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PERCHLORIC ACID, A FLUOROGENIC SPRAY REAGENT FOR TRYPTOPHAN, TRYPTAMINE, PEPTIDES CONTAINING TRYPTOPHAN AND OTHER 3-SUBSTITUTED INDOLES

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SUMMARY

When silica gel plates containing 3-substituted indoles including tryptophan, tryptamine and peptides containing tryptophan are sprayed with 70% perchloric acid, a specific strong yellow-orange fluorescence develops within a few minutes. As little as 40–850 pmole of 3-substituted indoles can be detected. Other indoles do not give this characteristic fluorescence.

INTRODUCTION

Various colour reactions are available for the detection of indole compounds on paper and thin-layer plates[1–3], but fluorogenic reagents such as ninhydrin[4], Procházka formaldehyde–acid reagent[1–6], *o*-phthalaldehyde[7–11] and gaseous formaldehyde[10,12–22] are more sensitive. Of these reagents, the most popular and sensitive are the Procházka reagent and gaseous formaldehyde but they are surpassed in sensitivity by the recently introduced glyoxylic acid vapour[23,24]. Unfortunately, none of these reagents is specific for indoles. For example, phenylethylamines such as catecholamines give similar fluorescent products[6,10,12,14–18,24].

Recently, a unique fluorogenic reaction for 3-(2-aminoethyl)indoles was reported which involves the derivatization of the amines with fluorescamine followed by treatment with strong acid[25]. This reaction is specific to 3-(2-aminoethyl)indoles that have an NH_2 group in the side-chain and it has been applied to the analysis of tryptophan in urine, blood and protein hydrolysates[25]. In the course of extending the reaction to the detection of compounds on thin-layer chromatographic (TLC) plates[26], it was observed that some N(side-chain)-substituted tryptophans and even simple indole derivatives gave intense yellow fluorescent spots when they were sprayed with

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70% perchloric acid (PCA), even before the treatment with fluorescamine. Although it is known that indoles fluoresce in strong acids such as perchloric[27,28], trifluoroacetic[29] and sulphuric acids[29] and that the acid-induced fluorescence has been used for fluorometric assay[30], the reactions have never been applied to the detection of tryptophan or related compounds on chromatograms.

EXPERIMENTAL

Reagents and materials

The sources of the indole derivatives used were as follows: indole, 2-methylindole, 3-methylindole (skatole, grade I, *ca.* 99%), 5-methylindole, 7-methylindole (grade II), 5-hydroxyindole, N-methyltryptamine, N-acetyl-L-tryptophan, L-tryptophan, tryptamine·HCl, 5-hydroxy-DL-tryptophan, 5-methoxy-DL-tryptophan, 5-hydroxytryptamine (serotonin) oxalate salt, 5-methoxytryptamine·HCl, N-acetyl-5-methoxytryptamine (melatonin), N-acetyl-5-hydroxytryptamine (N-acetylserotonin), N,N-dimethyltryptamine, tryptophol, 5-methoxytryptophol, 6-hydroxymelatonin, 5-hydroxytryptophol (*ca.* 98%), Gly–L-Trp, L-Pro–L-Trp, L-Leu–L-Trp, L-Phe–L-Trp·acetate·0.5 H_2O, L-Val–L-Trp, indole-3-acetic acid, 5-hydroxyindole-3-acetic acid dicyclohexylammonium salt, 3-indoleacetone (grade II), 5-methoxy-3-indoleacetic acid, DL-3-indolelactic acid (*ca.* 98%), 3-indolepyruvic acid, 3-indoleacetonitrile, isatin, indomethacin, indoxyl-β-D-glucoside (plant indican), indoxyl sulphate (urinary indican) potassium salt, kynurenic acid·0.5 H_2O, xanthurenic acid (*ca.* 99%), quinolinic acid (99–100%), picolinic acid (grade II), DL-kynurenine sulphate, 3-hydroxyanthranilic acid, 1-acetylindole-3-carboxaldehyde, 6-hydroxytryptamine (creatinine sulphate complex), 6-methyl-DL-tryptophan, 6-fluorotryptamine·HCl, DL-6-fluorotryptophan, DL-7-benzyloxytryptophan, 7-methyl-DL-tryptophan, 7-methyltryptamine, DL-4-fluorotryptophan and 4-methyl-DL-tryptophan from Sigma (St. Louis, Mo., U.S.A.); N-carbobenzoxy-L-tryptophan and 7-azatryptophan from Nutritional Biochemical (Cleveland, Ohio, U.S.A.); Gly–L-Trp–Gly and L-Lys–L-Trp–L-Lys from Schwartz/Mann (Orangeburg, N.Y., U.S.A.); 1,2-dimethylindole, 2,3-dimethylindole, 2,5-dimethylindole, 1-methylindole-2-carboxylic acid, 1-methyl-DL-tryptophan, 5-chloroindole, 5-chloroindole-2-carboxylic acid, ethyl indole-2-carboxylate, ethyl 2-ethoxy-5-hydroxy-3-indolecarboxylate, 1-methylisatin (98%) and 7-methoxyindole from Aldrich (Milwaukee, Wisc., U.S.A.); N-methylindole, N-ethylcarbazole, 2-phenylindole, carbazole, indole-5-carboxylic acid, 6-nitroindole, pyrrole, indole-β-carboxylic acid, indole-3-aldehyde, β-indolepropionic acid and β-indoleacrylic acid from Tokyo Kasei Kogyo (Tokyo, Japan); indole-2-carboxylic acid from Fluka (Buchs, Switzerland); 5-nitroindole and N-phenylcarbazole from Nakarai Chemicals (Kyoto, Japan); and L-Leu–L-Trp–L-Leu and Gly–Gly–L-Trp from Fox Chemical (Los Angeles, Calif., U.S.A.). Other test compounds were of reagent grade and were purchased from commercial sources.

