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Estimation of serum folate by a radioassay and a continuous-flow method, compared with an established microbiological tube assay

J. E. O'DONNELL, G. B. TENNANT, and B. M. JONES
Department of Haematology, University Hospital of Wales, Heath Park, Cardiff CF4 4XW, UK

There has been continued effort in recent years to improve the routine methods of serum folate estimation. The aim has been to make them simpler and more economical to perform while providing more reliable results. Methods have recently become available which claim a number of advantages over conventional bioassays. Here we compare two new methods, a radioisotope method and a fully automated microbiological assay, with a well-established microbiological tube assay.

Methods

ASSAY METHODS

The tube assay has been modified from that described previously (Tennant and Withey, 1972) by decreasing the phosphate buffer concentration to 0.075 M (Tennant *et al.*, 1974). Chloramphenicol (10 mg/l) was added as a solution of Chloromycetin (Parke Davis Ltd, Pontypool, Gwent) in ethanol. Assay medium was prepared in the laboratory (Baker *et al.*, 1959). A chloramphenicol-resistant strain of *Lactobacillus casei* subsp. *rhamnosus* (NCIB 11295), derived from NCTC 10302 (Tennant, 1977), was used for the tube assays. It has been shown that this strain gives results similar to those of other strains in more common use (Tennant *et al.*, 1974). The bacteria were subcultured into 'inoculum bottles' (Tennant *et al.*, 1974), and the cultures were grown for 24 hours at 37°C before being used, unwashed, to seed the assay medium. Standards were prepared by diluting a pteroyl glutamic acid (PteGlu) stock solution (10 mg/l) to give a range of 0.10 µg/l.

A fully automated continuous-flow method was used in which the growth response of *L. casei* (NCIB 11295) was estimated by measuring the rate of

reduction of 2, 3, 5, triphenyl tetrazolium chloride (Tennant, 1977). Standard PteGlu solutions were prepared as for the tube assay. BBL Folic Acid PGA Broth (Becton Dickinson Ltd, Wembley, Middlesex) was used in this method.

The radioassay kit (The Radiochemical Centre, Amersham, Bucks, UK) utilised ⁷⁵Se labelled folate (Johnson *et al.*, 1977). Supernatants were counted on a Wallac 300 gamma counter (LKB, South Croydon, Surrey, UK) and the results were calculated as recommended (Radiochemical Centre, 1976).

PREPARATION OF SERA

All sera were checked for sterility. Sodium ascorbate was added to sera (0.05 ml of a 100 g/l solution per ml of serum) before assay by the radioisotope method.

COMPARISON OF SERUM FOLATE RESULTS FROM THE THREE METHODS

Tube assay results were based on results from duplicate tubes at each of two dilutions (1/40 and 1/80) and results from the automated method on single peaks from neat and 1/2 diluted serum. The radioassay results were the mean of duplicate tubes. The three estimations on each serum were carried out simultaneously so that there could be no possibility of deterioration of samples between the assays.

COMPARISON OF ASSAY VARIATION

A control serum was assayed repeatedly by each method in each of eight batches. Pairs of tubes or single peaks were distributed throughout the batches by alternating them with specimens for routine assay. Comparison of the variation of the three methods was based on the results from individual tubes or peaks (at 1/40 dilution only in the tube assay). Estimation of the variation between the batches of each method were based on the means of the eight batches, thus excluding the in-batch variation.

Comparison of assay results

Serum folate results from the tube assay (x) were compared with those from the automated method (ya) (Fig. 1) (N = 62; r = 0.953, ya = 1.10x + 0.45) and the radioisotope technique (yr) (Fig. 2) (N = 66; r = 0.876; yr = 0.84x + 0.11). Table 1 shows the statistics obtained for the control serum in each batch together with the overall mean and the coef-

Fig. 1 Comparison of serum folate results from the AutoAnalyzer and the tube assay.

Fig. 2 Comparison of serum folate results from the radioassay and the tube assay.

