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THE ALKOXYACETIC ACIDS AS CALCIUM AND MAGNESIUM CHELATING AGENTS *IN VITRO*

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Abstract: Alkoxyacetic acids (AAAs) are known urinary metabolites of the corresponding ethylene glycol monoalkyl ethers with a wide range of industrial and domestic applications. Hemolysis is the principal toxic effect of AAAs in humans and animals. The mechanism of red-cell damage is not known. It is suggested that some disturbances in ion balance, mainly related to calcium are one of the reasons of hemolysis. No comparative studies in the available literature on the chelating properties of numerous AAAs in respect to calcium were found. Therefore, a comparison was made between chelating effects of five AAAs on calcium and magnesium *in vitro*. It was demonstrated that calcium was bound at lower AAAs concentrations than magnesium. The chelating effect of AAAs expressed by EC_{50} values was positively correlated with both pKa values and Log P values of the examined acids. The obtained data indicate that the acidity and hydrophilic properties are responsible for the chelating effect of AAAs on calcium and magnesium *in vitro*. These data do not provide an explanation for differences in the hemolytic activity of the examined compounds.

Keywords: chelators, calcium, magnesium, alkoxyacetic acids, hemolysis

Hemolysis is the principal effect of ethylene glycol monoalkyl ethers (EGAEs) acute poisoning in humans and laboratory animals (1). EGAEs theyself are not active hemolytic agents, but their metabolites, alkoxyacetic acids (AAAs) formed during metabolic activation in the liver, skin and testes are potent hemolysins (2, 3).

There are remarkable species differences for the hemolytic activity of these chemicals. Species whose red blood cells (RBCs) are sensitive, for example to butoxy-acetic acid (BAA), include rats, mice, rabbits, and baboons. RBCs of other species, such as pigs, dogs, cats, guinea pigs, and humans, appear to be resistant (1).

There are studies which indicate that younger rats and male rats are more resistant to the effects of EGAEs, and that RBCs from younger animals are less sensitive to the hemolytic action of BAA (4). *In vitro* studies with BAA showed that hemolysis of rat RBCs ensues after a lag period depending on the concentration of this metabolite (5). Also, it was noted that hemolysis of rat RBCs continues, even after BAA removal from the culture medium after an initial exposure (6).

The mechanism(s) of hemolytic action of AAAs was not elucidated. Some authors related it to the disturbances in ion balance, mainly to calcium concentration in red blood cells external environment (5).

Cell swelling and hemolysis were reduced by the addition of sucrose to the suspending media or by replacing external sodium with potassium. When calcium was absent in the suspending medium, or, when it was chelated by EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) or EDTA (ethylenediaminetetraacetic acid) in the presence of BAA, hemolysis was increased (5). Quinine, potassium channel blocker, prevented hemolysis induced by BAA in the presence of calcium, what implicates the calcium dependent Gardos channel involvement in this phenomenon (7). Also, spherocytosis and cell fragmentation induced by BAA were more pronounced in the lack of calcium. Addition of a little amount of calcium (0.05 mM) significantly reduced hemolysis, while the addition of magnesium had no effect. The dose-effect relationship between BAA concentration and hemolysis evaluated in the presence or absence of calcium demonstrated greater effect of BAA in the absence of calcium. A scorpion toxin, charybdotoxin, an inhibitor of the potassium channel activated by calcium, blocked the protective effect of calcium suggesting that the delay in the onset of hemolysis in the presence of calcium is due to potassium loss caused by this channel. It was suggested that hemolysis of RBCs requires external sodium and is associated with calcium uptake (5, 8).

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Calcium channel blockers such as verapamil or diltiazem attenuated the hemolytic effect of 2-butoxyethanol given *per os* to rats. Similarly, *in vitro* cell swelling, ATP depletion, and hemolysis of RBCs incubated with BAA were inhibited by calcium channel blockers (6). It was suggested that the protective effect of calcium channel blockers is not due to the prevention of calcium entry into the cell but to their action on the balance of other cations, i.e. sodium and potassium or membrane stabilization (5, 9). It seems that ATP depletion induced by BAA in RBCs (10, 11) is a result of an efflux of magnesium from the cell what might lead to the block of glucolysis.

