United States Patent [19]

Vandervoorde

[11] **3,852,415**

[45] **Dec. 3, 1974**

[54] **COMPOSITIONS FOR USE IN RADIOIMMUNOASSAY AS A SUBSTITUTE FOR BLOOD PLASMA EXTRACT IN DETERMINATION OF CARCINOEMBRYONIC ANTIGEN**

[75] Inventor: **Jacques Pierre Vandervoorde,** West Caldwell, N.J.

[73] Assignee: **Hoffman-La Roche, Inc.,** Nutley, N.J.

[22] Filed: **Oct. 13, 1972**

[21] Appl. No.: **297,565**

[52] **U.S. Cl.** ..... **424/1,** 23/230 B, 424/2, 424/12, 250/303

[51] **Int. Cl.** **A61k 27/04**

[58] **Field of Search** ..... 424/1, 2, 359, 177, 12; 23/230 B; 250/303

[56] **References Cited**

UNITED STATES PATENTS

3,697,638 10/1972 Hansen 424/1

3,720,760 3/1973 Bennich 424/1

OTHER PUBLICATIONS

Aach et al., Proc. Nat. Acad. Sci., U.S.A., Vol. 68, No. 5, p. 1056, May 1971.

Salmon et al., The Journal of Immunology, Vol. 104, No. 3, March 1970, pp. 665–667.

Salmon et al., The Journal of Immunology, Vol. 103, No. 1, July 1969, pp. 129–131.

Primary Examiner—Benjamin R. Padgett
Attorney, Agent, or Firm—Samuel L. Welt; Jon S. Saxe; Gerald S. Rosen

[57] **ABSTRACT**

A composition suitable for use as a substitute for blood plasma extract in producing antiserum titration curves and standard antigen inhibition curves for the determination of carcinoembryonic antigen in human plasma is disclosed.

7 Claims, 2 Drawing Figures

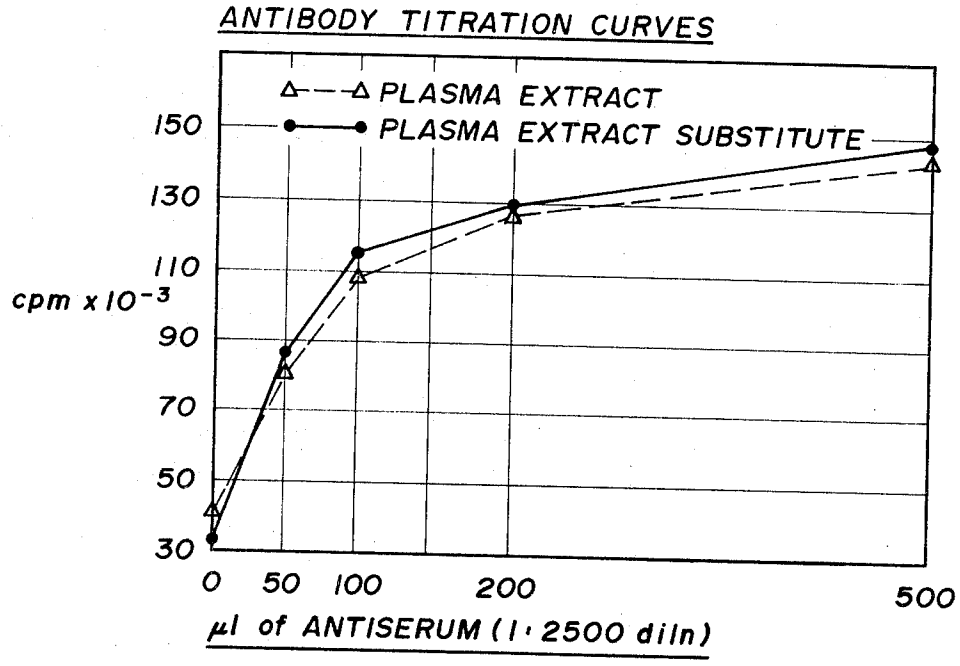
ANTIBODY TITRATION CURVES
PLASMA EXTRACT
PLASMA EXTRACT SUBSTITUTE
150
130
110
90
70
50
30
cpm x 10⁻³
0
50
100
200
500
µl of ANTISERUM (1·2500 diln)

