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DEVELOPMENT, VALIDATION AND APPLICATION OF AN ENZYME IMMUNOASSAY (EIA) OF ATRIOPEPTIN

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SUMMARY: A rapid, convenient, and sensitive enzyme immunoassay (EIA) for atriopeptin (AP) has been developed. The tracer-ligand for the assay is the 24-amino acid peptide, AP24, which has been covalently coupled to the tetrameric form of acetylcholinesterase (AChE) (EC 3.1.1.7). Tracer, unknown, and primary antibody are incubated in a 96-well microtiter plate precoated with secondary antibody. After washing, a colorimetric reaction is used to measure acetylcholinesterase activity. A direct linear correlation was obtained when comparing the conventional radioimmunoassay and the EIA by using the same primary antibody to assay: plasma samples (rat or human), HPLC column fractions, or atrial extracts. Besides being technically much less demanding and not requiring the use of the radioisotopes, the EIA is more sensitive than the radioimmunoassay and thereby lends itself to a "flash" same-day assay of samples.

The recent discovery that the cardiac atria synthesizes a potent hormone, atriopeptin (AP), that is a natriuretic-diuretic and vasodilator has profound implications in cardiovascular and renal physiology, pharmacology, and therapeutics (1,2). With the development of radioimmunoassays, it has become possible to study the release of AP into the circulation. Plasma levels of atriopeptin are elevated in experimental animals by volume expansion, vasoconstrictor agents, water immersion, and high salt diets (3-6). Elevated plasma AP levels have been reported in patients with congestive heart failure (7,8), paroxysmal atrial tachycardia (2,6,9), and end-stage renal disease patients (10). The combined use of high pressure liquid chromatography (HPLC) and radioimmunoassay has resulted in the demonstration that: a) the cardiac AP storage form is the 126-amino acid prohormone, b) the released circulating plasma form is the 28-amino acid peptide, AP28, (11) and c) that the right atrial stores of prohormone are selectively depleted during atrial stretch resulting in elevated AP28 blood levels (12).

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The current literature is replete with numerous studies pursuing the role of AP in physiological and pathophysiological states. The major analytical approach has been the use of radioimmunoassays which require the preparation or purchase of a [^{125}I]-labelled ligand. Some of the shortcomings of the radioimmunoassay (RIA) include: a) the continuous use of radioisotope, b) the short half life of the radioligand, c) the necessity for centrifugation, d) large numbers of samples become tedious to process, and e) the relatively long time period (usually 2-3 days) for completion of the assay. We report here the development, validation, and application of an enzyme immunoassay (EIA) which circumvents many of the problems encountered with RIA. This EIA is achieved by preparing an enzymatic tracer by the covalent coupling of rat AP24 with acetylcholinesterase from electric organs of electric eel Electrophorus electricus. Due to its very high turnover rate, acetylcholinesterase (AChE) can be detected very sensitively thus allowing a determination of atriopeptin at lower levels than that obtained with the corresponding RIA. The combined use of microtiter plates, solid phase separation and colorimetric enzyme assay allows complete automation of the method rendering it more suitable for routine analysis.

METHODS

Antibodies and reagents for immunoassays. The primary antiserum for rat AP24 (rAP24) employed in these studies was developed in a guinea pig using AP24 coupled to thyroglobulin (12). Also, data for human plasma extracts were acquired using an antibody developed against the human form of AP28 (purchased from Peninsula). The secondary antibody used for the coating of microtiter plates (EIA) was either affinity purified goat-antiguinea pig, (Sigma Chemical, St. Louis) or goat-antirabbit (Calbiochem) depending on which primary antibody was used. Unless otherwise stated, all reagents were from Sigma (St. Louis, MO). Microtiter plates (96 F Immunoplates I, with certificate) were purchased from Nunc (Denmark). For both the RIA and EIA, unlabelled AP24 or hAP28 (human AP28 differs from rat AP28 with a met for ileu substitution at position 12) was used as the competitor against either the iodinated or the enzyme coupled rAP24.

