The chelating agents are often used as active ingredients in the protective ointments because of their capacity of allergenic metal ions binding and reducing its penetration through the skin. The most effective ligands yet discovered preventing from metal ions induced dermatitis include ethylenediaminetetraacetic acid (EDTA), diphenylglyoxime and dimethylglyoxime and clioquinol (1). EDTA can be used in topical barrier creams as mono- to tetra-substituted salts or in the chelating complexes such as cyclohexane-1,2-diaminetetraacetic acid (CDTA) or diethylenetriaminepentaacetic acid (DTPA) (2, 3). Little is known about the influence of the additional chelating molecules introduced into the protective ointment containing with the primary chelating ligand on the efficacy of allergenic metal ions binding (4).

The aim of the study was to evaluate the effect of presence of selected amino acid ingredient on the chelation ability of Ni\(^{2+}\) or Co\(^{2+}\) ions of the protective ointment containing 10% disodium salt ethylenediaminetetraacetic acid (Na\(_2\)H\(_2\)EDTA\(\cdot\)2H\(_2\)O). Three amino acids (glycine — Gly, aspartic acid — Asp and histidine — His) in two concentrations and two pharmaceutical formulations were tested in the diffusion glass chamber with artificial membrane.

**EXPERIMENTAL**

The membrane made of the chemically modified cellulose (SPECTRAPORE) was applied to divide the diffusion glass chamber used in the in vitro test into the receptor and donor compartment. The solution containing 12 mg of Ni(NO\(_3\))\(_2\)\(\cdot\)6H\(_2\)O or 10 mg CoCl\(_2\)\(\cdot\)6H\(_2\)O dissolved in 20 mL of water rinsed the surface of the membrane and the pressure of peristaltic miniflow pump forced the solution circulation. The receptor compartment was filled with tested ointment. Every 60 min, the 100 mL aliquots were withdrawn from the receptor solution, diluted to the volume of 3 mL and used to determine the concentration of Ni\(^{2+}\) or Co\(^{2+}\) by the atomic absorption spectrophotometry (model AAS vario 6) at the wavelength of 240.7 nm for Co\(^{2+}\) and 232.0 nm for Ni\(^{2+}\). The experiments were run for 6 h, each ointment sample was analyzed three times.

The active chelating ingredients of each protective ointment, i.e., Na\(_2\)H\(_2\)EDTA\(\cdot\)2H\(_2\)O (10%) and one of the amino acids (Gly, Asp, His, 10% or 5%) were dissolved in the buffer of pH = 7.4 (prepared according to Polish Pharmacopoeia prescription), which was emulgated in the eucerin or hascobase with the application of unguator. The ointment without amino acid containing only 10% Na\(_2\)H\(_2\)EDTA\(\cdot\)2H\(_2\)O was also used in the test.

The statistical analysis was performed with the use of Statistica 7.0 computer program. The normal distribution of analyzed data was confirmed by Shapiro-Wilk test. The analysis of variance ANOVA and post hoc Tuckey test were applied to compare the differences in the chelation ability of Ni\(^{2+}\) or Co\(^{2+}\) between the specific pharmaceutical formulas of the protective ointments with different base formula or concentration of chosen amino acids.

**Keywords:** contact allergy dermatitis, protective ointment, chelation therapy

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A p value of < 0.05 was considered statistically significant.

