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SEASONAL VARIATIONS IN THE STABILITY OF MONOAMINES AND THEIR METABOLITES IN PERCHLORIC ACID AS MEASURED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The stability in acid medium of dopamine, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) was investigated. The stability of 5-HT and 5-HIAA was poor, but could greatly be improved by the addition of sodium bisulphite and disodium edetate. Under these conditions, dopamine, DOPAC, HVA, 5-HT and 5-HIAA showed good stability over 24 h at room temperature throughout the year when stored in capped vials. In uncapped vials, the stability of 5-HT and 5-HIAA was reasonable during the winter months, but was poor during the summer months.

INTRODUCTION

As a result of recent developments in the field of high-performance liquid chromatography (HPLC), it is now possible simultaneously to determine the concentrations of several monoamines and their metabolites in small samples of brain tissue (see, for example refs. 1–4). Most often, tissue homogenates are prepared in acid solution, since in such a medium monoamine catabolic enzymes are denatured and the stability of the catecholamines and their metabolites is very good. On the other hand, indoles such as serotonin (5-hydroxytryptamine, 5-HT) and its deaminated metabolite 5-hydroxyindoleacetic acid (5-HIAA) are subject to considerable degradation in acid medium[5–7].

Whilst such degradation usually is negligible when samples are prepared and kept on ice until manually injected into the HPLC system, the advent of automatic injection of samples has meant that the samples may stand for up to 15 h, often at room temperature (since not all autoinjectors are equipped with efficient cooling systems) before analysis. It is thus important to optimize the stability of the indoles when analysing samples by HPLC with automatic sample injection. The present study has therefore investigated the various protective agents that can be added to the perchloric acid homogenizing mixture in order to maximize stability. In addition, since it had been noted in previous years that the stability of indoles was worse in

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the spring and summer months than in the winter months, a stability study has been performed over a period of one year.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: dopamine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin oxalate, 5-hydroxyindoleacetic acid, epinine hydrochloride and dithioerythritol from Sigma (St. Louis, MO, U.S.A.); hexyl sodium sulphate from Research Plus (Bayonne and Denville, NJ, U.S.A.); methanol p.a., perchloric acid p.a., citric acid p.a., ascorbic acid p.a. and ethylenediaminetetraacetate disodium salt (disodium edetate) from Merck (Darmstadt, F.R.G.); and sodium bisulphite from Fisher (Fair Lawn, NJ, U.S.A.). All other reagents were standard laboratory reagents of analytical grade whenever possible.

Apparatus

The chromatographic system consisted of a Constametric III pump (LDC, Riviera Beach, FL, U.S.A.), a WISP Model 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.), a Guard-PAK precolumn with CN inserts (Waters) and a C_{18} reversed-phase column, which was either a Microsorb 3 μm (10 cm $\times$ 4.6 mm I.D., Rainin Emeryville, CA, U.S.A.) used for the analyses of all the samples of the first experiment and the samples run for the first nine months of the second experiment, or an Ultrasphere ODS 5 μm (15 cm $\times$ 4.6 mm I.D.) used for the analyses during the remaining months of the second experiment and for the analysis of the final experiment. (The Microsorb column deteriorated after constant usage over a long period, and no sufficiently efficient new Microsorb column was immediately available, so in consequence an Ultrasphere ODS was used in the later part of the study.) The peaks were detected either by electrochemical detection using an LC-3A detector (BAS Bioanalytical Systems, West Lafayette, IN, U.S.A.) equipped with a glassy-carbon working electrode at + 0.70 V *vs.* an Ag–AgCl reference electrode and a platinum auxiliary electrode, or by coulometric detection (ESA Coulochem, Bedford, MA, U.S.A., with voltages set to -0.05 and $+0.40$ V). Detector response was quantitated using a Model 3392 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The mobile phases consisted of 55 m*M* citrate buffer (pH 4.3), 1.5 m*M* hexylsulphate and 12% (v/v) methanol for the Microsorb columns, or 55 m*M* citrate buffer, 1.5 m*M* hexylsulphate and 16% (v/v) methanol for the Ultrasphere columns.

