A SYNOPSIS OF THE THESIS ENTITLED

STUDY ON IMPURITY PROFILING AND CHARACTERIZATION OF MAJOR DEGRADATION PRODUCTS FOR SELECTED ANTI-TUBERCULOSIS DRUGS.

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INTRODUCTION

Bacteria of Tuberculosis are killing human beings and animals since 1700s and years ago before them. During 1700s it was known as "the white plague" as patient gets pale due to TB and during 1800s it was known as "Consumption", as number of people died due to TB. After that the word Tuberculosis was coined by schonlein and now TB can be named as it affects organ like pulmonary TB, lymph node or extra pulmonary TB. The bacteria cause TB is mycobacterium tuberculosis was identified by German scientist Dr. Robert Koch on March 24, 1882, so that day is declared as world TB day.

TB is contagious disease; it can be transferred person to person or animal to human. A special ventilated room and good diet is necessary for TB patients. Earlier there was not proper diagnostic method for TB, the first method introduced for diagnosing TB was TB skin test after that various test was introduced but till date TB skin test is used along with Chest X-ray and TB blood test [1].

TB can be cured by completing course of antibiotics, rest and healthy diet. Antibiotics are prescribed depending on patient's profile of symptoms. Common therapy for TB is use of combination of antibiotics, e.g. Rifampicin and isoniazid along with streptomycin or ethambutol. This treatment is given since eighty years and still working without any change in it. Patient may have resistance to these antibiotics so drug resistant TB required different treatment, another type is multi drug resistant TB (Delamanide and Bedaquiline), TB along with HIV. For TB patients DOT (directly observed therapy) campaign is carried out by governments so that intentional or non intentional failure to take medicines can be avoided [2, 3].

Global TB status- 2019 [4]

World Health organization (WHO) provides every year global TB status from Africa, America, Eastern Mediterranean, Europe, south-east Asia and western Pacific region. In 2018, approximately 10 million people fell ill with TB globally. Drug resistant TB cases are increasing which is threat to public health. In 2018 half million cases of multidrug resistant TB were recorded. Three countries had largest share in global data which are India (27%), China (14%) and Russian federation (3%). These countries are included in END TB by 2020 campaign by WHO. European Region is about to achieve goal END TB by 2020.

Future Prospects [4]

Old antibiotics therapy, multidrug therapy and newer antibiotics, these are the treatment for TB although globally TB is killing millions of people every year. Vaccines are in pipeline so that TB risk can be reduced, new diagnosing techniques for simple and rapid diagnose of TB and therapy that reduce drug dosage course may we can see in future.

Classification of TB drugs [5]

Group A: Fluroquinolones: Levofloxacin, Moxifloxacin and Gatifloxacin

Group B: Second line injectable agents: Kanamycin,Amikacin and Capreomycin

Group C: Other core second line agents: Ethionamide/Proethionamide, Cycloserine/Terizidone, Linezolide, Clofazimine

Group D: add on agents: D1: Pyrazinamide, Ethambutol and High dose isoniazide **D2:** Bedaquiline, Delamanide **D3:** p-amino salicylic acid, Imipenam-cilastatin, Meropenam, Amoxiciline-clavulanate.

IMPURITY PROFILING [6-9]

Unwanted Chemicals or Related components are the impurities in pharmaceuticals which may remain with active pharmaceutical ingredients (APIs), or develop during stability testing, or develop during formulation or upon aging of both API and formulated APIs.

Presence of these impurities may affect efficacy and safety of the pharmaceutical product. For determination of related components, various analytical methodologies have been employed. There is immense need for developing a new analytical method for quality assessment of new emerging drugs.

According to ICH (*The international council for harmonization of technical requirements for registration of pharmaceuticals for human use*) guidelines Impurity is defined as "any component of medicinal product or new drug substance which is not the chemical entity defined as the active drug substance or an excipient in a drug product." Related component, related substance and related impurities are synonyms for the term impurities.

For impurity estimation there is analytical methods should be based on stability indicating methods to monitor the stability of pharmaceutical dosage form during the investigational phase, and once the drug is marketed, ongoing stability studies must be

performed. By performing stability indicating method, evidence is produced that how drug substance or drug product quality is speckled with time under the influence of variety of environmental conditions such as temperature, humidity and light, enables to establish a retest period or shelf life for a drug substance and recommended storage condition. Various methods are developed that can measure the amount of drug remaining and lost (presence of degradation products) or both. Approaches from several avenues can be employed to these methods for development.

