Fluorescence Reactions of Fluorescamine with Levodopa and Its Derivatives: Fluorescence Assay of 3-Methoxy-4-hydroxyphenylalanine in Levodopa Dosage Forms

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Abstract □ A simple fluorometric method for the quantitation of 3-methoxy-4-hydroxyphenylalanine, either alone or in the presence of levodopa, is presented. Fluorescence is developed by reaction with fluorescamine. The interactions of this reagent with levodopa and a number of its derivatives under various experimental conditions were studied. Negligible fluorescence was obtained with levodopa, dopamine, levodopa benzyl ester, and 6-hydroxydopamine.

Keyphrases □ Levodopa and derivatives—reaction with fluorescamine, fluorometric analysis for 3-methoxy-4-hydroxyphenylalanine □ Fluorescamine—reactions with levodopa and derivatives, fluorometric analysis for 3-methoxy-4-hydroxyphenylalanine □ Fluorometry—reaction of fluorescamine with levodopa and derivatives, analysis for 3-methoxy-4-hydroxyphenylalanine

Fluorescamine[1], 4-phenylspiro[furan-2(3*H*),1-phthalan]-3,3-dione, is a new fluorogenic reagent recently introduced (1) for the assay of primary amines in the picomole range. This compound reacts rapidly, in milliseconds, with primary amino groups to produce an intense fluorophore while excess fluorescamine is hydrolyzed in seconds to form nonfluorescent products. At present, the fluorescence assays of catecholamines, including levodopa and dopamine, in biological fluids (2, 3) are rather tedious and are sensitive to changes in reaction conditions. It is, therefore, desirable to investigate whether simpler fluorescence procedures may be achieved by the reaction of fluorescamine with catecholamines.

The present report deals with studies on the fluorescence reactions of fluorescamine with levodopa and a number of its derivatives under various experimental conditions. It is shown that 3-methoxy-4-hydroxyphenylalanine, and possibly other *O*-methylated catecholamines, can be assayed with a very simple procedure using fluorescamine.

EXPERIMENTAL

Materials—Levodopa[2], 3-methoxy-4-hydroxyphenylalanine[3], dopamine hydrochloride[4], 6-hydroxydopamine[5], and glycine[6] were used without further purification. Levodopa benzyl ester was synthesized in this laboratory and confirmed by elemental analysis (C, H, N) and IR. All other reagents were analytical grade, except acetone, which was spectrophotometric grade.

Fluorescence Development—Stock solutions of test compounds were prepared in 0.1 *N* HCl in concentrations about 20-fold in excess of that in the final reaction mixture. All glassware used was cleaned by presoaking in 20% nitric acid for at least 24 hr. One hundred microliters of each test solution was added to 1.5 ml of buffer solution (0.05 *M* sodium borate except where indicated) in a test tube and mixed thoroughly. While the test tube was held on a mixer[7], 0.5 ml of fluorescamine in acetone (30 mg/100 ml) was added rapidly and the resultant solution was immediately mixed vigorously for at least 30 sec. Rapid addition of the reagent and thorough mixing thereafter are essential. The fluorescence of the solutions was read on a spectrophotofluorometer[8] at an excitation wavelength of 390 nm and an emission wavelength of 480 nm.

When commercial levodopa capsules were assayed, the contents of four capsules were emptied, mixed, and weighed. An equivalent amount of one capsule was weighed and dissolved in 250 ml of water. The solution was vigorously shaken for 10 min and filtered. Samples not subjected to alumina separation were directly assayed using the volumes described previously. Other samples, after a fivefold dilution, were treated with alumina to remove levodopa; the general procedure of Anton and Sayre (4) was used except that disodium (ethylenedinitrilo)tetraacetate was omitted. About 96% of levodopa was removed after this procedure. After alumina treatment, 0.5 ml of the resultant solution was used for fluorescence development.

