Determination of Histamine by High-Performance Liquid Chromatography After Precolumn Derivatization with o-Phthalaldehyde-Sulfite

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Abstract

A fast and sensitive method was developed for in vivo determination of histamine in the brain microdialysate by reverse ion pair chromatography with electrochemical detection. The microdialysates were derivatized with o-phthalaldehyde and sodium sulfite, and separation was achieved using isocratic elution within 10 min. The separation was performed in an Agilent Eclipse Plus C18 column (3.0 x 150 mm, particle size 3.5 μm), and the mobile phase consisted of 100 mM monosodium phosphate (pH 6.0), 500 mg L⁻¹ OSA and 20% methanol (v/v). The linearity (R²) was found to be >0.999, with a range from 2 to 50 nM and excellent repeatability (relative standard deviation, 2.29–6.04%), and the limit of detection was 0.4 nM. This method was successfully applied to analyze the extracellular concentration of histamine in the hypothalamus of rats, with probe recovery calculated in vivo.

Introduction

Histamine (HA) is widely distributed and acts as a neurotransmitter in the mammalian brain. The cell bodies of the histaminergic neuron are mainly located in the tuberomammillary nucleus, which is a small region of the hypothalamus (HYP), and fibers are distributed widely throughout the brain (1). HA is responsible for many physiological functions including food intake, state of arousal, learning and reinforcement, temperature regulation, cardiovascular control, etc. (2). As a result, it is essential for us to learn the functions of HA in the brain. It is also useful for us to learn ways to prevent as well as treat the related diseases. Neurotransmitters are mainly effective in the extracellular space. The traditional tissue homogenate method can only analyze the whole content of neurotransmitter from both intracellular and extracellular space. However, the microdialysis method is used to obtain the neurotransmitters directly from the extracellular space in animals that are awake or under anesthetics for further analysis (3–5), and thus, it has been widely adopted.

Currently, there are many techniques for the determination of HA such as capillary electrophoresis (6), biosensors (7), gas chromatography–mass spectrometry (8), liquid chromatography–mass spectrometry (9–11), ion chromatography (12) and high-performance liquid chromatography (HPLC) (13). Among these approaches, HPLC is the most widely used because of its operational advantages (cost and repeatability). However, HA in the microdialysate is difficult to determine due to the low concentration and complex matrix in the samples (14). Furthermore, HA cannot be directly detected by the fluorescence detector (FLD) or electrochemical detector (ECD) without derivatization. In the previously research, HA was major detected with FLD after pre- and postcolumn derivatization. During the postcolumn derivatization phase, o-phthalaldehyde (OPA) was always...
used after the microdialysate separated in cation-exchange chromatography (15) or ion pair chromatography (16). The other method utilized was precolumn derivatization with 4-(1-pyrene) butyric acid N-hydroxysuccinimide ester (17) or 4-(1-pyrene)butanoyl chloride (18). The sample was then separated in a reverse phase column and detected with FLD. However, both of the two types of method have their limitations, the former method needs an additional pump while the latter method needs a harsh reaction condition (100°C for 90 min).

ECD is also a sensitive detector, and limit of detection (LOD) of which was several fmol, even amol (19). Currently, it has been extensively used in neuroscience field because of its extreme sensitivity, especially for the routine determination of monoamines (20). Thus, ECD could be an alternative method for determining HA, especially for much lower concentrations. After derivatization with OPA and β-mercaptoethanol, determination of HA with HPLC, coupled with ECD, had been previously established. This method was applied to determine HA in rat mast cells (21). To circumvent the limitations of this method (i.e., unpleasant odor and unstable derived products), an improved method was established and HA reacted with OPA-sulphite. This approach was successfully utilized to determine HA and N’-methylhistamine in brain homogenate (22). However, this method (LOD of 0.125 pmol with 100 μL injection) was not sufficient for determining of HA in the microdialysate. Determination of HA in the microdialysate is similar to homogenate, because the components are similar. However, the much lower concentration of HA in the microdialysate requires high resolution to avoid the interference of amino acids, which can also react with OPA-sulphite. Additionally, the volume of microdialysate is limited.

