Journal of Chromatography, 277 (1983) 217—227
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1777

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMEZINIUM IN HUMAN PLASMA

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(First received January 26th, 1983; revised manuscript received May 13th, 1983)

SUMMARY

A method with internal analogue standardization is described for the determination of 4-amino-6-methoxy-1-phenyl-pyridazinium methyl sulphate (ameziniummetilsulfate, Regulton®, LU 1631) in plasma. The method involves a cation-exchange filtration, a dequaternization reaction and subsequent determination by high-performance liquid chromatography. There is no interference by plasma components or amezinium metabolites found in plasma extracts. When using a 2-ml sample the lower limit of detection is 0.75 ng/ml; the lower limit of determination is about 2 ng/ml. Interassay coefficients of variation decrease from about 14% at the lowest concentration to about 5% at 20 ng/ml and above.

INTRODUCTION

Ameziniummetilsulfate* is a new antihypotensive agent [1] which differs from classic sympathomimetics by its completely novel structure and its unprecedented pattern of actions [2—5]. It has proved its value in the treatment of postural cardiovascular dysregulations in humans with a regimen of 10 mg b.i.d. [6—11]. The pharmacokinetic behaviour of amezinium has been described in detail for doses of 20 mg and above [12, 13] using a double-isotope derivatization technique with a lower level of determination of 3 ng/ml [14].

Since amezinium has been introduced into therapy a widespread interest in more specialized investigations has arisen in which determinations of amezinium concentrations in plasma play a salient role. Thus, the need has

*In this paper referred to as amezinium.

0378-4347/83/$03.00

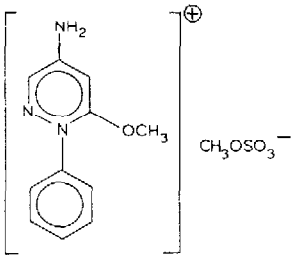
developed for a selective and sensitive method which can be practised in a well-equipped hospital laboratory or other. To this end, it should avoid the use of isotopes and highly sophisticated instrumentation and, rather, base itself on readily available techniques like high-performance liquid chromatography (HPLC).

This paper describes the development and evaluation of a selective determination method for amezinium in the lower nanogram range employing a cation-exchange filtration step prior to dequaternization by nucleophilic attack and subsequent separation and quantification by HPLC.

MATERIAL AND METHODS

Substances

Ameziniummetilsulfate. 4-Amino-6-methoxy-1-phenyl-pyridazinium methyl sulphate (ameziniummetilsulfate, LU 1631*) was first synthesized [1] in the Hauptlaboratorium of BASF Aktiengesellschaft. [methyl-^{14}C] Amezinium (specific activity: 336 MBq/mmole ≙ 9.08 mCi/mmole) was supplied by the Isotope Laboratory of BASF Aktiengesellschaft [1]. Being a pyridiazinium salt, amezinium is soluble and stable in water, methanol and ethanol; it decomposes, however, in alkaline media (pH > 8). The nature of the anion does not have any influence on the pharmacological action; it is only taken into account in so far as, in this study, all concentration data are based on the molecular weight 313 of the methyl sulphate, regardless of the chemical species actually handled.



Internal standard. 4-Amino-6-methoxy-1-*p*-tolyl-pyridazinium methyl sulphate (*p*-methyl-amezinium) was kindly supplied by Dr. Thyes, Hauptlaboratorium of BASF Aktiengesellschaft, who also provided in analytical grade quality 5-amino-2-phenyl-3(2H)-pyridazinone (LU 1724) and 5-amino-2-*p*-tolyl-3(2H)-pyridazinone (*p*-methyl-LU 1724), both products of the dequaternization procedure.

Other reagents. Cellulose phosphate (Whatman Grade P I; small ion capacity 3.7 mequiv./g dry weight) was used after a cycle of purification steps (washing with 1 *M* sodium chloride solution until supernatant is colourless: $\Delta E^{1\,cm}_{280-360\,nm} < 0.005$). The purified preparation can be stored without deterioration in the refrigerator at about 4° C for at least eight weeks.

All other reagents used were of the best commercially available quality.

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*Commercially available as Regulton®. Manufacturer: Nordmark-Werke, Uetersen, F.R.G.