Sources of impurities in pharmaceutical substance [6, 7]***:***

Impurities can be originated from various sources, phase of synthetic process and preparation of pharmaceutical dosage form. Impurities can originate either due to their synthetic route (Inherent Impurity) or due to Degradation (Degradant Impurity). Drug can be synthesized by several possible ways, and chances of developing a different impurity by same drug if they are produced by different sources.

Classification of impurity:

According to ICH Guidelines, Impurities can be classified as organic, inorganic and residual solvent. Organic impurity can arise from Starting materials, By-products, intermediates, degradation products. Inorganic impurities can arise from manufacturing process normally known and identified as reagents, ligands, catalyst, Heavy metal and other residual metal, Inorganic salt and other material (e.g. filter aids and charcoal).Residual solvents are impurities introduced with solvents, Inorganic or Organic Liquids used as a vehicle for the preparation of solutions or suspensions in the synthesis of a new drug substance.

The number of inorganic impurities and residual solvents are limited. These are easily identified and their physiological effects and toxicity are well known therefore the pharmacopeial limits and ICH guideline can assure that these impurities' harmful effect will not contribute in toxicity and side effects of drug substance. While for organic impurities, if drug is prepared from multi step synthesis route than there is increase in number and variety of impurities and also the variety of their structure are also unlimited depending on route and reaction conditions such as purity of the starting material, method of isolation, purification and storage conditions etc. in addition toxicity is unpredictable for this reason ICH guidelines set threshold limits above which identification of impurity is obligatory.

Sources of organic impurity:

Organic impurities may arise during manufacturing process or during storage of drug substance. The process related impurity can be derived from starting material, intermediates, reagents, ligands and catalysts used in chemical synthesis. Degradation product can be obtained from chemical degradation of drug substance or drug product under storage or stress condition. They may be identified or unidentified, volatile or non volatile and include the following:

1. Inherent Impurities

- Impurities originating from drug substance synthesis process: most of the drugs are chemically synthesized; chemical entities other than drug substance can be carried over to final drug product as trace level impurity. These chemical entities can be raw material, intermediates, solvents, chemical reagents, catalyst and by products. These impurities are referred to as process impurity. Aim to be achieved by identification of process impurity is to find the structure and origin of these impurities that will help in eliminating or reducing process impurities.

- Starting material and intermediates: final dosage form is prepared using starting materials and intermediates. In final product only those starting material and intermediates appear as impurity that remains unreacted throughout process and potentially survive in synthetic and purification process.

- Impurities in starting material: Impurities present in starting material will appear in final product after going through same chemical reaction that starting material has gone through; therefore knowledge of impurities in starting material is essential to figure out related impurities in final product.

- Reagents, ligands and catalyst: they are less found in APIs but may pose a problem as impurity. They are used in synthesis of drug product and can be carried over as trace level impurity.

- By product of synthesis: chemical reactions and side reactions are simultaneously carried out because chemical reactions are not 100% selective. Most common process impurities are by products formed by side reactions. Side reactions that can produce by products are incomplete reactions, overreaction, isomerisation, dimerisation, rearrangement, or unwanted reaction with chemical reagent or catalyst.

- Products of over-reaction: if synthesis is not selective in previous step, reagent attacks on intermediate not only at desired site.

- Products of side reactions: some of the side reaction is not avoidable and produce by product that can be carried in final product as trace level impurity. Those products should be identified and elucidated during impurity profiling.

2. Degradation Impurities

- Impurities originating from degradation of drug substance: impurities can also formed by degradation of end product. Degradation product formed during storage or aging of API or formulated APIs. According to ICH guidelines Degradation product can be defined as," molecule resulting from a chemical change in the substance brought about by overtime or due to the action of light, temperature, pH or water or by reaction with excipient and/or the intermediate container closure system. Forced Degradation or Stress Degradation method can be applied to produce atmosphere similar to long term storage condition. If there is no change in product at room temperature than temperature is gradually increased to 10^{o}C and degradation product is achieved but not more than 70^{o}C. Similarly acid/alkali, humidity and light conditions produce degradedness.

