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DETERMINATION OF PLASMA TESTOSTERONE BY MASS FRAGMENTOGRAPHY USING [3,4-^{13}C]TESTOSTERONE AS AN INTERNAL STANDARD

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SUMMARY

The combination of glass capillary gas chromatography—mass spectrometry is especially suitable for the recognition of compounds. The use of [3,4-^{13}C]testosterone as internal standard, mass fragmentography and isotope ratio measurement have been applied to the quantitative determination of testosterone in plasma. This paper describes the method, using *tert.*-butyldimethylsilylmethoxime and di-heptafluorobutyrate derivatives. The calibration graph in isotopic dilution is examined. The results obtained are compared with the results obtained by radioimmunoassay. The sensitivity of the method is judged from the lower limit of detection: 4.5 pg. The precision, and inter- and intra-assay are calculated.

INTRODUCTION

The need for specific, rapid determinations of steroid hormone levels in the clinical laboratory has led to the development of numerous analytical methods.

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A quantitative method has been developed for the estimation of testosterone in human plasma using the technique of combined gas chromatography—mass spectrometry (GC—MS) and a labelled standard. Sweeley et al. [1] and Hammar et al. [2] demonstrated the applicability of using a labelled analogue as the ideal internal standard to correct for losses of a substance under study in the initial isolation procedures. The usefulness of mass fragmentography in which GC—MS is used, in conjunction with isotopic dilution has been of great interest because of its high specificity and high sensitivity [3—10].

This paper describes a relatively simple method for the determination of plasma testosterone with high specificity and accuracy. After extraction and conversion into methoxime-*tert.*-butyldimethylsilyl (MO-TBDMS) or di-heptafluorobutyrate (DHFB) derivatives, five specific ions have been followed by the multiple ion detector unit of the mass spectrometer.

The sensitivity, precision and accuracy of the procedure were judged. The amounts of unlabelled testosterone in male and female plasma were compared with the results obtained by a radioimmunoassay method.

MATERIALS AND METHODS

Internal standard

[3,4-^{13}C]Testosterone was obtained from the Commissariat à l'Energie Atomique (Molécules Marquées), Saclay, France. The purity and the ^{13}C enrichment of this standard were confirmed by GC—MS and nuclear magnetic resonance (pulsed NMR and ^{13}C-NMR).

Gas chromatography—mass fragmentography

The first instrument used was a Hewlett-Packard 5798 GC coupled with the mass spectrometer V.G. 305.F. The second instrument was a quadrupole mass filter Hewlett-Packard 5970-A coupled with the Hewlett-Packard 5792-A chromatograph. The capillary column was directly connected to the mass spectrometer source, without a silanized glass line. The ionization was realized by electron-impact: 70 eV, ion source temperature 200°C.

The column was a fused-silica capillary column, 25 m × 0.21 mm, with bonded apolar phase (SE-54 type), from Hewlett-Packard. This column was programmed from 210°C to 245°C at 2°C/min, and from 245°C to 290°C at 8°C/min. The first part of the programme allows the chromatographic separation and the high temperature of the second part is concerned with washing the column before a new separation.

Sample preparation

A 2-ml volume of heparinized male plasma or a 4-ml volume of female plasma was added to 80 ng of [3,4-^{13}C]testosterone obtained by evaporation of an ethanolic solution of the labelled standard; 3 ml of distilled water and 0.05 ml of 1 *M* sodium hydroxide were added. After mixing, the diluted plasma was extracted with 20 ml and 10 ml of diethyl ether. After centrifugation, the upper layer was decanted and evaporated to dryness. The derivatives were formed by reacting the residue with reagents as described below. An additional purification of the residue is realizable before this reaction: the residue was

treated with 3 ml of a freshly prepared solution of digitonin (1.7 g in 50 ml of absolute ethanol) and the mixture was allowed to stand at 100°C for 4 min and at 30°C for 30 min. The ethanol was evaporated and the residue was washed with 3 ml of 0.14 *M* sodium chloride. Testosterone was extracted twice with 10 ml of light petroleum. After evaporation of this phase, the residue was treated to form derivatives.

