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THIN-LAYER CHROMATOGRAPHY OF HISTIDINE, HISTAMINE AND HISTIDYL PEPTIDES AT PICOMOLE LEVEL USING A UNIQUE FLUOROGENIC REACTION WITH FLUORESCAMINE

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SUMMARY

A thin-layer chromatography procedure has been devised for detecting histidine, histamine, histidyl peptides and related imidazole compounds; it is based on a unique fluorogenic reaction, in which the compounds are derivatized with fluorescamine, converted into different fluorescent products by heating in strong acid medium, and separated on silica gel plates with an appropriate solvent system. As little as 4 to 60 pmole of the histidine derivative could be detected under long-wave (366 nm) ultraviolet radiation. Other classes of compounds gave no fluorescent spots.

INTRODUCTION

Imidazole compounds have been detected with the Pauly reagent[1–3] or its modifications[3–8], although both the sensitivity and the specificity of diazonium-coupling reactions are inadequate for microanalysis of complex biological materials. About two decades after it was introduced as a fluorogenic reagent for the detection of glycine, histidine and tryptophan on paper chromatograms[9], *o*-phthalaldehyde (OPA) has gained wide recognition as a sensitive chromatographic reagent for histidine[10–13], histamine[10–13], histidyl peptides[13] and related imidazoles[12], with which it reacts to form unidentified fluorophores. The OPA methods are the most sensitive available for the chromatographic detection of imidazole compounds; however, they lack specificity, as OPA reacts with all compounds having a primary amino-group[11], and the formation of fluorophores is not limited to imidazole compounds[9–13]. Thus, until now, no specific reaction has been available for histidine derivatives on chromatograms. Recently, a new unique fluorogenic reaction of 2-(4-imidazolyl)ethylamines, such as histidine, histamine and histidyl peptides, with fluorescamine (a potent fluorogenic reagent for primary amines[14]) has been reported, permitting analysis for these compounds in the picomole range[15]. In the present investigation, a thin-

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layer chromatography (TLC) method based on this unique reaction has been developed for the microanalysis of histidine, histamine and histidyl peptides; its sensitivity is about 10 times better than that of previous TLC methods[10–13].

EXPERIMENTAL

Chemicals

Stock solutions (usually 10 m*M*) of the following compounds were prepared in water*: L-histidine, histidine thiohydantoin, DL-histidyl-DL-histidine, L-histidine ethyl ester hydrochloride (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.), L-histidinol dihydrochloride, imidazole (grade III), L-homocarnosine (4-aminobutyryl-L-histidine) sulphate, L-anserine (β-alanyl-L-1-methylhistidine) nitrate, L-carnosine (β-alanyl-L-histidine), L-1-methylhistidine, L-histidylglycine, L-histidyl-L-lysine hydrobromide, L-histidyl-L-tyrosine, L-histidyl-L-alanine, L-histidine methyl ester dihydrochloride, L-histidinol phosphate, L-histidine hydroxyamate, glycyl-L-histidylglycine, L-alanyl-L-histidine (containing 15% of ethanol), N-*tert.*-butoxycarbonyl-L-histidine, N-benzyloxycarbonyl-DL-histidine, N-benzyloxycarbonyl-L-histidylglycine, imidazol-4-ylacetic acid hydrochloride, 1-methylimidazole, 2-methylimidazole (Sigma, St. Louis, Mo., U.S.A.), L-3-methylhistidine, L-histidyl-L-leucine, L-histidyl-L-serine, angiotensin I (Schwartz/Mann, Orangeburg, N.Y., U.S.A.), histamine dihydrochloride, L-histidyl-L-phenylalanine (Mann, New York, N.Y., U.S.A.), N-α-acetyl-L-histidine hydrate, DL-2-methylhistidine dihydrochloride hydrate, DL-4-methylhistidine (Cyclo, Division of Travenol Labs., Los Angeles, Calif., U.S.A.), 1,4-methylhistamine [1-methyl-4-(2-aminoethyl)imidazole] dihydrochloride (grade A, Calibiochem, La Jolla, Calif., U.S.A.) and ophidine (β-alanyl-3-methylhistidine, free base; m.p. 248° (kindly supplied by Dr. J. Wolff, National Institutes of Health, Bethesda, Md., U.S.A.). Glucagon (Sigma) was dissolved in 0.1 *M* sodium borate buffer of pH 9.0 to a final concentration of 100 μ*M*. Other amino acids, peptides, diamines, polyamines, alkylamines, catecholamines, and primary amines were purchased from Sigma and were dissolved in water to make 10 m*M* stock solutions.

