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Electrochemical methods for determination of the protease inhibitor indinavir sulfate in pharmaceuticals and human serum

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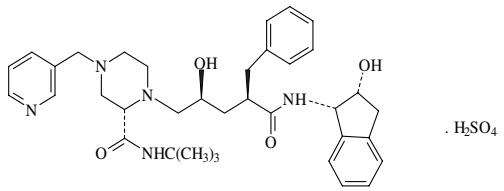
Indinavir sulfate is an inhibitor of the human immunodeficiency virus (HIV) protease. The aim of this study was to determine indinavir levels in serum and pharmaceuticals, by means of electrochemical methods using the hanging mercury drop electrode (HMDE). Indinavir exhibited irreversible cathodic waves over the pH range 2.00–12.00 in different supporting electrolytes. The current-concentration plot was rectilinear over the range from 8×10^{-7} M to 8×10^{-6} M with a correlation coefficient of 0.996 for differential pulse voltammetry (DPV) and 8×10^{-7} M to 1×10^{-5} M with correlation of 0.999 M for osteryoung square ware voltammetry (OSWV) in Britton-Robinson buffer at pH 10.00. The wave was characterized as being irreversible and diffusion-controlled. The proposed methods were fully validated and successfully applied to the determination of indinavir in capsules and spiked human serum samples with good recoveries. The repeatability and reproducibility of the methods as well as precision and accuracy (such as supporting electrolyte, serum samples) were determined. No electroactive interferences from the endogenous substances were found in serum samples.

1. Introduction

Indinavir sulfate [1(1*S*,2*R*),5(*S*)]-2,3,5-trideoxy-*N*-(2,3-dihydro-2-hydroxy-1*H*-inden-1-yl)-5-[2-[[(1,1-dimethylethyl)-amino]carbonyl]-4-(3-pyridinylmethyl)-1-piperazinyl]-2-(phenylmethyl)-D-erythro-pentonamide sulfate is an inhibitor of the human immunodeficiency virus (HIV) protease.
Indinavir has a low protein binding ranging from 60 to 70% in species and is extensively metabolized in all species by the cytochrome P450 system.



Indinavir sulfate

Indinavir has been determined by: spectrophotometry (Sarma et al. 2003; Erk 2004a; Sarma et al. 2002; Sankar 2002), capillary electrophoresis (Prado et al. 2004), liquid chromatographic (Jayewandene et al. 2001; Fizzano et al. 2000; Poirier et al. 1999) and HPLC (Suensson et al. 2000; Zhong et al. 1999; Li et al. 1999).
All the reported methods are laborious, time-consuming and require highly sophisticated instrumentation. Only one paper reported anodic voltammetry using a glassy carbon electrode (Erk 2004b). No analytical studies about the determination and reduction mechanism of indinavir in bulk form, pharmaceutical dosage forms and biological fluids have been reported. This led us to study its polarographic behavior in an attempt to develop a simple, sensitive and reliable method for its quantitative determination in pharmaceutical dosage forms and biological fluids and the results were promising. The high sensitivity attained adopting DPV and OSW techniques as an alternative substitute for HPLC.

2. Investigations, results and discussion

Cyclic, linear sweep and square wave voltammograms for 6×10^{-5} M solutions of indinavir for pH values within the range 2.0–12.00 (different buffer solutions) were recorded.
Indinavir was electrochemically reduced in a broad pH range using the hanging mercury drop electrode (HMDE). The cyclic voltammetric (CV) behavior of indinavir yielded one well-defined and sharp peak in Britton-Robinson buffer at pH 10.0 at −1477 mV. As pH decreased only one broad peak was observed (Fig. 1). Absence of cathodic peak on reverse sweep at pH 10.0 indicates irreversibility of the electrode process.
Due to the best-resolved signal obtained by CV, the effect of pH on peak potential and peak intensity were studied. The peak potential of the reduction process moved to a more negative potential and the reduction peak was getting sharper by raising the pH.
All obtained graphs were found to be similar. For this reason only a CV graph for the peak was given as Fig. 2a and 2b. The plot of the peak potential versus pH (Fig. 2a) showed two linear segments between 2.0 and 12.0. The linear segments can be explained by the following equations:

