Analytical Biochemistry 395 (2009) 86–90

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Horseradish peroxidase covalent grafting onto screen-printed carbon electrodes for levetiracetam chronoamperometric determination

M. Asunción Alonso-Lomillo *, Olga Domínguez-Renedo, Alba Hernández-Martín, M. Julia Arcos-Martínez

Department of Analytical Chemistry, Faculty of Sciences, University of Burgos, 09001 Burgos, Spain

ARTICLE INFO

Article history:
Received 30 June 2009
Available online 7 August 2009

Keywords:
Screen-printed electrodes
Enzymatic biosensors
Aryl diazonium salt
Levetiracetam
Pharmaceutical drugs

ABSTRACT

A new enzymatic electrochemical biosensor based on disposable transducers, namely screen-printed carbon electrodes, has been developed for the determination of the antiepileptic drug levetiracetam. Horseradish peroxidase was immobilized onto the carbon working electrode previously modified by an aryl diazonium salt. The formation of amide bonds between the amino and carboxylic groups of the enzyme surface, catalyzed by hydroxysuccinimide and carbodiimide, leads to the electrode functionalization. This orientated enzymatic modification results in high reproducibility, with an associated relative standard deviation of 6.21% for the slopes of several calibration curves in the calibration range from 0.10 to 0.83 mM. Experimental variables that can affect levetiracetam chronoamperometric response, such as hydrogen peroxide concentration, pH, and applied potential, were optimized to perform a selective determination. An average limit of detection of 1.75×10^{-5} M ($\alpha = \beta = 0.05$) was obtained. The biosensors were finally applied to the determination of levetiracetam in complex matrices such as pharmaceutical drugs, yielding successful results.

The second-generation antiepileptic drug levetiracetam (LEV,[1] (*S*)-α-ethyl-2-oxo-pyrrolidine acetamide) has been used for clinical purposes in a broad spectrum of applications since 2002. Approximately 76% of LEV is eliminated unchanged, with 95% of drug elimination occurring via urine [1]. The determination of LEV concentration might be of value in optimizing therapeutic effects while reducing adverse effects, especially for patients with renal impairment as well as for elderly and pediatric patients [2]. This fact points out the need for selective and sensitive methods of LEV analysis.

LEV has been analyzed by methods that require pretreatment of the samples such as solid-phase extraction, deproteinization by the addition of organic solvents, and formation of insoluble salts. In this way, high-performance liquid chromatography (HPLC) [3–6], gas chromatography (GC) [3], capillary electrophoresis (CE) [7], and HPLC–electrospray tandem mass spectrometry (HPLC–ESI–MS/MS) [2,8] techniques have been used. Also reported has been an electrochemical procedure based on the use of horseradish peroxidase (HRP), leading to a selective biosensor for monitoring LEV concentrations without any sample pretreatment [9].

Enzymatic electrochemical biosensors based on disposable transducers, such as screen-printed electrodes (SPEs), have become very attractive for environmental, clinical, and food analysis over the past 20 years [10,11]. The current tendency is to develop miniaturized systems with known characteristics such as versatility, relatively low cost of electrochemical instrumentation, and high sensitivity and selectivity that enable determination in situ and on-line.

Screen printing technology has made it possible to mass produce inexpensive, ready-to-use, disposable electrodes for use with electrochemical devices. Furthermore, SPEs increase the versatility of electrochemical techniques due to the wide range of their possible modifications.

HRP uses hydrogen peroxide to oxidize a wide variety of organic and inorganic one-electron donor compounds such as aromatic phenols, phenolic acids, indoles, amines, and sulfonates [12]. The mechanism of HRP electrochemical biosensors is well known. It involves the oxidation of native HRP by H_2O_2 to an intermediate compound, which is subsequently reduced by a substrate donor or directly at the electrode, regenerating the native enzyme [12–14]. Integration of the recognition element with the signal transducer has been shown to be the critical step for the success of biosensors. It has usually been achieved by modifying the surface of the transducer with a chemical layer that enables the immobiliza-

* Corresponding author. Fax: +34 947258831.
E-mail address: malomillo@ubu.es (M.A. Alonso-Lomillo).

