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9 February 1976

Radioimmunoassay for Colchicine in Plasma and Urine

Abstract. *A radioimmunoassay for the measurement of colchicine (in quantities as small as 0.05 nanogram) in plasma and urine was developed with the use of an antibody from immunized rabbits. After the intravenous injection of 2 milligrams of colchicine in seven subjects, the calculated zero-time concentration in the plasma was 2.9 ± 1.5 micrograms per deciliter, and the mean half-time in the plasma was 58 ± 20 minutes. Declining, but measurable, amounts of colchicine could be detected in urine up to day 9 after the drug was administered.*

Colchicine has been used in the therapy of the acute gouty attack for more than 150 years. It remains a drug of choice for control of the acute gouty attack (*1*) and is widely used as prophylactic therapy. If treatment begins within a few hours after the onset of the attack, more than 90 percent of patients respond within 12 to 48 hours (*2*). Colchicine given in small daily doses abolishes recurrences completely or reduces the frequency of attacks in 93 percent of patients with gout (*3*). However, the toxicity of this drug and methodologic problems resulted in little, or no, knowledge of pharmacokinetics of this agent. Plasma and urinary colchicine levels based on an isotopic dilution technique have been reported (*4*). ^{14}C-labeled colchicine was injected into volunteers, and plasma and urinary concentrations of the drug were determined by the radioactivity found in the colchicine zone on a thin-layer chromatographic system (*5*). Radioimmunoassay for colchicine (*6*) eliminates the need for an injected tracer and allows rapid analysis of small quantities of plasma or urine without purification. This assay should be of great use in toxicology, clinical pharmacology, and in the study of the physiology of microtubules (*7*).

Colchicine was conjugated to bovine serum albumin (BSA) through the ketone group on ring C by means of standard techniques (*8*). The only modification was an extraction with cold (4°C) chloroform to purify the colchicine oxime. Approximately 0.5 mg of the BSA conjugate was dissolved in 2.5 ml of saline and homogenized with 2.5 ml of complete Freund's adjuvant (Difco). Each of five New Zealand white rabbits received this mixture distributed among six subcutaneous injection sites at weekly intervals for 6 weeks, and then monthly. Binding of ^{3}H-labeled colchicine appeared in 6 weeks in one of the five rabbits; a usable titer appeared in 6 months. The colchicine-BSA conjugate was not characterized; the production of a usable antiserum was used as the end point of the procedure.

The radioimmunoassay depends on competition between unlabeled colchicine and ^{3}H-labeled-colchicine tracer for combination with binding sites on antibodies in the rabbit antiserum. Approximately 3000 dpm of ^{3}H-labeled colchicine (specific activity: 7.7 mc/mg, New England Nuclear) was used as the tracer. The tracer was dissolved in 0.5 ml of phosphate-buffered saline, *p*H 7.4, containing 0.01*M* EDTA and 0.01 percent merthiolate. From 5 to 200 μl of plasma, urine, or standard colchicine dissolved in control plasma or urine of the same volume as the unknown to be assayed was incubated for 24 hours at 4°C with the tracer and with 5 μl of rabbit antiserum, without agitation. The tubes were covered with aluminum foil to prevent exposure to ultraviolet light with the possible formation of varying quantities of the easily formed photoisomer lumicolchicine. The double antibody technique, in which we used 40 μl of sheep antiserum to rabbit γ-globulin and a second incubation of 16 hours at 4°C, resulted in reproducible separation of bound from free colchicine. After centrifugation, the supernatants containing the free colchicine were mixed with Aquasol (New England Nuclear), and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer. A typical standard curve (Fig. 1) permitted measurement of from 0.05 to 10 ng of colchicine per assay tube. Within this range, there was complete parallelism between the standard curve obtained with authentic colchicine and dilutions of urine and plasma samples. Assay of more than 200 μl of urine or 300 μl of plasma resulted in significant deviation from parallelism, and such quantities were not used. A variety of biologically active alkaloids—such as atropine, nicotine, yohimbine, berberine, brucine, and emetine—showed cross-reactivity of less than 0.001 percent. Lumicolchicine, produced by ultraviolet irradiation of colchicine and characterized by thin-layer chromatography (*5*), showed a cross-reactivity of 0.68 percent. Recent data suggest that a portion of plasma colchicine is weakly bound to serum proteins (*9*). For the following reasons it is likely that our radioimmunoassay measures all of the colchicine in plasma. (i) Significant binding to plasma proteins would be reflected in a lack of parallelism between standard curves and dilutions of plasma samples, and (ii) methylene chloride extraction, which rapidly breaks protein-steroid complexes by denaturation, did not result in increased concentrations of plasma colchicine.