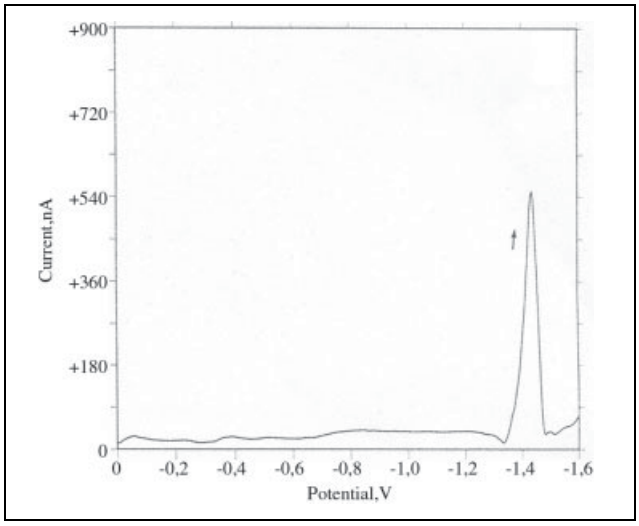


Fig. 1: Linear sweep voltammograms of 6×10^{-5} M indinavir in Britton Robinson buffer pH 10.00

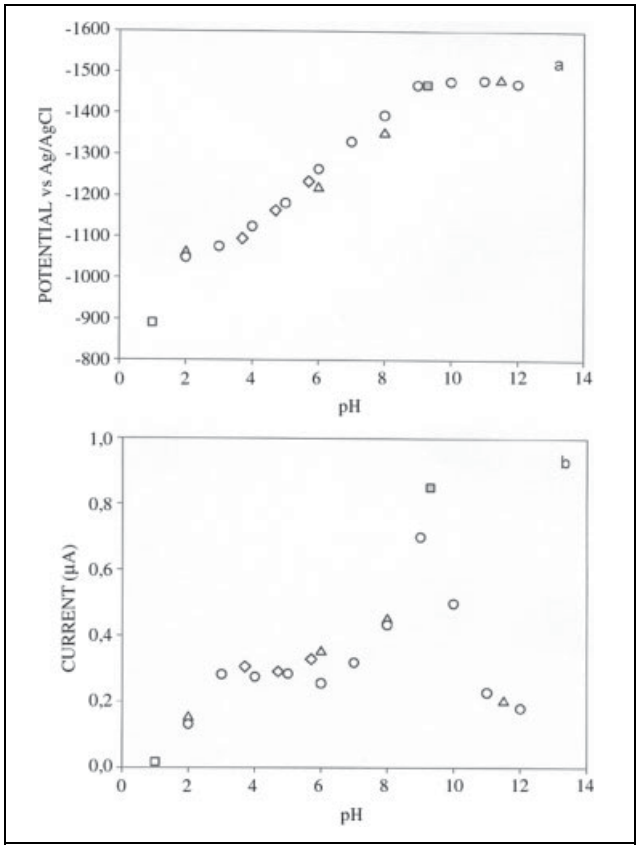


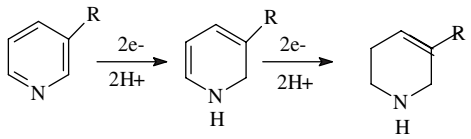
Fig. 2: Effect of pH on indinavir peak potential (a); and peak current (b); (□) 0.1 M H_2SO_4, (◇) acetate buffer, (△) phosphate buffer, (○) Britton-Robinson buffer, (■) Borate buffer solutions. Indinavir concentration 6×10^{-5} M, scan rate 100 mVs^{-1}

$$\mathrm{Ep(mV)} = 982.40 + 33.08\,\mathrm{pH} \quad (\mathrm{r} = 0.9991 \text{ between } 2.0 - 3.7) \qquad (1)$$

$$\mathrm{Ep(mV)} = 836.93 + 70.05\,\mathrm{pH} \quad (\mathrm{r} = 0.9993 \text{ between } 3.7 - 9.0) \qquad (2)$$

The linear segments become practically pH independent between 9.0 and 12.0. That indicates participation of a proton transfer in the electrode process. One of the intersection points of the curves (3.7) is close to the pK_a value of pyridine molecule which is pK_a about 5.0, is not different from the pK_a values reported for indinavir (htpp://www.emea.eu.int). Indinavir has another intersection point at 9.0. These intersection points can be explained by changes in protonation of the acid-base functions in the molecule.

