



Binding Behavior of Few Lanthanides and Heavy Metal ions with Proteins by Fast and Reliable Affinity Capillary Electrophoresis Method

HASSAN A. ALHAZMI* and MOHAMMED AL-BRATTY

Department of Pharmaceutical Chemistry, College of Pharmacy, Jazan University, Jazan,
Post code 45142, Saudi Arabia.

*Corresponding author E-mail: haalhazmi@jazanu.edu.sa

<http://dx.doi.org/10.13005/ojc/330619>

(Received: August 30, 2017; Accepted: September 20, 2017)

ABSTRACT

The interaction of two lanthanides Cerium (Ce^{3+}) and Gadolinium (Gd^{3+}) and two heavy metal ions Manganese (Mn^{2+}) and Zinc (Zn^{2+}) with various biological proteins Bovine Serum Albumin, Human Serum Albumin, β -lactoglobulin, Myoglobin and Ovalbumin were studied by using mobility shift affinity capillary electrophoresis. The used ACE method offers fast operation using shorter capillary, small injection volume, adequate short rinsing protocol and lower concentration of samples. The normalized difference of mobility ratio ($\Delta R/R_i$) was used to investigate the possible interaction. All interactions were summarized as $\Delta R/R_i$ chart and compared. For heavy metal ions, the interactions of Mn^{2+} with all proteins were significant with negative $\Delta R/R_i$ values except with Myoglobin (MB) as its $\Delta R/R_i$ close to zero (0.002 ± 0.03) and one of its $\text{cnf}(\Delta R/R_i)$ values intersect the zero line indicates insignificant interaction. Very weak interactions between Zn^{2+} and all proteins were detected with small $+\Delta R/R_i$ values and confidence intervals intersected the zero line. For lanthanides, Cerium (Ce^{3+}) showed weak interactions with BSA, HSA. Whereas, strong interactions were observed with MB and BLACT. Gd^{3+} showed almost insignificant interactions with most of the tested proteins except MB, it showed strong interaction with MB in a manner similar to Ce^{3+} . Influence of the coordination number of the metal ions and multiple binding sites within the proteins on the signs of $\Delta R/R_i$ values were discussed in details. Furthermore, the change in protein peak shape was found to be as evident for a possible conformational change for the proteins and their affinity to the metal ions.

Keywords: Affinity capillary electrophoresis, lanthanides, Heavy metal ions, Mobility shift, Protein-metal ion interaction.

INTRODUCTION

Metalloproteins

Proteins that have a specific metal ion as cofactor are called as Metalloproteins. A large number of proteins are metalloproteins and all these require metal ion for their function^{1,2}. Popular

protein-metal ion interactions are known to produce various crucial biological roles such as storage as ferritin for Fe^{3+} , metallothionein proteins for Zn^{2+} , Cu^{2+} and other heavy metals, transport as transferrin for Fe^{3+} and oxygen transport by binding to Fe^{2+} of heme metalloprotein, cofactor for enzymes e.g. selenium on glutathione peroxidase. Furthermore,

Cu^{2+} , Mn^{2+} , Fe^{2+} and Ni^{2+} can be incorporated to form active sites of antioxidant defense enzymes such as superoxide dismutases and Mg can be incorporated for hexokinase³⁻⁵. The toxicity of some of the heavy metals such as Hg^{2+} and As^{3+} can be manifested when they bind irreversibly to a variety of selenocystine enzymes⁶⁻⁸. The investigation of diagnostic biomarkers for several diseases such as metalloproteins is still growing¹. Furthermore, a substantial progress in producing organometallic complexes for several disorders such as cancer, inflammation, infection and neurological disorders have been achieved⁹⁻¹¹. The influences of metal ion partner should be investigated prior to developing new prodrugs. Therefore, the characterization of interactions between proteins and metal ions are important.

HSAB concept for study protein-metal ion interaction

The hard and soft acids-bases concept (HSAB) helps us to understand the interaction behaviors between metal ions and biological proteins^{12, 13}. In general, the metal ions interact with the protein binding sites of similar hardness or softness preferentially¹². Fig. 1 was successfully

drawn to summarize most of hard, borderline and soft acid metal ions and base functional groups. Many of base functional groups within Fig.1 are involved in the structure of the amino acids and hence protein.

Investigated proteins

Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), β -lactoglobulin (BLACT), Myoglobin (MB) and Ovalbumin (OVA) were selected for the study because of their important roles in various functions of the body and they are known to interact with metal ions. In mammals, Serum albumins have been found to be associated in the control of the biologically active concentrations of Ca^{2+} and Mg^{2+} ¹⁴. The reactivity and binding ability of BSA with a variety of monovalent and polyvalent metal ions such as Cu^{2+} , Ni^{2+} , Co^{2+} , Pt^{2+} , Cd^{2+} , Pb^{2+} has been investigated¹⁵⁻¹⁸ earlier. Similarly β -lactoglobulin was also reported to have affinity towards a number of metal ions such as Li^+ , Na^+ , Cu^{2+} and Zn^{2+} that also modulate its thermal aggregation.¹⁹⁻²¹ The interaction study of myoglobin with Co^{2+} , Zn^{2+} and Cu^{2+} was carried out by using