Once the concentration of HA and the probe recovery was determined, the concentration of HA in the brain extracellular can be calculated. Unfortunately, because the probe recovery was influenced by many factors (e.g., diameter, length, geometry and material of the membrane, the perfusion flow rate, the perfusate characteristics, the sample matrix characteristics, etc.) and thus the probe recovery determined in vitro was not accurate because the environment of the brain is difficult to imitate (23, 24). The zero-net flux method is a widely used in vivo calibration method. The analyte of interest is perfused at various concentrations through the probe (C_in), and the concentration of this analyte is then measured at the outlet of the probe (C_out). A plot of C_in – C_out versus C_in yields a straight line and the intercept of the x axis is the extracellular concentration of the chemical in the analyte (24, 25).

The goal of this research study is to develop a sensitive and fast method for determining HA in the microdialysate from HYP and to calculate the extracellular concentration of HA in the HYP with the zero-net flux method.

Experiment

Chemicals and solutions

In this research study, ultrapure water was prepared with a Millipore Elix system (Millipore, Bedford, MA, USA). HA, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium dihydrogen phosphate, dibasic sodium phosphate, OPA, sodium sulphite, borax, phosphoric acid (85%), sodium hydroxide, 1-octanesulphonic acid, and sodium salt (OSA) were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Tedla (Tedla Chemical, USA). Tryptophan (Trp), phenylalanine (Phe), lysine (Lys), leucine (Leu), isoleucine (Ile) and pentobarbital sodium were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

The stock solution of HA (0.01 M) was prepared in an artificial cerebrospinal fluid (aCSF) and stored at −20°C. The stock solution was stable in more than 1 month. Before the experiment, the solution was diluted with aCSF to the desired concentrations. The aCSF consisted of 145 mM NaCl, 2.70 mM KCl, 1.00 mM MgCl₂, 1.20 mM CaCl₂, 0.45 mM NaH₂PO₄ and 2.33 mM Na₂HPO₄, and pH was adjusted to 7.4 (26).

Microdialysis procedures

All animal experiments were carried out in accordance with protocols approved by the Animal Ethics Committee, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences (SYXK(E)2009-0051, No. 00006626).

On the day of the experiment, male Sprague-Dawley rats (250–350 g, Slac Laboratory Animal Co. Ltd, Changsha, China) were anesthetized with 1% pentobarbital sodium (intraperitoneal injection, 0.7 mL/100 g) and laid on a stereotaxic frame (Stoelting, Wood Dale, IL, USA). Their skulls were exposed after their scalps were removed. The guide cannula (CMA/11; Harvard Apparatus, Holliston, MA, USA) was implanted into HYP (2.0 mm posterior to bregma, 0.5 mm lateral from the midline and 7.2 mm below the superior surface of the skull). The guide cannula was fixed with screws and dental cement. After the surgery, the rats were housed individually in plastic cages (60 x 50 x 45 cm) for a week to allow the animals to recover.

On the day of the microdialysis experiment, a probe (CMA/11; Harvard Apparatus) was inserted into the cannula. The molecular cut-off for the probe was 6,000 Da, and the length of the membrane was 2 mm and made of cuprophane. The rats were then placed into the microdialysis bowl. The aCSF was delivered at a rate of 1.5 μL min⁻¹ using a 1 mL syringe (Hamilton 1000series; Harvard Apparatus) and a microinjection pump (KDS 101; KD Scientific, Holliston, MA, USA). Following a 2-h equilibration period, microdialysates were collected every 15 min (22.5 μL) and the samples were stored at −80°C for further analysis. All animals were sacrificed at the end of the experiment, and the placement of the guide cannula site was confirmed with histology methods.