FIG. 1

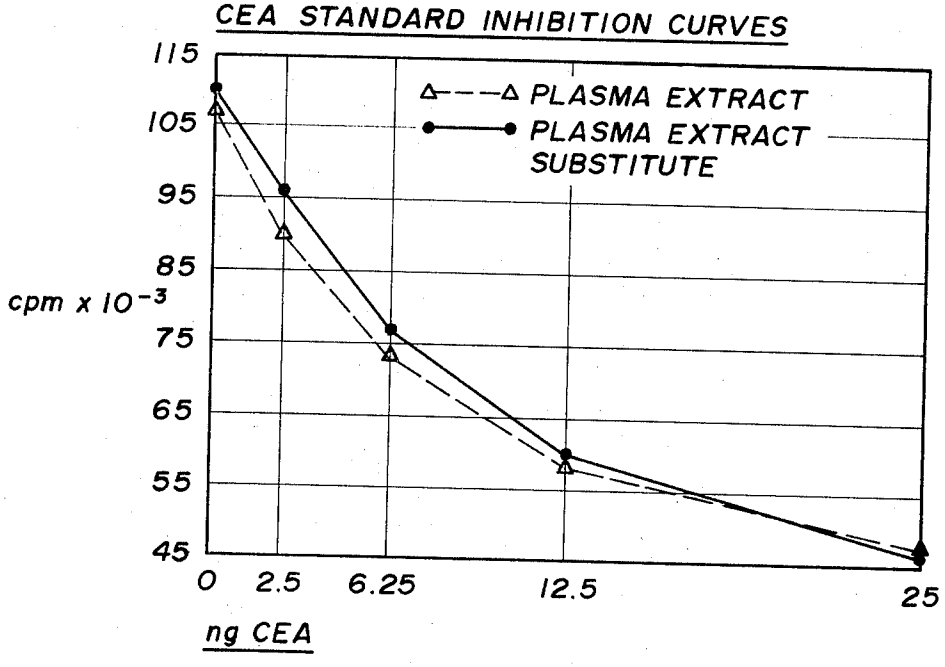
CEA STANDARD INHIBITION CURVES
PLASMA EXTRACT
PLASMA EXTRACT SUBSTITUTE
115
105
95
85
75
65
55
45
cpm x 10⁻³
0
2.5
6.25
12.5
25
ng CEA

FIG. 2

COMPOSITIONS FOR USE IN RADIOIMMUNOASSAY AS A SUBSTITUTE FOR BLOOD PLASMA EXTRACT IN DETERMINATION OF CARCINOEMBRYONIC ANTIGEN

BACKGROUND OF THE INVENTION

In order to accurately assay circulating carcinoembryonic antigen (CEA) in human blood, it is necessary to run antiserum titration curves and standard antigen inhibition curves for use in comparing against the test sample. The test sample is usually either blood serum or blood plasma. The antiserum titration curve is used to tell the activity of the antiserum and results from plotting the microliters of antiserum containing a known activity versus the counts per minute of radioiodinated CEA. The standard antigen inhibition curve is used to find the amount of CEA in a test sample and results from plotting nanograms of CEA versus counts per minute of radioiodinated CEA.

In performing the measurements required to develop the curves, it is necessary to use as a diluent medium a material which will permit consistent, reliable results. Prior to this invention, plasma extracted by glycoprotein solvents and dialysis from normal blood group Type A+ blood was used as the diluent medium. Such as extraction process is disclosed, for example, in U.S. Pat. No. 3,663,684, Example 5. While the results using this diluent are satisfactory, there is difficulty in obtaining sufficient quantities of normal plasma extract without an interfering amount of CEA.

There is therefore a need for a diluent medium which is economical and easy to obtain, permits consistent, reliable results and which further results in standard curves which are substantially identical to those resulting from the use of plasma extract from blood group Type A+ blood.

This latter property is important since it eliminates the need for developing new data which will be acceptable to the scientific community and governmental agencies. Such development is very time consuming and costly.

DRAWINGS

FIG. 1 shows antibody titration curves.

FIG. 2 shows standard inhibition curves.

