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Free Radical Formation in a Pharmaceutical Product Containing Bisoprolol Fumarate Stored Under Different Physical Conditions

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Abstract: The pharmaceutical product is a powdered tablets containing bisoprolol fumarate that is used in the treatment of circulatory system diseases. They were examined by X-band (9.3 GHz) electron paramagnetic resonance spectroscopy. The aim of this work was to determine the influence of the physical conditions of storage on the properties and content of free radicals in this pharmaceutical product. The product was subjected to a temperature of 50 °C, UVA radiation, and UVA radiation and then a temperature of 50 °C. The amplitude, integral intensity, linewidth of EPR lines, and g factor, were analyzed. Free radicals were formed in all tested samples; thus, the product containing bisoprolol fumarate should not be stored at a temperature of 50 °C, and it should be protected from UVA radiation, which is in line with the manufacturer's requirements. The content of free radicals in the examined product was highest after treatment at a temperature of 50 °C. The lowest free radical content characterized the product after the interaction of both UVA radiation and a temperature of 50 °C. EPR lines were not microwave saturated below a power of 70 mW, which indicates fast spin-lattice relaxation processes in the product. It has been demonstrated that free radical formation in the product containing bisoprolol fumarate depends on the type of physical factor.

Keywords: bisoprolol fumarate; thermal factor; UVA radiation; storage conditions; free radicals; EPR

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1. Introduction

Bisoprolol fumarate is used in the treatment of circulatory system diseases [1–26]. It is a β-blocker that blocks β-adrenergic receptors in the heart and vessels, lowering blood pressure and reducing the heart rate and the force of heart contractions. The drug is administered in cases of impaired contractile function of the left ventricle of the heart [1–9,21,22]. This drug reduces vascular spasms in hypertension, reduces the heart's need for oxygen in angina pectoris, and improves heart function in chronic heart failure [1–10]. The mechanism of action of bisoprolol is the inhibition of β_1-adrenergic receptors, which are mainly located in the heart [1,11,16–19,22,23]. The use of drugs that block β1 receptors stops the changes that occur when they are stimulated. It has been shown that bisoprolol may have a protective effect and prevent death or cardiovascular complications in patients after surgery, especially cardiac surgery [23].

Bisoprolol fumarate shows high selectivity towards β_1-adrenergic receptors [1,11,16–19,22,23]. Its high bioavailability can reach up to 90%. The absorption of the drug is independent

of food. About 30% of the drug binds to plasma proteins. The maximum blood pressure lowering effect is achieved approximately 14 days after starting treatment [20,21].

Bisoprolol fumarate can be eliminated from the body in an unchanged form (approximately 50% of the substance) or in the form of inactive metabolites in urine [20,21]. Bisoprolol fumarate is eliminated to the same extent by the kidneys and the liver; thus, there is no need to adjust the dosage in patients with hepatic impairment [20–23].

Another important drug used in circulatory system diseases, namely amlodipine besylate, is also worth mentioning here [27]. Amlodypine besylate, belonging to the group of dihydropyridine derivatives, is a calcium channel blocker used primarily to treat high blood pressure—hypertension and angina—chest pain. Amlodipine besylate has a direct relaxing effect on vascular smooth muscle. Amlodipine besylate can be used in patients of all ages, elderly or younger.

The assumption in this work is the need to store the product containing bisoprolol fumarate under conditions in which no free radicals are formed in the tested substance. The chemical structure of drug should not be destroyed [28–32]. Free radicals can change not only the structure but also the action of the drug. Elevated temperatures and UVA radiation have been taken into account in studies because these factors most often accidentally affect pharmaceutical products due to the environment during improper storage or transport. These studies were planned to emphasize the importance of proper storage conditions for pharmaceutical products, the disruption of which may lead to the formation of reactive free radicals in their structures, which has a negative impact on the use of drugs in medical therapy.

The primary aim of the work is to determine the effect of physical storage conditions on the properties and content of free radicals in a product containing bisoprolol fumarate. Free radicals generated under abnormal product storage conditions were investigated. A tool helpful in determining the optimal storage conditions for pharmaceutical products and substances is electron paramagnetic resonance spectroscopy, which is used to study free radicals [33–41]. Properly stored pharmaceutical samples should not produce EPR signals. Samples placed in a magnetic field that do not contain unpaired electrons do not absorb microwave radiation. Free radicals have unpaired electrons [41–49]. Pharmaceutical products and substances that absorb microwave radiation in a magnetic field contain unpaired electrons belonging to free radicals, and higher EPR signals are observed in samples with higher free radical contents. The EPR spectra of free radicals formed in bisoprolol fumarate under abnormal environmental conditions, such as elevated temperature and UVA exposure, have not been analyzed so far.

