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ANTISICKLING ACTIVITY EVALUATION OF 4 AROMATIC ALDEHYDES USING PROTON MAGNETIC RELAXATION

José E. Falcón Dieguez[1]
Centre of Biophysics and Medical Physics, University of Oriente,
Patricio Lumumba S/N, 90500, Santiago de Cuba, Cuba
and
The Abdus Salam International Centre for Theoretical Physic, Trieste, Italy,

Grisel del Toro García, Yamirka Alonso Geli and Manuel A. Lores Guevara
Centre of Biophysics and Medical Physics, University of Oriente,
Patricio Lumumba S/N, 90500, Santiago de Cuba, Cuba.

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[1] Junior Associate of ICTP. Corresponding author: falcon@cbm.uo.edu.cu; jfalcond05@yahoo.com

Abstract

The formation of a Shiff base aduct hemoglobin-aromatic aldehyde, has been reported as inhibitor of the hemoglobin S polymerization. Using the Proton Magnetic Resonance methodology, the polymerization kinetics can be studied and the delay time can be determined. Our studies *in vitro* show the inhibitor effect of the isovanillin, o-vanillin, m-hydroxybenzaldehyde and the p-hydroxybenzaldehyde, using molar ratio (hemoglobin S/compound) 1:1, 1:4 and 1:8. The td increment (expressed in percents) obtained for each one of the molar ratio was the following: isovanillin: 34±6% (1:1), 68±16% (1:4), o-vanillin: 26±10% (1:1), 63±20% (1:4), m-hydroxybelzaldehyde: 16±4% (1:1), 44±12% (1:4) and the p-hydroxybenzaldehyde: 10±3% (1:1), 32±8% (1:4). In the case of 1:8, the characteristic kinetics curve was not obtained. At the used concentrations, hemolytic activity was not found on the red blood cell. These results confirm the antisickling activity of these aromatic aldehydes, for a technique different to that reported in literature that also allows the quantification of concentration effect. The same ones will facilitate the study of the therapeutic usefulness of these compounds in the sickle cell anemia treatment.

INTRODUCTION

Sickle cell anemia, or homozygous sickle cell disease, is a genetic disorder, caused by a mutant hemoglobin (Hb). The hemoglobin S (HbS) result by an A-to-T mutation within the sixth codon of the β-globin coding region, the glutamic acid residues at the sixth position of two β-subchains are replaced by non-polar valine. This simple substitution causes a strong interaction between the Val-6(β) of a deoxi-Hb tetramer and a hydrophobic pocket of a second tetramer in the region Phe-85(β)/Leu-88(β),[1,2] causing intracellular formation of rigid polymers of deoxygenated hemoglobin (deoxyHb). The polymerization alters the shape and rigidity of the red blood cells (RBC) and triggers a sequence of pathogenic consequences[3]. In the search of a possible treatment, a great variety of compounds that inhibit the HbS polymerization due to direct interaction with the Hb (antisikling agents) has been reported. The formation of Shiff base of aromatic aldehydes with free amino groups in the Hb is very well-known.[4,5] The aromatic aldehydes interact with HbS producing a allosteric modulation to a high-affinity HbS molecule and inhibiting the intermolecular contacts that take place in the polymer. The most studied has been the 4-hydroxy-3-metoxybenzaldehyde (vanillin)[6,7] because of its properties and wide use in the food industry. Several method for kinetic polymerization study in HbS solutions have been developed.[8-13] In our laboratory the nuclear magnetic relaxation is used for the study of the polymerization process, using spontaneous deoxygenation. The water molecules associated with the Hb experiment variations in their mobility due to the polymers formation, causing a reduction of the relaxation time spin - spin (T2).[14-17] The kinetics of HbS polymerization are studied inside the erythrocytes or in solution with the variations that T2 experiment in the time. Through the kinetic study we can determine the delay time (td), the time in which the polymerization becomes critical and irreversible. Using the td variation it is possible to evaluate the action of a chemical agent on the HbS polymerization and their relationship with the concentration.[7,15] In the present work, the inhibitor effect of the polymerization of four aromatic aldehydes (isovanillin, o-vanillin, m-hydroxybenzaldehyde, p-hydroxybenzaldehyde) is evaluated (in vitro) to different molar ratios, using nuclear magnetic relaxation. In previous studies it is informed that the studied compounds show a low hemolytic activity on the RBC; for that reason a comparative study of the vainillina[7] with the aromatic aldehydes in study is developed.[18,19]

