GLC–Mass Fragmentographic Determination of Saccharin in Biological Fluids

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Abstract □ A specific and sensitive method is described for the determination of saccharin in biological fluids. The compound is extracted as its methyl derivative following a salt–solvent pair procedure and assayed by GLC with either flame-ionization or mass fragmentographic detection using ethylated or trideuteromethylated saccharin, respectively, as the internal standard for quantitation. Detector response was linear over concentrations of 50 ng/ml–10 μg/ml with multiple-ion detection mass fragmentography and from 2 μg/ml up to milligram levels with flame-ionization detection. Interference from endogenous substrates was never observed. Plasma kinetics and urinary elimination of saccharin in healthy human volunteers given the sweetener orally, acutely (50 mg/60 kg of body weight) or for 5 days (130 mg/60 kg of body weight/day divided over the three main meals), also are reported.

Keyphrases □ Saccharin—GLC–mass fragmentographic determination in human biological fluids, plasma kinetics and urinary excretion □ GLC—determination of saccharin in human biological fluids, plasma kinetics and urinary excretion □ Mass fragmentography—determination of saccharin in human biological fluids, plasma kinetics and urinary excretion □ Sweeteners, artificial—saccharin, GLC and mass fragmentographic determination of saccharin in biological fluids, humans

Saccharin, 1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide (I), is an artificial sweetener used in soft drinks, slimming diets, and diabetic foods. Recent toxicological studies (1) with this compound showed a higher incidence of bladder cancer in male rats fed during two-generation studies with a diet containing 5% I. Kinetic and distribution studies may be useful for investigating the causes of toxicity induced by exogenous compounds. However, the assay methods described in the literature for I determination are not suitable for studies in humans because of poor sensitivity or the use of labeled substrates (2, 3). Therefore, a specific and sensitive GLC–mass fragmentographic procedure was developed for the measurement of nanogram concentrations of I in biological fluids.

EXPERIMENTAL

Chemicals—Saccharin[1] (I) was used as the dihydrated sodium salt. Methyl iodide[2], ethyl iodide[2], trideuteromethyl iodide[3], and tetrahexylammonium hydrogen sulfate[4] also were used. All solvents were analytical reagent grade.

GLC—The chromatograph[5] was equipped with a flame-ionization detector[6]. The column was a glass tube, 2 m × 4 mm i.d., packed with 100–120-mesh Gas Chrom Q coated with 3% OV-17[7]. All newly prepared columns were conditioned at 280° for 1 hr without carrier gas flow and then for 24 hr with a carrier gas flow rate of 15 ml/min. During analysis, nitrogen was used as the carrier gas at a flow rate of 30 ml/min. Air and hydrogen flow rates were adjusted to give maximum detector response. The operating conditions were: column oven temperature, 210°; injection port heater temperature, 250°; and flame-ionization detector temperature, 250°.

[1] Lot X 17PS4632, Sherwin-Williams Co., Cleveland, Ohio.
[2] Merck, Darmstadt, West Germany.
[3] CEA, Gif-sur-Yvette, France.
[4] Hässle, Göteborg, Sweden.
[5] Carlo Erba Fractovap model G1.
[6] Carlo Erba model 20.
[7] Applied Science Laboratories, State College, Pa.

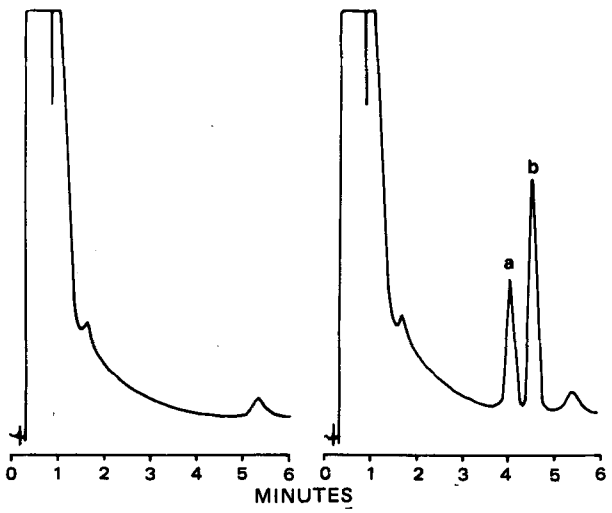

Figure 1—*Chromatograms obtained from control urine (left) and urine of a volunteer after I intake (right). Key: peak a, methylated I; and peak b, ethylated I (internal standard).*

