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Automation of the assay of folate in serum and whole blood

L. MILLBANK, R. E. DAVIS[1], MARY RAWLINS, AND A. H. WATERS
From the Department of Haematology, St Bartholomew's Hospital and Medical College, London

SYNOPSIS An automated system is described for the microbiological assay of serum and red cell folate concentrations using a chloramphenicol-resistant strain of *L. casei*. Both serum and whole-blood haemolysates are assayed directly without previous deproteinization. Results by this method are comparable to those obtained by standard manual folate assays. Reproducibility within and between assays is excellent. Special advantages of the method are its simplicity of operation, economy of reagents, and capacity for dealing with large numbers of specimens (80-100 per hour). The latter makes it especially suitable for providing a regional diagnostic service and for population surveys of nutrition.

The naturally occurring folate in serum and whole blood can be measured by microbiological assay with *Lactobacillus casei* provided that precautions are taken to protect the labile folate with ascorbic acid during assay (Toennies, Usdin, and Phillips, 1956; Baker, Herbert, Frank, Pasher, Hutner, Wasserman, and Sobotka, 1959; Herbert, 1961, 1966; Waters and Mollin, 1961). The clinical value of this assay is well established, not only in the differential diagnosis of patients with megaloblastomic anaemia, but also in assessing the folate nutritional status of patients in the absence of megaloblastomic anaemia (Herbert, 1962; Waters, 1963; Mollin and Hoffbrand, 1965).

The measurement of folate activity by microbiological assay with *L. casei* is a turbidimetric assay. Two different techniques have been used: an 'extraction' method using deproteinized serum (Baker *et al*, 1959; Waters and Mollin, 1961), and an 'aseptic addition' technique which avoids the need for protein precipitation (Herbert, 1961 and 1966). These methods all involve careful and repetitive pipetting, which is not only time-consuming and monotonous, but also self-limiting in terms of the number of specimens that can reasonably be handled in a diagnostic laboratory. Such techniques are ideally suitable for automation, but the need for sterilization and aseptic addition in microbiological assays poses a considerable problem. This has been overcome by the development of a chloramphenicol-resistant strain of *L. casei* by Davis, Nicol, and Kelly (1970), who described a semi-automated folate assay system using an autodiluter to set up the

[1]Present address: Department of Haematology, Royal Perth Hospital, Perth, Western Australia.

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assay, and a colorimeter fitted with a flow cell to measure growth in terms of optical density. Results with this system were comparable to those obtained by standard manual methods. These workers also outlined the principles of fully automating the folate assay using the Technicon AutoAnalyzer or Mecolab systems. The purpose of this paper is to describe in detail the methodology of the Mecolab folate assay system and to compare the results with those obtained by manual assay methods for serum folate (Waters and Mollin, 1961) and red cell folate (Hoffbrand, Newcombe, and Mollin, 1966).

Materials and Methods

APPARATUS

The Mecolab folate assay system[1] consists of a sampling unit, a sample transfer unit, autocolorimeter, and an analogue-digital converter with digital printout. The sampling unit, which is controlled by a Geneva wheel mechanism and served by a bank of autodilutors and dispensers, consists of an outer turntable holding 15 sample cups and an inner circular rack which holds the corresponding assay tubes (rimless 75 mm × 15·5 mm (3 in. × $\frac{5}{8}$ in.))[2]

A feature of the sample transfer unit is the high-speed mechanical stirrer which is essential to give a uniform suspension of the assay organism before the contents of the tubes are sampled into the flow cell of the autocolorimeter. This is a Chromoscan (Joyce Loebl MK11) which produces an electrical output that is passed to the analogue-digital converter and printed out on an arbitrary scale 0-1,000, each reading being accompanied by its tube number and rack number.

Ancillary apparatus consists of a suction pump which exhausts the autocolorimeter flow cell and flushes air through between readings.

