J. clin. Path., 1971, **24**, 18-22

Use of the 'Analmatic clinical system' in the microbiological assay of vitamin B_{12} and folic acid in serum

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SYNOPSIS The routine estimation of large numbers of serum folic acid and vitamin B_{12} levels using the Baird and Tatlock[1] Analmatic clinical system is described and the method evaluated.

Precision, accuracy, and carryover are shown to be at acceptable levels, and the advantages and disadvantages of the use of the system are discussed.

It is considered that with some modifications the Analmatic system offers a reliable and labour-saving method of carrying out considerable numbers of routine microbiological assays.

The problem of the routine estimation of 7,000 serum folic acid levels and 7,000 serum vitamin B_{12} levels each year in this laboratory has led us to consider whether the application of techniques of automatic analysis, successful in the biochemical field, might have a place in this area of the laboratory. The present manual methods, using *L. casei* for the estimation of serum folic acid, have been modified from those described by Waters and Mollin (1961), and those using *L. leichmannii* for the estimation of vitamin B_{12} have been based on a modification of the method of Rosenthal and Sarett (1952) by Spray (1955).

Some reduction in the labour involved in these assays can be achieved by using the aseptic addition method of Herbert (1966) and the use of *L. casei* resistant to chloramphenicol (Davis, Nicol, and Kelly, 1970). In the first instance, however, it was decided to investigate automation of both the vitamin B_{12} and folic acid estimations, including protein precipitation, and using the normally sensitive lactobacillus. The analytical steps involved are more suited to a discrete analyser than to the continuous flow analysers more widely used in biochemistry. For this reason the Analmatic clinical system (Baird and Tatlock) was chosen although, in fact, the same machine could lend itself to techniques using chloramphenicol-resistant *L. casei*, and it is hoped to pursue further investigations of folic acid estimation by this method.

The Analmatic clinical system is a discrete serum analyser operating on the batch analysis principle. As used in the experiments which follow, it handles batches of 100 specimens, which are placed in the racks of the instrument in plastic cups. The equipment is modular and consists of a processing module in which the specimens are measured, diluted, treated with the appropriate reagents, and prepared for colorimetry. The reaction mixtures are then successively fed through the colorimeter which is of the double-beam type and the signals from the latter, after appropriate amplification and conversion, are printed out directly. The printer module is equipped with scaling and linearizing facilities so that the results may be printed out directly in concentration terms.

The particulars of each patient and the results of the microbiological assays are punched into cards and read into an IBM 1130 computer, which is programmed to store the information contained and to produce a report on a line printer. This report is sent back to the clinician concerned.

[1]Messrs Baird and Tatlock Ltd, Higham Lodge, Blackhorse Lane, London E.17.

Received for publication 13 July 1970.

Estimation of Serum Vitamin B_{12}

MATERIALS

Acetate-cyanide buffer

Five ml stock acetate buffer (0·4 M, *p*H 4·6) and 2 ml 0·1% sodium cyanide solution made to 100 ml with triple glass-distilled water.

B_{12} assay medium

Difco-Bacto B_{12} assay medium (USP).

Stock culture

A freeze-dried preparation of *L. leichmannii* (ATCC 7830).

Inoculum
Ten ml of Difco-Bacto-micro-inoculum broth was inoculated with one ampoule of the culture and incubated at 37°C for 24 hours. The culture was washed three times with sterile triple-distilled water and resuspended in triple-distilled water to a final optical density of approximately 0·04 using a 625 nm green filter. One drop of this suspension was used as inoculum.

Standards
Cytamen solutions were prepared in sterile triple-distilled water to contain 0, 125, 250, 375, 500, 625, 750, 1,000, 1,250, 1,500 pg/ml.

METHOD
Plastic sample cups were filled with 1·0 ml of test serum, two cups being used per patient. The first syringe was set to extract 0·5 ml serum from the sample cup, and the second syringe to deliver 4·5 ml acetate-cyanide buffer into the corresponding tube to dilute and mix thoroughly with the 0·5 ml of serum.

Standard solutions were prepared in the range 0-1,500 pg/ml and 1 ml of each solution was put in each of two sample cups and treated exactly as a test serum.

Tubes were capped and autoclaved at 15 psi for 2½ minutes to precipitate protein and centrifuged in a BTL Analmatic 100 centrifuge permitting a load of 100 tubes to be processed at one time. The third syringe was set to extract 1 ml supernatant from the tubes and deliver 4 ml B_{12} assay medium into another tube to dilute and mix with the supernatant.

