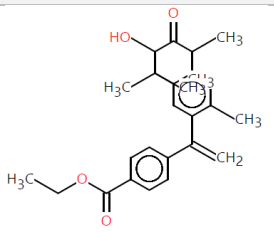
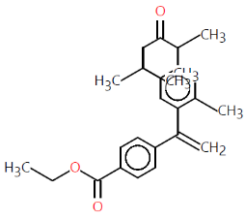
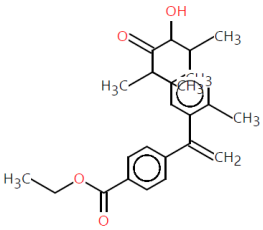
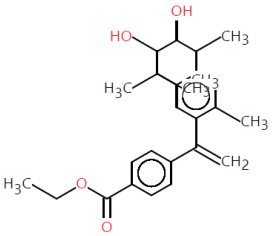
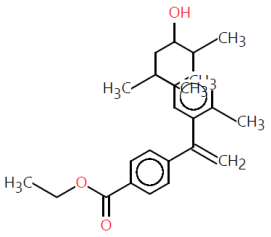
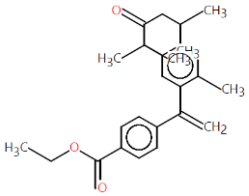
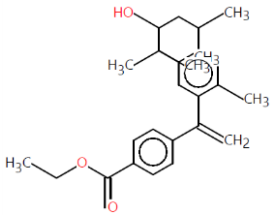
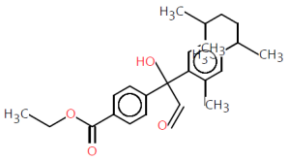
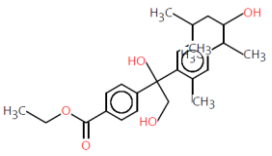
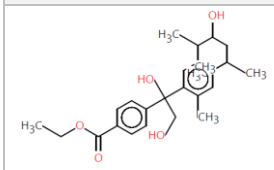
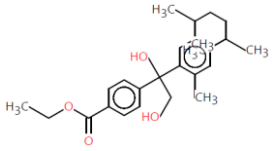
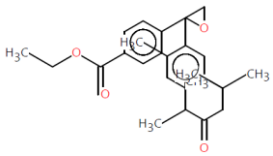
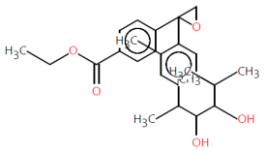
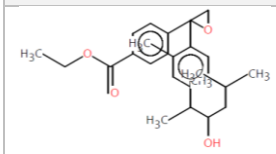
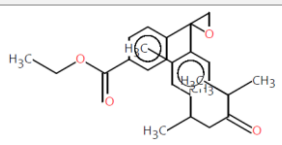
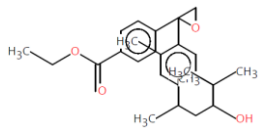
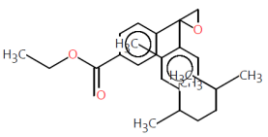
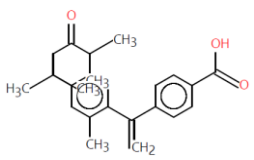
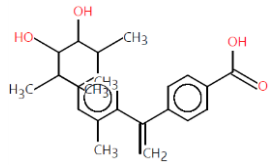
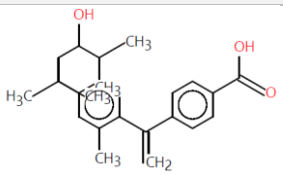
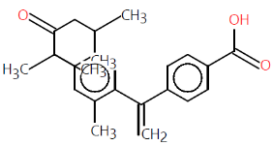
			
25	**26**	**27**	**28**
			
29	**30**	**31**	**32**
			

Table 20. Numbers and structure of predicted metabolites of compound E2 obtained after *in vivo* rat metabolism simulator.

1	2	3	4
			
5	**6**	**7**	**8**
			
9	**10**	**11**	**12**
			
13	**14**	**15**	**16**
			
17	**18**	**19**	**20**
			
21	**22**	**23**	**24**
			
25	**26**	**27**	**28**

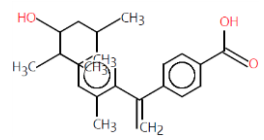
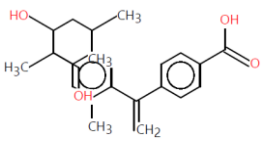
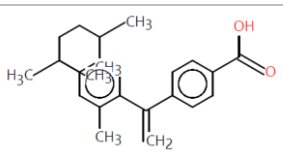
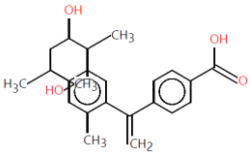
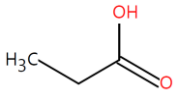
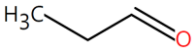
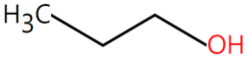
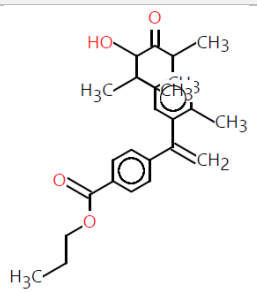
			

Table 21. Numbers and structure of predicted metabolites of compound E3 obtained after *in vivo* rat metabolism simulator.

1	2	3	4
			
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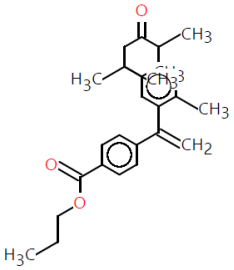	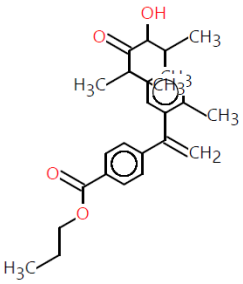	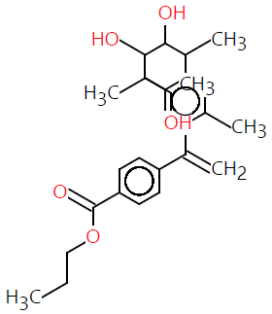	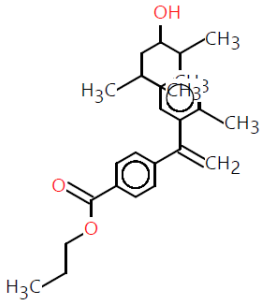
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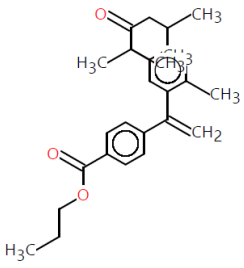	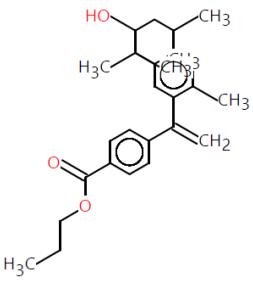	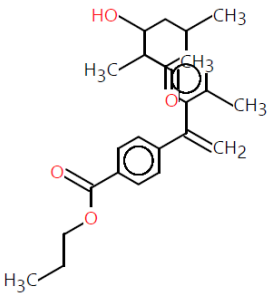	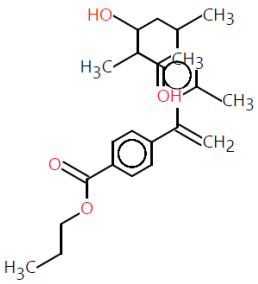
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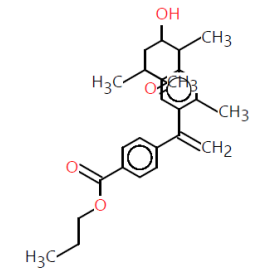	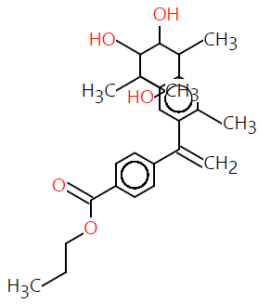	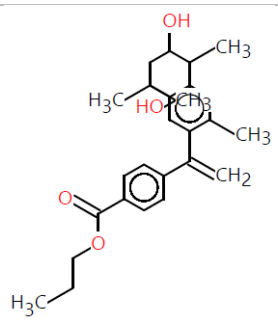	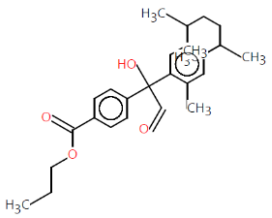
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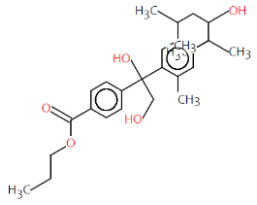	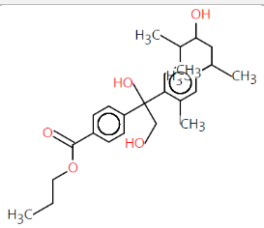	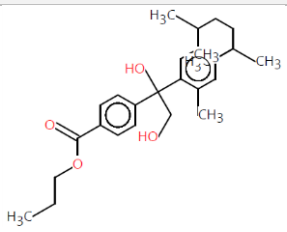	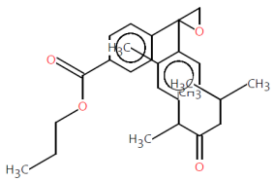
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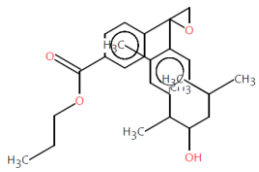	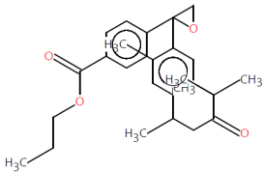	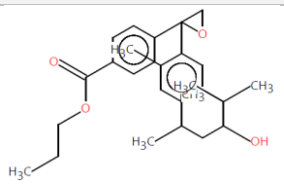	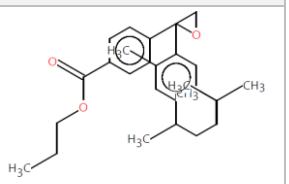
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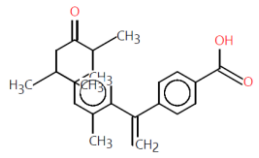	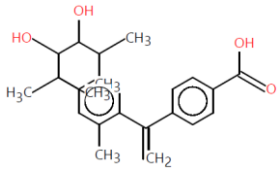	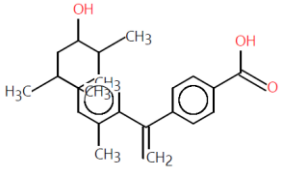	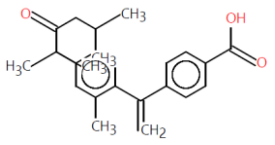
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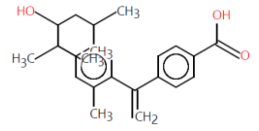	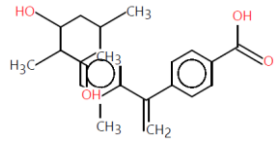	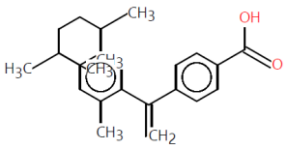	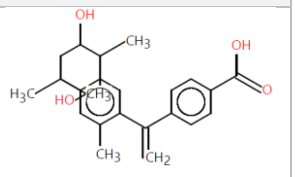

Table 22. Numbers and structure of predicted metabolites of compound E4 obtained after *in vivo* rat metabolism simulator.