Human plasma was supplied by the Transfusion Services of the Municipal Hospitals Mannheim and Ludwigshafen.

Preparation of spiked samples and determination of the nominal concentration

A standard solution of amezinium in human plasma is prepared from an aqueous stock solution (5 μg/ml) the exact concentration of which is determined spectrophotometrically (λ_{max} = 289 nm; ϵ = 15,100 cm^2 mmol^{-1}). From this standard solution containing 50.48 ng/ml the series of spiked samples with amezinium concentrations listed in Table VI is made by diluting with human plasma. The dilution steps are verified by weighing, the concentrations corrected according to the result of this weighing and taken as the basis for subsequent calculations as nominal concentrations. The human plasma samples thus prepared are divided into several series of aliquots of 2.0 ml and frozen at −25° C until analysis.

HPLC equipment

The equipment used comprises a Rheodyne sample injector, Type 7125, a high-pressure pump unit Waters Type 6000A, an HPLC column, Merck-Hibar® (250 × 4 mm), filled with LiChrosorb RP-8/7 μm (Merck), and a UV detector, Jasco-Uvidec-100 III, operated at 280 nm. The mobile phase is a mixture of acetonitrile—water (25:75, v/v) which is filtered (Teflon Fluorophore filter) and degassed. The flow-rate is 2 ml/min at about 20—28 MPa.

Analytical procedure

Ion-exchange columns of cellulose phosphate in 2-ml disposable syringes with the outlet closed with cotton wool are prepared (bed volume 0.9—1.1 ml) and washed twice with 2 ml of water. After addition of 44.84 ng of internal standard the plasma sample (0.1—2 ml; 2 ml throughout method evaluation) is applied to the column. The columns suspended in suitably sized tubes are centrifuged for about 10 min at 1000 *g*, washed with two consecutive 2-ml portions of water, and the unchanged amezinium and internal standard eluted with 1 ml of 1 *M* NaCl, centrifuging each time.

To this eluate 0.1 ml of 10 *M* NaOH is added and kept for 30 min at room temperature. Extraction of the resulting pyridazinones is performed by shaking with 4 ml of methylene chloride for 10 min. The organic layer is evaporated to dryness in a stream of nitrogen (temperature < 30° C). The residue is taken up in 100 μl of mobile phase and the maximum volume possible injected into the HPLC system.

Data recording and data reduction

The chromatogram is registered by means of a recorder and the region enclosing substance and internal standard is simultaneously digitized and stored on a magnetic tape cassette (A/D converter, Type BASF-TKL, with a cassette unit MFE 5450, Messrs. Fey, Munich, F.R.G.). The relevant chromatographic parameters (retention times, peak heights, and peak height ratios) are calculated by means of the GC evaluation program of Caesar and Klier [15] in a version adjusted to the requirements of an internal analogue standardization on the Honeywell-Bull Computer (BASF Data Processing Centre) via terminal.

Calculations

Amezinium concentration is calculated using peak height ratios according to the formula

$$c_A = \frac{m_A}{V} = \frac{k \cdot m_{St} \cdot Q}{V}$$

where c_A = amezinium concentration; m_A = the amount of amezinium in the assay; V = volume of sample; m_{St} = the amount of internal standard in the assay; Q = peak height ratio amezinium to internal standard; and k = proportionality factor between peak height ratio and mass ratio ($k = \frac{h_{St}}{h_A}$ for $m_{St} = m_A$).

The proportionality factor k is calculated for each series of analyses from three calibration samples of different concentrations done in duplicate. Possible systematic deviations in terms of an increase are to be judged as amezinium losses.

The data are summarized and tabulated, and various statistical parameters and tests based on the experimental data calculated using a Wang 2200.

To examine statistically the results to be discussed, the following calculations are carried out with confidence levels of 99%: nominal versus actual value comparisons for estimation of recoveries; testing of the regression coefficient for deviation from 1; testing of the ordinate intercept for deviation from 0 by t-test [16]; and linearity test of the calibration line by F-test [16]. Regression and correlation analysis are performed according to general instructions [17].

To estimate accuracy and precision of the method the mean values and their standard errors are determined for each concentration in all series of analyses. In the calculation of the quotients and differences (in order to determine accuracy) standard errors are calculated by error propagation.

