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SYNTHESIS OF ^{14}C-RADIOACTIVELY LABELED DERIVATIVES OF GOSSYPOL AND THE STUDY OF THEIR PHARMACOKINETICS

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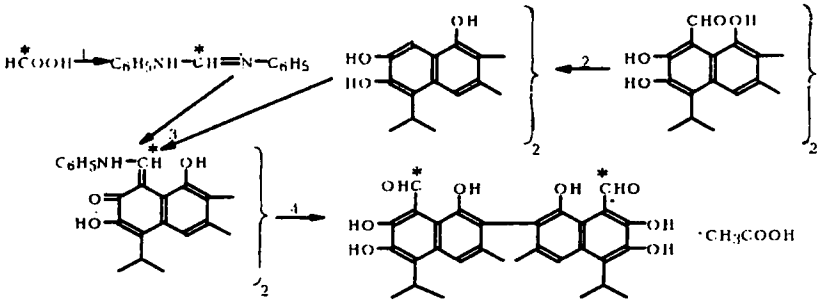
The method of obtaining radioactively labeled ^{14}C-gossypol-acetic acid has been improved: from it have been synthesized ^{14}C-derivatives of gossypol and their complexes with N-polyvinylpyrrolidone. A comparative study has been made of the pharmacokinetics of batriden, megosin, and their water-soluble complexes.

The growing arsenal of drugs created from gossypol (Gp) makes the study of their kinetics particularly urgent since it is impossible to develop the principles of the effective and safe use of drugs without taking into account the laws of their absorption, distribution, and accumulation in the various organs and their biotransformation and elimination from the organism.

The radioisotope method, in combination with the radiochromatography of labeled compounds, has proved to be the most effective for these purposes. To obtain radioactively labeled samples of Gp and its derivatives with high specific activities, we have improved the method of synthesizing ^{14}C-gossypol, which is performed in several stages [1-5].

In the development of a model synthesis of Gp it was established that the yield of diphenylformamide (DPFA) obtained in the first stage of the process (Scheme 1) rose from 12.67 to 16.42% if the product was recrystallized not from ordinary, but from absolute, ethanol, since the presence in the ethanol of even slight amounts of water exerts a hydrolyzing action on the DPFA [6].



Scheme 1. Synthesis of ^{14}C-gossypol-acetic acid.

The reaction of apogossypol (apo-Gp) with DPFA (stage 3) must be carried out under reduced pressure at 170°C for 3 h [1]. In this process, any air penetrating through the capillary creates the risk of oxidation of the apo-Gp, which is an unstable substance rapidly changing in the air at room temperature. Performing the reaction producing dianilinegossypol (DAGp) in an atmosphere of nitrogen excluded the possibility of oxidation of the apo-Gp, which, in its turn, led to an increase in the yield of DAGp from 49.30 to 60.40%. In stage 4, the gossypol-acetic acid (GAC) formed from DAGp by the method of [1] must be recrystallized twice. With the introduction of the above-described changes, it was sufficient to crystallize the GAC obtained once: TLC in system 1 then showed its high purity.

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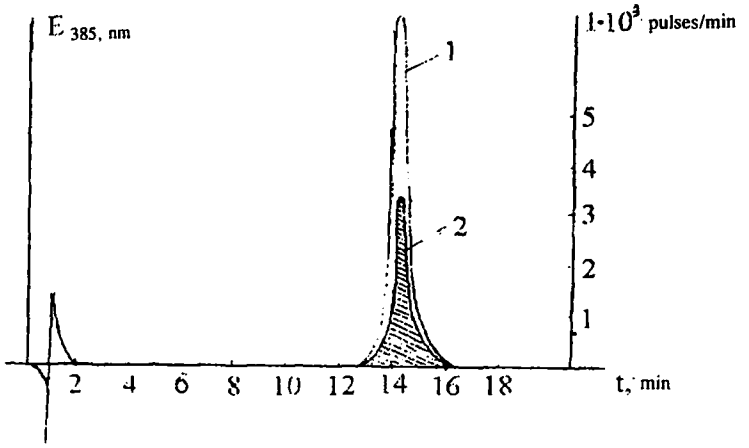


Fig. 1. Radiochromatogram of ^{14}C-GAC: *1*) extinction; *2*) radioactivity.

