Fluorometric Determination of 2,3-Bis(*p*-methoxyphenyl)imidazo[1,2-*a*]pyrimidine in Biological Materials

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Abstract □ Interest in 2,3-bis(*p*-methoxyphenyl)imidazo[1,2-*a*]-pyrimidine as a potent, orally active, anti-inflammatory agent required a method for its determination in serum, urine, and feces to permit studies of its 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 0.2 μg/ml, 0.3 μg/ml, and 1.2 μg/100 mg of drug in serum, urine, and feces, respectively. The overall mean recovery and the standard deviation from biological samples are 99.8 ± 5.1%. The procedure has been successfully applied to absorption studies in the dog.

Keyphrases □ 2,3-Bis(*p*-methoxyphenyl)imidazo[1,2-*a*]pyrimidine—fluorometric analysis, biological materials, anti-inflammatory agent □ Anti-inflammatory agents—2,3-bis(*p*-methoxyphenyl)-imidazo[1,2-*a*]pyrimidine, fluorometric analysis, biological materials □ Fluorometry—analysis, 2,3-bis(*p*-methoxyphenyl)imidazo-[1,2-*a*]pyrimidine, anti-inflammatory agent, biological materials

In a continuing search for nonsteroidal anti-inflammatory drugs, 2,3-bis(*p*-methoxyphenyl)imidazo[1,2-*a*]pyrimidine (I), together with some structurally related compounds, was found to be a highly potent agent (1). Studies of the absorption, metabolism, and excretion of I required a method for its determination in serum, urine, and feces.

Previous reports described fluorometric procedures for the determination of indoxole [2,3-bis(*p*-methoxyphenyl)indole] (2) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3) in biological materials. These methods were successfully used in investigations in animals and humans (3–6).

Exploratory studies showed that I possessed useful fluorescence characteristics. Therefore, a simple, rapid, sensitive, and specific fluorometric procedure was developed for the determination of I 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 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 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 were made with a spectrophotofluorometer[5] equipped with a xenon lamp dc power supply[6], a compact xenon arc lamp (150 w)[7], a potted photomultiplier tube (1P28)[5], and a two-axis recorder[8].

Table I—Lower Limits of Detection for I in Rat and Dog Serum, Urine, and Feces

Specimen	Detection Limit[a]
Rat serum	0.06 μg/ml
Dog serum	0.18 μg/ml
Rat urine	0.30 μg/ml
Dog urine	0.24 μg/ml
Rat feces	1.20 μg/100 mg
Dog feces	0.88 μg/100 mg

[a] Based on a sample response twice that of the appropriate specimen blank.

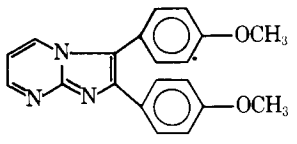
Standards—A stock solution of I was prepared by dissolving 5–10 mg in 100 ml of 95% ethanol with shaking. Working standards were prepared by dilution of the stock solution with 95% ethanol to give final concentrations of approximately 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 μg/ml. No special storage conditions were required since I is photochemically stable in acidic, neutral, or basic ethanolic solutions for at least 24 hr.

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 with 1 ml of 37% formaldehyde 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 10 min and then centrifuged for 10 min at 2000 rpm. A 1-ml aliquot of the ethyl acetate layer was transferred to a separate glass-stoppered centrifuge tube and evaporated to dryness in a stream of nitrogen. The residue was reconstituted in 5 ml of 95% ethanol for fluorometric analysis.

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, and 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, and 1 ml of water, 0.05 ml of 1 *N* aqueous sodium hydroxide, and 3 ml of ethyl



I

[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] 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.

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

Specimen	I Added[a]	I Found[b]	Recovery, %
Water	0.50	0.49	98.0
	1.00	0.98	98.0
	1.99	2.00	100.5
		Mean ± *SD*	98.8 ± 1.4
Rat serum	0.50	0.55	110.0
	1.00	0.95	95.0
	1.99	2.00	100.5
		Mean ± *SD*	101.8 ± 7.6
Dog serum	0.50	0.49	98.0
	1.00	1.03	103.0
	1.99	2.00	100.5
		Mean ± *SD*	100.5 ± 2.6
Rat urine	0.50	0.52	104.0
	1.00	1.03	103.0
	1.99	1.98	99.5
		Mean ± *SD*	102.2 ± 2.4
Dog urine	0.50	0.48	96.0
	1.00	1.00	100.0
	1.99	2.02	101.5
		Mean ± *SD*	99.1 ± 2.8
Rat feces	5.0	4.3	86.0
	10.0	9.8	98.0
	19.9	20.7	104.0
		Mean ± *SD*	96.0 ± 9.2
Dog feces	5.0	4.7	94.0
	10.0	10.0	100.0
	19.9	20.6	103.5
		Mean ± *SD*	99.1 ± 1.8

[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.