All test compounds were dissolved in 0.2 *M* sodium borate buffer (pH 9.0) whenever possible or a mixture of ethanol and the borate buffer (9:1) to make 1 m*M* stock solutions. The same solvents also were used to prepare dilute solutions.

Distilled-in-glass solvents, including chloroform, isopropanol, *n*-butanol, ethyl acetate, benzene, dioxane, methanol and *n*-hexane, were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.), ammonia solution (29.5% as NH_3) and

n-propanol from J. T. Baker (Phillipsburg, N.J., U.S.A.), acetic acid (Phix buffer grade) from Pierce (Rockford, Ill., U.S.A.), ethanol from USPHS S.S.C. (Perry Point, Md., U.S.A.) and PCA (70–72%) from Fisher Scientific (Fair Lawn, N.J., U.S.A.).

Pre-coated silica gel glass plates (silica gel 60, 20 × 20 cm, 0.25-mm layer, without fluorescence indicator; E. Merck, Darmstadt, G.F.R.) were used without any treatment. Samples were applied with volumetric micro-pipettes (Microcaps; Drummond, Broomall, Pa., U.S.A.). PCA solution was sprayed with an aerosol gun (Quixpray Kit, Pierce)*.

Solvent systems for separation of indole derivatives

(A) Chloroform–isopropanol–water (2:7:1).
(B) *n*-Butanol–acetic acid–water (5:2:3).
(C) Ethyl acetate–isopropanol–water (4:5:1).
(D) Chloroform–ethanol–acetic acid (13:6:1).
(E) Benzene–dioxane–methanol (5:3:2).
(F) *n*-Hexane–*n*-propanol–ammonia solution (2:7:1).

Thin-layer chromatography

A 1-μl aliquot of the standard solution, 1 m*M* or less, was applied 1.5 cm from the lower edge of a 20 × 20 cm plate with a 1-μl Microcap pipette and air-dried. Ascending chromatography was performed in a glass chromatographic tank at room temperature. After brief air-drying with a hair-dryer, the plate was sprayed with 70% PCA for 5 sec and the fluorescence was observed in the dark under long-wave ultraviolet (UV) light. After development with alkaline solvents, it was necessary to spray the plate for 10 sec.

RESULTS

Effect of concentration of PCA on the formation of fluorophores

The fluorescence intensity of representative indoles increased with increasing concentrations of PCA and all gave an intense yellowish fluorescence immediately after spraying the 70% PCA solution (Table I). The fluorescence was stable for at least 30 min.