Tubes were capped, autoclaved at 10 psi for 10 minutes, inoculated with 1 drop of *L. leichmannii* inoculum, and incubated for 24 hours at 37°C in the water bath.

After shaking the tubes were sampled automatically, their optical density estimated on the Analmatic colorimeter, and the results printed out on the automatic printer.

Duplication of each test in the trial allows estimations on only 40 patients, together with the appropriate standards, to be done on each rack.

For routine purposes single specimens could be estimated allowing 80 to 90 patients' sera per rack.

Estimation of Serum Folic Acid

MATERIALS
Phosphate buffer
0·1 M Sorensen phosphate buffer *p*H 6·1; ascorbic acid was added to give a final concentration of 100 mg%.

Folate assay medium
Single-strength folic acid assay pteroyl-glutamic acid broth, BBL Division of BioQuest, Maryland, USA.

Stock culture
A freeze-dried preparation of *L. casei* (ATCC 7469) was used.

Inoculum
Ten ml Difco-Bacto micro-inoculum broth was inoculated with one ampoule of culture and incubated at 37°C for 30 hours. The culture was washed three times in triple-distilled water and resuspended in triple-distilled water to a final O.D. of 0·06 using a 625 nm green filter. One drop of this suspension was used as inoculum.

Standard solutions
Highly purified crystalline pteroyl-glutamic acid was dissolved in an alkaline alcohol solution at *p*H 7-8 and stored in a refrigerator in the dark. Dilutions were made containing 1·25, 2·5, 3·75, 5·0, 7·5, 10·0, and 12·5 ng/ml.

METHOD
The method used was the same as for the estimation of serum vitamin B_{12}, using 0·5 ml serum and adding 4·5 ml phosphate buffer. After centrifugation 0·5 ml supernatant was added to 3·5 ml folate single-strength medium, and the tubes were then inoculated with one drop of the washed *L. casei* suspension. Standard solutions were prepared and treated exactly as the specimens.

Evaluation of Analmatic System for Microbiological Assays

CARRYOVER
Carryover can occur where solutions from different specimens take a common path. There are three points to be considered: (1) total carryover, (2) cross-contamination, and (3) sample diluent contamination.

Cross-contamination occurs when the sampling probe enters each cup of serum, some of which will adhere to the probe and may be deposited in the next specimen.

The sample can be contaminated by the diluent when a sample is drawn into the probe and is washed out by diluent into the reaction tube, but any diluent adhering to the probe may be deposited in the next sample cup.

Cross-contamination and sample contamination by the diluent are features of the sampling procedure of automatic analysing machines, therefore only this

procedure was tested and no comparison was made between manual and automated methods.

Determination of total carryover

Two pools of serum were used. Serum A had a high B_{12} value and serum B had a low value. The first three sample cups were filled with serum A and the second three sample cups were filled with serum B, that is, sera A1, A2, A3 and B1, B2, B3 with measured concentration a1, a2, a3, and b1, b2, b3. The carryover from specimen A3 to specimen B1 is given by $K = \frac{b1 - b3}{a3 - b3}$

(b3 is assumed to be the true value for serum B as it is preceded by two specimens of equal concentrations and the effect of carryover should be negligible).

B_{12} carryover

Ten tests were carried out by the manual and by the automated methods to estimate B_{12} concentration and the K values for carryover calculated.

	K Values	
	Automated	*Manual*
	0·1063	0·0419
	0·0482	0·0519
	−0·0060	0·0942
	0·0297	0·1194
	0·0350	0·0320
	0·0527	0·0654
	0·0138	0·3778
	0·0569	0·0484
	0·0000	0·0058
	0·0136	0·0599
Average	0·0403	0·0897

The mean carryover for the automated method was thus about 4% while that for the manual method was 9%. This was to be expected because in the case of the automated method the sample was taken into the sample probe and was washed out by the addition of buffer through the same tube. After precipitation and centrifugation the supernatant is taken up and washed out by medium. The transmission tubing in the system is made of a PTFE material with non-wetting properties to minimize carryover.

In the manual method, however, the same pipette was used for sampling serum, and the buffer was added later. In the same way supernatants were sampled using the same pipette and were added to tubes already containing media.

The automated method is, therefore, potentially more accurate than the manual. It also eliminates human error by the additions of exact preselected volumes.

Folic acid carryover

Ten tests for folic acid were carried out by both methods, and the K values calculated.