[1] *Abbreviations used:* LEV, levetiracetam; HPLC, high-performance liquid chromatography; GC, gas chromatography; CE, capillary electrophoresis; ESI–MS/MS, electrospray tandem mass spectrometry; HRP, horseradish peroxidase; SPE, screen-printed electrode; SPCE, screen-printed carbon electrode; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; $N_2C_6H_4NO_2$ BF_4^-, 4-nitrobenzenediazonium tetrafluoroborate; NBu_4BF_4, tetrabutylammonium tetrafluoroborate; LOD, limit of detection; RSD, relative standard deviation.

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doi:10.1016/j.ab.2009.08.004

tion of the enzyme [15]. Electrogenerated polypyrrole membranes, which have proved to be a very successful strategy for the development of enzyme-based sensors, have already been described for the entrapment of the enzyme HRP in the design of LEV biosensors [9].

During recent years, monolayer strategies for enzyme immobilization have become increasingly popular because they provide controlled and oriented recognition interfaces [15,16]. The electrochemical grafting of diazonium salts [17], which has been studied extensively [18], has also been applied to SPEs [19–25]. The procedure described by Alonso-Lomillo and coworkers [25], which involves the covalent immobilization of an enzyme onto screen-printed carbon electrodes (SPCEs) by means of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), has been followed for the development of a new HRP-based biosensor for LEV determination.

Thus, LEV becomes the substrate of the enzymatic reaction at the electrode surface, implying the formation of a product that can be monitored by chronoamperometry. To develop a selective and sensitive sensor, the experimental variables that strongly affect its chronoamperometric response were evaluated. It is usually most efficient to estimate the effects of several variables simultaneously [26]. In this way, applied potential (E_{ap}), pH, and concentration of H_2O_2 ($C_{H_2O_2}$) were assessed by means of the experimental design methodology [26,27]. The performance of the developed biosensors was checked in terms of reproducibility, repeatability, and application to complex matrices such as pharmaceutical drugs.

Materials and methods

Reagents

Several inks were used in the fabrication of SPEs, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink), and Electrodag 452 SS (dielectric ink) supplied by Acheson Colloiden (Scheemda, Netherlands).

All solutions were prepared with water purified with a Milli-Q device that provided resistivity of 18.2 MΩ cm. Nitrogen (99.99%) was used to remove dissolved oxygen.

4-Nitrobenzenediazonium tetrafluoroborate ($N_2C_6H_4NO_2$ BF_4^-, Sigma, Steinheim, Germany) was dissolved in acetonitrile, 0.1 mol dm^{-3} tetrabutylammonium tetrafluoroborate (NBu_4BF_4, Sigma–Aldrich, Steinheim, Germany).

HRP solutions (0.0015%, w/v, EC 1.11.1.7, Sigma) in 10 mM phosphate buffer (pH 6) were used.

NHS (Aldrich, Steinheim, Germany), and EDC (Fluka, Steinheim, Germany) solutions were prepared in 10 mM phosphate buffer (pH 6).

Stock standard solutions of LEV (kindly donated by UCB Pharma SA) were prepared by dissolving the appropriate amount in water.

As supporting electrolyte, 0.05 M phosphate buffer ($NaH_2PO_4{\cdot}2H_2O$, Panreac, Barcelona, Spain) and 0.1 M KCl (Merck, Darmstadt, Germany) solution was used. To adjust the pH value, 1 M NaOH (J.T. Baker, Deventer, Netherlands) was used.

Apparatus and software

SPEs were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke.

Electrochemical measurements were made with a μAutolab II electrochemical system with GPES software (Eco Chemie, Utrecht, Netherlands).

The pH of the solutions was measured with a Crison model 2002 pH meter (Barcelona, Spain).

Data analysis was processed with a STATGRAPHICS Plus software package for the experimental design process [28], PROGRESS for the robust regression [29], and DETARCHI for the limit of detection (LOD) [30].

Methods

Manufacturing of SPEs and electrochemical pretreatment

A DEK 248 screen-printing system, screen polyester mesh, and polyurethane squeegees were used to fabricate the electrodes, as described previously [25]. Briefly, sequential layer deposition was performed on a polyester film (0.5 mm thickness), obtaining 44 screen-printed configurations of three electrodes (working, reference, and counter electrodes) per film. 4 mm^2 carbon working electrodes were design.

