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Pharmacological Studies with Vinblastine in the Dog[1]

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SUMMARY

Tritiated vinblastine was prepared by catalytic exchange and its metabolism was studied in dogs. Plasma levels of drug fell in biphasic mode with initial and secondary phase half-lives of 17 to 38 min and 3 to 5 hr, respectively. Between 28.6 and 79.1% of plasma tritium was precipitable with cold trichloroacetic acid and thus was presumably protein bound. Blood leukocytes had levels of intracellular tritium between 2.4 and 11.8 times those of the coincident plasma samples. Over a 9-day period, urinary excretion accounted for 12.1 to 16.8% and fecal excretion accounted for 30.1 to 36.1% of the administered radioactivity. Ratios of biliary to plasma radioactivity varied between 7.3 and 56.9, with unchanged vinblastine being the major component (46.8 to 80.7%) in the bile.

INTRODUCTION

The alkaloid VLB[4] (Velban) has been a standard agent in cancer chemotherapy for many years, yet knowledge of its pharmacological disposition has remained rather sketchy in comparison with what is known of many other less useful drugs (4). Bioassay of plasma VLB levels, based on inhibition of the growth of cells in culture (10), and measurements in rats of the clearance curves of radioactivity derived from tritiated drug (1) have suggested that this agent leaves the circulation rapidly and is excreted preferentially in the bile. A major limitation to extensive studies, especially in humans, has been the difficulty of preparing adequate amounts of radiolabeled *Vinca* alkaloids; this problem, however, has been overcome recently by 2 groups of investigators (7, 8, 11). In the present work, VLB was prepared with tritium label using catalytic exchange, and some features of its metabolic fate were explored in dogs. In agreement with earlier studies in other species, there was a biphasic clearance from the plasma, with the initial phase being rapid; fecal excretion predominated. Furthermore, the peripheral blood leukocytes were able to concentrate the drug intracellularly. These data have appeared in preliminary form (5).

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[4] The abbreviation used is: VLB, vinblastine.

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MATERIALS AND METHODS

Animals and Tissues. Dogs of mixed breed weighing 10.7 to 24.5 kg were used in this study. They were maintained during the course of the experiments in metabolism cages equipped with a screen to separate the urine and feces. Blood samples were collected by venipuncture in heparinized Vacutainers for metabolic studies, and in EDTA for leukocyte counts and differentials. The plasma was separated by centrifugation at 1600 $\times$ *g* for 5 min. Leukocytes were separated from whole blood by sedimentation with 3% (w/v) dextran, lysis of the contaminating erythrocytes by 20 sec of exposure to distilled water before restoration of isotonicity, and centrifugation. Packed cell volumes were measured by centrifuging leukocyte suspensions in Shevky-Stafford and McNaught tubes; corrections for trapped interstitial medium were not carried out.

Cannulation of the Bile Duct. The animal was premedicated with atropine sulfate, and anesthesia was induced with sodium thiamylal and maintained with a mixture of oxygen, halothane, and nitrous oxide. The biliary system was exposed by a right paramedian incision, cholecystectomy was performed, and a siliconized rubber tube (Silastic 602-305; internal diameter, 1.98 mm, external diameter 3.18 mm; Dow-Corning Co., Newton, Mass.) with a siliconized Dacron mesh tab (6 mm wide extending 10 mm above the tube, glued to it) was inserted into the common bile duct via a choledochotomy incision by a modification of a method previously described (14). Thus the proximal arm of the cannula was placed retrograde and the distal end was inserted orthograde into the common bile duct. Silk ligatures (3-0) were used to seal the wall of the common bile duct around the cannula at this point, as well as to attach the Dacron mesh tab to the duodenum for stabilization. The orthograde arm of the cannula ran through the duct orifice and into the duodenal lumen for several cm before being brought through the duodenal well, which was inverted around the cannula. A duodenopexy was performed around a stab incision through the lateral abdominal wall where the catheter exited. The abdomen was closed in a routine manner, and the dog recovered from the anesthesia uneventfully. Following surgery, the dog was allowed free access to food and water, and was not restrained within the cage.

