An assessment of an automated fluorimetric blood phenylalanine technique for phenylketonuria screening and for accurate estimations

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SYNOPSIS An assessment of the automated fluorimetric technique for the estimation of phenylalanine, using blood collected and stored on filter paper, indicated that its accuracy and precision compared favourably with those of other methods, including the bacteriological inhibition assay. The method appeared to offer advantages, both in the detection of phenylketonuria and for the more accurate determinations required in the further investigation and control of known cases.

The recent report of the Medical Research Council Working Party on Phenylketonuria (1968) emphasized the need to introduce a blood phenylalanine screening method for detecting the disease in the newborn, now that the inadequacy of urine testing with Phenistix\(^1\) has been realized. Of the methods assessed in the report, the bacteriological inhibition assay of Guthrie (1961) seemed most satisfactory and it was suggested that, in future, the screening programme in Britain should be based on this test. It is unfortunate that only the bacteriological assay could be recommended because it may be impossible for all centres to adopt this method. It seems important, therefore, to investigate other suitable approaches to mass screening for phenylketonuria. This paper describes our recent experience of the automated fluorimetric technique for blood phenylalanine (Hill, Summer, Pender, and Roszel, 1965) using blood collected and stored on filter paper. The method was assessed both as a screening procedure for phenylketonuria, and as a method for accurate amino acid estimation.

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Materials and Methods

Blood phenylalanine was determined by the bacteriological inhibition assay (Guthrie, 1961) using a manufacturer's kit\(^2\). Blood was taken from the heel and collected onto the absorbent paper supplied.

Plasma phenylalanine was estimated by an ion exchange chromatography method using the Technicon amino acid analyzer\(^3\). Plasma was obtained by centrifuging heparinized venous blood and deproteinizing with picric acid (Stein and Moore, 1954). The protein-free supernatant was chromatographed using conditions suggested by Hacket, Mathias, and Pennington (1965).

The procedure used for the automated fluorimetric phenylalanine method was essentially that of Hill et al (1965). The determination is based on the formation of a fluorescent complex when phenylalanine is heated with ninhydrin in the presence of a dipeptide. In the place of

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\(^1\) Ames Company, Slough, England.
\(^2\) Mast Laboratories, 38, Queensland Road, Liverpool, England.
\(^3\) Technicon Instruments Co. Ltd, Hanworth Lane, Chertsey, Surrey, England.
the L-leucyl-L-alanine used in the original method, the cheaper dipeptide, glycyI-L-leucine (Sigma), was substituted (Hill, Summer, and Hill, 1967). With the latter, adequate sensitivity was obtained with a concentration of 80 mg/100 ml. The Technicon mark II fluorimeter replaced their earlier model and sufficient fluorescence was obtained with one 40 ft heating coil, compared with two coils in the published method. The 'flow-through' time of the method was reduced to 15 minutes. A type II sampler was employed with a 1:1 sampling to water wash time and a sampling rate of 40/hour.

For phenylalanine estimation by the fluorimetric method, plasma was diluted 1:50 with glass-distilled water. For whole blood analysis, blood was spotted onto Whatman no. 3 chromatography papers and allowed to dry. It was found that a disc of 3/8 in. diameter, punched from a spot, held 0-02 ml of blood. The discs were placed in AutoAnalyzer cups and were soaked in 1-0 ml of water to give a blood dilution of 1:50. Before analysis the cups were capped, inverted twice, and the discs were removed. The concentration of phenylalanine in the eluate was found to reach a maximum when the disc had been soaked in water for 10 minutes. This agreed with observations in the original method.

Comparisons of plasma phenylalanine concentration estimated by the fluorimetric and amino acid analyzer methods were carried out on specimens received in the laboratory from phenylketonurics on dietary treatment. These were sometimes several weeks' old before analysis by the fluorimetric technique. They were stored frozen in this laboratory but were often transported to us in an unfrozen state. For the comparison of the three methods, fresh bloods covering a range of phenylalanine concentrations were prepared by bleeding two phenylketonurics and mixing each blood, in varying proportions, with that from a normal of the same blood group.

Results

PRECISION AND ACCURACY OF THE METHODS

The precision of the filter paper collection and elution technique, for the estimation of blood phenylalanine, was assessed by carrying out the procedure on 25 spots of the same blood. Discs were punched from the blood spots and eluted, as described earlier. For comparative purposes, repeated analyses of a bulk dilution of the same blood were made (2 ml to 100 ml of water) and of individual dilutions in the sample cup in which 20 µl of blood was pipetted into 1-0 ml of water.

