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SPECIFIC DETECTION OF PRIMARY CATECHOLAMINES AND THEIR 3-O-METHYL DERIVATIVES ON THIN-LAYER PLATES USING A FLUORIGENIC REACTION WITH FLUORESCAMINE

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SUMMARY

Two specific and sensitive methods have been developed for the detection of primary catecholamines, their 3-O-methyl derivatives and other related phenylethylamines. The reaction is based upon the unique fluorescence produced by the treatment of their fluorescamine derivatives with perchloric acid (PCA). In method I, compounds are separated on plates and reacted by dipping the plates in acetone–*n*-hexane (1:4) containing fluorescamine. In method II, compounds are first derivatized with fluorescamine at the origin and then separated. When sprayed with 70% PCA, the fluorescamine derivatives appear as bluish fluorescent spots. The detection limit varied from 5 to 800 pmole.

INTRODUCTION

Primary catecholamines and their 3-O-methyl derivatives have been detected colorimetrically with various phenolic reagents [2,6-dibromoquinone-4-chlorimide[1,2], 2,6-dichloroquinone-4-chlorimide[3,4], Folin reagent[5], iron(III) chloride[2], phosphomolybdate[2,6], 4-aminophenazone–potassium hexacyanoferrate(III)[7] and diazonium salts of *p*-nitroaniline[1,3,8–11], sulphanilic acid[12–14] and other aromatic amines[4,7]], catechol reagents (Doty's reagent[15] and phloroglucinol[16]) and ninhydrin reagents[1,12]. Apart from the specific colorimetric detection of norepinephrine as noradrenochrome with potassium hexacyanoferrate(III)[9,17,18], these colorimetric spray reagents lack both sensitivity and specificity. On the other hand, more sensitive detection of primary catecholamines and their 3-O-methyl derivatives has been achieved fluorimetrically

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with formaldehyde in hydrochloric acid (Procházka reagent)[19], gaseous formaldehyde[20–26], glyoxylic acid vapour[27], ethylenediamine[3,9,22,28–30] and trihydroxyindole methods[13,22,31–33]. Despite their high sensitivity, these fluorimetric methods lack specificity[13,19–29]. This is also the case with the analyses of the above biogenic amines with 1-dimethylaminonaphthalene-5-sulphonyl chloride (Dns-Cl)[34] and fluorescamine[35], which are fluorigenic labelling reagents for primary amines.

In the course of the thin-layer chromatographic (TLC) detection of tryptophan, tryptamine, peptides with N-terminal tryptophan and other indoleamines as their fluorescamine derivatives, which give intense yellowish fluorescence upon spraying with perchloric acid (PCA) solution, we found that the fluorescamine derivatives of 3,4-dihydroxyphenylethylamines and 3-methoxy-4-hydroxyphenylethylamines, having an $-NH_2$ group in the side-chain, give an intense bluish fluorescence under the same conditions[36]. In this investigation, the above finding has been extended to the specific TLC detection of primary catecholamines and their 3-O-methyl derivatives by using 2-(2-nitrophenylsulphenyl)-3-methyl-3-bromoindolenine (BNPS-skatole) as a selective reagent for the degradation of interfering indoleamines[37,38].

EXPERIMENTAL

Reagents and materials

The sources of catecholamines and related compounds used were as follows: DL-α-methylnorepinephrine·HCl from Regis Chemical (Morton Grove, Ill., U.S.A.), L-β-3,4-dihydroxyphenylalanine (L-DOPA), 3-hydroxytyramine (dopamine)·HCl, 5-hydroxydopamine·HCl (*ca.* 95%), 6-hydroxydopamine·HBr, L-norepinephrine·HCl, DL-normetanephrine·HCl, tyramine·HCl, DL-octopamine·HCl, O-methyl-L-tyrosine·HCl, DL-*p*-chlorophenylalanine, DL-2-methyl-3-(3,4-dihydroxyphenyl)alanine (DL-α-methyl-DOPA), L-3-methoxytyrosine (L-3-O-methyl-DOPA), DL-4-hydroxy-3-methoxymandelic acid (vanillylmandelic acid), 3-hydroxy-4-methyl-α-ethylphenethylamine·HCl, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid), 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid), DL-3,4-dihydroxymandelic acid (grade I), bis-(4-hydroxy-3-methoxyphenylglycol) piperazine salt, L-phenylalanine, L-tyrosine, DL-synephrine, 3-methoxytyramine·HCl (grade II), DL-epinephrine, DL-metanephrine·HCl, L-ephedrine·HCl and DL-norephedrine·HCl from Sigma (St. Louis, Mo., U.S.A.), and N-methyldopamine and 4-O-methyldopamine from Research Plus Labs. (Denville, N.J., U.S.A.). Other test compounds used were of analytical-reagent grade and purchased from commercial sources. All test compounds were dissolved in 0.01 *N* hydrochloric acid solution to make 2 m*M* stock solutions. The same solution was also used to prepare dilute sample solutions.

