United States Patent Office 3,236,601 Patented Feb. 22, 1966

3,236,601

METHOD OF DETECTING UNCONJUGATED CATECHOL AMINE METABOLITES

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No Drawing. Filed Nov. 4, 1964, Ser. No. 409,010
21 Claims. (Cl. 23—230)

This is a continuation-in-part of application Serial No. 214,812, filed on August 6, 1962, now abandoned.

The present invention relates to a new and useful method for the qualitative detection and quantitative determination of unconjugated catechol amine metabolites and means for effecting said method.

More particularly, this invention is concerned with a test method useful in the diagnosis of pheochromocytoma and employs body fluids, such as urine, as the test medium.

Pheochromocytoma is usually characterized by the presence of a chromaffin cell tumor. Such a tumor, usually benign and being an adrenal medullar tumor, generally proves fatal if untreated. Early detection and removal of this tumor can effect a complete, or at least partial, reversal of any temporary structural changes which may have occurred in the circulatory system. A cardinal symptom of pheochromocytoma is hypertension with all of its associated manifestations. Simple hypertension, however, is a common condition encountered in daily medical practice; even when properly controlled, this requires continued treatment. Therefore, early and accurate diagnosis of pheochromocytoma is essential in order that prompt corrective therapy may be received by persons afflicted therewith, rather than mere maintenance treatment for simple hypertension.

In the practice of the present invention, the screening for pheochromocytoma is accomplished by a specific and reliable test which can be quickly and easily performed without complicated instruments and apparatus or specially trained personnel.

Heretofore, the detection of pheochromocytoma was effected by at least three conventional methods, i.e., the use of ganglionic-blocking agents to elicit a rise or fall in blood pressure; quantitative means of estimating urinary output of catechol (pressor) amines, such as epinephrine (adrenalin) and nor-epinephrine (nor-adrenalin), both of which increase in the presence of actively secreting tumors; or colorimetric tests for estimating the vanilmandelic acid of urine. Unfortunately, the minute quantities of epinephrine and nor-epinephrine present in the urine and plasma necessitated the use of elaborate bioassay of fluorimetric techniques for their determination. The catechol amines are also unstable in urine. Their decomposition with time can result in misleading and inaccurate assay results. The prior art vanilmandelic acid test involves the exercise of a high degree of scientific skill, tedious, time-consuming procedures and the preparation and use of highly unstable reagents. In spite of the numerous attempts to overcome some of the foregoing difficulties, none has been satisfactorily suitable in clinical practice.

In view of the foregoing, it is an object of the present invention to provide a reliable, quick, and inexpensvie test to aid in the diagnosis of pheochromocytoma.

It is a further object of this invention to provide a process to aid in the diagnosis of pheochromocytoma which does not expose the patient to personal, physical hazards attendant in drug-induced rise and fall of blood pressure.

The invention also contemplates a test method which can accurately determine the presence of unconjugated catechol amine metabolites, such as nor-metanephrine and/or metanephrine.

It is a further object of this invention to provide a visibly distinct colorimetric means of detecting the presence of chemical agents which are indicative of pheochromocytoma.

It is still a further object to provide a means for comparing the amounts of said agents present in the suspect fluid with a color chart signifying varying concentrations of said agents.

Other objects and advantages will become apparent from the following description taken in conjunction with the disclosure and examples.

Recent biochemical advances have shown that a marked increase of catechol amine metabolites, such as metanephrine and/or nor-metanephrine, exists in persons with pheochromocytoma. Such phenolic amine compounds are produced by the metabolism of catecholamines which were originally produced by the tumor cells of the adrenal glands. These metabolites can be present either in the hydroxyl (unconjugated) form or in the salt (conjugated) form.

It has now been discovered that the concentrations of catechol amine metabolites, such as metanephrine and/or nor-metanephrine, in urine can be determined by effective, precise and simple means. Generally, what is contemplated is a colorimetric ion exchange process for the detection of the presence of the unconjugated forms of such metabolites in body fluids, particularly urine.

