Correlation of Plasma 4,5-Bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile Levels with Biological Activity

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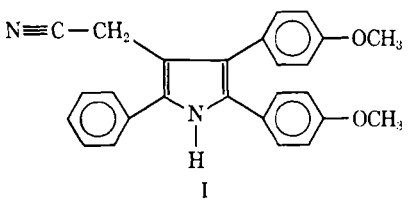
Abstract □ Investigations with 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile, a potent anti-inflammatory, antiarthritic, and antipyretic drug, were undertaken to: (*a*) develop a simple, rapid, sensitive, and specific method of analysis for the intact drug in biological matrixes; (*b*) determine the relationship between plasma drug concentrations, administered dose, and anti-inflammatory activity in polyarthritic rats; (*c*) compare the normalized plasma or serum drug concentrations in rats, dogs, and man when an equivalent oral dose was administered; and (*d*) estimate the plasma or serum drug disappearance half-life in rats, dogs, and man after single-dose oral drug administration. The results indicated that plasma drug concentrations in the polyarthritic rat, as measured by fluorometric techniques, were dose related. Biological activity, expressed as mean percentage inhibition of arthritis, was related to the logarithm of the average plasma drug concentration (micrograms per milliliter) in a dosage interval at the equilibrium state. After single-dose oral drug administration, at calculated equivalent dose levels (milligrams per kilogram), drug concentrations found in the plasma or serum of human subjects were greater than those found in rats or dogs. The plasma drug disappearance half-life found in man ($t_{1/2}$ = 1.5–2.0 hr.) was more similar to dogs ($t_{1/2}$ = 1.5–2.0 hr.) than to rats ($t_{1/2}$ = 11.4 hr.).

Keyphrases □ 4,5-Bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile—correlation of plasma levels with biological activity □ Plasma levels, 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile—correlated with biological activity □ Spectrophotofluorometry—analysis, 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile in plasma

In a continuing search for nonsteroidal anti-inflammatory drugs, 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile[1] (I) (1) was found to be one of the most potent, orally active agents, based on the hind-paw edema assay in rats, of a large number of structurally related compounds.

To study the absorption, metabolism, and excretion of these agents in animals and man, a method for drug analysis applicable to plasma, serum, urine, and feces was needed. Preliminary explorations showed fluorometry to be a generally useful tool for the determination of substituted pyrroles in this series. Since Compound I was selected for extensive biological evaluation (2), a simple, rapid, sensitive, and specific fluorometric method of analysis for this compound in biological matrixes was developed. The present study was undertaken to: (*a*) determine the relationship between plasma I concentrations, administered dose, and anti-inflammatory activity in polyarthritic rats; and (*b*) compare the normalized I concentrations in circulation and the I disappearance half-lives in plasma or serum of rats, dogs, and man after single-dose oral drug administration.



I

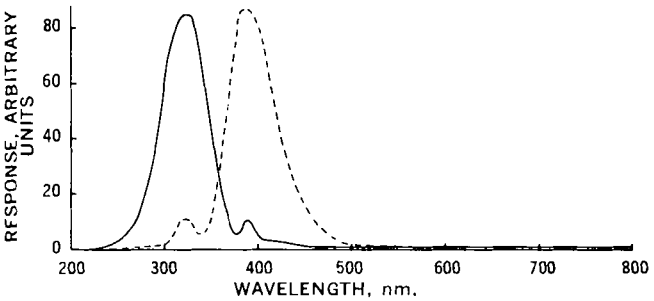


Figure 1—*Excitation* (———) *and fluorescence* (- - -) *spectra of I in benzene (excitation wavelength 325 nm., fluorescence wavelength 385 nm., meter multiplier setting 0.1, 0.287 mcg./ml.).*

EXPERIMENTAL

Reagents and Materials—The Compound I used in this study was synthesized[2]. Aqueous sodium hydroxide (1 *N*) was stored in glass containers. A solution of 2,5-diphenyloxazole[3] in 95% ethanol (0.1 mcg./ml.) was utilized as a spectrophotofluorometric standard. All solvents were reagent grade and were used as supplied. The purity of each lot of benzene was determined by measuring the fluorescence response at 385 nm. when excited at 325 nm. Acceptable lots, stored in glass containers, showed no detectable fluorescence at these wavelengths and a 0.1-m. multiplier setting. All pipets and centrifuge tubes were thoroughly prerinsed with alcohol or acetone and air dried.

