Microbiological Assay for Purine Analogues with *Lactobacillus casei*

By M. J. HARBER AND J. L. MADDOCKS

*K.R.U.F. Institute of Renal Disease, Welsh National School of Medicine,
The Royal Infirmary, Cardiff*

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INTRODUCTION

Hitchings *et al.* (1950) utilized the growth requirements of *Lactobacillus casei* in their search for antimetabolites that block nucleic acid synthesis. 6-Mercaptopurine (6-MP) markedly inhibited the growth rate of *L. casei* and possessed powerful antitumour activity (Skipper, 1954; Bieseke, 1954) and immunosuppressive properties (Schwartz, Stack & Dameshek, 1958; Calne, 1960). Azathioprine is an imidazole derivative of 6-MP which is rapidly converted to 6-MP *in vivo* by a non-enzymatic hydrolysis in the presence of sulphhydryl groups (Elion, Callahan, Bieber, Hitchings & Rundles, 1961). Both 6-MP and azathioprine are used extensively in the treatment of patients with malignant disease, organ transplants and auto-immune disorders. Drug dosage is usually adjusted according to the peripheral white cell count because no method of assay is available for estimating therapeutic blood levels. In this communication we present a microbiological method of estimating nanogram quantities of azathioprine and 6-MP in aqueous solution.

METHODS

Basal medium. A modification of the folic acid assay medium described by Waters & Mollin (1961) was used. Excess folic acid (20 ng/ml) was included in the medium but purines and pyrimidines were omitted. Buffering capacity was increased by addition of 0.2 M-Sørensen's phosphate buffer, pH 6.6 (500 ml/l), and acid-hydrolysed vitamin-free casein (Difco) was used instead of enzyme-hydrolysed casein. A slight precipitate appeared on autoclaving but cleared on cooling.

Lactobacillus casei ATCC 7469 was maintained on agar slopes prepared by adding a mixture of 5 mg/l of each of adenine sulphate, guanine hydrochloride and uracil to the single-strength medium solidified with 2 % (w/v) Bacto-Agar (Difco). A stock liquid culture in 10 ml of single-strength medium with added purines and pyrimidines was sub-cultured daily. The inoculum was prepared by washing the bacteria from a 24 h broth culture twice with sterile NaCl (0.9 %, w/v) and finally resuspending in NaCl to E_{560} of 0.05 units.

Standard solutions. Imuran azathioprine for injection (Burroughs Wellcome & Co., Dartford, Kent) in vials containing the equivalent of 50 mg free drug as the sodium salt was used. Mercaptopurine B.P. (6-MP; 100 mesh B.S.S.) was obtained from Burroughs Wellcome & Co. Due to the limited solubility of both drugs in water, 50 mg of each was dissolved in 20 ml of 0.1 M-NaOH and then diluted immediately to 500 ml with distilled de-ionized water to give 100 µg drug/ml. Stock aqueous solutions of azathioprine and 6-MP in the range 20 to 2000 ng/ml were prepared. These solutions were stored at 4 °C and used within 24 h of preparation.

Fig. 1. Growth inhibition of *Lactobacillus casei* by azathioprine and 6-mercaptopurine.

Assay procedure. Two ml of double-strength basal medium were added to 2 ml of each stock solution (in triplicate), plus four water blanks, to give standards containing 10 to 1000 ng/ml. The assay tubes were closed with aluminium caps and autoclaved at 10 lb/in² for 10 min. When cool each tube except one blank was inoculated with 2 drops of *Lactobacillus casei* from a Pasteur pipette (50 drops/ml) and incubated at 37 °C for 18 h. Growth was dispersed on a vortex mixer, and turbidity at 560 nm was measured in a Unicam S.P. 600 spectrophotometer.

RESULTS

Growth inhibition commenced at approximately 50 ng of azathioprine or 6-MP/ml. The regression line $y = a + bx$ was fitted to the data for each individual assay in the range 50 to 1000 ng/ml. The value x represents $\log_{10}$ concentration minus the mean $\log_{10}$ concentration. Good correlation between growth level and drug concentration was consistently obtained. The mean correlation coefficient (r) for seven azathioprine assays was -0.965 and for eleven 6-MP assays was -0.985 . Typical growth inhibition curves for azathioprine and 6-MP are shown in Fig. 1. The correlation coefficients for the data presented are -0.992 for azathioprine and -0.974 for 6-MP. The residual standard deviations were 0.006 and 0.009 respectively.

DISCUSSION

It is surprising that no microbiological assay technique for azathioprine or 6-MP has been described previously because the *Lactobacillus casei* model played a major role in the development of both drugs. Following the discovery of 6-MP as a powerful antimetabolite many microbiological studies were undertaken with large concentrations of drug (up to 240 µg/ml), but they were designed mainly to further understanding of the mechanism of action of 6-MP (Balis *et al.* 1957) and were qualitative rather than quantitative.

The microbiological assay reported here is considerably more sensitive than both u.v.

spectroscopy and the colorimetric estimation for 6-MP described by Loo & Michael (1958), which allow detection in the microgram range only. The sensitivity is equalled by a fluorimetric assay for purine-6-sulphonate, an oxidation product of 6-MP (Finkel, 1967), but the microbiological technique has the advantage of utilizing basic laboratory equipment only. Although greater accuracy might be desirable the degree of error involved is probably no greater than in the majority of microbiological assay methods.

Studies with radioisotopes by Burroughs Wellcome & Co. (1969) indicated that the peak level of 6-MP in human plasma following oral administration of therapeutic doses of azathioprine (1 to 3 mg/kg) is unlikely to exceed 1 μ g/ml. This is the limit of sensitivity of the fluorimetric method of Finkel (1967) when used to estimate 6-MP in plasma. The microbiological method described should be sufficiently sensitive for the estimation of pharmacological levels of 6-MP in biological fluids if coupled with a suitable extraction procedure.

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