something less than the absolute surface area of the injected mass. The apparent surface area of the injected mass is not known.

The possibility of controlling blood levels of phenobarbital for an extended period of time by regulation of particle size and size distribution of a single intramuscular dose is evident from these data. There was much variability in the individual blood level data obtained with each particle suspension during the first 3–6 hr., but it was not attributed to the dissolution and release pattern from the injected dose. This variability was probably due to a lower concentration of the drug in the body of the dog and its rapid distribution to the various tissues, leaving very little drug in the blood at the time of sampling. Based on the mean intravenous blood level estimate, the "apparent volume of distribution" was approximately 8.8 l.; since the blood volume in dogs weighing between 10 and 15 kg. is about 7% (700–1050 ml.) of body weight, the greater part of the drug must be in other tissues of distribution. As more drug is absorbed from the intramuscular suspensions, the relative standard error becomes smaller.

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Spectrofluorometric Assay of Apomorphine in Brain Tissue

W. KENT VAN TYLE and A. M. BURKMAN

Abstract □ A fluorometric technique has been developed that accurately and reliably measures submicrogram quantities of apomorphine in brain tissue. The assay involves an immiscible solvent extraction procedure which is highly efficient and makes possible the detection of apomorphine in concentrations of 0.1 mcg./g. tissue. The fluorometric assay is about 40 times more sensitive than the spectrophotometric method currently in use.

Keyphrases □ Apomorphine—spectrofluorometric analysis of submicrogram quantities in brain □ Spectrofluorometry—analysis of submicrogram quantities of apomorphine in brain

The increasing interest in apomorphine and related aporphines as centrally active behavioral and emetic stimulants has generated many questions concerning the CNS distribution of these compounds (1, 2). Using a spectrophotometric assay, Kaul *et al.* (3–7) studied apomorphine organ distribution and metabolism but were unable to estimate reliably the CNS levels due to the insufficient sensitivity of their method. Although a variety of techniques have been devised for the compound's determination (8, 9), none provides the sensitivity required to determine submicrogram quantities of apomorphine in small volumes of biological material.

The observation that crystalline apomorphine emits a bluish fluorescence under UV light and the acknowledged fact that fluorometry can be an extremely sensitive assay technique suggested the possibility of using a fluorometric method for apomorphine determination.

This report describes the development of such a procedure for estimating microquantities of apomorphine.

EXPERIMENTAL

Spectral Characteristics and Effect of Solvent—The output of the spectrophotofluorometer[1] was fed simultaneously into a photomultiplier microphotometer and an X-Y recorder[2]. A fused quartz cell (10.5 × 10.5 × 46 mm.) was used for all samples, with instrument slit arrangement number 5.

[1] An Aminco-Bowman spectrophotofluorometer equipped with a 1P21 potted photomultiplier tube assembly was used for all fluorescence measurements.

[2] Spectrophotofluorometer, microphotometer, and X-Y recorder were manufactured by American Instrument Co., Inc., Silver Spring, Maryland.

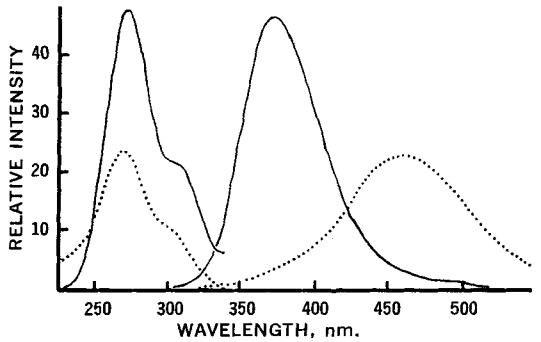

Figure 1—*Fluorescence spectra of apomorphine in ethyl acetate (0.33 mcg./ml. maximum, solid lines) and water (1 mcg./ml., dotted lines).*