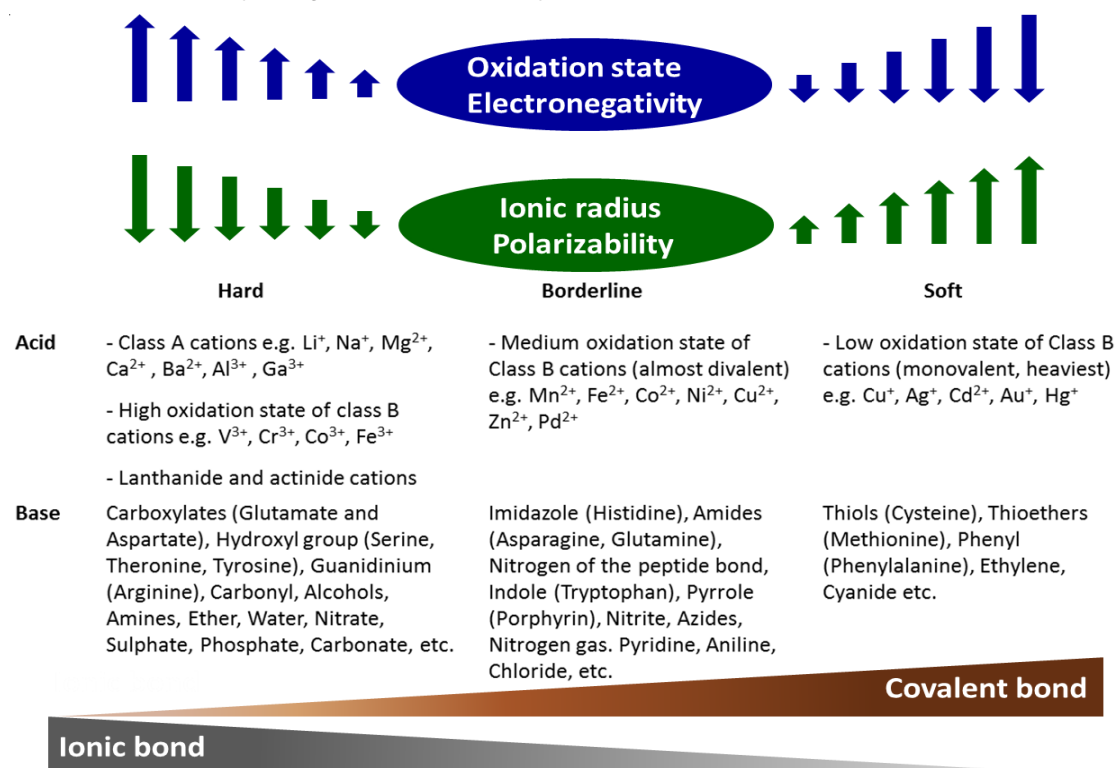


Fig. 1. Possible complex formations between acid metal ions and base ligands based on Hard and Soft Acids-Bases (HSAB) theory

UV-Visible spectroscopy by Tang *et al*²²., whereas, ovalbumin was also reported to have significant interactions with various metals such as Cu²⁺, Ni²⁺ ions²³ and Cd²⁺ ions²⁴ that was investigated by Immobilized metal ion affinity chromatography (IMAC), equilibrium dialysis method respectively as well as by affinity capillary electrophoresis¹².

Investigated metal ions

The behaviors of two lanthanide ions Cerium (Ce²⁺) and Gadolinium (Gd²⁺) and two heavy metal ions Manganese (Mn²⁺) and Zinc (Zn²⁺) on some biological proteins are not studied yet by using mobility shift- affinity capillary electrophoresis (mobility shift-ACE). Therefore, they were selected for study owing to their importance in current and future drug therapy. The lanthanide series of elements are categorized as hard acids and they form ionic or electrostatic bonds with other elements largely. Various studies related to the interaction of Ce²⁺ with biological proteins have been carried out^{25, 26}. The other lanthanide Gd²⁺ is toxic in biological systems as it competes with Ca²⁺ ions for all the processes that need Ca²⁺ for proper functioning, probably due to the fact that both ions have similar degree of hardness. It is extensively used to prepare contrast agents for Magnetic Resonance Imaging (MRI). Gd²⁺ interactions with various proteins and amino acids are studied nowadays and this idea is used to prepare protein based contrast agents recently²⁷⁻²⁹.

On the other hand, the heavy metal ion Mn²⁺ is bound to metalloproteins, mainly glutamine synthetase present in astrocytes^{30, 31} and acts as a cofactor for various enzymes inside the body. Similarly Zn²⁺ is known to interact with a variety of enzymes and amino acids like cysteine, histidine (-N), aspartate (-O), and glutamate (-O) residues³² and some hormones like Prolactine³³. It also plays role as a cofactor for various enzymes to act properly.

Mobility shift-affinity capillary electrophoresis

Affinity Capillary Electrophoresis (ACE) is a free solution Capillary Electrophoresis (CE) method³⁴. In this method the change in electrophoretic migration time or peak (area or height) for one of the reactants (an analyte or a ligand) before and after binding is used to investigate the interaction^{35, 36}. ACE has

received wide popularity in recent years being used in biological and pharmaceutical research³⁷⁻³⁹. Recently, ACE was effectively utilized for the investigation of the interaction between ovalbumin isoforms as well as few other proteins and various metal ions^[40, 41].

In mobility shift-ACE, the sample for injection contains an analyte and an EOF marker while the running buffer contains a ligand in varying concentration. Under the electric field, two peaks will be detected which are corresponding to the EOF marker and the analyte. The migration time of the analyte (e.g. protein) peak can be changed after binding to a ligand (e.g. metal ion). Therefore, this parameter is used for binding calculation. The use of an EOF marker is important to avoid calculation errors due to the possible change of the EOF during experiments due to some spontaneous and uncontrolled factors such as fluctuation on room temperature, possible metal ion and protein adsorptions on capillary wall.