PREPARATION OF ASSAY ORGANISM

The chloramphenicol-resistant strain of *L. casei* (ATCC7469 = NCIB8010) was developed from that used in the conventional folate assay method by passage through increasing concentrations of chloramphenicol. A stock solution of 0·1% chloramphenicol base BP in 1% ethanol was prepared. Increasing concentrations of chloramphenicol were added to universal bottles containing 5 ml of double-strength Lactobacillus broth AOAC (Difco-0901-15) and the final volume made up to 10 ml. Each concentration was prepared in triplicate. Chloramphenicol was added in the following increments: 0·1 to 1·0 μg/ml; thence 0·5 to 10 μg/ml; 1 to 20 μg/ml; 5 to 100 μg/ml; 10 to 300 μg/ml. In the event of poor growth at any stage, the organism was subcultured back into the same concentration from which it was taken and incubated until satisfactory growth was achieved. The organism was suitable for use in the assay when tolerant to a chloramphenicol concentration of 300 μg/ml. In the assay chloramphenicol was used at a concentration of 10 μg/ml. The differential between the two concentrations of chloramphenicol was necessary for vigorous growth in the assay.

The stock culture was maintained in liquid form on the highest concentration of chloramphenicol that it would tolerate and subcultured every 10 days. The stock culture was incubated until it reached an optical density of at least 1·2 before being stored at 4°C.

PREPARATION OF INOCULUM

On the day before the assay a tube of broth containing 100 μg/ml chloramphenicol was inoculated with 0·2 ml of suspension from the stock culture and incubated at 37°C. The inoculum was adjusted to an optical density within the range 0·825-0·875. Below this range the incubation time was lengthened and above this the blank was too high for accuracy. The optical density of the inoculum was adjusted by spinning down the culture, decanting the maintenance medium, and adding single-strength assay medium. These manipulations were simplified due to the presence of chloramphenicol which made aseptic technique unnecessary. If the blank was found to be too high under these conditions, it could be reduced by washing once or twice with 10 ml single-strength medium before adjusting the optical density.

ASSAY MEDIUM

Difco dry-mix folic acid casei medium was used. Double-strength medium was prepared according to the manufacturer's instructions except that no autoclaving was necessary. This was boiled and diluted to single strength with glass-distilled water. To each litre of single-strength medium was added 10 mg of chloramphenicol base BP (10 ml chloramphenicol stock solution); 250 mg of ascorbic acid was added to protect the labile folate during assay.

The reservoir of medium was supported on a magnetic stirrer and cooled to 4°C in a container of crushed ice throughout the setting-up period for the assay.

PREPARATION OF STANDARDS

A stock solution containing 150 mg pteroylglutamic acid[3] per litre in alkaline solution was stored at 4°C in a dark bottle. Under these conditions it could be kept for up to six months. A

[1]Joyce, Loebl and Co. Ltd, Gateshead, England.

[2]Disposable glass tubes from C. E. Payne and Sons, Ltd, London, SW4.

[3]Sigma crystalline 99-100 % purity.

Contents of Tube	Tube No.							
	1	2	3	4	5	6	7	8
Folic acid solution (150 ng/ml)	0·5	1·0	2·0	4·0	6·0	8·0	10·0	12·0
Water					to 100 ml			
Folate concentration (ng/ml)	0·75	1·5	3·0	6·0	9·0	12·0	15·0	18·0

Table I *Preparation of folic acid solutions for the standard curve*

working solution for each assay was made by diluting 1 ml of the stock solution up to 1 litre with glass-distilled water giving a concentration of 150 ng/ml. Standard dilutions were prepared from this according to Table I. Glass-distilled water was used throughout. Sample cups (see below) were filled with the standard solutions and capped. An extra cup of the highest standard was set up as an optical density check on the growth of the standard curve (see below).

PREPARATION OF SPECIMENS

Serum

Specimens of clotted blood were collected in specially washed sterile glass universal bottles. Serum separated from clotted specimens was decanted into 2 ml polystyrene sample cups (Technicon) containing approximately 2·5 mg of ascorbic acid and the cups were covered with polythene caps. Each specimen was given a serial number, placed in an aluminium freeze-file and deep frozen until assayed. In this way several hundred specimens could be stored in the minimum of space.

