

Simultaneous Determination of Torasemide and Its Major Metabolite M5 in Human Urine by High-Performance Liquid Chromatography–Electrochemical Detection

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Abstract

A high-performance liquid chromatographic method with electrochemical detection is described for the simultaneous determination of the loop diuretic 1-isopropil-3-[4-(3-methylphenylamino)-3-pyridinesulphonyl]urea (torasemide) and its major metabolite M5 in human urine. The assay is simple, fast, and easy. It requires a sample cleanup consisting of a solid-phase extraction under acidic conditions followed by chromatographic separation with a C18 μ Bondapack column. The use of a water–acetonitrile mobile phase (80:20, v/v, pH 3) ensures total separation from urine-interfering substances, and both compounds can be quantitated amperometrically at a glassy carbon electrode set to +1300 mV versus Ag–AgCl. The method demonstrates linearity for both the parent drug and the metabolite over a wide concentration range (up to 7 μ g/mL) and reproducibility with relative standard deviation lower than 2% in intraday and 5% in interday assays. The mean extraction recoveries are 78% for M5 and 60% for torasemide, and the limits of quantitation are 1 and 8 ng/mL, respectively. The method developed is applied to the analysis of healthy volunteers' urine samples collected at different time intervals after the oral ingestion of a single dose of 10 mg torasemide, and the results obtained are in agreement with the pharmacokinetic profile of torasemide.

Introduction

1-Isopropil-3-[4-(3-methylphenylamino)-3-pyridinesulphonyl]urea (torasemide) is the most active representative of the novel anilinopyridine sulphonylurea derivatives class diuretics. It has been found to have effects on water and electrolyte excretions. Because of its site of action blocking the sodium and chloride reabsorption at the loop of Henle, it can be classified by definition as a loop diuretic. Also, its diuretic profile closely resembles that of loop diuretics. However, in contrast to the typical loop diuretics

of the furosemide type, torasemide shows some advantages by its substantially longer biological half life, longer duration of action, and much less pronounced kaliuretic and phosphatoretic effects. It combines the long duration of action of the thiazides with the features of a high-ceiling loop diuretic. Urinary dose-response curves have shown torasemide to be five times as potent as furosemide (1). Furthermore, the nearly complete bioavailability (approximately 90%) (2) simplifies the change from intravenous to oral treatment, because almost the same efficacy can be achieved following both forms of administration. Torasemide is suitable for a broad spectrum of different indications that are effective for the treatment of hypertension in the very low dose of 2.5 mg and up to the treatment of high-grade renal failure with the high dose of 200–400 mg.

Torasemide is rapidly absorbed following oral administration (a peak plasma concentration is achieved within the first hour) and metabolized by the hepatic cytochrome P450 system with up to 25% of an intravenous dose appearing in the urine as unchanged drug (1,3–7). This diuretic undergoes different hydroxylations, oxidation, and reduction to produce five metabolites (whose formation scheme can be observed in Figure 1). The most important metabolites are M1, M3, and M5. The total amount of torasemide and metabolites recovered in urine after a single dose following the oral route of administration are 21% torasemide, 12% M1, 2% M3, and 34% M5 (8).

Because the present trend is to use doses of diuretics as low as possible because of its high potency and in order to minimize adverse effects, methods able to detect concentrations in the nanograms-per-milliliter range are necessary. Electrochemistry provides a sensitive detection for both the parent drug and the metabolite, and it constitutes a good alternative to the photometric detectors used in some works reported in literature (9–11).

Torasemide as well as the rest of the diuretics group are considered forbidden substances in sports. Therefore, it is necessary to develop an analytical method for the urine control of this diuretic in the doping laboratories.

This study describes a solid-phase extraction procedure fol-

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lowed by a high-performance liquid chromatographic (HPLC) method with amperometric detection for the simultaneous determination of torasemide and its major metabolite in humans (M5). The method is applied to the determination of both compounds in urine samples obtained from two different healthy volunteers after the oral ingestion of a single dose of 10 mg torasemide.

