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Analysis of (*R*)-4-oxo-5-phosphononorvaline (MDL 100,453) in rat plasma and brain dialysate using liquid chromatography after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate ☆

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Abstract

A liquid chromatographic (LC) method with precolumn derivatization and fluorescence detection has been developed for the quantitation of (*R*)-4-oxo-5-phosphononorvaline (MDL 100,453), which is a selective antagonist of *N*-methyl-D-aspartate receptor, in rat plasma and brain dialysate. The plasma samples were deproteinized with acetonitrile and then derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). The brain dialysis samples were dried in vacuum, reconstituted with borate buffer, and derivatized with AQC. The derivatized MDL 100,453 was analyzed by LC with a Nova-Pak C_{18} column at 32 °C using a gradient mobile phase. Detection was accomplished by fluorescence with excitation at 250 nm and emission at 395 nm. This analytical method was used to follow the time course of drug concentrations in rat plasma and brain dialysate after intravenous (i.v.) bolus injection of MDL 100,453 or a combination of i.v. bolus injection and i.v. infusion.

Keywords: 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; Brain dialysate; Liquid chromatography; MDL 100,453; *N*-Methyl-D-aspartate receptor; Rat plasma

1. Introduction

MDL 100,453 is a competitive antagonist of the action of glutamate at the *N*-methyl-D-aspartate receptor (NMDA) [1–3]. This receptor complex contains an integral calcium-permeable ion pore. Excessive or persistent activation of this receptor, such as occurs during cerebral ischemia, leads to calcium overload and neuronal death. MDL 100,453 can antagonize NMDA-induced calcium influx into cultured neurons and has shown biochemical and behavioral evidence of central NMDA antagonism following systemic administration to rodents [4]. Currently, this compound is

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being developed for the prevention of neurological damage after stroke and head trauma. In order to study the pharmacokinetics of MDL 100,453 and optimize the dosage regimen to achieve a steady-state drug concentration, an analytical methodology is needed to determine the drug concentration in animal plasma and brain. This report describes a liquid chromatographic (LC) procedure combined with precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) to form a fluorescent derivative for the quantitation of MDL 100,453 in rat plasma and brain dialysate which was obtained by using the technique of in vivo brain microdialysis [5,6]. AQC is a novel derivatization reagent that can rapidly react with both primary and secondary amino acids and form very stable derivatives for quantitative analysis [7–9]. The derivatization scheme of MDL 100,453 with AQC is illustrated in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

MDL 100,453 was obtained from Marion Merrell Dow Research Institute (Cincinnati, OH, USA). HPLC grade acetonitrile was purchased from Baxter (Burdick and Jackson division, Mushegon, MI, USA). An AQC derivatization kit was purchased from Waters (Milford, MA, USA). Sodium acetate (trihydrate) was purchased from Fluka (Ronkonkoma, NY, USA), and triethylamine and phosphoric acid 85% were purchased from EM Science (Cherry Hill, NJ, USA). Ethylenedinitrilo tetraacetic acid, disodium salt (EDTA) was from Curtin Matheson Scientific (Houston, TX, USA). Water was purified with a millipore Milli-Q water system (Bedford, MA, USA). Reagent grade chemicals were used throughout this study.

2.2. LC equipment

The LC system consisted of a Waters 625 LC solvent delivery system equipped with a column heater, a Waters 717 autosampler and a Waters 470 scanning fluorescence detector. A Waters Millennium 2010 workstation was used to control system operation and collect and analyze data.

2.3. Dosing experiment

Microdialysis probes were made using a concentric design. Each probe consisted of a 15 mm, 25 gauge stainless steel tube connected to PE-20 tubing, which served as the inlet. The outlet consisted of a fused silica capillary (75 μm i.d. × 150 μm o.d., Polymicro Technologies, Phoenix, AZ, USA) passed through the wall of the PE-20 tubing and into a 4 mm length of sealed dialysis tubing taken from a Filtral 16 hemodialyzer (CGH Medical, Inc., Lakewood, CO, USA). The AN.69 dialysis membrane was composed of polyacrylonitrile–sodium methalyl sulfonate copolymer, with an outer diameter of 260 μm. A piece of PE-10 tubing was attached to the silica capillary where it exits the probe. All junctions were glued using 2-ton epoxy (Devon Bearings, Inc., Brooklyn Hts., OH, USA).

