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SPECTROPHOTOMETRIC AND BIOASSAY METHODS FOR THE ESTIMATION OF ERYTHROMYCIN FORMULATION

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Abstract: Erythromycin is a naturally occurring chemotherapeutic agent used in the treatment of aerobic gram positive cocci, bacilli and a few gram negative organisms. In view of its resurgent use due to resistance to anti-bacterial chemotherapy, the study sets out to provide simple, sensitive and cost-effective analytical and bioassay techniques involving UV-spectrophotometric and Microbiological methods. Method validation was by means of a precision and accuracy assays. Ten brands of erythromycin formulation comprising, seven tablets and three suspension dosage forms were assayed by UV spectrophotometric at the λmax of 285nm and bioassay methods. Thin Layer Chromatographic (TLC) method was used to establish the identity of the active ingredient in the formulations. The spectrophotometric results, expressed as a percentage of stated amounts of erythromycin were 79 - 120%w/w, which shows that 70% of the test samples conformed to the official stated standard. The bioassay determination gave MICs ranging from 0.5-2µg/ml for all the samples, 0.5µg/ml and 1.0µg/ml for reference erythromycin against *S. aureus* and *B. subtilis*, respectively. Thus, 80% of the samples met the bioassay requirement. TLC fingerprint gave Rf values ranging from 0.71 - 0.79 for 80% of the samples while the remaining 20% gave no change in colour, elution nor increase in size of the principal spot. Hence, no single method could be adequate in the determination of the quality of pharmaceutical formulations.

Keywords: UV-Spectrophotometry; Microbiological assays; Analytical methods: Erythromycin Formulation

INTRODUCTION

Erythromycin known chemically as (3R*, 4S*, 5S*, 6R*, 7R*, 9R*, 11R*, 12R*, 13S*, 14R*)-4-[(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl -6- [3,4,6-trideoxy-3-(dimethylamino)– β– D -xylohexopyranosyl] oxy] oxacyclotetradecane-2,10-dione (Fig.1). Erythromycin is produced by a strain of *Saccaropolyspora erythraea* (formerly, *Streptomyces erythreus*) and belongs to the macrolide group of antibiotics. Macrolides are a group of antibiotics that belong to the polyketide class of natural products and whose activity stems from the presence of a large macrocyclic lactone (macrolide) ring to which one or more deoxy sugars, usually cladinose or desosamine, may be attached. The lactone rings are usually 14, 15, or 16-membered [1, 2]. Erythromycin is effective against gram positive and gram negative bacteria by binding to the 23S rRNA molecule of the bacterial ribosome blocking the exit of the growing peptide chain of sensitive microorganisms [3]. It is useful in the treatment of sinusitis, otitis externa, oral infection, cholera, respiratory tract infections, syphilis, non-gonoccocal urethritis, diphtheria and whooping cough prophylaxis and Q fever in children [4, 5].

Several methods have been proposed for the analysis of erythromycin. Dehouck et al. [6] reported the reversed-phase HPLC analysis of erythromycin and benzoylperoxide in acne gel. Leal et al. [7] analyzed erythromycin and other six macrolide antibiotics by HPLC using a C-18 column, a mobile phase consisting of phosphate buffer (pH 2.5) and acetonitrile and monitored the wavelengths in a range of 204-287nm. Hilton et al. [8] used HPLC-electrospray MS in combination with solid phase extraction (SPE) for the detection of several antibiotics contaminated in water including erythromycin. Flurer et al. [9] proposed micellar electro-kinetic chromatography for the determination of β-lactam antibiotics, aminoglycoside, clindamycin phosphate and erythromycin stearate using borate buffer containing sodium dodecyl sulfate as a background electrolyte. Spectrophotometry using complex formation was also proposed for the analysis of erythromycin in formulations [10]. Gas–liquid chromatography has been used for the quantitative analysis and separation of erythromycin in mixtures containing erythromycin A (EA), erythromycin B (EB), erythromycin C (EC), anhydrous erythromycin A, erythrolosamine and propionyl erythromycin using

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flame-ionization detection (FID) and similarly, EA and EB were separated and quantified in the presence of EC, AEA and ESM in erythromycin tablets [11]. Quantitative analysis has been successfully performed in a variety of matrices including raw material, pharmaceutical dosage forms, biological fluids and various tissues [12].

