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Determination of 5-hydroxy-*N*-methyl-2-pyrrolidone and 2-hydroxy-*N*-methylsuccinimide in human plasma and urine using liquid chromatography–electrospray tandem mass spectrometry

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Abstract

A method for simultaneous determination of 5-hydroxy-*N*-methyl-2-pyrrolidone (5-HNMP) and 2-hydroxy-*N*-methylsuccinimide (2-HMSI) was developed. These compounds are metabolites from *N*-methyl-2-pyrrolidone (NMP), a powerful and widely used organic solvent. 5-HNMP and 2-HMSI were purified from plasma and urine by solid-phase extraction using Isolute ENV+ columns, and analysed by liquid chromatography coupled to a mass spectrometer fitted with an atmospheric pressure turbo ion spray ionisation interface in the positive ion mode. The method was validated for plasma and urine concentrations from 0.12 to 25 μg/ml. The recoveries for 5-HNMP and 2-HMSI in plasma were 99 and 98%, respectively, and in urine 111 and 106%, respectively. For 5-HNMP and 2-HMSI, the within-day precision in plasma was 1–4 and 3–6%, respectively, and in urine 2–12 and 3–10%, respectively. The corresponding data for the between-day precision was 5 and 3–6%, respectively, and 4–6 and 7–8%, respectively. The detection limit for 5-HNMP was 4 ng/ml in plasma and 120 ng/ml in urine. For 2-HMSI, it was 5 ng/ml in plasma and 85 ng/ml in urine. The method is applicable for analysis of plasma and urine samples from workers exposed to NMP. © 2001 Published by Elsevier Science B.V.

Keywords: 5-Hydroxy-*N*-methyl-2-pyrrolidone; 2-Hydroxy-*N*-methylsuccinimide

1. Introduction

N-methyl-2-pyrrolidone (NMP; structural formula C_5H_9NO; CAS number 872-50-4, boiling point 202°C at 101.3 kPa) is a widely used organic compound. NMP dissolves most polymers and catalyses a numerous polymerisation reactions [1]. It is used as a solvent or formulating agent in the petrochemical and agricultural industry and as a stripping and cleaning agent in the microelectronics industry. The use of NMP as a substitute for other solvents of higher inherent toxicology, such as chlorinated solvents, is increasing. NMP has also been suggested as a skin penetration enhancer in transdermal therapy [2,3].

NMP is readily absorbed in the respiratory [4] and gastrointestinal tract [5] and through the skin [6] and is eliminated from the body, mainly by biotransformation to other compounds. A metabolic pathway has been suggested (Fig. 1), where NMP is metabolised through a chain of reactions to 5-hydroxy-*N*-

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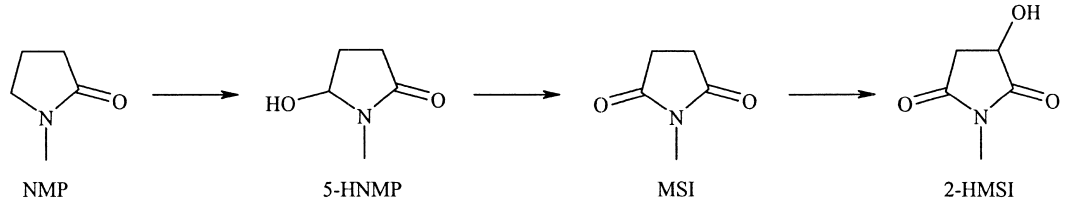

Fig. 1. Scheme of the major metabolic pathway for NMP.

methyl-2-pyrrolidone (5-HNMP), *N*-methylsuccinimide (MSI) and 2-hydroxy-*N*-methylsuccinimide (2-HMSI) [5]. Only a small fraction, about 1–2%, is eliminated as NMP [4,5].

NMP has been reported to be irritating to the respiratory system and eyes, and to cause headache even at low exposure levels [7]. This was not confirmed in a study on healthy volunteers experimentally exposed in a chamber [4]. Animal studies indicate that NMP may be a reproductive toxic compound [1,8–10]. There is also a case report describing a stillbirth after occupational exposure to NMP [10]. Thus, development of methods to assess the exposure to NMP is important.

