Blood and Urine Levels of Antitumor Agents Determined with Cell Culture Methods[1]

CHARLES G. SMITH, JOSEPH E. GRADY, AND FLOYD P. KUPIECKI

(The Upjohn Company, Biochemistry Research, Kalamazoo, Michigan)

SUMMARY

Blood and urine level distributions of several antitumor agents, administered I. V. or P. O. to human subjects, have been determined using cell culture assay and chromatography methods. Reproducible blood and urine levels could be determined after administration of amethopterin,[2] 5-fluorouracil, 6-mercaptopurine or streptovitacin A. Peak blood levels with these agents were 0.4–1.0 μg/ml (streptovitacin A, Ameth, and 6-MP) or 45 μg/ml (5-FU). Although cytotoxicity could be demonstrated in blood specimens after the administration of 5-FUDR, vinblastine, or cyclophosphamide, the data were not sufficiently reproducible for quantitative assay purposes. No drug activity was observed in blood following I. V. administration of actinomycin D or nitrogen mustard, in spite of the high cytotoxicity of these agents. In general, cytotoxic activity was observed in the urine specimens of patients after administration of all of the above agents.

Data on the recovery of antitumor agents added to whole human blood and serum *in vitro*, and the inherent cytotoxicity of human blood specimens *per se*, are also presented.

There is a paucity of data in the literature on blood and urine level distribution of antitumor agents, due most likely to the lack of highly sensitive bioassay methods for measuring the concentrations of biologically active materials at the low doses generally tolerated by the host. In the present study, cell culture bioassay procedures were used to determine the levels of several clinically useful or experimental agents in blood and urine after administration of the drugs to human subjects. The application of chromatographic methods using cell culture bioautography to demonstrate the similarity of the cytotoxic activity with the agent administered is illustrated in suitable cases. Preliminary reports of these studies have appeared elsewhere (5, 11).

METHODS

The basic cell culture methods were described previously by Smith *et al.* (12) and Grady *et al.* (7). All assays were carried out in Eagle's medium (2) fortified with 10% calf serum. Aliquots (0.4 ml) of blood and urine samples were added to the above basal medium (4.0 ml) at 0 time, before cells had become attached to the glass. Standard curves were determined in Eagle's medium for each assay as follows: the agent under study was added to Eagle's medium containing 10% calf serum plus either (a) 10% pooled human serum (Microbiological Associates, Inc.); (b) 10% of the serum from individual patients before treatment with the antitumor agent but following administration of ancillary medication; (c) no added human serum; or (d) the urine specimen obtained before treatment. With this control regimen, the effects of pooled human serum, individual human serum specimens (experimental), and urine on the dose-response curves of individual drugs were determined. Control tubes which contained specimens of blood and urine from the individuals under investigation, but no added agent, were included to test for inherent cytotoxicity in the subject's blood or urine. The cytotoxicity of ancillary medicaments used in the study was also measured to determine the effects, if any, on the cell culture assay. These protocols are illustrated in Table 1 using data obtained with streptovitacin A (5). The standard error of this assay procedure has been estimated at 10–25% (10, 12).

Blood samples[3] were centrifuged after clotting at room

[1] This work was supported in part by Contract Nos. SA-43-PH-1933 and PH43-62-168, Cancer Chemotherapy National Service Center, National Institutes of Health, Washington, D. C.

[2] The abbreviations used are: 5-FU, 5-fluorouracil; 5-FUDR, 5-fluorodeoxyuridine; 6-MP, 6-mercaptopurine; Ameth, amethopterin; VLB, vincaleutoblastine; 5-FUR, 5-flurouridine.

Accepted for publication July 22, 1964; revised October 16 1964.

[3] Specimens used in these investigations were provided by Drs. F. J. Ansfield (University of Wisconsin), B. W. Brooks (The Upjohn Company), Franklin H. Cox, (Borgess Hospital, Kalamazoo, Michigan), H. V. Demissianos (The Upjohn Company), J. B. Field (Mt. Sinai Hospital, Los Angeles), T. Hall (Children's Cancer Research Foundation), C. Heidelberger (University of Wisconsin), P. S. Rutherford (Borgess Hospital, Kalamazoo, Michigan), A. R. Stough (McAlester, Oklahoma), R. W. Talley (Henry Ford Hospital), and C. C. Tan (Sloan-Kettering Institute).

temperature and the resulting sera were quick-frozen and shipped in Dry Ice for cell culture assay. Specimens were frozen in all cases and were stored at −10°C. while awaiting assay. Urine specimens were frozen immediately after collection (usually voided) and stored in this condition until assay. The drugs administered by clinicians[3] and assayed in our laboratories included actinomycin D, Ameth, cyclophosphamide, 5-FUDR, 5-FU, 6-MP, nitrogen mustard, streptovitacin A, and vinblastine.

