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# HPLC method for analyzing new compounds – analogs of an antineoplastic drug

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Over the past decade, there has been a significant scientific knowledge increment in the field of oncology. Thus, the interest in discovering of new drugs and therapeutic approaches with a potential in oncology has risen. Recently, the group of synthetic retinoids is used in oncology. The third generation retinoid Bexarotene is used in the treatment of various types of cancer, for instance cutaneous T-cell lymphoma, breast cancer, advanced lung cancer, Kaposi sarcoma, prostate cancer, and other. In literature, analyses of Bexarotene include thin-layer chromatography. Moreover, gas chromatography-mass spectrometry has been used to determine the compound and its metabolites. In our previous work, we synthesized a structural analog of Bexarotene. The purpose of the present work is to describe the development and validation of a HPLC method for the analysis of the new compound.

**Key words:** oncology, HPLC, third-generation retinoids, Bexarotene

## INTRODUCTION

Retinoids are a class of chemical compounds structurally related to vitamin A and comprise natural and synthetic analogs. The relation between vitamin A and cancer diseases was first noted in the 1920s when experimentally induced vitamin A deficit was shown to lead to hyperplastic, metaplastic and dysplastic tissue changes such as preneoplastic lesions [1, 2-5].

Recently, it has been shown that retinoids can exert effects on certain fully transformed, invasive, neoplastic cells, leading to a suppression of proliferation. Nowadays, Bexarotene is the first in a novel class of pharmacologic agents, the RXR-selective retinoids or rexinoids, used in the treatment of oncological diseases [6]. The ability of retinoids to bind to different nuclear receptors and the formation of heterodimers cause a variety of effects. Thus, they can be used in the treatment of various cancer diseases [7, 8].

Bexarotene (brand name: Targretin) is approved by the U.S. Food and Drug Administration (FDA) (in late 1999) and the European Medicines Agency (EMA) (in early 2001) for the treatment of cutaneous T-cell lymphoma (CTCL). Its chemical structure is shown in figure 1.

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

Most methods for HPLC analysis of Bexarotene, reported in the literature, have been developed to evaluate Bexarotene in biological samples. Therefore, they are not suitable for routine analysis. Insufficient information on HPLC analysis of the substance Bexarotene and its derivatives necessitates the search for appropriate chromatographic conditions for analysis.

The present study describes a reversed-phase HPLC-UV analysis of new Bexarotene hydrazide-hydrazone derivatives.

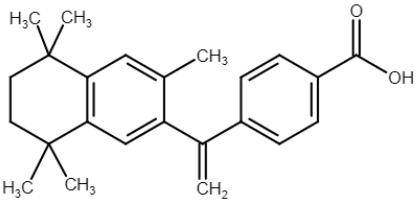


**Fig. 1.** Structure of Bexarotene - 4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl)ethenyl] benzoic acid.

Hydrazide and hydrazone derivatives were synthesized and their structures were confirmed by their spectral data. To determine the newly obtained structures a detailed analysis of the FT-IR spectra was performed. We analyzed the starting compound for synthesis - Bexarotene, its methyl ester and the newly synthesized hydrazone analog. Analysis of the compounds was performed in the range 4000-500 $cm^{-1}$.

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## EXPERIMENTAL

Bexarotene (99.99%, Fluorochem); methyl alcohol (99.99%, HPLC grade, Fisher Chemical), acetonitrile (99.99%, HPLC grade, Fisher Chemical), water (HPLC grade, Fisher Chemical), formic acid (99-100% A.R., CHEM-LAB).

### *Infrared spectroscopy*

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

### *HPLC analysis*

The development of a modification of the Bexarotene analysis approaches consists of the selection of suitable chromatographic conditions, determination of the appropriate wavelength for detection and selection of the mobile phase.

Chromatographic analysis was performed with a High-performance liquid chromatographic - Thermo Scientific UltiMate 3000 Analytical LC System. It was equipped with a quaternary pump (Thermo Scientific Dionex UltiMate 3000 LPG-3400SD Quaternary Pump), an automatic injector (Thermo Scientific Dionex UltiMate 3000 Autosampler), a variable wavelength vibration detector (VWD) and a diode array detector (Thermo Scientific Dionex UltiMate 3000 DAD-3000 Diode Array Detectors).

Bexarotene is poorly water soluble with a maximum solubility of about 10-50 μM in pure water. In this study, the stock standard solution of Bexarotene was prepared by dissolving an appropriate amount of the substance in a precisely defined volume of methanol. The solution was stored at +4 °C. Working standard solutions of defined concentrations were prepared by dilution of the stock solution, again in methanol.

