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Simplified procedure for measurement of serum dehydroepiandrosterone and its sulfate with gas chromatography–ion trap mass spectrometry and selected reaction monitoring

Michael A. Zemaitis*, Patricia D. Kroboth

Department of Pharmaceutical Sciences, University of Pittsburgh, School of Pharmacy, 904 Salk Hall, Pittsburgh, PA 15261, USA

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Abstract

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) are endogenous steroids that have recently been widely publicized as potential treatments for many disorders. This paper describes a gas chromatographic–ion trap mass spectrophotometric assay with selected reaction monitoring for measurement of DHEA and DHEAS levels. The hormones and internal standard (5-androsten-3β-ol-16-one methyl ester) are extracted from serum with Oasis solid-phase extraction tubes. The extracted steroids are dissolved in methanol and injected into a Finnigan GCQ ion trap mass spectrometer. In the selected reaction mode, both DHEA and DHEAS can be identified and quantified in a single injection. No derivatization or expensive deuterated internal standards are required.

Keywords: Steroids; Dehydroepiandrosterone

1. Introduction

Dehydroepiandrosterone (DHEA) and its sulfated metabolite DHEAS are endogenous steroids secreted by the adrenal cortex in response to adrenocorticotropin (ACTH) [1]. While DHEAS is the most abundant circulating steroid hormone in humans [1,2], relatively little is known about its physiological function or that of DHEA. It is well known, however, that concentrations of both hormones decrease during adult life [3,4]. Studies in humans of the effects of these hormones have been generally conducted in only a few subjects and often only either in men or in women. Many of the studies have been conducted in animals or in in vitro systems. Research to date has suggested beneficial associations between DHEA and/or DHEAS concentrations and body composition [5], immune function [6], and cardiovascular disease [7]. In vitro studies also show effects on the γ-aminobutyric acid (GABA)–benzodiazepine receptor [8]. Currently, DHEA is classified as a food supplement and therefore available to be taken without medical supervision. In order to evaluate the safety and efficacy of administered DHEA it is necessary to accurately measure serum levels of DHEA and its primary metabolite DHEAS. Radioimmunoassay (RIA) has been the most popular method of analysis and several companies manufacture kits for DHEA and DHEAS (DHEA/S) analysis. Because of differences in RIA kits, DHEA/S levels and ''normal ranges'' can vary widely depending on the method of analysis [9–11]. In order to improve

*Corresponding author.

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accuracy and specificity in DHEA/S analysis, several methods based on gas chromatographic separation and mass spectral or electron-capture detection have been published [10–15].

Although there are differences in the published gas chromatography–mass spectrometry (GC–MS) analytical methods, all require several steps in sample preparation and require separate analysis of DHEA and DHEAS. In these methods, DHEA is extracted from a biological sample into an organic solvent. The DHEAS in the remaining aqueous layer is chemically or enzymatically hydrolyzed and subsequently extracted as free DHEA. The extracts are then evaporated and one of several chemical derivatives of DHEA is produced for injection into the GC–MS system. Accordingly, two injections are necessary to measure DHEA and DHEAS in the same sample. In the method reported herein, a single solid-phase extraction (SPE) of serum is employed and both DHEA and DHEAS are analyzed in the same injection. This selectivity and simplicity is primarily due to the ion trap technology available in the Finnigan GCQ GC–MS used in these studies. With this ion trap instrument, selected reaction monitoring (SRM) (MS–MS) can be used to identify and quantify DHEA/S even in the presence of other hormones and endogenous chemicals extracted during the single SPE. Moreover, an analogue of DHEA is used as an internal standard eliminating the need to synthesize or purchase expensive stable isotope derivatives of DHEA/S to use as internal standards.

2. Experimental

2.1. Chemicals and reagents

Methanol and acetonitrile were purchased from Burdick and Jackson (Muskegan, MI, USA). DHEA and DHEAS were purchased from Sigma (St. Louis, MO, USA). 5-Androsten-3β-ol-16-one methyl ester (internal standard) was purchased from Steraloids (Wilton, NH, USA). Analyte stripped serum (heavy charcoal strip) was obtained from Biocell Labs. (Rancho Dominguez, CA, USA). Oasis SPE cartridges were purchased from Waters (Milford, MA, USA). These cartridges contain a copolymer designed to extract a wide range of acidic, basic and neutral compounds.