Table I compares the mean phenylalanine concentration and the precision obtained with the three methods of dilution. Precision is expressed by calculating the coefficient of variation: 

\[
\text{Coefficient of Variation} = \frac{1 \text{ standard deviation}}{\text{mean concentration}} \times 100.
\]

The results of the bulk dilution were presumed to measure the inherent variation in the automated analytical technique. Compared with this, both methods of individual dilution showed more variation but the blood disc elution technique was only slightly less precise than the pipette dilution.

The accuracy of the fluorimetric method was assessed by measuring the recovery of phenylalanine added to normal blood. Table II compares the results obtained when the bloods were diluted by pipette with those found on the same specimens using filter paper disc elution. The average recovery with the elution method was 96% and this appeared reasonably constant up to levels of 20 mg/100 ml. With the pipette dilution recoveries were generally higher and were greater than 100% at three of the concentrations.

As a further test of the accuracy of the fluorimetric technique, the results of plasma phenylalanine analyses by this method (using a 1 to 50 pipette dilution) were compared with values

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**Table I**  Precision of the fluorimetric phenylalanine assay using various dilution techniques

<table>
<thead>
<tr>
<th>Method of Dilution</th>
<th>No. of Observations</th>
<th>Mean Phenylalanine Concentration (mg/100 ml)</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ml blood to 100 ml water</td>
<td>23</td>
<td>12.2</td>
<td>2.3</td>
</tr>
<tr>
<td>0-02 ml blood to 1-0 ml water</td>
<td>30</td>
<td>11.3</td>
<td>2.8</td>
</tr>
<tr>
<td>3/8 in. diameter blood disc eluted</td>
<td>25</td>
<td>11.3</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**Table II**  Recovery of phenylalanine added to whole blood using the fluorimetric assay

<table>
<thead>
<tr>
<th>Phenylalanine Added (mg/100 ml)</th>
<th>Using Blood Disc Elution</th>
<th>Using 1:50 Dilution by Pipette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine Concentration</td>
<td>Recovery (%)</td>
<td>Phenylalanine Concentration</td>
</tr>
<tr>
<td>(mg/100 ml)</td>
<td></td>
<td>(mg/100 ml)</td>
</tr>
<tr>
<td>1-3</td>
<td>102</td>
<td>1-2</td>
</tr>
<tr>
<td>5</td>
<td>6-4</td>
<td>6-6</td>
</tr>
<tr>
<td>10</td>
<td>10-7</td>
<td>11-6</td>
</tr>
<tr>
<td>15</td>
<td>15-7</td>
<td>16-4</td>
</tr>
<tr>
<td>20</td>
<td>19-8</td>
<td>21-0</td>
</tr>
</tbody>
</table>

*1Each figure represents the mean of four determinations.*
found by the ion exchange method on the same samples (Fig. 1). With stored plasma specimens results using the fluorimetric technique were nearly always higher. However, when the analyses were carried out on fresh samples there was better agreement between the methods. Figure 2 compares blood phenylalanine levels found by a combination of the disc elution and automated fluorimetric techniques with plasma values obtained by the ion exchange method. There was an acceptable correlation between these two estimations of phenylalanine concentration.

NORMAL RANGE OF BLOOD AND PLASMA PHENYLALANINE IN INFANTS
Blood was taken from the heel of 60 infants, aged 1 week, and collected onto absorbent filter paper. Blood phenylalanine was determined on these samples by both the fluorimetric and bacterial inhibition methods. In Table III the ranges of phenylalanine concentration found are compared with those reported for the automated fluorimetric technique using blood collected in capillary tubes and diluted by pipette, also for plasma levels by the ion exchange method. The published results for the ion exchange technique have the lowest mean and the smallest standard deviation of all the methods. The agreement between the two fluorimetric techniques was good, although the blood disc elution method has a slightly wider range than that in which the blood was diluted by pipette. Of the methods considered, the bacterial inhibition assay had the highest mean concentration and standard deviation.