The above mentioned data suggest that AAAs can exert their hemolytic action via chelating calcium and magnesium in biological media. The individual AAAs may differ in respect to binding potency of these ions. Our previous studies *in vivo* and *in vitro* showed an increase of hemolytic activity of EGAEs and AAAs, respectively, with the growth of their pKa and Log P values (12-14).

The aim of the present study was to determine the capacity of five AAAs, consecutive homologous compounds, to calcium and magnesium binding in no biological system *in vitro*. For comparative purposes EGTA and EDTA were used as reference chelating agents of calcium and magnesium, respectively. Further, the pKa values of AAAs were determined and their Log P values were calculated for the assessment of relationship between physicochemical properties and chelating effects of these chemicals on calcium and magnesium.

EXPERIMENTAL

Chemicals: Methoxyacetic acid (MAA) and ethoxyacetic acid (EAA) were purchased from Sigma-Aldrich Ltd., Poland, while propoxyacetic acid (PAA), butoxyacetic acid (BAA), and pentoxyacetic acid (PEAA) were obtained from the Chair of Technology and Biotechnology of Drugs, Medical College, Jagiellonian University in Kraków. These last acids were synthetized in the raction of doubled amount of the corresponding alkoxides with chloroacetic acid. The reaction mixture was dissolved in water, extracted with an organic solvent, acidified and extracted again with methylene chloride. After evaporation of the organic solvent alkoxyacetic acids were distilled under reduced pressure. Next, these acids were additionally washed in basic environment. The identity of obtained acids was confirmed by means of both spectral ^{1}H-NMR) and elementary analysis.

Other chemicals were supplied by Sigma-Aldrich Ltd., Poland. A deionized water was purchased from Pointe Scientific (Poland).

Experimental design: The basic solutions, which concentrations are given in parentheses, used in experiments were as follows: $CaCl_2$ (4.0 mM), $MgCl_2$ (2.78 mM), AAAs (10.0 mM and 100.0 mM for chelating of calcium and magnesium, respectively), EGTA (2.0 mM) and EDTA (2.0 mM).

Chelating reaction in solutions containing constant quantities of calcium (1.0 mM) or magnesium (0.695 mM) and an increasing amount of AAAs in the range of 0.0-7.5 mM and 0.0-75 mM, respectively, were performed at room temperature. The reaction mixtures contained DMSO (dimethyl sulfoxide, final concentration 20%) were diluted with deionized water. Final volumes of these mixtures containing calcium or magnesium were 100 µL or 200 µL, respectively. The optimal reaction time was set experimentally at 15 min. Free calcium and magnesium in reaction mixtures were determined by means of diagnostic kits, i.e. Calcium (Pointe Scientific, Poland) and Magnesium (BioSystems, Spain).

Also, chelating properties of EGTA and EDTA in relation to calcium and magnesium, respectively, were evaluated in the same manner. In these experiments AAAs were replaced by EGTA or EDTA in equal quantity of 0-1.5 mM.

Determination of pKa values: The pKa values of AAAs were measured by a potentiometric titration. A sample of 50 mL of any AAA (10 mM) was treated with 0.5 mL portions of NaOH solution (100 mM) till complete neutralization of the acid and pH was measured by means of CP-315 pH-meter (Elmetron, Poland). The pKa value was read on the titration curve for a weak acid and strong base expressed as a relationship between log [salt]/[acid] and pH. According to Henderson-Hasselbalch equation, when [salt] is equal to [acid] then pKa = pH.

Calculation of Log P values: The Log P values of AAAs were calculated by means of a computer program PALLAS.

Statistical analysis: All values are expressed as mean ±S.D. of at least three to five determinations. The relationship between calcium or magnesium concentrations and levels of each chelating agent was used for the calculation of effective concentration (EC_{50}) of examined compound. The EC_{50} values were termed as the compound concentrations required for reduction of calcium or magnesium levels by 50%. These values were calculated on the basis of regression equations. Also a regression

analysis between the EC_{50} values of AAAs and their pKa or Log P (except Log P for PEAA) was conducted. The linear regression analysis was performed by means of a computer program STATISTICA version 6.0 PL.