2.2. Stock solutions

Stock solutions of DHEA, DHEAS, and the internal standard 5-androsten-3β-ol-16-one methyl ester (AME) of 1 mg/ml were prepared in methanol. Each stock was diluted 1:10, 1:100 and 1:1000 resulting in solutions of 100 ng/μl, 10 ng/μl and 1 ng/μl. These solutions were stored at −30°C and were brought to room temperature and used, as needed, to prepare standards and control solutions.

2.3. Standard solutions, control solutions and internal standards

In order to match the serum matrix of patient samples, standards and controls were prepared in Hypo-Opticlear (Biocell Labs.). This product is delipidized human serum which has been ''stripped'' of many hormones by charcoal filtration. The lot used was screened by the procedure reported in this paper and by RIA. It contained less than 0.5 ng/ml of DHEA and 20 ng/ml of DHEAS. Appropriate amounts of methanolic solutions of DHEA (1 ng/μl) and DHEAS (100 ng/μl) were added to 10×75 mm glass tubes and the methanol was evaporated. One-half ml of stripped serum was added to each tube. The tube was vortexed for 15 s and sonicated for 5 min to assure dissolution and distribution of DHEA/S. By this method standards were prepared containing per ml DHEA/DHEAS – 2/400 ng, 5/1000 ng, 10/2000 ng, 20/4000 ng, 30/6000 and 50/10 000 ng. Control solutions were prepared containing per ml DHEA/DHEAS 3/600 ng, 15/3000 ng, 40/8000 ng. The internal standard stock solution contained 50 ng/μl of AME. Ten μl of this solution was added to one-half ml of all standards, controls and unknown samples. The final concentration of internal standard in each serum sample was therefore 1000 ng/ml. Prior to SPE, 0.5 ml of 0.01 *M* phosphate buffer (pH=7) was added to each sample.

2.4. Instrumentation and conditions

The instrument used in these studies was a Finnigan GCQ GC–MS system. This instrument is an

ion trap mass spectrometer capable of carrying out SRM. This operation mode holds a selected parent ion in the ion trap and refragments it via collision with helium. A daughter ion spectrum can then be recorded. This form of MS–MS allows a high degree of specificity for a selected analyte even when it is present in a complex matrix such as biological fluid extracts. All injections were done in the splitless mode with a dwell time of 0.7 min before the purge valve was turned on. The mass spectrometer was tuned daily and was run at 200 V above the autotune voltage in the SRM mode with an automatic gain control value of 100. Electron ionization and positive ion detection were used. Data acquisition was begun 3 min after injection. From 3 min to 11.4 min, the parent mass was set at 270 with a notch width of 1 a.m.u. and a collision energy of 0.8. From 11.4 min until the end of the run the parent masses were set at 288 and 302 (dual SRM) with a notch width of 1 and a collision energy of 0.8. Daughter ion spectra were recorded from m/z 195 to m/z 320.

Separation of the three analytes was accomplished with a J&W Scientific (Folsom, CA, USA) DB-5MS capillary column (30 m×0.25 mm I.D.; 0.25 μm film thickness). GC conditions were: injector temperature, 270°C; transfer line temperature, 280°C; ion source temperature, 175°C; the GC oven temperature was programmed from 130°C (1.0 min hold) to 280°C (3.0 min hold) at a rate of 15°C/min. Helium was used as the carrier gas delivered at a constant velocity of 40 cm/s. The injection port liner was made of quartz and was packed with a CarboFrit inert liner (Restek, Bellefonte, PA, USA).

2.5. Sample preparation

To 0.5 ml of spiked stripped serum was added 10 μl of AME in methanol (50 ng/μl) and 0.5 ml of 0.01 *M* phosphate buffer (pH=7). Samples were mixed and sonicated for 5 min to ensure complete mixing. One-ml SPE tubes (Oasis HLB, Waters) were primed with 1 ml of methanol followed by 1 ml of water. The sample was loaded and drawn through the cartridge at a rate of 1 ml/min. The cartridge was washed with 1 ml of 5% methanol in water and allowed to dry for 20 min under vacuum; analytes were eluted with two 0.6 ml portions of acetonitrile. The acetonitrile was evaporated under a stream of nitrogen and the sample was reconstituted in 50 μl of methanol. Three μl of sample were injected into the GC system.

