ELSEVIER

Journal of Photochemistry and Photobiology B: Biology 34 (1996) 143–148

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY B:BIOLOGY

Chemical instability of 5-aminolevulinic acid used in the fluorescence diagnosis of bladder tumours

Mercedes Novo, Gereon Hüttmann, Heyke Diddens

Medizinisches Laserzentrum Lübeck, D-23562 Lübeck, Germany

Received 5 June 1995; accepted 15 December 1995

Abstract

Aqueous solutions of 5-aminolevulinic acid (ALA) prepared for intravesical instillation in the framework of a clinical study on the fluorescence diagnosis of urothelial bladder cancer were found to be unstable. This chemical instability of ALA was studied in aqueous solution of 37 °C as a function of concentration, pH and reaction time. Our investigations showed that the reaction of ALA is an irreversible process, which yields at least two reaction products in the pH range studied (pH lower than 8): the 2,5-(β-carboxyethyl)dihydropyrazine and the 2,5-(β-carboxyethyl)pyrazine. As a result of these studies, the conditions for the preparation of ALA solutions to be used for intravesical instillation were optimized: solutions of ALA in phosphate-buffered saline at a concentration of 0.18 M (3 g of ALA in 100 ml) neutralized to pH 5, were prepared and stored on ice until use. Solutions prepared under these conditions were stable and were used for fluorescence diagnosis of bladder tumours with successful results. The effect of the pH and the composition of the urine on the extent of the reaction of ALA and on the nature of its reaction products formed during instillation was investigated by comparing the urine of patients before and immediately after instillation of ALA.

Keywords: Chemical instability; 5-Aminolevulinic acid; Fluorescence diagnosis; Bladder tumours; Cancer

1. Introduction

5-aminolevulinic acid (ALA) has been found to induce the formation of photosensitizing concentrations of endogenous protoporphyrin IX (PPIX) in certain types of cell and tissue [1,2]. Because of advantages such as tissue specificity, rapid clearance of induced photosensitizer and possibility of topical administration of ALA-induced PPIX compared with exogenous photosensitizers, ALA has been extensively used in the last years in both pre-clinical and clinical studies on photodynamic therapy (PDT) and fluorescence diagnosis.

Our group is involved in different clinical studies on photodynamic therapy and fluorescence diagnosis with ALA-induced PPIX, conducted in collaboration with several departments of the Medical University in Lübeck [3,4]. In all these clinical studies, ALA is applied to the tissue at high concentrations and is usually neutralized to a pH well tolerated by the tissue [5]. During the preparation of aqueous solutions of ALA under the conditions used for instillation into the human bladder, the solutions turned from colourless to yellow within minutes as they were buffered to neutral pH. Furthermore, the colour of the solutions became more intense with time and their pH decreased significantly even in the presence of a phosphate buffer. These changes revealed the occurrence of a chemical reaction of ALA which is not desirable for clinical application owing to the practical problems derived and, more important, owing to the potential toxicity of the reaction products and the possible decrease in the efficacy of the instillation. Experiments were therefore performed to study the reaction of ALA in aqueous solution as a function of different parameters (pH, concentration, temperature and time) in order to determine the conditions under which ALA is stable, to find out whether the reaction is reversible and to characterize the reaction products.

It is well known that in vivo ALA undergoes an enzymatically induced dimerization to give porphobilinogen in the biosynthetic pathway to haem [6]. However, in the absence of the corresponding enzymes, the spontaneous dimerization of ALA yields different cyclic products. Granick and Manzerall [7] reported the dimerization of ALA to 2,5-(β-carboxyethyl)dihydropyrazine (CHPY) in alkaline solution. Later, Franck and Stratmann [8] showed that in alkaline solution the condensation of two molecules of ALA leads to two products in the ratio of 1:10, the minor product being identified as porphobilinogen and the predominant product as the unstable and difficult-to-isolate CHPY. Recently, Butler and George [9] identified three condensation products of ALA in aqueous solution depending on the reaction condi-

PII S1011-1344(96)07285-5

tions: the CHPY, which is formed at moderate pH under anaerobic conditions and yields 2,5-(β-carboxyethyl)-pyrazine (CPY) by aerial oxidation, and pseudo-porphobilinogen, obtained in strongly alkaline conditions together with the dihydropyrazine. The data of these workers indicated that no porphobilinogen is formed in the non-enzymatic cyclic dimerization of ALA.

