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Establishment of Simple Colorimetric Method of Analysis Artesunate in Tablets

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Abstract: *Artesunate (ARTS) is a readily available anti-malarial in combination therapy, the standard method used to determine the authenticity of ARTS tablets involves high performance liquid chromatography (HPLC). In many countries, resources to purchase and maintain such equipment are expensive and not always available. Primary aromatic amine was treated with sodium nitrite and hydrochloric acid for diazotization reaction followed by coupling with Artesunate at pH 4, 6, and 8 medium to form a yellow colored azo dye compound which exhibits maximum absorption (λ_{max}) at 420 nm. These experiment was repeated twice for Artesunate tablets. The colorimetric method can be used to obtain a rapid visual assessment of tablet authenticity. The method can also be used to quantify the drug content of tablets, when used in conjunction with a spectrophotometer. The optimum reaction conditions and other analytical parameters were evaluated.Three samples of Artesunate (raw material, Amipharma tablet, and Shanghai tablet) have been analyzed by different chemical methods. The Artesunate samples were authenticated using FTIR spectrum, the results was good and showed similarity for two Artesunate tablets (Amipharama Artesunate and Shanghai Artesunate). HPLC, and TLC analysis of Artesunate Raw Material has been measured with good retention time value.*

Keywords*: Artesunate; diazonium salt; Malaria*

مستخـلص

يعتبر عقار الارتيسيونيت Artesunate من اشهر الادوية المتداولة لمحاربة ومعالجة مرض الملاريا. حيث تعتبر تقنية كروموتغرافيا السائل عالية الضغط HPLC هي الطريقة القياسية للتعرف علي عقار الارتيسيونيت بالأقراص الا ان هذه التقنية مكلفة وغير متوفرة في العديد من الدول.

عومل أمين أروماتي أولي مع نتريت الصوديوم وحامض الهيدروكلوريك لأزوتته(diazotization) تبع ذلك اجراء تفاعل ازدواج مع عقار الارتيسيونيت في وسط تفاعل منظم عند اس هيدروجيني 8,6,4 لتكوين صبغة الأزو ذات اللون الاصفر التي تظهر امتصاص أقصي (λmax) عند 420 nm. تم تكرار التجربة اعلاه مرتين باستخدام أقراص عقار الارتيسيونيت. يمكن استخدام طريقة التحليل اللوني(colorimetric method) للتأكد من وجود مادة الارتيسيونيت بالأقراص, كما يمكن تقدير كمية العقار بقرص الارتيسيونيت وذلك باستخدام تقنية الطيف الضوئي spectrophotometric. وقد تم تعيين الظروف المثلي التي تؤثر على التفاعل اللوني والعوامل التحليلية الأخرى.

تم تحليل ثلاثة عينات من عقار الارتيسيونيت هي خام Artesunate, اقراض الارتيسيونيت انتاج كل من شركة أميفارما , و شركة شنقهاي بعدة طرق كيميائية.تم تحليل عينات عقار الارتيسيونيت باستخدام FTIR, حيث أوضح طيف الاشعة تحت الحمراء لأقراص عقار الارتيسيونيت المتحصل عليه من مصنعي شنغهاي و أميفارما علي نتائج متشابهة. تحليل خام عقار الارتيسيونيت بكروموتوغرافيا السائل الضغط العالي HPLC و الطبقة الرقيقة TLC أعطت نتائج مطابقة لقيمة معامل الاعاقة القياسية.

I. Introduction

Malaria is a life threatening parasitic diseases transmitted by Anopheles mosquitoes. The parasites multiply within red blood cells, causing symptoms similar to regular influenza that include headache, fever, anemia, chills, flu-like illness, and in severe cases, coma (cerebral malaria) and death [1].It is a common and serious tropical disease which continues to be a major public health problem world-wide causing disease and death throughout most of the developing world. Malaria is not just a disease commonly associated with poverty, but it is also a cause of poverty and a major hindrance to economic development [2-3].Artesunate (Fig.1) is an ideal antimalarial agent for the treatment of severe malaria, including cerebral malaria.It is a white crystalline powder with melting point range of 132–135^{o}C and slightly soluble in water [4].

