GLC Determination of Probenecid in Biological Fluids

ANTHONY G. ZACCHEI▲ and LINDA WEIDNER

Abstract □ A highly specific and quantitative GLC method for the determination of probenecid in biological fluids is described. The method is adaptable to automation with an automatic sample injector and a peak area integrator. The procedure involves the addition of an internal standard (*N*-dibutyl analog of probenecid) to plasma or urine samples followed by extraction at pH 1 into benzene. The extracted acids are converted to methyl esters for GLC analysis by reaction with ethereal diazomethane. The sensitivity of the method is such that 250 ng. of material can be detected in 1 ml. of plasma. GLC–mass spectrometry techniques confirmed that the developed method measures the parent drug probenecid.

Keyphrases □ Probenecid in biological fluids—GLC analysis □ GLC—analysis, probenecid in biological fluids

The use of the uricosuric agent, probenecid (I), for the treatment of gout and for the inhibition of the active transport of many substances necessitated the development of a rapid and sensitive quantitative method for the detection of I. Various spectrophotometric methods (1–3) have been reported; however, these methods lack the required specificity and sensitivity. A recently described GLC method (4) has several disadvantages; specifically, no internal standard is employed and the sensitivity is about 2 mcg./ml.

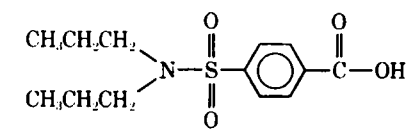
This report describes a very sensitive and specific GLC method for probenecid determinations, under conditions where only probenecid was administered, using an internal standard (*N*-dibutyl analog of probenecid, II). The method has the added advantage of being adaptable to automation.

EXPERIMENTAL

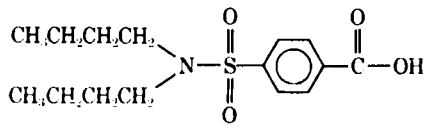
Reagents and Chemicals—The chemicals and reagents used were: probenecid, the *N*-dibutyl analog of probenecid, benzene, methylene chloride, and 0.5 *M* diazomethane (generated from *N*-nitroso-*N*-methylurea).

Instrumentation—*GLC*—A gas chromatograph[1] equipped with a flame-ionization detector and a 183 × 6-mm. glass column containing 1% OV-17 (phenyl methyl silicone gum) on Supelcoport, 90–100 mesh, was used. The column, detector, and injection port temperatures were 225, 240, and 245°, respectively. Helium was used as the carrier gas at a flow rate of 48–50 ml./min.

A gas chromatograph[2] equipped with a flame-ionization detector and a 183 × 6-mm. glass column containing 1% OV-17 on Supelcoport, 80–100 mesh, was employed for the automated GLC analysis. The column, detector, and injection port temperatures were 209, 276, and 265°, respectively. Helium was used as a carrier gas at a flow rate of 50 ml./min. Injections were performed automatically[3]. Peak areas were calculated automatically by an integrator[4].



I



II

GLC–Mass Spectrometry—All mass spectra were obtained on a mass spectrometer[5] using the GLC inlet with a 183 × 6-mm. glass column packed with 1% OV-17 on Supelcoport, 80–100 mesh. The gas chromatograph was operated isothermally at 230° with a helium flow rate of 30 ml./min. The mass spectrometry ionizing and accelerating potentials were 70 ev. and 3.5 kv., respectively. The source and injection port temperatures were both 270°.

Measurement of I in Biological Samples—Compound I was determined in biological samples as follows. One milliliter of plasma or 3.0 ml. of urine was added to a 50-ml. glass-stoppered centrifuge tube containing 15 mcg. of internal standard (in 1.0 ml. of phosphate buffer, pH 7), 1 ml. of 2 *N* hydrochloric acid, and 25 ml. of benzene. The tube was shaken for 10 min. and centrifuged, and at least 20 ml of the organic phase was transferred to a similar tube containing 2 ml. of 0.1 *N* sodium hydroxide. After shaking for 5 min., the tube was centrifuged and the organic phase was removed by aspiration. The pH of the aqueous phase was adjusted to 1 by the addition of 0.2 ml. of 2 *N* HCl, and the free acids were extracted into 5 ml. of methylene chloride (Vortex, 1 min.). After centrifuga-