RESULTS

The effects of different concentrations of MAA on calcium or magnesium levels are shown in Figure 1a and 1b. As demonstrated in this figure calcium was more effectively bound by MAA than magnesium. Similar effects were observed in case of other acids (Table 1). The EC_{50} values show differences in the ability of particular acids to chelate calcium or magnesium (Table 2). This ability decreased with an increase in the molecular weight of the examined acids.

The pKa and Log P values of the examined acids increased with the growth of their molecular weight (Figure 2a and 2b). The EC_{50} values of AAAs positively correlated with their pKa values (Figure 3a and b). The correlation between EC_{50} values of AAAs and their Log P values in case of calcium was statistically insignificant (Figure 4a), whereas in case of magnesium was significant (Figure 4b).

The binding ability of MAA, the most active compound, calcium or magnesium was 5.6- and 120-times lower in comparison with EGTA and EDTA, respectively.

DISCUSSION

Studies on the mechanism of hemolysis induced by BAA in rat RBCs appear to indicate that colloid osmotic lysis may occur when RBCs are incubated with BAA and that external sodium is necessary for this effect. Additionally, it was found that external calcium causes a delay in the onset of BAA-induced hemolysis (5). It is speculated that initially calcium has a protective effect via the activation calcium-dependent potassium channel which facilitates the loss of potassium thereby compensating the osmotic effect of increased sodium level within the cell. Subsequently, calcium may have other harmful effects through activation of proteases and externalization of phosphatidylserine in the cell membrane (5).

At present, little is known about the potential capacity for AAAs in respect to calcium and magnesium binding. In the available literature there is a lack of comparative studies on the chelating properties of numerous AAAs.

The results obtained in the present study indicate that AAAs chelate calcium and magnesium *in vitro*. As was expected, calcium was bound at lower AAA concentrations than magnesium. It is due to greater chemical activity of calcium in comparison with magnesium.

Contrary to expectations, the AAAs capacity for calcium and magnesium binding correlated negatively with their pKa and Log P values. These indicate that AAAs acidity and hydrophilic properties are crucial features for their chelating action *in vitro*. It was found that the acidity of examined chemicals decreased with their lipophilicity. It is likely that the structure parameter such as inductive effect, apart from solubitlity, partition coefficient, and ionization, may be responsible for differences in AAAs chelating activity on calcium and magnesium. The alkyl moiety represents an electron donor system, which exerts inductive effect on carboxylic group and lead to a decrease in acid strength together with increasing chain length. It seems that pKa and Log P have other sense for hemolytic activity of AAAs *in vivo*. Our previous studies *in vivo* and *in vitro*

Table 1. The effect of AAAs and other chelators on calcium and magnesium levels *in vitro*.

Compound	Calcium			Magnesium		
	Regression equation	r	p≤	Regression equation	r	p≤
MAA	Y=0.929-0.112x	-0.973	0.001	Y=1.002-0.014x	-0.966	0.001
EAA	Y=1.099-0.145x	-0979	0.001	Y=0.951-0.011x	-0.960	0.001
PAA	Y=1.153-0.110x	-0.977	0.001	Y=0.980-0.003x	-0.861	0.001
BAA	Y=1.029-0.072x	-0.960	0.001	Y=0.744-0.001x	-0.898	0.001
PEAA	Y=1.079-0.031x	-0.939	0.001	Y=0.741-0.001x	-0.930	0.001
EGTA	Y=0.905-0.593x	-0.953	0.001	NE		
EDTA	NE			Y=0.585-0.605x	-0.958	0.001