The need to select a drug product over another has become very important to the health care providers because it helps to predict therapeutic efficacy of the drug product [13]. The increasing use of erythromycin in the treatment of various cases of microbiological infections due largely to increase in the resistance of gram-positive and gram negative strains, and slow bactericidal action, has necessitated the need for the development of a robust chemical assay and bioassay methods for the estimation of erythromycin in different dosage forms. Although the effectiveness of the HPLC and other techniques highlighted are not in doubt, the high cost and complex operations involved are major shortcomings. It is therefore, pertinent that the utilization of rather simple, less expensive and sensitive techniques employed in this study could be useful in a resource limited environment.

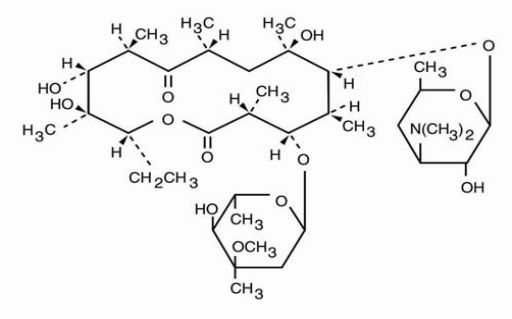

Fig.1: The Chemical Structure of Erythromycin

MATERIALS AND METHODS

Reagents/Chemicals/Organisms:

Methanol, dibasic potassium phosphate buffer pH 8, chloroform, distilled water, ethylacetate, erythromycin tablets and suspension, erythromycin reference standard (Merck, UK), Mc Farland standard of microorganisms (*S. aureus*, *B. subtilis*), disinfectants, Mueller Hinton broth, and Mueller Hinton agar. Ten brands of erythromycin comprising seven brands of tablet and three brands of suspension were sourced from pharmacies in Yenagoa and environs, South-south, Nigeria and were coded A to J. Their batch and official registration (NAFDAC) numbers and the address of the manufacturer for each brand as well as their corresponding manufacturing and expiry dates were duly documented.

Equipment:

Analytical balance (Shimadzu, Japan), pipettes, beaker, TLC plates, capillary tubes, Iodine tank, measuring cylinder, pH meter, spatula, volumetric flasks, mortar and pestle, 1cm quartz cell, UV/VIS spectrophotometer (Thermo corporation, England), wire loop, needle and syringe, tubes agar plates, autoclave, and incubator..

Weight uniformity test:

Ten tablets of each brand of erythromycin were accurately weighed one after the other using an analytical balance and the respective weights were recorded. The average weights, weight variation, standard deviation and percentage deviation of the samples were calculated. The three erythromycin test suspensions were weighed individually by placing an empty beaker in a balance, the balance was tarred and each of the suspension for each sample was emptied into the beaker, respectively, and the weight was recorded. The average weights, weight variation and percentage deviation were calculated for the samples.

Identification analysis

Powdered erythromycin tablet equivalent to 3mg was weighed into a beaker and 1ml of methanol was added to obtain a concentration equivalent to 3mg/ml. The mixture was filtered and the resultant clear supernatant was used as test solution. Standard solution of USP erythromycin was prepared by dissolving 3mg of standard erythromycin in 1ml of methanol to obtain a solution of 3mg/ml. Both standard and test solution of sample A were spotted on TLC plate using a capillary tube and allowed to dry. A solvent system consisting of a mixture of chloroform and methanol (85:15) in 10ml was prepared in an unlined chromatographic chamber and the plate was placed in the chamber until the solvent front has moved to about 7.5cm. The plate was removed from the chamber after which the solvent front was marked and the solvent was allowed to evaporate. The dried plate was placed in an iodine tank and it was observed for elution. The R_f values for both standard and test solutions were calculated as the distance moved by erythromycin divided by the distance moved by solvent front. The R_f value obtained from standard erythromycin solution was compared to that obtained from erythromycin test solution. This procedure was repeated for samples B to J used in this study.