Experimental

Reagents, chemicals, and standard solutions

Torasemide and 1-isopropil-3-[4-(3-carboxyphenylamino)-3-pyridinesulphonyl]urea (M5) as well as tablets containing 10 mg of torasemide were kindly supplied by Boehringer Mannheim (Mannheim, Germany). All sorbent extraction cartridges used were from Varian (Barcelona, Spain). HPLC-grade solvents were purchased from Lab-Scan (Dublin, Ireland), and water was obtained from the Milli-RO and Milli-Q Waters systems (Millipore Corp., Milford, MA). All of the reagents used were from Merck (Bilbao, Spain) and were of suprapure quality.

Stock solutions of 1000- $\mu\text{g/mL}$ torasemide and M5 were prepared in pure methanol and stored in the dark under refrigeration in order to avoid possible decomposition. Stock solutions of both compounds are stable in these conditions up to at least three years (12).

Drug-free human urine samples obtained from healthy volunteers were stored at -20°C and thawed to room temperature prior to analysis.

Spiked urine samples were prepared from the aliquots of drug-free urine at room temperature and doped with different concentrations of torasemide and M5.

Urine samples were collected at different time intervals from two healthy volunteers (male and female) after the oral ingestion

of a single dose of 10 mg torasemide, and aliquots were frozen at -20°C until analysis.

Instrumentation and chromatographic conditions

The chromatographic system used for the analytical separation consisted of a Model 2150-LKB HPLC pump (Pharmacia, Barcelona, Spain) with a Rheodyne Model 7125 injector (20- μL loop volume) (Pharmacia). The amperometric detection was carried out with an electrochemical detector (ED) (PAR Model 400) equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain). It was operated at +1300 mV versus an Ag–AgCl electrode in the DC mode with a 5-s low-pass filter time constant and a current range between 0.2 and 100 nA. Chromatograms were recorded using an LKB Model 2221 integrator (Pharmacia, Barcelona, Spain) with an attenuation of 8 mV/s at a 0.5-cm/min chart speed. Separation of the compounds was performed on a 125Å $\mu\text{Bondapak C18}$ 10- μm column (30-cm \times 3.9-mm i.d.) (Waters Association, Barcelona, Spain) preceded by a $\mu\text{Bondapak C18}$ precolumn module (Waters). Temperature was kept constant at $30^\circ\text{C} \pm 0.1^\circ\text{C}$ by using a Waters TCM temperature control system.

The mobile phase consisted of an acetonitrile–water mixture (20:80) containing 5mM potassium dihydrogenphosphate–phosphoric acid. The pH was adjusted to 3 and the buffer served as the supporting electrolyte. The $\mu\text{Bondapak C18}$ column head-pressure was 89 bar at a flow rate of 1.75 mL/min, and the injection volume was 20 μL .

The voltammetric study of M5 was made using an Eco Chemie Autolab voltammetric analyzer coupled to a Metrohm Model VA 663 three-electrode stand (Gomensoro, Madrid, Spain). The auxiliary electrode was a platinum rod, the reference electrode a saturated calomel, and the working electrode a 3-mm-i.d. Metrohm glassy carbon. Measurements of pH were carried out with a Radiometer (Copenhagen, Denmark) digital pH-voltmeter using a combined glass–Ag–AgCl(s)–KCl Ingold gk2301c.

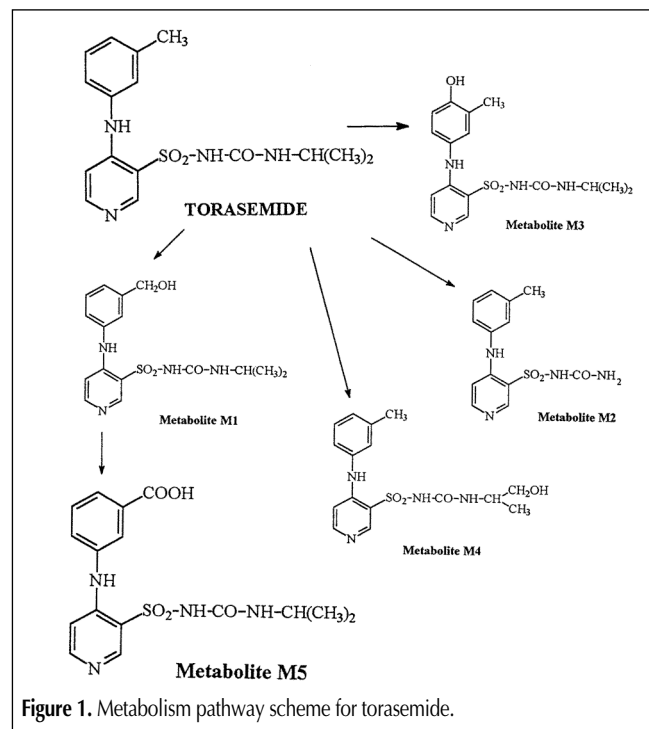
Evaporation of urine extracts to dryness was carried out with a Zymark TurboVap LV evaporator (Barcelona, Spain).