These previous studies showed that ALA reacts in aqueous solution to yield several condensation products. However, the controversial results obtained by the different workers show the complexity of the reaction of ALA, typical of carbonyl group reactions [10]. The conditions (concentration, temperature etc.) necessary for this reaction to take place were not investigated, so that the stability range of ALA is not yet defined. Furthermore, it must be proved that the products of the reaction of ALA after long reaction times (6 h) at high temperatures (70–100 °C) are the same as those which form in the presence of urine and at much milder conditions, e.g. during the instillation into the bladder of patients (1–2 h at 37 °C). Therefore the reaction of ALA in the present study has been investigated in aqueous solution under the latter conditions in order to identify the reaction products and to estimate the stability of ALA as a function of pH and concentration during the times relevant for instillation. This study was performed using the UV–visible absorption spectroscopy technique.

2. Experimental details

Aqueous solutions of ALA at different pH and concentration values were prepared by dissolving the appropriate amount of 5-aminolevulinic acid hydrochloride (Merck) in bidistilled water and adding NaOH (Merck, p.a.) to obtain the required pH. All solutions were buffered using a ten-times concentrated Dulbecco's phosphate-buffered saline (Gibco BRL) to obtain the phosphate and salts concentrations usually used for biological media (total concentration of phosphates, 9.6 mM; concentration of additional salts, 0.140 M). Saline without bivalent cations was used to avoid the formation of ALA precipitates in basic media. HCl (Merck) was added if necessary to acidify the solutions of ALA. Anaerobic solutions were prepared by bubbling nitrogen in the aqueous solution for 1 h before adding ALA.

A clinical study on the fluorescence diagnosis of bladder tumours [3] was performed on ten patients using solutions of ALA prepared under optimized conditions derived from the studies in aqueous solution. For the first two patients, solutions of ALA at pH 5.5 were used. The good results obtained in these cases, i.e. no irritation or damage of the bladder tissue and a pronounced fluorescence of ALA-induced PPIX, led to a further decrease in the pH to pH 5.0. The concentration of the ALA solutions was 0.18 M in all cases and a volume of 50 ml was instilled. The instillation time was 1 h for the first two patients and 2 h for the other eight patients. Samples of urine were collected before and immediately after the instillation of ALA into the bladder of the patients and were kept on ice until their analyses. Note that the samples of urine collected after the instillation of ALA (ALA–urine) contained, in addition to the urine produced during the instillation, the ALA which was not taken up by the bladder cells and the products of the non-enzymatic condensation of ALA formed during the instillation. Dilute solutions (1/100) of the urine collected after the instillation of ALA were prepared immediately after collection and were also kept on ice. The urine collected before the instillation was diluted 200 times to allow the measurement of the absorption spectra.

pH was measured with a WTW pH meter (model 521) equipped with a combined pH electrode (WTW type E56). Absorption spectra were measured with a UV–visible spectrometer Lambda 14P from Perkin–Elmer.

3. Results and discussion

3.1. Reaction of 5-aminolevulinic acid in aqueous solution

Stock solutions of ALA at pH 7.3–7.4 and different concentrations (0.005 M, 0.18 M and 0.30 M) were prepared and kept at 37 °C. The two more concentrated solutions turned yellow when being neutralized and their pH decreased rapidly. 3 min after preparation, aliquots of the stock solutions were taken to obtain 5 mM dilute solutions of pH 7.3, whose absorption spectra are shown in Fig. 1(a). The spectrum of the 5 mM stock (full curve) is identical with the absorption spectrum of ALA, whereas the spectra corresponding to the 0.18 M and 0.30 M stock solutions show a new band at about 350 nm, as well as changes in the form of the spectrum at shorter wavelengths. These changes in colour, pH and absorption spectra of the concentrate stocks reflect the occurrence of a chemical reaction of ALA. Furthermore, this reaction is largely dependent on the initial concentration of ALA, but it cannot be attributed to a simple aggregation process, since the spectral changes are permanent after dilution of the concentrated stocks.

