COMPARISON OF THE *IN VITRO* HEMOLYTIC EFFECTS PRODUCED BY ALKOXYACETIC ACIDS ON HUMAN AND RAT ERYTHROCYTES

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Abstract

Objectives: The alkoxyacetic acids (AAAs) are urinary metabolites of alkoxyethanol solvents. It is well documented that these chemicals can cause acute hemolytic anemia in humans and laboratory animals. There are scarce data on the relative hemolytic activity of these acids. Likewise, information is lacking on the relationship between their hemolytic activity and physicochemical properties. The aim of this study was to compare the hemolytic activity of five AAAs in red blood cells (RBCs) derived from donors' blood and male Wistar rats. Moreover, the possible relationship between lipophilic and hemolytic activity of AAAs was also investigated. Materials and Methods: The RBCs washed in TRIS buffer, pH 7.4, were adjusted to a packed cell volume (PCV) of about 20% and incubated in a water bath at 37°C for 0–3 h in the presence of different concentrations of AAAs. The hemolytic effects, in terms of the changes in RBCs, PCV, mean corpuscular volume (MCV) and free hemoglobin (HGB free) in incubation medium, were evaluated. Based on the dose-response relationship for RBCs, PCV and MCV, the effective concentration values (EC ⁵₀) and their 95% confidence intervals (95% CI) were calculated. The octanol-water partition coefficient (log P) and distribution coefficient (log D) of AAAs were computed using PALLAS software. The correlation between log P and log D values for AAAs at pH 7.4 and their EC ⁵₀ was analyzed. Results: Human RBCs were 1.9–3.1 times more resistant to the hemolytic activity of AAAs than rat erythrocytes. Also, the hemolytic activity of individual AAAs did not differ considerably; the maximum differences ranging from 2.0 to 3.3. The EC ⁵₀ values of AAAs highly correlated with their log P and log D values. Conclusions: The relatively small differences between the hemolytic effects of AAAs on rat and human erythrocytes may be associated with the strong acidity and relatively similar lipophilicity of these chemicals.

Key words: Human erythrocytes, Rat erythrocytes, Alkoxyacetic acids, Hemolysis, Relationship between lipophilic and hemolytic activity

INTRODUCTION

Ethylene glycol monoalkyl ethers (EGAEs), especially 2-methoxyethanol (ME), 2-ethoxyethanol (EE), 2-propoxyethanol/2-isopropoxyethanol (PE/IPE) and 2-butoxyethanol (BE) are extensively produced chemicals [1,2] with a wide range of industrial and domestic applications [3]. The presence of EGAEs in paints, lacquers, dyes, and cleaning agents has generated considerable interest in their toxicity. In recent years, EGAEs, particularly ME and EE, have been progressively replaced with propylene glycol ethers [4,5], which are less toxic [6].

It is well documented that these chemicals can cause hemolytic anemia in humans [7–9] and in various laboratory animals [10–12]. Male rats treated with EGAEs...
demonstrated a time- and dose-dependent increase in packed cell volume (PCV) and mean corpuscular volume (MCV), suggesting an early swelling of erythrocytes. Subsequent hemolysis of erythrocytes led to a time- and dose-dependent decrease in the number of circulating red blood cells (RBCs) and total hemoglobin concentration (HGB). These effects of EGAEs were accompanied by a significant increase in the number of circulating reticulocytes and plasma hemoglobin concentration [13,14]. Although EGAEs are known to produce acute hemolytic anemia in rat, mouse, hamster, rabbit and baboon, their effects on humans, as well as pig, dog, cat and guinea pig, were found to be markedly less pronounced. Similar differences in the effects of butoxyacetic acid (BAA), BE metabolite, on the hematological parameters were observed in vitro, in the studies on various mammals, including humans [11].

EGAEs can produce systemic toxicity, including spermatoxic, teratogenic, and hematological effects, following oxidation to their corresponding aldehydes and alkoxyacetic acids (AAAs) by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), respectively [13,15]. These enzymes can metabolize EGAEs in the liver, skin and testis [13,16–18]. The hematotoxicity studies of equimolar doses of BE, butoxyacetic aldehyde, and BAA revealed that the effects of all the three chemicals did not differ significantly, which indicates that the interconversion of these chemicals is an efficient metabolic process in vivo [13].

The data mentioned above suggest that AAAs can directly exert their hemolytic effect on RBCs. Individual AAAs may differ with respect to their hemolytic activity. However, little is known on the hemolytic strength of these acids or the relationship between their physicochemical properties and hemolytic activity.