Before modifying the SPCEs, a mechanical cleaning was carried out, sanding the counter and working electrode surfaces with thin grain sandpaper. Then the working electrode surface was activated by recording 20 cycle voltammograms between +2 and −2 V versus screen-printed Ag/AgCl reference electrode at a scan rate of 100 mV s^{-1} in a 0.1-M KCl solution [24,25].

SPCE surface modification

Electrochemically pretreated electrodes were immersed in a 3 mmol dm^{-3} solution of $N_2C_6H_4NO_2$ BF_4^- in acetonitrile and 0.1 mol dm^{-3} NBu_4BF_4. The grafting process was conducted by sweeping the potential between +800 and −400 mV versus screen-printed Ag/AgCl reference electrode at a scan rate of 200 mV s^{-1} [25]. Two sequential scans were performed.

Reduction of the 4-nitrobenzene groups to aminobenzene groups was followed by cyclic voltammetry between +200 and −1800 mV versus screen-printed Ag/AgCl reference electrode at a scan rate of 200 mV s^{-1}, two scans, in a deoxygenated 9:1 water/ethanol solution and 0.1 mol dm^{-3} KCl [25].

HRP electrode modification was achieved by modifying of the 4-aminobenzene-functionalized electrodes by the formation of amide bonds between the amino and carboxylic groups of the enzyme surface catalyzed by EDC and NHS [31]. The working electrode was washed using 10 mmol dm^{-3} phosphate buffer (pH 6), and then 5 μl of the HRP solution was dropped over it. Next, 2 μl of a 20 mmol dm^{-3} NHS solution and 2 μl of a 40 mmol dm^{-3} EDC solution in 10 mmol dm^{-3} phosphate buffer (pH 6) were added. It was left to react for 90 min at room temperature. The biosensor was finally washed in a stirred 50 mmol dm^{-3} phosphate buffer and 0.1 mmol dm^{-3} KCl solution at pH 7 for 1 min to eliminate the enzyme not covalently attached. The biosensor was stored in a blank buffer solution at 4 °C.

Results and discussion

LEV is a pyrrolidine derivative that can act as a substrate donor for the reduction of the HRP previously oxidized by H_2O_2. This reaction product is susceptible to suffering an electrochemical reaction at the electrode surface that can be followed by chronoamperometry.

The production of the biosensor for LEV determination implies several steps. The first step, after SPCE fabrication, is the modification of the working electrode by an aryl diazonium salt that is going to be the binding point for the enzyme.

Fig. 1A shows the cyclic voltammograms recorded for the electrochemical grafting of 4-nitrobenzenediazonium salt at the SPCE. The broad irreversible cathodic peak, located at 0.35 V versus screen-printed Ag/AgCl, is assigned to the generation of aryl radi-