	K Values	
	Automated	*Manual*
	0·0426	0·0254
	0·0000	0·0000
	0·0186	0·0000
	0·0261	0·1579
	0·0417	−0·0357
	0·0857	0·0574
	−0·0294	0·0435
	0·0000	0·1477
	0·0286	0·0865
	0·0000	0·0426
Average	0·0305	0·0654

Thus, to express this in perhaps simpler terms, the mean carryover in the automated method is of the order of 3%, while in the manual method this is doubled.

Cross-contamination

This was measured using a radioisotope method because of its accuracy, ease of operation, and its sensitivity. The object was to measure the cross-contamination from one sample cup to the next. Pooled serum with added human serum albumin labelled with ^{131}I was used; 1 ml serum was placed in each cup thus: cup no. 1* 2* 3* 4 5 6 7* 8* 9* 10 etc (*radioactive serum). The sampling procedure was carried out in the usual way. The contents of cups 4 and 10 and tubes 3 and 9 were retained for counting. The contents of cups 4 and 10 were made up to the same volume as tubes 3 and 9, ie 5 ml, to avoid geometric errors in counting.

Tube No.	*Count/20 Min in Tubes*	*Cup No.*	*Count/20 Min in Cups*	*Cross-contamination*
3	716×10^3	4	905	$6{\cdot}317 \times 10^{-4}$ ml
9	752×10^3	10	534	$3{\cdot}479 \times 10^{-4}$ ml
15	738×10^3	16	1043	$7{\cdot}063 \times 10^{-4}$ ml

$$\text{Cross-contamination} = \frac{\text{activity in cup 4}}{\text{activity in tube 3}} \times \text{vol sample dispensed.}$$

Vol specimen in sample cup = 1 ml
Depth of probe in specimen = 10 mm
Vol sample taken = 0·5 ml
Dilution = 1/10

Sample diluent contamination

One ml serum was placed in each of 10 sample cups. Acetate-cyanide buffer labelled with $Na^{131}I$ was used as diluent. The sampling procedure was carried out. The contents of all the cups and tubes were measured for radioactivity. The contents of the cups were made up to 5 ml to avoid geometric errors in counting.

	Count/20 Min in Tubes	*Count/20 Min in Cups*	*Sample Diluent Contamination*
1	$139{\cdot}9 \times 10^4$	216	$6{\cdot}947 \times 10^{-4}$ ml
2	$1{,}427 \times 10^4$	65	$2{\cdot}046 \times 10^{-4}$ ml
3	$1{,}449 \times 10^4$	25	$0{\cdot}7762 \times 10^{-4}$ ml
4	$1{,}454 \times 10^4$	107	$3{\cdot}311 \times 10^{-4}$ ml
5	$1{,}487 \times 10^4$	15	$0{\cdot}4538 \times 10^{-4}$ ml
6	$1{,}485 \times 10^4$	60	$1{\cdot}818 \times 10^{-4}$ ml
7	$1{,}449 \times 10^4$	28	$0{\cdot}8716 \times 10^{-4}$ ml
8	$1{,}461 \times 10^4$	51	$1{\cdot}687 \times 10^{-4}$ ml
9	$1{,}476 \times 10^4$	104	$3{\cdot}171 \times 10^{-4}$ ml
10	$1{,}481 \times 10^4$	106	$3{\cdot}221 \times 10^{-4}$ ml

Volume serum in cups = 1 ml
Depth of probe in specimen = 10 mm
Volume sample taken = 0·5 ml
Dilution = 1/10

The above figures show that cross-contamination and sample diluent contamination are extremely small.

PRECISION

Precision defines the 'scatter' to which any particular result may be subject. In this study this was evaluated by the technique outlined by Broughton, Buttolph, Gowenlock, Neill, and Skentelbery (1969).

Precision of vitamin B_{12} estimation

Three pooled sera were used of low, medium, and high values of B_{12}. Twenty repeat analyses were carried out by both methods over a period of 10 days. The samples were placed at random among the daily batch. The standard deviations from mean were calculated (Table I).

Except for the case of low values of B_{12}, the coefficient of variation and standard deviations from the means are less for the automated method than for the manual. These figures suggest that the automated method is at least as precise as the manual.

Precision of folic acid estimation

Three pooled sera were used and analysed over a period of 10 days by both methods (Table II).

The coefficient of variation and the standard deviations from the mean are less for the automated method than for the manual, except at the medium concentration where no significant difference is shown.