Mass Spectrometry—Mass spectra were recorded on an instrument coupled with a gas chromatograph[8] and used under the following conditions: energy of the ionization beam, 70 ev; ion source temperature, 290°; accelerating voltage, 3.5 kv; and trap current, 60 μamp. Mass fragmentography was carried out at 70 ev by focusing the mass spectrometer[9] on the ions at *m/z* 197 and 133 for methylated I and at *m/z* 200 and 136, characteristic of the spectrum of trideuteromethylated I. In both

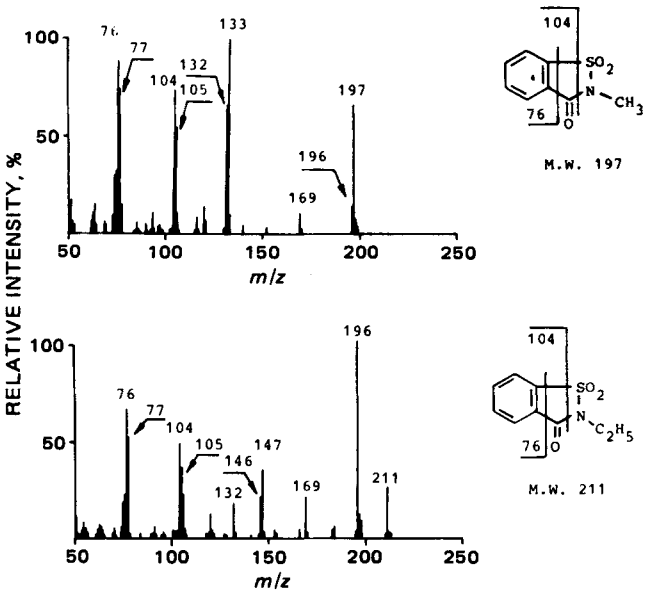

Figure 2—*Mass spectra of methylated I (top) and ethylated I (bottom).*

[8] LKB model 9000.
[9] LKB model 2091-B equipped with a computer system (model 2130) for data acquisition and calculation.

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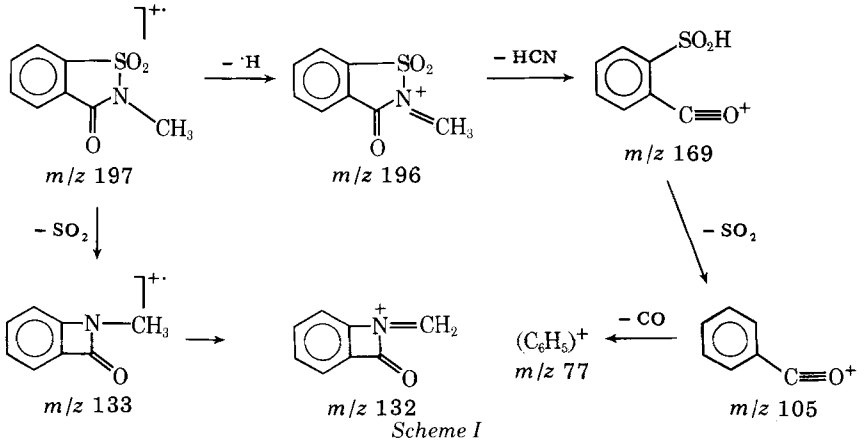


Scheme I

cases, the GLC conditions were as described, except that helium was the carrier gas.

Extraction and Derivative Formation—To 1 ml of plasma or urine, either 20 μg of ethylated I or 500 ng of trideuteromethylated I as the internal standard was added, depending on the expected concentration range and the type of detector used (flame ionization or mass fragmentography). The samples were deproteinized by the addition of 100 μl of trichloroacetic acid and then centrifuged at 4000×*g* for 20 min. The supernates were transferred to hermetically sealable glass tubes bearing a polytef disk in the inner part of the cap and were extracted with 5 ml of 0.5 *M* methyl iodide–methylene chloride solution after the addition of 70 μl of 10 *N* NaOH and 50 μl of 0.1 *M* tetrahexylammonium hydrogen sulfate solution in 0.1 *N* NaOH. Extraction was carried out for 2 hr by shaking at 60° in subdued light.