Scheme: Possible reduction mechanism of indinavir (Grimshaw 2000)



For indinavir the slope of Ep pH plot is −33 mV per unit over the pH 2.0–3.7 and is −70 mV per unit over the pH 3.7–9.0 and then becomes pH independent, which suggests that three protons and electrons are involved in the reduction mechanism. Considering the above results and bearing in mind the electrochemical behavior of pyridine, the parent moiety of indinavir at HMDE we may assume that the reduction process is located on the pyridine moiety in the molecule (Grimshaw 2000) (Scheme). Electron transfer reduction of pyridines in both acid and alkaline solution generates a protonated radical anion. This rapidly accepts a further electron and a proton to give dihydropyridines. Further reaction of the 1,2-dihydropyridine leads to a tetrahydropyridine. We may assume that the reduction mechanism of indinavir involves the total gain of 4 electrons like the pyridine molecule. We could not obtaine the fourth electron and proton, probably because of the differences in the molecular structure of indinavir and pyridine molecules.

The effect of pH on the peak current at HMDE was also studied with all supporting electrolytes and pH values. The ip versus pH curves (Fig. 2b) show that the peak current is maximum at pH 9.0 Britton-Robinson buffer and pH 9.3 borate buffers. However we selected pH 10.0 Britton-Robinson buffer due to the good shape of peak.

The reduction peak is displayed to more negative potential values when the scan rate increases, what is a characteristic behavior of irreversible processes. The absence or ill-defined shape of the anodic peak related to the reduction peak with a 48 mV negative shift in the cathodic peak potential confirmed the irreversibility of the reduction process in this media. Scan rate studies were also carried out to assess whether the processes at HMDE were under diffusion or adsorption control. When plotting ip versus square root of the scan rate ($\upsilon^{1/2}$ mV s^{-1}) a linear relationship was observed as with diffusion-controlled electrode process according to the following equation:

$$\mathrm{ip}(\mu\mathrm{A}) = 0.062\,\upsilon^{1/2}\,(\mathrm{mV\,s^{-1}}) - 0.14 \quad (\mathrm{r} = 0.990 \text{ (between } 5 - 1000\,\mathrm{mV\,s^{-1}}) \qquad (3)$$

For a diffusion current the plot of log ip as a function of log υ should have a slope of 0.5 and for a purely adsorption current a slope of 1.0. The observed slope value 0.57 indicates a diffusion controlled electrode process (Laviron 1980) in a solution containing 6×10^{-5} M indinavir at pH 10.0. The equation obtained is:

$$\log \mathrm{ip}(\mu\mathrm{A}) = 0.57 \log \upsilon\,(\mathrm{mV\,s^{-1}}) - 1.49 \quad (\mathrm{r} = 0.990 \text{ (between } 5 - 1000\,\mathrm{mV\,s^{-1}}) \qquad (4)$$

Tafel plot was obtained with a scan rate of 5 mV s^{-1} beginning from a steady-state potential in Britton-Robinson

Table 1: Regression data of the calibration lines for quantitative determination of indinavir by DPV and OSWV in supporting electrolyte and human serum samples

	DPV		OSWV	
	Supporting Electrolyte	Serum	Supporting electrolyte	Serum
Measured potential (V)	0.75	0.72	0.79	0.76
Linearity range (M)	8×10^{-7}–8×10^{-6}	8×10^{-7}–8×10^{-6}	8×10^{-7}–1×10^{-5}	8×10^{-7}–1×10^{-5}
Slope ($\mu A\ M^{-1}$)	6.57×10^4	6.87×10^4	6.40×10^4	8.39×10^4
Intercept (μA)	−0.032	0.057	−0.032	0.026
Correlation coefficient	0.996	0.999	0.999	0.997
SE of slope	1.91×10^3	1.84×10^3	1.49×10^3	2.89×10^3
SE of intercept	0.0086	0.0083	0.0084	0.0163
LOD (M)	1.26×10^{-7}	1.43×10^{-7}	1.57×10^{-7}	1.28×10^{-7}
LOQ (M)	4.21×10^{-7}	4.75×10^{-7}	5.23×10^{-7}	4.28×10^{-7}
Repeatability of peak current (RSD %)	0.55	0.23	0.44	0.81
Repeatability of peak potential (RSD %)	0.16	0.17	0.16	0.17
Reproducibility of peak current (RSD %)	1.45	0.93	0.72	1.52
Reproducibility of peak potential (RSD %)	0.15	0.17	0.16	0.16

buffer at pH 10.0. From the slope of the linear part αn was found to be 0.47. The Exchange current density (I_0) is 2.82×10^{-12} A cm^{-2} for this system.