This process comprises contacting the body fluid with an ion exchange means capable of adsorbing unconjugated catechol amine metabolites. These unconjugated metabolites are selectively adsorbed on the ion exchange means. Thereafter the addition of a mild alkalizing agent to the ion exchange means removes any residual body fluids from the adsorbed metabolites. Further, this alkalizing agent removes any undesirable substances from the adsorbed metabolites which interefere with color development resulting when a color developer is subsequently passed through the ion exchange means. This improves the concentration of desired metabolites at one area of the ion exchange material in contrast to contaminating substances being concentrated at different areas of the ion exchange material. The color developer is one which effects a visible color formation in the presence of adsorbed unconjugated catechol amine metabolites. The formation of a discernible color indicates a positive test for these unconjugated catechol amine metabolites and, if they are present in sufficient quantities, effectuates a test clearly positive for pheochromocytoma. The sensitivity of the present test is such that the intensity of the color produced can be correlated to the concentration of unconjugated catechol amine metabolites present.

The table below indicates the colors observed using a p-nitrobenzene diazonium salt as the color developer for various concentrations of urinary unconjugated catechol amine metabolites and the diagnosis obtained therefrom.

TABLE

Color	Salmon Pink to Faint Rose	Faint Rose to Deep Rose	Deep Rose to Purple
Amount of Unconjugated Catechol Amine Metabolites Present.	Trace 1–2 micrograms/ml.	5–10 micrograms/ml.	10–200 micrograms/ml.
Diagnosis	Normal	Pathological	Pathological.

Normal urines, containing 1–2 micrograms of the unconjugated catechol amine metabolites per ml. of urine, produce a faint rose color upon testing by this method. Where a pathological condition exists, i.e. where as high as 200 micrograms of the unconjugated catechol amine metabolites are present per ml. of urine, the color de-

veloper produces a deep rose to purple color. Standard urines containing known amounts of unconjugated catechol amine metabolites, such as metanephrine and normetanephrine, can be used to produce colors which, when duplicated in the form of comparison color charts, provide color standards to enable one to determine and detect a pathological or normal urine accurately and quickly by comparing the color developed in the ion exchange material with the color standards.

Any suitable ion exchange material can be employed so long at it is capable of retaining the desired unconjugated catechol amine metabolites for subsequent treatment with a color developer to produce a desired positive color change. When metanephrine and/or nor-metanephrine are to be detected, cationic ion exchangers and particularly weakly acidic cationic ion exchangers, are preferred. Exemplary cationic ion exchangers are the carboxylic and sulfonate resins. The cationic ion exchange materials are also preferably used in the salt (sodium) form rather than in the acid (hydrogen) form. When anionic unconjugated catechol amine metabolites, such as vanilmandelic acid are to be detected, anionic ion exchangers are preferred. The weakly basic anionic ion exchangers are most preferred for this form of the invention. Exemplary anionic ion exchangers are diethylaminoethyl cellulose and resins containing quaternary ammonium groups. The present invention preferably employs cationic ion exchangers.

In carrying the invention into practice, one can employ an ion exchange resin in granular form contained within a small glass column approximately 5 mm. in diameter. The resin in this column is supported with a substance such as glass wool or a paper disc. The resin is generally slurried in water in a concentration of about 30 g. of resin in 100 cc. of water and poured into the column to form a layer approximately one-half inch deep.

The urine sample, for example, is brought into contact with the appropriate ion exchange material. The unconjugated catechol amine metabolites present in the urine then enter into an ion exchange reaction with the ion exchange material and become absorbed by such material. The ion exchange material is then preferably contacted with water at the point where the urine sample was added. The water tends to wash away some of the residual urine and urine constituents which could interfere with subsequent color development.

The ion exchange material is then contacted with a mild alkalizing agent, such as tris (hydroxymethyl) aminomethane, sodium acetate and the like having a pH of about 8–10.5. This mild alkalizing agent aids in substantially completely separating interfering substances from the adsorbed unconjugated catechol amine metabolites. Sufficient mild alkalizing agent should be employed to maintain a pH of about 8–10.5 in the portion of the ion exchange material containing the unconjugated catechol amine metabolities. It has been found that about two drops of 0.1 M sodium acetate are sufficient to maintain the desired pH of about 8–10.5 for a 1 ml. sample of urine in an ion exchange resin column about 5 mm. in diameter and 0.5 inch deep.