Preparation and separation of fluorophores

All the dispensing procedures described below were carried out with Eppendorf pipettes (Bio-Rad Labs., Richmond, Calif., U.S.A.) unless otherwise specified. Histidine and related compounds were first derivatized with fluorescamine (Pierce, Rockford, Ill., U.S.A.) and then converted into new fluorophores as follows. To 10 μl of 10 m*M* aqueous sample in a disposable polyethylene micro test-tube (capacity 1.5 ml; Brinkman Instruments, Westburg, N.Y., U.S.A.), plus 40 μl of 0.2 *M* sodium borate buffer of pH 9.0, was added rapidly 50 μl of fluorescamine solution in acetonitrile (20 mg/100 ml), with vigorous shaking on a Vortex-type mixer. After allowing the mixture to stand for 5 min at room temperature, 50 μl of 2.0 *N* hydrochloric acid were added, and the tube was tightly capped. The mixture was incubated in a water bath at 80° for 1 h, then 1 μl of the cooled reaction mixture (equivalent to 0.67 nmole of original compound) was applied to a pre-coated silica gel TLC plate (silica gel 60; 20 × 20 cm; 0.25-mm layer; E. Merck, Darmstadt, G.F.R.) with a

* Distilled and deionized water was used throughout this work.

volumetric micro-pipette (Microcaps, Drummond, Broomall, Pa., U.S.A.). With glucagon, 100 μl of a 100 μM solution was used for the labelling with fluorescamine, and, after acid treatment, 2 μl of reaction mixture (equivalent to 0.83 nmole of glucagon) was used for TLC. The acid-induced fluorophores were developed by ascending chromatography with the following solvent systems in glass chromatographic tanks.

I, *n*-Butanol–acetic acid–water (5:2:3).
II, *n*-Butanol–acetic acid–water (4:1:5, upper phase).
III, Ethyl acetate–methanol–water (60:25:10).
IV, Chloroform–methanol–acetic acid (6:2:2).
V, Benzene–dioxan–acetic acid (2:5:3).
VI, Isopropanol–acetic acid–water (6:2:2).

After brief drying under a stream of cold air from a hair-dryer, the plate was examined, in the dark, under a long-wave (366 nm) ultraviolet (UV) lamp.

Micro-procedure for better sensitivity

A 4-μl portion of 0.2 *M* sodium borate buffer of pH 9.0 was applied to the wall of a polypropylene micro centrifuge-tube (D20 tube, capacity 0.4 ml; Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) with a 10-μl Hamilton syringe; 1 μl of aqueous sample was added with a 1-μl Microcaps pipette. After the buffered sample had fallen to the bottom of the tube, 5 μl of fluorescamine solution in acetonitrile (20 mg/100 ml) was rapidly added with a 10-μl Hamilton syringe while the tube was vigorously shaken on a Vortex mixer. After 5 min at room temperature, 5 μl of 2.0 *N* hydrochloric acid were added to the fluorescamine derivatives with a 10-μl Hamilton syringe, and the tube was tightly capped, then heated at 80° for 1 h on a water bath. The entire volume (15 μl) was then carefully applied to a pre-coated silica gel plate under a stream of hot air from the hair-dryer, avoiding unnecessary diffusion of the sample; after complete evaporation of the solvent, the chromatogram was developed with solvent II, and the fluorescent spots were detected in UV radiation.