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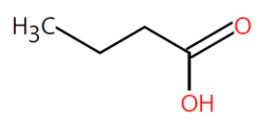	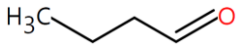	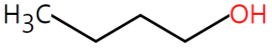	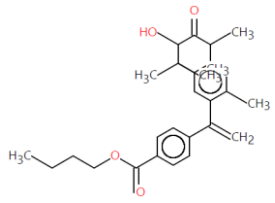

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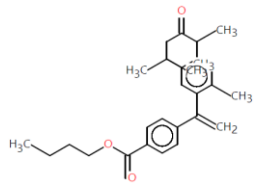	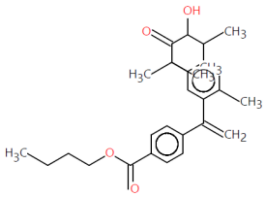	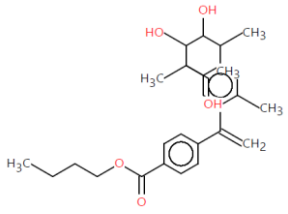	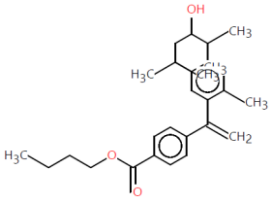
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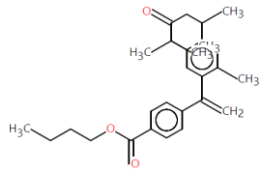	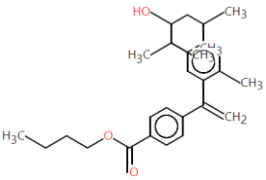	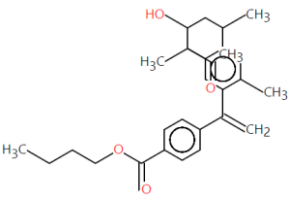	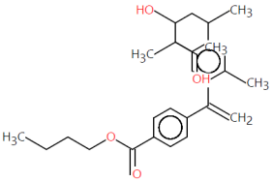
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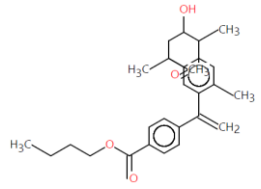	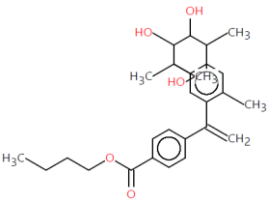	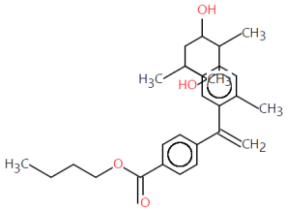	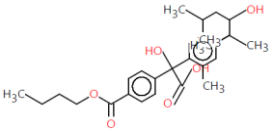
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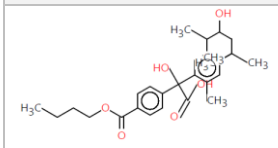
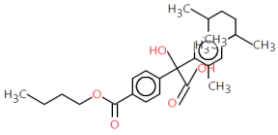
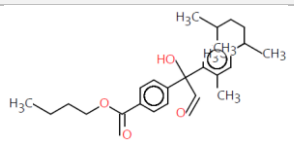
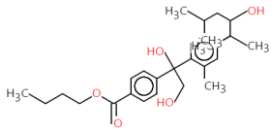
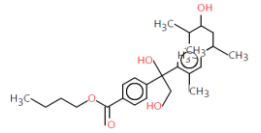
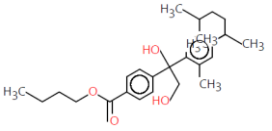
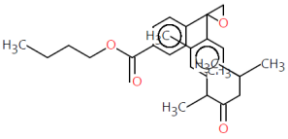
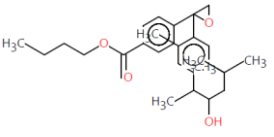
			
21	**22**	**23**	**24**
			
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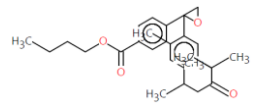	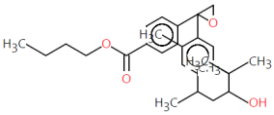	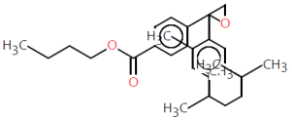	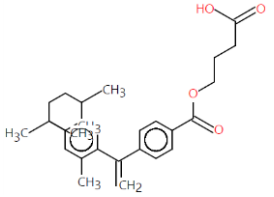
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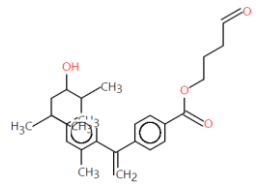	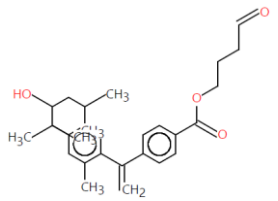	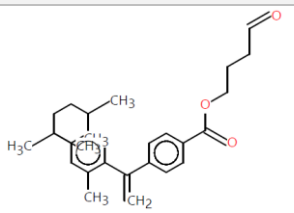	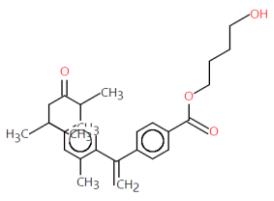

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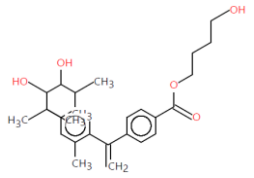	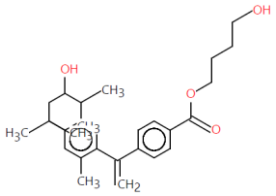	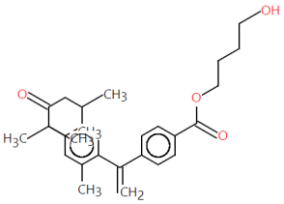	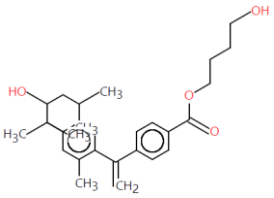
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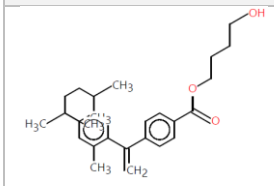	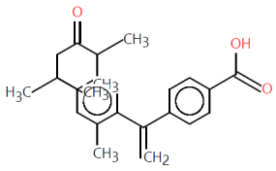	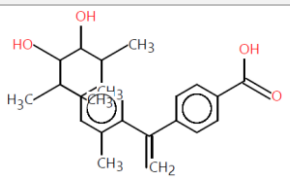	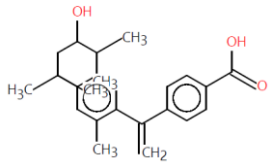
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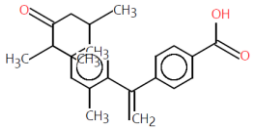	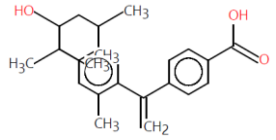	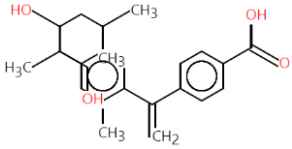	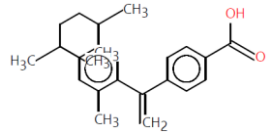
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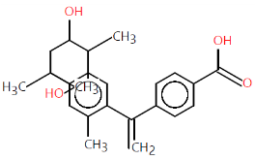	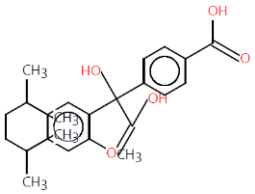	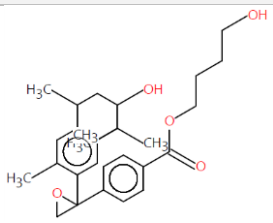	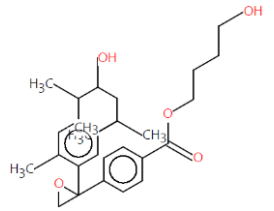
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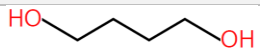
			

The data presented indicate that metabolites derived from Bexarotene esters partially overlap with those derived from Bexarotene. This is due to the metabolic transformation (hydrolysis) of the esters to Bexarotene and the alcohol used to synthesize the esters. This makes it possible for them to be used as prodrugs due to their ability to release Bexarotene after oral administration.

Ester metabolites other than those of Bexarotene are derived from biotransformations prior to hydrolysis. It can also be noted that more complex esters, or those with a larger molecular weight, yield a greater number of metabolites.

The alcohol obtained as a result of hydrolysis also undergoes biotransformation. Alcohols are known to be metabolized by the enzyme alcohol dehydrogenase to aldehydes and then to carboxylic acids. Methanol is initially metabolized to formaldehyde (HCHO) and then

to formic acid (HCOOH), which cause severe acidosis and lead to CNS and optic nerve damage. Ethanol has a direct narcotic effect on the CNS. After metabolism, acetaldehyde (CH_3CHO) and then acetic acid (CH_3COOH) are obtained, as a result of which it is possible to reach metabolic acidosis. Methanol and propanol are more toxic than ethanol, both alcohols having serious negative effects on the gastrointestinal tract and CNS. The effects of propanol on humans resemble those of ethanol, but with 2-4 times stronger action. It is metabolized to propanoic acid (C_2H_6COOH), from which metabolic acidosis can also occur. Butanol irritates the eyes, respiratory tract, and skin with prolonged exposure, although acute oral or parenteral exposure to large therapeutic doses in humans has no adverse effects. It is safe enough for use in cosmetics. According to literature data, in most cases, butanol is rapidly metabolized to carbon dioxide. In the liver metabolism simulation, butanoic acid (C_3H_9COOH) is produced.

The large range of possible metabolites predisposes to more pronounced pharmacological or toxic effects, which necessitates the need to conduct additional studies on the biological activity of the resulting metabolites.

4.1.1.1. DNA and protein binding

The characterization of the obtained Bexarotene derivatives includes the determination of their ability to form active metabolites, as well as their potential interaction with DNA and proteins. The information obtained is essential for the study of the biological potential of the newly synthesized compounds and their safety profile.

Based on the analysis, the mechanisms by which the metabolites of Bexarotene and the newly obtained compounds can potentiate liver damage through specific reactions of interaction with biological macromolecules (DNA and proteins) in the liver have been identified and summarized. The ability to bind the resulting metabolites of Bexarotene and its esters E1, E2, E3 and E4 to DNA and proteins are presented in Tables 23 and 24, respectively.

Based on the analysis, it is believed that the structure of Bexarotene and its esters have the ability to form metabolites that can bind to both DNA and proteins.

DNA-binding structures may induce genotoxicity. When bound to proteins, it is possible for protein conjugates to have a direct effect on the cell, by disrupting its basic functions, or to act indirectly and also lead to damage.

Table 23. Binding of Bexarotene and Bexarotene esters metabolites to DNA.

DNA binding	**Structural alert**	No alert found	Epoxides, Aziridines, Thiiranes and Oxetanes
	Mechanistil alert	-	*Alkylation, direct acting epoxides and related*
	Mechanistic domain	-	S_N^2
Metabolite №	***Bex***	1-11	-
	E1	1-18, 24-32	19-23
	E2	1-14, 21-28	15-20
	E3	1-19, 25-32	20-24
	E4	1-22, 28-46, 49	23-27, 47, 48

Table 24. Binding of Bexarotene and Bexarotene esters metabolites to proteins.

Protein binding	**Structural alert**	No alert found	Aldehydes	Ketones	Epoxides, Aziridines and Sulfuranes
	Mechanistic alert	-	*Schiff base formation with carbonyl compounds*	*Addition to carbon-hetero double bond*	*Ring opening S_N^2 reaction*
	Mechanistic domain	-	*Schiff base formation*	*Nucleophilic addition*	S_N^2
Metabolite №	***Bex***	2-4, 6, 8, 10, 11	7, 9	1, 5	-
	E1	2, 6, 7, 9, 11, 13, 14, 16-18, 25, 26, 28-32	1, 10, 12, 15	3-5, 8, 19, 21, 24, 27	19-23
	E2	1, 3, 7, 8, 10, 12-14, 22, 23, 25-28	2, 11	4-6, 9, 15, 18, 21, 24	15-20
	E3	1, 3, 7, 8, 10, 12, 14, 15, 17-19, 26, 27, 29-32	2, 11, 13, 16	4-6, 9, 20, 22, 25, 28	20-24

E4	1, 3, 7, 8, 10, 12, 14-18, 20-22, 28, 33, 34, 36, 37, 39, 40, 42-46, 49	2, 11, 13, 19, 29-31	4-6, 9, 23, 25, 32, 35, 38, 41	23-27, 47, 48	

As a result of this metabolism, Bexarotene gives 11 metabolites, none of which can bind to DNA, but 4 of them can bind to proteins.

The methyl ester of Bexarotene (E1) yields 32 metabolites, 5 of which can bind to DNA and 15 to proteins.

Analogous to E1, ethyl ester (E2) gives 28 metabolites - 6 of them can bind to DNA and 14 to proteins. Propyl ester (E3) yields 32 metabolites. 4 bind to DNA and 15 to proteins and the butyl ester (E4) gives 49 metabolites of which 7 bind to DNA and 22 to proteins.

In all predicted metabolites of the newly synthesized esters with DNA binding potential, the binding mechanism is via an S_N2 reaction. Binding to proteins occurs by Schiff base formation, nucleophilic addition and by S_N2 reaction.

Ester E4 shows the highest metabolic activity. It forms the largest number of metabolites that bind to DNA and proteins.

4.1.2. Rat liver S9 metabolism simulator

The liver S9 metabolism simulator is defined by the US National Library of Medicine's ‚IUPAC Glossary of Terms Used in Toxicology' as‚the supernatant fraction obtained from an organ homogenate (usually liver) by centrifugation at 9000 g for 20 minutes in an appropriate medium. This fraction contains a mixture of unfractionated microsomes and cytosol'. The microsomal component of the S9 fraction contains a wide variety of enzymes that metabolize xenobiotics. Typically used for *in vitro* ADME phase I and II metabolism studies.

Unlike liver microsomes, which contain only the subcellular fraction of the endoplasmic reticulum (containing mostly cytochrome P450 enzymes or uridine 5'-diphospho-glucuronosyltransferase), S9 fractions also contain cytosolic enzymes such as aldehyde oxidase, xanthine oxidase, sulfotransferases, methyltransferases, N-acetyl transferases and glutathione transferases.