DESCRIPTION OF THE INVENTION

According to this invention, a substitute for the plasma extract of blood type A+ blood has been developed. This substitute is a buffer composition containing as the buffer, a salt of an organic acid and a strong inorganic base, a preservative, a protein to coat glass tubes, a base and water.

In order to insure that the buffer is of proper composition to make it suitable for use in forming the standard antiserum titration curve the concentrations and amounts of ingredients as well as pH must be adjusted so the curve is substantially identical to that resulting when plasma extract from blood group Type A+ is used.

The substitute plasma extract composition, when used to make a standard inhibition curve must produce a curve which is substantially identical to that resulting when plasma extract from blood group Type A+ is used.

Surprisingly, it has been found that the same composition can be used as a plasma extract substitute when

running the titrations for both of the standard curves discussed.

The composition of plasma extract substitute which fulfills this criteria is described herein for a 1,000 ml. sample, 10 ml. of which are used for each point in the curve when making the curve.

The concentration of the organic acid used in the plasma extract substitute composition is operable within very narrow limits and depends upon the pK range of the organic acid used. Generally, a pK of from about 5 to 6 is operable, this range includes the pK's of polybasic acids. For purposes of this invention, however, only the pK_1 and pK_2 are significant. A preferred pK is in the range of about 2 to 3. Other criteria for choosing a suitable organic acid are that it must form water soluble salts with alkali metals, and it must cause the pH of the composition to be near neutral on the acid side.

Typical, suitable, organic acids are the tetraacetic acid compounds, particularly ethylenediamine tetraacetic acid (EDTA) which has a pK_1 of 2.0 and a pK_2 of 2.7. Other similar organic acids are suitable, for example, diethylenetriamine pentacetic acid.

Preferred alkali metal salts are the disodium and dipotassium salts.

The concentration of the organic acid should be equivalent in function to 1.28–1.32 gm./liter of disodium EDTA. Preferably, 1.3 gm./liter is required. In the event a tenfold concentration increase of the plasma extract substitute per liter is made, then the equivalent to 13.0 gm./l. of disodium EDTA is used. As used herein disodium EDTA means the disodium salt of ethylenediamine tetraacetic acid with two molecules of water of hydration, i.e., disodium EDTA dihydrate.

Other increases or decreases in concentration of the plasma extract substitute can be used but are not sufficiently accurate for practical purposes when making the standard curves.

Since CEA has a tendency to adhere to glass, it has been found necessary to use a small amount of proteinaceous material to coat the glass receptacles containing the diluent medium and prevent CEA from adhering. Such proteinaceous material must be inert with respect to the antibody-antigen reactions present when the curves are developed. A typical suitable material is bovine serum albumin (BSA). The BSA is commercially available as a 30 percent aqueous solution and is suitable in this form for use in the invention, also BSA in the form of a dry powder is suitable. The amount used in forming the compositions of this invention is 70 μ liters/liter or 21 mg./liter when dry powder is used. In the event an increase in concentration of the buffer is desired then 700 μl./l. or 210 mg./liter is used.

If the receptables are a material other than glass or glass coated with a non-reactive coating, e.g., Teflon, then there is no need for the proteinaceous material.

A small amount of a preservative should be used to prevent growth or microorganisms, particularly fungi. It has been found that either 0.17 gm./l. of sodium azide or a functionally equivalent amount of other preservatives, e.g., potassium sorbate, is suitable for the compositions of this invention. In the event the more concentrated plasma extract substitute is used, then 1.7 gm./l. of preservative is required. The invention is not limited to the use of the specific preservatives named herein since other functionally equivalent materials or

mixtures thereof are suitable and would be obvious to the skilled artisan.

Finally, sufficient base is used to adjust the pH of the plasma extract substitute to 6.45–6.55, preferably 6.5. The base used should be a base of the same metal forming the salt with the acid. For example, 1M sodium hydroxide is used when disodium EDTA is the organic acid salt. When the more concentrated medium is used, the pH is adjusted similarly to about 6.25 since upon dilution it then approaches the preferred 6.5. The amount of base used varies depending upon the pH of the composition prior to its addition. Generally, however, approximately 10 ml. of 1 M base are added.