Using the same dilution procedure, the absorption spectra of the three samples were measured 1 h after preparation of the stocks (Fig. 1(b)). After this time interval, the spectrum of the 5 mM stock solution already differs from that of ALA, showing analogous changes to the spectra of the concentrated solutions after reaction for 3 min, which can be explained as above. However, a new band at about 275 nm becomes distinct in the spectra corresponding to the concentrated stocks after reaction for 1 h. For a given initial concentration of ALA, the ratio of the absorbance at 275 nm to that at 350 nm increases with increasing reaction time, although the decrease in the concentration of ALA due to the reaction causes a decrease in the absorbance at 275 nm and has no effect on the absorbance at the 350 nm band. These facts can only be interpreted by the contribution of at least two different absorbing species to the experimental absorption spectra, so

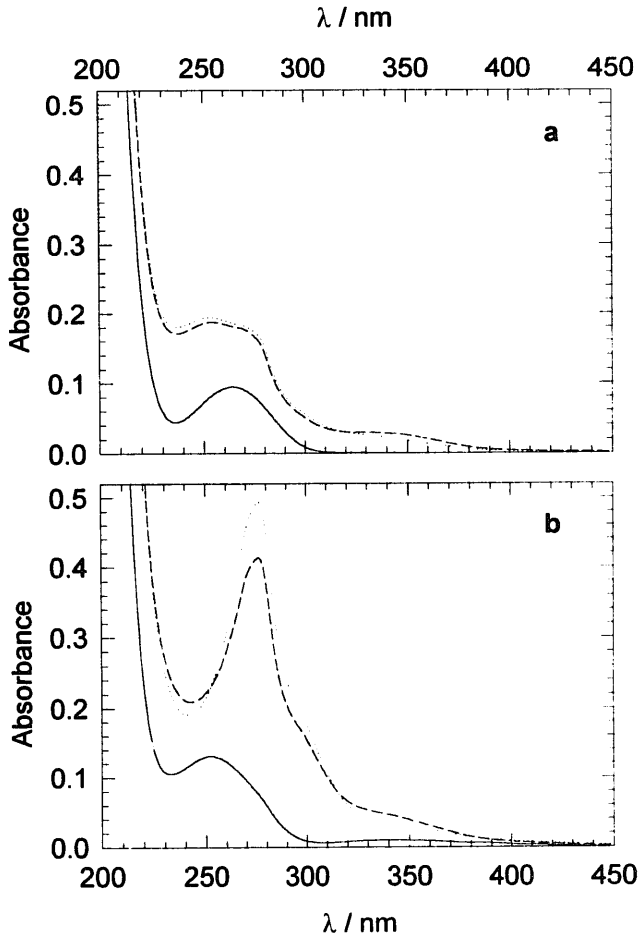


Fig. 1. Absorption spectra of ALA solutions (5 mM; pH 7.3) which were prepared from ALA stocks of pH 7.3–7.4 at three different concentrations, namely 5 mM (———), 0.18 M (– – –) and 0.30 M (· · ·), after reaction (a) for 3 min at 37 °C and (b) for 1 h at 37 °C.

that the absorption band at 275 nm must be due to a second product of the reaction of ALA. Studying the effect of oxygen on the absorption spectra of a reacting solution of ALA allowed us to assign these two absorption bands to the reaction products identified by Butler and George [9]. The absorption spectrum of a solution of ALA reacting under anaerobic conditions (Fig. 2) shows an intense band at about 350 nm and a shoulder at the low-wavelength absorption band, which must be attributed to the dihydropyrazine CHPY, the only product of ALA reaction in the absence of oxygen. When a small amount of oxygen was allowed to enter into the stock solution, the absorption spectrum changed significantly (Fig. 2), decreasing the absorbance at the 350 nm band as a new band appears at about 275 nm, as observed for the aerated solutions. These facts are perfectly explicable by the oxidation of CHPY to give CPY, so that the concentration of CHPY decreases as CPY is formed, this product being responsible for the absorption band at 275 nm which increases in intensity as the concentration of CPY becomes higher.