RESULTS

Specificity and sensitivity

When fluorescamine-labelled samples were heated in 0.67 *N* hydrochloric acid at 80° for 1 h and then subjected to TLC, histidine, histamine, histidyl peptides and related compounds gave a yellowish-green to green fluorescence on silica gel under UV radiation; other amino acids, peptides containing no histidine residues, catecholamines, di- and poly-amines and other common primary amines did not fluoresce, nor did N-substituted histidines and histamines or peptides with histidine in the C-terminal or in the internal positions. Two compounds (3-methylhistidine and 1,4-methylhistamine) gave a light blue to bluish-green fluorescence in acidic media and a blue fluorescence in neutral media. Histidine derivatives with free aminoethyl side-chains (except for L-1-methylhistidine and histidine hydroxamate) could be detected in amounts of 60 pmoles or less by using the micro-procedure (see Table I). These results indicate that, as well as a free amino-group in the side-chain, an unsubstituted nitrogen in position 1 of the imidazole ring is necessary for a compound to

TABLE I

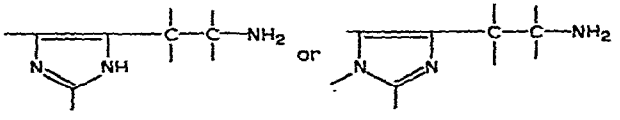
LIMITS OF DETECTION OF HISTIDINE, HISTAMINE, HISTIDYL PEPTIDES AND RELATED COMPOUNDS

Compound	*Limit of detection* (pmole)*	*Compound*	*Limit of detection* (pmole)*
Histidine and related compounds		*N-terminal histidine peptides*	
L-Histidine	20	L-His-L-Leu	40
L-1-Methylhistidine	>1000	L-His-Gly	7
DL-2-Methylhistidine dihydrochloride hydrate	40	L-His-L-Lys hydrobromide	60
L-3-Methylhistidine	20	L-His-L-Tyr	20
DL-4-Methylhistidine	7	L-His-L-Ala	20
L-Histidine methyl ester dihydrochloride	4	DL-His-DL-His	20
L-Histidine ethyl ester hydrochloride	6	L-His-L-Ser	20
L-Histidine hydroxamate	300	L-His-L-Phe	7
L-Histidinol dihydrochloride	40	Glucagon	40
L-Histidinol phosphate	40	N-benzyloxycarbonyl-L-His-Gly	>1000
Histidine thiohydantoin	>1000	*C-terminal histidine peptides and related compounds*	
N-α-Acetyl-L-histidine hydrate	>1000	L-Ala-L-His	>1000
N-tert.-butoxycarbonyl-L-histidine	>1000	L-Anserine nitrate	>1000
N-benzyloxycarbonyl-DL-histidine	>1000	L-Carnosine	>1000
Histamine and related compounds		L-Homocarnosine sulphate	>1000
Histamine dihydrochloride	60	Ophidine	>1000
1,4-Methylhistamine dihydrochloride**	40	*Sandwiched histidine peptides*	
		Gly-L-His-Gly	>1000
		Angiotensin I	>1000
		Imidazole and related compounds	
		Imidazole	>1000
		Imidazole-4-acetic acid hydrochloride	>1000
		1-Methylimidazole	>1000
		2-Methylimidazole	>1000

* Values were obtained by the micro-procedure described under Experimental, which included the preparation of fluorescamine derivatives, heat treatment in acid and separation on silica gel 60 plates, with *n*-butanol–acetic acid–water (4:1:5, upper phase) as mobile phase.

** The enzymic decarboxylation product of 3-methylhistidine.

be fluorescent in the reaction. Thus, the positive response is restricted to 2-(4-imidazolyl)ethylamines having the following structures:



Separation of histidine compounds

The R_F values obtained with the six solvent systems are listed in Table II. Most of the histidine derivatives gave single fluorescent spots; however, esters of