2.6. Calibration curves

Stripped serum was spiked with appropriate amounts of DHEA/S to produce standards that contained 2/400, 5/1000, 10/2000, 20/4000, 30/6000 and 50/10 000 ng of DHEA/DHEAS per ml. These calibration standards along with a sample of blank stripped serum were then subjected to the procedure described above. For assay validation, a calibration curve was generated from triplicate measurements of the standards each day for three days. Each day two separate control solutions containing 3/600, 15/3000 and 40/8000 ng/ml DHEA/S were run in triplicate for a total of six determinations per control. The average response factor for the triplicate standard curves was used to calculate the DHEA/S concentrations in the controls (see Section 2.7).

2.7. Calculations

Calibration curves were constructed by plotting the abundance ratio of [m/z 270 (DHEA)]/[m/z 270 (AME)] or the abundance ratio of [m/z 199 (DHEAS)]/[m/z 270 (AME)] as a function of serum steroid concentration. The response factor for each standard was calculated from the following formula: peak area of analyte×amount of internal standard/peak area of internal standard×amount of analyte. The average response factor along with the three known parameters (internal standard amount, peak areas for analyte and internal standard) was used to calculate the analyte concentration. The back-calculated values for the controls were used to measure the accuracy and variability of the procedure.

3. Results and discussion

The principle advantage of this assay is its simplicity – one extraction and one injection. The single extraction is accomplished with Oasis SPE cartridges (Waters). During assay development, these cartridges were found to offer the best balance between retain-

ing the non-polar DHEA and AME and the polar DHEAS. It was also noted that it was not necessary to derivatize DHEA in order to obtain acceptable chromatography. This is also critical to the success of the assay, since derivatizing a mixture of DHEA and DHEAS (with perfluoroacylating reagents) may result in sulfate displacement from some DHEAS with subsequent perfluoroacyl formation leading to erroneously low DHEAS levels and erroneously high DHEA levels [16,17]. Although one would expect DHEA and AME to chromatograph well on a DB-5 capillary column, the polar, less volatile sulfate ester would not be expected to chromatograph well. However when DHEAS is injected directly, it consistently produces three sharp reproducible peaks (in intensity and retention time). It appears that at the elevated temperature of the GC injection port, a reproducible decomposition occurs in which the elements of sulfuric acid are removed and one of three isomers is formed with different locations of double bonds in the A and B steroid rings. Such a reproducible decomposition occurs with the progestin ethynodiol diacetate; in the hot GC injection the compound loses the elements of acetic acid from the 3 position producing two isomers and a reproducible double peak in the chromatogram which is characteristic and readily quantified [18]. For DHEAS, the third isomer to exit the GC system is the predominate one. The decomposition pattern is reproducible within a given run, and is reproducible between runs if the injection port liner is replaced regularly with a clean one. To measure the consistency of the decomposition, the relative areas of peaks one and two (compared to peak three) were determined from one of the triplicate standard curves ($N=18$). Peak one was $16.2\pm2.1\%$ of peak three and peak two was $23.2\pm1.3\%$ of peak three (mean$\pm$S.D.). Fig. 1 is a chromatogram of an extracted standard containing DHEA, AME and DHEAS. Peaks A, B and C are the three isomers formed from DHEAS, peak D is AME and peak E is DHEA. It should also be noted that the internal standard elutes between the DHEAS peaks and the DHEA peak making it useful for quantifying both steroids.