RESULTS

Table 2 presents a summary of the ctyotoxicity of agents studied under standard test conditions, minimal concentrations recovered when added to whole blood *in vitro*, the calculated blood level in man at the dose administered (assuming a blood volume of 4.0 liters) and the concentration of substance actually found. The blood levels observed were much lower than those calculated, assuming complete absorption and distribution of blood. If Ameth, 5-FU, and vinblastine were distributed in total body water (40 liters), the dilution can be explained for these agents on this basis. Rapid elimination from the body does not seem to be the answer in most cases since the rate of appearance in the urine and total amount recovered cannot account for most of the drug administered. Metabolic degradation or conjugation cannot be ruled out and is known to occur with 5-FU, 5-FUDR (9), and 6-MP (3). Blood levels of nitrogen mustard and actinomycin D could not be demonstrated in this study in several patients although activity in urine was seen. The levels of 5-FUDR and VLB were much lower than anticipated. The situation with respect to 5-FU and 5-FUDR is discussed in more detail below. Although cyclophosphamide is inactive in the standard cell culture test, it showed cytotoxic activity in the blood of 1 human subject in this study, and cytotoxicity was previously reported in animals (6). This activity cannot be reported in terms of micrograms per milliliter since the standard is inactive *per se*.

TABLE 1

PROTOCOLS USED IN BLOOD AND URINE ASSAYS

CONTROL COMBINATION	ID_{50} (μg/ml)	
	Exp. 1	Exp. 2
1. Streptovitacin A in H_2O	0.031	0.021
2. Streptovitacin + T_0 blood[a]	0.036	0.020
3. Streptovitacin + pooled serum	0.038	0.020
4. Streptovitacin + urine	0.019	0.017
5. Amytal[b]	300	
6. Vesprin[b]	3	

[a] Post-Amytal-prestreptovitacin specimen from patient, 10% by volume.

[b] Ancillary medicaments.

The distribution of biologic activity in the blood of 3 patients given Ameth and 5 patients receiving 6-MP is presented in Table 3. These data show a peak blood level for amethopterin of 0.3 μg/ml after a single dose of 10 mg P.O. After the administration of a 400-mg dose of 6-MP P.O., a peak blood level of 0.9 μg/ml was observed. Urine specimens collected 8 hr. after drug administration contained 8.6, 12.9, and 17.5 μg/ml of amethopterin in 3 patients, accounting for 54, 35, and 37%, respectively, of the dose administered. Urine specimens from 3 patients receiving 6-MP contained 7.6, 19.5, and 24.4 μg/ml at 8 hr., which represented a maximum of 4% of the dose administered. These data confirmed previous studies (3, 8).

The distribution of biologic activity in the blood of 2 patients given 15 mg/kg of 5-FU I.V. is shown in Chart 1. A 2d dose of 15 mg/kg was administered to patient A at 14 hr. Recovery of bioactivity in urine specimens from these patients is shown in Table 4.

A comparison of 5-FU distribution in blood and urine was made on specimens obtained from a human subject to whom C^{14}-labeled drug (9) was administered and drug distribution determined by biologic and radiochemical assays; these data are presented in Table 5. Studies (9) have shown that 5-FU is converted to 5-FUR and 5-FUDR

TABLE 2

SUMMARY OF BLOOD LEVEL DATA

AGENT	STD, ID_{50} (μg/ml)	DETECTED IN SERUM[a] (μg/ml)	DOSAGE		MAX. BLOOD LEVEL (μg/ml)	
			(mg/kg)	Route	Calc.[b]	obs
Actinomycin D	10^{-3}–10^{-5}	5×10^{-3}	0.007	I.V.	0.1	$<5 \times 10^{-3}$
Amethopterin	5×10^{-3}	0.02	0.15	P.O.[c]	2.5	0.4
Cyclophosphamide	>100	—	5.7	I.V.	100	+
5-Fluorouracil	0.75	5	15	I.V.[c]	250	45
5-Fluorodeoxyuridine	0.005	0.05	30	I.V.	500	15
6-Mercaptopurine	0.05	0.25–0.5	5.7	P.O.[c]	100	1
Nitrogen mustard	0.15	100	0.4	I.V.	17	<100
Streptovitacin A	0.02	0.1	0.36	I.V.[c]	6	1
Vinblastine	10^{-6}	0.05	0.2	I.V.	35	<0.05

[a] Concentration detectable in control serum after addition of drug to freshly-drawn whole blood, clotting 2 hr. at 25°C. and centrifuging.

[b] Assuming uniform distribution in 4 liters of blood.

[c] Indicates a minimum of 3 patient-experiments.