TABLE II

R_F VALUES OF HISTIDINE, HISTAMINE, HISTIDYL PEPTIDES AND RELATED COMPOUNDS*

Compound	*R_F × 100 value in solvent system***					
	I	*II*	*III*	*IV*	*V*	*VI*
L-Histidine	65	49	76	84	62	81
Histamine dihydrochloride	74	70	86	97	78 (69)	80
DL-2-Methylhistidine dihydrochloride hydrate	63 (75)	40 (46)	75, 62	76 (86)	24	79
L-3-Methylhistidine	54 (66)	23	14 (11)	32	6 (3)	49
DL-4-Methylhistidine	63 (52)	44 (18, 24, 31)	78	87 (6, 10)	57	81
L-Histidine methyl ester dihydrochloride	64, 81	47, 78	76, 87	86, 99	63, 87	82, 88
L-Histidine ethyl ester hydrochloride	65, 85 (70, 78)	48, 86	76, 90	85, 99	62, 89	83, 88
L-Histidine hydroxamate	65	47	76	86	63	82
L-Histidinol dihydrochloride	76 (53)	76 (21)	86 (2)	97	79 (70)	87
L-Histidinol phosphate	57	26	45	12	11	71
1,4-Methylhistamine dihydrochloride	55 (65)	22 (40)	2 (14, 18)	30	1	27
L-His-L-Leu	80	75	78	95	85	88
L-His-Gly	66	52	52	86	72	81
L-His-L-Lys hydrobromide	56 (72)	26 (58)	18	20	5	75 (88)
L-His-L-Tyr	75	70	79	92	19	87
L-His-L-Ala	68	57	53	90	75	84
DL-His-DL-His	56, 58	27, 29	44	22, 35	6	71, 74
L-His-L-Ser	62 (58)	43 (34)	50	76	59 (49)	82
L-His-L-Phe	76	70	80	96	83	88
Glucagon	60	18	7 (0, 44, 46)	7 (1)	2	92

* Conditions as in Experimental.

** Values in parentheses are for trace spots, usually with transient reddish-orange fluorescence.

histidine were partially degraded to histidine by the acid treatment. Also, L-histidine hydroxamate was completely hydrolyzed to histidine. Although DL-histidyl-DL-histidine showed two fluorescent spots almost equal in intensity, this was probably due to contamination; its fluorescamine derivative also gave two spots. As shown in Table II, additional spots showing slight fluorescence were sometimes observed. Usually, these exhibited very weak reddish-orange fluorescence, which disappeared within 5 min under the UV lamp. Fluorophores produced from fluorescamine derivatives by acid were well separated in compact spots and produced stable intense fluorescence in acidic solvent systems; in contrast, fluorescence intensities gradually decreased after development in neutral solvent systems. Alkaline solvents diminished the acid-induced fluorescence markedly, and could not be used.

DISCUSSION

Recently, Edvinsson *et al.*[13] reported on the TLC of histidine, histamine and histidyl peptides on silica gel, using an OPA spray technique; the minimum detectable amount varied from 0.1 to 0.3 μg for the various histidyl peptides in spot tests. The reaction was unspecific in that tryptophyl peptides, indolamines and catecholamines in submicrogram amounts also gave fairly intense OPA-induced fluorescence[13]. Earlier workers[10,11] reported better sensitivities for imidazole compounds with OPA reagent (Table III). The method described here could be used to detect 4 to 60 pmoles of histidine, histamine and histidyl peptides after preparation of fluorescamine derivatives, heat treatment in acid and TLC separation; this represents a remarkable improvement in sensitivity over all published methods[10–13]. Further, the proposed method is specific for 2-(4-imidazolyl)ethylamines; other aromatic compounds do not interfere. The structures of the fluorophores produced from the fluorescamine derivatives of 2-(4-imidazolyl)ethylamines by treatment with acid have not yet been ascertained. The acid-induced fluorescence may be due to thermal re-arrangement products of non-fluorescent lactones, but not the lactones themselves, because the acid-produced fluorescence appears after the initial fluorescence of the fluorescamine derivatives has completely decayed[15].

TABLE III

COMPARISON OF SENSITIVITY OF PROPOSED METHOD WITH THOSE OF VARIOUS OPA METHODS AFTER TLC

Compound	*Limit of detection (pmole)* *			
	Proposed method	*OPA method*		
		Shelley and Juhlin[10]	*Turner and Wightman*[11]	*Edvinsson et al.*[13]
Histidine	20	—	220–440	640**
Histamine	60	680	54–270	540**
Histidyl peptide	7–60	—	—	410–1100**

* Values for the OPA methods have been recalculated from the values (in μg) reported in the literature.

