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Simultaneous determination of pregnenolone and 17α-hydroxypregnenolone by semi-micro high-performance liquid chromatography with an immobilized cholesterol oxidase as a pre-column reactor: Application to bovine adrenal fasciculata cells

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ABSTRACT

A method for the simultaneous determination of pregnenolone and 17α-hydroxypregnenolone by high-performance liquid chromatography with an immobilized cholesterol oxidation enzyme reactor was developed. Pregnenolone and 17α-hydroxypregnenolone were converted to progesterone and 17α-hydroxyprogesterone, respectively, by the immobilized enzyme packed into the reactor column, and could thus be monitored by UV absorption at 240 nm. The calibration curves for pregnenolone and 17α-hydroxypregnenolone were linear in the range of 0.4–10 and 0.3–10 μg/ml with a correlation coefficient of 0.9993 and 0.9998, respectively. The detection limit at a signal-to-noise ratio of 3 was 0.12 and 0.08 μg/ml for pregnenolone and 17α-hydroxypregnenolone, respectively. The conversion rate of pregnenolone to progesterone and 17α-hydroxypregnenolone to 17α-hydroxyprogesterone was 90.6% and 99.3%, respectively. Intra-day and inter-day precision (in terms of percentage coefficient of variation) were less than 9.3%, with accuracy greater than 94.8%. This method was successfully applied to the simultaneous determination of pregnenolone and 17α-hydroxypregnenolone secreted into the culture medium of bovine adrenal fasciculata cells and of both analytes produced within the cells.

1. Introduction

Pregnenolone and 17α-hydroxypregnenolone are the main precursors for gonadal and adrenal steroid hormone biosynthesis. Pregnenolone is initially synthesized from cholesterol by the specific cytochrome P450 side chain cleavage enzyme (P450scc), and then catalyzed by 17α-hydroxylase to form 17α-hydroxypregnenolone. Tachikawa et al. have reported on the influence of bioactive cytokines and other drugs upon steroidogenesis in bovine adrenal fasciculata cells stimulated by adrenocorticotropin (ACTH) [1]. The simultaneous determination of pregnenolone and 17α-hydroxypregnenolone, which are involved in the early steps of steroid synthesis, is useful for investigating steroidogenesis in adrenal fasciculata cells. To date, determination of pregnenolone and 17α-hydroxypregnenolone has mainly been based on the use of gas chromatography–mass spectrometry (GC–MS) [2,3] and immunoassay [4,5]. However, the GC–MS method requires a derivatization procedure, while immunoassay is difficult to optimize for simultaneous determination of both analytes. Direct measurement of pregnenolone and 17α-hydroxypregnenolone by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is not feasible, because the molecules do not absorb UV radiation. An efficient method which combines the enzymatic conversion of cholesterol to cholest-4-ene-3-one by cholesterol oxidase followed by analysis of the oxidized product by HPLC has been reported [6,7] and successfully applied to the determination of cholesterol in human monocyte-derived macrophages [6] and oxysterols in human erythrocyte membranes [7]. In previous studies, we demonstrated that an immobilized enzyme reactor (IMER) can be used not only for post-column reaction detection [8,9] but also as a pre-column tool [10,11]. HPLC combined with IMER allows the on-line enzymatic conversion of pregnenolone and 17α-hydroxypregnenolone to the UV-detectable cholest-4-ene-3-one automatically and with ease. In the present study, we

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have constructed a UV-detection system using an immobilized cholesterol oxidase enzyme reactor/semi-micro HPLC approach. We have applied the method to the simultaneous determination of pregnenolone and 17α-hydroxypregnenolone, both within bovine adrenal fasciculata cells and secreted into the culture medium by these cells.