2. Materials and Methods

2.1. Samples

Free radicals formed in the pharmaceutical product containing bisoprolol fumarate ($C_{18}H_{31}NO_4 \cdot 1/2C_4H_4O_4$) as the active component as well as auxiliary components that do not exhibit pharmacological activity. The chemical structure of bisoprolol fumarate is presented in Figure 1 [4]. Industrially produced, coated tablets containing 5 mg of bisoprolol fumarate in a powdered form were tested. The applied research method required samples in a powdered form. The obtained results concern the generation of free radicals in the pharmaceutical substance inside the tablet. Using EPR spectroscopy, information was obtained on the influence of thermal factors and UVA radiation on the substance contained in the tested tablets.

The pharmaceutical product was stored under the following physical conditions:

- Temperature of 50 °C (30 min);
- UVA radiation (30 min);

– UVA radiation (30 min) and then a temperature of 50 °C (30 min).

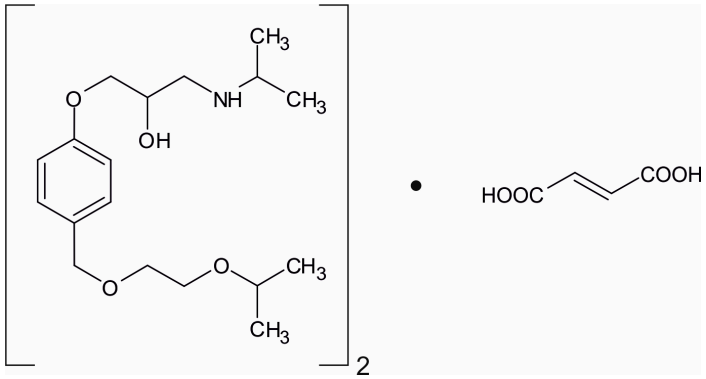


Figure 1. Chemical structure of bisoprolol fumarate [4].

The examination of the influence of a temperature of 50 °C on the tested pharmaceutical product containing bisoprolol fumarate was performed to show how dangerous it can be to accidentally expose this product to such high temperatures during storage or transport, for example, by leaving it on a windowsill, where on a sunny day, the temperature can reach 50 °C or even 60 °C. The research results show how important it is to adhere to the correct storage conditions for pharmaceutical products. The research method used in this work shows the generation of free radicals in the product as a result of exposure to an elevated temperature and UVA radiation. UVA radiation was chosen in the study because most of this radiation reaches the Earth's surface. Pharmaceutical products are therefore mainly exposed to the effects of this radiation.

In the examination using a thermal treatment, a professional thermal programmable sterilizer with the possibility of having continuous temperature control during heating from Memmert Firm (Schwabach, Germany) was used. The pharmaceutical product was incubated at a constant temperature of 50 °C in a chamber with dry hot air circulation. The air circulation provided a uniform temperature distribution in the chamber.

A thin layer of powder of the tested pharmaceutical product about 1 mm thick was left in Petri dishes with a diameter of 9 cm, and it was exposed to UVA radiation with a wavelength of 315–380 nm emitted by a Medisun 250 lamp from the Schulze & Bohm Firm (Brühl, Germany). The Medisun 250 lamp is equipped with four radiators with a power of 40 mW each. The distance of this product to the lamp was 30 cm.

For EPR measurements the tested samples were placed in thin-walled glass tubes with an outer diameter of 3 mm. The masses of the samples in the tubes were determined using a scale from Sartorius (Göttingen, Germany). The mass of the samples in the glass tubes was in the range 0.02–0.04 g.

2.2. EPR Measurements

The electron paramagnetic resonance studies of the pharmaceutical product containing bisoprolol fumarate were performed using an X-band (9.3 GHz) spectrometer with magnetic modulation of 100 kHz produced by the Radiopan Firm (Poznań, Poland) and the numerical data acquisition system Rapid Scan Unit of the Jagmar Firm (Kraków, Poland). The total power of the microwaves produced by klystron is 70 mW. Microwave power was set in the range 2.2–70 mW by using appropriate attenuation. The relationship between attenuation and microwave power is given by the formula [39,40]:

$$\text{attenuation [dB]} = 10 \lg[M_o/M] \quad (1)$$

where: M_o—the total microwave power produced by klystron (70 mW), and M—microwave power used during the measurement of the EPR spectrum.