Instrumentation—A two-speed reciprocating shaker[4] was used for shaking the samples in the horizontal position. All fluorometric measurements were made with a spectrophotofluorometer[5] equipped with a xenon lamp d.c. power supply[6], a compact xenon arc lamp (150 w.)[7], a potted photomultiplier tube (1P28)[5], and a two-axis recorder[8].

TLC—All chromatography was conducted on thin layers (250 μ) of silica gel GF_{254}[9], ascendingly developed in a solvent system composed of 20% (v/v) ethyl acetate in cyclohexane. The separated materials were visualized by: (*a*) irradiation of the plates with short wavelength (254 nm.) and long wavelength (366 nm.) UV lamps, or (*b*) spraying with 50% (v/v) aqueous sulfuric acid and heating at 110° for 20 min. Under these conditions, intact I had an R_f value of 0.26.

Preparation of Animals—In studies designed to determine the utility of the analytical methodology, Spartan[10] male rats, weighing 192 ± 9 g. each, were fasted for 16 hr. prior to single-dose oral drug administration. Drug-treated animals received 52.1 mg. I/kg. body

[1] U-24,568.

[2] The Research Division of The Upjohn Co. (1).
[3] Pilot Chemicals, Inc., Watertown, Mass.
[4] Eberbach & Sons, Ann Arbor, Mich.
[5] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[6] Sola Electric Co., Elk Grove, Ill.
[7] Engelhard Hanovia, Inc., Newark, N. J.
[8] Autograf, F. L. Moseley Co., Pasadena, Calif.
[9] Brinkmann Instruments, Inc., Long Island, N. Y.
[10] Spartan Research, Haslett, Mich.

Table I—Comparison of Fluorescence Responses[a] Observed in Benzene Extracts of Plasma, Serum, Urine, and Fecal Specimens from Various Species[b]

Biological Matrix	Rat	Dog	Man
Plasma	6–15(50)	5–8(4)	1–6(14)
Serum	5–10(50)	3–7(4)	1–4(3)
Urine	61–226(5)	57–79(4)	7–28(14)
Feces	1320–2950(5)	N.D.[c]	N.D.[c]

[a] Range of fluorescence responses, expressed as millimicrogram I equivalents per milliliter or gram of original sample aliquot. [b] Values in parentheses indicate the number of animals or subjects. [c] N.D. = not determined.

weight, contained in 0.5 ml. polysorbate 80 USP, by gastric intubation. All animals were allowed free access to food and water. At predetermined time intervals from 0 to 24 hr., five rats per time interval were sacrificed and exsanguinated *via* the dorsal aorta. Blood specimens were withdrawn and centrifuged. The serum was harvested and stored at −18°.

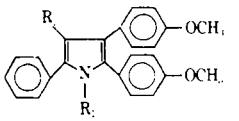
Four beagle dogs, weighing 10.2 ± 0.5 kg. each, were fasted for 16 hr. prior to single-dose oral drug administration. Two dogs received 30 mg. I/kg. body weight in polysorbate 80, and the two remaining animals served as vehicle-treated controls. All animals were allowed free access to food and water. Blood specimens were withdrawn from the jugular vein at 0, 1, 2, 3, and 6 hr. after drug administration. The serum was harvested and stored at −18°.