Procedure

In the first experiment, performed in May, 0.1 mol/l perchloric acid solutions were prepared containing 2.5 μmol/l of DA, epinine, 5-HT and 5-HIAA and 0.5 μmol/l of DOPAC and HVA. In addition, these solutions contained either (a) no additions; (b) sodium bisulphite (60 mg/l); (c) disodium edetate (100 mg/l); (d) ascorbic acid (50 mg/l); (e) dithioerythritol (100 mg/l); (f) ascorbic acid (20 mg/l) plus disodium edetate (100 mg/l); and (g) sodium bisulphite (50 mg/l) plus disodium edetate (50 mg/l). The solutions were divided into three sets, all samples being kept in capped 1.5-ml conical plastic centrifuge tubes (Eppendorf, Hamburg, F.R.G.).

One set was allowed to stand at room temperature (+24°C with a range of ±3°C) and was analysed immediately after preparation and then after 1, 6 and 20 days. The second set, kept in a refrigerator (+4°C), was analyzed after 20 days and samples from the third set kept in a deep-freeze at −18°C were analyzed after 20 days.

In the second experiment, a stock solution containing the monoamines and their metabolites at the above concentrations in 0.1 mol/l perchloric acid plus 60 mg/l sodium bisulphite and 100 mg/l disodium edetate was prepared. Aliquots of 250 μl of the stock solution were transferred into 1.5-ml Eppendorf microtubes, capped and stored frozen at −70°C. On each assay occasion, samples were thawed and the contents divided into three equal volumes, each part being transferred to an insert plastic tube for the WISP (40 mm × 4 mm I.D.; AB Cerbo, Trollhättan, Sweden). One of the samples was analysed forthwith, and the other two samples were left on the laboratory bench, one tube capped and the other uncapped. These samples were analysed approximately 24 h later. In the final experiment, samples (both capped and uncapped) were kept for 24 h at room temperature either in the dark or illuminated by two fluorescent lamps (40 W, positioned 135 cm above the samples) before analysis of the monoamine and monoamine metabolite contents.

RESULTS

The stabilities of dopamine, HVA, DOPAC, 5-HT, 5-HIAA and the internal standard epinine in perchloric acid solutions were determined after storage of samples (in capped tubes) for 1, 6 and 20 days at room temperature (+24°C) and for 20 days at either +4°C or −18°C. The concentrations were in each case compared with freshly thawed standard solutions (stored at −70°C) and the data expressed as % monoamine (or metabolite) remaining. Storage at −70°C for at least 6 months did not significantly affect the concentrations of any of the compounds in the standard solution (data not shown). Dopamine was stable (*i.e.* amount of compound remaining >90%) under all the conditions used (see Fig. 1), as was epinine (data not shown). At +4°C and −18°C, DOPAC was stable under all conditions except for samples to which dithioerythritol (100 mg/l) had been added, where a 40% decrease in the concentration was found after 20 days under both storage conditions. After 20 days at room temperature, the DOPAC in perchloric acid solutions without extra additions ("no additions") was 75% of control, whereas that in ascorbic acid was only 20% of control. Similar results were found for HVA (data not shown).

The indoles, 5-HT and 5-HIAA, were not particularly stable in perchloric acid solutions without additions (Fig. 1A). Addition of ascorbic acid (50 mg/l) worsened the stability of both compounds (Fig. 1B). Addition of sodium bisulphite (60 mg/l) was without effect on the stability of 5-HIAA, but worsened the stability of 5-HT (Fig. 1C). Improved stabilities of 5-HT and 5-HIAA were obtained by the additions of either disodium edetate (100 mg/l) or dithioerythritol (100 mg/l) (data not shown), but the best stability for all the solutions tested was obtained by the addition of sodium bisulphite (50 mg/l) plus disodium edetate (50 mg/l) to the perchloric acid solution (Fig. 1D). Under these conditions, <10% loss of dopamine, DOPAC, HVA, 5-HT, 5-HIAA or epinine was found when the capped samples were kept at room temperature for 24 h (Fig. 1D). Thus, disodium edetate plus sodium bisulphite were used as the additives for the remaining experiments described below.

