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Short communication

Determination of hexafluoroisopropanol, a sevoflurane urinary metabolite, by 9-fluorenylmethyl chloroformate derivatization

M. Buratti[a,*], C. Valla[a], D. Xaiz[a], G. Brambilla[b], A. Colombi[b]

[a]*Istituti Clinici di Perfezionamento, Laboratorio di Tossicologia Professionale, Via Commenda 10, 20122 Milan, Italy*
[b]*University of Milan, Faculty of Medicine, Department of Occupational Health, S. Paolo Hospital, Via Di Rudini 8, 20142 Milan, Italy*

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Abstract

A reversed-phase HPLC method with fluorescence detection for the quantification of hexafluoroisopropanol (HFIP) in urine is presented. HFIP, a metabolite of the inhalation anesthetic sevoflurane, is excreted mainly in urine as glucuronic acid conjugate. After enzymatic hydrolysis of the glucuronate, primary amino groups of interferent urinary compounds are blocked by reaction with *o*-phthalic dicarboxaldehyde and 3-mercaptopropionic acid, followed by labeling of HFIP with 9-fluorenylmethyl chloroformate. The derivatization reaction proceeds in a water–acetonitrile (1:1) solution at room temperature with a borate buffer of pH 12.5 as a catalyst. A stable fluorescent derivative of HFIP is formed within 5 min. The HFIP–FMOC derivative is separated by reversed-phase chromatography with isocratic elution on an octadecyl silyl column (33×4.6 mm, 3 μm) and guard column (20×4.0 mm, 40 μm), at 35 °C, and detected by fluorescence detection at an excitation wavelength of 265 nm and an emission wavelength of 311 nm. The method detection limit is 40 pg, per 10-μl injection volume, corresponding to 16 μg/l of HFIP in urine. The among-series relative standard deviation is <6% at 200 μg/l (n=6). As a preliminary application, the method was used to detect HFIP concentration in the urine of two volunteers exposed for 3 h to an airborne concentration of sevoflurane in the order of 2 ppm.

Keywords: Derivatization, LC; Hexafluoroisopropanol; Sevoflurane

1. Introduction

The fluorinated inhalation anesthetics, including desflurane, enflurane, halothane, isoflurane, and sevoflurane, are commonly used for the management of patients undergoing surgery. Among them sevoflurane has recently assumed a prominent role, because of some advantages, such as a low blood/gas partition coefficient that makes induction of anesthesia and awakening more easy to control. In humans, sevoflurane undergoes biotransformation rapidly to the primary metabolites fluoride and hexafluoroisopropanol (HFIP). HFIP is a halogenated short-chain alcohol, which is excreted in urine as glucuronide conjugate [1]. Urinary HFIP has been recently suggested as a valuable biomarker for the monitoring of occupational exposure of medical staff exposed to low concentration of airborne sevoflurane in operating rooms [2]. A few reports are available on the determination of HFIP in urine, all of them employing gas chromatography (GC) with head-

*Corresponding author. Tel.: +39-02-545-6025.
E-mail address: marina.buratti@unimi.it (M. Buratti).

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space procedures and flame ionization detection (FID) or mass spectrometry (MS) [2–4]. However, GC–FID is a somewhat insensitive technique, while MS detectors are relatively expensive. We wanted a easy method which would allow a rapid quantization of HFIP with a high degree of simplicity, and high-performance liquid chromatographic (HPLC) techniques appeared to fulfil these requirements. Recently, Huang et al. [5] reported a HPLC procedure for the determination of low-molecular-mass (C1–C4) alcohols in aqueous samples based on precolumn derivatization with 9-fluorenylmethyl chloroformate (FMOC). In this paper, we describe the derivatization process and separation conditions for the analysis of HFIP in urine by means of off-line precolumn double derivatization with *o*-phthalic dicarboxaldehyde/3-mercaptopropionic acid [6], and FMOC, followed by reversed-phase HPLC separation and fluorescence detection, which allow the routine determination of urinary HFIP at the ng/ml concentration level.