The mobility shift-ACE was selected in this work due to many advantages. It is very sensitive method since a very weak interaction can be detected by the migration time change of 0.1 s^{40, 41, 12}. Furthermore, the small change in the migration time is more obvious when the overall charge of a protein is changed by a metal ion and other surrounding anions. It allows to work with very small amount of an analyte, e.g. at the level of limit of detection (LOD) since the binding investigation is based only on the migration time. On the other hand, ligands with no UV absorption can be used. It has the ability for simultaneous investigation of interactions of a number of analytes with metal ions within one sample (mixture or even impure) due to its high separation power. Recently, this method has been used for the screening of interaction of an analyte (protein) with a ligand (metal ion)³⁵. The screening was based on using of a high concentration of a ligand to achieve the saturation³⁸⁻³⁹. Recently, Alhazmi *et al* has successfully published a mobility shift-ACE platform chart for a wide screening interactions between five proteins and 28 metal ions¹². However, behaviors of many element ions with the selected proteins are still not studied by mobility shift-ACE.

MATERIALS AND METHODS

Chemicals and reagents

Human serum albumin (HSA, 97%), Bovine serum albumin (BSA, 99%), β -Lactoglobulin (BLACT, 85% bovine milk), Myoglobin (MB, 90%) and Ovalbumin (OVA, 98%) were procured from Sigma-Aldrich (Steinheim, Germany). Metal ions Cerium (III) chloride (CeCl_3 , 99.99%), Gadolinium (III) chloride (GdCl_3 , 99.99%), Manganese (II) chloride (MnCl_2 , 99.99%) and Zinc chloride (ZnCl_2 , 99.99%) were also procured from Sigma-Aldrich (Steinheim, Germany). Acetanilide was purchased from Fluka (Steinheim, Germany), whereas, Disodium Ethylene diamine tetraacetic acid dihydrate ($\text{EDTA-Na}_2 \cdot 2\text{H}_2\text{O}$) and sodium hydroxide (NaOH) were obtained from Riedel de Haen (Hannover, Germany). Concentrated hydrochloric acid (Conc. HCl) was obtained from Merck (Darmstadt, Germany) and double distilled water was prepared in our laboratory.

Apparatus and instrumentation

A recently developed, very fast and reliable mobility-shift ACE method was used for investigation of metal ion interactions with the selected proteins. The electrophoretic separations were carried out on Agilent CE system (model G1600A; Agilent Technologies, Germany) that consists of an autosampler, a diode array detector and a capillary cooling system. Normal air plug was utilized to apply high pressure. Bare fused silica capillaries were purchased from Polymicro Technologies (Phoenix, USA) having I.D. 50 μm , total length 31 cm (short capillary) with an effective length of 22 cm. Rotilabos-syringe filters were purchased from Carl Roth (0.22 μm , CME, Karlsruhe, Germany). The pH of the buffer solutions used was adjusted by using Mettler Toledo pH-meter (FE20/EL20, Carl Roth, Germany). The collected data were interpreted by using the installed ChemStation software (Agilent). Finally, the binding calculations and their statistical analysis were done on Microsoft EXCEL™ (Microsoft Corporation, version 2013) using the mobility ratios of protein before and after addition of metal ions in running buffer.

Rinsing protocol

The conditioning of new capillaries were performed at a pressure of 1 bar first by using 1 N sodium hydroxide for 20 min. and then with double-

distilled water for 10 min. At the start and end of each working day, capillaries were flushed again at 2.5 bar by using 0.1 N NaOH for 10 min. followed by water for 5 min. At the beginning of each run, capillaries were rinsed at 2.5 bar for 2.5 min. with a solution contains 0.1N sodium hydroxide water and 0.1 M EDTA, for 1 min. and running buffer for 1.5 min. After each screening, the capillary was rinsed at 2.5 bar with 0.1 N NaOH solution for 10 min. followed by water for 5 minutes to remove adsorbed traces from proteins.

Separation conditions

Normal mode in which anode was present at inlet and cathode was present at outlet, was selected for the separations. Temperature maintained was 23°C and voltage applied was 10 kV. Hydrodynamic injections of samples were carried out at 50 mbar for 4.5 s, except for MB, as it was injected for 1.5 s since the short injection time of 1.5 s improved the electrophoretic separation between the EOF marker and MB. Running buffer was injected after the injection of samples at 50 mbar for 2.5 s to push the samples. A total of twelve runs were performed for the screening of each metal-protein interaction. Six runs were conducted for protein alone and six for proteins along with metal ions.

Preparation of solutions

Tris buffer was prepared with concentration of 20 mmol/L having pH 7.4. For the preparation, 2.42 g of tris was dissolved in 200 ml double distilled water. The pH 7.4 was adjusted with HCl and volume was made up to 1 L with double distilled water. Acetanilide was used as EOF marker as it remains neutral at pH 7.4^{41, 42}. The pKa of Acetanilide is 0.5 as the non-bonded electrons of nitrogen get delocalized through the resonance of the conjugative system. 37.5 mg of acetanilide was dissolved in 50 ml of tris buffer followed by sonication to get a concentration of 750 $\mu\text{g/ml}$. All the metal ion and protein solutions were prepared fresh in tris buffer every working day. The protein solutions were prepared in definite concentrations in tris buffer separately. Various concentrations of proteins were, BSA (20 $\mu\text{mol/L}$), HSA (20 $\mu\text{mol/L}$), BLACT (50 $\mu\text{mol/L}$), MB (60 $\mu\text{mol/L}$) and OVA (50 $\mu\text{mol/L}$) by dissolving an amount of 33 mg of BSA, 33.18 of HSA, 23 mg of BLACT, 25.8 mg of MB and 53.5 mg of OVA in a mixture of stock solution of acetanilide (5 ml) and making up the volume to 25 ml with tris buffer. Before carrying out

the experiments, all protein solutions were diluted three times in order to reduce band broadening as well as protein adsorption. The solutions of metal ions were prepared in the same tris buffer of concentration 20 mmol/L and pH 7.4 to obtain the concentrations of 25, 100 and 250 $\mu\text{mol/L}$.