Certified Bexarotene standard substance (20.0 mg) was weighed on an analytical balance. Then it was transferred to a 20 ml-volumetric flask containing 10 ml of methanol. The prepared stock solution has a final concentration of 1.0 mg/ml. Working standard solutions were prepared from the stock solution in a concentration range of 1.0 to 50.0 μg/ml. Five independent chromatographic analyses were performed for each working solution.

The synthesized product was weighed (50.0 mg) into a beaker; 10 ml of methanol was added and sonicated for 10 min. The solution was filtered through a membrane filter (0.22 μm) into a 50 mL volumetric flask containing 10 ml of methanol and the residue was washed with three portions of 10 ml of solvent. The volume of the flask was brought to the mark with methanol to give a 1 mg/ml solution. From this stock solution, test samples were prepared by appropriate dilutions.

In order to achieve good separation of substances under isocratic conditions, solvent mixtures such as formic acid, water, and acetonitrile in various combinations and ratios were tested as mobile phases. Following a number of studies, chromatographic analysis was performed using a mobile phase consisting of 80% of acetonitrile and 20% of 1% formic acid, using the AQUASIL C18 analytical column (150 mm × 4.6 mm, 5 μm) protected with a pre-AQUASIL C18 (10 mm × 4.6 mm, 5 μm). The column temperature was 30 °C. The feed rate of the mobile phase was 0.8 ml/min. The injection volume was 20 μl.

The UV detection of Bexarotene was performed at 260 nm. A wavelength of 260 nm was chosen for the present analysis because it is reported to produce less noise, which facilitates the quantification of the presence of Bexarotene in the mixture. The analysis was performed in an isocratic mode with a total duration of 17.5 min.

The applicability of the method was demonstrated by conducting a validation procedure. According to the requirements of ICH Q2 (R1) Validation of Analytical Procedures we determined the following analytical parameters:

- linearity of the standard curve;
- precision;
- accuracy;
- limit of detection (LOD) and limit of quantification (LOQ).

For the statistical analysis of the results, embedded Excel features and ANOVA compression analysis were used.

### *Linearity of the standard curve*

Linearity is the range of concentrations of the determined component in which the detector signal is linear in relation to its concentration. A standard solution of Bexarotene (1.0 mg/ml) was prepared to construct the standard curve. Six working solutions were obtained by diluting it at concentrations of 1.0, 10.0, 20.0, 30.0, 40.0, 50.0 μg/ml. Samples were injected five times. The linearity was estimated by the standard rule equation and the correlation coefficient R2. The calibration curve of the standard Bexarotene solution in a concentration range of 1.0 - 10.0 μg/ml is presented in figure 2.

### *Precision*

Precision is a parameter to show the similarity of results. A quantitative measure of precision is the standard deviation (SD), calculated on the basis of n measurements, as well as the relative standard

deviation (RSD). Repeatability (consecutive five-fold analysis at three concentration levels, within the day, under the same analytical conditions, by one operator) and reproducibility within the laboratory (sequential five-fold analysis of three concentration levels on different days) were evaluated. The results are presented in table 1.

### *Accuracy*

Accuracy is determined by calculating the bias value, as well as the relative percent deviation (b%) taking into account the real value of the standard substance of each of the analyzed samples. A six-fold injection was performed at the six concentration levels. The results are presented in table 2.

HPLC methods have been widely used for testing mixtures of substances. Without the development of an effective liquid chromatography method, it would not be possible to analyze mixtures of substances obtained during synthesis.

Different mobile phases were tested to optimize the analytical performance. In order to achieve sharp peaks and an appropriate separation of the components, several experiments with various compositions of solvents and various flow rates were conducted.

## RESULTS AND DISCUSSION

The three new Bexarotene analogs were synthesized according to scheme 1.

In the first step, one of the most versatile esterification methods, namely the reaction of the acid chloride with alcohol, was used to prepare Bexarotene methyl ester.

By the direct interaction of thionyl chloride with Bexarotene in methanol, an esterification process takes place in which Bexarotene reacts with thionyl chloride to form acyl chlorides. The acid chloride obtained *in situ* is extremely reactive and reacts with methanol to form the corresponding methyl ester. The methyl ester thus formed is isolated and then introduced into the next step of hydrazone synthesis - preparation of Bexarotene hydrazide.

The preparation of the hydrazide is based on the interaction of the intermediate methyl ester with hydrazine.

