Fluorometric Determination of Trifluoromethyl-Substituted 2,3-Bis(4-methoxyphenyl)indoles in Biological Materials

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Abstract □ Interest in the 5-, 6-, and 7-trifluoromethyl-substituted 2,3-bis(4-methoxyphenyl)indoles as potent, orally active anti-inflammatory agents required a method for their determination in serum, urine, and feces to permit studies of their absorption, metabolism, and excretion. A simple, rapid, sensitive, and specific procedure was developed based on an ethyl acetate extraction of alkaline specimens and subsequent fluorometric analysis of ethanolic solutions of the extract residues. The method is sensitive to 0.1 μg/ml, 0.5 μg/ml, and 0.7 μg/100 mg of these compounds in serum, urine, and feces, respectively. Overall mean recoveries and standard deviations of the 5-, 6-, and 7-trifluoromethyl-substituted compounds from biological samples were 102.2 ± 3.0, 102.2 ± 5.4, and 100.5 ± 5.7%, respectively. The procedure was applied successfully to absorption studies with 2,3-bis(4-methoxyphenyl)-7-(trifluoromethyl)indole in the dog.

Keyphrases □ 2,3-Bis(4-methoxyphenyl)indoles, trifluoromethyl substituted—fluorometric analysis, serum, urine, and feces □ Fluorometry—analysis, trifluoromethyl-substituted 2,3-bis(4-methoxyphenyl)indoles in serum, urine, and feces □ Anti-inflammatory agents—trifluoromethyl-substituted 2,3-bis(4-methoxyphenyl)-indoles, fluorometric analysis in serum, urine, and feces

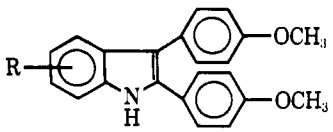
In a continuing search for nonsteroidal anti-inflammatory drugs, the trifluoromethyl-substituted 2,3-bis(4-methoxyphenyl)indoles were found to be potent orally active agents (1). Studies of their absorption, metabolism, and excretion required a method for their determination in serum, urine, and feces.

Previous reports described fluorometric procedures for the determination of indoxole [2,3-bis(4-methoxyphenyl)indole, I] (2), 4,5-bis(4-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3), and 2,3-bis(4-methoxyphenyl)imidazo[1,2-*a*]pyrimidine (4) in biological materials. These methods were successfully used in investigations in animals and humans (3–7).

Exploratory studies showed that the 5-, 6-, and 7-trifluoromethyl-substituted 2,3-bis(4-methoxyphenyl)indoles (II, III, and IV, respectively) possessed useful fluorescence characteristics. Therefore, a simple, rapid, sensitive, and specific fluorometric procedure was developed for their determination in biological materials.

EXPERIMENTAL

Reagents and Materials—Aqueous sodium hydroxide (1 *N*) was stored in glass containers. Ethyl acetate was equilibrated with an equal volume of water prior to use in the extraction step. A solution



I: R = H
II: R = 5-CF_3
III: R = 6-CF_3
IV: R = 7-CF_3

Table I—Excitation and Fluorescence Maxima for 2,3-Bis(4-methoxyphenyl)indoles in 95% Ethanol

Compound	Excitation Maximum, nm	Emission Maximum, nm
I[a]	320	420
II	330	415
III	325	415
IV	325	415

[a] Reference 2.

Table II—Effect of Extraction Time on Recovery of II[a] from Rat Serum

Extraction Time, min	II Found, μg/ml	Recovery, %
5	1.85	74.3
10	2.03	81.5
15	2.26	90.7
20	2.48	99.6
25	2.50	100.5
30	2.50	100.5

[a] The amount of II added was 2.49 μg/ml in all cases.

of quinine sulfate[1] (2.25 μg/ml in 0.1 *N* aqueous sulfuric acid) was utilized as a spectrophotofluorometric standard. All pipets and centrifuge tubes were thoroughly rinsed with alcohol or acetone and air dried prior to use.

Instrumentation—A blender[2] and/or a homogenizer[3] were used in the preparation of fecal specimens for extraction. A two-speed reciprocating shaker[4] was utilized for sample extractions in the horizontal position. Fluorometric measurements[5] were made with a spectrophotofluorometer[6] equipped with a xenon lamp dc power supply[7], a compact xenon arc lamp[8] (150 w), a potted photomultiplier tube[9], and a two-axis recorder[10].

Standards—Stock solutions of II–IV were prepared by dissolving 5–10 mg in 100 ml of 95% ethanol with shaking. Working standards were prepared by dilution of the stock solutions with 95% ethanol to give final concentrations of approximately 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 μg/ml. Standard solutions were stored in the dark since II, III, and IV solutions are susceptible to photochemical decomposition.