- Genotoxic and carcinogenic impurity in drug substance and product: Genotoxic compound have potential to damage DNA at any level of exposure and that such damage may lead/contribute to tumor development. A threshold of toxicological concern (TTC) value of 1.5 µg/day intake of Genotoxic impurity is considered to be associated with an acceptable risk for most pharmaceuticals.

- Enantiomeric Impurity: The majority of therapeutic chiral drugs used as pure enantiomers are natural products. The high level of enentio selectivity of their biosynthesis excludes the possibilities of the presence of Enantiomeric impurities. While for synthetic chiral drugs, racemates that are marketed are pure enantiomer than antipode is considered as an impurity. The reason for presence can be either the incomplete enentio selectivity of the syntheses or incomplete resolution of the enantiomers of the racemates. Although ICH guidelines exclude Enantiomeric impurities and pharmacopeias consider them as an ordinary impurity. The single Enantiomeric chiral drug is now considered as an improved chemical entity that may offer a better pharmacological profile and an increased therapeutic index with a more favorable reaction profile.

In general, an individual API may contain all of the above mentioned types of organic impurities varying from negligible to significant level.

Requirement for control of impurity

Impurities may possess unwanted pharmacological and toxicological effect that may suppress the benefits of drug administration. Impurities will have disastrous efficacy, different bioavailability, adverse effect or toxic effect. In case of chiral impurity one isomer may produce the desired therapeutic effect while another is therapeutically inactive or in worst cases produce unwanted effect.

Pharmacopeial status

Quality of chemically active substance with respect to organic impurities is controlled by a set of tests with in a pharmacopeial monograph. Individual monographs are periodically updated to keep place with scientific progress and regulatory development. Following the revised ICH Q3(A) (R2) impurity testing guideline major pharmacopeias will continue publishing new or revised relevant monographs and general chapters. Active substance containing an organic impurity not detected by relevant pharmacopeial test prescribed below is not of pharmacopeial quality unless the amount and nature of impurity are compatible with GMP.

Two general chapters (<466> and <1086>) of the US pharmacopeia (USP) deal with organic impurity testing. Concept and definitions are clearly described although different terminology from that of ICH is used. Until now, one of three types of tests in bulk pharmaceutical chemicals is ordered,

1) A Chromatographic purity test coupled with a non-specific assay.

2) A chromatographic purity- indicating method that also serves as an assay.

3) A specific limit test for known impurities, a procedure that requires reference standards for these impurities.

Table1. Limits for Impurities in degraded products of drug

Degradation product Impurity	Limits
Each identified degraded product	Not more than 1.0%
Each unidentified degraded product	Not more than 0.5%
Total degraded product	Not more than 2.0%

The European Commission decided that the principles and terminology of revised ICH Q3A should be implemented in the European Pharmacopeia (EP) monographs of the active substance; both new and already published. A general chapter concerning the control of impurity is introduced in fifth edition, while the revision of monograph entitled substance for pharmaceutical use has also been done.

ICH Guidelines [8,9]

According to ICH each impurity should be identified with respect to chemistry and safety aspect. Chemistry aspects include structure identification, reporting and quantitation using suitable analytical procedure. While safety aspects include process of gathering and evaluating the data concern the biological safety of an impurity (qualification). Individual listed impurity, limited with specific acceptance criterion are referred to as specified and they can be either identified or unidentified. Unspecified impurities are limited to general acceptance criterion.

Control of organic impurity

Control of organic impurity in new drug substance is based on maximum daily dose (MDD) and total drug intake (TDI). Table 4.2 and 4.3 provide ICH threshold for control of organic impurity.

Table 2 Thresholds for degradation products in new drug product

	Maximum daily dose	**Thresholds**
Reporting Thresholds	≤1 g	0.1%
	>1 g	0.05%
Identification Thresholds	<1 mg	1.0% or 5µg TDI, Whichever is lower
	1 mg -10 mg	0.5% or 200 µg TDI, whichever is lower
	>20 mg-2 g	0.2% or 2 mg TDI, whichever is lower
	>2 g	0.10%
Qualification Thresholds	<10 mg	1.0 % or 50µg TDI, Whichever is lower
	10 mg-100mg	0.5% or 200µg TDI, Whichever is lower
	>100 mg-2 g	0.2% or 3 mg TDI, Whichever is lower
	>2 g	0.15%