Formation of derivatives

The methoxime-*tert.*-butyldimethylsilyl derivative [8, 11—14] was formed by reacting the residue with 0.3 ml of methoxyamine · HCl (2%) in pyridine (MOX) (Pierce) for 1 h at 60°C. After evaporation, 0.2 ml of a freshly prepared mixture of TBDMS (2.5 mmol TBDMS-trifluoromethanesulphonate (Fluka), 5 mmol imidazole in 25 ml of dimethylformamide) was added. After 1 h at 40°C [11], the MO-TBDMS derivative was washed with 2 ml of water, and was extracted with 0.05 ml of hexane prior to gas chromatography.

Di-heptafluorobutyrate derivative [15] was formed by reacting the extract with 0.2 ml of heptafluorobutyric anhydride for 1 h at 60°C. After evaporation under a stream of nitrogen, the derivative was dissolved in 0.05 ml of hexane.

Radioimmunoassay

Plasma testosterone was determined in duplicate by a radioimmunoassay method used every day in our laboratory. The antibody was directly towards testosterone but reacted also to a small amount of 5α-dihydrotestosterone. The purification of testosterone from plasma was realized by chromatographic separation prior to radioimmunoassay using Celite microcolumns [16].

Isotope dilution—mass fragmentography

The two spectrometers were equipped with a multiple ion detector—peak matcher. The detector was focused on the ions at *m/e* 374 (M − 57), 375, 376, 377 and 378 for MO-TBDMS testosterone [17, 18] and at *m/e* 680 (M), 681, 682, 683 and 684 for DHFB testosterone.

The quantitative analysis and the concentration of the plasma testosterone used the equation of Pickup and McPherson [19]:

$$R_{kl} = \frac{(x/y)\,(P_k/E) + (Q_k/F)}{(x/y)\,(P_l/E) + (Q_l/F)} \quad \text{or} \quad R_{kl} = \frac{(x/y)P_kF + Q_kE}{(x/y)P_lF + Q_lE}$$

where R_{kl} is the ratio between the peaks at (M) and (M + 2) obtained in the recordings, E and F are the molecular masses of natural and labelled material, x is the mass of the natural material and y the mass of labelled compound. P_k, Q_k and P_l, Q_l are the relative intensities of the peaks at (M) and (M + 2) of the natural and the labelled compounds. These four coefficients were calculated using pure natural and labelled testosterones.

RESULTS AND DISCUSSION

Purity of [3,4-^{13}C] testosterone internal standard

The mass spectra of the MO-TBDMS and DHFB derivatives of natural and labelled testosterones are shown in Fig. 1. The presence of non-labelled