Microdialysis probes with a 4 mm semipermeable membrane were implanted stereotaxically into the right medial prefrontal cortex of male Sprague-Dawley rats (250–300 g) anesthetized with sodium pentobarbital, 55 mg kg^{-1}, i.p. Following an overnight recovery period, PE-50 tubes were inserted surgically into the carotid artery and jugular vein under halothane anesthesia. Immedi-

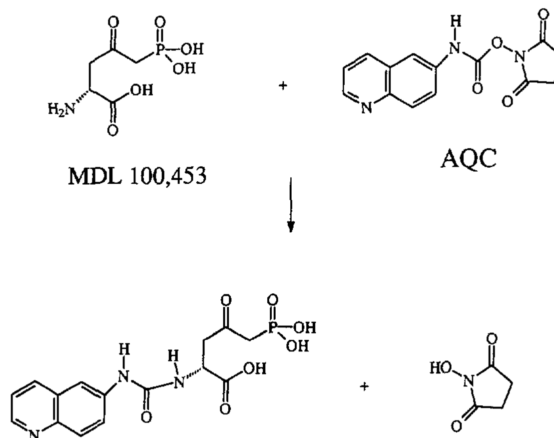

Fig. 1. Derivatization scheme of MDL 100,453 with AQC.

ately following the catheterizations, each rate was connected to a dual channel swivel with a spring tether to allow free movement within a 12-in circular Plexiglass container. The microdialysis probes were attached to inlet and outlet tubes and perfused at a flow rate of 2 μl min^{-1} using a Harvard 22 pump (Harvard Apparatus, Inc., South Natick, MA, USA) with Dulbecco's phosphate-buffered saline adjusted to 1.2 mM calcium chloride, pH 7.3. MDL 100,453 was dissolved in 0.9% saline with a 1 M equivalent of sodium hydroxide. Rats ($n = 3$) were injected intraveneously with a 236.8 μmol kg^{-1} bolus of MDL 100,453 via the jugular catheter. Following the i.v. bolus dose of MDL 100,453, dialysate fractions were collected in round-bottom microvials (6 mm × 32 mm) using a CMA/170 refrigerated fraction collector (CMA/Microdialysis, Stockholm, Sweden) at 5, 10, 20, 40, 60, 100, and 120 min with samples volumes ranging from 10 to 40 μl. Dialysates were frozen at −70 °C until assayed. A volume of 250 μl of blood was sampled from the carotid catheter into heparinized tubes at the 2, 10, 20, 30, 60, 90 and 120 min time points. The blood samples were then centrifuged for 5 min at 3000 rev min^{-1} and 5 °C. The plasma was frozen at −70 °C until assayed.

An independent experiment was conducted to determine the in vitro recovery of MDL 100,453 across the dialysis probe membrane. Three separate probes were immersed in a 6 μM solution of MDL 100,453, while colleting 20 min dialysate samples from the probe outlet tubing. Concentrations of MDL 100,453 in the dialysates were compared to the original 6 μM solution to calculate an average in vitro recovery of 11.1 ± 1.8% ($n = 3$). This in vitro probe recovery value does not necessarily reflect the actual in vivo recovery of MDL 100,453 across the dialysis membranes implanted in brain tissue. However, using the in vitro recovery value of 11%, we can estimate that actual brain concentrations of drug may be roughly ninefold to tenfold higher than dialysate concentrations.

A second experiment was performed using a combination of i.v. bolus injection at 88.6 μmol kg^{-1} and a concomitant i.v. infusion at 3.6 μmol kg^{-1} min^{-1} to male Sprague-Dawley rats ($n = 3$). The i.v. infusion was terminated at 80 min. The blood samples were taken at specified time points before and after the i.v. infusion was terminated. All blood samples were collected into heparinized tubes and then centrifuged for 5 min at 3000 rev min^{-1} and 5 °C. The plasma was frozen at −70 °C until assayed.