RESULTS

Cation-exchange filtration step

One of the most important steps in the proposed method of determination is the use of phosphorylated cellulose as cation-exchanger onto which amezinium is adsorbed from plasma solutions. Owing to the hydrophilic nature of the polymer matrix, it can be desorbed under mild conditions; with 1 *M* sodium chloride solution recoveries of more than 90% are obtained if the elution volumes are equal to or greater than the bed volume of the exchanger. By this technique separation of amezinium and its metabolites, none of which carries a positive charge [18], can be accomplished readily. Furthermore, the greater part of interfering organic material from plasma is removed. In order to exploit these advantages fully, care has to be taken to minimize background from the reagents and especially from the exchanger material which is not of analytical grade. It had been shown that a simple although extensive washing procedure yields exchanger material suitable for analysis in the lower nanogram range provided that not more than 1 ml of exchanger is used for one determination, otherwise quantification is seriously hampered by background noise during HPLC. This poses some restrictions to the scope of this step which become

TABLE I

RECOVERY OF AMEZINIUM AFTER CATION-EXCHANGE SEPARATION

Amezinium concentration = 191.0 ng/ml plasma.

Plasma volume (ml)	Percolate (%)	Wash water (%)	Eluate	
			(%)	(ng)
1	<1	<1	91.4	174.6
2	10.4	15.1	70.5	269.3
3	32.8	15.6	50.1	287.1

evident from Table I, where extraction yields obtained with radioactive amezinium are summarized. The recovery in the eluate of amezinium from plasma is volume-dependent, ranging from 90% with 1 ml of plasma to 50% with 3 ml of plasma. This appears to be due to the limited although relatively high capacity of the exchanger material which is used up by exchangeable material from the plasma. Accordingly, the loss during this step is accounted for by those amounts of amezinium appearing in the percolate and the wash waters. Unfortunately, for reasons specified above, this cannot be circumvented by the use of more exchanger material. Therefore, with this limitation of exchanger volume no gain in sensitivity of the method can be achieved using 3 ml instead of 2 ml of plasma.

Dequaternization

Since during the first step involved in this method no marked concentration can be achieved which would be a necessary prerequisite for a further purification and quantification by HPLC, use was made of one prominent property of the amezinium molecule, its reaction with nucleophilic agents to yield the corresponding pyridiazinone which is easily extracted by organic solvents. From the nucleophiles tested we finally chose NaOH which, within a reasonable reaction time at room temperature, produced the pyridazinone in high yields with a minimal amount of interfering by-products, as can be seen

TABLE II

YIELDS OF THE DEQUATERNIZATION STEP (INCLUDING EXHAUSTIVE EXTRACTION WITH CH_2Cl_2)

Concentration = 242.4 ng of LU 1631 per ml.

Final NaOH concentration (*N*)	Reaction time (min)	Yield (%)
0.1	30	29.7
0.1	120	49.5
1.0	30	83.5
5.0	30	93.3

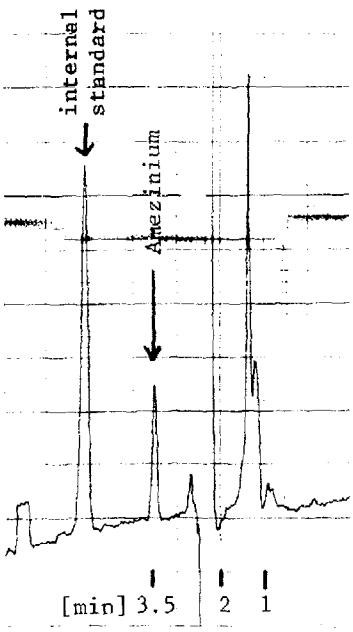

Fig. 1. Chromatogram of the dequaternization products of 10.56 ng of amezinium and of 44.84 ng of internal standard, isolated from 2 ml of plasma.

from Table II and Fig. 1. The chemical nature of the reaction product was verified by a comparison with the authentic compound with regard to retention times in HPLC, R_F values in thin-layer chromatography with different mobile phases, and UV spectrum.