Table 3 Impurity Thresholds in New drug Substances

Maximum Daily Dose	**Reporting Thresholds**	**Identification Thresholds**	**Qualification Thresholds**
≤2 g/ Day	0.05%	0.10% or 1.0 mg TDI, Whichever is lower	0.15% or 1.0mg TDI, whichever is lower
>2 g /Day	0.03%	0.05%	0.05%

*TDI- Total daily intake

SELECTION OF DRUGS [10-19]

- No Impurity profile and degradation product characterization Details available for some drugs in Literature
- Few new drug molecules are selected, no Impurity and Stability data available for new entity.
- Therefore, effective approaches and strategies are urgently needed to develop Impurity profile for selected anti-tuberculosis drugs. Those drugs are selected for stability study and impurity profiling.

The drugs selected are,

1. Terizidone
2. Bedaquiline
3. Rifabutin

OBJECTIVE OF THE STUDY

The objective of the proposed research is to develop an Impurity profile for selected Tuberculosis drugs, develop Method and validate that method according to ICH Guidelines.

- Development and Validation of simple, sensitive and reliable stability indicating assay methods for selected drugs as per ICH guidelines.
- Characterization of degradation products.
- Elucidation of Degradation pathways of the selected drugs.
- Estimation of Impurities present in APIs.
- Isolation and characterization of degradation products/impurities found in APIs
- Correlation of degradation behavior of drug with found impurities in API

MATERIALS AND METHODS

Procurement of drug

TRZ was procured from Macleods Pharma, Dahej, Gujarat.

BDQ was procured from Dishman Pharmaceuticals, Ahmedabad, Gujarat.

RFBT was procured from Lupin pharma., Dabhasha, Gujarat.

Preliminary study of APIs

Identification of drug was done by **Infrared Spectroscopy:** IR graph can identify which group is present in drug chemical structure. Presence of additional group helps to check drug purity. All drugs showed group which is presence in chemical structure of drug, no additional group found in any drug IR graph **DSC:** Differential scanning calorimetry can measure drug melting point, results obtained can show a range or exact melting point of drug, additional peak in graph shows presence of additional substance or salt. All drugs showed melting point which is reported in literature. **UV spectroscopy**: Each drug has its particular ʎmax if it contains chromophore group, due to presence of chromophore group drug has typical UV spectroscopic graph. Here all three drugs showed ʎmax which is reported for them.

Purity check

Drug purity was checked by **DSC** and **HPLC:** HPLC method developed for drugs to check that drug is not showing additional peak in chromatogram. All drugs peak purity and no additional peaks were found in chromatogram.

Stability study for each drug was cried out using HPLC instrument. Stability study was done in acid, alkali, peroxide, UV light, heat and water using HPLC. Drug was studied for individual Degradant, sample was prepared and injected in HPLC instrument, PDA detector can detect degradation product and drug peak. Degradation products were separated from drug peak using HPLC instrument.

Degradation product identification

Degradation product obtained in HPLC instrument was subjected to LC-MS instrument to obtain m/z ratio of degradation product. From m/z ratio of degradation product molecular weight would be obtained, predicting structure from molecular weight helps to identify degradation products. **NMR**: Nuclear magnetic resonance helps to identify organic elements present in which positions. NMR helps to build a chemical structure of degradation product.

STABILITY STUDY

Terizidone

Instrumentation

Terizidone stability study was completed using Waters binary HPLC instrument with PDA detector manual 20µL injector. **Mobile phase**: 30Mm ammonium acetate buffer pH

4.0±0.5 using glacial acetic acid and acetonitrile in gradient mode. Stability of TRZ was studied in acid, alkali, peroxide, neutral, light and thermal condition. Drug was degraded in every condition individually and checked for stability using HPLC instrument. Drug chromatogram and degraded sample chromatogram were compared for degradation of TRZ. Degradation peaks were separated from drug peak by developing stability study method.

Acid degradation

Drug acid degradation study was carried out in 1N HCl at room temperature. Drug was kept in acid sample for 3days; samples were taken in between to check drug degradation.

Alkali degradation

Drug alkali degradation study was carried out in 0.5N NaoH at room temperature; drug was kept in 0.5N NaoH for 1 hour. Acid and alkali samples were neutralized with alkali and acid respectively, after that injected in HPLC instrument.