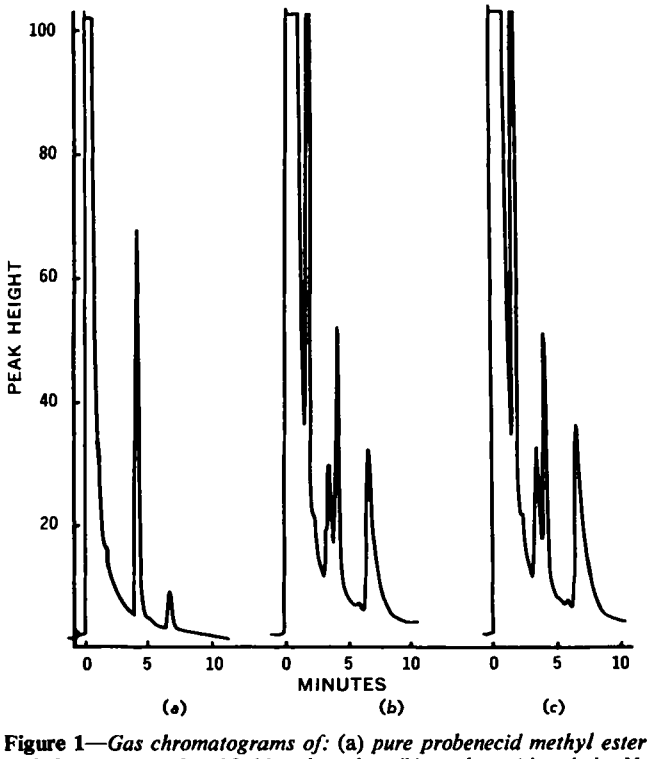


Figure 1—*Gas chromatograms of:* (a) *pure probenecid methyl ester and the corresponding* N*-dibutyl analog,* (b) *probenecid and the N-dibutyl analog added to control plasma and carried through the GLC method, and* (c) *material obtained from biological specimens after the addition of the internal standard. Five microliters was injected out of a total volume of 100 μl. of ethyl acetate.*

[1] Packard model 7400.
[2] Hewlett-Packard model 5750.
[3] With a Hewlett-Packard 7670A automatic sampler equipped with a Hamilton 7005 5-μl. syringe set for maximum injection.
[4] Hewlett-Packard 3370B.
[5] LKB-9000S.

Table I—Recovery of Probenecid from Plasma

Amount Added, mcg.		Amount Recovered[a]						Mean ± *SD*
60.0	A	62.1	61.3	59.2	59.3	61.1	61.7	60.78 ± 1.24
	B	103	102	99	99	102	103	
45.0	A	44.8	44.5	45.5	46.1	45.6	44.7	45.20 ± 0.63
	B	99	99	101	102	101	99	
30.0	A	29.9	28.9	31.0	29.6	29.0	29.9	29.72 ± 0.76
	B	100	97	103	99	97	100	
15.0	A	15.0	15.4	15.4	15.1	15.0	15.2	15.18 ± 0.18
	B	100	102	102	101	100	101	
7.5	A	7.25	7.17	7.69	7.38	7.24	7.36	7.34 ± 0.18
	B	97	96	103	98	97	98	
5.0	A	4.83	5.20	4.82	5.28	4.68	5.05	4.98 ± 0.24
	B	97	104	96	106	94	101	
1.0	A	0.95	1.10	0.91	0.89			0.96 ± 0.09
	B	95	110	91	89			
0.5	A	0.55	0.53	0.46	0.48			0.51 ± 0.04
	B	110	106	92	96			
0.25	A	0.30	0.21	0.27	0.27	0.30		0.27 ± 0.03
	B	120	84	108	108	120		

[a] Values in the A rows represent micrograms recovered; values in the B rows represent percent recovery.

tion, the aqueous phase was removed by aspiration and the methylene chloride phase was transferred to a 13-ml. conical centrifuge tube and then treated with 100 μl. of an ethereal solution of diazomethane. After 5 min., the tube was placed in a warm water bath in a hood; all solvent and unreacted reagent were allowed to evaporate under nitrogen. The residue was dissolved in 100 μl. of ethyl acetate and appropriate aliquots, usually 5 μl., were injected into the gas chromatographs. The retention times of the methyl esters of I and the *N*-dibutyl internal standard (II) were 4.6 and 6.6 min., respectively.

Standard water, urine, and plasma curves were constructed by plotting the peak height ratios (I/II) or peak area ratios (I/II) *versus* weight ratios (I/II). The peak height ratio obtained from an unknown was then used to determine the amount of I present. The standard samples were run simultaneously with the unknown samples as previously described. Recoveries of I added to plasma or urine in amounts of 0.2–60 mcg. ranged from 84 to 120% for analyses performed over several months. The mean recovery was 100.55 ± 6.37% over the entire concentration range.