MATERIALS AND METHODS

Reagents

The reagents were obtained from commercial suppliers and of analytic quality: ethanol (Laboratory Alpha Aesar, Germany); sodium heparin (Sigma laboratory, Germany); NaCl, Na_2HPO_4, and KH_2PO_4 (Panreac laboratory, Spain), isovanillin (Fluka laboratory, Switzerland), o-vanillin, m-hydroxybenzaldehyde, and p-hydroxybenzaldehyde (Aldrich laboratory, Germany).

Preparation of the hemoglobin samples and solutions

The HbS samples were obtained though whole blood samples taken from sickle cell patients in the clinical laboratory of the Hospital General, "Juan Bruno Zayas", in Santiago de Cuba. The heparinized

whole blood was centrifuged (3500 rpm, 10 min), the plasma and buffy coat were eliminated by decantation. The RBCs were washed three times with phosphate buffer saline (PBS) (pH=7.4).[20] The RBC was hemolyzed for freezing. The membrane remains were eliminated by centrifugation (3500 rpm, 10 min) and decantation. The Hb concentration was determined by the cianomethahemoglobin method[21] using a spectrophotometer Ultrospec III (Pharmacia, Germany). Finally the Hb solution was conserved at 4°C until the experiment day. The hemoglobin A (HbA) samples were kept from voluntary donors at the Blood Bank "Renato Guitart Rosell" of Santiago de Cuba. All the blood samples used were obtained with the informed consent.

Knowing the Hb concentration the compound mass necessary to establish the molar ratio Hb:compound (1:1, 1:4, and 1:8) was calculated. The compounds were dissolved in hydroalcoholic solutions (500 μL of ethanol and 500 μL of PBS)

Nuclear magnetic relaxation measurements

400 μL of hemoglobin plus 20 μL of hydroalcoholic solution (using the concentration needed in order to establish the molar ratio studied) were placed in a nuclear magnetic resonance tube. [7,14,15]

2s were measured with the Relaxometer Giromag 01® (4 MHz) using Hahn pulse sequences (90°-τ-180°), at 36°C.[16,17] All curves obtained from the polymerization kinetic were adjusted to determine the td value using Microcal Origin 5 (USA).

The influence of the compounds in the HbS polymerization kinetics and the effect of the concentration were evaluated using equation 1.[7] The pH(7.4), the HbS concentration and the temperature (36ºC) was controlled.

$$\%Vtd = \frac{td(mr) - td(pat)}{td(pat)} \times 100 \qquad (1)$$

td(mr): delay time for the molar ratio; *td(pat):* delay time for the pattern sample.

Study of hemolytic activity

The hemolytic effect has been determined in vitro in RBC,[7,18,19] for all compounds in study from samples of voluntary blood donors' (normal RBC) and sickle homozygous SS (SS RBC). The spectrophotometric method was used.[20,22] The molar ratios studied were 1:1, 1:4, 1:8, and 1:10[6,7] and the hemolytic activity was reported in hemolysis percent (% H). A comparative study of the %H average caused by the vainillina,[7] its isomers (isovanillin and o-vanillin)[18] and the benzaldehydes (m-hydroxybenzaldehyde and p-hydroxybenzaldehyde) was carried out,[19] using the means comparison with the test of multiple ranges and the analysis of simple variance ANOVA (Statgraphics Bonus 2.1) with a significant 95% level.

RESULTS

Antisickling action

In Figure 1 the temporal behavior of typical T2 in samples of HbS and HbA is shown. As it can be seen through the temporal behavior T2, under the established experimental conditions, it can follow the polymerization kinetics of HbS, obtaining the sigmoidal characteristic curve described in the literature.[10-17,23] Which is contrary to the obtained under the same conditions with HbA. A typical property of this kinetics is the existence of a delay time (td) previous to the polymerization of deoxyHbS molecules.