Plasma and atrial extracts. Rat blood samples (0.15 ml) were collected in 1/10 volume of 0.11M sodium citrate and centrifuged. Rat plasma were frozen immediately and stored at-70° until assayed. Normal human plasma was prepared by drawing blood into chilled syringes containing protease inhibitors such that the final concentration in the blood was 5mM EDTA, 11 mM Na citrate, 1 μg/ml pepstatin, 62.5 KIU/ml Aprotinin, and 10 μM PMSF. The blood was then centrifuged at 4000 x g, 10 min at 4°. Plasma was pipetted into polypropylene tubes, kept on ice and spiked or not with human AP28. Patient blood samples were drawn into EDTA vacutainer tubes (3.6 mM in blood), and centrifuged at 4000 x g, 10 min, 4°. Plasma was stored in polypropylene tubes at -70°. Prior to extraction, samples were thawed and centrifuged at 4000 x g, 10 min, 4°. Atrial peptide (AP) was extracted from human plasma using Waters' Sep-Pak octadecylsilyl cartridges. The cartridges were activated and washed with first 6 ml of 100% HPLC grade methanol (MEOH), and then 5 ml of distilled, deionized water. Two or three mls plasma was pushed through the cartridges at about 1 drop per second. The cartridges were washed with 11 ml water and then 0.5 ml 80% MEOH, 4 mM triethanolamine acetate buffer, pH4 (eluant). Polypropylene collection tubes were placed under the cartridges, and AP was eluted into them with 3 ml eluant, 1 ml at a time with 2 min pauses between each ml. Samples were chilled on ice, lyophilized until dry, and

reconstituted in 300 µl assay buffer. RIA recovery of hAP28 from normal plasma was 75%. Cardiac atria were removed from anesthetized rats. The frozen atria were pulverized in liquid nitrogen, extracted in 1.0M acetic acid, heated to 100° for 10 min, homogenized, and centrifuged (28,000 x g for 20 min). The supernatant was stored at -70° until assayed.

EIA apparatus. EIA was performed by using TITERTEK microtitration equipment, including an automatic plate washer (Microplate washer 120), an automatic dispenser (Autodrop), and a spectrophotometer (Multiskan MC) from Flow Laboratories (Helsinki, Finland).

Purification and measurement of AChE. Acetylcholinesterase (EC 3.1.1.7) was purified from the electric organ of electric eel Electrophorus electricus by one-step affinity chromatography as described by Massoulie et al. (13). The characteristics of this preparation are detailed in Pradelles et al. (14). The tetrameric form of the enzyme (G_4 form) was prepared from the crude purified preparation by incubation with trypsin (1 µg/ml) for 18 hours at 25° in 0.1M phosphate buffer pH 7.0 (trypsin/AChE {w/w}, 1/2000). This mixture, which contains both G_4 and asymmetric forms in equivalent quantities, was used without further purification for the preparation of conjugates. Details of the molecular forms of AChE are reviewed in Massoulié et al. (15). AChE activity was measured using the method of Ellman et al. (18) as described in Pradelles et al. (14).

