A Microbiological Assay for Estimating Concentrations of Camptothecin Lactone in Mouse Tissues

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The marked sensitivity of the yeast *Hansenula wingei* NRRL Y-2340 strain 5 to a new antineoplastic alkaloid served as the basis for a logarithmic-ratio microbiological assay which has been developed for the estimation of concentrations of camptothecin lactone (or cytotoxic equivalents) in tissues of mice. The utility of this assay has been demonstrated by presenting data which show the concentrations of camptothecin lactone (or cytotoxic equivalents) detected in various tissues of mice at intervals following intraperitoneal injection of a 1 or 0.5 LD_{10} dose of this drug.

Camptothecin lactone (designated NSC 100880 by the Cancer Chemotherapy National Service Center, National Cancer Institute), a plant alkaloid isolated from *Camptotheca acuminata*, has

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
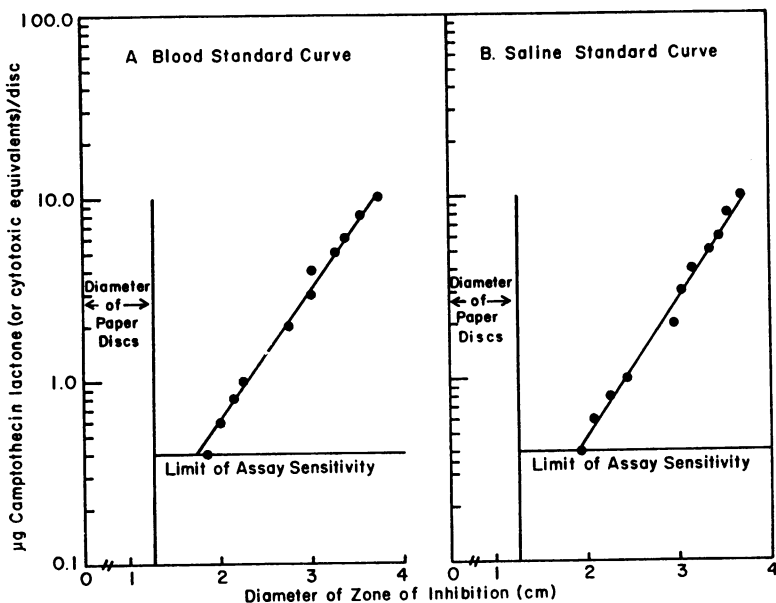

FIG. 1. *Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of camptothecin lactone in tissues of mice. Assay microorganism: Hansenula wingei NRRL Y-2340 strain 5. (A) Blood standard curve: index of precision, 0.07. (B) Saline standard curve: index of precision, 0.06.*

been reported to have strong antileukemic and antitumor activity (4) and is scheduled for clinical evaluation.

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logarithmic-ratio microbiological assay which has been developed for the determination of the distribution and concentrations of camptothecin lactone (or cytotoxic equivalents) in the tissues of mice which have been injected with nonlethal doses of this new antineoplastic alkaloid.

MATERIALS AND METHODS

Previously described methods (2) were used to find an appropriate microorganism for the assay of camptothecin lactone. From more than 300 species of bacteria, yeasts, and filamentous fungi tested for sensitivity to this compound, *Hansenula wingei* NRRL Y-2340 strain 5 was selected as the assay organism. This yeast was maintained on agar slants of Sabouraud Dextrose Agar (Difco). For the preparation of seeded agar assay plates (90 by 15 mm, no. 1029, Falcon Plastic, Inc., Los Angeles, Calif.), stationary cultures of *H. wingei* were grown for 16 to 18 hr at 27 C in Sabouraud dextrose broth. 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 mμ) in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.). This suspension was diluted 1:50 with saline, and 20 ml of this suspension was added to 1 liter of cooled (50 to 55 C) Sabouraud Dextrose Agar. Five ml of the inoculated agar was added to the previously described plates containing 8 ml of congealed sterile agar.