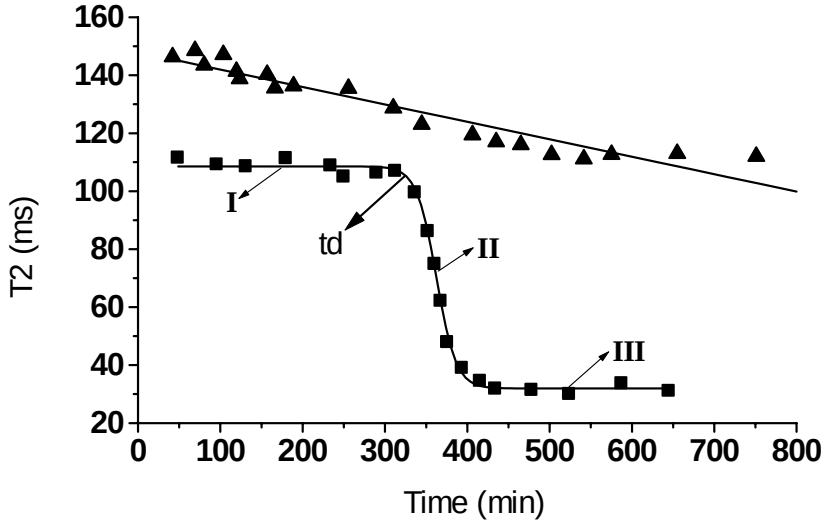

Figure 1. Temporal behavior of the spin-spin relaxation time (T2) (90°-τ-180° (Hahn), 4 MHz, 36 ºC), measured with the Relaxometer *Giromag* 01®, in HbA solution (▲) and HbS (■). The continuous lines represent the lineal and sigmoidal fit corresponding to the HbA and HbS samples respectively. In HbS the three stages correspond with: the beginning of reversible molecular agglutination (I), the irreversible polymerisation of HbS (II), and the end of polymerisation with the formation of micro domains (III).

Three different molar ratio (1:1, 1:4 and 1:8) were studied in order to evaluate the effect of the isovanillin, o-vanillin, m-hydroxybenzaldehyde and p-hydroxybenzaldehyde on the polymerization kinetics of HbS. In the figures 2, 3, 4 and 5 the curves obtained in one of the experiments realized for each compound are shown. An effect inhibitor of the polymerization expressed through the td increment (concentration dependent) was seen in the four aldehydes.

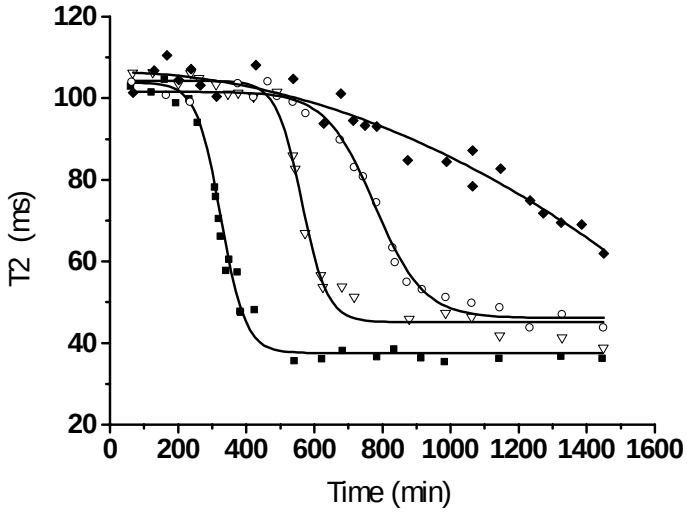

Figure 2. Polymerization kinetics of HbS solutions for the samples pattern and tried with different isovanillin concentrations. Curves of the temporal behavior of the spin-spin relaxation time T2 measured with the Relaxometer *Giromag* 01® (90°-τ-180° (Hahn), 4 MHz, 36 ºC). ■ Pattern (HbS solution), △ Molar ratio 1:1, ○ Molar ratio 1:4 y ♦ Molar ratio 1:8.