ARTS is activatedin vivo by hydrolysis, to dihydroartemisinin, the activemetabolite of the drug. Artemisinin is a sesquiterpenelactone isolated from Artemisiaannua, a herbthat has been used traditionally in China for manyyears in the treatment of malaria[5 -7].

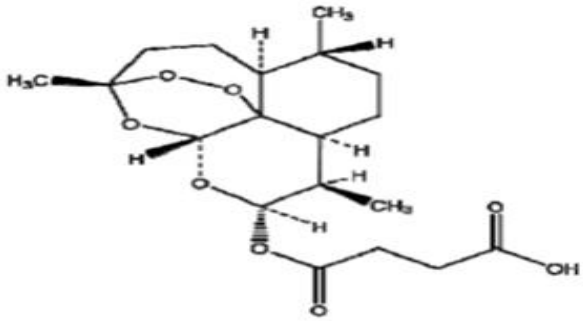

Fig.1 structure of Artesunate

The main procedure used to determine the authenticity of ARTS tablets included high performance liquid chromatography (HPLC). As ARTS is not stable in gas chromatography analysis, this technique is not convenient for characterizing the intact structure of this sesquiterpene endoperoxide [8]. In many countries, resources to purchase and maintain HPLC are not always available, therefore, they are particularly vulnerable to the growing problem of counterfeit ARTS as well as other drugs.ARTS does not possess reactive groups like Antimalarial such as Chloroquine and Sulfadoxine.In this work ARTS raw materials was authenticated using HPLC, FTIR, and Thin layer chromatography (TLC) before used as control samples. The aim of study is to establisha selective, simple, rapid and accurate colorimetric test to determine ARTS in tablets.

II. Materials and Methods

Solvents and chemicals

The following drug materials were procured; pure Artesunate powder (Amipharma company, and Shanghai International Trading Co. Ltd., Sudan), All reagents were prepared with distilled water and were of analytical reagent grade.

Instrumentation

The equipment's used are: Electronic balance (Metler Toledo, P31- Min 0.01 g), UV-visible spectrophotometer (Model UNICO 2100), HPLC (LC-2010HT) SHIMADZU consisted of a Hewlett Packard 1050 system, fitted with auto sampler, quaternary pump and variable wavelength detector, and FTIR was tested by BUCK SCINTIFIC 500 infrared spectrometer of Equinox Companies.

Preparation of diazonium salt

18.8 mmol (1.75g) of pure aniline was dissolved in mixture of 2.5 ml of concentrated Hydrochloric acid and 10 ml water, the mixture was cooled below 5°C and diazotized by adding a solution of sodium nitrite 9.41 mmol (0.65g/1.5ml water) with constant stirring, after 5 minutes, 2.625g of ARTS in 5 ml buffer 4 was added slowly and with stirring to the diazonium salts, A yellow precipitate was formed and filtered through Buchner funnel, washed with 25ml cold water and dried on a sheet of filter paper. The above steps were repeated using buffer 6 and 8.

Quantitative determination of Artesunate in genuine and counterfeit tablets

One tablet of ARTSwas transferred to 13×100 mm test tubes. A standard curve was prepared with samples containing 0 (blank), 2.5, 50, 100 mg of analytical grade Artesunate. 5 ml buffer solutions at pH 4 was added, the tubes gently swirled and the samples were allowed to sit at room temperature for 20 min. Then 1.5 ml of diazonium salt was added and the tubes were gently swirled. After 5 min, a distinctive yellow color appeared in the genuine Artesunate tablet samples. For quantitative analysis, the yellow reaction product was extracted from the water insoluble tablet excipients by adding 15 ml of ethyl acetate and transferred the mixture to 250 ml glass beaker (see Fig.2), then vigorously the mixture was stirred. After phase separation, the upper organic phase was transferred to 13×100 mm borosilicate glass tubes and the absorbance measured at 420 nm. Absorbance measurements were 0 to the blank. Artesunate content for each tablet was determined from the standard curve.