When a serum extract is injected into the GC–MS system in the full scan mode, DHEA/S are barely detectable or are obscured by other steroids such as cholesterol. However, in the SRM mode, the parent

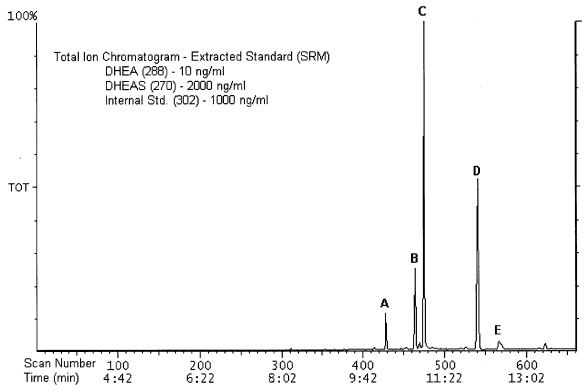

Fig. 1. Chromatogram of an extracted serum standard containg DHEA, DHEAS and AME. Peaks A, B and C are derived from DHEAS, peak D is AME and peak E is DHEA.

ions of DHEA/S and AME are stabilized in the ion trap and subsequently refragmented to produce daughter ion spectra. This method results in specific total ion chromatograms that include only peaks from DHEA/S or AME (Fig. 1).

Figs. 2–4 show the full scan spectra (A) and daughter ion spectra (B) for DHEA, DHEAS and AME, respectively. For DHEA, the parent ion ($m/z=288$) is the base peak in the full scan mode. The daughter spectrum of 288 has a base peak of 270 that was subsequently used for quantification of DHEA. For the ''desulfated'' isomers of DHEAS, 270 is the base peak and represents removal of the elements of sulfuric acid from the 3 position and introduction of a double bond into the steroid ring system. Each of the three peaks attributable to DHEAS have the same full scan spectrum, as would be expected from three steroid isomers that differ only by location of two double bonds in the A and B rings. The daughter ion spectra of each isomer are also alike with $m/z=199$ as the base peak. The intensity of the 199 peak in isomer C (Fig. 1) was used for quantification. The full scan spectrum of the internal standard AME reveals a parent ion at $m/z=302$ with a base peak of 270. The daughter ion spectrum has a base peak of 270 that was subsequently used for quantification of DHEA and DHEAS.

Extraction recovery for DHEA/S was calculated by comparing peak area ratios between DHEA or DHEAS and internal standard obtained from a pure

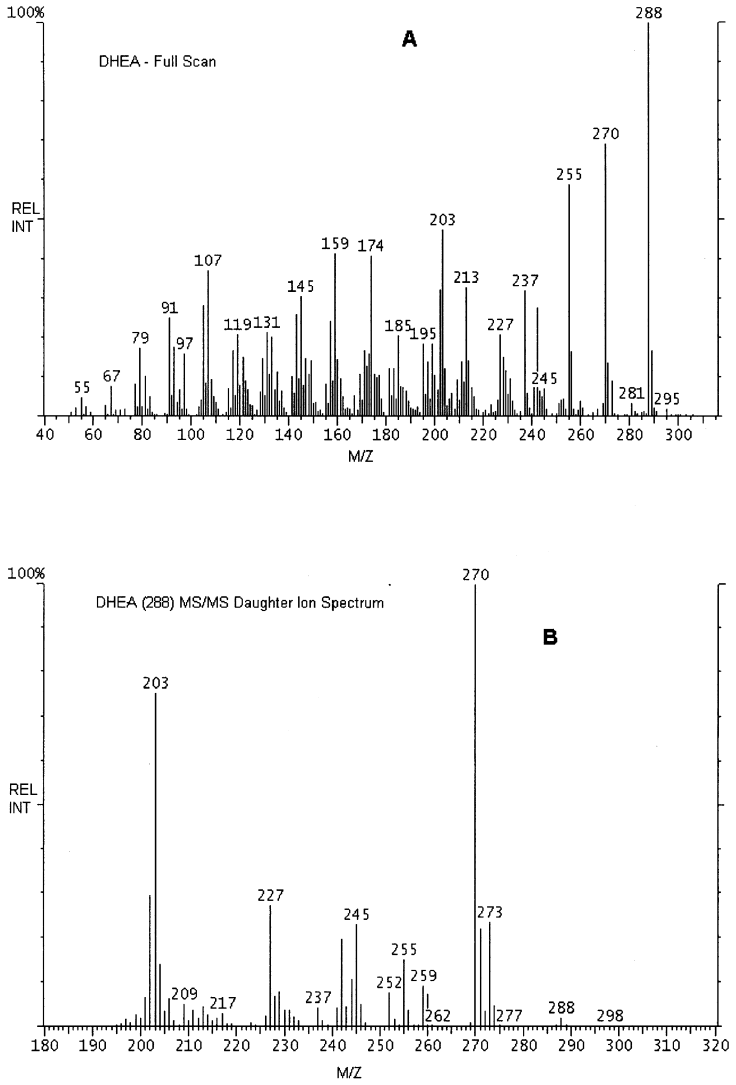

Fig. 2. The full scan spectrum (A) and MS–MS daughter ion spectrum (B) of DHEA.

