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Analytical derivatizations of volatile and hydrophilic carbonyls from aqueous matrix onto a solid phase of a polystyrene–divinylbenzene macroreticular resin

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Abstract

Extraction and derivatization of carbonyls to benzyloximes, pentafluorobenzyloximes or 2,4-dinitrophenylhydrazones is simplified and reaction times are substantially reduced by simultaneous sorption and derivatization from aqueous solution onto a solid phase. In this reaction a macroreticular polystyrene–divinylbenzene resin acts as a sorbent and catalyst to allow simultaneous extraction and derivatization of hydrophilic and lipophilic aldehydes and ketones from simple as well as complex matrices including plasma. Conversion to the 2,4-dinitrophenylhydrazones or pentafluorobenzyloximation at ambient temperature requires 10 and 20 min, respectively. These reaction conditions correspond to at least a 6-fold reduction in reaction times for derivatization of the reactive aldelhydes and a 36–72-fold reduction for preparation of derivatives for the slower reacting ketones. © 1997 Elsevier Science B.V.

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1. Introduction

Determination of carbonyls is important in studies of lipid peroxidations [1–3], biotransformation of hydrocarbons [4,5], as well as atmospheric photo-oxidation these compounds [6], and finally for investigation of possible toxins formed by advanced oxidation techniques used to prepare drinking water [7]. Since many of these analytes do not possess a chromophore, fluorophore or electrophore, analytical derivatization is a common step in measurement of these analytes [10–15]. Yu et al. recently reviewed the two standard reactions used in such determinations: pentafluorobenzyloximation (PFBoximation) and formation of the 2,4-dinitrophenylhydrazones. The former is typically used in sample preparation for gas chromatographic analysis and the latter for analysis by high performance liquid chromatography.

Regardless of the derivatizing agent or instrumental technique used, sample preparation and analytical derivatization is time consuming. Oximations at ambient temperature require a minimum of 2 h for the derivatization of simpler aldehydes and as long as 24 h for the reaction of ketones such as acetone or methy ethyl ketone [4–9]. Oximation of more complex carbonyls is a post-extraction technique requiring anhydrous pyridine and either elevated temperature or reaction times of more than 12 h [15,16].

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The classical method for preparation of 2,4-dinitrophenylhydrazones in biological fluids containing protein (e.g., plasma) requires a minimum reaction time of 3 h at ambient temperature [10,11] as well as liquid–liquid extraction. In order to reduce reaction times [10] for derivatization of carbonyls from urine with 2,4-dinitrophenylhydrazine, reaction temperatures are raised to 40–70°C. A reaction time of only 1 h for derivatization of carbonyls in aqueous solution required a temperature of 40°C [12]. Subsequent isolation on solid phase appeared to be complicated by low breakthrough volumes which required a second and a third cartridge to adsorb analytes that were not completely adsorbed from the first or second cartridge.

It is apparent that there is a need to simplify and speed up sample preparation procedures for the determination of carbonyls. Reactions on a macroreticular polystyrene–divinylbenzene resin (XAD-2) appear to have characteristics of simplicity and speed not found in existing techniques. An example is the oximation of prostaglandin E_2 (PGE_2) for determination by gas chromatography–mass spectrometry in which use of XAD-2 allowed rapid, simultaneous extraction and derivatization [17]. Either O-pentafluorobenzyloximes (PFBoximes) or O-benzyloximes (Bzoximes) of this analyte were obtained in high yield within 20 min by adsorption and derivatization directly from plasma. The reaction appeared to require co-adsorption of analyte and reagent restricting the technique to determination of readily sorbed compounds. This excluded application to the determination of polar and hydrophilic compounds.

Determination of polar carbonyls is important. They constitute approximately 50% of the carbonyls found in normal and peroxidized rat liver microsomes [18–20]. These include propanal, pentanal, butanone and butanal, as well as the hydroxyalkenals which are soluble to moderately soluble in water [18–20]. Other water soluble and potentially toxic carbonyls, such as formaldehyde, glyoxal and methylgloxal acid are all formed from the oxidation of organics under a variety of conditions [7,8].