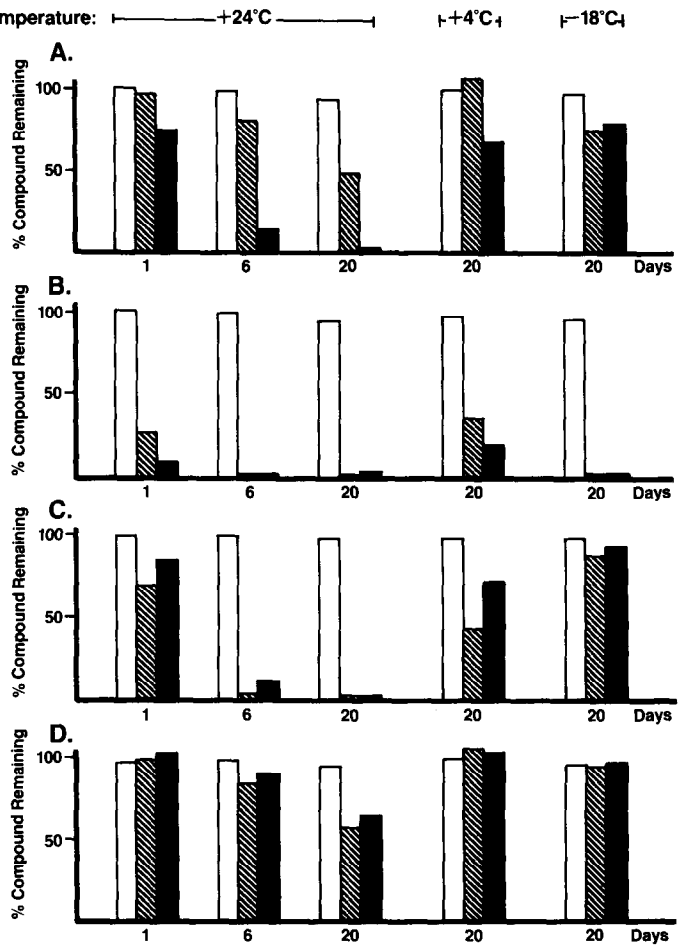

Fig. 1. Stability of 2.5 μmol/l dopamine (□), 5-HT (▨) and 5-HIAA (■) in 0.1 mol/l perchloric acid solution either: (A) without further additions; (B) plus 50 mg/l ascorbic acid; (C) plus 60 mg/l sodium bisulphite; and (D) plus both 50 mg/l sodium bisulphite and 50 mg/l disodium edetate. Results are given as the concentrations remaining in the samples as a percentage of the concentration in freshly thawed samples after the storage times (in capped tubes) and temperatures indicated in the figure. The experiments were undertaken in May.

The stabilities of the monoamines and metabolites were determined over the course of 1 year, by comparing the concentrations in samples (both capped and uncapped) that had been kept at room temperature for 24 h with those in freshly thawed samples. The stability of epinine and dopamine in the capped samples showed little or no instability during the year, the median values being essentially 100% (Fig. 2). The uncapped samples, however, showed consistently increased concentrations of both dopamine and epinine to about 120% of control (Fig. 2). Since the epinine and dopamine curves shown in Fig. 2 are very similar, the most likely explanation for this increase is that there is a 20% concentrating effect on the uncapped samples due to evaporation. The effects of such evaporation can be compensated for by expressing