2. Experimental

2.1. Materials

HFIP, *o*-phthalic dicarboxaldehyde (OPA), 3-mercaptopropionic acid (MPA), FMOC, and β-glucuronidase/sulfatase, type H-2, from *Helix pomatia*, were obtained from Aldrich (Milan, Italy). Acetonitrile, hydrochloric acid (12 *M*), acetic acid (99.5%), boric acid, sodium acetate, and sodium hydroxide were purchased from Carlo Erba (Milan, Italy). All chemicals were of analytical purity or HPLC grade.

The octadecyl silyl column (33 mm×4.6 mm I.D., 3 μm particle size) Supelcosil C_{18} and the Supelguard C_{18} guard column (20 mm×4.0 mm I.D., 40 μm particle size) were obtained from Supelco (Milan, Italy).

2.2. Instrumentation

Incubations of the hydrolysis reactions was performed in a Thermolyne Dri-Bath (PBI, Milan, Italy). HPLC separation was carried out using a Waters LC Module 1 plus instrument (Waters, Milan, Italy) equipped with a HP-1046A fluorimetric detector (Hewlett-Packard, Cernusco, Italy). Data acquisition and elaboration were by means of Millenium-2010 software (Waters).

2.3. Urine sample storage

The urine for the method development was collected from healthy volunteers in the laboratory. The urine was not filtered and no preservatives were added to the urine. As soon as possible after collection, 1-ml aliquots were separated and stored in polyethylene disposable tubes at −20 °C until analysis. Before analysis, frozen samples were conditioned at 37 °C for 15 min, with frequent stirring.

2.4. Standard solutions and calibration curves

Standard solutions of HFIP were prepared at the time of analysis by diluting the alcohol in acetonitrile: concentration was adjusted to 399 μg/ml (2.38 μmol/ml). Working standard solutions (0.4, 0.8, and 1.6 μg/ml; 2.4, 4.8, and 9.6 nmol/ml) were freshly prepared in water.

2.5. Derivatizing solutions

Sodium acetate buffer (0.5 *M*) was prepared by dissolving sodium acetate in water and titrating to pH 5 with diluted acetic acid (0.5 *M*). For enzymatic hydrolysis, a diluted solution of β-glucuronidase/sulfatase juice in acetate buffer was prepared, corresponding to β-glucuronidase activities of 800 U/ml. Sodium borate buffer (pH 12.5, 0.5 *M*) was prepared by dissolving boric acid in water and titrating to the required pH with sodium hydroxide solution (5 *M*). OPA (50 mg/ml), MPA (50 μl/ml) and FMOC solutions (2.5 mg/ml, 10 m*M*) were prepared in acetonitrile.

2.6. Derivatization of calibration curves

For calibration purposes, freshly prepared working standard solutions (400 μl), were added with 190 μl of water, 200 μl of borate buffer, and 700 μl of FMOC. The derivatization reaction was driven at room temperature for 5 min; the reaction mixture was then acidified by adding 40 μl of 6 *M* hydro-

chloric acid. Aliquots of these solutions (20 μl) were injected into the HPLC system.

2.7. Analytical procedure for urine samples

To release HFIP from its conjugated form with glucuronic acid, an enzymatic hydrolysis step was employed. Urine samples (400 μl), added with 160 μl of β-glucuronidase/sulfatase solution, were incubated for 16 h at 37 °C. After cooling, reagents for derivatization purpose were added to the test tube: 30 μl of 2 *M* NaOH, 200 μl of borate buffer (to obtain pH values around 12), 30 μl of OPA, 30 μl MPA, and 700 μl of FMOC were added sequentially. Derivatization reaction was driven at room temperature for 5 min; the reaction mixture was then acidified by addition of 40 μl of 6 *M* hydrochloric acid (final pH<3). Aliquots of this solution (20 μl) were injected into the HPLC system. The presumed derivatization product resulting from reaction between FMOC and HFIP is shown in Fig. 1.

For the analysis of HFIP in an unknown sample, the retention time was compared with that of an external standard and the method of peak height measurement was used for quantitative assessment, using calibration graphs constructed by plotting the height of FMOC–HFIP elution peak versus the concentration of HFIP in a series of aqueous standard solutions processed as described above. Urinary HFIP concentrations were calculated comparing integrated peak height counts of HFIP derivative obtained from the unknown urine sample with that from the aqueous standard calibration curve.