Methods for monitoring NMP in air have been described [4,11,12]. However, due to the extensive percutaneous absorption of NMP, methods for monitoring air levels may underestimate the exposure. Methods for biological monitoring of the exposure have the potential to overcome this problem.

Our group has previously reported a method for the analysis of NMP itself in urine and plasma [4] and also methods for the analysis of the different proposed metabolites in both urine and plasma [13–15]. We have also showed that there is a good correlation between air levels of NMP and the metabolites in urine and plasma for exposed human subjects [16]. For 5-HNMP, an air level of 50 mg NMP/m^3 corresponded approximately to 45 μmol/l in plasma and 120 mmol/mol creatinine in urine. However, the analysis of the metabolites use gas chromatography coupled to a mass spectrometer (GC–MS) [13,15]. Furthermore, the analysis of 5-HNMP required derivatisation. These methods are therefore rather time consuming. In this study, we present a simple and rapid method for the analysis of 5-HNMP and 2-HMSI in plasma and urine using liquid chromatography coupled to a tandem mass spectrometer (LC–MS–MS).

2. Experimental

2.1. Apparatus

Analyses were performed with a Perkin-Elmer Series 200 liquid chromatography system with autosampler (Applied Biosystems, Norfolk, CT, USA), coupled to an API 3000 LC–MS–MS (Applied Biosystems/MDS-SCIEX, Toronto, Canada). The column used was a Genesis C_{18} (50×2.1 mm), with a particle size of 4 μm (Jones, Lakewood, CO, USA). For the work-up procedure, Isolute ENV+ (the sorbent is a hyper cross-linked hydroxylated polystyrene–divinylbenzene copolymer) solid-phase extraction (SPE) columns were used (200 mg, 6 ml; IST, Hengoed, UK). The disposable SPE columns were used with a VacElut SPS 24 (Varian, Harbor City, CA, USA), connected to a aspirating pump. After the washing step, water was removed from the columns with a Model 3E-1 centrifuge (Sigma, Deisenhofen, Germany).

2.2. Chemicals

5-HNMP, tetra-deuterium labelled 5-HNMP ([$^2H_4$]-5-HNMP) and tri-deuterium labelled 2-HMSI ([$^2H_3$]-2-HMSI) were synthesised by Synthelec (Lund, Sweden). 2-HMSI was from Aldrich (Milwaukee, WI, USA). Methanol and ethyl acetate were from LabScan (Dublin, Ireland), 99.5% ethanol from Kemetyl AB (Haninge, Sweden) and glacial acetic acid from Merck (Darmstadt, Germany).

2.3. Sampling and storage

Blood samples were collected by venipuncture in 10 ml evacuated tubes (Venoject, Terumo Europe, Leuven, Belgium) containing sodium heparin. The blood was allowed to cool to room temperature after

sampling, and then centrifuged at 1500 *g* for 10 min. The plasma was stored in 10-ml test tubes at −20°C until analysis.

Urine samples were stored without any pre-treatment in 10-ml polyethylene test tubes at −20°C until analysis.

2.4. Preparation of standards

Stock solutions were prepared by addition of 25 mg 5-HNMP or 2-HMSI, to 25 ml of water. Standard solutions in desired concentrations (2.50–250 μg substance/ml for plasma standards 5.0–1000 μg substance/ml for urine standards) were prepared by further dilution of the stock solutions in water. Urine and plasma standards containing 5-HNMP and 2-HMSI (0.125–12.5 μg/ml for plasma standards and 0.25–50 μg/ml for urine standards), were prepared by adding 50 μl of the standard solutions to 1 ml of urine or plasma.

For the determination of the recovery, standard solutions of both compounds were prepared in water containing 0.5% acetic acid.

2.5. Work-up procedure

The 5-HNMP and 2-HMSI were extracted using Isolute ENV+ solid-phase extraction columns previously conditioned with 5 ml of methanol followed by 10 ml of water. Aliquots of 50 μl internal standard solutions containing 140 μg/ml [$^2H_4$]-5-HNMP and 40 μg/ml [$^2H_3$]-2-HMSI were added to 1.0 ml of plasma or urine. The samples were applied on the columns followed by a washing step with 1.0 ml of water. The columns were dried for 5 min by air suction and then centrifuged at 1500 *g* for 10 min. The analytes were then extracted from the columns with 2 ml of an 80:20 (v/v) mixture of ethyl acetate and ethanol. The extract was evaporated under a nitrogen stream and then re-suspended in 1.0 ml of water containing 0.5% acetic acid, before transferred into injection vials. The samples were stored at −20°C until analysis.