An MCM 101 recorder of microwave frequency and a magnetic induction meter with an NMR probe from the EPRAD Firm (Poznań, Poland) were used.

The following parameters of the first-derivative EPR spectra were analyzed: amplitude (A), integral intensity (I), linewidth (ΔB_{pp}), resonance magnetic induction (B_r), and g factor. Both the amplitude (A) and integral intensity (I) of EPR lines, depend proportionally on the content of free radicals in the tested sample [35–40]. Magnetic interactions effect the linewidth (ΔB_{pp}) of EPR lines [35–40]. The g factor was calculated as [35–38]:

$$g = h\nu/\mu_B B_r \quad (2)$$

where: h—Planck constant, ν—microwave frequency, μ_B—Bohr magneton, and B_r—resonance magnetic induction.

Ultramarine with sulfur paramagnetic centers was used as the reference [50,51]. The free radical content (N) in the examined preparations containing bisoprolol fumarate was determined according to the following formula [35,39,40]:

$$N = n_u\ (I_p W_u)/(I_u W_p m) \quad (3)$$

where: n_u—the number of paramagnetic centers in ultramarine as the reference, I_u—integral intensity of the EPR line of ultramarine, I_p—integral intensity of EPR line of the tested product, W_u—receiver gain for the EPR line of ultramarine, W_p—receiver gain of the EPR line of the tested product, and m—mass of the sample.

The total differential method was used to determine measurement errors. The maximum errors of the measured quantities were determined. Parameter changes smaller than the maximum errors were not included in the results discussion.

The computer programs spectroscopic programs for recording and analyzing spectra from Jagmar (Kraków, Poland), LabVIEW 8.5 from National Instruments (Austin, TX, USA), Origin (OriginLab, Northampton, MA, USA), and Microsoft Excel 2019 (Redmond, WA, USA) were used in the EPR studies.

3. Results and Discussion

3.1. Free Radical Formation in the Examined Pharmaceutical Product Stored Under Different Physical Conditions

EPR studies conducted in this work indicated that the chemical structure of the tested pharmaceutical product containing bisoprolol fumarate changes under the influence of a temperature of 50 °C and UVA radiation. A change in structure also occurred when both of these physical factors acted on the product. After exposing this product to a thermal factor in the form of a temperature of 50 °C, UVA radiation, and after initial exposure to UVA radiation and then to a temperature of 50 °C, EPR lines were obtained for the tested drug samples. The appearance of EPR spectra with a spectroscopic splitting factor g of 2 is evidence of the formation of unpaired electrons in the product during its exposure to the above-mentioned factors for 30 min. A characteristic feature of free radicals is the presence of unpaired electrons in their molecules and the possession of EPR signals [33–41].

Free radicals were formed in the examined pharmaceutical product during storage under the conditions considered. Figure 2 presents the contents (N) of free radicals in the tested pharmaceutical product containing bisoprolol fumarate depending on the effect of the physical factor. The contents (N) of free radicals generated by the action of a temperature of 50 °C, UVA radiation, and by the action of UVA radiation and then a temperature of 50 °C were compared.