In addition to the concentration dependence, the occurrence of the reaction of ALA was found to be strongly dependent on the pH. When ALA is dissolved in water without

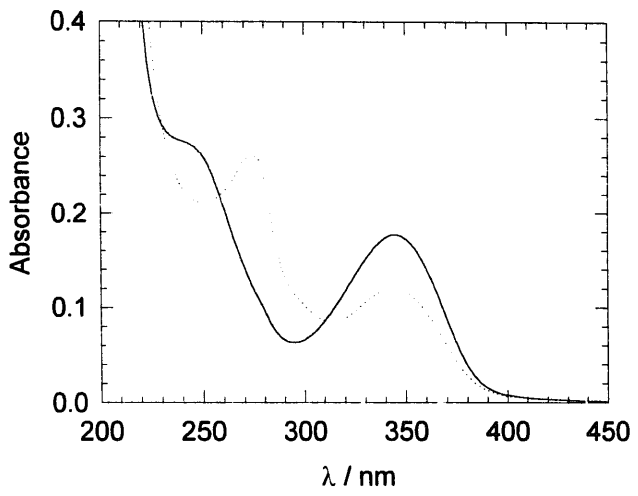


Fig. 2. Absorption spectra of ALA solutions (4.6 mM; pH 7.3), which were prepared from a deareated stock of pH 7.9 and concentration 0.27 M reacting at room temperature: ———, after reaction for 5 min; · · ·, after letting some oxygen enter into the stock.

adding NaOH, the acid solution obtained (0.3 M; pH 2) is stable, showing an absorption spectrum which is invariable with time and has no band at wavelengths longer than 320 nm. The reaction of ALA in the pH range relevant for clinical use was studied by preparing stock solutions of ALA of the same concentration (0.18 M) and different pH values (5.2, 6.3 and 7.4), which were left to react at 37 °C for several hours. It was observed that, although the same concentration of phosphate buffer was present in all three solutions, the acidity of the stock at pH 5.2 did not vary during the reaction, whereas the pH values of the other two stocks decreased significantly, as indicated in Fig. 3(a). Since the absorbances of the stock solutions both at 350 nm and at 275 nm were too high to be measured, the absorbance of the CHPY was detected at 400 nm instead of 350 nm as a function of the reaction time (Fig. 3(a)). Dilute solutions (5 mM; pH 7.3) were thus prepared from each stock at different reaction times in order to measure the absorbance at 275 nm (Fig. 3(b)). Since neither ALA nor CPY absorbs at 400 nm, the absorbance measured at this wavelength is proportional to the concentration of CHPY formed in the reaction of ALA. The data in Fig. 3(a) show that the concentration of CHPY increases very rapidly in the first 10–15 min of reaction at any pH, indicating that the rate of formation of this product is high. However, about ten times more product is formed during that reaction time in the pH 7.4 stock solution than at pH 5.2, and two times more than in the stock of pH 6.3. This suggests that the rate of formation of CHPY is strongly dependent on the pH and this may be due to the different reactivities of the protonated and deprotonated forms of ALA and/or to an acid–base catalysis of the reaction. At longer reaction times the behaviour of the 400 nm absorbance is different for the three stock solutions. It increases slowly with increasing reaction time in the solution at pH 5.2, and in contrast decreases slightly in the pH 6.3 stock and significantly in the pH 7.4 stock. These different variations in the concentration of