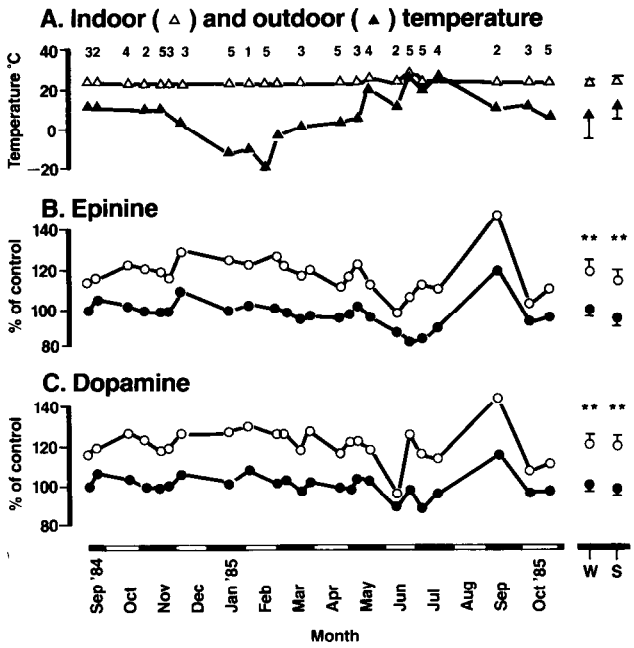

Fig. 2. Stability of 2.5 μmol/l dopamine and epinine over 1 year. Samples were stored for 24 h at room temperature either capped (●) or uncapped (○) and the concentrations of epinine (B) and dopamine (C) expressed as a percentage of the concentration in freshly thawed samples. The indoor (△) and outdoor (▲) temperatures are given in (A), together with the climatic conditions, rated on the following scale: 1, snow; 2, rain; 3, cloudy; 4, cloudy with sunny periods; and 5, sunny. The sample points for the periods 17/9 1984–28/2 1985 + 7/10–25/10 1985 and for the period 6/3–10/9 1985 were grouped together as W ("winter") and S ("summer"), respectively, and the values given as medians ± q. Significant differences between the samples stored in closed and open tubes: *, $2P < 0.05$; **, $2P < 0.01$; NS, not significant ($2P > 0.10$) (Wilcoxon's Matched Pairs Signed Ranks test).

the data using epinine as an internal standard. In this way, it was found that the stability of dopamine, DOPAC and HVA in both capped and uncapped samples was about 100% over 24 h at room temperature throughout the year (Fig. 3A–C).

The stability of 5-HT and 5-HIAA in capped samples was about 100% and 90%, respectively, over 24 h at room temperature, throughout the year (Fig. 3D–E). For the uncapped samples, however, the stabilities of these indoles showed seasonal variation, with reasonably good stabilities being found in the period mid september–late february (W), but with poor stability being found in the period early march–early september (S) (Fig. 3 D–E). Chromatograms undertaken during the S period for the freshly thawed samples and for the samples (both capped and uncapped) stored at room temperature for 24 h are given as examples in Fig. 4. For the samples stored uncapped for 24 h, three extra peaks were found on the chromatogram (beside the DOPAC, 5-HIAA and HVA peaks) (Fig. 4C), whereas no such peaks were found for the capped samples (Fig. 4B).

The effect of storage of both capped and uncapped samples at room temperature for 24 h under conditions of either constant light or constant darkness (as opposed to the samples shown in Fig. 3, where the light:dark cycle was seasonally