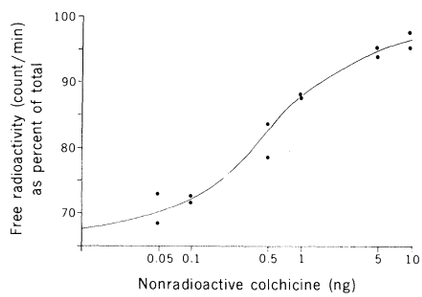

Fig. 1. A typical standard curve for the radioimmunoassay of colchicine.

Seven adult male patients between the ages of 45 and 66 years were studied (with informed consent). All patients had no evidence of hepatic or renal disease; one had a history of gout, but was on no medication, and another patient with a history of gout had a mild attack, with symptoms relieved by the colchicine used in the study. Colchicine (2 mg) was infused within 30 seconds into an antecubital vein in such a way that there was no infiltration into subcutaneous tissues. Blood was drawn into tubes containing dry heparin at 0, 1, 3, 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes. On the day of colchicine administration, urine was collected for the first 2 hours so that we could examine the acute excretory phase (*10*); collection was continued for the

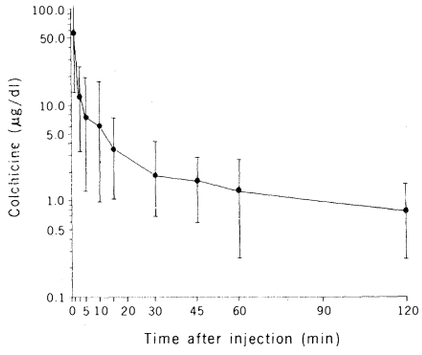

Fig. 2. The disappearance of colchicine in the plasma. The curve was constructed from the means and range of individual values in seven adult male patients without hepatic or renal disease. Colchicine (2 mg) was injected intravenously at zero time.

next 22 hours, for completion of a 24-hour collection. In order to examine the possibility of prolonged circulating levels of colchicine and of prolonged excretion of stored colchicine, plasma levels were assayed at 8:00 a.m. for 4 to 10 days, and 24-hour urinary collections were assayed in three patients for 7 to 10 days. All plasma and urinary samples were collected and stored in containers impervious to ultraviolet light. The concentrations of colchicine in the plasma for each patient were plotted on semilogarithmic paper to give a curve showing its disappearance in plasma. After a phase of rapid decline (the rapid mixing phase), plasma colchicine concentrations declined in a reasonably linear fashion, after 15 to 30 minutes until the end of the initial 2-hour observation period. The slope of this line and the zero-time intercept were calculated for each patient. From these values, the apparent volume of distribution and the half-time in the plasma (the time necessary to reach half the initial, calculated zero-time concentration) were calculated by standard methods (*11*).

A composite curve of the disappearance of colchicine from the plasma was constructed from the mean and range of individual values in seven patients (Fig. 2). As was expected, values in the initial 5 minutes were very variable, ranging from 12 to 2000 ng/ml. At 120 minutes, plasma colchicine, ranging from 4.4 to 19.6 ng/ml, was easily measured in all patients by this method. Plasmas of the control subjects were not different from the zero blanks. The calculated zero-time colchicine concentrations, apparent volumes of distribution, and plasma half-times are presented in Table 1. In five of the seven patients, detectable colchicine concentrations (0.46 to 2.6 ng/ml) were still present 24 hours after administration of the drug. In some patients, colchicine could be detected as late as the morning of day 7. From 128 to 720 μg of colchicine was excreted in the urine in the first 24 hours after administration of the drug (Fig. 3). Of particular interest was that this assay could be used to detect significant excretion of colchicine concentrations ranging from 2 to 10 μg/day from day 4 to day 9 after administration.

