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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MEASUREMENT OF GUANIDINO COMPOUNDS OF CLINICAL IMPORTANCE IN HUMAN URINE AND SERUM BY PRE-COLUMN FLUORESCENCE DERIVATIZATION USING BENZOIN

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SUMMARY

High-performance liquid chromatographic microanalyses for guanidino compounds in human physiological fluids have been accomplished by means of a pre-column fluorescence derivatization method using benzoin. The guanidino compounds in urine or deproteinized serum after ultrafiltration are converted to the fluorescent derivatives with benzoin in an alkaline medium, and the derivatives are separated simultaneously within 25 min on a reversed-phase column (μBondapak Phenyl) with a linear gradient elution of methanol in aqueous mobile phase (pH 8.5). The method permits the quantitative determination of guanidinosuccinic acid, methylguanidine, taurocyamine and guanidinobutyric acid at concentrations of as low as 8—78 pmol/ml in human urine and serum.

INTRODUCTION

Several guanidino compounds occur in human physiological fluids and tissues. Among the biogenic guanidino compounds, guanidinosuccinic acid and methylguanidine have been considered to be uraemic toxins in uraemic syndrome since these compounds accumulate in the body fluids of uraemic patients [1—3] and give rise to a complex of symptoms similar to uraemia [4, 5]. Recently, taurocyamine also has been suspected as a uraemic toxin since it is increased in uraemic rabbit brain and induces uraemic convulsions [6]. The concentrations of the above three guanidino compounds in normal human serum are generally too low to be quantified by the conventional chromatographic methods including ion-exchange high-performance liquid chromatography (HPLC) [6—11], thin-layer [12, 13], paper [14, 15] and gas [16, 17] chromatography.

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We previously developed a fluorimetric method for the selective determination of guanidino compounds based on their reaction with benzoin in an aqueous methyl cellosolve—potassium hydroxide solution in the presence of β-mercaptoethanol (to stabilize the fluorescent products) and sodium sulphite (to suppress blank fluorescence) [18, 19]. The fluorescent derivatives of the guanidino compounds (excluding guanidine) produced by this benzoin reaction, corresponding to the chemical structures of 2-substituted amino-4,5-diphenylimidazoles [20], were separable by reversed-phase HPLC as reported previously [21], though the reaction gave a small by-product peak identical to the benzoin derivative of guanidine in the chromatogram. Moreover, the sensitivity of that HPLC method enabled the detection of guanidino compounds at the femtomole level. Thus, the pre-column derivatization method with the benzoin reaction may be useful for the HPLC quantification of biogenic guanidino compounds, particularly at very low concentrations in biological samples.

On the other hand, the HPLC method utilizing the benzoin reaction in post-column detection, which was recently described in a separate paper [22], did not allow the reliable quantification of methylguanidine, guanidinosuccinic acid and taurocyamine in normal human sera because the sensitivity of the on-line post-column detection was limited by the dilution of the HPLC column eluate with the reagent solutions. However, the method [22] as well as other post-column derivatization methods (using 9,10-phenanthraquinone [9, 10] or ninhydrin [11] as fluorogenic reagent) permits the quantification of guanidino compounds at the picomole level.

The purpose of the present research was to establish a simple and rapid HPLC method utilizing the benzoin pre-column fluorescence derivatization technique [21] for the microanalysis of those guanidino compounds especially implicated as uraemic toxins in human serum and urine. Phenylguanidine (PG), which is not present in human physiological fluids, was used as an internal standard.

EXPERIMENTAL

Chemicals and solutions

Deionized and distilled water was used. Taurocyamine was kindly supplied by Prof. A. Mori (Institute for Neurobiology, Okayama University Medical School, Okayama, Japan). Tris(hydroxymethyl)aminomethane (Tris) and benzoin (both from Wako, Osaka, Japan) were recrystallised from aqueous methanol (60%, v/v) and absolute methanol, respectively, to remove fluorescent impurities. Other chemicals were of reagent grade. Diaflo membranes (UM 05) used for ultrafiltration were obtained from Amicon (Lexington, MA, U.S.A.). A standard solution of guanidino compounds was prepared in 0.01 *M* hydrochloric acid. The reagent solutions used for the fluorescence derivatization were prepared as described previously [21].