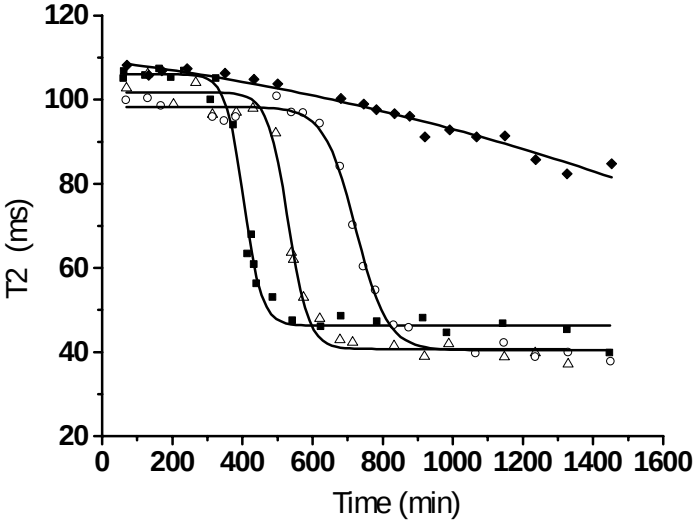

Figure 3. Polymerization kinetics of HbS solutions for the samples pattern and tried with different o-vanillin concentrations. Curves of the temporal behavior of the spin-spin relaxation time T2 measured with the Relaxometer *Giromag* 01® (90°-τ-180° (Hahn), 4 MHz, 36 ºC). ■ Pattern (HbS solution), △ Molar ratio 1:1, ○ Molar ratio 1:4 y ♦ Molar ratio 1:8.

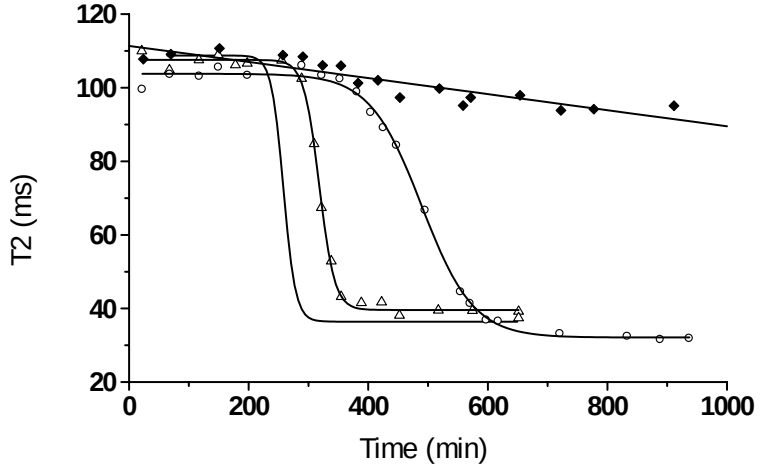

Figure 4. Polymerization kinetics of HbS solutions for the samples pattern and tried with different m-hydroxybenzaldehyde concentrations. Curves of the temporal behavior of the spin-spin relaxation time T2 measured with the Relaxometer *Giromag* 01® (90°-τ-180° (Hahn), 4 MHz, 36 ºC). ■ Pattern (HbS solution), △ Molar ratio 1:1, ○ Molar ratio 1:4 y ♦ Molar ratio 1:8.