mixture of DHEA/S (25/5000 ng) and 1000 ng of internal standard with the ratio obtained upon extraction of DHEA/S (25/5000 ng/ml) from 1 ml of serum and its addition to 1000 ng of internal standard. Extraction recovery for AME was calculated by comparing the peak area ratio of AME to DHEA obtained for a pure mixture of AME (100 ng) and DHEA (100 ng) with the ratio obtained upon extraction of AME (100 ng/ml) from 1 ml of serum and its addition to 100 ng of DHEA. Recovery was calculated to be 91% for AME, 85% for DHEA and 84% for DHEAS.

The validity of the assay procedure was established through experiments designed to study the accuracy and precision of the method. The six-point calibration curves were linear with reproducible slopes, x intercepts of approximately zero and correlation coefficients (r)>0.994. The calculated concentrations for the triplicate determinations of two sets of controls are presented in Table 1. As can be

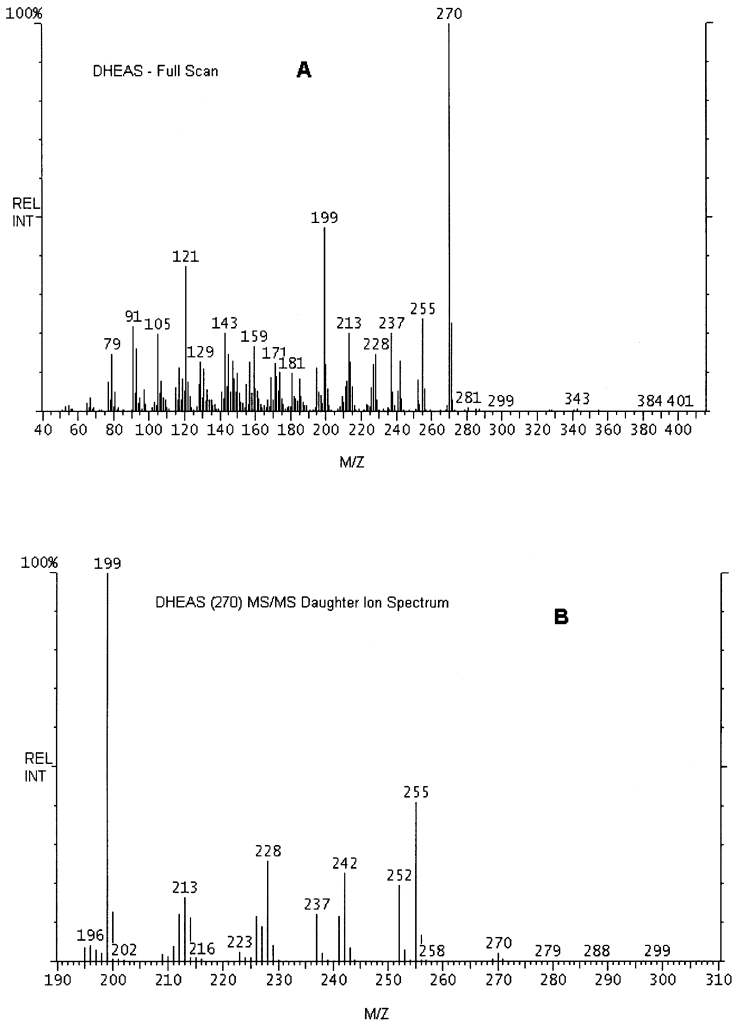

Fig. 3. The full scan spectrum (A) and MS–MS daughter ion spectrum (B) of DHEAS.

seen from these data, the mean concentrations at each level were no more than ±10% from the respective target concentrations and the coefficients of variation (S.D./mean×100) were all less than 15%. The low end of the standard curve was selected to simulate typical low serum values in humans, however, the limit of detection for the assay is significantly lower. One series of standards and controls were run using only 0.1 ml of sample. By adding only 100 ng of AME, and increasing the multiplier voltage to 300 V above the autotune voltage, these samples were analyzed adequately. Accordingly, this procedure could easily be modified to measure small concentrations of DHEA/S in human or animal biological fluids or tissue homogenates.