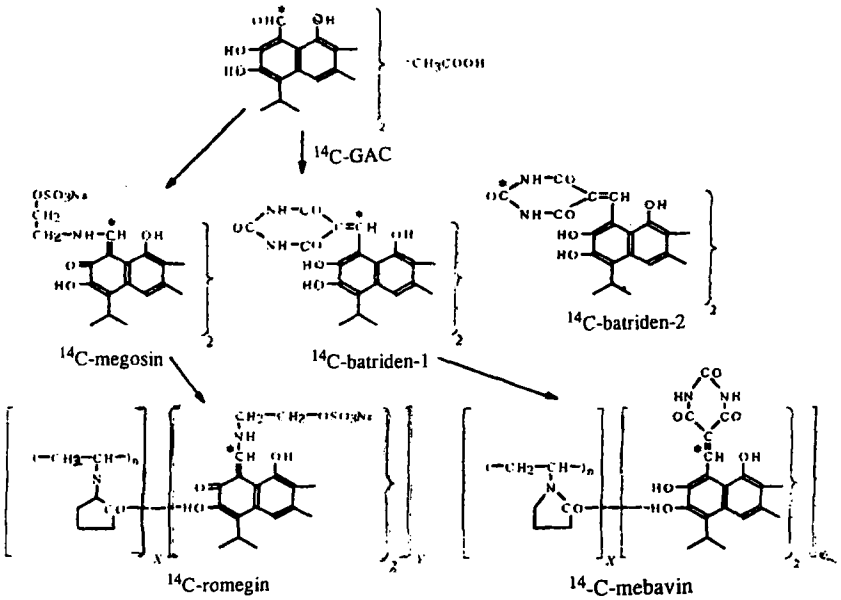
The improvement in the method has led to a 60% increase in the yield of desired product. This should undoubtedly lead to an increase in the degree of inclusion of the initial label into the final products in the synthesis of ^{14}C-Gp. By the same method, using ^{14}C-HCOOH (FA) we have synthesized ^{14}C-gossypol-acetic acid (^{14}C-GAC) with a specific activity of 1.82 μCi/mg. The degree of inclusion of the initial radioactivity in the final product was 1.76%, while in [1] it was 1.1%.

The identification and the determination of the radiochemical purity of the ^{14}C-GAC were achieved by TLC and HPLC. The graphical illustration of the results of the radiometry of the eluent from the chromatography of the ^{14}C-GAC by HPLC (Fig. 2) showed the radiochemical purity of the labelled compound.

Mastery of the method of synthesizing ^{14}C-GAC radioactively labeled in the aldehyde groups has permitted the synthesis of practically any radioactively labeled drugs from gossypol that are necessary for pharmacokinetic and other investigations. Thus, we have synthesized ^{14}C-megosin and ^{14}C-batriden-1 with fairly high specific radioactivities (Scheme 2). Zonal analysis of radiochromatograms of the compounds obtained [7] showed their high degrees of radiochemical purity.

We have obtained water-soluble complexes of Gp and its derivatives with N-polyvinylpyrrolidone (PVP) [8, 9], and this has enabled their toxicity to be reduced and their bioavailability to be increased. For the performance of pharmacokinetic investigations we synthesized a complex of ^{14}C-batriden with PVP — ^{14}C-mebavin — and a complex of ^{14}C-megosin with PVP — ^{14}C-rometin.

For batriden we obtained two radioactive forms: ^{14}C-batriden-1, with the label in the gossypol part of the molecule, and ^{14}C-batriden-2, with the label in the barbituric acid residues (Scheme 2).



Scheme 2. Radioactively labeled gossypol derivatives.

The preparation of ^{14}C-batriden-2 gave rise to the necessity for performing comparative pharmacokinetic investigations of batriden labeled in different parts of its molecule, with the aim of elucidating features of the biotransformation of the drug in the animal organism [5, 10, 11, 13].

The pharmacokinetics of the compounds described above were studied in experiments on mice: the main pharmacokinetic parameters and constants were determined, the distribution and binding of the drugs at the tissue, cellular, and subcellular levels were investigated, and the pathways and dynamics of their elimination from the organism were traced.

