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FREE RADICALS IN THERMALLY STERILIZED *ACIDUM BORICUM* AND OPTIMIZATION OF THIS PROCESS *

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Abstract: Free radicals formation in the *acidum boricum* (AB) during thermal sterilization process was examined by an X-band (9.3 GHz) electron paramagnetic resonance (EPR) spectroscopy. *Acidum boricum* was sterilized according to the pharmacopea norms at temperatures 160°C (120 min), 170°C (60 min), and 180°C (30 min). Free radicals (~10^{17} spin/g) were thermally formed in these drug. The free radicals system revealed complex character, and the asymmetrical EPR spectra were measured. Mainly oxygen free radicals exist in the tested heated AB. Slower spin-lattice relaxation processes exist in AB sterilized at 160, 170 and 180°C. AB may be sterilized at temperatures 160, 170 and 180°C. For AB thermal sterilization at temperature 170°C is recommended. Free radicals concentrations changes during storage of the examined AB, and probably interactions with oxygen molecules may be responsible for this effect.

Keywords: EPR spectroscopy, free radicals, *acidum boricum*, thermal sterilization

Free radicals are very reactive species and they may be responsible for toxic effects in human organism (1). Free radicals appear in the materials exposed to interactions of the external physical factors, such as high temperature or irradiation (2-10). Both the above mentioned factors are used to sterilization of drugs (11, 12). Because of rupturing of chemical bonds, it is expected that sterilized drugs may contain unpaired electrons. Conditions of sterilization process should be so chosen that free radicals will not be produced or their amount will be as low as possible. The temperature of sterilization or the dose of radiation should be strong enough to kill microorganisms in drugs, but their values should give the lowest free radicals concentrations in the samples.

The aim of this work was to determine free radical properties and concentrations in the exemplary thermally sterilized *acidum boricum* (AB). The influence of sterilization conditions on the free radicals formations in this drug was tested. The normative temperatures and times of heating of AB were used. The application of electron paramagnetic resonance spectroscopy (EPR) to optimization of thermal sterilization process of AB was proposed. The lowest amounts of free radicals are formed in drugs at the best conditions of thermal sterilization.

EXPERIMENTAL

Samples

Free radicals in AB sterilized at different conditions according to the pharmaceutical norms (11) were examined. The AB powdered samples were heated at temperature 160°C for 120 min, 170°C for 60 min and 180°C for 30 min. Sterilization was performed in hot air oven with air circulation.

Chemical structure of AB is shown in Figure 1 (13). AB is used as a drug for inflammation of the

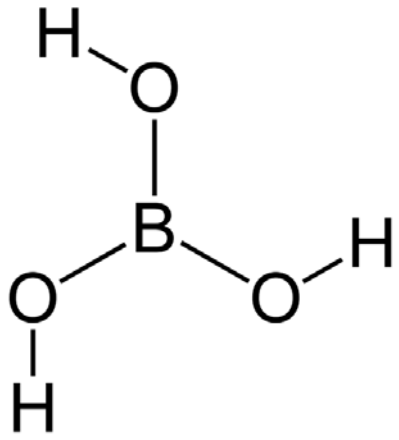

Figure 1. Chemical structure of *acidum boricum* (13)

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skin and the external urogenital organs, eczema, burns, bruises, edema, epidermal damage (14-17). AB is applied externally for washing the skin with liquid 2-3 times a day. The fluid can also be used for rinses. It can not be used in infants and children up to 11 years, as well as when extensive open wounds and cuts occur (14-17). AB can cause side effects: long-term use on broken skin or mucous membranes may cause rashes, redness, peeling, and systemic toxicity (especially in infants and young children), whose symptoms include vomiting, diarrhea, convulsions, damage internal organs (liver, kidney) (14-17).

EPR measurements

Free radicals were examined by the use of electron paramagnetic resonance (EPR) spectroscopy. For EPR measurements the powdered samples of original and sterilized AB were placed in the thin wall glass tubes with the external diameter of 3 mm. Mass of these samples located in the tubes were measured. EPR signals were not observed for the empty tubes. Free radicals properties and their concentrations in the samples were tested.

