Available online on 15.05.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

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Development and Validation of UV-Visible Spectrophotometric method for simultaneous estimation of Etoposide and Picroside-II in bulk and pharmaceutical formulation

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ABSTRACT

Aim: To develop and validate a simple, precise, accurate, and sensitive UV-visible spectrophotometric method for the simultaneous estimation of Etoposide (ETO) and Picroside-II (PK-II) in a bulk and pharmaceutical formulation according to the ICH guidelines.

Methods: The absorption spectra of ETO and PK-II were carried out over the range of 200-800 nm, and absorption maxima were determined. Multiple calibration standards were prepared of both the drugs separately, and absorbance were recorded at respective λmax. Calibration curve were plotted and the linear responses were studied. Various analytical method validation parameters viz. accuracy, precision, LOD, LOQ, robustness and ruggedness were calculated using QC standards.

Results: The absorption maxima of ETO and PK-II were found to be 208 nm and 265 nm respectively. Linearity range for ETO and PK-II were found to be 1-7 µg/ml and 1-35 µg/ml with correlation coefficient 0.999 and 0.999. The intra-day and inter-day study shows percent relative standard deviation in between 0.11 to 1.08 and 0.12 to 1.38. LOD and LOQ were found to be 0.1321 µg/ml and 0.4003 µg/ml for ETO whereas 0.1616µg/ml and 0.4897µg/ml for PK-II. The total percent recovery of ETO and PK-II were found to be 99.09 and 99.68 respectively.

Conclusion: The simple, precise, accurate, and sensitive UV-visible spectrophotometric method for the simultaneous estimation of Etoposide (ETO) and Picroside-II (PK-II) in a bulk and pharmaceutical formulation was developed and validated.

Keywords: UV-visible spectrophotometry, simultaneous estimation, Etoposide and Picroside-II.

Article Info: Received 25 March 2019; Review Completed 05 May 2019; Accepted 08 May 2019; Available online 15 May 2019

Cite this article as:

Bhusari S, Borse G, Wakte P, Development and Validation of UV-Visible Spectrophotometric method for simultaneous estimation of Etoposide and Picroside-II in bulk and pharmaceutical formulation, Journal of Drug Delivery and Therapeutics. 2019; 9(3):257-262 **http://dx.doi.org/10.22270/jddt.v9i3.2868**

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INTRODUCTION

Etoposide**[Fig.1]** is a semi-synthetic podophyllotoxin derived from the roots of *Podophyllumpeltatum*[1].It is a well-known anti-cancer agent used intravenously or orally[2-3]. Etoposide when administered orally shows poor and variable bioavailability which ranges from 25 to 75%[4-6].Several attempts are being made by the researchers across the globe to achieve consistent and improvised oral bioavailability of the Etoposide[7-12]. Recently, a plant based oral bioavailability enhancer has been developed for the Etoposide by the Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Briefly, Picroside-II; **[Fig. 2]** a phytochemical from rhizomes of Picrorrhizakurroa[13,] when administered with Etoposide, is found to enhance oral bioavailability of Etoposide consistently by 35%[14]. Considering therapeutic and commercial importance of combination of Etoposide and Picroside-II, it was envisaged that development of UV-Visible spectrophotometric method for simultaneous estimation of Etoposide and Picroside-II will be worth.