Collection and Storage of Specimens—Blood samples were allowed to clot at room temperature (27°) for 1 hr and then were centrifuged at 2000 rpm for 10 min, and the serum was harvested. Urine specimens were collected, and 1 ml of 37% formaldehyde was added for each 50 ml. All serum, urine, and fecal specimens were stored at −18° until analyzed.

Extractions—In a 15-ml glass-stoppered centrifuge tube were placed 1 ml of serum or urine, 0.05 ml of 1 *N* aqueous sodium hydroxide, and 3 ml of ethyl acetate. The mixture was shaken in a horizontal position for 20 min, avoiding exposure to direct sunlight, and then centrifuged at 2000 rpm for 10 min. A 1-ml aliquot of the ethyl acetate layer was transferred to a glass-stoppered centrifuge tube and

[1] Matheson, Coleman, and Bell, Milwaukee, Wis.
[2] Model PB-5A, Waring Products Corp., Winsted, Conn.
[3] Virtis 45, Virtis Co., Gardiner, N.Y.
[4] Eberbach & Sons, Ann Arbor, Mich.
[5] Identical results were obtained with a Hitachi Perkin-Elmer (model MPF-2A) spectrophotofluorometer, Perkin-Elmer Corp., Norwalk, Conn.
[6] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[7] Sola Electric Co., Elk Grove, Ill.
[8] Engelhard Hanovia, Inc., Newark, N.J.
[9] 1P28, Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[10] Autograf, F. L. Moseley Co., Pasadena, Calif.

Table III—Lower Limits of Detection[a] for II–IV in Rat and Dog Serum, Urine, and Feces

Sample	II	III	IV
Rat serum, μg/ml	0.06	0.04	0.08
Dog serum, μg/ml	0.04	0.04	0.12
Rat urine, μg/ml	0.20	0.28	0.14
Dog urine, μg/ml	0.30	0.48	0.22
Rat feces, μg/100 mg	0.46	0.42	0.28
Dog feces, μg/100 mg	0.62	0.74	0.64

[a] Based on sample response two times that of the appropriate blank.

evaporated to dryness in a stream of nitrogen. The residue was reconstituted in 5 ml of 95% ethanol and stored in the dark until analyzed.

For rat feces, the frozen specimen was weighed and placed in the homogenizer[3]. A volume of water equivalent to twice the sample weight was added, and the mixture was homogenized at high speed for 1 min. A 100-mg aliquot of the slurry was weighed into a 15-ml glass-stoppered centrifuge tube; then 1 ml of water, 0.05 ml of 1 *N* aqueous sodium hydroxide, and 3 ml of ethyl acetate were added. The extraction was completed as described for serum and urine.

For dog feces, the total specimen was weighed and placed in the blender[2]. A volume of water equivalent to twice the sample weight was added, and the mixture was homogenized at high speed for 1 min. An aliquot (about 20 ml) was removed and homogenized[3] at high speed until a fine slurry was produced. A 100-mg aliquot was weighed into a 15-ml glass-stoppered centrifuge tube; then 1 ml of water, 0.05 ml of 1 *N* aqueous sodium hydroxide, and 3 ml of ethyl acetate were added. The extraction was completed as described for serum and urine.

Fluorometric Analysis—The spectrophotofluorometer was standardized with the quinine sulfate solution (60% transmission at 0.3 multiplier setting[9]), using the excitation and emission maxima for the specific compound to be determined (Table I). Fluorescence intensities of the samples were measured at these wavelength settings.

Table IV—Recovery of II from Aqueous Solution and Biological Materials

Sample	II Added[a]	II Found[b]	Recovery, %
Water	0.50	0.50	100.0
	1.00	0.99	99.0
	2.00	1.99	99.5
		Mean ± *SD*	99.5 ± 0.5
Rat serum	0.50	0.49	98.0
	1.00	1.01	101.0
	2.00	2.00	100.0
		Mean ± *SD*	99.7 ± 1.5
Dog serum	0.50	0.50	100.0
	1.00	1.05	105.0
	2.00	2.05	102.5
		Mean ± *SD*	102.5 ± 2.5
Rat urine	0.50	0.55	110.0
	1.00	1.04	104.0
	2.00	2.04	102.0
		Mean ± *SD*	105.3 ± 4.2
Dog urine	0.50	0.53	106.0
	1.00	1.01	101.0
	2.00	1.98	99.0
		Mean ± *SD*	102.0 ± 3.6
Rat feces	4.99	5.01	100.4
	9.98	10.14	101.6
	19.96	20.74	103.9
		Mean ± *SD*	101.9 ± 1.8
Dog feces	4.99	4.89	98.0
	9.98	10.43	104.5
	19.96	20.51	102.7
		Mean ± *SD*	101.7 ± 3.4

[a] Values for water, serum, and urine are expressed as micrograms per milliliter. Values for feces are expressed as micrograms per 100 mg. [b] Corrected for appropriate specimen blank.