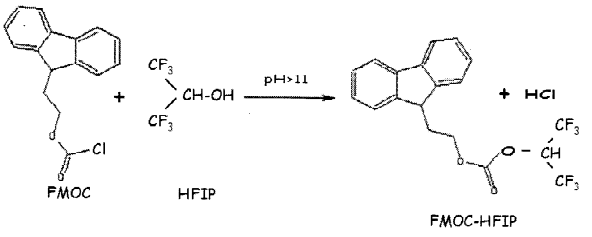

Fig. 1. Schematic representation of the reaction between 9-fluorenylmethyl chloroformate (FMOC) and hexafluoroisopropanol (HFIP) to give the presumed derivatization product (FMOC–HFIP).

2.8. Chromatographic conditions

Chromatographic separation was obtained by isocratic elution on a reversed-phase C_{18} column, at a flow-rate of 2.0 ml/min and at a temperature of 35 °C. The optimum composition of the mobile phase was found to be a mixture of acetonitrile–tetrahydrofuran–water (48:3:49, v/v). Fluorimetric detection was performed at an excitation wavelength of 265 nm, monitoring the emission at 311 nm.

2.9. Preliminary application

To establish the suitability of the proposed procedure for the monitoring of occupational exposure, the urinary excretion of metabolism-derived HFIP was studied in two volunteers exposed for 3 h to sevoflurane airborne concentrations of about 2 ppm. Spot urine samples were collected at the end of the exposure period.

3. Results

3.1. Chromatographic separation

Representative chromatograms obtained from an aqueous standard solution derivatized with FMOC alone are shown in Fig. 2A, referred to an aqueous calibration standard solution corresponding to urinary concentrations of HFIP=800 μg/l. However, chromatograms obtained from urine samples obtained from sevoflurane unexposed subjects derivatized by means of FMOC alone, presented a large number of big peaks, eluting very close to the one of HFIP (Fig. 2B). The preliminary addition of OPA and MPA reagents, which react with primary amines and amino acids, before the addition of FMOC, allowed one to eliminate the majority of these unwanted peaks, related to physiologically excreted compounds, and to obtain a notable reduction in the width of the chromatographic front. In Fig. 3 the elution patterns obtained after the double derivatization process with OPA–MPA and FMOC are shown; the chromatogram from a unexposed subject is depicted in Fig. 3A and Fig. 3B shows the chromatogram from a volunteer exposed to sevoflurane (sevoflurane airborne concentration=2 ppm; HFIP=

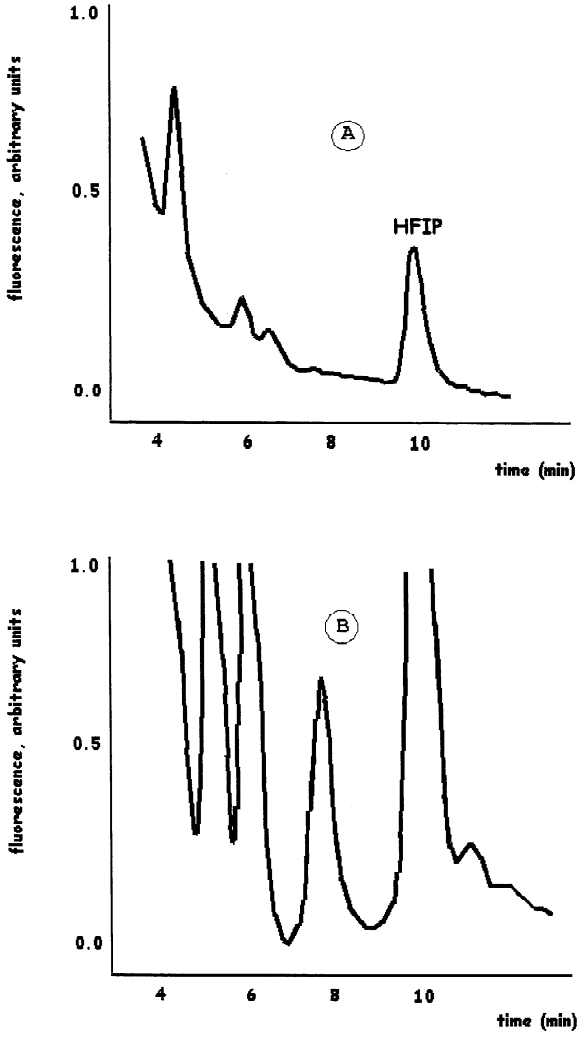

Fig. 2. Representative chromatograms of elution profiles obtained by one-step derivatization with FMOC. (A) Aqueous calibration solution (HFIP=800 μg/l). (B) Urine from a unexposed subject (expected concentration of HFIP<16 μg/l).