Distilled-in-glass solvents, *n*-butanol, benzene, dioxane, ethyl acetate, acetone, methanol and *n*-hexane, were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Fluorescamine, 2-(2-nitrophenylsulphenyl)-3-methyl-3-bromoindolenine (BNPS-skatole) and acetic acid (Phix buffer grade) were purchased from Pierce (Rockford, Ill., U.S.A.), ethanol (USPHS) from S.S.C. (Perry Point, Md., U.S.A.) and perchloric acid (PCA, 70–72%) from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Glass plates pre-coated with silica gel (silica gel 60, 20 × 20 cm, 0.25-mm layer, without fluorescent indicator) (E. Merck, Darmstadt, G.F.R.) were used throughout without any treatment. Samples were applied with a volumetric micro-

pipette (Microcaps; Drummond, Broomall, Pa., U.S.A.). Spraying procedures were performed with an aerosol gun (Quixpray Kit, Pierce).

Spot test for the evaluation of specificity of the reaction

An aliquot (1 μl) of 1 m*M* solutions of various compounds in 0.01 *N* hydrochloric acid solution was spotted on a silica gel 60 plate and buffered by spotting 1 μl of 0.2 *M* phosphate buffer (pH 8.0). The sample was subjected to reaction with fluorescamine by the pre-dipping method[39] as described below (method II) and viewed under longwave UV light after spraying with 70% PCA.

Selective degradation of interfering indoleamines by treating with BNPS-skatole

To an aliquot of 10 μl of a 2 m*M* stock solution of compound in 0.01 *N* hydrochloric acid were added 10 μl of a 10 m*M* BNPS-skatole[37,38] solution in acetic acid, and the mixture was allowed to stand at room temperature for 30 min.

Thin-layer chromatography

A 1-μl aliquot of the BNPS-skatole reaction mixture, equivalent to 1 nmole of the original compound, was applied with a micro-pipette 1.5 cm from the lower edge of a plate, air-dried and analysed by one of the two methods described below.

Method I. Detection after separation of native compounds. The spotted plate was developed with an appropriate solvent system and compounds were derivatized with fluorescamine by a modification of the dipping method[40]. The plate developed with a neutral solvent system was dried at 110° for 5 min (the plate developed with an acidic solvent system was dried at 110° for 15 min), sprayed with 0.2 *M* phosphate buffer (pH 8.0), re-dried at 110° for 10 min and dipped for 30 min in an acetone–*n*-hexane (1:4) solution containing fluorescamine (10 mg per 100 ml). The plate was then sprayed with 70% PCA for 5 sec and fluorescence was observed under a longwave UV lamp in the dark.

Method II. Detection after separation of fluorescamine derivatives. The compounds spotted on the plate were buffered by spotting 1 μl of 0.5 *M* phosphate buffer (pH 8.0), air-dried and derivatized at the origin with fluorescamine by the pre-dipping method[39]. The lower 2 cm of the plate was dipped in the fluorescamine solution for 30 min, and then the plate was dried without heating. After separation of the fluorescamine derivatives formed, the plate was briefly air-dried, sprayed with 70% PCA for 3–5 sec and the fluorescence was observed.

RESULTS

Effect of PCA concentration on fluorescence

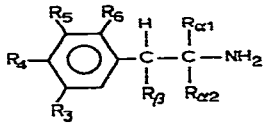
When fluorescamine-labelled primary catecholamines and their 3-O-methyl derivatives on silica gel plates were sprayed with various concentrations of PCA solution for a given time (5 sec), the intensity of the PCA-induced fluorescence increased with increasing concentrations of PCA up to the maximal fluorescence with 70% PCA.