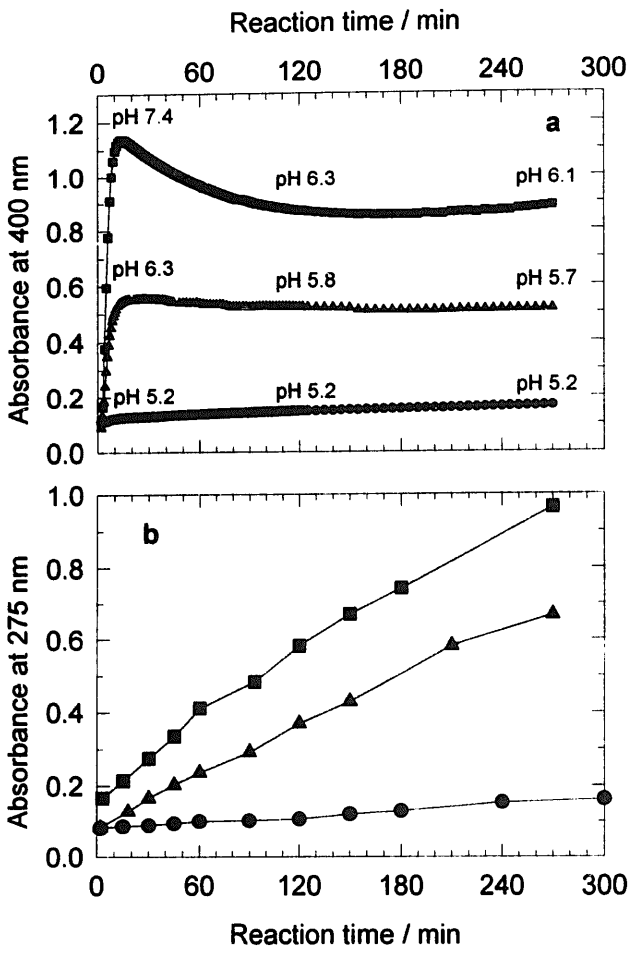
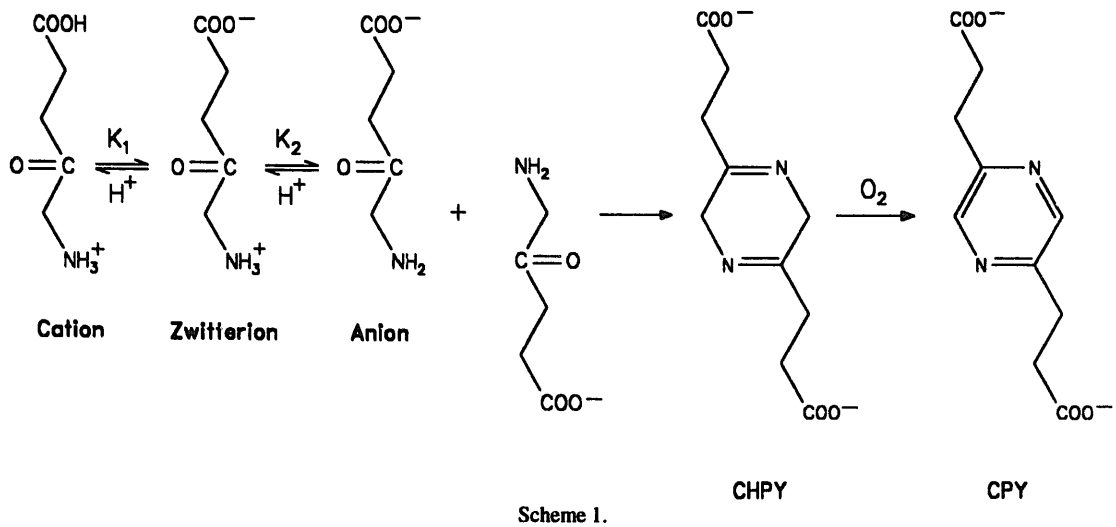


Fig. 3. Time dependence of the reaction of ALA at 37 °C for solutions of concentration 0.18 M and different initial pH values. (a) Absorbances at 400 nm vs. reaction time of ALA stock solutions of pH 5.19 (●), pH 6.31 (▲) and pH 7.44 (■). The pH of the stock solutions is indicated at three reaction times. (b) Absorbances at 275 nm measured for the dilute solutions (5 mM; pH 7.3) which were prepared from those stocks at different reaction times.

CHPY as a function of reaction time can be explained by the further reaction of CHPY to give CPY, whose rate would depend on the concentration of CHPY and therefore would be higher as the pH of the stock increases. The data of the 275 nm absorbances (Fig. 3(b)) are in keeping with this interpretation. The rate of increase in the absorbance measured as the slope of the curves is ten and 20 times higher for the solutions of pH 6.3 and pH 7.4 respectively, than for the pH 5.2 stock. This indicates that the rate of formation of CPY increases strongly with increasing pH.

The potential reversibility of the reaction of ALA was checked by comparing the absorption spectra of two acid diluted solutions of ALA: one prepared from an acid and stable stock solution and the other from a reacting stock (data not shown). These spectra were very different; the former coincided with the absorption spectrum of ALA at low pH, whereas the latter showed the presence of the reaction products of ALA. Hence, the reaction of ALA is not reversible by acidification.