For the correlation studies, Spartan[10] male rats (200–280 g.) were made polyarthritic by intradermal injection into the tail of 0.5 mg. of dead *Mycobacterium butyricum* (Difco) in 0.1 ml. mineral oil on Day 1. On Day 15, rats with severe arthritis were visually scored. The polyarthritic animals were divided into two groups and weighed. On Day 16, the test group, divided into four subgroups, received the drug orally, b.i.d., at levels of 0, 2.4, 6.8, and 11.4 mg./kg. in 0.5 ml. polysorbate 80. After 28 doses (Day 29), the arthritic scores were again determined. Beginning with the 29th (last) dose, plasma drug concentrations were measured at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr. Five rats per dosage group per time interval were used for the determination of plasma drug concentrations. Forty-five rats per dosage group were used for the determination of plasma inflammation units, visual scoring of inhibitory effects on inflammation, and effects on body weights.

Drug Administration to Man—Two normal human male volunteer subjects were fasted for 16 hr. prior to drug administration. Subjects E.G. and D.K. received single oral doses of 1.333 and 0.524 mg. I/kg. body weight in polysorbate 80, respectively. Food was withheld for an additional 4 hr. Blood specimens were withdrawn at 0, 1, 2, 3, 4, 6, 8, and 24 hr. after drug administration. All plasma specimens were harvested and stored as previously described.

Plasma Inflammation Units Assay—The assay method for the determination of plasma inflammation units in the adjuvant-induced polyarthritic rat was described previously by Glenn and Kooyers (3).

Table II—Excitation Maxima, Fluorescence Maxima, and TLC Mobilities of 4,5-Bis(*p*-methoxyphenyl)-2-phenylpyrrole Analogs



R_1	R_2	Excitation Maximum, nm.	Fluorescence Maximum, nm.	TLC Mobilities, R_f[a]
H	H	335	395	0.35
H	$-CH_2-C\equiv N$	325	385	0.26
H	$-CH_2N(CH_3)_2$	325	390	0
CH_3	H	320	390	0.41
CH_3	$-CH_2-C\equiv N$	320	380	0.30
CH_3	$-CH_2-N(CH_3)_2$	315	380	0

[a] All thin-layer chromatograms were ascendingly developed in a solvent system composed of 20% (v/v) ethyl acetate in cyclohexane.

Table III—Recovery of I from Aqueous Solution and Rat Specimens

Biological Matrix	Added, mcg./ml.	Found, mcg./ml.	Recovery, %
Water[a]	0.105	0.102	97.1
	0.263	0.253	96.2
	0.526	0.520	98.9
		Mean ± *SD* =	97.4 ± 1.38
Rat plasma[b]	0.102	0.094	92.2
	0.255	0.291	114.1
	0.510	0.523	102.6
		Mean ± *SD* =	102.9 ± 11.0
Rat serum[c]	0.102	0.090	88.2
	0.255	0.247	96.9
	0.510	0.525	102.9
		Mean ± *SD* =	96.0 ± 7.4
Rat urine[d]	1.052	1.034	98.3
	2.630	2.659	101.1
	5.260	5.086	96.7
		Mean ± *SD* =	98.7 ± 2.2
Rat feces[e]	10.52	9.39	89.3
	26.30	25.22	95.9
	52.60	52.44	99.7
		Mean ± *SD* =	95.0 ± 5.3

[a] Observed values corrected for a water blank of 0.010 mcg./ml. [b] Observed values corrected for a plasma blank of 0.014 mcg./ml. [c] Observed values corrected for a serum blank of 0.010 mcg./ml. [d] Observed values corrected for a urine blank of 0.23 mcg./ml. [e] Observed values corrected for a feces blank of 2.95 mcg./g.

Fluorometric Assay—Place 2 ml. of serum or plasma in a glass-stoppered centrifuge tube. Add 0.05 ml. 1 *N* aqueous sodium hydroxide and 5 ml. benzene, and shake in the horizontal position for 20 min. Avoid exposure to direct sunlight. Centrifuge for 10 min. at 2000 r.p.m. Transfer a 4-ml. aliquot of the benzene layer to a fresh glass-stoppered centrifuge tube and store in the dark until assay. By using the same excitation and emission maxima as was used for I, the fluorometer is standardized with a solution of 2,5-diphenyloxazole in 95% ethanol (75% transmission; 0.1-m. multiplier setting). The excitation maximum for I in benzene is at 325 nm. and the emission maximum is at 385 nm. (Fig. 1). The concentration of I is determined from a standard curve with appropriate corrections for the serum or plasma blank.