Preparation of human physiological fluids

Serum and urine specimens were obtained from healthy volunteers in our laboratory and from patients with various diseases in hospital (Chidoribashi Hospital, Fukuoka, Japan).

Dialysed serum. The ultrafiltration conditions were examined using dialysed serum. A 10-ml aliquot of normal human serum was dialysed against 6 l of water for 24 h at 4° C using a cellophane tube (Wako).

Serum sample solution. A 300-μl aliquot of serum was mixed with 300 μl of water or a standard solution of guanidino compounds, 200 μl of 2.5 nmol/ml PG and 100 μl of 0.9 *M* hydrochloric acid. The mixture was placed in an Amicon dialysing device (5-ml cell volume) and then ultrafiltered through a UM 05 membrane under nitrogen gas at a pressure of 3 kg/cm^2. A 200-μl aliquot of the filtrate was used for the fluorescence derivatization.

Urine sample solution. A 50-μl aliquot of urine was mixed with 550 μl of water or a standard solution of guanidino compounds, 200 μl of 10 nmol/ml PG and 100 μl of 0.9 *M* hydrochloric acid. A 200-μl aliquot of the resulting mixture was used for the fluorescence derivatization.

Fluorescence derivatization

A 200-μl aliquot of the sample solution was placed in a test tube, to which were added 100 μl each of 4.0 m*M* benzoin solution (in methyl cellosolve) and an aqueous solution containing 0.1 *M* β-mercaptoethanol and 0.2 *M* sodium sulphite, and 200 μl of 2.0 *M* potassium hydroxide with cooling in ice—water. The mixture was heated in a boiling water-bath for 5 min, cooled in ice—water for approx. 1 min, and then 200 μl of an aqueous acidic solution containing 2.0 *M* hydrochloric acid and 0.5 *M* Tris—hydrochloric acid buffer (pH 9.2) were added. A 100-μl aliquot of the final mixture was used for HPLC.

HPLC apparatus and conditions

The HPLC system consisted of a Hitachi 635 high-pressure pump, a Shimadzu SIL-1A syringe-loading sample injector and a Jasco FP-110 HPLC fluorescence spectrophotometer equipped with a xenon lamp. The fluorescence of the eluate was monitored at 425 nm emission against 325 nm excitation. The column was μBondapak Phenyl (particle size 10 μm; 300 × 3.9 mm I.D.; Waters Assoc., Milford, MA, U.S.A.). This column can be used for more than 300 analyses with only a small decrease in the theoretical plate number. For the separation of the benzoin derivatives of guanidino compounds on the column, a linear gradient elution with a methanol concentration between 50% and 80% (v/v) in the aqueous mobile phase containing 10% (v/v) 0.5 *M* Tris—hydrochloric acid buffer (pH 8.5) was carried out during 25 min at a constant flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

The following biogenic guanidino compounds have been demonstrated in human urine and serum [23] (abbreviation in parentheses); guanidine (G), methylguanidine (MG, δ-guanidinovaleric acid (GVA), guanidinoacetic acid (GAA), guanidinopropionic acid (GPA), guanidinobutyric acid (GBA), guanidinosuccinic acid (GSA), arginine (Arg), N-α-acetylarginine (AArg), homoarginine (HArg), taurocyamine (TC), argininosuccinic acid (ASA), creatine (Cr) and creatinine (Crn). Of these, TC, GSA, GBA and MG could be separated and quantified fluorimetrically by the present HPLC method.

Fig. 1 shows typical chromatograms obtained with sera from a patient with renal failure and from a normal subject, and with a normal urine. The benzoin derivatives corresponding to the guanidino compounds and PG as an internal standard in the samples are separated within 25 min on the μBondapak Phenyl column with linear gradient elution of methanol in aqueous mobile phase (pH 8.5) under the HPLC conditions described in the experimental section. All the peaks produced by the derivatization with benzoin (except for the peaks eluted around the time of the void volume) can be readily identified on the basis of their retention times in comparison with the standard compounds and also by co-chromatography of the standards and the sample with a different eluent, i.e. using a lower methanol concentration (40—70%), than that used for the recommended procedure. This elution provided better separation of the peaks but their elution was delayed; the complete separation needed 45 min.

