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APPLICATION OF ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY FOR EXAMINATION OF FREE RADICAL SCAVENGING PROPERTIES OF INSULIN ANALOGS

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Abstract: The performed study reflects the innovatory application of EPR spectroscopy which enabled to detect antioxidant properties of insulin analogs. Despite of scientific importance this research indicates the additional benefits from the implementation of insulin therapy in diabetic patients. The two rapid-acting insulin analogs (insulin lispro and insulin aspart), together with two long-acting recombinant analogs of regular human insulin (insulin detemir and insulin lantus) and three recombinant mixtures of analogs (biphasic insulin lispro 25/75 – BILis 25, biphasic insulin aspart 30/70 – BIAsp 30, biphasic insulin aspart 50/50 – BIAsp 50) were examined by X-band (9.3 GHz) electron paramagnetic resonance (EPR) spectroscopy. The kinetics of insulin interactions with free radicals was determined. The antioxidative insulin properties were characterized by the ability to quench free radicals. As a result, the decrease of EPR signal of the free radical reference appears. DPPH (1,2-diphenyl-2-picrylhydrazyl) was used as the paramagnetic reference in this study. The four groups of insulins in terms of interactions with free radicals were found and their interactions with DPPH decreased as follows: I group (rapid-acting insulin analog, insulin lispro, analog mixtures, BIAsp 50, BIAsp 30) > II group (rapid-acting insulin analog, insulin aspart) > III group (analog mixtures, BILis 25) > IV group (long-acting insulin analogs, insulin detemir, insulin lantus). In conclusion, insulin interactions with free radicals depend on the type of insulin.

Keywords: insulin analogs, antioxidant, free radicals, DPPH, EPR spectroscopy

Insulin analogs are commonly used in intensive antidiabetic therapy. Insulin is mainly used in type 1 diabetes mellitus as well as in type 2 diabetic patients as an add-on therapy to the non-insulin antidiabetic medications (1, 2). The rapid-acting human insulin analogs have a more rapid subcutaneous absorption, faster onset and shorter duration of action than regular human insulin. Their use improves postprandial glycemic control and decreases the risk of hypoglycemia (3). The long-acting analogs of insulin are more stable in the solution and have a longer half-life than neutral protamine Hagedorn (NPH) insulins. Moreover, the mentioned analogs are characterized by a relatively flat action profile, which reduces variability of glycemic values and decreases the incidence of hypoglycemia. In consequence, the long acting insulin analogs provide a more effective, safer and physiologic insulin replacement therapy in comparison with NPH insulins (4, 5). The therapy with premixed insulin analogs is one of the possible models of intensive insulin therapy (6) providing basal and postprandial coverage with lower number of daily injections (7). When compared with basal insulin analogs, premixed insulin analogs demonstrate higher reduction of HbA_{1C} value (8). Despite the fact that all of the above mentioned therapeutic agents belong to homogenous pharmacological category of insulin analogs, their molecular structure and composition as well as physicochemical properties are diverse, which may influence the efficacy of the management process (9). The presence of free radicals in insulin samples could modify their pharmacodynamics and also pharmacokinetic properties.

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Therefore, the aim of this study was to determine the interactions between different types of insulins and free radicals in terms of their antioxidative properties. Electron paramagnetic resonance (EPR) spectroscopy was used to observe the insulin effect on unpaired electrons in a model free radical source such as DPPH. The performed spectroscopic analyzes were innovatory. The EPR tests with DPPH were already applied both in pharmacy (10-12) and medicine (13-15), but they have not been conducted for insulins so far. It is proposed to apply the EPR results practically.

EXPERIMENTAL

Studied samples

The following insulins were examined: the rapid-acting insulin analogs – insulin lispro (Humalog®: 100 U/mL, Eli Lilly and Co.), and insulin aspart (NovoRapid®: 100 U/mL, Novo Nordisk); the long-acting insulin analogs – insulin detemir (Levemir®: 100 U/mL, Novo Nordisk) and insulin glargine (Lantus®: 100 U/mL, Sanofi-Aventis); the premix insulin analogs – insulin premix containing 30% soluble insulin aspart and 70% protamine-crystallized insulin aspart – BIAsp 30 (NovoMix 30®: 100 U/mL, Novo Nordisk), insulin premix containing 50% soluble insulin aspart and 50% protamine-crystallized insulin aspart – BIAsp 50 (NovoMix 50®: 100 U/mL, Novo Nordisk), insulin premix containing 25% insulin lispro solution and 75% insulin lispro protamine suspension – BILis 25 (Humalog Mix 25®: 100 U/mL, Eli Lilly and Co.).