In summary, our radioimmunoassay can be used to detect colchicine in small volumes of plasma and urine. After we reported our method (*6*), Boudene and others (*12*) reported in brief a radioimmunoassay of colchicine based on antibodies to a conjugate of *N*-deacetylthiocolchicine and protein. However, since their assay was 100 times less sensitive than ours, it was not clinically or pharmacologically useful. Our results are comparable to data obtained by a method in which radioisotope was given to patients by infusion, followed by a cumbersome procedure requiring extraction and chromatography. In our previous studies, the half-time of colchicine in plasma was 19.3 minutes, and the calculated zero concentration was 1.7 μg/dl. The higher values obtained with radioimmunoassay reflect either patient variability or differences in method. Since the radioimmunoassay does not require purification or chromatography, presumably there is both decreased cleavage of the ring C methoxyl group and decreased conversion to lumicolchicine. The older method required persistence of the labeled methoxyl group for the assay of plasma and urinary colchicine; therefore, loss of this labile group would result in falsely low values.

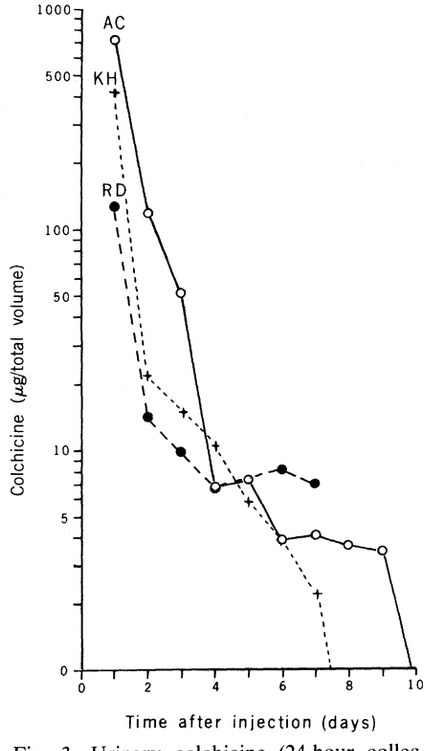

Fig. 3. Urinary colchicine (24-hour collections) in three adult male patients without hepatic or renal disease after intravenous injection of colchicine (2 mg).

Table 1. Plasma colchicine dynamics.

Subject	Calculated zero-time concentration (μg/dl)	Apparent volume of distribution (liter/kg)	Half-time (min)
R.D.	5.5	0.57	90
W.H.	3.8	0.61	49
A.C.	3.6	0.76	37
G.E.	2.5	0.94	65
K.H.	1.1	2.36	75
J.R.	1.9	1.72	52
C.K.	2.2	0.98	35
Mean ± standard deviation			
	2.9 ± 1.5	1.13 ± 0.66	58 ± 20

By the previous method, plasma colchicine could not be detected later than 2 hours after administration of the drug. The radioimmunoassay enabled the detection of low concentrations of drug in the plasma from 24 to 96 hours after the drug was given. In addition, we confirmed observations (*10*) that significant amounts of colchicine can be found in the urine up to 10 days after administration. Such prolonged excretion despite a short plasma half-time implies tissue binding. We have shown that colchicine is concentrated in peripheral leukocytes (*13*) and can be measured for a longer period of time in these cells than in plasma. The pharmacokinetics demonstrated by these studies are similar to those of other agents. For example, digoxin is excreted predominantly in unchanged form by the kidney (*14*), has a high degree of tissue binding, and is excreted in the urine for at least 7 days after a single intravenous dose (*14*).

Our findings explain several observed clinical phenomena. Intravenous colchicine had a brief trial as a chemotherapeutic agent in the treatment of inoperable bronchogenic carcinoma (*15*). When treatment was given for a short period and was then interrupted by a 3-week rest period, there was little or no hematologic or gastrointestinal toxicity. When similar doses were administered from one to three times per week without a rest period, major toxicity was seen, indicating a cumulative effect. In addition,

colchicine is effective in the treatment of about three-quarters of all attacks of gout without significant rebound after cessation of therapy (*16*), although other modes of therapy, such as adrenocorticotrophic hormone, may be followed by recurrent attacks.