Absorption and Excretion Studies—Drug (I), in capsule form or in saline solution as the sodium salt, was administered to purebred beagle dogs of both sexes. Drug (I) in gelatin capsules was administered to normal human subjects. Probenecid was administered to the dogs at a dose of 19 mg./kg. as the free drug or in combination with pivampicillin (33 mg./kg.) and ampicillin (23.3 mg./kg.). Similar dosage formulations were administered to human subjects.

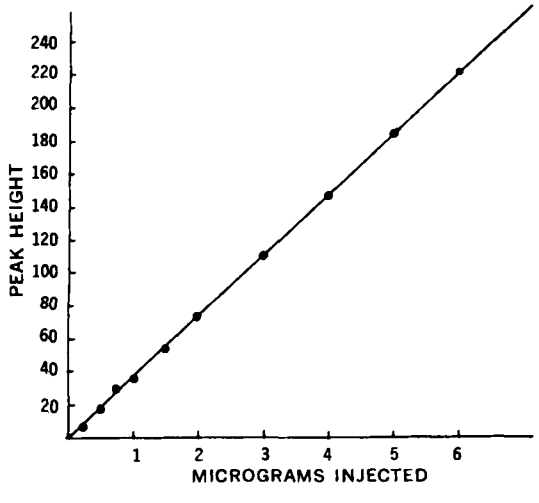


Figure 2—*Relationship between peak height and amount injected. Five microliters was injected in each case.*

Animals and human subjects were fasted prior to drug administration. Urine was collected from animals housed in individual metabolism cages and was frozen as collected in dry ice-cooled containers. Blood was collected in heparinized syringes, and the plasma was separated by centrifugation and frozen until assayed. The plasma and urine samples were analyzed as previously described.

RESULTS AND DISCUSSION

Figure 1 presents gas chromatograms of the following samples: (*a*) pure probenecid methyl ester and the corresponding *N*-dibutyl analog, (*b*) probenecid and the *N*-dibutyl analog added to control

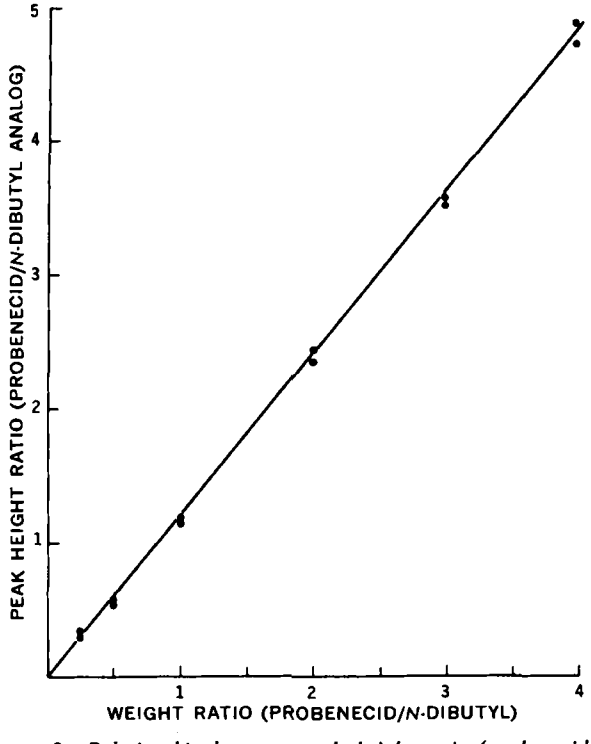


Figure 3—*Relationship between peak height ratio (probenecid/N-dibutyl analog) and weight ratio (probenecid/N-dibutyl analog). Five microliters was injected in each case out of 100 μl. following appropriate recovery (water, plasma, or urine).*