The aim of this study was to compare the hemolytic activity of five AAAs, consecutive homologous compounds, on RBCs derived from healthy donors and male Wistar rats. In addition, the log of dissociation constant (pKa) as well as the partition coefficient (Log P) and the distribution coefficient (Log D) of AAAs were calculated in order to assess the relationship between the physicochemical properties and the hemolytic activity of these acids.

MATERIALS AND METHODS

Chemicals

Methoxyacetic acid (CAS: 625-45-6; MAA) and ethoxyacetic acid (CAS: 627-03-2; EAA) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), while propanoyacetic acid (CAS: 54497-00-6; PAA), BAA (CAS: 2516-93-0), and pentoxyacetic acid (PEAA) were synthetized.

Synthesis of PEAA, BAA, and PAA

The three AAAs were obtained using a modified method of Rule et al. [19]. Sodium was dissolved in the relevant alcohol, and 0.5 of stoichiometric amount (calculated for sodium) of chloroacetic acid was added. The reaction mixture was refluxed, the products were purified by distillation under reduced pressure and by several washings, according to their physicochemical properties. Their structures were confirmed by 1H-NMR spectral analysis.

PEAA. Sodium (4.6 g, 0.2 mol) was dissolved in stirred hot n-pentyl alcohol (50 ml), and then chloroacetic acid (9.45 g, 0.1 mol) dissolved in n-propanol (25 ml) was added dropwise. The thick slurry was refluxed with stirring for 2 h. On the next day, water (100 ml) was added and the solution was extracted twice with diethyl ether (50 ml). The aqueous layer was acidified with 25% H₂SO₄ solution. The product was extracted with ether, the ether layer was dried with anhydr. Na₂SO₄, and ether was evaporated under vacuum. The residue was distilled at reduced pressure, b.p. 230–233°C. Finally, the product was purified by dissolving in methylene chloride (CH₂Cl₂), extraction into alkaline solution of 2% NaOH, acidification with 25% H₂SO₄ and extraction into CH₂Cl₂. After evaporation of CH₂Cl₂, the pure product was obtained. Yield 1 g.

BAA. Obtained in a similar way as PEAA, starting from n-butanol (75 ml), b.p. 153–156°C. Yield 4.6 g.

PAA. Obtained in a similar way as PEAA, starting from n-propanol (75 ml), b.p. 180–186°C. Yield 3 g.

Calculation of pKa, Log P and Log D values

For AAAs, the pKa, Log P and Log D values, i.e. the log of dissociation constant, octanol-water partition coefficient, and distribution coefficient, respectively, were calculated using PALLAS software [20].
Red blood cell suspensions

Heparinized blood samples derived from healthy donors or male Wistar Kr: (WI)WUBR rats (10–12 weeks old) were centrifuged at 1000xg for 10 min before removal of plasma and buffy coat. The RBCs were washed three times with a buffer, pH 7.4, containing 10 mM TRIS, 140 mM NaCl, 2 mM CaCl₂, 4 mM KCl, 10 mM, and 0.1% bovine albumin [21]. The cells were then adjusted to a packed cell volume (PCV) of about 20% and incubated in a water bath at 37°C for 0–3 h in the presence of different concentrations of AAAs (see Tables 1 and 2).

Table 1. EC₅₀ values for AAAs calculated from RBC, PCV, and MCV changes in human erythrocytes in vitro

<table>
<thead>
<tr>
<th>Hematological parameter</th>
<th>AAAs</th>
<th>Concentration (mM)</th>
<th>EC₅₀ (mM)</th>
<th>95% CI</th>
<th>Ratio</th>
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<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td></td>
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<tr>
<td>RBCs</td>
<td>MAA</td>
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<tr>
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<td>PEA</td>
<td>4.0</td>
<td>16.0</td>
<td>ND</td>
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</table>

Ratio: EC₅₀ for MAA to EC₅₀ for other AAAs.
ND — not determined, because the EC₅₀ values exceeded the concentration range used in the experiment.
CI — confidence interval.

