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Reference value for the ALAD enzyme activity ratio in men based on the improvement of each analytical step of the colorimetric method and the active/non-active lead concept

Aim: The ALAD enzyme activity ratio, which is highly sensitive to and specific for lead inhibition, can be used to diagnose lead-exposed individuals, but a reference value for it has not yet been established. Therefore, we aimed to establish a reference value for the ALAD activity ratio by improving each analytical step of the colorimetric method described by Mitchell et al.

Materials and methods: Activators, pH, hemolyzing agents, and Ehrlich's reagent were evaluated to obtain the optimal conditions for the restoration of lead-inhibited ALAD enzyme activity.

Results: $ZnCl_2$ and DTT were the preferred activators, the pH of sodium phosphate buffer was adjusted to 6.2, and distilled water was used as a hemolyzing agent. CV for the imprecision of non-activated ALAD, activated ALAD, and the activity ratio was decreased from 5.1%, 3.7%, and 7.1% to 2.6%, 1.9%, and 1.9%, respectively. Linearity improved from 20-fold dilution to 80-fold. Higher activated absorbances were observed at moderately high lead concentrations (12.5-50 μg dL^{-1}).

Conclusion: The reference value for the ALAD activity ratio based on 137 healthy men was 0.32-0.64. We think that the activity ratio could be a useful marker for screening lead-exposed individuals. It is also suggested that lead could behave as both an active and non-active metal.

Key words: ALAD, reference value, activity ratio, active lead

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Kolorimetrik metodun her bir analitik basamağının iyileştirilmesinden sonra belirlenen ALAD enzim aktivitesi oranının erkeklerdeki referans aralığı ve aktif-pasif kurşun kavramı

Amaç: Kurşun inhibisyonuna oldukça duyarlı ve özgül olan ALAD enzim aktivitesi oranı kurşuna maruz kalmış bireylerin teşhisinde kullanılabilir, fakat bunun referans aralığı henüz oluşturulmamıştır. Bu nedenle Mitchell ve arkadaşları tarafından geliştirilen kolorimetrik metodun her bir analitik basamağını iyileştirerek ALAD enzim aktivitesi oranının referans aralığını belirlemeyi amaçladık.

Yöntem ve gereçler: Kurşun ile inhibe olan ALAD enzim aktivitesinin tekrar aktif olması için gereken en iyi koşulları belirlemek için aktivatörler, pH, hemolize edici ajanlar ve Ehrlich's reaktifi değerlendirildi.

Bulgular: Aktivatör olarak $ZnCl_2$ ve DTT tercih edildi, sodyum fosfat tamponun pH'sı 6,2'ye ayarlandı ve hemolize edici ajan olarak distile su kullanıldı. Aktif olmayan ALAD, aktif olan ALAD ve aktivite oranının tekrarlanabilirliğinin değişim katsayıları sırasıyla % 5,1'den % 2,6'ya, % 3,7'den % 1,9'a ve % 7.1'den % 1,9'a düştü. Lineerite 20 kat dilüsyondan 80 kata yükseldi. Hafif yüksek kurşun konsantrasyonlarında, 12.5-50 μg dL^{-1}, daha yüksek aktive olmuş absorbanslar saptandı.

Sonuç: 137 sağlıklı erkek bireyden elde edilen ALAD aktivite oranının referans aralığı 0,32-0,64'dü. Aktivite oranının kurşuna maruz kalmış bireylerin taranmasında faydalı olabileceğini değerlendirdik. Ayrıca kurşunun hem aktif hem de inaktif bir metal gibi davranabileceği önerilebilir.

Anahtar sözcükler: ALAD, referans değer, aktivite oranı, aktif kurşun

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Introduction

δ-Aminolevulinic acid dehydratase (ALAD; EC 4.2.1.24) is a cytosolic enzyme in the heme biosynthetic pathway that catalyzes the condensation of 2 molecules of 5-aminolevulinic acid to form 1 molecule of the monopyrrole porphobilinogen. It is one of the most useful markers for evaluating lead exposure, because its activity is extremely sensitive to and specific for blood lead concentration (1-4). Atomic absorption spectroscopy, the gold standard test for blood lead analysis, requires technical experience for routine use and special instrumentation, and is not cost-effective. Therefore, a practical method for detecting both acute and chronic lead poisoning is necessary for use as a metabolic marker in the laboratory assessment of lead status. For this purpose the ALAD enzyme activity determination method should be improved (5-7) and a reference value for the ALAD activity ratio should be established (8).