EPR measurements

Interactions of the tested insulins with free radicals were examined by the use of electron paramagnetic resonance spectroscopy with microwaves of frequency of 9.3 GHz from an X-band. DPPH (1,2-diphenyl-2-picrylhydrazyl) as the paramagnetic reference was the model source of free radicals. Free

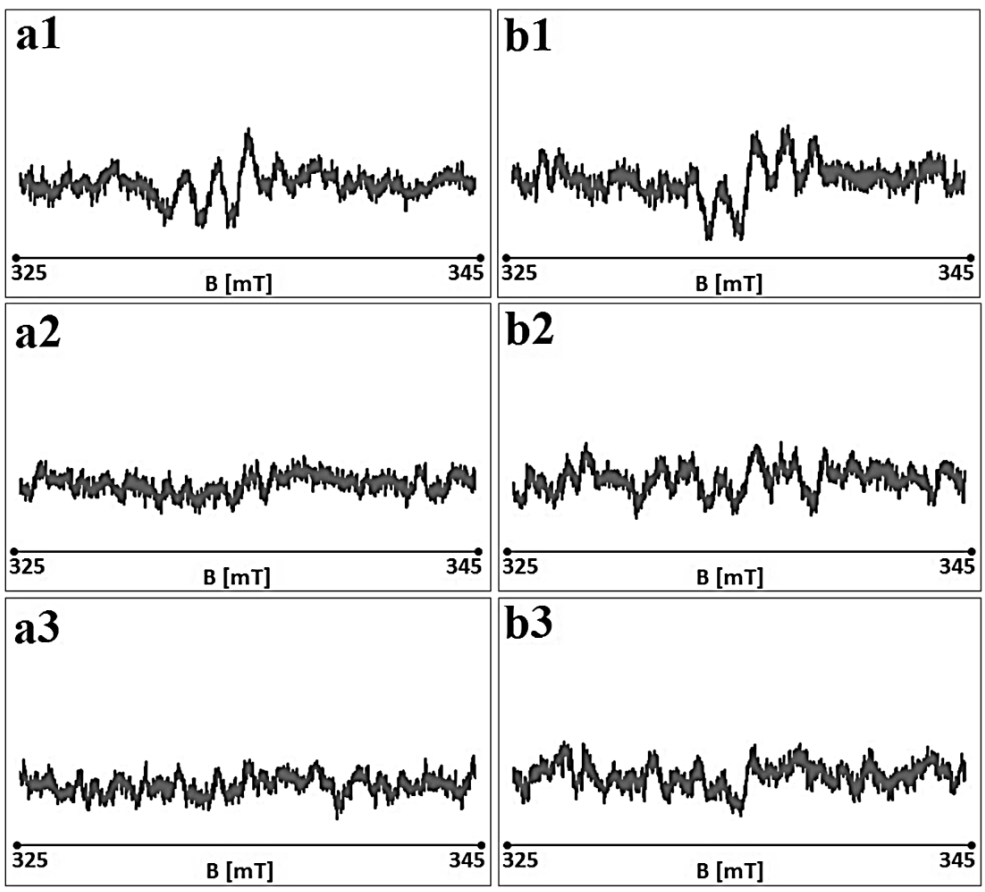

Figure 1. EPR spectra of DPPH free radicals interacting with the fast interacting insulin analogs, insulin lispro (a1, a2, a3), and insulin aspart (b1, b2, b3), during 5 min (a1, b1), 30 min (a2, b2) and 60 min (a3, b3). **B** is the magnetic induction of the field produced by electromagnet of the EPR spectrometer

