Acta Poloniae Pharmaceutica – Drug Research, Vol. 72 No. 6 pp. 1177–1181, 2015 ISSN 0001-6837
Polish Pharmaceutical Society

INTERACTIONS OF INSUMAN COMB 25 INSULIN WITH FREE RADICALS – KINETICS EXAMINATION BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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Abstract: Insuman Comb 25 insulin's interactions with free radicals were examined in this study. The interaction of recombinant biphasic isophane insulin, Insuman Comb 25, with free radicals was assessed by continuous wave electron paramagnetic resonance (EPR) spectroscopy with microwaves of 9.3 GHz frequency. The model free radical molecule – DPPH (1,1-diphenyl-2-picrylhydrazyl) was used. EPR spectra of DPPH in contact with the tested insulin and EPR line of paramagnetic DPPH as the free radical with unpaired electrons localized on nitrogen (N) atoms, were compared. The aim of this study was to check the hypothesis about scavenging activity of Insuman Comb 25 insulin against free radicals. The EPR spectra were recorded numerically by EPR spectrometer of Radiopan (Poznań, Poland) and the Rapid Scan Unit of Jagmar (Kraków, Poland). Amplitudes (A) of EPR lines of DPPH and g-factor were analyzed. Amplitudes (A) of DPPH decreased upon contact with analyzed insulin, what confirmed its antioxidative character and scavenging activity against free radicals. The kinetics of interactions of DPPH with Insuman Comb 25 insulin was tested. Amplitudes (A) of the spectra of DPPH in contact with the insulin decreased with increasing time of interactions, and after 40 min were stabilized. It was pointed out that EPR spectroscopy may be used as the tool in pharmacy of antioxidative drugs.

Keywords: Insuman Comb 25 insulin, scavenging activity, free radicals, DPPH, EPR spectroscopy

Diabetes mellitus is associated with the developing risk of chronic complications (1) and deterioration of quality and shortening of life span (2, 3). Insulin therapy improve metabolic control and bring a reduction incidence of long term diabetic complications (3).

Insulin therapy is implemented in the management of patients with diabetes of all types. The need for insulin depends upon the impaired balance between insulin biosynthesis, secretion and insulin resistance (4). The use of insulin preparations is recommended for all patients with diabetes type1 (T1D) (5). The primary treatment goal of T1D is achieving and maintaining near-normoglycemia through intensive insulin therapy, avoiding acute complications, and preventing long-term complications (microangiopathy and macroangiopathy), as well as facilitating as close as possible to a normal life (6). The progressive nature of type 2 diabetes (T2D) requires clinicians to systematically evaluate patients and unfortunately first- and second-line antidiabetic agents such as metformin and the sulfonylureas do not prevent the characteristic decline in β cell function associated with T2D. Insulin replacement therapy can therefore quickly become a necessity in some patients (7). Insulin therapy is indicated for patients with T2D in whom glycemic targets were not achieved with two or more antidiabetic agents and for those who suffer from severe hyperglycemia as indicated by fasting plasma glucose (FPG) levels higher than 250 mg/dL, HbA1c concentration higher than 10% and/or symptoms of hyperglycemia (8). Available on pharmaceutical market insulin formulations are characterized by dif-

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ferent pharmacological properties with respect to time of onset, peak activity and duration of action (5).

Actually, five main types of insulin formulations are available on pharmaceutical market: regular insulin, NPH, rapid-acting analogs, basal analogs, and pre-mixed insulin that meets the different needs of patients and response to management (9, 10). Biosynthetic human insulins are still one of the most commonly used in clinical practice. Among them, biphasic premixed insulins that incorporate the combination of short or rapid-acting insulin with its intermediate-acting, cover both postprandial glucose excursion as well as basal insulin needs simultaneously. Insulin mixtures provide convenience to patients needing a simple insulin treatment plan (11, 12). As compared with basal insulin alone, premixed regimens usually tend to diminished HbA1c to a greater extent (13).

Taking into account that less costly alternative to insulin analogs - human insulins (14) - are still present on pharmaceutical market, being one of the most commonly prescribed by the clinical practitioners, we decided to evaluate the antioxidant properties of one of the human premixed formulation i.e., Insuman Comb 25, using electron paramagnetic resonance method. The interactions of Insuman Comb 25 insulin with the model free radicals (DPPH) were examined. Our study concentrated on kinetics of these interactions.