The results of a study of the general topography of the distribution of ^{14}C-batriden-1 in the animal organism showed that, with both the enteral and the parenteral routes of administration, the drug exhibited a high hepatotropicity. Of fundamental importance in characterizing batriden as immunosuppressive is the considerable accumulation and prolonged circulation of the drug in the organs of the immunity system — the lymph nodes, the spleen, and the thymus. In minute amounts, batriden penetrates into the lungs, heart, muscles, testes, and brain.

Repeated administration of ^{14}C-batriden-1 showed its capacity for accumulating in the organism, which must be taken into account in the performance of long courses of immunosuppressive therapy.

In an investigation of the dynamics and pathways of excretion of ^{14}C-batriden-1 from the mouse organism on its intraperitoneal injection it was established that in the course of 10 days 70.64% of the total dose of radioactivity injected was eliminated with the feces. During the same period, only 3.26% of the radioactivity was excreted with the urine. In the exhaled air the radioactivity remained at the background level. The elimination of batriden from the organism by an extrarenal pathway characterizes it as suitable to propose as an immunosuppressor for kidney transplantation since in this case it may be expected that nephropathology will not lead to marked changes in the circulation of the drug in the organism.

A radiochromatographic analysis of extracts of the feces revealed one product of the biotransformation of the drug the radioactivity of which amounted to about 20% of the total radioactivity eliminated from the organism. However, the overwhelming bulk of the ^{14}C-batriden-1 was excreted from the organism in the unchanged state. In order to elucidate features of the biotransformation of batriden in experiments on mice, we made a pharmacokinetic study of ^{14}C-batriden-2 and a comparative analysis of the pharmacokinetics of ^{14}C-batriden-1 and ^{14}C-batriden-2.

A topographical chart of the distribution of ^{14}C-batriden-2 in the mouse organism is similar to that on the administration of ^{14}C-batriden-1: with respect to the degree of maximum accumulation of radioactivity, the organs form the same sequence. Calculation of the pharmacokinetic parameters and constants of ^{14}C-batriden-2 in the blood and the dynamics and pathways of excretion of the label from the organism showed the absence of any appreciable difference in the pharmacokinetics of ^{14}C-batriden-1 and ^{14}C-batriden-2. The fact that on the formation of a radioactive metabolite the pharmacokinetics of ^{14}C-batriden-1 were identical with that of ^{14}C-batriden-2 shows that the biotransformation of batriden takes place without cleavage of the bonds between the gossypol moiety and the barbituric acid residues.

To obtain information on the molecular mechanism of the action of batriden, in an individual series of experiments we investigated features of the intracellular distribution and the binding of ^{14}C-batriden-1 with macromolecules of the subcellular structures of hepatocytes. When the results obtained were compared with literature reports on the intracellular distribution of ^{14}C-gossypol and ^{14}C-megosin in hepatocytes [12, 14, 15], their tendency to inclusion in the mitochondrial and microsomal fractions and relatively weak binding with the hepatocyte nuclei were found. A study of the binding of ^{14}C-batriden-1 with the macromolecules of a liver homogenate, of mitochondria, of microsomes and of blood plasma showed that the overwhelming bulk (60-80%) of the radioactivity was bound with lipids, about 20% with proteins, and the least with the RNA and DNA of the liver homogenate (5.8 and 0.5%, respectively).

Thus, the results obtained permit the assumption that interaction with the cell nucleus does not play an important role in the mechanism of the action of batriden. The biological effect is most probably due to the membranotropic properties of the drug. Thanks to its high affinity for lipids, batriden accumulates in the lipid layer of cell membranes (mitochondrial, plasmatic) and, on binding with their protein components, including enzymes, localized in these membranes, exerts an influence on their functional activity.

A comparison of the pharmacokinetic parameters of ^{14}C-batriden and ^{14}C-mebavin showed a sharply rising rate of absorption of mebavin (by almost 2 orders of magnitude). The apparent volume of distribution of the drug and its clearance diminished considerably; the time of its disappearance from the blood plasma decreased by a factor of 2. In the case of mebavin, the general topography of the organ distribution of radioactivity changed only slightly; however, a more pronounced accumulation and prolonged circulation of the label in the immunocompetent organs — the lymph nodes and the spleen — showed an increasing specificity of the action of the drug.