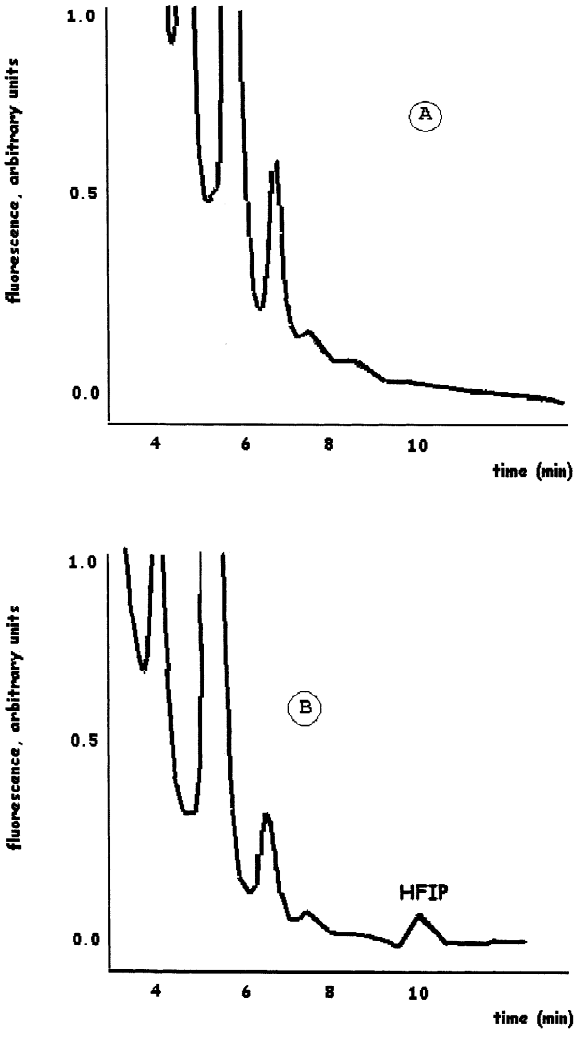

Fig. 3. Chromatographic elution profile obtained from urine samples after the double derivatization with OPA–MPA and FMOC. (A) Urine of a unexposed subject (the same urine sample used in Fig. 2B). (B) Urine from a sevoflurane exposed volunteer (HFIP=190 μg/l).

190 μg/l). The bulk of endogenous unidentified components is chromatographed within 5 min. No components from the biological matrix were found to interfere with the elution of the metabolite of interest. The retention time of FMOC–HFIP was 10 min. FMOC–HFIP eluted as sharp and symmetrical peak, sufficiently separated from main contaminants, and other FMOC derivatives. The entire run, before next sample could be injected, required 12 min. The method was employed in the evaluation of over 200 urine samples, examined over a period of 2 months, without significant methodological problems.

3.2. Optimization of the derivatization conditions

Analytical parameters have been optimized to obtain a maximum yield of FMOC–HFIP derivative,

and to achieve effective HPLC separation. The reaction time, the proportion of acetonitrile–water, the pH and temperature of the solution were varied around the expected optimal values [5]. The FMOC derivatization reaction progressed quickly in an alkaline environment, and a pH value in the range 11–13 was required to obtain consistent results. Reaction temperature (range, 18 to 60 °C) and reaction time (range, 2 to 90 min) did not influence at any appreciable degree the derivatization yield. There was some dependency of the reaction yield on the composition of the reaction mixture, in that at least 50% acetonitrile must be present in the reaction mixture to assure full solubilization of OPA–MPA and FMOC reagents and their derivatives. The optimal conditions were found to be: a pH value around 12, derivatization reaction driven at room temperature for 5 min, reaction solution consisting of water–acetonitrile (1:1, v/v), and acidification to pH≤3 after derivatization completion. The acidification step prevents the decomposition of FMOC–HFIP derivative, and allows for stability of the fluorescent derivative of at least 72 h, when stored at room temperature in the dark.