ACCURACY

Accuracy is a measure of the ability of the method to reproduce the correct result for a specimen. In this study this was evaluated using specially prepared dilutions of the absolute standard used in the

	Low B_{12}		*Medium B_{12}*		*High B_{12}*	
	Automated	*Manual*	*Automated*	*Manual*	*Automated*	*Manual*
Mean	278·25	276·25	613·25	561·75	981·0	965·75
SD	64·03	51·96	90·0	108·6	140·7	208·8
Coefficient of variation	22·79%	18·8%	14·66%	19·33%	14·34%	21·62%

Table I *Vitamin B_{12} estimations*

Time span = 10 days
No. of analyses = 20

	Low Folic Acid		*Medium Folic Acid*		*High Folic Acid*	
	Automated	*Manual*	*Automated*	*Manual*	*Automated*	*Manual*
Mean	3·76	3·30	6·51	6·92	17·85	15·60
SD	0·4662	0·7115	1·283	1·339	2·508	3·972
Coefficient of variation	12·4%	21·67%	19·71%	19·35%	14·06%	25·63%

Table II *Folic acid estimations*

Time span = 10 days
No. of analyses = 20

Serum	*Result from Laboratory A (ng/ml)*	*Result from Laboratory B (ng/ml)*	*Automated Result (ng/ml)*	*Manual Result (ng/ml)*
8672	6·8	4·0	10·0	13·8
8686	7·2	7·5	8·2	13·0
Wellcomtrol	7·2	—	9·8	11·5

Table III *Accuracy of folic acid estimation*

methods themselves, and commercial sera to which values have been ascribed by the manufacturers.

Accuracy of folic acid estimation

Wellcomtrol sera were analysed for folic acid by both methods (Table III).

All values for folic acid on the control sera were higher than the values given by the manufacturers. However, there is quite a variation in results from laboratories A and B, especially in the case of serum 8672. The values for the sera analysed by the automated methods are closer to those stated for the control sera and for this reason this method can be regarded as more accurate than the manual method in our hands.

Accuracy of vitamin B_{12} estimation

Standard solutions were prepared containing known concentrations of cytamen—245, 500, and 1000 pg/ml—and these were assayed by both methods.

Solution	*Automated (Average)*	*Manual (Average)*
245 pg/ml	235 pg/ml	260 pg/ml
500 pg/ml	450 pg/ml	600 pg/ml
1000 pg/ml	1070 pg/ml	1200 pg/ml

These small series of tests suggest that the automated method for assaying B_{12} gives results which are not less accurate than those obtained by the manual method.

One hundred specimens of patients' sera were assayed for B_{12} and folic acid by the manual and automated methods. The results by the two methods were compared by calculating correlation coefficient and drawing regression lines.

There was a good correlation between B_{12} concentrations measured by the manual method and by the automated method ($r = 0{\cdot}9384$; $y = 0{\cdot}9485x + 0{\cdot}2900$). There was also good correlation between folic acid concentrations measured by both methods ($r = 0{\cdot}9696$; $y = 0{\cdot}9170x + 0{\cdot}3301$).

Discussion

A system capable of performing sampling and dilution mechanically is potentially capable of improving the precision and accuracy of microbiological assays. The Baird and Tatlock Analmatic system is one in which all samplings and dilutions are carried out automatically by the machine. The assessments have shown that the potential improvement is realized and that the automated method is more accurate than the manual one. Carryover from one specimen to the next is reduced and the precision of the pipetting shows the advantages of mechanical reproducibility. The values estimated by the automated technique correlate closely with those obtained by manual methods, and the Analmatic system can thus be said to have much to contribute in this field of analysis.

There are, however, several drawbacks using this system, notably the following:

(*a*) The speed of sampling on this system was slow. However, since this work was completed a simple adaptor has been provided to allow two rows to be sampled at once, thus increasing the operating speed.

(*b*) The tubes must be shaken thoroughly before the optical density can be read. This has to be done by corking the tubes and shaking manually. Gentle agitation of the racks is not sufficient to resuspend the growth of Lactobacillus from the bottom of the tubes, especially when growth is heavy. Shaking during incubation would result in a much reduced growth of Lactobacillus, as this organism requires relatively anaerobic conditions which do not obtain during shaking.

(*c*) Since in this laboratory a normal batch consists of 150 to 200 specimens as well as standards, larger racks containing perhaps 200 tubes would simplify operations, especially if tests are not carried out in duplicate. This would require a larger water bath for incubation. Many laboratories, however, may find the existing capacity sufficient.

With these modifications the Analmatic machine offers a reliable method of dealing with considerable numbers of microbiological assays with acceptable accuracy and precision, and a considerable saving in labour.

This work was carried out with the assistance of a Royal Victoria Hospital research fellowship.

The prototype Analmatic clinical system used in this work was generously presented by Messrs Baird and Tatlock Ltd.

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