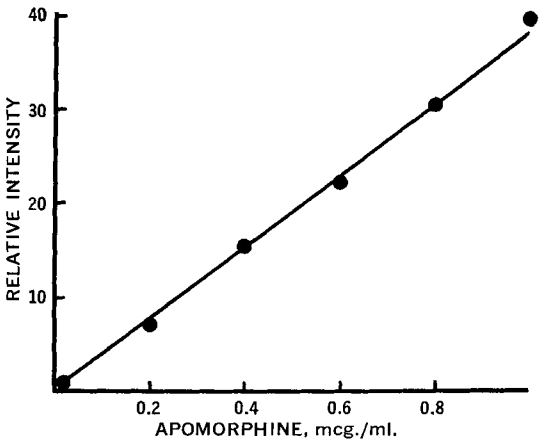

Figure 2—*Linear relation of fluorescence intensity to concentration of apomorphine in the extracted aqueous standard.*

The spectral characteristics of apomorphine were determined in both neutral distilled water and ethyl acetate[3], according to the method described by Udenfriend *et al.* (10). The aqueous solution contained 1 mcg. of apomorphine base/ml., and the ethyl acetate solution was prepared by extracting 5 ml. of aqueous solution with 15 ml. of ethyl acetate[4].

Effect of Concentration—The relation of fluorescence intensity to concentration of apomorphine base was determined by preparing serial dilutions of a 1.0-mcg./ml. aqueous apomorphine solution. The range of concentrations investigated was 0.01–1.0 mcg./ml. water. A 5-ml. aliquot of the aqueous standard was extracted for 2–3 min. with 15 ml. of ethyl acetate, and the fluorescence of the ethyl acetate extract was measured using the excitation and emission maxima for apomorphine fluorescence in this solvent (270/370 nm.).

Extraction Procedure for Brain—A modification of the extraction procedure suggested by Kaul *et al.* (4) for the estimation of apomorphine in biological materials was employed in this investigation. Not more than 1 g. of tissue to be assayed was homogenized in 3–4 ml. of 0.1 *N* HCl and 1 ml. of ethyl acetate in a homogenizer[5] equipped with Pyrex microhomogenization flasks. The acid homogenate was quantitatively transferred to a volumetric flask, and sufficient 0.1 *N* HCl was added to make a total volume of 10 ml. A 5-ml. aliquot of the acid homogenate was transferred to a 50-ml. glass-stoppered centrifuge tube and was shaken for 2–3 min. with 5 ml. of ethyl acetate The sample was centrifuged, the ethyl acetate was aspirated and discarded, and the previous wash step was repeated with another 5-ml. portion of ethyl acetate which was similarly discarded. The ethyl acetate washes significantly reduced the fluorescence of tissue blanks. Under acidic conditions the protonated apomorphine remained in the aqueous phase (pKa 8.9). Following aspiration of the second ethyl acetate wash, the pH of the homogenate was adjusted to 7.0 ± 0.5 by the addition of solid sodium bicarbonate; the pH was verified on the pH meter[6] equipped with a combination electrode[7]. The apomorphine present in the sample was extracted by shaking with 15 ml. of ethyl acetate for 2–3 min., the mixture was centrifuged, and the organic phase was withdrawn with a Pasteur pipet. Fluorescence intensity of the ethyl acetate extract was determined using the 270/370-nm. wavelengths.

Appraisal of Method—To determine the ability of the assay procedure to quantify apomorphine accurately in the presence of potentially interfering biological materials, addition–recovery experiments were performed with acidified pigeon brain homogenates. Two whole pigeon brains were homogenized in sufficient 0.1 *N* HCl to make 125 ml. of a stock homogenate containing approximately 30 mg. of tissue/ml. The sample was prepared by diluting 1.0 ml. of neutral apomorphine HCl solution containing 100 mcg. of base/ml. with sufficient acidified brain homogenate to make 100 ml. of sample. Five 5-ml. aliquots of the sample solution were assayed according to the described procedure along with a 5-ml. aliquot of the brain homogenate containing no apomorphine. The tissue blank thus determined was subtracted from the sample fluorescence intensity.

