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THE STABILITY OF 5–AMINOLEVULINIC ACID AND ITS ESTER DERIVATIVES

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Abstract: Application of 5–aminolevulinic acid (ALA) and its esters for treatment of cancer and other disorders became a rapidly developing branch of photodynamic diagnosis and therapy. For clinical use, solutions of ALA must be buffered to physiological pH. Unfortunately, in such conditions they are unstable. Two molecules of ALA condense and 2,5–(β–carboxyethyl)–dihydropyrazine and 2,5–(β–carboxyethyl)–pyrazine are formed. Although, numerous eleborations relate to stability of ALA alone very little or none is known about the stability of ester derivatives of ALA. The present investigations have comprised the stability of ALA and its esters at 37°C in respect of pH, concentration and time of reaction. The studies showed that ALA esters alike ALA undergo dimerisation at pH near 5. Moreover, it is possible that at pH>5.5 hydrolysis of the esters occurs, but this elaboration do not comprise explanation of this phenomenon.

Keywords: Photodynamic therapy, ALA derivatives, stability

In the last 25 years, a photodynamic method of diagnosis and therapy of tumours, has become a very promising and fast developing modality of cancer treatment (1). It relies on interaction of three separately inactive components (2) like photosensitive and tumour selective dye, light exciting the dye and oxygen solved in the environment of a tumor selective dye, light exciting the dye and oxygen solved in the environment of a tumor tissue (3).

Photodynamic Diagnosis (PDD) employs photosensitiser ability to emit its previously absorbed energy what results in red fluorescence of a sensitised tissue at a wavelength of about 400 nm. Irradiation at a wavelength of about 630 nm causes excitation of the dye which next transfers energy to oxygen. It results in producing a highly reactive form of oxygen, i.e. a singlet oxygen that interacts with other extra– and intracellular structures. Cancer cells are destroyed in that process called Photodynamic Therapy (PDT) (3).

There are two ways of introducing a photosensitiser into tumor cells. First one relies on administration of readymade dye. Second way, intensively developed in the last 15 years, relies on application of 5–aminolevulinic acid (2, 4, 5). All nucleated cells are able to enzymatic conversion of ALA to protoporphyrin IX that plays a role of endogenous photosensitiser (6).

Photodynamic therapy with ALA uses metabolic disorders of tumour cells. For some types of cancers, activity of porphobilinogen deaminase i.e. an enzyme, which catalyses formation of uroporphyrinogen from porphobilinogen, is higher that in healthy tissue. It results in faster synthesis and gathering of porphyrins in diseased cells. Furthermore, activity of ferrochelatase, the enzyme responsible for incorporation of iron into protoporphyrin IX, in cancer cells is lower (1, 7). Thus, incomplete heme cannot be secreted from the cell.

One of the most significant disadvantages of exogenous photosensitisers is their retention causing long–term skin photosensitivity. Patients underwent such a therapy, have to avoid light exposure up to six weeks (2, 7). Application of ALA photosensitizes skin for no longer than 24–48 h. (2, 4, 5, 7). Unfortunately, hydrophilicity of ALA gives its poor penetration into a tissue. That drawback limits the use of 5–aminolevulinic acid for treating only superficial lesions (1, 7). To avoid this problem, the conception of ALA pro–drug has been developed towards derivatives that are pharmaceu-

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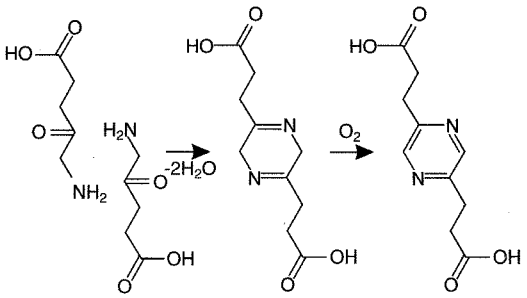
tically inactive and better penetrate cancer tissues. Local enzymes, at the place of destination, carry conversion of derivatives into their active forms (7, 8). On this basis, numerous ester derivatives of ALA have been synthesised. Due to additional hydrocarbon chain, esters are more lipophilic than the parent drug. After penetration into the cell, derivatives of ALA are hydrolysed by unspecific esterases (7, 8, 9).

