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An enzymatic cycling method for the determination of serum total bile acids with recombinant 3α-hydroxysteroid dehydrogenase☆

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Abstract

A highly sensitive enzymatic cycling method was developed for the serum total bile acids assay. We constructed a prokaryotic expression system to prepare the recombinant 3α-hydroxysteroid dehydrogenase in place of the natural enzyme and for the first time used it in the total bile acids assay. The production rate of thio-NADH correlated with the bile acids concentration and was measured by the change of absorbance at 405/660 nm. The enzymatic cycling method could detect 0.22 μmol/L total bile acids in serum. Within-run and between-run imprecisions were 1.2–3.7% and 2.3–4.8%, respectively. The calibration curve for total bile acids in serum was linear between 0.5 and 180 μmol/L. This method was free from interference by bilirubin, hemoglobin, ascorbate, and lactate dehydrogenase. In conclusion, serum total bile acids could be measured by the enzymatic cycling method with recombinant 3α-hydroxysteroid dehydrogenase as the tool enzyme.

Keywords: Bile acids; Enzymatic cycling method; 3α-Hydroxysteroid dehydrogenase

Bile acids are a group of metabolic products from cholesterol. They have a common structure of α-hydroxyl group at carbon 3. It is well known that the determination of total bile acids (TBA) in serum is a sensitive test for the evaluation of liver function. Several techniques can be used for the determination of TBA, such as gas chromatography, high-performance liquid chromatography, radioimmunoassay, and enzymatic methods. Of the previous enzymatic methods, 3α-hydroxysteroid dehydrogenase (3α-HSD, EC1.1.1.50) is used to reversibly catalyze the oxidoreduction of bile acids at carbon 3 and the product of NADH generated from concomitant reduction of NAD^+ is measured by ultraviolet spectrophotometry or fluorometry. However, these methods are unsuitable for routine use in clinical laboratory due to the requirement of extraction procedures from a large volume of serum, expensive equipments or radioisotopes.

3α-HSD, the tool enzyme used for the determination of serum TBA in enzymatic methods, can be obtained from *Comamonas testosteroni* when it is cultured in a steroid-containing medium [1]. In 1970s, enzymatic method for TBA assay was first generated using the highly purified 3α-HSD from *Pseudomonas*/*C. testosteroni* [2]. In this method, the change of absorbance at 340 nm from the increase of NADH was measured by ultraviolet spectrophotometry. The second generation of the enzymatic assay was an enzymatic end-point spectrophotometric method in which NADH-diaphorase-nitrotetrazolium blue was used as an indicator and the

☆ *Abbreviations:* TBA, total bile acids; 3α-HSD, 3α-hydroxysteroid dehydrogenase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; R-1, the first reagent; R-2, the second reagent.

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yielded diformazan was measured spectrophotometrically at 540 nm [3]. This method was yet not sensitive enough and the yielded diformazan stained the colorimetric cups. The third generation of the enzymatic TBA assay additionally coupled 3-oxo-5-β-Δ4-dehydrogenase [4], which improved the sensitivity but the disadvantage of staining cups by the produced diformazan remained.

In order to simplify the procedures and increase the sensitivity of the TBA assay further, we developed a new enzymatic cycling method which was performed in the presence of NADH, thio-NAD^+, and 3α-HSD, and measured the increase of absorbance at 405/660 nm from thio-NADH produced in the reaction at 37 °C. Other assays using the enzymatic cycling method have been developed for the determination of various substances [5–7].

Previously, 3α-HSD was directly purified from *C. testosteroni* [8], but the purification procedure was complicated and the recovery efficiency was low. In this study, we cloned the 3α-HSD gene from *C. testosteroni* isolated from pond mud and expressed the cloned gene in *Escherichia coli.* The recombinant 3α-HSD could efficiently catalyze the reversible oxidoreduction reaction of 3α-hydroxysteroids including bile acids, thereby could be used in the enzymatic cycling method for the TBA assay. Here we for the first time reported the simple, specific, and sensitive method by using the recombinant 3α-HSD as the tool enzyme for the measurement of TBA in serum.