2. Materials and methods

2.1. Materials and reagents

Pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone and bile salts (mixture of approximately 50% sodium cholate and approximately 50% sodium deoxycholate) were products of Sigma–Aldrich (St. Louis, MO, USA). ACTH (i.e., adrenocorticotropin) was obtained from the Peptide Institute (Osaka, Japan). Trilostane [(4α,5α,17β)-4,5-epoxy-3,17-dihydroxyandrost-2-ene-2-carbonitrile] was obtained from Toronto Research Chemicals (Ontario, Canada). Krebs-Ringer-4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (KRH buffer; pH 7.4), used as an incubation medium, contained the following: 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 5.6 mM glucose, and 0.5% bovine serum albumin. Tissue culture instruments were obtained from Falcon Plastic (Cockeysville, MD, USA). Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (Ham) were from Life Technologies (Grand Island, NY, USA). Fetal bovine serum was obtained from Nacalai Tesque (Kyoto, Japan). Cholesterol oxidase (cholesterol:oxygen oxidoreductase, EC 1.1.3.6) from *Cellulomonas* sp. was obtained from Sigma–Aldrich. Aminopropyl controlled-pore glass (aminopropyl-CPG, 1400 Å pore diameter, 120–200 mesh) was purchased from CPG (Lincoln, Park, NJ, USA). Methanol and acetonitrile of HPLC grade were obtained from Wako (Osaka, Japan). All other chemicals used were of analytical-reagent grade.

2.2. Preparation of immobilized cholesterol oxidase and enzyme reactor

The procedure for preparation of immobilized cholesterol oxidase and IMER was described previously [12]. In brief, 0.5 g of aminopropyl-CPG was activated with 10 ml of 0.1 M phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde. The suspension was stirred under aspirator vacuum for 1 h at room temperature. The aldehyde-activated aminopropyl-CPG was filtered on a glass filter to remove the excess glutaraldehyde, and thoroughly washed with distilled water. The aldehyde-activated glass was transferred to the reaction container, and 10 ml of 0.1 M phosphate buffer (pH 6.0) containing cholesterol oxidase (550 units) was added. The reaction was allowed to continue with occasional shaking for 2 h on ice, and subsequently for 2 h at room temperature. After the reaction, the glass coupled to the enzyme was filtered on a glass filter, and then washed with 0.1 M phosphate buffer (pH 7.0) and with distilled water. The Schiff base double bond and the residual aldehyde groups were reduced with 10 ml of 0.1 M phosphate buffer (pH 4.5) containing 1.0% sodium tetrahydroborate for 1 h at room temperature. The immobilized cholesterol oxidase thus obtained was filtered on the glass filter, and then washed with distilled water. The preparation was packed into a stainless steel column (10 mm × 4 mm i.d.) and used as a pre-column IMER.

2.3. Standard solutions

Standard stock solutions of pregnenolone, 17α-hydroxypregnenolone, progesterone and 17α-hydroxyprogesterone were prepared by dissolving each of the analytes in 2-propanol to a concentration of 1 mg/ml. Standard working solutions were prepared from standard stock solutions by dilution with acetonitrile containing bile salts, and the final concentration of bile salts was adjusted to 1%.

2.4. Isolation and primary culture of bovine adrenal fasciculata cells

Bovine adrenal glands were kindly provided by the Center of Iwate Livestock Industry. Adrenal fasciculata cells were prepared by collagenase digestion as previously described [1]. The isolated cells were suspended in DMEM/Ham's F-12 (1:1) containing 10% fetal bovine serum, 3 μM cytosine arabinoside and an antibiotic mixture (100 units/ml penicillin, 100 μg/ml streptomycin and 0.3 μg/ml amphotericin B), and were maintained as a monolayer culture in 35 mm dishes at a density of 2×10^6 cells. The cells were cultured at 37 °C in a CO_2 incubator (95% air/5% CO_2).

2.5. Reaction with ACTH and trilostane

After 4 days of culture, the cells were washed twice with prewarmed KRH buffer and then incubated with or without 1 nM ACTH and/or in the presence or absence of 10 μM trilostane in 2 ml of KRH buffer for 1.5 h at 37 °C. The reaction was terminated by transferring the medium to tubes in an ice-cold bath.

