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Purification of Carbonic Anhydrase from *Capoeta umbla* (Heckel, 1843) Gills and Toxicological Effects of Some Metals on Enzyme Activity

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Abstract

In this study, *in vitro*effects of some metal ions (Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+})on cytoplasmic carbonic anhydrase(CA, EC 4.2.1.1) from *Capoeta umbla* gill was investigated. CA was purified from the gills of *C. umbla* for the first time. It was purified with the Sepharose-4B-L-Tyrosine Sulphanilamide affinity chromatography method. The overall purification was approx. 31.69-fold with a yield of 53.33%, and a specific activity of 326.73 EU/mg proteins. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) showed a single band corresponding to a molecular weight of approx. 29 kDa. The constants of the enzyme inhibitor complex (K_i) and 50% inhibitory values (IC_{50}) for metal ions were determined by Lineweaver-Burk graphs and plotting activity % vs. [I], respectively. The $K_i$constants and IC_{50} values were 0.012 ± 0.0135 and 0.136 mM for Fe^{3+}, 0.019 ± 0.0113 and 0.191 mM for Cd^{2+}, 0.041 ± 0.0075 and 0.289 mM for Pb^{2+}, and 0,120 ± 0.034 and 0.924 mM for Ni^{2+}. It was determined that Fe^{3+}, Cd^{2+} and Pb^{2+} inhibited the enzyme competitively while Ni^{2+} inhibited the enzyme noncompetitively. The potential inhibitor for *C. umbla* gill CA was found as Fe^{3+} from these results.

Keywords:*Capoeta umbla*, Carbonic anhydrase, Gills, Metal toxicity

Introduction

Metals are natural trace components of the aquatic environment, but their levels have increased due to industrial, agricultural and mining activities (Kalay and Canlı, 2000). This situation may be hazardous for living systems, especially aquatic organisms, including specific enzymes. It is well-known that enzymes catalyse almost all chemical reactions in the metabolism of the living systems. These chemical substances including pollutants, pesticides, drugs and metal ions influence metabolism at low concentrations by decreasing or increasing enzyme activities (Ekinci *et al.*, 2007).Specifically, some enzymes including carbonic anhydrase (CA) are considered drug- and chemical-targeted enzymes. For example, CA is expressed in almost all the tissues of living things. Because of these, the enzyme, which has great importance in the balance of pH and respiration in various tissues, is a known target enzyme for different substances (Coban *et al.*, 2008; Söyüt and Beydemir, 2011).

CA is a zinc metalloenzyme catalysing the reversible hydration of CO_2 to produce H^+ and HCO_3^- (Supuran, 2010). CA plays key roles in a wide variety of physiological processes involving CO_2 and $HCO3^-$. In animals the various CA isozymes are found in many different tissues and are involved in a number of different physiological processes, including bone resorption, calcification, ion transport, acid–base transport, and a number of different metabolic processes such as biosynthetic reactions (gluconeogenesis, lipogenesis, and ureagenesis). In algae and plants they play an important role in photosynthesis (Ivanov *et al*, 2007; Cannon *et al.*, 2010; Zhang *et al.*, 2010; Lionetto *et al.*, 2012).

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The vertebrate gas exchange organ, the gills is essentially composed of a highly complex vasculature, surrounded by a high surface area epithelium that provides a thin barrier between a fish's blood and aquatic environment. Fish gills are the first point of contact between metals and impurities in water. Metals can bind to gill of fish and corrupt the ion-regulatory and respiratory functions of the gills (Playle, 1998). CA, abundantly present in gill epithelial cells, is assumed to play a role in these processes (Perry and Laurent, 1990; Evans *et al.,* 2005). The erythrocyte CA is the primary and possibly the only site of HCO_3^- dehydration in fish, forming CO_2 which diffuses across the respiratory gill epithelium into the ventilatory water. A small portion of the CO_2 is hydrated by cytoplasmic branchial CA, supplying HCO_3^- and H^+ for Na^+/H^+ (NH_4^+) and Cl^-/HCO_3^- apical ionic exchangers. In view of these processes regulating acid–base and NaCl balance, the cell-specific localization of CA in fish gills has been investigated (Henry *et al.,* 1988; Sender *et al*., 1999) and a large interspecies variability has been observed (Conley and Mallatt, 1988).