The composition is prepared by mixing all the ingredients except the base in about 800 ml. deionized or distilled water. Following this, the solution is brought to the preferred pH, 6.5 with the base and the solution is made to 1,000 ml. with deionized or distilled water. (In the case of a tenfold concentration increase, the pH is brought to 6.25.) The resulting solution is stable at room temperature (20°–25°C.) or 4°C. for several weeks.

As used herein carcinoembryonic antigen (CEA) includes all antigenic material which is specific to antibodies of carcinoembryonic antigen. Typical CEA antigen materials are disclosed in U.S. Pat. No. 3,663,684 and U.S. Pat. Application Ser. No. 133,404, filed Apr. 2, 1971 now U.S. Pat. No. 3,697,638.

The following Examples illustrate the invention.

EXAMPLE 1

Preparation of Plasma Substitute

1.3 Grams of EDTA·Na_2·$2H_2O$ (3.7 mM) is dissolved in about 800 ml. of water. 0.17 Grams of sodium azide and 70 μl. of 30 percent aqueous BSA are added to the solution. The pH is adjusted to 6.5 with 1M sodium hydroxide and the volume made up to 1,000 with deionized or distilled water. This solution is stable at 4°C. or at room temperature for several weeks.

EXAMPLE 2

Preparation of Concentrated Plasma Substitute

13.0 Grams of EDTA·Na_2·$2H_2O$ (37 mM) is dissolved in about 800 ml. of water. 1.7 Grams of sodium azide and 700 μl. of 30 percent aqueous BSA are added to the solution. The pH is adjusted to 6.25 with 1M sodium hydroxide and the volume made up to 1,000 ml. with deionized or distilled water. The pH of this stock solution, upon diluting tenfold to make the working solution, approaches 6.5. This stock solution is stable for several weeks at 4°C. or at room temperature.

EXAMPLE 3

Preparation of Plasma Extract

1 Ml. of normal blood group Type A+ plasma is diluted with 4 ml. of saline solution in a test tube. 5 Ml. of 1.2 M perchloric acid is added to each tube. The mixtures are centrifuged for 20 minutes at 1,000 × g. The supernates are decanted in dialysis bags which are then sealed. The bags are placed in a dialysis bath and dialyzed for 18 hours against 60 volumes of deionized or distilled water which is changed several times during the dialysis. A final 3 hour dialysis against 60 volumes of 0.01 M ammonium acetate buffer, pH 6.8 is performed. The extracts are then transferred to disposable test tubes.

EXAMPLE 4

Antiserum Titration Curve with Plasma Extract

Graded amounts, i.e., 50, 100, 200 and 500 μl of antiserum to CEA which is diluted 1 to 2,500 in a buffer comprising 9 volumes of borate buffer (pH 8.4) and 1 volume of blood group Type A+ plasma are added to four test tubes, each containing 10 ml. of plasma extract prepared as in Example 3. Only water was added to one of the test tubes for a zero measurement. The mixtures were incubated for 0.5 hour at 45°C. about 3 nanograms of I^{125} CEA having 150,000–200,000 cpm. were then added to each tube and the mixture incubated for 0.5 hour at 45°C. 5 Ml. of zirconyl phosphate gel (pH 6.25) was then added to each tube. The tubes were centrifuged at 1,000 × g for 5 minutes at room temperature. The supernatant was then discarded and the gel precipitate was resuspended in 10 ml. of ammonium acetate solution (0.1 M, pH 6.25). The gel was then separated by centrifugation at 1,000 × g for 5 minutes and assayed for gel bound I^{125}. The results are shown in FIG. **1.** The assay indicates the activity of the antiserum, knowledge of which is needed for use in the radioimmunoassay for CEA.