Extraction of the pyridazinones

As shown in Table III, extraction from the alkaline sodium chloride solution can be accomplished by a series of organic solvents. For obvious reasons use of methylene chloride in a ratio of 4:1 is recommended to achieve a high recovery in a volatile organic phase. It will be shown later that methylene chloride in spite of its marked polarity will produce acceptable background levels even for the lower nanogram range aimed at. Therefore, no attempts to further purify the extract, for example by a cycle of back- and re-extraction, are necessary.

TABLE III

YIELD OF DEQUATERNIZATION PRODUCT FROM SALINE MEDIUM BY EXTRACTION WITH FOUR VOLUMES OF ORGANIC SOLVENT

Concentration = 242 ng of LU 1724 per ml.

Solvent	Extraction yield (%)
Cyclohexane	0
Diethyl ether	37.5
Ethyl acetate	64.9
Dichloromethane	81.8

Suitability of the chromatographic method

It can be seen from Fig. 1 that the chromatographic method adopted here is suited for perfect separation of the dequaternization products of amezinium and the internal standard during a 10-min chromatographic run. There is no interference by material originating from plasma or cellulose phosphate. The baseline remains satisfactory provided that not more than 1 ml of cellulose phosphate is used (Fig. 2a). In this case a background noise is observed simulating an apparent concentration of amezinium which will be quantified later. Furthermore, Fig. 2b—d clearly demonstrates by means of three representative examples with plasma of individuals treated with amezinium that no compounds show up with retention times being the same or similar to that of the dequarternized internal standard. Thus, all chromatographic requirements for the application of the method are met.

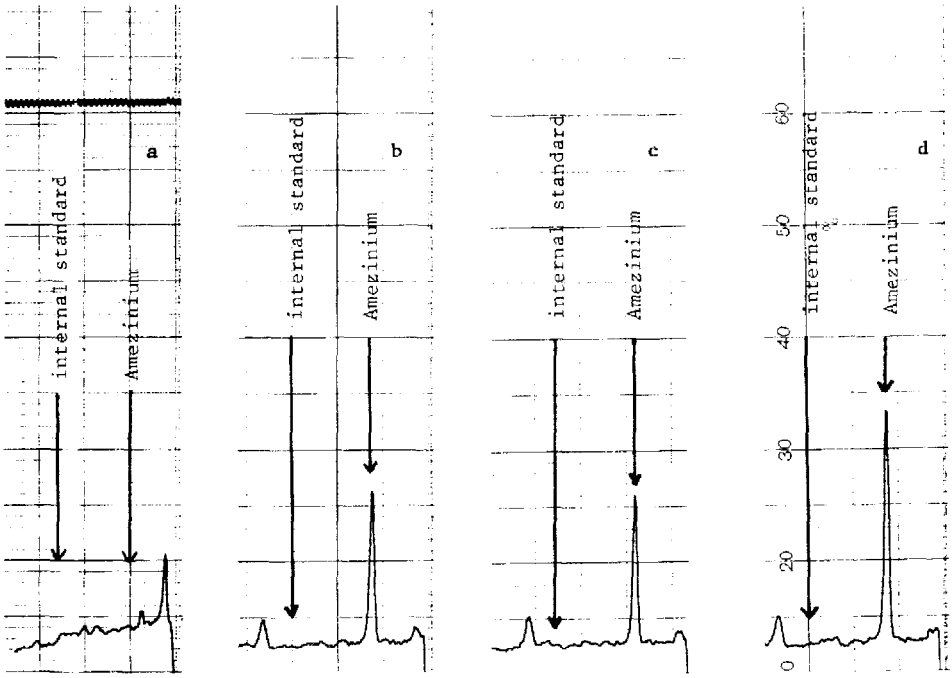

Fig. 2. Chromatograms obtained from (a) 2 ml of blank plasma, and (b—d) plasmas of three volunteers, after repetitive oral application of amezinium (10 mg b.i.d.), carried through the analytical procedure: (b) volunteer A, day 1, 8.00 p.m., (c) volunteer B, day 6, 8.00 p.m., (d) volunteer C, day 11, 8.00 a.m.

Suitability of the internal standard

In a concentration range of 2—20 ng per injection the dequaternization products of amezinium and the internal standard (mass ratio 1:1.093) show identical behaviour with respect to possible adsorption losses in chromatography and peak form in elution which can be seen from the constancies of peak height ratios: they average around 1.45 with a variation coefficient of 2.6%.