A study of the distribution of ^{14}C-mebavin in cell fractions of mouse hepatocytes showed that, in contrast to ^{14}C-batriden-1, the degree of binding of the drug with mitochondria and microsomes was approximately 2 times less, which may be an explanation of the lower toxicity of mebavin.

Mebavin's solubility in water leads to an increased amount of unbound drug in the blood plasma, which may be of importance in the manifestation of the effect of this immunosuppressor. While for ^{14}C-batriden-1 the proportion of unbound radioactivity in the blood plasma was 16.45%, for ^{14}C-mebavin it was 28.44%.

The dynamics of the elimination of ^{14}C-mebavin from the animal organism was considerably accelerated. While 50% of the intraperitoneally injected ^{14}C-batriden was excreted in 4 days, half of the ^{14}C-mebavin injected was eliminated from the organism in one day. After a week, only 68.59% of the radioactivity of ^{14}C-batriden-1 had suffered excretion, while 96.60% of the ^{14}C-mebavin was excreted in the same period

Thus, the faster and more complete excretion of ^{14}C-mebavin from the organism favorably distinguishes this drug from the initial batriden, since it decreases its cumulativeness and, correspondingly, the manifestation of toxic effects, while its solubility in water considerably raises its bioavailability

The pharmacokinetics of ^{14}C-megosin and of its complex with PVC — ^{14}C-rometin were studied. The results of the experiments showed that ^{14}C-megosin exhibited a higher bioavailability than ^{14}C-rometin (periods of half-absorption 0.75 and 2.95 h, respectively). ^{14}C-Megosin penetrated into the blood circulation system not only faster but also to a greater degree. At equal doses (recalculated to the ^{14}C-megosin content) the concentration of ^{14}C-megosin in the blood was 1.2-1.5 times higher than on the injection of ^{14}C-rometin: only at the end of four days after injection did their concentrations in the blood become equal because of the faster elimination of ^{14}C-megosin and the prolonged circulation of ^{14}C-rometin (periods of half-elimination from the blood 27.7 and 38.49 h, respectively).

Differences were also observed in the degrees of binding of the drugs by the blood plasma proteins: the proportion of bound ^{14}C-megosin was 77%, and of ^{14}C-rometin 71%. It may be assumed that PVP so envelopes the megosin molecule (and also the batriden molecule) that it blocks possible sites of the binding of these gossypol derivatives with the plasma proteins. And the acquisition by the complexes of the properties of water solubility additionally led to a rise in the free fraction of these drugs (^{14}C-rometin by 6%; ^{14}C-mebavin by 12%) in comparison with the initial megosin and batriden.

The general topographies of the organ distributions of ^{14}C-megosin and its polymer complex were similar. Their maximum accumulations were found in the liver, the blood and lymph, and the thymus, their concentrations in these organs being close. Only for the liver was a clear difference in the accumulation of the drugs observed: on the administration of ^{14}C-megosin its accumulation in the liver was twice as great as that of ^{14}C-rometin on the administration of the latter. It may be concluded from these results that, in the first place, on the introduction of ^{14}C-megosin the liver actively "extracts" the absorbed and circulating drug from the systemic blood circulation, as a result of which the rate of its elimination from the blood is higher than that of ^{14}C-rometin (27.7 as compared with 38.49 h); in the second place, the detoxication of ^{14}C-megosin proceeds correspondingly faster (and we shall also see this below); and, in the third place, ^{14}C-rometin is characterized by a lower hepatotropicity, with retention of the same concentrations in the immunocompetent organs.

In a study of the excretion of these drugs from the organism it was established that in the course of 7 days after the administration of ^{14}C-megosin 84% of the radioactivity was eliminated (82.5% in the feces and 1.5% in the urine); in the same time, only 72% of the radioactivity of ^{14}C-rometin was excreted (52.2% in the feces, and 19.8% in the urine). The results of the investigations confirmed the exclusive role of hepatobiliary excretion in the elimination of ^{14}C-megosin. At the same time, the increase in the amount of the radioactivity of ^{14}C-rometin excreted in the urine correlates with the results of a study of the pharmacokinetics of ^{3}H-PVP; the polymer accumulates to the greatest degree (after the liver) in the kidneys and is eliminated from the organism exclusively (95%) in the urine [16].

