

Degradation Mechanism and Stability of 5-Aminolevulinic Acid

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ABSTRACT: The physiological substance and precursor of the heme synthesis 5-aminolevulinic acid (ALA) is a promising prodrug for photodiagnosis and photodynamic therapy of epithelial tumors, particularly in urological and gynecological tissues. For the clinical use of this substance, a chemically stable and sterile drug formulation is required. In the present study, degradation mechanism of ALA in aqueous solution and possibilities to improve its stability were examined. A capillary electrophoretic method was developed that was suitable for the quantification of ALA and of two degradation products. The intermediate degradation product was 2,5-dicarboxyethyl-3,6-dihydropyrazine, which was further oxidized to 2,5-dicarboxyethylpyrazine. The structures of the degradation products were proven by ¹H and ¹³C nuclear magnetic resonance spectroscopy. ALA degradation was very efficiently inhibited by adjusting the pH of the aqueous solution to a value <5 and by purging with nitrogen. Additives such as antioxidants did not improve the ALA stability. These results demonstrated that low pH ALA aqueous solution may be one possible dosage form to be considered for market introduction. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association *J Pharm Sci* 89: 1335–1341, 2000

Keywords: 5-aminolevulinic acid; drug stability; capillary electrophoresis; photosensitizers

INTRODUCTION

5-Aminolevulinic acid (ALA) is the precursor of protoporphyrin IX (PP IX) in the heme biosynthesis pathway and has been suggested for photodiagnosis and photodynamic therapy of epithelial cancer because PP IX can function as photosensitizer. However, no commercial product for diagnosis or therapy of cancerous tissues of inner organs, such as bladder or uterus, is presently available, although ALA products are under clinical evaluation. The development of stable ALA formulations for clinical use requires knowledge

of degradation mechanisms and kinetics of the compound. For this, stability-indicating analytical tools must be developed.

ALA belongs to the class of α -amino ketones, which dimerize readily under alkaline conditions.¹ The formed dihydropyrazines can further oxidize to pyrazines. The degradation pathways of ALA have already been studied to some extent (Figure 1). Under alkaline conditions, the formation of 2,5-dicarboxyethyl-3,6-dihydropyrazine (DHPY),^{2–6} porphobilinogen^{3,7} and pseudoporphobilinogen^{3–5,7} via an open-chain dimeric ketimine has been previously postulated. Further, the oxidation of DHPY to 2,5-dicarboxyethylpyrazine (PY) in the presence of oxygen has been reported.^{3–5,7,8} Nonetheless, the published results on ALA degradation mechanisms and products are conflicting. This conflict can be as-

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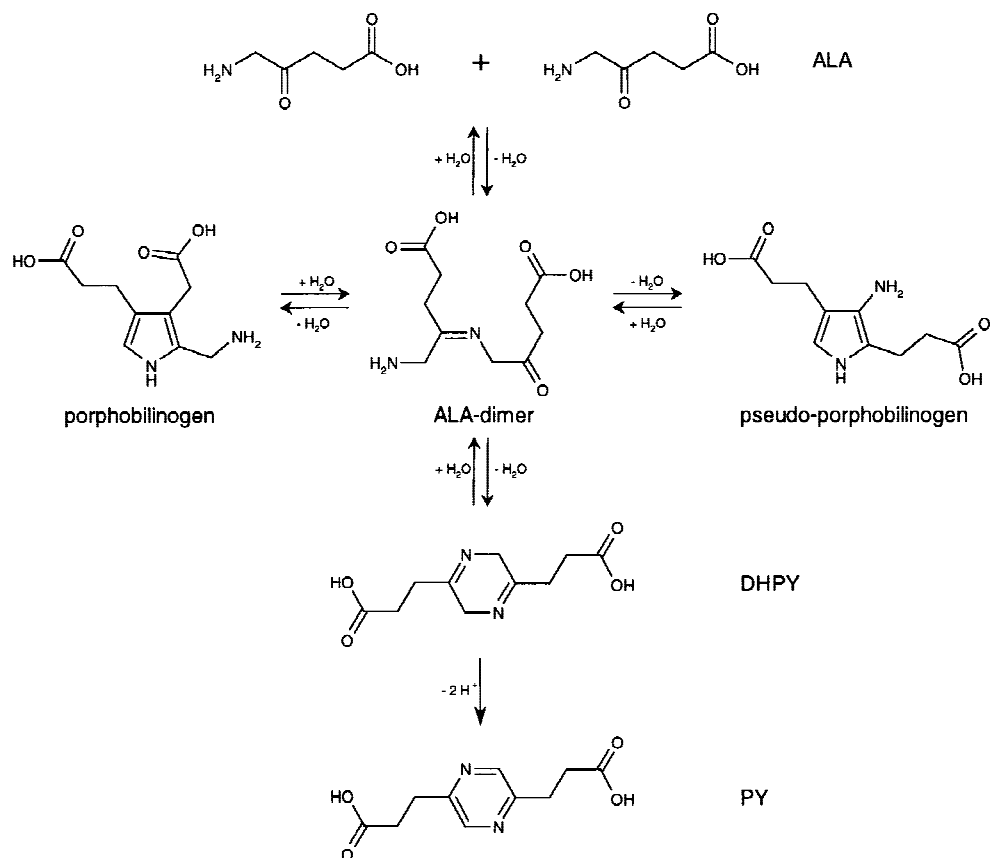


