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MEDICAL DIAGNOSTIC METHOD

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ABSTRACT OF THE DISCLOSURE

Method for detection of phenylalanine in blood by paper chromatography utilizing as the developing medium about 0.3 to 1.0% of ninhydrin dissolved in the upper layer formed by allowing a butanol-aqueous acetic acid mixture to stand.

This invention relates to a novel means for diagnosing pathological conditions. More particularly, it is concerned with a diagnostic method for determining abnormal amounts of phenylalanine in the blood of humans, especially the new-born.

About 30 years ago, it was discovered that the reason for mental retardation in some children was a defect in their phenylalanine metabolism. Such children came to be known as phenylketonuric (PKU) babies. PKU babies appear to be normal at birth, but progressively degenerate between about 4 months and 24 months of age to levels of severe to moderate mental deficiency. Estimates indicate the incidence of PKU to range from one in every 40,000 up to as high as one in every 20,000 births.

Whether or not babies are phenylketonuric is determined by analyzing their blood or urine for the presence of phenylalanine, an amino acid. It is known that normal babies have a phenylalanine in blood or urine content of from 1 to 3 mg./100 ml., while the same fluids in PKU babies may have phenylalanine contents of 20 to 30 times this level.

Fortunately, if diagnosed early enough, PKU babies can be put on special diets, low in phenylalanine, which allow their normal development. It readily can be seen that saving just one of these children from a life of institutionalization can save the taxpayer a large amount of money, some estimates ranging up to one hundred thousand dollars. In addition to this cost, there would be avoided the loss to the community in terms of productive capacity, calculated as the loss of earning capacity of these persons for four decades or more of adult productive life. And, of course, the value of proper treatment to the child and to his parents is immeasurable.

Many municipal and state governments, recognizing the benefits to be obtained, are enacting legislation requiring the screening of babies for phenylketonuria during the first few months ol life. And now, in New York, under a new law, it will be mandatory to screen babies for the presence of this metabolic defect during their early days of life, even before they leave the hospital.

Up to now, there have been several methods employed for screening the urine or blood of babies for PKU: Testing urine with ferric chloride is the oldest, the best known and the most widely used test. This is a rapid method and not too expensive, but has the shortcoming that the baby must be 7 to 8 days old before the phenylalanine level is high enough in the blood to "spill" over into the urine. During this time, there is the tendency in some for damage to the brain to occur. Recently, a pathologic test for phenylketonuria has been developed using a bio-plate assay known as "inhibition assay." This test uses whole blood of newly-born babies, and overcomes the shortcomings of the urine test methods, but tends to give false positives (perhaps because of the presence of other interfering factors in the blood). The bio-assay method is rather expensive to run, requiring sterile techniques, and technicians conducting the test have to be fairly highly trained. Two other methods employed for screening and diagnosis of fluids for phenylalanine involve the use of snake venom L-amino acid oxidase, which is rather hard to obtain and is expensive, and a photometric method for determining phenylalanine, which requires specialized instruments.

There has now been found a convenient and economical means for determining the content of phenylalanine in the blood of humans, especially infants. This new method is based on a chromatographic separation technique, employing an absorbent carrier in strip form. The method is rapid, requires inexpensive reagents, and can be carried out by technicians without specialized training. With this method, it is found that pathological levels of phenylalanine in suspect serum can be detected conveniently and rapidly, the time required for a complete test being of the order of 70 to 75 minutes.

It is, accordingly, a principal object of this invention to provide a means for determining the presence of abnormal amounts of phenylalanine in blood rapidly and economically.

It is a further object to provide a means for screening all babies for the presence of abnormal amounts of phenylalanine in their blood before they leave the hospital so that effective treatment immediately can be rendered minimizing the possibility of future mental retardation.

These and other objects of the instant invention readily apparent to those skilled in the art are accomplished through practice of the process which comprises:

A diagnostic method for determining phenylalanine in suspect blood comprising:

(a) spotting serum of said blood on an absorbent carrier in strip form;

(b) developing chromatographically the nonvolatile components of said serum by contacting said absorbent carrier with a developer consisting of from about 0.3 to about 1.0% of ninhydrin dissolved in the separated upper layer which forms by mixing butanol and aqueous acetic acid and allowing the mixture to stand; and

(c) drying said absorbent carrier in the air whereby the presence of phenylalanine in said blood is confirmed by the formation of a colored zone.

The terms employed herein and in the claims are well known to those skilled in the art of paper chromatography. By the term "spotting" is meant the technique of applying to an absorbent carrier strip a minute drop of a liquid which contains nonvolatile components to be separated by chromatography. Ordinarily, the volatile components of the serum are evaporated before development of the chromatogram. By "absorbent carrier," it is meant to contemplate generally paper which can, for example, be made of cellulose, polyvinyl chloride, or from modified forms of celluose or even from glass fibers, although it is preferred to use paper made from cellulose. By the term "developing chromatographically," is meant the technique of separating the nonvolatile substituents of the mixture spotted on the carrier by causing a liquid phase to move through the absorbent carrier during which separation of the nonvolatiles takes place. In the practice of this method, the movement of the developer front edge may be downward (descending paper chromatography), upward (ascending paper chromatography), or even horizontal (circular, disk or radial paper chromatography). It is especially convenient for less-skilled technicians to use ascending paper chromatography in the instant method. Ninhydrin, also known as 1,2,3-indantrione hydrate, is a readily available reagent useful for the detection of free amino and carboxyl groups in proteins, amino acids and peptides, yielding a color, usually blue, under the proper conditions. The term "developer" is contemplated to mean a solvent containing ninhydrin which is brought into con-

tact with the absorbent carrier in strip form at some place remote from the test spot.