A stock solution of camptothecin lactone was prepared in sterile saline and was suitably diluted so that, when 0.08 ml of the various dilutions was added to
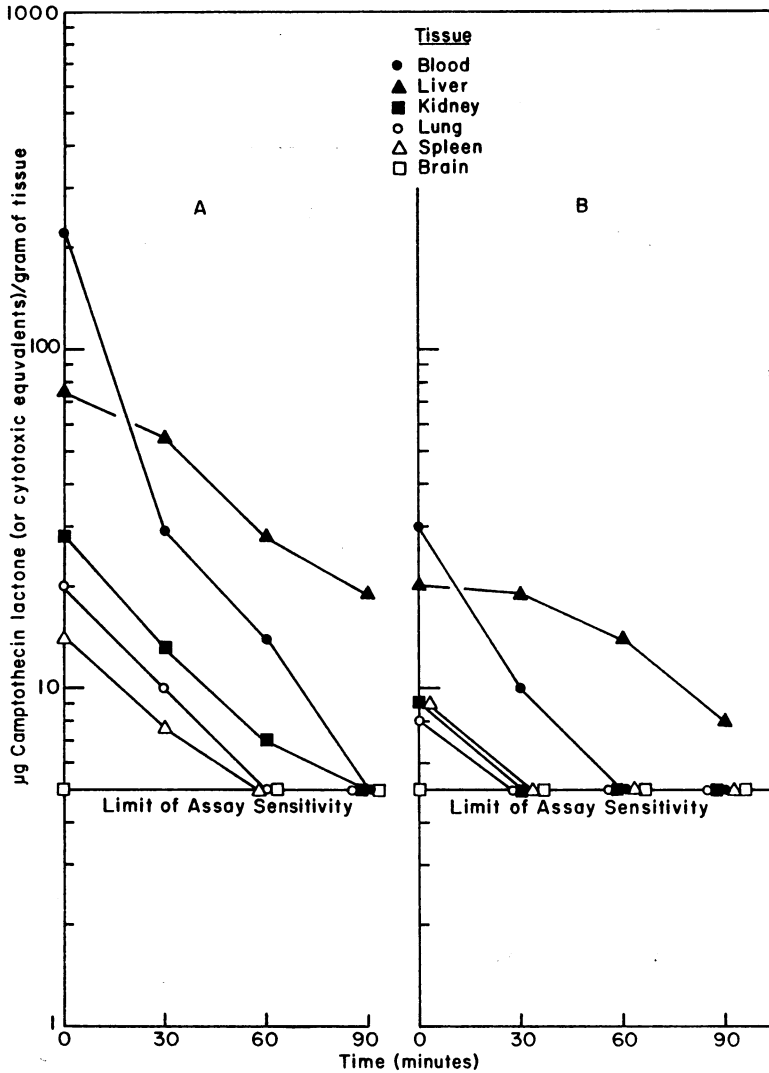

FIG. 2. *Concentrations of camptothecin lactone detected in tissues of mice by microbiological assay. Each point represents the mean drug level of five mice. Assay microorganism: Hansenula wingei NRRL Y-2340 strain 5. (A) Animals were injected with a single LD$_{10}$ intraperitoneal dose (158 mg/kg) of drug. (B) Animals were injected with a single 0.5 LD$_{10}$ intraperitoneal dose (79 mg/kg) of drug.*

filter paper discs (1.27 cm in diameter, no. 740-E, Schleicher and Schuell Co., Keene, N.H.), the following concentrations (μg of camptothecin lactone per disc) were obtained: 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.

An additional stock solution of drug was prepared in which the drug was dissolved in heparinized whole mouse blood. Additional paper discs were impregnated with 0.08 ml of this solution and appropriate dilutions thereof to yield the concentrations per disc indicated previously. After the addition of the graded concentrations of drug to the discs, the latter were promptly and securely placed on the surface of the seeded agar plates. Drug concentrations per disc and unknown samples were prepared in triplicate. Each plate contained a maximum of three discs. Two of these discs contained either experimental samples (blood or tissue homogenates) or standard curve solutions of different concentrations. The third disc contained an empirically selected concentration of 1.0 μg of camptothecin lactone per disc, which allowed for correction of plate-to-plate variation in zone sizes. The plates were incubated for 15 to 18 hr at 27 C.
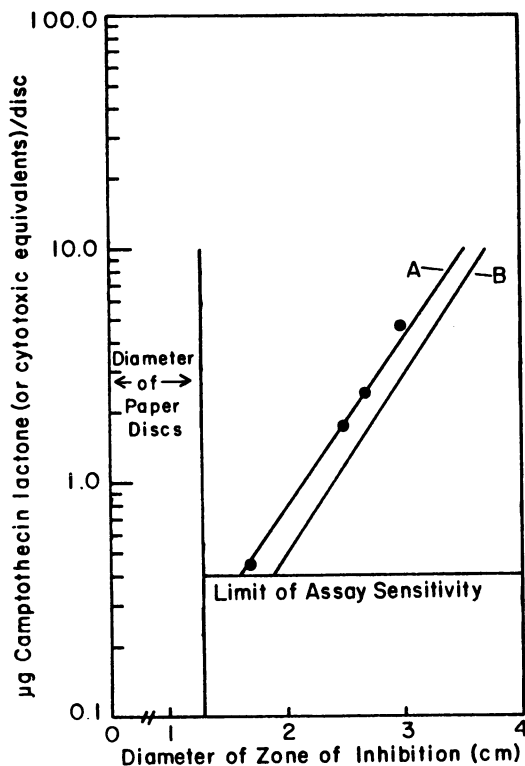

FIG. 4. *Analysis of the accuracy of the logarithmic-ratio microbiological assay for camptothecin lactone.*

The resulting zones of inhibition on the plates were measured and corrected as previously reported (3). The corrected mean diameters of the zones of inhibition surrounding the discs, which contained known concentrations of drug, were plotted on semilogarithmic graph paper, the zone sizes on the arithmetic scale and drug concentrations per disc on the logarithmic scale. Standard curves were constructed through the points thus obtained by the method of least squares. Drug concentrations in the experimental samples were obtained by reading the drug concentrations on the ordinate of the blood standard curve which corresponded to the size of the corrected zones of inhibition surrounding the discs impregnated with the tissue preparations.