Table 1 Variation of the control serum

Batch	Tube assay				AutoAnalyzer				Radioassay			
	N	Mean	SD	CV(%)	N	Mean	SD	CV(%)	N	Mean	SD	CV(%)
1	23	1.74	0.102	5.9	22	1.48	0.108	7.3	19	1.28	0.162	12.7
2	34	2.19	0.064	2.9	27	1.82	0.152	8.4	20	1.18	0.116	9.9
3	14	1.13	0.036	3.2	21	1.29	0.128	9.9	20	0.74	0.114	15.4
4	22	1.47	0.055	3.7	21	1.53	0.138	9.0	19	0.82	0.183	22.4
5	36	1.45	0.091	6.2	22	1.45	0.141	9.7	20	1.13	0.210	18.7
6	29	1.88	0.133	7.1	29	1.38	0.148	10.8	19	1.06	0.167	15.7
7	20	1.32	0.073	5.6	21	1.20	0.103	8.6	19	1.12	0.148	13.2
8	32	1.14	0.033	2.1	22	1.39	0.123	8.9	20	1.13	0.086	7.7
Overall mean and CV (%)		1.58		4.6		1.44		9.1		1.06		14.5
CV between the means of each batch (%)			24.3				12.9				17.4	

ficients of variation of the batch means by the three methods.

Comparison of cost

The time taken to perform the radioisotope method by the suggested protocol (Radiochemical Centre, 1976) and the tube assay were similar. Both methods involved a day's work from reception of the samples to calculation of the results although neither allowed the assay to be completed during one day. The practical size of a tube assay batch was potentially much larger than one with the radioisotope method as used. The automated method involved less than

half the technician time of the other two methods.

The cost of consumable materials used in assaying 100 samples (with necessary standards and controls) are set out in Table 2. The difference in the cost of consumables was small between the two microbiological methods. The cost of these methods was reduced by preparing medium in the laboratory. The cost per sample of using radioisotope kits was between 10 and 30 times greater than with the automated technique. A direct comparison of these techniques with the tube assay was not made because the cost of cleaning glassware was difficult to assess fairly.

The capital cost of installing these methods would

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Table 2 Cost of consumables in folate assays November 1976

Item	Cost in pence per 100 samples				
	Tube assay*		AutoAnalyzer*		Radio-assay
	BBL	UHW	BBL	UHW	
Assay medium	1000	91	333	31	—
Buffer	48	48	26	26	—
Ascorbate, Tween, chloramphenicol, TTC	8	8	13	13	9
Medium additions	—	—	84	84	—
Sampler tips	—	—	50	50	105
Maintaining inoculum	38	3	337	33	—
Folate assay kits	—	—	—	—	11000
Counting vials	—	—	—	—	37
Total cost per sample	10.9	1.5	8.4	2.4	111.5

*Assay medium = BBL Folic acid PGA broth (BBL) or medium prepared in the laboratory (UHW).

vary according to situation so that no precise estimate can usefully be made. However, it may be noted that while dilution and incubation equipment were relatively simple, facilities for the washing and preparation of glassware to a high standard were essential for successful results with the tube assay. The automated method was performed on basically standard AutoAnalyzer equipment and an automatic gamma counter was necessary for use with the radioisotope kit.

Discussion

The serum folate results were generally comparable using the three methods. The isotope method gave results which were somewhat lower than those of the microbiological methods. The trend shown by the slope of the regression line was borne out by the results from the control serum which gave consistently lower values with the radioassay. A minor part of this difference would be due to the 1/20 dilution of the sample by the sodium ascorbate solution in the radioassay. More fundamentally, differences in the competitive binding of the various forms of folate by the porcine serum binder (Mantzou *et al.*, 1974) made obligatory the use of a 5 methyl tetrahydropteroyl glutamic acid secondary standard; insufficient was supplied with the kits for a meaningful comparison to be made with the PteGlu standard used in the *L. casei* assays.

The inbatch variations of the manual methods reflect the number of dilution stages. They were lower in the tube assay with a single dilution stage than in the radioisotope method which has five dilution stages and a decanting of supernatant. The high variation between batch means in the tube assay

was probably due to differences in the inoculum cultures from day to day. Growing the inoculum in continuous culture exerts greater control over the physiological state of the organism, and the variation between batch means was consequently lower with the automated technique. The variation between batch means with the radioassay was, surprisingly, little less than with the tube assay. This may reflect differences between kits or in the way reagents were made up on different days.