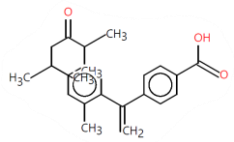
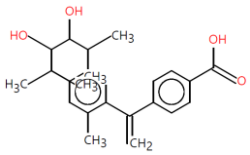
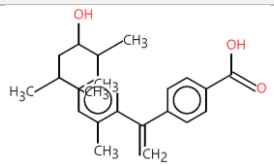
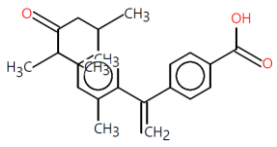
The simulation of metabolism due to the liver S9 fraction is imperative because of the possibility of formation of metabolites different from those obtained in the liver *in vivo*

simulation. The resulting metabolites from the simulation of liver S9 metabolism of Bexarotene and its esters E1, E2, E3 and E4 are presented in Tables 25, 26, 27, 28 and 29, respectively.

The obtained data show that in the liver S9 metabolism simulation Bexarotene gives 5 metabolites. Bexarotene esters give a greater number of metabolites – E1 gives 12, and E2, E3 and E4 give 14 metabolites each.

In the metabolism of Bexarotene esters, hydrolysis is observed to Bexarotene and the alcohol used for synthesis. This further supports the hypothesis that Bexarotene esters can be administered as prodrugs.

Table 25. Numbers and structure of predicted metabolites of Bexarotene obtained after rat liver S9 metabolism simulator.

1	2	3	4
			
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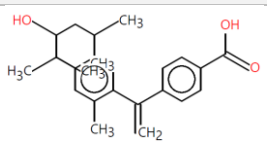
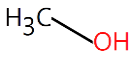
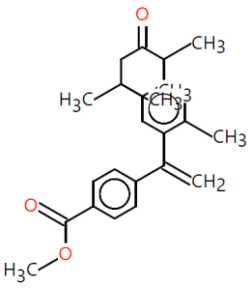
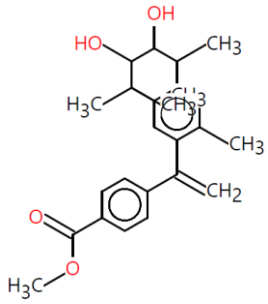
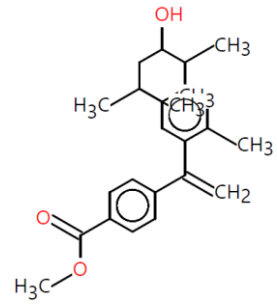
			

Table 26. Numbers and structure of predicted metabolites of compound E1 obtained after rat liver S9 metabolism simulator.

1	2	3	4
			
5	**6**	**7**	**8**

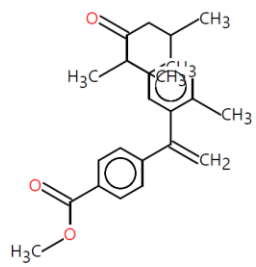
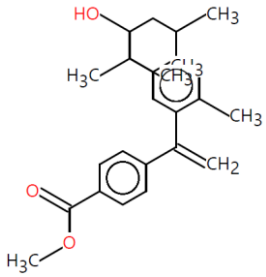
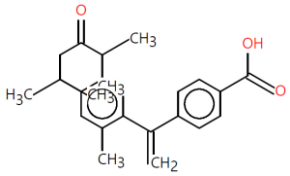
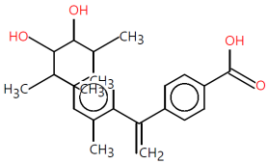
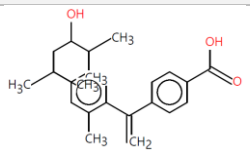
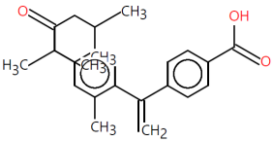
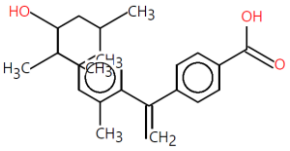
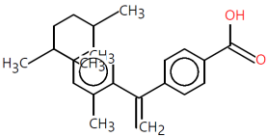
			
9	**10**	**11**	**12**
			

Table 27. Numbers and structure of predicted metabolites of compound E2 obtained after rat liver S9 metabolism simulator.

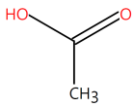
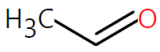
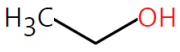
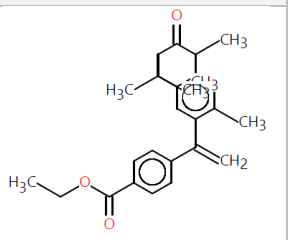
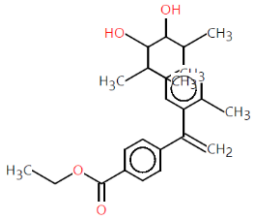
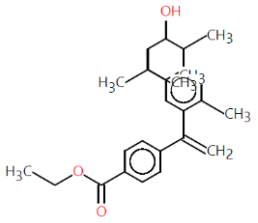
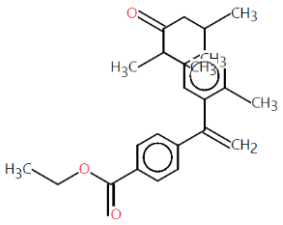
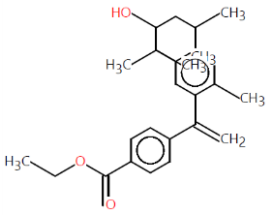
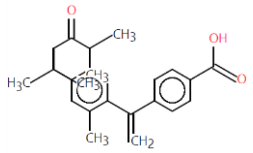
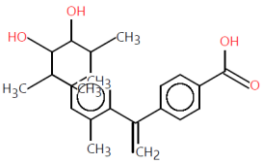
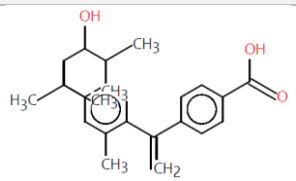
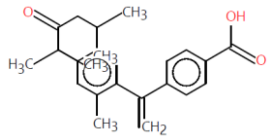
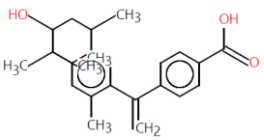
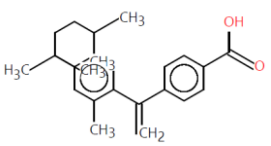
1	**2**	**3**	**4**
			
5	**6**	**7**	**8**
			
9	**10**	**11**	**12**
			
13		**14**	
			

Table 28. Numbers and structure of predicted metabolites of compound E3 obtained after rat liver S9 metabolism simulator.

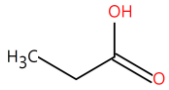
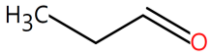
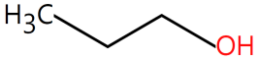
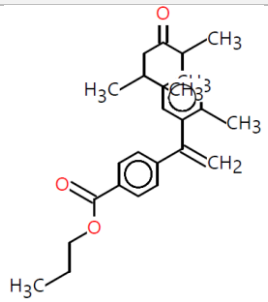
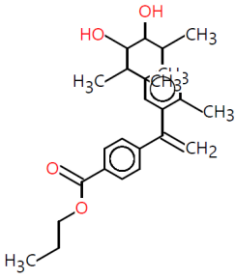
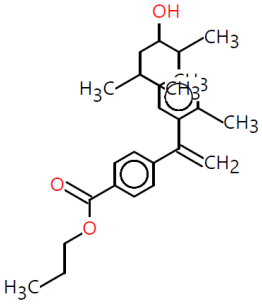
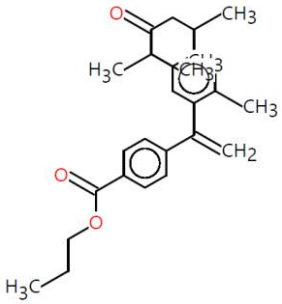
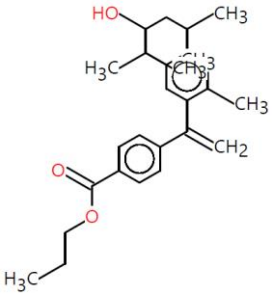
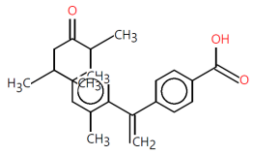
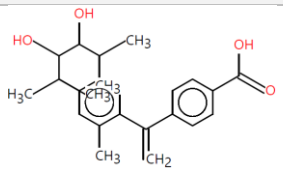
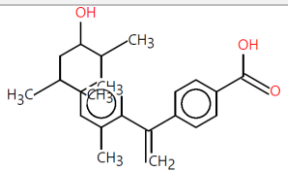
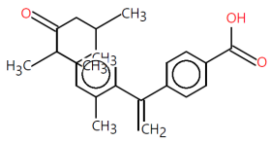
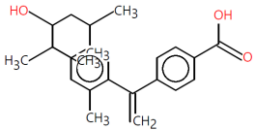
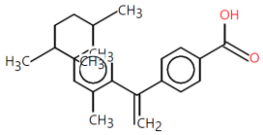
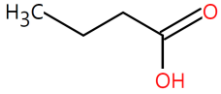
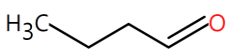
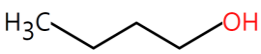
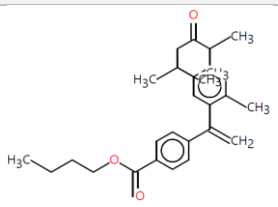
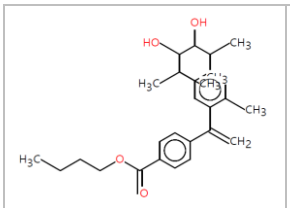
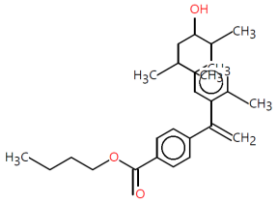
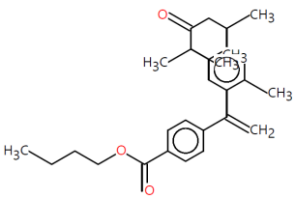
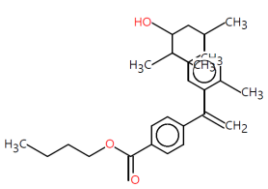
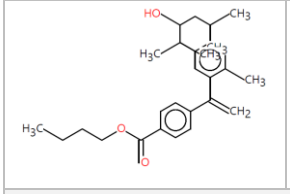
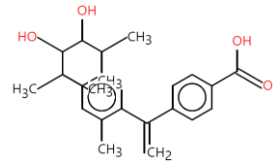
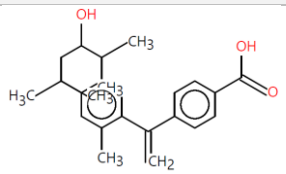
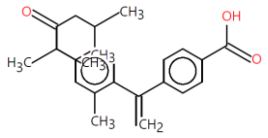
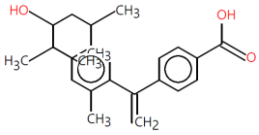
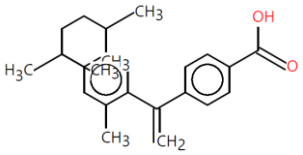
1	2	3	4
			
5	**6**	**7**	**8**
			
9	**10**	**11**	**12**
			
13		**14**	
			

Table 29. Numbers and structure of predicted metabolites of compound E4 obtained after rat liver S9 metabolism simulator.

1	2	3	4
			
5	**6**	**7**	**8**

			
9	**10**	**11**	**12**
			
13		**14**	
			

In E1, Bexarotene is metabolite № 12, and in E2, E3, and E4, Bexarotene is metabolite № 14. After the release of Bexarotene, it is also metabolized to the same metabolites obtained in the simulation of Bexarotene metabolism. The remaining metabolites of the esters were obtained as a result of reactions preceding the hydrolysis.

4.1.2.1. DNA and protein binding

An analysis was made for the possibility of DNA and protein binding of the metabolites obtained in the liver S9 metabolism simulator. The ability to bind the resulting metabolites of Bexarotene and its esters E1, E2, E3 and E4 to DNA and proteins are presented in Table 30.

Table 30. Binding of Bexarotene and Bexarotene esters metabolites to DNA and proteins.