2.6. Analysis

Sample aliquots of 10 μl were injected into the system. The mobile phase was a water–methanol gradient, containing 0.5% acetic acid. Initially, the mobile flow was 100% water for 0.5 min. Then a linear gradient to 100% methanol was applied in 0.5 min and the mobile phase was kept at 100% methanol for 3 min after which the column was conditioned in 100% water for 4 min. The mobile flow-rate was 0.2 ml/min.

The atmospheric pressure turbo ion spray interface was set to 550°C, the ion spray voltage to 5500 V and the declustering potential was 30 V. For 5-HNMP, analysis after collision induced fragmentation of the precursor ion at *m/z* 115.7 was performed at *m/z* 57.7 [collision energy (CE) 31 V] and 84.7 (CE 19 V). For the deuterium-labelled 5-HNMP the precursor ion at *m/z* 119.7 was fragmented to *m/z* 60.7 (CE 31 V) and 87.7 (CE 19 V). For 2-HMSI, analysis after collision induced fragmentation of the precursor ion at *m/z* 129.7 was performed only at *m/z* 57.7 (CE 31 V) while *m/z* 60.7 (CE 31 V) was chosen after the fragmentation of the precursor ion of [$^2H_3$]-2-HMSI at *m/z* 132.7. The peak area ratios between the analytes and the internal standards were used for quantification.

3. Results and discussion

3.1. Work-up procedure

SPE columns were chosen for work-up since it has previously been reported that liquid–liquid extraction give low extraction recoveries [13,14] for 5-HNMP and 2-HMSI. Several different disposable columns (Isolute ENV+, C_{18} and C_8) were tested for the extraction of 5-HNMP and 2-HMSI in urine, and also several different elution solvents. Initially, different columns were tried with a mixture of water–methanol as elution solvent. For the ENV+ column this gave a yield close to 100% for 2-HMSI but only about 40% for 5-HNMP. The other columns gave lower recoveries.

Elution with organic solvent was thereafter tested. This procedure has the disadvantage that an evaporation step must be added between the SPE and the analysis. The same three columns as above were tried with different ethyl acetate–ethanol ratios. Pure ethyl acetate gave recoveries between 90 and 99% for 5-HNMP in all columns while the recovery for

2-HMSI varied between 20 and 45%. For the C_8 and C_{18} columns, when using 20% ethanol in ethyl acetate as elution solvent, the recoveries were >93% for 5-HNMP but only between 20 and 85% for 2-HMSI. Isolute ENV+ columns and a mixture of 80:20 of ethyl acetate and ethanol gave best results. This conditions gave also high recoveries for plasma and were therefore chosen for the work-up procedure.

Nine plasma samples and ten urine samples from different human subjects, were spiked with a low and high concentration of 5-HNMP and 2-HMSI, before work up using the ENV+ columns (0.125 and 6.25 μg/ml in plasma; 0.25 and 25 μg/ml in urine). They were analysed and compared with water samples with the corresponding concentrations of 5-HNMP and 2-HMSI. Recoveries and the precisions in the recoveries are shown in Table 1. The high precisions found between samples from different subjects indicate that the effects from the biological matrices are small.

There is no need for hydrolysis of plasma or urine since there is no conjugation with glucoronic acid or sulphate for either 5-HNMP or 2-HMSI in NMP exposed subjects [5].

3.2. Chromatography and mass spectrometry

Chromatographic columns of different lengths and packing were tested. The best result, when comparing both peak shape and retention time, was achieved with a Genesis C_{18} column (50×2.1 mm). With a retention time of about 2 min for both 2-HMSI and 5-HNMP and almost symmetrical peaks, this was by far the best column. Longer columns all gave more or less tailing peaks and high retention times while little differences were found between different stationary phases. Typical chromatograms are shown in Figs. 2 and 3. There were small peaks appearing in the chromatograms from the blank samples, both for 5-HNMP and 2-HMSI. However, since the ratios between the major fragments for both compounds deviated strongly from that of standards it can be assumed that the peaks originate from other compounds with the same retention time.

