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No Effect of Diltiazem on the Hepatic Clearance of Indocyanine Green in the Rats

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In order to investigate the effect of the pretreatment with various doses of diltiazem (DTZ) on the pharmacokinetics of indocyanine green (ICG) at steady state, especially the hepatic blood clearance due to the change of hepatic blood flow, the following experiments were carried out with ICG, a hepatic function test marker, not metabolized in liver and only excreted in bile. The intravenous bolus injection (3,780 μg/kg) and the constant-rate infusion (10,100 μg/kg/hr) of ICG into the left femoral vein were made in order to check the steady-state plasma concentration (C_{ss} of 10 μg/ml) of ICG at 20, 25 and 30 min. Following a 90-min washout period, the intravenous bolus injection (108, 430, 860 and 1,720 μg/kg) and the constant-rate infusion (108, 433, 866 and 1,730 μg/kg/hr) of DTZ into the right femoral vein were made and the achievement of the steady-state plasma levels (C_{ss} of 50, 200, 400 and 800 ng/ml) of DTZ were conformed at 60, 70 and 80 min. During the steady state of DTZ, the intravenous bolus injection (3,780 μg/kg) and the constant-rate infusion (10,200 μg/kg/hr) of ICG into the left femoral vein were made and also the steady-state plasma concentration of ICG was checked at 20, 25 and 30 min. The plasma concentrations of DTZ and ICG were determined using a high performance liquid chromatographic technique. At the steady state, the hepatic blood clearance of ICG was obtained from the plasma concentration and blood-to-plasma concentration ratio (R_B) of ICG. The pretreatment with various doses of DTZ did not influence the plasma concentrations, R_B and plasma free fraction (f_p) of ICG. So the hepatic blood clearance of ICG was independent of concentration of DTZ. The hepatic blood clearance of ICG could be affected by both hepatic bood flow and hepatic intrinsic clearance. But there was no change of the hepatic blood clearance of ICG between the control and the DTZ-pretreated rats in this study. So it may be suggested that DTZ does not influence hepatic blood flow.

Key words : Diltiazem, Indocyanine green, Steady-state hepatic blood clearance

INTRODUCTION

Diltiazem (DTZ), a calcium channel blocker (Sato *et al.*, 1971) with a benzodiazepine nucleus is widely used in the treatment of a variety of cardiovascular diseases (Nagao *et al.*, 1982; Narita *et al.*, 1983; Chaffman and Brogden, 1985) such as hypertension, angina pectoris and arrhythmia. Its primary *in vitro* action involves the inhibition of slow channel transmembrane calcium flux in cardiac and vascular smooth muscle (Zelis and Schroeder, 1980; Flaim and Zelis, 1982; Isanta *et al.*, 1987) and thus it has influences on the *in vivo* hemodynamics and electrophysiologics (Bourassa *et al.*, 1980; Stone *et al.*, 1980; Antman *et al.*, 1980). The antihypertensive mechanism of DTZ is considered to be due mainly to its vasodilatory action based on its calcium antagonistic property (Nagao *et al.*, 1982).

Thus, DTZ would be expected to increase hepatic blood flow (Flaim and Zelis, 1980, 1982; Isanta *et al.*, 1987) as well as to reduce peripheral vascular resistance secondary due to an arterial vasodilatory effect (Stone *et al.*, 1980). Only recently have efforts been focused on the possibility that the profound cardiovascular effects of DTZ might result in significant alterations in hepatic blood flow and thereby in the pharmacokinetic characteristics of DTZ itself (Isanta *et al.*, 1987). It has been reported that DTZ inhibits oxidative drug metabolism (Bauer *et al.*, 1986). However, the effect of DTZ on hepatic blood flow is still controversial. Indocyanine green (ICG) clearance does not change during DTZ therapy in humans (Bauer *et al.*, 1986). But, the effect of the concentration of DTZ on the systemic clearance of ICG at steady state was not revealed in their work.