DNA binding	**Structural alert**	No alert found	***Protein binding***	**Structural alert**	No alert found	Aldehydes	Ketones
	Mechanistic domain	-		**Mechanistic domain**	-	*Schiff base formation with carbonyl compounds*	*Addition to carbon-hetero double bonds*

	Mechanistic domain	-		Mechanistic domain	-	*Schiff base formation*	*Nuclephilic addition*
Metabolite №	***Bex***	1-5	***Metabolite №***	***Bex***	2, 3, 5	-	1, 4
	E1	1-12		***E1***	1, 3, 4, 6, 8, 9, 11, 12	-	2, 5, 7, 10
	E2	1-14		***E2***	1, 3, 5, 6, 8, 10, 11, 13, 14	2	4, 7, 9, 12
	E3	1-14		***E3***	1, 3, 5, 6, 8, 10, 11, 13, 14	2	4, 7, 9, 12
	E4	1-14		***E4***	1, 3, 5, 6, 8, 10, 11, 13, 14	2	4, 7, 9, 12

Based on the analysis, we can conclude that the structure of Bexarotene and its esters have the ability to form metabolites that can bind to proteins, but not to DNA.

When bound to proteins, it is possible that the protein conjugates can have a direct effect on the cell, by disrupting its basic functions, or act indirectly and again lead to damage.

None of the metabolites obtained from the simulation of rat liver S9 metabolism of Bexarotene and its esters could bind to DNA. Of the 5 metabolites of Bexarotene obtained, 3 cannot bind to DNA, and two of them can bind to proteins by nucleophilic addition.

Eight of the E1 ester metabolites were unable to bind to proteins. The remaining 4 can bind to proteins by nucleophilic addition.

In the case of E2, E3 and E4 esters, it was observed that 9 of the obtained metabolites could not bind to proteins, 1 could bind by Schiff base formation and 4 – by nucleophilic addition.

E2, E3 and E4 esters show the highest metabolic activity. They form the largest number of metabolites that bind to proteins.

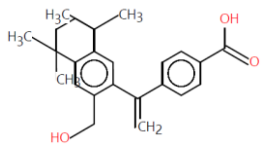
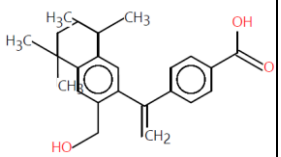
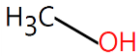
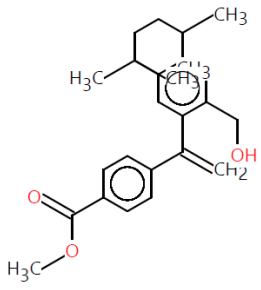
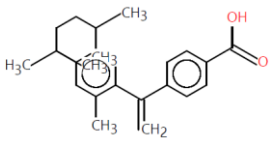
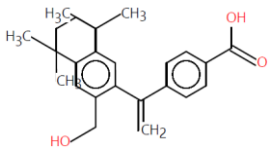
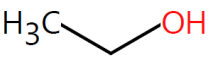
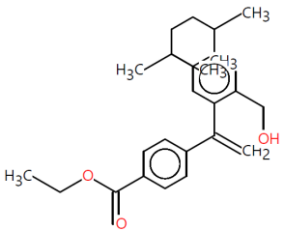
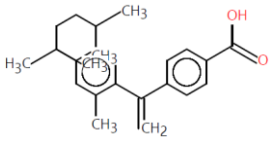
4.1.3. Skin metabolism simulator

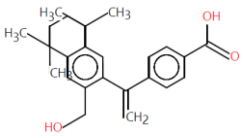
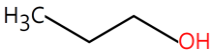
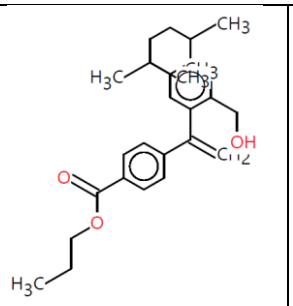
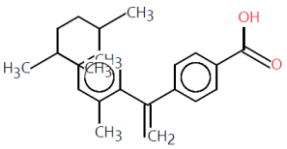
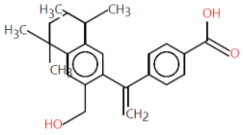
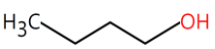
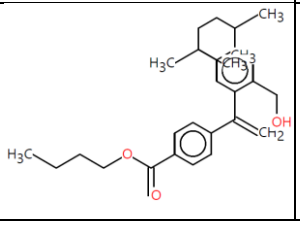
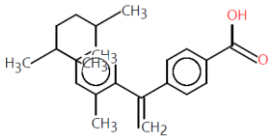
The skin, like other organs in the human body, contains many enzymes that are capable of metabolizing endogenous and exogenous substances. Cutaneous metabolism, in turn, can affect a number of processes, including skin toxicity, absorption, maintenance of homeostasis, delivery of dermal dosage forms, and efficacy. Because of the potential wide-ranging effects that skin metabolism can affect, interest in it is growing. This, in turn, leads to the development of *in vitro* methods for predicting the potential of various substances to produce skin metabolites.

The main indication of Bexarotene in therapy is its application for the treatment of CTCL in the form of a 1% gel. Therefore, it is of particular importance to investigate the possibilities of skin metabolism of the newly synthesized Bexarotene analogues.

Therefore, an analysis was performed to determine the potential skin metabolism of Bexarotene and its esters E1, E2, E3 and E4. The obtained data are presented in Table 31.

Table 31. Numbers and structure of predicted metabolites of Bexarotene and its esters E1, E2, E3 and E4 obtained after skin metabolism simulator.

№	1	2	3	4
Bex		-	-	-
E1				
E2				

E3				
E4				

The obtained data shows that in the simulation of skin metabolism, Bexarotene gives 1 metabolite and its esters give 4 metabolites each. Metabolite № 1 is the same for both Bexarotene and its esters. Again, a hydrolysis process was observed, releasing Bexarotene (metabolite № 4) and the alcohol used to synthesize the ester. This confirms the possibility of dermal application of Bexarotene esters as prodrugs according to the QSAR Toolbox.

4.1.3.1. DNA and protein binding

An analysis was made for the possibility of DNA and protein binding of the metabolites obtained in the skin metabolism simulator. The ability of the metabolites of Bexarotene and its esters E1, E2, E3 and E4 to bind to DNA and proteins are presented in Table 32.

Table 32. Binding of Bexarotene and Bexarotene esters metabolites to DNA and proteins.

DNA binding	**Structural alert**	No alert found	***Protein binding***	**Structural alert**	No alert found
	Mechanistic alert	-		**Mechanistic alert**	-
	Mechanistic domain	-		**Mechanistic domain**	-
Metabolite №	***Bex***	1	***Metabolite №***	**Bex**	1
	E1	1-4		**E1**	1-4
	E2	1-4		**E2**	1-4

	E3	1-4			**E3**	1-4
	E4	1-4			**E4**	1-4

Based on the analysis, it is believed that the structure of Bexarotene and its esters have the ability to form metabolites that cannot bind to either DNA or proteins.

4.2. Prediction of oral activity of Bexarotene, Bexarotene metabolites and newly synthesized esters using Molinspiration software

4.2.1. Determination of oral activity by Molinspiration software

The ideal drug molecule must have certain physicochemical properties that satisfy Lipinski's rule. The latter predicts the possibility that a biologically active molecule can be taken orally. According to Lipinski's rule, a molecule must have a molecular mass < 500 daltons; octanol-water partition coefficient (miLogP) < 5; < 5 hydrogen bond donors; < 10 hydrogen bond acceptors; polar surface area of 140 A˚; < 10 rotating connections.

If a compound does not violate more than one rule, then it will have good pharmacokinetic properties and increased bioavailability in the body's metabolic process.

Determination of the pharmacokinetic properties of Bexarotene and the newly synthesized Bexarotene esters was done using Molinspiration software. The results of the analysis are presented in Table 33.

The rate of absorption depends on the lipophilicity of the drug molecule. Lipophilicity is expressed as the logarithm of the ratio of the drug that distributes in the organic phase to that in the aqueous phase, or ‚LogP'. There are both physical and mathematical methods for measuring LogP. In this case, LogP is calculated using the methodology developed by Molinspiration as the sum of fragment-based contributions and correction factors. The method can handle practically all organic and most organometallic molecules.

The molecular polar surface is calculated as the sum of the fragment contributions. Polar fragments with oxygen O- and nitrogen N-atoms are considered. Polar surface area has been shown to be a parameter characterizing drug absorption, including intestinal absorption, bioavailability, colon adenocarcinoma (Caco-2) permeability, and blood-brain barrier penetration.

Table 33. Pharmacokinetic parameters of Bexarotene and newly synthesized Bexarotene esters obtained using Molinspiration software.

Property	Bex	E1	E2	E3	E4
miLogP	6.81	7.07	7.44	7.95	8.41
TPSA	37.30	26.30	26.30	26.30	26.30
Molecular mass	348.49	362.51	376.54	390.57	404.59
Number of H^- acceptors	2	2	2	2	2
Number of H^- donors	1	0	0	0	0
Number of violations	1	1	1	1	1
Number of rotatable bonds	3	4	5	6	7
Molecular volume	348.76	366.29	383.09	399.89	416.69

An increase in molecular mass leads to a decrease in permeability in the intestine and in the central nervous system. Therefore, compounds with larger molecular mass are less likely to be orally active.

The number of rotatable bonds is a measure of molecular flexibility. The bioavailability of drugs depends on it. A rotatable bond is defined as any non-ring single bond bonded to a non-terminal heavy (ie, non-hydrogen) atom. Amide C-N bonds are not considered because of their high rotational energy barrier.

After the study, it was found that according to Lipinski's rule, all compounds give 1 violation. It follows that both Bexarotene and the newly synthesized esters are orally active, i.e. have good pharmacokinetic indicators and increased bioavailability in the body's metabolic process.

4.2.2. Determination of bioactivity by Molinspiration software

Drug-likeness can be defined as a complex balance of different molecular properties and structural features that determine whether a given molecule is similar to a known drug. These properties, mainly hydrophobicity, electron distribution, hydrogen bonding characteristics, size and flexibility of the molecule and of course the presence of various pharmacophoric

characteristics affect the behavior of the molecule in a living organism, including bioavailability, transport properties, protein affinity, reactivity, toxicity, metabolic stability and many others.

The variety of possible drug targets (each requiring a different combination of matching molecular features) is so vast that it is possible to find a common denominator for all of them and express the similarity of the drug molecule by a single ‚magic number'. Simple counting criteria (such as molecular weight limits, logP, or number of hydrogen bond donors or acceptors) also have relatively limited applicability and are only useful for discarding obvious non-drugs. The higher the value, the better a compound binds to the particular receptor or enzyme.

Again, an analysis of the bioactivity of Bexarotene and the newly synthesized Bexarotene esters was performed using Molinspiration software. The results of the analysis are presented in Table 34.

Table 34. Bioactivity results of Bexarotene and newly synthesized Bexarotene esters obtained using Molinspiration software.

Property	**Bex**	**E1**	**E2**	**E3**	**E4**
GPCR ligand	0.47	0.32	0.28	0.30	0.33
Ion channel modulator	0.14	0,04	0.06	0.05	0.07
Kinase inhibitor	0.01	-0.06	-0.11	-0.09	-0.07
Nuclear receptor lignad	0.92	0.71	0.70	0.68	0.68
Protease inhibitor	0.02	-0.10	-0.13	-0.11	-0.07
Enzyme inhibitor	0.39	0.24	0.20	0.23	0.25

From the obtained data, it can be seen that Bexarotene has the greatest biological activity of all the compounds. However, the esters themselves have activity against the receptors and enzymes listed in Table 34. In such a case, the esters do not fully meet the definition of prodrugs. On the other hand, the activity of esters against the same receptors and enzymes would increase the activity of the drug. From Section 4.1. we found that esters release Bexarotene after metabolism, so it follows that the administration of Bexarotene in the form of a pro-drug (ester)

should be at lower doses and probably less frequently. This, in turn, will increase patient 'compliance'.

4.3. Prediction of absorbtion, distribution, metabolism, excretion and toxicity of Bexarotene and newly synthesized Bexarotene esters using PreADME/Tox software

Predicting the pharmacokinetic and toxicological profile of molecules is essential in drug development, saving time, cost and unnecessary animal testing. The obtained results of the *in silico* analysis are described in Table 35.

Table 35. Pharmacokinetic and toxicological parameters of Bexarotene and newly synthesized Bexarotene esters using PredADME/T software.

