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The use of *o*-phthalaldehyde as a spray reagent for the thin-layer chromatographic identification and quantitation of bufotenin and 5-methoxy-N:N-dimethyltryptamine

The use of *o*-phthalaldehyde (OPT) for the fluorometric determination of 5-hydroxyindole derivatives such as serotonin (5-HT) and 5-methoxytryptamine was described by MAICKEL AND MILLER[1], who also reported on the nonspecificity of OPT and compared the relative fluorescence of several 3:5 substituted indoles. This reagent was subsequently used for the quantitative estimation of 5-HT in biological material: in rat brain by MAICKEL *et al.*[2] and in human urine by KORF AND SEBENS[3]. A 0.02 % solution of OPT in acetone was used as a spray reagent for the detection of imidazoles and indoles by AURES *et al.*[4]. These authors considered that the amino group and 5 substitution of indoles were necessary for the fluorescence with this reagent. They also reported that a preliminary spray with acetic acid or sodium hydroxide was necessary for optimal fluorescence. However there is no report on the use of this reagent for 5-hydroxy- or methoxy-N-methylated tryptamines.

In view of the very high sensitivity reported for serotonin (0.025 μg) and in our search for a more specific and sensitive spray reagent for bufotenin, we examined the effectiveness of OPT as a spray reagent for the qualitative identification and quantitative determination of N-methylated indolealkylamines on thin-layer chromatograms, and also to determine the structural requirements for this reaction.

Experimental

In a preliminary experiment bufotenin, N-methylserotonin (NMS) and 5-methoxy-N:N-dimethyltryptamine (5-MeO DMT) were treated with OPT according to the method of MAICKEL AND MILLER[1]. At a 0.05 μg level the fluorescence readings at F_{max} (activation 360 mμ) for the three 5-hydroxy compounds, (serotonin, N-methylserotonin and bufotenin) were 0.59, 0.46, 0.62 respectively. The 5-methoxy compound, 5-MeO DMT, gave at the same 0.05 μg level a reading of 3.47, a value very similar to that reported for 5-methoxytryptamine[1]. The fluorescence readings obtained at various concentration levels are shown in Table I. Thus N-methylated amines show the same sensitivity as the primary amines. The standard reference compounds were spotted on a silica gel thin-layer chromatography (TLC) plate and run in one dimension with $CHCl_3$–CH_3OH–NH_3 (12:7:1). After development (solvent front 14 to 15 cm) the plates were dried and sprayed with OPT (10 mg OPT, 100 mg cysteine in 5 ml methanol and 5 ml HCl). The plate was then heated at 100° for 15 min and observed under UV light. Intense fluorescence ranging from yellowish-green to blue was noted in several cases.

Fluorescence maxima of the spots. A variety of substituted indoles with different substituents in the 3, 5 and 6 positions showed positive reactions with OPT. The sensitivity of this reagent for different compounds and also the differences in fluorescence maxima were studied. The fluorescence maximum was determined by eluting the fluorescent spot on the TLC plate with 2 ml of 6 *N* HCl and taking the eluate after centrifugation. The HCl eluate was then read on an Aminco Bowman Spectrofluorom-

TABLE I

FLUORESCENCE READINGS SHOWING LINEARITY BETWEEN CONCENTRATION *vs.* FLUORESCENCE

Compound	*Concentration (μg)*					
	2.0	*1.5*	*1.0*	*0.5*	*0.25*	*0.05*
5-HT	27.35	20.56	16.21	7.66	3.71	0.59
NMS	22.54	18.02	11.30	5.37	2.72	0.47
Bufotenin	22.35	16.36	13.95	7.11	3.21	0.62
5-MeO DMT	89.36	61.37	44.37	23.57	13.51	3.47

TABLE II

REPLICATE FLUORESCENCE READINGS (TEST-BLANK) OF 1 μg SPOTS

Compound	*Readings*		
	1	*2*	*3*
5-HT	0.87	0.79	0.68
NMS	0.46	0.45	0.36
Bufotenin	0.78	0.68	0.66
5-MeO DMT	4.38	4.33	4.28

eter at activation 360 mμ. A blank spot was simultaneously extracted for the blank reading. While the fluorescent spots showed a characteristic maximum, the blank did not show any in that region. With the spray reagent we used we found that the fluorescence maxima were higher by 10 mμ than those obtained in solution with MAICKEL AND MILLER's[1] method. When cysteine was eliminated from the spray reagent, the maximum was the same. However, we noted that the peak extended over a 10 mμ range and that the reading had to be taken at the precise maximum.

Quantitative TLC analysis. The quantitative reproducibility of the fluorescence on a TLC plate was then examined by spotting replicate samples of the same concentration and reading the fluorescence units after developing the plate in one solvent system as described above. Replicate analyses of 1-μg spots of serotonin, N-methylserotonin, bufotenin, and 5-MeO DMT are shown in Table II. A linear relationship between concentration and fluorescence was found in the range between 0.1 and 1.0 μg. In a typical case the quantitative estimation of a standard mixture of serotonin, N-methylserotonin, bufotenin (N:N-dimethylserotonin) and 5-MeO DMT after two-dimensional TLC showed a recovery of 85 %. The results are summarized in Table III.