Specificity and sensitivity of the PCA reagent

Of the many mono-substituted indoles tested, the characteristic yellow fluorescence was observed with 3-substituted indoles, including tryptophan, tryptamine and peptides containing tryptophan. Exceptions were indole-3-aldehyde and indole-β-carboxylic acid (Table II). Other mono-substituted indoles did not fluoresce at the 1-nmole level, but a few compounds with a carboxyl or phenyl group at the C-2 position gave a strong blue fluorescence before and after the PCA spray. Of the di-substituted indoles tested, no fluorescence was observed with 1-acetylindole-3-carboxaldehyde, 1,2-dimethylindole and 2,5-dimethylindole. In contrast, 1-methyl-

* Should be carried out in a well ventilated hood because even a trace amount of gaseous PCA may be irritating.

TABLE I

EFFECT OF PCA CONCENTRATION ON FLUORESCENCE

A 1-μl aliquot of a 1 m*M* solution in 0.2 *M* sodium borate buffer (pH 9.0) was spotted on a silica gel 60 plate, air-dried and sprayed with various concentrations of PCA. The plates were irradiated with a long-wave UV lamp and the fluorescence was observed. The intensity of fluorescence is designated as follows:

−, negative; tr, trace; +, weak; ++, strong; +++, very strong.

Compound	*Concentration of PCA (%)*							
	25		*40*		*55*		*70*	
	0–2 min	*5–10 min*	*0–2 min*	*5–10 min*	*0–2 min*	*5–10 min*	*0–2 min*	*5–10 min*
L-Tryptophan	−	−	−	−	−	−	+++	+++
Tryptamine·HCl	−	−	−	++	+	++	+++	+++
N-Acetyl-L-tryptophan	−	−	−	−	tr	+	+++	+++
N-methyltryptamine	−	−	−	++	++	++	+++	+++
3-Methylindole	+	+	+++	+++	+++	+++	+++	+++

indole-2-carboxylic acid and 5-chloroindole-2-carboxylic acid fluoresced, probably owing to the carboxyl group at the C-2 position. Although 3-substituted indoles typically gave yellowish fluorescent spots, white spots were observed with some 5-hydroxy and 5-methoxy derivatives. Also, no fluorescence was observed with 3-substituted indoles that contained substituents in the C-2 position (except indomethacin) or fluorine in the C-4 or C-6 position. Metabolites of tryptophan such as kynurenic acid, kynurenine and 3-hydroxyanthranilic acid also gave white spots. Numerous other compounds tested, including steroids, bile acids, catecholamines, their 3-O-methyl derivatives, amino acids other than tryptophan, peptides containing no tryptophan, di- and polyamines and alkylamines, did not fluoresce. Of the compounds that do fluoresce, the minimal detectable amount ranged from 40 to 850 pmole.

In addition to fluorescence, the PCA spray also gave rise to characteristic visible colours with some indoles, notably 3-indoleacetone (reddish pink), 3-indoleacetonitrile (faint blue) and indoxyl sulphate (faint blue).

The R_F values of the reactive compounds obtained with the six solvent systems are given in Table III.

DISCUSSION

Of the many compounds tested, only the 3-substituted indoles typically gave a yellowish fluorescence at room temperature within a few minutes after spraying with 70% PCA. The PCA spray is simpler than the Procházka[1–6], gaseous formaldehyde[10,12–22] and glyoxylic acid vapour[23,24] reagents, which require heating (80–100°, 30–60 min) to produce fluorescent products and are not specific to indoles[6,10,12,14–18,24]. Metz[31] and Hara and Takeuchi[32] used PCA for the detection of certain steroids and bile acids. In the present reaction, however, steroids and bile acids do not produce any detectable fluorescence at the 1 nmole level because the plates are not heated.

The yellowish fluorescence obtained with PCA is also obtained with hydrochloric, sulphuric and phosphoric acids but not with acetic and trifluoroacetic acids.

TABLE II

LIMITS OF DETECTION OF VARIOUS INDOLES AND RELATED COMPOUNDS

Values after TLC using silica gel 60 plates developed with solvent system A. Spray, 70% PCA for 5 sec. Yellow-orange fluorescence was usually observed unless specified in parentheses as follows: P, purple; YG, yellowish green; GP, greyish purple; GB, greyish blue; B, blue; GRB, greenish blue.