Mitchell et al. (9) proposed a reliable and quantitative indicator of lead exposure: the ratio of activated to non-activated ALAD activity. We preferred to use this simple ratio as the non-activated/activated ALAD activity ratio. The activity ratio described here is more practical, because results do not require normalization by use of hemoglobin or hematocrit concentrations, units of enzyme activity do not need to be calculated, and activation of ALAD provides an internal positive control for the test (9).

The present study was undertaken to determine the optimal conditions for the rapid and accurate measurement of ALAD enzyme activity by focusing on the sources of error in the method. After completing our modification, the method was first tested on blood samples supplemented with different concentrations of lead standard, and then the active/non-active lead concept was considered. The reference value for the ALAD activity ratio was established for men. Its establishment may increase the probability of early identification of lead-exposed individuals due to the advantages of the ratio and its widespread adoption as a quick screening test.

Materials and methods

Chemicals

Na_2HPO_4 $12H_2O$ (Merck, 6345), NaH_2PO_4 $2H_2O$ (Merck, K 4972473), trichloroacetic acid (TCA, Merck, Art810, 7423226), mercuric chloride ($HgCl_2$, Merck, 4417), hydrochloric acid (HCl, Riedel de Haen, 30721), reduced glutathione (GSH, Sigma, G 4251), dithiothreitol (DTT, Sigma, D 5545), zinc chloride ($ZnCl_2$, Sigma, Z 4875), *p*-dimethylamino benzaldehyde (Sigma, D 8904), Triton X-100 (Fluka, 93420), aminolevulinic acid hydrochloride (ALA HCI, Sigma, A 3785), and glacial acetic acid (GAA, Riedel de Haen, 27225) were used for the preparation of reagents.

Activators

Separately, 0.1 mol L^{-1} of $ZnCl_2$ and DTT, and 0.5 mol L^{-1} of GSH were prepared in distilled water.

Buffer systems

A 1-mol L^{-1} solution of both dibasic sodium phosphate (Na_2HPO_4 $12H_2O$) and monobasic sodium phosphate (NaH_2PO_4 $2H_2O$) buffer was prepared and mixed to obtain a sodium phosphate buffer of pH 6.2, and 50 mmol L^{-1} of ALA HCl was prepared in pH 6.2 sodium phosphate buffer.

Regular Ehrlich's reagent

Twenty grams of *p*-dimethylamino-benzaldehyde was dissolved per liter of 6 N HCl.

Modified Ehrlich's reagent

In about 600 mL of GAA was dissolved 20 g of *p*-dimethylamino-benzaldehyde, and then 100 mL of distilled water and 100 mL of 9 mol L^{-1} perchloric acid were added. This mixture was diluted to 1000 mL with GAA.

TCA containing mercuric chloride reagent

In distilled water, 100 g L^{-1} of TCA containing 100 mmol L^{-1} of $HgCl_2$ was prepared.

The activators, ALA phosphate buffer, and Ehrlich's reagents were prepared daily. pH of the buffers was monitored and recorded for each experiment.

Blood sampling

To minimize personal effects, lithium heparinized whole blood specimens were collected from the researchers during method improvement. Specimens taken from healthy non-medicated male volunteers that had no occupational or other unusual contact with lead and presented to the laboratory for periodical check-up were used in the reference range

study. Blood samples were obtained by venipuncture into lithium-heparinized vacutainer tubes, then placed in ice water and assayed within 5 h of collection. Informed consent was obtained from each volunteer.

Measurement of modified ALAD enzyme activity

ALAD enzyme activity was measured using a modification of the previously described method (9) for whole blood. This method is based on the ratio of non-activated and activated forms of ALAD enzyme activity according to the quantity of porphobilinogen measured with a Philips AU 530 spectrophotometer.