ADME/T parameters	**Bex**	**E1**	**E2**	**E3**	**E4**
Absorbtion					
HIA	97.93	100	100	100	100
Caco-2	22.220	27.559	42.081	43.436	44.676
Skin permeability	-0.7892	-0.7898	-0.7696	-0.7324	-0.7089
Distribution					
PPB	100	100	100	100	100
BBB	1.8619	9.2775	11.216	13.846	15.833
Metablism					
CYP3A4 inhibitor	-	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP3A4 substrate	Substrate	Substrate	Substrate	Substrate	Substrate
CYP2C19 inhibitor	-	-	-	-	-
CYP2C9 inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP2D6 inhibitor	-	-	-	-	-
CYP2D6 substrate	-	-	-	-	-
Renal excretion					
MDCK	0.0775394*	0.0733903*	0.0656403*	0.0989707*	0.0927979*

Toxicity					
Ames test	+	+	+	+	+
Carcinogenicity in rats	-	+	-	-	-
Carcinogenicity in mice	-	-	-	-	-
hERG Inhibition	Medium	Medium	Medium	Medium	Medium

Human intestinal absrobtion (HIA): Low absorbtion 0.00 – 20.00 %; Moderate absorbtion 20.00 – 70.00 %; Excellent absorbtion 70.00 – 100.00 %;

Caco-2 cell permeability: High permeability > 70.0 nm/sec; Medium permeability 4.0 - 70.0 nm/sec; Low permeability < 4.0 nm/sec;

Skin permeability: values vary from -3.00 to 6.00;

Plasma Protein Binding (PPB): Strong connection > 90.0%; Weak connection < 90.0%

Blood Brain Barrier (BBB): High CNS absorbtion > 2.00; Intermediate CNS absrobtion 0.10 ÷ 2.00; Low CNS absrobtion < 0.10

MDCK: Low permeability < 25.0 nm/sec; Medium permeability 25.0 ÷ 500.0 nm/sec; high permeability > 500.0 nm/sec

Ames test: Positive: mutagenic; Negative: non-mutagenic.

Carcinogenicity: Positive: carcinogenic; Negative: non-carcinogenic.

The absorption, distribution, metabolism, excretion and toxicity of the newly synthesized molecules were evaluated. To assess the absorption profile, two main predictive models were analyzed, that of permeability in colon adenocarcinoma (Caco-2)-derived cells and the rate of human intestinal absorption (HIA). Caco-2 cells have several mechanisms for drug transport across the intestinal epithelium and are mainly used as an *in vitro* model to evaluate the absorption of oral drugs. The HIA is the sum of the bioavailability and absorption of the test compound, estimated from the ratio of excretion or cumulative excretion in urine, bile, and feces. From the obtained data, it can be seen that the intestinal absorption after oral administration for the esters is excellent (100%). All molecules were observed to have an average cellular permeability, with permeability increasing with increasing molecular mass.

To predict the distribution profile, the ability to bind to plasma proteins and the ability to pass through the blood-brain barrier were evaluated. The degree of binding to plasma proteins is of utmost importance, since the process of its distribution from the body affects the

availability, action and subsequent efficacy of the drug and must be taken into account when determining the appropriate dosage. Thus, all analyzed compounds were found to have a high affinity for plasma proteins (100%).

A good *in vivo* distribution is assumed to have molecules with lower binding to plasma proteins. Pharmacologically active is the unbound drug because in free form such molecules can diffuse across biological membranes and reach their specific targets.

Penetration through the blood-brain barrier is an important pharmacokinetic parameter for the analysis of potential drugs to be considered for the treatment of CNS diseases.

Among the analyzed compounds, it was observed that Bexarotene has the lowest degree of absorption through the blood-brain barrier. All newly synthesized Bexarotene esters have a high degree of absorption through the blood-brain barrier, most of them with values above 10.00. Treatment of CNS tumors with Bexarotene ester should be more effective than with Bexarotene because the ester acts as a pro-drug and after crossing the blood-brain barrier, Bexarotene will be released. This will provide an opportunity to expand knowledge about the action of Bexarotene in the treatment of other diseases, such as Alzheimer's disease, Parkinson's disease and schizophrenia.

The metabolism of esters is assessed by their ability to inhibit four enzymes of the cytochrome P450 (CYP450) complex, which consists of a family of liver enzymes responsible for the metabolism of endogenous and xenobiotic substances in the human body. CYP450 is responsible for the metabolism of approximately 90% of xenobiotics in the human body, including drugs, which is mainly carried out by CYP3A4, CYP2C19, CYP2C9 and CYP2D6. Inhibition of some of these enzymes indicates the potential of the tested drug to interact with the metabolism of other drugs that use the same biotransformation pathway, making them accumulative and therefore toxic to patients.

Of the compounds analyzed, all are CYP2C9 inhibitors and only Bexarotene is not a CYP3A4 inhibitor.

CYP2C9 constitutes about 18 % of the cytochrome P450 protein in liver microsomes. About 100 drugs are metabolized by CYP2C9, including drugs with a narrow therapeutic index such as warfarin and phenytoin and other commonly prescribed drugs such as acenocoumarol, tolbutamide, losartan, glipizide, and some nonsteroidal anti-inflammatory drugs.

CYP3A4 metabolizes about 30% of prescription drugs. Substrates for the isoenzyme include acetaminophen, codeine, cyclosporine (ciclosporin), diazepam, and erythromycin. The enzyme also metabolizes some steroids and carcinogens. The data obtained show that Bexarotene and its esters are also substrates for CYP3A4.

It follows that compounds that are inhibitors of the relevant isoenzymes should not be administered with other drugs or xenobiotics substrates for the cytochrome P450 isoenzymes due to the risk of increasing their plasma concentrations.

To analyze the ester excretion profile, the MDCK cell permeability model was evaluated, used to predict drug excretion. All compounds had low permeability to MDCK cells in an *in silico* assay, suggesting that these molecules would have a long renal excretion time.

The toxicity of the newly synthesized esters should also be analyzed, especially parameters such as carcinogenicity, mutagenicity and cardiotoxicity. The most commonly used antineoplastic drugs are mutagenic and teratogenic in *in vivo* tests.

The model for predicting mutagenicity of esters is the Ames test. It was developed in 1972 and uses strains of *Salmonella typhimurium* bacteria. The latter are used to assess whether the tested compounds can generate damage to bacterial DNA. In this model, all tested compounds were confirmed to have a mutagenic effect. Further *in vitro* and *in vivo* studies are needed to demonstrate mutagenicity and to verify whether the therapeutic benefits of these newly synthesized esters outweigh the risks of their use.

When assessing toxicity, the carcinogenicity of the newly synthesized esters must also be analyzed, since many antineoplastic agents lead to the development of secondary cancers. Of the esters analyzed, only E1 showed carcinogenic activity in rats, while none of the newly synthesized compounds showed carcinogenic activity in mice. This indicates a relevant degree of safety for compounds E2, E3 and E4.

Another parameter in evaluating new drug compounds is cardiotoxicity. Inhibition of the hER gene leads to impaired expression of potassium channels and the subsequent occurrence of heart problems. In some cases, a fatal outcome is possible. The analyzed compounds were observed to have a medium risk in inhibiting the hER gene and therefore were not potentially cardiotoxic derivatives according to *in silico* tests.

After evaluating the pharmacokinetic profile of the newly synthesized esters, it was observed that the molecules presented promising intestinal absorption, distribution, metabolism, excretion and toxicity characteristics. Compounds E2, E3 and E4 are seen to have the most promising ADME/T *in silico* profile, showing high intestinal absorption and a satisfactory distribution profile. All three compounds have little renal excretion. Compound E1 showed high toxicity, due to mutagenic and carcinogenic action, probably due to the hydrolysis of E1 to Bexarotene and methanol, described Section 4.1. Compounds E2, E3 and E4 show lower toxicity. The ADME/T profile of compounds E2, E3 and E4 makes them potential CNS antineoplastic agents.

5. GENERAL TOXIC EFFECTS OF SUBSTANCES OBSERVED *IN VIVO* IN RATS AFTER ADMINISTRATION OF BEXAROTENE AND ETHYL ESTER OF BEXAROTENE

The similar pharmaceutical profiles of the newly synthesized Bexarotene esters make it possible to study them *in vivo*. The performed QSAR analysis for the determination of metabolic activity, described in Section 4.1., showed that after metabolism the esters are hydrolyzed to Bexarotene and the alcohol used for their synthesis. This gives us reason to use the ethyl ester of Bexarotene in *in vivo* studies because the ethanol released due to hydrolysis is the least toxic compared to the alcohols derived from the other esters, namely methanol, propanol and butanol.

The total toxicity assay by parts confirmed the conclusions drawn from the *in silico* studies described in Sections 4.2 and 4.3 for the newly synthesized Bexarotene esters.

After administration of ethyl ester of Bexarotene at a concentration of 16.19 mg/ml within 30 minutes, staggering and lack of coordination was observed in one of the rats. In the group treated with a higher dose of ethyl ester, 24.28 mg/ml, two of the rats showed impaired gait and staggering, and another showed hind leg dragging, slowed movements, and difficulty breathing, but preserved exploratory activity.

This side effect was not observed in the Bexarotene-treated group. This effect is probably due to the possibility of esters crossing the blood-brain barrier, as described in section 4.3. This property of theirs would enhance the effect of ethanol hydrolyzed compounds on the CNS.

By the sixth hour after administration, a significant improvement in the locomotor activity of the affected rats was observed.

At the end of the study period, no mortality was reported in the rats.

5.1. Effects of Bexarotene and newly synthesized ethyl ester of Bexarotene on body masses in experimental rodents

The change in body mass in the experimental animals is one of the main indicators for evaluating the toxicological properties/characteristics of the substance under investigation. The results of are presented in Figure 22.

An increase in body weights was observed in rats in all experimental groups. This is an indicator of normal physiological status and absence of suffering in rodents.

In the control group, no statistically significant changes were observed in the studied indicator. In the experimental animals treated with Bexarotene and its ethyl ester, there was a significant increase in body weights. The change in the group treated with ethyl ester of Bexarotene at a dose of 500 mg/m^2 body surface area is the most statistically reliable. ($p < 0.01$).

The peculiarities in the dynamics of body weights point to a low risk of toxicity with a single administration of the retinoid and its newly synthesized ester.

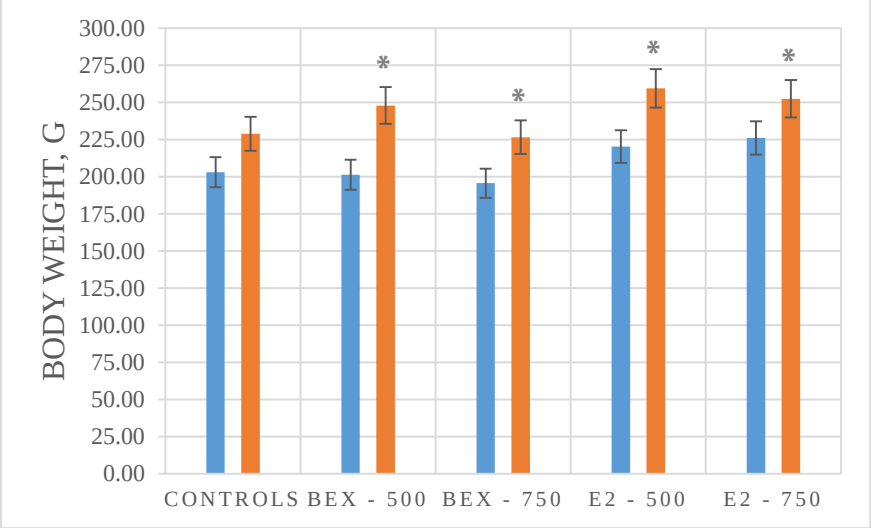


Figure 22. Effects on body weights in rats 72 hours after single administration of Bexarotene and ethyl ester of Bexarotene at doses of 500 and 750 mg/m^2 body surface area (* $p < 0.05$ vs. Controls).

Tracking the change in body weights and reporting the difference in the indicator at the end of the experiment compared to the beginning in individual groups is presented in Figure 23.

A significant increase in the index was observed in the groups treated with Bexarotene and ethyl ester of Bexarotene at a dose of 500 mg/m^2 body surface area vs. controls ($p < 0.01$). In the rats treated with higher doses of Bexarotene and its ethyl ester (750 mg/m^2), the effect was less pronounced, and the values of the indicator were close to those of the controls.

Administration of Bexarotene and its ethyl ester at lower doses resulted in a significant change in body weights. When comparing the dynamics in the indicator between the two doses, the trend is preserved. The change in body weights in the group treated with Bexarotene

750mg/m^2 bw was 33.60 % less than that in the Bexarotene 500 mg/m^2 body surface area group, and at an administered dose of 750 mg/m^2 ethyl ester the change was 32.80% less than the 500 mg/m^2 dose (p = 0.0066).

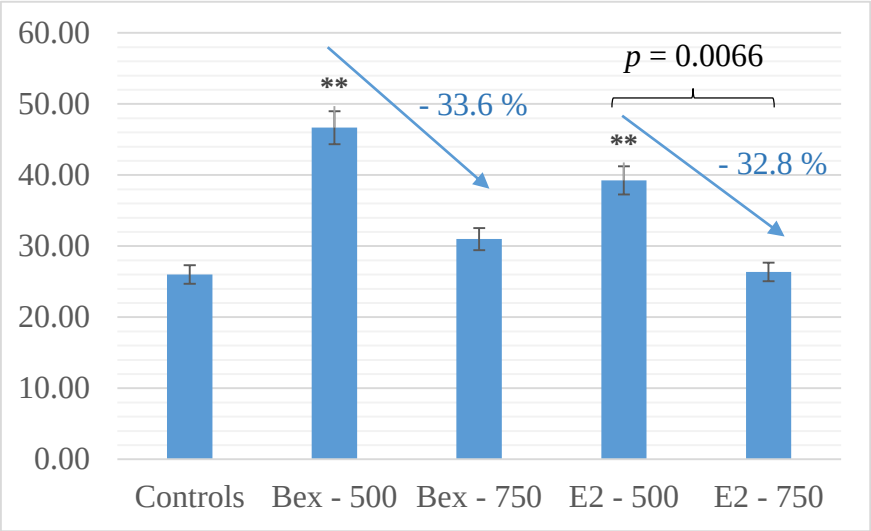


Figure 23. Change in body weights in rats 72 hours after a single oral administration of suspensions of Bexarotene and ethyl ester of Bexarotene at doses 500 and 750 mg/m^2 body surface area - difference finish - start (* $p < 0.05$ vs. Controls; ** $p < 0.01$ vs. Controls).