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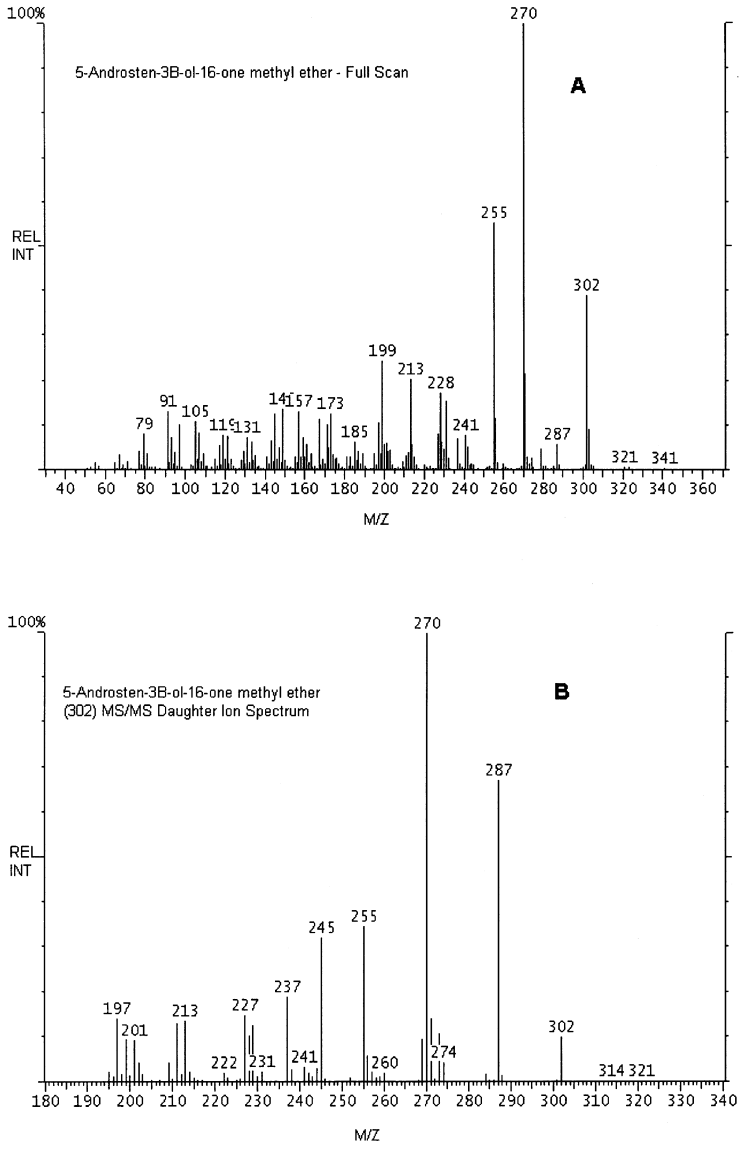

Fig. 4. The full scan spectrum (A) and MS–MS daughter ion spectrum (B) of 5-androsten-3β-ol-16 one methyl ester.

Table 1
Calculated concentrations of DHEA/S serum controls (mean±S.D.) on each of three separate days

Study	DHEA (ng/ml)			DHEAS (ng/ml)		
	Low	Medium	High	Low	Medium	High
I (N=6)	3.3±0.5	14.7±1.9	37.4±3.4	642±58	3211±424	8096±631
II (N=6)	3.1±0.4	14.9±1.4	37.1±1.9	659±21	2966±192	7269±347
III (N=6)	3.0±0.4	15.3±1.9	38.7±1.1	651±29	2981±101	7323±281

Duplicate samples were each run in triplicate (N=6). Each standard curve was also run in triplicate and all points were used to construct a composite standard curve each day. Target values for DHEA/S were: Low=3.0/600 ng/ml; Medium=15.0/3000 ng/ml; High=40/8000 ng/ml.

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