RESULTS AND DISCUSSION

Binding calculation

The mobility ratios R_i and R_f of the test protein were calculated with and without interaction with ligand metal ions respectively, using an EOF marker, by the equation.1 $R = t_{eof}/t_{prot}$ where t_{eof} is the migration time for EOF marker (Acetanilide) and t_{prot} is the migration time for test protein. The normalized difference between the mobility ratios $(R_i - R_f)/R_f$ or $\Delta R/R_f$, equation.2 were used to represent the interaction results.

The confidence interval of $\Delta R/R_f$ ($cnf\Delta R/R_f$) was calculated by equation.3:

$$cnf(\Delta R/R_f) = (\Delta R/R_f) \pm \left(t_{\alpha/2, n_1+n_2-2} \cdot \sigma_{total} \cdot \sqrt{\frac{2}{n_1 \cdot n_2}} \right) / R$$

For a given degree of freedom, $t_{\alpha/2}$ is the t-value at probability 0.975 ($\alpha/2 = 0.025$). The n_1 and n_2 are the two data numbers of the series to estimate R_i and R_f . The value n_1+n_2-2 is the degree of freedom.

$$\text{Total standard deviation, } \sigma_{total} = \sqrt{\frac{2 \cdot (f_1 \cdot \sigma_1^2 + f_2 \cdot \sigma_2^2)}{(f_1 + f_2)}}$$

where f_1 and f_2 are the numbers of freedom ($n-1$) whereas σ_1 and σ_2 are the standard deviations of the data of two series for R_i and R_f , respectively.

Interaction of metal ions with proteins

The binding behavior of each group of metals (Table.1) on the selected proteins, BSA, HSA, BLACT, MB and OVA was studied under physiological pH 7.4. Two Lanthanide group metal ions (Ce^{3+} and Gd^{3+}) and two heavy metal ions (Mn^{2+} and Zn^{2+}) were examined. As we know that multiple sites are exhibited by most of proteins for different metal ions, therefore, each metal ion was used in high concentrations for fast interaction screening with protein in order to attain saturation^{41, 42}. Metal ion concentrations of around 250 $\mu\text{mol/L}$ was proved to be suitable for all the tested proteins except for BLACT and MB, because of their intensive change in peak shapes that were not able to get integrated when they are used in concentrations above 100 $\mu\text{mol/L}$ for BLACT^{41, 42} and above 25 $\mu\text{mol/L}$ for MB. Six repeated runs were performed for each solution and the migration time data were used for the calculation of $\Delta R/R_f$ values and their confidence intervals to measure the interaction strength using equations 1, 2 and 3. All obtained $\Delta R/R_f$ values and their $cnfs$ are summarized in Table.1. Indeed, it is difficult to compare between metal ions by using this Table. Hence, $\Delta R/R_f$ chart (Fig.2) was successfully created to make comparisons easier Table.1.

Table. 1: $\Delta R/R_f$ values with their confidence intervals of all investigated protein-metal ion interactions

Metal ions		Proteins				
		BSA	HSA	BLACT	MB	OVA
Heavy Metals	Mn^{2+}	-0.067 \pm 0.008	-0.068 \pm 0.006	-0.135 \pm 0.019	0.002 \pm 0.003	-0.098 \pm 0.015
	Zn^{2+}	0.013 \pm 0.003	0.005 \pm 0.007	0.013 \pm 0.003	0.009 \pm 0.002	0.008 \pm 0.003
Lanthanides	Ce^{3+}	-0.016 \pm 0.031	-0.082 \pm 0.081	-0.125 \pm 0.007	-0.392 \pm 0.027	-0.034 \pm 0.066
	Gd^{3+}	-0.007 \pm 0.021	-0.00002 \pm 0.011	0.008 \pm 0.002	-0.102 \pm 0.021	-0.012 \pm 0.037

The negative and positive values of $\Delta R/R_f$ are of great importance as far as the preliminary evaluation of protein-metal ions interactions is concerned. It gives insight into the coordination of metal ion bound with the residues of protein and surrounding anions. For example, the estimated initial charge of BSA in the running buffer, is -18 at pH 7.4³⁶ in absence of metal ion ligand whereas, when a metal ion ligand is added in the buffer, the charge of BSA generally decreases to ≤ -18 (less negative) that leads to increased electrophoretic mobility resulting in a positive $\Delta R/R_f$ value as in case of BSA-Zn²⁺. On the other hand, some metal ions especially those which have high coordination numbers and are bound to BSA could further coordinate with some of the anions around and that will cause an increase in the charge of BSA ≥ -18 (more negative). In these cases, the electrophoretic mobility would be decreased resulting in a negative $\Delta R/R_f$ value as in case of BSA- Mn²⁺ (see Figure. 2).