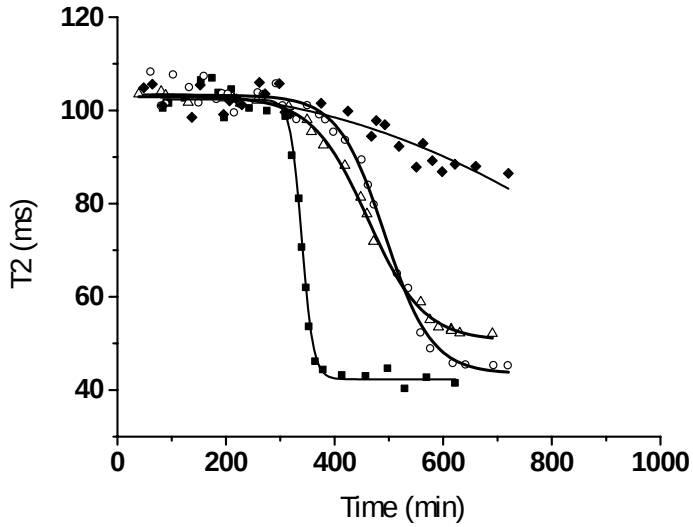

Figure 5. Polymerization kinetics of HbS solutions for the samples pattern and tried with different p-hydroxybenzaldehyde concentrations. Curves of the temporal behavior of the spin-spin relaxation time T2 measured with the Relaxometer *Giromag* 01® (90°-τ-180° (Hahn), 4 MHz, 36 ºC). ■ Pattern (HbS solution), △ Molar ratio 1:1, ○ Molar ratio 1:4 y ♦ Molar ratio 1:8.

The %Vtd average for the molar ratios 1:1 and 1:4 is shown in Table 1. The %Vtd of the molar ratio 1:8 is not reported, because as it can be observed in figures 2, 3, 4 and 5 for the molar ratio 1:8 for each compound, the td could not determined. This could be due to the saturation of the intermolecular binding sites in the polymer, and to the presence of a compound excess that would cause the polymerization inhibition by a balanced exchange among free molecules in the solution and those connected to the Hb.

Table 1. Effect of the aromatic aldehydes on the td of the HbS polymerization, expressed through the percentage of td variation (% Vtd) for the molar ratio 1:1 and 1:4.

Compound	**%Vtd (1:1)**	**%Vtd (1:4)**	**n**
Isovanillin	34 ± 6	68 ± 16	*7*
o-Vanillin	26 ± 10	63 ± 20	*7*
m-Hydroxybenzaldehyde	16 ± 4	44 ± 12	*7*
p-Hydroxybenzaldehyde	10 ± 3	32 ± 8	*7*

The statistical analysis reported non significant differences (p=0.05) of the inhibiting effect among isomers, isovanillin and o-vanillin; as well as m-hydroxybenzaldehyde and p-hydroxybenzaldehyde. While comparing the vanillin isomers (iso and orto) with the benzaldehyde isomers (m-OH and p-OH), the vanillin isomers presented a bigger antisickling effect ($p < 0.05$).

Previous studies informed that the hemolytic effect of the vanillin,[7] isovanillin,[18] o-vanillin,[18] m-hydroxybenzaldehyde[19] and 3-hydroxybenzaldehyde,[19] on the RBCs of voluntary donors, was not significant (p=0.05) for each molar ratio used (1:1, 1:4, 1:8 and 1:10). In the study on the SS RBC, for each compound, statistically significant differences (p=0.05) were not reported among the molar ratios.[7,18,19] The average % H oscillated in an interval from 0.2±0.1 to 0.5±0.2% and of 0.1±0.1 to 0.8±0.7%, for the cells of voluntary donors and SS, respectivamente.[7,18,19] Only vanillin, increased moderate and significantly ($p < 0.05$) the hemolysis to the relationship 1:10 on cells SS,.[7] The average % H caused by the five aromatic aldehydes to different concentrations on the RBC is shown in figure 6. Differences statistically significant were not obtained (p=0.05) in the hemolytic activity among the aldehydes for each one of the molar ratios and or cellular groups. The hemolytic effect produced to these concentrations did not exceed 3%, having a bigger variability in the erythrocytes SS.