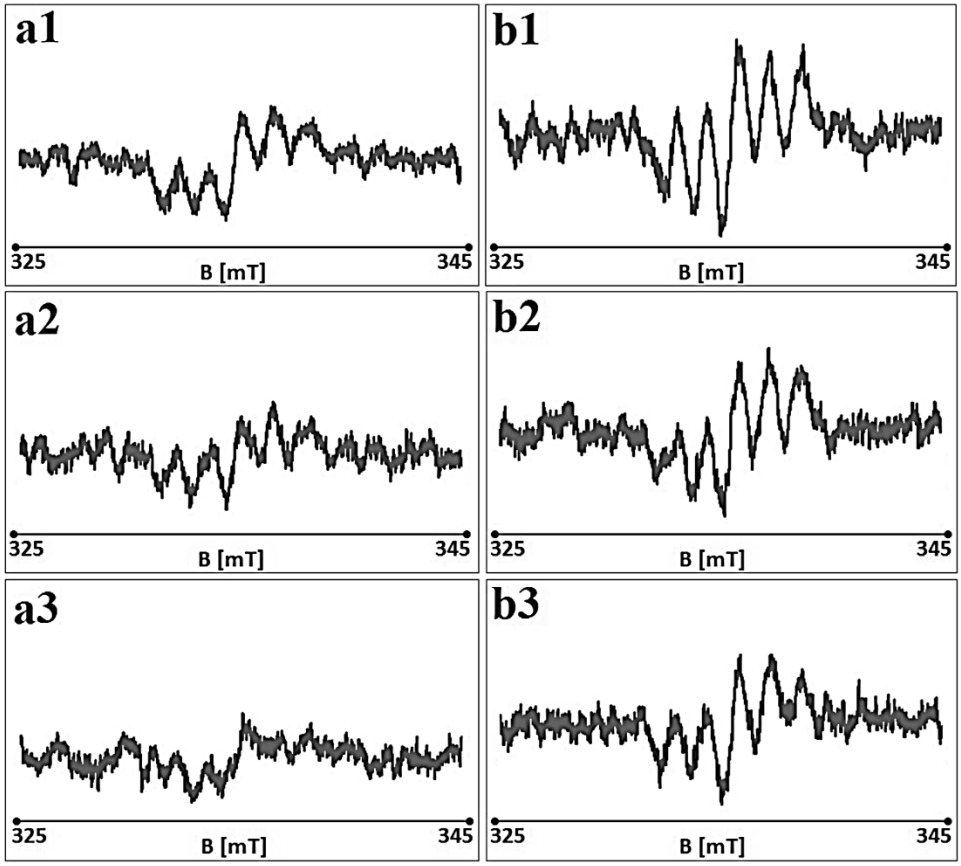

Figure 2. EPR spectra of DPPH free radicals interacting with human analogs insulin, detemir (a1, a2, a3), and glargine (b1, b2, b3), during 5 min (a1, b1), 30 min (a2, b2) and 60 min (a3, b3). **B** is the magnetic induction of the field produced by electromagnet of the EPR spectrometer

radicals with unpaired electrons located on nitrogen atoms existed in this reference (16, 17). The samples of DPPH in 10% ethyl alcohol solution were prepared and the EPR line of the model free radicals was measured. Next, the EPR line of DPPH in contact with the tested insulin was measured. The EPR line of DPPH was compared with EPR lines of DPPH which interacted with the tested insulins. As the result of interactions of DPPH and insulin the EPR line of DPPH decreased. This decrease of spectral line was the effect of decreased number of unpaired electrons in the used model of free radicals – DPPH, which stood for the decreased number of free radicals in it. The antioxidative properties of the tested insulins are proportional to quenching of free radicals and the EPR signals of DPPH. The quenching of free radicals by insulins is responsible for the decrease of amplitude (A) of EPR line of DPPH. In our measurements, the amplitude (A) was the most important parameter in achieving the aim of the analysis. In EPR spectroscopy, the absorption of microwaves by paramagnetic sample was measured (16, 17). Free radicals of DPPH absorbed microwaves, therefore, unpaired electrons of the model free radicals were excited to the higher energy levels. This energy was returned by unpaired electrons in relaxation processes and the electrons came back to the lower energy levels. Free radicals of DPPH were quenched by interactions with insulin and the lower number of unpaired electrons were excited by microwaves to the higher energy levels, while the lower EPR signals for DPPH were observed. The tested samples (DPPH and insulins) were placed in the thin-walled glass tubes with the external diameter of 1 mm. The empty tubes were free of EPR signals.