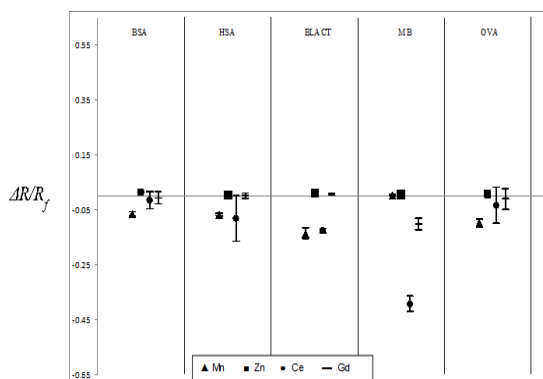


Fig. 2. $\Delta R/R_f$ chart showing interactions of metal ions with various biological proteins

Heavy metal ions

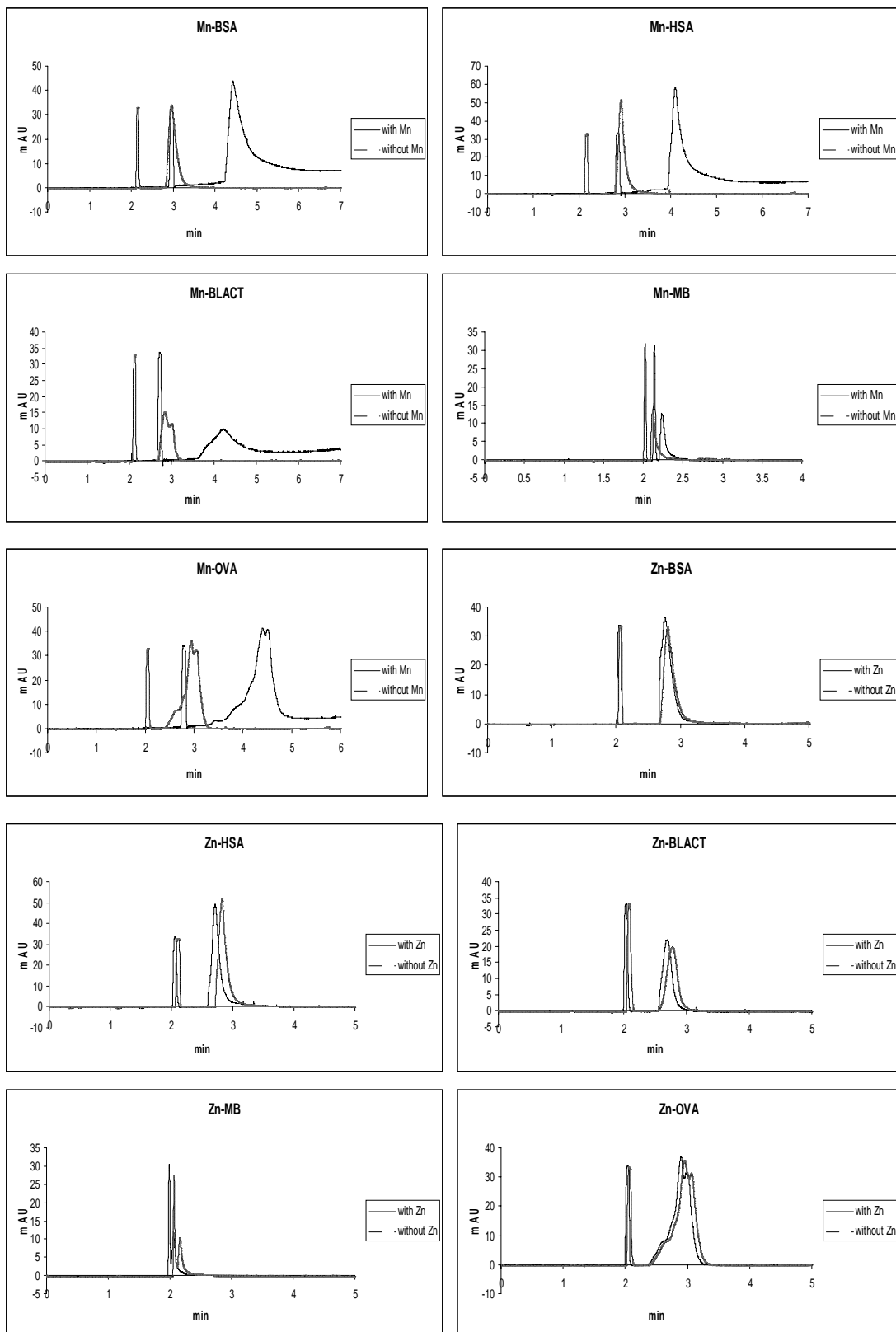
The interactions of heavy metal ions (Mn²⁺ and Zn²⁺) selected for study were found to be different for different proteins. For Mn²⁺ the interaction was in negative direction in most of the cases except Myoglobin (MB). The interaction of MB was not significant as their $\Delta R/R_f$ value < 0.01 and closed to zero and one of its *cnf*($\Delta R/R_f$) values intersect the zero line (0.002 ± 0.003). With other proteins, Manganese ion was found to interact comparatively more and led to the decrease in the electrophoretic mobility and hence negative values were obtained. Strongest interaction was observed in case of BLACT ($\Delta R/R_f \pm cnf = -0.135 \pm 0.019$) followed by OVA > HSA \geq BSA. On the other hand the other investigated ion Zn²⁺ showed very weak or could be insignificant interactions with all the

tested proteins as their confidence intervals intersected the zero line. Interestingly all the small $\Delta R/R_f$ values in case of Zinc ion was found to be positive for all the proteins. This indicates that, Zinc ions could decreased the overall charge of proteins and made it less negative, therefore, increased the electrophoretic mobility that resulted into positive $\Delta R/R_f$ values.