EXAMPLE 5

Antiserum Titration Curve with Plasma Extract Substitute

The identical procedure of Example 4 was followed except that 10 ml. of the composition of Example 1 was used in place of the 10 ml. of plasma extract. The results are shown in FIG. **1.**

EXAMPLE 6

Antiserum Titration Curve with Plasma Extract Substitute

5 ml. of the product of Example 2 were diluted to 50 ml. with deionized or distilled water and utilized as the diluent following the identical procedure of Example 5. The resulting curve is identical to that of Example 5 and shown in FIG. **1.**

EXAMPLE 7

Standard Inhibition Curve with Plasma Extract

100 μl. of antiserum of CEA was added to each of five test tubes containing 10 ml. of the plasma extract of Example 3. Graded amounts of CEA, i.e., 0, 2.5, 6.25, 12.5 and 25 ng., were added to the tubes and the mixture incubated for 0.5 hour at 45°C. about 3 ng. of I^{125}-CEA with 150,000–200,000 cpm were added to each tube and the mixture incubated for 0.5 hour at 45°C. 5 Ml. of zirconyl phosphate gel (pH 6.25) was then added to each tube. The tubes were centrifuged at 1,000 × g for 5 minutes at room temperature. The supernatant was then discarded and the gel precipitate was resuspended in 10 ml. of ammonium acetate solution (0.1 M, pH 6.25). The gel was then separated by centrifugation at 1,000 × g for 5 minutes and assayed for gel bound I^{125}. The results are shown in FIG. **2.** The curve is used to find the amount of CEA in a plasma extract sample.

EXAMPLE 8

Standard Inhibition Curve with Plasma Extract Substitute

The identical procedure of Example 7 was followed except that 10 ml. of the composition of Example 1 was

used in place of the 10 ml. of plasma extract. The results are shown in FIG. **2.**

EXAMPLE 9

Standard Inhibition Curve with Plasma Extract Substitute From Example 2

5 ml. of the product of Example 2 were diluted to 50 ml. with deionized or distilled water and utilized as diluent following the identical procedure of Example 8. The resulting curve is identical to that of Example 8 and shown in FIG. **2.**

The slightly higher level of radioactivity of the curve using the plasma extract at the zero value indicates the presence of a very small but measurable amount of CEA in the plasma extract.

I claim:

1. A diluent composition suitable for forming an antibody to CEA titration curve or a CEA standard inhibition curve substantially identical to that resulting when blood plasma is used as the diluent, said composition having a pH 6.45–6.55 and comprising per liter; an amount of a salt of an organic carboxylic acid with an alkali metal which is equivalent to 1.3 grams of disodium ethylenediamine tetraacetic acid dihydrate, 0.17 grams of an antimicrobial preservative, 70 μl. of a 30 percent w/w aqueous solution of bovine serum albumin, sodium hydroxide, and sufficient water to make 1 liter.

2. A diluent composition suitable for forming an antibody to CEA titration curve or a CEA standard inhibition curve substantially identical to that resulting when blood plasma is used as the diluent, said composition having a pH 6.45–6.55 and comprising per liter; 1.3 grams of disodium ethylenediamine tetraacetic acid dihydrate, 0.17 grams of an antimicrobial preservative, 70 μl. of a 30 percent w/w aqueous solution of bovine serum albumin, sodium hydroxide, and sufficient water to make 1 liter.

3. The composition of claim **2** wherein the preservative is sodium azide.

4. A diluent composition suitable for forming an antibody to CEA titration curve or a CEA standard inhibition curve substantially identical to that resulting when blood plasma is used as the diluent, said composition having a pH 6.25 and comprising per liter; 13.0 grams of disodium EDTA dihydrate, 1.7 grams of an antimicrobial preservative, 700 μl. of 30 percent w/w aqueous BSA, sodium hydroxide, and sufficient water to make 1 liter.

5. The composition of claim **4** wherein the preservative is sodium azide.

6. In a method of making an antibody to CEA titration curve by forming graded amounts of antiserum to CEA by dilution with a buffer, incubating in a diluent, adding radiolabelled CEA, incubating, adding zirconyl phosphate gel, separating the resulting precipitated gel bound with radiolabelled material, assaying it for radioactivity and recording the results on a graph as counts per minute vs. μ liters of antiserum, the improvement which comprises utilizing as the diluent, the composition of claim **1.**

7. In a method of making a CEA standard inhibition curve by adding antiserum to CEA to graded amounts of CEA in a diluent, incubating the mixture, adding radiolabelled CEA, incubating, adding zirconyl phosphate gel, recovering the resulting gel precipitate bound with radiolabelled material, assaying it for radioactivity and recording the results on a graph as counts per minute vs. nanograms of CEA, the improvement which comprises utilizing as the diluent, the composition of claim **1.**

* * * * *