UV Assay method for determination of Artesunate in bulk and tablet

Two tablets were crushed into powder in a porcelain mortar with pestle. ARTS powder equivalent to 0.25g was weighed into 25 ml volumetric flask. A quantity of buffer solution at pH 4 was added to Artesunate powder to 10 ml mark and allowed to stand for 20 minutes to hydrolyses the drug. The sample was filtrated with

a sintered glass filter into 25 ml volumetric flask, then the absorbance of this solution was measured at two wavelength 242 and 420 nm before diazotized by adding 10 ml of equivalent diazonium salt to filtered solution. The resultant solution was allowed to stand for 5 minutes to form a yellowish solution. The amount of the final mixture solutions formed was assayed spectrophotometerically at 420 nm.

FTIR Assay method of ARTS

0.008g of raw material ARTS and ARTS with diazonium saltwere mixed with 0.15g KBr, then the IR spectra was recorded[9].

HPLC method for determination of ARTS

Twenty tablets were weighed and finely powdered. An accurately weighed powdered sample equivalent to 100 mg of ARTS was transferred to 20 ml volumetric flask; 15 ml of mobile phase was added and the flask was ultrasonicated for 5 min. The volume was then made up to the mark with mobile phase and solutions was filtered through Whatman filter paper No.41. one ml of the filtrate was transferred to 10 ml volumetric flask,1 ml of standard stock solution was added and volume was made up to the mark using mobile phase to get final concentration of 500 µg/ml of ARTS (500µg/ml of ARTS as internal standard). After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the tablet sample solution was injected. Chromatogram was obtained and peak areas were recorded. The peak area ratio of Artesunate to the internal standard was calculated and the amount of Artesunate present in sample was estimated.

Thin layers Chromatography

The ARTSdrop was spotted on pre-coated silica gel plates. The mobile phase used for separating of ARTSconsisted of methanol, toluene, ethyl acetate and glacial acetic acid as mobile phase in ratio of 1:4.5:4:0.1 v/v. spot was visualized by derivatization with an acidified 4-methoxybenzaldehyde in methanol-water[9].

III. Results and discussion

The colorimetric analysis showed that was really composed or not, from Fig.2. The yellow colour indicated that Artesunate was composed.

Fig.2 ARTS tablet with diazonium solution, and the ethyl acetate extract

The specificity and simplicity of this colorimetric method (ARTS - benzene diazonium salt test) will certainly be useful in detecting counterfeit Artesunate.Simple UV method has become necessary for the assay of this drug because, UV unlike HPLC is simple, rapid and readily available in malaria endemic areas of the world. This will also help to checkmate influx of fake and adulterated products into the drug market and reduce the burden of malaria[10]. In order to assay Artesunate by UV method, it is necessary to involve it in a reaction process that would break the endo peroxide ring and introduce a least one double bond in the molecule.The UV-visible scan of the Artesunate reaction mixture showed UV-absorption with the λ_{max} at 420 nm and 242 nm, in Table1, six samples were investigated under UV spectrophotometer, four samples were mixed with diazonium salt, and two samples without diazonium salt in different wavelength at 242 nm and 420 nm, from results showed that the absorbance values were increased when Artesunate mixed withdiazonium salt (raw material at 242 nm equal 0.294, raw material at 420 nm equal 0.557, Amipharama tablet at 420 nm equal 0.418, Shanghai tablet at 420 nm equal0.417 because colour increased the intensity which made absorbance increased in UV spectrophotometer. Artesunate pure has white colour, the absorbance values were at 242 nm equal 0.146, and at 240 nm equal 0.126.

Table1 UV of ARTS in different types and different wavelengths at pH 4

Samples	Raw material	Raw material	Raw material + diazonium	Raw material + diazonium	Amipharama tablet + diazonium	Shanghai tablet + diazonium
Absorbance	0.126	0.146	0.457	0.294	0.418	0.417
λ (nm)	420	242	420	242	420	420

The absorbance values of ARTS samples with diazonium salt at wavelength 420 nm showed that the raw material has the higher absorbance value equal 0.457, more than Amipharama tabletequal 0.418, and Shanghai tablet equal 0.417 respectively which return to that the ARTS tablets did not pure for their additive comparable to raw material.The results obtained from the present study show that UV absorption of ARTS could be employed for the assay of the drug especially in poorly equipped laboratories like those found in most developing countries. The proposed method is sensitive and reproducible.