EPR spectra of DPPH were numerically detected with electron paramagnetic resonance spectrometer with magnetic modulation of 100 kHz produced by RADIOPAN (Poznań, Poland) and the RAPID

SCAN UNIT of JAGMAR (Kraków, Poland). Microwave frequency was measured by MCM101 recorder of EPRAD (Poznań, Poland). EPR spectra of DPPH and DPPH interacting with insulins, were measured as the first-derivative lines. The acquisition of the individual EPR line was done during the time of 1 second. The EPR spectra of DPPH were measured with the low microwave power of 2.2 mW to avoid microwave saturation of the resonance curves. The total microwave power produced by klystron of the EPR spectrometer was 70 mW. The microwave power of 2.2 mW corresponded to the attenuation of 15 dB at the exit. The following parameters of the EPR spectra of DPPH were determined, amplitudes (A), and g-factors. Amplitudes (A) of the EPR spectra increased with increasing amount of free radical contents in the samples (16, 17). The most important for DPPH are dipolar interactions between free radicals which broaden EPR lines. The g-factor of free radicals of DPPH was determined from the electron paramagnetic resonance condition in the following way (16, 18):

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

where: h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – induction of resonance magnetic field. The g-factor depends on localization of unpaired electrons in the sample (17, 18).

The accuracy of amplitude (A) of EPR line of DPPH was ± 0.01 a.u. The microwave frequency (ν) and the induction of magnetic field (B), were measured with the accuracy of ± 0.0002 GHz, and ± 0.01 mT, respectively. The accuracy of g-value equalled ± 0.0002. The errors for the spectral physical values were determined by the use of total differential method. Total differential takes into account the errors of all the factors affecting the determined value. Differential of the function contains its derivatives with respect to all factors.

The experimental errors for the parameters of EPR spectra were very low, because the parameters of the EPR spectra of DPPH were analyzed by the professional spectroscopic programs of JAGMAR (Kraków, Poland) and LabVIEW 8.5 of National Instruments (USA).

RESULTS AND DISCUSSION

Free radicals are key factors responsible for the development of diabetic vascular complications. The mechanisms underlying the diabetic endothelial dysfunction may involve several biochemical pathways with an increase in glucose concentration providing the initial metabolic disorders (19, 20). Potential mechanisms by which hyperglycemia can lead to excessive free radical formation include direct autoxidation of glucose, activation of lipooxygenase, stimulation of glycation pathways, activation of kinase C activity, intracellular activation of