Preparation of AP24-AChE conjugates. Rat AP24 was coupled to AChE by the intermediary of heterobifunctional reagent N-succinimidyl-4 (N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), using a procedure described for the conjugation of antibody or antigens to enzymes (17,18). This method involves the reaction of a thiol group (previously introduced into AP24 in the N-terminal part) with a maleimido group incorporated into the enzyme after reaction with SMCC. A thiol group was introduced into AP24 by reaction of the primary amino group of the peptide (N-terminal residue) with S-acetyl mercaptosuccinic anhydride (SAMSA) using a method derived from that described by Ishikawa et al. (17). 100 µl of a 17 mg/ml SAMSA solution in anhydrous dimethylformamide was added to 200 µg of AP24 dissolved in 100 µl of 0.1M borate buffer (pH 8.5). The mixture was allowed to react for 30 min at 25°, and 50 µl of 0.1M Tris/HCl buffer (pH 7.4) and 300 µl of 0.17M hydroxylamine buffer (pH 7) were then added. After a further 2 hrs incubation at 25° the peptide was purified on Sep-pak C18 cartridges (19). The methanol was lyophilized, and the peptide was redissolved in 0.1M phosphate buffer (pH 6) containing 5 mM EDTA. The concentration of AP24 in this solution was measured by RIA and its thiol content was determined colorimetrically (412 nm) after reaction with 0.5 mM 5-5'-dithiobis-nitrobenzoic acid (DTNB) as described by Ellman (20). These measurements revealed that 0.8 thiol groups were incorporated per molecule of AP24. In order to incorporate the maleimido groups into AChE, the AChE was first treated with N-ethyl-maleimide to block any thiol groups, and then the N-hydroxysuccinimide function of SMCC reacted with the primary amino groups of the enzyme. 30 µl of a 12.5 mg/ml solution of N-ethyl maleimide dissolved in 0.1M phosphate buffer (pH 7) was added to 300 µl of G_4-AChE preparation. After 30 min incubation at room temperature, 30 µl of SMCC (5.6 mg/ml in anhydrous dimethylformamide) was added, and the mixture was reacted for a further 30 min at 30°C. The various molecular forms of AChE were then isolated by chromatography on a Biogel A 1.5 m column (30 x 1 cm). Fractions corresponding to G_4 and asymmetric forms of the enzyme were pooled and AChE concentrations determined enzymatically. Conjugation of thiolated AP24 with maleimido-AChE was carried out by mixing activated enzyme (G_4 or asymmetric forms) immediately after its isolation on Biogel A 1.5 m column with a large excess of AP24-SH (AP24/AChE molecular ratio 50/1). After incubation for 3 hours at 30°, the unreacted AP24 was removed by molecular sieve chromatography on a Biogel A 15 m column (1.6 x 90 cm). All conjugates were either stored frozen at -20° or were lyophilized and kept at 4° until use. No loss of activity was observed during the coupling. No significant modification of the binding properties of conjugates has been noted under these storage conditions over a six-month period.

Enzyme immunoassay. EIA was performed in the following assay buffer: phosphate buffer (0.1M pH 7.4) containing 0.4M NaCl, 1mM EDTA, 0.1% bovine serum albumin, and 0.001% sodium azide. EIA for AP24 was performed using the procedure already described for eicosanoids (14). This is a classical competition immunoassay based on the use of a peptide-AChE conjugate as tracer. Immunoreaction takes place in microtiter plates coated with a

second antibody so that separation between bound and free fractions of the tracer occurs in the same time that specific immunoreaction between primary and secondary antibody occurs. The EIA was performed using 96 well microtiter plates that had previously been coated with purified goat anti-guinea pig IgG (or goat anti-rabbit IgG) secondary antibody. The plates were coated with a solution containing 2.5 μg/ml in 50 mM phosphate buffer (pH 7.4). A volume of 200 μl of the IgG was added to each well and allowed to bind at room temperature overnight. The next day the plates were washed, removing any unbound antibody, and stored with 300 μl EIA buffer/well. These plates were then stable at 4° for several weeks. The day of the assay, the plates were washed with 10 mM phosphate buffer pH 7.4 containing 0.05% Tween-20. The total incubation volume was 150 μl. A standard curve was generated using either rat AP24 or human AP28 and added to the microtiter plates in 50 μl aliquots. Samples were then added and brought up to a volume of 50 μl with EIA buffer containing 0.01% NaN_3. The primary antibody was added at a final concentration of $1:1/3 \times 10^6$ for GP4(AP24) or $1:10^5$ Peninsula antibody (for human plasma assays) also in 50 μl aliquots. The plates were then incubated at 4°. Depending on the sensitivity needed for the assay, the enzyme was added immediately, 4 hours later, or the next day. Following an overnight incubation with the enzyme, the plates are washed, and 200 μl of Ellman's reagent is added to each well. Ellman's reagent consists of 20 mg acetylthiocholine iodide and 21.5 mg 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB), in 100 ml of distilled water (pH 7.4). The acetylcholinesterase degrades the acetylthiocholine to acetate and thiocholine. When dithiobisnitrobenzoate reacts with the thiocholine, a yellow color is formed and can be read at 414 nM. The enzymatic reaction was allowed to proceed under mild agitation at room temperature until read by a spectrophotometer (usually 1 or 2 hrs later).