Increasing the pH of the reaction mixture containing ARTS and diazonium salt resulted in precipitation of the colored reaction product. This yellow precipitant is extractable into ethyl acetate. Absorbance measurements of the ethyl acetate fraction shows increased intensity with increased reaction pH (Fig.3)

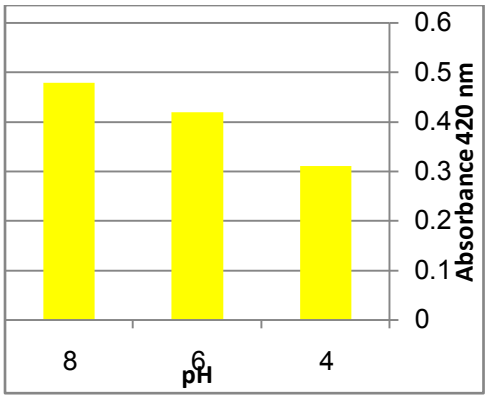

Fig.3 The Absorbance of extractethyl acetate of ARTS–diazonium at different pH

Under the same assaypH conditions, other antimalarial compounds were compared in order to evaluate assay specificity. At pH equal 6, Artemisinin develops a yellow color, while Primaquine develops an orange color. Sulfadoxine gives a strong yellow color when pH is increased to 8. Although less intense at lower pH, ARTS is the only drug from the compounds tested that develops a yellow color at pH 4.

The optimum time of coupling reaction between diazonium salt and Artesunate in buffer 4 was determined by recording the absorption of the reaction mixture at 420 nm in different time intervals. Fig. 4 shows that 20 minutes was required for maximum color intensity development and the yellow color was stabled after 30 minutes.

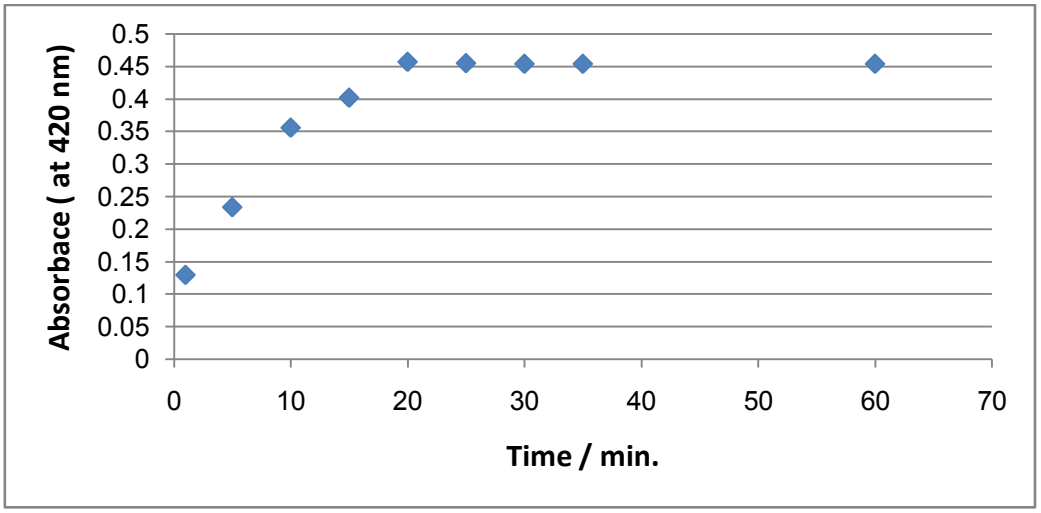

Fig. 4 Absorbance versus Time / min of coupling reaction at pH 4 IR analysis of Artesunate

The FTIR spectra of ARTS presented here was analyzed by two different laborites, and the frequencies of the modes are fully consistent with earlier studies of Artesunate, and with the vibrational data reported for some 1, 2, 4 trioxanes by Jefford and co-workers Sohrabi[11].

