Measurement of serum folate: experience with ⁷⁵Se-selenofolate radioassay

IAN JOHNSON, HUGH GUILFORD, AND MICHAEL ROSE¹

From the Haematology Department, St George's Hospital, Tooting, London SW17 and The Radiochemical Centre, Amersham, Bucks

SUMMARY Three hundred and eighty-two serum samples were assayed for folate content by a standard *Lactobacillus casei* microbiological assay and by radioassay using the ⁷⁵Se-selenofolate radioassay kit from the Radiochemical Centre, Amersham. Results showed encouraging concordance. Explanations were discovered for the few disparities. The radioassay procedure is simple and an assay batch can be completed in one working day.

We wish to document our results from measurements of serum folate levels by both microbiological assay and radioassay.

Samples

Three hundred and eighty-two successive samples of clotted blood, submitted over a 25-week period to the haematology laboratory at St George's Hospital for serum folate assay, were separated and stabilised for purposes of radioassay by the addition of about 5 mg of sodium ascorbate per millilitre of serum. Ascorbic acid was added to the samples for microbiological assay. The folate content of these samples was measured both by a radioassay method and by microbiological assay. Each assay was conducted in ignorance of the result obtained by the other method by another operator.

Forty-five blood samples in sequestrene were assayed for their red cell folate content. Samples for microbiological assay were prepared as a 1:10 dilution of whole blood in 1% aqueous ascorbic acid, with a drop of Tween 80 solution. Those samples for radioassay were prepared as a 1:8 dilution in glass-distilled water and, after complete haemolysis, sodium ascorbate was added to a final concentration of 5 mg/ml.

Methods

MICROBIOLOGICAL FOLATE ASSAY

The *Lactobacillus casei* method of Waters and Mollin (1961) was used for serum folate assay. Red

cell folate was assayed by the method of Hoffbrand *et al.* (1966). Sera were assayed in 25 batches in triplicate. Whole blood samples were assayed in the same number of batches in duplicate. These techniques had been employed for clinical diagnostic purposes for 10 years at St George's Hospital without remarkable problems.

FOLATE RADIOASSAY

The folate radioassay is based on the general principles of competitive protein binding analysis, except that it sets out to measure total circulating folates rather than one specific analogue. N⁵-Methyltetrahydrofolate in solution in caprine serum is used for standardisation in a range of concentrations from about 0.5 to 16 µg/l. Goat's serum is used as the vehicle for N⁵-methyltetrahydrofolate in the standard curve as it has virtually no endogenous folate content nor folate binder (Mantzos *et al.*, 1974). This avoids the need for charcoal adsorption which produces untoward alterations in the serum composition. Duplicate aliquots of 200 µl of each standard or sample are pipetted into polypropylene assay tubes, diluted with lysine buffer, pH 10.5, and vortex mixed. All tubes are heated for 5 minutes in a boiling water bath to denature endogenous binding protein, and then cooled to ambient temperature. To each clear solution is added 100 µl of a solution of ⁷⁵Se-selenofolate in a 0.1M phosphate-EDTA-albumin buffer. The radio-selenium label is incorporated into a cysteine moiety which replaces glutamate to produce pteroyl-l-methylselenocystein ⁷⁵Se. Then 100 µl of porcine serum in the same buffer is added. Porcine serum provides plentiful and avid folate binding proteins (Mantzos, 1975). The folate binding behaviour of porcine serum is more reproducible than milk or

¹Correspondence: Dr M. S. Rose, Haematology Department, St George's Hospital, SW17

Received for publication 6 December 1976

milk extracts. After vortex-mixing, the tubes are incubated at ambient temperature for 30 minutes. A suspension of albumin-coated charcoal (200 μ l) is added. This charcoal reagent is prepared by mixing 5 parts charcoal with 1 part bovine serum albumin in 10 parts distilled water. After centrifugation the albumin-coated charcoal is resuspended in 0.1M phosphate-EDTA-buffer containing 0.1% bovine albumin. This is desiccated, and the 200 μ l of reconstituted charcoal dispensed into each mixture is coated with 5 mg of albumin. The tubes are centrifuged at $>1000 g$ for 15 minutes. The supernates containing protein-bound folate are decanted into counting tubes, leaving 'free' folate adsorbed on the charcoal.