EXPERIMENTAL

Insulin sample

In this work, Insuman Comb 25® (suspension for injection, Sanofi-Aventis) was studied. Insuman Comb 25 insulin, produced by recombinant DNA technology, is a biphasic isophane insulin suspension consisting of 25% dissolved insulin and 75% crystalline protamine insulin.

DPPH – the model free radical molecule

DPPH (1,1-diphenyl-2-picrylhydrazyl), which is the model free radical molecule (15, 16) was used to examine scavenging activity of Insuman Comb 25 insulin against free radicals. Chemical structure of DPPH is shown in Figure 1 (15, 16). Unpaired electron localized on nitrogen (N) atom was responsible for its paramagnetic character and for its EPR signal (15, 16).

EPR measurements - the apparatus conditions

Electron paramagnetic resonance measurements were performed by EPR spectrometer working at the X-band of microwaves with 9.3 GHz frequency. Microwave frequency (ν) [±0.0002 GHz] was obtained by MCM101 recorder produced by EPRAD (Poznań, Poland). EPR spectrometer with continuous waves and magnetic modulation of 100 kHz produced by Radiopan (Poznań, Poland) was used. Numerical acquisition of EPR spectra of DPPH was done by the Rapid Scan Unit of Jagmar (Kraków, Poland). The time of acquisition of the single line was 1 second. The total microwave power produced by klystron was 70 mW. Attenuation of 15 dB resulted with microwave power of 2.2 mW during the measurements. This low microwave power provided guarantees of the microwave saturation absence in the signals.

Detection of EPR spectra of DPPH

The first-derivative EPR spectra of 10% ethyl alcohol solution of DPPH and DPPH in contact with the Insuman Comb 25 insulin, were measured. The tested samples in the thin walled glass tubes with external diameter of 1 mm were located in magnetic field in the resonance cavity of the EPR spectrometer. These empty tubes did not give EPR signals in the used experimental conditions (receiver gain, microwave power, magnetic field).

The EPR spectrum of DPPH – the model free radical molecule in 10% ethyl alcohol solution was shown in Figure 2a. Interactions of Insuman Comb 25 insulin with DPPH quenched its EPR signal. This effect resulted from Insuman Comb 25 insulin scavenging activity against DPPH free radical molecules, and the antioxidant properties of this insulin.

EPR analysis

The kinetics of interactions of Insuman Comb 25 insulin with DPPH was examined. The changes in the EPR line of DPPH during interaction with Insuman Comb 25 insulin by 5 min up to 60 min were determined. The changes of amplitudes (A) of the EPR line of DPPH with increasing of time (t) of interaction of Insuman Comb 25 insulin with DPPH

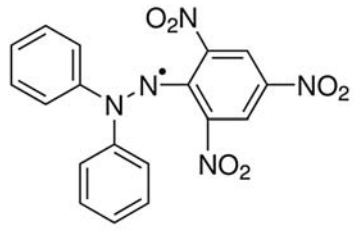

Figure 1. Chemical structure of DPPH molecule with unpaired electron (•) localized on nitrogen (N) atom (15, 16)