3.3. Calibration, recovery, reproducibility and storage

Calibration curves from aqueous or enriched urine standards were linear in the interval 50–3200 μg/l (0.3–19.0 μmol/l) for the studied metabolite. The calibration curve was described by the equation $y=9.6+187x$. When concentration of HFIP derivative exceeded the linearity range, samples were adequately diluted with mobile phase and reinjected. The limit of detection (LOD) was obtained from calibration curves (four different concentrations in the range 200–2000 μg/l, five determinations for each point), by use of the intercept (a) and standard error of its estimate S.E.(a) of the regression line for HFIP concentrations versus signal [7]. The limit of detection, calculated from $y=a+3$S.E.(a), resulted in $LOD_{HFIP}=16$ μg/l (0.09 μmol/l), corresponding to 80 pg of HFIP in a 20-μl injection (0.48 pmol). No matrix effects were observed when comparing the slopes of a standard addition line, obtained from enriched urine, and a calibration graph constructed using aqueous HFIP standard solutions under the same separation and detection condition. In order to evaluate the overall recovery of the procedure, calibration plots were built up by injecting standard solutions of HFIP prepared in water or in urine (added amounts in the range 200–1000 μg/l). The aqueous sample is considered to have 100% recovery. The average recoveries from urine can be obtained from the slope ratio of linear regression equations (urine to water). Overall percent recoveries higher than 95% were consistently obtained. The repeatability (precision within a run of ca. 5 h, expressed as relative standard deviation, RSD) of the method, determined by analysis of eight aliquots of a enriched urine (HFIP=200 μg/l), was RSD<7%. The reproducibility (between-day precision) among different assays on the same samples during a period of 2 months was RSD<13% ($n=11$). During the development of the procedure and up to now, more than 500 injections have been made on the same HPLC column without any observed column aberration. The C_{18} pre-column was changed every 100 injections, as a general rule to insure adequate protection and improve lifespan of the analytical column.

Various storage conditions were examined to minimize the loss of the urinary metabolite before instrumental analysis. Untreated urine samples were stored at −18 °C for 4 weeks, without significant modifications of metabolite concentrations. The hydrolyzed mixture appears to be stable for 24 h in sealed vials when stored in refrigerator. Derivatized samples could be stored at room temperature, in the dark, for 72 h without appreciable modifications of signal intensity.

3.4. Applicability of the analytical method

With the purpose of a initial trial to test the suitability of the analytical method for the biological monitoring of exposure to airborne sevoflurane, urinary excretion values of HFIP were assessed in two volunteers, which were exposed for 3 h to air contaminated with 2 ppm of sevoflurane. The presence of HFIP was evidenced in both samples, urinary HFIP concentrations being, respectively, 190 and 302 μg/l.

4. Discussion

For the determination of HFIP, gas chromatographic procedures are the only ones available. However, GC–FID is somewhat insensitive, while the management of GC–MS could be relatively exacting, when a small number of samples is analyzed in a run. A purpose of the present study was to devise a easy and reliable method to be adopted for routine determination of HFIP in human urine. To this goal, HPLC methods appeared well suited. However, the absence of an UV-absorbing or fluorescent chromophore in the HFIP molecule prevented the sensitive detection of native analyte by HPLC; in addition, the high polarity of the metabolite of interest in conjunction with the complex urine matrix represented a difficult analytical challenge. These problems have been circumvented by double derivatization prior to chromatographic analysis. FMOC, a reagent widely used for precolumn derivatization of primary and secondary amino groups [8], and also capable of forming stable ester-bond derivative with hydroxyl groups [9], and low-molecular-mass aliphatic alcohols in aqueous samples [5], was exploited for the determination of HFIP in urine. Analytical parameters have been optimized to obtain a maximum yield of FMOC–HFIP derivative, and to achieve effective HPLC separation. At first, derivatization with FMOC was studied using phosphate buffer (pH 12, 60 m*M*) as catalist, needed for removal of hydrogen ions from the hydroxyl group of the alcohol as suggested in the paper of Huang et al. [5]. Due to the water insolubility of FMOC, the reaction mixture requires a high proportion of acetonitrile (acetonitrile–water, 50:50, v/v), resulting in precipitation of the phosphate buffer. Experiments carried out to determine the kind and concentration of buffer able to maintain the required pH of the derivatization medium, finally indicated borate buffer 0.5 *M* at pH 12.5 as the one adequate for urinary HFIP derivatization. Optimum stability and yield of the FMOC–HFIP derivative were achieved after derivatization and acidification. All these features made the FMOC–HFIP derivative very interesting for the purpose of our study. However, chromatograms obtained from urine samples derivatized by means of FMOC alone, presented a large number of potentially interfering peaks, eluting close to the HFIP peak (Fig. 2B). Actually, FMOC reacts rapidly and under mild conditions with both primary and secondary amino acids in biological fluids [10]. Hence, many of the usual constituents of urine, giving FMOC derivatives, could greatly contribute to the complexity of the observed elution profile. To overcome this problem, primary amino groups were blocked by reaction with OPA and MPA, followed by labelling of hydroxyl groups with FMOC. The improvement of elution profiles, in the absence of any additional purification step, is clearly appreciable by comparison of the chromatogram obtained after the double derivatization with OPA–MPA and FMOC (Fig. 3A) with the one obtained with one-step FMOC derivatization (Fig. 2B). As a result of the double derivatization process, the bulk of the unwanted peaks disappeared and a cleaner chromatographic profile, free from interferences, was obtained.