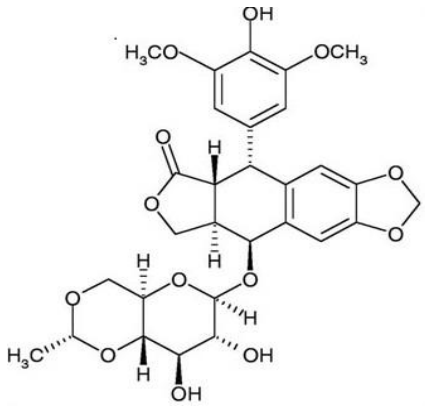

Figure 1: Chemical structure of Etoposide

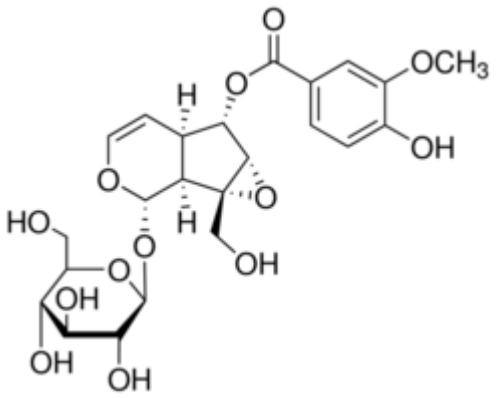

Figure 2: Chemical structure of Picroside-II

MATERIALS AND METHODS

Chemical and Reagents

Picroside-II (purity 98% by HPLC) was obtained as gift sample from Natural Products Chemistry Division of Indian Institute of Integrative Medicine (CSIR), Jammu. Etoposide, was purchased from TCI chemicals, India. Methanol (distilled) was used as solvent for preparation of diluents.

Instruments Used

A UV-visible double beam spectrophotometer with spectra manager software UV-530, Jasco was used for multi component analysis. Quartz cells having 3 cm length along with 1 cm path length were used for spectral measurement. Weighing balance (Vibra HT, Essae) with internal calibration mode was used for the accurate weighing.

Preparation of standard stock solution

The standard stock solution having concentration 1000 µg/ml(Stock-I) of each ETO and PK-II were prepared separately by dissolving accurately weighed 5 mg of API in 5 ml Methanol. Stock-I solution of both the bulk drug were further suitably diluted with solvent system Methanol to achieve the solution of concentration 100 µg/ml (Stock-II) and 10 µg/ml (Stock-III).Similarly, the standard stock solutions of combined dosage form of ETO and PK-II were prepared having the concentrations 1000 µg/ml, 100 µg/ml and 10 µg/ml.

Determination of wavelength of maximum absorbance (λmax)

The standard stock solution of ETO and PK-II having concentration 10µg/ml (Stock-III) were scanned separately in UV range from 200-800 nm against reference sample Methanol and spectrum were recorded. The λmax were determined of both bulk drug. In order to achieve accuracy the above process was repeated 4 times.

Preparation of calibration curve

Calibration curve were defined by diluting the stock-II standard solution of both bulk drug i.e. ETO and PK-II to achieve the seven different calibration standards i.e. 1, 2, 3, 4, 5, 6, and 7 µg/ml for ETO and 1, 5, 10, 15, 20, 25, 30, and 35 µg/ml for PK-II. Each calibration standard was scanned at pre-defined λmax i.e. 208 nm and 265 nm of ETO and PK-II respectively using fixed wavelength measurement mode. The absorbance at respective wavelength were noted of various calibration standards. The concentration vs. absorbance graph were plotted using Excel program of Microsoft Office 2010separately. Above mentioned procedure was repeated five times to obtain reproducible results.

Development of Simultaneous Equation

To determine both drugs by the technique of simultaneous equation method (vierodt's method), sample should contain two absorbing drugs each of which absorbs at the λmax different from the other **[Table 1]**.

The concentration of both the can be obtained by formula -

Cx = (A2ay1 – A1ay2) / (ax2ay1 - ax1ay2)

Cy = (A1ax2– A2ax1) / (ax2ay1 - ax1ay2)

Where,

λ1: Wavelength maxima for ETO

λ2: Wavelength maxima for PK-II

ax_1 and ax_2: Absorptivity of ETO at 208 nm and 265 nm

ay_1 and ay_2: Absorptivity of PK-II at 208 nm and 265 nm

A1: Absorbance of ETO at 208 nm

A2: Absorbance of PK-II at 265 nm

CX and CY: the concentration of ETO and PK-II respectively in the diluted sample.

Method Validation

The developed UV method for the estimation of ETO and PK-II in bulk drug and pharmaceutical formulation was validated as per the ICH guidelines. Various parameters like linearity and range, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were analysed using pre-defined calibration standards or quality control standards as described below [15-16].

Linearity and Range

Linearity was evaluated by linear regression analysis and calculated by least square method. The calibration curves shows correlation between absorbance and concentration level within the concentration range of 1-7 µg/ml for ETO and 1-35µg/ml for PK-II. Plots were subjected to linear regression least square analysis. R square value was important factor for establishing linearity. The interval between upper and lower concentration limit with acceptable linearity was reported to be the range of the proposed UV method.