UV Spectrophotometric analysis:

Preparation of buffer solution: Potassium di-hydrogen orthophosphate (0.2M) was prepared by dissolving 27.22g of potassium di-hydrogen orthophosphate in 1000ml of distilled water while 0.2M NaOH was prepared by dissolving 8g of NaOH in 1000ml of distilled water. Afterward, 250ml of 0.2M potassium di hydrogen orthophosphate and 250ml of 0.2M NaOH were mixed together to give a volume of 500ml and the pH of the resulting solution was adjusted to 8.

Preparation of erythromycin stock solution: Standard erythromycin (0.5g) was accurately weighed and transferred into a beaker were it was dissolved with some dibasic phosphate buffer pH 8 and methanol (1:1). The resultant solution was transferred into a 50ml volumetric flask and it was made up to mark with the buffer solution to give a stock concentration of 10mg/ml. A portion of the solution was scanned between 280-300nm.

Calibration curve for erythromycin by UV spectrophotometry: From the stock solution (10mg/ml) the following concentrations of erythromycin were prepared using a micro-pipette; 1.0µg/ml, 2.0µg/ml, 3.0µg/ml, 4.0µg/ml, 5.0µg/ml and 6.0µg/ml. The absorbance of these concentrations were measured and recorded at 285nm. The absorbance versus erythromycin concentrations were plotted using Microsoft excel 2007 version to obtain the standard curve.

Precision and accuracy: The precision and accuracy of the UV-spectrophotometric method were determined by performing five replicate analyses on the standard erythromycin solutions at three different concentrations (i.e. 1µg/ml, 5µg/ml and 10µg/ml). The In-between day precision was evaluated by running these concentrations five times within-run while the intra-day precision was performed by replicate analyses on the three drug concentrations for a period of five days with fresh solutions on each day.

Preparation of test sample: A weight equivalent to 500mg of powdered erythromycin of brand A was weighed and transferred into a beaker and dissolved with some dibasic phosphate buffer pH 8 and methanol (1:1) and filtered. The resultant solution was transferred into a 50ml volumetric flask and it was made up to mark with the buffer solution to give a stock concentration of 10mg/ml. From the prepared stock solution, concentrations of 1µg/ml and 6µg/ml were prepared using a micro-pipette and there UV absorbance were measured at the λ_{max} of 285nm. This procedure was repeated for brands B to J. The absorbance was extrapolated on the calibration curve and the percentage content of each of the test sample was calculated.

Biological assay:

The microbiological assay of ten brands of erythromycin sample A to J was determined by subjecting each of the brands to susceptibility testing against reference organisms, *S. aureus and B. subtilis* [14].

Preparation of antibiotic stock solution:

All the materials needed for this procedure were autoclaved at 121°C for 15mins including 100ml volumetric flask. 128mg of pure erythromycin powder was weighed into a sterile weighing bottle and transferred into the 100ml standard volumetric flask, it was dissolved with methanol by shaking and made up to the 100ml mark with sterile water to give a concentration of 1280µg/ml. This procedure was repeated for 128mg equivalent weight of all brands in the two dosage form (tablet and suspension) used in this study.