It can be concluded that there are no symptoms of toxicity based on body weight after oral administration of Bexarotene and its ethyl ester.

5.2. Effects of Bexarotene and ethyl ester of Bexarotene on basic biochemical parameters

5.2.1. Liver enzymes

The liver is the main metabolic organ involved in the biotransformation of both endogenous substrates and xenobiotics, including drugs. Elevated levels of some biochemical markers reflect its damage. An example of this is the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

An increase in the serum levels of these enzymes gives us information about liver damage, but is not always enough to accurately diagnose the problem. Although AST is not liver-specific because it is contained in various organs, such as liver, brain, heart, etc., it can be

used as an indicator of liver damage. On the other hand, elevated ALT values can be an indicator of liver damage. Its serum levels increase mainly in hepatitis and inflammation of the liver.

The ratio of the values of the two enzymes is of great informative value, such as whether the condition is acute or chronic. It also gives an indication of the disease, although it cannot be diagnosed by the AST / ALAT ratio alone.

AST / ALAT ratio < 1 – non-alcoholic fatty liver disease.

AST / ALAT ratio = 1 – acute viral hepatitis or drug toxic effect.

AST / ALAT ratio > 1 – cirrhosis.

AST / ALAT ratio > 2 – alcoholic liver disease.

Determining liver toxicity is an important part of refining the safety level of newly synthesized drug molecules, as the liver is the main metabolizing organ. Conducting experiments on animals is an integral part of the initial toxicological screening and provides information on the onset and extent of damage to this vital organ.

Changes in the concentrations of the AST enzyme of the experimental animals after a single treatment with Bexarotene and its ethyl ester at the indicated doses are presented in Figure 24.

Minor changes in AST enzyme concentrations were observed in the exposed groups compared to the control.

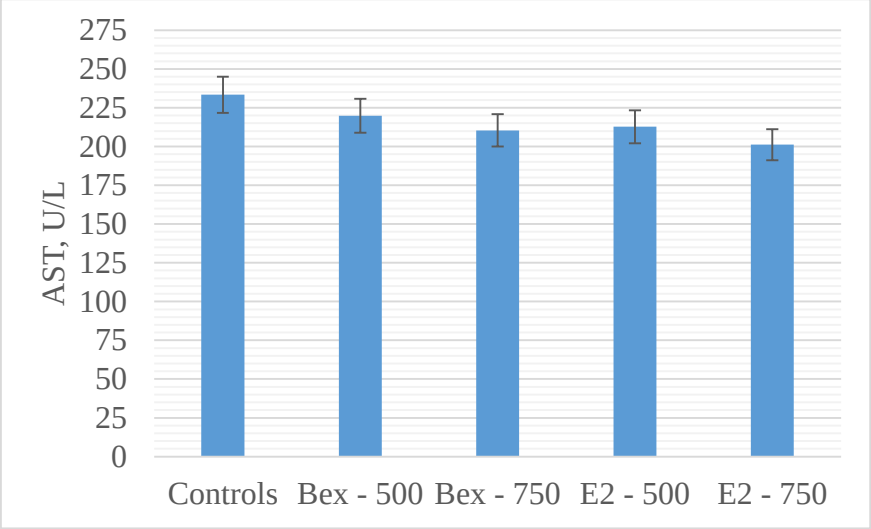


Figure 24. Changes in the concentrations of the AST enzyme of experimental animals after a single treatment with Bexarotene and its ethyl ester at doses of 500 and 750 mg/m^2 body surface.

The changes in ALT enzyme concentrations of experimental animals after a single treatment with Bexarotene and its ethyl ester at the indicated doses are presented in Figure 25.

In the rodents treated with 500 mg/m^2 ethyl ester of Bexarotene, a significant decrease in the indicator was observed compared to the controls (p = 0.012), while no statistically significant changes were reported in the other groups. The difference between Bexarotene and its ethyl ester in doses of 500 mg/m^2 body surface area has a confidence level of p = 0.013, while at the doses of 750 mg/m^2 body surface area one is missing.

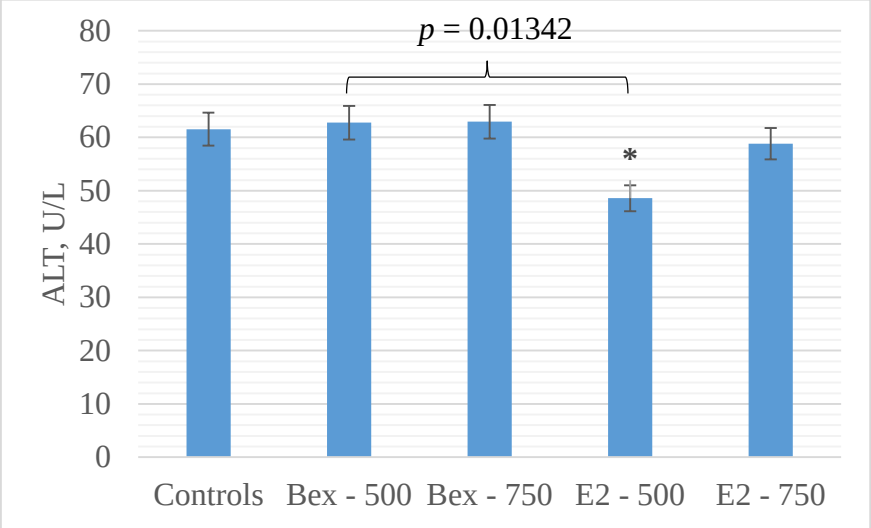


Figure 25. Changes in the concentrations of the AST enzyme of experimental animals after a single treatment with Bexarotene and its ethyl ester in doses of 500 and 750 mg/m^2 body surface (* $p < 0.05$ compared to Controls).

The peculiarities in the dynamics of these liver biochemical markers point to a low risk of hepatotoxicity after a single administration of the retinoid and its newly synthesized ester. There are no literature data on the presence of such organ toxicity of newly synthesized Bexarotene esters. For the first time, such a pilot study is conducted in our experiments.

5.2.2. C-reactive protein

C-reactive protein (CRP) is a cyclic, acute-phase protein synthesized by the liver in the presence of inflammation. It is released within 4 to 6 hours after the initiation of the inflammatory process. Peak concentrations are reached after 36 to 50 hours. CRP is a reliable early marker of inflammatory response.

The changes in the CRP concentrations of the experimental animals after a single treatment with Bexarotene and its ethyl ester at the indicated doses are presented in Figure 26.

Minor variations in CRP concentrations were observed in the exposed groups. The peculiarities in the dynamics of this indicator also point to a low risk of toxicity with a single administration of the retinoid and its newly synthesized ester.

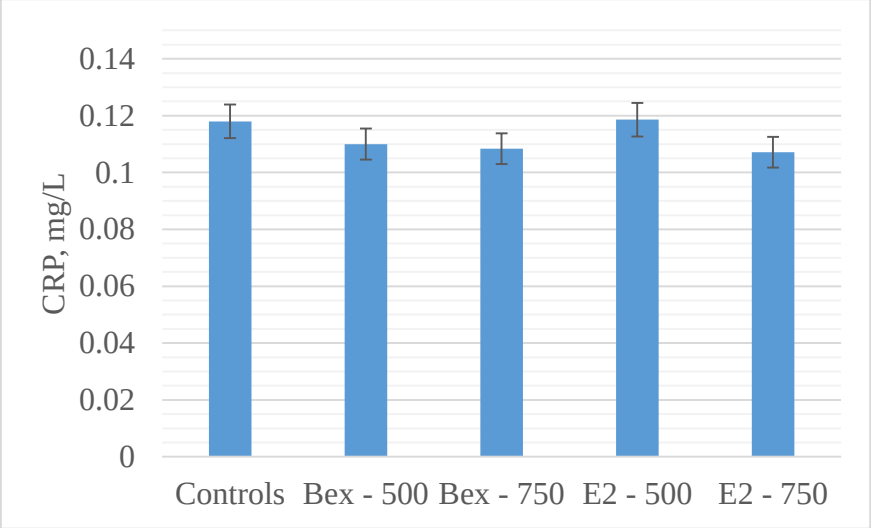


Figure 26. Changes in CRP concentrations of experimental animals after a single treatment with Bexarotene and its ethyl ester at doses of 500 and 750 mg/m^2 body surface.

5.3. Macroscopic examination of the organs after a single administration of Bexarotene and ethyl ester of Bexarotene

Macroscopic examination of organs is one of the main and oldest methods of research and diagnosis. It is performed by dissecting a corpse to determine the cause and manner of death and to assess any disease or injury that may have been present.

The internal inspection of the corpse begins with its dissection. This is intended to allow the examiner to assess anatomic placement and relationship between adjacent organs. Taking and storing organ samples allows a macroscopic assessment of the shape, weight, size, elasticity, consistency and even color of the organs. This would give us information about the possibly occurring acute toxic effects of administered doses of newly synthesized drugs.

In the macroscopic examination of the organs during dissection of the rats 72 hours after oral administration of Bexarotene and ethyl ester of Bexarotene in doses of 500 and 750 mg/m^2 body surface area the following changes are observed:

- Bloating of the intestines and stomach;
- Hematomas of the lungs.

Gastrointestinal problems may be due to physiological factors, while haematomas are likely to have resulted from cauterization of the rats during administration of the solutions. In Appendix № 1, Figures 1 to 5, images from the macroscopic examination are presented.

In conclusion, we can say that Bexarotene and its newly synthesized ethyl esters do not significantly affect body weight and do not have organ toxicity. The compiled safe pharmacological profile of the newly synthesized ethyl ester makes it possible to conduct more in-depth studies on its pharmacokinetic and pharmacodynamic characteristics.

V. CONCLUSIONS

As a result of our research, the following conclusions can be drawn:

1. A synthetic method for obtaining ester products of the antineoplastic drug Bexarotene was developed, with the help of which four new esters were obtained.
2. A method was developed to monitor the reaction process using thin-layer chromatography.
3. The newly obtained compounds were structurally characterized using melting point, IR spectroscopy, UV-VIS spectroscopy and HPLC.
4. A new HPLC method was developed for the determination of Bexarotene and its derivatives alone and in mixtures.
5. A theoretical model was applied to evaluate the potential liver and skin metabolic activity of Bexarotene and its newly synthesized derivatives. It was found that:
   - Four of the theoretical metabolites of Bexarotene have the possibility of binding to proteins, two of them through the formation of Schiff bases and two through nucleophilic addition.
   - Bexarotene derivatives show potential for binding to DNA and proteins, which may be the cause of generation of toxic effects.
6. A theoretical model was applied to evaluate the pharmacokinetic characteristics of Bexarotene and the newly synthesized esters and their bioactivity.
   - Bexarotene esters show good pharmacokinetic characteristics according to Lipinski's rule compared to Bexarotene. A biological activity identical to that of Bexarotene has also been determined.
   - Newly synthesized esters are characterized by good ADME/Tox properties. Their ability to cross the blood-brain barrier makes it possible to treat CNS-related diseases.
7. An *in vivo* model was applied to determine general toxic effects of Bexarotene and ethyl ester of Bexarotene.
   - The results show no liver toxicity of ethyl ester of Bexarotene at a dose of 750 mg/m^2.

VI. CONTRIBUTIONS

- The synthetic approach developed in the ‚Pharmaceutical Chemistry' department for obtaining new ester derivatives of Bexarotene represents a contribution to the chemistry of synthetic retinoids.

- A method for monitoring the reaction process using thin-layer chromatography has been successfully developed.

- Structural characterization of the newly synthesized compounds was successfully carried out by means of melting point, IR spectroscopy, UV-VIS spectroscopy and HPLC.

- A newly developed HPLC method was successfully applied for the determination of Bexarotene and its derivatives alone and in mixtures.

- With the help of theoretical approaches, the possibilities for metabolic activation of Bexarotene, its metabolites and derivatives were successfully identified, including some of their pharmacokinetic characteristics and bioactivity were determined.

- The general toxic effects *in vivo* on the liver of Bexarotene and the ethyl ester of Bexarotene have also been experimentally evaluated.