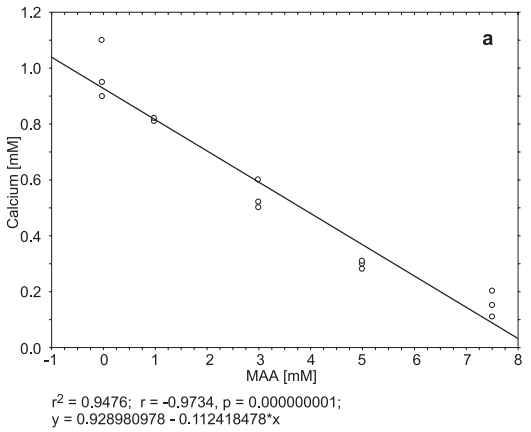
NE, not examined; r – correlation coefficient; p – significance level.

showed an increase of hemolytic activity of EGAEs and AAAs, respectively, with the growth of their Log P and pKa values (12-14). The results obtained in the present study do not provide an explanation for differences in the hemolytic activity of examined chemicals. On the other hand, the differences in the testicular toxicity produced by EGAEs are dependent on the physicochemical properties and structural characteristics of these chemicals. The testicular toxicity expressed by the histological changes and the decrease in testicular weight were diminished with increasing chain length (15). Also, mutagenic-

Table 2. The EC_{50} values of AAAs and other chelators.

Compound	Calcium	Magnesium
	EC_{50} [mM]	EC_{50} [mM]
MAA	3.80 ± 0.189	46.80 ± 3.880
EAA	4.10 ± 0.298	54.90 ± 3.130
PAA	5.90 ± 0.293	210.80 ± 7.140
BAA	7.30 ± 0.502	396.50 ± 5.950
PEAA	18.70 ± 0.475	393.50 ± 7.480
EGTA	0.68 ± 0.014	NE
EDTA	NE	0.39 ± 0.011

NE, not examined.
The mean ± S.D. values are given.



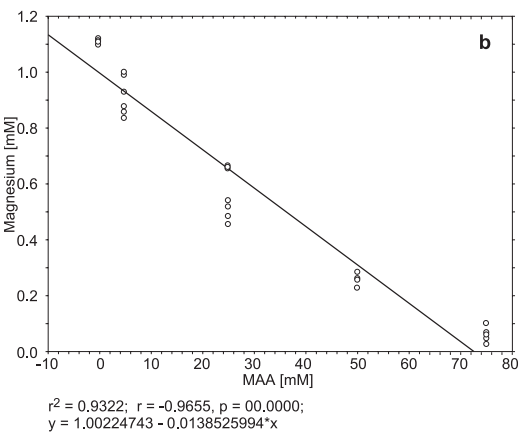
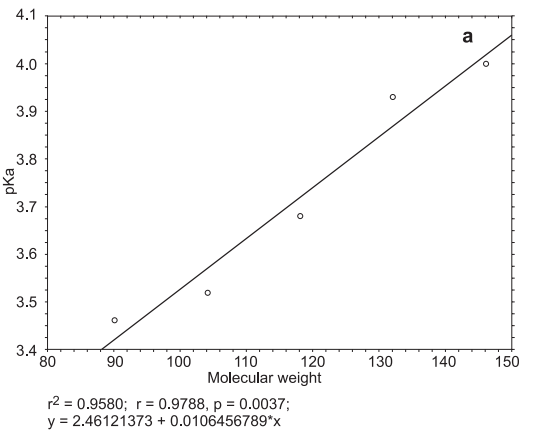


Figure 1. The effect of MAA on calcium (a) and magnesium (b) levels *in vitro*.



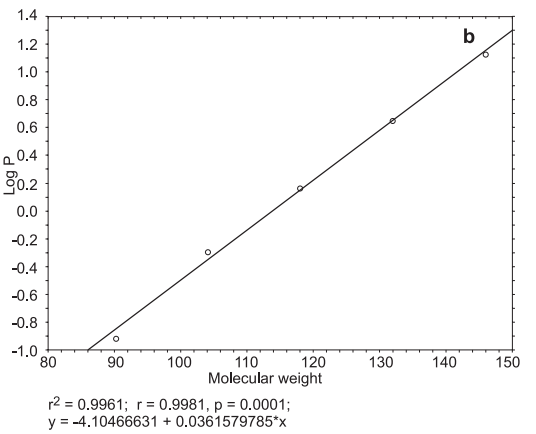
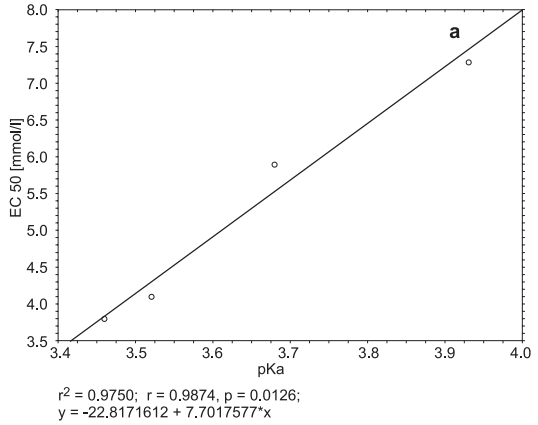