Specificity of the fluorigenic reaction

When fluorescamine derivatives of various compounds on silica gel were

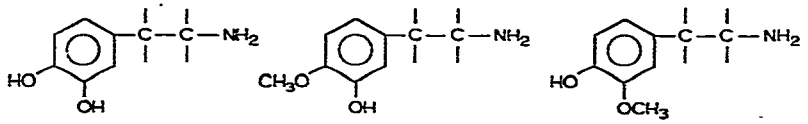
TABLE I

DETECTED PHENYLETHYLAMINES AND THEIR CHEMICAL STRUCTURES



Compound	$R_{\alpha 1}$	$R_{\alpha 2}$	R_β	R_3	R_4	R_5	R_6
L-DOPA	H	COOH	H	OH	OH	H	H
L-3-O-Methyl-DOPA	H	COOH	H	OCH_3	OH	H	H
DL-α-Methyl-DOPA	CH_3	COOH	H	OH	OH	H	H
Dopamine	H	H	H	OH	OH	H	H
5-Hydroxydopamine	H	H	H	OH	OH	OH	H
6-Hydroxydopamine	H	H	H	OH	OH	H	OH
4-O-Methyldopamine	H	H	H	OH	OCH_3	H	H
L-Norepinephrine	H	H	OH	OH	OH	H	H
DL-α-Methylnorepinephrine	H	CH_3	OH	OH	OH	H	H
DL-Normetanephrine	H	H	OH	OCH_3	OH	H	H
3-Methoxytyramine	H	H	H	OCH_3	OH	H	H

sprayed with 70% PCA, only a few classes of compounds showed fluorescence while most lost their fluorescence. Apart from the indoleamines such as tryptamine and tryptophan, which have been shown to exhibit yellowish fluorescence with the spray, compounds which gave fluorescence in amounts of less than 1 nmole were L-DOPA, L-3-O-methyl-DOPA, DL-α-methyl-DOPA, dopamine·HCl, 5-hydroxydopamine·HCl, 6-hydroxydopamine·HBr, 4-O-methyldopamine, L-norepinephrine·HCl, DL-α-methylnorepinephrine·HCl, DL-normetanephrine·HCl and 3-methoxytyramine·HCl (Table I). The colours of their PCA-induced fluorescence were bluish in all instances. The above results indicates that the 3,4-dihydroxy-(I), 3-hydroxy-4-methoxy-(II) or 3-methoxy-4-hydroxyphenylethylamine (III) skeleton is required for this particular fluorigenic reaction. 4-Methyl-α-ethyl-*m*-tyramine sometimes gave a similar bluish fluorescence, which developed gradually in a few minutes after spraying. On the other hand, the following related compounds showed no fluorescence at the 1-nmole level: DL-epinephrine, DL-metanephrine·HCl, N-methyldopamine, DL-synephrine, L-ephedrine·HCl, DL-norephedrine·HCl, epinine·HCl, tyramine·HCl, DL-octopamine·HCl, L-tyrosine, O-methyl-L-tyrosine·HCl, L-phenylalanine, DL-*p*-chlorophenylalanine, vanillylmandelic acid, homovanillic acid, homoprotocatechuic acid, DL-3,4-dihydroxymandelic acid and bis-(4-hydroxy-3-methoxyphenylglycol). Other common amino acids, peptides containing no tryptophan, amino sugars,



(I) (II) (III)

histamine, diamines, polyamines and alkylamines gave no fluorescence at the 1-nmole level.

TLC of native compounds and successive derivatization with fluorescamine followed by detection with 70% PCA (method I)

Neither the native compounds nor fluorescamine derivatives were displaced or extracted by the fluorescamine solution during the fluorescamine staining by the modified dipping method[40]. Interfering indoleamines were completely destroyed by the BNPS-skatole treatment while primary catecholamines were scarecely degraded. O-Methyl primary catecholamines were stable to the reagent. Table II shows the separation of primary catecholamines, their O-methyl derivatives and related phenylethylamines, including the compounds which did not form fluorescent derivatives in this procedure.

TABLE II

R_F VALUES OF PRIMARY CATECHOLAMINES, THEIR 3-O-METHYL DERIVATIVES AND RELATED PHENYLETHYLAMINES OBTAINED WITH METHOD I

Conditions as in Experimental. Solvent systems: A, *n*-butanol–acetic acid–water (5:2:3); B, benzene–dioxane–acetic acid (4:5:1); C, ethyl acetate–ethanol–acetic acid (3:2:1); D, benzene–acetone–methanol (1:2:1); E, *n*-butanol–ethanol–water (2:1:1).

Compound (1 nmole)	*R_F × 100 value in solvent system*				
	A	*B*	*C*	*D*	*E*
L-DOPA	50	0	21	1	5
Dopamine·HCl	58	2	18	3	4
5-Hydroxydopamine·HCl	51	4	9	1	2
6-Hydroxydopamine·HBr	57	1	ND**	ND**	3
L-Norepinephrine·HCl	54	2	18	5	3
DL-Normetanephrine·HCl	60	4	18	26	19
3-Methoxytyramine·HCl	62	6	17	13	22
DL-α-Methyl-DOPA	52	0	31	1	50
L-3-O-Methyl-DOPA	55	1	19	2	38
4-Methyl-α-ethyl-*m*-tyramine·HCl*	76	21	ND**	ND**	53
Tyramine·HCl*	66	6	30	16	31
DL-Octopamine·HCl*	62	5	31	31	21
O-Methyl-L-tyrosine·HCl*	61	2	24	2	45
DL-*p*-Chlorophenylalanine*	67	2	27	3	50
L-Phenylalanine*	61	2	25	3	46
L-Tyrosine*	56	1	25	2	43

* Compounds whose bluish green fluorescence of fluoresamine derivatives disappears on spraying with 70% PCA.