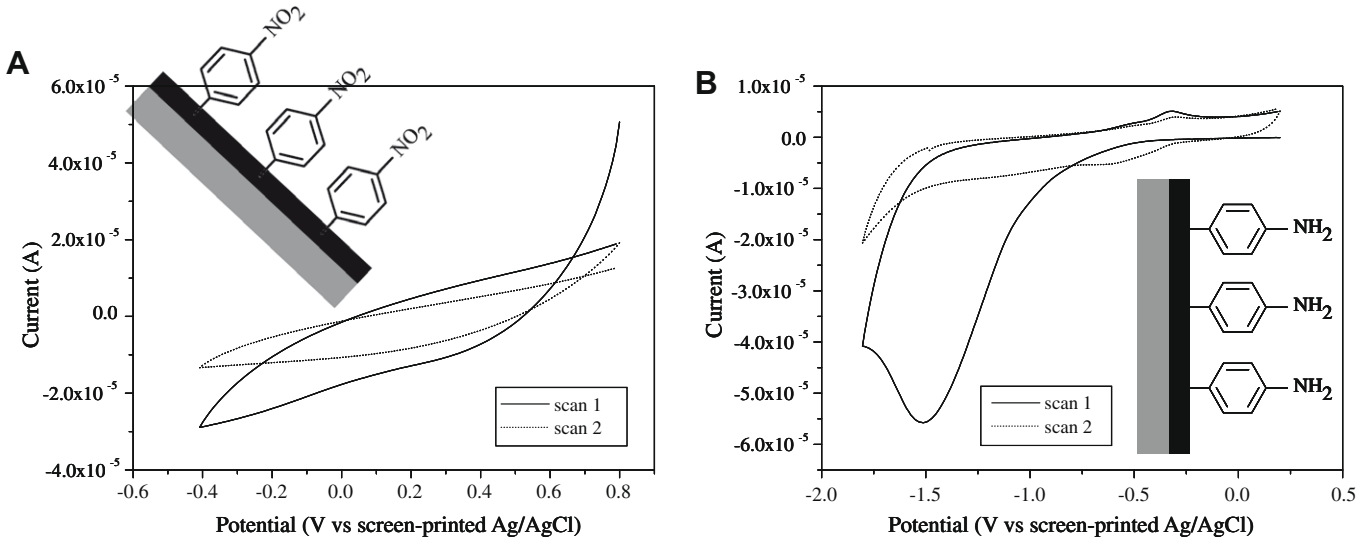

Fig. 1. Cyclic voltammograms corresponding to the electrochemical grafting of a diazonium salt at the SPCE using a 3 mmol dm^{-3} solution of $N_2C_6H_4NO_2$ BF_4^- in acetonitrile and 0.1 mol dm^{-3} NBu_4BF_4 (A) and following reduction of the 4-nitrobenzene groups to aminobenzene groups in a deoxygenated 9:1 water/ethanol solution and 0.1 mol dm^{-3} KCl (B). Scan rate = 200 mV/s.

cals that couple to the electrode surface, leading to a strongly bonded organic layer [17,32].

To attach the HRP enzyme covalently, following the carbodiimide chemistry, the NO_2 groups were transformed into NH_2 groups [32] as shown in Fig. 1B. This chemical modification of the carbon surface not only improves the properties of the composite [33] but also allows the functionalization of the NH_2 groups with HRP as described above, with the HRP-modified electrodes being performed for LEV determination.

Then experimentation was carried out to determine the relationship between the experimental variables and the chronoamperometric response, obtained by the LEV biosensor, as quickly and surely as possible with the best possible precision. This relationship is a known mathematical model that adequately represents changes in the response within the zone of interest.

The experimental variables that can define the selectivity of the biosensors using chronoamperometry are the constant applied potential, pH of the buffer solution, and H_2O_2 concentration, which may reach inhibition of the activity of HRP when using high levels [34]. Therefore, these three variables were optimized by means of a 2^3 central composite design. This optimization process arranges the factors and their interactions according to their influence on the recorded current [26,27].

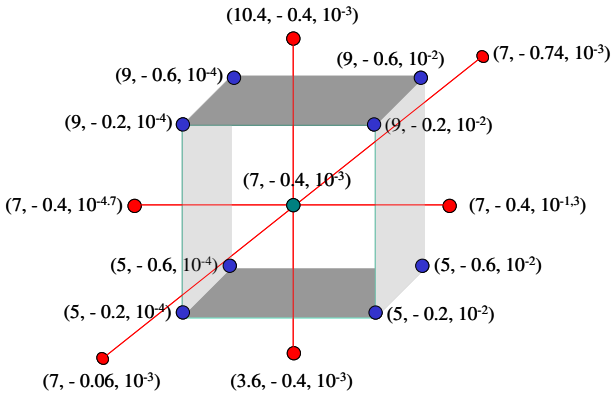

Fig. 2. Definition of the experimental domain.

The experimental domain (Fig. 2) is defined by the values corresponding to the high (+) and low (−) levels and to the central point (0) for each factor:

pH (+) = 9.0	E_{ap} (+) = −0.2 V	$C_{H_2O_2}(+) = 1.0 \times 10^{-2}$ M
pH (−) = 5.0	E_{ap} (−) = −0.6 V	$C_{H_2O_2}(-) = 1.0 \times 10^{-4}$ M
pH (0) = 7.0	E_{ap} (0) = −0.4 V	$C_{H_2O_2}(0) = 1.0 \times 10^{-3}$ M

Then the experiments corresponding to all of those possible combinations, bearing in mind the three replicates in the central point necessary to estimate the residual value, were carried out.