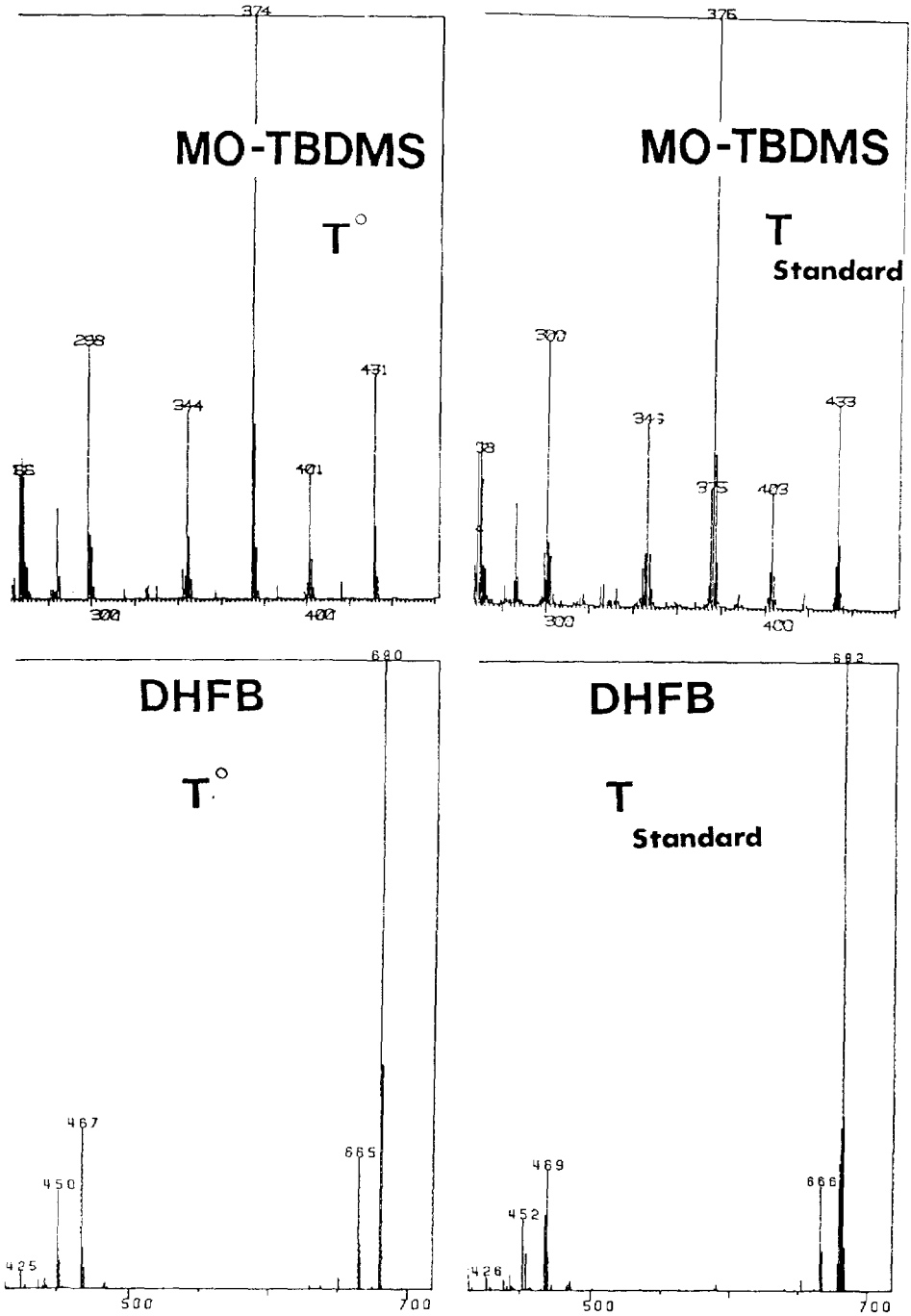

Fig. 1. Mass spectra of the MO-TBDMS and the DHFB derivatives of natural testosterone (T^0) and labelled testosterone ($T_{Standard}$).

testosterone in the standard is verified by calculation using the isotopic molecular ions. [3,4-^{13}C] Testosterone is a mixture of 80% of the bis-^{13}C compound (T**), 17% of the mono-^{13}C compound (T*) and 3% of the non-labelled compound (T^0). This is confirmed by high field NMR.

TABLE I

MEAN VALUES OF THE FOUR COEFFICIENTS FOR THE TWO DERIVATIVES

	MO-TBDMS derivatives		DHFB derivatives	
	Coefficient	C.V. (%)	Coefficient	C.V. (%)
Magnetic spectrometer (n = 22)				
P_k	0.72	0.64	0.71	0.3
Q_k	0.024	8.4	0.029	7.7
P_l	0.056	3.0	0.042	0.6
Q_l	0.62	2.0	0.61	1.1
Quadrupole mass filter (n = 11)				
P_k	Not measured		0.71	1.2
Q_k			0.014	5.5
P_l			0.046	2.8
Q_l			0.62	0.6

The presence of T^0 in the internal standard requires relatively complicated correction techniques for the observed peak height in quantitative analysis. The existence of T^0 in the standard increases Q_k in comparison with Q_l, and the ordinate for null abscissa.

The four coefficients P_k, P_l, Q_k and Q_l were determined using the peak heights of the molecular ions measured with the two spectrometers (Table I).

Standard curves

Standard curves $R_{kl} = f(x/y)$ used for the estimation of testosterone in male and female plasma have a linear part for small amounts of T^0 [4, 19—21]. The ratio R_{kl} between (M) and (M + 2) increases with increasing amounts of T^0.