Probe recovery of HA in vivo (zero-net flux method)

The zero-net flux method was utilized to determine the extracellular concentration of HA in HYP of rats. During the microdialysis procedure as previously described, the aCSF spiked with known concentrations of HA (C_in, 0, 5, 10, 15 and 20 nM) was perfused via a liquid switch (CMA; Harvard Apparatus). At each level, the HA flux across the probe’s membrane was allowed to equilibrate for 15 min. Two dialysate samples were collected (each 15 min, 22.5 μL). The net HA flux (out-in) was measured at every perfusate concentration and the regression analysis was used to calculate the actual concentration of extracellular HA in the HYP.

Apparatus and chromatography

Chromatography was performed with a Dionex Ultimate 3000 HPLC system which consisted of a biocompatible isocratic pump (ISO-3100BM), an autosampler (WPS 3000 TBSL) and an ECD (Coulochem III). The column was Agilent Eclipse Plus C18 (3.0 x 150 mm, particle size 3.5 μm) and the injection volume was 10 μL. The mobile phase consisted of 100 mM monosodium phosphate (pH 6.0), 500 mg L⁻¹ OSA and 20% methanol (v/v), the flow rate was set to 0.35 mL min⁻¹ and the temperature was set to 35°C for the column
and 4°C for the autosampler. Potentials were set to 250/550 mV for ECD1/ECD2 of the analysis cell (Model 6011 coulometric, dual electrode cell).

**Derivatization procedure**

The derivatization reagent was prepared with the following procedure. First, the stock solution was prepared by dissolving 17 mg of OPA into 0.5 mL methanol and then by adding 0.5 mL 1.25 M sodium sulfite and 4 mL 0.1 M of borax (pH 10.4). The stock solution (final concentration: OPA, 25 mM; sulfite, 125 mM) was kept at 4°C. The stock solution was prepared every 3 days. The working solution was prepared by adding 1 mL stock solution to 4 mL 0.1 M of borax (pH 10.4). During the analysis, 20 μL HA standard solutions or samples were mixed with 2 μL working solution.

**Method validation**

Linear equation was calibrated with standard solutions prepared at six different concentration levels (2.0, 5.0, 10.0, 15.0, 20.0 and 50.0 nM) in aCSF. To investigate the matrix effect, the linear equation was also calibrated in solutions prepared in the microdialysate. Calibration curves were constructed by plotting the peak areas of HA-OPA-sulfite (y, nA min) against the concentration (x, nM).

The standard solutions with HA (5.0 nM and 10.0 nM) were added to the microdialysates from the HYP for calculation of precision and recovery. The intra- and interday variability for the HA was assayed in triplicate on the same day as well as on three consecutive days, respectively.

**Results**

**Optimization of derivatization conditions**

The volume ratio between the microdialysate and the derivatization reagent was set to 10:1 during the derivatization procedure. The derivatization conditions for HA with OPA-sulfite were similar to that of the amino acids. The main factors affecting the derivatization yields were the pH value of the buffer, reaction time, temperature and concentration of OPA and sodium sulfite. The pH value was optimized to 10.4 according to the previously reports (27, 28). To obtain the best derivatization condition, various concentrations of OPA and sodium sulfite (0.05–25 mM, OPA: sulfite = 1:1, c/c) were tested with microdialysate (see Figure 1A). From Figure 1, one is able to see that the peak areas were stable at the concentration of OPA when ranging from 1 to 25 mM. Thus, 1 mM OPA was deemed sufficient for HA in the microdialysate to react completely. Considering the differences among the various samples, the final concentration of OPA was set to 5 mM.

With the optimized concentration of the derivatization agents (5 mM OPA and sulfite), the variation of derivatization time (1, 3, 5, 25 and 45 min) was further tested with the microdialysate (see Figure 1B). The results indicated that the reaction was completed in 1.5 min, and the peak area was stable between the reaction time 1.5 and 10 min. Thus, the reaction time was set to 1.5 min.