The benzoin reaction does not give fluorescent derivatives of biological substances having no guanidino moiety in the molecule such as amines, amino acids, sugars, lipids, keto acids, steroids, vitamins and nucleosides, as demonstrated previously [18, 19]. In addition, some biological compounds tested (e.g. tyrosine, tryptophan, vitamin A, adenosine, guanosine and thymidine) which have weak native fluorescence do not interfere with either the detection or separation of the peaks of the biogenic guanidino compounds in

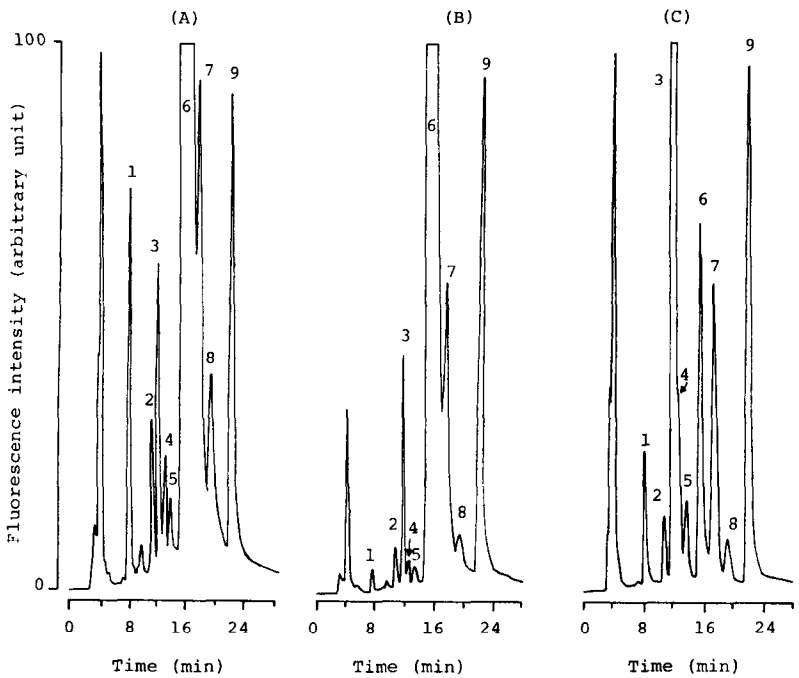

Fig. 1. Chromatograms of the benzoin derivatives of guanidino compounds in serum from a patient with renal failure (A) and from a normal subject (B), and in a normal urine (C). The samples were treated as described in the experimental section. Peaks: 1, GSA; 2, TC; 3, GAA, Cr and Crn; 4, GPA and AArg; 5, GBA; 6, Arg, ASA, GVA and HArg; 7, G and by-products produced from the other guanidino compounds during the derivatization [21]; 8, MG; 9, PG (internal standard).

the chromatograms, because they are co-eluted around the time (3.0—5.0 min) of the void volume.

The chromatographic condition recommended is one of the results of the investigations for the complete separation of TC, GSA and MG which are the compounds of most interest in the toxic manifestation of uraemia, while the basic separation conditions for the derivatives of the guanidino standards were previously described [21]. However, the established HPLC method did not permit the separation of some arginyl-containing oligopeptides in serum from the biogenic guanidino compounds. The benzoin derivatives of several oligopeptides tested with one or two arginyl residues [e.g., tuftsin, angiotensins I, II and III, bradykinin, luteinizing hormone releasing hormone (LHRH) and

