A microbiological assay for the measurement of methotrexate in biological fluids

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SUMMARY A microbiological assay for the measurement of methotrexate has been designed, using a chloramphenicol resistant mutant of *Lactobacillus casei* as the test organism. The method is simple, precise and inexpensive. Results are not significantly different from those obtained using a methotrexate radioassay kit employing a competitive protein binding technique. However, where patients are being treated with co-trimoxazole, the competitive protein binding technique is unsuitable for measuring methotrexate levels due to the cross reactivity of the binder. This is not a problem with the microbiological assay described here, which is able to measure methotrexate in the presence of large doses of trimethoprim.

The methods were not affected by the presence of seven commonly encountered antibiotics. These had negligible effect on either method up to concentrations of 100 mg/l.

Methotrexate, 4-amino-10 methyl pteroylglutamic acid, is a cytotoxic drug which has wide clinical use in the treatment of neoplastic disease.¹⁻⁴ It is also considered a most effective agent in the treatment of severe psoriasis.⁵ Methotrexate achieves its cytotoxic effect by acting as a spurious substrate for dihydrofolate reductase which prevents the reduction of dihydrofolate to tetrahydrofolate.⁶ This in turn blocks the formation of 5, 10-methylene tetrahydrofolate and thus synthesis of thymidine and DNA. Recently there has been extensive use of high dose methotrexate therapy followed by rescue with 5-formyl tetrahydrofolate (folinic acid). This means that high concentrations of the drug can be attained with free intracellular methotrexate in the rapidly dividing tumour cells which ensures the maximum cytotoxic effect. 5-formyl tetrahydrofolate can be metabolised to 5, 10-methylene tetrahydrofolate without the need for dihydrofolate reductase and is therefore able to bypass the block.

The two most severe side effects of high dose methotrexate treatment are myelosuppression and renal failure which have been reported to have a 6% mortality.^{7,8}

The use of high dose methotrexate requires careful monitoring of the patient's serum concentrations of the drug and a number of methods have been designed for this purpose.⁹⁻¹³ One commonly used method is based on competitive protein binding,¹²

but this suffers from cross reactivity with drugs such as trimethoprim that bind strongly to the bacterial dihydrofolate reductase used in the assay.

There are occasions when patients are treated with both methotrexate and trimethoprim (co-trimoxazole) and in these patients the competitive protein binding assay gives erroneous results. Although the manufacturers of the kit claim a cross reactivity of only 15%, in our hands the cross reactivity has been much higher than this.

We describe here a microbiological method for the measurement of methotrexate using a chloramphenicol resistant strain of *Lactobacillus casei* (ATCC 7469) as the test organism which is simple, precise and unaffected by the presence of trimethoprim.

Material and methods

The method is essentially a modification of the automated microbiological folate method described by Davis *et al*¹⁴ and utilises the same chloramphenicol resistant strain of *L casei* as the test organism, and the same assay medium.

STANDARDS

A stock solution was prepared by taking 1 ml of methotrexate solution (Lederle) containing 1 mg/ml and diluting this to 1000 ml (1 mg/l) with distilled water. This solution was found to be stable at 4°C for at least six months. On the day of the test the

Table 1 Preparation of methotrexate standards for the microbiological assay

Distilled water (ml)	5.0	4.75	4.62	4.5	4.25	4.0	3.75	3.5	3.0	2.5
Methotrexate working solution (ml)	0	0.25	0.38	0.5	0.75	1.0	1.25	1.5	2.0	2.5
Concentration (ng/l)	0	50	75	100	150	200	250	300	400	500
or (nmol/l)	0	0.11	0.17	0.22	0.33	0.44	0.55	0.66	0.88	1.1

stock solution was further diluted 1/1000 giving a concentration of 1 µg/l. This working solution was used to prepare the final standard solutions as shown in the protocol (Table 1).

CONTROL SERUM

A serum control was prepared by adding a known quantity of methotrexate to pooled sera. The sample was divided into aliquots and lyophilised. On the day of the assay it was reconstituted with distilled water and suitable dilutions made.

ANTIBIOTICS

The sensitivity of the chloramphenicol resistant *L. casei* was tested against a series of methotrexate control samples, to which, the following antibiotics were added at a concentration of 100 mg/l: cefotaxime, streptomycin, tetracycline, amoxycillin, benzylpenicillin and gentamicin. These samples were also tested by the isotopic assay.

Aqueous solutions of the same antibiotics at concentrations of 10 mg/l were treated as unknown samples and assayed by the microbiological assay.

To one methotrexate control serum trimethoprim was added to a concentration of 4 mg/l and assayed by both methods.

LEUCOVORIN (LEDERLE "FOLINIC ACID")

Various concentrations of leucovorin were added to aqueous methotrexate solutions containing 10, 1 and 0.1 µmol of methotrexate respectively. These were assayed by both methods.

ISOTOPIC ASSAY KIT

The competitive protein binding kit was obtained from the New England Enzyme Centre, Boston, US and was used in accordance with the manufacturer's recommendations.