Materials and methods

Materials

Plasmid pET-15b was from Novagen (Germany). *Taq* DNA polymerase, restriction enzymes, and *E. coli BL21(DE3)pLysS* were purchased from Promega. Ampicillin, chloramphenicol, deoxynucleoside triphosphate, isopropyl-1-thio-β-D-galactopyranoside (IPTG), and protein molecular weight markers were obtained from SABC (Beijing, China). Steroids, bilirubin, thio-NAD^+, and NADH were from Sigma (St. Louis, MO). Probond Resin was from Invitrogen (Carlsbad, CA). Hemoglobin prepared from hemolyzed human erythrocytes was used for the interference test. All other chemicals were of the highest quality commercially available.

Bacteria strains and growth condition

3α-HSD-producing *C. testosteroni* was isolated from pond mud in Beijing. It was grown at 30 °C in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). *E. coli* DH5 α and *BL21(DE3)pLysS* were grown in LB medium at 37 °C. Chloramphenicol was added to the medium to 34 μg/mL for growing *BL21(DE3)pLy sS.*

Overexpression and purification of recombinant 3α-HSD

The 3α-HSD gene was amplified by PCR from the genomic DNA of *C. testosteroni* and was inserted into the *Nde*I/*Bam*HI restriction sites of pET15b vector. The expressed 3α-HSD protein had a tag of $(His)_6$ sequence followed by a thrombin-cleavage site at its N-terminal. The host bacteria *E. coli BL21(DE3)pLysS* containing the recombinant pET15b vector were grown to an optical density of 0.6 at 660 nm in LB medium containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol, and the overexpression of 3α-HSD was induced by the addition of IPGT to a final concentration of 1 mmol/L. By using the Pharmacia AKTA Protein Purifier system, the recombinant enzyme was purified in a Ni^{2+}–Sepharose column and eluted by applying a linear gradient of imidazole (0–0.5 mol/L) in 20 mmol/L phosphate buffer, pH 7.5, and 0.5 mol/L NaCl. Fractions containing purified 3α-HSD were pooled, desalted, and dissolved in 0.1 mol/L Tris–Cl buffer, pH 9.0.

Enzyme activity

The enzyme activity of the recombinant 3α-HSD was determined in an enzyme reaction by the change of absorbance of NADH at 340 nm [9]. One unit of enzyme activity was defined as the amount of the enzyme that oxidized 1 μmol/L of androsterone per minute under the assay conditions.

Protein determination

Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

Reagents for the TBA assay and application to automatic analyzer

Coupled reactions in the TBA assay using enzymatic cycling method. The cyclic reactions in this TBA assay are shown in Fig. 1. Bile acids reversibly change to its oxidized form catalyzed by 3α-HSD, and the oxidoreduction of bile acids occurs repeatedly in the presence of thio-NAD^+ and NADH. TBA concentration can be derived from the amount of thio-NADH (reduced form of thio-NAD^+) which is measured from the change of absorbance per minute at 405/660 nm.

Reagents. The first reagent (R-1) contained 1.5 mmol/L thio-NAD^+ in 0.1 mol/L glycine (pH 4.0), and the second reagent (R-2) contained 1.5×10^4 U/L 3α-HSD and 2 g/L NADH in 0.2 mol/L diethanolamine buffer (pH 9.0).

The assay was performed in a 7170 automatic analyzer (Hitachi). The assay condition was optimized for a rate assay by measuring the change of absorbance at 405/660 nm for 5 min at 37 °C from a mixture containing sample 4.0 μL, R-1 300 μL, and R-2 100 μL.

Procedures of the TBA assay. Sample 4.0 μL and R-1 300 μL were mixed and incubated at 37 °C for 5 min, and R-2 100 μL was then added to initiate the reaction. The absorbance at 405 nm (primary wavelength) and 660 nm (secondary wavelength) was measured at regular intervals for up to 5 min. The absorbance at 660 nm was subtracted from the absorbance at 405 nm. The increase in the rate of absorbance between sixth minute and eighth minute was calculated by linear regression analysis.

Optimal conditions for the enzymatic cycling method

Optimal studies were performed for each of the components in the TBA assay using Hitachi 7170 automatic analyzer.