2.4. Preparation of plasma standards

Stock solution was prepared by dissolving an appropriate amount of MDL 100,453 in water to form a concentration of 2750 μg ml^{-1}. This stock solution was then diluted with water to obtain spiking solutions of 2200, 1650, 1100, 550, 110 and 11 μg ml^{-1}. The plasma standards (250, 200, 150, 100, 50, 10 and 1 μg ml^{-1}) were prepared by spiking 100 μl of the drug-fre rat plasma with 10 μl of the stock and the spiking solutions.

2.5. Preparation of brain dialysis standards

Stock solution was prepared by dissolving an appropriate amount of MDL 100,453 in water to form a concentration of 15 μg ml^{-1}. The spiking solutions were prepared by diluting 25, 50, 75 and 100 μl of the stock solution to 1 ml with water. An aliquot of 10 μl of the spiking solutions was mixed with 40 μl of brain dialysis blank to form brain dialysis standards (300, 225, 150 and 75 ng ml^{-1}).

2.6. AQC derivatization

The plasma standard or plasma sample was mixed by vortexing with an equal volume of acetonitrile in a 500 ml polypropylene microcentrifuge tube. The solution was centrifuged for 5 min at 1700 rev min^{-1}. A 10 μl aliquot of the clear supernatant was transferred to a round-bottom vial (6 mm × 32 mm) and treated with 70 μl of the borate buffer (0.2 M, pH 8.8). To this solution, a 20 μl aliquot of AQC reagent (3 mg ml^{-1} in acetonitrile) was added to react with MDL 100,453 for 2 min.

The brain dialysis standard or sample was taken to dryness under vacuum with a Savant Speedvac concentrator (Farmingdale, NY, USA).

Table 1
Gradient profile of LC

Time (min)	Mobile phase (%)	
	A	B
Plasma		
0	100	0
0.5	96	4
18	95	5
19	0	40
35	0	40
Brain dialysate		
0	100	0
18	97	3
19	0	40
35	0	40

Key: A, 140 mM sodium acetate buffer containing 3 μM EDTA and 17 mM triethylamine, pH 6.0. B, acetonitrile.

The dried standard or sample was reconstituted in a 30 μl of the borate buffer (0.2 M, pH 8.8). A 20 μl aliquot of the AQC reagent was added to react with MDL 100,453 for 2 min.

2.7. *Chromatographic analysis*

Separations were carried out on a Waters Nova-Pak C_{18} column (150 mm × 3.9 mm) at 32 °C using a gradient mobile phase with a flow rate of 1.0 ml min^{-1}. The detailed gradient profiles are described in Table 1. The mobile phase A was prepared by dissolving 190.4 g of sodium acetate (trihydrate) in 1l of water. To this solution were added 10 mg of EDTA and 23.7 ml of triethylamine, and followed by titration to pH 6.0 with phosphoric acid to obtain a concentration mobile phase A. A volume of 100 ml of this concentrated mobile phase A was diluted to 1000 ml with water to obtain mobile phase A. The latter was then filtered through a 0.45 μm filter and sparged with helium before use. Mobile phase B was acetonitrile. Fluorescence detection was accomplished with the excitation wavelength set at 250 nm and the emission wavelength set at 395 nm. The injection volume was 10 μl.

The peak area of MDL 100,453 obtained from the standard solutions was plotted against the standard concentration to obtain a first-order calibration curve ($y = ax + b$). The concentration of MDL 100,453 in the rat plasma and brain dialysate was calculated based on the peak area of MDL 100,453 obtained against the calibration curve.

3. Results and discussions

3.1. *LC analysis*

Due to the lack of a chromophore in MDL 100,453, the quantitation of this compound in rat plasma and brain dialysate requires a chemical derivatization step to enhance its detectability. In addition, AQC derivatization greatly increases the retention of MDL 100,453 from the void volume in reverse-phase LC for quantitative analysis. The derivatization of MDL 100,453 with AQC was completed within 2 min. Fig. 2 shows representative chromatogrms of a rat plasma blank and a spiked plasma sample. Fig. 3 shows the chromatogram of a rat plasma sample obtained 60 min after an i.v. bolus injection (88.6 μmol kg^{-1}) and concomitantly started i.v. infusion (3.6 μmol kg^{-1} min^{-1}) of MDL 100,453. Under the chromatographic conditions described, most of the derivatization byproducts and endogenous unknown peaks eluted after 12 min, and they were completely washed out from the column with 40% acetonitrile. The pH of the mobile phase was critical for the separation of MDL 100,453 from the endogenous contaminants. Lowering the pH of the mobile phase A from 6.0 to 5.0 slightly increases the retention of MDL 100,453, but it also causes interference resulting from endogenous products.