Extraction procedure for urine samples

Urine was subjected to a solid-phase extraction step prior to chromatographic analysis for the purpose of sample cleanup and enrichment. A C2 disposable solid-phase cartridge was first conditioned with 1 mL methanol. Then, 1 mL phosphoric acid (0.075M) was added, and before drying a 1 mL urine–0.5 mL phosphoric acid solution was loaded onto the column. The sample was aspirated and rinsed with 1 mL phosphoric acid and 1 mL deionized water. Subsequently, 1 mL dichloromethane and 1 mL chloroform were passed through. Then, the cartridge was allowed to dry for 10 s and elution of the isolated was performed with 2×0.5 mL of pure methanol. The combined eluates were evaporated to dryness under a gentle stream of nitrogen at 40°C . The solid residue was redissolved in 100 μL of the mobile phase and injected onto the column.

Recovery

The recovery of torasemide and M5 were evaluated at two different concentration levels (50 ng/mL and 2 $\mu\text{g/mL}$) by comparing the peak areas of aqueous solutions containing known



amounts of M5 and torasemide before and after the solid-phase extraction procedure. Each sample was analyzed five times.

Precision and limit of quantitation

The intraday precision of the method was evaluated by analyzing on the same day five replicates of spiked urine samples at a 1- $\mu\text{g/mL}$ concentration of torasemide and M5 against a calibration curve. The interday precision was assessed by analyzing spiked urine samples at a concentration of 1 $\mu\text{g/mL}$ on different days against a calibration curve. The precision was given by inter- and intraday relative standard deviation.

Calibration graphs were prepared by chromatographing urine samples doped with known amounts of torasemide and M5. Peak areas of torasemide and M5 were plotted against the concentrations. Data obtained were treated by unweighed least-squares regression analysis.

The limit of quantitation was defined as the sample concentration of torasemide or M5 resulting in a peak area of ten times that of the signal-to-noise ratio.

Selectivity

In order to evaluate the selectivity of the method, urine samples obtained from two healthy volunteers (male and female) taken at different time intervals after the single-dose administration of torasemide were subjected to the assay procedure, and the retention times of the endogenous compounds in the urine were compared with those of torasemide and its metabolite.

Results and Discussion

HPLC of torasemide and M5

In previous studies we reported the electrochemical properties of torasemide (13) and the development of an HPLC–ED system for its determination in pharmaceuticals and urine samples (14). The method was adequate for the separation and determination of the parent drug, but when urine samples collected at different time intervals were injected in the chromatographic system, other peaks could be observed in the chromatograms. Because of its concentration profiles and data obtained from bibliographic references, we considered the possibility of attributing these peaks to torasemide metabolites.

These metabolites were not commercially available, and only the major one (M5) could be obtained from the pharmaceutical company Boehringer Mannheim.

Upon the basis of the oxidation of M5, an HPLC–ED system was optimized for its separation and determination. In order to choose the optimum potential value for the amperometric detection, hydrodynamic voltammograms of the compound were obtained. In a first attempt, M5 was run with the mobile phase usually employed for torasemide (water–acetonitrile, 65:35, pH 5.5) (14), but the retention time for the compound was so low that it eluted with the injection peak. By decreasing the pH of the mobile phase to a value of 3.0, a longer retention of M5 on the C18 column was obtained, which allowed for the separation from torasemide and made possible the recording of the hydrodynamic voltammograms (as can be observed in Figure 2). An oxidative

potential of 1300 mV was chosen as the working potential, because it was the minimum potential necessary for providing a reproducible oxidation with the maximum sensitivity for both compounds without increasing the background current.