The organic phase (4 ml) was transferred to conical glass tubes and evaporated to dryness under a gentle nitrogen stream in a water bath at 35°. The residue was resuspended in 50 μl of methylene chloride, and the tetrahexylammonium salt was precipitated by the addition of 2 ml of *n*-hexane. The tubes were capped and centrifuged for 5 min at 4000×*g*. The supernatant fraction (1.8 ml) was transferred to conical glass tubes and evaporated to dryness under a nitrogen stream at 35°. The residue was dissolved in 50 μl of methylene chloride, and a 1–5-μl aliquot of this solution was injected for either GLC or mass fragmentographic analysis. Addition of I to drug-free plasma and urine samples at concentrations

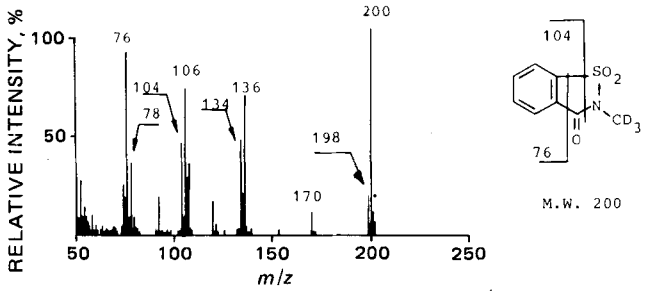


Figure 3—*Mass spectrum of trideuteromethylated I.*

Table I—Precision of I Assay Method in Human Plasma and Urine

Parameter	Concentration of I in Plasma[a] 0.05 μg/ml	0.5 μg/ml	5 μg/ml	Concentration of I in Urine[b] 5 μg/ml	50 μg/ml	500 μg/ml
$\bar{X}$	0.0487	0.498	5.08	4.87	51.5	517.2
SD	0.0045	0.014	0.23	0.22	1.9	11.1
CV, %	9.2	2.8	4.5	4.5	3.7	2.2
n	4	4	4	4	4	4

[a] Determination by multiple-ion detection mass fragmentography. [b] Determination by flame-ionization detection GLC.

Table II—Plasma I Kinetic Parameters Determined in Four Healthy Volunteers Given a Single Oral Dose of 50 mg/60 kg of Body Weight

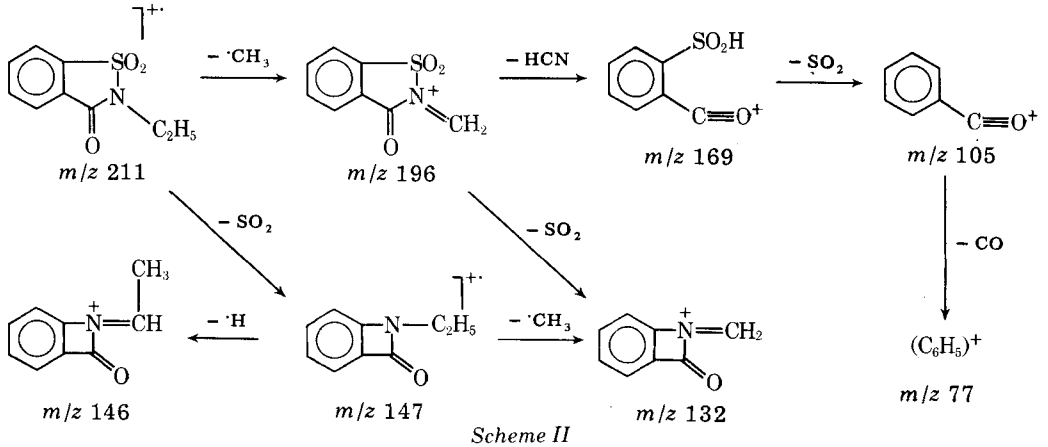
Parameter[a]	$\bar{X} \pm SE$
K_a, min^{-1}	0.045 ± 0.008
$t_{1/2}$, min	121 ± 16
AUC, μg/ml hr	5.02 ± 1.17
Peak value, μg/ml	1.95 ± 0.47
Time of peak concentration, min	30–60

[a] K_a = rate constant of absorption, $t_{1/2}$ = half-life, and *AUC* = area under the curve.