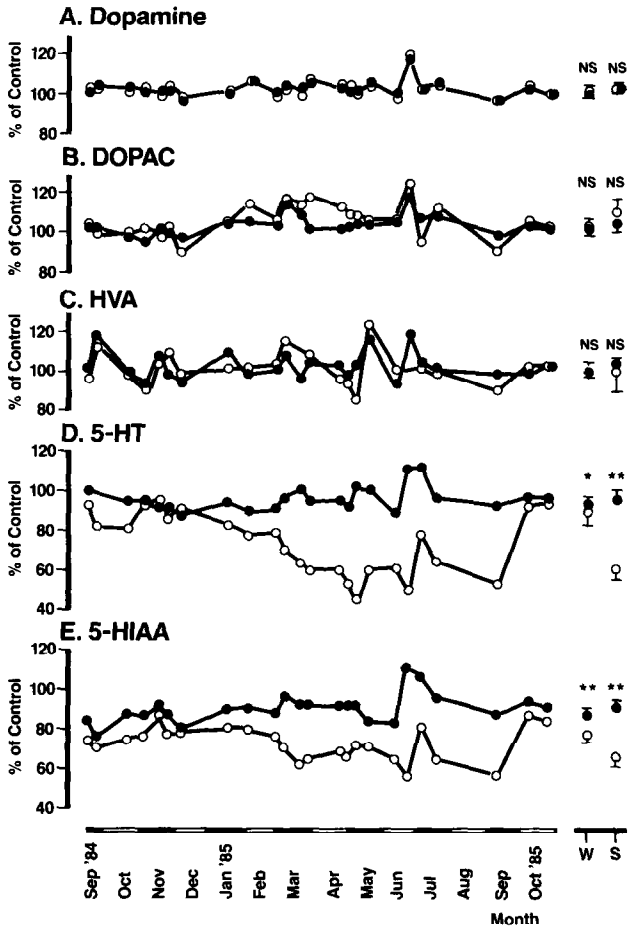

Fig. 3. Stability of (A) 2.5 μmol/l dopamine; (B) 0.5 μmol/l DOPAC; (C) 0.5 μmol/l HVA; (D) 2.5 μmol/l 5-HT and (E) 2.5 μmol/l 5-HIAA over 1 year. Samples were stored for 24 h at room temperature either capped (●) or uncapped (○), and the concentrations (having compensated for the effects of evaporation by using epinine as the internal standard) expressed as a percentage of the concentration in freshly thawed samples. For explanation of W, S and the statistics used, see legend to Fig. 2.

dependent) were assessed during the W period. The pattern of stabilities for the monoamines and their metabolites were the same when kept under constant light as when kept under constant darkness (Table I).

DISCUSSION

It is well known that in aqueous solutions catecholamines, indoleamines and their metabolites can undergo degradation due to oxidation, which can be further enhanced in the presence of metal ions (see, for example refs. 5–9). Therefore, addition of antioxidants such as sodium bisulphite and/or metal chelating agents such as disodium edetate is a procedure used in many laboratories. In the present study,

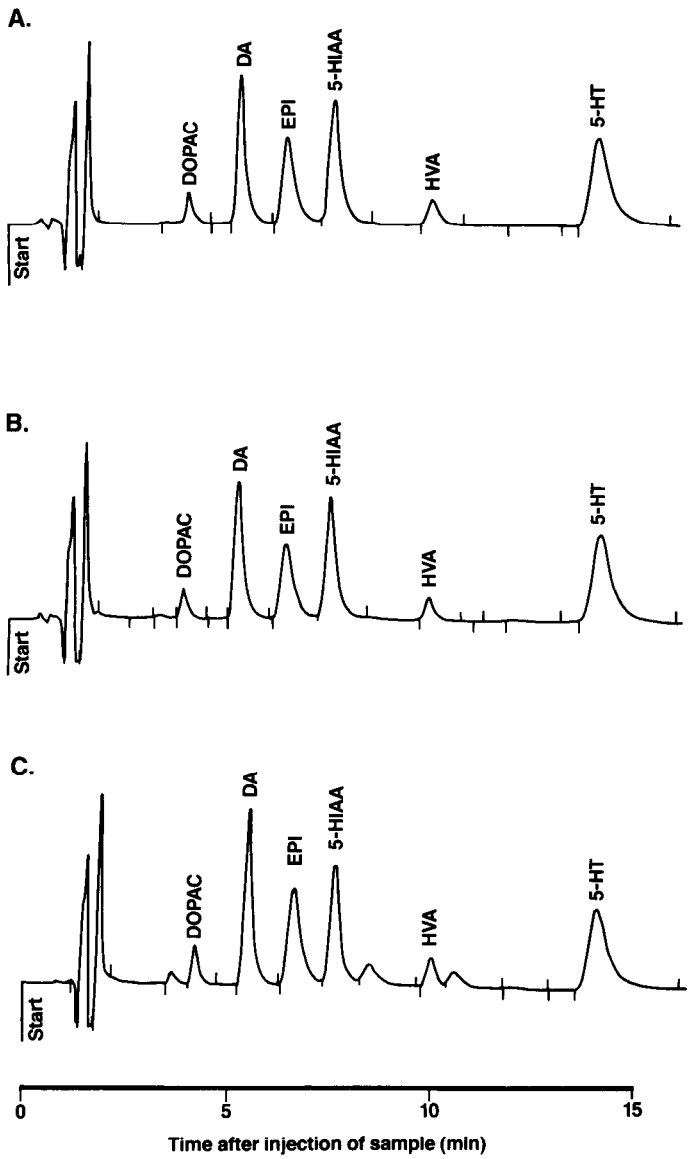

Fig. 4. Example chromatograms of (A) freshly thawed standard solutions (containing 2.5 μmol dopamine, 0.5 μmol/l DOPAC, 0.5 μmol/l HVA, 2.5 μmol/l 5-HT, 2.5 μmol/l 5-HIAA and 2.5 μmol/l epinine as internal standard), and standard solutions left for 24 h at room temperature either capped (B) or uncapped (C). The chromatograms shown in the figure were run in July.