As a result of this approach, a precursor product for the synthesis of the corresponding hydrazone derivatives of Bexarotene is obtained. It is applied in the next step of the synthesis without isolation.

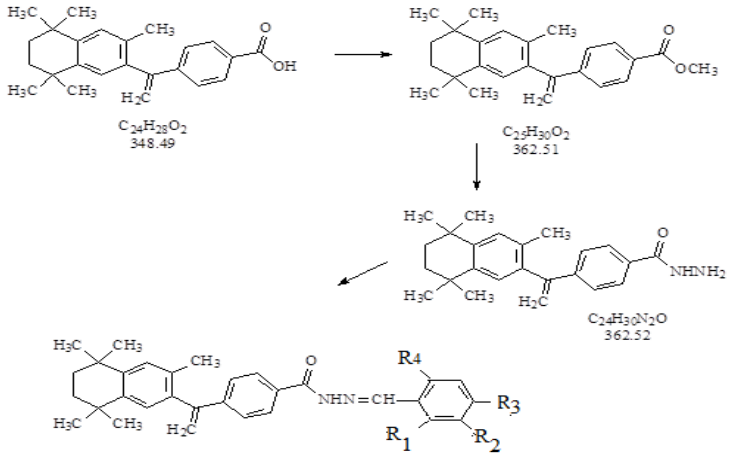
After the procedure described the partner aldehydes were incorporated and three hydrazone derivatives of Bexarotene were obtained.

The following derivatives were obtained:

The V1 compound is a hydrazone derivative of 3-chlorophenyl benzaldehyde and Bexarotene - 3-chlorophenyl-methylidene-4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2 yl)ethenyl] benzohydrazide. In its structure the substituents are as follows: R2- Cl, R1=R3=R4=H.

The V2 compound is a hydrazone derivative of 4-(trifluoromethyl) benzaldehyde and Bexarotene - 4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl)ethenyl]-N'-[(E)-[4-(trifluoromethyl)phenyl] methylidene]benzohydrazide. In its structure the substituents are as follows: R3=CF3, R1=R2=R4=H.

The V3 compound is a hydrazone derivative of 2,6-dichlorobenzaldehyde and Bexarotene –2,6-dichlorophenyl-methylidene-4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl)ethenyl] benzohydrazide In its structure the substituents are as follows: R1=R4=Cl, R2=R3=H.




**Scheme 1.** General scheme of Bexarotene analogs synthesis.

The structures of Bexarotene, its methyl ester and the newly synthesized analogs were confirmed by IR analysis.

The valent C-H oscillations in the mono substituted benzene derivatives are expressed as a triplet at about 3100 $cm^{-1}$.

Referring to literature data, the spectrum of the ν(C=N) band in the hydrazone structure is found in the range of 1609-1585 $cm^{-1}$. Similar bands were described for the newly obtained compounds and were not observed in the parent Bexarotene structure.

After the structures were elucidated, chromatographic analysis was performed.

We have developed an HPLC analysis method by which we aimed to detect the presence of unreacted amount of Bexarotene in the newly synthesized products and also the possible side products and impurities resulting from the synthesis. This will permit optimizing of the reaction conditions, as well as increasing the yield of the resulting synthesis.

Bexarotene was subjected to chromatographic analysis. As a result, following chromatograms were obtained (figures 3, 4, 5).

Only one peak was eluted at a retention time of 7.07 min. It indicated the presence of Bexarotene in the sample (figure 3).

Chromatograms of Bexarotene at different concentration levels are presented in figure 4.

The peak obtained by Bexarotene assay and the calibration curve are shown in figure 5.

The results obtained show that linearity (R2 = 0.9998) was achieved in the target concentration range of 1.0 - 50.0 μg/ml.

The repeatability and reproducibility of the chromatographic method are presented in table 1.

The limit of quantification is calculated on the basis of signal-to-noise ratio (S/N ≥ 10). In our experiment, the limit of quantification of Bexarotene is 0.5 μg / ml.

When the chromatograms of Bexarotene and its methyl ester are compared it is clearly visible that two peaks are distinct. The peak eluted at a retention time of 13.83 min is consistent with an ester. The results are presented in figure 6.

The retention times of the obtained Bexarotene analogs were determined (table 3). Their chromatograms are represented in figures 7, 8, 9.

The $V_1$ compound is a hydrazone derivative of 3-chlorophenyl benzaldehyde and Bexarotene - 3-chlorophenyl-methylidene-4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2 yl)ethenyl] benzohydrazide.