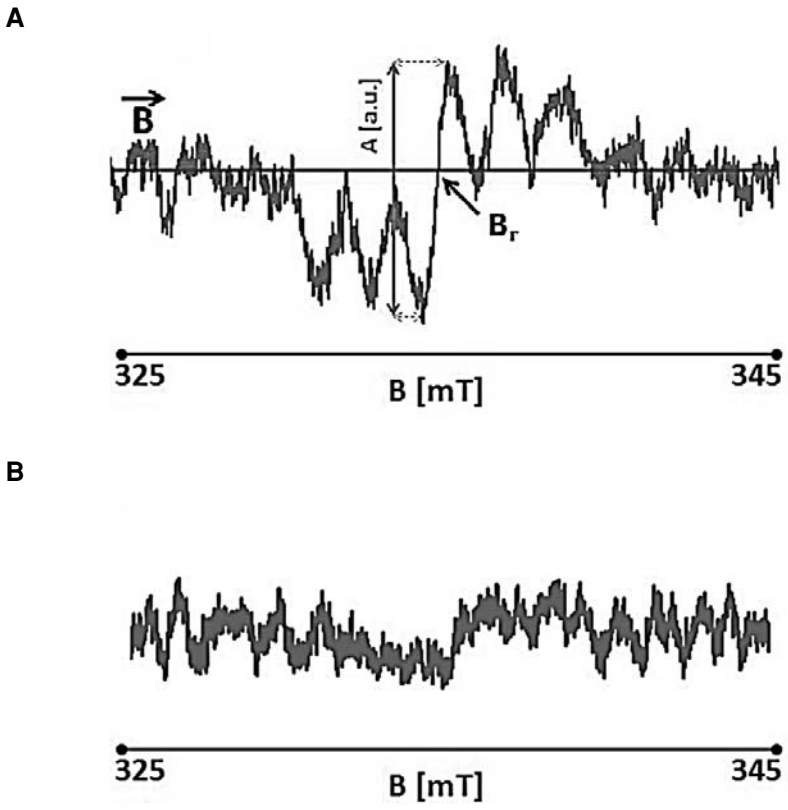

Figure 2. The first-derivative EPR spectrum of DPPH used as the model free radical molecule, where **B** = magnetic induction of the field produced by electromagnet, $\mathbf{B_r}$ = the resonance magnetic induction, **A** = amplitude (a), and DPPH interacting with the Insuman Comb 25 insulin during 60 min (b), respectively

were obtained. The decrease of amplitude (A) of EPR line of DPPH was proportional to the interactions of Insuman Comb 25 insulin with DPPH.

g-Factor depended on unpaired electron localization for EPR line of DPPH – the model free radical molecule was determined. The following formula in calculation was used (17-19): $g = h\nu/\mu_B B_r$, where: h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – induction of resonance magnetic field. Microwave frequency (ν) was measured, and the resonance magnetic induction (B_r) was determined from the EPR line (Fig. 2a).

The following accuracies of the determined spectral parameters were obtained: [± 0.01 a.u.] for amplitudes (A), and [± 0.0002] for g-factors. The errors for the spectral parameters were determined by the method of the total differential, which respected the errors of all the measured physical values.

EPR spectra of DPPH were measured and analyzed by professional spectroscopic programs of Jagmar (Kraków, Poland) and LabVIEW 8.5 of National Instruments (USA).

RESULTS AND DISCUSSION

Different composition and physicochemical properties of human insulin, influence the efficacy of the management process. Interactions between free radicals and insulins in terms of antioxidative properties of the former ones could modify pharmacodynamic and pharmacokinetic profile of insulin action. The results of our present study revealed that EPR spectra of DPPH free radical molecule changed after contact with Insuman Comb 25 insulin. The EPR spectra of DPPH interacting with this insulin for different times of interactions are shown in Figure 3. The influence of Insuman Comb 25 insulin on EPR spectra of DPPH confirmed our hypothesis about scavenging activity of this insulin against free radicals. The EPR spectra of DPPH and DPPH in contact with Insuman Comb 25 insulin after 60 min of their interactions, were compared in Figure 2 a,b, respectively. It is visible that the insulin quenched the EPR line of DPPH.

The results of kinetics analysis is presented in Figure 4, as the influence of time (t) of interactions with Insuman Comb 25 insulin on amplitude (A) of the EPR spectrum of DPPH free radicals. It was observed that the amplitudes (A) decreased with increasing time of interaction and after 40 min its value stabilized. The decrease of amplitude (A) reflected the scavenging properties of the model free