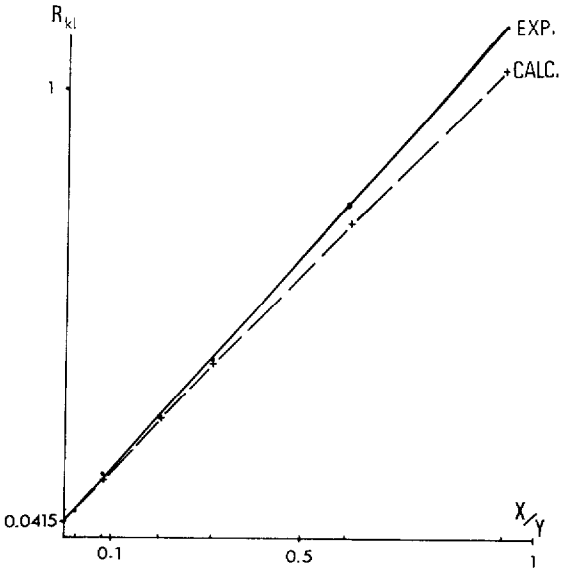

Fig. 2. Standard curves for determination of testosterone in the range of x/y = 0—1.

The Pickup—McPherson equation allows the mathematical approach of isotopic dilution, showing the real nearness of the experimental and the calculated curves (Fig. 2). There is a good agreement between the two curves for small values of x/y.

Nineteen standard curves were realized with the magnetic spectrometer during three years and three standard curves with the quadrupole mass filter. These 22 curves have a relative standard deviation of 4%. The equation of the middle curve for the magnetic spectrometer is

$$R_{kl} = \frac{(x/y)\,(13.47) + 0.48}{(x/y) + 11.55}$$

This curve is linear for x/y in the range 0—0.5, and the linear regression equation is $R_{kl} = 1.115\,(x/y) + 0.0447$. For $x/y = 0$, the experimental value of R_{kl} is the same as that of the calculated one.

The quadrupole mass filter gives the standard curve defined by

$$R_{kl} = \frac{(x/y)\,(15.33) + 0.30}{(x/y) + 13.37}$$

The slope of the standard curve has a constant value for the range of x/y = 0—0.5 (Table II). These results show that isotope dilution is obligatorily used

TABLE II

SLOPES OF STANDARD CURVES FOR THE RANGE x/y = 0—0.5

x/y					
0	0.001	0.01	0.1	0.2	0.5
Magnetic spectrometer					
1.163	1.162	1.161	1.143	1.123	1.068
Quadrupole mass filter					
1.145	1.145	1.143	1.128	1.111	1.064

for a good correspondence between the experimental and the calculated standard straight lines. The slope of the curve depends on the four coefficients (Q_k, Q_l, P_k and P_l) and is given by the equation

$$(R_{kl})' = \frac{EF(P_kQ_l - Q_kP_l)}{(P_lF\,x/y + Q_lE)^2}$$

The most important part of the curve is near $x/y = 0$, where the slope is

$$(R_{kl})'_0 = \frac{F}{E} \cdot \frac{(P_kQ_l - Q_kP_l)}{(Q_l)^2}$$

and the ordinate to origin is $R_{kl_0} = Q_k/Q_l$. Consequently, the first stage in developing an isotope dilution assay is the selection of a suitable labelled standard. This standard must be relatively pure, without non-labelled

compound, to give a small coefficient Q_k. Under these conditions, the standard curve is linear near the small x/y values, the ordinate to the origin is minimum, and the slope is then a stable value $(R_{kl})' = P_k F/Q_l E$.

Blanks and accuracy

Water blanks gave no measurable peak with the highest sensitivity of the multiple ion detector. The accuracy of the method was investigated by adding known amounts of natural testosterone to the plasma aliquots from a pool. The regression curves (Table III) indicate that the slopes are not significantly different from unity.

TABLE III

ACCURACY OF THE METHOD

Four series of measurements were performed with different additions of T**.

	Regression line*
14 measurements on 6 points T** = 36 ng (magnetic spectrometer)	$Z = 0.988W + 0.113$ ($r = 0.99$)
16 measurements on 8 points T** = 32 ng (magnetic spectrometer)	$Z = 0.998W + 0.195$ ($r = 0.99$)
10 measurements on 5 points T** = 43 ng (magnetic spectrometer)	$Z = 0.994W + 0.006$ ($r = 0.99$)
10 measurements on 5 points T** = 59 ng (quadrupole mass filter)	$Z = 1.001W + 0.038$ ($r = 0.99$)

*Z = experimental value, W = T** added.