The radioactivity in each counting tube is quantified in a gamma counter set for selenium-75. A 5-point standard curve is constructed by plotting counts for each of the duplicate standards against the N⁵-methyltetrahydrofolate concentration of each. The folate level of each of the unknown samples can be interpolated from this curve.

These reagents, and detailed protocol for their use, can be obtained in kit form from The Radiochemical Centre, Amersham.

Results

REPRODUCIBILITY

Human serum controls based on N⁵-methyltetrahydrofolate were included in all the assays in this study. The between-assays variation for these sera was as follows (7 batches of kits; 2 operators): Serum (a) Mean value ($n = 16$) 3.4 μ g/l, CV = 6.6% Serum (b) Mean value ($n = 14$) 6.0 μ g/l, CV = 12.7% The within-assay variation on 10 replicates, mean value 3.37 μ g/l, was 4.0%. (This sample was not one of the controls used to determine inter-assay variation.)

SERUM FOLATES

The distribution of values from these two methods is shown in the correlation diagram in Figure 1. The correlation coefficient is 0.82. A few of these points show a significant disparity between the biological and radio assay. Of these, the ringed results were from subjects who, upon enquiry, were discovered to be receiving either antimicrobial or cytotoxic preparations at the time of sampling. The bioassay result would thus be expected to be falsely

Fig. 1 Results of serum folic acid assay by radioisotope dilution method and by microbiological method from 382 samples. The ringed results were on samples known to be from subjects taking broad-spectrum antibiotics.

depressed. The correlation coefficient for duplicate samples upon radioassay 'in-batch' is 0.97.

RED CELL FOLATES

The correlation between bioassay and radioassay values for a small number of samples run in a preliminary study of red cell folate determination using the radioassay are shown in Fig. 2; the correlation coefficient is 0.64, which clearly leaves much to be desired. The correlation coefficient for duplicate samples upon radioassay 'in-batch' is 0.96.

Fig. 2 Results of red cell folate assay by both methods on 45 samples. The ringed results were from two samples taken from a subject receiving cytotoxic treatment.

Discussion

Microbiological assays for serum folate have been used to identify clinical states due to folate depletion since their advent (Tepley and Elvehjem, 1945). Although a red cell folate level is a more reliable determinant of folate status, being less susceptible to short-term fluctuation than serum folate levels (Chanarin, 1969), serum folate determinations provide a valuable adjunct to clinical diagnosis.

Radioisotopic assays for folate measurement have been explored since folate binders were identified in milk (Ghitis, 1967) and in animal (especially porcine) sera (Mantzou *et al.*, 1974). A variety of methods have been documented (Waxman *et al.*, 1971; Rothenberg *et al.*, 1972; Dunn and Foster, 1973; Mantzou, 1975) recently, all of which suffer from the shortcomings of scintillation counting associated with the use of tritiated folic acid, which appears to prohibit their widespread application for clinical

diagnostic purposes (Coulson and Rose, 1974; International Society of Haematology, London, 1975). Further development of a method, using a gamma-emitting radiofolate adhering to basic principles of saturation analysis, has yielded a simple, reliable, and effective method which has a number of advantages compared with the *L. casei* assay.

(i) It is not susceptible to the caprices of the organism, which occasionally undergoes mutation with alterations in growth patterns. Results are not influenced by contaminating organisms and antibiotics, nor by most drugs which the subject may have been taking at the time of sampling. Large doses of methotrexate, however, may give rise to circulating levels in serum sufficient to raise the apparent folate level artificially.