2.1. Validation of the procedure

Validation of the procedure for the quantitative assay of the drug was examined by evaluation of the limit of detection (LOD), limit of quantification (LOQ), repeatability, reproducibility, precision, accuracy and recovery. The regression equations associated with the calibration curves (Table 1) exhibited good linearity for both techniques that supported the validation of the proposed procedure.

LOD and LOQ were calculated on the peak current using the following equations (Riley and Rosanske 1996, Swartz and Krull 1997).

$$\text{LOD} = 3\,s/m; \quad \text{LOQ} = 10\,s/m \tag{5}$$

where s is the standard deviation of the peak current (three runs) and m the slope of the calibration curve. The LOD and LOQ values are also shown in Table 1. Both LOD and LOQ values confirmed the sensitivity of the proposed methods.

Repeating five experiments on 8×10^{-6} M indinavir for both techniques tested the repeatability (1 day) and reproducibility (over a week) of peak potential and peak current. The results are shown in Table 1.

The repetition of sample analysis after a 96 h period did not show any significant and appreciable change in assay results.

2.2. Analysis of Crixivan® capsules

The developed procedure was successfully applied to the determination of indinavir sulfate in its pharmaceutical formulation. There was no need for any extraction step prior to the drug analysis. The accuracy of the methods was determined by its recovery during spiked experiments. In order to detect interactions of excipients the standard addition technique was applied to the same preparations, which were analyzed. The results demonstrate the validity of the proposed methods for the determination of indinavir sulfate in capsule.

The percentage recovery of the drug in capsules, based on the average of five replicate measurements was found to be 99.7% ± 0.51 for DPV 100.0% ± 0.8 for OSWV. The mean percentage recovery showed no significant excipient interferences, thus the procedure was able to assay indinavir in the presence of excipients and hence it can be considered specific. The results were in good agreement with the label claim and the proposed techniques were sufficiently accurate and precise in order to be applied to pharmaceutical dosage forms.

Table 2: Assay results from indinavir capsules (Crixivan®) and mean recoveries in spiked capsules

	DPV	OSWV
Labeled claim (mg)	200.00	200.00
Amount found (mg)[a]	199.34	200.04
RSD %	1.27	0.65
Bias %	0.33	−0.20
Added (mg)	20.00	20.00
Found (mg)[a]	20.07	20.16
Recovery %	100.36	100.80
RSD % of recovery	0.51	0.81
Bias %	0.36	−0.80

[a] Each value is the mean of 5 experiments

2.3. Analysis of spiked human serum

Indinavir was successfully determined in spiked human serum samples by applying the optimized procedure without any prior extraction steps. Acetonitrile was tried as a serum precipitating agent. No extraction steps other than the centrifugal protein separation were required prior to the assay of indinavir. Figs. 3a and 3b illustrate DP and OSW voltammograms obtained from spiked serum samples at different concentrations of indinavir following the optimized conditions. Obtained recovery results of spiked human serum samples are given in Table 3.

$$\text{For DPV } I(\mu A) = 6.87 \times 10^4\, C(M) + 0.057 \quad n = 5\ (r = 0.9987) \tag{6}$$

$$\text{For OSW } I(\mu A) = 8.39 \times 10^4\, C(M) + 0.026 \quad n = 7\ (r = 0.9970) \tag{7}$$

The estimated detection limits for both methods are shown in Table 1. The amount of indinavir in serum was calculated from the related linear regression equation for both techniques. The precision and accuracy of indinavir in serum were assessed from five replicates at 6×10^{-6} M. Good recoveries of indinavir were achieved from serum (Table 3). The LOD, LOQ, repeatability and reproducibility of peak current and peak potential are also shown in

Table 3: Application of the DPV and OSWV methods to the determination of indinavir in spiked human serum samples