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In order to investigate the effect of the pretreatment with various doses of DTZ on the hepatic blood clearance of ICG at steady state due to the change of hepatic blood flow, the following experiments were carried out. ICG was chosen because it is a hepatic blood flow marker and is not metabolized in the liver but only excreted in the bile (Paumgartner *et al.*, 1970; Pang and Rowland, 1977). ICG is easy to handle and is widely accepted for hepatic blood flow measurement in the past 30 years (Caesar *et al.*, 1961; Daneshmend *et al.*, 1981; Kraft *et al.*, 1991). So, in this study, the hepatic blood clearance of ICG during the steady state was estimated after loading and i. v. infusion of ICG in the rats. Following the washout period, the steady state of DTZ was achieved after loading and intravenous infusion of DTZ in the same rats. After reaching the steady state of DTZ, the hepatic blood clearance of ICG during the steady state was estimated after loading and intravenous infusion of ICG and the intluence of various concentrations of DTZ on the hepatic blood clearance of ICG was observed. And also this experiment was accomplished in order to study the effect of the pretreatment with various doses of DTZ on the change of hematocrit and plasma protein binding of ICG.

MATERIALS AND METHODS

Materials

DTZ was a gift from Hanil Pharmaceutical Co. (Seoul, Korea). ICG was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Heparin (5000 IU/ml) was a product from Green Cross Pharmaceutical Co. (Seoul, Korea). Imipramine, the internal standard (IS) for HPLC analysis, was provided by Hwan-In Pharmaceutical Co. (Seoul, Korea). HPLC-grade acetonitrile and methanol were obtained from J. T. Baker Chemical Co. (Philipsburg, NJ, U.S.A.). High-purity water (Milli Q Filtration System, Millipore Corporation, Bedford, MA, U.S.A.) was used throughout the study. Other chemicals and solvents were analytical or HPLC grade, without further purification.

Animals

Male Sprague-Dawley rats (6 weeks of age, Dae Han Laboratory Animal Research Center Co., Eumsung, Korea) were acclimated to laboratory conditions for at least 2 weeks and used for the experiments. The rats, weighing 200~250 g and aged 8~10 weeks, were used in this study. Rats freely received a diet (Jeil Diet, Daejeon, Korea) and tap water. Rats were randomly divided into two groups and they were housed individually over 2 weeks in a temperature of 18~20°C and the relative humidity of 50~60%.

Infusion design

The experiment was designed as listed in Table I and the dosage regimen was established as listed in Table II. The initial dose (X_0) and the maintenance dose (k_0) of ICG and DTZ were determined by the following equations (1) and (2), respectively, and the pharmacokinetic parameters of ICG and DTZ were calculated by a standard method (Gibaldi and Perrier, 1982). The X_0 and k_0 were determined to achieve the steady-state plasma concentrations (C_{ss}) of DTZ to be approximately 50, 200, 400 and 800 ng/ml and Css of ICG to be approximately 10 μg/ml.

$$X_0 = V_{d,\beta} \cdot C_{ss} \quad (1)$$

$$k_0 = Cl_t \cdot C_{ss} \quad (2)$$

where $V_{d,\beta}$ and Cl_t are the volume of distribution at the terminal phase and total body clearance, respectively.

Animal treatment

Each rat was fasted overnight before the commencement of the experiment. Each rat was kept in supine position during the whole experimental period. After anesthetizing rat with ether, one catheter (PE-50 polyethylene tubing, Clay Adams, Parsippany, NJ, U.S.

Table I. Experimental design for the achievement of the steady-state plasma levels of indocyanine green and diltiazem

	Time (min)				
	0 →	30 →	120 →	200 →	230
Loading and *i. v.* infusion of ICG	Sampling at 20, 25 and 30 min				Sampling at 220, 225 and 230 min
Wash-out period					
Loading and *i. v.* infusion of DTZ			Sampling at 180, 190 and 200 min		

Table II. Dosage regimen for the achievement of the steady-state plasma concentration of indocyanine green (ICG) and diltiazem (DTZ)

	X_0[a] (μg/kg)	k_0[b] (μg/kg/hr)	$V_{d,\beta}$[c] (ml/kg)	Cl_t[c] (ml/kg/min)	Expected C_{ss}
ICG	3,780	10,100	378	16.8	10.0 (μg/ml)
DTZ	108	108	2,150	36.1	50.0 (ng/ml)
	430	433	2,150	36.1	200 (ng/ml)
	860	866	2,150	36.1	400 (ng/ml)
	1,720	1,730	2,150	36.1	800 (ng/ml)

[a]$X_0=V_{d,\beta} \cdot C_{ss}$
[b]$k_0=Cl_t \cdot C_{ss}$
[c]Quoted from the data of Lee *et al.* (1992, 1993).