Haemolysates

Specimens of whole blood were collected in Staynes sequestrene tubes. Haemolysates were prepared for assay using an autodilutor to dilute blood 1/20 with 1% ascorbate water. A trace of Tween 80 (0·03 ml of 10% Tween 80 per 100 ml of medium) was added to the ascorbate water to assist haemolysis. The final volume of haemolysate was 1 ml which was dispensed directly into sample cups and stored deep frozen until assay. The haemolysate was assayed directly without preliminary deproteinisation.

Blanks were not required for the determination of whole-blood folate using the Chromoscan MKII autocolorimeter with a 620 mμ filter when the final dilution was 1/2,000.

PROCEDURE

Setting up

The reservoir of assay medium was inoculated with 0·1 ml per litre of inoculum prepared as described above and after thorough mixing was stirred continuously by a magnetic stirrer. The inlet tube of the autodilutor was transferred to the reservoir and the autodilutor flushed with at least 75 ml of inoculated assay medium before starting. Of each sample (carried on the outer turntable), 0·05 ml was taken up automatically and diluted with 4·95 ml of inoculated assay medium and then dispensed into the assay tubes carried on racks in the centre of the turntable. If required, higher dilutions could be obtained by adjusting the autodilutor to withdraw smaller aliquots. However, a separate standard curve should be set up for each dilution in order to compensate for minor differences in calibration of the autodilutors.

As each cycle of the turntable was completed the central rack was removed by hand and replaced with another containing empty tubes. In this way duplicate or multiple assays of each specimen could be set up. Standards were set up in quadruplicate and test in duplicate. In practice the standards were run through in duplicate at the beginning and end of the assay. Extra cups containing distilled water were placed before and after the standards to wash the sampling pipette and avoid carry-over of folic acid.

Incubation

As the racks of assay tubes were filled, they were stored at 4°C until all were completed. All racks containing standards and specimens should start incubation at a uniform temperature if the same standard curve is to be used throughout a large batch. These precautions assume lesser importance in smaller batches where the setting up time does not exceed 30 minutes.

After incubation overnight, all racks were shaken by hand next morning. Incubation was terminated when the highest standard reached a preselected optical density, namely, 800-850 on the arbitrary scale of the instrument. Extra tubes of the highest standard were included so that optical density checks could be carried out approaching the estimated time of completion of incubation (approximately 20 hours).

Reading

After incubation all racks were left for at least 30 minutes at a temperature of 4°C before reading. Reading was fully automatic. The racks were placed on the turntable of the sample transfer unit. The tubes were stirred by the mechanical stirrer and the uniform suspension was sampled into the flow cell of the autocolorimeter at intervals of 15 seconds. The reading was printed on paper tape together with the tube and rack number.

Calculation of results

Standard folic acid solutions were set up in quadruplicate. The average optical density readings for each concentration can then be plotted

on linear graph paper to obtain a standard curve from which the test results can be determined, or alternatively, as in the present study, a computer can be used to calculate the results. A linear interpolation programme was used in association with an Olivetti Programma 203. In the particular programme used (STO 904) there was only storage for six standards including the lowest or zero point of the curve. The other points selected were 1·5, 3·0, 6·0, 9·0, and 18·0 ng/ml. The wide interval between 9 and 18 ng/ml was possible because the curve was approximately linear between these points. However, an additional standard was set up at 15 ng/ml in case the top standard at 18 ng/ml grew beyond the upper limit of the optical density range of the instrument. The standards were first entered into the programme. The optical density readings of the standards were used as a back check on the programme before the optical density readings of the specimens were entered. The programme calculated folate concentration in ng/ml.

In the case of whole blood folate, results were finally expressed as red cell folate concentration by the formula (Hoffbrand *et al*, 1966):—

$$\text{Red cell folate} = \frac{\text{whole blood folate} - \text{serum folate}\left(1 - \frac{\text{PCV}}{100}\right)}{\frac{\text{PCV}}{100}}$$

MANUAL ASSAY METHODS

To evaluate the Mecolab automated folate assay system serum and red cell folate concentrations were also assayed by the conventional manual methods used in this laboratory—serum folate (Waters and Mollin, 1961, 1963) and red cell folate (Hoffbrand *et al*, 1966) using Difco dry-mix folic acid casei medium.