The availability of a specific and sensitive method for the measurement of colchicine, not requiring the administration of a radioisotope, should lead to important clinical uses. Whether plasma and urinary concentrations are different in gouty patients on colchicine prophylaxis who have recurrent attacks as opposed to those who do not is not yet known. Our method should be helpful in monitoring patient compliance. Finally, since colchicine is under study in clinical situations apart from its use in acute gout—for example, in hereditary Mediterranean fever (*17*) and in hepatic cirrhosis—a method for the study of the clinical pharmacology of this agent should assume increasing practical importance.

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Gibbons and Their Territorial Songs

Abstract. *Discovery of the great call of the Javan gibbon and finding an enclave of the agile gibbon in Kalimantan permit for the first time a comparison of vocalizations among all major taxa of* Hylobates. *The songs are stereotyped, constant throughout the interrupted areas of distribution of each taxon, and are sexually divocal.*

Recently we succeeded in tape-recording wild populations of the rare silver gibbon of Java, whose unique territorial song has not previously been described. This was during our travels in Southeast Asia to hear all species of gibbons in the natural state in order to compare their vocalizations. We believe such criteria (*1*) might solve the problem of how many species make up the genus *Hylobates* of man's fourth closest relatives. We also discovered throughout the genus that the male's and female's contributions to the territorial song are entirely different, that these songs are stereotyped, and that they are consistent throughout the entire range of each species, even those whose distribution is interrupted by intrusions of another species. Further, we found that the familiar vocalizations of the agile gibbon, hitherto known only from western Malaysia and Sumatra, also prevail over a large section of central Kalimantan as well as in extreme southern Thailand; at the latter place, south of Yala, we had earlier misidentified their songs (*2*). We tape-recorded in the wild all the isolated populations of gibbons (there is a total of 14 such populations variously separated by rivers and straits) except those of the concolor gibbon, which we could not find in the portions of its range we visited, in Laos. Also, we did not observe the populations of siamangs and lar gibbons in western Malaysia, whence excellent recordings were already available.

Audiospectrograms of the territorial songs of gibbons, which we label as if they are all species of equal rank, are displayed in Fig. 1. We have incorporated a few recordings, superior to ours, from colleagues as indicated. A glance at Fig. 1, each line of which reads like a musical staff, shows that the scores are so long and complex (some involving polyphony) that they deserve a more detailed description, which we will offer after introducing the manner and time of delivery and apparent function of the territorial song.

Each pair of gibbons daily advertises its territory by loud singing accompanied by gymnastics—a show of force. The female's great call dominates the half-hour morning bout. It is a brilliant theme lasting 20 seconds or more, repeated every 2 to 5 minutes. It swells in volume after soft opening notes, achieves a climax in pitch, intensity, or rapidity (at which time the gymnastics occur), then subsides. The male's shorter phrases, varying according to the species, either appear at appointed times during the great call, follow it as a coda, are interspersed between great calls (the female's opening notes command his silence during her aria), or are broadcast from his sleeping tree during a predawn chorus (*3*). The male begins this chorus as a simple phrase after which he is silent for a quarter-minute while listening to his neighbors reply in kind. During the next 45 minutes or so until dawn, the male gradually adds to and embellishes the phrase to make it an elaborate, varying, brief song. The female gibbon can utter the short calls of the male; the male, however, never sings the great call. Subtle differences characterize individuals (*3*), and often the subadult joins the bout of great calls (Fig. 1, line 6). Thus, territorial singing of the family broadcasts precise information on the species and sex of particular individuals, the area occupied by the parents, and the presence of a junior ready to form a new pair.

The siamang's great call (Fig. 1, top) is of barks alternating with booms resonated in a vocal pouch which the animal blows up like a balloon. Despite the bark's prominent harmonic, its pitch apparent to the human ear is that of the lower of the two parallel tones, lower also than the male's scream which is uttered at each of the two accelerations by the female. The song ends in a polyphonic tour de force marked by a rise in pitch of the female's bass line to harmonize with the thunderous pouch booms of the male.

The concolor gibbon's dialogue, at least in the zoo pair whose recordings are shown in Fig. 1, commences with a series of short phrases by the male to be interrupted by the great call, which reaches a climax at the highest pitch uttered by any gibbon (not markedly high in this particular, lethargic female), then subsides in a birdlike twitter. The male responds with a much longer, extemporized phrase than before.

Most of the morning singing of the Javan gibbon, *Hylobates moloch*, is a fe-