A.) was cannulated into the left femoral artery for blood sampling and the other catheters into the left and right femoral veins for drug administration. After recovery from anesthesia, the X_0 (3,780 μg/kg) and the k_0 (10,100 μg/kg/hr) of ICG were administered via the left femoral vein. A 150 μl aliquot of blood sample was withdrawn into heparinized tubes via the left femoral artery at 20, 25 and 30 min after X_0 to check whether or not the steady-state plasma concentration (C_{ss} of 10 μg/ml) of ICG was achieved. Following a 90-min washout period (no drug was administered), the X_0 (108, 430, 860 and 1,720 μg/kg) and the k_0 (108, 433, 866 and 1,730 μg/kg/hr) of DTZ were administered via the right femoral vein. A 300 μl aliquot of blood sample was withdrawn via the left femoral artery at 60, 70 and 80 min after X_0 to check whether or not the steady-state plasma levels (C_{ss} of 50, 200, 400 and 800 ng/ml) of DTZ were achieved. After the achievement of the steady state of DTZ, the X_0 (3,780 μg/kg) and the k_0 (10,100 μg/kg/hr) of ICG were administered via the left femoral vein. A 150 μl aliquot of blood sample was withdrawn via the left femoral artery at 20, 25 and 30 min after X_0 to check whether or not the steady-state plasma concentration of ICG was achieved. The plasma was separated immediately by centrifugation at 10,000 g for 2 min (H-31, Kokusan, Tokyo, Japan). During the experiment, the rat body temperature was maintained close to 37°C using a heating pad (CFP 8185, Bioscience, Sheerness, Kent, U.K.) with a rectal temperature probe connected to a temperature controller. Plasma samples were stored below -20°C until HPLC assay.

Hematocrit

The average hematocrit was determined from the first and last blood samples according to the modified microhematocrit method. The fresh arterial blood was pipetted into a heparinized capillary tube (φ_{int} 1.1~1.2×75 mm, Superior^R, Paul Marienfeld KG, Bad Mergentheim, W. Germany) at the height of 60 mm. The end of one side of the capillary tube was sealed with silicon wax (Superior^R). This was centrifuged for 2 min at 10,000 g. The proportions of plasma and red cells were determined by International Microcapillary Reader (Hawksley & Sons Limited, Lancing, Sussex, England).

In vitro blood-to-plasma concentration ratio (R_B) of ICG

A conventional *in vitro* method was performed as follows. After the administration of heparin at a dose of 0.1 ml/100 g body weight (100 units/100 g body weight), whole blood was collected via the carotid artery from each rat in the control and the DTZ-pretreated groups. Aliquots (10 μl) of normal saline containing various amounts of ICG were added to 1 ml of whole blood. The samples were incubated for 30 min at 37°C and at a rate of 75 oscillations per minute (opm). After separation by centrifugation at 10,000 g for 2 min, the concentrations of ICG in plasma were then assayed. R_B value was calculated by dividing the blood concentration by the plasma concentration. At the same time, the hematocrit was determined. ICG concentrations in the red blood cells (RBC, C_{RBC}) were calculated according to the following equations on the basis of mass balance:

$$C_{RBC}=\frac{C_b-(1-Hct) \cdot C_P}{Hct} \quad (3)$$

$$C_b=C_P[1-Hct+(C_{RBC}/C_P) \cdot Hct] \quad (4)$$

where C_{RBC}, C_b and C_P are the concentrations of ICG in RBC, whole blood and plasma, respectively, and Hct is the hematocrit fraction.