Even after subjecting amezinium and internal standard to the entire

TABLE IV

CONSTANCY OF PEAK HEIGHT RATIOS AFTER ENTIRE ANALYTICAL PROCEDURE

n = 5, 2-ml samples.

Mass ratio amezinium/ *p*-methyl-amezinium (ng/ng)	Peak height ratio	
	$\bar{x} \pm s_x$	C.V. (%)
100.96/348.57	0.4011 ± 0.0113	2.8
50.48/174.28	0.4143 ± 0.0169	4.1
10.09/ 34.86	0.4010 ± 0.0146	3.6

procedure, including the cation-exchange step, dequaternization, extraction of products, and chromatography, the peak height ratios for a mixture of amezinium and internal standard at low, intermediate and high concentrations remain constant, suggesting closely identical behaviour during analysis (Table IV).

Peak height ratios, indeed, are a suitable parameter for the measurement of amezinium concentration after internal standardization with its methyl analogue. The calibration line obtained is linear within the range of about 2.4—25 ng of LU 1724 per injection (F-test); its ordinate intercept of Q = +0.015 significantly differs from 0, but it can be neglected due to its low value.

The reproducibility of HPLC analysis is satisfactory showing variation coefficients of 2.1% at 2.4 ng/injection and 0.4% at 24 ng/injection, the correlation coefficient covering the entire range being $r \geqslant 0.999$).

Overall yield of entire procedure

If amezinium is subjected to the analytical procedure described above, about 45—55% of it finally arrives at the quantification step, a value to be regarded as satisfactory taking into account the numerous stages where losses can occur. It is in reasonable accordance with the value of about 48% which can be calculated from the yields of individual steps.

Evaluation of the method

Within a period of five weeks a number of plasma samples of different known amezinium concentrations were subjected in duplicates to the method described above. In Table V the results of these test series are listed versus the nominal concentrations.

Recovery. Table VI gives data on the recovery of added amezinium which represents a measure of the accuracy of the results if selectivity of the method is given.

Within the concentration range 1—20 ng/ml differences of —1% to 26% between actual and nominal concentrations are found with a surplus of 26% at the lowest concentration being significant. A look at the absolute differences reveals that this observation may be explained by a background of about 0.2 ng/ml which, of course, should turn up quite distinctly in blank plasma determinations which will be discussed later. Taking this background (0.23 ± 0.053 ng/ml) into account, recoveries listed in Table VI in parentheses can be esti-

TABLE V

RESULTS OF METHOD EVALUATION

The individual values represent the means of duplicate determinations.

Nominal concentration (ng/ml)	Series 1 (ng/ml)	Series 2 (ng/ml)	Series 3 (ng/ml)	Series 4 (ng/ml)	Series 5 (ng/ml)	Series 6 (ng/ml)	Series 7 (ng/ml)	Series 8 (ng/ml)	Series 9 (ng/ml)	Series 10 (ng/ml)
1.02	1.3577	1.4074	1.6643	0.9838	1.1367	1.2664	1.5381	1.2927	1.2810	0.9645
2.04	1.8373	2.7079	2.1404	2.3451	1.6333	2.1336	2.5051	2.2953	2.3225	2.1733
5.07	5.8465	5.7691	4.8627	5.5744	4.2761	5.4808	5.5163	5.2451	4.4850	4.9003
10.08	10.4940	10.3359	9.2317	10.8760	8.5279	11.8495	10.0574	10.1769	8.2407	9.9308
19.75	21.6532	19.6030	19.2135	21.7584	21.6997	20.3430	20.9790	20.3594	19.4870	19.9366

TABLE VI

EVALUATION OF AMEZINIUM METHOD: DATA ON ACCURACY AND PRECISION

Values in parentheses are corrected for background of blank plasma samples (0.229 ± 0.053 ng/ml; $\overline{x} \pm s_{\overline{x}}$).