The reaction ratio of OPA, sodium sulfite, and HA was 1:1:1 (22), but HA can also react with OPA without thiol or sulfite as described previously (15, 16). To prevent the latter reaction and to get the major product of HA-OPA-sulfite, different ratios between sodium sulfite and OPA were investigated. The concentration of OPA was fixed at 5 mM, and concentrations of sodium sulfite were gradually changed to 1, 3, 5, 25 and 50 mM. The result indicated that peak areas of HA in the microdialysate did not have significant differences between these groups under different derivatization conditions (data were not shown), which was also in accordance with previous reports (22). This was most likely because even when reacted with the least sodium sulfite, its level was much in excess of HA and other compounds with the amine group, such as amino acids.
Stability of the four groups was also investigated. Peak areas of all
groups were gradually decreased in 12 h in 4°C (see Figure 1C). The
more sodium sulfitie in the derivatization reagent, the more stable the
derivatization product produced. Thus, the ratio of OPA and sulfitie 
was set at 1.5 and the product was stable in 7 h (<2% was degradation).

Optimization of separation conditions

The retention time of HA–OPA-sulfitie was similar to some hydrophilic
amino acids’ derivative products in the reverse chromatography. Addi-
tionally, some hydrophobic amino acids would increase the analysis 
time. An ion pair reagent could improve the separation, for example, 
OSA could increase the retention time of the amino compounds that
contain positive charge and reduce the retention time of the com-
pounds that contain negative charge or no charge. There are several 
key parameters affecting the retention time in ion pair chromatogra-
phy including pH value, concentration of the ion pair reagent and 
the organic reagent (29). In this research study, different pH values 
(4.5–6.0) were tested for separation with 100 mM phosphate and 
400 mg L−1 OSA in 15% methanol (see Figure 2A). To protect the 
column, the maximum pH value was set to 6.0 because the silica-based 
packing has some solubility in the pH > 6 aqueous mobile phase. To 
avoid the interference of the other metabolies, some hydrophobic 
amino acids with large capacity factors were simultaneously investigated
with HA including Trp, Phe, Lys, Leu and Ile. The capacity factors of 
the amino acids were reduced as pH values increased, while the capacity 
factor of HA was slightly affected. This was most likely due to the 
absence of a carboxy group. To separate HA from the other amino 
acids and shorten the separation time, the pH value 6.0 was selected.

Different concentrations of ion pair reagent OSA were investigated 
with 100 mM phosphate (pH 6.0) in 20% of methanol. The capacity 
factor of HA was 4.2, and the values were larger than 10.0 for the 
amino acids investigated (Trp, Phe, Lys, Ile and Leu) when no OSA 
was added. The results with OSA range from 300 to 600 mg L−1
and are shown in Figure 2B. During the concentration of OSA in-
creased from 0 to 600 mg L−1, the capacity factor of HA was approx-
imately stable, but the capacity factor of the amino acids was decreased. 
While OSA was added in the mobile phase, the sulfo 
group in OSA interacts with the solid phase C18, then the retention 
times of the amino acids were reduced. However, the imine group in 
the HA–OPA-sulfitie contains positive charge, thus, the retention time 
of HA was stable while the OSA was added. In the end, 500 mg L−1 
OSA was selected, as this concentration was enough to separate HA 
from the other amino acids.

The type of buffer is also important in the mobile phase. Two dif-
ferent buffer solutions (phosphate and citric acid) were investigated in 
this research study (pH 6.0). HA (100 mM) was completely separated 
from the other compounds in phosphate buffer; however, it was not 
well separated in citrate buffer. Although citrate has more buffer ca-
pacity than phosphate at the pH value 6.0, phosphate was chosen in 
the end.

The retention times of all the compounds decreased following the 
increase of methanol in the mobile phase. Then, 20% methanol was 
selected because HA can be well separated and the separation time 
was also acceptable.

Optimization of detection conditions

To optimize the detecting potential, the ECD-1 was set to 0 mV and 
ECD-2 was optimized from 0 to 750 mV with a step of 50 mV (see 
Figure 3A). From Figure 3, it is evident that HA did not oxidize 
under 250 mV and the responses increased slightly when the potential 
was higher than 550 mV. Thus, the detection potential was set to 
250 mV for ECD-1 and 550 mV for ECD-2. The ECD-1 was used 
to decrease the noise from the other chemicals, and ECD-2 was used 
for the analysis.