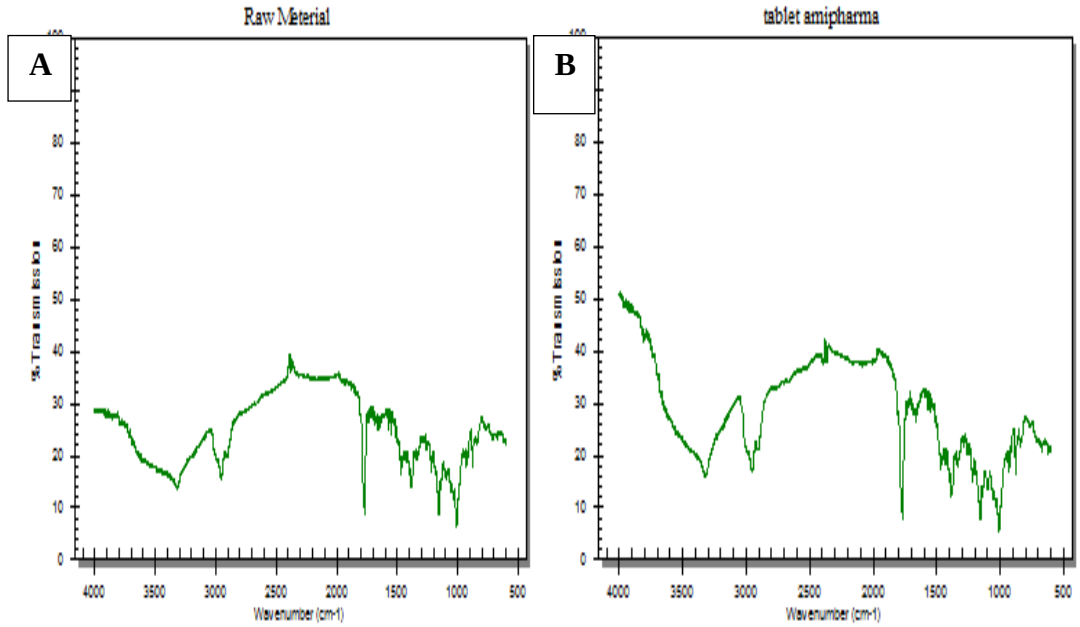

Fig.4 FTIR spectra of (A) Artesunate raw material, (B)Amipharama Artesunate tablet

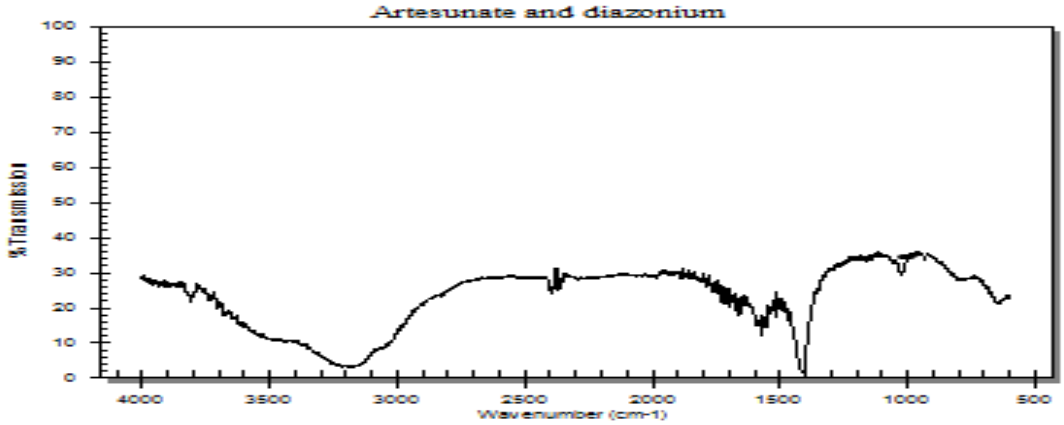

Fig.5 Artesunate raw material with diazonium

The FTIR spectra of pure Artesunate showed characteristic bands at 3279 cm^{-1} (O-H stretching vibrations), 2947 cm^{-1} (Fermi resonance of the symmetric CH3 stretch with overtones of the methyl bending modes), 1093 cm^{-1} (C-O stretching), 890-820 cm^{-1} (O-O-C stretching in boat/twist form), 825cm^{-1} (O-O stretching in boat/twist form) and 1420-1310 cm^{-1} (C=O). It indicates the properties of the O-O-C component, respectively representing the 1, 2, 4- trioxane ring [11].

Fig.5 showed that the FTIRof the yellow product of coupling reaction between Artesunate and diazonium salt, the spectra obtained showed similarity to pure Artesunate presented in Fig.4 except peak at 2947 cm–1 it was disappeared. The N=N stretching vibration of symmetrical trans azo compound is forbidden in the IR spectroscopy. Unsymmetrical azo- benzene absorbed at 1420 cm^{-1}. The band are weak because of the nonpolar nature of the bond.

