NOTES

A TURBIDIMETRIC BIOASSAY METHOD FOR DETERMINATION OF MYCOPHENOLIC ACID

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Mycophenolic acid has been known as an antifungal and antibacterial antibiotic, and recently this substance was shown to exert significant antiviral and antitumor activities[1,2,3].

Microbioassay by an agar cup-plate method has been done for estimation of mycophenolic acid in body fluids and urine of mice by the method described by FLOREY *et al.*[4] and the present authors[5]. But this method cannot be used for an accurate quantitative determination of mycophenolic acid since the inhibitory zones produced were relatively obscure.

In an attempt to find a better method, optimum experimental conditions were investigated for the turbidimetric determination[6] of mycophenolic acid using *Candida albicans* as the assay organism. The sensitivity of *C. albicans* to the agent was found to be affected by the components and the pH of the medium, incubation period, and inoculum size. Particularly, the activity of mycophenolic acid was highly dependent on the pH of medium. The optimum response was obtained by using a medium containing 0.45 % yeast extract and 2.0 % glucose in M/10 Na_2HPO_4-M/20 citric acid (MCILVAINE) buffer at pH 3.2 by inoculating 10^4 cells per ml of assay medium of the test organism, and incubating for 16 hours at 30°C. By using this standard response line, it is possible to determine a concentration as low as about 0.04 mcg/ml of mycophenolic acid.

The newly developed method has been found to give highly reproducible results.

Turbidimetric Assay Procedure

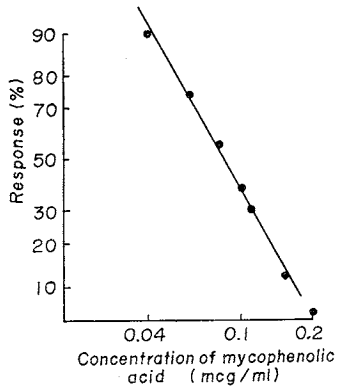
The test organism is *C. albicans* # 8, which is maintained by monthly transfer on SABOURAUD's agar slants by incubation at 27°C for 2 days. A suspension of the test organism for inoculation is prepared by suspending in sterilized saline the cells of adequate quantity from a fresh slant culture which had been incubated at 37°C for 24 hours. Optical density of this suspension is measured in a spectrophotometer (Hitachi Model 101) at 470 mμ and its living cell content is adjusted to 1.0×10^6 cells/ml by using a calibration curve.

The medium for the assay of mycophenolic acid has the following composition : 2.0 % glucose and 0.45 % yeast extract (Difco), in M/10 Na_2HPO_4-M/20 citric acid (MCILVAINE) buffer at pH 3.2 to make final amount of 100 ml of the medium. Yeast extract is dissolved in the buffer described above at the rate of 0.5 % and autoclaved at 110°C for 15 minutes, and 20 % glucose solution is made separately with the same buffer, sterilized by filtration and stored in a refrigerator. Immediately prior to experiments, both solutions are mixed at ratio of 9 : 1.

A standard stock solution of mycophenolic acid, which had been isolated from *Penicillium compactum* and recrystallized is prepared to give a concentration 100 mcg/ml by dissolving 10 mg of mycophenolic acid in about 0.1 ml of sterile 0.5 N NaOH solution and then adding the sufficient sterile distilled water to give a volume of 100 ml. This stock solution may be used for as long as one month if the solution is stored below 5°C. At the time of the test, this stock solution is diluted further in sterile distilled water to give concentrations of 2.0, 1.0, 0.8, 0.6 and 0.4 mcg/ml.

The assay procedure is carried out as follows : the cell suspension is added to the medium in an amount of 1 % so as to contain 1.0×10^4 cells per ml of assay medium and 4.5 ml of this inoculated medium is distributed into a series of sterile plugged test tubes of 18 × 180 mm followd by the addition of 0.5 ml of standard dilutions of

Fig. 1. Typical standard response line in the turbidimetric assay of mycophenolic acid.



mycophenolic acid, unknown solution and sterile distilled water (control). This test culture is incubated at 30°C for 16 hours.

At the end of incubation 0.1 ml of 37 % formaldehyde solution is added to each tube and shaken well to disperse the cells. The optical density of the cell suspension is determined photometrically at 470 mμ and served as a measure for population. Per cent response is calculated from the optical density of each test tube relative to that of a control. The per cent responses obtained from the standard solutions are plotted against the logarithm of the concentration of mycophenolic acid on log-probability paper. The best straight line is fitted through the points. The potency of sample is estimated from this standard response line after its per cent response had been determined.

A typical standard response line is shown in Fig. 1.

Accuracy of the Method

Experiments were designed to test for accuracy of the method. This was done by culturing simultaneously 12 tubes in line with the intention of the present standard method for each of the concentrations of 0.06, 0.08 and 0.10 mcg/ml. The results are shown in Table 1.

The potency in the table indicates the value calculated from randomly selected sets of three tubes, one of each concentration, regarding the 0.08 mcg/ml concentration as the test sample. Based on an average of 12 assays, the potency of the samples was 97.3 % of the theoretical value and its 95 % confidence limits (2×standard error) was approximatly ±5.1 %. When three tubes were used for one test solution, this confidence limits were restricted to approximatly ±4.0 %.

Table 1. Accuracy of the method

Tube	Growth (%)			Calculated potency	
	0.06 mcg/ml	0.08 mcg/ml	0.1 mcg/ml	mcg/ml	%*
1	76.2	61.9	46.3	0.079	98.8
2	80.0	63.7	50.0	0.078	97.5
3	75.0	60.7	47.8	0.079	98.8
4	73.0	63.0	48.7	0.076	95.0
5	71.0	63.0	50.7	0.075	93.8
6	77.5	65.2	50.0	0.079	98.8
7	79.0	64.8	46.3	0.076	95.0
8	77.5	66.3	48.7	0.077	96.3
9	77.5	65.0	46.0	0.076	95.0
10	71.0	59.8	47.6	0.076	96.3
11	83.7	65.0	47.5	0.082	102.5
12	75.5	62.5	50.2	0.080	100.0
Mean	76.4	63.4	48.3	0.078	97.3
Standard error	3.68	1.96	1.65		2.54
Approximate 95% confidence limits (2×standard error)	±7.36	±3.92	±3.30		±5.08

* Calculated potency/theoretical potency (0.08 mcg/ml)×100.

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