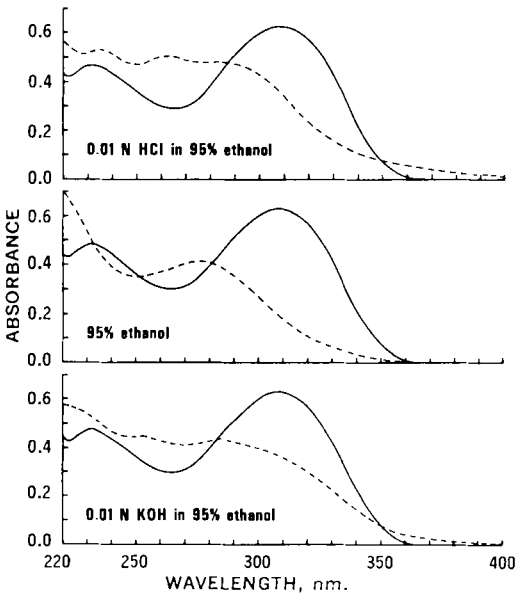


Figure 2—*UV absorption spectra of I in acidic, neutral, and basic ethanol (9.18 mcg./ml.) at time zero (—) and 24 hr. (- - -) after exposure to direct sunlight.*

Table IV—Comparison of I Concentrations in Rat Serum, Dog Serum, and Human Plasma (Micrograms per Milliliter) after Single-Dose Oral Drug Administration in Polysorbate 80

Species:	Rat	Dog M65-204 ♂	Dog 5272 ♀	Man (E.G.)	Man (D.K.)
Dose, mg./kg.:	52.1	30.0	30.0	1.333	0.524
Specimen Analyzed:	Serum	Serum	Serum	Plasma	Plasma
Hours					
1	1.36 ± 0.14[a]	0.30	1.29	0.140	0.083
2	1.38 ± 0.32	2.38	1.22	0.263	0.113
3	1.18 ± 0.31	1.16	0.79	0.203	0.065
6	0.99 ± 0.07	0.33	0.27	0.070	0.013
$\int_0^6 C \cdot dt$[b]	6.59	5.50	4.51	0.915	0.346
$\bar{c}$[c]	1.10	0.92	0.75	0.153	0.058
$\bar{c}_{eq}$[d]	0.021	0.031	0.025	0.115	0.111
Ratio[e]	1.00	1.47	1.19	5.48	5.29

[a] Mean ± *SEM* for five rats per time interval. [b] Integrated area under the serum or plasma level curve over the 0–6-hr. time interval, expressed as micrograms hour per milliliter, where C = concentration of I in micrograms per milliliter and t = time in hours. [c] Average serum or plasma drug concentration in micrograms per milliliter obtained by dividing integrated area by 6. [d] Average serum or plasma drug concentration equivalent to a 1-mg./kg. dose of I, expressed as micrograms per milliliter. [e] Average serum or plasma concentration ratio compared to the rat.