In the present study, CA, which is very important and ubiquitous in metabolism, was purified from *C. umbla* gill by a fast and simple technique. Additionally, in vitro inhibition effects of some metals (Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+}) *C. umbla* gill CA were investigated.

Materials and Methods

Chemicals

Sepharose-4B, protein assay reagents and chemicals for electrophoresis were purchased from Sigma–Aldrich. All other chemicals were of analytical grade and obtained from Merck.

Preparation of the Homogenate

Fish samples were caught from Murat River (Bingöl, Genç).Gill samples were taken from each fish. Gills samples were washed three times with 0.9% NaCI, an isotonic saline solution. The gill cells were lysed by immersion in liquid nitrogen (approximately -163 °C). The lysed sample was transferred to a buffer solution (50mM Tris–HCl + 0.1 MNa_2SO_4,pH 8.0)andcentrifuged at 4°C, 15000 g for 60 min(Le Trang *et al*., 1983).The plasma and precipitate were removed. Supernatant was used in further studies.

Purification of CA from *C. umbla* Gills by Affinity Chromatography

CNBr activated Sepharose-4B was washed with ddH_2O. After that, tyrosine was attached to the activated gel as a spacer arm and finally diazotized sulphanilamide clamped with tyrosine molecule as ligand. The homogenate was applied to the prepared Sepharose-4B-L-Tyrosine Sulphanilamide affinity column equilibrated with 25 mM Tris–HCl/0.1M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22mM Na_2SO_4 (pH 8.7). *C. umbla* gill CA was eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3). All procedures were performed at 4°C(Bayram *et al*., 2008). The enzyme used this *in vitro* experiment section was dialyzed.

Measurement of CA Activity

CA activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson(Wilbur and Anderson, 1948).CO_2-hydratase activity as an enzyme unit (EU) was calculated by

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using the equation (t_0-t_c/t_c) where t_0 and t_c are the times for pH change of the non-enzymatic and enzymatic reactions, respectively.

Protein Determination

Protein levels werespectrophotometrically measured at 595 nm according to Bradford's method(Bradford, 1976), with bovine serum albumin being used as a standard.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The control of enzyme purity was carried out using Laemmli's procedure(Laemmli, 1970) in 3% and 8% acrylamide concentrations for running and stacking gel, respectively. SDS (10%) was added to the gel solution. The gel was stabilized in a solution containing 50% propanol + 10% TCA + 40% distilled water for 30 min. Staining was performed for about 2 h in a solution of 0.1% Coommassie Brilliant Blue R-250 + 50% methanol + 10% acetic acid. Finally, washing was carried out in a solution of 50% methanol + 10% acetic acid + 40% distilled water until the protein bands were cleared.

***In vitro* Inhibition Assays**

The effects of increasing concentrations of Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+}on *C. umbla* gill CA activities were determined colorimetrically using CO_2-hydratase assay. The metals were also tested in the hydratase activity assay in triplicate at each concentration used. Different concentrations of metals were examined in preliminary assaysand as described in the literature (Vitale *et al.*, 1999). Enzyme activities were measured in the presence of different concentrations of Fe^{3+}(0.06, 0.10, 0.12, 0.13 and 0.26 mM), Cd^{2+}(0.07, 0.09, 0.14, 0.21 and 0.35 mM), Pb^{2+}(0.20, 0.29, 0.32, 0.36 and 0.43 mM)and Ni^{2+}(0.32, 0.65, 0.97, 1.13 and 1.29 mM). Control enzyme activity in the absence of a metal was taken as 100%. For each metal, an activity % vs. inhibitor concentration tube was drawn using conventional polynominal regression software (Microsoft Office 2010, Excel). Metal concentrations that produced 50% inhibition (IC_{50}) calculated from graphs (Söyüt and Beydemir, 2011).

To determine $K_i$constants, three different inhibitor concentrations were tested: 0.04, 0.12 and 0.26 mM for Fe^{3+}; 0.07, 0.14 and 0.21 mM for Cd^{2+}; 0.20, 0.32 and 0.43 mM for Pb^{2+} and 0.45, 0.65 and 1.27 mM for Ni^{2+}. *P*-nitrophenylacetate was used as a substrate. The substrate concentrations were 0.15, 0.3, 0.45, 0.6 and 0.75 mM in these experiments. The Lineweaver-Burk curves obtained were used for the determination of $K_i$and inhibitor type (Lineweaver and Burk, 1934).

