Microbiological Assay and Tissue Distribution of 5-Diazouracil in Mice

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Received for publication 1 February 1971

A sensitive, precise microbiological assay has been developed for the determination of tissue distribution of 5-diazouracil, a potential antitumor and antimicrobial agent.

5-Diazouracil (2,5-dioxo-5-diazopyrimidine; designated NSC 23519 by Cancer Chemotherapy National Service Center, National Cancer Institute) has been reported to have antineoplastic activity against a variety of experimental neoplasms (8), to be effective in the chemoprophylaxis of poliomyelitis virus in mice (4), and to inhibit cell division in bacteria and yeast (7). Initial trials in humans with a variety of different tumor types yielded no chemotherapeutically successful results (1). However, 5-diazouracil has been reported to be effective in protecting mice which were experimentally infected with gram-negative bacteria (6) frequently found to be responsible for infections in acute leukemia patients (2). Figure 1 shows the structure of 5-diazouracil.

We have developed a microbiological assay for the determination of the distribution of 5-diazouracil in the tissues of mice which have been injected with therapeutically realistic doses of the drug, based on earlier toxicity studies.

Previously described methods were used to select an appropriate microorganism for the assay of 5-diazouracil (5). A strain of Escherichia coli derived from E. coli ATCC 9637 resistant to 1 mg of methotrexate (MTX) per ml and designated E. coli/MTX was selected as the assay organism because of its sensitivity to the drug. A simple glucose-salts medium was used for culture maintenance, for inocula preparation, and for the preparation of the assay plates. It consisted of 1% NH₄Cl, 0.73% K₂HPO₄, 0.3% KH₂PO₄, 0.012% MgSO₄ and 2% glucose (prepared separately and added aseptically). When needed, 1.5% agar was added. For the preparation of seeded agar plates (90 by 15 mm; no. 1029; Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.), stationary cultures of E. coli/MTX were grown for 16 to 18 hr at 37 C in the glucose-salts medium. Cells from these cultures were collected and washed by centrifugation in saline (0.85% NaCl), resuspended in saline, and adjusted to 20% light transmittance (660 nm) in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). The suspension was diluted 1:200 in saline, and 10 ml of this suspension was added to 1 liter of cooled (50 to 55 C) glucose-salts agar medium. Six-milliliter samples were dispensed into previously described petri plates. E. coli/MTX is a stable mutant, and the addition of MTX to the assay plates is not required.

A stock solution of 5-diazouracil was prepared in sterile saline and appropriately diluted. When 0.08 ml of the various dilutions was added to filter paper discs (1.27 cm in diameter; no. 740-E; Schleicher and Schuell Co., Keene, N.H.), the following concentrations were obtained (micrograms of 5-diazouracil per disc): 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0. An additional 5-diazouracil stock solution was prepared in which the drug was dissolved in freshly drawn mouse blood and diluted with saline. Previously described filter paper discs were impregnated with 0.08 ml of the respective solutions containing graded concentra-
tions of the drug. These discs were placed on the surface of each seeded agar plate and pressed down securely with flamed forceps. All plates and drug concentrations per disc were prepared in triplicate. Each individual plate contained a maximum of three discs. Two discs contained, individually, either experimental samples or standard curve solutions of different concentrations. A third control disc containing an empirically selected concentration (1.0 μg of 5-diazouracil per disc) was added to each plate, which allowed for correction of plate-to-plate variation in zone sizes. All plates were incubated simultaneously at 30°C for 18 to 22 hr with plates which contained discs impregnated with body fluids or tissue homogenates from mice which received 5-diazouracil. The resulting zones of inhibition on the triplicate standard plates were measured and corrected as follows. If the mean diameter of all of the control disc zones was greater than that of an individual control disc zone, the difference was added to all of the zones on that plate. Conversely, if the average diameter of the control disc zones was less than that of an individual control disc, the difference was subtracted from all the zones on that plate. The mean diameter of these corrected zones was determined for each drug concentration. Standard curves were constructed through the points thus obtained by the principle of least squares.

In all in vivo experiments, BDF₁ mice (mixed sexes, 18 to 22 g in weight) were used. Drug was administered intraperitoneally in 0.85% NaCl at a dose of 30 mg/kg. Immediately, one group of five mice was bled by cardiac puncture and killed, and liver, lungs, heart, brain, spleen, and kidneys were removed. Saline homogenates of these tissues were prepared in an Omni-Mixer (Ivan Sorvall, Norwalk, Conn.) and assayed. This procedure was repeated with additional groups of five mice at various time intervals.

Typical standard inhibition curves obtained with 5-diazouracil, dissolved in saline or in heparinized whole mouse blood, are shown in Fig. 2. The indexes of precision (1) of the respective curves are 0.03 (saline) and 0.04 (blood). Figure 3 represents graphically the levels of drug detected in the blood and spleens of mice which were injected intraperitoneally with a 0.5 LD₁₀ dose (30 mg/kg) of drug. All other tissues failed to yield detectable concentrations of 5-diazouracil. Relatively high concentrations of 5-diazouracil

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**Fig. 2.** Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of 5-diazouracil. Assay microorganism, Escherichia coli ATCC 9637/methotrexate. (A) Saline standard curve; index of precision, 0.05. (B) Blood standard curve; index of precision, 0.06.
were detected in mouse blood and spleens. Peak levels were observed immediately in the blood and spleen tissues of mice (approximately 12.5 μg/ml and 4.1 μg/g, respectively) after single-dose intraperitoneal injection of a 0.5 LD_{10} dose and then dropped rapidly. These results agree with the blood levels in mice reported earlier (8), as measured by a colorimetric technique.

This work was supported by contract no. PH43-65-594 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health.

LITERATURE CITED