RESULTS AND DISCUSSION

Solution Stability of 4,5-Bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile—Standard I has absorption maxima ($\lambda_{max}^{ethanol}$) at 231 and 308 nm. with molar absorptivities (ϵ) of 19,900 and 26,900, respectively. During the routine UV determination of absorptivities in 95% ethanol, 0.01 *N* HCl in 95% ethanol, and 0.01 *N* KOH in 95% ethanol, spectral changes were observed between 0 and 24 hr. (Fig. 2) which could not be reversed by neutralization with acid or base. Changes indicative of a degradation were observed also in the fluorescence excitation spectra. TLC showed two zones with R_f values of 0.15 and 0 in addition to intact I, confirming degradation. Subsequent experiments demonstrated that a photochemical degradation was involved, since I was stable in acidic, neutral, and basic ethanol for at least 24 hr. when stored in the absence of direct sunlight. Similar investigations conducted with aqueous suspensions, benzene solutions, and polysorbate 80 solutions indicated that I was stable for at least 24 hr. in the absence of direct sunlight. As a precaution, all specimen extracts were stored in the dark until assayed. The stability studies conducted on the acidic aqueous suspension and polysorbate 80 solutions indicated that I should be stable in the stomach. TLC of rat plasma extracts provided additional evidence that the intact compound is absorbed.

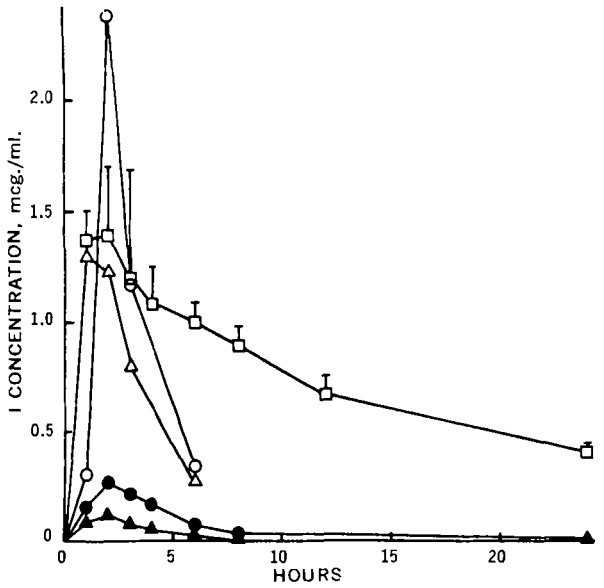


Figure 3—*Plasma or serum concentrations of I in rats, dogs, and man after single-dose oral drug administration in polysorbate 80. Key:* □—□, *rats, 52.1 mg./kg.;* ○—○, *Dog M65-204 ♂, 30 mg./kg.;* △—△, *Dog 5272 ♀, 30 mg./kg.;* ●—●, *man (E.G.), 1.333 mg./kg.; and* ▲—▲, *man (D.K.), 0.524 mg./kg.*

Assay Sensitivity and Specificity—The maximum fluorometric sensitivity of I in benzene is 0.005 mcg./ml. using a meter multiplier setting of 0.003. However, the lower limits of detection of I in extracts of rat plasma, serum, urine, and feces are 0.03 mcg./ml., 0.02 mcg./ml., 0.46 mcg./ml., and 5.9 mcg./g. of the original sample aliquots, respectively. These values are based on a sample response two times that of the appropriate blank (Table I). Under the assay conditions described, a linear relationship between fluorescence response and concentration is obtained for I in benzene over the range of 0–0.3 mcg./ml. Direct quantification from a standard curve has been adequate. The method detects any material that is extractable into benzene under alkaline conditions and fluoresces at 385 nm. when excited at 325 nm. Excitation, fluorescence, and TLC data (Table II) show the general applicability of the method to the determination of similarly substituted pyrroles. Interference by endogenous materials in most specimens is very low, as indicated by the lower limits of detection cited here. However, metabolic transformations in I could give rise to circulating materials responding to the assay. Accordingly, excitation spectra were routinely obtained on plasma and serum extracts as a check on specificity; metabolic changes would be expected to result in altered UV spectra and, therefore, fluorescence spectra. No such changes were observed. Moreover, TLC examination of extracts of plasma and serum specimens from drug-treated rats, dogs, and humans showed that greater than 90% of the total fluorometric response was in the zone corresponding to the intact drug (R_f 0.26).