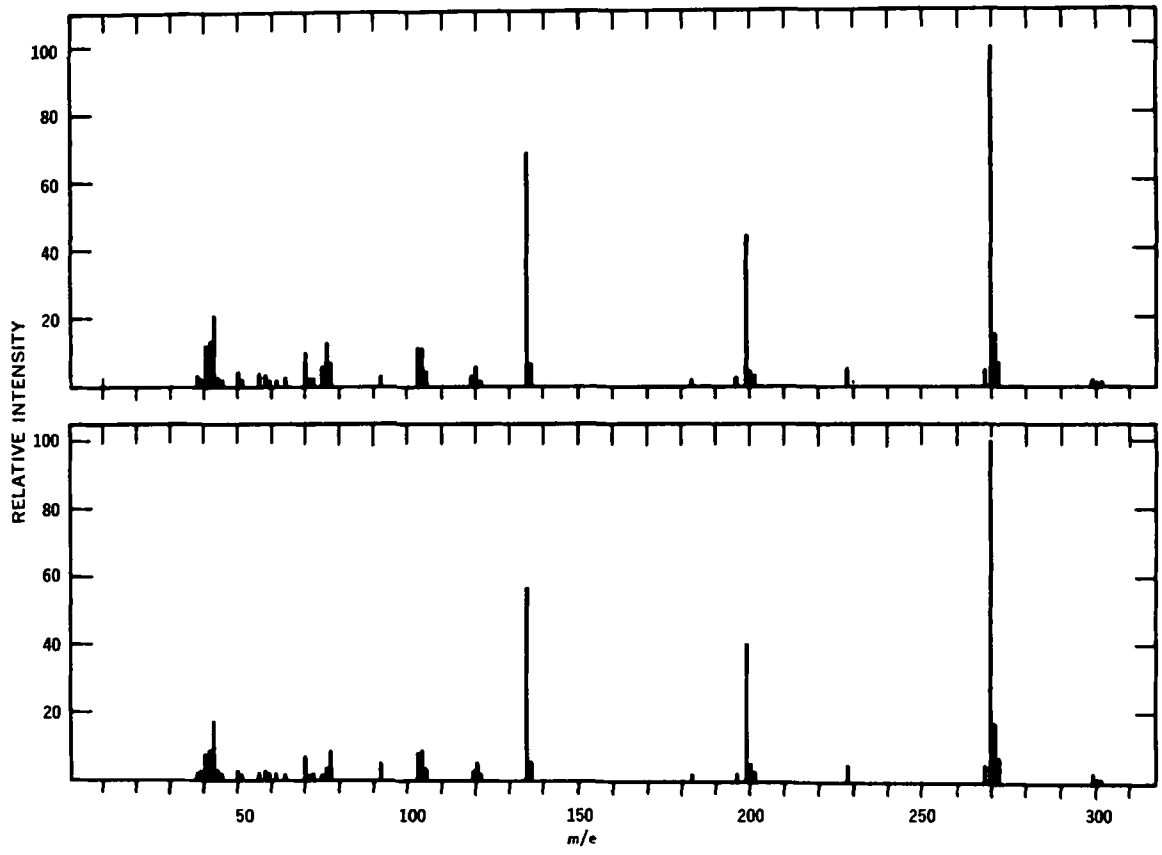


Figure 4—*Mass spectra for probenecid methyl ester (top) and the material obtained from dog plasma (bottom).*

plasma and carried through the GLC procedure, and (*c*) material obtained from biological specimens after the addition of the *N*-dibutyl internal standard. As stated previously, the methyl ester of probenecid had a retention time of 4.6 min. and the corresponding internal standard had a retention time of 6.6 min. Gas chromatograms of control samples did not exhibit any peaks with retention similar to probenecid methyl ester or the methyl ester of the internal standard. A linear relationship is observed when peak height (or peak area) is plotted *versus* amount injected (Fig. 2). A typical standard peak height ratio (I/II) *versus* weight ratio (I/II) is presented in Fig. 3. The slope of the straight line is 1.20. Values for the slope ranged from 1.03 to 1.25, depending upon specific analysis over 6 months. A summary of the recovery results of I obtained with dog plasma in the range of 0.2–60 mcg. added to the initial tube is presented in Table I. As evident, the GLC procedure for probenecid in biological fluids is very sensitive and exhibits a high degree of precision and accuracy.

Table II—Plasma Levels of Probenecid following Administration of Probenecid to Dogs (Intravenous or Oral, 19 mg./kg.)