The rapidity of the XAD-2 supported reaction as well as the very high sorption capability of this phase suggested that even polar compounds could be derivatized under conditions used to isolate and oximate the more lipophilic PGE_2. Consequently, the XAD-2 reaction was studied as a means for rapid isolation of hydrophilic carbonyls from aqueous matrix as their PFBoximes or their 2,4-dinitrophenylhydrazones.

2. Experimental

2.1. Reagents

O-Pentafluorobenzylhydroxylamine hydrochloride ($PFBONH_2 \cdot HCl$) and O-benzylhydroxylamine hydrochloride ($BzONH_2 \cdot HCl$) were purchased from Sigma (St. Louis, Mo, USA). 2,4-Dinitrophenylhydrazine (2,4-DNPH) was obtained from Eastman Kodak (Rochester, NY, USA) and was recrystallized from ethanol. Solvents and pure analytes were purchased from the usual commercial suppliers, such as Aldrich (Milwaukee, WI, USA), Fisher (Toronto, Canada), BDH (Toronto, Canada) and Caledon (Georgetown, Canada). The XAD-2 resin was purchased from Aldrich and was cleaned of fines, carbonate and organic contaminants by methods previously described [16,17].

2.2. Synthesis of analytical standards for the Bzoximes and PFBoximes

A 20-ml volume of 0.1 *M* phosphate buffer at pH 3.1 containing 1 mmol of carbonyl and 1.1 mmol of reagent were added to 500 mg XAD-2 that had been pre-wetted with 500 μl. The reagent was not completely dissolved. The reaction mixture was ultrasonicated for 30 min during which time the reagent was completely dissolved and/or transferred to the surface and reacted with the carbonyl. The water was removed by aspiration and the XAD-2 was first washed three times with 5 ml of 0.01 *M* HCl and then dried under a slow stream of nitrogen. The oximes or the 2,4-dinitrophenylhydrazones were eluted from the resin with two 10 ml washes of pentane, which were then combined. Where products were found to be impure the reaction mixture was purified by column chromatography on silica gel.

2.3. Analytical derivatizations of carbonyls from aqueous or plasma solutions using benzyl or pentafluorobenzyl hydroxylamine hydrochloride

2.3.1. Method A

A 300-mg quantity of XAD-2 was weighed into a 16×100 mm screw cap vial and pre-wetted with 300 μl of acetonitrile. Four ml of phosphate buffer at pH 3.1, and containing reagent, was added. In the initial experiments the amount of reagent varied from 1 to 4 mg. One ml of a solution containing the carbonyls was added to this mixture. The reaction mixture was shaken for 20 min at ambient temperature. The resin was isolated by aspiration and washed three times with 5 ml of 0.01 *M* HCl with the water being removed by aspiration. The resin was dried as above and the derivatives were eluted with 2 ml of pentane. A 1–2 μl aliquot was injected for GC–ECD analysis. For HPLC analysis of the Bzoximes, the derivatives were eluted with 5 ml of acetonitrile and a 25–50 μl aliquot was injected. For analysis at lower concentrations only the PFBoximes were prepared and in this case the pentane was carefully evaporated at 35°C to avoid losses of the volatile derivatives (i.e. those of formaldehyde, acetaldehyde, acetone and propanal). The residue was then reconstituted in 100 μl iso-octane.

2.3.2. Method B

A 1-ml aliquot of a solution containing the analytes was added to 4 ml of 0.1 *M* phosphate buffer at pH 3.1 containing $PFBON_2 \cdot HCl$. In the initial experiments the amount of reagent varied from 0.1 to 4 mg, but 0.5 mg was used in the final conditions. The solution containing analyte and reagent at pH 3.1 was maintained at ambient temperature for 10 min and then added to 300 mg XAD-2 pre-wetted with acetonitrile as described above. After shaking for an additional 10 min the resin was isolated by aspiration, air dried and then the derivatives were eluted with 2 ml iso-octane. An aliquot of this was injected directly for GC–ECD analysis as described above. Alternatively, the derivatives were eluted with pentane which was then evaporated at 35°C to avoid losses of the volatile derivatives (i.e. those of formaldehyde, acetaldehyde, acetone and propanal) and the residue was reconstituted in 100 μl iso-octane.