EPR spectra of AB were measured by an X-band (9.3 GHz) electron paramagnetic resonance

Table 1. Amplitude (A), linewidths (ΔB_{pp}) and g-factor of EPR spectra of the tested thermally sterilized *acidum boricum*. Data for the EPR spectra measured 15 minutes, 2, 8, 10, 13, 16, 22, 32 and 40 days after sterilization.

Sample (Times after sterilization)	Parameters of sterilization								
	160°C/120 min			170°C/60 min			180°C/30 min		
	A [a. u.] [±0.1]	ΔB_{pp} [mT] [±0.02]	g [+0.0002]	A [a. u.] [±0.1]	ΔB_{pp} [mT] [±0.02]	g [+0.0002]	A [a. u.] [±0.1]	ΔB_{pp} [mT] [±0.02]	g [+0.0002]
15 min	0.8	1.02	1.8822	0.9	0.69	1.8821	1.0	0.55	1.8820
2 days	0.7	0.69	1.9953	1.0	0.63	1.9950	1.0	0.46	1.9946
8 days	0.7	0.69	1.9937	0.9	0.72	1.9941	1.1	0.35	1.9940
10 days	0.8	0.70	1.9940	1.0	0.95	1.9937	0.9	0.62	1.9940
13 days	0.3	0.46	2.0001	0.2	0.62	1.9979	0.1	1.00	1.9977
16 days	0.3	0.46	1.9947	0.2	0.27	1.9961	0.2	0.77	1.9962
22 days	0.9	0.73	1.9994	1.2	0.81	1.9993	0.9	0.54	1.9996
32 days	1.0	0.69	1.9924	1.3	0.74	1.9931	0.8	0.58	1.9929
40 days	0.3	0.31	1.9960	0.2	0.57	1.9964	0.2	0.65	2.0011

Table 2. Parameters A_1/A_2 and B_1/B_2 of EPR spectra of the tested thermally sterilized *acidum boricum*. Data for the EPR spectra measured 15 minutes, 2, 8, 10, 13, 16, 22, 32 and 40 days after sterilization.

Sample (Times after sterilization)	Parameters of sterilization					
	160°C/120 min		170°C/60 min		180°C/30 min	
	A_1/A_2 [±0.02]	B_1/B_2 [±0.02]	A_1/A_2 [±0.02]	B_1/B_2 [±0.02]	A_1/A_2 [±0.02]	B_1/B_2 [±0.02]
15 min	0.73	1.03	1.00	0.62	0.94	0.67
2 days	0.67	0.42	0.81	1.00	0.60	1.88
8 days	1.03	0.92	1.02	1.10	0.91	0.99
10 days	0.44	0.82	1.57	1.77	0.89	0.77
13 days	0.62	0.80	0.92	0.69	0.87	1.71
16 days	0.63	0.86	1.33	2.45	1.37	0.74
22 days	0.60	0.76	0.5	1.48	1.10	0.65
32 days	1.12	1.11	0.66	0.83	0.64	0.78
40 days	1.69	0.67	1.20	0.51	0.82	1.31