HPLC analysis of Artesunate

HPLC method has been developed for quantification of Artesunate in combined tablet formulation. The validation procedure confirms that this is an appropriate method for their quantification in theplant material and formulation. It is also used in routine quality control of the raw materials as well as formulations containing any or all of these compounds.

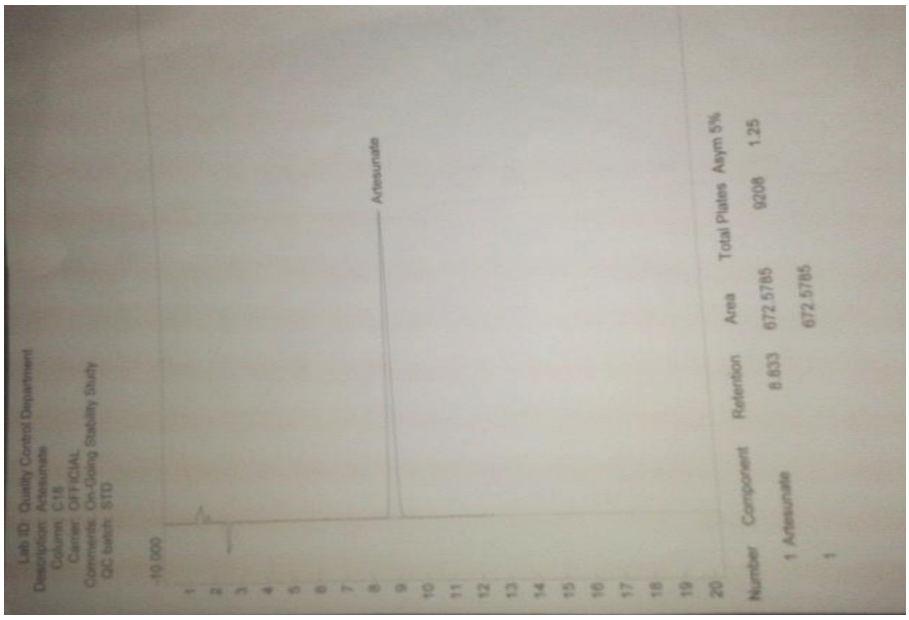

Fig.6 HPLC of ARTS Raw material

The HPLC analysis was performed on the LCGC Quails C8 (5 µm, 250 mm X 4.6 mm) column in isocratic mode, at 300^{o}C temperature using a mobile phase consisting of Acetonitrile: phosphate buffer (70:30, v/v) at a flow rate of 0.8 ml/min. The detection was carried out at 221nm for Artesunate. The retention time of Artesunate raw material was 8.833 which was high more than Artesunate raw material studied by (P.S.Jain, 2013) with value [12].

ARTS raw material was authenticated by using TLC. Fig.7 showed that Rf of ARTS in methanol, toluene, ethyl acetate and glacial acetic acid as solvent system equal 0.51, which was agree with standard value 0.57.

Fig.7 TLC of ARTS Raw material

IV. Conclusion

The coupling reaction between ARTS and diazonium salt at room temperature and pH equal 4 provides accurate, quick, and simple method to identify counterfeit Artesunate tablets. The yellowproductexhibited under a pH ranged from 4 to 8 at room temperature. Although a quantitative
assay requires a balance, a filter photometer and extraction with ethyl acetate, while a qualitative assay can be obtained in the field without the need for organic solvent extraction or the use of sophisticated equipment. The qualitative method is sensitive enough to require only less than onetablet assuming 50 mg ARTS per tablet. This derivatization method is also being investigated by our laboratory for the detection of ARTS and Artemisinin in biological fluids
using HPLC.

From the spectral analysis it can be concluded that the information obtained from FT-IR spectra of pure Artesunate in single or in combination with other drugs could be used to ascertain the presence of these drugs in formulations because no two functional groups have the same vibrational frequency and fingerprint.
Spectrophotometric methods for the analysis of Artemisinin, Primaquine, and Sulfadoxine and Artesunate in formulation has been developed, which takes reasonable time to complete.

Acknowledgments

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