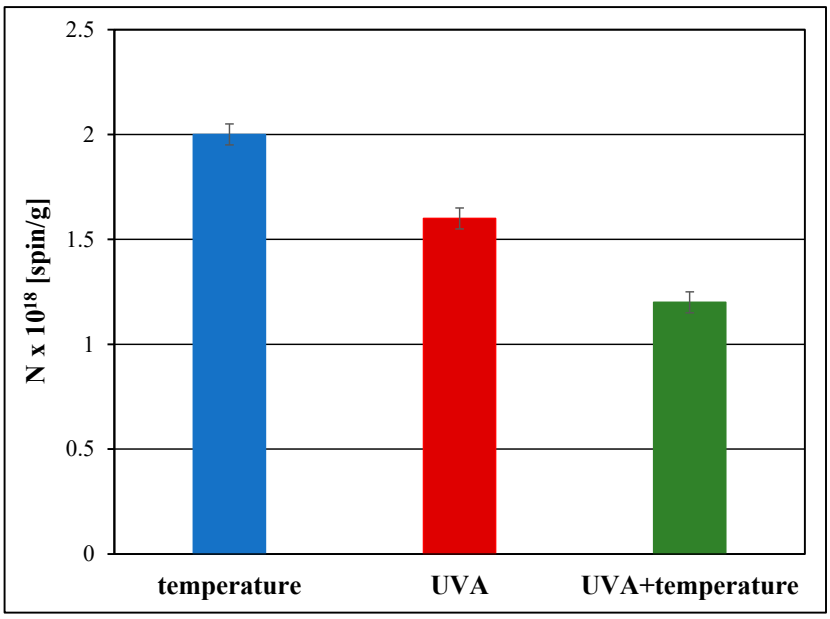


Figure 2. The free radical content (N) in the product containing bisoprolol fumarate, formed by the action of a temperature of 50 °C, UVA radiation, and by the action of UVA radiation and then a temperature of 50 °C. The time of action of each factor on the pharmaceutical product was 30 min. The free radical contents were determined based on the analysis of EPR spectra recorded at a microwave power of 2.2 mW.

The contents (N) of free radicals in the product containing bisoprolol fumarate are of the order of 10^{18} [spin/g] (Figure 2). The contents (N) of free radicals in the pharmaceutical product containing bisoprolol fumarate was highest after exposure of the product to a temperature of 50 °C. The lowest content (N) of free radicals was generated in the product with bisoprolol fumarate by exposure to UVA radiation and then an elevated temperature of 50 °C. Degradation probably occurs during exposition of the product to both a temperature of 50 °C and UVA radiation, but the recombination reaction between the radicals leads to a reduction in their quantity in the final product.

The examined product containing bisoprolol fumarate was most sensitive to thermal factors. At a higher temperature, the highest number of chemical bonds are broken, and the highest number of free radicals appear. The groups OH, NH, and CH_3 are probably released under the influence of heat and radiation energy. This problem requires further qualitative studies of free radicals. The complex shape of the EPR spectra indicates the occurrence of several groups of free radicals in the tested substance. The EPR spectra are superpositions of several overlapping lines. The UVA radiation range is not so strong as the interaction with the thermal factor. A relatively lower number of chemical bonds are broken, and the free radical content takes on a lower value than in the thermally treated product. The action on the tested product was successively determined with UVA radiation and then a temperature of 50 °C. Even though both factors generate free radicals, they did not ultimately result in the generation of more free radicals in the product. The content of free radicals in the product containing bisoprolol fumarate was lower after the action of both a temperature of 50 °C and UVA radiation than after individual factors, a temperature of 50 °C or UVA radiation, indicating the recombination of free radicals. Regardless of the acting factor, due to the content of free radicals formed in the order of 10^{18} [spin/g], the examined pharmaceutical product containing bisoprolol fumarate should be protected from a temperature of 50 °C and UVA radiation.

The generation of free radicals, free radical reactions, and reactions with oxygen molecules should not occur during the storage of drugs because these reactions may result

in structural changes in the substance molecules. The indicated structure changes based on the formation of unpaired electrons in the tested substance. The EPR method, which is used to examine paramagnetic substances [35,37,40], demonstrated the presence of free radicals in the tested samples; however, taking into account the reactivity of free radicals, modifications to the structure of the compounds constituting this substance should be expected. The product containing bisoprolol fumarate should not be stored with access to UVA radiation and should not be exposed to a temperature of 50 °C.

3.2. Comparison of the EPR Spectral Parameters for the Examined Pharmaceutical Product Stored Under Different Physical Conditions

The EPR spectra of free radicals generated in the product containing bisoprolol fumarate at a temperature of 50 °C and by UVA radiation differ in their parameters. The parameters of the EPR spectra of the tested product treated at a temperature of 50 °C, with UVA radiation, and with UVA radiation and then a temperature of 50 °C are shown in Tables 1–3, respectively. The tables include the parameters of the EPR spectra recorded at various sample microwave powers: 2.2 mW, 7 mW, and 55 mW.

Table 1. EPR spectra parameters (A, I, ΔB_{pp}) of the product containing bisoprolol fumarate exposed to a temperature of 50 °C, recorded with different microwave powers (M). A, I, ΔB_{pp}—amplitude, integral intensity, and linewidth of EPR lines, respectively.

Parameters of the EPR Spectra	M [mW]		
	2.2	**7**	**55**
A [a. u.] [±0.02]	5.41	10.22	27.02
I [a. u.] [±0.04]	25.96	54.06	181.22
ΔB_{pp} [mT] [±0.04]	2.14	2.30	2.59

Table 2. EPR spectra parameters (A, I, ΔB_{pp}) of the product containing bisoprolol fumarate exposed to UVA radiation, recorded with different microwave powers (M). A, I, ΔB_{pp}—amplitude, integral intensity, and linewidth of EPR lines, respectively.