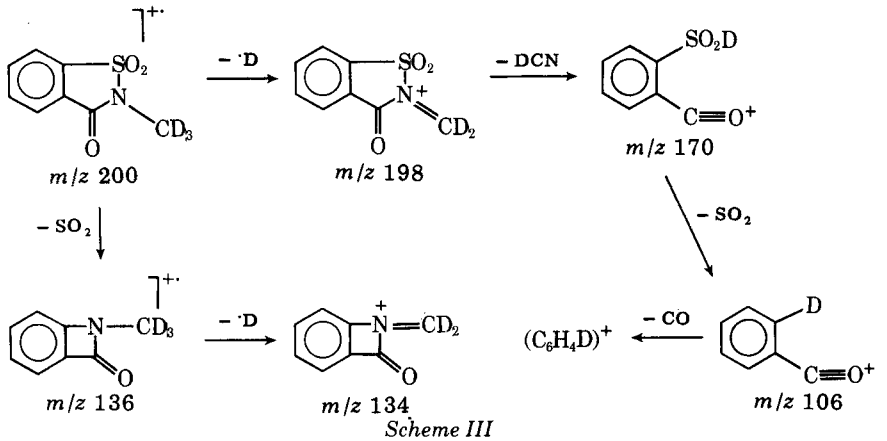
ranging from nanograms to micrograms resulted in overall recoveries of 51 ± 4 and 58 ± 3%, respectively.

Preparation of Ethylated I and Trideuteromethylated I—Ethylated I and trideuteromethylated I were prepared according to the described salt-solvent pair procedure by extracting I from water with either 0.5 *M* ethyl iodide or 0.5 *M* trideuteromethyl iodide–methylene chloride solutions.

Studies in Humans—In the single-dose experiment, four healthy male volunteers ingested a 0.1% solution of I in water (50 mg/60 kg of body weight) after an overnight fast. Venous blood samples were withdrawn 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min after I intake. Urine



Scheme II



Scheme III

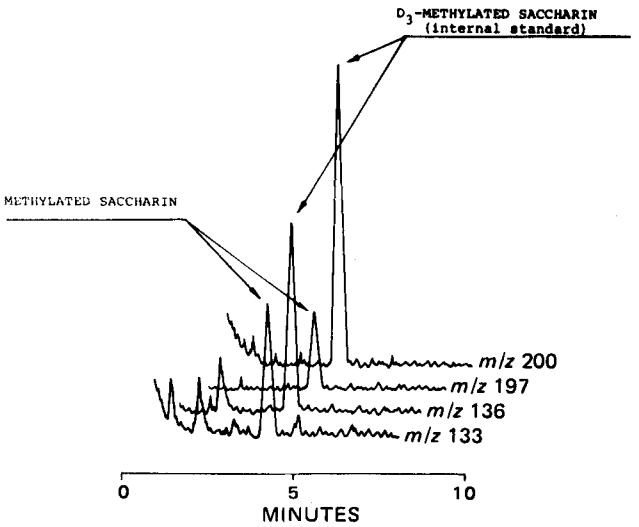


Figure 4—*Mass fragmentogram of a plasma extract from a human volunteer after I intake.*

samples were collected at selected intervals up to 24 hr.

In the repeated-dose experiment, another four healthy male volunteers were given I orally at a dose of 130 mg/60 kg of body weight/day for 5 days. These subjects were free to divide the amount of I into three doses daily to be taken between 7 am and 8 pm with meals. On Day 5, plasma and urine samples were collected at regular intervals over 24 hr.

Data Analysis—Plasma kinetic parameters of I were determined by the method of residuals (4), and the values of the area under the curve (*AUC*) were determined by the trapezoidal rule (5).

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms obtained from the urine extracts of human volunteers before and after intake of saccharin (I). Peak a corresponds to methylated I, and peak b corresponds to an ethylated I structural analog chosen as the internal standard.