A color developer is then passed into contact with the ion exchange material near the point where the urine sample contacted the ion exchange material. Aqueous solutions of salts of diazotized p-nitroaniline are suitable as the color developer. Preferred color developer are the fluoroborate and p-toluene sulfonate salts of diazotized p-nitroaniline. About 0.05 to 0.1 mg. of color developer are utilized in each test when quantities of urine, ion exchange resin and mild alkalizing agent described above are employed. Other useful color developers are p-diazosulfanilic acid; 2,6-dichloroquinonechlorimide; 4-diazo - N - monoethyl-o-toluidine fluoroborate; 4-diazo-N,N-diethylaniline fluoroborate; 4-diazo-N-ethyl-N-(beta-hydroxyethyl)-aniline and the like.

When an ion exchange material is employed as a resin in a column form in the present invention, the column or support should be so arranged that a urine solution can percolate easily and uniformly through it. The pH of the urine sample is preferably maintained below 7.0 prior to passage through the column. Thus, as the urine passes through the column, the ion exchange resin allows non-ionic materials to pass through but selectively adsorbs the desired ionic unconjugated catechol amine metabolites at the top of the column. The conjugated materials also pass through the resin and do not enter into the color reaction. The unconjugated materials remain bound to the resin at the ion exchange reactive sites. When a cationic ion exchange material is employed, cationic unconjugated catechol amine metabolites, such as metanephrine and nor-metanephrine, are adsorbed by the ion exchange material. Any anionic catechol amine metabolites, such as vanilmandelic acid, even though unconjugated, are not adsorbed and pass through the column. When an anionic ion exchange material is employed, the unconjugated anionic catechol amine metabolites are selectively adsorbed, and the cationic materials pass through the column.

After the urine sample has passed through the column, mild alkalizing means are used to wash through both the urine residues which have been retained at the top of the column and the phenolic acids which might impede the color development. Adding a mild alkalizing agent at this point, in such a small quantity as 1 ml. is effective. Since this mild alkalizing solution is so small in volume it is concentrated in the uppermost portion of the resin means, so that when a visible color is subsequently formed, it may be found within this narrow area.

Following this step, a solution of a color developer, such as a salt of diazotized p-nitroaniline, is added. Preferably, this color developer is used in about a 0.1% concentration.

In addition to utilizing alkalizing and color developing solutions, it is further contemplated that one can use impregnated bibulous discs. In such a procedure, bibulous material, such as filter paper sheeting, is immersed into a suitable impregnating solution. The alkalizing solution is prepared in a concentration sufficient to deposit enough alkalizing material on the bibulous carrier so as to maintain the pH at about 8–10.5. The color developing solution is prepared so as to contain between about 0.05 to 0.10 mg. of color developer per disc for use in a 5 cc. column. The alkalizing and color developing solutions are individually employed to impregnate separate portions of bibulous material. After drying, the sheets are cut into the desired shape and size. In use a disc is placed on top of the resin column and several drops of water are added on top of the disc to leach the reagent from the disc and cause it to percolate through the column.

In addition to the good results obtained by the above described procedure, a further embodiment produces an even more acute color formation. If the urine sample is first treated with a small volume of an acidic substance, such as HCl, and then heated to boiling for several minutes, this results in a hydrolysis of any conjugated catechol amine metabolites to the unconjugated forms which in turn are adsorbed by the ion exchange material and form a color with the color developer.

Instead of employing the ion exchange material in column form obtained through use of granular ion exchange resins, ion exchange paper can be used to form a column. The ion exchange paper can be prepared directly from ion exchange materials, such as cellulose containing ion exchange reactive sites. Alternatively, it can be prepared by impregnating ion exchange material into previously prepared paper or by mixing the ion exchange material with paper pulp prior to formation of paper from such pulp. A sheet of ion exchange paper can be rolled into a tight roll and this roll used as an

ion exchange column. The test sample and reagent liquids can pass down through the paper column or the paper columns can be dipped into the liquid sample and liquid reagent solutions. The liquids thus can pass up through the paper column by absorption and capillary action. Still another form of the invention involves dipping a narrow unrolled sheet of ion exchange paper into the test and reagent liquids. Alternatively a column could be prepared by stacking discs of ion exchange paper.