PROCEDURE FOR THE MICROBIOLOGICAL ASSAY

A suitable quantity of double strength assay medium¹⁴ was diluted with an equal volume of distilled water. To each litre of single strength medium was added 10 mg chloramphenicol, 0.6 ml of an overnight culture of the test organism with an absorbance of 0.7, and 7.5 ml of 2 µg/l folic acid solution. The inoculated medium was mixed continuously on a magnetic stirrer.

Suitable dilutions were made of the test sera in

distilled water. These will vary depending on the dosage of methotrexate administered and the period of time elapsed since the last dose. We have found that a range of dilutions from 1/50 through to 1/10 000 satisfactory, however where very large doses of methotrexate are used, higher dilutions may be necessary.

Fifty microlitres of the methotrexate standards, the serum dilutions and the control serum dilutions (in duplicate) were placed into plastic disposable tubes and diluted 1/100 by adding 4.95 ml of the prepared medium to each and then incubated at 37°C for 16 h. After incubation, the contents of the tubes were mixed and the absorbance read in a spectrophotometer fitted with a flow cell.

Calculation

- (i) From the absorbance of the standards the lowest methotrexate concentration which inhibits the growth of the test organism was determined.
- (ii) Similarly the highest dilution in the test and control tube at which inhibition occurs was found.
- (iii) Multiplying the concentration in (i) above by the serum dilution obtained in (ii) gave the result in

Regression line of competitive protein binding assay against microbiological assay

ng/l. This can be converted to nmol/l by multiplying by 2.2/1000.

Results

Sixteen sera and three CSF samples from hospital patients being treated with methotrexate were assayed by both methods and the results are shown in Table 2. A regression line of competitive protein binding assay against microbiological assay is shown in the Figure. The correlation coefficient was 0.99 (<0.001). The mean of the microbiological assay was 511.3 nmol/l and the mean of the isotopic assay was 513.8 nmol/l. There was no significant difference between the means (paired t test, $t = 0.64$).

The methotrexate control sample to which trimethoprim had been added was assayed by both methods, as were serum samples from four patients being treated with trimethoprim (Septrin) and methotrexate. Results are shown in Table 3.

The results of the methotrexate control samples to which the various antibiotics were added are listed in Table 4. Aqueous solutions at concentrations of 10 mg/l of the antibiotics showed inhibition equivalent to <1 nmol/l of methotrexate when assayed by the microbiological assay.

Methotrexate concentrations of 10 μ mol were shown to be unaffected by up to 250 mg/l of leucovorin in either method. Similarly neither method was affected by 100 mg/l of leucovorin with methotrexate concentrations of 1 μ mol, or 0.1 μ mol.

One sample was assayed 20 times in 5 different batches by the microbiological method. Results

Table 2 Comparison of results from two methods for measuring methotrexate

		Microbiological assay (nmol/l)	Competitive protein binding assay (nmol/l)
Sera	1	112.7	90.2
	2	28.2	24.2
	3	2.6	3.5
	4	101.0	110.0
	5	10.6	13.2
	6	21.1	20.9
	7	197.1	176.0
	8	7.0	8.3
	9	56.3	55.0
	10	3.2	5.2
	11	12.3	12.5
	12	154.0	130.9
	13	24.6	28.8
	14	1815.0	1793.0
	15	536.3	475.0
	16	3.3	4.0
CSF	1	10.6	18.7
	2	1460.0	1518.0
	3	11.0	14.5

Correlation coefficient 0.99 ($p < 0.001$).

Table 3 Comparison of results from two methods for measuring methotrexate in the presence of trimethoprim

Sample	Microbiological assay (nmol/l)	Competitive protein binding assay (nmol/l)
1	66	1144
2	308	1188
3	193	880
4	58	660
Control serum A	98	101
Control serum B	96	924

Both control sera contained 100 nmol/l methotrexate and in addition control sera B contained 4 mg/l trimethoprim.

ranged from 275–344 nmol/l, with a mean of 308.9, SD of 24.6 and coefficient of variation of 7.9%.

Discussion

The concurrent treatment with co-trimoxazole and methotrexate in some patients can create difficulties in monitoring methotrexate concentrations by protein binding methods. The cross reactivity of trimethoprim with the protein binding assay was found to be considerably greater than the 15% claimed by the manufacturer (Table 3). Trimethoprim was added to a methotrexate control serum at a concentration of 4 mg/l. This concentration was used as it is representative of peak serum concentrations of trimethoprim found in patients receiving co-trimoxazole. An expected result of 100 nmol/l of methotrexate yielded a recovery of 96% by the microbiological assay and 924% by the protein binding assay. Serum from patients on both methotrexate and co-trimoxazole demonstrated similar discrepancies with the protein binding assay (Table 3).