Figure 1. Condensation products of ALA according to the literature (refs 2–9).

cribed to the variety of stability test conditions and analytical methods used. Very importantly, most of the studies did not elucidate the structure of the degradation products.

In addition to dimerization, ALA can also undergo keto–enol tautomerism.^{4,9} Ultraviolet (UV) and nuclear magnetic resonance (NMR) spectroscopic investigations suggested that a small amount (i.e., <0.3%) of enolic ALA is formed at pH 7.8.⁴ Isosbestic points of the keto and enol forms were found at 215 and 285 nm,⁹ and enolization at C₅ occurred faster than at C₃.⁴

The knowledge acquired thus far on ALA stability remains essentially qualitative because of the variety of possible reactions that may occur simultaneously and the difficulty to isolate intermediate degradation products. Increased ALA stability in aqueous solutions of low pH (pH of 5.5 or lower) and at low temperature was deduced from changes in the yellowish color and UV spectrum.¹⁰ Furthermore, the degradation rate also depended on the ALA concentration.^{6,11}

The aim of the present study was to identify and quantify ALA degradation products and deg-

radation rate in aqueous solutions in view of developing a simple dosage form for clinical use. Quantitation of ALA itself has been limited to a variety of high-performance liquid chromatography (HPLC) methods involving derivatization reactions, similar to those used for amino acid assays.^{12–14} Attempts for direct ALA quantitation are scarce. Typically, ion-exchange chromatography^{15,16} and ion-pairing chromatography¹¹ have been used, whereas reversed-phase HPLC (RP-HPLC) appears unsuitable for this highly polar compound with insufficient retention in a RP-HPLC column. Alternatively, an efficient method for the determination of charged substances is capillary electrophoresis (CE). A CE method using micellar electrokinetic chromatography has already been described for concomitant quantitation of ALA and porphobilinogen in biological samples.¹⁷

EXPERIMENTAL SECTION

Materials

5-Aminolevulinic acid hydrochloride was purchased from Sochinaz (Vionnaz, Switzerland).

Pyrazine, pyrazine carboxylic acid, levulinic acid, $\text{Na}_2\text{B}_4\text{O}_7$, NaH_2PO_4 , Na_2HPO_4 , and NaOH were from Fluka Chemie (Buchs, Switzerland) and cetyl trimethylammonium hydrogen sulfate was from Merck (Darmstadt, Germany). Double-distilled water was used throughout.

Capillary Electrophoresis (CE)

CE was performed using a P/ACE 5010 CE instrument (Beckman, Fullerton, CA) equipped with an on-column UV detector or a diode array detector. A fused silica capillary (eCAPTM Capillary Tubing, Beckman) with dimensions of 37 cm (effective length of 30 cm) $\times$ 50 μm i.d. (375 μm o.d.) was used for separation, and "Gold-Nouveau" software (Beckman) was used for data acquisition. Samples were injected hydrodynamically (0.5 psi, 3 s). The running buffer was 50 mM borate buffer of pH 9.4. The voltage was set at 20 kV, and the temperature kept at 30 $^\circ\text{C}$. Analytes were detected by UV absorbance at 200 nm. The capillary was rinsed with 0.1 N NaOH for 2 min prior to each analysis. The buffer solutions were filtered through a 0.45- μm pore size filter (Schleicher/Schuell, Dassel, Germany) and degassed by ultrasonication. The electroosmotic flow (EOF) was measured by injection of acetone. Partially degraded ALA in 200 mM phosphate buffer of pH 7.4 was used for optimization of the CE method.