Table 2. EC₅₀ values for AAAs calculated from RBC, PCV, and MCV changes in rat erythrocytes in vitro

<table>
<thead>
<tr>
<th>Hematological parameter</th>
<th>AAAs</th>
<th>Concentration (mM)</th>
<th>EC₅₀ (mM)</th>
<th>95% CI</th>
<th>Ratio</th>
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<td>RBCs</td>
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<td>6.0</td>
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<td>4.1–4.6</td>
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<td>MAA</td>
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<td>16.0</td>
<td>15.9</td>
<td>14.2–17.3</td>
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<td>15.0</td>
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</tbody>
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Abbreviations as in Table 1.
demonstrated that the hemolytic activity of MAA and other AAAs, reflected by a decrease in RBCs and PCV and an increase in MCV, was the most pronounced effect at 3 h of incubation. The increase in HGB_free in an incubation medium of human and rat erythrocytes was most evident at 2 and 3 h of incubation (Fig. 2). The hematological changes observed during the experiment did not correlate with AAAs concentration, while those referring to RBC, PCV, and MCV showed a nonlinear correlation.

Red blood cell parameters

Erythrocyte suspension was sampled at 0, 1, 2, and 3 h of incubation, and RBC count (10^6/μl), PCV (%), and MCV (fl) were determined using COBAS MICROS analyzer (Roche, Palo Alto, CA, USA). Also, free hemoglobin concentration (HGB_free, g/dl) in the incubation medium was quantified colorimetrically using Drabkin’s reagent. The quality of hematological analyses was tested systematically using standard human blood CBC-3D Hematology Control (R & D System Inc., Minneapolis, USA). The day-to-day repeatability, as the precision of measurement for RBC, PCV, and MCV in blood, equaled 4.2%, 4.5%, and 4.4% (n = 30), respectively.

Statistical analysis

The results are presented as means ±SD for three independent experiments with duplicate determinations. Data were analyzed by two-way analysis of variance with repeated measures on one factor (MANOVA). To compare the erythrocyte samples treated with different AAAs concentrations and the control sample at each of the time-points, one-way analysis of variance (ANOVA) followed by Dunnett test was used. Based on the dose-effect relationships at 3 h of experiment, expressed as the percentage of initial values, nonlinear regressions with 95% CI, and effective concentration (EC_{50}) values were calculated. The SPSS package version 12.0 (SPSS Inc., Chicago, IL, USA) was used for the analysis. The EC_{50} values were the concentrations of AAAs required for a 50% decrease in the initial RBC and PCV values, or a 50% increase in the initial MCV value. Then the correlation between Log D and Log P values of AAAs at pH 7.4 and their EC_{50} were assessed. The calculations were performed using STATISTICA 6.0 PL.

RESULTS

The effects of different concentrations of MAA on RBC count and PCV and MCV values in rat erythrocytes, expressed as the time-dependent variables, are shown in Figure 1. Similar effects on human and rat erythrocytes were observed for all the other AAAs. These data demonstrated that the hemolytic activity of MAA and other AAAs, reflected by a decrease in RBCs and PCV and an increase in MCV, was the most pronounced effect at 3 h of incubation. The increase in HGB_free in an incubation medium of human and rat erythrocytes was most evident at 2 and 3 h of incubation (Fig. 2). The hematological changes observed during the experiment did not correlate with AAAs concentration, while those referring to RBC, PCV, and MCV showed a nonlinear correlation.
based on the dose-effect curves, were gradually decreasing from MAA to PEAA. The EC₅₀ values for MAA and EAA, based on PCV changes in human erythrocytes, as well as EC₅₀ for all AAAs, based on MCV changes, exceeded the concentration range used in the experiment. Similar results were obtained for EAA, PAA, and BAA; the changes being reflected by PCV and MCV values for rat erythrocytes. In general, the hemolytic activity of AAAs increased with their increasing molecular weight. Human RBCs were relatively more resistant to the hemolytic activity of AAAs than rat RBCs. The EC₅₀ values in human erythrocytes were 1.9–3.1 times as high as in rat RBCs. Also, the differences between the hemolytic activity of individual AAAs were small. They did not usually exceed 86x151 with AAAs concentration in an incubation medium, especially in rat erythrocytes (Fig. 3). The changes in MCV for human erythrocytes did not correlate significantly with the concentrations of BAA and PEAA. The EC₅₀ values,

The values represent mean ±SD from three independent experiments with duplicate determinations.

*P ≤ 0.01 significantly different from control (0 mM AAAs).

Open circles 0 mM AAAs (control sample), closed circles 20 or 5 mM MAA, open triangles 20 or 5 mM EAA, closed triangles 20 or 5 mM PAA, open squares 10 or 5 mM BAA, closed squares 10 or 5 mM PEAA.

**Fig. 2.** The effect of methoxyacetic acid (MAA), ethoxyacetic acid (EAA), propoxyacetic acid (PAA), butoxyacetic acid (BAA), and pentoxyacetic acid (PEAA) on HGB concentration in the incubation medium of erythrocytes isolated from the blood of humans (a) and male rats (b). The erythrocytes were incubated with different concentrations of the examined acids for 1, 2, and 3 h.