Parameters of the EPR Spectra	M [mW]		
	2.2	**7**	**55**
A [a. u.] [±0.02]	6.00	10.84	27.81
I [a. u.] [±0.04]	20.23	42.51	180.81
ΔB_{pp} [mT] [±0.04]	1.84	1.98	2.55

Table 3. EPR spectra parameters (A, I, ΔB_{pp}) of the product containing bisoprolol fumarate exposed to UVA radiation and then treated at a temperature of 50 °C, recorded with different microwave powers (M). A, I, ΔB_{pp}—amplitude, integral intensity, and linewidth of EPR lines, respectively.

Parameters of the EPR Spectra	M [mW]		
	2.2	**7**	**55**
A [a. u.] [±0.02]	4.86	10.05	27.21
I [a. u.] [±0.04]	15.74	38.60	174.20
ΔB_{pp} [mT] [±0.04]	1.80	1.96	2.53

The values of amplitudes (A), integral intensities (I), and linewidths (ΔB_{pp}) of EPR lines of the product containing bisoprolol fumarate for the measurements with a microwave power of 2.2 mW are compared in Figure 3a–c. The results obtained for the product exposed

to a temperature of 50 °C, UVA radiation, and UVA radiation and then a temperature of 50 °C were taken into account.

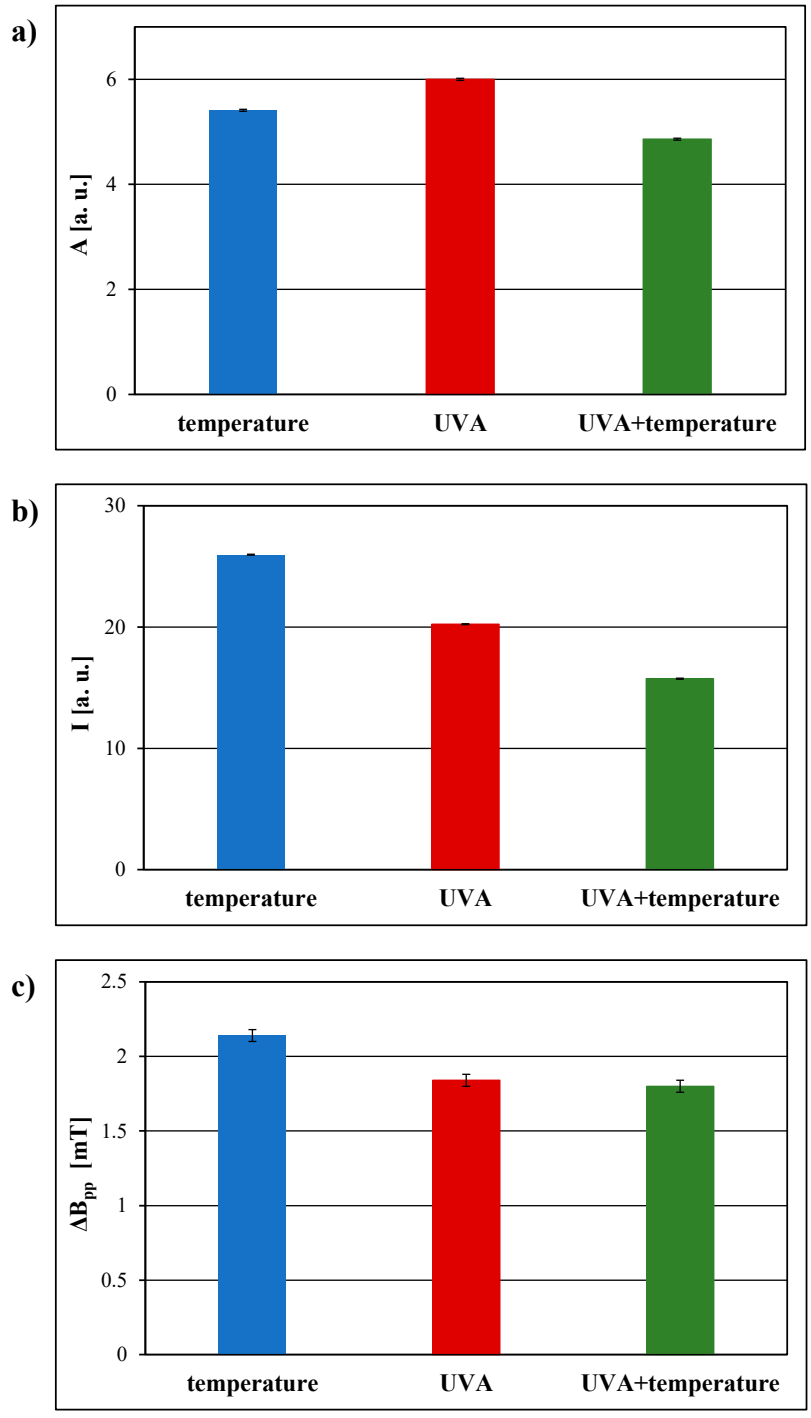


Figure 3. Comparison of amplitudes (A) (**a**), integral intensities (I) (**b**), and linewidths (ΔB_{pp}) (**c**) of EPR lines of the product containing bisoprolol fumarate, for the measurements with a microwave power of 2.2 mW, for the product exposed to a temperature of 50 °C, UVA radiation, and UVA radiation and then a temperature of 50 °C. The time of action of each factor on the pharmaceutical product was 30 min.

The highest amplitude value (A) of the EPR line at a microwave power of 2.2 mW was obtained after exposure of the product with bisoprolol fumarate to UVA radiation (Figure 3a). The lowest amplitude value (A) of the EPR line at a microwave power of 2.2 mW was obtained after exposure of the product with bisoprolol fumarate to UVA radiation