2.4. Analytical derivatizations of carbonyls in aqueous or plasma solution with 2,4-dinitrophenylhydrazine

A 1-ml aliquot of a solution containing the analytes was added to a mixture of 300 mg XAD-2 pre-wetted with 300 μl of acetonitrile and 4 ml of 1.0–1.5 *M* HCl containing 2,4-DNPH. Use of varying amounts of reagent was studied but the final conditions required 4 μ*M* of reagent. The resulting reaction mixture was shaken for 10 min at ambient temperature. The resin was isolated by filtration using a Supelco Vacuum Module and a cartridge fitted with a fritted polystyrene disk. A water aspirator was used to supply suction. The resin was washed with distilled water until the washings were neutral. This usually required 4×5 ml of distilled water. The resin was air dried by briefly drawing air through the reactor/sorbent bed and then sealing the cartridge and keeping it under vacuum. The derivatives were eluted with 5 ml acetonitrile and a 25-μl aliquot was injected for HPLC analysis.

2.5. Instrumentation

The PFBoximes were determined on a Hewlett–Packard (H–P) 5790 gas chromatograph (GC) equipped with a pulse linearized ECD and a J&W fused capillary column DB-1, 30 m×0.25 mm with film thickness of 0.25 mm. A J&W telescopic on-column injector was used. The output of the detector was recorded and integrated by a Millennium (Waters) data handling program. The carrier gas was hydrogen with a linear velocity of 62 cm/s at 180°C. The make-up gas was 10% methane in argon at a flow-rate of 15 ml/min. The temperature was programmed from 110 to 290°C at 4°C/min.

The Bzoximes and 2,4-dinitrophenylhydrazones were determined by high performance liquid chromatography (HPLC) on a Waters symmetry column (octylsilica) using gradient elution. (Note: In this study, derivatization with $BzONH_2 \cdot HCl$ was used as a model with the single objective of proving the principal of reaction of polar carbonyls on XAD-2.

For reasons of speed in sample preparation the analysis of these derivatives was limited to HPLC.) The HPLC consisted of a 712 Waters Intelligent Sample Programmer, a Waters 600 E multi-solvent delivery system, a Waters 994 programmable photodiode array detector and a Waters 5200 printer/plotter. The flow-rate was 1 ml/min and the gradient for both Bzoximes and 2,4-dinitrophenylhydrazones began at 35% acetonitrile in water and increased in a linear gradient to 80% acetonitrile in water in 20 min.

Table 1
Equilibrium concentrations and yield of benzyloximation as a function of molecular weight

Carbonyl	C_{eq} (μM)	% Yield in 20 min reaction time
Acetone	243	75
3-Pentanone	108	100
4-Heptanone	0	100
5-Nonanone	0	100
Formaldehyde	269	130
Acetaldehyde	253	75
Propanal	96	85
Nonanal	0	100

Initial concentrations of carbonyls 333 μM.

3. Results and discussion

3.1. Selection of analytes

The carbonyls with one to three carbon atoms are formed during oxidations in biological, environmental or industrial processes and were used as models for polar, hydrophilic carbonyls. The C4–C9 group of compounds served as models for carbonyls of intermediate and decreasing polarity and water solubility. Carbonyls with more than 9 carbon atoms were not studied. Such molecules would, a priori, be expected to be highly sorbed and, based on previous work, would be expected to react quickly [17].

3.2. Analytical derivatizations with benzylhydroxylamine hydrochloride

Derivatization with $BzONH_2 \cdot HCl$ was the first model studied because this reagent is relatively water soluble. At an initial concentration of 1.6 mg/ml, the equilibrium concentration (C_{eq}) of reagent in the aqueous phase was C_{eq}=8 mg/ml. Accordingly, analyte molecules in the aqueous phase and on the solid would be exposed to reagent thus increasing the possibility of reaction. If derivatization with this best case reagent were not observed then further investigations would not have been warranted.

Carbonyls with one to three carbon atoms were poorly sorbed from water onto XAD-2 but sorption increased rapidly with molecular weight (Table 1). Despite poor sorption of these analytes, yields of more than 70% could be obtained for the simultaneous sorption and Bzoximation. Reaction yield for Bzoximation of aetone increased with the amount of reagent used (Table 2). There was a slight decrease in yield when 8 mg were used.