Interaction of metal ions with proteins had an interesting effect on peak shape and intensity and it changed with the interaction. The change in the protein peak shape is also due to the conformational change of the protein¹². Manganese ion (Mn²⁺) interaction was significant and in negative direction in case of proteins BSA, HSA, BLACT and OVA and it was evident from the change in peak shape and intensity in their corresponding electropherograms (Fig.3). Peaks corresponding to the complexes Mn-BSA and Mn-HSA were observed to be higher in intensity and broader in shape, whereas, in case of BLACT, peak with metal ion was broader but significantly lesser in intensity owing to the good interaction of Mn²⁺ with BLACT. Mn²⁺ did not show any significant interaction with MB and it was confirmed by the peaks obtained with and without metal ion as there was no any significant change in shape or intensity was observed. If we consider the electropherogram of Mn²⁺-OVA, splitting of peak was observed in case of protein with metal ions, which is because of the separation of two isoforms OVA-A and OVA-B. Zinc ions (Zn²⁺) displayed no significant interaction with all the proteins and the electropherograms displayed no significant change in peak shape or intensity in all the cases.

Lanthanides

The interactions of lanthanides Cerium (Ce³⁺) and Gadolinium (Gd³⁺) with various proteins were found to be interesting, especially with the Myoglobin (MB) protein. Ce³⁺ showed weak interaction with BSA, HSA, and OVA and the $\Delta R/R_f$ values were typically in the negative direction for all the proteins and their confidence intervals cross the zero lone. Whereas, strong interactions were observed between Ce³⁺ ions and MB showing $\Delta R/R_f$ values in negative direction (-0.392 ± 0.027) as well as with BLACT ($\Delta R/R_f = -0.125 \pm 0.007$). This



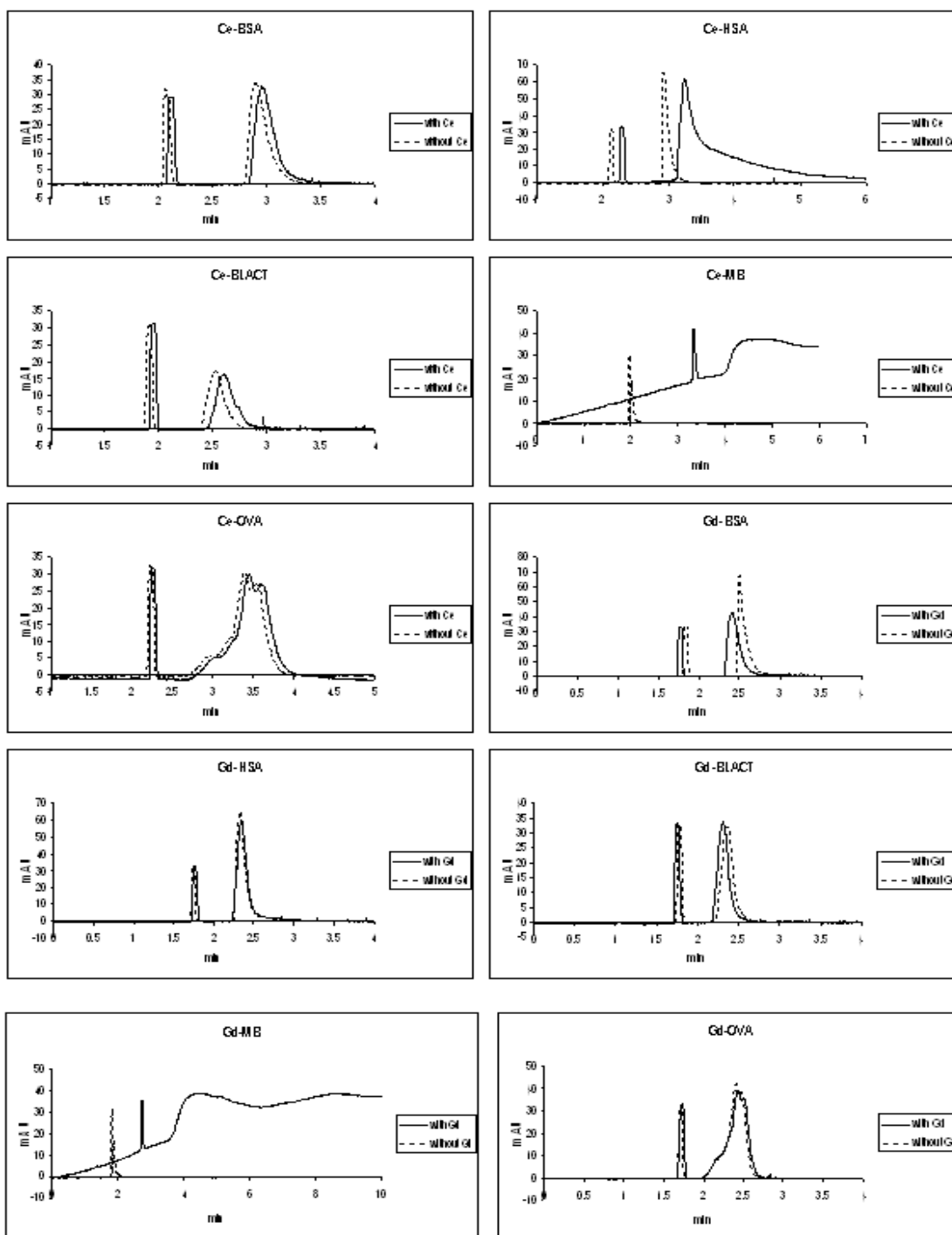


Fig. 3. Typical electropherograms of EOF marker (acetanilide, 1st migrated peak) and proteins (2nd migrated peak) BSA, HSA, BLACT, MB and OVA without (solid line) and with (dotted line) Mn^{2+} , Zn^{2+} , Ce^{3+} and Gd^{3+} in the running buffer. *Separation conditions*: capillary I.D. 50 μm and total length 31 cm with effective length 22 cm, buffer tris (pH 7.4) 20 mmol/L, sample injection for 4.5 s at 50 mbar (for MB 1.5 s) followed by buffer (2.5 s), voltage 10 kV, UV 214 nm, capillary cartridge temperature at 23°C. Rinsing at 2.5 bar using solution containing 0.1 N NaOH and 0.1 M EDTA for 2.5 min, water for 1 min followed by tris buffer for 1.5 min. Extra