Technique	Medium	Indinavir added (M)	n	Indinavir	Average found (M)	RSD % recovery	Bias %
DPV	Serum	6×10^{-6}	5	5.9877×10^{-6}	99.796	0.70	0.20
OSWV	Serum	6×10^{-6}	5	5.9910×10^{-6}	99.850	1.72	0.15

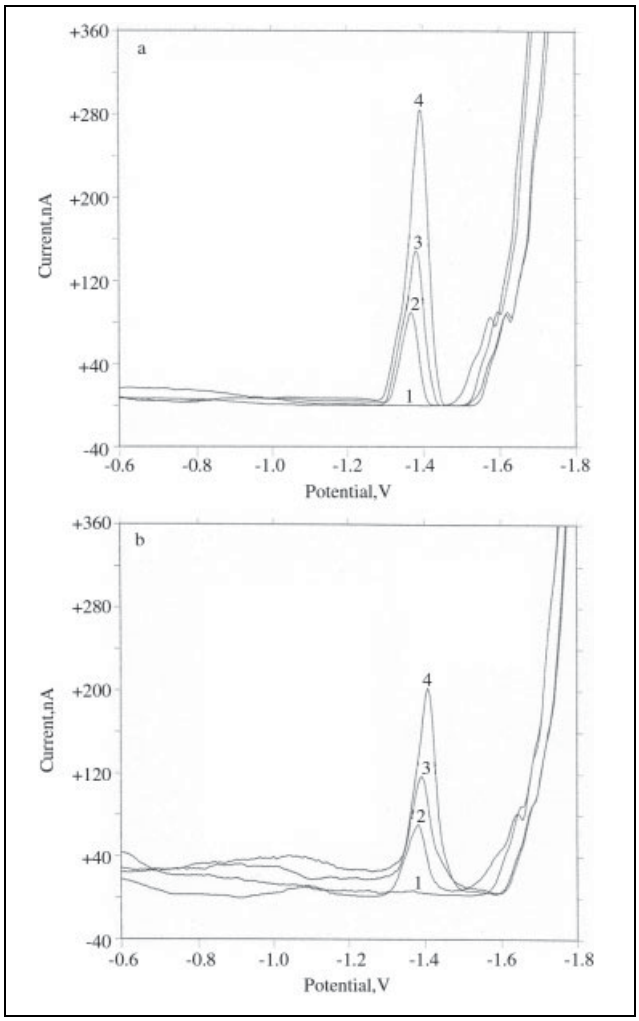


Fig. 3: Differential pulse (a) and Osteryoung square wave (b) voltammograms in Britton-Robinson buffer at pH 10.00 obtained for the determination of indinavir in spiked serum. (1) Blank (2) 8×10^{-7} M (3) 1×10^{-6} M (4) 2×10^{-6} M indinavir extract in Britton Robinson buffer at pH 10.00

Table 1. Typical DPV and OSWV curves of indinavir are given in Figs. 3a and 3b; no reduction compounds and no extra noise peak present in biological material peak occurred in the potential range where the analytical peak appeared.

Samples were stable over a period of approximately 8 h. No significant changes were observed in the peak currents and potentials between the first and last measurements.

3. Experimental

3.1. Apparatus

The cyclic, linear sweep, DP (differential pulse) and OSW (osteryoung square wave) voltammetric experiments at a hanging mercury drop electrode (HMDE) were performed using a BAS 100 W electrochemical analyzer. A three electrode cell systems incorporated HMDE as working electrode, an Ag/AgCl (3 M KCl) reference electrode: all potentials are quoted relative to this electroda. A platinum-wire auxiliary electrode was also used. The pH value of the solutions was measured using a Model 538 pH meter (WTW, Austria) using a glass electrode with an accuracy of ± 0.05 pH and calibrated with standard buffers at room temperature.

Operating conditions for DPV were: pulse amplitude: 50 mV; pulse width: 50 ms, sample width: 17 ms; pulse period: 200 ms, scan rate: 20 mVs^{-1} and OSW conditions were: pulse amplitude: 25 mV; frequency: 15 Hz; potential step: 4 mV.

Cyclic voltammetry: The initial and final potential were variable, depending on the pH value and the cut-off the electrolyte. Scan rate measurements in the range 5–1000 mV s^{-1} were carried out.