First, 50 µL of lithium-heparinized whole blood was hemolyzed in 950 µL of distilled water, and then reagents were added to the test tubes in ice water, in the order shown in the Table. Samples were incubated for 60 min at 37 °C (with and without activators) in sodium phosphate buffer. The reaction was stopped and deproteinized by adding 1000 µL of TCA containing $HgCl_2$ in ice water, and then it was agitated and left for 3 min. The supernate was obtained by centrifugation at 1200 × g for 3 min and was then mixed with an equal volume of regular Ehrlich's reagent. Absorbance was determined at 555 nm against a reagent blank (1000 µL of distilled water, and 1000 µL of regular Ehrlich's reagent). The activity ratio was calculated by dividing the absorbance of non-activated samples by that of activated samples.

Table. Order of the reagents added to the test tubes.

Reagents	Activated µL	Non-activated µL
Hemolysate	500	500
$ZnCl_2$, 0.1 M	100	-
DTT, 0.1 M	100	-
Distilled water	-	200
Sodium phosphate buffer	200	200
ALA-phosphate buffer 0.05 M	100	100

$ZnCl_2$: zinc chloride; DTT: dithiothreitol

Analytical accuracy of the previous method

Firstly, the ALAD enzyme activity measurement method described by Mitchell et al. was evaluated in order to reveal its analytical accuracy and locate the sources of error in the method. The Mitchell et al. test procedure was as follows: 100 µL of heparinized whole blood, 600 µL of Triton X-100, 100 µL of GSH, 100 µL of $ZnCl_2$, and 100 µL of KPO_4 (pH 6.7) buffer containing aminolevulinic acid were added one at a time and proceeded as above for activated samples. An additional 200 µL of Triton X-100 was used instead of 100 µL of GSH and 100 µL of $ZnCl_2$ for non-activated samples. We monitored the absorbance spectrum of the final reaction that occurred between porphobilinogen and modified Ehrlich's reagent for 60 min. The imprecision of the method was evaluated via 20 replicates of the same specimen, and its linearity was examined with 10-, 20-, 40-, and 80-fold dilutions.

Reaction between Ehrlich's reagent and porphobilinogen

To improve the analytical accuracy of the method we began with the last step and compared the use of regular Ehrlich's reagent and modified Ehrlich's reagent by evaluating the absorbance of activated and non-activated ALAD enzyme, in addition to the ratio. All of the volumes were prepared as duplicates and divided into 2 tubes, just before adding Ehrlich's reagent. One was studied with regular and the other with modified Ehrlich's reagent.

Stopping the reaction of ALA to porphobilinogen conversion

All of the volumes were duplicated again and volumes were divided into 2 tubes, just before adding TCA in ice water.

Hemolysis

Distilled water and Triton X-100 were compared with each other as hemolyzing agents.

Activators

One by one, 100 µL of each activator (GSH, DTT, and $ZnCl_2$) was added to the test tubes to compare their individual effects.

pH

Finally, the effects of pH changes in restoring the activity of the lead-inhibited ALAD enzyme were evaluated. During the last step (just before incubation) 100 µL of sodium phosphate buffer and 100 µL of distilled water were used instead of 200 µL

of sodium phosphate buffer. In this way the final pH was increased from 6.41 to 6.53.

Inhibition studies

Serial dilutions were made with 4000 μg dL^{-1} of lead standard. Final blood lead concentrations were 2000, 500, and 125 μg dL^{-1} before the hemolysis of 50 μL of heparinized whole blood with 950 μL of distilled water. The effects of adding lead before and after hemolysis were evaluated. The samples were prepared as above to obtain final concentrations of 100, 25, and 6.25 μg dL^{-1} for hemolyzed samples, which were incubated at 37 °C for 90 min, both before and after hemolysis. We compared each absorbance and ratio with those of the non-inhibited results of the same sample.

After identifying the optimal conditions for ALAD inhibition by the lead standards, the linearity of inhibition was evaluated by adding serial dilutions of the lead standards after hemolysis. All samples were incubated at 37 °C for 150 min. The final concentrations of lead were 100, 50, 25, 12.5, and 6.25 μg dL^{-1}. In addition, linearity and inhibition were evaluated in samples that were not pre-incubated.

Analytical accuracy of the modified method

We evaluated the analytical performance of the modified method by examining its imprecision and linearity. Eighteen replicates, and 40-, 80-, and 160-fold dilutions of the same specimen were measured.