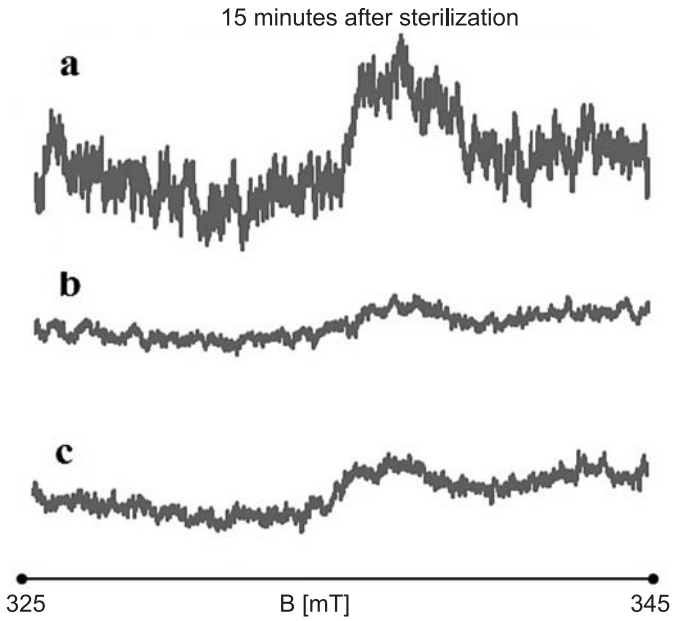

Figure 2. EPR spectra of *acidum boricum* sterilized at 160°C (120 minutes) (a), 170 °C (60 minutes) (b), and 180°C (30 minutes) (c). The measurement was done 15 minutes after sterilization with microwave power of 2.2 mW

spectrometer with magnetic modulation of 100 kHz produced by Radiopan (Poznań, Poland). To avoid microwave saturation, EPR lines were recorded at low microwave power of 0.7 mW and high attenuation of 20 dB.

For the original and sterilized AB samples the line shape and the parameters of the EPR spectra were analyzed. For the studied samples the following parameters of EPR spectra were determined: g-factors, amplitudes (A), integral intensities (I), and line widths (ΔB_{pp}). Amplitude and integral intensity are dependent on free radicals concentration in the samples (18). Line width depends on magnetic interactions in the samples (18).

g-Values were calculated from the equation of resonance condition according to the formula (18):

$$g = h\nu / \mu_B B_r$$

where: h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – resonance magnetic field.

Microwave frequency (ν) was directly measured by MCM101 recorder produced by Eprad (Poznań, Poland). The B_r values were obtained from the EPR spectra.

Effect of microwave power in the range of 2.2-70 mW on EPR spectra were examined. Changes of A, I and ΔB_{pp} with microwave power were obtained. Spin-lattice relaxation processes in the samples were characterized by observation of microwave saturation of their EPR lines. Power of microwave saturation of EPR lines increases with fastening of spin-lattice relaxation processes (18).

The parameters A_1/A_2 and B_1/B_2 of line shape of the EPR spectra were analyzed. The values of A_1, A_2, B_1 and B_2 are presented in Table 2.

The line shape parameters A_1/A_2 and B_1/B_2 were determined for the EPR spectra recorded in the range of microwave power 2.2-70 mW. The changes of the shape of EPR spectra with microwave power were tested. The shape of EPR spectra changes with the increasing of microwave power for the samples with the several types of free radicals (18, 19). The complex character of the free radicals system of the sterilized samples was checked.

Concentrations of free radicals (N) in the studied samples were compared. The concentration was determined as the value proportional to the integral intensity (I) of EPR spectrum (18, 20). The I values were obtained by double integration of the first-derivative EPR spectra. Ultramarine was used as the reference for concentration of free radicals. The integral intensities of the EPR lines of the examined AB and the integral intensity of the ultramarine line were compared. The second reference – a ruby crystal ($Al_2O_3:Cr^{3+}$) was permanently placed in a resonance cavity. For each sample and for the reference - ultramarine, the EPR line of a ruby crystal was detected. The same receiver gain and the same microwave power were used. The free radicals concentration (N) was determined as follows:

$$N = N_u[(W_uA_u)/I_u]/[I/(WAm)]$$

where: N_u - the number of paramagnetic center (1.2×10^{19} spin) in the ultramarine reference, W and W_u - the receiver gains for sample and ultramarine, A and A_u - the amplitudes of ruby signal for the sample and ultramarine, I and I_u - the integral intensities for the sample and ultramarine, m - the mass of the sample.

RESULTS AND DISCUSSION

The performed electron paramagnetic resonance and infrared studies indicate that these spectroscopic methods are very useful in the process of AB preparation. The process of production of AB should be accompanied by the minimal free radicals formation and their chemical structures should be unchanged. These two aspects, low contents of free radicals and the pure chemical units, are the important condition to proper interaction of the drugs in the human organism during pharmacotherapy.