Results

SERUM FOLATE CONCENTRATIONS MEASURED BY MANUAL AND AUTOMATED METHODS

There was good correlation ($r = 0{\cdot}940$) between serum folate concentrations measured by the manual and automated methods (Fig 1). However, there was some displacement of the regression line ($y = 0{\cdot}931x + 0{\cdot}207$) indicating a slightly lower value (mean 5%) by the automated method. This could be explained on the basis of dilution drift (see below), because the final serum dilution used in the manual method was 1/80 while that used in the automated method was 1/100.

WHOLE BLOOD FOLATE CONCENTRATIONS MEASURED BY MANUAL AND AUTOMATED METHODS

Using the automated method it was unnecessary to deproteinize the haemolysate before assay (Fig 2).

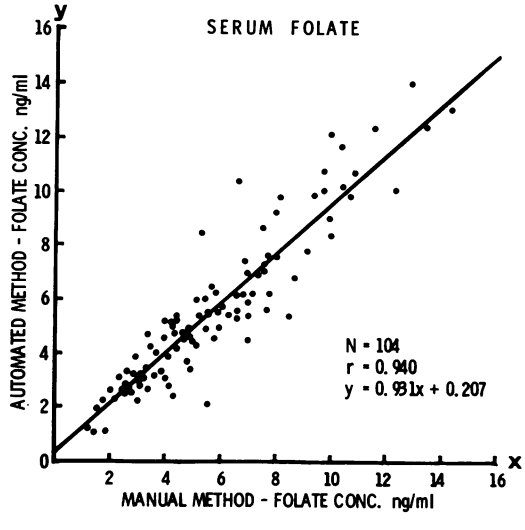

Fig 1 *Correlation of serum folate concentrations measured by the automated and manual methods*

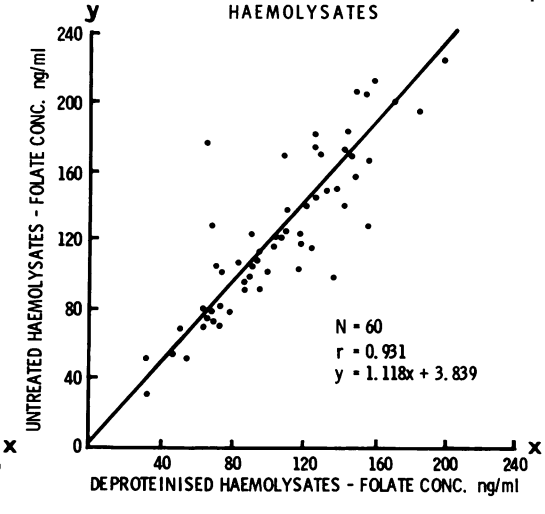

Fig 2 *Correlation of the folate concentrations of untreated and deproteinized haemolysates measured by the automated method.*

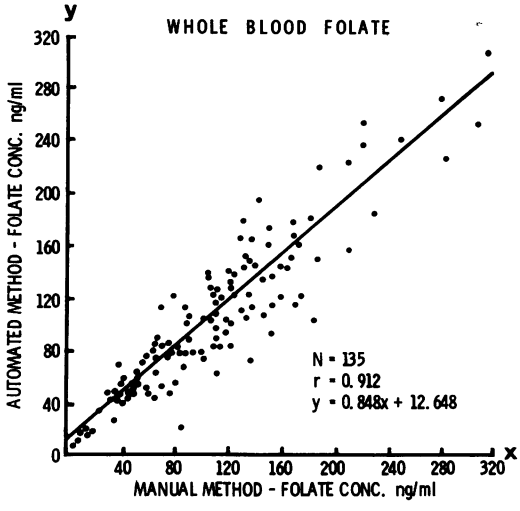

Fig 3 *Correlation of whole blood folate concentrations measured by the automated and manual methods.*

in the manual method. There was a good correlation between whole blood folate concentrations (by the automated method) of deproteinized and untreated haemolysates ($r = 0{\cdot}931$; $y = 1{\cdot}118x + 3{\cdot}839$), with a tendency for the untreated specimen to give a higher result (Fig 2).