** Obtained by spot tests.

Alternative procedures for the TLC of these histidine derivatives might involve the separation of the amines in their intact forms, with subsequent derivatization with fluorescamine and successive acid treatment, or separation of the fluorescamine derivatives, followed by acid treatment. The derivatization of primary amines with fluorescamine on developed TLC plates has been achieved by dipping the plates in a solution of fluorescamine in acetone–hexane (1:4) (see ref. 16); this offers an approximately 10-fold improvement in sensitivity compared with the previous spraying method[17]. The pre-labelling of primary amines with fluorescamine at the origin of TLC plates has also been achieved at the picomole level, either by pre-development or by pre-dipping[18] using the same solvent for fluorescamine as used in the dipping method[16]. The optimal concentration of hydrochloric acid for acid treatment of the fluorescamine derivatives of 2-(4-imidazolyl)ethylamines was reported[15] to be about

0.67 *N*. When fluorescamine-labelled primary amines on silica gel 60 plates were sprayed with various concentrations of hydrochloric acid and heated at 80° for 30 min in an electric oven, 0.5 *N* hydrochloric acid gave the highest yield of the acid-induced fluorescent products from the fluorescamine derivatives of 2-(4-imidazolyl)-ethylamines (Table IV); this is in good agreement with results obtained in solution[15].

TABLE IV

ACID-INDUCED FORMATION OF FLUOROPHORES FROM FLUORESCAMINE DERIVATIVES

In obtaining these results, 0.1 ml of a 10 m*M* aqueous solution of the authentic compound was mixed with 0.1 ml of 0.2 *M* sodium borate buffer of pH 9.0 and allowed to react with 0.1 ml of fluorescamine solution in acetonitrile (20 mg/100 ml), with vigorous shaking on a Vortex mixer. For catecholamines, 0.2 *M* phosphate buffer of pH 8.0 was used instead of the borate buffer to avoid fluorescence quenching due to chelation of borate by the catechol grouping. A 1-μl aliquot (equivalent to 3.3 nmoles of compound) was spotted on to a silica gel 60 plate, air-dried and sprayed with hydrochloric acid of various normalities; the plate was then heated at 80° for 30 min in an electric oven. The intensity of fluorescence observed in the dark under 366-nm radiation is designated as follows: ++, very strong; +, strong; Tr, trace; −, negative.

Fluorescamine derivative of	*Hydrochloric acid concentration (N)*			
	0.1	*0.5*	*1.0*	*2.0*
L-Histidine	−	++	+	Tr
Histamine dihydrochloride	−	++	+	Tr
L-His-L-Leu	−	++	+	Tr
L-Tryptophan	−	−	−	−
Tryptamine hydrochloride	−	−	−	−
L-Trp-L-Ala	−	−	−	−
DL-Norepinephrine hydrochloride	−	−	−	−
DL-Normetanephrine hydrochloride	−	−	−	−
3,4-Dihydroxyphenylalanine (DOPA)	−	−	−	−
Dopamine hydrochloride	−	−	−	−
3-Methoxytyramine hydrochloride	−	−	−	−
L-Alanine	−	−	−	−
L-Arginine hydrochloride	−	−	−	−

The fluorescamine derivatives of tryptophan, tryptamine and tryptophyl peptides have been reported to fluoresce in strong acid[19], and their chromatographic detection at the picomole level has been achieved by spraying with 70% perchloric acid[20]. In fact, acid-induced fluorescence was also observed with fluorescamine-labelled 3-(2-aminoethyl)indoles after spraying with 0.5 *N* hydrochloric acid; however, it appeared only during the early stages of heating at 80° (usually within 5 min) and disappeared completely after prolonged heating (30 min), when the acid-induced fluorescence of fluorescamine-labelled 2-(4-imidazolyl)ethylamines was at a maximum. Although the sensitivity of the method involving spraying with 0.5 *N* hydrochloric acid was poorer than that of the micro-procedure described under Experimental, the spray method would be useful for the specific detection of histidine-related compounds among the many fluorescent spots due to other primary amines on TLC plates.

Thus, two types of chemical species, 3-(2-aminoethyl)indoles[19,20] and 2-(4-

imidazolyl)ethylamines[15], can now be specifically detected at the p.. ..mole level by using fluorescamine, both on TLC plates and in solution. The development of a specific method for analysis of other biogenic aromatic ethylamines, such as catecholamines, by the same general methods as in the present work is in progress.

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