Neutral degradation

Drug was kept in water for 24 hour at room temperature to know the effect of water on TRZ.

Oxidative degradation

TRZ was kept in 6% Hydrogen peroxide to initiate oxidation of TRZ at room temperature.

Photolytic and Thermal degradation

Drug was kept under UV light in photo chamber and another sample was kept in hot air over at 80^0C to know effect of temperature. Both the study was done for 28days.

Bedaquiline

Instrumentation

Bedaquiine stability study was completed using Waters binary HPLC instrument with PDA detector manual 20µL injector. **Mobile phase**: 30mM Disodium hydrogen phosphate buffer pH 2.5±0.5 using ortho phosphoric acid and methanol in gradient mode. Stability of BDQ was studied in acid, alkali, peroxide, neutral, light and thermal condition. Drug was degraded in every condition individually and checked for stability using HPLC instrument. Drug chromatogram and degraded sample chromatogram were compared for degradation of BDQ. Degradation peaks were separated from drug peak by developing stability study method.

Acid degradation

BDQ was kept in 1N HCl at 80^0C for 75minutes to study the effect of acid on BDQ.

Alkali degradation

BDQ was kept 3N NaoH at 80^0C for 5hrs to study the effect of alkali on BDQ

Oxidative degradation

BDQ was kept in 12% hydrogen peroxide at 80^0C for 5hrs to check oxidative effect of hydrogen peroxide on BDQ

Neutral degradation

BDQ was kept in water for 3hrs at 80^0C to check the effect of water on BDQ.

Photolytic and thermal degradation

BDQ was kept under UV light for 28 days to check any degradation due to UV light. BDQ was kept in hot air oven at 80^0C for 28 days to check effect of temperature on BDQ.

Rifabutin

Instrumentation

Rifabutin stability study was completed using Waters binary HPLC instrument with PDA detector manual 20µL injector. **Mobile phase**: 30mM ammonium acetate buffer pH 4.0±0.5 using glacial acetic acid and acetonitrile in gradient mode. Stability of RFBT was studied in acid, alkali, peroxide, neutral, light and thermal condition. Drug was degraded in every condition individually and checked for stability using HPLC instrument. Drug chromatogram and degraded sample chromatogram were compared for degradation of RFBT. Degradation peaks were separated from drug peak by developing stability study method.

Acid degradation

RFBT was kept in 0.5N HCl for 24hour at room temperature to check any reaction between drug and acid.

Alkali degradation

RFBT was kept in 0.1N NaoH for 2 hrs at room temperature to check any reaction between drug and alkali.

Oxidative degradation

RFBT was kept in 30% hydrogen peroxide for 1 hr at 80^0C to check drug is oxidized.

Neutral degradation

RFBT was kept in water at 80^0C for more than 24hours to check any effect of water on RFBT.

Photolytic and thermal degradation

Drug was kept under UV light to check any effect of UV light to degrade RFBT. Drug was kept in hot air oven at 80^0C for study of effect of temperature on RFBT.

KINETIC STUDY

After stability study completed drug kinetics study were done in different condition using different temperature, time and concentrations of Degradant.

Terizidone

For TRZ degradation kinetic study acid, neutral and oxidative conditions were selected. For acid kinetic study, 0.1N, 0.3N and 0.5N concentrations were studied at 25^0C, 60^0C and 80^0C at 10minutes interval till 50minutes. For neutral degradation drug was kept in water at 25^0C, 60^0C and 80^0C at 5minutes interval till 25minutes. For oxidative kinetic study, 1%, 3% and 5% peroxide was studied at 25^0C, 40^0C and 60^0C at 10minutes interval till 50 minutes. Drug degradation kinetic study was not completed in alkali medium as drug is sensitive for alkaline medium and forming degradation products with drug peak. Drug is not degrading in photolytic and thermal condition so degradation kinetic study was required for these conditions.