Radioimmunoassay. Radiolabelled AP24 was prepared by iodination with the chloramine-T method. Monoiodo-[^{125}I]-AP24 was separated from the unreacted AP24 and the diiodo peptide by reverse phase HPLC using a μBondpak C-18 column and a linear gradient of acetonitrile/water with 0.05% trifluoroacetic acid. [^{125}I]-AP24 fractions were stored in RIA buffer at -20°. A radioimmunoassay for rat AP24 or human AP28 was developed using a secondary antibody immunoprecipitation technique (12). These assays were performed in 12 x 75 glass tubes at 4° in a total volume of 300 μl. The assay buffer was 100 mM phosphate buffer (pH 7.4) containing 0.25% bovine serum albumin, 0.065% sodium azide, and 3% polyethylene glycol. Nonspecific binding was determined by incubations containing [^{125}I]-AP24 (10,000 cpm per tube), and secondary antibody (goat anti-guinea pig) in RIA buffer. The primary antibody dilution was 1:300,000 and the secondary antibody dilution was 1:10,000 which produced a binding of 40% of total counts. The assay was incubated overnight (4°). The next day 1 ml of 0.25% bovine serum albumin was added to each tube and centrifuged at 3,000 rpm for 30 min to pellet the bound AP24. The pellet is then counted with a 10-well Micromedic gamma counter. Human plasma RIAs were performed by incubating first the primary antibody 1:33,333 with sample aliquots and standards in RIA buffer overnight at 4°.The radiolabelled AP24 is then incubated for 4 hours at room temp, followed by the addition of the secondary antibody (1:10,000) for 2 hours also at room temp. Otherwise, the assay was the same as that for AP24.

RESULTS AND DISCUSSION

In order to establish the utility of the newly developed enzyme immunoassay, a number of criteria were essential for validation. Experiments were therefore designed to establish the linearity and sensitivity of the EIA and to compare the EIA to the conventional radioimmunoassay (RIA) using the same primary antiserum.

The EIA standard curve was concentration dependent with 50% binding at 30 pg AP24/tube and minimal detectability at 3 pg/tube (Fig 1). The EIA was about two to three times more sensitive than the RIA even though less primary antibody (one-third) was required.

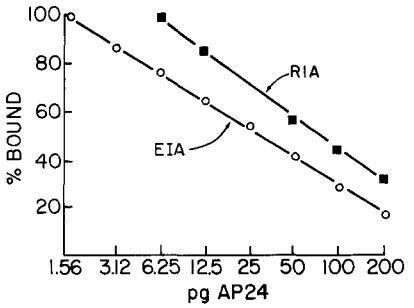


Figure 1. Atriopeptin standard curves generated in the radioimmunoassay or EIA. A standard curve using rat AP24 was generated in both the EIA and RIA using either AChE-AP24 or [^{125}I]-AP24 as the labelled competitor. The cold peptide, AP24, was added to each assay, in 50 μl aliquots followed by the primary antibody and the labelled competitor. The assays were incubated overnight at 4° and analyzed the next day, either by centrifugation and counted on a gamma counter (RIA), or acetylthiocholine and DTNB were added and read on a spectrophotometer at 415 nM (EIA).

Furthermore, in the EIA assay, if the addition of the enzymatic conjugate was delayed by either 4 or 24 hrs, the sensitivity (at 50% binding) is increased to 15 pg or 10 pg respectively. The 24-hour delay assay had a limit of detection of one pg; this enhanced sensitivity with the EIA therefore allowed analysis of unconcentrated samples (e.g., basal rat plasma levels or cardiac ventricular extracts) which fall below the detectable level of the RIA. The specificity of the assay was not modified by the use of the enzymatic tracer since identical cross reactivities were measured in both the RIA and EIA (Table 1) using the same primary antiserum. The intraassay and the interassay variation was 2% and 3% respectively.

TABLE 1

CROSS REACTIVITIES OF VARIOUS ATRIAL PEPTIDES IN THE EIA VS. RIA

	% of rat AP24	
	EIA	RIA
AP21	100	100
AP24	100	100
rAP28	74	73
hAP28	37	49
AP126	100	100

Standard curves were generated using either rAP21 (r=rat), rAP28, hAP28 (h=human), or rAP126 (rat prohormone) as the cold competitor to displace the enzyme linked AP24 or the ^{125}I-AP24. Cross reactivities were determined by comparing the 50% bind of each peptide to that of AP24 and expressing it as a percentage of rAP24 immunoreactivity.