Statistical procedures

All statistical analyses were performed using SPSS v.15.0 for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to verify the hypothesis of normal distribution. Comparisons between 2 groups were made using the Mann-Whitney U test to establish significance. P values < 0.05 were considered significant.

Results

The absorbance of both activated and non-activated ALAD enzyme activity was stable only between 10 and 25 min. Although absorbance increased during the first 10 min and decreased 25 min later, the ratio was stable between 10 and 50 min (Figure 1a). The coefficients of variation (CV) for the 20 replicates of non-activated samples, activated samples, and the ratio were 5.1%, 3.7%, and 7.1%, respectively (Figure 1b). The ratio was linear up to 20-fold dilution (Figure 1c).

Regular Ehrlich's reagent was preferred because the differences between ratios were not significant (P = 0.412) and the preparation of the reagent selected was more practical. TCA and ice water were considered to be enough to stop the reaction, because there was no significant difference between the results (P = 0.716).

Distilled water was examined as an alternative to Triton X-100 as the hemolyzing agent. Although the ratios for Triton X-100 and distilled water were similar, the absorbances of both activated and non-activated samples were higher in specimens hemolyzed with Triton X-100. CVs of activated and non-activated samples for Triton X-100 and distilled water were 10.2% and 9.6%, and 4.6% and 4.2%, respectively. The optimal hemolysis obtained with 20-fold dilution with distilled water was also proven by microscopic analysis.

Dual combinations of activators (DTT, $ZnCl_2$, and GSH) showed additive or complementary restoration of activity (Figure 2). Using these combinations of activators we can expect a more accurate evaluation of lead exposure than that obtained by using only one of them. Based on these results we decided to use DTT as an activator instead of GSH. $ZnCl_2$ and DTT were used for the maximum restoration of ALAD activity. The effect of 10-min incubation at 37 °C also was examined after adding $ZnCl_2$. The absorbance of activated samples with and without additional incubation was not significant (P = 0.614); therefore, we did not use an additional incubation step for activation with zinc.

The pH of each analytical step was as follows; hemolysate, 7.43; $ZnCl_2$, 7.40; DTT, 7.30; sodium phosphate buffer, 6.65; $ALAPO_4$ buffer, 6.41 (reaction pH). Although the absorbance of non-activated and activated samples was similar, activated samples had greater absorbance at pH 6.41; as a result, the ratio was slightly lower in these samples (Figure 3). pH 6.41 was preferred instead of pH 6.53 due to the higher absorbance of activated samples.

Although the samples were 10-fold more concentrated with lead, the absorbance of activated and non-activated ALAD, and the activity ratio were similar for the samples incubated at 37 °C for 90 min prior to hemolysis (Figure 4a). For samples incubated