Accuracy

The accuracy was determined by means of recovery studies by evaluating % mean recovery of both the drugs. The known concentrations of drug were added at the different level viz. 80%, 100%, and 120% level. The pentaplicate of three different pre-defined concentration solution of both the drugs i.e. ETO (1.2, 3.4, and 6.8 µg/ml) and PK-II (1, 20, and 35 µg/ml) were prepared. The absorbance was measured at wavelength 208 nm and 265 nm wavelength for ETO and PK-II respectively. The above method was perform three times.

The percent recovery was calculated by formula -

%RC= (SPS-S/SP) × 100

Where,

SPS = Amount found in the spiked sample

S = Amount found in the sample

SP = Amount added to the sample

% RC = Percent recovery

Precision

The repeatability of method was checked by statistical evaluation. Intraday and inter-day variations were studied. Five different solution of both the drug were prepared for ETO (1.2, 3.4, and 6.8 µg/ml) and PK-II (1, 20, and 35 µg/ml) and analysed at morning, afternoon and evening time of three consecutive days. Deviation in the results was calculated in terms of % RSD (% relative standard deviation).

Robustness

Robustness was determined by changing the wavelength ±1nm from 208nm for ETO and 265nm for PK-II. Middle level quality control sample of ETO (3.4 µg/ml) and PK-II (20µg/ml) was prepared and analyzed at pre-defined wavelength. The results were calculated in terms of % RSD.

Ruggedness

Ruggedness study of the method was carried out by analyzing triplicate samples of ETO (3.4 µg/ml) and PK-II (20 µg/ml) using two different instruments (V-530, Jasco and BA-UV-2600, Bioage). Results were expressed in terms of % RSD.

Limit of Detection (LOD)

The LOD of the developed UV method was determined by formula -

LOD=3.3×SD/S

Where,

SD= Standard deviation of Y-intercepts

S= Slope of calibration curve

Limit of Quantitation (LOQ)

The LOQ of the developed UV method was determined by formula -

LOQ= 10×SD/S

Where,

SD= Standard deviation of Y-intercepts

S= Slope of calibration curve

Estimation of ETO and PK-II content in pharmaceutical formulation

In-house formulation of ETO was prepared by using bio enhancer PK-II with pharmaceutically accepted excipients. The formulation contain ETO and PK-II in ratio of2:1.Weighed the quantity of powder equivalent to 2 mg of ETO and 1mg of PK-II and dissolved in 1 ml of methanol using ultra sonication and the solution was filtered using 0.22 µm filter. Filtered solution was suitably diluted to get concentration in ratio 2:1 (ETO: PK-II) and analyzed for drug content using simultaneous equation method.

RESULTS AND DISCUSSION

Determination of wavelength of maximum absorbance (λmax)

Identification of maximum absorbance wavelength is prerequisite for quantitative UV analysis. Solution with absorbance value less than 1 were considered to be appropriate for the determination of wavelength having maximum absorbance. Considering the above mentioned point determination of λmax of ETO and PK-II solution of 10 µg/ml concentration each were carried out by full scan mode of UV-Visible spectrophotometer. The full scan mode was processed by Jasco UV software and λmax were determined. The λmax was found to be 208nm and 265nm for ETO and PK-II **[Fig. 3 and Fig. 4]**respectively. The overlain spectra of both drugs shown in **Fig. 5**.