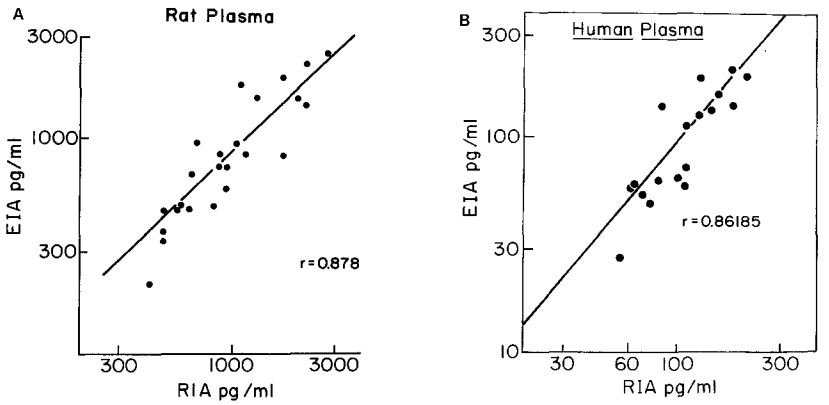
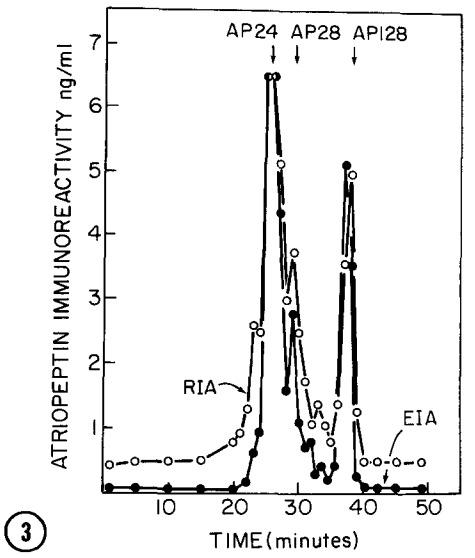


Figure 2. Comparative immunoassay of rat plasma and human plasma.

Figure 2A. Aliquots of either 20 or 50 μl of conscious rat plasma were added to the RIA or the 24-hour delay EIA. The rat plasma samples were obtained from untreated controls or rats with surgical aortovenous fistulas in order to have samples with a wide range of AP blood levels. The immunoreactivity of each sample was plotted from the two assays, and a linear regression was performed. A line was generated with r=0.87828 and a slope of 0.8549. The EIA was able to detect samples that were too dilute for the RIA and were not plotted.

Figure 2B. Human plasma samples were obtained from normals or patients undergoing cardiac bypass surgery. Aliquots were then added to both the 24-hour delay EIA or the RIA. In both cases, the primary antibody was directed against hAP28 (purchased from Peninsula) and the labelled competitor was either [^{125}I]-AP24 (RIA) or AChE-AP24 (EIA). A line was generated with a slope of 0.9958 and an r value of 0.86185.

Analysis of plasma samples was essential for understanding the role of atrial peptides in physiological processes. Extraction of plasma on octadecasilyl (ODS) columns permitted quantitative assessment of levels where recoveries (i.e., radiolabelled AP ligand or of unlabelled rAP24 or human AP28 spiking of samples) were carefully calculated. On the other hand, in experimental animal studies where changes in plasma AP levels were compared to control plasma samples taken from the same animal during various manipulations (e.g., volume expansion, vasoconstrictors, salt loading, etc.), direct measurement of plasma samples (without extraction) can rapidly give relative responsiveness. We collected a number of rat plasma samples under a variety of conditions designed to give a wide range of AP blood levels. These unextracted plasma samples obtained from conscious rats were measured in both assays, resulting in a direct linear correlation (Fig 2A). Similarly, a range of human plasma samples were collected. Unanesthetized human plasma AP levels were considerably lower than those obtained from anesthetized rats. Thus, all human plasmas were concentrated on ODS columns, and analysis of the reconstituted samples gave comparable values when analyzed by both the EIA and the RIA (Fig 2B).