Thus, 5 ml of phosphate buffer–KCl solution was placed into the electrochemical cell. The corresponding potential was applied, and once a steady-state current was reached, the corresponding volume of H_2O_2 solution was added. A reduction current was recorded. LEV was then added, registering the chronoamperometric current of a 0.45 mM solution as the response variable for the analysis.

Fig. 3 shows the results obtained for this experimental design in terms of contours of estimated response surface. From this optimization process, the following optimum values for the experimental variables in the LEV determination were derived:

E_{ap} = −0.4 V	$C_{H_2O_2} = 1.0 \times 10^{-3}$ M	pH = 7.0

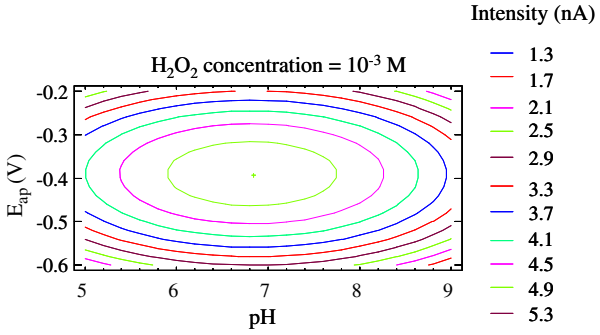

Fig. 3. Contours of estimated response surface for the 2^3 central composite design performed for the optimization of the experimental variables.

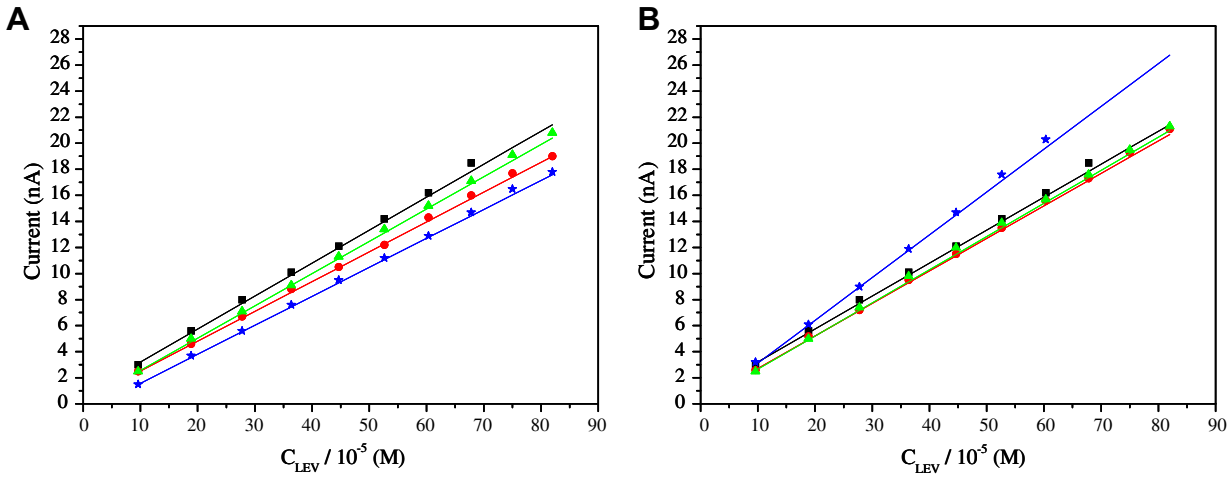

Fig. 4. Experimental points and linear regressions obtained from the calibration curves recorded under optimum conditions for checking the reproducibility (A) and repeatability (B) of the developed biosensor for LEV determination.

Table 1
Detection limits ($\alpha = \beta = 0.05$) based on linear regressions between the concentration, in the range from 0.10 to 0.83 mM, and the current.