RESULTS AND DISCUSSION

Table I shows the approximate net fluorescence readings obtained when the levodopa compounds[9] were separately mixed with fluorescamine at pH ~9.2. Both glycine and 3-methoxy-4-hydroxyphenylalanine reacted with the reagent to give significant fluorescence, whereas all other compounds gave readings only slightly higher than the blank (blank fluorescence = 0.3–0.4). Since it has been shown (1) that fluorescamine reacts indiscriminately with the primary amino group, whether it is present in a simple amine, an amino acid, a peptide, or a protein molecule, the inability of these compounds to form fluorophores with fluorescamine was thought to be due to a lack of reaction with the reagent rather than to the production of nonfluorescent reaction products.

At an alkaline pH of 9.2, the oxidation of unprotected catecholamine molecules to form indole-type compounds is extremely rapid. Therefore, it is possible then that the primary amino group in these compounds is effectively destroyed prior to reaction with fluorescamine. Careful deoxygenation of reagent solutions by flushing with nitrogen produced significant fluorescence from le-

[1] Fluram, Roche Diagnostics, Nutley, NJ 07110
[2] Supplied by INTERx Research Corp., Lawrence, KS 66044
[3] Supplied by Dr. A. Pletscher, Hoffmann–La Roche Co., Basel, Switzerland.
[4] Schwarz/Mann, Orangeburg, N.Y.
[5] Pfaltz and Bauer, Inc., Flushing, N.Y.
[6] J. T. Baker, through VWR Scientific, Buffalo, N.Y.
[7] Vari-Whirl mixer, Van-Lab, VWR Scientific, Buffalo, N.Y.
[8] Aminco-Bowman, Silver Spring, Md.
[9] The weak fluorescence (see Table I) developed with levodopa, dopamine, 6-hydroxydopamine, and levodopa benzyl ester might be partly attributed to impurities present in these samples. The readings, therefore, represent upper limits of fluorescence that could be developed with these compounds.

Table I—Approximate Fluorescence Readings of Dopa Compounds after Reaction with Fluorescamine

Compound[a]	Net Fluorescence
Levodopa	0.13
Dopamine	0.11
Levodopa benzyl ester	0.13
6-Hydroxydopamine	~0
3-Methoxy-4-hydroxyphenylalanine	2.90
Glycine	6.86
Catechol	~0

[a] All in a final concentration of about .1 μg/ml.

vodopa and dopamine solutions. However, these readings were highly sensitive to prevailing experimental surroundings and were not consistently reproducible. Unsuccessful attempts have been made to inhibit this possible oxidation by adding a metal chelating agent [disodium (ethylenedinitrilo)tetraacetate, up to 1% in the final reaction solution] or an antioxidant (sodium metabisulfite, up to 3% in the final reaction solution) or by substituting the borate buffer with a sodium bicarbonate buffer at the same pH. Helling and Bollag (5) reported that Pb^{+2} complexes with catechols to form insoluble salts. Addition of a 10-fold excess of lead acetate to a 1-μg/ml levodopa solution did not increase the fluorescence intensity. The fluorescence readings of reaction systems involving levodopa, dopamine, levodopa benzyl ester, and 3-methoxy-4-hydroxyphenylalanine were not significantly altered in the 7.8–9.2 pH range when a sodium borate–hydrochloric acid buffer was employed.

The intense fluorophore formed between fluorescamine and 3-methoxy-4-hydroxyphenylalanine under the present experimental conditions is in sharp contrast to the very weak ones obtained with levodopa and dopamine. The fluorescence spectra for the fluorophore are shown in Fig. 1. 3-Methoxy-4-hydroxyphenylalanine is a major metabolite of levodopa in animals and humans. In the rat, it demethylates to levodopa (6); because of its longer biological half-life compared to the parent compound in humans (7), there is intense interest in studying the feasibility of using 3-methoxy-4-hydroxyphenylalanine as a timed-release form of levodopa in the treatment of parkinsonism.