The $V_2$ compound is a hydrazone derivative of 4-(trifluoromethyl) benzaldehyde and Bexarotene-4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl)ethenyl]-N'-[(E)-[4-(trifluoromethyl)phenyl]methylidene] benzohydrazide.

The $V_3$ compound is a hydrazone derivative of 2,6-dichlorobenzaldehyde and Bexarotene – 2,6-dichlorophenyl-methylidene-4-[1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl) ethenyl]benzohydrazide.

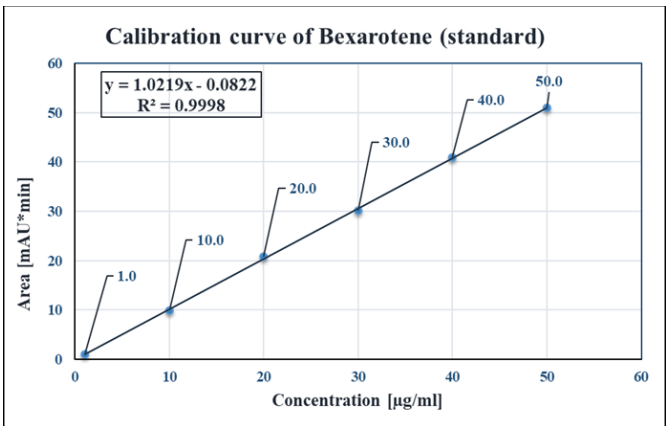



**Fig. 2**. Calibration curve of standard Bexarotene solutions in the concentration range of 1.0 - 50.0 μg/ml.

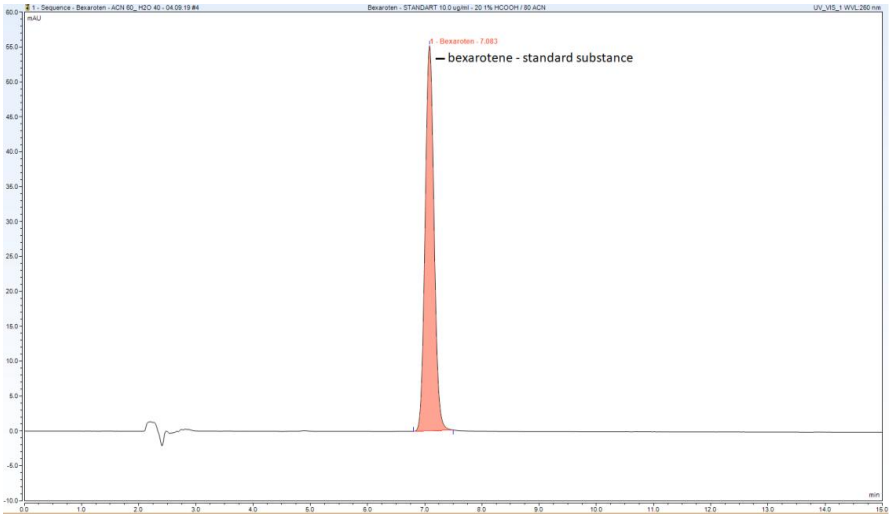



**Fig. 3.** Chromatogram of a standard solution of Bexarotene..

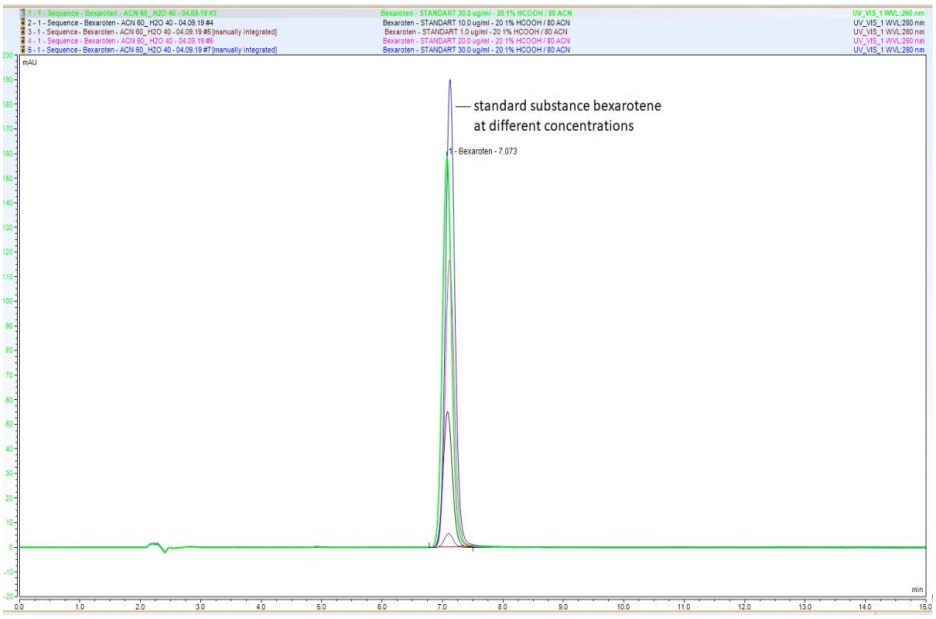



**Fig. 4.** Chromatograms of Bexarotene standard substance measured at different concentration levels.

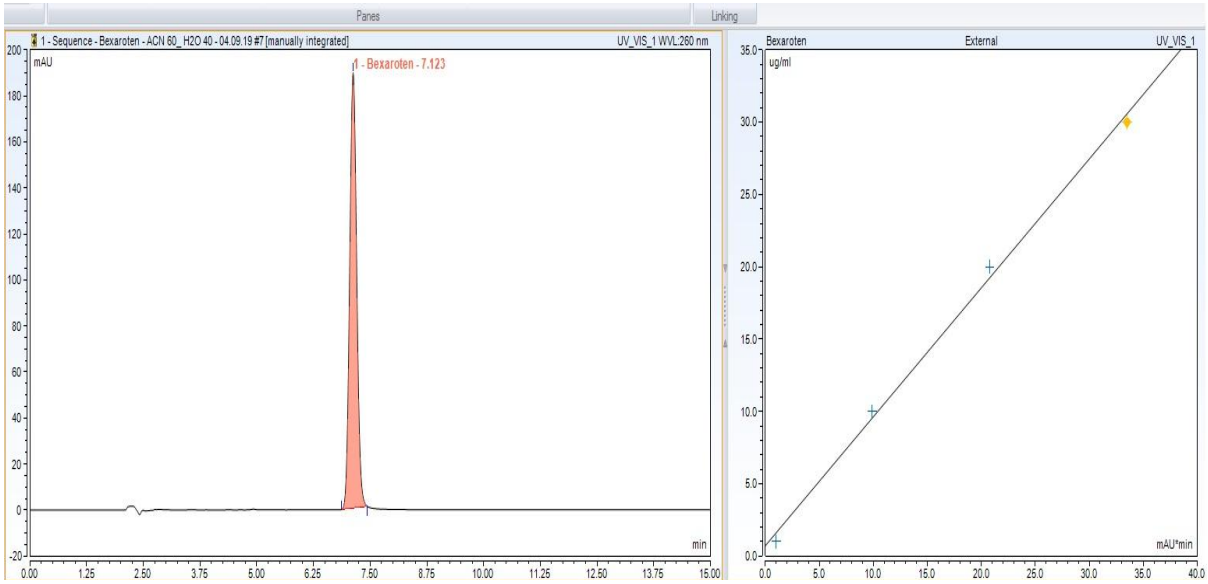