ASSESSMENT OF TECHNIQUE FOR DETECTING PHENYLKETONURIA
An assessment of the blood disc elution fluorimetric technique, the bacterial inhibition assay, and the ion exchange method, in detecting phenylketonuria, was attempted by analysing 14 samples prepared by mixing phenylketonuric and normal blood in varying proportions. Figure 3 shows the results and the normal range for each method. With the fluorimetric analysis on whole blood and the ion exchange method on plasma, all test specimens had a
An assessment of an automated fluorimetric blood phenylalanine technique

Fig. 3 Phenylalanine estimations on specimens prepared by mixing blood from normal and phenylketonic subjects.

- Bacteriological = blood concentration by bacteriological inhibition assay.
- Fluorimetric = blood concentration by automated fluorimetric method using the blood disc elution technique.
- Ion exchange = plasma concentration by the ion exchange chromatographic technique.

△ = blood from phenylketonic patient 1
○ = blood from phenylketonic patient 2
The broken lines indicate the lower and upper limits of the normal range for each method.

phenylalanine concentration above the normal range. Using the bacterial inhibition blood assay, three test specimens fell within the normal range for the method and one gave a borderline value.

Discussion

We have attempted to assess the precision and accuracy of the automated fluorimetric phenylalanine assay, particularly the modification in which blood is taken onto filter papers and a punched-out disc, containing dry blood, is eluted with distilled water. Our results (Table 1) suggested that this technique was only slightly less precise than when blood was diluted for analysis by pipette. It is probable that greater variation would occur when the blood is actually collected from a baby's heel. However, our normal range for the fluorimetric method was very little wider than that found by Hsia, Berman, and Slatis (1964) using blood taken into capillary tubes and diluted in the laboratory (see Table III).

Experiments to assess the fluorimetric technique by estimating the recovery of added phenylalanine, and by a comparison with the ion exchange method, demonstrated that acceptable standards of accuracy could be achieved. The greatest discrepancies occurred when comparing plasma phenylalanine levels by the two methods using rather old specimens (Fig. 1). There was a tendency then for the fluorimetric method to give increased results, particularly at high phenylalanine concentrations. With fresh plasma there was a much better correlation between the values obtained with both methods.

The satisfactory correlation between blood phenylalanine concentrations determined with the fluorimetric technique, using filter paper collection and elution, and plasma levels by the ion exchange method (Fig. 2) indicated that the former should be quite accurate enough for estimation for the further investigation and dietary control of phenylketonuric patients. It is extremely convenient to collect and transport blood on filter paper and there is an added advantage that, when dry, constant results are obtained with the fluorimetric phenylalanine assay for at least 11 weeks. It is much more difficult to preserve unprocessed blood, or plasma, for this length of time.

We compared the effectiveness of the three methods in detecting phenylketonuria (Fig. 3) because it seemed possible that the bacteriological inhibitions assay, which is known to respond to phenylalanine metabolites (Guthrie, 1961), may be more sensitive than techniques which are more specific for phenylalanine. In fact, the bacteriological assay appeared the least satisfactory of the techniques we investigated. This can probably be ascribed to the poorer precision and accuracy of the bacterial assay, because both the mean value and standard deviation, for normal infants, were higher than those of the other two methods. The ion exchange method, estimating plasma phenylalanine concentration, produced the clearest distinction between the test samples and the normal range, but large scale screening by this method would be impractical. Our results suggested that the fluorimetric technique, also, offers a sensitive method for phenylketonuria detection.

Collection of blood on filter paper and analysis by the fluorimetric method offers a simple procedure which should cover all phenylalanine estimations required in the routine hospital laboratory. Experience has shown that, for screening, the sampling rate may be increased to 60/hour. Thus a single machine could handle about 120,000 tests/year. We have found the fluorimetric technique very suitable for large-scale screening. Its main advantage over the bacteriological assay is the ease of scanning the recorder chart for high peaks, compared with the care necessary when assessing bacterial growth zones. The principal disadvantage of the fluorimetric method lies in the high cost of the apparatus (approximately £3,000). However,
compared with the bacterial assay, this may be offset by the lower cost of labour and materials, and we estimate that, if used full time for phenylketonuria screening, the outlay in equipment could be saved in two years. If the apparatus is used less for screening it is, of course, available for other fluorimetric analyses.

The authors acknowledge the helpful advice of Dr F. J. W. Lewis, Dr S. T. Crowther, and Mr M. Bywater, and the technical assistance of Miss Mary Lightfoot.

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