Figure 2. The relationship between pKa (a) or Log P (b) values of AAAs and their molecular weight.



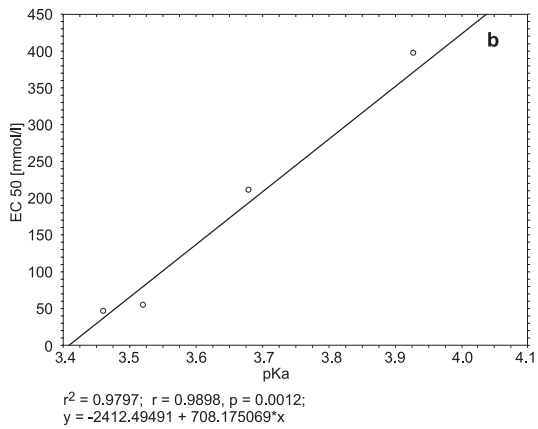
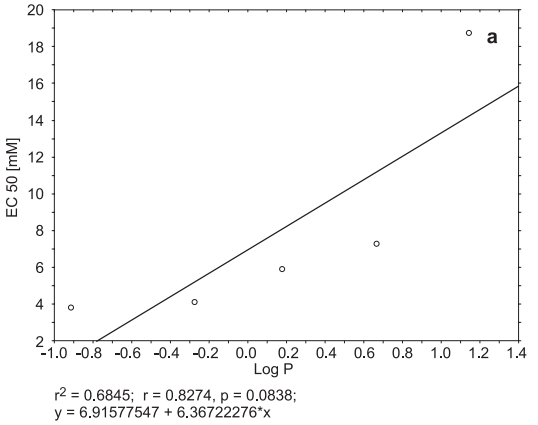


Figure 3. The correlation between EC_{50} values of AAAs in relation to calcium (a) and magnesium (b) and their pKa values.



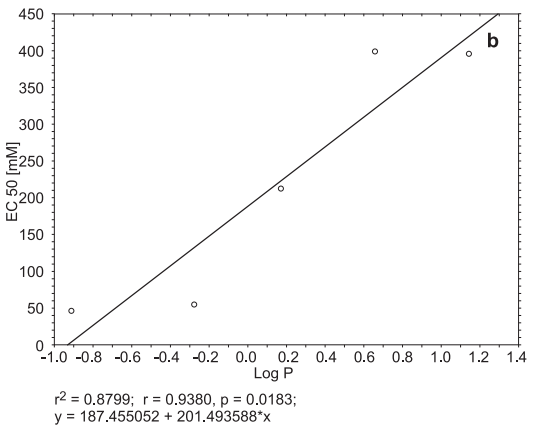


Figure 4. The correlation between EC_{50} values of AAAs in relation to calcium (a) and magnesium (b) and their Log P values.

ity of some EGAEs and their metabolites increased with reduced chain length, while cytotoxicity increased with the length of the alkyl groups (16).

Our results and those of others exemplify appropriate end-points for demonstrating compound differences in calcium and magnesium chelating activity and other toxic effects, as well as their association with physicochemical properties.

In conclusion, it is clear that there are considerable differences between individual AAAs in activity of calcium and magnesium binding. This activity decreased with the growth of lipophilicity and drop of acidity. The reason for the differences in chelating activity between these chemicals may be the structure parameters such as inductive effects and physicochemical properties.

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