The first stage of examination of the sterilized AB is to check its paramagnetic or diamagnetic character. Our EPR analysis shows that AB is diamagnetic before sterilization. This result is the confirmation of the chemical purity of the tested samples which were taken as the representative AB. The EPR spectra are observed only for paramagnetic samples, which contain unpaired electrons, for example unpaired electrons of free radicals (18). The paramagnetic samples located in the resonance cavity of the electron paramagnetic resonance spectrometer absorb microwaves of the proper frequency fitted to the energy levels of the unpaired electrons in magnetic field. The absorbed energy increases with increasing of the amount of unpaired electrons in the paramagnetic samples (18). Chemical structure of the analyzed AB (Fig. 1) (13) indicates that the absence of unpaired electrons is expected in the original samples. The ruptured chemical bonds, so also the unpaired electrons, were not detected in the original AB, because for all the tested samples EPR spectra were not obtained.

Paramagnetism appears in the analyzed AB during the thermal sterilization process. The EPR

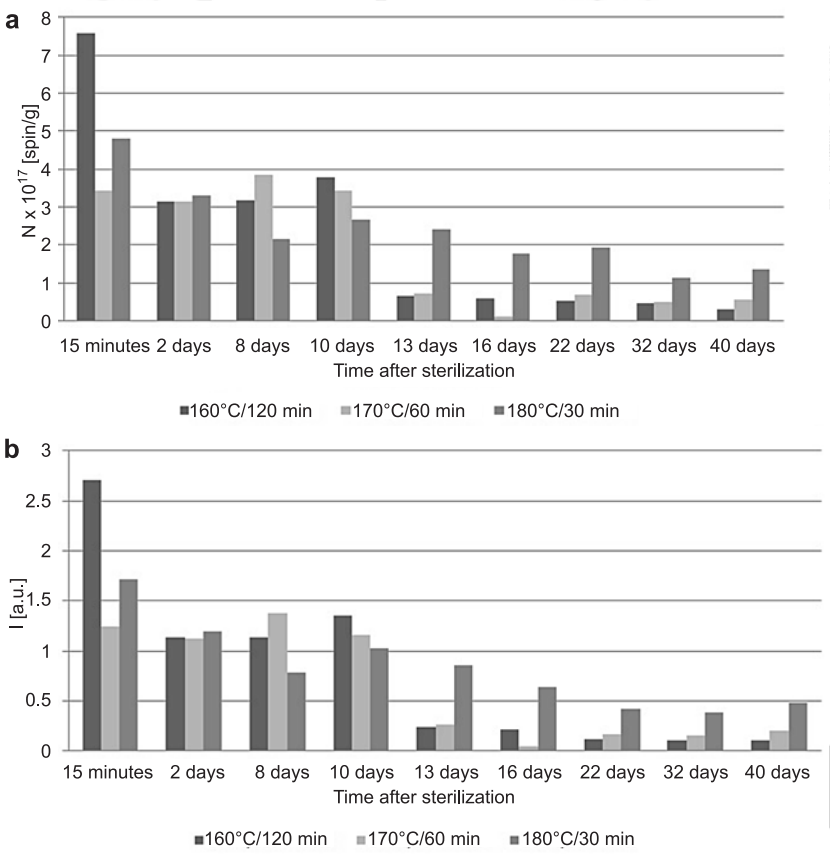

Figure 3. Change of free radical concentration (N) and integral intensity (I) in the storage thermal sterilized *acidum boricum*

spectra were obtained for AB (Fig. 2) sterilized at 160, 170 and 180°C.

The parameters of the spectra: amplitudes (A) and line widths (ΔB_{pp}) and g-factor depend on the sterilization conditions (Tab. 1).

It can be seen that independently on the sterilization conditions free radicals are formed in the analyzed AB. The sterilizations conditions were chosen according to the pharmaceutical norms (11), but the transformation from dia- to paramagnetic form of this drug is clearly visible. This aspect of the unexpected paramagnetism of the thermally sterilized AB is not included in the norms (11). The existence of free radicals in the sterilized drugs is one of the main problems, which should be resolved. Free radicals may be responsible for the dangerous interactions in tissues (1), so their concentrations in the substances, which contacts with them should be minimized. We propose to search the conditions of thermal sterilization, such as temperature and time, which give in the effect the lowest free radicals concentrations in the AB after sterilization. These examination may be done in laboratories by the use of electron paramagnetic resonance spectrometer, or in the future in the industrial firms before sterilization of the individual AB samples by the EPR spectrometer of the smaller dimensions. The EPR spectroscopic analysis is proposed as additional to examination of microorganisms presence in the drugs.