Substitution in indole ring	*Compound*	*Limit of detection (pmole)*
None	Indole	> 1000
N-1	N-Methylindole	> 1000
C-2	2-Methylindole	> 1000
	2-Phenylindole	250 (B)*
	Indole-2-carboxylic acid	250 (B)*
	Ethylindole-2-carboxylate	200 (B)*
C-3	3-Methylindole	200
	Indole-3-acetic acid	150
	3-Indoleacetone	150
	DL-3-Indolelactic acid	200
	3-Indolepyruvic acid	200
	3-Indoleacetonitrile	200 (YG)
	β-Indolepropionic acid	200
	β-Indoleacrylic acid	200
	Indole-3-aldehyde	> 1000
	Indole-β-carboxylic acid	> 1000
	Indoxyl-β-D-glucoside	400
	Indoxyl sulphate potassium salt	200 (P)
	L-Tryptophan	150
	Tryptamine·HCl	75
	Tryptophol	150
	N-Acetyl-L-tryptophan	150
	N-Methyltryptamine	150
	N,N-Dimethyltryptamine	40
	N-Carbobenzoxy-L-tryptophan	200
	L-Trp–Gly	150
	L-Trp–L-Ile	200
	L-Trp–L-Glu	200
	Gly–L-Trp	150
	L-Pro–L-Trp	40
	L-Leu–L-Trp	100
	L-Phe–L-Trp·acetate	200
	L-Val–L-Trp	200
	Gly–L-Trp–Gly	100
	L-Lys–L-Trp–L-Lys	100
	L-Leu–L-Trp–L-Leu	300
	Gly–Gly–L-Trp	300
C-5	5-Methylindole	> 1000
	5-Hydroxyindole	> 1000
	5-Nitroindole	> 1000
	5-Chloroindole	> 1000
	Indole-5-carboxylic acid	> 1000
C-6	6-Nitroindole	> 1000
C-7	7-Methylindole	> 1000
	7-Methoxyindole	> 1000

(Continued on p. 172)

TABLE II *(continued)*

Substitution in indole ring	*Compound*	*Limit of detection (pmole)*
N-1, C-2	1,2-Dimethylindole	> 1000
	1-Methylindole-2-carboxylic acid	300 (B)*
N-1, C-3	1-Methyl-DL-tryptophan	500
	1-Acetylindole-3-carboxaldehyde	> 1000
C-2, C-3	Carbazole	> 1000
	Isatin	> 1000
	2,3-Dimethylindole	> 1000
C-2, C-5	2,5-Dimethylindole	> 1000
	5-Chloroindole-2-carboxylic acid	250 (B)*
C-3, C-4	4-Methyl-DL-tryptophan	150
	DL-4-Fluorotryptophan	> 1000
C-3, C-5	5-Hydroxyindole-3-acetic acid DCA** salt	350 (P)
	5-Methoxy-3-indoleacetic acid	500 (GB)
	5-Hydroxy-DL-tryptophan	250 (GP)
	5-Methoxy-DL-tryptophan	200 (GB)
	5-Hydroxytryptamine oxalate salt	500 (GP)
	5-Methoxytryptamine·HCl	100 (B)
	N-Acetyl-5-methoxytryptamine	150
	N-Acetyl-5-hydroxytryptamine	850 (P)
	5-Methoxytryptophol	200
	5-Hydroxytryptophol	500
C-3, C-6	6-Methyl-DL-tryptophan	200
	6-Hydroxytryptamine	200
	6-Fluoro-DL-tryptophan	> 1000
	6-Fluorotryptamine·HCl	> 1000
	6-Hydroxymelatonin	400 (GP)
C-3, C-7	7-Methyl-DL-tryptophan	1000
	7-Methyltryptamine	200
	7-Benzyloxytryptophan	1000
N-1, C-2, C-3	N-Ethylcarbazole	> 1000
	N-Phenylcarbazole	> 1000
	1-Methylisatin	> 1000
C-2, C-3, C-5	Ethyl 2-ethoxy-5-hydroxy-3-indolecarboxylate	> 1000
N-1, C-2, C-3, C-5	Indomethacin	250
Related compound	Pyrrole	> 1000
	7-Azatryptophan	200 (B)*
	Kynurenic acid	2 (B)*
	Xanthurenic acid	> 1000
	Quinolinic acid	> 1000
	Picolinic acid	> 1000
	DL-Kynurenine sulphate	350 (GRB)*
	3-Hydroxyanthranilic acid	100 (B)*

* Native fluorescence.
** Dicyclohexylammonium.