Tandem mass spectrometry using collision in-

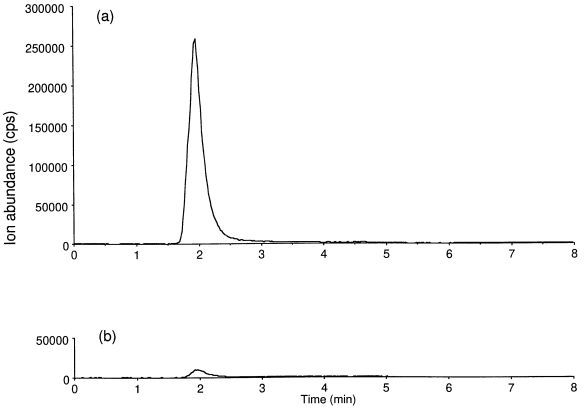

Fig. 2. Chromatograms showing a urine sample with a low concentration (0.25 μg/ml, *m/z* 84.7) of 5-HMNP (a) and a blank urine sample (b).

Table 1
Within-day precision and recoveries for the overall method of 2-HMSI and 5-HNMP in plasma and urine from non-exposed subjects

Compound	Matrix	Concentration (μg/ml)	Fragment *m/z*	Recovery (%)	Precision in recovery (%)	Within-day precision (%)	Number of samples
5-HNMP	Plasma	0.125	57.7	95	5	5	9
5-HNMP	Plasma	0.125	84.7	99	4	4	9
5-HNMP	Plasma	6.25	57.7	100	1	1	9
5-HNMP	Plasma	6.25	84.7	101	2	1	9
5-HNMP	Urine	0.25	57.7	109	10	10	10
5-HNMP	Urine	0.25	84.7	118	12	12	10
5-HNMP	Urine	25.0	57.7	105	3	2	10
5-HNMP	Urine	25.0	84.7	112	5	2	10
2-HMSI	Plasma	0.125	57.7	100	7	6	9
2-HMSI	Plasma	6.25	57.7	97	3	3	9
2-HMSI	Urine	0.25	57.7	113	12	10	10
2-HMSI	Urine	25.0	57.7	100	2	3	10

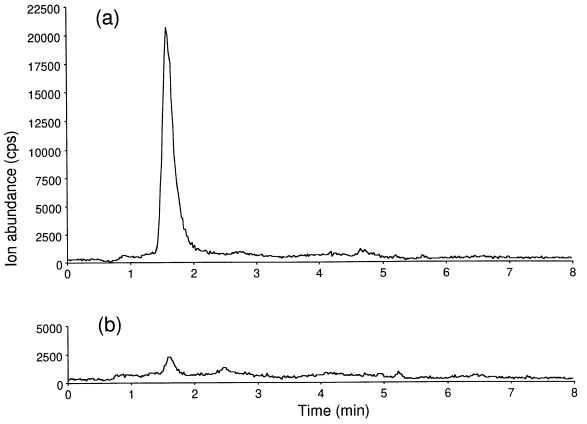

Fig. 3. Chromatograms showing a urine sample with a low concentration (0.25 μg/ml, *m*/*z* 57.7) of 2-HMSI (a) and a blank urine sample (b).

duced fragmentation was performed on 5-HNMP and 2-HMSI using high temperatures, 550°C and a water–methanol mixture to ionise the analytes in atmospheric pressure. For 5-HNMP, fragmentation was performed on the precursor ion *m*/*z* 115.7 yielding in fragmentation to ions with different *m*/*z* (Fig. 4). When the mass spectrometer was initially tuned, it was tuned with an automatic program which chose the mass fragment *m*/*z* 57.7 and this mass number was also chosen in our analysis during this work. When the fragmentation later was investigated manually, *m*/*z* 57.1 gave the best intensity. Thus, a gain in sensitivity is expected if the analysed mass fragment is changed to *m*/*z* 57.1. Similar fragmentation of the internal standard precursor ion *m*/*z* 119.7, gave fragments predominantly with *m*/*z* 87.7 and *m*/*z* 60.7. For 2-HMSI, fragmentation was performed