Results

The *C. umbla* gill CA was purified by Sepharose 4B-L-tyrosine-sulfanylamide affinity chromatography and purity was confirmed by SDS-PAGE (Figure 1). The enzyme was purified 31.69-fold with a yield of 53.33% with a specific activity of 326.73 EU/mg protein from *C. umbla* gill (Table 1).

In addition to purification of the enzyme, we investigated the *in vitro* effects of Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+}on fish gill CA activity. All metal showed inhibitory effects on the enzyme. IC_{50} values of Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+} were0.136, 0.191, 0.289 and 0.924 mM, and K_i constantswere 0.012 ± 0.0135, 0.019 ± 0.0113, 0.041 ± 0.0075 and 0.120 ± 0.034 mM, respectively (Table 2).While Fe^{3+}, Cd^{2+} and Pb^{2+} showed competitive inhibition,Ni^{2+} showed

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noncompetitive inhibition (Figure 2, Figure3).According to these results, Fe^{3+} has higher inhibition effects than Cd^{2+}, Pb^{2+} and Ni^{2+}.

Discussion

Severalecological changes occur in water because of human activities, including agricultural, industrial and municipal wastes. The pollution of the aquatic environment with heavy metals has become a serious health concern because of their toxicity and accumulation by organisms (Mendil *et al.,* 2010). Some metals, such as Cd, Hg and Pb, have no known essential role in living organisms, and are toxic at even low concentrations. But at low levels, some metals such as Cu, Co, Zn, Fe and Mn are essential for enzymatic activity and severalbiological processes. The essential metals also become toxic at high concentrations (Bryan, 1976).For example, many enzymes contain Zn^{2+} with in the structures, such as CA (Lionetto *et al.,* 2012).

CAs, the zinc-binding enzyme family, has important roles in a number of tissues for living physiology, for example, pH and CO_2 homeostasis (Soyut et al., 2008). CO_2, produced in fish tissues, is hydrated rapidly by CA enzyme, converted into HCO_3^-, and transported in the blood (Geers and Gros, 2000; Esbaugh *et al.,* 2005; Esbaugh and Tufts, 2006;Supuran, 2008). Approximately 98% of the transported and stored CO_2 is in HCO_3^- form. At the respiratory epithelium, erythrocytic CA catalyses the rapid dehydration of $HCO_3^-$to molecular CO_2, which then diffuses passively into the ventilatory water stream. Moreover, the CO_2/HCO_3^-system constitutes one of the most important physiological buffers for acid–base regulation(Doğan, 2006; Alp *et al*., 2010;Ekinci and Beydemir, 2010).

Up to now, CA has been purified from many different tissues including human erythrocytes (Bayram et al., 2008; Şentürk et al., 2009), fish gills (Bone et al., 1995; Skaggs and Henry, 2002; Hisar et al., 2004; Ceyhun et al., 2010), fish erythrocytes (Bülbül et al., 2003; Hisar et al., 2005; Doğan, 2006), rainbow trout brain and liver (Söyüt and Beydemir, 2008; Söyüt et al., 2008). Although, CA and the inhibitory effects of many metals on CA have been studied in most fish tissues, no study has been done on *C. umbla* gill CA, yet.

For this reason, we investigated *in vitro*effects of some metals, Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+}, on CA enzyme from *C. umbla* gill.It was purified with the Sepharose-4B-L-Tyrosine Sulphanilamide affinity chromatography method. The overall purification was approx. 31.69-fold with a yield of 53.33%, and a specific activity of 326.73 EU/mg proteins. The enzyme purity and subunit molecular weight (29 kDa) were determined by SDS-PAGE electrophoresis method (Figure 1). Additionally, the four metals (Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+}) dose-dependently inhibited *in vitro* CA activity. IC_{50} values for Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+} were 0.136, 0.191, 0.289 and 0.924 mM, and K_i constants were 0.012 ± 0.0135, 0.019 ± 0.0113, 0.041 ± 0.0075 and 0.120 ± 0.034 mM, respectively (Table 2). While Fe^{3+}, Cd^{2+} and Pb^{2+} showed competitive inhibition,Ni^{2+} showed noncompetitive inhibition (Figure 2, Figure 3). According to these results, Fe^{3+} has higher inhibition effects than Cd^{2+}, Pb^{2+} and Ni^{2+}.