Specificity

The specificity is the most important property of an analytical method. In this instance, it is based on the selective isolation procedure: high resolution of the capillary column, and the highly specific ion detector. The derivatives of possible interfering steroids such as epitestosterone and dehydroepiandrosterone did not interfere in the assay (Fig. 3).

Precision

The precision was calculated from duplicate measurements of 35 plasmas covering the x/y range of 0.03—4. The intra-assay coefficients of variation obtained were near 2.8%. The 22 standard curves have a standard deviation of 4% (inter-assay S.D.).

Limit of detection

The sensitivity of the determination procedures described here is judged on

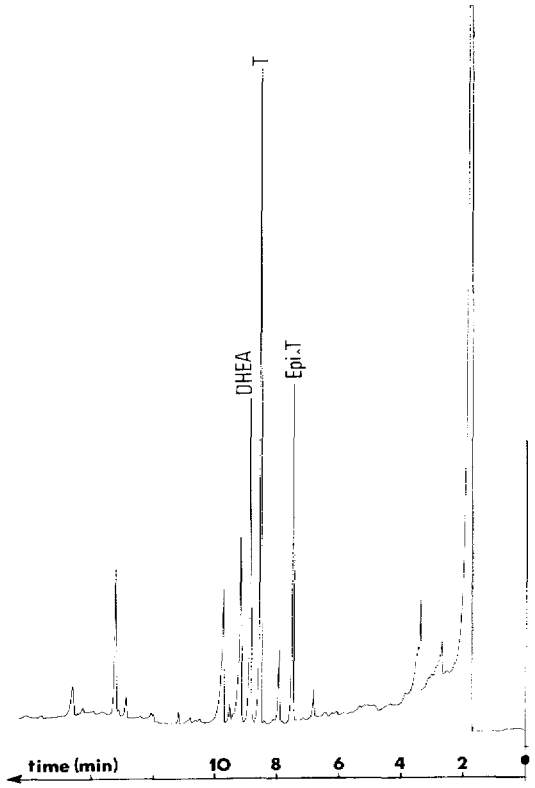

Fig. 3. Gas chromatogram. DHEA did not interfere with testosterone. DHEA = dehydroepiandrosterone, Epi-T = epitestosterone, T = testosterone.

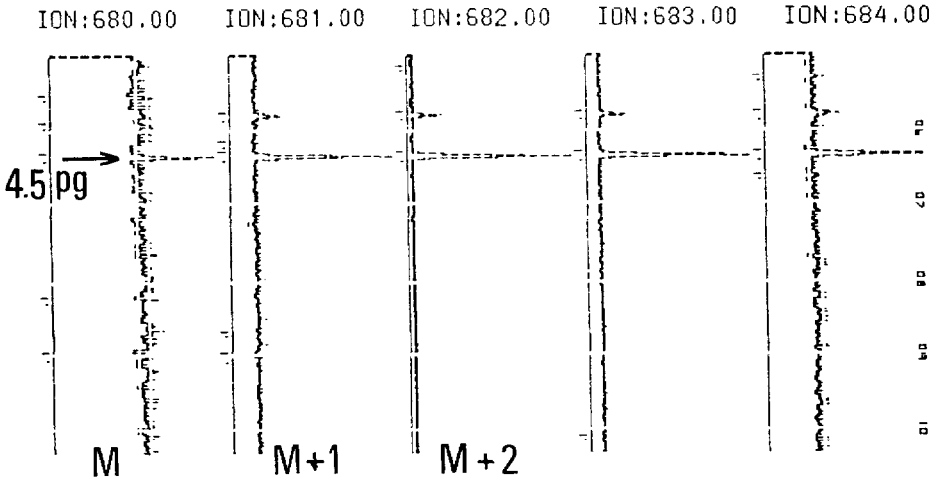

Fig. 4. Gas chromatogram of 4.5 pg of natural testosterone, for 60 pg of labelled standard.

the basis of the signal-to-noise (s/n) ratio. The lower limit of detection is near 4.5 pg of testosterone (Fig. 4) for a peak intensity of $s/n = 4.2$ [22].