VII. LIST OF PUBLICATIONS AND PARTICIPATIONS

1. DISSERTATION RELATED PUBLICATIONS

1. Yana Koleva, Svetlana Georgieva, Nadya Agova, **Ivelin Iliev** – Molecular Properties and Bioactivity Score of Newly Synthesized Derivatives of Bexarotene, New Industries, Digital Economy, Society – Projections of the Future II – Booklet of the 58th Science Conference of Ruse University, Bulgaria, 2019, p26-31
2. Nadya Agova, **Ivelin Iliev**, Emiliya Georgieva, Nely Ermenlieva, Svetlana Georgieva - Antibacterial activity of 4-isopropyl-phenyl-methylidene-4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl)ethenyl] benzo hydrazide, Varna Medical Forum, 2021, April 1, p. 336-370
3. Nadya Agova, **Ivelin Iliev**, Silviya Stamova, Svetlana Georgieva - Approaches for hydrazone synthesis with potential biological activity, Academic journal Management and education - June 2021, p. 137-141
4. **I. R. Iliev**, Y. K. Koleva, S. F. Georgieva - Influence of skin metabolites of the newly synthesized derivative of bexarotene and paracetamol on the potential antitumor effect, Bulgarian Chemical Communications, Volume 54, Special Issue B2 (pp. 15-19) 2022.

2. OTHER PARTICIPATIONS

1. Conference with International Interest ,New Industries, Igital Economy, Society – Projections Of The Future‘, Razgrad – 2019.
2. International Scientific Conference ‘Education, Science, Economy and Technologies’, Burgas - September 1, 2020 and 24-25. June 2021.
3. IX-A scientific session for teachers and students of Medical College - Varna - 26-31. March 2021.
4. International Asian Congress on Contemporary Sciences-V - 01-02. June 2021.
5. National program European scientific networks (Drug Molecule) - 30. June 2021.
6. Ninth International Conference ‘Modern Trends in Science’ - 15-19. September 2021.
7. Seventh Pharmaceutical Business Forum and Scientific and Practical Conference ,Digital solutions and innovations in pharmaceutical practice and education - challenges and opportunities‘ - 22-23. October 2021.
8. International Conference On Innovative Studies Of Contemporary Sciences, Tokyo summit - 15. January 2022.
9. 4th International Congress Of Multidisciplinary Studies In Medical Sciences –18-20. February 2022.
10. Ankara Congresi - 02. April 2022.
11. International Conference ,Education, Science, Economics and Technologies‘ – Burgas - 23. June 2022.
12. Scientific Conference ,Sea and Health’ – 20. May 2022.
13. ‘80 years of Medical College – Varna’ - tenth scientific session with international participation - 21-22. October 2022.
14. Eighth Pharmaceutical Business Forum and Scientific-Practical Conference ,Pharmacists and doctors - united to support the patient‘ - 28–29. October 2022.

VIII. FINANCING

Project №. 20008/2020 – ‚Science‘ Fund of the MU - Varna ‚Investigation of the toxicity of Bexarotene hydrazones using *in vitro* and *in vivo* models‘ with the project leader Assoc. Prof. Svelana Fotkova Georgieva, Ph.D., Grassroots organization: Faculty of Pharmacy, MU-Varna.

IX. APPENDICES

APPENDIX № 1

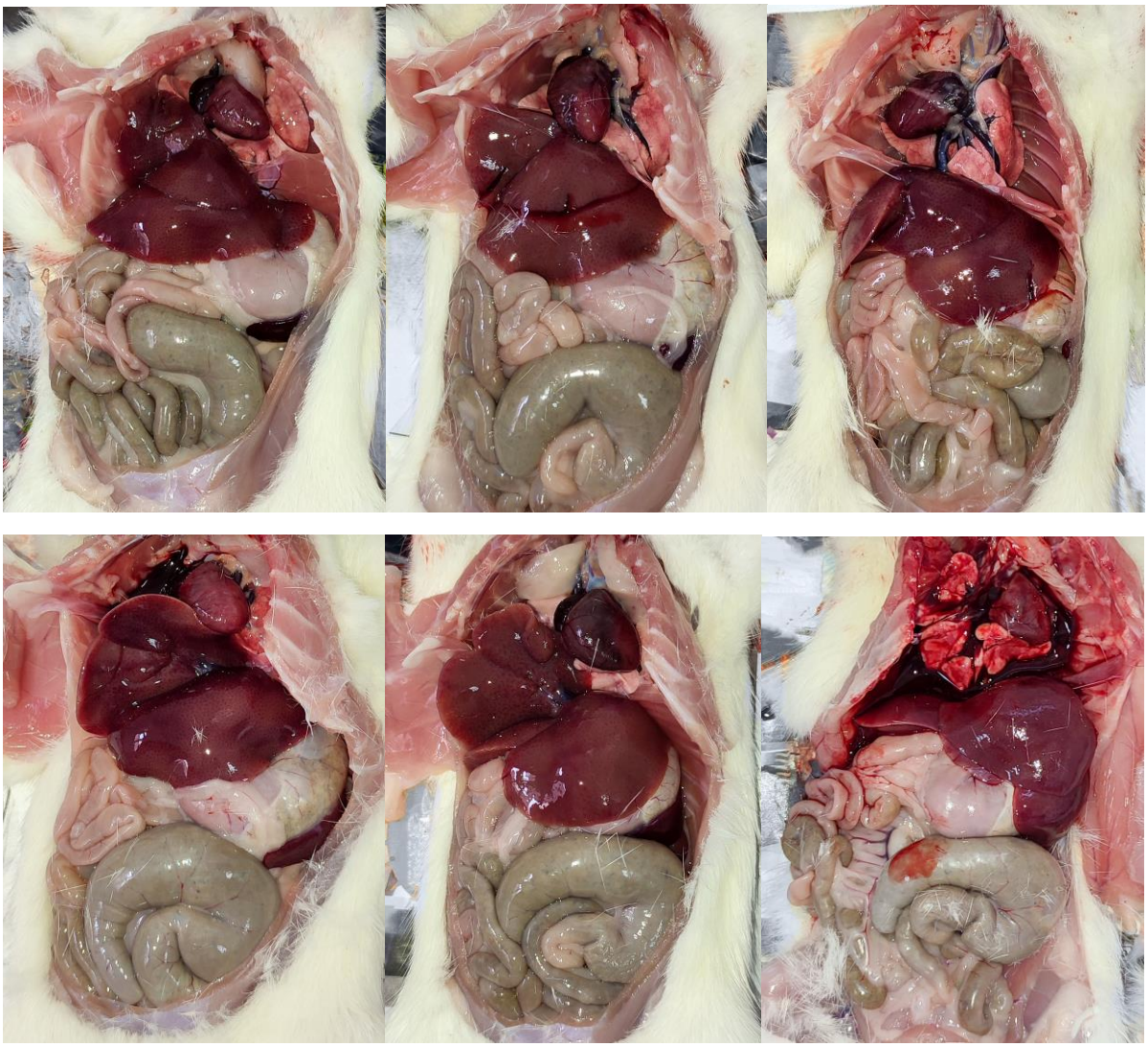


Figure 1. Macroscopic view of control group treated with 25% aqueous ethanol solution.

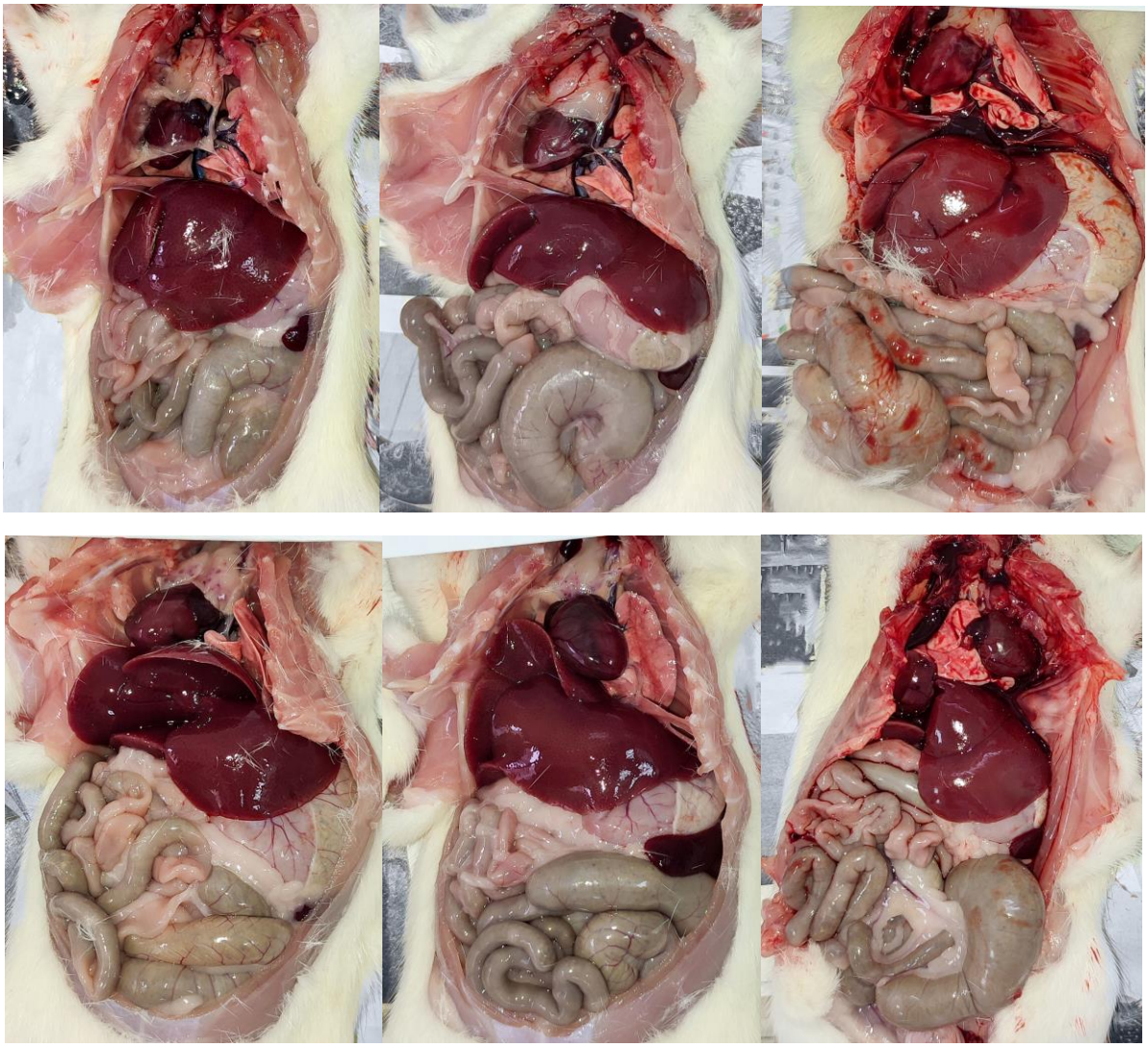
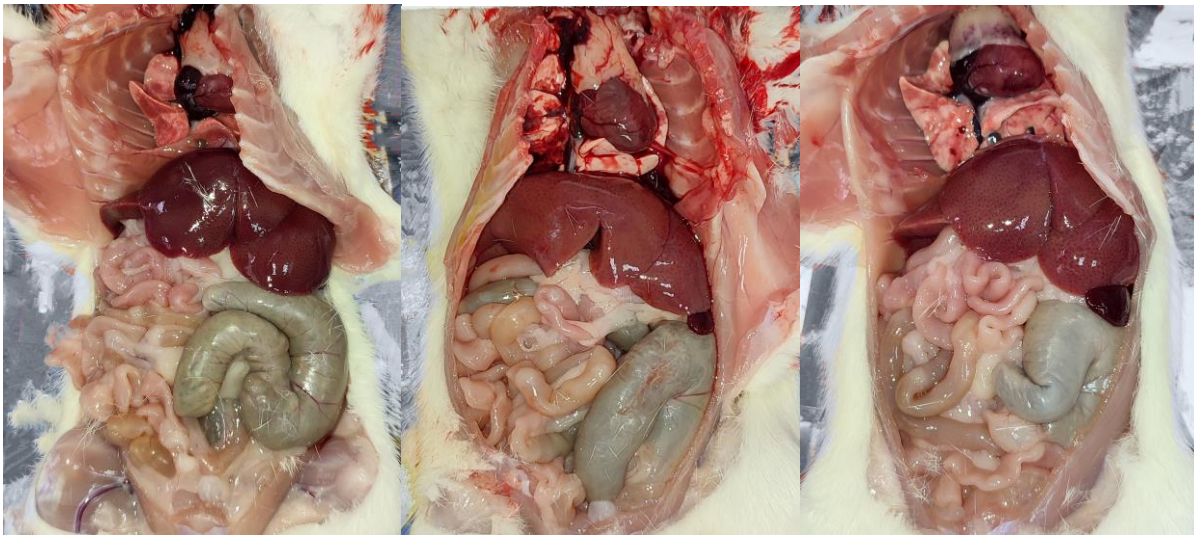


Figure 2. Macroscopic view of rats treated with a dose of 500 mg/m^2 Bexarotene.