Since a pilot study indicated that the initial concentration of MDL 100,453 in rat plasma after i.v. bolus injection was less than 1 μmol ml^{-1}, a calibration curve for the quantitation of MDL 100,453 was, thus, prepared for the range 5–1200 nmol ml^{-1}. Within this concentration range, the calibration curve showed good linearity with an average correlation coefficient of 0.9996. The accuracy and precision were demonstrated by a recovery study of MDL 100,453 from four different rat plasma levels over a 2-day period. The

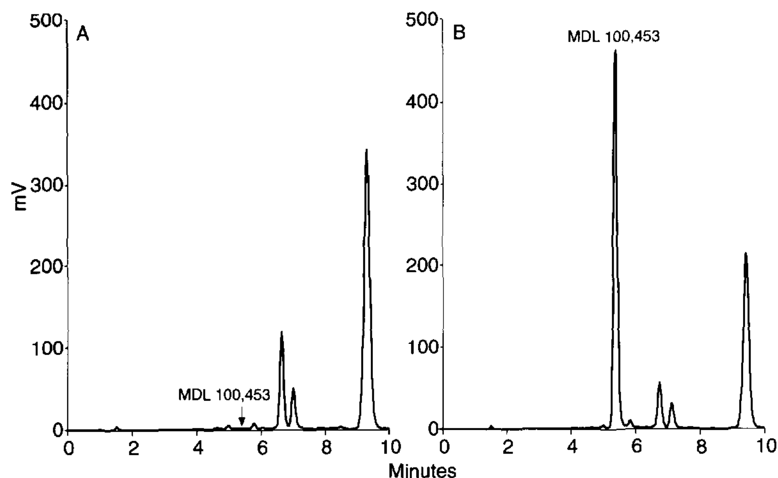

Fig. 2. LC chromatograms of (A) of rat plasma blank, and (B) a spiked sample (900 nmol ml^{-1}).

results are summarized in Table 2. The recovery of MDL 100,453 ranged from 101.8 to 113.3% with a relative standard deviation of 2.6–5.5%. The obtained stability data showed that MDL 100,453 was stable in rat plasma for up to 7 months at −10 °C (Table 3).

The gradient profile for the analyis of MDL 100,453 in brain dialysis samples was similar to that for the analysis of plasma samples with only a slight modification. This modification was to achieve a baseline separation of the MDL 100,453 from an endogenous unknown compound that eluted right before MDL 100,453. Fig. 4 shows the chromatograms of a blank and a spiked rat brain dialysis sample. Fig. 5 shows the chromatogram of a brain dialysis sample collected 40 min after an i.v. bolus injection of 236.8 μmol kg^{-1}. The brain dialysis samples needed to be dried and reconstituted in borate buffer before AQC derivatization to achieve maximum concentration for LC analysis. No sample clean-up step was necessary to achieve quantitative results. The calibration curve for brain dialysate showed correlation coefficient of 0.9862.

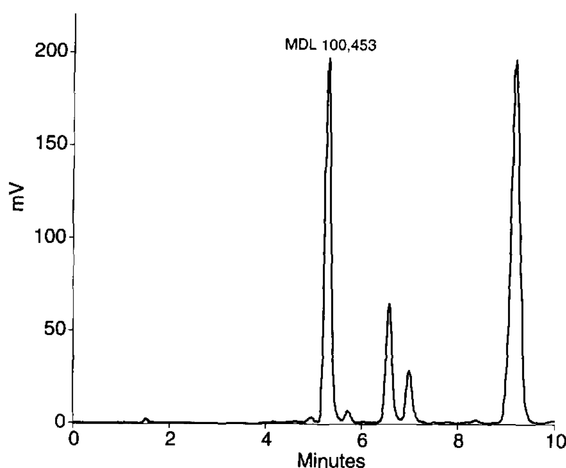

Fig. 3. LC chromatogram of rat plasma obtained 60 min after an i.v. bolus injection (88.6 μmol kg^{-1}) followed by i.v. infusion (3.6 μmol kg^{-1} min^{-1}).

