An enzyme inhibition assay for 2,4-diamino-5-(3'4'-dichlorophenyl)-6-methylpyrimidine (DDMP, NSC 19494)

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SUMMARY An enzyme inhibition method for the determination of serum levels of DDMP is described. This has proved to be a simple, practical, and reliable method for the clinical monitoring of patients.

DDMP is one of several diaminopyrimidines which inhibit the enzyme dihydrofolate reductase (DHFR, EC 1.5.1.4) (McCormack and Jaffe, 1969). It has antitumour activity in man and is currently under trial in several centres for the treatment of various tumours including hypernephroma, carcinoma of the bronchus, and intracerebral tumours (Murphy *et al.*, 1954; Price *et al.*, 1975; Price and Hill, 1976; Miller *et al.*, 1976). Experimental evidence suggests that an antitumour effect is unlikely unless the extracellular DDMP level is around 10^{-5} M (Hill *et al.*, 1973). This produces severe bone marrow suppression in man (Miller *et al.*, 1976) which can be prevented by the administration of folinic acid (leucovorin, NSC 3590) (Price *et al.*, 1975; Price and Hill, 1976; Miller *et al.*, 1976). DDMP also causes skin rashes, headaches, nausea, and dyspepsia in some patients (Murphy *et al.*, 1954; Miller *et al.*, 1976), effects which appear to be independent of its effect on folate metabolism. The drug has a long and variable duration of action with a reported half-life of 55 hours in dogs (Stickney *et al.*, 1973) which may cause accumulation and side effects related to the serum level (Miller *et al.*, 1976). It is, therefore, desirable to monitor serum levels of the drug during treatment, both to ensure that a therapeutic level is being achieved and also to show whether any untoward symptoms are likely to be drug-induced.

Assays for determining the serum levels of DDMP have been described using a solvent extraction followed by: (a) thin layer chromatography and fluorimetric scanning (Simmons and DeAngelis, 1973); (b) thin layer chromatography and UV

absorbance scanning (DeAngelis *et al.*, 1975); and (c) gas liquid chromatography (Cridland and Weatherley, 1977). All of these methods give good results but need a fairly elaborate workup of clinical samples, and equipment which is expensive and not universally available. The purpose of this paper is to describe an enzyme inhibition assay which can be performed using simple equipment without an undue loss of sensitivity. The method is essentially a modification of that of Bertino and Fischer (1964) for the assay of methotrexate, which exploits the DHFR-catalysed reduction of dihydrofolate to tetrahydrofolate in a NADPH-linked reaction (Blakley, 1969). The addition of DHFR inhibitors to the assay system leads to a reduction in rate which is related to the amount of inhibitor added.

Material and method

The K_i of methotrexate for DHFR is approximately 10^{-13} M (Jackson *et al.*, 1976), so that the inhibition of the enzyme is approximately stoichiometric until the inhibited rate is reduced to about 20% of the uninhibited rate. In the case of DDMP, the K_i estimated in the system described below by the method of Dixon (1953) is approximately 10^{-9} M. The inhibition is competitive with dihydrofolic acid, and a straight line calibration curve is obtained by plotting the amount of inhibitor added versus the reciprocal of the initial reaction rate ($1/v$) (Dixon, 1953).

The degree of inhibition of DHFR produced by an equimolar aqueous solution of DDMP is greater than that produced by an equimolar solution in human serum, probably due to protein binding of the drug. This factor is allowed for in the assay method by one of two equivalent methods:

(A) The calibration curve can be set up using pretreatment serum as diluent for the DDMP standards. (B) If no pretreatment serum is available, the calibration curve may be made with a standard DDMP solution in water or serum and an individual correction made for each patient by the following method:

The serum sample is assayed directly and the apparent level (x $\mu\text{g/ml}$) determined. 10 μl of aqueous DDMP solution (100 $\mu\text{g/ml}$) is then mixed with 990 μl of the sample, which is re-assayed (level y $\mu\text{g/ml}$). In the absence of protein binding the difference in level ($y - x$) would be expected to be 1 $\mu\text{g/ml}$. Hence the true level of DDMP in the original sample is $x/(y - x)$ $\mu\text{g/ml}$. This method depends on the proportion of free DDMP in the assay system being constant over the range of levels encountered. It is shown below that this is the case.

MATERIAL

All reagents were of AnalaR grade and were purchased from BDH Limited, Poole, Dorset, UK or from Hopkin and Williams Limited, Romford, Essex, UK. DDMP was kindly supplied by Dr A. Bye of the Wellcome Foundation. Dihydrofolic acid was obtained from Sigma, London Limited and NADPH from Boehringer Mannheim, Lewes, Sussex, UK. DHFR was prepared from rat livers by the method of Bertino and Fischer (1964). This preparation was stable for up to 12 months at -40°C .