PCA is preferred because it reacts faster and the fluorescence is more stable. The failure of trifluoroacetic acid to induce fluorescence may be due to its higher volatility. Weaker acidity would apply to acetic acid.

The results in Table I are consistent with those of Tauber[27], who reported that fluorescence was not observed for 3-substituted indoles in solution when the PCA

TABLE III

SEPARATION OF 3-SUBSTITUTED INDOLES AND RELATED COMPOUNDS

A 1-μl aliquot of 1 mM stock solution, equivalent to 1 nmole, was spotted on silica gel 60 plates. After development the plates were air-dried and sprayed with 70% PCA solution for 5 sec. Plates developed with alkaline solvent F were sprayed with the reagent for 10 sec.

Compound	*$R_F \times 100$ value in solvent system**					
	A	*B*	*C*	*D*	*E*	*F*
3-Methylindole	96	96	90	97	93	91
Indole-3-acetic acid	91	94	89	92		28
5-Hydroxyindole-3-acetic acid DCA** salt	89					
3-Indoleacetone	95	95	90	92	69	90
5-Methoxy-3-indoleacetic acid	90	67	87	94	88	28
DL-3-Indolelactic acid	47	67	44	92 (26)	2	24
3-Indolepyruvic acid	36	94	88	92	0	21
3-Indoleacetonitrile	95	95	89	95	67	90
Indomethacin	91	88	88	93	79	83
Indoxyl-β-D-glucoside	88	71	85	45	50	20
Indoxyl sulphate	79	65	84	20	18	40
L-Tryptophan	45	61	34	5	1	22
Tryptamine·HCl	13	69	8	15	2	68
5-Hydroxy-DL-tryptophan	32					
5-Methoxy-DL-tryptophan	21	58	27	2	0	21
5-Hydroxytryptamine oxalate salt	38					
5-Methoxytryptamine·HCl	14 (38)	70	26 (7)	17	4, 8	70 (65)
N-Acetyl-L-tryptophan	56	74	52	57	7	27
N-Methyltryptamine	6	59, 68	4	15	2	67
N,N-Dimethyltryptamine	7	57	4	12	8 (86)	90
N-Acetyl-5-methoxytryptamine	93	86	86	95	74	88
N-Acetyl-5-hydroxytryptamine	92					
Tryptophol	95	93	90	95	85	88
5-Methoxytryptophol	95	89	89	95		88
6-Hydroxymelatonin	73					
5-Hydroxytryptophol	95	90	88	81		
N-Carbobenzoxy-L-tryptophan	79		80	92	44	40
Gly-L-Trp	18	59	9	1	0	17
L-Pro-L-Trp	10	59	5	2	1	17
L-Leu-L-Trp	49	73	33	13	2	33
L-Phe-L-Trp·acetate	52	72	41	15	4	31
L-Val-L-Trp	40	71	23	11 (8)	0	29
Gly-L-Trp-Gly	11	53	4	1	0	14
L-Lys-L-Trp-L-Lys	0	23	0	0	0	2
L-Leu-L-Trp-L-Leu	63	85	59 (46)	27 (33)	5	55, 62
Gly-Gly-L-Trp	10	53	3	1	0	12
Kynurenic acid	56	62	51	11	3	37
DL-Kynurenine sulphate	41	57	23	3	2	0, 21
3-Hydroxyanthranilic acid	10	41	4	0 (2)	0	0

* Values in parentheses indicate minor fluorescent spots.

** Dicyclohexylammonium.

concentration was less than 30%. This is why fluorescamine-labelled 3-(2-aminoethyl)-indoles and peptides that contain amino-terminal tryptophan could be specifically assayed with 22% PCA without interference from N-substituted tryptophans and

tryptamines and peptides containing tryptophan within the chain or at the carboxyl terminus[25].

The present fluorogenic PCA spray seems to be well suited for the specific and highly sensitive detection of tryptophan and its major metabolites as well as peptides containing tryptophan. Recently, high-performance liquid chromatographic methods have been developed for the analyses of tryptophan metabolites[33–35], involving assays based on the native fluorescence of the indole skeleton. It now appears possible that a more specific and sensitive method can be developed based upon the fluorogenic reaction with PCA.

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