Table III—Plasma Levels of I in Four Human Volunteers Given 130 mg/60 kg of Body Weight Orally for 5 Days

Hour	I Concentration, μg/ml
0800	ND[a]
1000	0.35 ± 0.12
1200	0.13 ± 0.07
1400	0.28 ± 0.05
1800	0.07[b]
2000	0.23 ± 0.07
2200	ND
2400	ND
AUC, μg/ml hr	2.24 ± 0.39

[a] Not detectable (<0.05 μg/ml). [b] Only one value available.

Table IV—Urinary I Excretion by Four Healthy Volunteers Given a Single Oral Dose of 50 mg/60 kg of Body Weight

Time Interval	I Concentration	
	Milligrams	Percent of Dose
1000–1200	15.06 ± 2.58	25.6 ± 4.7
1200–1400	10.08 ± 3.49	17.1 ± 6.3
1400–1600	9.62 ± 2.97	16.0 ± 4.9
1600–1800	4.32 ± 0.83[a]	7.4 ± 1.7[a]
1800–2000	3.55 ± 1.36	6.1 ± 2.4
2000–2400	2.76 ± 0.63	4.7 ± 1.2
2400–1000	2.80 ± 0.37	4.8 ± 0.7
Excretion, 24 hr	47.14 ± 1.62	79.9 ± 4.3

[a] Only three values available.

The chemical identity of these peaks was verified by combined GLC–mass spectrometry, and the resulting mass spectra are shown in Fig. 2. The mass spectrum of methylated I showed a prominent molecular ion at *m/z* 197; other intense fragments were present at *m/z* 133 (base peak), 132, 105, 104, 77, and 76, which arose as proposed in Scheme I. The mass spectrum of ethylated I was also consistent with the proposed structure, a molecular ion being present at *m/z* 211 and other intense fragments as shown in Scheme II.

For I determinations at nanogram concentrations, a mass fragmentographic procedure with trideuteromethylated I as the internal standard was used. Trideuteromethylated I has similar physicochemical properties to methylated I, resulting in an identical retention time. The use of such an internal standard reduces column adsorption losses and, consequently, increases the sensitivity.

Figure 3 shows the mass spectrum of trideuteromethylated I. For this compound, the assigned chemical structure was confirmed by the characteristic pattern of fragmentation (Scheme III), which was similar to that of methylated I. Therefore, stable isotope dilution mass fragmen-

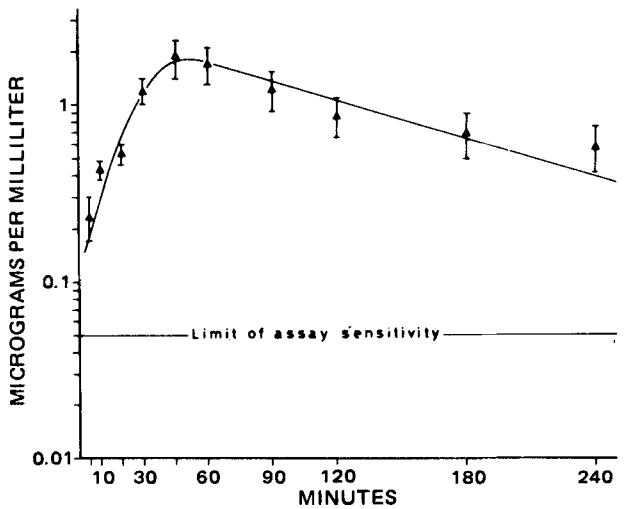


Figure 5—*Plasma I kinetics in four healthy volunteers given a single oral dose of 50 mg/60 kg of body weight.*

Table V—Urinary I Excretion by Four Human Volunteers Given 130 mg/60 kg po for 5 Days

Time Interval	I Concentration, mg
2000–0800	13.13 ± 2.30
0800–1200	20.03 ± 1.68
1200–1600	37.44 ± 7.31
1600–2000	29.06 ± 9.31
Excretion, 24 hr	89.03 ± 15.01
Percent of administered dose	80.35 ± 14.08

tography was carried out by focusing the instrument on the molecular ions of methylated I and trideuteromethylated I occurring at m/z 197 and 200, respectively, and on the fragment ions at m/z 133 and 136, which arise from the loss of sulfurous anhydride from the molecular ions. Figure 4 shows a typical mass fragmentogram obtained from a human plasma extract after I intake.