After the establishment of the working potential, different extraction procedures were assayed for the simultaneous isolation of M5 and torasemide from the urine matrix.

Initially, liquid–liquid extraction using different organic solvents (i.e., hexane, dichloromethane, chloroform, diethyl ether, and ethyl acetate) was assayed by adjusting the pH of the aqueous phase to different values in order to cover the entire range (from acidic pH at approximately 1.0 to alkaline pH at 13.0). Also, several salts (NaCl, CaCl_2 , Na_2SO_4 , and NaClO_4) were tried for the salting out effect. As expected, only the use of relatively polar organic solvents (i.e., ethyl acetate and diethyl ether) at a low pH (< 5) made possible the simultaneous extraction of M5 and torasemide, because both are acidic compounds. The recovery for these initial assays was evaluated from a standard aqueous solution of M5 (1 $\mu\text{g/mL}$) using ethyl acetate as the organic solvent (Table I).

Once the optimum pH for the extraction was determined (pH 3.00), urine spiked with M5 was extracted under these conditions, but too many interferences from endogenous compounds were observed in the chromatogram. The optimum pH was used again with different solvents and solvent combinations because we were

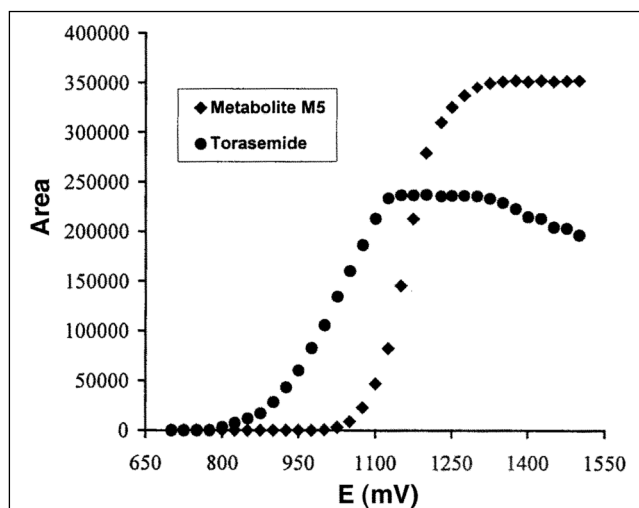


Figure 2. Hydrodynamic voltammograms of torasemide (●) and M5 (■): 5- $\mu\text{g/mL}$ concentration for both compounds, water–acetonitrile mobile phase (65:35, 5mM $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$, pH 3), and 1-mL/min flow rate.

Table I. Influence of the pH of the Aqueous Phase in the Percentages of Recovery for Torasemide and Its Major Metabolite M5 Using Liquid–Liquid Extraction with Ethyl Acetate

pH of the aqueous phase	%Recovery for M5
2	97.23
3	95.94
4	52.55
5	8.07
6	0.00

trying to find one that provided an extract free from interfering materials, although part of the compound was not extracted. After several attempts and changing even the pH, no successful method was found.

Then, solid-liquid extraction was applied. Different cartridges were used: C18, CH, LRC, PH, C8, CN, and C2. The latter provided the best retention for M5 using acidic conditions (14) and washing it with dichloromethane and chloroform (as described in the Materials and Methods section). Although combinations of liquid-liquid and solid-liquid extractions were assayed, no improvements were achieved.

Linearity and limit of quantitation

In urine, the peak area of torasemide and M5 varied linearly with concentration over the 9-ng/mL to 7- μ g/mL (torasemide) and 2-ng/mL to 7- μ g/mL (M5) range.

The slopes of the fitted straight lines for the calibration curves were $9.9 \times 10^2 \pm 0.7 \times 10^2$ and $116 \times 10^2 \pm 6 \times 10^2$ area/(μ g/mL) with a correlation coefficient of the linear regression analysis of 0.9997 and 0.9998 for torasemide and M5, respectively.

The limits of quantitation were 8 and 1 ng/mL for torasemide and M5, respectively.