Determination of MIC using broth dilution method:

Twelve (12) tubes containing 5ml each of Mueller Hinton broth was prepared with the 1st tube being double strength concentration and 16ml of water was autoclaved at 121°C for 15min. The tubes were labelled from the first tube being double strength to the 12th tube where the 11th and 12th tube were designed as positive and negative controls. 4ml from stock solution (1280µg/ml) of pure erythromycin was transferred into the tube containing 16ml sterile water to obtain a concentration of 256µg/ml. 5ml of the 256µg/ml solution was transferred into tube 2 and mixed and this was repeated up to tube 10 where 5ml of the resulting solution was discarded. 0.1ml of standardized suspension of *S. aureus* was inoculated into each of the tubes with exception of tube 12, which served as negative control. This procedure was carried out in duplicate and the tubes were incubated at 37°C for 24hrs, then the tubes were observed for turbidity as evidence of growth. This procedure was also carried out with *B. subtilis* and for all the brands of erythromycin used in this study. The minimum inhibitory concentration MIC of the pure erythromycin is the least concentration of the tube without any evidence of growth. The MIC of the standard erythromycin for each of the two organisms was compared with those of the brands. The MIC results were then interpreted according to the CLSI guidelines for MIC breakpoints for each of the organism [15].

Determination of MIC using agar dilution method:

Mueller Hinton agar plates containing 128µg/ml, 64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml and 0.25µg/ml of pure erythromycin were prepared aseptically. The plates were labeled according to their concentrations and also divided into 4 portions and 2 parts were inoculated with standardized suspension of *S. aureus* while the remaining part was inoculated with *B. subtilis*. All the inoculated plates were incubated at 37°C for 24hrs and the plates were observed for growth. This procedure was carried out for all brands of erythromycin. The MIC is the plate with the least antibiotic concentration that showed no visible growth. The MIC of the standard erythromycin for each organism was compared with those of the brands. The MIC results were then interpreted according to the CLSI guidelines for MIC breakpoints for each of the organisms.

Statistical analysis:

Results were expressed as mean ± SD and CV/RSD (%) for the precision and accuracy assays and statistical analysis were carried out using GraphPad InStat Software version 2 and Microsoft Excel windows 7 (Microsoft Corporation, USA).

RESULTS

Weight Uniformity Tests:

The percentage deviation of each tablet from the average weight for samples A–J ranged from -2.76 to 3.39%.

Identification test:

The R_f value, which is calculated as the ratio of the distance moved by drug sample against the distance moved by the solvent front of the reference standard of erythromycin and the test samples A to J are shown in Table 1.

UV-spectrophotometric method:

Standard curve for erythromycin: The calibration curve for the standard erythromycin was linear over a concentration range of 1.0 to 6.0 µg/ml with the regression line equation obtained as y = 0.188x - 0.056 (R^2 = 0.995).

Precision of the analytical method: The coefficient of variation, a measure of precision, was < 1% for in-between run and was < 10% for the inter-day run, which is a measure of reproducibility of the analytical method (Table 2). Also, the relative error (%), an indicator of accuracy was within 4%.

Percentage purity for erythromycin test samples: The % purity for samples A–J determined by UV spectrophotometry using the regression equation obtained from the standard curve (Table 3). Samples A–J showed % purity, which ranged from 79.1 to 129.4%.

Biological assay method:

Microbiological assay results showing inhibition or no inhibition of the test organisms, *S. aureus* and *B. subtilis* for the erythromycin standard and test samples A to J are shown in Table 4. Table 5 gives the summary of the minimum inhibitory concentration of erythromycin test samples A to J.

Table.1: R_f values of standard erythromycin and test samples A to J

Sample code	Distance moved by solvent front (cm)	Distance moved by drug sample (cm)	R_f value
A	7.50	5.60	0.75
B	7.50	5.60	0.75
C	7.50	5.80	0.77
D	7.50	5.30	0.71
E	7.50	5.80	0.77
F	7.50	5.90	0.79
G	7.50	5.80	0.77
H	7.50	5.80	0.77
I	7.50	-	-
J	7.50	-	-
Standard	7.50	6.00	0.80

Table.2: Precision studies for ciprofloxacin (n=5)

	Expected conc.(µg/ml)	Observed mean conc. ± SD (µg/ml)	Coefficient of variation/relative error (%)
In-between run	1.0	0.96 ± 0.003	0.34
	5.0	4.80 ± 0.038	0.79
	10.0	10.50 ± 0.05	0.48
Inter-day run	1.0	0.92 ± 0.039	4.2
	5.0	5.30 ± 0.47	8.8
	10.0	9.50 ± 0.72	7.6
Accuracy	1.0	1.02 ± 0.04	4
	5.0	4.95 ± 0.15	3
	10.0	10.2 ± 0.36	3.5