Table V—Recovery of III from Aqueous Solution and Biological Materials

Sample	III Added[a]	III Found[b]	Recovery, %
Water	0.49	0.50	102.0
	0.98	0.98	100.0
	1.97	1.96	99.4
		Mean ± *SD*	100.5 ± 1.4
Rat serum	0.49	0.48	97.9
	0.98	0.99	101.0
	1.97	1.96	99.4
		Mean ± *SD*	99.4 ± 1.6
Dog serum	0.49	0.47	95.9
	0.98	0.97	98.9
	1.97	1.98	100.5
		Mean ± *SD*	98.4 ± 2.3
Rat urine	0.49	0.53	108.1
	0.98	1.02	104.1
	1.97	1.99	101.0
		Mean ± *SD*	104.4 ± 3.6
Dog urine	0.49	0.53	110.2
	0.98	1.05	107.1
	1.97	1.98	100.5
		Mean ± *SD*	105.9 ± 5.0
Rat feces	5.03	5.03	100.0
	10.05	9.90	98.5
	20.10	22.21	110.5
		Mean ± *SD*	103.0 ± 6.5
Dog feces	5.03	4.95	98.4
	10.05	9.55	95.0
	20.10	22.81	113.4
		Mean ± *SD*	102.3 ± 9.8

[a] Values for water, serum, and urine are expressed as micrograms per milliliter. Values for feces are expressed as micrograms per 100 mg. [b] Corrected for appropriate specimen blank.

Drug concentrations were calculated from a standard curve (working standards) with appropriate corrections for serum, urine, or feces blanks.

Animal Preparation—In studies designed to determine the utility of the method, three male beagle dogs, 12.5 ± 1.2 kg, were fasted for 16 hr prior to single-dose oral drug administration. Two dogs received approximately 20 mg of IV/kg in polysorbate 80, and the remaining animal served as a vehicle-treated control. All animals were fasted for an additional 4 hr after drug administration and were then allowed free access to food and water.

Blood specimens were withdrawn from the jugular veins at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr postadministration. Urine specimens were collected at predetermined time intervals during the first 24 hr after drug administration. All specimens were stored at −18°.

TLC—All chromatography was conducted on thin layers (250 μm) of silica gel F_{254}[11], ascendingly developed in 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 II, III, and IV had R_f values of 0.22, 0.30, and 0.44, respectively.

RESULTS AND DISCUSSION

Earlier studies with indoxole (2) and 4,5-bis(4-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3) showed those compounds to be susceptible to photochemical degradation. Similar behavior was found for II–IV. Therefore, as a precaution, all specimen extracts were stored in the dark until analyzed.

Fluorescence responses for II–IV were compared with those for indoxole (I), using the excitation and emission maxima of the latter (Table I). On a molar basis relative to a value of unity for I, responses for II, III, and IV were 1.89, 2.69, and 1.67, respectively. A preferential

[11] Brinkmann Instruments, Westbury, N.Y.

Table VI—Recovery of IV from Aqueous Solution and Biological Materials

Sample	IV Added[a]	IV Found[b]	Recovery, %
Water	0.51	0.49	96.0
	1.01	1.04	102.9
	2.02	2.04	100.9
		Mean ± *SD*	99.9 ± 3.6
Rat serum	0.51	0.46	90.2
	1.01	1.00	99.0
	2.02	2.11	104.4
		Mean ± *SD*	97.8 ± 7.8
Dog serum	0.51	0.50	98.0
	1.01	1.07	105.9
	2.02	2.11	104.4
		Mean ± *SD*	102.7 ± 4.2
Rat urine	0.51	0.40	96.0
	1.01	1.00	99.0
	2.02	2.14	105.9
		Mean ± *SD*	100.3 ± 5.1
Dog urine	0.51	0.46	90.2
	1.01	1.04	102.9
	2.02	2.26	111.8
		Mean ± *SD*	101.6 ± 10.9
Rat feces	5.1	5.4	105.8
	10.1	10.3	101.9
	20.2	20.2	100.0
		Mean ± *SD*	102.5 ± 3.0
Dog feces	5.1	5.1	100.0
	10.1	9.4	93.0
	20.2	20.3	100.4
		Mean ± *SD*	97.8 ± 4.2

[a] Values for water, serum, and urine are expressed as micrograms per milliliter. Values for feces are expressed as micrograms per 100 mg. [b] Corrected for appropriate specimen blank.

electronic distribution in the resonance states of the trifluoromethyl-substituted compounds is suggested.

