

The evaluation of effect of selected metal ions on the efficiency of passive and active transport of imipramine

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Summary

Aim. The aim of the study was to evaluate the effect of zinc as well as magnesium or copper ions on the efficacy of passive transport of imipramine hydrochloride in *in vitro* model. According to results from passive transport, the next aim of the studies was to check the efficiency of active transport of imipramine hydrochloride in the presence or absence of zinc ions.

Method. The passive transport study was conducted in specially designed capsules, while CaCo-2 cell lines were used in active transport evaluation. Zinc, magnesium and copper content was determined by F-AAS method. The analysis of imipramine hydrochloride was performed using HPLC method.

Results. Mean concentrations of zinc, magnesium, and copper ions obtained in this experiment were as follows: 2.98, 1.34 and 3.52 mg/L, respectively. The presence of zinc ions increased the efficiency of active transport of imipramine hydrochloride by 39%. Furthermore, the transport of zinc ions in the presence of imipramine hydrochloride was 27% greater than that of the zinc-containing solutions without imipramine hydrochloride. The extending of the time of experiment from 30 to 60 minutes resulted in an increase in transport efficiency of more than 10% in both cases.

Conclusions. The efficiency of passive and active transport of imipramine hydrochloride is influenced by the presence of Mg, Zn and Cu ions. The passive transport of imipramine hydrochloride after 90 minutes of experiment was the most effective in the presence of copper and zinc ions. Further studies conducted on the CaCo-2 cell line indicated a clear positive interaction of imipramine – zinc.

Key words: depression, imipramine, transport

Introduction

Scientific papers have showed that excessive activity of the glutamatergic system plays a significant role in the pathogenesis of affective disorders. An important mechanism of antidepressant activity is inhibition of the N-methyl-D-aspartate receptor (NMDA) – the key receptor of this system. NMDA is an ionotropic receptor composed of protein subunits forming an ion channel permeable to Ca^{2+} , Na^+ and K^+ ions [1]. In addition to the binding site of the agonist (glutamic acid), there are also binding sites for many substances modulating NMDA activity, such as magnesium(II) ion binding site responsible for blocking of ion channel and zinc(II) ion binding site responsible for inhibition of NMDA receptor activation [2]. Another ionotropic receptor for the glutamatergic system is the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) whose activation is associated with antidepressant activity [3]. Zinc(II) ions modulate the activity of this receptor, which, depending on the concentration, increase its activity or completely block this ion channel. In many studies, the pathogenesis and treatment of depression was therefore related to the concentration of elements such as zinc, magnesium, copper, and iron [4–9]. The modification of glutamatergic transmission through the effect on the NMDA receptor are perceived as the chances of improving the effectiveness of the pharmacotherapy of depression. Stanisławska et al. [10] showed that women with higher levels of Mg and Zn ions in serum had fewer depressive symptoms. The results of a study by Ranjbar et al. [11] indicate that concurrent zinc supplementation with antidepressants improves their efficacy compared to placebo or standard drug therapy alone. By contrast, a comparative analysis of copper ions concentrations in serum of patients is attempted to demonstrate the relationship between the concentration of this element in the blood and depressive symptoms and anxiety [12–18]. Zinc(II) and Magnesium(II) ions belong to important modulators of glutamatergic transmission and may therefore play an important role in the pathogenesis of affective disorders [3, 19–22].

Imipramine hydrochloride (IMI) antidepressants from the group of tricyclic antidepressants of the first generation was chosen as a model drug for evaluation of the effect of selected metal ions in passive and active transport of this compounds. Poleszak et al. [4] showed increased antidepressant activity in the forced swim test in mice administered simultaneously with IMI and magnesium. Studies on the effect of imipramine therapy on magnesium and zinc levels were also conducted [7, 11]. According to research by Siwek et al. [5], zinc supplementation increases the effectiveness and speed of initial therapeutic response to IMI treatment in patients who have not previously responded to antidepressant pharmacotherapy. In addition, zinc ions are present in high concentrations in the brain (average is around 150 μM), and the area in which the largest amounts of this element are found is the hippocampus [23].

Evaluation of the efficiency of transport of active substances by artificial membranes and cell monolayers is a key step in the drug discovery and development of the potential drug. These studies are particularly important for drugs absorbed after oral administration. Considering the fact that imipramine with zinc was shown to have a positive effect on depression we decided to explore their mutual interactions at the membrane transport stage.