Table.3: Percentage purity of Samples A to J

Sample code	Absorbance		Observed conc. (mg/ml)		Mean Observed conc. (µg/ml)	Percentage purity (%)
	1µg/ml	6µg/ml	1µg/ml	6µg/ml		
A	0.15	0.98	1.09	5.47	3.28	93.7
B	0.17	1.30	1.20	7.21	4.21	120.3
C	0.15	1.00	1.09	5.62	3.36	96.0
D	0.18	1.20	1.26	6.68	3.97	113.4
E	0.18	0.90	1.26	5.10	3.18	90.7
F	0.19	1.40	1.31	7.74	4.53	129.4
G	0.16	1.10	1.15	6.15	3.65	104.3
H	0.18	0.90	1.26	4.82	3.04	86.9
I	0.13	0.80	0.99	4.55	2.77	79.1
J	0.13	0.80	0.99	4.55	2.77	79.1

Table.5: Summary of minimum inhibitory concentration (MIC) of erythromycin test samples A to J on the test organisms, *S. aureus* and *B. subtilis*

Sample code	M I C		Remarks
	S. aureus	*B. subtilis*	
A	0.5	1	Passed
B	0.5	1	Passed
C	0.5	0.5	Passed
D	0.5	2	Intermediate
E	0.5	1	Passed
F	0.5	0.5	Passed
G	0.5	1	Passed
H	2	2	Failed
I	0.5	1	Passed
J	2	2	Failed

Table.4: Inhibitory effect of erythromycin standard and test sample A to J on *S. aureus* and *B. subtilis*

S/N	Conc. (µg/ml)	A		B		C		D		E		F		G		H		I		J		Standard	
		Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs	Sa	Bs
1	64	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	32	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	1	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	+	–	–	+	+	–	–
8	0.5	–	+	–	+	–	–	–	+	–	+	–	–	–	+	+	+	–	+	+	+	–	+
9	0.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	0.125	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MIC		0.5	1	0.5	1	0.5	0.5	0.5	2	0.5	1	0.5	0.5	0.5	1	2	2	0.5	1	2	2	0.5	1

Key: (-): inhibition; (+): no inhibition; Sa: *S. aureus*; Bs: *B. subtilis*; MIC: Minimum inhibitory concentration

DISCUSSION

UV spectrophotometry and bioassay techniques were employed in analyzing ten samples of erythromycin tablet and suspension in this study. The weight variation test carried out showed that all the samples of erythromycin passed the test because not less than an individual tablet or suspension was expected to deviate more than 5% from the average weight [14]. In this study, it was only sample G that had one tablet deviating more than 5% from the mean, which means all the samples except sample G conformed to the weight uniformity test. The implication of a tablet deviating by more than 5% from the average weight is an indication of an increase in the quantity of the active ingredient above permissible average and this could result in higher plasma concentration of such tablet beyond the maximum safe concentration when administered. It is therefore important to carry out the uniformity of weight test in order to assess the uniformity of the content of the active ingredient in each unit dose.

The TLC identification technique for the respective samples showed elution, colour change, change in size of principal spot and R_f values ranging from 0.71-0.79 except samples I and J, which did not show any elution, colour changes or increase in size of principal spot as compared with reference standard that gave R_f value of 0.80. All the samples fell within the range ≤ 0.9 point from the standard except samples I and J. This could be as a result of presence of impurities in these drug samples.