The elimination of the radioactivity of ^{14}C-rometin partly via the gastrointestinal tract and partly via the kidneys may witness a process of biotransformation of the drug in the organism.

Thus, on the basis of the experimental results obtained one may conclude that there is a change in the pharmacokinetic parameters of ^{14}C-megosin on its administration in the form of the complex with PVP, ^{14}C-rometin. The absorption of the drug is retarded and its circulation in the blood is prolonged, with an increase the proportion of the drug not bound with the blood proteins; while the former bioavailability for the lymphatic system and the thymus is retained, the hepatotropicity of ^{14}C-rometin is considerably lowered, and its prolonged action leads to some retardation of excretion from the organism.

On generalizing the investigation performed as a whole, it is impossible not to mention the high informativeness of the radioisotope method in biological studies of Gp derivatives. It was possible to study these compounds experimentally both under tissue conditions and under cellular and subcellular conditions. A comparative analysis of the pharmacokinetic parameters

of ^{14}C-batriden-1- and ^{14}C-batriden-2, containing the label in different parts of the molecule, permitted us to show indirectly that the biotransformation of batriden in the organism takes place without cleavage of the bond between the carbon atom of an aldehyde group of Gp and a carbon atom of barbituric acid.

The creation of water-soluble complexes of Gp derivatives with polymers for medicinal purposes has enabled the bioavailability of the drugs and the specificity of their action to be raised, their toxicity to be lowered, and their circulation in the organism to be shortened or, conversely, to be prolonged: in one word, their pharmacological indices to be modeled.

EXPERIMENTAL

The individuality of the compounds synthesized was checked by TLC on Silufol-254 plates in the systems: 1) acetone–benzene–acetic acid (1:9:0.6); 2) benzene–dioxane (9:1); 3) acetone–benzene–water–acetic acid (4:1:1:0.1); 4) acetone; 5) acetone–DMFA–acetic acid (18:1:1); 6) acetone–toluene (7:3).

Spot reagent: 1% solution of phloroglucinol in 2 N HCl in ethanol.

The radiochemical purity of the compounds was determined by the zonal analysis of their radiochromatograms and by HPLC (^{14}C-GAC) on a Perkin-Elmer-601 chromatograph: temperature 20°C, 5 mm × 0.5 m, Ostadeil Silix 2 reversed phase: in gradient elution, as the weak phase we used 10% acetic acid (AA) and, as the strong eluent, ethanol. Under identical chromatographic conditions, labeled and pharmacopeial specimens of GAC had the same retention time — 14 min.

The radiometry of samples was conducted on a Rack-Beta 2 liquid radiospectrometer (LKB, Sweden) using type ZhS-8 scintillation liquid.

The synthesis of ^{14}C-GAC included four stages:

Preparation of ^{14}C-DPFA. Aniline (92 ml) was added to a mixture of ^{14}C-FA (270 mCi), 98% FA (16 ml), and 85% orthophosphoric acid (0.12 ml), and the resulting reaction mixture was heated at 150°C with distillation of the water formed in the reaction for 3 h. Then the temperature was slowly raised to 250°C and the excess of aniline was distilled off. The product was cooled, filtered off, and recrystallized from absolute ethanol. This gave 14.09 g (16.42%) of ^{14}C-DPFA; mp 136-138°C.

Preparation of apo-Gp. A mixture of 50 ml of 40% NaOH solution and 10 g of GAC was heated at 95°C for 30 min and was then cooled in an ice bath and brought to a weak acid reaction with conc. H_2SO_4. The apo-Gp that precipitated was extracted with ether, the extract was dried over anhydrous Na_2SO_4, and the ether was distilled off under vacuum. The residue was dissolved in 50 ml of benzene and precipitated with heptane. Yield 6.12 g (76.26%). TLC in system 1: R_f 0.3.

Preparation of ^{14}C-DAGp. A mixture of 5.48 g of apo-Gp and 14.09 g of ^{14}C-DPFA was heated in a current of nitrogen under vacuum (160 mm Hg) at 170°C for 3 h. Then it was cooled, and 20 ml of acetone was added. The ^{14}C-DAGp formed was filtered off and washed with acetone. Yield 4.78 g (60.4%). TLC in system 2: R_f 0.6.