There was a good correlation ($r = 0{\cdot}912$) between whole blood folate concentrations measured from deproteinized haemolysates by the manual method and from untreated haemolysates by the automated method (Fig 3). As in the case of serum folate concentrations, there was some displacement of the regression line ($y = 0{\cdot}848x + 12{\cdot}648$) indicating a slightly lower value (mean 2·6%) by the automated method. This could also be explained on the basis of a dilution drift (see below), because the final whole blood dilution used in the manual method was 1/800 while that used in the automated method was 1/2,000.

Type of Specimen	*Dilution*	*N*	*Range (ng/ml)*	*Mean*	*SD*	P
Serum	1/100	134	1·2-18	5·9	3·1	< 0·01
Serum	1/200	134	0·7-16·2	4·6	2·1	
Haemolysate	1/100	83	18-248	126	56·5	< 0·01
Haemolysate	1/200	83	14-204	101	44·8	

Table II *Comparison of serum and whole blood folate concentrations assayed at two dilutions by the automated method*

EFFECT OF DILUTION ON ASSAY RESULTS

To investigate the performance of the assay at higher dilutions a second autodilutor was adjusted to deliver 0·025 ml of serum or haemolysate in 5 ml of assay medium, giving a dilution of 1/200 (equivalent to a final dilution of 1/200 for serum and 1/4,000 for whole blood). This was run in parallel with the usual 1/100 dilution (equivalent to final dilutions of 1/100 for serum and 1/2,000 for whole blood). Standard curves were set up at each dilution. Both serum and whole blood folate concentrations showed a downward drift of approximately 20% between dilutions of 1/100 and 1/200 (Table II). The drift was observed with two different batches of medium (Difco folic acid casei medium with added ascorbic acid).

REPRODUCIBILITY OF THE AUTOMATED METHOD FOR ASSAYING SERUM FOLATE CONCENTRATION

Multiple assays on the same serum in the same assay

Two turntables were each loaded with 13 aliquots of the same pooled serum. Each turntable was run through twice as in the usual assay procedure giving 52 separate determinations on the same serum at a 1/100 dilution. The coefficient of variation was 3·2% (mean serum folate concentration 5·2 ng/ml).

Correlation between duplicate estimations in the same assay

In practice the serum folate concentration is expressed as the mean of two separate assay tubes. To determine the agreement between duplicate tubes of the same serum the combined coefficient of variation was calculated and found to be 1·5% for paired observations on 62 sera, with folate concentrations ranging from 1·9 to 17·0 ng/ml.

Inter-assay variation

There was good correlation between independent double determinations of the serum folate concentration of 26 sera in different assays ($r = 0{\cdot}996$; $y = 0{\cdot}995x + 0{\cdot}14$). The mean serum folate concentrations of these sera covered a range from 2·4 to 10·2 ng/ml.

Discussion

The Mecolab folate assay system is an automated flow system which has greatly simplified

the procedure of serum and whole blood folate assays. The use of a chloramphenicol-resistant strain of *L. casei* (Davis *et al*, 1970) eliminates the need for sterilization or aseptic addition. Chloramphenicol base BP is more convenient to use than the sodium succinate which is relatively inactive unless first autoclaved in nutrient medium. When used in lactobacillus broth AOAC and autoclaved at 15 psi for 15 min the activity of chloramphenicol sodium succinate approximates to the base equivalent. Whole serum and haemolysates can be assayed directly without preliminary deproteinization which results in considerable saving in time and manipulations.

In principle, an autodilutor is used to dilute serum and haemolysates with inoculated medium containing ascorbic acid which is necessary to protect the labile folate during assay. The flow system is interrupted at this stage and the assay tubes, which are held in special circular racks, are transferred to a hot room at 37°C. The assay is incubated at 37°C for approximately 20 hours, until the top standard reaches a fixed optical density. After incubation the racks of assay tubes are placed on the sample transfer unit. A special feature of this unit is the high speed mechanical stirrer which is necessary for producing a uniform suspension of the assay organism before it is sucked through the autocolorimeter. Optical density is read after a 15-second interval which allows stable conditions to prevail in the flow cell and the reading is printed out through an analogue-digital converter on to paper tape. Reading is very rapid and fully automatic, thus avoiding a monotonous and time-consuming aspect of the manual method. Finally, the optical density readings are converted to folate concentrations. This was done in the present study by an Olivetti Programma 203, but could also be done by plotting a standard curve and calculating the results of the test specimens from this.

