Colorimetric method for estimating methylmalonic acid in urine

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The increased urinary excretion of methylmalonic acid in vitamin B₁₂ deficiency has attracted attention and several studies have suggested that it has clinical value as a test of vitamin B₁₂ deficiency (Cox and White, 1962; Bashir, Hinterberger, and Jones, 1966).

Chromatographic methods of estimation have been used but are either time consuming or only semi-quantitative over a narrow range of methylmalonic acid levels. Giorgio and Plaut (1965) described a quantitative colorimetric method using the coupling of methylmalonic acid with diazotised p-nitroaniline after purification of urine by ion exchange resin. The resin they used (Dowex 3) is no longer available.

As the rapid detection of methylmalonic aciduria has potential use in the clinical laboratory in patients in whom multiple haematonic deficiencies may be present, it was decided to try to develop a suitable method using this colour reaction.

**PRINCIPLE**

Acidified urine is saturated with ammonium sulphate and extracted with ethanol/ether. The extract is passed through a strongly basic ion exchange resin column. Methylmalonic acid is eluted with hydrochloric acid and coupling with diazotised p-nitroaniline is carried out on an aliquot of the effluent.

**MATERIALS**

Urine is collected for 24 hours after 10 g of valine has been administered orally (Gompertz, Jones, and Knowles, 1967); 10 ml of concentrated hydrochloric acid may be used as a preservative.

- Diethyl ether AR and ethanol AR
- 0.1N HCl Ammonium sulphate (GPR)
- Deacidite FF (IP) < 200 mesh, chloride form. (Permutit Co. Ltd.)

**DIAZO REAGENT** requires aqueous sodium nitrite, 0.5% (W/V), p-nitroaniline, 750 mg per litre in 0.2N HCl, and aqueous sodium acetate, 0.2M.

- Sodium nitrite solution, 4 ml, and 15 ml p-nitroaniline solution are mixed and cooled in an ice bath and 4 ml sodium acetate solution is added.
- Molar acetate buffer pH 4.3 is prepared from sodium acetate AR solution and acetic acid AR mixed in the ratio 16:35 : 33:90.
- 3N sodium hydroxide aqueous.

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Measurement of mean corpuscular and packed cell volumes with a Coulter cell counter

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The demonstration by Mattern, Brackett, and Olson (1957) that the Coulter cell counter could be used for cell sizing has been confirmed by many others (Douglas and Atkinson, 1960; Grant, Britton, and Kurtz, 1960; Brecher, Jakobek, Schneiderman, Williams, and Schmidt, 1962; Nevius, 1963; Lushbaugh and Lushbaugh, 1965).

A small computer is now available from Coulter Electronics Ltd., Dunstable, Bedfordshire, England, which can be attached to Coulter cell counter models B and F. This summarizes the size of the pulses produced by the cells over a period during the actual count, divides by the number of cells, including correction for coincidence, and displays the mean corpuscular volume (MCV). An additional computer, used in conjunction with the above, will calculate the packed cell volume (PCV) from the MCV and the total red cell count and display this value. These results are available at the end of the counting period for the total cell count, not additional time or calculations being required.

We have tested these attachments for calculating the MCV and PCV and the purpose of this communication is to record our assessment of their performance.

MATERIALS AND METHODS

The attachments for measurement of the PCV and MCV were loaned by Coulter Electronics Ltd., together with a Coulter cell counter model F. The apparatus was installed by the manufacturers, calibrated and ready for use.

The red cell count was performed using a 1 in 50,000 dilution of the blood in phosphate-buffered isotonic saline formulated as follows:

- NaCl: 8g
- KH₂PO₄: 0.2g
- KCl: 0.2g
- Na₂HPO₄: 1.15g
- DI sod. versenate: 0.2g
- Water to: 1 litre

The pH of this solution was 6.8

One hundred different samples of blood were counted from patients suffering from a variety of anaemias (megaloblastic, other macrocytic anaemias, haemolytic anaemias, microcytic, hypochromic, and symptomatic anaemias) as well as from patients with polycythaemia and normal persons. The MCV and PCV recorded by the machine were noted.

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**TABLE**

**RECOVERIES OF METHYLMALONIC ACID ADDED TO URINE**

<table>
<thead>
<tr>
<th>No. of Estimations</th>
<th>Methylmalonic Acid Added (mg)</th>
<th>Methylmalonic Acid Found Mean ± 2SD (mg)</th>
<th>Mean Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>250</td>
<td>180±15</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>175</td>
<td>130±10</td>
<td>74</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>79±9</td>
<td>79</td>
</tr>
</tbody>
</table>

**SUMMARY**

A colorimetric method for the estimation of methylmalonic acid in urine is described. The method is suitable for use in clinical pathology laboratories.

Acidified urine is saturated with ammonium sulphate and extracted with ethanol and ether, and the extract is purified using a strongly basic ion exchange resin. The methylmalonic acid is eluted and a colour developed by coupling with diazotised p-nitroaniline.

I should like to thank Dr. J. S. Swale for much valuable discussion, Dr. Kressman of Permutit Co. Ltd. for samples of various ion exchange resins, and The Dow Chemical Co. for resin samples.

REFERENCES