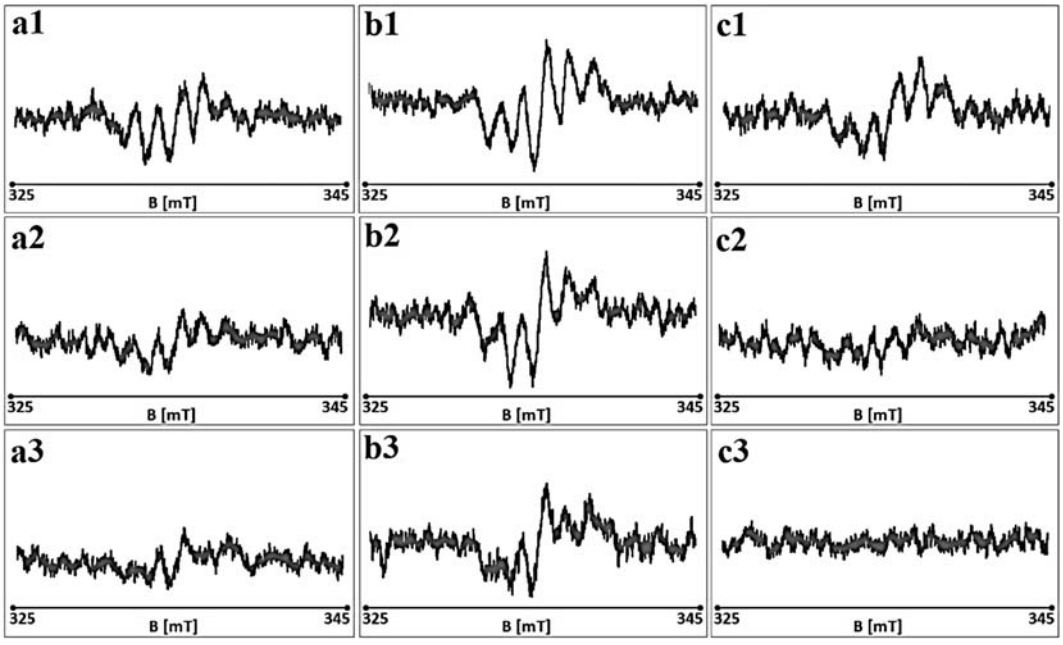

Figure 3. EPR spectra of DPPH free radicals interacting with mixed type insulin, BILis 25 (a1, a2, a3), BIAsp 30 (b1, b2, b3), and BIAsp 50 (c1, c2, c3), during 5 min (a1, b1, c1), 30 min (a2, b2, c2) and 60 min (a3, b3, c3). **B** is the magnetic induction of the field produced by electromagnet of the EPR spectrometer

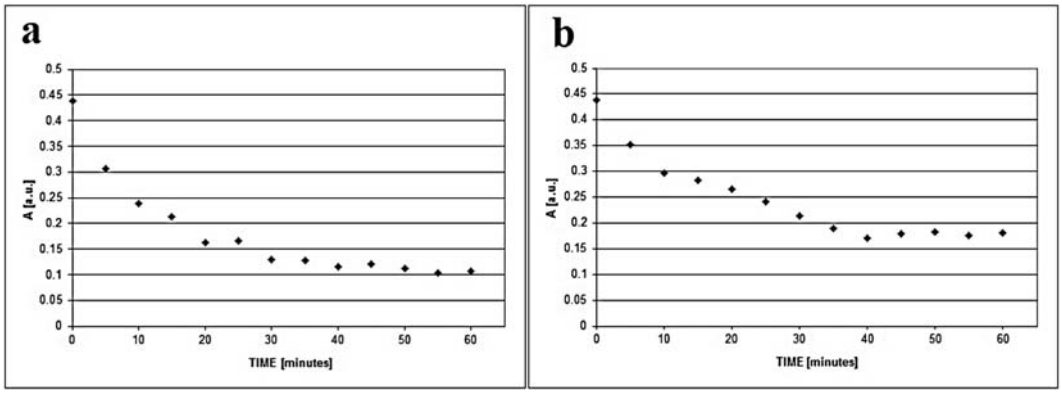

Figure 4. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with the fast interacting insulin analogs, insulin lispro (a), and insulin aspart (b), with time of interaction (t)

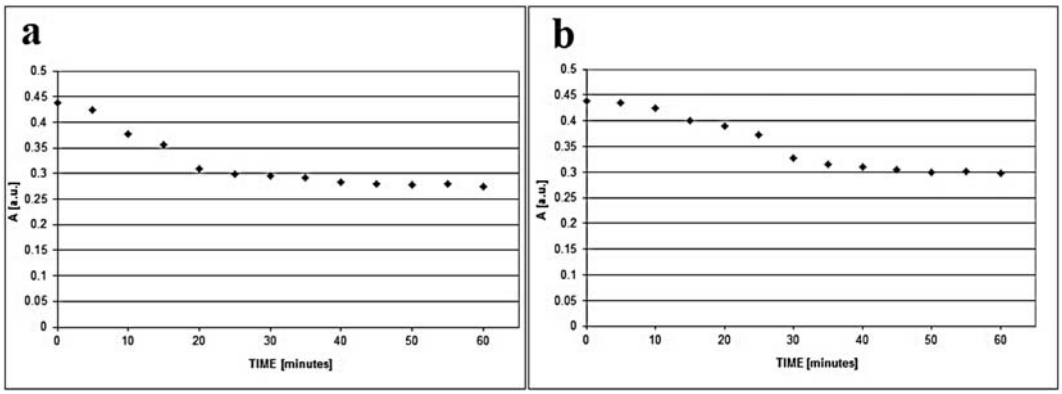

Figure 5. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with human analogs insulin, insulin detemir (a), and insulin glargine (b), with time of interaction (t)