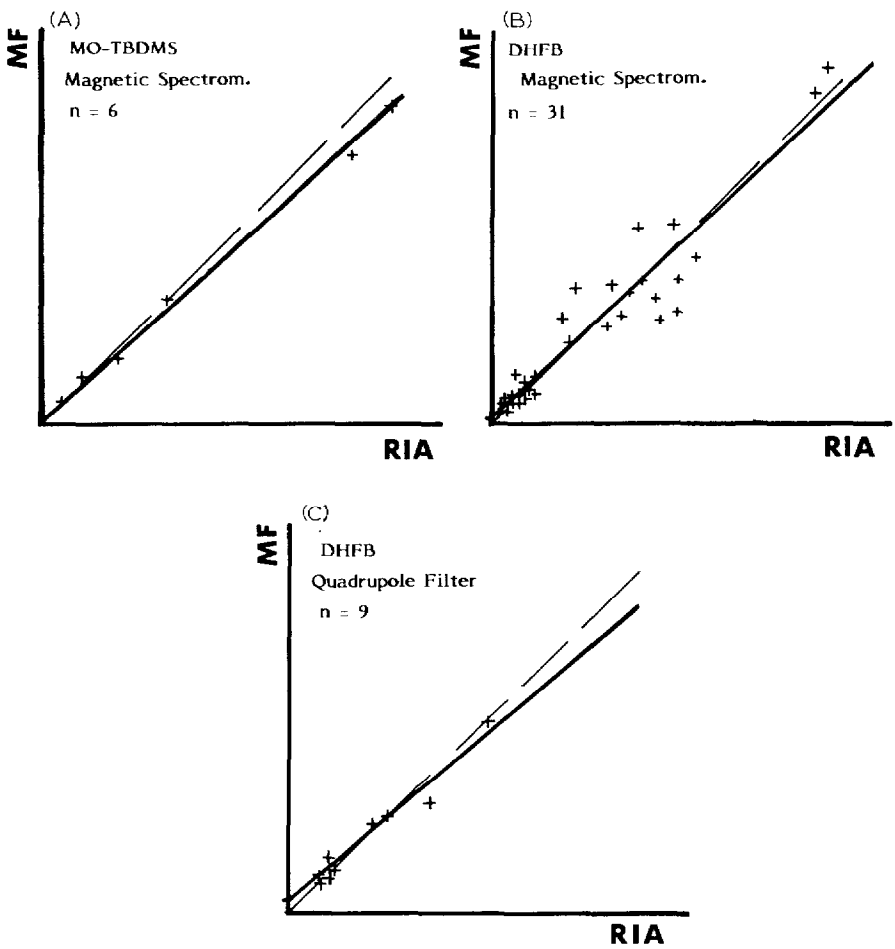

Fig. 5. Comparison between the mass fragmentography (MF) and the radioimmunoassay (RIA) methods: three series of measurements were made using the two spectrometers. (A) $Y = 0.916X - 0.057$ (nmol/l), (B) $Y = 0.961X + 0.519$, (C) $Y = 0.831X + 0.830$.

Comparison of results by isotopic dilution—mass fragmentography (ID—MF) and radioimmunoassay

The amounts of testosterone were measured in duplicate. For ID—MF and radioimmunoassay, three series of measurements were made: (1) six plasmas with MO-TBDMS derivative and (2) 31 samples with DHFB derivative for the two spectrometers, and (3) nine samples (DHFB derivative) for the quadrupole mass filter. The correlations between testosterone concentrations obtained by radioimmunoassay and by ID—MF are shown in Fig. 5. This comparison of results demonstrates that the radioimmunoassay appreciably over-estimates the true value, in accordance with previous reports [23, 24].

In summarize, the ID-MF method described here affords a sensitive and reliable technique to measure plasma testosterone. An internal standard labelled with ^{13}C in the molecular skeleton allows isotopic dilution without losses of it as deuterated standards.

The use of the Pickup—McPherson equation allows a good understanding

of the ID—MF phenomena, especially if the internal standard contains an appreciable amount of non-labelled compound.

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