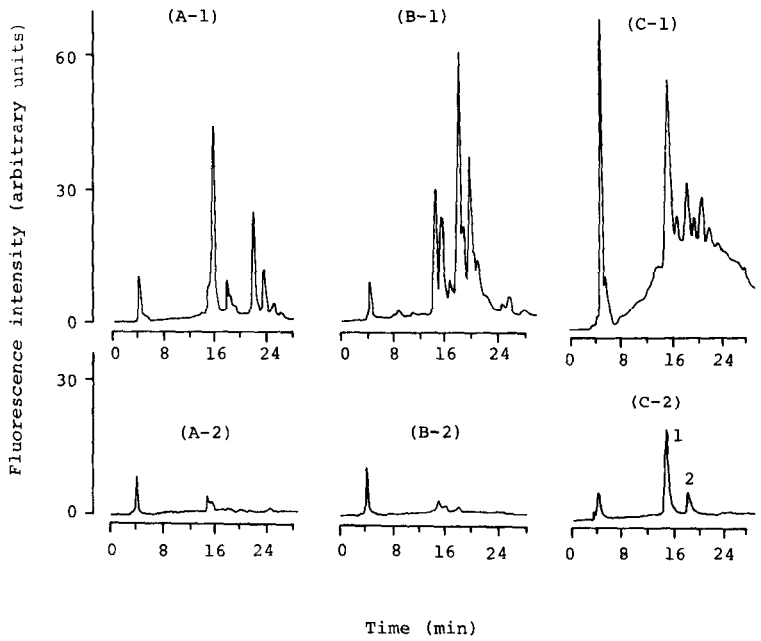

Fig. 2. Effects of ultrafiltration with a UM 05 membrane on removal of peptides and proteins from the peptide solutions (A and B) and a dialysed serum (C). A-1 and B-1 (without ultrafiltration): aliquots (200 μl) of two solutons of peptides (A-1, 2 nmol/ml each of tuftsin and angiotensin III, molecular weight 500—1000; B-1, 2 nmol/ml each of angiotensin II, bradykinin, angiotensin I, LHRH and neurotensin, molecular weight 1000—2000) were directly used for the fluorescence derivatization. A-2 and B-2 (with ultrafiltration): aliquots (900 μl) of the peptide solutions used for A-1 and B-1 were ultrafiltered with a UM 05 membrane, then 200-μl portions of the filtrates were used for the fluorescence derivatization. C-1 (by deproteinization with perchloric acid): to 300 μl of dialysed serum, 250 μl each of the peptide solutions used for A and B and 100 μl of 4 *M* perchloric acid were added. After centrifuging at 800 *g* for 10 min, 200 μl of the supernatant were neutralized with 100 μl of 2 *M* potassium carbonate. A 200-μl portion of the resulting supernatant was used for the fluorescence derivatization. C-2 (by deproteinization with ultrafiltration): to 300 μl of the same dialysed serum as that for C-1, 250 μl each of the peptide solutions used for A and B and 100 μl of 0.4 *M* hydrochloric acid were added. The mixture was then ultrafiltered with a UM 05 membrane. A 200-μl portion of the filtrate was used for the fluorescence derivatization. All the chromatograms were obtained with the same sensitivity range of the detector. Peaks: 1, arginine; 2, by-product; other peaks were from peptides and/or proteins.

TABLE I

RECOVERIES OF GUANIDINO COMPOUNDS ON ULTRAFILTRATION USING A DIAFLO UM 05 MEMBRANE

Conditions*	Percentage recovery** [mean (C.V.)]					Recovery ratio*** [mean (C.V.)]			
	GSA	TC	GBA	MG	PG	GSA	TC	GBA	MG
Three-fold dilution, 0.1 *M* HCl	74 (10)	65 (13)	80 (8)	75 (7)	73 (6)	1.02 (7)	0.90 (7)	1.10 (5)	1.03 (2)
Two-fold dilution, 0.1 *M* HCl	76 (9)	68 (13)	67 (8)	65 (11)	60 (6)	1.25 (4)	1.12 (8)	1.11 (4)	1.08 (6)
Three-fold dilution, H_2O	7 (26)	69 (19)	56 (20)	75 (13)	30 (31)	0.23 (26)	2.37 (16)	1.92 (10)	2.61 (16)
Two-fold dilution, H_2O	5 (38)	67 (9)	58 (9)	52 (18)	36 (11)	0.15 (27)	1.87 (6)	1.61 (6)	1.45 (19)

*Three-fold dilution: to 300 μl of the dialysed serum, 300 μl each of a standard solution of the guanidino compounds (PG, 0.25 nmol/ml; others, 1.0 nmol/ml each) and 300 μl of water or 0.3 *M* hydrochloric acid were added. The mixture was then ultrafiltered with a UM 05 membrane. Two-fold dilution: to 500 μl of the dialysed serum, 300 μl of the standard solution of the guanidino compounds and 200 μl of water or 0.45 *M* hydrochloric acid were added. The mixture was then ultrafiltered with a UM 05 membrane.