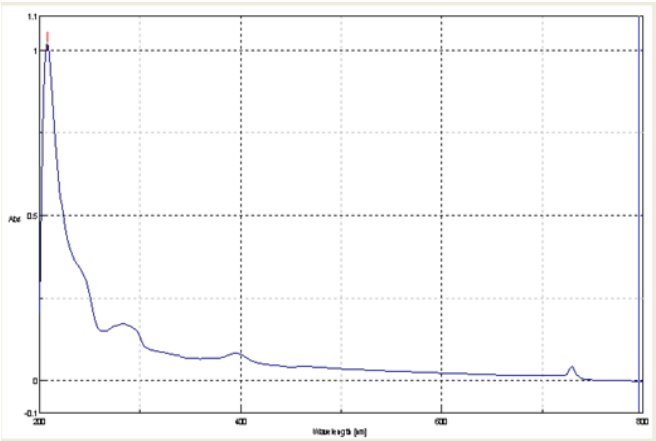

Figure 3: UV-Spectrum of ETO

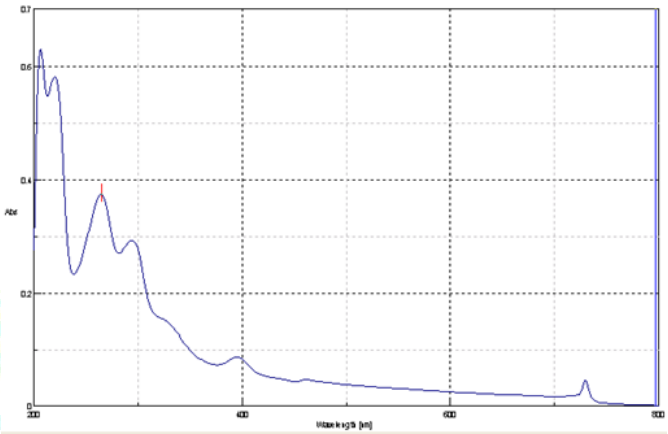

Figure 4: UV-Spectrum of PK-II

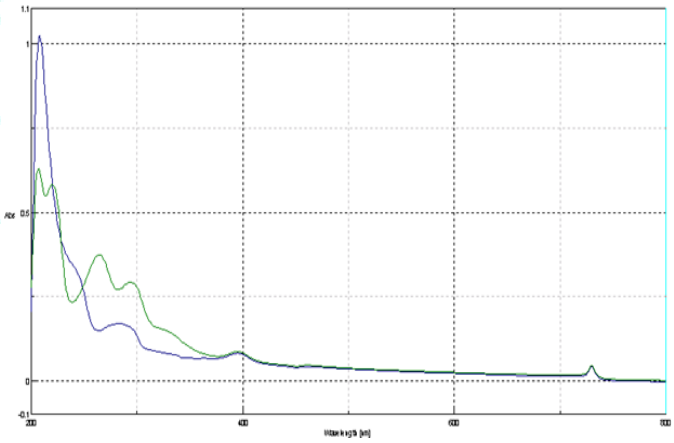

Figure 5: Overlap spectra of ETO and PK-II

Preparation of calibration curve

Quantification of unknown samples by UV-Visible spectrophotometer or any other instrumental method of analysis requires reproducible calibration curve and a mathematical equation representing correlation between concentration and the response. Considering the utility of quantitative analysis of ETO and PK-II, calibration curve for both drug were developed using seven different calibration standards. The absorbance of different calibration standards at wavelength 208 nm and 265 nm for ETO and PK-II respectively were recorded by fixed wavelength mode. Calibration curve was repeated five times.

Method validation

Linearity and Range

Linearity and range are the key parameters of analytical method which demonstrates the limit within the intended method to be used for its optimum performance. Considering the importance of linearity and the range, seven points calibration curve of ETO between the range 1-7 µg/ml and PK-II between the range 1-35 µg/ml were plotted. The concentrations and the respective mean absorbance values of ETO and PK-II are mentioned in **Table 1**. Calibration curve were subjected to least square regression analysis yielded an equation; y = 0.1238X + 0.0034 and y = 0.0252x + 0.0059with correlation coefficient and for ETO and PK-II respectively **[Fig. 6 and Fig. 7]**. The linearity study revealed that the developed UV method was found to be linear adherence to the system of Beers Law over the concentration range of 1 to 7 µg/ml for ETO and 1 to 35 µg/ml for PK-II.