The aim of the study was to investigate the interaction of selected bioelements (Zn, Cu, Mg) and IMI at the stage of passive transport. The passive transport study was conducted in specially designed capsule experiment. According to results from passive transport, the next aim of research was the evaluation of the effect of zinc on active transport of imipramine hydrochloride in *in vitro* model. Therefore, thermostat device Transcell-2017 designed for the experiment was then used, which utilized the CaCo-2 epithelial cell monolayer for the analysis of the active transport.

Material and methods

Reagents

Anhydrous zinc chloride and anhydrous magnesium chloride were purchased from Sigma-Aldrich, St. Louis, USA. Sodium chloride was purchased from Alfa Aesar[®], Kandel, Germany and anhydrous copper(II) chloride from POCh SA, Gliwice, Poland. IMI was from Jelfa SA, Jelenia Gora, Poland. Four times distilled water with conductivity of less than 1 $\mu\text{S}/\text{cm}$ was obtained on HLP 5 (Hydrolab Poland). Zinc(II), magnesium(II) and copper(II) ion content standards at a concentration of 1 g/L were purchased from the District Office of Measures in Lodz, Poland.

Reagent solutions preparation – passive transport

For IMI passive transport study $2 \cdot 10^{-2}$ mol/L IMI solution was prepared in sodium chloride solution at a physiological concentration of 0.9%. Zinc(II) chloride, magnesium(II) chloride and copper(II)chloride solutions at a concentration of $2 \cdot 10^{-2}$ mol/L were also prepared by dissolving the corresponding salts of these metals in 0.9% sodium chloride solution. By diluting certified zinc(II), magnesium(II) and copper(II) reference materials also in physiological saline, standard solutions used for F-AAS assays were prepared. Calibration curves were determined for zinc(II) in the range 0.1–1 mg/L, for magnesium(II) in the range of 0.075–0.3 mg/L and for copper(II) in the range of 0.1–1 mg/L.

In the passive transport study, the proprietary Teflon single chamber capsules (Figure 1) were used.

Two capsule openings were covered with a semi-permeable membrane made of regenerated cellulose (Serva, VISK-ING® Dialysis Tubing, MWCO 12000–14000). Approximately 4 mL of solution obtained by mixing in a 1:1 volume ratio: IMI + 0.9% NaCl, IMI + Zn²⁺, IMI + Cu²⁺, IMI + Mg²⁺, Zn²⁺ + 0.9% NaCl, Cu²⁺ + 0.9% NaCl, Mg²⁺ + 0.9% NaCl, respectively, were introduced into the capsules. Capsules, after being filled with test solutions of known concentration, were placed in 250-mL flasks containing 0.9% NaCl solution and placed in a Gastroel-2014 apparatus and then incubated at a temperature of 37°C for 90 min [24]. Samples of the test solution in a 2-mL volume were taken from the flasks after 15, 30, 45, 60, 75, and 90 minutes. Elements in the obtained samples were determined by F-AAS, while imipramine was determined by RP-HPLC.

Reagent solutions preparation – active transport

Active transport studies were performed with a solution of 120 mg/L IMI and a 25 mg/L zinc(II) chloride solution in physiological saline. By diluting zinc(II) salts also in physiological saline solution, the zinc standard solution used for F-AAS assays was prepared. Imipramine standard solutions for RP-HPLC assays were prepared by dissolving IMI in physiological saline and a standard curve was prepared in the range of 0.0625–1 mg/mL.

Active transport

Colon epithelial cells (colorectal carcinoma, CaCo-2 ATCC HTB-37) were cultured in Eagle's Minimum Essential Medium (EMEM) with 15% FBS, and antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL) (ATCC, Manassas, VA, USA). Mycelial cultures were maintained at 37°C in a humidified atmosphere that contained 5% CO₂ in 75 cm² Falcon-type flask at density 5·10⁵ cells. Cell morphology was investigated daily by a microscope (Olympus, Tokyo, Japan). Using the Trypan Blue Exclusion Test (Thermo Fisher Scientific, Waltham, MA, USA), the cell viability during culturing was assessed. In all cultures, the cells showed the correct shape. The percentage of cells that adhered to the substrate was 95–100% each time. For the final experiments, CaCo-2 cells were seeded onto PET membranes (0.4 µm pore size) inside SPL Life Sciences 6-well Insert system (SPL Life Sciences, Korea) at a density of 2·10⁴ cells/well. The cells were grown for 21 days with medium changed every 2–3 days. Using