*In vitro*effects of some heavy metals on enzymes, such as intestinal and branchial CA and Na^+–K^+–ATPase,which play a key role in salt and osmoregulation and acid–base balance in the teleost fish, was studied. The early work of Christensen and Tucker (1976) demonstrated CA inhibition by heavy metals for the first time in fish. The study was carried out on red bloodcells CA of the teleost *Oncorhynchus mykiss*. Erythrocyte CA, which represents the mostabundant pool of the enzyme in fish, appeared significantly *in vitro* inhibited by several heavy metals, such as, Cd^{2+}, Cu^{2+}, Ag^+, and Zn^{2+} (Lionetto *et al.,* 2012).In another study,CA activities in gill and

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intestinal of European ell (*Anguilla Anguilla*) homogenates were significantly inhibited by Cd^{2+}. The gill CA was much more sensitive to heavy metal as compared to the enzyme activity in the intestine, as observed by comparing the IC_{50} values (gill IC_{50}: 0.01 mM and intestine IC_{50}: 0.036 mM)(Lionetto *et al*., 2000). The effects of ammonia and urea on branchial CA enzyme which plays a key role in ionregulation, osmoregulation and acid-base balance of rainbow trout (*Oncorhynchus mykiss*) were investigated. CA activities of the groups exposed to ammonia and urea were measured at 1, 2 and 3 h. The inhibitory effects of ammonia and urea on gill CA activities started immediately after the exposures. The differences between the initial CA activities for the controls werenot significant ($P > 0.01$). The CA activities were significantly ($P < 0.01$) inhibited both in ammonia and urea group. However, the ammonia was inhibited more than urea since there weresignificant differences between the final values of gill CA activities (Hisar *et al*., 2004). Another study demonstrated *in vitro*and *in vivo*effects of the pesticides, deltametrine, diazinon, propoxur and cypermethrin on the activity of rainbow trout gill CA. The four pesticides dose-dependently inhibited *in vitro*CA activity. IC_{50} values for deltametrine, diazinon, propoxur and cypermethrin were 0.137, 0.267, 0.420 and 0.460 µM, respectively. *In vitro*results showed that pesticides inhibit fish gill CA activity intherank order of deltametrine> diazinon> propoxur> cypermethrin. Besides, *in vivo*studies of deltametrinewere performed on CA activity of rainbow trout gill. Fish gill CA was significantly inhibited at three concentrations (0.25, 1.0 and 2.5 µg/L) at 24 and 48 h(Ceyhun *et al*., 2010).

Söyüt and Beydemir (2011) investigated the *in vitro* effects of Co^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+}on chemical-targeted CA enzyme from rainbow trout kidney. The enzyme was purified with a specific activity of 17.285 EU/mg and 31.7% yield and approximately 1800-fold using simple affinity purification method. Molecular weights of the subunit and native enzyme were estimated as 28.7 kDa and 26.9 kDa via SDS-PAGE and Sephadex-G 200 column, respectively. Inhibitory effects of Co^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+}on CA activity were determined using the esterase method under *in vitro* conditions. $IC_{50}$and K_i values were calculated for metals. K_i values for Co^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+} were 0.035, 1.2, 34.8, 103 and 257 mM from Lineweaver–Burk graphs, respectively. Consequently, *in vitro* inhibitionrank order was determined as $Co^{2+}>Zn^{2+}>Cu^{2+}>Cd^{2+}>Ag^{+}$. Alım *et al.* (2014) investigated the inhibition effects of some metal concentrations (Ag^{+}, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}) on the activity of CA from tuna (*Thunnus thynnus* Linnaeusi, 1758) gill, *invitro*.The tuna gill CAwas obtained by affinity chromatography with a yield of 31%, a specific activity of 1062 EU/mg protein and a purification of 44.06-fold. The purity of the enzyme was confirmed by SDS-PAGE. Inhibitory effects of metals on CA activity were determined at different concentrations using the hydratase method under *in vitro* conditions. Consequently, *in vitro* inhibition rank order was determined as $Ag^{+}>Cu^{2+}>Pb^{2+}>Zn^{2+}>Cd^{2+}> Co^{2+}$. From these results, they showed that Ag^{+} is the most potent inhibitor of CA enzyme.

