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RADIOIMMUNOASSAY OF URINARY FREE CORTISOL.(U)
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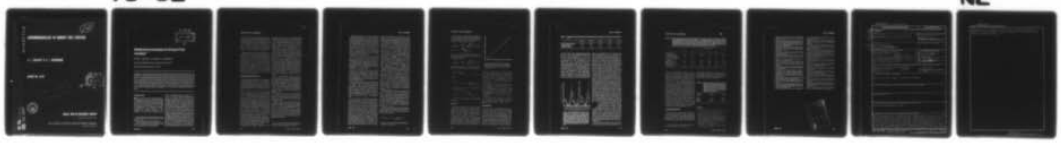

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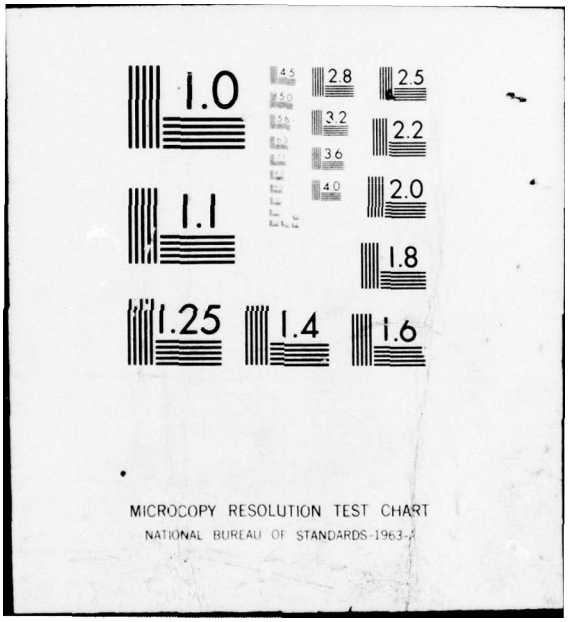
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RADIOIMMUNOASSAY OF URINARY FREE CORTISOL

R. L. SOKOLOFF & R. L. HILDERBRAND

REPORT NO. 76-62

NAVAL HEALTH RESEARCH CENTER
SAN DIEGO, CALIFORNIA 92152

NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND
BETHESDA, MARYLAND

Radioimmunoassay of Urinary Free Cortisol[a]

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Received for publication October 13, 1976

Abstract. The radioimmunoassay for urinary free cortisol described in this paper is simple, rapid, and reproducible. The method uses a commercially available antibody preparation and is performed in two steps. The first step includes an extraction and a column purification to remove materials antigenically similar to cortisol from the urine. The second step is the radioimmunoassay using dextran-coated charcoal to separate bound and unbound cortisol. ^{3}H-cortisol is added prior to any mechanical manipulation to allow calculation of analytical recovery for the purification procedure. The coefficient of variation for interassay determinations was a maximum of 10.3% and for intraassay determinations a maximum of 5.7%. Analytical recovery averaged 97.7%. One technician can analyze 100 samples per week.

INTRODUCTION

TISSUE cortisol levels as a function of psychological or physical stress have been of particular interest in numerous investigations (8, 22, 24, 27, 28). Levels have been determined by fluorometry (7, 30, 31), gas chromatography (4, 19) and gas chromatography-mass spectrometry (25, 32), competitive protein binding assay (CPB) (3, 14, 18, 20, 23), and most recently, radioimmunoassay (RIA) (2, 6, 9, 13, 14, 15, 26, 29). RIA determination of cortisol in human serum or plasma is simplified by the relatively low concentrations of antigenically similar materials which are present, and by use of specific antibody preparations which are commercially available. Diurnal variation studies of cortisol excretion using indwelling venous catheters (10, 11, 33) have demonstrated episodic excretion of cortisol. This creates a problem in the interpretation of results from a single venipuncture in human factors research and may indicate a need for the use of indwelling venous catheters in certain studies (22, 27). Urinary free cortisol (UFC) has been used as an indicator of adrenocortical function for many years and in addition is used in the human factors research to relate

[a] Presented before the Laboratory Section, American Public Health Association, Miami Beach, Florida, October 19, 1976.