The main value, which is interested for the sterilized AB, is the free radicals concentration (N) and integral intensity (I). Free radicals concentra-

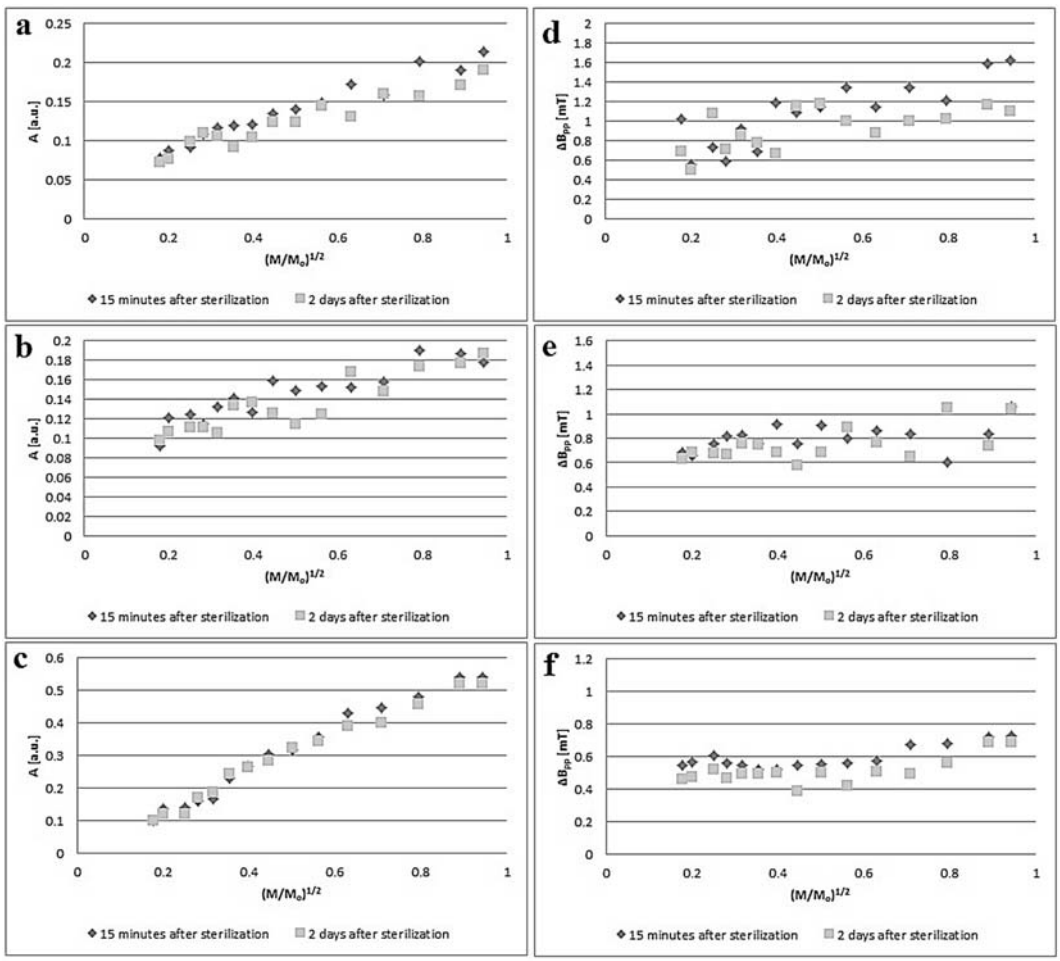

Figure 4. Influence of microwave power (M) on amplitude (A) (a, b, c) and linewidth (ΔB_{pp}) (d, e, f) of EPR spectra of *acidum boricum* sterilized at 160°C (120 minutes) (a, d), 170°C (60 minutes) (b, e), and 180°C (30 minutes) (c, f). The measurement was done 15 minutes and 2 days after sterilization. M is microwave power used during the measure of the EPR spectrum. M_o is the total microwave power produced by klystron (70 mW)

tions for the tested thermally sterilized AB are compared in Figure 3.