sorbitol pathways as well as activation of NADPH oxidases (21). Moreover, the decreased potential of the extracellular and intracellular antioxidant capacity has been reported as a factor responsible for enhancing the oxidative state in diabetic patients (22). The experimental and clinical studies have revealed that excessive free radicals expression trigger insulin resistance, independently of the presence of other risk factors, such as obesity, impaired fasting glucose and metabolic syndrome (23, 24). Our present study has indicated that all the applied insulin analogs interact with free radicals and demonstrate antioxidant properties *in vitro*. The quenching of free radicals by insulins was observed as a decrease of amplitude (A) of the EPR lines of the reference – DPPH with g-factor of 2.0036 (Figs. 1-6).

DPPH was a well-defined free radical reference. Electron paramagnetic resonance (EPR) spectroscopy was used as the sensitive physical method to examine interactions of the sample with free radicals. Decrease of the EPR signal of paramagnetic DPPH indicated unmistakable interactions of the sample with free radicals. For the samples which do not interact with free radicals EPR signal of DPPH remains unchanged. The obtained g-value (2.0036) confirmed the chemical purity of the reference sample. Unpaired electrons exist in its structure on nitrogen (N) atoms. The interactions of unpaired electrons with the magnetic moment of nitrogen nuclei and structure of the energy levels in DPPH, decided about the g-values. The measured g-value comply with the theoretical one for DPPH, which confirmed that the model was properly used (17). The advantages of DPPH as the paramagnetic model in examination of insulin interactions with free radicals were a stabile chemical structure and a strong EPR line. The changes of the clearly visible EPR signal of DPPH after interactions with insulins were easy to determine, therefore we were able to achieve

the high accuracy of our measurements. The accuracy of the used EPR spectrometer and the additional equipment (recorder of microwave frequency, and NMR magnetometer) played also an important part in obtaining precise results. The quenching of the EPR lines of DPPH decreased with the time of interactions with particular insulins, finally being saturated. EPR spectra of DPPH in contact with the analyzed analogs of insulins during 5, 30, and 60 min are shown in Figures 1-3. The EPR signals of free