Structure Elucidation

PY was synthesized by dissolving 1.5 g of ALA in 5 mL of 5 N KOH and subsequent addition of 100 mg of HgCl_2 . The mixture was filtered, and the filtrate was acidified with HCl , whereby PY precipitated.

DHPY was prepared by dissolving ALA in 5 N NaOH while purging with nitrogen. This procedure converted ALA into DHPY with a yield of ~95%, as assessed from the peak surfaces in the electropherogram.¹⁸ The electropherogram revealed the absence of ALA and PY.

The identity of PY in dimethyl sulfoxide (DMSO) and of DHPY in 5 N NaOH was confirmed by ^1H and ^{13}C NMR spectroscopy (Bruker DRX500) at 295 K. Carbon spectra were recorded with broadband proton decoupling. Carbon assignments were supported by DEPT-135 spectra.

Stability Testing

ALA stability in solution was examined under various storage conditions, as indicated in Fig-

ures 2–4. The degradation products were quantified as described previously.¹⁸

RESULTS AND DISCUSSION

CE for Assaying ALA and Degradation Products

The CE method developed and optimized here was found suitable for the determination of ALA and its main degradation products in aqueous solutions of pH <9.4. When ALA was dissolved in water and the pH was adjusted to ~8 with 2 N NaOH , three compounds could be separated by CE (Figure 5). The substance with the lowest effective mobility was identified as ALA. The amphoteric compound ALA (pK_a values of 4.0 and 8.2²) exists as anion at pH 9.4 and consequently migrates to the anode. However, because of the prevailing effect of the EOF, ALA was detected at the cathodic side of the capillary. In addition to ALA, two degradation products with anionic properties were detected in the electropherogram. Kinetic investigations revealed that the second peak corresponded to an intermediate component, which further converted into a final degradation product (third peak). The higher effective mobility of the degradation products suggests, for example, a higher acidity of these substances compared with ALA. This increased acidity of the degradation products is consistent with the divalent

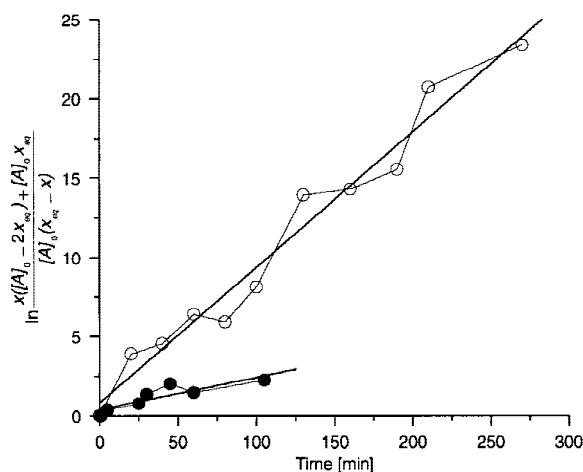


Figure 2. ALA degradation following reversible second-order reaction. Conditions: 0.1 g mL^{-1} of ALA was dissolved in 0.6 N NaOH , pH 5.5 (●) or 200 mM Na_2HPO_4 , pH 7.4 (○); 50 $^\circ\text{C}$. The regression parameters are: $y = 0.086x + 0.805$; $R^2 = 0.96773$ (for pH of 7.4), and $y = 0.020 + 0.37505x$; $R^2 = 0.87604$ (for pH of 5.5).