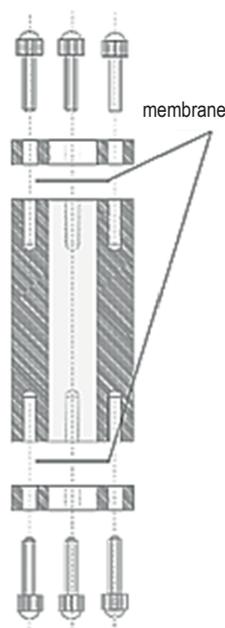


Figure 1. Capsule diagram for passive transport study

a Millicell ERS-2 Epithelial Volt-Ohm Meter electrical resistance system (Millipore, Merck, Darmstadt, Germany), the integrity of the colon epithelial cell monolayer was determined by measuring the trans-epithelial electrical resistance (TEER). CaCo-2 cells with TEER values above $800 \Omega \cdot \text{cm}^2$, indicating tightness of the junctions between intestinal epithelial cells, were used in the experiments.

The insert system with CaCo-2 cells was placed in the container so that their layer was in contact with physiological saline solution. A solution containing zinc ions, a solution of IMI and a solution of IMI with zinc (supernatant) was applied to the surface of the CaCo-2 cell monolayer. Each variant was incubated at 37°C for 30 and 60 min (maximum cell survival time determined experimentally) in a Transcell-2017 apparatus (device designed for the purpose of the experiment). Supernatant samples (above monolayer of CaCo-2 cells) and filtrate (below CaCo-2 cell monolayer) were sampled for analysis of zinc content (F-AAS) and imipramine (RP-HPLC).

Determination of the tested elements by F-AAS

Using an iCE 3000 Spectrometer (Thermo Scientific, UK), zinc(II), magnesium(II) and copper(II) content was determined by flame atomic absorption spectrometry (F-AAS) [25, 26]. The method was tested with the use of the certified reference material – Virginia Tobacco leaves (CTA-VTL-2). Table 1. shows the performance of the atomizer.

Table 1. Parameters of the spectrometer during the analysis of zinc(II), magnesium(II) and copper(II) by F-AAS

Parameters	Zn	Mg	Cu
Type of flame/fuel	Acetylene/Air		
Wave length [nm]	213.9	285.2	324.8
Slot width [nm]	0.7	0.2	0.7
Measurement time [s]	2s		
Curve fitting type	A linear least-square fit		
Coefficient of determination	$R^2 = 0.9986$	$R^2 = 0.9994$	$R^2 = 0.9976$
Concentration range [$\mu\text{g/g}$]	0.1-1	0.075-0.3	0.1-1

IMI determination by RP-HPLC

The solutions containing IMI were evaporated (Büchi evaporator, Germany) under pressure of 200 mBa at 40°C down to dryness. The concentrated analyte was dissolved in methanol transferred through Whatman No. 3 filter paper. The extracts were quantitatively dissolved in 1.5 mL of methanol and subjected to separation by

RP-HPLC using the Hitachi HPLC (Merck, Japan) equipped with a pump type L-7100, the Purospher® RP-18 (4 × 200 mm, 5 μm) column kept at 25°C, and UV detector operated at $\lambda = 275$ nm. The isocratic separation was applied with mobile phase as follows: methanol at flow rate of 1 mL/min. The quantitative analysis of IMI was performed using a calibration curve with the assumption of the linear size of the area under the peak and the concentration of the reference standard. Quantification was done by measuring peak area with reference to the standard curve derived from five concentrations (0.0625–1 mg/mL). The results were expressed in mg/L.

Statistical analysis

The statistical analysis of the results was performed by One-Way ANOVA with Tukey's multiple-comparison *post-hoc* test (GraphPad Instat 3). The difference between the means at $p \leq 0.05$ was considered statistically significant.

Results

The use of the procedure developed for the samples preparation for the analysis as well as the adoption of F-AAS method to evaluate the amount of bioelements and RP-HPLC method to evaluate the amount of imipramine hydrochloride (IMI) allowed for their accurate and rapid determination.