**Fig. 5.** Chromatogram of Bexarotene and a calibration curve.

**Table 1.** Evaluation of the repeatability and reproducibility of the chromatographic method

| Substance | Concentration [µg/ml] | In-Day Analysis | | Intra-Day Analysis | |
|---|---|---|---|---|---|
| | | SD | RSD [%] | SD | RSD [%] |
| Bexarotene | 1.00 | 0.0003231 | 0.0320 | 0.0001250 | 0.0125 |
| | 20.0 | 0.0002119 | 0.0011 | 0.0025532 | 0.0127 |
| | 50.0 | 0.0005001 | 0.0010 | 0.0002876 | 0.0006 |

The calculated RSD values are eligible and fall within the range of 0.0006 - 0.0320%.

**Table 2.** Evaluation of the accuracy of the chromatographic method

| Substance | Concentration [µg/ml] | bias | b [%] |
|---|---|---|---|
| Bexarotene | 1.00 | 0.00035 | 0.035 |
| | 10.0 | 0.00500 | 0.050 |
| | 20.0 | 0.00100 | 0.005 |
| | 30.0 | 0.00120 | 0.004 |
| | 40.0 | 0.01700 | 0.043 |
| | 50.0 | 0.00480 | 0.010 |

The calculated values of [b%] are eligible and fall within the range of 0.004 - 0.050%.