The ion exchange paper is preferably used, however, in a different manner. A barrier ring produced from wax, paraffin or other hydrophobic material, is formed on the surface of a sheet of ion exchange paper. The barrier ring must not extend through the paper. This surface barrier ring has an internal diameter of about ⅛ to ½ inch and is employed to prevent drops of liquid placed on the paper within such ring from flowing along the surface of the paper. In this form of the test, drops of urine sample, mild alkalizing agent and color developer are successively placed within the barrier ring. These drops then are absorbed by the paper and the liquids flow into the paper and then radially outward through the paper beyond the barrier ring. When unconjugated catechol amine metabolites are present in detectable amounts, a visible colored ring will appear on the paper. The diameter of this colored ring will be greater than the diameter of the barrier ring. The amount of unconjugated catechol amine metabolites in the test sample can be determined from comparison of the intensity of the colored ring with color standards representing known amounts of the metabolites.

The following example is illustrative of the use of ion exchange means in column form and treatment with mild alkaline and color developing solutions.

Example 1

Four glass columns 5 mm. in diameter were filled to a predetermined height with an ion exchange resin supported with a glass wool or paper disc. The sodium salt form of a methacrylate cationic ion exchange resin containing carboxylic groups and having a size of 200 to 400 mesh was poured into the column forming a layer about ½ inch deep. This resin is marketed under the trade name "Amberlite IRC–50" by Rohm and Haas Company. Four 1 ml. samples of urine containing, respectively, 0, 1 microgram, 5 micrograms and 10 micrograms of added unconjugated catechol amine metabolites (metanephrine) were individually passed through the four separate resin columns. The pH in each column was maintained at about 8.0 to 10.5 by the dropwise addition of 0.1 M sodium acetate. Following this a 0.1% solution of p-nitrobenzene diazonium p-toluene sulfonate was added to each column. After 10–30 seconds no color was observed in the column containing no unconjugated catechol amine metabolites (a normal urine), a visible salmon pink color was noted in the column containing one microgram of the metabolites, a faint rose color appeared in the column containing 5 micrograms of the metabolites, and a deep rose color appeared in the column which contained 10 micrograms of the metabolites.

The following example demonstrates the use and the method of preparation of discs.

Example 2

A mild alkalizing aqueous solution was prepared by dissolving 20 gm. of tris (hydroxymethyl) aminomethane in 100 ml. of water. Filter paper sheets (Whatman 3 MM) were immersed into the aqueous solution which was adjusted so that, upon drying, about 10 mgm. of alkalizing material remained in each disc subsequently made therefrom. The sheets, dried at 70° C.–100° C. for 10–20 minutes in a forced air oven, were cut into discs of 5 mm. diameter.

For the second type of disc, filter paper as above was similarly impregnated with an aqueous color developing solution of p-nitrobenzene diazonium p-toluene sulfonate. Such discs resulted in an impregnation of an amount of color developer equivalent to 0.05–0.10 mgm. per disc. These sheets were then subjected to air drying in an oven for 10–20 minutes at 70° C.–100° C. and cut into the desired size and shape for testing in a 5 mm. diameter glass column.

Single alkalizing discs were then inserted into each of four glass columns containing a ½ inch layer of cation exchange resin (sodium cycle). Each column was separately marked and into the first was placed a 1 ml. normal urine sample. Through the second column was passed a 1 ml. urine sample containing a known 1 microgram added amount of unconjugated catechol amine metabolites. The third column was contacted with a 1 ml. urine sample which contained 5 micrograms of added unconjugated catechol amine metabolites, while to the fourth column a 1 ml. sample urine was added which contained 10 micrograms of added unconjugated catechol amine metabolites. The assembled columns were then each washed with 1–2 drops of water. Next, a color developer impregnated disc was placed on the top of each column and was also washed with 1–2 drops of water. Within 10–30 seconds, positive color reactions were noted in the columns containing those urines which contained the unconjugated catechol amine metabolites duplicating color shades and intensities as described in Example 1. No observable color reaction was detected in the case of the normal urine containing no added catechol amine metabolites.

The following example demonstrates the use of another formulation in the preparation of discs.

Example 3

The method of preparation of the discs previously described was duplicated using a mild alkalizing solution of 0.1 M sodium acetate for one series of discs and a color developer of p-nitrobenzene diazonium fluoroborate for the second set of discs. Four urine samples as described in Example 2 were again placed into four glass columns and the method of testing was duplicated. The results were the same as in Example 2.