2.6. Sample preparation

Steroids produced in the medium (i.e., the extracellular fraction) were enriched by solid phase extraction. The reaction medium (1.8 ml) was immediately loaded on a Bond Elut C_8 cartridge (50 mg, 1 ml, Varian, Inc., Palo Alto, CA, USA) that had been preconditioned by washing with 1 ml of methanol and 1 ml of water, and then, following loading, the cartridge was washed with 2 ml of water to remove polar substances in the culture medium. Next, the steroids retained on the cartridge were eluted with 285 μl of acetonitrile. Bile salts (15 μl; 20%, w/v) were added to the eluate to a final concentration of 1%, and the mixture was then centrifuged at 10,000 rpm for 2 min. The supernatant was used as the sample solution. Steroids within the cells (i.e., the intracellular fraction) were prepared by disrupting the cells with zirconia beads. The suspension was then centrifuged at 3000 rpm for 10 min at room temperature. The supernatant obtained was loaded onto the cartridge and the steroids were then eluted from the cartridge in a manner similar to that described above.

2.7. Apparatus and procedure

A schematic diagram of the pre-column IMER/semi-micro reversed-phase HPLC system for the simultaneous determination of pregnenolone and 17α-hydroxypregnenolone is shown in Fig. 1. Three Shimadzu LC-10A pumps (Kyoto, Japan), two 'six-ported' switching valves (FCV-12AH, Shimadzu), a column oven (CTO-10A, Shimadzu) and an automatic sample injector (SIL-10AD, Shimadzu) were controlled by a system controller (SCL-10A, Shimadzu). Three different solutions, that is, two carrier streams (i.e., 0.05 M phosphate buffer (pH 7.0) and water) and a mobile phase, were pumped through the individual pumps. Sample solutions containing unknown concentrations of pregnenolone and 17α-hydroxypregnenolone were injected through the automatic sample injector in a sample volume of 5 μl. The IMER was thermostated at 40 °C in a column oven and pre-equilibrated with a carrier stream containing phosphate buffer (pH 7.0) prior to sample injection. The sample solution was passed via the carrier stream containing phosphate buffer (pH 7.0; pump A), and was pumped at a flow rate of 0.3 ml/min. The solution was then passed through a trap column

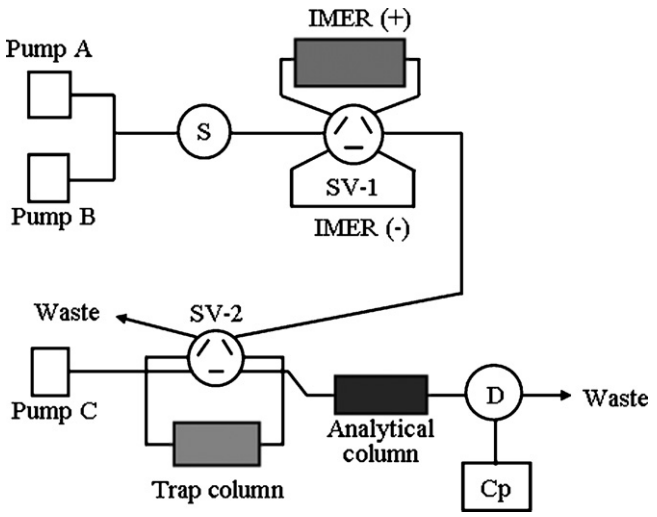

Fig. 1. Schematic diagram of the pre-column IMER/semi-micro HPLC system for the determination of pregnenolone and 17α-hydroxypregnenolone. IMER = immobilized enzyme reactor; pump A = 0.05 M phosphate buffer, pH 7.0; pump B = water; pump C = mobile phase (acetonitrile:water, 50:50); S = sample injector; SV = switching valve; D = detector; Cp = computing integrator.

(Guard Cartridge Capcell pak MF Ph-1, 10 mm × 2 mm i.d., Shiseido, Tokyo, Japan). After 5 min, the switching valve (SV) on this line (SV-2) was switched, and the enzymatically converted progesterone and 17α-hydroxyprogesterone were back-eluted from the trap column using a mobile phase (acetonitrile:water = 50:50), which was pumped at a flow rate of 0.15 ml/min (pump C). Progesterone and 17α-hydroxyprogesterone were subsequently separated on an analytical column (Capcell pak C1, 150 mm × 2 mm i.d., type UG 120, Shiseido), and detected by UV absorption at 240 nm with a photodiode array detector (SPD-M10A, Shimadzu). The trap column and analytical column were thermostated at 40 °C in the same column oven as the IMER. Chromatographic data were recorded and evaluated using CLASS M10A software (Shimadzu). During the period of separation and detection, the IMER and the trap column were washed with water (pump B) then buffered with phosphate buffer (pH 7.0; pump A), to prepare for the next analysis.