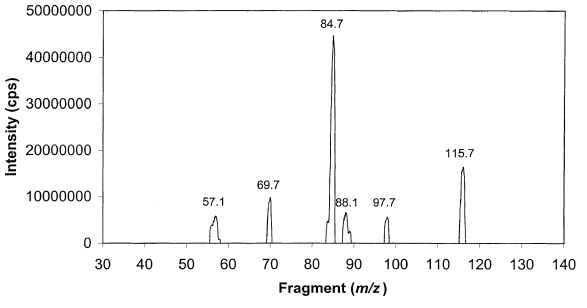

Fig. 4. Mass spectrum showing the fragmentation pattern for 5-HNMP, where *m*/*z* 115.7/84.7 is the dominating fragment.

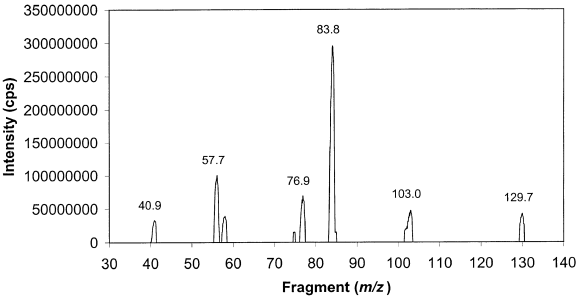

Fig. 5. Mass spectrum showing the fragmentation pattern for 2-HMSI, where *m*/*z* 129.7/57.7 is the best fragment.

on the precursor ion *m*/*z* 129.7 (Fig. 5). For the deuterium-labelled internal standard, analysis was performed on fragment *m*/*z* 132.7/60.7. The high temperature was necessary for good ionisation of 2-HMSI.

3.3. Quantitative analysis

3.3.1. Calibration graph

The concentration range in plasma was 0.12–12.5 μg/ml for both 5-HNMP and 2-HMSI, and in urine the range was 0.25–50.0 μg/ml. Samples containing more than 12.5 and 50.0 μg/ml, respectively, were diluted ten times with water before work up. Standard curves with $r>0.99$ were obtained by the peak area ratios between the analyte peaks and the internal standard peaks from samples spiked with known concentrations.

3.3.2. Detection limit

Plasma and urine samples were collected from nine and ten volunteers, respectively, who were presumed to be non-exposed to NMP or its metabolites. Internal standards were added and the samples were analysed according to the method described in Sections 2.5 and 2.6. The detection limit was calculated as reported by Miller and Miller [17], as the concentration corresponding to the mean plus three times the standard deviation of the peak area ratios with the same retention time as the analytes, 5-HNMP and 2-HMSI. The detection limit was 4 ng/ml for 5-HNMP in plasma and 120 ng/ml in urine (*m*/*z* 84.7). For 2-HMSI in plasma, the detection limit was 5 ng/ml and in urine, 80 ng/ml.

3.3.3. Precision

The within-day precision of the method was determined by analysis of nine different plasma and ten different urine samples spiked to 0.12 and 6.2 μg/ml for both 5-HNMP and 2-HMSI in plasma and to 0.25 and 25.0 μg/ml in urine. The coefficients of variation (C.V.) were determined for the peak area ratio between 5-HNMP and 2-HMSI and their corresponding internal standard peak. The results are presented in Table 1.

The between-day precision was studied by analysis of one plasma and one urine sample, spiked to a known concentration of 5-HNMP (1.0 and 6.2 μg/ml for plasma and 1.0 and 25.0 μg/ml for urine) and 2-HMSI (1.0 and 6.2 μg/ml for plasma and 1.0 and 25.0 μg/ml for urine samples). The plasma samples were analysed nine times over a period of 3 weeks and the urine samples nine times over a period of 2.5 months. The results are shown in Table 2.