Consequently, we investigated the *in vitro*effects of the metals, Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+}, on the activity of *C. umbla* gill CA. Carbonic anhydrase was purified from the gills of *C. umbla* for the first time.$K_i$and IC_{50} parameters of these metals were determined. It is clear that Fe^{3+} is the most potent inhibitor for CA enzyme. $K_i$and IC_{50} graphs show that Fe^{3+}, Cd^{2+}, Pb^{2+} inhibit CA in a competitive manner and Ni^{2+} inhibit CA in anoncompetitive manner (Figure 2, Figure 3, Table 2). According to $K_i$constants, the best inhibitor for *C. umbla*gill CA is Fe^{3+}.Our results are in agreement with others reported about teleost fish in the literature. *C. umbla*, living in riversand lakes, is consumed highly by the local residents. The rising industrial and settlement areas near the revers cause a great danger for the creaturesliving in watery areas. The required precautions should be taken immediately.

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Table 1. Summary of purification procedure for *C. umbla* gill CA enzyme by a Sepharose-4B-tyrosine-sulfanilamide affinity column chromatography.

Purification steps	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification Factor
Homogenate	300	60	29.10	1746	18000	10.309	100	1
Sepharose-4B-L tyrosine-sulphanilamide affinity chromatography	2141.67	6	4.897	29.382	9600	326.73	53.33	31.69

Table 2. The results of the activity of CA; K_i, IC_{50} values and inhibition types

Inhibitor	IC_{50} (mM)	K_i(mM)	Inhibition type
Fe^{3+}	0.136	0.012±0.0135	Competitive
Cd^{2+}	0.191	0.019±0.0113	Competitive
Pb^{2+}	0.289	0.041±0.0075	Competitive
Ni^{2+}	0.924	0.120±0.034	Noncompetitive

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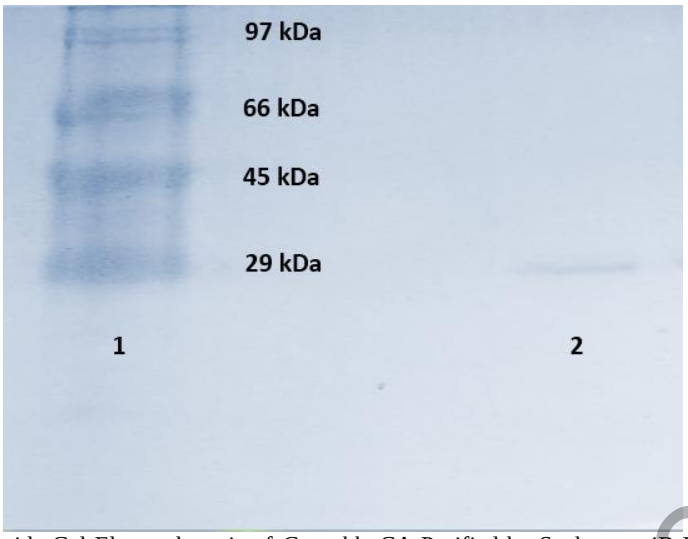

Figure 1. SDS-Polyacrylamide Gel Electrophoresis of *C. umbla* CA Purified by Sepharose 4B-L-Tyrosine-Sulphanilamide Affinity Gel Chromatography (1: Standard proteins. 2: *C. umbla* gill CA)