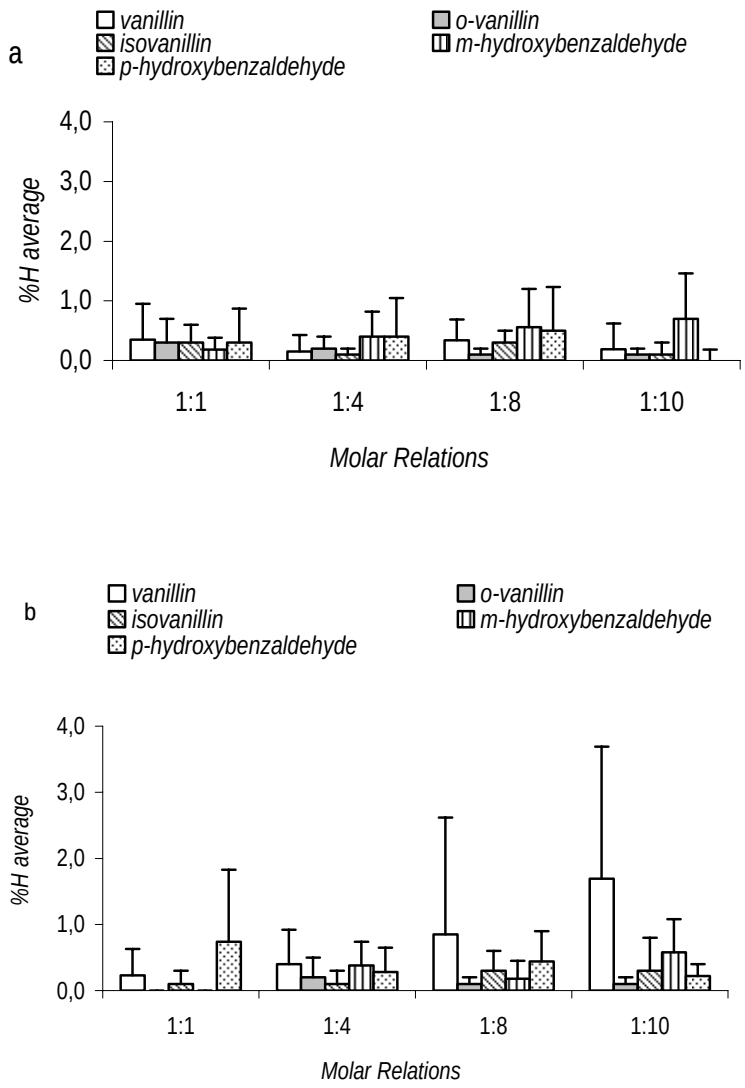

Figure 6. Hemolytic activity of five aromatic aldehydes on RBC.[7,18,19] The data represent the average of all tests for molar ratio and their respective standard deviation. a: hemolysis percent average (% H) on RBC from voluntary donors. b: hemolysis percent average (% H) on RBC from SS. Differences statistically significant were not obtained (p=0.05) in the hemolitic activity among molar ratio, among cellular groups, or among aldehydes. The hemolitic effect provoked to these molar ratios doesn't exceed 3%, having bigger variability in the SS RBC.

DISCUSSION

The kinetics of HbS polymerization constitute an essential factor in the clinical severity of sickle cell anemia.[3,29] The time average during which the human RBC are in hypoxic circulation is near 15 seconds. [30] If the td of the HbS polymerization was bigger than the hypoxic time in the circulation, the RBCs could complete the oxygenation-deoxygenation cycle before the intracellular polymerization accelerated and the sickling occurred. As a consequence the interactions between the RBC and the endothelial cells (endothelial adhesion),[24-26] the vascular obstruction, as well as the vasooclusive crises

frequency[27,28] could be diminished. That's why, some of the main therapeutic strategies is the chemical modification of the Hb inside the RBC, to diminish the polymerization degree and with it the subsequent sickling, which is the main modulator of sickle cell physiophatology.[31,32]

In previous studies[4,5] the antisikling activity of various aromatic aldehydes was reported in function of two parameters: the percent of Hb modification and the influence on the oxygen affinity, expressed through the partial pressure (P50). Later, on other authors reported new studies on the moderate antisickling activity of the vanillin.[6,7] In this work are reported the in vitro effect of four of these aromatic aldehydes on the HbS polymerization kinetics, as well as their dependence with the concentration, using the proton magnetic relaxation.