and a temperature of 50 °C (Figure 3a). At a microwave power of 2.2 mW, the integral intensity value (I) of the EPR line of the product containing bisoprolol fumarate was found to decrease in the following order: product exposed to 50 °C > product exposed to UVA radiation > product exposed to UVA radiation and 50 °C (Figure 3b). For a microwave power of 2.2 mW, the highest EPR linewidth value (ΔB_{pp}) was obtained for the product exposed to a temperature of 50 °C (Figure 3c). Similar but lower EPR linewidths (ΔB_{pp}) were obtained for the product after exposure to UVA radiation and for the product exposed to UVA radiation and a temperature of 50 °C (Figure 3c).

Changes in the width (ΔB_{pp}) of the EPR line of the product containing bisoprolol fumarate with the change of the type of the acting physical factor (Figure 3c) indicate structural changes in the tested substance. Changes in the chemical structure and the distance between unpaired electrons lead to changes in the magnitude of dipole interactions and thus to changes in the EPR linewidth of free radicals.

Large values of EPR linewidth (ΔB_{pp}), above 1 mT (Tables 1–3, Figure 3c), may indicate strong dipole interactions in the product containing bisoprolol fumarate. Dipole interactions between unpaired electrons increase with the decreasing distance between them [37]. It can therefore be assumed that the distances between unpaired electrons in the product containing bisoprolol fumarate are small, which allows their interactions. This is evidenced by high contents of free radicals, in the order of 10^{18} [spin/g] (Figure 2), generated at a temperature of 50 °C and by UVA radiation.

The values of the amplitude (A), integral intensity (I), and linewidth (ΔB_{pp}) of the EPR line of the product containing bisoprolol fumarate exposed to the action of physical factors depend on microwave powers (Tables 1–3). The effect of microwave power in the range of 2.2–70 mW on the amplitude (A), integral intensity (I), and linewidth (ΔB_{pp}) is presented in Figures 4–6, respectively.