In the case of aldehydes, yields did not appear to be related to the amount of reagent when 2 mg or more were in the reaction mixture, but when less reagent was used the yield was reduced. As in aqueous based derivatizations, aldehydes reacted with oximating agents at a much faster rate than ketones. The solid phase supported reaction, however, was much faster for oximation of both classes of carbonyls and allowed derivatizations of hydrophilic ketones in the 20 min reaction time. This is not readily achieved by techniques currently in the literature. These results suggested XAD-2 supported reaction of carbonyls was not necessarily limited to lipophilic carbonyls but could also be used to rapidly isolate and oximate hydrophilic carbonyls.

3.3. Analytical derivatizations with pentafluorobenzylhydroxylamine hydrochloride

Derivatization with $PFBONH_2 \cdot HCl$ from water onto XAD-2 represented a more difficult problem. Formaldehyde, acetaldehyde, propanal and acetone, all partition into the aqueous phase to a very high degree. In contrast, $PFBONH_2 \cdot HCl$ was almost completely sorbed onto the surface. As a result, analytes and reagent were predominantly present in different phases. Despite this, high yields of PFBoximes of both aldehydes and ketones were obtained in a reaction time of 20 min.

These results were unexpected because of the disparate distributions of reagent and analyte. How-

Table 2
Percentage yield of carbonyl Bzoxime as a function of total weight of $BzONH_2 \cdot HCl$

Weight in mg of $BzPNH_2 \cdot HCl$	Yield of the Benzyloxime±relative standard deviation			
	Acetone	Formalaldehyde	Acetaldehyde	Propionaldehyde
8	73±5	ND	76±7	75±13
4	85±4	129±0.9	72±4	75±13
2	64±1.7	136±3	74±10	72±9
1	45±4	136±3	73±9	66±4
0.5	10[a]	ND	ND	ND

[a] Method B=derivatization carried out with 1 mg $BzONH_2 \cdot HCl$ using method 2.
ND=Not done.

ever, while the C1–C3 analytes partitioned substantially into the aqueous phase, there was a small but measurable adsorption for acetaldehyde, propanal and acetone. It is possible that there was also some small, immeasurable adsorption of formaldehyde. In addition, $PFBONH_2 \cdot HCl$ may have been present in the aqueous phase but at low concentrations. If the rate was sufficiently fast, then even small concentrations of analyte and reagent in a common phase would push the reaction to completion. Regardless of mechanism, it is apparent that carbonyls can be isolated and derivatized via XAD-2 supported reaction regardless of their structure.

Under these reaction conditions, however, a minimum of 2 mg of $PFBONH_2 \cdot HCl$ were required whereas the aqueous based derivatization utilized 0.5 mg. The use of 2 mg of reagent was considered a drawback to the solid phase reaction because higher amounts of reagent would increase the background and could degrade chromatographic columns. The approach to reducing the amount of $PFBONH_2 \cdot HCl$ was based on the understanding that oximation is a two step process. The first step is formation of the hydroxylamine and the second is dehydration to the oxime [21]. Recovery of the PFBoximes of ketones from aqueous matrix shows that even for the slow reacting analytes, such as acetone, the hydroxylamine intermediate can be formed in the aqueous phase.

Formation of the intermediate in aqueous phase prior to sorption would require less reagent than the total solid phase reaction, since both reagent and analyte would be in the same phase. Moreover, the hydroxylamine would be more lipophilic than either starting material and would be rapidly sorbed. The relatively hydrophobic environment of the XAD-2 surface could enhance dehydration to the oxime. Providing that the first step was fast, this two stage reaction would result in rapid formation of PFBoxime at lower concentrations of the reagent.

The data in Table 3 demonstrate that the procedure in reaction B requires only 500 μg $PFBONH_2 \cdot HCl$ to produce quantitative yield of the PFBoximes of both aldehydes and ketones. These data indicated that XAD-2 supported reactions can be used to rapidly and easily oximate all carbonyls regardless of (i) the degree of analyte sorption onto this solid phase and (ii) the structure of the analyte, as both ketones and aldehydes are derivatized and extracted in the short time frame. And with the two step procedure, these characteristics are achieved without penalty of excess reagent.