it was found that whilst dopamine, epinine, DOPAC and HVA were stable under most of the conditions tested, the indoles 5-HT and 5-HIAA were not stable in acid solution. This instability was in fact worsened by the addition of ascorbic acid (Fig. 1B), so this compound is certainly not to be recommended in this regard, especially as it also results in a significant broadening of the electrochemically detected solvent

TABLE I

STABILITY OF MONOAMINES AND THEIR METABOLITES STORED FOR 24 h EITHER CAPPED OR UNCAPPED AT ROOM TEMPERATURE UNDER CONDITIONS OF EITHER CONSTANT DARK OR LIGHT

Values are medians (with the ranges in parentheses) of three experiments, performed October–November 1985, of the concentrations (having compensated for the effects of evaporation by using epinine as internal standard) as percentages of the concentrations in freshly thawed samples. The initial concentrations were: dopamine 2.5 μmol/l; DOPAC 0.5 μmol/l; HVA 0.5 μmol/l; 5-HT 2.5 μmol/l; 5-HIAA 2.5 μmol/l.

	Concentrations (% of Control) under conditions of:			
	Constant darkness		*Constant light*	
	Capped	*Uncapped*	*Capped*	*Uncapped*
Dopamine	100 (99–100)	99 (96–100)	100 (99–100)	100 (99–100)
DOPAC	101 (101–102)	101 (100–106)	103 (101–105)	104 (101–105)
HVA	103 (102–103)	102 (100–104)	103 (103–104)	103 (102–105)
5-HT	98 (93–98)	94 (93–98)	93 (91–98)	90 (83–94)
5-HIAA	91 (89–92)	80 (78–86)	91 (89–92)	82 (81–85)

front[10]. Sodium bisulphite, when taken on its own, did not improve the stability in acid medium of the indoles, in agreement with a previous study[7]. On the other hand, the addition of sodium bisulphite in combination with disodium edetate resulted in a good stability of the indoles (Fig. 1D) as well as for dopamine, epinine, DOPAC and HVA.

Most stability studies reported in the literature have not taken into account the effect of possible seasonal variations. In the present study, it was found that whilst dopamine, DOPAC, HVA, 5-HT and 5-HIAA were stable at room temperature for 24 h throughout the year when stored capped, samples stored uncapped for 24 h showed considerable instability of 5-HT and 5-HIAA during the "summer" months (March–September) but not during the "winter" months (Fig. 3). This seasonal dependence could not be ascribed to variations in the ambient temperature, which in the laboratory was rather constant throughout the year (Fig. 2A). However, the length of the day:night cycle varies considerably during the year in Sweden, raising the possibility that the instability of the 5-HT and 5-HIAA found during the "summer" months is light-induced, especially since it is known that 5-HIAA is subject to photodecomposition in bright daylight[6,10,11]. However, the amount of incident light falling on the uncapped samples (which were stored in opaque vials) was rather small. In addition, there was no difference between the stabilities of the uncapped samples stored (during the "winter" period) under conditions of constant fluorescent light as when stored in constant darkness (Table I) (although it can be argued that the fluorescent light may not have the same effects on indole stability as bright daylight). However, both these arguments taken together would suggest that photosensitivity cannot *per se* explain the "summer" instability of the indoles in uncapped samples found in the present study. It is possible that agents more prevalent in the atmosphere in summer, such as pollen, etc., may also contribute to the seasonal instability of the indoles. Regardless of the explanation, however, the data presented here underline the importance of ensuring that samples are capped when stored at

room temperature (*i.e.* in uncooled automatic injectors) prior to HPLC analysis of their indole content.

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