Table V—Plasma Levels of I in Adjuvant-Induced Polyarthritic Rats after Multiple Oral Doses of the Drug in Polysorbate 80

Hours[c]	I Plasma Concentrations (mmcg./ml.)[a] ± *SEM*[b] 2.4 mg./kg.	6.8 mg./kg.	11.4 mg./kg.
0	0[b]	37 ± 6	77 ± 16
1	37 ± 6	137 ± 31	234 ± 50
2	59 ± 13	135 ± 19	219 ± 42
3	41 ± 7	174 ± 18	280 ± 33
4	58 ± 13	139 ± 22	236 ± 41
6	26 ± 5	124 ± 20	272 ± 36
8	0[b]	151 ± 9	247 ± 55
12	0[b]	77 ± 12	138 ± 24
24	0[b]	0[b]	37 ± 5
$\int_0^{12} C \cdot dt$[d]	276.0	1528.0	2686.5
$\bar{c}$[e]	23.00	127.33	223.88

[a] mmcg./ml. = millimicrograms per milliliter. [b] *SEM* = standard error of mean. All values corrected for an average plasma blank of 10 mmcg./ml. [c] Time after last dose of drug on Day 29. [d] Integrated area under the plasma level curve over the 0–12-hr. dosage interval, expressed as millimicrograms hour per milliliter, where C = concentration of I in millimicrograms per milliliter and t = time in hours. [e] Average plasma drug concentration in millimicrograms per milliliter obtained by dividing integrated area by 12. [f] Plasma I concentration less than 20 mmcg./ml.

Table VI—I Inhibitory Effects in Adjuvant-Induced Polyarthritic Rats after Multiple Oral Doses of the Drug in Polysorbate 80[a]

Dose of I, mg./kg. orally, b.i.d.	Initial Body Weight, g.	Final Body Weight, g.	Change in Body Weight[b], g.	Initial Score	Final Score	Percent *I*[c]	Plasma Inflammation Units	Percent *I*[c]
0	240	248	8 ± 6.7	8.0 ± 0	7.5 ± 0.3	—	51 ± 5.8	—
2.4	239	258	19 ± 9.7	8.0 ± 0	5.8 ± 0.6	23	39 ± 5.4	24
6.8	235	278	43 ± 5.8	8.0 ± 0	3.8 ± 0.6	49	22 ± 3.4	57
11.4	240	279	39 ± 10.7	8.0 ± 0	2.9 ± 0.4	61	19 ± 2.8	63

[a] Values reported are mean ± *SEM* for 45 rats per group. [b] Animals receiving effective nonsteroidal anti-inflammatory agents showed improvement in body weight gain. This serves as another index for the determination of the effectiveness of anti-inflammatory drugs, since untreated adjuvant-induced polyarthritic control rats usually lose weight. The slight weight increase in vehicle-treated controls may be attributable to increased absorption of dietary nutrients or a slight anti-inflammatory effect. [c] Percent *I* = percent inhibition of arthritis as measured by the respective assays.

Recovery Experiments—Known amounts of I in 95% ethanol were evaporated to dryness in centrifuge tubes, and water or specimens of rat plasma, serum, urine, or feces were added. The samples were thoroughly mixed and extracted, and the extracts were analyzed fluorometrically. The results (Table III) indicated that recoveries were essentially quantitative and compared favorably with those obtained from simple aqueous samples. An overall mean recovery ± standard deviation of 98.0 ± 6.2% was obtained.

Comparison of Circulating Drug Levels in Rat, Dog, and Man—Studies designed to determine the utility of the fluorometric method for measurement of intact drug in rat serum, after single-dose oral administration of 52.1 mg./kg. body weight, showed that peak serum drug concentrations of 1.38 ± 0.32 mcg./ml. were attained within 2 hr. (Fig. 3). Some indication of biological variation in absorption may be seen in the standard errors of the mean serum drug concentrations. By using the data between 2 and 24 hr. after drug administration, the serum drug disappearance half-life was estimated graphically to be 11.4 hr. These results indicated that the fluorometric method was suitable for measuring plasma drug concentrations in rats at a dosage level of at least 5 mg./kg.