Standard erythromycin was scanned within the UV-VIS region for the maximum wavelength (λ_{max}) of absorption, which was found to be 285nm. This λ_{max} of erythromycin made it possible to determine the concentrations of erythromycin in the test samples with little or no interference. The calibration curve was linear over the concentration range of 1.0µg/ml to 6.0µg/ml and the regression coefficient (R^2=0.995) allowed for accurate determination of the concentration and hence percent purity of test samples A to J. The coefficient of variation (%), an indicator of precision and the relative error (%), a measure of accuracy of the analytical method, which were evaluated by replicate analyses of the pure drug solution at three different concentrations within working range, indicate high precision and accuracy of the method. The inter-day precision, which is a measure of the reproducibility of the method with coefficient of variation being less than 10% shows that the method was highly reproducible. The UV spectrophotometric method was therefore sensitive and precise.

The assay of Samples A–J by UV spectrophotometric method gave results that showed that not all the samples fell within the BP limits. The BP [14] specifies that erythromycin tablet or suspension must not be less than 90% and not more than 110% of the active ingredient. This clearly indicates that not all the samples contain the required amount of the active ingredients as specified by the BP. Samples A, C, E, G and H fell within the BP range while samples B, D, F, I, and J fell outside the BP range. The amount of active ingredient of 79% in sample I and J was far below the minimum stated requirement and could be said to be substandard while the amount of the active principles in samples B, D and F were above the upper limit of the BP range and could also be said to be substandard on account of overage. The implication of overage of this nature is grave since drug products are potential poison and therefore when administered at dosages exceeding their limits may predispose patients to adverse drug reactions. The failure of samples I and J to meet the quality requirement may pose great danger to the health of children as these samples are suspension, which are usually recommended for children. Again, the suboptimal amount of samples I

and J could lead to bacterial resistance to erythromycin. The findings of suboptimal amount and overage in the test samples may stem from under incorporation of active ingredient or over-incorporation of active principles to probably beat the accelerated stability testing, poor formulation, and poor storage facilities.

The result from the bioassay showed that samples A, B, C, E, F, G and I passed because they gave MIC of 0.5µg/ml for *S. aureus* and 1µg/ml for *B. subtilis*, sample D gave intermediate MIC of 0.5µg/ml for *S. aureus* and 2µg/ml for *B. subtilis* while sample H and J gave MIC of 2µg/ml for both organisms. The designation of either passed, intermediate or failed was based on comparison with standard erythromycin, which gave MIC of 0.5µg/ml for *S. aureus* and 1µg/ml for *B. subtilis* [15]. Samples H and J that failed and sample D, which gave intermediate result might be because of human error, the sub-standard nature of the brand or inadequate storage condition at the level of manufacturing and distribution, or presence of gross contaminants in the brands.

On comparing the spectrophotometric and bioassay methods, it was observed that sample J failed to meet the stated requirement for both methods while sample I passed the bioassay method, it failed to meet the stated standard using spectrophotometric analysis. TLC fingerprinting further confirms the near absence of erythromycin in samples I and J. The finding indicates that TLC fingerprint is useful in identification of the active ingredient of a drug formulation. The UV spectrophotometric method though adequate in ascertaining the purity of the erythromycin tablet and suspension, bioassay technique could be useful in detecting the real time activity of the drug.

The finding in this study shows that UV spectrophotometry and bioassay methods though relatively inexpensive and simple compared to HPLC assay methods [16, 17] are effective in the determination of the quality and quantity of active substances in erythromycin tablet and suspension. Based on the results obtained from these methods, it is very important to combine various simple, precise, and sensitive methods of analysis to authenticate the quality of drug samples because of error and limitation of some of the analytical methods. When a drug conforms to standards as stated in the official monograph, it gives assurance of the quality and predicts therapeutic efficacy as well as safety of the drug. It is therefore necessary for both manufacturers and regulatory bodies to utilize more than one analytical method in the determination of the quality of active drug in pharmaceutical preparations.

CONCLUSION

In conclusion, erythromycin can be successfully analyzed using UV spectrophotometry and bioassay methods. The use of various simple, precise, and sensitive methods in combination for the determination of active drug in pharmaceutical formulation is very essential to authenticate the analytical processes especially in resource limited environment. Therefore, no single method applied in isolation is sufficiently accurate in providing enough data or information on the quality of a drug product.

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