Excitation and emission spectra were obtained on assayed samples of mouse and pigeon brain following intravenous apomorphine administration to ascertain whether any naturally occurring fluorescent materials were being carried through the extraction to influence the total fluorescence intensity attributed to apomorphine. Male albino mice and adult domestic pigeons received apomorphine HCl in isotonic saline in doses of 10 and 1 mg./kg., respectively. The spectra thus obtained were compared with those of authentic apomorphine in ethyl acetate.

RESULTS AND DISCUSSION

The spectral characteristics for the fluorescence of apomorphine in neutral distilled water and ethyl acetate are presented in Fig. 1. The primary excitation peak for apomorphine was found to be 270 nm. in both solvents. A lesser excitation peak appeared in both solvents at 305 nm. The position of the emission peak, however, was solvent dependent and was 460 nm. in distilled water and 370 nm. in ethyl acetate. The fluorescence intensities of the emission spectra of solvent blanks were in the range of 0.2–0.3 unit. All wavelength determinations reported are observed values and were not corrected for instrumental variation. The intensity of apomorphine fluorescence was also solvent dependent, and it was found that the compound's fluorescence in ethyl acetate was about seven times that seen in water.

The virtues of ethyl acetate as an extraction solvent for apomorphine have been amply documented (4, 9) so that examination of this solvent's suitablity as a fluorescence vehicle seemed appropriate. However, since water might also represent an acceptable fluorescence medium, it was necessary to assess its relative utility as well. The decision to compare these solvents at a neutral pH was influenced by two observations. Preliminary studies on the pH-dependent character of apomorphine fluorescence in water revealed that intensity plateaus to a maximum value over the pH range of 3–8. Determinations in neutral water ensured optimum sensitivity. A pH of 7 was particularly appropriate inasmuch as Kaul *et al.* (4) established that ethyl acetate "effects complete extraction of the base from an aqueous solvent at pH 6–8. . . ."

Ethyl acetate was ultimately adopted as the fluorescence medium because of the greater assay sensitivity associated with its use.

As indicated in Fig. 2, the relation of fluorescence intensity to apomorphine concentration in ethyl acetate is linear over the range of concentrations investigated. It should be reiterated that a 5-ml. sample of apomorphine standard was extracted with 15 ml. of ethyl acetate and the fluorescence of this ethyl acetate extract was determined. Therefore, the concentrations indicated in Fig. 2 refer to those of the aqueous standard from which the apomorphine was extracted.

Addition–recovery experiments and spectral analyses of brain extracts revealed that the extraction procedure is highly efficient at removing apomorphine from brain tissue with minimal contamination of interfering fluorescent materials of biological origin. The percent recovery ± standard error of added apomorphine to

[3] Spectral quality ethyl acetate (Matheson, Coleman and Bell, Norwood, Ohio) was used in all procedures requiring this solvent.
[4] If virtually all of the apomorphine originally in the water solution were partitioned into ethyl acetate, the maximum attainable concentration of apomorphine base in ethyl acetate would be 0.33 mcg./ml.
[5] Virtis "45."
[6] Corning model 12.
[7] Corning Semimicro.

pigeon brain homogenate was found to be 98.2 ± 1.0. Likewise, fluorescence spectra of extracts from brains of mice and pigeons which had been administered apomorphine systemically were identical to spectra obtained for authentic apomorphine in ethyl acetate.

Although several procedures were reported for the quantitative determination of apomorphine, none possesses adequate sensitivity for studies of the compound's CNS distribution. Kaul *et al.* (4) reported that the spectrophotometric assay was unable to detect the presence of apomorphine in tissue homogenates in concentrations less than 4 mcg./g. Preliminary investigations of the compound's CNS distribution in mice using the fluorometric assay reported here indicate that concentrations of apomorphine as low as 0.1 mcg./g. brain tissue can be readily determined. As a result of the increased sensitivity of this fluorometric procedure, studies of apomorphine disposition in brain are now being productively pursued.

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Synthesis and Biological Evaluation of Some 4-Arylazopyrazoles

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Abstract □ A variety of derivatives of N^1-(4-methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles and N^1-hippuryl-4-arylazo-3,5-dimethylpyrazoles were prepared. Preliminary biological data also are described.