3.2. Pharmacokinetic study

The LC method developed has been used to support pharmacokinetic studies of MDL 100,453 in rat. Both plasma and brain dialysate results following an i.v. bolus injeection to three rats at 236.8 μmol kg^{-1} are shown in Fig. 6. Plasma concentrations of MDL 100,453 peaked at 832.7 ± 160.6 (standard deviation) nmol ml^{-1} within the first 2 min following the i.v. bolus injection. The plasma levels proceeded to decay in a non-linear fashion. The latter, more linear portion of the graph (30–120 min) was used to calculate a plasma half-life ($t_{1/2}$) of 31 min. After a brief distribution period in the first 5 min, maximal dialysate levels of MDL 100,453 were attained 10 min after drug administration. Dialysate

Table 2
Inter-day and intra-day accuracy and precision data for MDL 100,453 in rat plasma

	Concentration prepared (nmol ml^{-1})			
	49.69	199.37	395.53	798.97
Analysis day 1				
1 [a]	54.66	196.53	419.87	875.84
2 [a]	58.97	205.39	377.43	826.10
Mean concentration (nmol ml^{-1})	56.84	200.98	398.65	850.97
Recovery (%)	114.4	100.8	100.8	106.5
Analysis day 2				
1 [a]	58.74	215.15	395.86	868.69
2 [a]	52.82	194.73	425.27	849.93
Mean concentration (nmol ml^{-1})	55.80	204.96	410.59	859.31
Recovery (%)	112.3	102.8	103.8	107.6
Mean recovery (%)	113.3	101.8	102.3	107.0
Relative standard deviation	5.4	4.6	5.5	2.6

[a] Concentration found (nmol ml^{-1}).

Table 3
Stability of MDL 100,453 in rat plasma stored at −10 °C

Day	Sample 1		Sample 2		Sample 3	
	Concentration (nmol ml^{-1})	Percentage of initial concentration	Concentration (nmol ml^{-1})	Percentage of initial concentration	Concentration (nmol ml^{-1})	Percentage of initial concentration
0	244.6	100.0	128.1	100.0	26.6	100.0
1	214.0	87.5	127.7	99.7	25.7	96.6
4	232.4	95.0	101.2	89.0	23.6	88.7
5	226.4	92.6	107.8	84.2	26.5	99.6
230	252.8	103.4	131.2	102.3	27.8	104.5

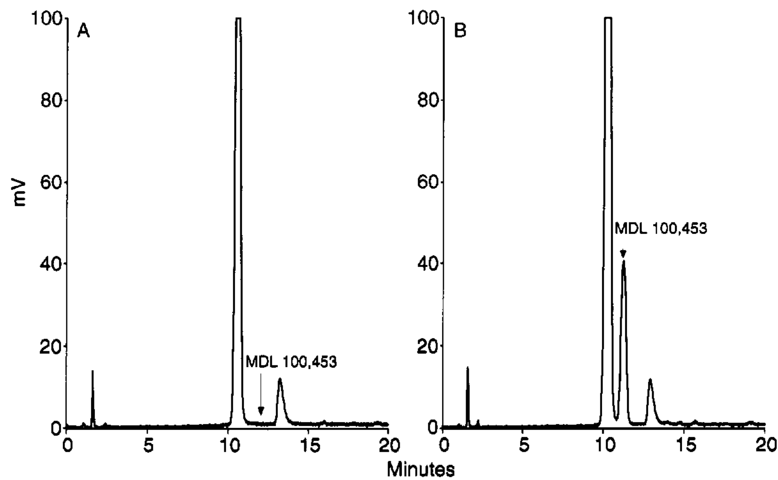

Fig. 4. LC chromatograms of rat brain dialysate blank (A) and a spiked sample (75 nmol ml^{-1}) (B).

concentrations remained stable at approximately 1 nmol ml^{-1} until the 40 min time point, after which the levels began to decay with a $t_{1/2}$ of 34 min, calculated using the last five data points (40–120 min).