**The values were calculated from the results of five analyses using a new UM 05 membrane each time.

***The recoveries of the guanidino compounds on each ultrafiltration were divided by that of PG (internal standard).

neurotensin] are eluted at retention times between 14 and 28 min with two or more fluorescent peaks due to cleavage of peptide bonds during the fluorescence derivatization, as shown in chromatograms A-1 and B-1 of Fig. 2.

An ultrafiltration technique with a UM 05 membrane, which excludes substances larger than approx. 500 daltons, was thus employed to remove such interfering peptides present in samples. Fig. 2 shows the efficacy of the ultrafiltration on the removal of the peptides and serum proteins. The oligopeptides tested (molecular weight 500—2000) in 0.1 *M* hydrochloric acid were not present in the ultrafiltrate (Fig. 2, A-2 and B-2). When the dialysed serum was ultrafiltered, no peak (other than those of arginine and a by-product of arginine due to the fluorescent derivative of guanidine) appeared in the chromatogram (Fig. 2, C-2), even though oligopeptides such as tuftsin, angiotensins I, II and III, bradykinin, LHRH and neurotensin were added to the dialysed serum before the ultrafiltration. The observation of the arginine peak in this chromatogram means that the endogenous arginine still remains in the serum even after the dialysis since the arginine content is generally very high in human serum.

On the other hand, in the chromatogram (Fig. 2, C-1) obtained with the same dialysed serum but which was deproteinized with perchloric acid, there are still several peaks corresponding to the peptides and an enormous broad peak probably due to small proteins not removed by the deproteinization. Thus, deproteinization with an acidic precipitating reagent such as perchloric acid is not suitable for clean-up of serum samples.

Table I shows the recoveries of the guanidino compounds added to the dialysed serum and taken through the ultrafiltration step. Because of the basic nature of the guanidino compounds, acidification of the serum sample gave fairly good recoveries (60—85%) with coefficients of variation (C.V.) of 6—13% for the guanidino compounds tested, though low recoveries for the guanidino compounds especially for GSA and PG were observed when the same sample was diluted two- or three-fold with water. Additionally, the coefficients of variation of their recoveries, depending on the lot number of the membrane, can be reduced by comparing the recovery of each guanidino compound to that of the internal standard, as shown in Table I. Consequently, in the procedure recommended, the serum samples spiked with the internal standard were diluted three-fold with hydrochloric acid (final concentration 0.1 *M*) and then ultrafiltered.

The ultrafiltration procedure was not necessary for normal urine because the determined values of the guanidino compounds in the urine treated or untreated by ultrafiltration were identical within the error of the assay. However, some other urines, particularly from patients with diseases such as proteinuria, may require ultrafiltration. We therefore checked the protein content of each urine sample by a simple test using protein-test paper (Wako) before proceeding with the HPLC.

Calibration curves for both serum and urine, which were constructed by plotting the ratios of net peak heights of the spiked guanidino compounds against the peak height of the internal standard, were linear in the relationship between the ratios and the amounts of the guanidino compounds added to urine or serum. The correlation coefficients (r) of all the curves were greater than 0.997 and no change of the slopes in the graphs was observed depending

on the urine or serum used. In the curves, the ratios of GSA, TC, GBA and MG against the internal standard (PG: 0.5 nmol per 0.3 ml of serum and 2.0 nmol per 50 μl of urine) were 0.24, 0.50, 0.54 and 0.27, respectively, for the concentration of 0.6 nmol per 0.3 ml of serum, and 0.19, 0.40, 0.42 and 0.20, respectively, for the concentration of 2.0 nmol per 50 μl of urine.

The above results indicate that the present internal standard method permits the quantitative determination of the biogenic guanidino compounds in serum and urine.