TABLE 3

BLOOD LEVELS OF AMETHOPTERIN AND 6-MERCAPTOPURINE AFTER ORAL ADMINISTRATION

AGENT	PATIENT	BLOOD LEVEL (μg/ml) AT VARIOUS TIMES (hr).							
		0.5	1.0	1.5	2	4	6	8	12
Amethopterin (10 mg)	1	0.02	0.33	0.36	0.41	0.36	—[a]	0.09	—
	2	0.24	0.24	0.20	0.24	0.13	—	0.01	—
	3	0.48	0.28	0.22	0.28	0.32	—	0.04	—
6-Mercaptopurine (400 mg)	4	0.81	0.79	0.79	0.78	0.61	—	—	—
	5	0.79	0.85	0.80	0.81	0.82	—	—	—
	6	0.62	0.65	0.59	<0.25	<0.25	<0.25	<0.25	<0.25
	7	0.94	0.74	1.0	0.78	0.65	0.78	—	0.43
	8	0.40	0.27	0.32	—	<0.25	0.48	<0.25	<0.25

[a] No analyses were performed on these samples. No cytotoxicity was observed in any of the pretreatment specimens.

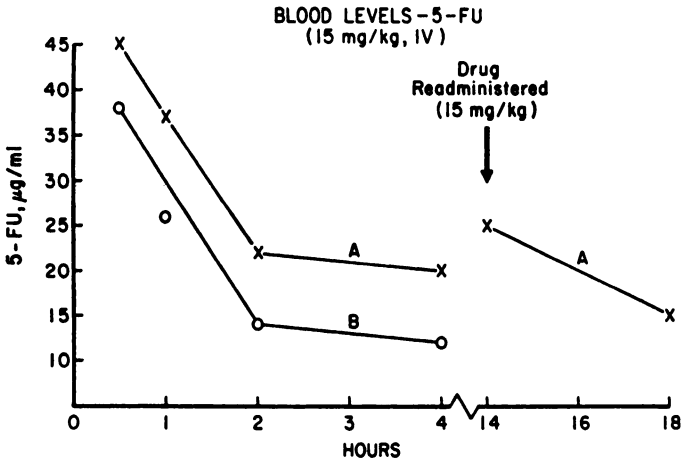

CHART 1—The distribution of biologic activity in the blood of 2 patients given 5-fluorouracil. Recovery of bioactivity in urine specimens from these patients is shown in Table 4.

TABLE 4

DISTRIBUTION OF 5-FLUOROURACIL (5-FU) IN URINE

Collection Interval (hr.)	5-FU (μg/ml)
0–0.5	3700
0.5–1	1950
1–2	100
2–4	50
4–14	100
14–18[a]	875

[a] Second dose administered at 14 hr.

in animals. Since the latter 2 compounds are approximately 100 times more cytotoxic in the cell culture test than 5-FU (13), the total biologic activity measured with 5-FU as the standard would be approximately 3 times the actual concentration (by weight) of 5-FU *per se* if only 1% of each nucleoside were present. It is felt that the greater concentration of 5-FU in 3 of the blood specimens and 2 of the urine specimens, when measured biologically, can be attributed to the high cytotoxicity of 5-FUR and 5-FUDR. Paper chromatography (14) of these specimens showed the presence of 5-FU by bioassay, but neither

TABLE 5

COMPARISON OF 5-FLUOROURACIL[a] LEVELS BY BIOLOGIC AND RADIOCHEMICAL ASSAYS

SAMPLE	5-FU (μg/ml)	
	Cytotox	Radio.[b]
Blood (min.)		
30	38	21
60	26	9.3
120	14	6.2
260	12	5.7
Urine (hr.)		
3.5	280	223
7.5	117	2.8
12–24	35	0.2

[a] After 15 mg/kg C^{14}-labeled 5-fluorouracil (5-FU), I.V.
[b] Courtesy Dr. C. Heidelberger.

5-FUR nor 5-FUDR could be demonstrated because of the small amount present. It should be emphasized that maximal agreement between biologic and radiochemical determinations was observed at the earliest sampling times (Table 5), presumably before major metabolism of 5-FU occurred.

The assay of 5-FUDR in human sera was not nearly as satisfactory as that for 5-FU, because the former, although markedly cytotoxic to mammalian cells *in vitro*, is cleaved rapidly *in vivo* to 5-FU (9). In this case, a lower bioactivity is observed than would be predicted from the dose of 5-FUDR given.