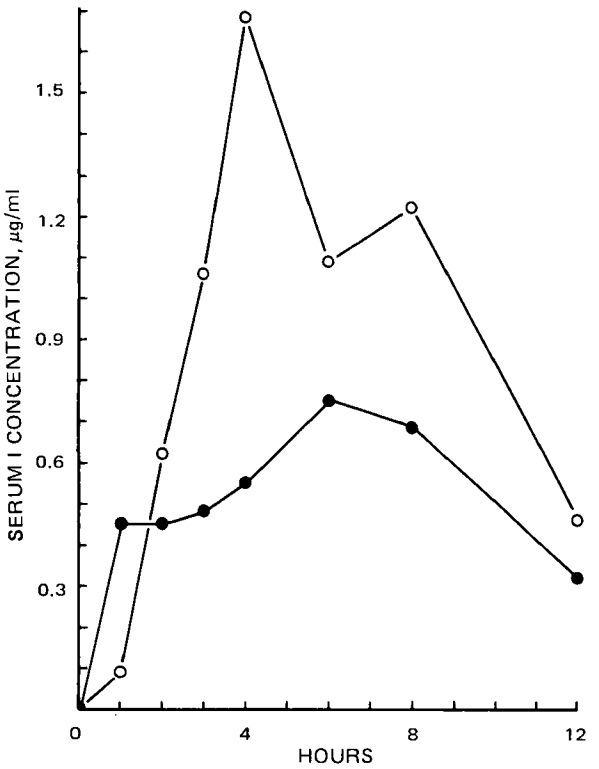


Figure 1—*Serum concentrations of I in dogs after single-dose oral drug administration in polysorbate 80. Key:* ○, *Dog BL-73 (male), 43.3 mg/kg; and* ●, *Dog 366 (male), 37.3 mg/kg.*

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 meter multiplier setting)[5], using the excitation (370 nm) and emission (470 nm) maxima for I. Fluorescence intensities of the extract residue solutions were measured at these wavelength settings. Compound I concentrations were calculated from a standard curve (working standards) with appropriate corrections for serum, urine, or feces blanks.

Preparation of Animals—In studies designed to determine the utility of the analytical methodology, three male beagle dogs, 10.5 ± 1.4 kg, were fasted for 16 hr prior to single-dose oral drug administration. Two dogs received ~40 mg of I/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, and 12 hr after drug administration. 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}[9], ascendingly developed in a solvent system of acetone–*n*-heptane–concentrated ammonium hydroxide (200:200:1 v/v). 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 0.18.

RESULTS AND DISCUSSION

Earlier studies with indoxole (2) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3) showed those compounds to be susceptible to photochemical degradation. By contrast, I is stable in acidic, neutral, or basic ethanolic solutions in the presence of light for at least 24 hr. Therefore, no special precautions are necessary in the storage of specimen extracts.

The maximum fluorometric sensitivity for I in 95% ethanol is 0.005 μg/ml (meter multiplier setting of 0.003)[5]. 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 emission of I. Calculated lower detection limits for I, based on sample responses twice those of the appropriate blanks, are shown in Table I. In serum, urine, and feces, I may be detected at levels of ≥0.2 μg/ml, ≥0.3 μg/ml, and ≥1.2 μg/100 mg, respectively.

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

To determine recoveries from biological samples, known amounts of I 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. Recoveries were quantitative (overall mean and standard deviation of 99.8 ± 5.1%), justifying the use of a simple standard curve in routine analysis as described previously (Table II).

Utility of the method was established by a limited study in dogs. Following oral administration of approximately 40 mg of I/kg, peak serum I levels of 0.75–1.7 μg/ml were observed between 4 and 6 hr (Fig. 1). Measurable concentrations were present throughout the 12-hr sampling interval. These results are similar to those reported for indoxole (2, 5) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3) in dogs.