Concentrations of the guanidino compounds in human serum and urine were analysed by the present method (Tables II and III). The concentrations of GSA and MG in normal serum are very low compared to those in normal urine. However, the levels in sera from patients with uraemia or chronic renal failure undergoing haemodialysis are dramatically increased, as has also been demonstrated in other reports [1—3, 22]. On the other hand, there are no significant differences in the TC and GBA concentrations in the serum between uraemia and other diseases, though both compounds are slightly increased in sera from these patients. The concentration values of the individual guanidino compounds in serum and urine are in good agreement with the data obtained by the post-column fluorescence derivatization methods using 9,10-phenanthra-

TABLE II

CONCENTRATIONS OF GUANIDINO COMPOUNDS IN SERA FROM HEALTHY ADULTS AND PATIENTS

Sex	Guanidino compound (nmol/ml)			
	GSA	TC	GBA	MG
Healthy adults				
F	0.27	0.45	0.11	ND*
F	0.36	0.16	0.04	0.28
M	0.20	0.58	0.05	ND
M	0.10	0.33	0.05	0.13
F	0.34	0.38	0.04	0.12
M	0.49	0.33	0.08	0.22
M	0.23	0.40	0.06	ND
M	0.38	0.43	0.06	0.24
M	0.25	0.42	0.04	0.08
M	0.46	0.43	0.06	ND
Patients**				
M (a)	10.83	2.56	0.32	6.66
M (b)	7.75	1.34	0.46	1.85
F (b)	9.10	0.84	0.43	6.63
M (c)	0.76	3.31	0.43	0.20
F (d)	1.53	0.48	0.30	0.28
M (e)	1.53	0.61	0.52	0.76
F (e)	1.61	1.31	0.36	0.34

*ND, not detected.

**Disease: (a) uraemia; (b) chronic renal failure; (c) gastritis with haemorrhage; (d) stomach cancer; (e) arteriosclerosis.

TABLE III

CONCENTRATIONS OF GUANIDINO COMPOUNDS IN URINE FROM HEALTHY ADULTS

Sex	Urine volume in a day (ml)	Guanidino compounds (μmol/day)			
		GSA	TC	GBA	MG
M	800	40.7	0.4	4.0	19.0
M	880	34.7	9.2	11.5	14.6
M	860	33.6	ND*	3.8	16.6
M	1230	52.5	1.2	4.9	29.9
M	1160	21.3	12.7	12.1	29.3
M	1800	33.1	ND	4.5	23.3
F	720	37.6	0.4	7.1	6.4
F	1030	59.7	7.7	8.1	17.9
F	920	35.5	7.3	15.5	13.7
F	780	32.3	1.2	3.9	10.0

*ND, not detected.

quinone [9, 10], ninhydrin [11] or benzoin [22] with HPLC. But the post-column derivatization methods do not allow the concentrations of GSA, MG and TC in normal sera to be determined. Their concentrations (100—400 pmol/ml) can be determined by the post-column detection methods provided the serum samples are concentrated.

As demonstrated in the Introduction, the present pre-column derivatization method is about 50 times more sensitive than the post-column derivatization method [22] utilizing the benzoin reaction in both methods, even in the analysis of guanidino compounds in normal sera. The sensitivity of determination by post-column reaction may be affected by various factors (other than the column efficiency) such as dilution with the reagent solutions, diffusion in the reactor system and/or the limiting conditions of the derivatization on-line to the chromatograph.

The lower limits of detection for the guanidino compounds determined by the proposed method are 16, 8, 8 and 12 pmol/ml in serum, and 78, 29, 30 and 60 pmol/ml in urine for GSA, TC, GBA and MG, respectively. These values correspond to amounts of approx. 50—100 fmol in an injection volume suitable for this HPLC method, giving a signal-to-noise ratio of 2.

The coefficients of variation in repeatability assays (n = 10) of the guanidino compounds [the compound and its concentration (nmol/ml) in parentheses] in serum are 5.4% (GSA, 0.49), 6.4% (TC, 0.33), 6.1% (GBA, 0.08) and 3.3% (MG, 0.22), and for urine are 4.2% (GSA, 39.4), 4.7% (TC, 10.5), 3.6% (GBA, 13.1) and 3.1% (MG, 16.6).

The present HPLC method with the benzoin pre-column derivatization technique necessitates a clean-up procedure for serum or protein-rich samples but gives a satisfactory sensitivity in the quantitative analysis of the biogenic guanidino compounds especially with suspicion of uraemic toxin; the sensitivity allows the use of less than 300 μl of serum or urine. This method is also rapid and simple to perform and can therefore be applied for routine use.

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