2.8. Calibration and assay validation

The calibration curve for the assay was performed, in triplicate, by subjecting the peak areas for the spiked concentrations of pregnenolone and 17α-hydroxypregnenolone to least-squares linear regression analysis. The limits of detection (LOD) and the limit of quantitation (LOQ) were estimated in terms of the concentration of pregnenolone or 17α-hydroxypregnenolone giving a signal-to-noise ratio of 3 for LOD and 10 for LOQ. Intra- and inter-day precision and accuracy were evaluated by assaying four replicates of two different concentration levels of standard samples for pregnenolone and 17α-hydroxypregnenolone (0.4 μg/ml and 2 μg/ml, respectively). Intra-day precision was evaluated by calculating the coefficients of relative standard deviation (RSD) in analyzing four replicates of two different concentration levels within 1 day; four replicates of two different concentration levels were analyzed within 1 week to determine inter-day precision. Accuracy was expressed as the mean percentage of the concentration observed divided by the nominal concentration. Recovery was estimated by dividing the peak area observed in which a sample was added to the culture medium and then treated with the solid phase extraction, by the peak area observed in which a sample was directly injected to the IMER–HPLC. Recovery was also estimated for two different concentration levels of standard samples for both pregnenolone and 17α-hydroxypregnenolone (0.5 μg/ml and 1 μg/ml, respectively). Each test was repeated three times.

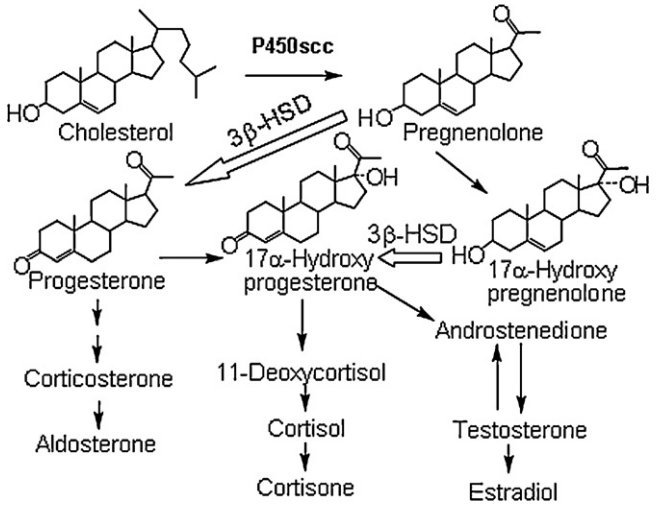

Fig. 2. Biosynthetic pathway of adrenal steroid hormones.

3. Results and discussion

The biosynthesis of adrenal steroid hormones starts with the cleavage of the side chain of cholesterol to form pregnenolone, and pregnenolone subsequently undergoes hydroxylation to form 17α-hydroxypregnenolone. The former reaction is catalyzed by P450scc, and the latter reaction by 17α-hydroxylase. Pregnenolone or 17α-hydroxypregnenolone is converted by 3-β-hydroxysteroid dehydrogenase to give progesterone or 17α-hydroxyprogesterone, respectively. The biosynthetic pathway of adrenal steroid hormones is illustrated in Fig. 2.