Plasma protein binding of ICG

Blood was collected into heparinized tube via the carotid artery and plasma was obtained by centrifugation. *In vitro* binding of ICG to rat plasma proteins was determined in the control and the DTZ-pretreated groups by an equilibrium dialysis technique. Dialysis was performed against isotonic Sörensen phosphate buffer (pH 7.4) at 37°C. A 2 ml aliquot of the Sörensen phosphate buffer (pH 7.4) was pipetted into one side of semipermeable membrane (M.W. cut off: 50,000, Spectrum Medical Industries, Inc., Los Angelis, CA, U.S.A.) and the same volume of plasma into the other side (Multidialysis Cell, Sankyo Plastic, Tokyo, Japan). A 50 μl aliquot of 4 mg/ml ICG solution was spiked to plasma side. Then each cell was placed in a water-bath at 37°C and shaken for 24 hr at 2.8 cm amplitude and 80 opm. The concentrations of ICG in plasma and the Sörensen phosphate buffer were measured. The binding value was corrected for volume change by the method of Tozer *et al.* (1983). The amount of adsorbed ICG to the membrane and/or the surface of the dialysis unit was very little (less than

1%) and therefore was neglected.

HPLC assay of DTZ

The concentrations of DTZ were determined in plasma using a HPLC technique. The extraction procedure was used with the modified method of Goebel and Kolle (1985). A 50 μl aliquot of the internal standard (2 μg/ml of imipramine) dissolved in methanol was evaporated by a centrifugal evaporator (CVE-200D, Tokyo Rikakikai Co., Tokyo, Japan). A 100 μl aliquot of rat plasma was added to the residue and vortex-mixed for 10 s with a vortex mixer (G-560, Scientific Industries, Inc., Bohemia, NY, U.S.A.). The sample was then extracted with 3 ml of t-butyl methyl ether by vortexing for 5 min. After centrifugation, the upper layer was pipetted into another tube. Subsequently, the organic layer was back-extracted with 100 μl of 0.01 M hydrochloric acid by vortexing for 1 min. Each phase was separated with centrifugation at 1,200 g for 5 min. The aqueous layer was evaporated under nitrogen gas in centrifugal evaporator. The residue was reconstituted with 100 μl of the mobile phase and a 20 μl aliquot was injected directly onto the reversed-phase column (Novapak C_{18}, 15 cm × 3.9 mm, 4 μm particle size, Waters, Millipore Co., Milford, MA, U.S.A.). The mobile phase, acetonitrile : methanol : 0.04 M ammonium bromide : triethylamine (40 : 24 : 36 : 0.1, v/v/v/v), was adjusted to pH 6.3 with 2 N hydrobromide with a pH meter (Model 7, Corning Limited, Halstead, Essex, England). The flow rate was 1 ml/min and the eluent was monitored at 237 nm. The limit of quantitation for DTZ was estimated to be approximately 10 ng/ml. The intra-day and inter-day coefficients of variation of DTZ for plasma samples were less than 9.3 % and 8.9%, respectively.

HPLC assay of ICG

The concentrations of ICG were determined in plasma using a HPLC technique. The extraction procedure was used with minor modification of the method of Dorr and Pollack (1989). Briefly, a 50 μl aliquot of rat plasma was mixed with a 60 μl aliquot of the internal standard (2 μg/ml of 1-naphthyl acetic acid) dissolved in acetonitrile to deproteinize the sample and vortexed for 5 min. After centrifugation, a 10 μl of the supernatant was injected directly onto the reversed-phase column (μBondapak C_{18}, 25 cm × 4.6 mm, 10 μm particle size, Waters, Millipore Co., Milford, MA, U.S.A.). The excitation wavelength of a fluorescent detector (LS-5 with LC flow cell, Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) was set at 243 nm and the emission wavelength at 372 nm. The mobile phase was a mixture of (0.05 M phosphate buffer with 1% triethylamine) : acetonitrile (100 : 50, v/v) and the pH was adjusted to 4.0. The flow rate was 1.3 ml/min. The limit of quantitation for ICG was estimated to be approximately 100 ng/ml. The intra-day and inter-day coefficients of variation of ICG for plasma samples were less than 9.7% and 8.3%, respectively.