3.3.4. Application and comparison with other methods

Urine samples ($n=161$ for 5-HNMP and $n=156$ for 2-HMSI) from a toxicokinetic study of ten NMP exposed human volunteers and plasma samples ($n=16$) from two NMP exposed workers were analysed for 5-HNMP and 2-HMSI by the methods described by Jönsson and Åkesson [13,15] and by the methods presented in this paper. Comparing the two methods, by plotting results from one method against the other, shows good correlation for both urine and plasma samples and for both metabolites. However, the results were slightly lower when analysed with LC–MS–MS compared to GC–MS. Urine shows linear regression slopes of 0.85 and 0.79 for 5-HNMP and 2-HMSI, respectively (Fig. 6). The

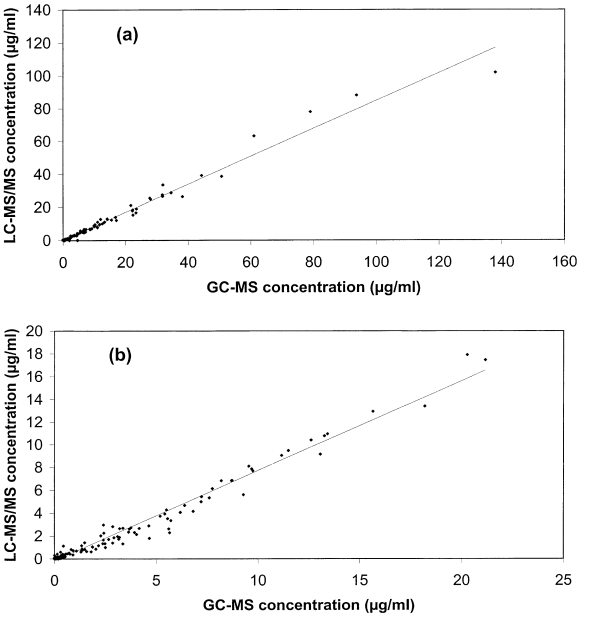

Fig. 6. Comparison between 161 and 156 determinations of 5-HNMP (a) and 2-HMSI (b), respectively, in urine, by the method described here (LC–MS method; y-axis) and the GC–MS method described earlier by Jönsson and Åkesson [13] (GC–MS; x-axis). The equation of 5-HNMP (a) is $y = 0.85x + 0.004$, $r^2 = 0.98$. The results from 5-HNMP was obtained using the mass fragment m/z 115.7/84.7. For 2-HMSI (b) the equation is $y = 0.79x - 0.16$, $r^2 = 0.98$.

Table 2
Between-day precision of plasma and urine samples, spiked with different concentrations of 2-HMSI and 5-HNMP

Compound	Matrix	Concentration (μg/ml)	Fragment m/z	Between-day precision (%)	Number of samples
5-HNMP	Plasma	1.00	57.7	5	10
5-HNMP	Plasma	1.00	84.7	5	10
5-HNMP	Plasma	6.25	57.7	3	10
5-HNMP	Plasma	6.25	84.7	5	10
5-HNMP	Urine	1.00	57.7	12	11
5-HNMP	Urine	1.00	84.7	6	11
5-HNMP	Urine	25.0	57.7	4	11
5-HNMP	Urine	25.0	84.7	4	11
2-HMSI	Plasma	1.00	57.7	6	10
2-HMSI	Plasma	6.25	57.7	3	10
2-HMSI	Urine	1.00	57.7	8	11
2-HMSI	Urine	25.0	57.7	7	11

equation of the regression line for 5-HNMP in plasma was $y = 0.85x + 0.04$ with a correlation coefficient (r) of 0.99 and equation of the regression line for 2-HMSI in plasma was $y = 0.78x - 0.05$ with $r = 0.98$. The deviation of the results in the urine samples, can be explained by the interval of 10–12 months, between the analyses of method 1 (GC–MS) and the method here presented. A 5% loss has been seen for 5-HNMP when stored at −20°C over a period of 5 months, while for 2-HMSI, there was a loss of 9% over a period of 8 months [13]. No degradation have previously been found in plasma for either 5-HNMP or 2-HMSI when stored at 4°C for 3.5 months, and at −20°C for 2.5 months, respectively [15]. On the other hand, the plasma samples in this study were analysed by LC–MS–MS 24–28 months after the analysis by GC–MS. It is possible that some degradation may occur during that period of time.

4. Conclusion

We present here a simple and fast method for the simultaneous determination of 5-HNMP and 2-HMSI in plasma and urine. The method has high precision, excellent recovery and detection limits that are sufficiently low for the determination of 5-HNMP and 2-HMSI in plasma and urine from subjects exposed to NMP. Thus, this method may be used for biological monitoring of exposure to NMP.

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