The highest free radicals concentrations were obtained for AB sterilized at 160°C during 120 min. The lowest free radicals concentrations were obtained for AB sterilized at temperature of 170°C during 60 min, so these temperature and time of sterilization may be proposed for this drug.

Free radicals concentrations formed during the sterilization process of AB are not stable during the storage of these drugs after heating (Fig. 3). This effect should be taken to account in the practical applications of drugs storage. It is possible that the interactions of free radicals of the sterilized AB with oxygen molecules are responsible for the evolution of the concentrations of unpaired electrons in the samples (Fig. 3). The interactions of free radicals with paramagnetic oxygen molecules O_2 were observed for other organic samples (21-23). The paramagnetic organic samples interact with oxygen molecules *via* their unpaired electrons.

It was shown that free radicals in the analyzed sterilized AB have the specific properties. The main feature of the paramagnetic system in the heated AB is its complex character, which is responsible for the complex shape of the unsymmetrical EPR spectra. The EPR parameters A_1/A_2 and B_1/B_2 changes with time of storage (Table 2), so it can be concluded that several groups of free radicals were formed during thermal sterilization of AB.

It is the expected effect, because the different chemical bonds may be ruptured at the used temperatures. The chemical structure of the tested AB (Fig. 1) (13) points out that mainly free radicals with unpaired electrons localized on oxygen atoms are formed. The oxygen free radicals are responsible for the obtained apparent g values (Table 1).

The continuous microwave saturation of the EPR lines (Fig. 4) indicates the homogeneous broadening of these lines (18). It is characteristic for the samples with homogenous distribution of the free radicals in their molecular units. The spin islands do not exist in the heated AB. This feature is the confirmation of the well performed sterilization of AB, this process was interacting in the whole volume of the drug samples.

Microwave power (M/M_o) effect on amplitudes (A) of the EPR lines of AB sterilized at 160, 170, and 180°C (Fig. 4 a, b, c) points out the fast spin-lattice relaxation processes in the samples. The microwave saturation of the EPR lines are not observed, the amplitudes increase with the increase of microwave power in the used range of its values (up to 70 mW) (Fig. 4 d, e, f). For the fast relaxing systems, unpaired electrons excited by microwaves fast come back to the ground energy levels (18). Similar fast spin-lattice relaxation processes were observed for thermally sterilized clarithromycin (6), neomycin (8) and sisomicin (8).

Spectroscopic analyses performed in this work show that EPR methods may be proposed as the additional ones to obtain the best conditions of the thermal sterilization of AB. The aim of the EPR measurements is to choose the optimal temperature and time of sterilization of the individual AB from the proposed by the pharmaceutical norms. EPR spectroscopy may be applied to compare chemical structure and free radicals in the original and thermally treated AB. Sterilization process should not modify the chemical structure of AB and should not form free radicals in its samples.

CONCLUSIONS

The performed EPR studies of the thermally sterilized AB indicate that:

1. Free radicals are formed in AB during thermal sterilization at temperatures 160°C (120 min), 170°C (60 min) and 180°C (30 min), as evidenced by EPR spectra.
2. Free radicals in the sterilized AB reveal the following properties:
   - complex character of free radicals system with the complex shape of EPR spectra;
   - mainly oxygen free radicals exist in the tested heated AB;
   - homogeneous broadening of EPR lines, proved by the continuous microwave saturation of the resonance signals, and
   - fast spin-lattice relaxation processes exist in AB sterilized at 160, 170 and 180°C for 120, 60 and 30 min, respectively.
3. Free radicals concentrations in AB (~10^{17} spin/g) depend on the temperature and time of sterilization. The highest free radicals concentrations characterize thermally sterilized AB at 180°C for 30 min.
4. Free radicals concentrations change during storage of the examined AB, and probably interactions with oxygen molecules may be responsible for this effect.

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