**Fig. 3.** Non-linear regressions with 95% confidence intervals and effective concentration (EC₅₀) values for methoxyacetic acid (MAA) calculated from changes in RBCs (a), PCV (b), and MCV (c) of erythrocytes isolated from rat blood, depending on acid concentration. The erythrocytes were incubated with different concentrations of MAA for 3 h.
the ratios of 2.0 and 3.3 in human and rat erythrocytes, respectively (Tables 1 and 2).

The Log P, Log D and pKa values of the examined acids gradually increased with the growing carbon atom number in alkyl moiety (Table 3).

Both in the case of human and rat erythrocytes, the correlations between EC\textsubscript{50} of AAAs and their Log P and Log D values were usually significant (Figs. 4 and 5).

**DISCUSSION**

Hemolysis is the principal effect of acute poisoning with EGAEs in various species. It was postulated that AAAs, which form during the metabolic activation of EGAEs in the liver, skin, and testis, are potent hemolytic agents [10,13,22]. There are remarkable species differences in the hemolytic activity of EGAEs in vivo and AAAs in vitro.

RBCs from healthy blood donors were less susceptible to the hemolytic activity of BAA [11] and other AAAs in vitro [23] than the rat RBCs. Also, erythrocytes from the patients with hemolytic anemia exhibited particular resistance to the hemolytic effect of BAA [24]. Nevertheless, the sub-hemolytic changes expressed as RBC deformability and swelling, as well as their greater sphericity and osmotic fragility were observed when rat erythrocytes in vitro were exposed to a 100-fold lower concentration of BAA than were the human RBCs [21].

The results obtained in the present study indicate that AAAs exert a relatively weak hemolytic effect both on human and rat erythrocytes. The hemolytic effect was expressed as a decrease in RBCs and PCV and an increase both in MCV and HGB\textsubscript{free} in incubation medium. For each of the AAAs, the EC\textsubscript{50} quotients for human and rat erythrocytes were within the range of 1.9–3.1. Also, the differences in the hemolytic activity of individual AAAs were of a similar order. The relative strength of the hemolytic effect of the examined acids, expressed as the ratio of EC\textsubscript{50} for MAA to EC\textsubscript{50} for the other AAAs, ranged between 1.0–2.0 and 1.0–3.3 for human and rat erythrocytes, respectively. It seems that the differences in the effects of AAAs on RBCs derived from various species may be associated with the mechanism of red cell damage. Human RBCs

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>Log P (pH = 7.4)</th>
<th>Log D (pH = 7.4)</th>
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<tr>
<td>MAA</td>
<td>3.54</td>
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<td>0.10</td>
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<td>PAA</td>
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<td>0.61</td>
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<td>BAA</td>
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<tr>
<td>PEAA</td>
<td>4.28</td>
<td>1.63</td>
<td>-1.06</td>
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</table>

**Table 3.** Acidity (pKa), partition coefficient (Log P) and distribution coefficient (Log D) values for AAAs

**Fig. 4.** Correlations between Log D (a) or Log P (b) and EC\textsubscript{50} values for alkoxyacetic acids (AAAs) acting on human erythrocytes.

**Fig. 5.** Correlations between Log D (a) or Log P (b) and EC\textsubscript{50} values for alkoxyacetic acids (AAAs) acting on rat erythrocytes.
have a much lower permeability to certain anionic molecules other than bicarbonates and chlorides. Some anions such as lactates and anionic amino acids are transported by specific transport systems in human RBCs [25]. The target of AAAs activity could be the protein(s) crucial to membrane integrity or membrane transport. For example, a transport function disrupted by a detergent anion could allow a passive leak of sodium and potassium ions from the cells. The loss of these ionic gradients may create an unbalanced osmotic force due to hemoglobin, which may result in osmotic lysis of RBCs [26].

The mechanisms responsible for species differences in RBC sensitivity to AAAs remain unknown. It was suggested the differences in the structure, biochemistry and physiology of erythrocyte membrane might be a causal factor. The disruption of the membrane permeability to sodium and potassium ions was found to lead to osmotic swelling and subsequent hemolysis of erythrocytes. Since AAAs cause swelling and hemolysis of erythrocytes, expressed respectively as elevated MCV and HGB₉₀, it is presumed that the erythrocyte membrane is the target of AAAs action. This assumption gives rise to two major questions, namely, what is the exact target of AAAs in erythrocyte membrane, and what role does it play in the species-specific differences in sensitivity to AAAs. Literature search failed to identify any structural, biochemical, or physiological features, such as protein and lipid composition of the membrane, Na⁺/K⁺–, and Ca²⁺/Mg²⁺–ATPases, etc. as the critical effects of AAAs activity [11].