Statistical analysis showed a close correlation between serum and whole blood folate concentrations measured by the automated and standard methods used in this laboratory (Waters and Mollin, 1961; Hoffbrand *et al*, 1966). There was also excellent reproducibility of results within and between assays. However, there was a dilution drift of approximately 20% when serum and haemolysates were assayed at a dilution of 1/200 instead of the usual 1/100 dilution. This could also explain the slightly lower (approximately 5%) serum and whole blood folate concentrations obtained by the automated method which was set up at a higher dilution than the manual method. A similar stimulatory effect of serum has been reported by Cowan (1969) using the whole serum aseptic addition assay described by Herbert (1961, 1966).

There is an increasing demand for serum and whole blood folate assays, not only in diagnostic haematology, but also as a screening test of folate status in patients with gastrointestinal disease, malnutrition, and in the whole range of conditions associated with increased folate requirement. However, a diagnostic laboratory, even if specially orientated towards carrying out microbiological assays, cannot meet these requirements, and it has been the practice in this country to refer specimens for folate assays to special laboratories where this test is carried out. A busy laboratory using a manual assay method can cope with approximately 200 specimens per week, involving two trained technicians working full time. However, the Mecolab system can assay 80-100 specimens in duplicate per hour, which gives it a tremendous capacity over a normal working day. This makes the Mecolab automated folate assay system especially suitable for providing a regional diagnostic service based on a central hospital. This system is also suitable for large group studies, especially nutritional population surveys, and for research into folate metabolism involving the assay of large numbers of specimens.

A similar automated assay system based on the Technicon AutoAnalyzer has been described by Davis *et al* (1970), but the present discrete sample system is simpler to operate than the continuous flow system. However, both of these automated systems involve a considerable capital outlay, and where either finance or the work load is limited a semi-automated system based on an autodilutor and a colorimeter fitted with a flow cell is a reasonable compromise (Davis *et al*, 1970).

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References

Baker, H., Herbert, V., Frank, O., Pasher, I., Hutner, S. H., Wasserman, L. R., and Sobotka, H. (1959). A microbiologic method for detecting folic acid deficiency in man. *Clin. Chem.*, **5**, 275-280.

Cowan, J. D. (1969). Ph.D. Thesis, University of London, pp. 94 and 166.

Davis, R. E., Nicol, D. J., and Kelly, A. (1970). *J. clin. Path.*, **23**

Herbert, V. (1961). The assay and nature of folic acid activity in human serum. *J. clin. Invest.*, **40**, 81-91.

Herbert, V. (1962). Experimental nutritional folate deficiency in man. *Trans. Ass. Amer. Phycns*, **75**, 307-320.

Herbert, V. (1966). Aseptic addition method for *Lactobacillus casei* assay of folate activity in human serum. *J. clin. Path.*, **19**, 12-16.

Hoffbrand, A. V., Newcombe, B. F. A., and Mollin, D. L. (1966). Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. *J. clin. Path.*, **19**, 17-28.

Mollin, D. L., and Hoffbrand, A. V. (1965). *Ser. Haematol.*, **3**, 1.

Spray, G. H. (1969). Estimation of red cell folate activity. *J. clin. Path.*, **22**. 212-216.

Toennies, G., Usdin, E., and Phillips, P. M. (1956). Precursors of the folic acid-active factors of blood. *J. biol. Chem.*, **221**, 855-863.

Waters, A. H. (1963). Ph.D. Thesis, University of London.

Waters, A. H., and Mollin, D. L. (1961). Studies on the folic acid activity of human serum. *J. clin. Path.*, **14**, 335-344.

Waters, A. H., and Mollin, D. L. (1963). Observations on the metabolism of folic acid in pernicious anaemia. *Brit. J. Haemat.*, **9**, 319-327.