Keyphrases □ 4-Arylazopyrazoles—synthesis and biological evaluation as antimicrobial agents □ Antimicrobial agents, potential—synthesis and biological evaluation of 4-arylazopyrazoles □ N^1-(4-Methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazole derivatives—synthesis and biological evaluation as antimicrobial agents □ N^1-Hippuryl-4-arylazo-3,5-dimethylpyrazole derivatives—synthesis and biological evaluation as antimicrobial agents

The interest in the synthesis and biological evaluation of pyrazole derivatives has been renewed by the fact that 1-carbamoyl-3-methyl-4-(2-chloro-4-nitrophenylhydrazono)-2-pyrazolin-4,5-dione possesses anti-*Trichinella spiralis* activity (1).

This report includes the syntheses of N^1-(4-methoxyphenylsulfinyl) - 3,5 - dimethyl - 4 - arylazo-, N^1 - hippuryl-3,5-dimethyl-4-arylazo-, and N^1-hippuryl-3-methyl-4-arylazo-5-phenylpyrazoles. The preparation of others was recently described (2, 3).

The syntheses of N^1-(4-methoxyphenylsulfinyl)-3,5-dimethyl-4-arylazopyrazoles and N^1-hippuryl-4-arylazo-3,5-dimethylpyrazoles were achieved by the condensation of 2,3,4-pentanetrione-3-arylhydrazones (4) with 4-methoxyphenylsulfinylhydrazine (5) and hippurylhydrazine (6), respectively. N^1-Hippuryl-4-arylazo-3-methyl-5-phenylpyrazoles were similarly prepared from 1-phenyl-2-arylhydrazono-1,2,3-butanetrione (7) and hippurylhydrazine (6).

BIOLOGICAL RESULTS

4-Arylhydrazono-1-carbamoyl-3-methyl-2-pyrazolin-5-ones (2) and 3,5-dimethyl-4-arylazo-5-phenyl-N^1-carbamoylpyrazoles (3) were tested for antimicrobial activity (8) against *Staphylococcus aureus* (No. 20390), *Klebsiella pneumoniae* (No. 1200), *Pseudomonas aeruginosa* (No. 1320), *Escherichia coli* (No. 12140), *Trichophyton mentagraphytes* (No. 17410), *Candida albicans* (No. 3470), and *Mycobacterium tuberculosis* (No. H37RV). They were found inactive.

Anti-*Trichinella spiralis* activity of N^1-(4-methoxyphenylsulfinyl)- and N^1-hippuryl-4-arylazopyrazoles was determined by using the method of Garg (1). All these compounds were essentially inactive.

The IR spectra of representatives of all the pyrazoles showed characteristic bands of —C=C—N=N in the range 1480–1540 cm.$^{-1}$, aryl C=C in the range 1580–1670 cm.$^{-1}$, substituted phenyl in the range 690–730 cm.$^{-1}$, and —NH in the range 2700–3400 cm.$^{-1}$ (broad). UV spectra of the representatives showed $\lambda_{max.}^{EtOH}$ between 237–246 and 327–347 nm. These data are summarized in Table I.

EXPERIMENTAL[1]

3-Arylhydrazono-2,3,4-pentanetriones (4), 2-arylhydrazono-1-phenyl-1,2,3-butanetriones (7), hippurylhydrazine (6), and 4-methoxyphenylsulfinylhydrazine (5) were prepared by earlier described procedures.

2-Arylhydrazono-1,3-diphenyl-1,2,3-propanetriones—These were obtained by coupling diazotized anilines with 1,3-diphenyl-1,3-propanedione under conditions used previously (9).

***N*^{1}-(4-Methoxyphenylsulfinyl)-3,5-dimethyl - 4 - arylazopyrazoles: General Procedure**—A solution of 4-methoxysulfinylhydrazine (0.005 mole) in alcohol (15 ml.), containing a few drops of concentrated sulfuric acid, was added to the appropriate 2,3,4-propane-

[1] Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer Infracord using KBr phase. UV spectra were measured with a Bausch and Lomb Spectronic 505 spectrophotometer.