During both GLC with flame-ionization detection and mass fragmentography, no interference due to endogenous substrates was observed. Good linearity in detector response also was found over a concentration range of 50 ng/ml–10 μg/ml with multiple-ion detection mass fragmentography and from 2 μg/ml up to milligram levels for GLC with flame-ionization detection. The precision (percentage coefficients of variation) was calculated from the standard deviation/mean × 100. Assays were made in quadruplicate at six concentrations of I, thus covering the range expected in *in vivo* experiments. The results summarized in Table I refer to determinations from human plasma and urine.

The validity of this method for *in vivo* determinations was tested by studying the plasma kinetics and urinary elimination of I in healthy volunteers after single (50 mg/60 kg of body weight) or repeated (130 mg/60 kg of body weight/day for 5 days divided over the three main meals) oral doses.

Figure 5 shows plasma I kinetics in human volunteers after acute administration. The compound was absorbed rapidly through the GI tract, reaching peak plasma values between 30 and 60 min. Elimination of I followed a monoexponential decay pattern with a calculated half-life of 121 ± 16 min at the times considered (Table II).

Table III reports the plasma I levels determined in the volunteers given repeated oral doses. Concentrations were higher close to the time of I intake, *i.e.*, breakfast, lunch, and dinner. The *AUC* was about half that after a single dose of 50 mg/60 kg of body weight, although the daily dose was almost 2.5 times larger. However, these differences probably arose from differences in absorption since I was taken with food and not in a fasting condition and since the total daily dose was divided *ad libitum*, an experimental condition that may be considered closer to the real situation.

Urinary I excretion by human volunteers given a single oral dose is reported in Table IV. Compound I was eliminated within a few hours of administration, with ~60% of the dose being excreted unchanged by 6 hr, and reached 79.9% at 24 hr. Excretion of I after repeated administration is shown in Table V. Following this schedule of treatment, the 24-hr excretion again amounted to ~80% of the daily dose. These data are in good agreement with previous reports indicating that I is excreted in the urine rapidly and almost completely in humans and laboratory animals (6, 7). This observation is consistent with the relatively short plasma half-life of I, which is poorly metabolized.

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Size Exclusion Chromatography of Liposomes on Different Gel Media

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Abstract □ Uncharged and negatively and positively charged liposomes of egg lecithin were prepared by sonication and chromatographed on three different gel media. The column effluent was investigated by turbidimetric measurements. The operational parameters were selected to obtain baseline separation of the liposomes. Liposome peaks were fractionated and identified by their K_{av} (distribution coefficient) values. Baseline separation into two fractions was obtained with cross-linked dextran gel, and three fractions were obtained with cross-linked agarose gel.

Keyphrases □ Liposomes—synthesized and fractionated using size exclusion chromatography on different gel media □ Size exclusion chromatography—of liposomes on different gel media □ Fractionation—size exclusion chromatography of liposomes on different gel media □ Chromatography, size exclusion—liposomes, synthesized and fractionated on cross-linked agarose and cross-linked dextran gels

Parenteral and oral dosage forms with increased drug selectivity are achieved by linking drugs to carriers. Such forms are of pharmaceutical importance, and several concepts using liposomes have been suggested (1, 2). Liposomes are formed when phospholipids are allowed to swell in aqueous media. When suitably dispersed, the liposomes consist of a series of concentric lipid bilayers, which alternate with aqueous compartments. The bilayers and aqueous compartments can entrap lipid-soluble and water-soluble substances, respectively.

Size exclusion chromatography[1] (3) commonly is used to separate liposome-entrapped substances from nonentrapped material (4–6), but there has been little evaluation of different gel media for their ability to separate liposomes of different sizes. Huang (7) described the separation of vesicles of homogeneous size by using size exclusion chromatography on a gel with 4% agarose. Chen and

[1] Gel filtration traditionally is used to describe separation according to size on soft permeation media in aqueous solution (3). However, the term size exclusion chromatography is now recommended by the International Union of Pure and Applied Chemistry.

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