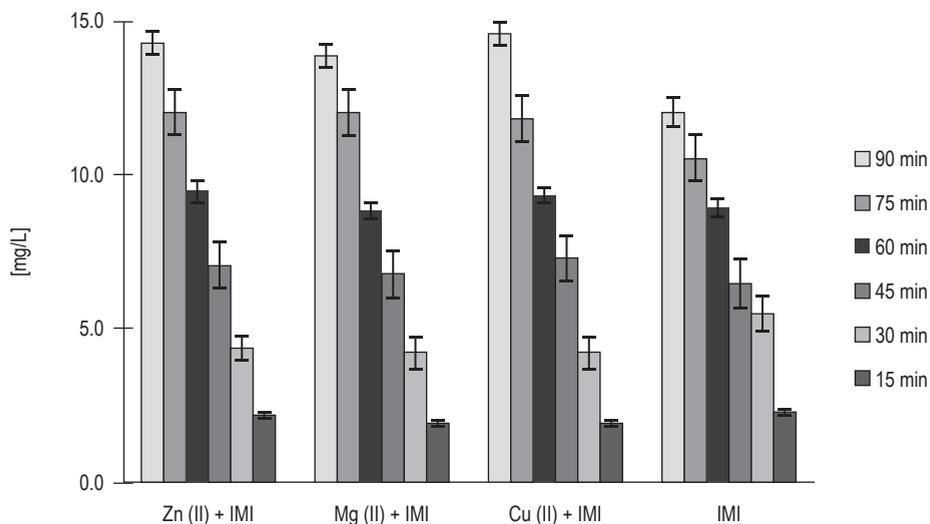


Figure 2. Chart of change in concentration [mg/L] of imipramine hydrochloride in 0.9% NaCl solution for studies in the presence of zinc(II), magnesium(II) and copper(II) ions and imipramine hydrochloride, depending on the time of experiment

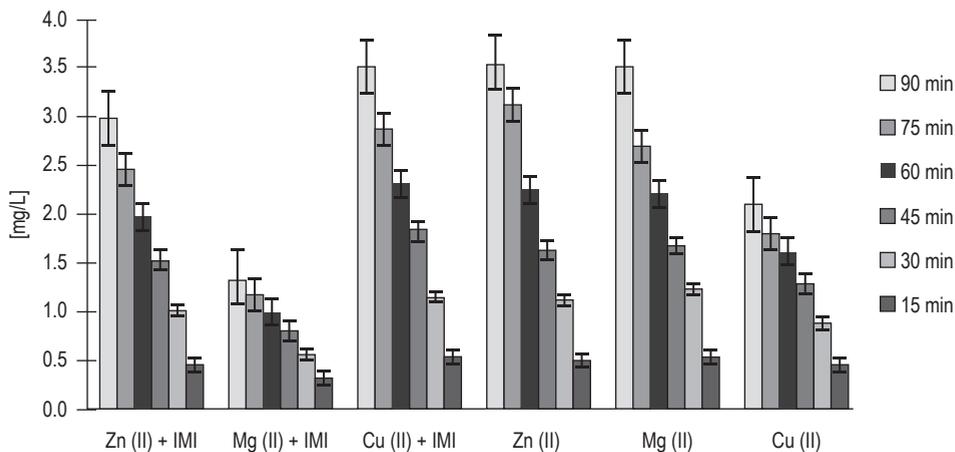


Figure 3. Graph of zinc(II), magnesium(II) and copper(II) concentration changes [mg/mL] for research conducted in the presence of imipramine hydrochloride and for metals according to the time of experiment

The experiment was performed in two *in vitro* models (passive and active transport) to assess the effect of selected metal ions on the transport of IMI.

In the model of passive transport, the efficiency of IMI transport through semipermeable membrane was investigated in the first variant, and the efficiency of diffusion of this drug after the addition of zinc(II), magnesium(II) or copper(II) ions in the second variant (Figure 2).

It was determined that the mean concentration of IMI after 90 min of the experiment was 12.11 mg/L, while the mean concentrations of the drug tested in the presence of Zn(II), Mg(II) or Cu(II) ions were 14.32, 13.83 and 14.59 mg/L, respectively. There were statistically significant differences between IMI vs. IMI + Cu(II) concentrations. Passive transport of IMI after 90 min was most effective for tests in the presence of Cu(II) ions.

In the next step of the study, the effect of the presence of IMI on the efficiency of diffusion of zinc(II), magnesium(II) and copper(II) ions by semipermeable membrane (Figure 3) was evaluated.

The analysis began with an experiment on solutions containing only ions of the tested metals. There were statistically significant differences between Cu(II) vs. Zn(II) and Cu(II) vs. Mg(II) concentrations after 90-min incubation. Cu(II) concentration was significantly lower than Zn(II) and Mg(II) concentrations. The concentrations of the tested metals were then determined in solutions containing also the test drug. Mean concentrations of zinc(II), magnesium(II) and copper(II) ions obtained in experiments conducted in the presence of IMI after 90 min were as follows: 2.98 mg/L, 1.34 mg/L and 3.52 mg/L, respectively.