** ND = Not detected.

TLC of fluorescamine derivatives and successive detection with 70% PCA (method II)

All of the compounds listed in Table I were successfully derivatized with fluorescamine at the origin of the plate by the pre-dipping method[39]. Chromatography of the fluorescamine derivatives of these compounds gave single, bluish green fluorescent spots which changed to bluish fluorescent spots upon spraying with 70% PCA. The R_F values of the fluorescamine derivatives obtained with four solvent systems are given in Table III.

TABLE III

R_F VALUES OF FLUORESCAMINE DERIVATIVES OF PRIMARY CATECHOLAMINES, THEIR 3-O-METHYL DERIVATIVES AND RELATED PHENYLETHYLAMINES OBTAINED WITH METHOD II

Conditions as in Experimental. Solvent systems: F, ethyl acetate–*n*-hexane–methanol–water (60:20:25:10); G, chloroform–isopropanol–water (2:8:1); H, *n*-butanol–acetic acid–water (5:2:3); I, benzene–dioxane–acetic acid (2:5:1).

Compound (1 nmole)	*R_F × 100 value in solvent system*			
	F	*G*	*H*	*I*
L-DOPA	26	45	84	80
Dopamine·HCl	70	84	91	89
5-Hydroxydopamine·HCl	1	75	ND**	0
6-Hydroxydopamine·HBr	69	83	ND**	ND**
L-Norepinephrine·HCl	59	81	90	87
DL-Normetanephrine·HCl	67	83	92	90
3-Methoxytyramine·HCl	69	83	93	92
DL-α-Methylnorepinephrine·HCl	66	83	93	92
DL-α-Methyl-DOPA	19	ND**	87	ND**
L-3-O-Methyl-DOPA	27	45	84	75
4-Methyl-α-ethyl-*m*-tyramine·HCl*	69	88	95	96
4-O-Methyldopamine	69	NA***	NA***	NA***
Tyramine·HCl*	71	87	94	92
DL-Octopamine·HCl*	68	87	94	90
O-Methyl-L-tyrosine·HCl*	39	45	83	81
DL-*p*-Chlorophenylalanine*	45	46	84	77
L-Phenylalanine*	44	46	84	82
L-Tyrosine*	35	46	85	77

* Compounds whose bluish green fluorescence of fluorescamine derivatives disappears on spraying with 70% PCA.

** ND = Not detected.

*** NA = Not analysed.

Sensitivity of the methods

The limits of detection of the primary catecholamines, their O-methyl derivatives and DOPAs in the two methods are given in Table IV. Although these values were obtained by omitting the BNPS-skatole treatment before TLC analysis, similar results were also obtained when the procedure included the BNPS-skatole treatment. Generally, O-methyl derivatives were detected with higher sensitivity than the corresponding dihydroxy derivatives, which are sensitive to oxidation. Substitution of a methyl group at the α-position (the carbon atom to which the $-NH_2$ group in the side-chain is bound) decreased the sensitivity of detection.

DISCUSSION

The sensitivities of the present methods are comparable to those of other fluorimetric methods[9,22,26,27,31]. A higher sensitivity may be obtained by adding an antioxidant to the solvent systems, especially when using primary catecholamines and DOPAs which have a polyhydroxybenzene moiety that is sensitive to oxidation. In addition to degradation of compounds, incomplete derivatization of primary amino

TABLE IV

R_F VALUES AND DETECTION LIMITS OF THE POSITIVE PHENYLETHYLAMINES AFTER TLC

TLC performed on silica gel 60 plates (Merck).