Kits based on competitive binding for measuring methotrexate which use a dihydrofolate reductase derived from bacteria can be expected to give spuri-

Table 4 Comparison of results from two methods for measuring methotrexate in the presence of various antibiotics

Antibiotic	Microbiological assay (nmol/l)	Competitive protein binding assay (nmol/l)
Cefotaxime	99	100
Tetracycline	99	98
Streptomycin	99	100
Benzyl penicillin	111	108
Amoxil	99	95
Gentamicin	110	99
Trimethoprim	96	924

All samples contained 100 nmol/l of methotrexate and 100 mg/l of each antibiotic, except for trimethoprim which was used at a concentration of 4 mg/l.

ous high results in the presence of normal therapeutic levels of trimethoprim. Trimethoprim is specifically directed against the bacterial dihydrofolate reductase used as a binder in this assay and competitive binding protein kits are therefore unsuitable for use in the presence of this agent.

Aqueous solutions of seven commonly encountered antibiotics at concentrations equivalent to peak serum levels had no inhibitory effect on the microbiological assay.

L casei appeared to be particularly sensitive to inhibition by methotrexate and the method was found to be capable of detecting as little as 0.5 nmol of this drug. The serum dilutions required for the microbiological assay result in concentrations of trimethoprim and other antibiotics tested which were too low to affect growth of the test organisms (Table 4). In a preliminary study, the addition of leucovorin to a limited range of methotrexate concentrations revealed similar results by both methods with only limited interference seen in the lower methotrexate range.

In the lower range results were marginally less than those obtained by the competitive protein binding assay (Figure); however there was no statistically significant difference between the means of a series of samples (Table 2).

The use of a chloramphenicol-resistant strain of *L casei* as the test organism, greatly simplified the procedure and had the advantage that aseptic manoeuvres were not necessary, which permits the use of automated diluting systems. The microbiological inhibition assay using *L casei* has been used in this laboratory for over three years.

It takes longer to obtain results using this method than the radioisotope dilution assay as overnight incubation is required. However, the microbiological assay has been shown to give valid results of methotrexate and unlike the radioisotope dilution assay is unaffected by the presence of trimethoprim. The test is cheap and can be run for approximately one sixth of the cost of the radioisotope dilution assay in our laboratory.

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References

- ¹ Djerassi I, Abir E, Rover GL, Treat CL. Long term remissions of childhood acute leukaemia: Use of infrequent infusions of methotrexate; supportive roles of platelet infusions and citrovorum factor. *Clin Pediatr* 1966;**5**:502-9.
- ² Jaffé N, Frei E, Watts H, Traggis D. High dose methotrexate in osteogenic sarcoma: A 5-year experience. *Cancer Treat Rep* 1978;**62**:259-64.
- ³ Truman S, Coleman M, Silver RT. Treatment of resistant phase lymphoma with "high dose" methotrexate and folinic acid rescue. *Proc Am Assoc Cancer Res* 1976;**17**:302.
- ⁴ Djerassi I, Rominger CJ, Kim JS, Turchi J, Suvansri U, Hughes D. Phase 1 study of methotrexate with citrovorum factor in patients with lung cancer. *Cancer* 1972;**30**:22-6.
- ⁵ Baker H, Fry L, Pegum JS. In: Rook A, ed. *Recent advances in dermatology*. London: Churchill-Livingstone, 1973.
- ⁶ Paxton JW. High dose methotrexate therapy—An area of uncertainty. *Aust NZ J Med* 1979;**9**:722-32.
- ⁷ Von Hoff DD, Penta JS, Helman LJ, Slavik K. Incidence of drug related deaths secondary to high-dose methotrexate and citrovorum factor administration. *Cancer Treat Rep* 1977;**61**:745-7.
- ⁸ Pitman SW. Clinical trial of high-dose methotrexate with citrovorum factor. Toxicological and therapeutic observations. *Cancer Chemother Rep* 1975;**62**:381-3.
- ⁹ Kinkade JM, Vogler WR, Dayton PG. Plasma levels of methotrexate in cancer patients as studied by an improved spectrophotofluorometric method. *Biochem Med* 1974;**10**:327-50.
- ¹⁰ Noble WC, White P, Baker H. Assay of therapeutic doses of methotrexate in body fluids of patients with psoriasis. *J Invest Dermatol* 1975;**64**:69-76.
- ¹¹ Raso V, Schreiber R. A rapid and specific radioimmunoassay for methotrexate. *Cancer Res* 1975;**35**:1407-10.
- ¹² Myers CE, Lippman ME, Eliot HM, Chabner BA. Competitive protein binding assay of methotrexate. *Proc Natl Acad Sci (Wash)* 1975;**72**:3683-6.
- ¹³ Falk FC, Clark DR, Kalman SM, Long TF. Enzymatic assay for methotrexate in serum and cerebrospinal fluid. *Clin Chem* 1976;**22**:785-8.
- ¹⁴ Davis RE, Nicol DJ, Kelly A. An automated method for the measurement of folate activity. *J Clin Pathol* 1970;**23**:47-53.

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