Hours	mcg. Probenecid/ml. Plasma[a]					
	A		B		C	
0.08	74.4	108.6	77.4	102.9	0.73	0.6
0.25	69.6	95.7	70.8	82.8	10.9	8.4
0.50	57.3	81.6	67.5	85.5	24.3	18.4
1.0	53.3	81.0	54.0	74.1	27.5	18.3
2.0	44.2	69.0	46.5	63.8	32.7	18.8
3.0	39.3	59.7	40.5	56.5	29.3	63.0
4.0	34.1	54.0	39.0	46.6	24.3	62.7
6.0	28.5	45.0	30.7	42.5	23.2	53.2
8.0	22.8	38.3	29.3	39.3	21.8	45.6
24.0	3.0	10.5	3.7	12.3	3.6	13.9
$t_{1/2}$	5.5	8.0	6.0	9.8	7.6	9.4

[a] Group A, dogs received probenecid intravenously, 19 mg./kg.; Group B, dogs received probenecid intravenously, 19 mg./kg., and ampicillin intravenously, 23.3 mg./kg.; and Group C, dogs received probenecid orally, 19 mg./kg., and pivampicillin orally, 33.3 mg./kg.

This GLC method was applied to numerous samples of biological origin, specifically with respect to dog and human urine and plasma. The samples analyzed also contained ampicillin and pivampicillin, neither of which had any effect on the GLC assay of probenecid. Typical probenecid levels in dog plasma are seen in Table II.

Confirmation of specificity of analysis was obtained when representative unknown biological specimens were analyzed by combined GLC–mass spectrometry using the technique previously described. Figure 4 presents a comparison of the mass spectrum of the synthetic methyl ester of probenecid with the mass spectrum of the material isolated from biological origin following oral administration of drug (I). The details of the fragmentation pattern of probenecid were reported previously (4–6); the mass spectra were identical in all respects. The molecular ion (*m/e* 299) represents ~2% of the intensity of the base peak (*m/e* 270), which results from cleavage of the side chain beta to the nitrogen. The characteristic metastable ions at 192.6, 173.8, and 146.8 were observed.

CONCLUSION

A sensitive, specific, and accurate GLC method for the determination of probenecid in biological fluids is presented. The method is adaptable to automation with the use of an automatic sample injector and a peak area integrator. The use of an internal standard eliminates any errors in quantitation as a result of sample manipulation (7). The sensitivity of the method is such that 250 ng. of material can be detected in 1 ml. of plasma. No interference in the GLC method was observed with ampicillin or pivampicillin. GLC–mass spectrometry results from various analyzed samples confirmed the specificity and identity of probenecid.

REFERENCES

(1) E. K. Tillson, *J. Pharmacol. Exp. Ther.*, **111**, 385(1954).

(2) K. H. Beyer, *Arch. Int. Pharmacodyn. Ther.*, **48**, 97(1954).

(3) P. G. Dayton, J. F. Yu, W. Chen, L. Burger, L. A. West, and A. B. Lutma, *J. Pharmacol. Exp. Ther.*, **159**, 278(1963).

(4) K. Sabih, C. D. Klaassen, and K. Sabih, *J. Pharm. Sci.*, **60**, 745(1971).

(5) P. G. Dayton and J. Perel, *Ann. N.Y. Acad. Sci.*, **179**, 399 (1971).

(6) A. M. Guarino, W. D. Conway, and H. M. Fales, *Eur. J. Pharmacol.*, **8**, 224(1969).

(7) H. M. McNair and E. J. Bonelli, "Basic Gas Chromatography," 5th ed., Varian Aerograph, Walnut Creek, Calif., 1969, pp. 150, 151.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 6, 1973, from the *Merck Institute for Therapeutic Research, West Point, PA 19486*

Accepted for publication July 30, 1973.

The authors thank Carl Ziegler for supplies of the *N*-dibutyl analog of probenecid.

▲ To whom inquiries should be directed.

5-Fluorouracil and Derivatives in Cancer Chemotherapy III: *In Vivo* Enhancement of Antitumor Activity of 5-Fluorouracil (FU) and 5-Fluoro-2′-deoxyuridine (FUDR)

JOHNSON JATO* and J. J. WINDHEUSER▲

Abstract □ The antitumor activity of 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR) has been enhanced in mice in the presence of deoxyribose donors such as 2′-deoxyuridine (UDR). Based upon *in vitro* data, it appears that the enhancement is due to the conversion of 5-fluorouracil to 5-fluoro-2′-deoxyuridine in blood and the stabilization of 5-fluoro-2′-deoxyuridine due to the presence of 2′-deoxyuridine.