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[c] The views presented in this paper are those of the authors. No endorsement by the Department of the Navy has been given or should be inferred.

corticosteroid excretion to human performance (24). Although the specificity of determination of UFC increased with the advent of the CPB assays and has increased even more with RIA, the wide spectrum of urinary steroids, including cortisol conjugates (4, 21) may necessitate additional steps to remove interfering constituents.

This report describes a method for determining UFC which we have found to be reliable. It uses a simple chromatography step to remove cross reacting materials and offers the convenience of using a commercial antibody preparation. The procedure has been applied to over 1,000 urine specimens as part of an ongoing program to correlate biochemical constituent values to occupational stress and performance levels among Naval personnel (e.g. 28). The correlation of the cortisol levels to stress and performance will be reported elsewhere.

MATERIALS AND METHODS

Buffer Solution. Prepare a 0.125 M borate, 0.11 M NaCl buffer, pH 7.6. This is stable at room temperature for at least two weeks.

Gelatin Solution. Prepare on day of use by dissolving 100 mg gelatin (Sigma) in 100 ml buffer solution at 30 C. Cool and maintain at 0–4 C.

[1, 2, 6, 7-^{3}H]-Cortisol Solutions. Prepare the stock solution by first evaporating the solvent from an ampoule containing 1 mCi [1, 2, 6, 7-^{3}H]-cortisol (Amersham-Searle) with the aid of N_2 gas. The ^{3}H-cortisol is redissolved in 4.2 ml 10% ethyl acetate in *iso*-octane. One milliliter aliquots are each subjected to column chromatography as described below, and the eluted radiocortisol in the fractions containing 40% ethyl acetate in *iso*-octane, is pooled in a 15-ml culture tube by successively adding a fraction and evaporating the solvent with the aid of N_2 gas. The ^{3}H-cortisol is redissolved in 10 ml methylene chloride and stored at −20 C.

During column purification aliquots are subjected to liquid scintillation determinations in order to calculate the recovery. Recovery of the radiolabel in the 40% ethyl acetate fraction should exceed 90%.

Radiocortisol working solution is prepared just prior to use by evaporating the methylene chloride from 0.6 ml ^{3}H-cortisol stock solution and redissolving the radiocortisol in 100 ml buffer solution. Triplicate 200 μl aliquots are removed for subsequent liquid scintillation determinations ($DPM_{extraction,\ 200\ \mu l}$ in "Calculations").

Cortisol Standard (with ^{3}H-Cortisol Solution), 100 ng/ml. Dissolve 25 mg cortisol (Calbiochem) in 100 ml methanol and prepare a 1 μl/ml cortisol solution by making serial dilutions with methanol in benzene solution (1 : 9, v/v). Transfer 9 ml to a volumetric flask, add 1,000 ng ^{3}H-cortisol in methylene chloride (determined with specific radioactivity data supplied by the manufacturer), and dilute to 100 ml with methanol in benzene. Triplicate 200 μl aliquots are subjected to liquid scintillation counting in order to check the specific radioactivity of the standard ($SR_{standard}$). The standard is stored as 4 ml aliquots in culture tubes at −40 C.

Anti-cortisol Antibody Solution. The contents of a "100 test" vial[d] containing lyophylized anti-cortisol antibody (RIA of California, 17688 Patterson, Perris, CA 92370) are dissolved in 6 ml buffer solution. Aliquots of 0.5 ml are stored in 15-ml culture tubes at −40 C. This is sufficient for over 600 equilibration mixtures. In determining optimum concentrations we referred to Zettner's Method 1 (34).

Immediately prior to use, the frozen antibody solution in one tube is thawed and diluted to 10 ml with cold gelatin solution. The solution is maintained in an ice-water bath throughout use.

Column Chromatography. The chromatography system is described by Abraham, et al. (1). In brief, celite is heated at 1000 F for 16 to 18 h, cooled, and mixed very thoroughly with ethylene glycol : water (80 : 20), using 0.5 ml of stationary phase for each gram of celite. This column material can be stored in a sealed container at room temperature for up to 3 weeks without performance decrement.