The present study provides a simple and rapid fluorometric method for the determination of the purity of 3-methoxy-4-hydroxyphenylalanine in the presence of impurities such as nonmethylated catecholamines and other nonamino catechol compounds. Figure 2 shows a fluorescence calibration plot for 3-methoxy-4-hydroxyphenylalanine. Good linearity was obtained up to a concentration of 1 μg/ml of drug in the final reaction mixture. This calibration plot holds even up to a concentration of 10 μg/ml without any significant self-quenching. A 0.1-μg/ml solution gives a fluorescence reading twice that of the blank. The present method compares favorably with the ferricyanide oxidation method (3) for the assay of 3-methoxy-4-hydroxyphenylalanine in the presence of

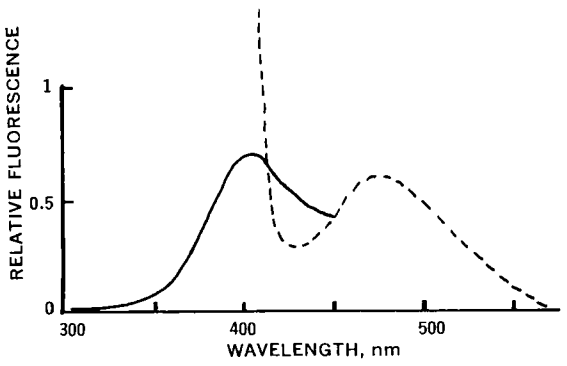

Figure 1—*Excitation (---) and emission (——) spectra (uncorrected) of fluorophore formed between 3-methoxy-4-hydroxyphenylalanine and fluorescamine. Emission wavelength = 480 nm, excitation wavelength = 390 nm, and 3-methoxy-4-hydroxyphenylalanine = 0.29 μg/ml of final reaction mixture.*

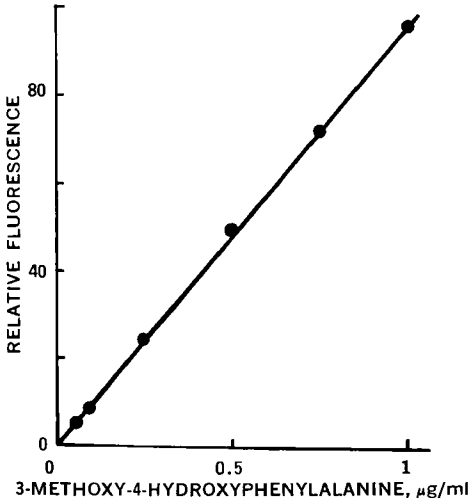

Figure 2—*Calibration plot for the reaction of 3-methoxy-4-hydroxyphenylalanine with fluorescamine.*

levodopa and dopamine in terms of simplicity, rapidity, and sensitivity to 3-methoxy-4-hydroxyphenylalamine. However, in the latter method, the interfering fluorescence due to the same catecholamines is less than that found in the present method.

The utility of the present technique can be illustrated in the assay of impurities in levodopa dosage forms. Watson (8), using a GLC assay, recently showed that 3-methoxy-4-hydroxyphenylalanine is the exclusive impurity in commercial levodopa dosage forms. Capsules of levodopa[10] were assayed for 3-methoxy-4-hydroxyphenylalanine using the present assay, both with and without a prior separation step involving alumina. The content of 3-methoxy-4-hydroxyphenylalanine in these capsules had been determined previously to be 0.35% by the GLC technique (8). Because of the relatively large errors involved in integrating the small peak generated by 3-methoxy-4-hydroxyphenylalanine in the proximity of the parent peak of levodopa, the standard deviation for this GLC-determined value was estimated to be at least 0.05%[11]. Using the present fluorometric method with prior alumina separation, the 3-methoxy-4-hydroxyphenylalanine impurity content was determined to be 0.43 ± 0.03% (mean ± standard deviation, four measurements). Without prior alumina separation, duplicate determinations gave values of 0.52 and 0.52% for 3-methoxy-4-hydroxyphenylalanine in the commercial levodopa capsules. Thus, it is evident that the present fluorometric method is accurate in determining the content of 3-methoxy-4-hydroxyphenylalanine in levodopa dosage form when an alumina separation step is employed before fluorescence development.