Table 1 – Linearity study for ETO and PK-II

Sr. No.	Conc. (µg/ml)	Absorbance of ETO at 208 nm	Conc. (µg/ml)	Absorbance of PK-II at 265 nm
1	1	0.1302	1	0.0335
2	2	0.2505	5	0.1326
3	3	0.3803	10	0.2618
4	4	0.4893	20	0.5168
5	5	0.6219	25	0.6344
6	6	0.7378	30	0.757
7	7	0.8802	35	0.8911

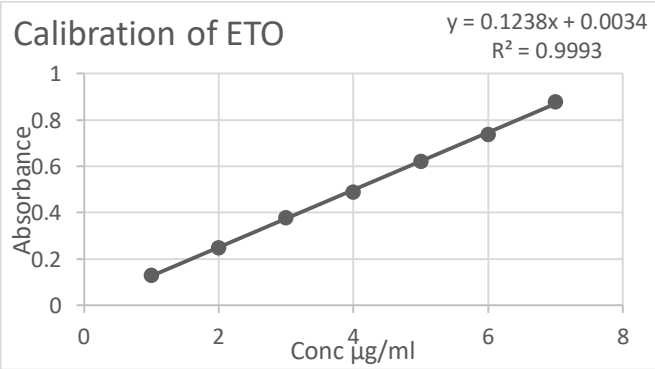

Figure 6. Calibration curve for ETO

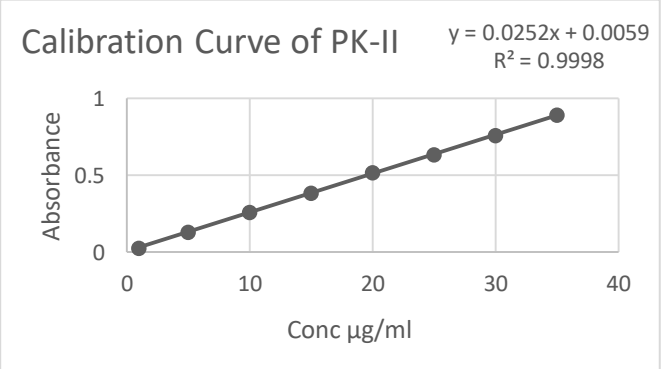

Figure 7. Calibration curve for PK-II

Accuracy

Accuracy is the measure of closeness of the experimental value to the actual amount of the substance in the matrix. Accuracy is to be established over the entire calibration range of the analytical method so that at any point of determination, results obtained would be reliable. UV method for ETO and PK-II, accuracy was established by recovery studies. Mean recovery of ETO was found to be 100.09, 98.12, and 99.06 and of PK-II was found to be 100.15, 98.32, and 100.59 at 80 %, 100% and 120% standard addition respectively. % RSD were found to be less than 2for the ETO and PK-II, recovery studies are shown in **Table 2**. The results of accuracy studies, determined that the developed UV method is highly accurate as the percent recovery was found to be between 97 to 100%.

Table 2– Recovery studies for ETO and PK-II

Origin level(µg/ml)	ETO			Origin level (µg/ml)	PK-II		
	Concentration (%)	% Recovery	% RSD		Concentration (%)	% Recovery	% RSD
1.2	80	100.09	0.18	1	80	100.15	0.98
3.4	100	98.12	1.90	20	100	98.32	0.14
6.8	120	99.06	1.85	35	120	100.59	1.77

Precision

Precision is a measure of degree of scatter, expresses the reproducibility of the measurements. It is expected that an analytical method should generate reproducible outcomes. Precise analytical method leads to accurate results. Considering the importance of reproducible and accurate results, intra-day and inter-day precision of developed UV method were established at 1.2, 3.4 and 6.8 µg/ml concentration levels of ETO and at 1, 20, and 35µg/ml concentration levels of PK-II. The results were expressed in terms of mean absorbance values, percent assay and % RSD for the intra-day and inter-day precision study, demonstrated in **Table 3** and **Table 4**respectively for ETO and PK-II. Percentage RSD values of intra-day precision

study were found to be between 0.23 and 0.70 for ETO and between 0.11 and 1.08 for PK-II whereas those of inter-day precision study were between 0.24 and1.05 for ETO and between 0.17 and 1.38 for PK-II. % RSD values were less than 2, demonstrated the precision of developed UV method.