[d] The vial contains antibody which is sufficient for 100 determinations on plasma when the procedure recommended by RIA of California is used (5, 6).

Disposable pipettes (5.0 ml) containing a 3-mm glass boiling bead at the bottom are packed with 400 mg of column material by vacuum aspiration. The prepared columns with sample added are eluted twice with 3.5 ml of 10% ethyl acetate in *iso*-octane and then eluted once with 3.5 ml of 20%, 30%, and 40% ethyl acetate in *iso*-octane, successively. ^{3}H-cortisol was recovered in our work in the 40% elution step. All solvents are prepared immediately prior to use and columns are used only once.

Dextran-coated Charcoal. Prepare on the day of use. Five grams Norit A (Amend Drug and Chemical Co.) and 0.5 g Dextran-70 (Pharmacia) are suspended in 85 ml water in a 150-ml beaker with the aid of an overhead stirrer for 3 h. During the final hour and throughout use, the suspension is maintained in an ice-water bath.

Liquid Scintillation Counting. Glass counting vials, prepared for liquid scintillation counting, contain 200 μl sample and 10 ml Aquasol (New England Nuclear). The counting efficiency is determined for each assay through the use of an internal ^{3}H-toluene standard (ICN).

Unless otherwise specified, all chemicals are 'Baker Analyzed' Reagent Grade from J. T. Baker, and are used without further purification. Semi-automatic samplers (Gilman) and repeat pipetors (Oxford) may be used throughout the procedure. All glassware is prepared by washing with Acationox solution (Scientific Products), rinsing ten times with deionized water, and drying at 60 C.

Assay Procedure. Each extraction mixture is prepared by placing a urine aliquot equivalent to 1/125 of an average hourly volume into a 15-ml culture tube, adding buffer to increase the total volume to 1 ml, and then adding 500 μl ^{3}H-cortisol working solution and 5 ml methylene chloride. Each tube is covered with a teflon-lined cap, shaken vigorously for 5 min, and centrifuged 2 min at 2,000 × g. The aqueous layer is aspirated and discarded, and 0.5 g anhydrous Na_2SO_4 is added. The mixtures are shaken for 2 min and centrifuged 2 min; 3 ml aliquots are transferred to disposable 13 × 100-mm test tubes and the methylene chloride is evaporated by placing the tubes in a 50–55 C bath under a stream of air. The cortisol is redissolved in 1 ml 10% ethyl acetate in *iso*-octane solution and subjected to column chromatography as described above. The cortisol is eluted in the 40% fraction which is collected in a 13 × 100-mm test tube. The solvent is evaporated with the aid of N_2 gas and a 50–55 C bath, and the cortisol is redissolved in 1.7 ml buffer solution. A 200 μl aliquot is removed from each solution and placed in a corresponding counting vial for subsequent liquid scintillation determination ($DPM_{column,\ 200\ \mu l}$). Each equilibration mixture is prepared by transferring another 200 μl aliquot to a 10 × 75-mm test tube, placing the tube in an ice-water bath, adding 200 μl antibody solution, subjecting to vortex mixing, and transferring the tube to a 3 C shaker bath.

Reference equilibration mixtures are prepared by placing 5, 10, 20, 30, 50, 70, 90, 110, 130, 150, and 170 μl aliquots of cortisol standard (with ^{3}H-cortisol) solution in 13 × 100-ml test tubes, evaporating the solvent with the aid of N_2 gas, redissolving the cortisol in 1.7 ml buffer solution, and then transferring a 200 μl aliquot and adding antibody solution as described above. Another 200 μl aliquot of each cortisol standard in buffer solution is transferred to a counting vial for liquid scintillation determination ($DPM_{standard,\ 200\ \mu l}$).