3.1. Specificity

Simultaneous determination of pregnenolone and 17α-hydroxypregnenolone using the pre-column IMER/semi-micro reversed-phase HPLC system was performed as described in Section 2.7. Fig. 3 shows a typical chromatogram obtained using standard solutions. The detection times for progesterone enzymatically converted from pregnenolone and for 17α-hydroxyprogesterone enzymatically converted from 17α-hydroxypregnenolone were

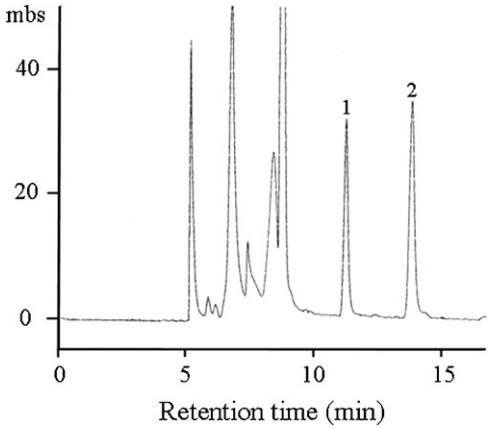

Fig. 3. Typical chromatogram of standard preparations using the pre-column IMER/HPLC system. Peaks: 1 = 17α-hydroxypregnenolone; 2 = pregnenolone.

Table 1
Precision and accuracy of HPLC analysis of pregnenolone and 17α-hydroxypregnenolone.

Analyte	Nominal concentration (μg/ml)	Intra-day (n = 4)		Inter-day (n = 4)	
		Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)
Pregnenolone	0.4	94.8	6.3	96.3	9.3
	2.0	97.9	5.2	98.6	8.0
17α-Hydroxypregnenolone	0.4	98.5	7.5	98.1	5.5
	2.0	99.3	3.6	98.8	6.3

Table 2
Analytical recoveries of pregnenolone and 17α-hydroxypregnenolone from the incubation medium (KRH buffer).

Analyte	Spiked (μg/ml)	Expected (μg/ml)	Detected (μg/ml)	Recovery (%)
Pregnenolone	0.5	3.0	3.29	109.7
	1.0	6.0	6.09	101.5
17α-Hydroxypregnenolone	0.5	3.0	3.15	105.0
	1.0	6.0	5.40	90.0

approximately 11.2 and 13.9 min, respectively. The time for one measurement was 15 min.

Detection times were comparable to those for the standard solutions of progesterone and 17α-hydroxyprogesterone, and the detected peaks were ascertained by diode array spectral analysis. There was no UV absorption at 240 nm for pregnenolone or 17α-hydroxypregnenolone before enzymatic conversion. Matrix-specific interfering peaks from the reaction medium were not observed, and the addition of 10–500 μM trilostane to the reaction medium did not interfere with the simultaneous determination of pregnenolone and 17α-hydroxypregnenolone.

3.2. Linearity

Peak area (y, mV s) was measured and plotted against the concentration (x, μg/ml) of pregnenolone or 17α-hydroxypregnenolone. The calibration curves were linear in the range of 0.4–10 and 0.3–10 μg/ml for pregnenolone and 17α-hydroxypregnenolone, respectively. The regression equations of the calibration curves were $y = 39.25x - 2.49$ ($r^2 = 0.9993$) and $y = 33.67x - 1.85$ ($r^2 = 0.9998$) for pregnenolone and 17α-hydroxypregnenolone, respectively.

3.3. Sensitivity

The LODs at a signal-to-noise ratio of 3 were 0.12 and 0.08 μg/ml for pregnenolone and 17α-hydroxypregnenolone, respectively, while those at a signal-to-noise ratio of 10 were 0.4 and 0.3 μg/ml, respectively.

3.4. IMER efficiency and stability

Calibration curves for progesterone and 17α-hydroxyprogesterone were also obtained by analyzing standard solutions with the IMER(−) line. By comparison with the regression equations for pregnenolone and 17α-hydroxypregnenolone obtained with the IMER(+) line, the conversion rates of progesterone and 17α-hydroxyprogesterone were calculated to be 90.6% for pregnenolone and 99.3% for 17α-hydroxypregnenolone. The IMER is extremely stable for repeated use (greater than 1000 sample injections), and therefore can be used without any significant decrease in activity.

3.5. Accuracy and precision

Table 1 shows the intra- and inter-day accuracy and precision (RSD) for progesterone and 17α-hydroxyprogesterone. The intra-day precision ranged from 5.2% to 7.5%, and the inter-day precision ranged from 5.5% to 9.3%. The accuracy ranged from 94.8% to 99.3%. Both precision and accuracy were within the acceptable ranges.