Concentration level	M5	Torasemide
50 ng/mL	77.1% \pm 3.2%	59.3% \pm 4.1%
2 μ g/mL	78.3% \pm 2.7%	60.1% \pm 3.9%

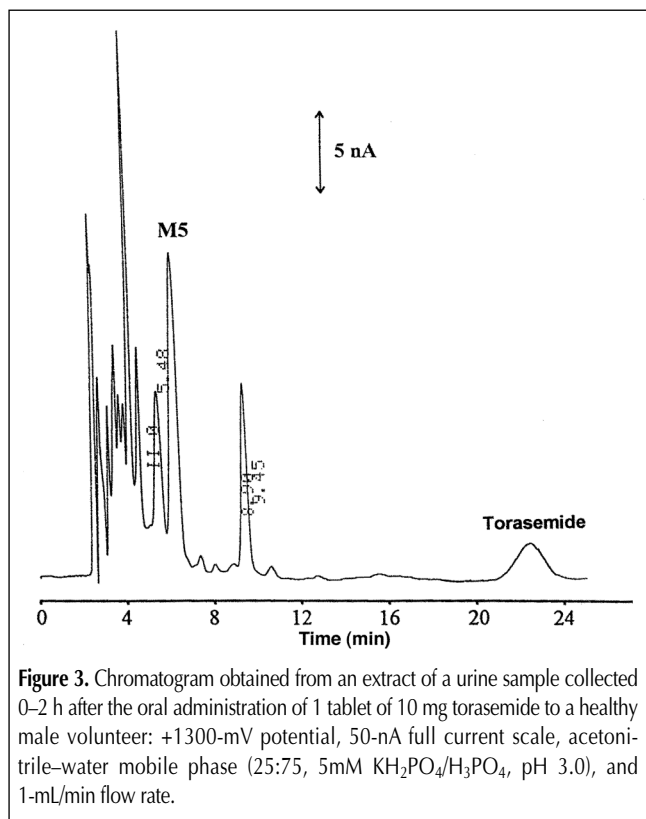


Figure 3. Chromatogram obtained from an extract of a urine sample collected 0–2 h after the oral administration of 1 tablet of 10 mg torasemide to a healthy male volunteer: +1300-mV potential, 50-nA full current scale, acetonitrile–water mobile phase (25:75, 5mM $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$, pH 3.0), and 1-mL/min flow rate.

Extraction recovery

The mean extraction recoveries obtained were approximately 60% and 78% for torasemide and M5 (Table II).

Precision

The relative standard deviation for within-run precision that was calculated from replicate ($n = 5$) determinations of the same urine was 2.62% and 1.51% for torasemide and M5, respectively, at a concentration level of 1 μ g/mL. The corresponding values for between-run precision were 5.61% and 4.92% for torasemide and M5, respectively.

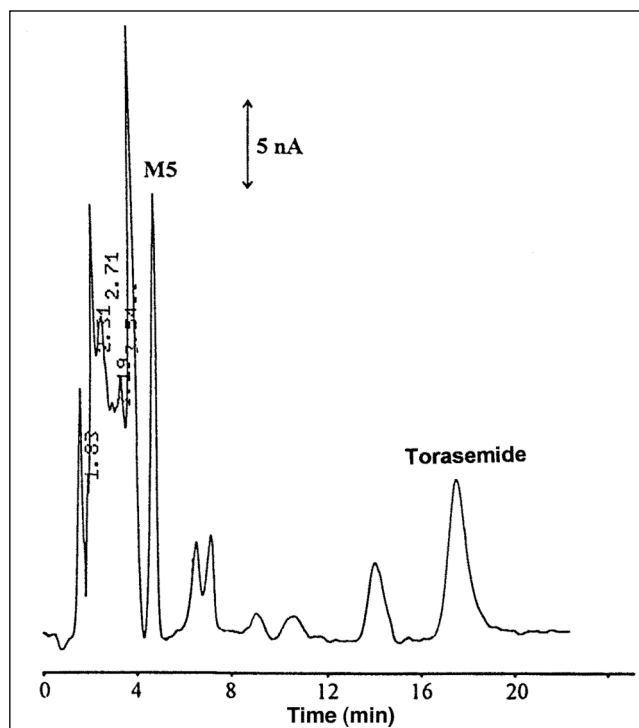


Figure 4. Chromatogram corresponding to an extract of a urine sample 8–24 h after the oral administration of 10 mg torasemide to a healthy male volunteer: +1300-mV potential, 50-nA full current scale, acetonitrile–water mobile phase (20:80, 5mM $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$, pH 3.0), and 1.75-mL/min flow rate.