Common detect cells for ECD are coulometric and amperometric 
cells. In this study, both the coulometric (6011 cell) and amperometric 
cells (5041 cell) were investigated in this HPLC system for HA. Under 
the same column and sample, the signal of the coulometric cells was 
about ten times that of the amperometric cells, with a 0.002-inch gask-
et. The ratios between the signal and noise of these two cells were al-
most the same, which can be seen from the normalization of the HA 
peak (see Figure 3B). The coulometric cells were selected in the end, 
because it can easily reach equilibrium and need less maintenance.

With the optimized parameters, HA was detected and completely 
separated from the other compounds. Typical chromatograms of the
standard solution and the microdialysate are shown in Figure 4.

Method validation

The linearity of the detector response to the standards was determined.
During the analysis, a range of concentrations (2–50 nM) of HA was 
selected for the detection. The statistical results are presented in 
Table 1. Linear correlation coefficients (R²) were found to be greater 
than 0.999 in both the aCSF and the microdialysate. The membrane

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Figure 2. Optimization of separation conditions. (A) Effect of pH on the capacity 
factor of the HA and some hydrophobic amino acids. Mobile phase: 100 mM 
phosphate and 400 mg L−1 OSA in 15% methanol. (B) Effect of OSA 
concentration on the capacity factor of the HA. Different concentrations of 
OSA were investigated with 100 mM phosphate (pH 6.0) in 20% of methanol.
of the microdialysis probe can block the large molecules, while the small molecules (e.g., inorganic salt, amine acids and neurotransmitters) could pass through the membrane due to the concentration gradient. Additionally, concentration of the inorganic salt in the microdialysate was much higher than other metabolites. Thus, the matrix effect was limited because of the similar environment of the aCSF and the microdialysate.

The LOD at a signal-to-noise of 3:1 was 0.4 nM, and the limit of quantification (LOQ) at a signal-to-noise of 10:1 was 1.3 nM. The intraday and interday precision of the assay were between 2.29% and 6.04% (relative standard deviation, RSD), respectively. The recoveries of HA were between 97.4 and 98.9% (microdialysis samples spiked with 10 and 5 nM HA).

**Determination of the basal level of HA with probe recovery calibrated in vivo**

The recovery was interference by several parameters including diameter, length, geometry and material of the membrane, the perfusion flow rate, the perfusate characteristics and the sample matrix characteristics, etc. (23). Recovery *in vitro* cannot be used for *in vivo* calibration with accuracy because of the effect of the brain environment (24), which was impossible to imitate accurately. Probe recovery calibrated *in vivo* could avoid the limitations, and thus, the zero-net flux method was one method which has been widely used. Figure 5 presents this method of four rats. The slope of the line was the recovery of the probe calculated *in vivo*, which was higher than calculated *in vitro*. The average recovery of the probe determined *in vivo* is 49.2 ± 11.4% (*n* = 4). The average concentration of extracellular HA of rat HYP was 8.2 ± 1.3 nM (*n* = 4).

**Discussion**

Fast scan cyclic voltammetry can be used for the determination of HA (30); however, it was difficult to be oxygenated on the glassy carbon electrode or the metal electrode in DC mode. Thus, pre-column derivatization was used to detect HA by ECD, e.g., OPA-sulfite derivatization was used for determination of HA in the brain homogenate (22). The procedure needs to be optimized for the detection of HA in the microdialysate because concentrations of HA and amino acids are different from the brain homogenate. The concentration of HA in the microdialysate is very low, and to avoid the dilution of the sample, the volume ratio between the microdialysate and the derivatization

![Figure 3](image-url) Optimizations of detection conditions. (A) Hydrodynamic voltammograms of OPA-sulfite derivatives. Mobile phase: 0.1 M phosphate, 500 mg L$^{-1}$ OSA, pH 6.0, containing 20% (v/v) methanol. (B) Chromatograms (HA standard) from coulometric cell and amperometric cell, signals 1 and 2 were from the amperometric cell and coulometric cell, respectively.