3.6. Recovery

Recovery was determined by comparing the peak area of the standard solutions without solid phase extraction to that of standard solutions spiked into KRH buffer at two different concentration levels and then extracted by solid phase extraction. As shown in Table 2, all recovery values for pregnenolone and 17α-hydroxypregnenolone ranged from 90.0% to 109.7%. In future, the accuracy of recovery determination will be further improved by the use of an adequate internal standard.

3.7. Applications to studies using bovine adrenal fasciculata cells

In the adrenal fasciculata, ACTH binds its receptor on the cell surface to stimulate steroidogenesis. The first step in the production of steroid hormones involves the cleavage of cholesterol, which is converted to pregnenolone by the enzyme cytochrome P450scc [13]. Pregnenolone is then converted to progesterone by the enzyme 3-β-hydroxysteroid dehydrogenase. Essentially the steroid hormones are catalyzed by enzyme cascades. Trilostane, a modified steroid molecule, is a competitive inhibitor of 3-β-hydroxysteroid dehydrogenase. In vitro, trilostane inhibits the conversion of pregnenolone to progesterone but does not alter the conversion of cholesterol to pregnenolone or of progesterone to corticoid hormones [14]. As shown in Table 3, the culture medium (extracellular fraction) of bovine cells stimulated with ACTH in the presence of trilostane for 1.5 h, contained 0.449 ± 0.015 μg/ml pregnenolone and 0.334 ± 0.087 μg/ml 17α-hydroxypregnenolone (n = 3 for

Table 3
Simultaneous determination of pregnenolone and 17α-hydroxypregnenolone secreted from or found intracellularly in bovine adrenal fasciculata cells.

Sample	Additions		Amount determined (μg/ml)	
	ACTH	Trilostane	Pregnenolone	17α-Hydroxypregnenolone
Extracellular				
1	−	−	<DL	<DL
2	−	+	<DL	<DL
3	+	−	<DL	<DL
4	+	+	0.449 ± 0.015	0.334 ± 0.087
Intracellular				
1	−	−	<DL	<DL
2	−	+	<DL	<DL
3	+	−	<DL	<DL
4	+	+	0.343 ± 0.110	0.158 ± 0.053

Values are mean ± SD (n = 3); <DL, below detection limit.

both). When the cells were not stimulated with ACTH, or were stimulated with ACTH in the absence of trilostane, the peaks for progesterone and 17α-hydroxy progesterone were less than the LOD. Addition of trilostane enabled the determination of pregnenolone and 17α-hydroxypregnenolone by inhibiting steroidogenesis. Similarly, adrenal fasciculata cells (intracellular fraction) stimulated with ACTH in the presence of trilostane for 1.5 h contained 0.343 ± 0.110 μg/ml pregnenolone and 0.158 ± 0.053 μg/ml 17α-hydroxypregnenolone ($n = 3$ for both). When the cells were not stimulated with ACTH, or were stimulated with ACTH in the absence of trilostane, the peaks for progesterone and 17α-hydroxy progesterone were less than the LOD. Thus, we suggest that the present method is suitable for the investigation of early steroidogenesis in adrenal fasciculata cells.

4. Conclusion

The pre-column IMER/semi-micro HPLC system developed in this study is a novel UV-detection method for the simultaneous determination of pregnenolone and 17α-hydroxypregnenolone. The enzymatic conversion of pregnenolone and 17α-hydroxypregnenolone to progesterone and 17α-hydroxyprogesterone by an immobilized cholesterol oxidase reactor, followed by the analysis of the oxidized product by semi-micro reversed-phase HPLC was successfully achieved. The total run time per sample was within 15 min. The method was then successfully used to determine the amounts of pregnenolone and 17α-hydroxypregnenolone secreted into the culture medium (extracellular fraction) of bovine adrenal fasciculata cells and the amounts of both analytes produced within the cells (intracellular fraction). Trilostane inhibited 3-β-hydroxysteroid dehydrogenase in the cells, and thereby enabled the simultaneous determination of pregnenolone and 17α-hydroxypregnenolone. This method was suitable for the investigation of the initial step in steroidogenesis in adrenal fasciculata cells.

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