RESULTS

The study showed that the presence of 10% of one of the chosen amino acids in the protective ointment containing 10% Na₂H₂EDTA·2H₂O enhanced the chelation ability of Ni²⁺ for both eucerin (p = 0.034) and hascobase vehicle (p = 0.021) as well the chelation ability of Co²⁺ (p = 0.026 and p = 0.019 for eucerin and hascobase, respectively). The effect of amino acids on the Ni²⁺ or Co²⁺ chelation ability of the tested protective ointment is shown in Figures 1–4. The same relation was observed in the presence of 5% amino acids in the analyzed protective ointments chelated Ni²⁺ (p = 0.037 and p = 0.032 for the eucerin and hascobase, respectively) as well Co²⁺ (p = 0.028 and p = 0.033 for the eucerin and hascobase, respectively). The optimal Ni²⁺ and Co²⁺ ions chelation effect of Ni²⁺ and Co²⁺ ions was observed for the protective ointment containing hascobase, 10% Na₂H₂EDTA·2H₂O and 10% histidine which was found to chelate 42.4 ± 2.18% of Ni²⁺ and 45.19 ±
3.68% of Co²⁺ after 6 h of diffusion test, whereas the ointment containing 10% Gly or 10% Asp chelated 35.29 ± 1.39% of Ni²⁺ and 33.19 ± 2.72% of Co²⁺ or 21.4 ± 1.93% of Ni²⁺ and 19.87 ± 2.68% of Co²⁺, respectively. The ointment containing only 10% Na₂H₄EDTA·2H₂O without amino acid supplement was found to chelate 20.19 ± 1.39% of Ni²⁺ and 17.53 ± 2.39% of Co²⁺. For all tested pairs of ointment the differences in the chelation ability of Ni²⁺ and Co²⁺ were statistically significant (p < 0.05) with the exception of the pair hascobase/hascobase + 10% Asp in the ointment. Moreover, the ointment containing eucerine, 10% Na₂H₄EDTA·2H₂O and 10% histidine appeared to be optimal for chelating Ni²⁺ and Co²⁺ ions. After 6 h lasting diffusion test, the ointment with 10% histidine and 10% Na₂H₄EDTA·2H₂O emulsified in the eucerine was found to chelate 44.12 ± 2.64% of Ni²⁺ and 43.11 ± 2.37% of Co²⁺, whereas the ointments containing 10% Gly or 10% Asp chelated 37.12 ± 2.42% of Ni²⁺ and 34.11 ± 3.07% of Co²⁺ and 24.12 ± 2.74% of Ni²⁺ and 22.42 ± 3.02% of Co²⁺, respectively. The ointment containing only 10% Na₂H₄EDTA·2H₂O without amino acids chelated 18.62 ± 1.74% of Ni²⁺ and 19.42 ± 2.02% of Co²⁺. There was no statistically significant difference in chelation efficacy of Ni²⁺ and Co²⁺ between the ointment without amino acid and the ointment with Asp. For the all remaining pairs of the analyzed ointments the differences were statistically significant.

For all pairs of the tested ointments differing only by the concentration of chosen amino acid (5% or 10%), statistically significant higher ability of chelation of Ni²⁺ and Co²⁺ was shown for the ointments containing 10% of chosen amino acid. The ointment with 5% His and 10% Na₂H₄EDTA·2H₂O chelated only 27.12 ± 2.44% of Ni²⁺ and 29.23 ± 1.72% of Co²⁺ for eucerin formula and 28.12 ± 1.94% of Ni²⁺ and 23.42 ± 2.31% of Co²⁺ for hascobase formula. The effect of the base used in the pharmaceutical formulas appeared to be statistically insignificant in all tested pairs of ointments.

**DISCUSSION AND CONCLUSION**

Many chelating agents able to chemically bind Ni²⁺ and Co²⁺ metal ions have been investigated for their capacity to protect against the development of metal allergenic contact dermatitis. Most of them were used in the topical protective barrier creams, however, its usage in some situations may be limited because of their toxicity, especially after systematic dosage (5). Little is known about the efficacy of chelating allergenic metal ions by the protective ointments containing endogenous, natural chelating ligands such as aminoacids. EDTA was often used in topical pharmaceutical formulations in several forms as mono-, di-, tri-, and tetra-substituted salts (6). The barrier cream containing 10% Na₂H₄EDTA·2H₂O was shown to be the most effective in the chelation of allergenic metal ions. The higher concentrations of Na₂H₄EDTA·2H₂O or tetra-substituted salt (CaNa₂EDTA) appeared to have fair effectiveness in chelating metal ions. (7, 8). In the present study, the elaboration of a chemical formula of the protective ointment with 10% Na₂H₄EDTA·2H₂O and one of three amino acids (Gly, Asp, and His) in two concentrations (5% or 10%) was performed with the application of the diffusion chamber equipped with artificial membrane and two pharmaceutical bases (hascobase or eucerine). The protective ointment containing 10% Na₂H₄EDTA·2H₂O and 10% histidine was found to have the greatest chelation ability of Ni²⁺ and Co²⁺ ions. Moreover, as it appeared in this study, the protective ointment with 10% His was more effective in chelating Ni²⁺ and Co²⁺ than the ointments with Asp and Gly because of several reasons. The value of logarithm of equilibrium constant (pK) for Na₂H₄EDTA·2H₂O and Ni²⁺ and Co²⁺ ions depends on the pH value according the Ringbome curve. Histidine creates a pH of approximately 7.6 which is an optimal value for the most effective kinetics of the chelation reaction of the analyzed metal ions. Histidine, as a basic amino acid, probably binds iron in the epidermis more effectively than the acidic or neutral ligand such as Asp or Gly because the epidermis is slightly acidic (9, 10).