Synthesis of Tritiated VLB. VLB sulfate (25 mg) was

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dissolved in a mixture of trifluoroacetic anhydride (0.5 ml) and tritiated water (100 mCi; 0.1 ml), and the solution was kept at −20° for 2 days. After removal of the solvent in a vacuum, the free base was purified by preparative thin-layer chromatography on Merck Silica Gel HF_{254}, using chloroform:methanol (95:5, v/v), converted to the crystalline sulfate, and recrystallized from ethanol. The yield was 15 mg with a specific activity of 1.08×10^8 dpm/mg. This material was indistinguishable from authentic VLB on the basis of the accumulation of metaphase figures in growing cultures of S180 cells. Prior to its injection into animals, the tritiated VLB was repurified by thin-layer chromatography. There was no evidence for exchange of the tritium in VLB with water.

Metabolic Studies. VLB (Eli Lilly Company, Indianapolis, Ind.) was administered i.v. to 4 dogs at a dose of 0.15 mg/kg, together with 21.2 to 60.2×10^6 dpm of tritiated alkaloid. The dogs were maintained for up to 9 days in metabolism cages. Blood, urine, bile, and stool samples were collected and processed as described above. In the case of the stools, the samples were homogenized with 0.5 N NaOH. Radioactivity was assayed with a Packard Tri-Carb liquid scintillometer (counting efficiency for most tritium samples, except stool and bile, was close to 20%) using automatic external and internal standardization to correct for quenching. In some experiments, samples were also plated out on stainless steel planchettes and counted in windowless mode with a Nuclear-Chicago gas flow counter.

Chromatography. This was carried out in ascending fashion on 0.5 mm layers of Merck Silica Gel HF_{254} using chloroform:methanol (95:5, v/v) and methanol as the solvents. The R_F values for VLB in these 2 systems were, respectively, 0.22 and 0.57. For the assay of the amounts of free VLB, samples were either applied directly to the plates (urine only) or extracts were prepared with 2 volumes of benzene at pH 9, concentrated with a stream of air; then the spotting was performed. After development, the plates were scraped and counted in a liquid scintillation counter.

Biological Studies. Changes in levels of the blood cells were followed in the 4 treated dogs. Leukocytes were counted in a Model A Coulter counter, and 100 cell differentials were done on Wright's stained air-dried blood films.

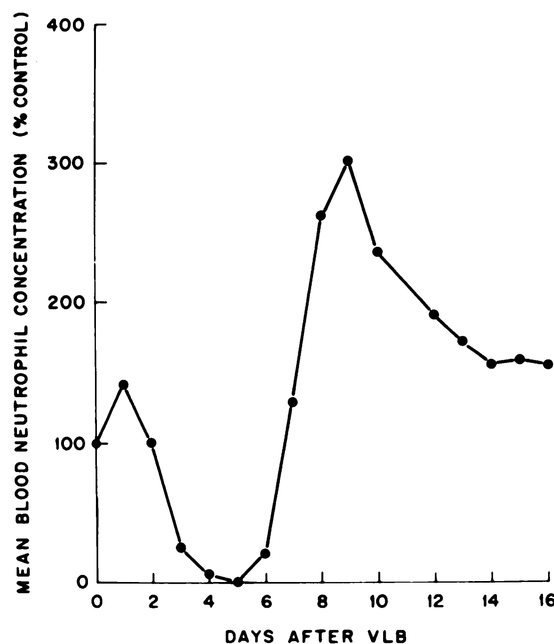

Chart 1. Mean blood neutrophil concentrations in 4 dogs following treatment with VLB (0.15 mg/kg) expressed as percentage of the value on the day of administration. After Day 3, the *points* represent the means of the values for 3 dogs.