Table 3 – Intra-day precision for ETO and PK-II

Concentration (µg/ml)	ETO			Concentration (µg/ml)	PK-II		
	Mean	% Assay	% RSD		Mean	% Assay	% RSD
1.2	0.1556	99.76	0.70	1	0.0264	100.30	1.08
3.4	0.4437	100.39	0.25	20	0.5331	99.62	0.13
6.8	0.8718	98.62	0.23	35	0.9321	100.95	0.11

Table 4 – Inter-day precision for ETO and PK-II

Concentration (µg/ml)	ETO			Concentration (µg/ml)	PK-II		
	Mean	% Assay	% RSD		Mean	% Assay	% RSD
1.2	0.1558	99.87	1.05	1	0.0264	100.17	1.38
3.4	0.4410	99.77	0.62	20	0.5336	100.17	0.12
6.8	0.8701	98.43	0.24	35	0.9333	99.76	0.17

Robustness

Robustness is the ability of a method to resist the change in its performance in spite of small un-intentional change in method parameters like solvent composition, buffer strength, pH, ±1nm wavelength etc. Change may occur and hamper the performance, it is expected that such change should not alter the performance of the analytical method. Hence, robust analytical method is studied. Robustness of proposed UV method was established by scanning the sample solution for ±1nm wavelength from 208 nm for ETO and 265 nm for PK-II. Change in the wavelength by ±1nm did not affect the performance of developed method. The% RSD values were found to be between 0.88 and 1.48 for ETO and between 0.15 and 0.23for PK-II, shown in **Table 5**for ETO and PK-II respectively. Percentage RSD values were below 2 depict that the proposed UV method was robust in nature.

Table 5– Robustness study for ETO and PK-II

Concentration (µg/ml)	ETO			Concentration (µg/ml)	PK-II		
	λmax	Absorbance	% RSD		λmax	Absorbance	% RSD
3.4	207	0.4379	0.8810	20	264	0.5169	0.233
3.4	209	0.4359	1.4876	20	266	0.5213	0.153

Ruggedness

Ruggedness is the ability to resist the change in method performance in-spite of influential environmental factors like temperature, pressure, equipment, etc. Rugged analytical methods are free from environmental/external factors impact. The ruggedness of proposed UV method, for ETO and PK-II solutions were analysed by using two different UV-Visible spectrophotometers belongs to different laboratories. Sample analysis resulted into % RSD values between 0.63 and 1.81 for ETO and between 0.23 and 0.30 for PK-II. Results showed that the proposed UV method was rugged as % RSD values were less than 2, shown in **Table 6** of ETO and PK-II.

Table 6 – Ruggedness study for ETO and PK-II

Concentration (µg/ml)	ETO			Concentration (µg/ml)	PK-II		
	Instrument	Absorbance	% RSD		Instrument	Absorbance	%RSD
3.4	Jasco	0.4383	0.63	20	Jasco	0.5169	0.23
3.4	Bioage	0.4467	1.81	20	Bioage	0.5110	0.30

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

Generally, LOQ is the first calibration standard. LOQ represents the lowermost concentration that can be analysed. LOD represents the lowest quantity of substance that can be distinguished from the absence of that substance (a blank value) with a stated confidence level (generally 99%). LOD and LOQ of proposed UV method were found to be 0.1321 and 0.4003µg/ml for ETO whereas 0.1616 and 0.4897 µg/ml for PK-II, as shown in **Table 7** for ETO and PK-II. Lower LOQ values indicated that the proposed method would be sensitive enough to quantify the ETO and PK-II content of samples at its lower level.

Table 7 – LOD and LOQ for ETO and PK-II

Sr. No.	Parameter	ETO	PK-II
1	LOD	0.1321µg/ml	0.1616µg/ml
2	LOQ	0.4003 µg/ml	0.4897µg/ml

Estimation of Etoposide and Picroside-II content in pharmaceutical formulation

The developed UV method was successfully applied for estimation of ETO and PK-II content in pharmaceutical formulation. The ETO and PK-II content in the pharmaceutical formulation was found to be 101.36% and 100.91% respectively by simultaneous equation method.

CONCLUSION

The simple, precise, accurate, and sensitive UV- visible spectrophotometric method for the simultaneous estimation of ETO and PK-II in a bulk and pharmaceutical formulation was developed and validated. The recovery result confirms the accuracy of method. The proposed method was found to be robust and rugged in nature. Thus, it can be effectively applied for the estimation of ETO and PK-II in bulk and pharmaceutical formulation.

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