One of the main problems occurring in ALA application is its instability in solutions. For therapy of bladder cancer usually 3% 5–aminolevulinic acid hydrochloride is used (10, 12, 13). The pH of such a solution ranges from 2.5 to 2.9. Having in view patient's comfort, solutions are buffered closely to the physiological pH. In such conditions the loss of ALA is observed. The condensation of two molecules of ALA (that depends on concentration and pH) takes place (Scheme 1). This irreversible process commences with producing of 2,5–(β–carboxyethyl)–dihydropyrazine that further can be easily oxygenated yielding 2,5–(β–carboxyethyl)–pyrazine. Formation of a new compound can be observed as yellowing of the solution or spectrophotometrically as a considerable increase in absorbance in the range of 220–350 nm (10, 11, 12).

EXPERIMENTAL

The hydrochlorides of 5–aminolevulinic acid and its esters – methyl (m–ALA), butyl (b–ALA) and hexyl (h–ALA) – were synthesised at the Industrial Chemistry Research Institute in Warsaw. Solutions were prepared by dissolving an appropriate amount of the mentioned compounds in adequate phosphate buffer (PB) (Polskie Odczynniki Chemiczne). The 0.003 M and 0.03 M solutions of ALA have been examined in 0.25 M PB, 0.18 M ALA in 0.4 M PB according to Medac (13). The pH of PB and buffered ALA derivatives were checked with an electronic pH–meter Elmetron CPC–551. The device was calibrated with buffer solutions acquired from Merck. The required pH of ALA solutions was matched precisely with appropriate PB to make it possible a comparison of absorption bands in respect of various concentrations and the same pH. Samples were incubated at 37°C. For 10 min. before the measurements samples were aerated with capillaries to achieve possibly uniform oxygenated pyrazine (10, 11, 12). The absorption spectra were recorded with a Varian Cary 50 BIO spectrophotometer. To make absorption bands of various concentrations comparable, 0.003 M solutions of ALA and esters were examined in a 10 mm optic pathelength cuvette, 0.03 M in – 1 mm, and 0.18 M – in 1 mm then divided by 6.



Scheme 1. Condensation of two molecules of ALA [12].

Time between the start of ALA dissolving in a phosphate buffer and the first measurement was no longer than 2 min. The recordings of absorption spectra were made at 0, 5, 15, 30, 60, 180, and 360 min. The baseline correction with the corresponding PB was performed for each experiment. The changes of absorption spectra were examined at pH = 4.5; 5.5; 5.8; 6.3; 6.8; 7.0.

RESULTS

Absorption spectra and pH of ALA and esters

Table 1 shows pH values of 0.03 M aqueous solutions of ALA and its esters. Due to the presence of hydrocarbon chains, the pH of esters is higher than ALA's alone. No changes of the absorption bands were recorded just after solutions preparation and after 30 days (solutions kept at room temperature) – the data are not shown. For such pH, the examined compounds are stable. The obtained data coincide with the ones achieved by other authors (10, 11, 13).

The hydrocarbon chains, bounded to the oxygen of carboxylic group of ALA, do not influence the absorption spectrum, thus 5–aminolevulinic acid and its esters have identical bands with the maximum at 266 nm (the absorption band for ALA is shown in Figure 1A).

For 0.003, 0,03, and 0.18 M buffered solutions of ALA and its derivatives no relevant changes of pH value during 6–hour observation were measured. For 0.3 M ALA, the pH decreased from 6.33 to 5.88 during 37 days.