METHOD

Serum samples were obtained from patients and stored at -20°C until assayed.

Reagents

- A DDMP stock solution, 1 mg/ml (3.7 mmol/l), in 3.7 mmol/l lactic acid. This solution is diluted 1 in 500 before use.
- B Dihydrofolic acid, 1 mg/ml (2.3 mmol/l), in 0.25M β -mercaptoethanol (just sufficient 0.1M sodium hydroxide is added to dissolve).
- C NADPH, 2 mg/ml (2.4 mmol/l), aqueous solution.
- D Potassium phosphate buffer 0.15M pH 7.
- E DHFR preparation (specific activity 5×10^{-8} IU/ml, protein content 4.5 mg/ml).

Reagents B and C are made up on the day of use and stored on ice. Dihydrofolic acid should be protected from light. The following components are then added to a 1 cm cuvette:

Potassium phosphate buffer	2.4 ml
NADPH solution	0.1 ml
DHFR	0.1 ml (or a sufficient volume to

give 5×10^{-9}
IU of activity)

Sample (standard solution or undiluted serum) 0.1 ml

The absorbance at 340 nm is monitored in a Cary 16 recording spectrophotometer at 30° in a thermostatted cell housing (full scale deflection of 0.1 absorbance units) for 5-10 minutes, which gives a rate r_1 . This rate is due to oxidation of NADPH by endogenous components in the assay system. Dihydrofolic acid solution (0.1 ml) is then added as the substrate, and the rate is again measured (r_2). The rate of reaction due to DHFR (r_3) is then given by $r_3 = (r_2 - 0.964 r_1)$. The factor 0.964 is to correct for the change in volume in the cuvette on the addition of the dihydrofolic acid. The amount of DDMP added (in the range 0-200 ng) is then plotted against $1/r_3$ to produce a standard curve, and the levels of the unknowns are derived as already described.

Results

This method gives a linear standard curve, an example of which is shown in Figure 1. The coefficients of variation for the 0, 0.5, 1.0, 1.5, and 2.0 $\mu\text{g/ml}$ points are 4.3, 3.0, 2.0, 3.3, and 5.7%, respectively, giving a mean value of 3.7% and an expected accuracy of approximately $\pm 9\%$ using duplicate assays.

The linearity of the binding of DDMP is shown in Figure 2. Dilutions of DDMP were made in serum to give total levels of up to 2.5 $\mu\text{g/ml}$. The apparent level (x) was determined from a standard curve made

Fig. 1 A specimen standard curve for the enzyme inhibition assay. Means of 10 determinations are shown for each point. The error bars indicate ± 1 standard deviation.

Fig. 2 The apparent level (y) determined from dilutions of DDMP in the serum of a patient showing a linear relationship with the total level (x): $y = 0.558x + 0.015$; correlation coefficient = 0.995.

with aqueous dilutions. The linearity has been shown to extend up to at least 4 µg/ml in those patients in whom higher levels were encountered.

Validation: A total of 44 clinical samples from seven patients have been assayed both by this method and by solvent extraction followed by gas-liquid chromatography (Cridland and Weatherley, 1977). The linear regression analysis is as follows:

$$y = 0.91x + 0.09$$

$$r = 0.98$$

$$p = 0.01$$

where y = level determined by gas-liquid chromatography, x = level determined enzymatically, and r = correlation coefficient.

An illustration of the correlation between the two methods is shown in Figure 3.

Discussion

This paper describes a simple and reliable method for determining serum levels of DDMP. It exhibits an adequate degree of reproducibility and is particularly suited for patient monitoring in those centres where sophisticated equipment is not available, any recording spectrophotometer with adequate sensitivity being suitable. It has the disadvantage that any inhibitor of dihydrofolate reductase would affect the assay, and in particular any active metabolite of DDMP might be detected. These disadvantages do not seem to be important in practice, no significant differences being observed from the gas-liquid chromatography method even in patients who had been undergoing treatment for more than a year. It is possible that the same method might be satisfactory also for other diaminopyrimidines (eg, pyrimethamine or 2,4-diamino-5-(3',4'-dichloro-

Fig. 3 A comparison of serum DDMP levels determined by gas-liquid chromatography (x) and by enzyme inhibition (y) in successive samples taken from one patient over a period of four weeks: $y = 0.859x + 0.95$; correlation coefficient = 0.93.

phenyl)-6-ethylpyrimidine, DDEP) or for trimethoprim (using bacterial DHFR).

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