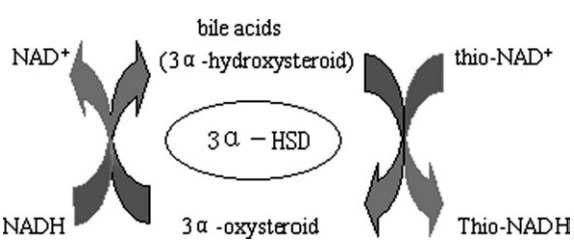

Fig. 1. Coupled oxidoreductions in the TBA assay using enzymatic cycling method.

Optimal pH. Optimal pH for the reaction system was evaluated by mixing R-1 (pH 4.0) and R-2 (pH 7.0–10.0) with the ratio of 3:1. Standard solution (50 μmol/L glycochenodeoxycholic acid) 4.0 μL mixed with R-1 300 μL was incubated at 37 °C for 5 min. The enzymatic cycling reaction was initiated by the addition of 100 μL of R-2, pH 7.0–10.0, and the increase of absorbance at 405/660 nm per minute was measured.

Optimal concentration of thio-NAD$^+$. Standard solution 4.0 μL mixed with R-1 300 μL containing 0.1–1.5 mmol/L thio-NAD$^+$ was incubated at 37 °C for 5 minutes. The enzymatic cycling reaction was started by the addition of 100 μL R-2, and the increase of absorbance at 405/660 nm per minute was measured.

Optimal concentration of NADH. Standard solution 4.0 μL mixed with R-1 300 μL was incubated at 37 °C for 5 min. The enzymatic cycling reaction was started by the addition of 100 μL R-2 containing 0.1–6.0 g/L NADH. The increase of absorbance at 405/660 nm per minute was measured.

Optimal concentration of 3α-HSD. Standard solution 5 μL mixed with R-1 300 μL was incubated at 37 °C for 5 min. The enzymatic cycling reaction was initiated by the addition of 100 μL R-2 containing 1000 U/L -3.0×10^4 U/L 3α-HSD. The increase of absorbance at 405/660 nm per minute was measured.

Assay evaluation

According to the NCCLS guidelines, linearity, analytical recovery, precision, and interference of the TBA assay were evaluated in Hitachi 7170 analyzer.

Linearity. A sample containing higher concentration of TBA was sequentially diluted with saline, and TBA in these samples was measured.

Recovery. The recovery studies were performed by mixing one volume of various bile acids solutions (50 μmol/L) with nine volumes of pooled serum sample containing 20.5 μmol/L TBA. Serum samples with saline instead of bile acids were measured as controls. Triplicate measurements of TBA in these samples were performed.

Within-run and between-run coefficients of variations. Three pooled serum samples containing 6.4, 10.2, and 50.7 μmol/L TBA (low, middle, and high serum TBA concentrations, respectively) were assayed repeatedly. In the within-run experiment, each sample was assayed for 20 times. In the between-run experiment, each sample was assayed daily for 20 consecutive days.

Interference by other substances. One volume of various amounts of bilirubin (0–850 μmol/L), hemoglobin (0–5 g/L), chyle (triglyceride 0–11.3 mmol/L), ascorbate (0-500 mg/L), and lactate dehydrogenase (0–20,000 U/L) was added to nine volumes of serum samples, and it was analyzed. Sera mixed with different anticoagulants including heparin, citric acid, and EDTA-K_2 were also used to evaluate the effect of anticoagulants on the TBA assay. Serum samples with saline instead of interfering substances and anticoagulants were measured as controls.

Results

Overexpression and purification of the recombinant 3α-HSD

The recombinant 3α-HSD could be purified in one step by using metal chelate chromatography, and the purity of the purified protein was greater than 99% as judged by 10% SDS–PAGE and Coomassie blue staining (Fig. 2). The coding sequence of 3α-HSD gene is 774 bp long, which code a protein of 258 amino acid residues with the predicted molecular mass of 26.4 kDa. On SDS–PAGE gel, the size of the recombinant 3α-HSD was about 29 kDa. After cleavage by thrombin, the size of the recombinant protein measured by mass spectrum was 26.4 kD. The total bacterial protein was 0.73 g/L of which 16.4% were the recombinant enzymes. Relative activity of the purified protein was 194.7 U/mg protein, we therefore used the purified protein without cleavage by thrombin for the enzymatic cycling reaction of TBA assay.