As it is shown in this work, using this method chemical compounds that act directly on the HbS polymerization can be evaluated. The literature informs that the aromatic aldehydes, through the formation of a Shiff base with free groups amino in the Hb, inhibit stereospecifically some of the intermolecular contacts that take place in the deoxy-HbS polymers, in coincidence with the presented results.

The %Vtd obtained shows a delay effect of the polymerization for these compounds, which are concentration dependent.

The effectiveness of antisickling agents that act in stereochemical inhibition will be given by the bond specificity and the competition between the binding sites in the HbS polymers. From the results it is corroborated that bigger substituents numbers in the aromatic ring cause a bigger steric impediment and as a consequence a greater retard in the HbS polymerization. The reaction of shiff base formation hemoglobin-aldehyde will be directly influenced by the position that occupy the substituents in the aromatic ring (steric and electronic effect), due to this the electronic cloud of the carbonyl group will be affected, favoring or not the addition reaction.

The aromatic aldehydes studied increased the polymerization td. Establishing a reactivity order, including the vanillin,[7] based on the % Vtd the results were the following: isovanillin ≈ o-vanillin> 3-hydroxybenzaldehyde> 4-hydroxybenzaldehyde> vanillin.

The vanillin and p-hydroxybenzaldehyde show the smallest antisickling effects. The weak electrondonors substituents in the para aromatic ring position (p-OH) diminish the carbonyl group (CO) reactivity for a nucleophilic addition reaction. These same substituents in the meta position are neuter (m-OH) or lightly electron-withdrawing (m-OCH3). In the case of the vanillin, the methoxy group (OCH3) is located in the meta position, with a negative inductive effect (- I) and a mesomeric positive (+M) effect, while the hydroxyl (OH) is located in the para position along with –I and a predominant +M. These effects provoke a decrease in the nucleophilic addition reactivity of the CO group carbon and thus the intermediate product is smaller due to the combination of electronic and steric effects, the activity was considered moderate and actually smaller than the rest of the studied compounds.

The Schiff base formation in the NH_2t region of the ß chains would increase the Hb oxygen affinity preventing the normal bond with the 2,3-diphosfoglicerate, a potent negative modulator in the

bond with the oxygen. The aromatic aldehydes that contain a hydroxyl group in ortho position (o-OH) have shown a potent effect in the oxygen union to the Hb.[4,5] This substituents can lead to these compounds toward the specific places in the Hb which influence strongly in the oxy-deoxy balance, favoring the oxygenated form. The isovanillin and o-vanillin were more reactive and they produced a similar effect in the polymerization inhibition, without differing statistically (p=0,05) when comparing the % Vtd total with their standard deviation.

One of the main clinical manifestations in SCA is the cronic hemolytic anemia,[33] that's why any compound that exacerbates hemolysis or modifies the rheologic properties, will have a marked negative effect in the course of the disease. These factors have been considered in the hemolytic activity studies of compounds with antisickling activity. The aromatic aldehydes presented in this work as inhibitors of the HbS polymerization td were previously studied and it was demonstrated that, as in SS RBC and in normal RBC, their hemolytic effects is low. For the variations among assays obtained, it should be kept in mind that the clinical state and hematology vary in an individual to another. The cellular morphological changes and the resistance to external factors will also be variable and dependent on individual characteristics, having a greater incidence in the case of the SS RBC. No significant differences were found when comparing the means using a multiple ranges test (p=0.05). Under the experimental conditions of the study, the % H average on the SS RBC and normal was smaller than 3% for all compounds; less than the reference value (10%) reported for this method.[22] In patients with SCA the intracellular polymerization of deoxyHbS molecules provoked membrane damages and caused the Hb liberation with up to 40 % hemolysis.[1,2]

Keeping in mind these factors, as well as the experimental methodology; it could be thought that the low percentage of hemolysis corresponds with the pharmacological action of these compounds (inhibition of the kinetics of HbS polymerization through the td increment), enhancing the antisickling activity reported for these aromatic aldehydes.

ACKNOWLEDGMENTS

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