3.4. Analytical derivatizations of carbonyls in aqueous or plasma solution with 2,4-dinitrophenylhydrazine

The XAD-2 supported reaction was also used to prepare 2,4-dinitrophenylhydrazones for HPLC analysis. Concentration of HCl and the amount of reagent both influenced yield. Optimal yield for derivatization of both aldehydes and ketones was achieved with 1.0 N HCl and 4 mmol of reagent. As in the case of $PFBONH_2 \cdot HCl$, the chromophoric reagent was also highly sorbed, but hydrophilic aldehydes and ketones were still recovered in quantitative yield. The solid phase technique reduced the reaction step of sample preparation for HPLC analysis from 3 h to 10 min. Moreover, as a solid phase

Table 3
Yield for PFBoximation as a function of weight, $PFBONH_2 \cdot HCl$ and sample preparation method

Carbonyl	Derivatization method			
	Method A		Method B	
	Weight of $PFBONH_2 \cdot HCl$	Yield	Weight of $PFBONH_2 \cdot HCl$	Yield
Acetone	4	87±6	1	100±2
	2	74±9	0.5	85±7
	1	64±7	0.1	64±5
Nonanone	4	78±5	1	96±6
	2	83±7	0.5	95±10
	1	76±5	0.1	72±8
Acetaldehyde	2	92±4	1	99±1
	1	88±6	0.5	98±2
	0.5	90±5	0.1	68±5
Propanol	2	89±7	1	92±7
	1	86±4	0.5	92±4
	0.5	86±4	0.1	71±4

Method A: Total solid phase reaction mixture.
Method B: Aqueous/solid reaction.
Amounts of all carbonyl were adjusted to allow for a minimum of a 4-fold molar excess of reagent.

technique, sample preparation was also considerably simplified by eliminating the liquid–liquid extraction step.

The scope of the reaction was tested by determining C-1 to C-9 carbonyls from plasma without precipitation of proteins or any other modification other than adjusting the acid concentrations. The yields were identical to those obtained for derivatization of these analytes from water which was used as a same day control. This indicated that even for polar, hydrophilic analytes the reaction was not hindered by the protein and lipid components of plasma.

The calibration curve was determined for compounds with greater than three atoms. These are not present in laboratory water or in plasma. As a result there are no background peaks in the analysis of carbonyls with more than three carbon atoms. The responses were linear on a four point calibration curve from 50–3 μg/ml. This was typified by the pair of relatively water soluble analytes, methyl ethyl ketone and butanal, $r \geq 0.999$ (these were used because they were not present in water). Repeated analyses at 3 μg/ml gave a relative standard deviation of 6%.

3.5. Analysis of carbonyls in clinical conditions which produce free radical oxidation

Solid phase analytical derivatization on XAD-2 was used to profile carbonyls in plasma. Samples were obtained from an individual with mitochondrial myopathy, a genetic defect which produces an impairment of oxidative phosphorylation. Because this is equivalent to an anoxic situation there is an increase in blood supply, and therefore oxygen, to the muscle. The combined impairment of oxygen utilization and an increased supply of oxygen results in an increase of lipid peroxide [22] and subsequent tissue damage. Treatment with anti-oxidants should prevent peroxidative damage to tissues.

Carbonyls, predominantly aldehydes, are long lived products of lipid peroxide that can circulate in the plasma [1] and their concentrations in tissue and/or plasma may reflect the degree of destruction of lipids caused by this process. The plasma car-

bonyls were determined to assess whether antioxidant treatment would alter the profile of these compounds in a patient with mitochondrial myopathy. Blood was collected before and after a month long treatment with a cocktail of anti-oxidant vitamins. The plasma was isolated and then analyzed for the carbonyl fraction by extraction–derivatization with 2,4-DNPH onto XAD-2. Compounds were identified by comparison of their retention times to those of synthesized 2,4-DNPH derivatives. Normal background concentrations of carbonyls were determined by analyzing plasma from healthy volunteers.