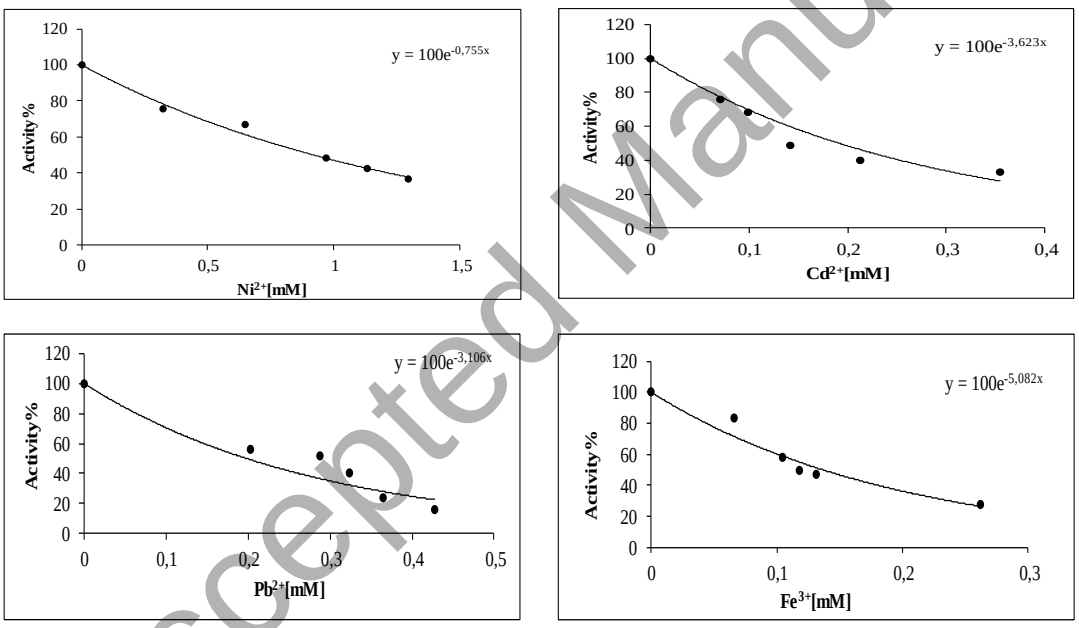

Figure 2. Activity%-[Metal] regression analysis graphs for *C. umbla* gill CA in the presence of four different metal concentrations.

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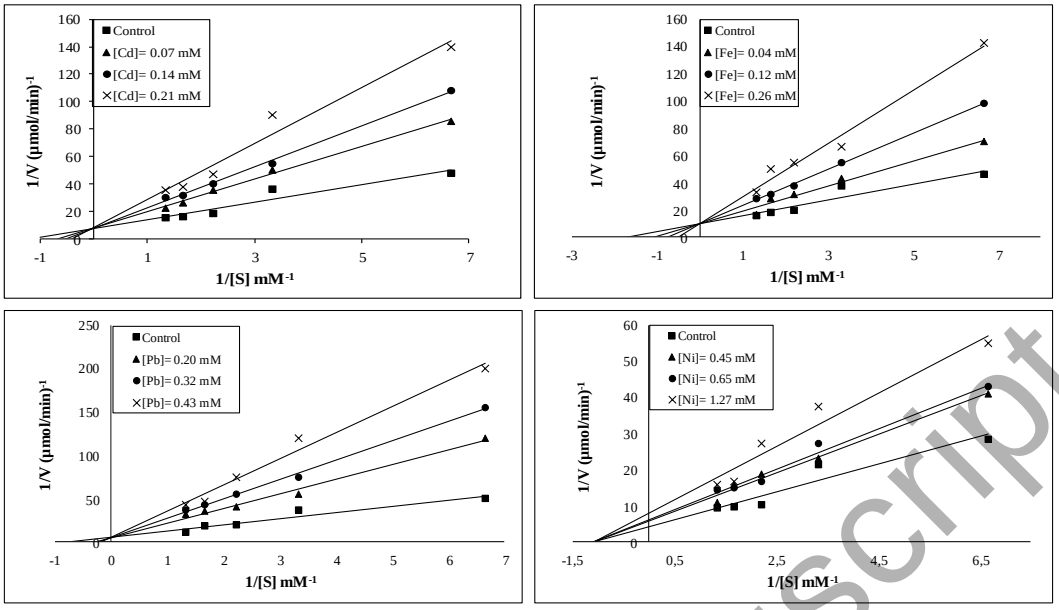

Figure 3. Lineweaver–Burk graph in 5 different substrate concentrations and in 3 different metal (Fe^{3+}, Cd^{2+}, Pb^{2+} and Ni^{2+}) concentrations for determination of K_i.