n	Nominal concentration (ng/ml)	Actual concentration (ng/ml, $\overline{x} \pm s_{\overline{x}}$)	Precision		Accuracy	
			S.D. (ng/ml)	C.V. (%)	Actual/nominal (%)	Actual − nominal (ng/ml)
10	1.02	1.29 ± 0.07 (1.06 ± 0.08)	0.22	17.22	126.4 ± 6.9* (104.0 ± 8.6)	0.27 ± 0.07 (0.04 ± 0.09)
10	2.04	2.21 ± 0.10 (1.98 ± 0.11)	0.31	13.96	108.3 ± 4.8 (97.1 ± 5.4)	0.17 ± 0.10 (−0.05 ± 0.11)
10	5.07	5.20 ± 0.17 (4.97 ± 0.18)	0.54	10.41	102.5 ± 3.4 (98.0 ± 3.5)	0.13 ± 0.17 (−0.09 ± 0.18)
10	10.08	9.97 ± 0.34 (9.74 ± 0.34)	1.08	10.79	98.9 ± 3.4 (96.7 ± 3.4)	−0.10 ± 0.34 (−0.33 ± 0.34)
10	19.75	20.50 ± 0.31 (20.27 ± 0.31)	0.97	4.72	103.8 ± 1.5 (102.7 ± 1.6)	0.75 ± 0.31 (0.52 ± 0.31)

*Significantly different from 100%.

mated which now do not deviate significantly from the expected values, the absolute deviation ranging between −0.33 and +0.52 ng/ml. Using this experimentally verified correction, accurate results are obtained which is confirmed by a correlation analysis between nominal and corrected actual values (individual data) with a regression coefficient of $b = 1.029 \pm 0.0126$ ($r = 0.995$) which is not significantly different from 1 at the 99% level of confidence.

Precision. Data on the precision of the entire analytical procedure are given in Table VI. It can be seen from this table that the coefficients of variation (C.V.) range from about 5% at 20 ng/ml to 18% at 1 ng/ml. This inter-assay variation is expectedly greater than the intra-assay variation which was estimated to increase from C.V. ≈ 4% at 20 ng/ml to C.V. ≈ 17% at 1 ng/ml. It is obvious that the precision of the method is unsatisfactory at the lowest concentration of 1 ng/ml.

If, in routine practice, a coefficient of variation of 15% is still accepted for duplicate determinations (this corresponds to a ratio of 1 to 1.25 in the individual determinations) we have to expect that about 5% of the analyses need to be repeated.

Following such a procedure, which has proved its value in routine practice of a number of analytical methods, only minor variations are found for the data obtained here with respect to accuracy. The risk of a falsification of statements resulting from outliers not eliminated is, however, minimized.

If the standard deviations are regarded as a function of the observed mean values, a linear relation may be assumed in first approximation for the concentration range up to 10 ng/ml. Regression analysis gives values of 0.1 ± 0.03 ng/ml for the ordinate intercept (absolute error) and of 0.09 ± 0.005 for the slope corresponding to a weighted relative error of about 9%. This linear function can be used as a weighting function in any fitting procedures where homogeneous variances are required. For values above 5 ng/ml the commonly used weighting function $1/c_A{}^2$ can be adopted without serious distortions of error structure.

Limit of determination and detection. The lower limit of determination (defined as that concentration which can still be determined with coefficients of variation of about 10%) is about 5 ng/ml according to the data discussed above with the use of 2-ml plasma samples . If one is prepared to accept a somewhat lower reliability corresponding to coefficients of variation of up to 15%, concentrations of down to 2 ng/ml can be estimated.

To determine the lower limit of detection of unchanged amezinium, human blank plasma is subjected to the analytical method described in this paper. A mean value of 0.23 ± 0.17 ng/ml ($\bar{x} \pm s_x$) results from the values obtained. If the lower limit of detection is defined as three times the standard deviation above the mean blank value, it is estimated at 0.75 ng/ml.

Experiences in routine practice

In the meantime the method described has been applied to the determination of amezinium concentrations in human plasma after application of 10 mg per os. It demonstrated its suitability to follow the time course up to 32 h after administration. Without any modification it can be used to determine amezinium in human urine at 25 ng/ml, the lowest concentration encountered

after 1 mg intravenous administration, a coefficient of variation of $< 3\%$ was found.

ACKNOWLEDGEMENT

The authors wish to thank Mrs. Ursula Kripp for her skilful and knowledgeable assistance.

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