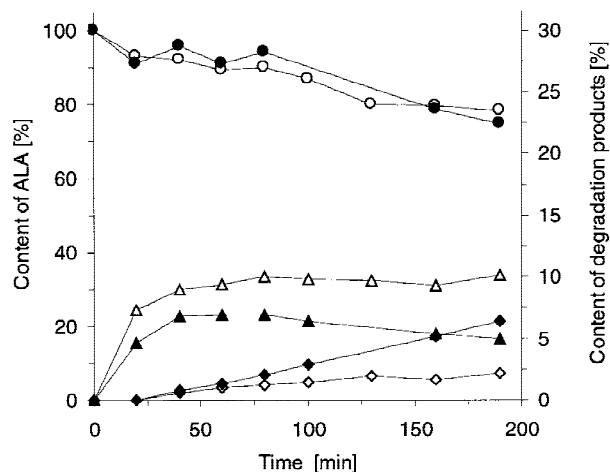


Figure 3. Degradation of 1.0 mg mL^{-1} ALA solutions ($\circ$, $\bullet$) either purged with nitrogen (open symbols) or oxygen (filled symbols). The right-hand y-axis gives the concomitant increase in the degradation products DHPY ($\triangle$, $\blacktriangle$) and PY ($\diamond$, $\blacklozenge$). Conditions: 200 mM Na_2HPO_4 pH 7.4; 50°C .

anions DHPY and PY shown in Figure 1. The peak assignments were confirmed by NMR structure analysis, as outlined later, and by using a synthesized PY standard, as described elsewhere.¹⁸ Under the present conditions the comigration of further unknown impurities with

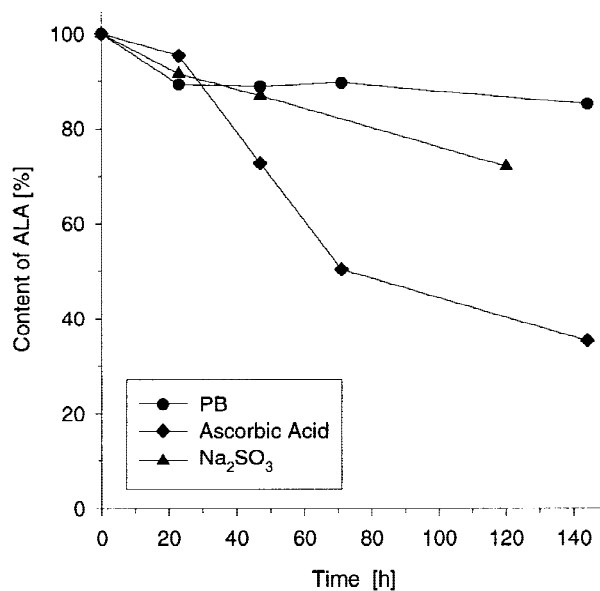


Figure 4. Influences of various additives on the degradation of ALA. Conditions: ALA dissolved in 200 mM NaH_2PO_4 , pH 7.4 (PB) at 25°C ; addition of 0.1% ascorbic acid and 0.1% Na_2SO_3 .

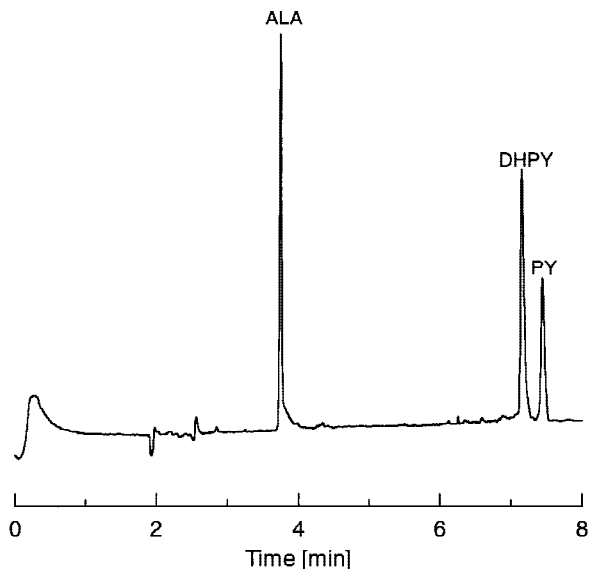


Figure 5. Electropherogram of partially degraded ALA solution. Separation conditions: 50 mM borate buffer, pH 9.4; 30°C ; 20 kV; uncoated capillary, 37/30 cm, $50 \mu\text{m}$; detection at 200 nm.

one of the three detected compounds was excluded with the aid of peak purity analysis by diode array detector.