Table 1. pH values of 0.03 M aqueous solutions of hydrochlorides of ALA derivatives

Compound name	pH
ALA	2.65–2.90
m–ALA	3.95–4.01
b–ALA	4.87–4.95
h–ALA	4.94

Stability of ALA solutions

The absorption spectra of 0.03 M ALA, at two different pH values, are shown in Figure 1. At pH = 4.5 (Figure 1A) no changes can be seen over the time of experiment (6 h). Figure 1B reveals intensive absorbance growth and change of a spectrum shape at pH = 7.0. The absorbance at 278 nm raised about 54 times for the above given experimental conditions with the maximum shift from 266 nm to 278 nm. The linear absorbance growth *vs.* time for these wavelengths was recorded (Figure 1B inserted graph). Lack of plateau suggests that a new product is formed that has considerably higher molar absorption coefficient.

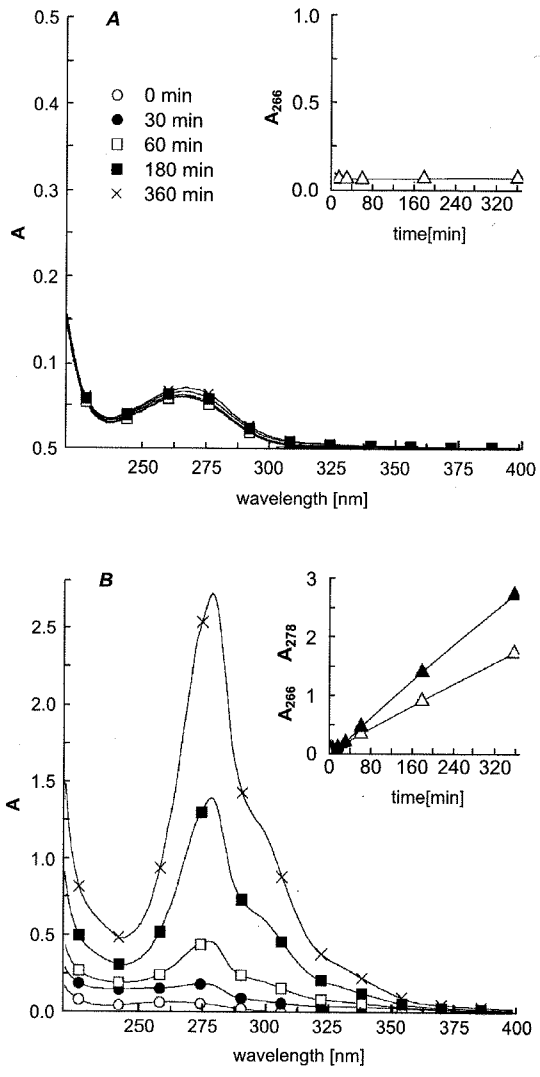


Figure 1. Changes of absorption spectra for 0.03 M ALA solution. A – pH 4.5; B – pH 7.0. Inserted graphs show absorbance *vs.* time at 266 (open triangles) and 278 nm (filled triangles).

At pH ≤4.5, independently of concentration, the investigated ALA solutions were stable (Figure 2). The solution of 0.18 M shows a rapid absorbance growth at pH>4.5. For the same experimental conditions, 0.03 M ALA was stable at pH ≤5.5. The most diluted solution (0.003 M) was stable at nearly the whole range of examined pH and only for the value of about 6.8, an insignificant absorbance growth and change of a spectrum shape were observed.

Stability of 0.03 M ALA derivatives

The aqueous solutions of 0.03 M ALA and esters (except h–ALA) are stable over 6 h at 37°C and at pH ≤5.5. At pH >5.5 the distinct absorbance growth at 278 nm was observed (Figure 3). Similar results for ALA have been revealed by Novo et al. (12).

The m–ALA shows a higher rate of absorbance growth than ALA and b–ALA. Repeated measurements confirmed such a tendency. Visually, except yellowing at higher pH, no other effects (i.e. turbidity) of ALA and m–ALA were observed.