The following example demonstrates a method of obtaining increased color senistivity.

Example 4

Four 1 cc. urine samples as described in Example 1 above and also containing some conjugated catechol amine metabolites were each treated with a small volume (one drop) of 3 N HCl and boiled for several minutes. Subsequently, identical methods of determination of the presence of catechol amine metabolites were effected as described in Example 1. The same colors as previously observed were obtained but were decidedly brighter and more intense due to hydrolysis and conversion of some conjugated metabolites to unconjugated metabolites.

The following example describes use of ion exchange paper for the novel process of detecting unconjugated catechol amine metabolites.

Example 5

Sheets of paper impregnated with cationic ion exchange material having carboxylic acid reactive sites and marketed under the trade name "Amberlite WA–2" by the Rohm and Haas Company were cut into 1 inch squares. This paper was previously converted to the sodium form by treating it with sodium hydroxide. In the center of each square a small ring of wax was applied. When one drop of urine was applied to the center of the ring it formed a small liquid sphere on the surface of the paper and then slowly diffused into the paper. Subsequent addition of one drop of 0.5 N tris (hydroxymethyl) aminomethane solution and one drop of p-nitrobenzene diazo-

nium fluoroborate, allowing each drop to diffuse completely before addition of the next drop, gave an excellent separation of unconjugated catechol amine metabolites, such as metanephrine and nor-metanephrine, from color contaminants in the urine. The color developed as a fairly sharp circle, concentric to the applied wax circle. This system was used to check out urines containing, respectively, 1, 2, 5 and 10 micrograms of added nor-metanephrine per ml. of urine. A faint pink rose circle developed with the sample containing 1 microgram of metabolite. At a concentration of 2 mircograms of metabolite/ml. of urine, it was easy to distinguish and record the pink rose color which developed. At metabolite concentrations of 5 and 10 micrograms/ml. of urine, the color development was intense and unmistakable.

An anionic ion exchange paper can even be used to detect cationic unconjugated catechol amine metabolites if a modified procedure is employed. This is described in the following example.

Example 6

A 1 inch square portion was cut from a sheet of diethylaminoethyl-modified cellulose anionic ion exchange paper. In the center of the square a small ring of wax was applied. A drop of urine containing 10 micrograms of added unconjugated nor-metanephrine/ml. of urine was placed in the center of the wax ring and it then diffused into the paper. Subsequently, a drop of 0.5 N tris (hydroxymethyl) aminomethane solution was added within the wax ring. A 0.1% solution of the fluoroborate salt of diazotized p-nitroaniline was added around the outer end of the paper. A purple ring formed which was concentric with the wax ring.

Other types of cationic ion exchange paper can also be used as described in the following examples.

Example 7

A 1 inch square portion was cut from a sheet of phosphate-modified cellulose cationic ion exchange paper. In the center of the square a small ring of wax was applied. A drop of urine containing 10 micrograms of added unconjugated nor-metanephrine/ml. of urine was placed in the center of the wax ring and allowed to diffuse into the paper. Subsequently a drop of 0.5 N tris (hydroxymethyl) aminomethane solution and one drop of 0.1% solution of fluoroborate salt of diazotized p-nitroaniline were added within the wax ring. This produced a good separation of the absorbed metabolite and formed a distinct purple colored band concentric with the wax ring.

Example 8

The procedure of Example 7 was followed using carboxymethyl cellulose cationic ion exchange paper. A distinct purple ring formed indicating strong concentration of the metabolites.

Example 9

The procedure of Example 7 was followed using "Amberlite WA–2" cationic ion exchange paper in the hydrogen form. A drop of 1 N tris (hydroxymethyl) aminomethane solution was also added to the center of the wax ring prior to the addition of the urine. The subsequent procedure was the same as above. A distinct and positive purple color formed but the separation was not as good as in the preceding examples.

In summary, the present invention is concerned with a new, rapid and accurate colorimetric ion exchange test for the determination of unconjugated catechol amine metabolites, such as nor-metanephrine and/or metanephrine, in body fluids, particularly urine. The test is effected by contacting a fluid to be tested with an ion exchange means, subjecting said ion exchange means to treatment with a mild alkalizing agent to maintain the pH of the ion exchange material in contact with the urine sample between about 8.0 and 10.5, and developing a color in the ion exchange means with a color developer, such as diazonium salt, thereby effecting a color formation in the presence of any unconjugated catechol amine metabolites, such as metanephrine and/or nor-metanephrine.