Keyphrases □ 5-Fluorouracil (FU)—antitumor activity enhanced by deoxyribose donors (2′-deoxyuridine), mice □ 5-Fluoro-2′-deoxyuridine (FUDR)—antitumor activity enhanced by deoxyribose donors (2′-deoxyuridine), mice □ Antitumor activity, 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR)—enhanced in presence of deoxyribose donors (2′-deoxyuridine), mice □ Cancer chemotherapy—enhancement of 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR) activity by deoxyribose donors (2′-deoxyuridine), mice

A previous article (1) reported the *in vivo* stabilization of 5-fluoro-2′-deoxyuridine (FUDR) and its formation from 5-fluorouracil (FU) in the presence of nucleosides. The speculation was made that these findings could have marked effects on the clinical use of 5-fluorouracil and 5-fluoro-2′-deoxyuridine in cancer chemotherapy. Following the synthesis of 5-fluoro-2′-deoxyuridine (2), it was shown that the nucleoside was 10^3 times as effective as 5-fluorouracil *in vitro* (3). However, the relatively high anticipated effectiveness of 5-fluoro-2′-deoxyuridine as compared to 5-fluorouracil has not been borne out in clinical use. It was suggested (4) that the lower activity observed was due to the *in vivo* cleavage of 5-fluoro-2′-deoxyuridine to the pyrimidine base and the phosphorylated sugar moiety. The stabilization of 5-fluoro-2′-deoxyuridine by 2′-deoxyuridine (UDR) in blood (1) led to the speculation that this approach could lead to a marked clinical enhancement of 5-fluoro-2′-deoxyuridine activity. This article reports the effect of combining 5-fluorouracil and 5-fluoro-2′-deoxyuridine with 2′-deoxyuridine in protecting mice against L-1210 leukemia and adenocarcinoma 755.

EXPERIMENTAL

Materials and Instruments—The following were used: 5-fluorouracil[1], 5-fluoro-2′-deoxyuridine[1], 2′-deoxyuridine[2], deoxycytidine[2] (CDR), and 5-trifluoromethyl-2′-deoxyuridine[3] (F_3TDR). All other substances used were either reagent grade or of the highest purity available.

A spectrophotofluorometer[4], a TLC scanner[5] modified to contain pendrive motor and range offset, and an *x-y* recorder were used.

***In Vitro* Analysis of 5-Fluorouracil and 5-Fluoro-2′-deoxyuridine**—Drugs and additives in known initial concentrations in blood were dialyzed and evaporated by the method of Windheuser *et al.* (5). The dry samples were dissolved in a constant volume of ethanol. The samples were applied to a fluorescent TLC plate, which had previously been activated at 110° and ruled to restrict the lateral motion of the spots to within the range of the scanner optic. The components were resolved by multiple-pass discontinuous development (6). The developer for the process was composed of ethyl acetate–ethanol–10% ammonium hydroxide (200:5:2). The dried plates were scanned in the direction of development for fluorescence quenching at an activation maximum of 280 nm. and a fluorescence maximum of 530 nm., using a spectrophotofluorometer[4] with a thin-film scanner attachment. Quantities of substances present in the blood were computed by appropriate comparison of sample and standard peak areas obtained by planimetry. The method exhibited sufficient sensitivity to detect 10 ng. of 5-fluorouracil and 5-fluoro-2′-deoxyuridine.

***In Vivo* Mouse Studies**—*L-1210 489 Leukemia*—A standardized suspension prepared from 7-day-old tumors from BDF_1 mice was injected intraperitoneally. The volume was adjusted to provide 1×10^6 cells/injection. Twenty-four hours after the transplant, the mice were divided into groups of 10 and each group was injected intraperitoneally for 7 days with one of the drug regimens listed in Table I.

The number of mice per group that died from the leukemia, as confirmed by autopsies, was recorded daily until all animals were dead. An average lifespan, that is, the average number of days that mice in each group survived after tumor transplant, was computed. The T/C value, which is the ratio of the lifespan of the experimental group to that of the control group, was calculated. An increase in lifespan representing the percent increase of the lifespan of each group relative to the control was determined from the T/C (Table I). The increase in lifespan is used as a measure of the effectivness of the drug regimen.

Adenocarcinoma 755—Donor BDF_1 mice bearing 10-day-old tumors were sacrificed, and the tumors were removed with sterile surgical instruments inside a tissue culture hood. Then the tumors

[1] Hoffmann–La Roche, Nutley, N. J.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Supplied by Ben Venue Laboratories, Bedford, Ohio, through Dr. William Wolberg, University of Wisconsin Hospitals, Madison, Wis.
[4] Aminco model 9-8106, American Instrument Co.
[5] American Instrument Co.