(ii) The time necessary for an operator to be involved directly with the microbiological assay of a batch of 45 samples is at least 7 to 9 hours spread over three days. Even making a generous allowance for the time of counting the radioactivity, the results from the radioassay can be available the same day the assay was run. Direct technical involvement is necessary for about 2.5 hours, assuming an auto-gamma counter is available. If a radioassay for vitamin B₁₂ is already installed with available counting apparatus there seems no clear reason to persist with microbiological methods for folate assay, unless the cost of radioassay materials becomes prohibitive.

A recent survey of commercial assay kits was highly critical of the quality of the products and the reliability of the results (Kubasik *et al.*, 1975). The performance, however, of the ^{75}Se -selenofolate kit under routine conditions at St George's Hospital illustrates that, for the purposes of serum folate assay, it is reliable, reproducible, and accurate. It is a rapid and simple procedure without the difficulties of β -counting which have bedevilled some of its antecedents.

Our preliminary findings, measuring red cell folate, suggest that the method lends itself to this purpose, and modifications are being explored to improve the reproducibility and correlation with bioassays (Johnson and Rose, 1977). While it is widely recognised that some drugs affect the microbiological assay (Reizenstein, 1965; Beard and Allen, 1967) we are in the process of determining more comprehensively which commonly administered drugs interfere with the microbiological assay (Rose and Johnson, in preparation).

We thank Mrs J. Tooze for performing the microbiological folate determination on the folate samples submitted during this study.

References

- Beard, M. E. J. and Allen, D. M. (1967). Effect of antimicrobial agents on the *Lactobacillus casei* folate assay. *Amer. J. clin. Path.*, **48**, 401-404.
- Chanarin, I. (1969). *The Megaloblastic Anaemias*. Blackwell, Oxford.
- Coulson, J. and Rose, M. S. (1974). Serum folate assay (correspondence). *Blood*, **44**, 764-765.
- Dunn, R. T. and Foster, L. B. (1973). Radioassay of serum folate. *Clin. Chem.*, **19**, 1101-1105.
- Ghitis, J. (1967). The folate binding in milk. *Amer. J. clin. Nutr.*, **20**, 1-4.
- 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.
- International Society of Haematology, London (1975). *Workshop on Folate Binding Proteins*. Proceedings of the European and African Division.
- Johnson, I. and Rose, M. S. (1977). Measurement of red cell folate: experience with ⁷⁵Se-selenofolate radioassay. *J. clin. Path.*, in the press.
- Kubasik, N. P., Volosin, M. T., and Sine, H. E. (1975). Comparison of commercial kits for radioimmunoassay: III. Radioassay of serum folate. *Clin. Chem.*, **21**, 1922-1926.
- Mantzou, J. D. (1975). Radioassay of serum folate with use of pig plasma folate binders. *Acta Haemat. (Basel)*, **54**, 289-296.
- Mantzou, J. D., Alevizou-Terzaki, V., and Gyftaki, E. (1974). Folate binding in animal plasma. *Acta Haemat. (Basel)*, **51**, 204-210.
- Reizenstein, P. (1965). Errors and artefacts in serum folic-acid assays. *Acta med. scand.*, **178**, 133-139.
- Rothenberg, S. P., da Costa, M., and Rosenberg, Z. (1972). A radioassay for serum folate: Use of a two-phase sequential-incubation, ligand-binding system. *New Engl. J. Med.*, **286**, 1335-1339.
- Tepley, L. J. and Elvehjem, C. A. (1945). The titrimetric determination of 'Lactobacillus casei factor' and folic acid. *J. biol. Chem.*, **157**, 303-309.
- Waters, A. H. and Mollin, D. L. (1961). Studies on the folic acid activity of human serum. *J. clin. Path.*, **14**, 335-344.
- Waxman, S., Schreiber, C., and Herbert, V. (1971). Radioisotopic assay for measurement of serum folate levels. *Blood*, **38**, 219-228.