In all experiments, BDF$_1$ mice (mixed sexes, 18 to 22 g) were used. With the exception of blood, tissues were prepared for assaying by homogenizing weighed portions (approximately 1 g) of the respective tissues in an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.) containing 2 ml of saline.

FIG. 3. *Parallel relationship between the saline standard curve for the logarithmic-ratio microbiological assay of camptothecin lactone and points from the assay of undiluted and diluted blood from mice injected with a single 0.5 LD$_{10}$ intraperitoneal dose of drug. (A) Undiluted blood, 1:2, 1:3, and 1:4 dilutions thereof. (B) Saline standard curve reconstructed from Fig. 1.*
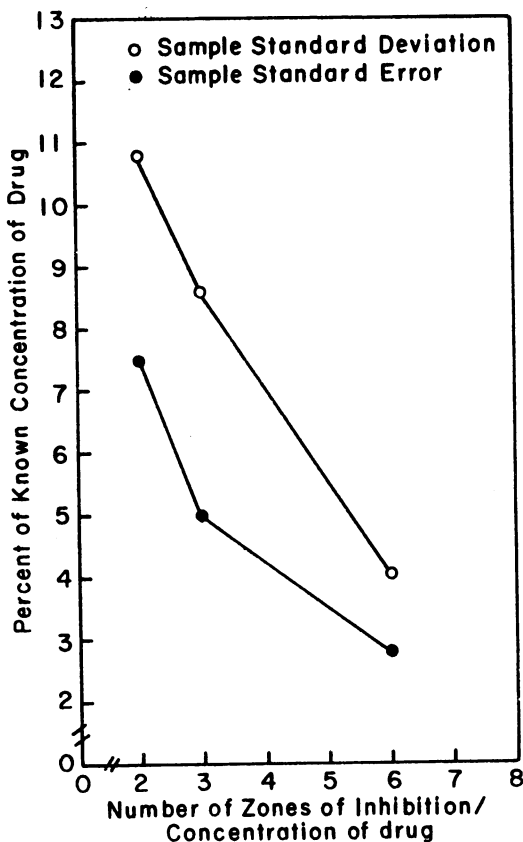
RESULTS AND DISCUSSION

H. wingei was selected as the microorganism for the assay of camptothecin lactone because of its

sensitivity to this inhibitor, its linear dosage response, and its rapid growth rate. Representative standard assay curves, obtained with camptothecin lactone dissolved in saline and in heparinized whole mouse blood, are shown in Fig. 1. In either diluent, concentrations as low as 0.4 μg/disc (5.0 μg/ml), which is <0.04 of 1 LD$_{10}$ dose of this drug, could be detected. The concentrations of camptothecin lactone detected in the tissues of mice, which were injected intraperitoneally with a 1 or 0.5 LD$_{10}$ dose of drug, are shown in Fig. 2. Maximal drug levels were detected immediately after the administration of drug; however, no drug was detected in brain tissue. In microbiological assays, it is assumed that the product being assayed in the experimental samples differs from the standard drug solution only by some dilution factor. This assumption was tested since dilutions of the unknown preparations, when assayed, should yield points which parallel the standard curve in a logarithmic-ratio assay (1). Data which substantiate this assumption are presented in Fig. 3.

The indices of precision (λ) for the blood and saline standard curves are 0.07 and 0.06, respectively, where λ equals the standard deviation of the points on the individual curve/slope of curve in millimeters between any two points which represent a 10-fold difference in the concentration of the assay compound. A linear relationship between drug concentrations and response is essential for microbiological assays (1). From the blood standard curve (Fig. 1A), the diameters of zones of inhibition, which resulted from five representative concentrations (0.4, 0.6, 0.8, 1.0, and 2.0 μg/disc) of camptothecin lactone, were used to calculate the sample regression coefficient (b) and the sample standard deviation of the regression coefficient (s_b). The values obtained for b and s_b were 1.08 and 0.12, respectively. A test of significance for b was conducted where $t = b/s_b$ or 9.0, which indicated a highly linear relationship between drug concentrations and diameters of zones of inhibition. An analysis of the accuracy of the assay method is presented in Fig. 4; the standard deviation (s) and standard error (s_x^-) are expressed as a percentage of a known concentration of drug, and are based on 2, 3, and 6 assay zones of inhibition per known concentration of drug. From this figure, the number of zones required to give a desired level of accuracy can be obtained. For example, three assay zones for each sample can be expected to yield a standard error not greater than 5.0%. Thus, 95% of all three-zone replicate assays should yield data with a standard error no larger than $2 \pm s_x^-$ or $\pm 10.0\%$. If data are needed with a standard error no larger than 2.8%, six assay zones would be required for each sample. The curves in Fig. 4 can be used with numerical values taken from any portion of the standard curves in Fig. 1, since a standard control disc, which allowed corrections for any plate-to-plate variation in zone sizes, was placed on each assay plate. Thus, all points on the standard curves are equally strong.

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