NOTES

Microbiological Assay for Estimating Concentrations of DL-Alanosine in Mouse Tissue

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A sensitive, precise microbiological assay was developed for the determination of tissue distribution of DL-alanosine, a new antitumor agent.

Alanosine is an antibiotic produced by *Streptomyces alanosinicus* n. sp. (ARCC 15710) which reportedly has both antiviral and antitumor activity (7). The structure of alanosine has been determined, and the agent has been identified (1) as L-2-amino-3- (N-nitroso) hydroxylamino-propionic acid (see Fig. 1). A method for the chemical synthesis of alanosine, both the D- and L-isomers, has been described (6). Alanosine has demonstrated marked antitumor activity against a transplanted fibrosarcoma induced by simian virus in newborn hamsters, although the antibiotic did not show any in vitro antiviral activity against the virus itself (7). It has also been reported to inhibit a variety of other experimental solid tumors (4) and leukemia L1210 in mice. Alanosine inhibits the synthesis of ribonucleic acid (RNA) adenine from formate and glycine in Ehrlich ascites tumor cells in vivo but does not affect the rate of RNA guanine synthesis (4). In studies with *Candida albicans*, it has been reported that L-alanosine depresses the activity of aspartate transcarbamylase and adenylosuccinate synthetase (3) and is an inhibitor of bacterial glutamic acid carboxylase (2).

For the in vivo evaluation of a new and potentially useful chemotherapeutic agent, it is essential to know the tissue distribution and concentrations of the drug. This report describes a logarithmic-ratio microbiological assay which has been developed for the determination of the distribution and concentrations of DL-alanosine (or cytotoxic equivalents), designated NSC 143647 by the National Cancer Institute, in the tissues of mice which have been injected with nonlethal doses of this new antineoplastic agent.

Previously described methods were used to

select an appropriate microorganism for the assay of DL-alanosine (5). A strain of *Escherichia coli* ATCC 9637 resistant to the antitumor antibiotic actinobolin (8) was selected as the assay organism because of its unique sensitivity. Biochemical studies thus far have yielded no explanation for the unique sensitivity of this bacterium. Glucose-salts medium was used for culture maintenance, inoculum preparation, and assay with the following modification. Stock cultures were maintained on this medium supplemented with 1 mg of actinobolin per ml. The medium 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. Broth cultures of *E. coli*/ACB were grown for 16 to 18 h at 37 C in the previously described medium for the preparation of seeded agar assay plates (100 by 15 mm, Lab-Tex Products). Cells from these cultures were collected and washed twice 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). A 0.2-ml amount of this suspension was added to 1 liter of cooled (50 C) glucose-salts agar medium supplemented with 10 µg of adenosine

FIG. 1. Structure of DL-alanosine.

per ml. Adenosine was added to the medium, because experiments (9) revealed that this nucleoside enhanced the sensitivity of *E. coli*/ACB to inhibition by DL-alanosine. No biochemical explanation is available at this time. A 6-ml amount of the inoculated agar medium was dispensed into the previously described petri plates.

DL-Alanosine was dissolved in saline (0.85% NaCl) and diluted in saline so that, when 0.08 ml of the various dilutions was pipetted onto filter paper disks, the following concentrations were obtained: 0.01, 0.02, 0.04, 0.06, 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 μg of DL-alanosine per disk. Another stock solution of DL-alanosine was prepared with the drug dissolved in freshly collected heparinized mouse blood, and diluted with saline. DL-Alanosine standard curves prepared with the drug dissolved in homogenates of mouse spleen, kidney, liver, and brain yielded results identical to those obtained when the drug was dissolved in blood. Previously described procedures (10) were used in assaying biological materials (see Fig. 2 and 3).

Determination of DL-alanosine concentrations in biological materials. BDF₁ mice weighing 18 to 22 g were administered a single dose, intraperitoneally, of 200 mg/kg (ca. 0.5 LD₅₀). Immediately after injection, and at 30, 60, 120, and 240 min, mice were sacrificed and bled by cardiac puncture; liver, lungs, brain, spleen, and kidneys were removed, weighed, homogenized in saline, and assayed.

DL-Alanosine concentrations (or cytotoxic equivalents) were detected in the blood, kidney, brain, and urine of the mice. Blood levels of 115 $\mu\text{g}/\text{ml}$ were detected immediately after drug administration but fell rapidly, and no drug was detected in the blood later than 2 h postinjection. The highest concentration of DL-alanosine was found in the urine (approximately 560 $\mu\text{g}/\text{ml}$ of body fluid) 30 min after drug administration. Assayable levels of DL-alanosine were found in the kidney and brain (approximately 100 $\mu\text{g}/\text{g}$ of tissue) immediately after injection and remained present through 4 h after injection. All other tissues failed to yield detectable concentrations of DL-alanosine.

At this time, only the DL-alanosine is availa-

FIG. 2. Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of DL-alanosine. Assay microorganism was *Escherichia coli* ATCC 9637/actinobolin. A, Blood standard curve; index of precision, 0.016. B, Saline standard curve; index of precision, 0.021.

FIG. 3. Concentrations of DL-alanosine detected in tissues and body fluids of mice by microbiological assay. Each point represents the mean drug level of five mice. Assay microorganism was *Escherichia coli* ATCC 9637/actinobolin. Mice were injected with a single intraperitoneal mice (200 mg/kg) of drug.

ble for evaluation. It would be interesting to know if D- or L-alanosine individually would give similar or different results. Studies of this aspect of the problem are planned.

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