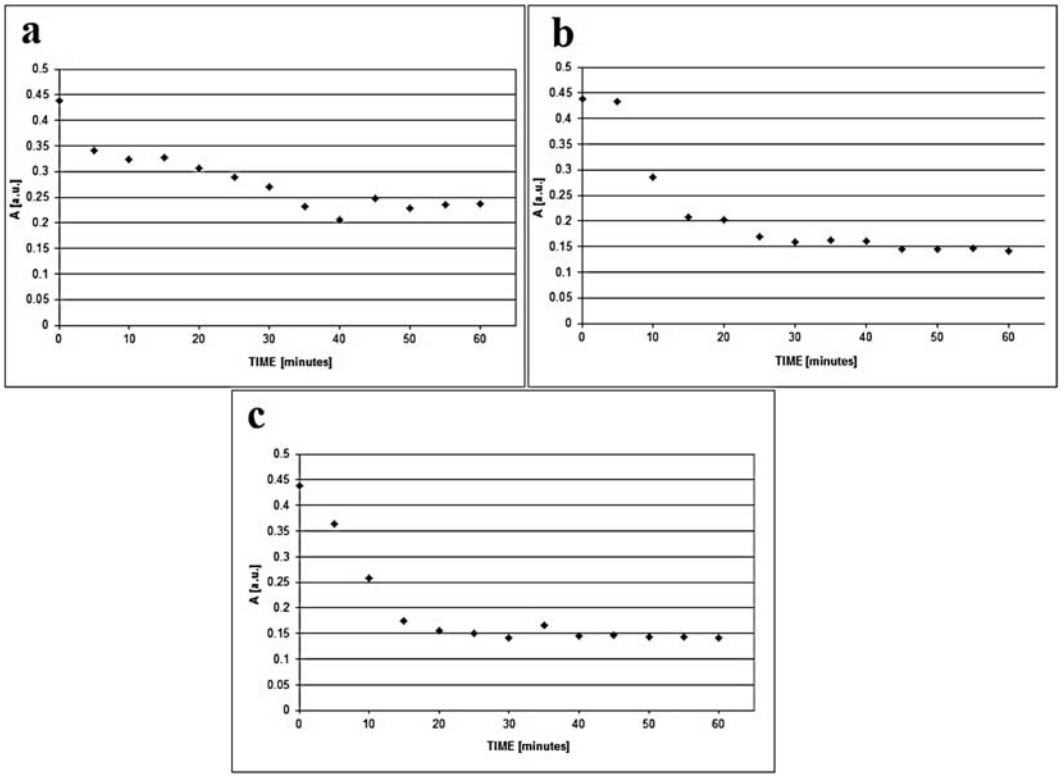

Figure 6. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with mixed type insulin, BILis 25 (a), BIAsp 30 (b), and BIAsp 50 (c), with the time of interaction (t)

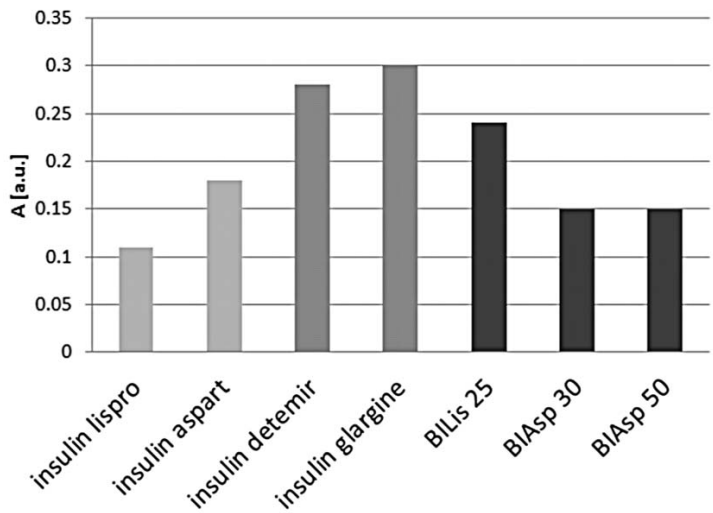

Figure 7. Comparison of the amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with the tested insulins after 60 min of interaction

radicals of DPPH decrease with increasing time. The amplitudes (A) of the EPR spectra of DPPH interacting with the examined insulins *versus* the time of interactions are presented in Figures 4-6. Amplitudes (A) of EPR lines of DPPH in contact with the particular insulins during 60 min are compared in Figure 7.

Taking the quenching of free radicals into account (Figs. 1-7), the insulins from the following four groups (I-IV) differ in amplitudes (A) [a.u. – arbitrary units] of DPPH EPR lines, I group, A, 0.11-0.15 a.u. (fast interacting analogs, insulin lispro, analog mixtures, BIAsp 50, and BIAsp 30); II group, A, 0.18 a.u. (fast-acting analogs, insulin aspart); III group, A, 0.24 a.u. (analog mixtures, BILis 25); IV group, A, 0.27-0.30 a.u. (long-acting insulin analogs, insulin detemir, insulin glargine).

The interactions of insulin samples with free radicals decrease as follows: I group (A, 0.11-0.15 a.u.) > II group (A, 0.18 a.u.) > III group (A, 0.24 a.u.) > IV group (A, 0.27-0.30 a.u.).

The antioxidative properties of the examined insulins decrease in a similar way to the presented relation for quenching of free radicals and amplitudes (A) of the reference - DPPH. The strongest interactions with free radicals were observed for the I group of insulin (fast interacting analogs, insulin lispro, analog mixtures, BIAsp 50, and BIAsp 30), because the lowest amplitudes (A, 0.11-0.15 a.u.) of EPR lines of DPPH in contact with these samples were obtained. The weakest interactions with free radicals were observed for the IV group of insulin (long-acting insulin analogs, insulin detemir, insulin glargine), because of the highest (A, 0.27-0.30 a.u.) EPR lines of DPPH in contact with these analyzed active substances.