may be attributed to the high coordination numbers of Ce^{3+} ion^{1, 8} that allows it coordinate with other surrounding ions present resulting in the increase in negative charge of the protein and decrease in the electrophoretic mobility. The other lanthanide metal ion investigated was Gd^{3+} that showed almost insignificant interactions with most of the tested proteins except MB. Gd^{3+} also showed strong interaction with MB in a manner similar to Ce^{3+} , and in this case the $\Delta R/R_r$ values were also in negative direction demonstrating that the negative charge of protein was increased by Gd^{3+} ions that led to the decrease in electrophoretic mobility. Hence various binding sites present on MB and further coordination with the surrounding anions should be taken into account when considering Ce^{3+} and Gd^{3+} based drugs. Interactions of Gd^{3+} with BSA, HSA, BLACT and OVA were very weak and the $\Delta R/R_r$ remained near the zero line with non-significant values. Again there is some possibility of interaction that was present there but it could not be detected by the instrument. Over all, the most significant interaction was observed between lanthanide metal ions Ce^{3+} and Gd^{3+} and MB, whereas, other tested proteins showed weak affinity towards these metal ions except BLACT that showed good interaction with Ce^{3+} also.

If we observe the change in peak shape and intensity in case of lanthanides with proteins, Cerium (Ce^{3+}) displayed insignificant change in peaks corresponding to Ce^{3+} -BSA, Ce^{3+} -BLACT and Ce^{3+} -OVA whereas for Ce^{3+} -HSA and Ce^{3+} -MB, the change was highly significant with broader peak in case of Ce^{3+} -HSA and broader and higher intensity peak for Ce^{3+} -MB with elevated baseline (the right side of peak was not touching the baseline). Gadolinium (Gd^{3+}) showed significant interaction with MB only and it was evident from the peak obtained corresponding to Gd^{3+} -MB that was much broader and elevated not touching the baseline. The significant change on MB peak shape could be due to the strong influence on the protein conformation. No significant change in peaks was obtained for other proteins as no significant interaction was observed between Gd^{3+} and other proteins (Figure. 3).

CONCLUSIONS

ACE is becoming more popular nowadays especially for the investigation of protein-metal ion interactions. In this work, an optimized and fast ACE method was developed successfully with lower sample concentration, smaller injection volume and a proper rinsing protocol. Several factors were further considered in order to improve the precision. These were sample pushing, shorter rinsing protocol, extra flushing after several consecutive runs and getting buffer solutions at inlet and outlet refreshed. In order to get good long term precision, 0.1 M EDTA was used in the rinsing protocol. The optimized conditions that were finalized was injection of sample at a pressure of 50 mbar for a period of 4.5 s that was pushed with the help of buffer at 50 mbar for a period of 2.5 s. Rinsing protocol was shortened with solutions of 0.1 N NaOH and 0.1 M EDTA at 2.5 bar for a period of 2.5 min. followed by water and running buffer for 1 min. and 1.5 min. respectively. Some extra flushing was also employed after completing every 60 runs, at the same pressure by using 0.1 N NaOH solution for 10 min. followed by water for 5 min. Buffer solutions were regularly refreshed to improve the precision of the results after every 30 consecutive runs. The $\Delta R/R_r$ values were found to be different for different metal ions. In some cases it was found to be significant and in positive or negative direction showing good interaction while in other cases the interaction was very weak and can be considered as non-significant. These could probably be owing to the capability of coordination of the metal ions and positions of their binding sites. For example, binding sites at the surface of the protein could facilitate coordination with the background anions leading to negative $\Delta R/R_r$, while binding sites inside the protein could facilitate coordination with potential ligands (amino acid residues) leading to positive $\Delta R/R_r$. The change in peak shapes of proteins after binding with metal ions have been successfully used to detect the conformational change of protein due to influence of metal ions.

ACKNOWLEDGEMENT

The authors are thankful to Prof. Dr. Hermann Wätzig, Institute of Medicinal and Pharmaceutical Chemistry, Braunschweig, Germany, for his valuable guidance throughout this research work.