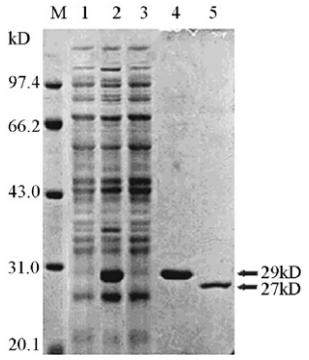

Fig. 2. Overexpression and purification of the recombinant 3α-HSD. M, Protein molecular weight marker. (1) Supernatant of bacterial lysate without IPTG induction; (2) supernatant of bacterial lysate after with IPTG induction for 5 h; (3) flow-through bacterial lysate from the column; (4) recombinant 3α-HSD eluted from nickel–Sepharose column; and (5) recombinant 3α-HSD after cleavage by thrombin.

Characterization of recombinant 3α-HSD

With androsterone as the substrate of enzymatic reaction and the incubation temperature of 37 °C, the optimal pH for the enzyme was 10.5 and 9.4 when the cofactors were NAD$^+$ and thio-NAD$^+$, respectively. The enzymatic reaction became faster at 37 °C and the $Q_{10\ (25–35\ °C)}$ was 1.7. The recombinant 3α-HSD could efficiently catalyze the 3α-hydroxyl dehydrogenation for androsterone and bile acids with the Km values between 4.2 and 51.1 mol/L. Metal ions were not required for the activity of the enzyme, EDTA had no effect on the activity, and Fe^{3+}, Fe^{2+}, and Zn^{2+} inhibited the enzyme activity. The recombinant 3α-HSD was stable at pH 9.0 and lower temperature. The enzyme remained active for 2 months at 4 °C and for at least 1 year at −20 °C without stabilizers. After incubation at 37 °C for 2 h, the enzyme still had 80% of its activity. The enzyme lost about 50% and 100% of its activity when it was stored for 30 min at 40 and 70 °C, respectively.

Time course of the enzyme reaction

Time course of the enzyme reactions in assays of a standard solution and two human serum samples was observed. As shown in Fig. 3, the reactions in all of these samples were linearized after the addition of R-2.

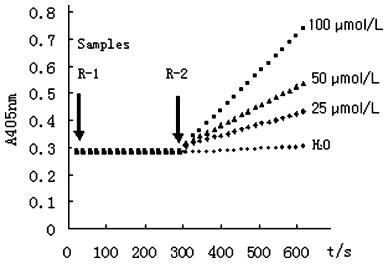

Fig. 3. Time course of the TBA assay.

Optimization of TBA assay

A standard solution (50 μmol/L glycochenodeoxycholic acid) was used for the assessment of optimal pH for the TBA assay. The highest absorbance was found when R-2 pH 9.0 was used (Fig. 4A).

The standard solution was used for the assessment of optimal concentrations of thio-NAD^+ and NADH in the TBA assay. To achieve adequate sensitivity and absorbance range required for serum TBA measurement, we chose the concentration of 1.5 mmol/L for thio-NAD^+ and 2.0 g/L for NADH in the reaction mixture (Figs. 4B and C).

The standard solution was also used for the assessment of optimal concentration of 3α-HSD for the TBA assay. The sensitivity was increased in proportion to the concentration of 3α-HSD (Fig. 4D). To achieve adequate sensitivity and absorbance range, we used the concentration of 1.5×10^4 U/L for 3α-HSD in the reaction mixture.

Assay evaluation

Lower range of detection

The minimum detection limit of the TBA assay was defined as mean + 3SD for the blank. Blank sample (0.9% NaCl) was measured 10 times, mean of the results was 0.1 μmol/L and the SD was 0.04 μmol/L. The minimum detection limit of the TBA assay was therefore estimated to be 0.22 μmol/L.

Linearity

Results of TBA concentration and the change of absorbance at 405 nm were linear when TBA content in samples ranged between 0.5 and 180 μmol/L (Fig. 5). The linear regression of the enzymatic cycling method (y) and TBA concentrations (x) yielded: $y = 9.9088x + 5.4222$, and the correlation coefficient was 0.9998.