Estimation of the hepatic blood clearance (Cl_h^b) of ICG

The steady-state plasma concentrations of ICG were estimated by averaging the final three samples obtained during each infusion. ICG appears to be removed from the systemic circulation of the rat entirely by the liver based upon biliary excretion (Klaassen and Plaa, 1969). Thus, hepatic plasma clearance (Cl_h^p) could represent the total body clearance. Utilizing standard pharmacokinetic techniques (Gibaldi and Perrier, 1982), Cl_h^p was estimated from the steady-state plasma concentrations ($C_{SS,FV}$) of ICG produced during the femoral infusion:

$$Cl_h^p = \frac{k_0}{C_{SS,FV}} \tag{5}$$

where k_0 is the rate of infusion of ICG. Since ICG removal rates are independent of blood or plasma data, it follows that

$$Cl_h^b \cdot C_b = Cl_h^p \cdot C_p \tag{6}$$

where Cl_h^b is the hepatic blood clearance based on blood concentrations (C_b).

$$\text{Thus, } Cl_h^b = \frac{Cl_h^p}{R_B} \tag{7}$$

Statistical analysis

The Student's paired t-test was used to assess statistical differences between the control period and each treatment period. The 0.05 level of probability was selected as the criterion of significance. All values are reported as mean ± standard deviation.

RESULTS AND DISCUSSION

Effect of DTZ concentrations on the R_B and plasma protein binding of ICG

The effect of DTZ concentrations on the R_B and plasma free fraction (f_p) of ICG is summarized in Table III. There were no significant differences of R_B and f_p of ICG between the control and the DTZ-pretreated rats. And also, negligible amount of ICG was distributed into erythrocytes. This result is well agreed with that of Daneshmend *et al.* (1981). Considering the moderate hepatic extraction ratio of ICG in the rats (Grainger *et al.*, 1983), alterations in unbound fraction may cause subsequent changes in the circulating concentrations of ICG which would influence the calculated values of transhepatic extraction as well as

Table III. Effect of diltiazem concentrations on the hematocrit (Hct), blood-to-plasma concentration ratio (R_B) and plasma free fraction (f_p) of indocyanine green[#]

	Control	Steady-state diltiazem concentration (ng/ml)			
		50	200	400	800
Hct	0.470±0.010	0.467±0.011	0.472±0.013	0.469±0.009	0.471±0.010
R_B	0.532±0.048	0.530±0.051	0.533±0.039	0.524±0.042	0.535±0.050
C_{RBC}/C_p[a]	0.004±0.001	0.004±0.001	0.004±0.001	0.005±0.001	0.004±0.001
f_p	0.025±0.002	0.027±0.003	0.029±0.003	0.021±0.004	0.024±0.003

[a]Calculated by equation (4) in the text.
[#]Mean±standard deviation (n=5~8).

hepatic and intrinsic clearances. An additional complication in assessing the effect of DTZ concentrations on ICG disposition is the binding of ICG to plasma proteins. ICG is bound significantly to plasma proteins and appears to be associated with α_1-acid glycoprotein as well as albumin (Baker, 1966); the precise contribution of each protein to ICG binding in the rat plasma is unknown. However, DTZ did not influence the plasma protein binding of ICG. It means that the change of hepatic plasma clearance of ICG reflect the change of both hepatic blood flow and hepatic intrinsic clearance.

Effect of DTZ concentrations on the hepatic blood clearance of ICG

The steady state of DTZ was achieved within 60 min in the plasma concentration-time profile after the X_0 and k_0 of various doses of DTZ (Fig. 1). Fig. 2 shows the steady-state achievement of ICG within 20 min in the plasma concentration-time profiles of ICG after the X_0 and k_0 of ICG in the control and after the steady-state achievement of 200 ng/ml of the DTZ-pretreated rats. The steady-state concentrations of ICG in the DTZ-pretreated rats were not significantly different from those in the control rats.

Table IV lists the effect of the different concentrations of DTZ on the steday-state hepatic plasma and blood clearance of ICG. There was no significant difference of the steady-state hepatic plasma and blood clearance of ICG between the control and the DTZ-pretreated rats.

Previous investigators have measured liver blood flow after dosing with calcium antagonists (Bauer *et al.*, 1986; Meredith, *et al.*, 1985; Freely, 1984): DTZ did not make uniformly change of the estimated liver blood flow but did make decrease in hepatic drug metabolism of humans (Bauer *et al.*, 1986). But, the relationship between the concentration of DTZ and the systemic clearance of ICG was not investigated. And also, as they pointed out in their work, the systemic clearance of ICG after the intravenous administration of ICG was apt to fluctuate.