To find further potential degradation products, the separation conditions were varied. Substances with higher effective mobility in borate buffer, which would be detectable late or in anodic direction, were searched for by addition of cetyl trimethylammonium hydrogen sulfate to reverse the EOF. In this mode, all anionic substances are expected in front of the EOF. When an acidic running buffer of pH 2 was used, the final degradation product PY was detected as a cation, which revealed its amphoteric character. Further degradation products were not found in these experiments. Porphobilinogen, which has been described as an ALA degradation product in the literature,^{3,7} was not found under the present experimental conditions. In the electropherogram, spiked porphobilinogen was selectively separated from the other components of the test solution.

Structure Elucidation

The final degradation product PY (third peak in the electropherogram) was synthesized by the method of Franck and Stratmann.³ The ^1H NMR spectrum confirmed the structure of the expected

pyrazine derivative (Table 1). The spectrum showed a singlet signal of the aromatic protons at 8.4 ppm and signals characterizing the methylene and carboxylic acid protons related to the propionic acid chain. Additionally, the structure of the degradation product PY was unambiguously identified from the ^{13}C data that displayed singlet and doublet aromatic carbon signals. The ^{13}C chemical shifts closely matched the calculated ones.

The intermediate degradation product DHPY was formed from ALA on treatment with NaOH during the process of PY synthesis (*vide supra*). The isolation of DHPY was, however, not achieved, but the DHPY structure could be confirmed by direct NMR spectroscopy of the strong alkaline solution. DHPY was unambiguously identified by the ^1H singlet signal at 3.58 ppm and the ^{13}C singlet at 53.04 ppm, both of which are related to the CH_2 groups at positions 2 and 5 of the dihydropyrazine ring (Table 1). Note that the carboxyl groups of DHPY, which was dissolved in NaOH, were deprotonated, whereas those of the PY, dissolved in DMSO, were not; this result explains the major differences in the shift of the alkyl chain resonance between DHPY and PY.

Kinetics of ALA Degradation

When ALA was stored in vigorously degassed (O_2 elimination) 200 mM phosphate buffer of pH 5.5 and 7.4 (measured pH values) at 50°C , ALA appeared to dimerize following a reversible second-order reaction, as determined from the time

course of ALA concentration decrease (Figure 2). The underlying kinetics can be described by:¹⁹

$$2\text{A} \xrightleftharpoons[k_{-1}]{k_1} \text{B}$$

$$\ln \frac{x([A]_0 - 2x_{eq}) + [A]_0 x_{eq}}{[A]_0(x_{eq} - x)} = \frac{2[A]_0([A]_0 - x_{eq})}{x_{eq}} k_1 t \quad (1)$$

where $[A]_0$ is the initial ALA concentration (0.1 g mL^{-1}), x is the reaction variable (decrease in concentration of ALA with time t), and x_{eq} is the decrease in ALA concentration under equilibrium conditions. Plotting the following expression versus time yielded straight lines:

$$\ln \frac{x([A]_0 - 2x_{eq}) + [A]_0 x_{eq}}{[A]_0(x_{eq} - x)} \quad (2)$$

With 0.1 g mL^{-1} ALA solutions, the ALA equilibrium concentrations determined by the described kinetics were 55% at pH 5.5 and 27% at pH 7.4 (Figure 2). Being typical for second-order reactions, ALA degradation depended on the initial concentration, with higher concentrations leading to faster degradation. At pH 5.5 and initial ALA concentrations of 0.3 and 0.1 g mL^{-1} , 13 and 3%, respectively, of ALA degraded within 45 min. The rather high initial ALA concentrations used in this study correspond to those used to date by our clinical partners for endometrium ablation.

For ALA solutions (1 mg mL^{-1}) that were either saturated with oxygen or purged with nitrogen, different degradation kinetics were observed at 50°C (Figure 3). Although the presence of oxygen did not affect the rate of ALA disappearance within the 3 h studied, it accelerated the conversion of DHPY to PY, resulting in a lower accumulation of DHPY. Considering longer reaction times, ALA and DHPY concentrations should become constant after reaching equilibrium in the absence of oxygen because azomethine formation is an equilibrium reaction. Under the influence of oxygen, however, DHPY is continuously removed, which is likely to result in continuing ALA degradation over longer time periods. Therefore, purging ALA solutions with nitrogen should help to stabilize ALA by slowing down oxidation of DHPY to PY.