Low recoveries (80–90%) of II and III from rat serum were obtained utilizing the 10-min extraction previously described for indoxole (2). A determination of the recovery of II from rat serum as a function of extraction time (Table II) established the need for a 20-min extraction and suggested that the trifluoromethyl-substituted analogs are more tightly bound to serum proteins than is the parent compound, indoxole.

The maximum fluorometric sensitivity for II–IV in 95% ethanol was 0.005 μg/ml (meter multiplier[9] setting of 0.003). However, the practical lower limits of detection in biological matrixes are controlled by the magnitude of the specimen blanks in addition to the inherent fluorescence emissions of the specific compounds. Calculated lower detection limits for II–IV, based on sample responses twice those of the appropriate blanks, are shown in Table III. Taken collectively, II–IV may be detected in serum, urine, and feces at levels of ≥0.1 μg/ml, ≥0.5 μg/ml, and ≥0.7 μg/100 mg, respectively.

Under the described conditions, a linear relationship between fluorescence response and concentration of II–IV was obtained over the 0–0.8-μg/ml range in 95% ethanol. Direct quantification from a standard curve was adequate.

To determine recoveries from biological samples, known amounts of II–IV in 95% ethanol were evaporated to dryness in centrifuge tubes, and water or specimens of rat or dog serum, urine, or feces were added. The samples were thoroughly mixed and analyzed by the standard procedure. Overall mean recoveries and standard deviations for II, III, and IV from biological samples were 102.2 ± 3.0% (Table IV), 102.2 ± 5.4% (Table V), and 100.5 ± 5.7% (Table VI). These results validate the use of simple standard curves in routine analyses as described previously.

The utility of the method was established by a limited study with IV in dogs. Following oral administration of approximately 30 mg of IV/kg, peak serum IV levels of 1.85–2.19 μg/ml were observed between

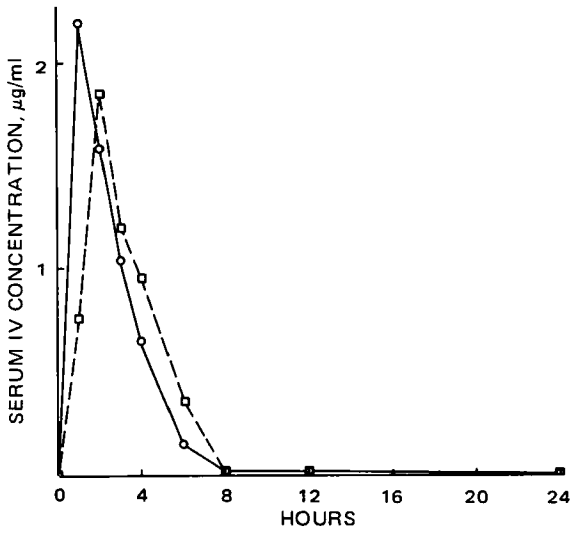


Figure 1—*Serum concentrations of IV in dogs after single-dose oral drug administration in polysorbate 80. Key:* ○, *Dog 478 (male), 20.9 mg/kg; and* □, *Dog 479 (male), 20.3 mg/kg.*

1 and 2 hr (Fig. 1). Measurable concentrations were present during the first 12 hr after drug administration. These results are similar to those reported for indoxole (2, 6), 4,5-bis(4-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3), and 2,3-bis(4-methoxyphenyl)imidazo[1,2-*a*]pyrimidine (4) in dogs.

The present method detects materials that are extractable from an alkaline solution with ethyl acetate and that possess excitation and fluorescence characteristics in 95% ethanol similar to the administered drug. Interference by endogenous materials in most specimens is very low, as indicated by the lower limits of detection (Table III). However, metabolic transformations could give rise to compounds responding in the assay. Accordingly, excitation spectra were routinely obtained on serum extracts as a check on specificity; metabolic changes would be expected to result in altered UV spectra and, therefore, fluorescence excitation spectra. No such changes were observed in the dog. Moreover, TLC of serum extracts showed that the major component (>85%) was similar to IV, confirming the measurement of intact drug. Thus, the method possesses adequate sensitivity and specificity for drug absorption studies in animals.

Based on fluorometric and TLC analyses, less than 1% of the dose was excreted in urine as intact IV by the dog during the 24-hr collection interval. Extensive metabolism and/or biliary secretion are indicated.

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