At pH ≥6.3 b–ALA became turbid after about 40 min, and measurement of real absorbance was difficult because of strong scattering of the light beam. The disturbing turbidity was reduced by dilution of b–ALA solutions just before measurement in adequate PB and subsequently the recorded data were multiplied by the factor equal to the degree of dilution. The absorbance at 278 nm for

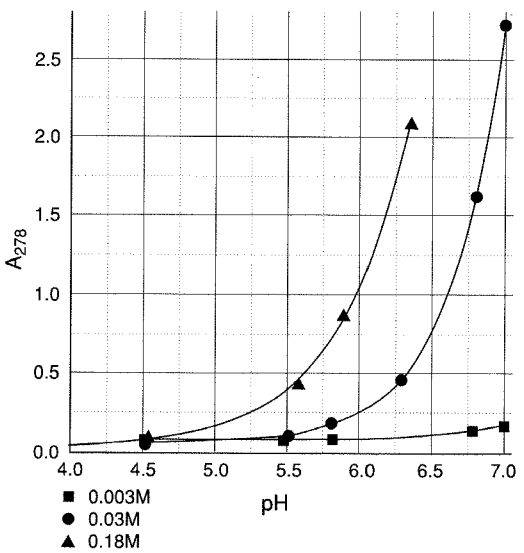


Figure 2. Dependence of absorbance on pH, for 0.18, 0.03, and 0.003 M ALA solutions. Absorbance was measured at 278 nm after 6 hours of incubation at 37°C.

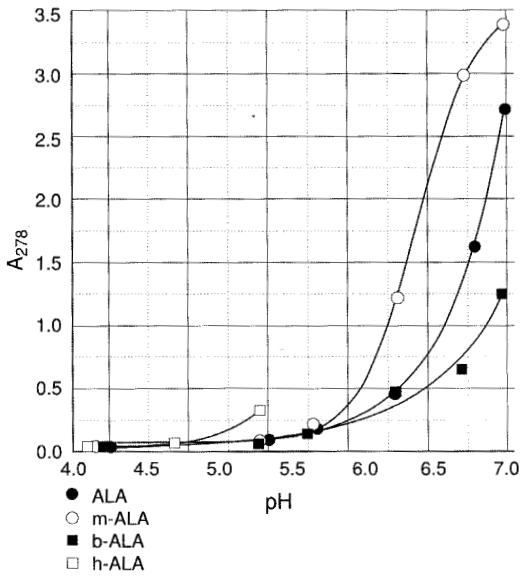


Figure 3. Influence of pH on absorbance of 0.03 M solutions of ALA derivatives. Absorbance measured at 278 nm, after 6 hours of incubation at 37°C. The curves for ALA, b–ALA and h–ALA were fitted with exponential growth function. The fitting for m–ALA was performed with sigmoidal function due to better interpolation in this case than with exponential growth fitting.

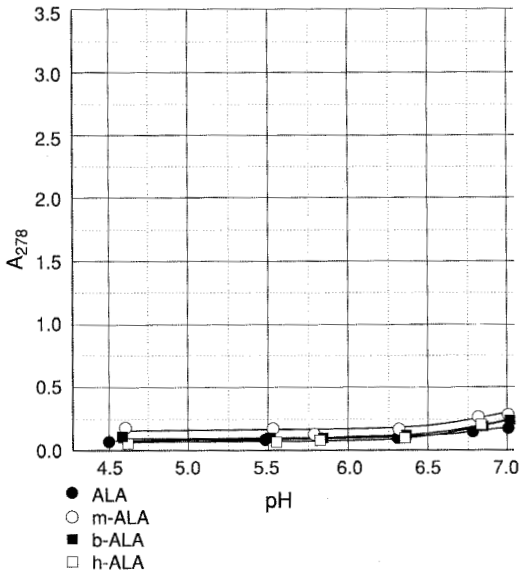


Figure 4. Influence of pH on absorbance of 0.003 M solutions of ALA derivatives. Absorbance was measured at 278 nm, after 6 hours of incubation at 37°C. The curves were fitted with exponential growth function.

b–ALA is slightly smaller than for ALA alone, but with the methods available in the laboratory it was impossible to determine if b–ALA was more stable than ALA.