It is advantageous, in carrying out the process of this invention, to use paper as the absorbent carrier in strip form, and to use as a developer, a mixture of butanol and aqueous acetic acid, which contains from about 0.3 to about 1.0% of ninhydrin by weight dissolved therein. The paper may be in the form of small or large strips, or small or large sheets, but it is especially convenient to use strips that are about one inch wide by five inches long. The type of paper employed is not critical to this invention. However, for best results, only the most highly purified analytical grades of filter paper ought to be used. These are widely available as filter paper, chromatographic grades, from laboratory supply houses, world-wide. It has been found that especially rapid results can be obtained if the paper used is that known by the tradename and number, "Whatman No. 4," which is manufactured by W. & R. Balston Ltd., and which may be obtained in the United States from H. Reeve Angel & Co., Inc., 52 Duane Street, New York 17, N.Y.

A convenient way for obtaining the solvent preferred for use in this invention is to mix by volume 2 parts of butanol, 5 parts of water and one part of acetic acid and to allow the mixture to stand until it separates into 2 layers. The upper layer is removed by decantation and this is used as the developing solvent, after adding ninhydrin thereto. While the amount of ninhydrin is not critical, best results in terms of rapidity and ease of interpretation are found to follow the use of about 0.8% by weight dissolved in the said upper layer obtained from the mixture of butanol and aqueous acetic acid.

While the method of this invention can be used without internal controls, it is eminently suited for use with a control, as by checking a suspect serum against a serum known to contain the normal amount of phenylalanine, which is from about 1 to about 3 mg. per 100 ml. of said serum. For rapid routine screening, however, the method of choice uses as a control a spot of serum containing a pathological amount of phenylalanine, 15 mg. per 100 ml. of serum. Development of the suspect serum parallel to the control spot can be utilized by relatively unskilled technicians to screen samples: if the color intensity of the phenylalanine spot in the suspect serum is equal to or greater than the control spot, the serum is positive and will be so reported. Normal serum will show only a trace of color due to phenylalanine.

The instant method is also useful to determine the phenylalanine content in adult sera. Use of the method is especially advantageous in the diagnosis of that condition known as prenatal phenylketonuria. For this, sera from expectant mothers are examined for pathological amounts of phenylalanine. If high levels are found, the mother can be put on a special diet to lower the level of phenylalanine in the blood. This minimizes the possibility for transfer of phenylalanine through the placenta into the blood stream of the foetus, in which brain damage might occur.

The method of this invention may be understood by reference to the following example which represents one of the embodiments by which it may be carried out. The invention is, however, not to be construed as being limited in any manner by the example.

EXAMPLE

A developer solution is prepared by mixing 2 liters of butanol, 5 liters of water and 1 liter of acetic acid thoroughly and allowing the mixture to separate into 2 layers. The upper layer is separated and to this there is added enough ninhydrin reagent to provide a concentration of 0.8% by weight.

Several sheets, 1 x 5 inches in size, of Whatman No. 4 filter paper (obtained from H. Reeve Angel & Co., Inc., 52 Duane Street, New York 7, N.Y.) are spotted one-half inch from the bottom narrow edge, near the right-hand edge, with 3 gammas of normal human blood serum containing 15 mg./100 ml. of phenylalanine. The control spots are allowed to dry.

To an eight ounce wide-mouth jar fitted with a screw cap and a downwardly suspended hook (on which to hang the paper) is added 10 ml. of the developing solution. To one of the paper strips is applied a spot of serum directly from a centrifuge capillary tube said spot being located one-half inch from the bottom narrow edge and to the left of the control spot. The amount spotted is selected to give a spot equal in diameter to the control spot on the right. The suspect serum spot is allowed to dry, then the paper is hung from the suspended hook into the jar with about an eighth of its lower edge dipping into the solvent (developer) layer. The developer is allowed to pass up the sheet for 40 to 45 minutes. The sheet is removed from the developer and is allowed to air dry for 30 to 45 minutes and the color intensity of the top, farthest-advanced, spot of the suspect sample is compared with the corresponding spot of the controlled serum. It is found that normal serum shows only a trace of color due to phenylalanine-ninhydrin complex and pathological serum shows an intensity of the phenylalanine spot equal to or greater than that developed from the control spot of serum.

What is claimed is:

1. A diagnostic method for determining phenylalanine in suspect blood comprising:
   (a) spotting serum of said blood on an absorbent carrier in strip form;
   (b) developing chromatographically the nonvolatile components of said serum by contacting said absorbent carrier with a developer consisting of from about 0.3 to about 1.0% by weight of ninhydrin dissolved in the separated upper layer which forms by mixing butanol and aqueous acetic acid and allowing the mixture to stand; and
   (c) drying said absorbent carrier in the air whereby the presence of phenylalanine in said blood is confirmed by the formation of a colored zone.

2. A diagnostic method for determining phenylalanine in suspect blood comprising:
   (a) spotting serum of said blood on an absorbent carrier in strip form;
   (b) developing chromatographically the nonvolatile components of said serum by contacting said absorbent carrier with a developer consisting of 0.8% by weight of ninhydrin dissolved in the separated upper layer which forms by mixing 2 parts of butanol, 5 parts of water and 1 part of acetic acid, all parts being by volume, and allowing the mixture to stand; and
   (c) drying said absorbent carrier in the air whereby the presence of phenylalanine in said blood is confirmed by the formation of a colored zone.

References Cited

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