The equilibration mixtures are maintained in a 0–3 C shaker bath for 18 h. Fifty microliters of the dextran-charcoal suspension are added, and followed immediately by vortex mixing. Each mixture is replaced in the bath 2–3 min, and then centrifuged 2 min at 0 C. A 200 μl aliquot of each supernatant is transferred to a corresponding scintilllation vial for liquid scintillation determination ($DPM_{equilibration,\ 200\mu l}$).

Calculations. Excretion of UFC (ng/min) can be calculated from the data by the following steps. For each assay, parameters m and b in the function

$$F_{equilibration} = m \times \frac{1}{\%\ bound} + b$$

= total cortisol (labelled plus unlabelled) in equilibration mixture

are determined by applying the method of least squares for a linear fit (17) to data collected from the reference equilibration mixtures. For each of these mixtures

$$\frac{1}{\%\text{ bound}} = 0.444 \times \frac{DPM_{standard,\ 200\ \mu l}}{DPM_{equilibration,\ 200\ \mu l}}$$

$$F_{equilibration} = \frac{DPM_{standard,\ 200\ \mu l}}{SR_{standard}}$$

$F_{equilibration}$ is estimated for the mixtures containing cortisol extracted from urine by applying the equation

$$\frac{1}{\%\text{ bound}} = 0.444 \times \frac{DPM_{column,\ 200\ \mu l}}{DPM_{equilibration,\ 200\ \mu l}}$$

followed by application of the first equation. Lastly, the excretion rate is determined:

$$\text{Rate (ng/min)} = 2.08 \times (F_{extraction} - F^{*}_{extraction})$$

where

$$F_{extraction} = 2.5 \times F_{equilibration} \times \frac{DPM_{extraction,\ 200\ \mu l}}{DPM_{column,\ 200\ \mu l}}$$

= total cortisol (labelled plus unlabelled) in extraction mixture

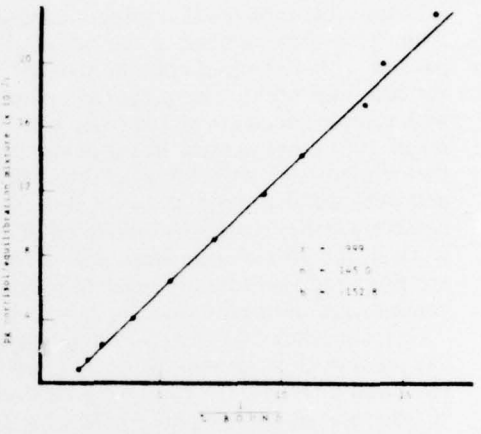
$$F^{*}_{extraction} = 2.5 \times \frac{DPM_{extraction,\ 200\ \mu l}}{SR_{working}}$$

= labelled cortisol added to extraction mixtures

RESULTS

The relationship between bound cortisol and cortisol present in an equilibration mixture may be established for each assay by a graph rather than by application of the method of least squares. The result from a typical determination is shown in Graph 1.

Interassay and intra-assay variabilities were determined on aliquots of pooled urine covering a range of average cortisol excretion rates. Each pool was prepared by combining individual urine specimens in amounts proportional to column excretion rates and freezing aliquots at −40 C. Interassay variability (Table 1) and intra-assay variability (Table 2) averaged 6.7% and 3.9%, respectively. Analytical recovery averaged 97.7% throughout the range of the test (Table 2).



Graph 1. ***Relationship between reciprocal percent bound cortisol and total cortisol (pg) in equilibration mixture based on results from reference equilibration mixtures.***

In another study, urine specimens were collected from 3 male subjects at 3 h intervals throughout a 78 h period. Excretion rates were highest for each subject during the interval 0300 through 0600 or 0600 through 0900 and were lowest during intervals immediately preceding these periods (Graph 2).

The 24 h excretion rate of UFC for 30 subjects averaged 25.0 μg. Results were lower for women than for men (Table 3) at a confidence level of .075 (t − value = 1.93).