Analytical recovery

Analytical recoveries of various bile acids were in the range of 87.5–117.1% (Table 1).

Coefficient of variations

The coefficient of variations (CVs) for within-run test were 1.2–3.7%, and those for between-run test were 2.3–4.8%.

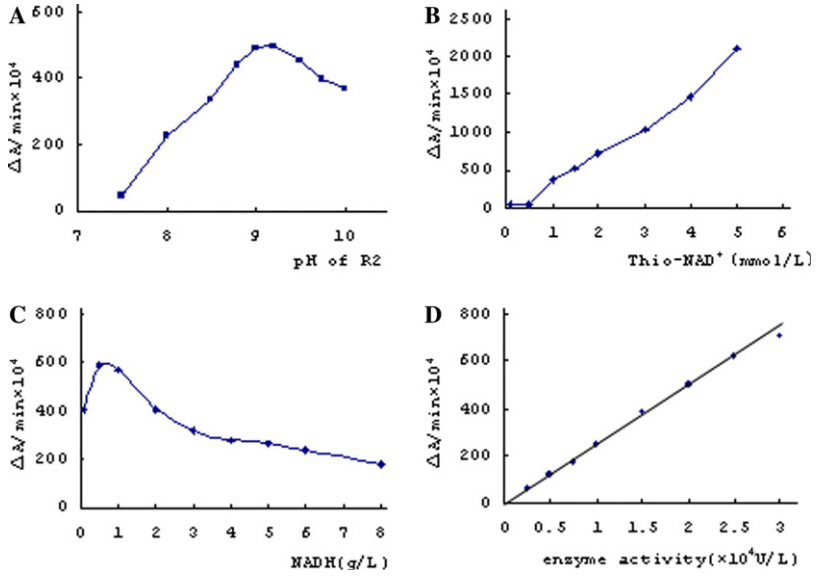

Fig. 4. Optimal conditions for the TBA assay. (A) Effect of pH on the TBA assay using 1.5 mmol/L thio-NAD^+, 2.0 g/L NADH, and 1.5×10^4 U/L 3α-HSD; (B) effect of thio-NAD^+ concentration on the TBA assay using 2.0 g/L NADH and 1.5×10^4 U/L 3α-HSD; (C) effect of NADH concentration on the TBA assay using 1.5 mmol/L thio-NAD^+ and 1.5×10^4 U/L 3α-HSD; and (D) effect of 3α-HSD activity on the TBA assay using 1.5 mmol/L thio-NAD^+ and 2.0 g/L NADH.

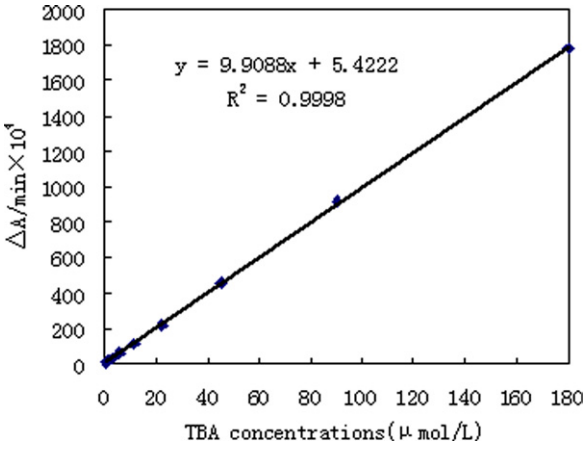

Fig. 5. The linearity of TBA assay.

Table 1
Analytical recoveries of various bile acids

Materials	Recovery rate (%)
Cholic acid	87.9
Deoxycholic acid	111.2
Taurodeoxycholic acid	117.1
Glycocholic acid	97.6
Taurocholic acid	88.9
Chenodeoxycholic acid	87.5
Glycochenodeoxycholic acid	96.7
Taurochenodeoxycholic acid	104.2

Interference by other substances

Various substances in blood that might interfere with the TBA assay were added to human serum (1:9 volume), and they were measured. Addition of up to 680 μmol/L bilirubin, 5.0 g/L of hemoglobin, 11.3 mmol/L triglyceride, 500 mg/L ascorbate, and 15,000 U/L lactate dehydrogenase did not affect the TBA assay. Effects of anticoagulants were also examined in the TBA assay. Addition of up to 2 g/L EDTA dipotassium, 200 mg/L sodium heparin or 5 g/L sodium citrate did not affect the TBA assay.