The data in Table 6 show the blood level distribution obtained in 2 patients (4 separate experiments) given streptovitacin A I.V. In this case, a good correlation was observed between patients at a dose of 0.2 mg/kg, where peak blood levels of 0.4–0.6 μg/ml were observed. When 0.36 mg/kg was administered, a blood level of approximately 1.0 μg/ml was found. The persistence of cytotoxic activity in the patient's blood for 24 hr. after administration of the drug should be noted. Measurable activity was still present in 1 patient even after 36 hr. Since this pattern was observed repeatedly, it is thought to be real. No explanation can be offered for the total

lack of cytotoxicity in the blood of patient 2 in the 30-min. sample.

Urine levels in patients receiving streptovitacin A are shown in Table 7. On chromatography (14) of urine specimens from these subjects, streptovitacin A was found on bioautography. Patient 4, who demonstrated a normal blood level, showed essentially no activity in the urine, which cannot be explained with the data in hand. From 10 to 15% of the streptovitacin administered was accounted for in the urine of 3 patients.

Determinations of the inherent cytotoxicity of human blood sera, collected at random both from hospitalized patients with and without cancer and from normal subjects, were made in order to determine the degree of interference which could be expected in such studies. In a series of 200 specimens, 15% significantly inhibited the growth of KB cells (25–50%) in the standard assay procedure at a concentration of 10% in the nutrient medium.

TABLE 6

BLOOD LEVELS OBSERVED WITH STREPTOVITACIN A

TIME OF SAMPLE	STREPTOVITACIN A (μG/ML)			
	Patient 1[a]	Patient 2		
		Exp. 1[a]	Exp. 2[a]	Exp. 3[b]
Min.				
0[c]	0	0	0	0
10	0.58	0.38	—	0.64
20	—	—	—	1.08
30	0.53	0.22	0.38	0
60	0.28	0.12	—	1.08
120	0.27	0.11	0.13	1.0
Hr.				
12	0.26	0.13	0.09	1.0
24	0.14	0.14	—	—
36	0.03	—	—	—

[a] 0.2 mg/kg in a single I.V. dose.
[b] 0.36 mg/kg in a single I.V. dose.
[c] Prestreptovitacin, post-Amytal (or Vesprin) specimen.

TABLE 7

URINE LEVELS AFTER STREPTOVITACIN A ADMINISTRATION

TIME OF SAMPLING	STREPTOVITACIN A (μG/ML)					
	Patient 1[a]	Patient 2			Patient 3[a]	Patient 4[b]
		Exp. 1[a]	Exp. 2[b]	Exp. 3[b]		
Min.						
0[c]	0	0	0	0	0	0
30	157	—	185	—	—	4.2
60	71	185	130	—	72	4.7
120	18	70	100	—	—	—
Hr.						
4–5	—	—	—	21	14	3.9
8	—	10	—	12	—	—
12	7.5	—	2	4	—	—

[a] 0.3–0.35 mg/kg in a single I.V. dose.
[b] 0.2 mg/kg in a single I.V. dose.
[c] Prestreptovitacin, post-Amytal (or Vesprin) specimen.

Three of four cytotoxic sera tested were inactivated by heating at 56°C. for 30 min. These data confirm those reported by other investigators (1, 4).

DISCUSSION

The data presented illustrate the value of cell culture bioassay technics in the determination of blood and urine levels of certain cytotoxic agents. In spite of marked sensitivity to agents such as actinomycin D, and calculated blood levels which should be well above the minimal detectable concentration, no cytotoxicity was found in serum after I.V. administration to humans. Whether this represents extremely rapid elimination or bioconversions cannot be determined from the above data. A combination of bioactivity and distribution of radioactivity would be most interesting in such a case. When the agent administered is converted to a more or less active compound, as in the case of 5-FU and 5-FUDR, total cytotoxicity is determined and expressed as the equivalent of the particular compound used as a standard. When sufficient activity in all of the components is present, quantitative papergram bioassays can be run to give the exact biologic equivalent of each component. Determining the activity of 1 compound, using another as a standard, is indeed dangerous unless one knows that their dose-response curves over the ranges being investigated are the same.

It is interesting to note that the inherent cytotoxicity present in certain human serum specimens can usually be removed by a moderate heat treatment. This allows the determination of heat-stable cytotoxic agents, like 5-FU and 6-MP, in serum even though the particular specimens may be cytotoxic *per se*. The value of using the patient's pretreatment specimen as a control and, where sufficient sample is available, constructing a standard curve in this patient's serum as well cannot be overemphasized. When the agent under study is sufficiently active, chromatography can be coupled with bioassay to give information on the identity of the cytotoxicity observed with the agent administered.

ACKNOWLEDGMENTS

The authors are indebted to the physicians noted who provided the specimens used in this study. The competent technical assistance of Mrs. M. E. Hall, Mrs. W. Newbre, and Mrs. D. Nidy is acknowledged. The excellent assistance and cooperation of Mr. M. R. Burch is especially appreciated. Papergram separations were performed by Mr. W. L. Lummis.

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