Preparation of ^{14}C-GAC. A suspension of 4.78 g of ^{14}C-DAGp in a mixture of 70 ml of ether and 70 ml of AA was cooled in an ice bath and was treated with 8.6 ml of conc. H_2SO_4 and, after 2 min, with 18 ml of water. The resulting product was filtered off and washed with water. The ^{14}C-GAC was dissolved in 80 ml of ether, and an equal volume of AA was added. The crystals that formed were filtered off and washed with AA. The yield of ^{14}C-GAC was 2.61 g (63.2%), mp 182-184°C, specific radioactivity (sp.r.) 1.82 μCi/mg. The degree of inclusion of the initial radioactivity was 1.76%. TLC in system 1: R_f 0.55.

Preparation of ^{14}C-Megosin. β-Aminoethyl hydrogen sulfate (0.28 g) was dissolved in a solution of 0.08 g of NaOH in 3.4 ml of absolute ethanol, and the mixture was boiled for 1 h. In parallel, 0.5 g of ^{14}C-Gp with a specific activity of 1.2 μCi/mg was dissolved with heating to 80°C in 7.2 ml of absolute ethanol. The solutions so obtained were combined, and heating was continued for 10 min. The mixture was cooled, and the precipitate was filtered off and washed with ethanol and then with DE. The yield of ^{14}C-megosin was 0.47 g, sp.r. 0.46 μCi/mg; TLC in system 3: R_f 0.8.

Preparation of ^{14}C-Batriden-1. Barbituric acid (0.3 g) was boiled in 10 ml of ethanol for 3 h. In parallel, ^{14}C-Gp (0.3 g) was dissolved in 7 ml of ethanol at 80°C. The solutions were combined and heating was continued. The resulting ^{14}C-batriden-1 was filtered off and washed with ethanol and then with DE. Yield 0.27 g, sp.r. 0.6 μCi/mg. TLC in system 5: R_f 0.8.

Synthesis of ^{14}C-Batriden-2. ^{14}C-Barbituric acid (^{14}C-BA; 46 mg) was boiled in 5 ml of 70% ethanol until dissolution was complete. In parallel, 0.82 g of BA was boiled in 28 ml of ethanol for 3 h, and 1.0 g of Gp was dissolved with heating

in 24 ml of ethanol. The solution of ^{14}C-BA was added to the Gp solution and, after the mixture had been boiled for 30 min, the part of the barbituric acid that had been dissolved in ethanol was added. After brief heating, the formation of ^{14}C-batriden-2 was observed. It was filtered off and washed with ethanol, with hot water, and with DE. Yield 0.86 g (60.6%), sp.r. 4.4 μCi/mg. TLC in system 6: R_f 0.7.

^{14}C-Mebavin was obtained as in [8]

^{14}C-Rometin was obtained as in [9].

The pharmacokinetics of the radiolabeled gossypol derivatives synthesized were studied in experiments on random-bred white mice weighing 20-22 g. The ^{14}C-gossypol derivatives were administered to the animals in a dose of 100 mg/kg, and their water-soluble complexes with PVP in a dose of 1000 mg/kg. For each drug experiment we used five animals. After the times investigated, the animals were decapitated; the blood was collected in centrifuge tubes moistened with heparin solution, and the plasma and the cellular elements of the blood were separated by centrifugation at 3000 rpm.

The organs were extracted and were freed from fat and connective tissue. Samples of the organs and tissues weighing 50-100 mg were subjected to hydrolysis with 1 ml of formic acid at 80°C for 2 h. Aliquots (0.5 ml) of the hydrolysates were added to scintillation bottles previously charged with 5 ml each of a toluene-Triton scintillator having the following composition: PPO, 5.0 g; POPOP, 0.125 g; toluene, 666.7 ml; Triton X-100, 333.3 ml.

The radioactivities of the samples were measured in a Beta-1 liquid scintillation radiometer. The level of accumulation of a drug under investigation in the organs and tissues was expressed as the number of pulses/min of the drug in 1.0 g of crude weight of the biotissue.

The dynamics and pathways of excretion of the compounds under study were investigated with the use of a hermetically sealed metabolic chamber. The collected excrements were hydrolyzed with formic acid and the radioactivities were measured.

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