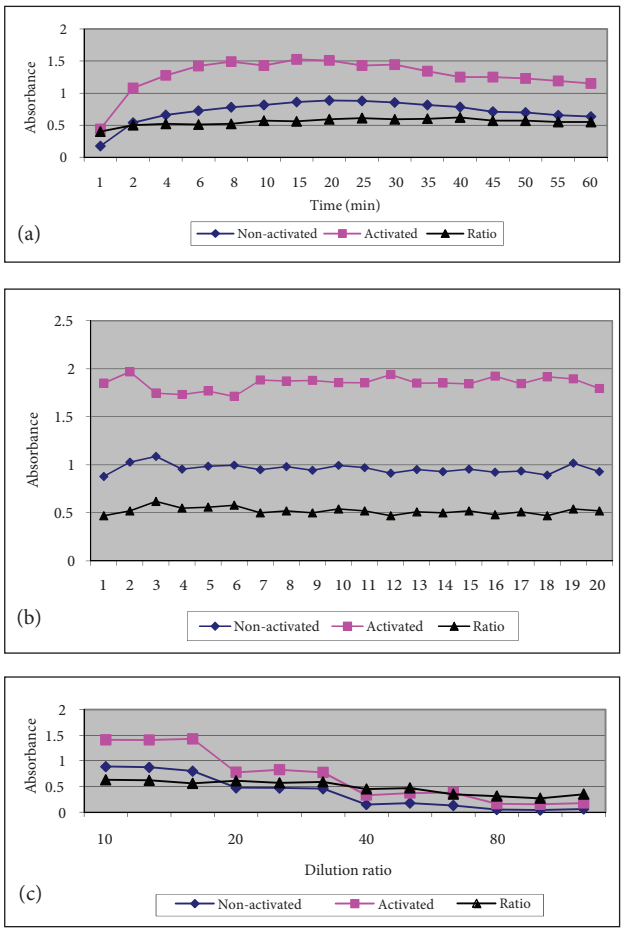

Figure 1. Analytical accuracy of the method before improvement.
(a) Stability of the color after modified Ehrlich's reagent was added. (b) Precision. (c) Linearity. All of the results were obtained from the same specimen.

after hemolysis, although the absorbance of the activated samples was similar, absorbance of the non-activated samples increased as the lead concentration decreased. It was also observed that moderately high lead concentration (12.5-50 μg dL^{-1}) could result in higher activated absorbance (Figure 4b).

The CVs of the 18 replicates, according to the modified method for non-activated samples, activated samples, and the activity ratio, were 2.6%, 1.8%, and 1.9%, respectively (Figure 5a). The ratio was linear up to 80-fold dilution (Figure 5b).

The reference value for the activity ratio obtained from 137 healthy men aged 19-36 years was 0.32-0.64. Minimum and maximum activity ratios were 0.38 and 0.78, respectively.

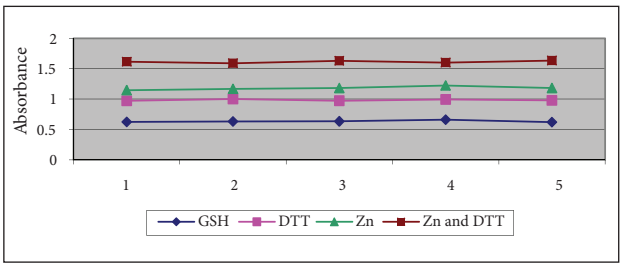

Figure 2. The effects of zinc, GSH, and DTT as an activator.

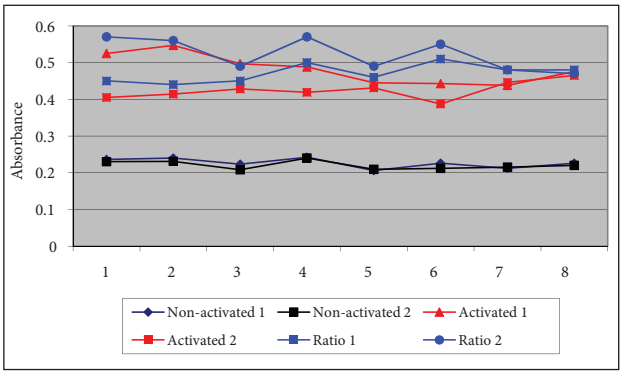

Figure 3. The effect of pH.
The numbers 1 and 2 indicate the results with pH 6.41 and pH 6.53, respectively.

Discussion

The present study demonstrates the improvement of the analytical accuracy of the previous method following some modifications. Mitchell et al. obtained CVs for imprecision of the ratio in lead-exposed and unexposed individuals (11.4% and 7.9%, respectively) (9). The result obtained with the present method was compatible with the data for unexposed individuals. After improvement of the method's analytical steps, CV of the absorbance of non-activated and activated samples, as well as the ratio decreased at least 2-fold (Figures 1b, 5a). The reasons for this analytical improvement may be explained by our selection of activators and pH.

The differences between the effects of the 3 activators and their combined effects on activation were evaluated. GSH and $ZnCl_2$ were used as an activator in the previous method, but the absorbance of the activated ALAD obtained with GSH was 2-fold lower than that of any other activators (Figure 2). As such, DTT was used instead of GSH as an activator to obtain maximum restoration. In light of the previous