3.2. Reagents

Indinavir sulfate and its pharmaceutical formulation were kindly provided by Merck Sharp & Dohme Pharm. Ind. (Istanbul, Turkey). Crixivan® capsules (Merck Sharp & Dohme Pharm. Ind., Istanbul, Turkey) were labeled to contain 200 or 400 mg per capsule. The 200 mg Crixivan® capsules were selected for this study. The inactive ingredients (anhydrous lactose and magnesium stearete, dyes, gelatin, titanium dioxide, silicon dioxide and sodium lauryl sulfate) are commercially available products (http://www.rxlist.com). All chemicals for preparation of buffers and supporting electrolytes were reagent grade (Merck or Sigma). Stock solutions of indinavir sulfate (1×10^{-3} M) were prepared in bi-distilled water and kept in the dark in a refrigerator.

All other stock solutions were also prepared in bi-distilled water and kept in the dark in a refrigerator in order to minimize decomposition. All experiments were performed at room temperature. Dissolved oxygen was removed by passing pure nitrogen (99.99%) through the solution for 3.0 min prior to electrochemical analyses. Five different supporting electrolytes, namely sulphuric acid (0.1 M), phosphate buffer (0.2 M, pH 2.0–11.5), acetate buffer (0.2 M, pH 3.7–5.7), borate buffer at pH 9.30 and Britton-Robinson buffer (0.04 M, pH 2.0–12.0) were prepared in doubly distilled water. Standard solutions were prepared by dilution of the stock solution with the selected supporting electrolyte to give solutions containing indinavir in the concentration range of 8×10^{-7} M to 1×10^{-5} M. The calibration curve for DPV and OSW analysis was constructed by plotting the peak current against the indinavir concentration between 8×10^{-7} and 8×10^{-6} and 8×10^{-7} and 1×10^{-5} M, respectively. The ruggedness and precision were checked at different days, within day ($n = 5$), and between days ($n = 5$) for three different concentrations. Relative standard deviations were calculated to check the ruggedness and precision of the method (Riley and Rosanske 1996; Swartz and Krull 1997).

The precision and accuracy of analytical methods are described in a quantitative fashion by the use of relative errors (bias %). One example of relative error is the accuracy, which describes the deviation from the expected results. Current-potential curves of sample solutions recorded after 96 h after preparation did not show any appreciable change in assay values.

3.3. Capsule assay procedure

Indinavir sulfate was determined in commercial capsules of Crixivan® by using voltammetric techniques. The content of ten capsules was weighed and powdered. A weighed portion of the powder content equivalent to 1×10^{-3} M of indinavir was transferred into a 50 mL-calibrated flask and completed to the volume with bi-distilled water. The contents of the flask were sonicated 15 min to affect complete dissolution. Appropriate solutions were prepared by taking suitable aliquots of the clear supernatant liquor and diluted with the selected supporting electrolyte. Voltammograms were recorded as in pure indinavir sulfate. The nominal content of the drug in capsules was determined referring to the regression equation.

3.4. Recovery studies

In order to exclude interferences by excipients, known amounts of the pure drug were added to the different preanalyzed formulations of indinavir sulfate and the mixtures were analyzed by the proposed techniques. After five repeated experiments, the recovery results were calculated using the calibration equation.

3.5. Analysis of spiked human serum

Serum samples, obtained from healthy individuals (after obtaining their written consent) were stored frozen until assay. An aliquot volume of serum sample was fortified with drug dissolved in bi-distilled water to achieve a final concentration of 1×10^{-3} M. Acetonitrile removes serum proteins more effectively, as the addition of 1 volume to 1.5 volumes of

serum is sufficient to remove of the proteins. After vortexing for 30 s, the mixture was centrifuged for 10 min at 5000 rpm for getting rid of serum protein residues and the supernatant was discarded. The concentration of indinavir was varied in the range of 8×10^{-7} M to 8×10^{-6} M for DPV and 8×10^{-7} M to 1×10^{-5} M for OSWV in human serum samples. These solutions were analyzed in the voltammetric cell containing Britton-Robinson buffer at pH 10.00. The amount of indinavir in spiked human serum samples for the recovery studies was calculated from the related calibration equation.

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