The acidic reaction mixture is directly injected into the analytical column, ensuring a satisfactory precision and allowing to achieve an improved sensitivity. More than 500 injections have been performed without any worsening of the separation performances of the column. The adoption of aqueous calibration curves for quantitation of HFIP urinary concentration represents a further procedure simplification. The calibration curve demonstrated good linear relationship between alcohol concentration and HPLC response, with correlation coefficient in the order of 0.997. The slopes of calibration curves obtained from different enriched urines or from aqueous solutions were very consistent and appeared to be independent from matrix influence. The main advantage of our method is that it is simple and straightforward, requiring little sample pretreatment. In addition, the method does not employ formal extraction (e.g., liquid–liquid, solid-phase) and the standards are diluted identical to the urine samples, thus controlling for potential errors in micropipetting. However, because the use of internal standardization could guarantee higher reliability, during the development of the analytical procedure, we checked many alcohols to find out an appropriate internal standard. But, we were unable to identify a suitable compound, matching a satisfying compromise in term of reactivity in the derivatization process, retention time and resolution, which, in

addition, should be never findable in urine. In the end, we resolved to adopt external standardization to measure the concentration of HFIP in unknown samples, also supported by the good performances of the method, with special reference to percent recovery and precision.

The reported limit of detection (in the low ng/ml range) is better that those previously reported for GC–FID procedures and close to the one reported for mass spectrometers, and can be considered adequate for the evaluation of airborne sevofluorane exposure as low as 0.5 ppm.

The developed method was applied to the determination of HFIP in the urine samples of two volunteers exposed to level of airborne sevoflurane in the order of 2 ppm. In these subjects, HFIP concentrations in spot urine samples collected after 3-h exposure, were found to be, respectively, 95 and 302 μg/l. These findings, being in good accordance with results of other authors who studied the urinary excretion of HFIP in operating room personnel exposed to airborne concentration of sevoflurane as high as 20 ppm, supported the suitability of the proposed method for the measurement of HFIP in urine [2,4].

In conclusion, biomonitoring of exposure to halogenated anesthetics by suitable biomarkers is important for the protection of the health of occupationally exposed subjects, because the toxicological relevance of low-dose exposure is so far unclear [11]. For the monitoring of medical staff exposed to low concentration of sevoflurane, the measurement of urinary excretion of HFIP is the suggested biomarker.

The method proposed here combines simple sample treatment and derivatization, high sample throughput and reduced analysis time together with sufficient sensitivity and acceptable reproducibility, similar to those obtainable with the more demanding GC–MS techniques. Its accessibility, ease of use, and low costs make this technique quite attractive, as it provides a procedure simple, robust and readily reproducible in other laboratories. As sevoflurane utilization increases, this assay should represent a valid tool for routine biological monitoring of occupational exposure of medical staff exposed to low concentration of airborne sevoflurane in operating rooms.

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