Compound	*Method I**		*Method II***	
	$R_F \times 100$	*Detection limit (pmole)*	$R_F \times 100$	*Detection limit (pmole)*
L-DOPA	50	80	26	75
DL-α-MethylDOPA	52	500	19	250
L-3-O-MethylDOPA	55	8	27	20
Dopamine·HCl	58	90	70	75
3-O-Methyldopamine·HCl	62	5	69	10
4-O-Methyldopamine			69	5
5-Hydroxydopamine·HCl	51	300	1	75
6-Hydroxydopamine·HBr	57	800	3	200
L-Norepinephrine·HCl	54	70	59	200
DL-α-Methylnorepinephrine·HCl			66	350
DL-Normetanephrine·HCl	60	20	67	20

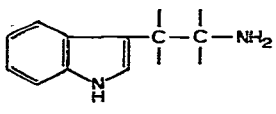
* Solvent system: *n*-Butanol–acetic acid–water (5:2:3).

** Solvent system: ethyl acetate–*n*-hexane–methanol–water (60:20:25:10).

groups also leads to decreased sensitivity. DL-α-Methyl-DOPA and DL-α-methylnorepinephrine were less fluorescent than L-DOPA and L-norepinephrine, respectively, both in the first step of staining with fluorescamine and in the second step of acidification. This may be explained by the steric hindrance of the α-methyl group, which affects access of fluorescamine to the adjacent -NH_2 group. In such cases, the sensitivity was improved by prolongation of the period of fluorescamine staining. Similar observations have also been reported with DL-α-methyltryptophan and L-tryptophan[36].

The specificity of the present fluorigenic reaction is unique, in that N-substituted phenylethylamines such as epinephrine and metanephrine are not detectable, unlike in other fluorimetric methods[9,22,26,27,31,32]. The present reaction should be useful when norepinephrine and/or normetanephrine are analysed in the presence of epinephrine and/or metanephrine, *e.g.*, as in the histochemical staining of noradrenergic fibres in tissues.

Since first reported by Udenfriend *et al.*[35], fluorescamine has been used extensively as a potent fluorigenic labelling reagent for amino acids, peptides, proteins and other primary amines. We have attempted so far to apply the general reagent as a tool for specific analyses of biogenic amines in view of its rapid reactivity and high sensitivity. So far, specific and sensitive analyses of three types of major biogenic amines, *viz.*, indoleamines (IV, tryptamines, tryptophans and peptides with N-terminal



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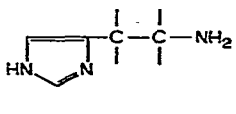
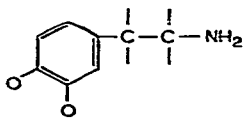


(V)



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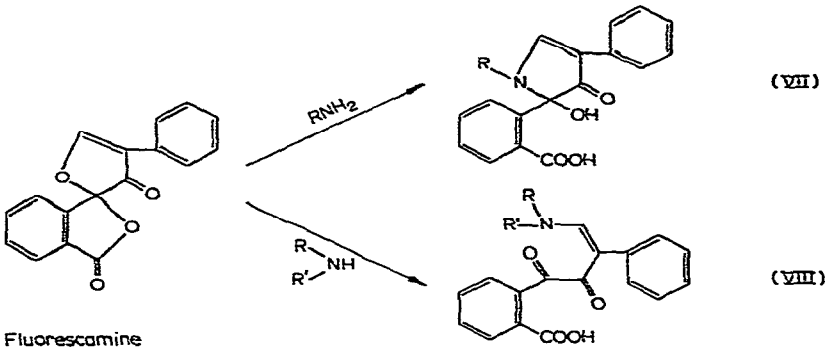

tryptophan)[36,41], imidazoleamines (V, histamines, histidines and peptides with N-terminal histidine)[42,43] and phenylethylamines (VI, primary catecholamines, their O-methyl and hydroxy derivatives and DOPAs) have been realized using fluorescamine. The structural characteristic common to these amines is an aromatic ethylamine whose $-NH_2$ group is unsubstituted. The essential requirement of the $-NH_2$ group suggests that the unique reactions are initiated by fluorescamine fluorophores (VII) of primary amines and not by aminoenone chromophores[44] (VIII) derived from secondary amines. Once their fluorescamine fluorophores (VII) have been formed, the differences in the structures of the individual amines, especially indole, imidazole and catechol moieties, are responsible for the specificity expressed in the respective reactions. For example, among many types of VII only those of type IV and VI show intense yellow and blue fluorescence, respectively, in strong acids[36,41]. If VII is heated in strong acids, only type V gives intense blue fluorescence[42,43]. The distinction of VI and IV may be simply achieved by the difference in colours of their fluorescamine fluorophores (VII) in strong acids or by the selective degradation of IV with BNPS-skatole to oxindole derivatives[45], whose fluorescamine fluorophores are not fluorescent in strong acids. Moreover, in the analysis of VI, the BNPS-skatole treatment eliminates the possible interference by 3-substituted indoles, which give yellowish fluorescence upon spraying with 70% PCA solution[46].

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