The use of ICG is based on the assumptions that it is entirely extracted by the liver and systemic clearance value is equivalent to liver blood flow (LBF). Although the use of ICG as a hepatic blood flow mark-

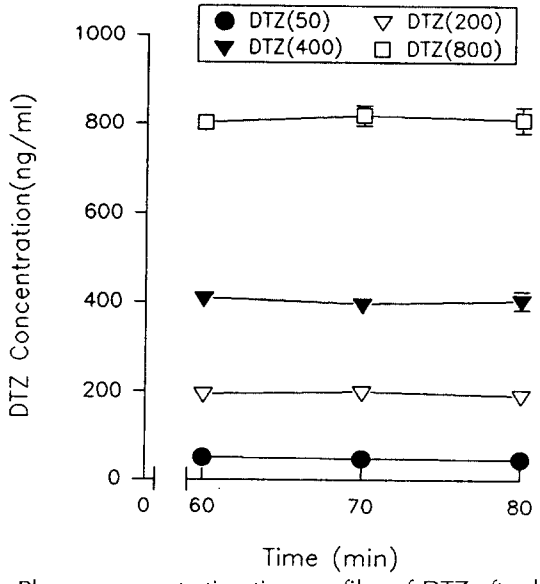

Fig. 1. Plasma concentration-time profiles of DTZ after loading and *i. v.* infusion of various doses of DTZ via the right femoral vein. The arabic numerals in the bracket represent the targeted steady-state concentrations of DTZ.

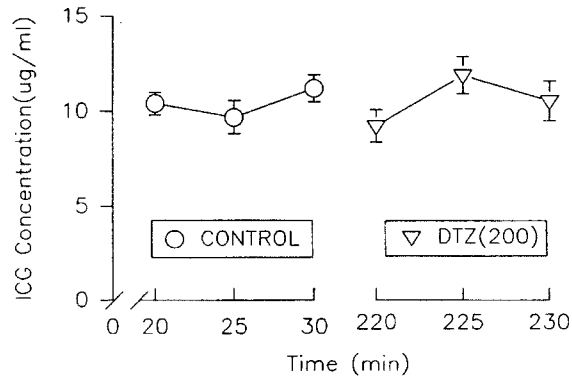

Fig. 2. Plasma concentration-time profiles of ICG after loading and *i. v.* infusion of ICG via the left femoral vein in the control and after the steady-state achievement of 200 ng/ml of the DTZ-pretreated rats.

Table IV. Effect of diltiazem concentrations on the hepatic plasma (Cl_h^p) and blood (Cl_h^b) clearances of indocyanine green[#]

	Control	Steady-state diltiazem concentration (ng/ml)			
		50	200	400	800
Cl_h^p	17.0±0.66	16.3±0.86	17.6±0.80	16.3±1.10	16.0±1.17
$Cl_h^{b,a}$	32.0±1.24	30.7±1.60	33.0±1.50	31.1±2.10	29.9±2.20

[a]Calculated by equation (7) in the text.
[#]Mean±standard deviation (n=5~8).

er was questioned recently (Skak and Keiding, 1987; Daemen *et al.*, 1989; Skerjanec *et al.*, 1994), it is usually employed in both human and animal research (Caesar *et al.*, 1961; Daneshmend *et al.*, 1981; Kraft *et al.*, 1991). But, ICG cannot be classified as a high extraction substrate in the rats, since the transhepatic extraction ratio is approximately 0.4 (McDevitt, *et al.*, 1977; Roberts *et al.*, 1986; Pollack *et al.*, 1990; Pollack and Brouwer, 1991). The systemic clearance of ICG is therefore a function of both the intrinsic ability of the liver to extract ICG from the systemic circulation (i.e., hepatic intrinsic clearance) and the rate of presentation of ICG to the liver (i.e., hepatic blood flow). But, ICG is not metabolized in the liver (Paumgartner *et al.*, 1970; Pang and Rowland, 1977). Therefore, if DTZ may affect hepatic blood flow, the steady-state hepatic blood clearance of ICG will be changed. But there was no change in the steady-state hepatic blood clearance of ICG between the control and the DTZ-pretreated rats in this study. So it suggests that DTZ does not influence hepatic blood flow.

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