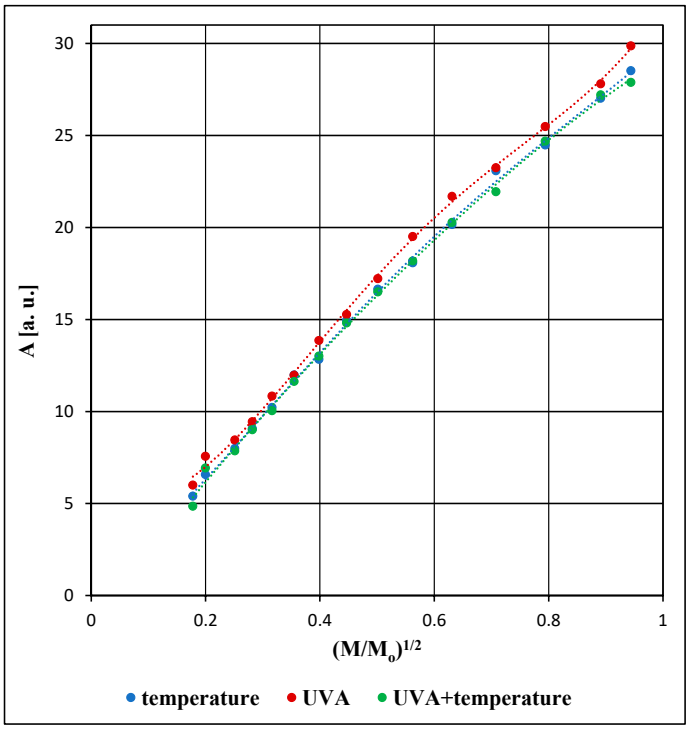


Figure 4. Comparison the effect of microwave power on the amplitude intensity (A) of the EPR spectrum of a product containing bisoprolol fumarate that was exposed to a temperature of 50 °C, UVA radiation, and UVA radiation and then a temperature of 50 °C. The time of action of each factor on the pharmaceutical product was 30 min. M—the microwave power used during measurement of the EPR spectrum. M_o—the total microwave power generated by the klystron (70 mW).

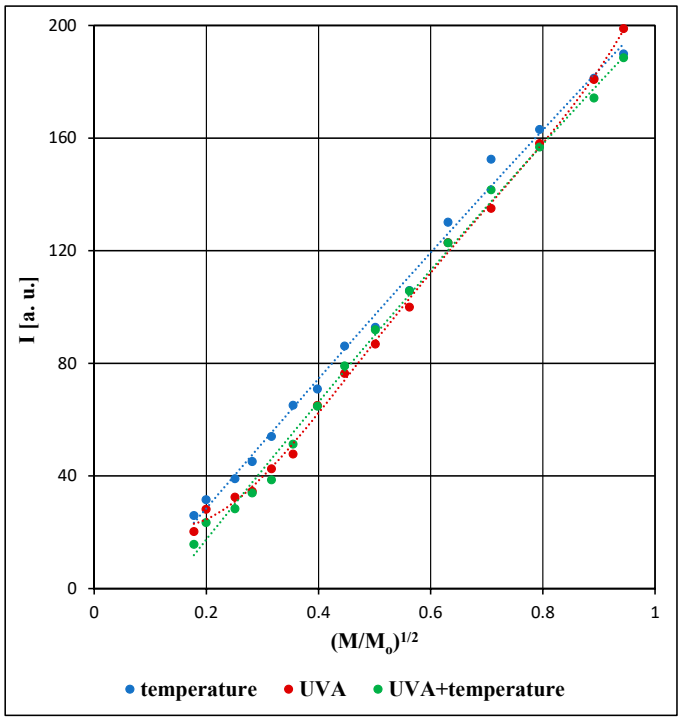


Figure 5. Comparison of the effect of microwave power on the integral intensity (I) of the EPR spectrum of a product containing bisoprolol fumarate that was exposed to a temperature of 50 °C, UVA radiation, and UVA radiation and then a temperature of 50 °C. The time of action of each factor on the pharmaceutical product was 30 min. M—the microwave power used during measurement of the EPR spectrum. M_o—the total microwave power generated by the klystron (70 mW).

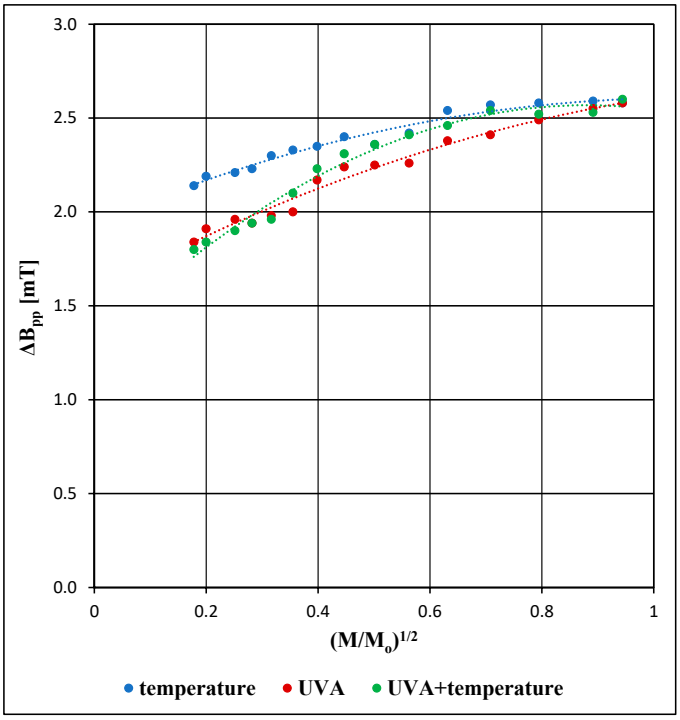


Figure 6. Comparison of the effect of microwave power on the linewidth (ΔB_{pp}) of the EPR spectrum of a product containing bisoprolol fumarate that was exposed to a temperature of 50 °C, UVA radiation, and UVA radiation and then a temperature of 50 °C. The time of action of each factor on the pharmaceutical product was 30 min. M—the microwave power used during measurement of the EPR spectrum. M_o—the total microwave power generated by the klystron (70 mW).