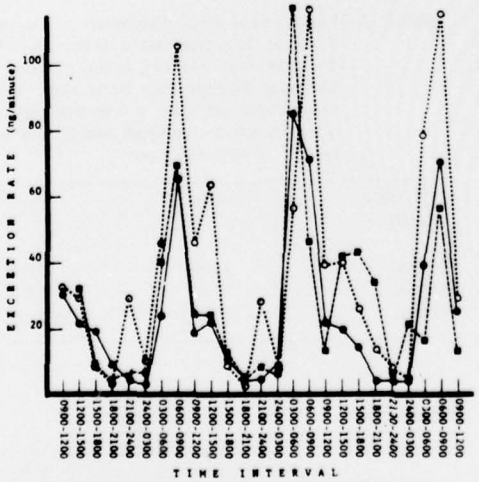
DISCUSSION

We have presented an RIA method for determining UFC which appears to have high specificity. The 24 h excretion rates (Table 3) as determined by the method are comparable to or lower than other published values (12, 18, 20, 23, 29). Four factors contribute to the reliability of the test. First, the commercial antibody preparation possesses unusually low cross-reactivity as determined by Campuzano (5, 6) for serum con-

TABLE 1—*Interassay Variability*. Samples were analyzed without replication 9 times over an 8 month period.

	Pool #1	Pool #2	Pool #3	Pool #4	Pool #5
Average Cortisol Excretion (ng/min-subject)	15.8	28.6	45.6	61.3	84.2
Coefficient of Variation	7.2%	6.3%	10.3%	4.7%	4.9%

stituents. Most other antibody preparations seem to cross-react appreciably with one or more other steroids (2, 9, 13, 14, 15, 26, 27). In some cases this may be due to contamination of the preparations with transcortin. Cross reactivity becomes a serious problem in the analysis of urine, considering that only 1% of serum cortisol is excreted as UFC (12). The remainder is excreted primarily as reduced metabolites which are conjugated to sulfuric and glucuronic acids (4, 12, 21). The multitudinous urinary substances are still being identified (21), therefore, complete determination of the cross-reactivity of an antibody preparation with individual urinary steroids and related metabolites is yet to be done. Second, potentially interfering substances are removed by extraction of the cortisol into methylene chloride and chromatography of the extracts on celite-ethylene glycol-H_2O columns. The columns are easily prepared and the ability of the columns to fractionate serum steroids has been demonstrated (1). In our preliminary experiments, elimination of the column chromatography step resulted in a 2–3 fold increase in determined UFC (Unpublished data). We think that the increase was due to impurities which are removed when chromatography is employed. Third, a single increment of ^{3}H-cortisol is added to the extraction mixtures to monitor procedural losses and to establish the reciprocal percent bound cortisol in the equilibration mixtures. Corrections are made for its presence in each mixture, and so the counting error can be minimized by adding a large amount of this ^{3}H-cortisol tracer. Fourth, and perhaps most unusual, a cortisol standard solution which contains radiocortisol is prepared and aliquots are stored frozen until use. The specific radioactivity of this solution is carefully measured prior to freezing the aliquots. Presence of the radiocortisol [illegible] method of correcting for adsorption, solvent evaporation or other changes [illegible] standard during prolonged storage [illegible] throughout the assay—a feature which is particularly useful for many types of longitudinal studies. Although we experienced difficulty in accurately pipeting small aliquots of the standard solution, the amount of cortisol present in the reference equilibration mixtures could later be precisely determined through knowledge of the specific radioactivity.



Graph 2. *Diurnal Variation of Cortisol Excretion*. Samples were collected from 3 healthy males (Ages 26–30) at 3 h intervals, volumes measured, and aliquots frozen for subsequent analysis. The subjects participated in their usual work-relax-sleep cycles and activities, except that sleep was interrupted in order to obtain urine samples. Each plotted line on the graph represents urinary cortisol values obtained from one of the three subjects.

Both serum and urinary free cortisol levels are related to the corticoadrenal activity

TABLE 2—*Intra-assay Variability and Analytical Recovery.* For each urine pool ten extraction mixtures were prepared as described; five contained 11.2 ng added cortisol. Each extraction mixture was analyzed for UFC. The analytical recovery of the added cortisol for each pool was estimated by subtracting the average value of the five mixtures without added cortisol from the average of the five mixtures with added cortisol. Deviations of the analytical recoveries from 100% are within the variabilities in the measurements shown in the table.