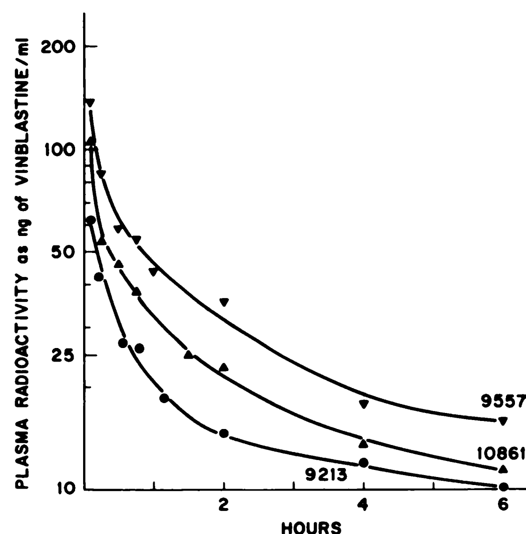

Chart 2. Levels of plasma radioactivity expressed as ng/ml of VLB equivalents, for 3 dogs receiving the drug at a dose of 0.15 mg/kg.

RESULTS

Biological Effects of VLB. The daily mean blood neutrophil concentration of the 4 dogs, expressed as a percentage of the value on the day VLB was administered, is shown in Chart 1. One dog died on Day 3, so that values after that day represent the means of 3 animals. Marked neutropenia by Day 3, nadir at Day 5, and recovery by Day 7, with an overshoot at Day 9, were evident. Similar hematological effects for unlabeled VLB at this dose have been reported (2).

Metabolic Studies. After administration of labeled VLB (0.15 mg/kg), the plasma curves for clearance of radioactivity (Chart 2) were obtained. Data are expressed as ng equivalents of VLB, although this was not the only chemical species present. A large number of data points were not gathered, but the evidence suggests a biphasic clearance of tritium label with half-lives in the range of 24 to 30 min and 4 to 7 hr for the initial and early (to 6 hr) secondary phases. In 1 dog, in which samples were collected for several days, there appeared to be a very slow ($t_{1/2}$, 70 hr) tertiary phase also. However, since this animal (S 12487) was the one that had undergone bile duct cannulation, it may not have represented a normal physiological situation. Further study

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of the plasma radioactivity disclosed that between 28.6 and 79.1% was precipitated with cold 5% (w/v) trichloroacetic acid, the amount increasing with time elapsed over the period 15 min to 24 hr after administration of drug. Exhaustive washing with cold acid slowly extracted much of this radioactivity. Of the nonprecipitable radioactivity, the bulk was extractable with benzene at pH 9 (2 volumes of solvent). This material was subjected to thin-layer chromatography to determine the levels of free VLB (Chart 3). Here, the biphasic nature of the clearance curves was even more apparent. Estimations of the values for the $t_{1/2}$ from these graphs were: initial, 17 to 38 min; and secondary, 3 to 5 hr. Thus, there was a pattern of rapid initial clearance followed by persistence of a low level of drug in the plasma. Apart from VLB and its metabolites in free or bound form in the plasma, radioactivity was also associated with the blood cellular elements. The ratio of the concentration of tritium in whole blood to that in plasma averaged 1.3 over the 1st 6 hr after injection of [^{3}H]VLB. This suggests that the cells are able to concentrate the drug or its metabolites. In the case of the white cells isolated by sedimentation, lysis, and washing, the intracellular levels of radioactivity were up to 11.8 times as great as those in the coincident plasma (Table 1). The relative resistance of this intracellular radioactivity to washing the cells suggests that it is bound to some receptor, perhaps microtubule protein. In those leukocyte samples examined, more than 80% of the intracellular radioactivity was in the form of VLB. It has been demonstrated previously that the platelets are able to concentrate and bind VLB in unchanged form (8, 13).