Table III. Concentrations of Torasemide and Its Major Metabolite M5 in Urine Collected from Two Different Volunteers at Different Time Intervals

Time interval (h)	Urine volume (mL)		M5 concentration (μ g/mL)		Torasemide concentration (μ g/mL)	
	Male*	Female†	Male	Female	Male	Female
0–2	750	840	0.55	0.44	0.84	0.81
2–8	1000	650	2.16	3.12	0.89	1.23
8–24	705	820	2.04	1.67	0.12	0.76
Total time (h)	Total volume (mL)		Total milligrams per percentage dose		Total milligrams per percentage dose	
0–24	2455	2310	4.01/40.1	3.77/37.7	1.60/16.0	2.10/21.0

* Male, healthy male volunteer.

† Female, healthy female volunteer.

Specificity

The cleanup procedure and the chromatographic system were applied to real urine samples obtained from healthy volunteers after the ingestion of torasemide tablets. The method allowed for the simultaneous extraction of M5 and unchanged torasemide from urine at different time intervals. A chromatogram corresponding to 0–2 h that was free from interfering substances at the elution times for the drug and its metabolite is shown in Figure 3.

When 8–24-h samples were run in the HPLC–ED system (using a 75:25 water–acetonitrile mobile phase, pH 3.0), M5 was not totally well-resolved from the rest, although it could be quantitated by the integrator. With this mobile phase M5 eluted at 6.2 min and torasemide at 22.5 min. This would be a very good system if the very small interferences at the beginning of the chromatogram could be eliminated. Assays were made washing the cartridge with diethyl ether, a 0.5M phosphoric acid–acetonitrile mixture, and a 0.5M phosphoric acid–methanol mixture. This kind of experiment provided good results in terms of clean solution extracts, but recovery for both compounds decreased drastically.

If the same extract corresponding with the 8–24-h urine samples was run with a weaker mobile phase (water–acetonitrile, 80:20, pH 3.0), M5 would be separated just enough from the interferences (9.35-min elution time), but torasemide eluted at 29 min (which is a very long time) and produced a broad peak.

In order to determine in the same run both torasemide and M5 in urine collected at any time interval, a water–acetonitrile mobile phase (80:20, pH 3) was required and the flow rate adjusted to 1.75 mL/min. With these conditions the retention time was 5.0 min for M5 and 17.1 min for torasemide (Figure 4).

Analytical applications

The cleanup procedure and the chromatographic system were applied to real urine samples obtained from healthy volunteers after the ingestion of torasemide tablets.

Table III shows the concentrations of M5 and torasemide found in urine obtained from two different volunteers collected at different time intervals after the oral administration of a single dose of 10 mg torasemide. The final percentages of drug and metabolite excreted (40% M5 and 16% torasemide) were in agreement with the values reported in literature (34% M5 and 21% torasemide) (10).

Discussion

The HPLC method and the cleanup procedure developed allowed for the determination of the diuretic and its metabolite M5 free from interferences from urine, but other peaks appeared in the chromatograms after M5 and before torasemide. They could be other metabolites of torasemide (probably M1 and M3), because the retention times of these compounds are between M5 and torasemide (15) and their concentration profiles resembled that of M5. However, because of the impossibility of getting standard metabolites, this could not be confirmed.

Conclusion

In a previous work we described an HPLC–ED assay for the determination of torasemide in tablets and human urine (14), but

this method was unable to determine the major metabolite of torasemide (M5), which eluted with the injection peak because of its high polarity. This fact led us to develop a chromatographic method able to determine in the same run M5 and torasemide.

The method proposed does not require lengthy sample preparation, requires a minimum amount of time and specimen, can measure both the parent drug and its major metabolite, and is easy to run so it can be set up in any laboratory engaged in therapeutic drug monitoring.

The chromatographic method with amperometric detection that was developed presented some advantages over other reported methods, particularly the low time required for the determination of both compounds as well as the low quantitation limits achieved.

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