Analyzing the concentration changes after 15, 30, 45, 60, and 90 min for zinc(II) and magnesium(II) ions in this study variant, lower values were confirmed in comparison with analogous trials carried out without drug. Whereas in case of copper(II), on the contrary, the resulting concentrations were higher when compared to solutions with the addition of IMI.

Discussion

Imipramine is a model antidepressant with known pharmacokinetics, but there are no studies describing the effect of metal ions on the transport performance of this drug. The amount of the elements delivered to the tissues in the human body is also affected by organic compounds with complexing properties. Such trends have been noted in the study of depression in which imipramine and zinc were used [11]. The inhibitory effects of magnesium and calcium on the absorption of tetracycline are commonly known. In addition, in the literature information about inhibition of levofloxacin absorption with the simultaneous presence of aluminum hydroxide can be found [27]. The similar effect of iron(II) sulphate(VI) on the absorption of ciprofloxacin and ofloxacin was also described [28]. Zinc, magnesium and copper ions are transported from food *via* passive transport, so it was interesting to investigate their effect on imipramine transport efficiency. Zinc from food is absorbed in the small intestine due to ZIP4 transporters and passive transport, similar to magnesium and copper [29–31].

The analysis of determined concentrations of metals showed statistically significant differences between Cu(II) + IMI vs. Mg(II) + IMI and Zn(II) + IMI vs. Mg(II) + IMI. The concentrations of Zn(II), Mg(II) and Cu(II) obtained in two variants of the experiment were also compared: the tested metals and the tested metals with the addition of IMI. The following statistically significant differences were found: Cu(II) vs. Zn(II) + IMI, Cu(II) vs. Mg(II) + IMI, Zn(II) vs. Mg(II) + IMI, Cu(II) vs. Cu(II) + IMI, Zn(II) vs. Zn(II) + IMI, and Mg(II) vs. Mg(II) + IMI. As a result of the metal assays, it has been shown that the addition of the tested drug improves the efficiency of Cu(II) passive transport. The efficiency of transport of zinc ions was slightly reduced in the presence of IMI. Transport of magnesium ions was significantly more effective in solutions without the addition of IMI.

Due to the importance of zinc for the treatment of depression, IMI alone, zinc ions and zinc ions with IMI were selected for the analysis of active transport. By analyzing the results of the tests performed for zinc, it was found that the transport of this element in the presence of IMI was 27% higher than in the case of analysis performed for solutions containing zinc alone without IMI. In addition, an increase in the experiment time from 30 to 60 minutes also resulted in an increase in transport efficiency of more than 10% (Table 2).

Table 2. The content of zinc determined in samples obtained from the active transport experiment with or without imipramine hydrochloride (IMI)

Sample	[mg/L]	SD	[mg/L]	SD
30 min				
Control cells	—	—	—	—
IMI + Zn	4.19	1.36	15.68	2.16
Zn	5.45	1.18	11.34	1.65
60 min				
IMI + Zn	2.71	0.67	18.20	1.21
Zn	4.66	0.56	13.41	1.01

— not detected; n = 3 repetitions; ≤ 0.05 ; – supernatant; – sample below the cell monolayer

A similar trend has been observed in transport studies of IMI alone. In this case, the addition of zinc ions increased the efficiency of active IMI transport by 39% and prolongation of the time of analysis resulted in a significant increase in the amount of IMI in the filtrate (Table 2 and 3).

Table 3. The content of imipramine hydrochloride (IMI) determined in samples obtained from the active transport experiment with or without zinc

Sample	[mg/L]	SD	[mg/L]	SD
30 min				
Control cells	—	—	—	—
IMI + Zn	38.03	3.87	6.92	2.27
IMI	26.17	3.06	4.18	0.77
60 min				
IMI + Zn	35.21	1.89	10.51	3.85
IMI	28.12	4.96	10.98	5.87

— not detected; n = 3 repetitions; ≤ 0.05 ; – supernatant; – sample below the cell monolayer

Conclusions

1. The efficiency of passive transport of imipramine hydrochloride through artificial membrane is influenced by the presence of analyzed metal ions, especially zinc and copper ions.
2. Based on presented studies, transport across CaCo-2 cell monolayer, both in the case of the analysis of the efficiency of the transport of IMI and zinc from solution, greater amounts of these compounds were determined when both substances

were used together. There is a clear positive IMI–zinc interaction, facilitating their transport through cells – primarily in active transport.

3. The transport of Zn is time-dependent. No such a trend has been observed for IMI.
4. The obtained results may suggest that simultaneous administration of IMI with zinc in *in vitro* conditions may increase the amount of these substances in tissues.

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