The present method possibly could be extended to assay for 3-methoxy-4-hydroxyphenylalanine in plasma and other biological fluids after it has been separated from catecholamines by alumina (4). Where the ratio of 3-methoxy-4-hydroxyphenylalanine to other catecholamines is high, alumina separation may not be required. For example, in the cerebrospinal fluids of parkinsonian patients receiving 3–6 g of levodopa daily, the average levodopa level was found to be 0.3–0.5 nmole/ml, whereas the level of 3-methoxy-4-hydroxyphenylalanine was 2–3 nmoles/ml (9). With this low concentration ratio of levodopa *versus* 3-methoxy-4-hydroxyphenylalanine, the present method could possibly be used directly to determine the concentration of the methylated metabolite in the presence of the parent drug without any significant interference.

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Systematic Identification of Drugs of Abuse I: Spot Tests

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Abstract □ More than 40 of the most commonly encountered street drugs were subjected to several spot tests. These tests were carried out in a special sequence leading to the construction of a flowsheet. Thus, with a limited number of simple tests, it is possible to identify tentatively or narrow down the drug. Since each drug investigated was subjected to all selected tests, whether such tests were developed for this type of compound or not, some unexpected and undocumented results were obtained.

Keyphrases □ Abuse drugs—systematic identification, spot tests, flow chart □ Drugs, abuse—systematic identification, spot tests, flow chart □ Spot tests—drugs of abuse, systematic identification

Spot tests still serve a valuable role in the identification of abused drugs today. Since many of the abused drugs are nonproprietary preparations and are prepared and marketed without any standards or quality control by any authority, they often contain substances other than what they are alleged to contain. Therefore, a fast simple battery of tests to provide preliminary information concerning the drug is needed.

In spite of the limitations of spot tests, such as the occurrence of false positives or false negatives, the lack of specificity, and the difficulty of interpreting some results, they still provide speedy answers to preliminary questions pertaining to these substances. The useful information provided by spot tests includes: *(a)* the definite absence of a compound or a group of compounds, and *(b)* the possible presence of a compound or compounds that belong to a certain group. The positive information provided by these tests will help in the selection of the specific confirmatory tests necessary.

The purpose of this work was to increase the usefulness of spot tests. More than 40 of the most common street drugs were subjected to nine different reagents. The results obtained provided a basis for the systematic classification of these drugs on an identification scheme. They also produced a number of unexpected and undocumented uses for conventional tests. Many false negatives and false positives were encountered and documented. These results increase the value of the tests once they are recognized and considered.

EXPERIMENTAL

Materials—The 43 drugs[1] investigated in this work are grouped in Table I according to their chemical nature and some of the results obtained in this work.

Preparation of Samples—The preparation of the standard references for the spot tests varied according to the test and the form of the drug provided. In general, the spot tests were performed directly on the powdered or liquid forms. For specific tests where no moisture is desired and where the drug has to be in a powdered form, solvents were removed from drugs available only in solution.

Generally, 1–2 mg was used for the tests; however, lysergide was detected in quantities as low as 5 μg. The tests were performed in 10-ml clear glass test tubes, and observation continued for approximately 30 min after the tests were completed.

Reagents and Procedures—The following reagents were prepared as indicated.

Mayer's Reagent (1)—This reagent consists of: mercuric chloride, 0.68 g; potassium iodide, 2.5 g; and distilled water to make 100 ml.

Dragendorff's Reagent (2)—Solution A consists of: bismuth subnitrate, 0.85 g; distilled water, 40 ml; and acetic acid, 10 ml. Solution B consists of: potassium iodide, 8.0 g; and distilled water, 20 ml. To prepare the concentrate, 5 volumes of A and 2 volumes of B are mixed. In this investigation, 20 ml of acetic acid was added to 10 ml of the concentrate, which was then diluted with 100 ml of distilled water (3).

Wagner's Reagent (4)—This reagent consists of: iodine, 1.27 g; potassium iodide, 2 g; and distilled water to make 100 ml. A small amount of the drug is dissolved in a few drops of 10% HCl. The reagent is then added dropwise to the acidic solution. A precipitate is

[1] Obtained from the United States Pharmacopeial Convention, Inc., the National Institute of Mental Health, and miscellaneous pharmaceutical and chemical companies.