For the prevention of a metal induced dermatitis, a chelating ligand used should be non-toxic, non-irritant, non-sensitizing and not adsorbed through the skin. In addition, it should form a complex with Ni²⁺ and Co²⁺ ions that is more stable than the nickel conjugate antigen and have more rapid complexation reaction kinetics with Ni²⁺ and Co²⁺, compared to the skin-protein-nickel/cobalt-conjugate antigen. Moreover, the ligand should be dispersed in a vehicle that is suitable for cutaneous application and has a pH value that favors Ni²⁺ and Co²⁺ ions complexation (more than 7 for basic ligand). The pH of the epidermis and dermis ranges between 4.2–6.5 and 7.2–7.3, respectively. Therefore, the region where the immunological reaction occurs is virtually neutral and it may be that a ligand which complexes Ni²⁺/Co²⁺ under such conditions is favorable, in comparison to ligands requiring pH conditions for metal complexation that are significantly higher than or significantly lower than pH = 7 (11).
The very important ingredient of the protective ointment is the buffer which ensures the binding of $\text{H}_3\text{O}^+$ ion liberated in the reaction of $\text{Na}_2\text{H}_2\text{EDTA}$ with allergenic metal ions and enhances the displacement of the reaction equilibrium to the right (10). All three chosen amino acids improved the chelation ability of $\text{Ni}^{2+}/\text{Co}^{2+}$ ions in comparison to the ointment with $\text{Na}_2\text{H}_2\text{EDTA}$ only, probably because of their buffering property. The buffering effect of histidine is enhanced by a basic character of this amino acid and the possibility of binding two $\text{H}_3\text{O}^+$ by histidine molecule. Amino acids themselves have the property of chelating the allergenic metal ions and thus, they play an active role in $\text{Ni}^{2+}/\text{Co}^{2+}$ removal by enhancing the reaction of metal ions binding by EDTA and by chelating $\text{Ni}^{2+}/\text{Co}^{2+}$.

In the normal human albumin, the N-terminal region comprised of the amino acid sequence N-Asp-Ala-His-Lys has been shown to be a strong binding site for ions of such transition metals like Co, Ni, Cu (12). It has been suggested that among these amino acids, histidine at position 3 in human albumin polypeptide chain is essential for copper and cobalt binding (13, 14). Thus, histidine as an active chelating ingredient of the protective ointment may play a role of alternative binding site for $\text{Ni}^{2+}/\text{Co}^{2+}$ which diminishes allergenic reaction in the epidermis.

In the present study, no statistical differences between the protective ability of ointment containing eucerin or hascobase have been established. It should be noticed that the vehicle alone cannot have a positive effect on the skin status and the reduction of allergy reaction. The choice of the vehicle in the chemical formula of the ointment depends on its galenic stability and type of emulsion created in the ointment. The general rule is that water in oil emulsion (w/o) is more effective against aqueous solution of irritant agents than o/w emulsion (15).

Based on the presented results it can be stated that the $\text{Na}_2\text{H}_2\text{EDTA}$ and histidine at 10% concentration were an active component of barrier creams prepared in the experiment.

REFERENCES