![Figure 4](image-url) Elution profiles of HA standard solution and the microdialysate after derivatization with OPA-sulfite. Chromatograph 1: standard amino acids solution (10 nM). Chromatograph 2: microdialysis sample from HYP.
A reagent was set to 10:1 during the derivatization procedure. The main factors affecting the derivatization yields including the pH value of the buffer, reaction time and concentration of OPA and sodium sulfite were optimized in this study.

Because of the amino acids can also react with OPA, an ion pair reagent was utilized to separate HA from the amino acids, whose concentrations were much higher than HA. Thus, the much lower concentration of HA in the microdialysate requires high resolution to avoid the interference of amino acids. Key parameters affecting the retention time including pH value, concentration of the ion pair reagent and the organic reagent were optimized.

Common detect cells for ECD are coulometric and amperometric cells. The compounds can completely react in the coulometric cells, and the signal is also enhanced. In this study, the coulometric and amperometric cell provide the same sensitivity. However, in the coulometric cell, the signal stability is considerably greater and the detector response is much more stable owing to a large electrode surface; only a small fraction is needed for the completion of the redox reaction, in which case partial electrode surface deactivation does not affect the overall response significantly (31). It is easy to operate, without routine maintenance which was important for the amperometric cell. Thus, the coulometric cell was chosen.

This established approach was successfully applied to determine HA in the HYP precisely with probe recovery calibrated in vivo. It can also be used for the routine determination of HA in vivo of the other animals or in different brain states.

**Conclusion**

Determination of HA in the brain microdialysate is still a challenging task, because of the low concentration, complex matrix, and small sample volume. In the current research, a simple and rapid HPLC method with an electrochemical determination approach was developed for the determination of HA in the microdialysates. Under the optimized parameters, HA reacted with OPA-sulfite completely in 1.5 min. The derivatization product of HA was well separated, and the total separation time was <10 min. The LOD was below 0.4 nM.

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**Table I. The Statistical Parameters of the Assay**

<table>
<thead>
<tr>
<th>Linear regression, correlation coefficient, LOD and LOQ (n = 3)</th>
<th>Linear equation</th>
<th>$y = 0.0258x + 0.0226^a$</th>
<th>$y = 0.0263x + 0.0753^b$</th>
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<tr>
<td>$R^2$</td>
<td>0.9991$^a$/0.9995$^b$</td>
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<td>Linear range (nM)</td>
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<td>LOD (nM)</td>
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<td>LOQ (nM)</td>
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<tr>
<td>Intra- and interday precision$^c$</td>
<td>Intraday (RSD, 5 nM/10 nM)</td>
<td>3.92/2.29%</td>
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<td></td>
<td>Interday (RSD, 5 nM/10 nM)</td>
<td>4.56/6.04%</td>
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<tr>
<td>Recovery of histamine$^d$</td>
<td>Basal (nM)</td>
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<td></td>
<td>Measured (nM)</td>
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<td></td>
<td>Recovery (%)</td>
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<td></td>
<td>Recovery (%)</td>
<td>97.4 ± 5.4</td>
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</table>

$^a$y, peak area (nA·min); $x$, concentration (nM), solutions were prepared in aCSF.

$^b$Solutions were prepared in microdialysate.

$^c$Assayed in sextuplicate on the same day and on three consecutive days.

$^d$Mean ± SD of six measurements.

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**Figure 5.** Zero-net flux of four rats. HYP HA levels corrected for in vivo probe recovery are represented by the x-intercepts of the plotted linear regression lines.
The proposed method was successfully applied to in vivo study of HA in HYP. The concentration of extracellular HA in the rat HYP was 8.2 ± 1.3 nM. This fast and sensitive approach can be used for the routine determination of HA in vivo of other animals or in different brain states of the rat/mice.

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