	Calibration 1	Calibration 2	Calibration 3
Sensibility $\times 10^{-5}$ (nA M^{-1})	0.248 ± 0.001	0.290 ± 0.001	0.249 ± 0.001
Intercept (nA)	0.073 ± 0.075	0.110 ± 0.072	0.213 ± 0.075
S_{yx}	0.103	0.099	0.104
Coefficient of determination (R^2)	0.999	0.999	0.999
Detection limit (M)	1.87×10^{-5}	1.52×10^{-5}	1.86×10^{-5}

Control experiments were carried out under the optimum conditions using bare electrodes and HRP-modified electrodes by adsorption without NHS and EDC. No analytical signal was obtained.

With the aim of checking the performance of the HRP-based biosensors for the LEV determination, precision in terms of reproducibility and repeatability, as well as LOD, was calculated.

Several calibration curves were carried out in the concentration range from 0.10 to 0.83 mM by different HRP-based biosensors (Fig. 4A). Current versus concentration regression parameters were optimally evaluated to eliminate the anomalous points that would alter intercept and slope values [14,29]. Then reproducibility was calculated in terms of relative standard deviation (RSD) of these slopes, yielding a value of 6.21% ($n = 4$ and $\alpha = 0.05$). In comparison with the previously reported electrochemical biosensor for LEV determination [9], the covalent immobilization of the enzyme improves the sensor reproducibility by more than 60%.

Likewise, successive calibration curves in the same concentration range were performed by a single HRP-based biosensor (Fig. 4B). RSD values of 14.04% for the first four calibration curves and 0.89% for the first three were obtained ($\alpha = 0.05$).

To calculate the LOD, taking into account the probability of false positive (α) and negative (β) results [35], several linear relations between the concentration and signal were set. Table 1 summarizes the calibration parameters, which have also been optimally evaluated as mentioned above, and the LODs calculated using DETARCHI [30].

Finally, the developed LEV biosensor was applied to the determination of this drug in pharmaceutical preparations, with Keppra tablets containing 500 mg of LEV.

A homogeneous LEV sample was weighted and, after pulverizing a tablet, was dissolved in 10 ml of water, ultrasonicated, and finally centrifuged.

Thus, the concentration of LEV found, 489.36 ± 30.09 mg ($n = 3$, $\alpha = 0.05$) by the standard addition of identical volume (100 µl) of a solution of LEV, 5×10^{-3} M, to a sample of the drug agrees with that given by the manufacturer (Table 2).

Conclusions

This work has described a new HRP-based biosensor for the determination of the antiepileptic drug LEV in synthetic and real samples without any pretreatment of the sample. The covalent immobilization of the enzyme over the electrochemical transducer leads to a more reproducible sensor than the previous one reported for this determination. In this way, an RSD value of 6.21% was obtained for the slopes of the four calibration curves recorded using different biosensors under the optimum experimental conditions. These conditions were evaluated using the experimental design methodology, which allows analysis of an experimental domain with the minimum number of experiments and takes into account the interaction between variables. The optimum experimental conditions for the chronoamperometric determination, found by the experimental design methodology, were pH 7, −0.4 V, and a concentration of 1 mM of an H_2O_2 solution. The biosensors were finally applied to the determination of LEV in pharmaceutical drugs, with Keppra tablets containing 500 mg of LEV that were dissolved in water. The obtained results showed good agreement with the theoretical results, providing evidence for the selectivity of the proposed sensor.

Table 2
Recoveries, biases, RSDs, and 95% confidence intervals for mean for LEV determination in pharmaceutical preparations using the developed HRP biosensor.

C_{LEV} (mg)	C_{LEV} found (mg)	Recovery (%)	Bias (%)	RSD (%)	95% confidence interval for mean
500	500.58	100.12	0.12	2.47	489.36 ± 30.09
	476.52	95.30	−4.70		
	490.96	98.19	−1.81		

Acknowledgments

The authors acknowledge funding via Junta de Castilla y León (BU022A07, BU003B08, and 9096827 E) and Ministerio de Ciencia e Innovación (TEC-2008/01367) and also acknowledge J.M. Trejo (Hospital General Yagüe, Burgos, Spain) for his kind collaboration. M.A. Alonso-Lomillo is funded by a Ramón y Cajal fellowship from the Spanish Ministry of Education and Science.

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