The present method detects materials that are extractable from an alkaline solution with ethyl acetate and that possess excitation and fluorescence emission characteristics in 95% ethanol similar to I. Interference by endogenous materials in most specimens is very low, as indicated by the lower limits of detection (Table I). 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 meta-

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

bolic 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 I, 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 I by the dog during the 24-hr collection interval. Extensive metabolism and/or biliary secretion are indicated.

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 19, 1975, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication April 10, 1975.

The authors thank Mr. S. R. Shaw and Mr. W. F. Liggett for technical assistance.

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Conformationally Constrained Analogs of Mescaline II

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Abstract □ The synthesis of methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-piperidyl) acetate is described. In addition, preliminary pharmacological data comparing the compound with mescaline are given.

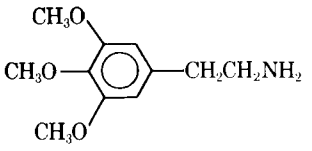
Keyphrases □ Mescaline—synthesis of conformationally constrained analog, methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-piperidyl) acetate, IR and NMR spectra □ Methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-piperidyl) acetate—analog of mescaline, synthesis, IR and NMR spectra

In an earlier publication (1), the synthesis of several conformationally constrained analogs of mescaline (I) was reported. This article reports the synthesis of methyl -2-(3,4,5-trimethoxyphenyl) -2- (2-piperidyl) acetate (II) as a modified mescaline analog. Compound II possesses the trimethoxyphenyl ring and the two-carbon chain of mescaline. The nonmethoxy analog of II, methylphenidate[1] (III), is a known central nervous system stimulant (2). It was anticipated that II would possess stimulant properties like mescaline and methylphenidate.

DISCUSSION

The synthetic route utilized for II was basically the same as that employed by Hartmann and Panizzon (3) for the synthesis of methylphenidate (Scheme I). The starting material, 3,4,5-trimethoxyphenylacetonitrile (IV), was synthesized according to the procedure of Telang and Smith (4).

Compound IV was reacted with 2-chloropyridine, using sodium hydride as the base, to form 2-(3,4,5-trimethoxyphenyl)-2-(2-pyridyl)acetonitrile (V). Compound V was hydrolyzed with sulfuric acid to form the amide VI. Methanolysis of VI gave the methyl ester VII. Catalytic hydrogenation of VII afforded the desired product II.



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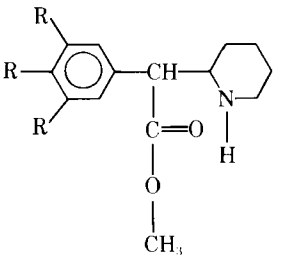

II: R = —OCH_3

III: R = —H

EXPERIMENTAL[2]

Pharmacology—The spontaneous activity of the test compound was compared to amphetamine, methylphenidate, and mescaline by means of activity chambers according to the procedure of Wolters *et al.* (1). The response of amphetamine, methylphenidate, and mescaline was reported previously (1).

The test compound at the 50-mg/kg dosage induced locomotor activity corresponding to that of saline in the first 40 min. Beyond 40 min, the rate of activity was comparable to mescaline, although the stereotyped behavior of mescaline scratching was not observed with the test compound at this dose.

Chemistry *2- (3,4,5-Trimethoxyphenyl) -2- (2-pyridyl)acetonitrile Hydrochloride (V-HCl)*—A solution of 75 ml of anhydrous dimethylformamide and 11.0 g (0.0531 mole) of IV was cooled to 0°. Then 3.0 g (0.060 mole) of sodium hydride was added. After the cessation of hydrogen evolution, a solution of 6.07 g (0.053 mole) of 2-chloropyridine in 50 ml of dimethylformamide was added drop-

[1] Ritalin Hydrochloride, Ciba Pharmaceutical Co., Summit, N.J.

[2] IR spectra were determined on a Perkin-Elmer model 337 spectrophotometer using potassium bromide pellets. NMR spectra were determined on the Varian model A-60A using tetramethylsilane as the internal standard and deuterochloroform as the solvent in approximately 30% concentration. The letter abbreviations used follow: s = singlet, 2s = two singlets, t = triplet, m = multiplet, and b = broad. Melting points were determined in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Microanalyses were performed by Alfred Bernhardt Mikroanalytishes Laboratorium, Fritz-Pregel-Strasse, West Germany.