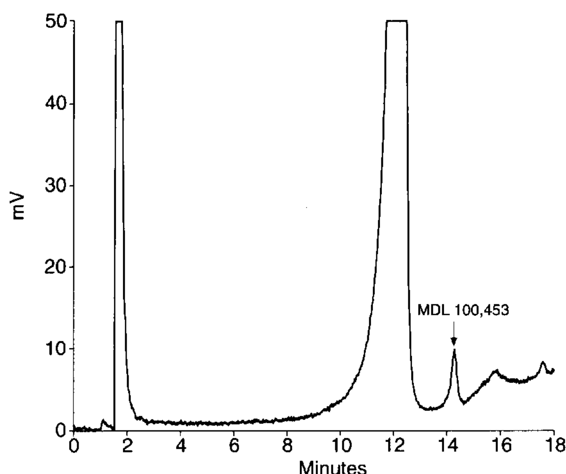

Fig. 5. LC chromatogram of rat brain dialysate obtained 40 min after an i.v. bolus injection (236.8 nmol ml^{-1}).

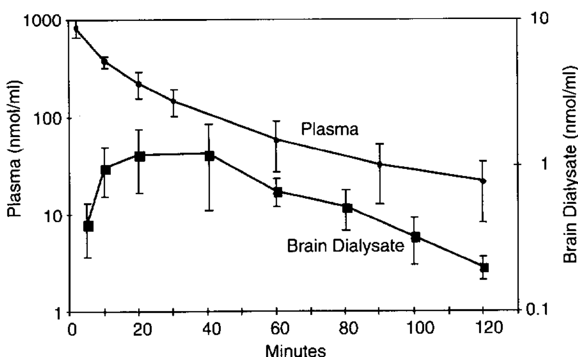

Fig. 6. Time course of mean (±SD) plasma and brain dialysate concentrations of MDL 100,453 after an i.v. bolus injection of 236.8 nmol ml^{-1} to rats ($n = 3$).

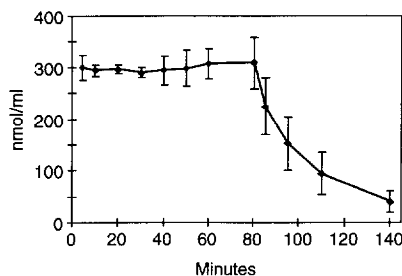

Fig. 7. Time course of mean (±SD) plasma concentrations of MDL 100,453 after i.v. bolus injection of 88.6 μmol kg^{-1} followed by i.v. infusion of 3.6 μmol kg^{-1} min^{-1} to rats ($n = 3$).

The plasma concentrations of MDL 100,453 in rats after dosing at 88.6 μmol kg^{-1} using i.v. bolus injection followed by i.v. infusion at 3.6 μmol kg^{-1} are shown in Fig. 7. In this experiment, the i.v. infusion was terminated at 80 min and the time course in Fig. 7 reflects this experiment. It is evident that the loading dose followed by an i.v. infusion was able to achieve steady-state levels at the very earliest time pointed sample. In this study, $t_{1/2}$ was 22 min for the plasma concentration for 80 min following i.v. loading dose and i.v. infusion. This $t_{1/2}$ value is in general agreement with the $t_{1/2}$ of 31 min observed in the previous i.v. bolus injection.

4. Conclusions

An LC method with AQC derivatization and fluorescence detection has been developed for the quantitation of MDL 100,453 in rat plasma and brain dialysate. The derivatization was simple and fast and the derivatized MDL 100,453 was stable for at least 1 day at room temperature to allow for quantitative analysis.

At the plasma concentrations obtained following an i.v. bolus injection of MDL 100,453, the drug enters the brain quickly with maximum levels attained in approximately 5–10 min. By comparing the $t_{1/2}$ of MDL 100,453 in brain dialysate versus plasma, we conclude that, after the first 30 min post-drug administration, brain and plasma concentrations of MDL 100,453 decay at a similar rate, yielding a $t_{1/2}$ of approximately 30 min.

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