In dogs, after single-dose oral administration of 30 mg. I/kg. body weight, peak serum drug concentrations of 1.29–2.38 mcg./ml. were observed at 1–2 hr. Disappearance of I from the serum was very rapid, and the half-life was estimated graphically to be 1.5–2.0 hr. Similarly in man, drug absorption and disappearance from the plasma were rapid. Peak plasma drug concentrations of 0.113 and 0.263 mcg./ml. were observed at 2 hr. following single-dose oral administration of 0.524 and 1.333 mg. I/kg. body weight, respectively. The plasma drug disappearance half-life was estimated to be 1.5–2.0 hr.

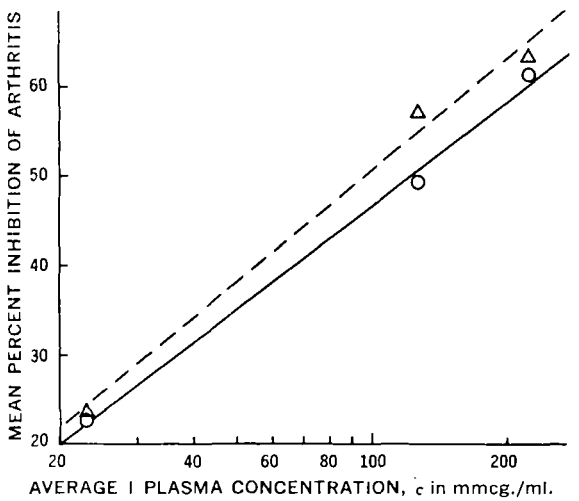


Figure 4—*Correlation of antiarthritic activity and plasma concentrations of I in rats. Adjuvant-induced polyarthritic rats received drug at levels of 0, 2.4, 6.8, and 11.4 mg./kg. p.o., b.i.d., in polysorbate 80. Mean percentage inhibition of arthritis was obtained from arthritic score* (○—○) *and plasma inflammation unit* (△—△) *measurements. Plasma drug concentrations are expressed as averages over a 12-hr. interval following the last dose.*

By assuming a linear relationship between average plasma or serum drug concentrations and the oral dose of drug administered to each species, all data were normalized to a 1-mg./kg. equivalent dose of I (Table IV). Analysis of the data available, which were obtained at the same sampling times during the 0–6-hr. time interval, indicated that: (*a*) 1.2–1.5 times more drug was found in the serum of dogs as compared to rats, and (*b*) 5.3–5.5 times more drug was found in the plasma of humans as compared to rat serum. Differences in absorption and distribution of the intact drug and/or vehicle, as well as differences in metabolic rates in the various species, may be responsible for the marked differences observed.

Correlation of Plasma Drug Levels with Biological Activity—Investigations in the rat indicated that biological activity was related to the logarithm of the oral dose of I administered. Experiments were designed to determine whether a correlation existed between: (*a*) plasma drug levels as measured by the fluorometric assay, (*b*) the dose of I administered, and (*c*) biological activity in the polyarthritic rat. In the chronic multiple-dose experiment, conducted with polyarthritic male rats, the results (Table V) indicated that the average drug levels at the equilibrium state ($\bar{c}$, expressed as micrograms per milliliter) were linearly related to the oral dose of I administered (D, expressed as milligrams I per kilogram body weight). The best-fit straight line, as determined by regression analysis, could be expressed by the equation $\bar{c} = 22.31D - 28.46$ (correlation coefficient = 0.99). Biological activity, as measured by plasma inflammation units or visual scoring, was linearly related to the logarithm of the oral dose (Table VI) and, hence, to the logarithm of the average plasma drug levels at the equilibrium state (Fig. 4). These results, which are consistent with earlier extensive studies on indoxole (4, 5), suggested that biological effects of I are directly related to plasma drug concentrations and that the fluorometric method could be used for the selection of species and doses for toxicologic investigations as well as measurement of plasma drug absorption and disappearance in man.

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