In order to determine the nature of the fluorescent condensation product the reaction was carried out using 5-MeO DMT (1.0 mg) and OPT under the experimental conditions described by MAICKEL AND MILLER[1]. The reaction mixture (1.5 ml) was then spotted (10 μl) on a silica gel TLC plate using 10, 5 and 1 μl and developed with chloroform, methanol and ammonia (12:7:1) as the solvent system. When viewed under UV light two intensely blue fluorescent spots were seen in all three dilutions. Both the spots showed a fluorescence maximum at 470 mμ (excitation 360 mμ). The reaction mixture was then brought to pH 10 with 10 % sodium hydroxide and extracted into ethyl acetate. The base also showed two spots on two-dimensional TLC

TABLE III

FLUORESCENCE IN SOLUTION[1] AND ON TLC, ACTIVATION 360 mμ

Compound	*Solution*			*TLC*				
	F_{max}	*Minimum estimated amount (μg)*	*Test blank*	*F_{max} with*		*Minimum detectable amount (μg)*		*Test blank*
				OPT	*OPT and L-cysteine*	*Qualitative*	*Quantitative*	
5-HT	480	0.05	5.4	482	491	0.02 (yellow)	0.05	4.0
Bufotenin	480	0.05	5.4	479	492	0.05 (yellow)	0.1	2.5
NMS	477	0.05	4.6	485	489	0.05 (yellow)	0.25	3.2
5-MeO DMT	470	0.05	27.6	470	478	0.05 (blue)	0.1	2.8
6-MeO Indole					441	1.0	[a]	[a]
6-BzO DMT					565	0.3	[a]	[a]
6-HT					420	0.5	[a]	[a]

[a] No characteristic maximum below 1 μg.

TABLE IV

R_F VALUES OF FLUORESCENT PRODUCTS OBTAINED WITH OPT

Compound (0.01 μg)	*Chloroform–methanol–NH_4OH (12:7:1)*		*Isopropanol–water–NH_4OH (17:3:1)*	
	Spot 1	*Spot 2*	*Spot 1*	*Spot 2*
5-HT	0.08	0.18	0.06	0.10
Bufotenin	0.31	0.49	0.14	0.23
NMS	0.08	0.18	0.04	0.08
5-MeO DMT	0.64	0.86	0.34	0.53

which were well separated from 5-MeO DMT. Furthermore, the OPT reaction product could be distinguished from the original 5-MeO DMT by the negative reaction with *p*-dimethylaminocinnamaldehyde. Serotonin (10 μg), N-methylserotonin (10 μg) and bufotenin (10 μg) showed a similar reaction with OPT (50 μl) and HCl (0.1 ml), the reaction mixture giving two spots on TLC with the same fluorescence maxima and approximately the same fluorescence readings. On a two-dimensional chromatogram of the reaction mixture the products of bufotenin and 5-MeO DMT were separated from each other and also from the products of serotonin and N-methylserotonin. The latter two compounds, however, did not separate in the two solvent systems tested (See Table IV and Fig. 1).

Results and discussion

OPT gives a fluorescent product on TLC plates with indole with substituents (OH, MeO, OzB) in 5 or 6 positions. The substituents in 3 position can be H or $CH_2CH_2NR_1R_2$ where $R_1 = R_2 = H$, $R_1 = H$, $R_2 = CH_3$ or $R_1 = H$, $R_2 = COCH_3$. For

qualitative detection, OPT can be used as a spray reagent to detect levels as low as 0.05 μg of bufotenin, N-methylserotonin and 5-MeO DMT. This could also be done after reacting the amine mixture with OPT and HCl and then using the fluorescent products for TLC. A typical reaction may be carried out as follows: the final concentrate of the amine mixture after removal of solvent from a biological sample containing at least 1 μg of the amines, is heated with 0.05 ml of 0.05 % OPT in methanol and 0.1 ml of conc. HCl for 30 min. 10 μl of this reaction mixture is then used for TLC. In view of the high sensitivity (0.005 μg) of this method at least for serotonin, N-methylserotonin, bufotenin and 5-MeO DMT, this quantitative TLC technique can be used either by elution of the spot or by fluorescence scanning for the estimation of these compounds in biological materials.