	URINE POOL					
	Blank	#1	#2	#3	#4	#5
Cortisol (ng) in extraction mixture	0.8	5.6	10.8	17.7	22.5	29.7
Coefficient of Variation	3.5%	3.5%	4.1%	2.9%	1.7%	5.5%
Cortisol (ng) in extraction mixtures with added cortisol	11.9	17.5	23.4	27.6	31.7	40.7
Coefficient of Variation	2.6%	2.4%	2.1%	2.8%	5.7%	2.5%
Difference (ng)	11.1	11.9	12.6	9.9	9.2	11.0
Analytical Recovery of added cortisol	99.1%	106.1%	112.2%	88.3%	82.3%	97.9%

and to the cortisol levels in the tissues. Secretion of cortisol by the adrenal gland is believed to be episodic: serum levels may undergo wide fluctuations within a brief period (4, 8, 10, 11, 16, 21, 33) and may even be affected by the sampling process itself (22, 27). A widely held assumption has been that cortisol is excreted in the urine of a particular individual at a rate which is proportional to the serum concentration and that cortisol metabolites maintain unvarying proportionality. Thus, an excretion measurement of urinary free cortisol during a given interval should be an index to the integrated serum level for that period. Although we observed a diurnal variation in the excretion rate of UFC[*] (Graph 1) *vis-a-vis* the circadian profile of serum cortisol measured by others (10, 11, 16, 33), recent evidence does suggest variations in the ratio of urinary cortisol metabolites (4, 21).

ACKNOWLEDGMENTS

We express our appreciation to Dr. Stephen Horvath and Ms. Helen Campuzano for the initial gift of lyophylized antibody preparation, to Mr. Richard Koch for his technical aid, and to Mr. Earl Edwards for his comments during the preparation of this manuscript. The project was funded by the Naval Medical Research and Development Command, No. MF51.524.002-5011.

TABLE 3—*Twenty-four Hour Excretion of Urinary Cortisol.* The rates were determined for 30 apparently healthy, active males and females. Twenty-four hour specimens were obtained over a five day period; aliquots were stored at −40 C and analyzed the following week.

CORTISOL (μg/24 h)	Female (n = 14)	Male (n = 16)
Mean	20.6	28.9
Median	17.4	27.4
Range	4.5–48.1	11.5–52.8

[*] We also observed a similar pattern in plots of μg cortisol/mg creatinine versus time interval.

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1. REPORT NUMBER: 76-62
2. GOVT ACCESSION NO.
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4. TITLE *(and Subtitle)*: Radioimmunoassay of Urinary Free Cortisol.
5. TYPE OF REPORT & PERIOD COVERED: Final rept.,
6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s): Roger L. Sokoloff, M.S.
Richard L. Hilderbrand, Ph.D.
8. CONTRACT OR GRANT NUMBER(s)

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Naval Health Research Center
San Diego, CA 92152
F51524
10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
MF51.524.002-5011

11. CONTROLLING OFFICE NAME AND ADDRESS
Naval Medical Research & Development Command
NNMC, Bethesda, Maryland 20014
12. REPORT DATE
28 September 1976
13. NUMBER OF PAGES

14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*
Bureau of Medicine and Surgery
Department of the Navy
Washington, D. C.
10p.
15. SECURITY CLASS. *(of this report)*
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15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

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Cortisol, Radioimmunoassay, Urine

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The radioimmunoassay for urinary free cortisol described in this paper is simple, rapid, and reproducible. The method uses a commercially available antibody preparation and is performed in two steps. The first step includes an extraction and a column purification to remove materials antigenically similar to cortisol from the urine. The second step is the radioimmunoassay using dextran coated charcoal to separate bound and unbound cortisol. ^{3}H-cortisol is added prior to any mechanical manipulation to allow calculation of analytical recovery for the purification procedure. The coefficient of variation for interassay

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