REFERENCES

1. Swart, C. *Anal Bioanal Chem.* **2013**, *405*, 5697–5723.
2. Mounicou, S.; Szpunar, J.; Lobinski, R. *Chem Soc Rev.* **2009**, *38*, 1119–1138.
3. Lodish, H.; Berk, A.; Matsudaira, P.; Kaiser, C.A.; Krieger, M. *Molecular Cell Biology*, **2003**, 5th edn. W. H. Freeman, New York.
4. Andreini, C.; Bertini, I.; Cavallaro, G.; Holliday, G.L.; Thornton, J.M. *J Biol Inorg Chem.* **2008**, *13*, 1205–1218.
5. Sun, H.; Chai, Z. *Annu Rep Prog Chem Sect A.* **2010**, *106*, 20–38.
6. Carvalho CML, Chew E, Hashemy SI, Lu J, Holmgren A, *J Biol Chem.* **2008**, *283*, 11913-11923.
7. Romero-Canelon, I.; Sadler, P.J. *Inorg Chem.* **2013**, *52*, 12276"12291.
8. Frezza, M.; Hindo, S.; Chen, D.; Davenport, A.; Schmitt, S.; Tomco, D.; Dou, Q.P. *Curr Pharm Des.* **2010**, *16*, 1813–1825.
9. Chen, D.; Milacic, V.; Frezza, M.; Dou, Q.P. *Curr Pharm Des.* **2009**, *15*, 777–791.
10. Hannon, M.J. *Pure Appl Chem.* **2007**, *79*, 2243–2261.
11. Desoize, B. *Anticancer Res.* **2004**, *24*, 1529-1544.
12. Alhazmi, H.A.; Nachbar, M.; Albishri, H.M.; El-Hady, D.A.; Redweika, S.; El Deeb, S.; Wätzig, H. *J Pharm Biomed Anal.* **2015**, *107*, 311–317.
13. Wiberg, N.; Wiberg, H. *Inorganic Chemistry*, **2001**, English Version, first edn. Academic Press, California.
14. Majorek, K.A.; Porebski, P.J.; Dayal, A.; Zimmerman, M.D.; Jablonska, K.; Stewart, A.J.; Chruszcz, M.; Minor, W. *Mol Immunol.* **2012**, *52*, 174–182.
15. Liu, H.; Shi, X.; Xu, M.; Li, Z.; Huang, L.; Bai, D.; Zeng, Z. *Eur J Med Chem.* **2011**, *46*, 1638–1647.
16. Sathyadevi, P.; Krishnamoorthy, P.; Jayanthi, E.; Butorac, R.R.; Cowley, A.H.; Dharmaraj, N. *Inorganica Chimica Acta.* **2012**, *384*, 83–96.
17. Gharagozlou, M.; Boghaei, D.M. *Spectrochim Acta A Mol Biomol Spectrosc.* **2008**, *71*, 1617–1622.
18. Klotz, I.M.; Urquhart, J.M.; Fiess, H.A. *J Am Chem Soc.* **1952**, *74*, 5537–5538.
19. Navarra, G.; Leone, M.; Militello, V. *Biophys Chem.* **2008**, *131*, 52-61.
20. Navarra, G.; Tinti, A.; Foggia, M.D.; Leone, M.; Militello, V.; Torreggiani, A. *J Inorg Biochem.* **2014**, *137*, 64-73
21. Beierlein, F.R.; Clark, T.; Braunschweig, B.; Engelhardt, K.; Glas, L.; Peukert, W. *J Phys Chem B.* **2015**, *119*, 5505–5517
22. Tang, Q.; Zheng, X.F.; Wang, J.Y.; Liu, Y.Y.; Yuan, Y.L. *Guang Pu Xue Yu Guang Pu Fen Xi.* **2009**, *29*, 1958-1961.
23. Sharma, S.; Agarwal, G.P. *Anal Biochem.* **2001**, *288*, 126-140.
24. Verma, S.R.; Arora, J.P.S.; Shankar, J.S.; Dutt, D.; Pal, C. *Water Air Soil Poll.* **1989**, *43*, 53-59.
25. Ali, M.; Kumar, A.; Kumar, M.; Pandey, B.N. *Biochimie.* **2016**, *123*, 117-129.
26. Kumar, A.; Ali, M.; Ningthoujam, R.S.; Gaikwad, P.; Kumar, M.; Nath, B.B.; Pandeya, B.N. *Radiation; J Hazardous Mat.* **2016**, *307*, 281–293.
27. Shenghui, X.; Jingjuan, Q.; Fan, P.; Mathew, C.; Jenny, Y. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology.* **2013**, *5*, 163–79.
28. Li, S.; Jiang, J.; Zou, J.; Qiao, J.; Xue, S.; Wei, L.; Long, R.; Wang, L.; Castiblanco, A.; White, N.; Ngo, J.; Mao, H.; Liu, Z.R.; Yang, J.J. *J Inorg Biochem.* **2011**, *107*, 111–118.
29. Caravan, P. *Acc Chem Res.* **2009**, *42*, 851-62.
30. Takeda, A. *Brain Res Rev.* **2003**, *41*, 79–87.
31. Holeysovska, H. *Collect Czech Chem Commun.* **1961**, *26*, 3074-3080.
32. Alhazmi, H.A.; El Deeb, E.; Nachbar, M.; Redweik, S.; Albishri, H.M.; El-Hady, D.A.; Wätzig, H. *J Sep Sci.* **2015**, *38*, 3629-3637.
33. Pace, N.J. Eranthie Weerapana E., *Biomolecules.* **2014**, 419-434.
34. Neubert, R.H.H.; Rüttinger, H.H. *Affinity Capillary Electrophoresis in Pharmaceuticals and Biopharmaceutics.* **2003**, Marcel Dekker Inc., New York.
35. Albishri, H.M.; El Deeb, S.; Al-Garabli, N.; Al-Astal, R.; Alhazmi, H.A.; Nachbar, M.; El-Hady, D.A.; Wätzig, H. *Bioanalysis.* **2004**, *6*, 3369-3392.
36. Wätzig, H.; Degenhardt, M.; Kunkel, A. *Electrophoresis.* **1998**, *19*, 2695-2752.
37. Chu, Y.; Avila, L.Z.; Gao, J.; Whitesides, G. *Acc Chem Res.* **1995**, *28*, 461-468.
38. Redweik, S.; Xu, Y.,; Wätzig, H. *Electrophoresis,* **2012**, *33*, 3316–3322.
39. Redweik, S.; Cianciulli, C.; Hara, M.; Xu, Y.; Wätzig, H. *Electrophoresis.* **2013**, *34*, 1812-1819.
40. Mironov, G.G.; Logie, J.; Okhonin, V.; Renaud, J.B.; Mayer, P.M.; Berezovski, M.V. *J Am Soc Mass Spectrom.* **2012**, *23*, 1232-1240.
41. Heegaard, N.H.H.; Nilsson, S.; Guzman, N.A. *J Chromatography B.* **1998**, *715*, 29–54.