The analysis of the interaction time of the insulins with free radicals pointed out the differentiation in the tested groups. The times after the amplitudes (A) of EPR lines in the following groups decreased to the constant values in the following manner: I group, (t_{min}, 20-40 min) (fast-acting insulin analogs, insulin lispro (40 min); analog mixtures, BIAsp 50 (20 min), and BIAsp 30 (30 min); II group, (t_{min}, 40 min) (fast-acting analogs, insulin aspart (40 min); III group, (t_{min}, 40 min) (analog mixtures, BILis 25 (40 min); IV group, (t_{min}, 35-40 min) (long-acting insulin analogs, insulin detemir (35 min), insulin glargine (40 min).

In the I group of insulins with the strongest antioxidative properties, fast interactions with free radicals were observed for two drugs, BIAsp 50 (t_{min} = 20 min) and BIAsp 30 (t_{min} = 30 min). The relatively slow interactions with free radicals were obtained for insulin lispro (t_{min} = 40 min). In the II group of insulins slow interactions with free radicals existed. The long time (t_{min}) was revealed in the case of insulin aspart (40 min). In the III insulins group slow interaction with free radicals (BILis 25 (t_{min} = 40 min)) was visible. In the IV group of insulins, insulin detemir (t_{min} = 35 min) interacted relatively faster with free radicals than insulin glargine (t_{min} = 40 min).

Taking into consideration all the examined insulins, relatively faster interactions with free radicals were obtained for BIAsp 50 (t_{min} = 20 min) and BIAsp 30 (t_{min} = 30 min).

The obtained results confirmed usefulness of an X-band (9.3 GHz) electron paramagnetic resonance spectroscopy in determining the interactions of insulins with free radicals. The application of DPPH as the paramagnetic reference in this study was chosen. This reference contained high amount of free radicals and strong EPR signals were measured for it, therefore, the quenching of its resonance line by insulin samples was clearly visible. Professional spectroscopic programs additionally affected the accuracy of the results. The decrease in values of the amplitude of DPPH EPR line and the kinetics of the changes with increasing time of DPPH – the insulin interactions illuminated the antioxidative properties of insulins.

EPR spectroscopy and model free radical DPPH sample were earlier documented in our previous study as an useful tool for assessing free radical expression and antioxidant properties of the biosynthetic human insulins of three groups: short-acting, intermediate-acting, and pre-mixed insulins (25).

CONCLUSIONS

Electron paramagnetic resonance studies of different insulins interacting with model free radicals proved that:

All the insulins actively interact with free radicals, and the quenching of DPPH free radicals was observed.

Interactions with free radicals, which resulted in quenching the EPR line of DPPH, depend on the type of insulin, and, therefore, the four insulin groups with different antioxidative properties were distinguished, I group (fast-acting analog, insulin lispro, analog mixtures, BIAsp 50, BIAs*p* 30); II group (fast-acting analog, insulin aspart); III group (analog mixture, BILis 25); IV group (long-acting analogs, insulin detemir, insulin glargine).

The interactions of insulins with free radicals resulted in the quenching amplitudes (A) of EPR

line of DPPH decrease in the following order, I group (A, 0.11-0.15 a.u. > II group (A, 0.18 a.u. > III group (A, 0.24 a.u. > IV group (A, 0.27-0.30 a.u.).

The insulins differ in time of interactions with free radicals. The relatively faster interactions with free radicals were characteristic for BIAsp 50, and BIAsp 30.

The usefulness of electron paramagnetic resonance spectroscopy and model free radical reference – DPPH in determining the interactions of insulins with free radicals was confirmed.

The results of our pioneering research revealing the antioxidative properties of insulin analogs should be carefully assessed and confirmed in human clinical studies. Moreover, future direction in EPR study concerning the interactions between free radicals and insulins should be related to the assessment of the influence of UV, storage time and storage temperature on free radicals' expression in opened vialled insulin samples. It may be of great importance in the verification of currently recommended storage conditions of punctured insulin vials used in different types of insulin delivery devices including pens, jet injectors and external insulin pumps.

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