Bedaquiline

For Bedaquiline degradation kinetic study acid, alkali and oxidative conditions were selected from stability study results. For acid kinetic study, 0.1N, 1N and 2N HCl concentrations were studied at 25^0C, 50^0C and 80^0C at 10minutes interval till 1 hr. For alkali study, 0.1N, 1N and 2N NaoH concentrations were used at 25^0C, 50^0C and 80^0C for 1 hour interval till 6hrs. For oxidative kinetic study 1%, 3% and 6% hydrogen peroxide was used at 25^0C, 40^0C and 60^0C at 10minutes interval till 1 hour. Response surface methodology was successfully applied to compare predicted value from Design expert ™ Software and actual obtained value. Drug is not degrading in neutral, photolytic and thermal condition so degradation kinetic study was required for these conditions.

Rifabutin

Rifabutin degradation kinetic study work is going on.

RESULT AND DISCUSSION

Terizidone

Terizidone stability study method was developed in 30mM ammonium acetate buffer pH 4.0±0.05 using glacial acetic acid and acetonitrile in gradient mode. Retention time for TRZ was 46±0.5minutes and complies with system suitability parameters.

TRZ was degraded to 35.09% in 0.5N HCl for 3 days at room temperature, 25.02% in 0.5N NaoH for 1hour at room temperature, 5.89% in 6% hydrogen peroxide for 24hour at room temperature and 100% degradation in water for 24hour at room temperature. Drug degraded in very negligible amount under photolytic and thermal conditions after 28days. Marketed formulation (Tericox) was also studied for stability study and assay. Average assay result was 99.02% which is in limit. Stability study results were very close to API results.

Degradation kinetic study was carried out in acid, neutral and oxidative condition. TRZ was followed first order kinetic study in acid and neutral condition. In oxidative medium zero order reaction is proceeding.

Bedaquiline

Bedaquiline stability study method was developed in 30mM disodium hydrogen phosphate buffer pH 2.5±0.05 using ortho phosphoric acid and methanol in gradient mode. Retention time for BDQ was 31.46±0.5minutes and complies with system suitability parameters.

BDQ was degraded to 74.95% in 1N HCl for 75minutes at 80^0C temperature, 53.49% in 3N NaoH for 5hours at 80^0C temperature, 35.07% in 12% hydrogen peroxide for 5hour at 80^0C temperature and 65.45% degradation in water for 3hour at 80^0C temperature. Drug is degrading in negligible amount in thermal and photolytic condition after 28days. As marketed formulation for BDQ is not available in India, synthetic mixture was prepared using common available excipient. Obtained stability result was very close to API results without interference of excipient.

Degradation kinetic study was carried out in acid, alkali and oxidative condition. BDQ was followed first order kinetic study in acid, alkali and neutral conditions.

Response surface methodology tool was applied to predict degradation kinetics parameters for different conditions. Degradation kinetic parameters were predicted from design expert software and compared with actual values obtained. Predicted and obtained values were close. Tool was applied for acid, alkali and oxidative degradation kinetic study.

Rifabutin

Rifabutin stability study method was developed in 30mM ammonium acetate buffer pH 4.0±0.05 using glacial acetic acid and acetonitrile in gradient mode. Retention time for RFBT was 10.2 ±0.5minutes and complies with system suitability parameters.

RFBT was degraded to 40.78% in 0.5N HCl for 24 hours at room temperature, 65.38% in 0.1N NaoH for 2hour at room temperature, 75.39% in 30% hydrogen peroxide for 1hour at 80^0temperature. Drug degraded in very negligible amount under neutral, photolytic and thermal conditions. Marketed formulation (Ributin) was studied for assay and stability study. Average assay result was 99.1% and stability study results were not so different than API results.

CONCLUSION

Reproducible and robust HPLC method methods were developed for drugs. HPLC methods were validated according to ICH guidelines. Other parameters of ICH guidelines were also studied like accuracy and precision.

Stability study was carried out and found that all three drugs are photo stable and thermal stable for more than 28days. Rifabutin is water stable at 80^0C for more than 24hours. All drugs are susceptible for acid, alkaline and oxidative conditions.

Kinetic study results showed that all drugs are following first order kinetic in Degradant, TRZ is following zero order in hydrogen peroxide medium.

WORK IN PROGRESS

Terizidone

- Isolation and characterization of DPs.

Bedaquiline

- Isolation and characterization of DPs.

Rifabutin

- Response surface methodology tool for degradation kinetic study
- Isolation and characterization of DPs.

- Data compilation and submission
- Publication of research work
- Thesis writing.

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