The results are summarized in Fig. 1a–c. Formaldehyde, acetaldehyde and acetone are the major carbonyls present in distilled water which was used as a laboratory blank. In plasma taken during the untreated condition, a compound with retention time corresponding to acetaldehyde was the predominant carbonyl. The presence of acetaldehyde, in a patient who did not drink ethyl alcohol, was unexpected. Appearance of this compound may be due to altered pathways of oxidations or peroxide in this condition. Alternatively, this may be a carbonyl of unknown structure, such as a hydroxylated alkenal. Due to its greater polarity, a hydroxyalkenal could elute at the same time as acetaldehyde. After antioxidant treatment, peak area of this compound was reduced to slightly above that found for acetaldehyde in distilled water, where the concentration of this alkenal was 1 μg/ml.

Plasma concentrations of acetone in samples of normal plasma varied from 8 to 10 μg/ml and were 5–6-fold higher than the background concentrations found in distilled water. Treatment with anti-oxidants had little effect on plasma concentrations of acetone but this compound is not formed by mechanisms involving oxidation by free radicals.

Similar data was obtained in an individual undergoing angioplasty. This is equivalent to reperfusion and there should be an increase in products resulting from free radical oxidation. Plasma was collected before and after angioplasty. There was a four-fold increase in a peak corresponding to acetaldehyde after the treatment. As in the case of the mitochondrial myopathy patient, there was no change in

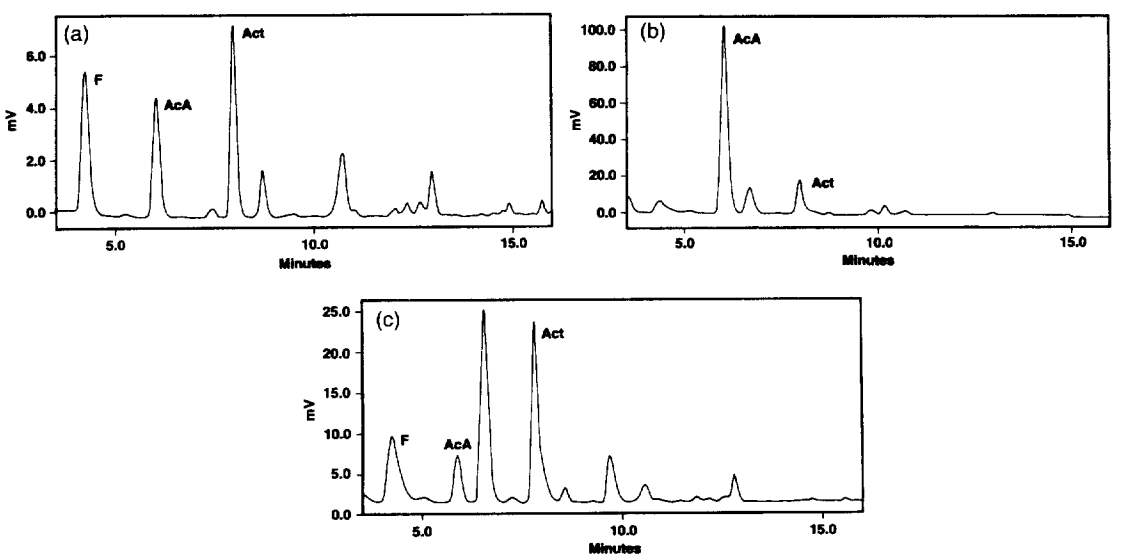

Fig. 1. (a) Trace of 2,4-dinitrophenylhydrazones of carbonyls found in distilled water. F=formaldehyde; AcA=acetaldehyde; Act=acetone. (b) Trace of 2,4-dinitrophenylhydrazones of carbonyls found in plasma of an individual with mitochondrial myopathy prior to antioxidant treatment. (c) Trace of 2,4-dinitrophenylhydrazones of carbonyls found in plasma from an individual with mitochondrial myopathy after antioxidant treatment.

the concentrations of acetone before or after the treatment.

4. Conclusions

The solid phase reaction provides the advantages of a solid phase sample preparation coupled over existing liquid-based derivatizations and extractions coupled with shorter preparation times. In the two step technique there is a 6-fold decrease in the reaction time for pentaflourobenzylation of aldehydes and a 36 to 72-fold decrease in the corresponding derivatization of ketones. For derivatizations with 2,4-dinitrophenylhydrazine at ambient temperature there is a 18-fold decrease in reaction time. Finally, this procedure traps the volatile and water soluble carbonyls as their derivatives, thus facilitating the determination of such analytes.

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