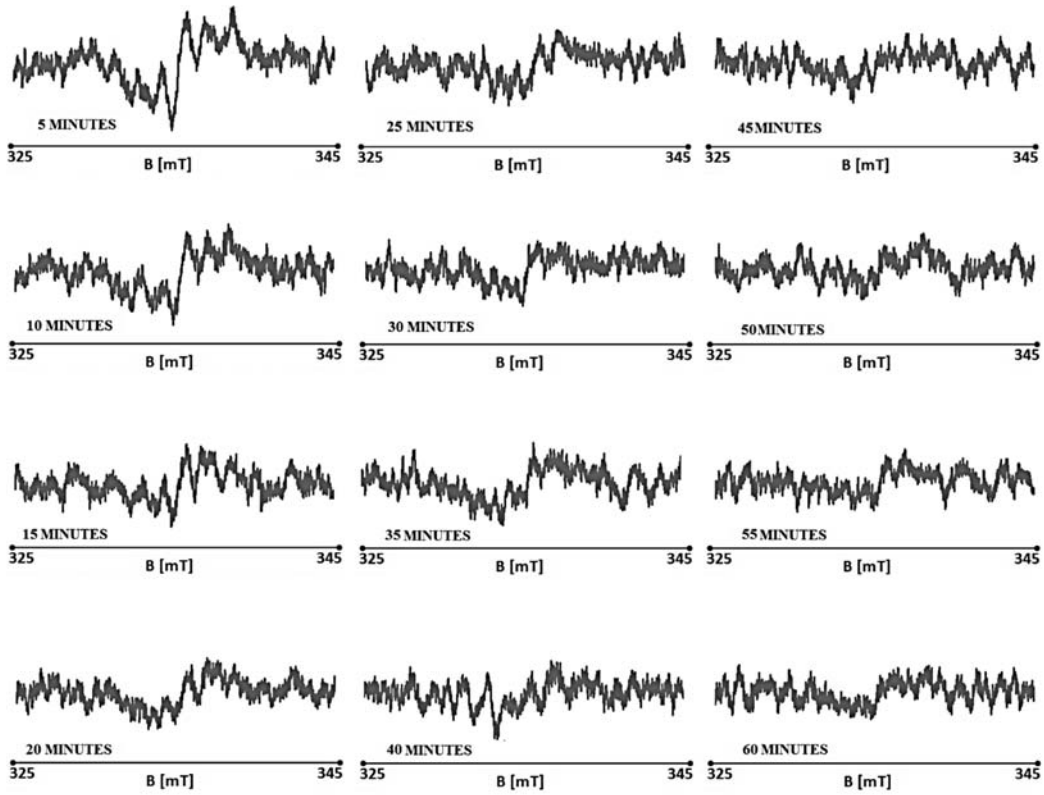

Figure 3. The EPR of DPPH interacting with the Insuman Comb 25 insulin depend on interaction time (t), respectively. **B** = magnetic induction

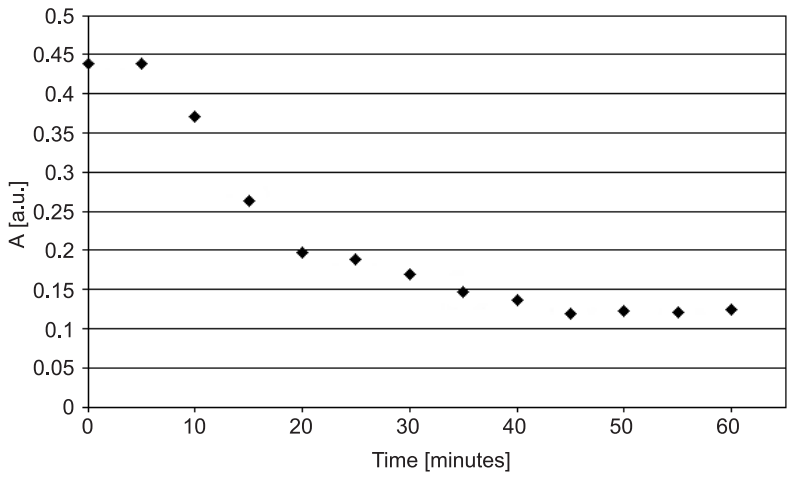

Figure 4. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH in contact with Insuman Comb 25 insulin depending on the increasing time of interaction

radicals. This effect increased with increasing of the time (t), and after 40 min the scavenging was saturated. The time of saturation of scavenging activity of Insuman Comb 25 insulin pointed out that interactions of this insulin with free radicals were fast.

Our examination confirmed antioxidant character of Insuman Comb 25 insulin.

The obtained results of our examinations are difficult to discuss with similar analyses due to the lack of the results of comparable examinations of insulins' antioxidative properties assessed by EPR method. Electron paramagnetic resonance spectroscopy was proposed as the innovatory experimental technique useful in the field of pharmacy. Free radical scavenging properties of biphasic human insulin, Insuman Comb 25, can point out the benefits of introduction of insulin therapy in type 2 diabetic patients who do not achieve therapeutic goals related to effective control of blood glucose. Antioxidative properties of exogenously delivered conventional human insulins may be of great importance in reducing oxidative stress *in vivo* and delay the progression of diabetic vascular complications.

CONCLUSIONS

Electron paramagnetic resonance (EPR) study pointed out the scavenging activity of Insuman Comb 25 insulin against free radicals. The kinetics of interactions of DPPH with Insuman Comb 25 insulin indicated that these interactions were fast and they stabilized after 40 min. The performed spectroscopic investigation by the use of microwaves proved antioxidant character of the applied insulin formulation.

Acknowledgments

This study was financially supported by Medical University of Silesia in Katowice, grant no. KNW-1-075/N/5/0.

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Received: 19. 06. 2015