The pH dependence of the reaction of ALA can be explained on the basis of the acid–base equilibria of this aminoacid (Scheme 1). The values of the corresponding acidity constants were determined spectrophotometrically and are $pK_1 = 4.05 \pm 0.05$ and $pK_2 = 8.3 \pm 0.1$. These values indicate that the zwitterion is the major species present in the pH range between 5 and 7.5, although significant amounts of the two other acid–base species exist depending on the acidity. Thus, at pH 5, about 10% of the molecules of ALA are cations whereas, at pH 7.3, about 10% of the ALA molecules are anions. According to these results, a scheme similar to that proposed by Butler and George [9] could explain the reaction of ALA, where the anion, a species with a deprotonated amino group, is the only one that is able to react with the ketone group of a neighbouring molecule to yield the cyclic dihydropyrazine (Scheme 1). For the condensation to occur, the amino group of ALA should be deprotonated. This explains the strong pH dependence of the reaction, since the concentration of the anion increases with the pH, as shown above. Therefore the solutions of ALA are only stable at such high acidities where the anion does not exist (note that,



Scheme 1.

although at pH 5.2 the concentration of that species is only 0.07% of the total concentration of ALA, some reaction does occur as shown in Fig. 3). Furthermore, as the anion reacts, the concentration of protons increases in the solutions of ALA in order to keep the equilibrium zwitterion–anion (see Scheme 1), explaining the decrease in the pH observed during the reaction of ALA. Independent of the mechanism of the reaction, the study of which is beyond the objectives of this work, it can be expected that the condensation of two molecules will be strongly influenced by the concentration, temperature and viscosity. Those parameters together with the acidity must be optimized and controlled in order to avoid the reaction of ALA used in clinical studies of PDT and fluorescence diagnosis.

3.2. *Reaction of 5-aminolevulinic acid during instillation in the human bladder*

On the basis of our results, conditions for the preparation of ALA solutions to be instilled into the bladder of patients for the fluorescence diagnosis of bladder tumours were optimized. Since the concentrations of ALA for clinical use should be high enough to induce the accumulation of detectable amounts of PPIX, a minimal concentration of 0.18 M (3 g of ALA in 100 ml of phosphate buffer) was used. The pH of the solutions was reduced to pH 5, which is near to the limit of tolerance of the bladder, since the pH range of human urine can vary from 4.8 to 8.4 [11]. Finally, the temperature should be controlled by cooling the stocks used for the preparation of the solution of ALA, preparing the mixture on ice and storing the resulting solution at low temperatures. Solutions of ALA prepared and kept under these conditions were stable for several days.

In order to study the reaction of ALA during instillation in the human bladder, absorption spectra and pH of the patients' urine collected before and immediately after the instillation were measured. The pH of urine collected before the instillation differed significantly among the different patients, varying from pH 5.1 to pH 7.7. A general decrease in pH was observed in the urine after the instillation of ALA. However, for those patients whose urine showed a pH higher than 6.5 before instillation, the pH of the ALA–urine was still higher than 6. This means that the urine produced during the instillation can significantly increase the pH of the solution of ALA, thus leading to a higher rate of non-enzymatic condensation of ALA.

Difference spectra were obtained by subtraction of the absorption spectra of urine collected before the instillation of ALA from those of the ALA–urine, previously normalized at short wavelengths. Fig. 4(a) shows a typical difference spectrum (dotted curve) compared with the absorption spectrum of a dilute solution obtained after reaction for 2 h at 37 °C of a 3% stock of ALA of pH 6.3 (full curve), which accounts for the reaction in aqueous solution under similar conditions (these two spectra have been normalized at 277 nm to allow comparison). The similarities between the difference spectra and that obtained in aqueous solution suggest that the pyrazine CPY is also formed by reaction of ALA in urine. The slight differences might be attributed to an effect of the medium or to further reaction of the CPY with constituents of the urine. The accuracy of the difference spectra is not high enough to detect the absorption of the CHPY.