Although the present invention has been described in conjunction with preferred embodiments, exemplary modifications and variations may be resorted to without departing from the scope and spirit of the invention, as will be readily understood by those skilled in the art. Such modifications and variations are considered within the purview and scope of the present invention and appended claims.

What is claimed is:

1. A process for the detection of unconjugated catechol amine metabolites in fluids which comprises the steps of contacting the fluid to be tested with an ion exchange means capable of adsorbing unconjugated catechol amine metabolites from a fluid containing the same; treating said ion exchange means with a mild alkalizing agent; and adding to said ion exchange means a color developer which effects a visible color formation in the presence of adsorbed unconjugated catechol amine metabolites.

2. A process according to claim 1 wherein the color formed by the color developer is compared to colors formed by known quantities of unconjugated catechol amine metabolites to determine the amount of unconjugated catechol amine metabolites present in the test fluid.

3. A process according to claim 1 wherein the unconjugated catechol amine metabolites to be detected are selected from the group consisting of metanephrine and nor-metanephrine.

4. A process according to claim 1 wherein the mild alkalizing agent has a pH of from about 8.0 to about 10.5.

5. A process according to claim 1 wherein the ion exchange means is a cationic ion exchanger.

6. A process according to claim 1 wherein the ion exchange means is a cationic carboxylic acid ion exchange resin in the sodium salt form.

7. A process according to claim 1 wherein the ion exchange means is cationic carboxymethylcellulose.

8. A process according to claim 1 wherein the ion exchange means is cationic phosphate-modified cellulose.

9. A process according to claim 1 wherein the ion exchange means is a cationic carboxylic acid ion exchange resin in the hydrogen form.

10. A process according to claim 1 wherein the ion exchange means is an anionic ion exchanger.

11. A process according to claim 1 wherein the ion exchange means is anionic diethylaminoethyl cellulose.

12. A process according to claim 1 wherein the ion exchange means is in the form of particles packed in a columnar form.

13. A process according to claim 1 wherein the ion exchange means is in the form of ion exchange paper.

14. A process according to claim 1 wherein the mild alkalizing means is tris (hydroxymethyl) aminomethane.

15. A process according to claim 1 wherein the mild alkalizing means is sodium acetate.

16. A process according to claim 1 wherein the color developer is p-nitrobenzene diazonium fluoroborate.

17. A process according to claim 1 wherein the color developer is p-nitrobenzene diazonium p-toluene sulfonate.

18. A process according to claim 1 wherein the test fluid is treated with hydrochloric acid and boiled prior to contacting it with the ion exchange means.

19. A process according to claim 1 wherein the mild alkalizing agent and the color developer are impregnated in separate bibulous sheets, said sheets being individually and separately placed in contact with the ion exchange material and water being passed through said bibulous sheets to leach out the mild alkalizing agent and color developer and bring them into contact with the ion exchange material.

20. A process for the detection of unconjugated catechol amine metabolites in fluids which comprises the steps in sequence of (1) contacting a fluid to be tested with an ion exchange means capable of adsorbing unconjugated catechol amine metabolites from a fluid containing the same; (2) treating said ion exchange means with a mild alkalizing agent at a pH of from about 8.0 to about 10.5, and (3) adding to said ion exchange means a color developer which effects a visible color formation in the presence of adsorbed unconjugated catechol amine metabolites.

21. A process for the detection in fluids of cationic conjugated catechol amine metabolites selected from the class consisting of metanephrine and nor-metanephrine which comprises the steps in sequence of (1) contacting the fluid to be tested with a cationic ion exchange paper capable of adsorbing said cationic unconjugated catechol amine metabolites from a fluid containing the same; (2) treating said cationic ion exchange paper with an aqueous solution of tris (hydroxymethyl) aminomethane at a pH of from about 8.0 to about 10.5, and (3) adding to said cationic ion exchange paper a color developer selected from the class consisting of p-nitrobenzene diazonium

fluoroborate and p-nitrobenzene diazonium p-toluene sulfonate which effects a visible color formation in the presence of said adsorbed unconjugated catechol amine metabolites.

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