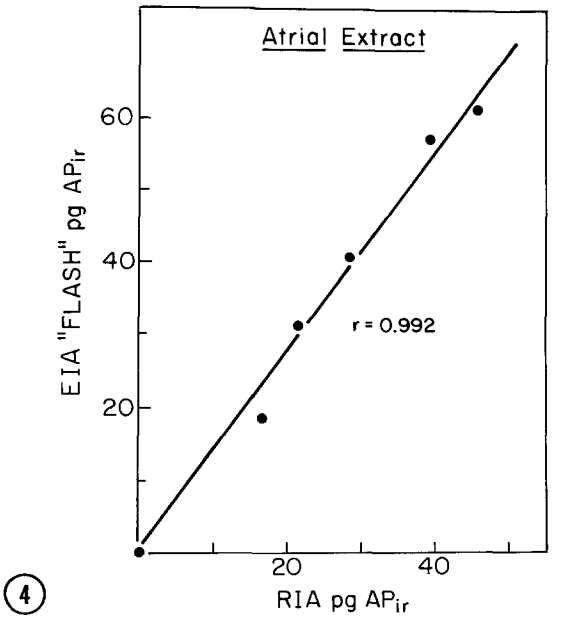


Figure 3. Comparison of the analysis of atrial extract by either "flash" EIA assay or RIA. Aliquots of the atrial extract ranging from 3 - 8 μl (of 1:1000 dilution) were added to both assays. The samples analyzed in the RIA were incubated overnight with the primary Ab and the [^{125}I]-AP24. The next day the assay was centrifuged, and the pellets were counted on a gamma counter. The samples analyzed by the EIA were incubated with the primary antibody for 2 hours at room temperature, followed by a 2-hour incubation with the AChE-AP24. The plates were then washed, acetylthiocholine and DTNB added, and read by the spectrophotometer.

Figure 4. EIA "flash" assay vs. RIA of the HPLC separation of atriopeptins. Approximately 100 ng of rAP24, rAP28, and rAP126 were injected onto a Vydac C_{18} reverse phase column. The samples were eluted off the column with a gradient of 40-60% acetonitrile 0.05% TFA over 30 minutes with a flow of 1 ml/min. One-minute fractions were lyophilized, reconstituted in 1 ml EIA buffer, and 25 μl was assayed in both the RIA (overnight incubation) and EIA "flash" assay 2-hour primary antibody, 2 hours with AP-AChE, followed by washing and spectrophotometric analysis.

One great advantage of the EIA is that a very rapid analysis (i.e., "flash" assay) of samples is possible. A good example of the utility of the flash assay is presented in Fig 3. A mixture of the C-terminal AP peptides and the prohormone was prepared and separated by reverse phase HPLC. Aliquots of each fraction were analyzed in both assays and generated comparable results (Fig 3). The capability of analyzing column fractions, for example, during a purification effort on the same day of separation, allows the rapid selection of possibly labile samples (in this case, in a solvent system of acetonitrile-trifluoroacetic acid). The disadvantage of the flash assay is that it is less sensitive than the longer disequilibrium assays and therefore requires somewhat higher levels of AP. The flash assay exhibited 50% bind of 55 pg compared to either 40 pg with no delay of the addition of the AChE-AP24, or 15 pg with a 4-hour delay of the enzyme. On the other hand, the "flash" assay is ideal to quantitate very high levels of AP as would be obtained in atrial extracts (Fig 4).

The EIA can be adapted for handling a large number of samples. The titer plates precoated with secondary antibody can be stored at 4° in large numbers for immediate use. Buffer and primary antibody can be uniformly delivered with a pipetting machine. Unknown aliquots or standards are usually separately hand pipetted because variable volumes are usually employed. Numerous commercial EIA spectrophotometer readers for 96 well titer plates are available which are extremely rapid and readily connected to computers for data reduction. In addition, the enzyme-linked AP24 is stable in a lyophilized form for several months and in an aqueous form for several weeks, unlike the iodinated form which must be made and purified once a month. In summary, the EIA assay for AP described here provides a number of advantages over the conventional [^{125}I]-ligand RIA. These include no radioisotopes, no radioactive disposal, no tube labelling, no centrifugation, a long-lasting ligand, great ease of automation, enhanced sensitivity, and of course any primary and appropriate secondary antibody can be used with the EIA ligand.

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