Despite the structural similarities of EGAEs and AAAs, the toxicity of these chemicals differs significantly. Whereas the main effects of the short-chain EGAEs (i.e. ME and EE) in laboratory animals are developmental and reproductive disorders [27–29], the predominant toxic effect of IPE and BE is acute hemolytic anemia [11,14,30,31]. Moreover, the long-chain EGAEs cause no reproductive or developmental effects in laboratory animals. It was also found that MAA and EAA were retarding growth and induced gross structural defects in rat embryos in vitro, while PAA and BAA were markedly less embryotoxic and produced only minor anomalies [32].

The studies of the structure-activity relationships for the \textit{in vitro} hematoxicity of AAAs in rat blood, conducted by Ghanayem et al. [33], indicate that the presence of the ether linkage and the length of alkyl chain are critical for the development of hemolytic activity. The hemolytic activity of these acids was found to be as follows: BAA > PAA = PEAA > EAA > MAA. Furthermore, it was noted that the hemolytic effect of AAAs was associated with a parallel increase in MCV and a decrease in blood ATP levels [33]. Currently, it is not known why erythrocyte swelling or ATP depletion is the primary effect of these acids.

The results obtained in the present study indicate that the effects of various AAAs on human and rat erythrocytes are qualitatively similar and comprise early swelling of the cells followed by hemolysis. They revealed small quantitative differences in hemolytic activity of individual AAAs. The examined compounds are relatively strong organic acids with pKa values within the range of 3.54–4.28. The overall ratio of AAAs, both ionized and nonionized, which depends on the pH of the aqueous phase in the incubation medium and the acidity (pKa), may be described as the distribution coefficient (Log D). Log D represents the effective lipophilicity of a chemical at a given pH, when both its intrinsic lipophilicity (Log P) and degree of ionization [34] are taken into account. The Log D values of the examined AAAs at pH 7.4 ranged between –2.81 and –1.67. These values correlated well with EC₅₀ values (non-linear regression: \( r_s = 0.978–0.987; P < 0.001 \)).

Similar results were obtained for Log P describing the intrinsic lipophilicity of the functional groups and carbon skeleton of the chemical. This parameter refers only to the equilibrium of the nonionized chemical between the aqueous phase and the organic phase [34], e.g. cell membrane lipids. In the present study, the Log P values of the examined acids were within a relatively narrow range of -0.36 to 1.63. Also, these values correlated well with EC₅₀ values (non-linear regression: \( r_s = 0.981–0.987; P < 0.001 \)).

Thus, the hemolytic activity of AAAs depends both on their effective (Log D) and intrinsic (Log P) lipophilicity. The acidity of the examined compounds was found to be decreasing with an increasing length of their alkoxy chain.
The alkyl moiety represents an electron donor system which exerts an inductive effect on AAAs carboxyl group and leads to a decreasing acid strength as the chain length increases. Thus, it is likely that the structure parameters and the inductive effect, which determine polarity and ionization, as well as the solubility in lipids may be responsible for the small differences in the hemolytic activity of individual AAAs.

The present results are consistent with our previous observations on the hemolytic effects of four EGAEs (2-methoxyethanol, 2-ethoxyethanol, 2-isopropanoylthanol, and 2-butoxyethanol [14,31]). On the other hand, they are not concordant with our recent finding of the chelating effects of AAAs on calcium and magnesium in vitro [35]. It has been suggested that the hemolytic activity of these acids is associated with a disturbed ionic balance, mainly regarding the calcium level in the external environment of red blood cells [36]. Considerable differences have been found between individual AAAs with respect to their binding of calcium and magnesium. This activity tended to decrease with a decreasing acidity of these chemicals [35].

To sum up, the present study revealed relatively small species differences in the hemolytic effect of AAAs on human and rat erythrocytes. Also, the hemolytic activity of individual AAAs did not differ considerably. A direct quantitative relationship was found between the physicochemical properties of AAAs and their hemolytic activity. It seems that the Log D and pKa values have a significant influence on AAAs hemolytic activity in vitro. The mechanisms responsible for the species differences in the sensitivity of red blood cells to AAAs require further investigations.

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