Because of very rapid and considerable turbidity of 0.03 M h–ALA solutions (even at pH = 5.5, after 30 min), the studies of this compound were made in limited range. The solutions of h–ALA at pH ≤4.5 did not become turbid even after storing them for 24 h at room temperature.

Stability of 0.003 M ALA derivatives

Tenfold lowering of concentration of ALA and esters solutions results in a considerable decrease in the reaction rate. Under the above determined experimental conditions, an insignificant absorbance growth can be seen at pH >6.8 (Figure 4). For such experimental conditions, stability of ALA and esters is comparable.

Estimation of ALA decay

The ALA solution (0.3 M; pH = 6.33) was incubated at 37°C for 885 h (about 37 days). Absorbance growth was recorded at 266 nm and 278 nm. The most significant reaction rate was during the first 50 h. The data coincide with those published by Gadmar et al., who estimated an effective ALA concentration on the basis of PPIX fluorescence in a cell line (14). Despite a very long period of incubation, the plateau did not appear. In order to exclude influence of evaporation of the solution during aeration, firstly, the temperature of the solution was reduced and aeration lasted up to 10 min. Next, the ratio A_{278}/A_{266} was determined that reached the asymptotic value about 1.57 (not shown). This was helpful to distinguish between the growth of absorbance as a result of course of reaction or the solvent evaporation. Also normalization of the spectra showed that the reaction stopped or its rate was very low (not shown).

Assuming that the whole ALA reacted and only pyrazine was produced, the growth of absorbance of about 160 times at 278 nm shows that no more than 20% of ALA was lost during the first 48 h.

DISCUSSION

The stability of the examined compounds depends on their concentration and pH as well. According to Medac Investigator's Brochure, optimal conditions for the treatment of bladder cancer are the concentration of ALA about 3% (0.18 M) and pH about 5.0 that corresponds to the lowest possible physiological pH of the urine. According to the mentioned elaboration, the ALA decay in such a solution over 5 h is not greater than 5% (13). We obtained results similar to those given above.

Esterification does not significantly influence stability of ALA derivatives. Due to more rapid

growth of absorbance, at pH >5.8, 0.03 M m–ALA seems to be less stable than ALA alone. The 0.003 M ALA and esters are stable at almost whole range of the examined pH. At pH between 6.8 and 7.0, a slight stability loss was detected.

The phenomenon of turbidity occurred only for 0.03 M solutions of b–ALA and h–ALA. Because of lack of proper methods, the process could not be definitely elucidated. The explanation may be the hydrolysis of ALA esters, that at low pH is a reversible reaction and equilibrium between de–esterification and re–esterification is obtained. At higher pH, the irreversible hydrolysis of esters takes place and alcohol is produced (15). The turbidity cannot be seen for m–ALA, due to very good solubility of methanol in water. The hydrolysis of b–ALA and h–ALA gives alcohols of very limited solubility in water what results in turbidity.

Little is known about mechanisms of hydrolysis of ALA–esters. The matter consists in optimum pH (near 8) when esterases show highest activity. Thus, it is very difficult to determine the factor influencing hydrolysis (pH and/or esterase).

Application of ALA esters can considerably suppress the disadvantages of photodynamic therapy. Firstly, the pH of their water solutions is higher than those of ALA. The second thing is that esters better penetrate tissues. Stability of ALA esters is similar to ALA's one but their concentrations can be lower to achieve the same effect. This can subsequently result in smaller loss of an active substance and pH is more suitable for therapy (7, 8). The optimal pH, when therapeutic solutions are relatively stable, is about 5.0–5.5. Despite insignificant loss of ALA during 6 h, the fresh solutions prepared just before application should be used.

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