Urinary and fecal excretion of radiolabeled drug was studied in 2 dogs over a 9-day period. The data shown in Chart 4 indicate that, while the urinary radioactivity reached a plateau within 4 days, fecal excretion continued at a relatively constant rate throughout the experiment, and thus obviously represented the major route of elimination. Of the urinary radioactivity, VLB accounted for a significant fraction whereas, in the stool samples, the amount of unchanged drug was very small (Table 2). In the dog with a cannulated bile duct (S 12487), levels of total radioactivity and of unchanged VLB could be compared in the coincident

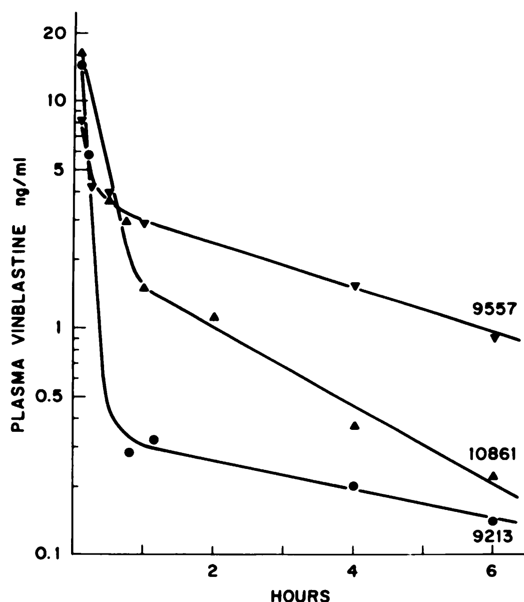

Chart 3. Levels of free, extractable, labeled VLB in the plasma of 3 dogs given drug at 0.15 mg/kg.

Table 1
Concentration of radioactivity by leukocytes

Time after VLB (hr)	Dog 9557 ng equivalents/ml	Dog 9557 Ratio, WBC:plasma	Dog S 12487 ng equivalents/ml	Dog S 12487 Ratio, WBC:plasma
0.25	272.6	3.2	238.5	2.4
1	269.4	6.2	441.4	5.9
4	209.0	11.8	474.0	10.1
24	211.9	7.6	292.8	7.2

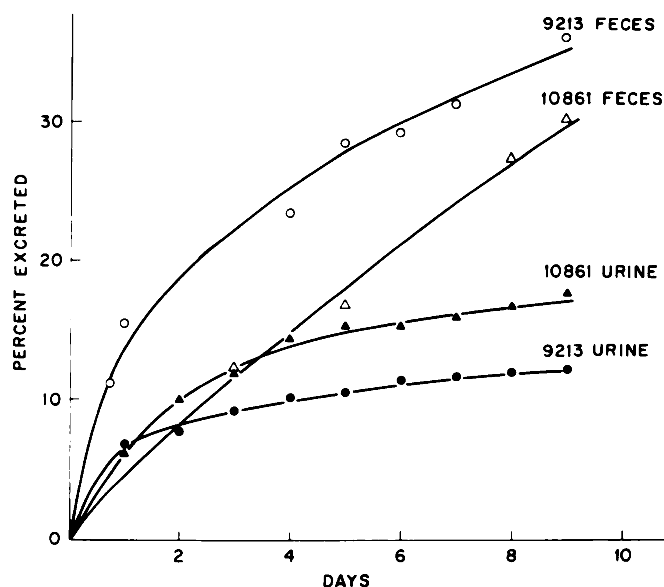

Chart 4. Excretion of radioactivity in the urine (O, Δ) and stools (●, ▲) of 2 dogs given labeled VLB at 0.15 mg/kg.

Table 2
Composition of excreted radioactivity

Time (days)	% unchanged VLB: Urine, Dog 9213	Urine, Dog 10861	Stools, Dog 9213	Stools, Dog 10861
1	46.9	72.4	2.7	[a]
2	57.9	70.1	[a]	[a]
3	56.8	71.7	[a]	8.2
4	35.4	66.1	8.5	[a]
5	22.0	11.1	3.5	2.5
6	16.3	6.2	1.0	[a]
7	15.6	2.2	[a]	[a]
8	2.6	1.8	[a]	2.6
9	0	3.6	1.3	2.4

[a] Stools were not passed on these days.