Stability of ALA in Solution Containing Various Potentially Stabilizing Agents

Optimization of the solution pH represents an efficient means to control ALA stability. The in-

Table 1. ^1H and ^{13}C NMR Spectra of DHPY and PY

DHPY (aq. NaOH) ^a			PY (DMSO) ^b		
	^1H	^{13}C		^1H	^{13}C
a	3.58	53.04	8.45	143.18	
b	—	175.34	—	152.90	
c ^c	2.10	35.63	2.96	32.12	
d ^c	1.96	35.69	2.67	29.05	
e	—	183.80	12.17	173.59	

^a ^1H shifts were referenced with respect to the water line (4.78 ppm at 295 K) and ^{13}C shifts relative to internal TSP (0 ppm). ^b Referenced to the solvent line (39.51 ppm for ^{13}C and 2.50 ppm for ^1H). ^c Assignments of c and d may have to be interchanged for both compounds and nuclei.

intermediate degradation product DHPY exists in a pH-dependent equilibrium with ALA. For illustration, when ALA was completely converted into DHPY by NaOH, ALA could be almost fully recovered after reacidification of the solution. Because higher pH values promote DHPY formation, lowering of the pH of unbuffered aqueous ALA solution (0.3 g mL^{-1} ; $\text{pH} = 1.7$) was expected to improve ALA stability. Indeed, when 0.3 mL^{-1} of ALA was dissolved in strong acids (HCl , H_2SO_4 , H_3PO_4 , HClO_4 ; all 1 N), no significant degradation was observed at 40°C during 6 weeks, whereas 5% ALA degradation was measured in pure water under the same storage conditions. Most obviously, however, such strongly acidic ALA solutions are not of relevance for clinical use, so that other strategies for stabilization need to be found. Furthermore, the 5% ALA degradation in a simple aqueous ALA solution without pH adjustment would not be acceptable for a commercial dosage form.

One possible approach to prepare stable and clinically useful ALA solutions consists of using nonaqueous, water-miscible solvents for ALA. ALA is sufficiently soluble in glycerol ($\sim 10\%$), DMSO ($\sim 10\%$), and propylene glycol ($\sim 5\%$). However, the immediate yellow appearance of these solutions when ALA is dissolved may be considered to indicate ALA degradation. Thus, this approach was not further pursued.

Additional stabilization approaches consisted of the addition of antioxidants, such as Na_2SO_3 and ascorbic acid (Figure 4). Unfortunately, none of these agents exerted a stabilizing effect. Ascorbic acid even accelerated the degradation, due to its presumably higher redox potential, so that it acted even as oxidizing agent. Finally, a variety of dosage forms, such as oil-in-water and water-in-oil emulsions, microemulsions, and hydrogels were evaluated for their importance for ALA stability. Disappointingly, none of the many dosage forms tested improved the ALA stability over that of purely aqueous acidic solutions.

CONCLUSIONS

The capillary electrophoretic method developed here provides a very efficient means to separate and quantify ALA and its main degradation products DHPY and PY in aqueous solutions. In contrast to previous studies, both DHPY and PY were isolated and their structure analyzed individually by NMR. Because the major objective of this

study was to develop a stable dosage form for clinical use, the determined degradation kinetics in aqueous solutions of pH 5.5 and 7.4 should be of high practical relevance. From the kinetic analysis, a reversible second-order reaction was determined, as was expected for the reversible dimerization reaction of ALA to the DHPY. The analysis revealed that aqueous ALA solutions of both pH 5.5 and 7.4 are not sufficiently stable for storage as a dosage form. Experiments at lower pH values suggested that pH values < 2 would be required to preserve the ALA stability for long-term storage of an aqueous solution. Other dosage forms for ALA studied included nonaqueous solutions, oil-in-water and water-in-oil emulsions, microemulsions, and hydrogels, as well as the addition of antioxidants to aqueous solutions. None of these approaches improved the ALA stability. In view of a future dosage form for ALA, we propose a two-component system with lyophilized ALA kept in one vial and the aqueous neutralizing liquid in a second vial. The final solution of pH 5.5–7.4 would have to be prepared a maximum of 1 h before use.

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