**Table 3.** Retention times of the Bexarotene analogs

| Bexarotene analog | Retention time (min) Bexarotene analog | Retention time (min) Bexarotene | Retention time (min) methyl ester of Bexarotene |
|---|---|---|---|
| *V1* | 7.450 | 7.00 | 13.92 |
| *V2* | 5.942 | 7.00 | 13.92 |
| *V3* | 6.703 | 7.00 | 13.92 |

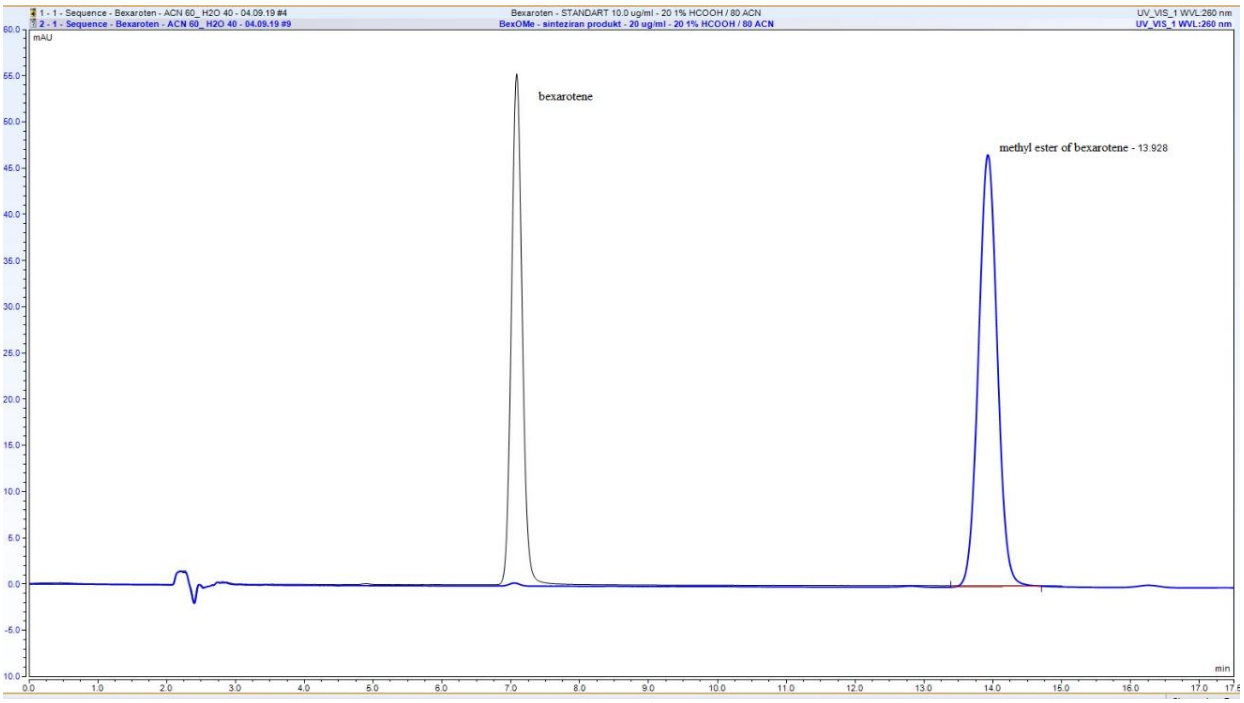



**Fig. 6.** Chromatograms of Bexarotene and of its methyl ester

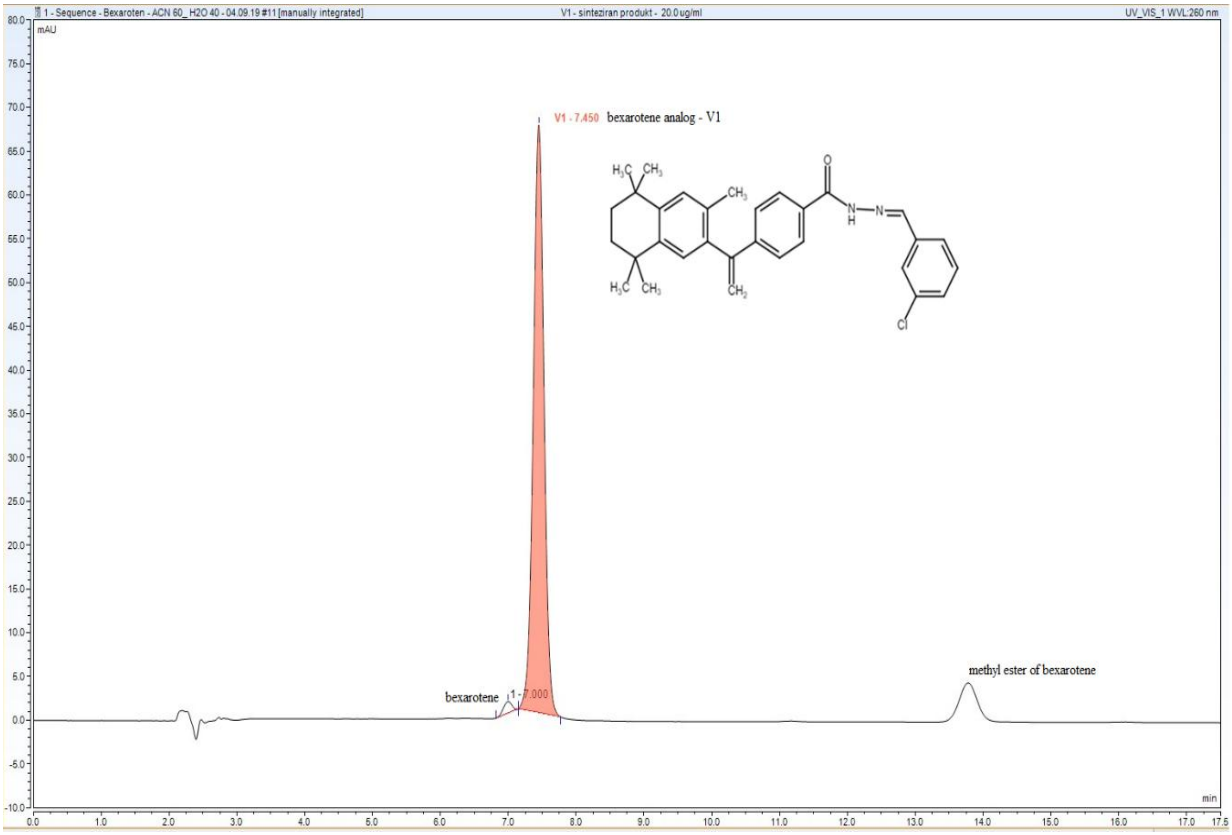



**Fig. 7.** Chromatogram of Bexarotene analog $V_1$.

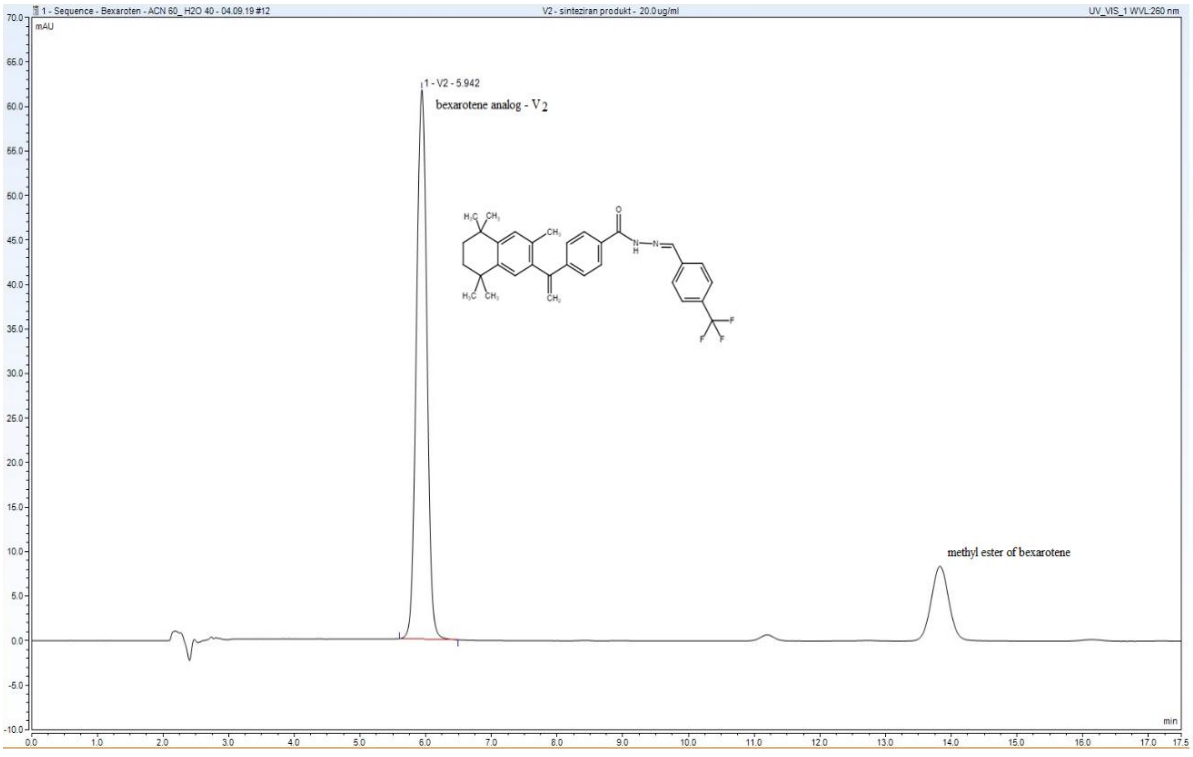



**Fig. 8.** Chromatogram of Bexarotene analog $V_2$.

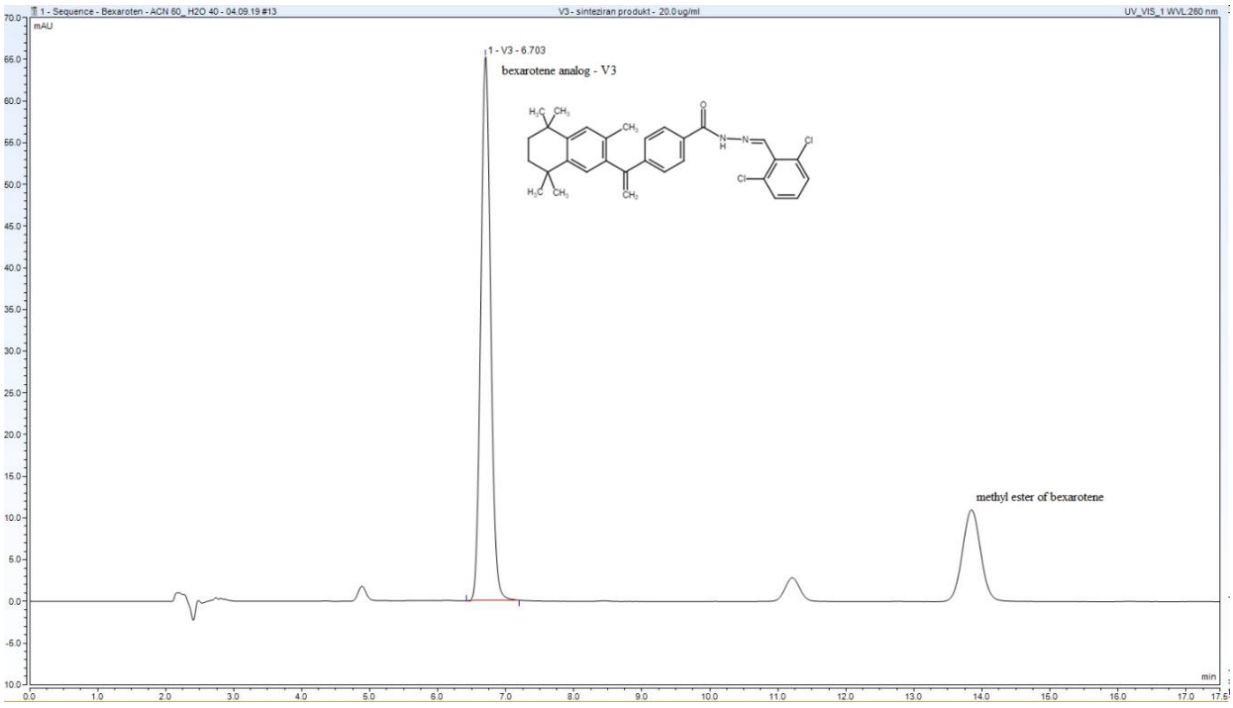



**Fig. 9.** Chromatogram of Bexarotene analog $V_3$.

## CONCLUSION

The HPLC method developed allows the composition of the resulting compounds to be analyzed.

As a result of the analysis it can be concluded that the samples obtained contain an insignificant amount of unreacted methyl ester.

In order to optimize the synthesis and to avoid the presence of unreacted methyl ester or aldehydes in the preparation of hydrazone derivatives of Bexarotene, a change in the duration of the synthesis is possible.

Using HPLC for testing the new compounds enabled us to conclude on the content of the samples obtained, the presence of unreacted starting substances and impurities, as well as to analyze newly obtained hydrazone analogs of the antineoplastic drug bexarotene.

The results show that the developed and validated chromatographic method for determining the concentration of bexarotene is fast, simple and suitable for routine analyses in daily laboratory practice. It is characterized by good linearity and high precision. The sample preparation and analysis time are relatively short; the cost of the method is relatively low.

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