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bile and plasma samples (Table 3). It is evident that major elimination of drug occurred through the biliary system. The ratios of biliary to plasma radioactivity, 7 to 57, are entirely compatible with those reported (10 to 50) for vincristine in the rat (11). Unchanged VLB accounted for the major proportion of biliary activity. This is in marked contrast to fecal radioactivity (Table 2), indicating that breakdown of VLB occurs in the gut.

In chromatograms of urinary, biliary, and fecal radioactivity, there was a maximum of 3 major areas of label in addition to VLB. One was at the origin, another was close to the solvent front, and a third, not present in all samples, that migrated just ahead of VLB (R_F 0.38 in 95:5 methanol: chloroform). The nature of these metabolites was not explored in detail.

DISCUSSION

In this study it is clear that, when VLB is given to dogs at a dosage level sufficient to produce leukopenia, the drug undergoes a rapid initial clearance phase, followed by slow elimination from the blood. A major factor in the rapid early disappearance is undoubtedly uptake of VLB by various tissue cells, notably those rich in microtubule protein or tubulin, which have an affinity for metaphase-arresting agents (3). Binding of *Vinca* alkaloids by tubulin is known to be a very rapid process (12). Earlier work has shown that platelets bind VLB avidly and, also, by virtue of subsequent slow release of drug, may act as a reservoir component (8, 13). The present work shows that leukocytes are also able to concentrate VLB, and since levels of radioactivity in whole blood exceeded the concentrations in the plasma by 30%, it appears likely that significant amounts of drug enter the erythrocytes, too. The secondary rise in plasma radioactivity and VLB that occurred at 24 hr may reflect release of drug from these reservoir tissues in response to a fall in circulating alkaloid.

A 2nd feature that must markedly influence the pharmacokinetics of VLB is the existence of a large component of the drug associated with plasma proteins. Interaction of the *Vinca* alkaloids with human serum proteins, notably the α- and β-globulins, has been studied *in vitro* by Donigian and Owellen (6). As much as 75% of VLB was bound by physiological concentrations of these serum proteins, a figure close to our own maximum of about 80% for dog plasma. This phenomenon must be a key factor in prolonging the circulation of the drug. It may also be related, together with intracellular binding of VLB, to the discrepancy between the low percentage of plasma radioactivity accounted for as free extractable VLB as compared with the bile and urine, where binding proteins and "reservoir cells" are absent.

A 3rd factor influencing the distribution of VLB is undoubtedly its mode of excretion. Biliary excretion is the major mechanism involved, as attested to by this and other studies with both VLB (1) and the related vincristine (11), although very significant amounts also are eliminated in the urine. Drug excreted in the bile is probably subject to reabsorption, since VLB is known to be effective when given p.o. (9). Thus, the capability exists for enterohepatic circulation of VLB and perhaps its metabolites. This must underlie the very prolonged fecal excretion of radioactivity. In the course of this process, most VLB would be degraded to metabolites by the combined action of the host and the intestinal flora.

This work serves to demonstrate the type of metabolic pattern for the *Vinca* alkaloids in dogs that has previously been delineated in rodents. Extension to humans, for whom the knowledge of the metabolism of VLB and vincristine is still incomplete, is the intent of future study.

Table 3
Levels of radioactivity and of free VLB in dog plasma, bile, and urine

Time (hr)	Radioactivity (μg VLB equivalents/ml)			VLB (μg/ml)		
	Plasma	Bile	Urine	Plasma	Bile	Urine
0.5	0.078	0.572		0.024	0.350	
1	0.075	2.320		0.022	1.872	
2	0.056	3.188			2.208	
4	0.047	1.900		0.013	1.165	
24	0.040	0.593	0.479[a]	0.004	0.317	0.419[a]
48	0.035	0.586	0.508[a]	0.003	0.246	0.390[a]
72	0.022	0.766	0.393[a]		0.237	0.315[a]

[a] The urine samples represent 0- to 24-, 24- to 48-, and 48- to 72-hr collections. Dog S 12487 was studied.

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