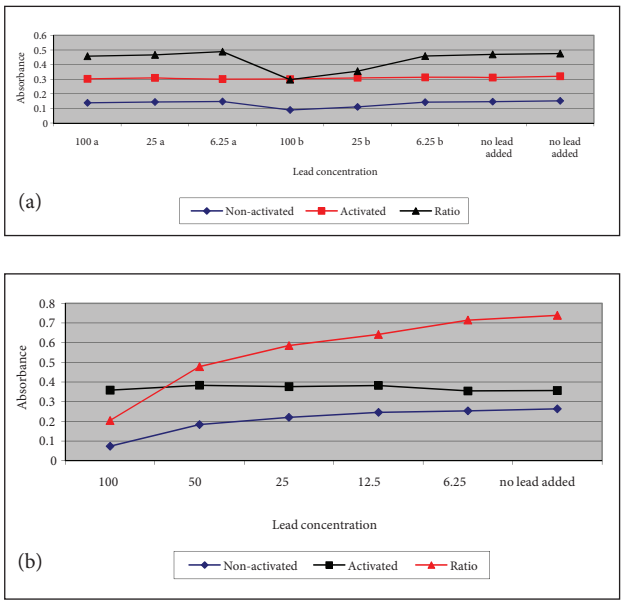

Figure 4. Lead-mediated ALAD inhibition. (a) Optimum conditions for lead-mediated ALAD inhibition. a: Incubated for 90 min at 37 °C before hemolysis; b: incubated for 90 min at 37 °C after hemolysis. (b) Linearity of ALAD inhibition. Incubated at 37 °C for 150 min after hemolysis, but ALA incubation was 60 min at 37 °C.

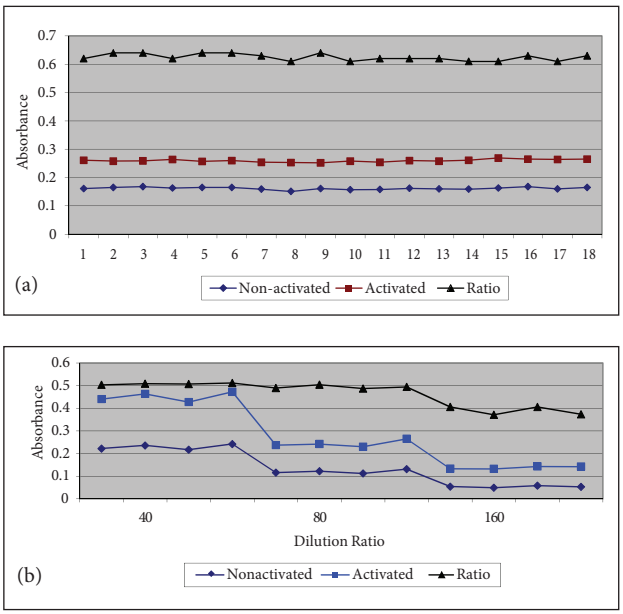

Figure 5. Analytical accuracy of the modified method. (a) Precision. (b) Linearity.

study (10), we considered that each agent alone was not sufficient to fully restore ALAD activity, because the combined treatment of 2 agents resulted in more effective restoration (Figure 2). $ZnCl_2$ was considered an essential activator (11,12).

Sodium phosphate buffer with a final pH of 6.41 consistently yielded activated ALAD values that were 15%-20% higher than those obtained with the same buffer with a final pH of 6.53. In erythrocytes of highly exposed subjects the concentration of lead in blood exceeded 80 µg 100 g^{-1} and the optimal pH for activity of the enzyme shifted from 6.8 to 6.0. These pH values were the original pH of the buffer solution used (13); therefore, we prepared sodium phosphate buffer of pH 6.2 to diagnose lead-exposed individuals accurately and to obtain precise results for healthy individuals.

The accuracy of the present method was also evaluated based on the lead-dependent inhibition of ALAD activity. In general, the absorbance of activated ALAD was similar, despite a gradual increase in the lead concentration of samples, but samples with lead concentrations between 12.5 and 50 µg dL^{-1} had slightly more activated absorbance. This could be explained by active and non-active lead terminology. If active lead binds to the ALAD irreversibly and non-active lead binds reversibly, activated ALAD absorbance may not decrease up to the high lead concentrations because of the prevention of ALAD inhibition by the connection of ALAD with non-active lead.

This is the first study to establish a reference value for the ALAD activity ratio in healthy men. The value obtained using the modified colorimetric method will provide an opportunity to diagnose lead-exposed individuals more accurately and practically. Further studies should be designed to confirm the active/non-active lead concept.

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