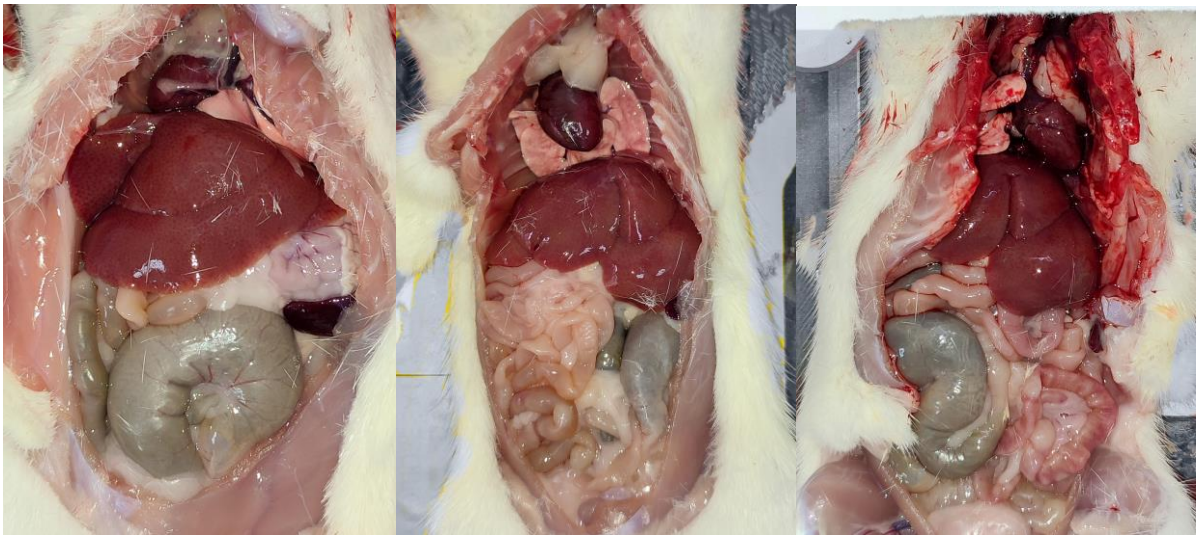
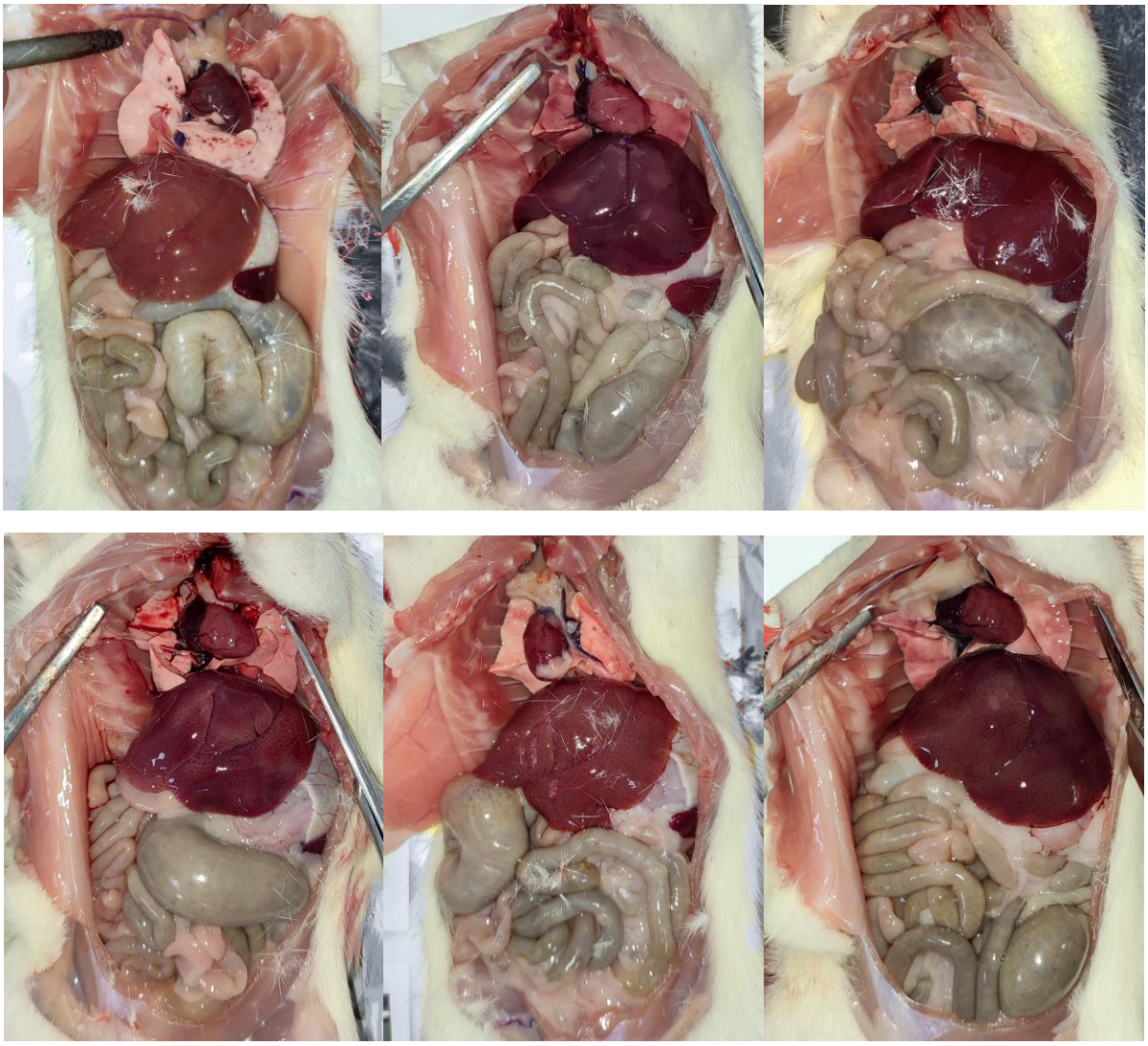


Figure 3. Macroscopic view of rats treated with a dose of 750 mg/m^2 Bexarotene.



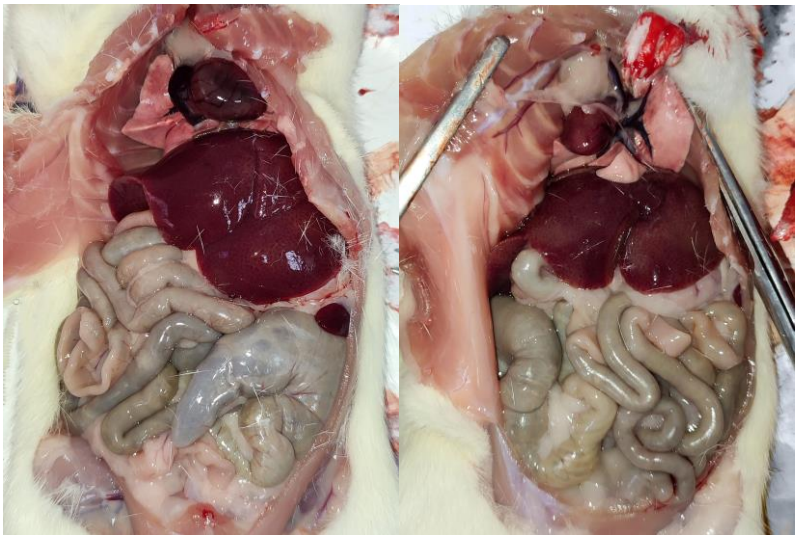
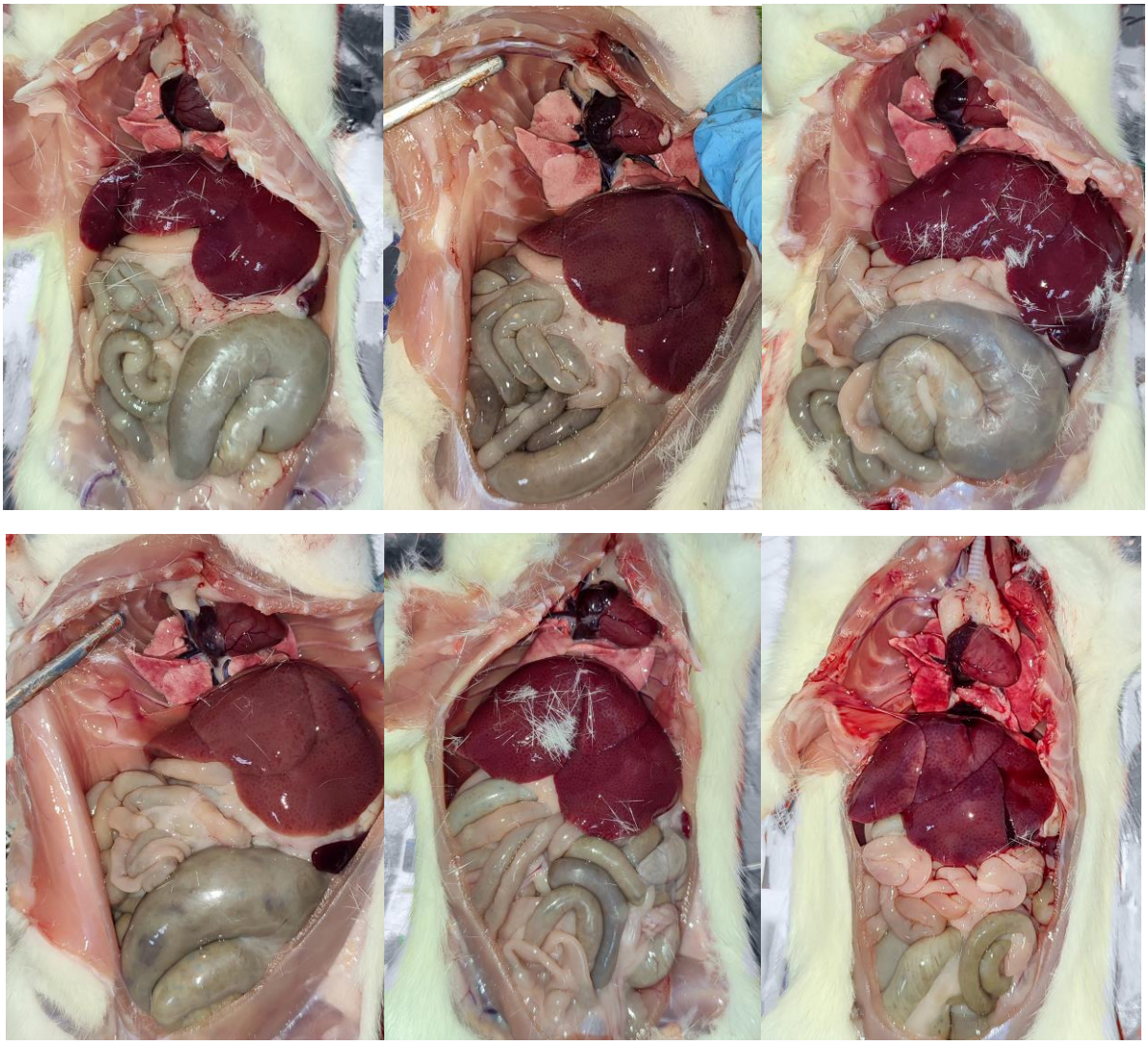


Figure 4. Macroscopic view of rats treated with a dose of 500 mg/m^2 ethyl ester of Bexarotene.



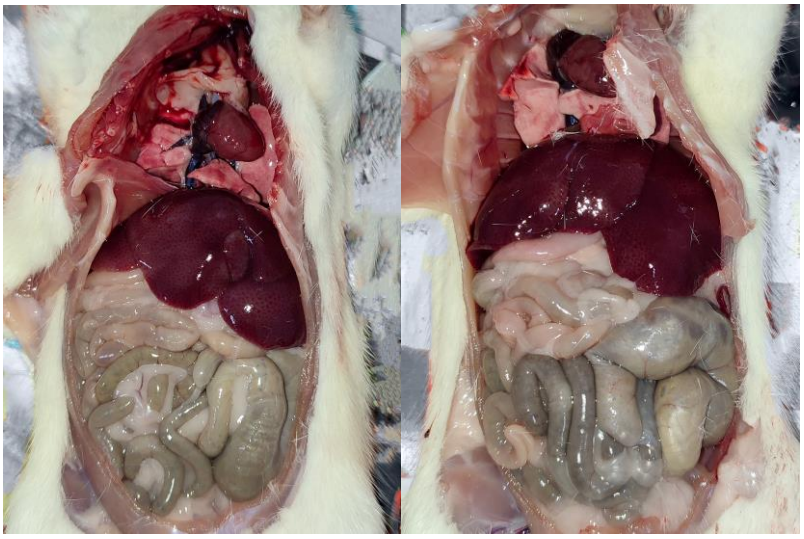


Figure 5. Macroscopic view of rats treated with a dose of 750 mg/m^2 ethyl ester of Bexarotene.