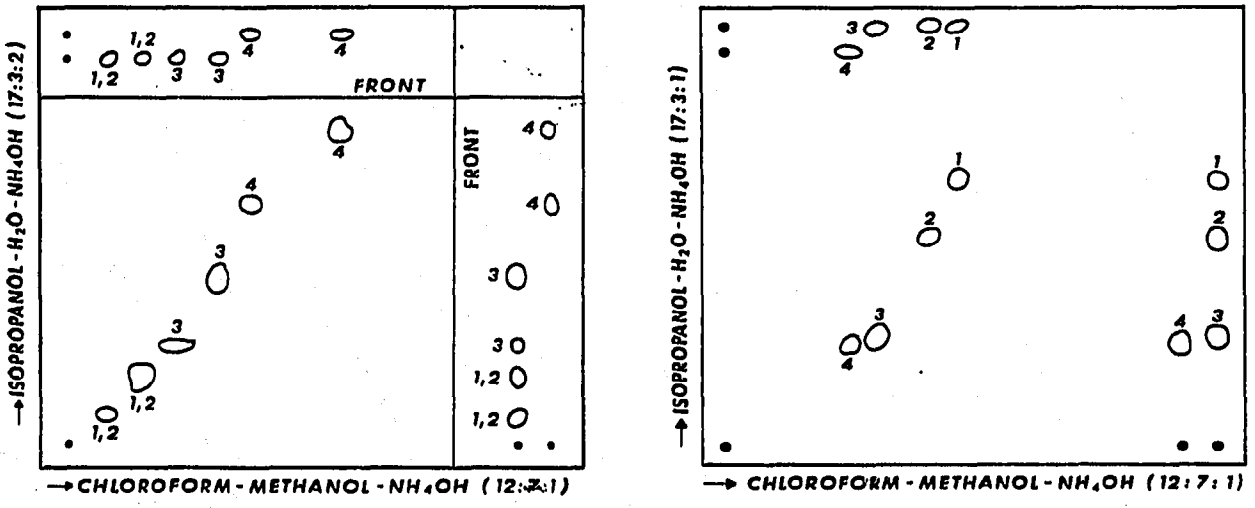

Fig. 1. Two-dimensional thin-layer chromatogram (Silica Gel G) of the reaction products of a mixture of (1) 5-HT, (2) NMS, (3) bufotenin and (4) 5-MeO DMT.

Fig. 2. Two-dimensional thin-layer chromatogram (Silica Gel G) of a mixture of (1) 5-MeO DMT (R_F 0.85, 0.64); (2) bufotenin (R_F 0.66, 0.57); (3) 5-HT (R_F 0.34, 0.41); and (4) NMS (R_F 0.33, 0.34).

We noticed that the fluorescence readings obtained in solution[1] were higher than the readings obtained for the same concentration by the elution of the fluorescent spots on TLC. If, however, the TLC spots corresponding to the compounds were scraped off and the silica gel treated with OPT and HCl, the readings were comparable. These methods using OPT are superior to other methods in that there is a separation of closely related 5-hydroxyindole derivatives. A typical chromatogram after two-dimensional TLC of a mixture of four compounds is shown in Fig. 2. We have used this method extensively in the qualitative identification and quantitative estimation of bufotenin in urine samples. Full details of this study will be published elsewhere.

6-Hydroxytryptamine gives a fluorescent spot with this reagent at a concentration level of 1.0 μg and fluorescence is at 410 mμ. The fluorescence reading is not much higher than the blank up to levels of 1.0 μg. Therefore, although 6-OH derivatives can be visualized by this reagent, it is not sensitive enough for quantitation. Thus, the fluorescence reaction by itself is not specific for indolealkylamines substituted in the 5-position, but the fluorescence maxima (470–480 mμ) appear to be specific for this group of compounds.

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Separation and detection of basic dyes by polyamide thin-layer chromatography

Basic dyes are not permissible for use as food additives now, but an analytical method for the compounds is necessary because there is the danger of them being used or mixed in foods and pharmaceutical formulations either erroneously or illegally. Thin-layer chromatography (TLC) using only silica gel plates has previously been applied to the analysis of basic dyes[1]. No results however, have proved entirely satisfactory. In this paper, therefore, an attempt at analysis was made using polyamide layers, which are frequently used for separation and identification of water and oil-soluble dyes[2–6].

Materials and methods

Adsorbent. Polyamide powder (obtained from E. Merck, Darmstadt, G.F.R.) was used as the adsorbent. Before use, it was washed with a volume of methanol equal to 3–5 times the volume of polyamide powder. After drying in air, it was further dried in vacuum in a desiccator containing calcium chloride.

Dyes. Each test solution of the basic dyes listed in Table I was prepared by dissolving 10 mg of each of them in 10 ml of 60% ethanol solution. Aliquots (μl) of this solution were used for TLC.

Developing solvents. (a) Benzene; (b) carbon tetrachloride; (c) methanol; (d) ethanol; (e) 28% ammonia–methanol (1:8); (f) benzene–methanol (5:1); and (g) carbon tetrachloride–methanol (4:1). All the solvents were of analytical grade.

Preparation of the thin layers. Glass plates (20 × 20 cm) were coated with a homogeneous slurry composed of 15 g polyamide powder and 50 ml of isopropanol by means of an applicator giving a thin layer approximately 250 μ in thickness. The coated plates were dried in air for 15 min and then at 60° for 30 min. They were kept in a desiccator over silica gel until required.