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In this manual fluorometric method, blood samples are used that have been impregnated on filter paper, a convenient collection technique that is widely used to screen newborns for phenylketonuria. The modified procedure, based on the method of McCaman and Robins [J. Lab. Clin. Med. 59, 885 (1962)], includes elution of phenylalanine from specimens on filter paper, removal of proteins by precipitation with trichloroacetate acid at 0°C, and then reaction with a ninhydrin-peptide reagent for color development. The standard curve is linear to at least 200 mg/L and the CV is 8.3% for a phenylalanine concentration of 27 mg/L. The modified procedure is suitable both for early screening for phenylketonuria and for monitoring blood phenylalanine of phenylketonurics during dietary therapy.

Additional Keyphrases: phenylketonuria • inherited disorders • screening • monitoring therapy

Phenylalanine determinations in blood are used both to detect and to monitor phenylketonuria (1–3). Most laboratories use one of two popular methods. The Guthrie microbial inhibition assay (4) is used widely for newborn screening but has been criticized because of its low specificity and variable results (2, 5, 6). The fluorometric method of McCaman and Robins (7) offers improved precision but except in high-volume laboratories this method has mainly been used as a confirmatory test when the Guthrie assay is positive. The more quantitative fluorometric methods are also preferred for monitoring phenylketonuric patients during dietary therapy, the main disadvantage being the requirement for liquid plasma or serum. Automated assay procedures based on fluorometry and utilizing blood specimens spotted on filter paper have been reported (8–10), but for practical reasons they are not useful in many laboratories. Adaptation of the fluorometric procedure (7) to provide a manual method utilizing filter-paper specimens seemed to be a very simple task, but several unforeseen problems had to be solved before results were satisfactory.

We report here a modified manual procedure that combines the accuracy of the fluorometric methods with the simplicity and convenience of collecting specimens on filter paper. We have used this quantitative manual method for more than one year with excellent results for both our screening and monitoring programs.

Materials and Methods

Equipment

Paper disc punch. A paper disc, 9/16 inch in diameter, will contain about 20 μL of whole blood when saturated.

Metabolic shaking incubator (GCA/Precision Scientific, Chicago, IL 60647).

Refrigerated centrifuge (Sorvall RC-2; Ivan Sorvall Inc, Norwalk, CT 06852).

Spectrophotometer (Amino SPF-125; American Instrument Co., Silver Spring, MD 20910).

Reagents

A reagent kit (Sigma no. 60F) for the fluorometric determination of phenylalanine contains standards and blood-collection cards (Sigma Chemical Co., St. Louis, MO 63178). All reagents used in the modified procedure except A and B are from this Sigma kit. Water was house-distilled and deionized.

A. Trichloroacetic acid (TCA), 250 g/L.

B. Succinate buffer, 1.63 mol/L, pH 5.9: Dissolve 19.25 g of succinic acid in 80 mL of water, adjust the pH with NaOH, 5 mol/L, at room temperature and dilute to 100 mL.

C. Mix 50 mL of this reagent B with 30 mL of NaOH, 2 mol/L.

D. Ninhydrin solution: Dissolve 27 mg of ninhydrin (preweighed, vial no. 60-127) in 1.67 mL of succinate buffer, 0.6 mol/L (no. 60-3). This solution is stable for more than a month at 4°C in a dark bottle.

E. Dipeptide solution: Dissolve 2 mg of L-leucyl-L-alanine (preweighed, vial no. 60-102) in 0.66 mL of succinate buffer (stock no. 60-3).

F. Ninhydrin-peptide reagent: Just before each run, mix 1.4 mL of the ninhydrin solution with 0.7 mL of the dipeptide solution. This amount is sufficient for 50 determinations.

G. Copper solution: Dilute 13 mL of copper concentrate (stock no. 60-6) to 130 mL with water. This is sufficient for 50 determinations and is stable for two weeks.

Filter Paper Standards

Normal heparinized blood is separated into 1-mL fractions. To each are added aliquots of phenylalanine solution, 4 g/L, so that the phenylalanine concentration ranges from 20 to 200 mg/L. After being mixed gently by inversion and allowed to stand for 30 min at room temperature, a 20-μL aliquot is withdrawn, spotted on the filter paper, and dried at room temperature; the paper is stored in a plastic envelope. No
autoclaving is necessary. The phenylalanine concentration of each spot equals the added phenylalanine plus that present in the original blood sample as determined by either the method of McCaman and Robins (7) or by our modified method.

Procedure

Punch paper discs from the whole-blood-impregnated specimen cards and the filter-paper standards. Place each disc in a 14-mL polypropylene tube.

Add 0.8 mL of water to each tube. One tube without a paper disc serves as the reagent blank and will be used to zero the fluorometer unless filter paper for standards and specimens is the same, in which case the paper blank may be used to zero the fluorometer.