The amplitudes (A) (Figure 4) and integral intensities (I) (Figure 5) of the EPR lines of the pharmaceutical product containing bisoprolol fumarate increased with increasing microwave power. This effect is due to the fast spin-lattice relaxation processes in the studied samples. Unpaired electrons excited by microwave radiation quickly transition to lower energy levels [34,35,37]. This phenomenon occurred regardless of the physical factor, for the product with bisoprolol fumarate stored at a temperature of 50 °C, exposed to UVA radiation, and exposed to UVA radiation and a temperature of 50 °C. The widths (ΔB_{pp}) of the EPR lines of the tested pharmaceutical product exposed to physical factors increased with increasing microwave power (Figure 6).

The obtained results confirmed that EPR spectroscopy can be used to assess the effect of storage conditions on free radicals in pharmaceutical products, taking into account physical factors, such as elevated temperature and UVA radiation. Storage conditions influence the EPR spectra parameters of products. Earlier the formation of free radicals under the influence of elevated temperature and UVA radiation in the pharmaceutical products was found by the EPR method in the case of *Ungentum ophthalmicum* [52], caffeic acid [53], and antibacterial ointments containing fusidic acid and neomycin [54,55].

The novelty lies in the study of the influence of thermal factors and UVA radiation on the pharmaceutical product, which has not been analyzed before from this perspective. Free radical formation in the product containing bisoprolol fumarate has not been tested previously. What is also new is that not only the effect of individual factors, such as an elevated temperature or UVA radiation, but both of these factors were examined. An important reason for research is to test a pharmaceutical product under conditions of exposure to abnormal external factors. This situation can occur unintentionally during the transport of the product or its storage. The average patients, employees of the pharmaceutical industry, and employees of the transport company should be aware of the threat of free radicals. The direct method of studying free radicals that we use—EPR spectroscopy—provides such knowledge.

There are no similar studies conducted by other researchers known in the scientific literature. We proposed examination of the formation of free radicals in pharmaceutical products to assess the stability of the drug under given conditions. Free radical formation under the physical factors indicates that structural changes occur.

4. Conclusions

The EPR research method showed that during storage, the pharmaceutical product containing bisoprolol fumarate should not be exposed to UVA radiation or a temperature of 50 °C because under these conditions, free radicals are formed. The obtained results are consistent with the manufacturer's recommendations according to which the temperature of storage should not exceed 25 °C, and this drug should be protected against ultraviolet radiation. It has been shown that exposure of the product with bisoprolol fumarate to the indicated negative factors results in the formation of free radicals in the tested product in the amount ~10^{18} [spin/g]. It has been clearly confirmed, by detection of free radicals, that this product is not stable at 50 °C or with exposure to UVA radiation.

The content of free radicals generated in the tested pharmaceutical product containing bisoprolol fumarate depends on the type of physical factor acting on it. The content of free radicals in the pharmaceutical product containing bisoprolol fumarate was highest after exposure to a temperature of 50 °C. The lowest content of free radicals was generated in the product with bisoprolol fumarate by exposure to UVA radiation and then a temperature of 50 °C.

The EPR lines of the pharmaceutical product containing bisoprolol fumarate exposed to a temperature of 50 °C, UVA radiation, and both UVA radiation and a temperature of

50 °C do not become microwave saturable up to a microwave power of 70 mW. The lack of microwave saturation of the EPR line is evidence for the occurrence of fast spin-lattice relaxation processes in this product. It was confirmed that EPR spectroscopy can be used to assess the effect of storage conditions on free radicals in pharmaceutical products. The limitation of this method is that it tests pharmaceutical products in powdered form, not in tablet form. Interesting future studies could examine free radicals in substances taken from different areas of the tablets exposed to external physical factors.

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Data Availability Statement: The EPR data obtained in this study are available upon request from the authors.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

EPR (electron paramagnetic resonance), NMR (nuclear magnetic resonance), UV (ultraviolet radiation).

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