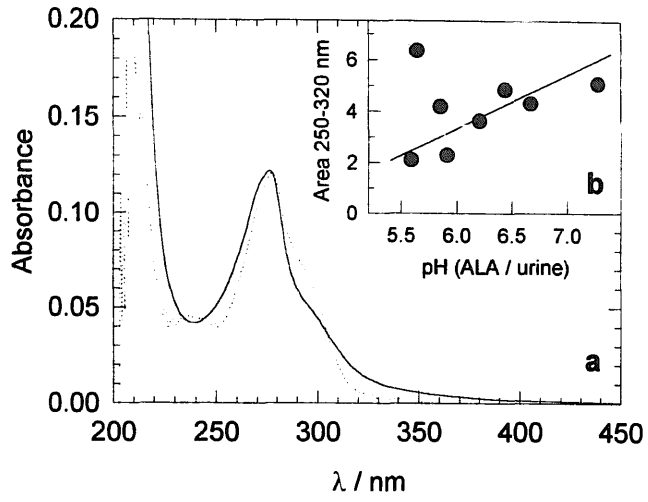


Fig. 4. (a) Absorption spectrum of a dilute aqueous solution of ALA (5 mM; pH 7.3) prepared from a stock of concentration 0.18 M and pH 6.31 (———), and difference spectrum obtained from the absorption spectra of the urine of a patient by substraction of the spectrum of the urine collected before the instillation of ALA from that of the ALA–urine (· · ·). The spectra were normalized at 277 nm to allow comparison. (b) Plot of areas between 250 and 320 nm of the difference spectra obtained as explained above vs. pH of the ALA–urine for the patients with 2 h of instillation.

The areas between 250 nm and 320 nm of those difference spectra were determined and a correlation with the pH of the ALA–urine was found (Fig. 4(b)). The absorption of the reaction product increased with increasing pH. This result shows again the necessity to control the pH not only before but also during the instillation in order to avoid the non-enzymatic reaction of ALA. These variations could be avoided by adding a sufficient amount of a suitable buffer to the solution of ALA, or by controlling the pH of the urine of the patients by means of a suitable diet.

Acknowledgments

Our special thanks go to Drs. M. Mosquera and F. Rodríguez Prieto from the University of Santiago de Compostela (Spain) for their critical review of the manuscript. M.N. thanks the Xunta de Galicia for a post-doctorate scholarship.

References

[1] J.C. Kennedy, R.H. Pottier and D.C. Pross, Photodynamic Therapy with endogenous protoporphyrin IX: Basic principles and present clinical experience, *J. Photochem. Photobiol. B: Biol.*, *6* (1990) 143–148.

[2] J.C. Kennedy and R.H. Pottier, Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy, *J. Photochem. Photobiol. B: Biol., 14* (1992) 275–292.

[3] K. Svanberg, I. Wang, S. Colleen, I. Idwall, C. Ingvar, R. Lundgren, R. Rydell, D. Jocham, A. Knipper, S. Thomas, H. Diddens, S. Bown, G. Gregory, S. Montán, I. Jonsson, S. Andersson-Engels and S. Svanberg, Clinical multi-colour fluorescence imaging of malignant tumours–initial experience, submitted to *Acta Radiologica*.

[4] H. Hörauf, G. Hüttmann, H. Diddens, B. Thiele and H. Laqua, Die Photodynamische Therapie (PDT) des Lidbasalioms nach topischer Applikation von Delta-Aminolaevulinsäure (ALA), *Ophthalmologe, 91* (1994) 824–829.

[5] R. Baumgartner, M. Kriegmair, R. Knuechel, H.G. Stepp, P. Heil and A. Hofstetter, Delta-ALA-assisted fluorescence detection of cancer in the urinary bladder, *SPIE, 2081* (1994) 74–80.

[6] A.M. del C. Batlle, Porphyrins, porfirias, cancer and photodynamic therapy, a model for carcinogenesis, *J. Photochem. Photobiol., 20* (1993) 5–22.

[7] S. Granick and D. Manzerall, Porphyrin biosynthesis in erythrocytes. II. Enzymes converting δ-aminolevulinic acid to coproporphyrinogen, *J. Biol. Chem., 232* (1958) 1119–1140.

[8] B. Franck and H. Stratmann, Condensation products of the porphyrin precursor 5-aminolevulinic acid, *Heterocycles, 15* (1981) 919–923.

[9] A.R. Butler and S. George, The nonenzymatic cyclic dimerisation of 5-aminolevulinic acid, *Tetrahedron, 48* (1992) 7879–7886.

[10] W.P. Jencks, Mechanism and catalysis of simple carbonyl group reactions, *Prog. Phys. Org. Chem., 2* (1964) 63–128.

[11] R.C. Weast (ed.), *CRC Handbook of Chemistry and Physics*, CRC Press, Boca Raton, Fl, 8th edn., 1987.

[12] M. Schmitt-Conrad, personal communication.