Present interest in new methods of folate estimation is due partly to a belief that microbiological assays suffer from certain drawbacks, including poor between centres correlation, unreliability (particularly because growth of the organism is subject to inhibition by antibiotics), and high interassay variation. Wider understanding of the bacteriology of the assay systems and uniformity of factors such as the strain of organism employed (Tennant *et al.*, 1974) will help to eliminate differences in the results from laboratory to laboratory. The effect of antibiotics on the growth of *L. casei* is well known, and affected specimens can be identified, and in most cases correct answers obtained, by using a two-point parallel line assay (Tennant and Withey, 1972; Scott *et al.*, 1974). The problem of antibiotics has been greatly reduced by the automated method (Tennant, 1977).

It appears that the advantages of using radioisotopes to measure serum folate have been somewhat exaggerated. The time involved and the accuracy obtained are roughly comparable to those of a normal microbiological tube assay but the cost is considerably higher.

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Letter to the Editor

Assay of antibacterial drugs using *Bacillus stearothermophilus*

In the description of the rapid microbiological gentamicin assay (Wahlig and Holt, 1976) it is suggested that the *Bacillus stearothermophilus* method could have wide applications. It is also suggested that the method is simple, accurate, and fast in both technical time and result. We have investigated this method and do not agree.

Sixteen specimens of serum spiked with known amounts of gentamicin were prepared from either known potency material or were samples used in previous quality control surveys: they ranged from 1.25 mg/l to 20 mg/l. The specimens were assayed by the acetylase method (Haas and Davies, 1973) or by the method described by the instructions in the Merck Refobacin-Test.

Using the same analysis as used in the Public Health Laboratory Service quality control survey (Reeves and Bywater, 1975) the mean per cent error plus two standard deviations was 33.3 for the microbiological test and 21.3 for the acetylase method. Thus the microbiological method would fall into the 'poor' group.

There were other problems using the high temperature method: (a) there was a tendency for the media to dry out; (b) getting the plates to float was not very easy; (c) the high humidity led to excess moisture being present and on several occasions the discs were washed off; (d) we found that the method took two hours to perform before incubation and considerable technical expertise was needed to set it up; (e) in our hands, no growth was visible for four hours in comparison with a total time to perform the acetylase method of 1½-2 hours.

We feel that this method cannot be recommended. It has little or no advantage over the acetylase method or other rapid

microbiological techniques (Shanson and Hince, 1977).

R. WISE

J. M. ANDREWS

Department of Medical Microbiology,
Dudley Road Hospital,
Dudley Road,
Birmingham B18 7QH,
UK

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The authors have commented as follows:

It is difficult to account for the wide range of error in the results of Drs Wise and Andrews using the high temperature method described by us; in our individual hands and in those of several experienced colleagues in both Germany and the UK, much greater accuracy has been regularly attained during the performance of many hundred, even thousands, of clinical assays by this procedure.

We are also surprised by the technical difficulties met by Drs Wise and Andrews. In our hands the plates, plastic of course, float readily on water, growth appears quite obviously well within three hours, and during this time the plates do not become excessively damp nor do the discs float off. The length of preparation time required by these workers seemed to us very excessive and may be attributable to their unfamiliarity with this method. The obvious advantages of the high temperature method are not mentioned in their letter.

When writing this paper we recognized that the method and its variations reported by us would probably appear unorthodox and even heretical to some workers: microbiological assay procedures, of which we have between us over 50 years' continuous clinical experience, are notoriously subject to personal views and prejudices. Nevertheless we felt that a simple and rapid method, however unusual in technique, which gave reliable and accurate results in our and other experienced hands, should be available for workers in the field.

R. J. HOLT

Department of Microbiology,
Queen Mary's Hospital for Children,
Carshalton, Surrey, UK
HELMUT WAHLIG
E. Merck, Darmstadt,
Germany