Place the tubes in a shaking incubator at 37 °C for 30 min, 110 cycles per minute. Cool all the tubes in an ice bath and add 0.2 mL of cold reagent A (TCA) to each tube. Mix and retain in the ice bath for 20 min. The samples must be kept at 0 °C.

Centrifuge to remove precipitated protein (Sorvall refrigerated centrifuge, 27 000 × g, 10 min). Transfer 0.2 mL of the supernate to a second 10 × 75 mm tube and add 80 μL of reagent C to each tube. Mix gently.

Add 40 μL of reagent F to each tube and mix gently. Incubate for 2 h at 60 °C. Cool the tubes in cold water for a few minutes and then add 2 mL of reagent G to each; mix gently.

Set the fluorescence to zero with the reagent blank (or paper blank) and read the fluorescence of the test samples and standards. Read within 45 min after adding reagent G (excitation wavelength, 365 nm; emission wavelength, 500 nm).

Results and Discussion

Linearity. The linear relationship between relative fluorescence intensity (FI) and sample phenylalanine concentration is demonstrated in Figure 1. The linear range is sufficient to distinguish normal from phenylketonuric patients. For samples containing phenylalanine exceeding 200 mg/L, smaller aliquots of the supernate may be used. The sample is diluted 0.2 mL with TCA, 50 g/L (a fivefold dilution of reagent A); the result is multiplied by the dilution factor to obtain the final concentration. We found good agreement between results by this modified procedure and the method of McCaman and Robins (7).

Elution. Either TCA, 1 g/L, or water is satisfactory for eluting phenylalanine from the paper; final fluorescence intensities are essentially the same. Elution is complete in less than 30 min in the shaking incubator at 37 °C. Less-stringent elution conditions have been reported (8), which may be satisfactory; comparison studies have not been performed. Analytical recovery of phenylalanine from the specimens impregnated on filter paper ranged between 95 to 100%.

Both the Guthrie and the chromatographic methods (9) specify that the paper specimens be autoclaved; however, this treatment is neither necessary nor desirable for our modified method. In fact, elution is incomplete when specimens containing high phenylalanine concentrations are autoclaved. Also, with autoclaved specimens the standard curve is not always linear. Therefore specimens on filter paper should not be autoclaved when our modified procedure is used.

Reproducibility

Three whole-blood specimens with different phenylalanine concentrations were repeatedly impregnated on filter paper and analyzed to determine the reproducibility of our procedure. Even at a phenylalanine concentration of 27 mg/L, the CV is <7.0% (Table 1).

Blood-Impregnated Paper Standards

Accuracy is improved if specimens of blood (or plasma) of known phenylalanine concentration impregnated on paper are used to prepare the standard curve. This assures that samples and standards are in the same matrix and are subjected to exactly the same reaction conditions.

There is essentially no difference in phenylalanine concentration determined from plasma or whole blood. The concentration of phenylalanine inside the erythrocyte is not significantly different from that of the serum. When adding phenylalanine to whole blood for preparation of the standards, allow time for the added phenylalanine to equilibrate with the erythrocytes; equilibration is complete in 15 min at room temperature.

Differences in the spreading of whole blood and plasma

\[ \begin{array}{|c|c|c|}
\hline
\text{Specimen} & 1 & 2 & 3 \\
\hline
\text{Mean, mg/L} & 27 & 51 & 95 \\
\text{SD} & 1.7 & 2 & 3.5 \\
\text{CV, %} & 6.3 & 3.9 & 3.7 \\
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\end{array} \]

Three whole-blood specimens containing different phenylalanine concentrations were spotted on filter paper. Twenty discs of each specimen were punched out for determination of phenylalanine by our procedure.
through filter paper have been reported (11, 12). We recommend that the type of sample, that is, whole blood or plasma, be consistent with the samples used to prepare the standard curve. Physiological changes in hematocrit will not cause large errors. For example, an increase in hematocrit as large as 25% (0.40 to 0.50) may result in a sample volume increase of 7% in the filter paper spot (11).

Clinical Application

The modified manual method, used in our laboratory for more than a year, has greatly simplified monitoring known phenylketonuric patients on dietary therapy. Parents of such patients who are having blood concentrations monitored at frequent intervals are taught to obtain the specimen by impregnating the designated spots on the filter paper after a finger stick. The specimen is then mailed to the laboratory and the parents are later telephoned by the physician if the phenylalanine value suggests a dietary change. A similar procedure is used by a physician’s nurse when a positive screening test suggests additional followup testing.

Our procedure is also suitable for screening of newborns. One technologist can easily complete 50–70 assays in 6 h. The current cost of reagents is less than 10¢ per determination.

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References