Discussion

Clinically, serum TBA has been widely used as one of the sensitive indicators for the evaluation of liver function. For example, increased serum TBA was found in almost all of the acute hepatitis patients. Serum TBA can also be used to distinguish between active stage and persistent stage of chronic hepatitis. It was increased in 89.2% of chronic active hepatitis, but was normal in chronic persistent hepatitis. In liver cirrhosis, serum TBA was higher than 30 μmol/L in all stages of the disease, even in patients without changes of bilirubin, alanine aminotransferase, and alkaline phosphatase [4]. In other hepatobiliary diseases such as intrahepatic and extrahepatic cholestasis, alcoholic hepatitis, and toxic hepatitis, serum TBA concentration usually increased to various extents.

The direct enzymatic method that measured reduced coenzyme or the coloring agent derived from the reduced coenzyme had some disadvantages. Because of the lower concentration of TBA in serum ($\leqslant$10 μmol/L in normal individuals), sensitivity of the previous assay methods was insufficient for clinical use. Although more samples used in an assay may improve the sensitivity, interference in the result by other substances may also occur. Nonspecific nitrotetrazolium blue reaction may happen in the coloring system affecting the accuracy of the assay. Furthermore, the resultant formazan contaminated the colorimetric cups and the instrumental tubes. An improved, sensitive, and simple method for automatic determination of serum TBA is therefore needed. Enzymatic cycling method has been used for the determination of several substances, such as myo-inositol and carnitine. In this study, we used this method and developed a novel colorimetric assay for the determination of serum TBA. A linear range up to 180 mol/L TBA can be obtained from this method. The within-run and between-run CVs were 1.2–3.7% and 2.3–4.8%, respectively.

In this method, the critical reagent is the enzyme 3α-HSD of which the purity and activity is closely related to the quality of the assay. This tool enzyme was obtained from *C. testosteroni* using the purification methods of chromatography and isoelectric focusing techniques. Consequently, the high price of this enzyme may limit the use of serum TBA as a routine examination in clinical practice. In this study, we tried to get the enzyme by using recombinant technology. We first isolated *C. testosteroni* from soil. 3α-HSD gene was amplified from the bacteria, inserted into prokaryotic expression plasmid pET-15b, and expressed in *E. coli* BL21(DE3)pLysS. The recombinant 3α-HSD with a (His)6 tag at the N terminal was successfully purified by a Ni^{2+}–Sepharose column. Characterization of the recombinant enzyme and the quality of TBA assay using this enzyme in this study indicate that this recombinant enzyme is completely suitable for the assay of serum TBA using enzymatic cycling method.

However, the TBA assay methods are unable to distinguish the species of bile acids in serum. 3α-HSD reversibly catalyzes the oxidoreduction of 3-hydroxyl on C19-, C21-, and C24-steroids to their corresponding 3-oxosteroids. Bile acids are a group of C24-steroids with a 3-hydroxyl group on carbon 3. Serum bile acids glucuronidated or sulfated at the 3-hydroxyl group are actually not measured by the enzymatic cycling method, therefore, the results of "serum total bile acids" obtained from this method should be treated with caution in some disease conditions. In normal individuals, about

10% of the total serum bile acids are sulfated [10]. In hepatobiliary diseases, the proportion of sulfated bile acids may be changed. For example, the proportion may be increased to 80% in obstructive jaundice and acute hepatitis [11]. Approximately 9% of the serum total bile acids are glucuronidated in normal samples [12], and similar proportion was reported in children with cholestasis [13]. Consequently, we should develop a relatively simple and cheap method for the measurement of bile acids profiles and their conjugates. In addition, standardization of the TBA assay and the material used as the standard are also important issues needed to be done in the future.

In conclusion, we have developed a novel colorimetric assay for the quantitative assay of serum TBA using enzymatic cycling method that is simple, specific, and sensitive. This method can be used in biochemical automatic analyzers available in most clinical laboratories.

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