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FLUORESCENCE-INDUCING PROCEDURES FOR USE IN HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A comparison is made of three methods which have been previously employed to induce fluorescence in compounds, with particular reference to high-performance thin-layer chromatographic (HPTLC) chromatoplates. In addition, it has been found that the simple procedure of exposing the chromatoplates to HCl, HBr, or HNO_3 room-temperature vapors, followed by heating, is sufficient to produce strong fluorescence in a wide variety of compounds. This brings to HPTLC the sensitivity advantages inherent in fluorescence as a visualization technique. In all cases, fluorescent spot intensity remained unchanged for at least two weeks.

INTRODUCTION

High-performance thin-layer chromatography (HPTLC) combines the advantages of ease of operation using inexpensive equipment, with higher resolution than has been possible using conventional TLC. It has been widely employed for qualitative and quantitative analysis of various mixtures[1].

Ultimately HPTLC, like other TLC, depends on the sensitivity of the visualization technique employed. UV and fluorescence permit the detection of compounds in nanogram to picogram amounts, provided, of course, the substances of interest respond. There are many compounds, however, for which these otherwise sensitive methods give only a weak or negligible response. Other commonly used methods rely on sprayed reagents to render chromatographed materials visible (*e.g.*, through charring, iodine absorption, selective reagents), but none of these is adequate for high-precision trace analysis. Any method of visualization, therefore, which can convert a broad range of substances to fluorescent products is potentially valuable to TLC, and especially, HPTLC.

Such a method was described by Segura and Gotto[2], who employed heated NH_4HCO_3 to produce fluorescence in many types of organic compounds on silica gel or alumina plates. Shanfield and co-workers[3,4] utilized a gaseous electrical discharge, in the presence of various activating gases at low pressure, to produce fluorescence in diverse organic compounds spotted on thin-layer silica gel plates. In addition to the high sensitivity which this method offers, it appears to give better reproducibility and more intense fluorescence for some compounds, and in a considerably shorter time than Segura's thermal method. A modification of the thermal method has been sug-

gested by Segura[5], in which a $ZrCl_4$ dip solution is used to intensify spot fluorescence.

The present work compares these procedures for their potential benefits to HPTLC. In addition, we report on another simple and rapid technique for inducing strong fluorescence in a number of organic compounds, namely exposure to inorganic acid vapors, followed by heating.

EXPERIMENTAL

Compounds, solvents, activating gases, inorganic acids

A number of organic compounds were evaluated by spotting them on chromatoplates in varying amounts. These were obtained from commercial and laboratory sources. All solvents used to dissolve these compounds for subsequent spotting were of analytical, spectral, or HPLC grade.

NH_4HCO_3, ammonium salts and other reagents, when employed, were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Activating gases used in the discharge chamber were nitrogen, methane and methyl amine. $ZrCl_4$ was obtained from Alfa Products (Danvers, MA, U.S.A.). Inorganic acid vapors were derived at room temperature from standard strength HCl (37%), HBr (40%), HNO_3 (70%), all analytical grade.

Thin-layer chromatoplates

Most of the experiments were performed on 10 × 10 cm HPTLC silica gel 60 plates (E. Merck, Darmstadt, G.F.R.). In order to eliminate fluorescent background, heating at 260°C for 4 h prior to use, was generally sufficient. Occasionally, it was necessary to immerse the plates in a 1% Ludox solution before heating. For comparison purposes, some experiments were done using the "Permakote" thin-layer silica gel plates described by Shanfield and co-workers[3,4]. The Permakote plates do not use any organic binder and are readily rendered fluorescence-free by chromic acid treatment.

Electrical discharge chamber

The high-voltage discharge chamber was the same as that described in Shanfield *et al.*'s paper[3]. The main components comprise a glass chamber for holding chromatoplates under partially evacuated conditions, with access for selected activating gases. A high-voltage, capacitive discharge is effected through wrapped metal foil on the exterior of the chamber.

Test procedures

Solutions containing substances to be evaluated were usually made up to a concentration of 1 μg per μl of solvent. Chromatoplates were spotted with a micropipette using 200 nl of solution in most cases. The solvent was allowed to evaporate at room temperature. From time to time, solvent alone was spotted side-by-side with solution to guard against solvent artifacts.

For discharge chamber experiments, spotted chromatoplates were placed in the chamber where the activating gas was controlled to a pressure of 5 Torr, and the high voltage applied for 3 to 7 min. After removal, the plate was heated in an oven at 130°C for 5 min. Fluorescence was observed under a 365-nm UV lamp (Ultraviolet

Products, San Gabriel, CA, U.S.A.) until its brightness stabilized. Spot intensity was estimated on a scale from 1 to 10 (1 = weak, 10 = strong).

Experiments using a $ZrCl_4$ solution to enhance fluorescence followed Segura's[5] suggested procedure, except that ethanol was used as a solvent instead of water. This reduced spot diffusion. A 20% solution of $ZrCl_4$ was used for dipping chromatoplates, followed by heating at 160°C for 5 min.

Segura's description of the thermal procedure was followed for experiments involving that method, *i.e.*, exposing spotted chromatoplates to the vapors of NH_4HCO_3 for 4 h at 130°C.

HPTLC chromatoplates (Merck) were exposed in gaseous discharge experiments using nitrogen, methane and methyl amine gases. Most of these gases were evaluated with low volatility materials present in the chamber: NH_4HCO_3, maleic anhydride, phthalic anhydride, ammonium formate, ammonium acetate, ammonium benzoate and sodium bicarbonate. Plate fluorescence was observed and estimated before and after heating at a temperature of 120°C for 10 min.

The inorganic acid vapor procedure consisted in exposing spotted chromatoplates to the vapors of HCl, HBr and HNO_3 at room temperature (24°C), for a period of 10 min. Subsequently, the plates were placed in an oven (air) at 160°C for 10 min. Fluorescent spots were evaluated immediately after an experiment. In no case did spot intensity diminish in less than two weeks of desiccator storage.

RESULTS AND DISCUSSION

Comparison of thermal, $ZrCl_4$ and gas discharge methods

Table I sets out a comparison of the thermal, $ZrCl_4$ and gas discharge methods for inducing fluorescence in a number of organic compounds spotted on HPTLC chromatoplates. A group of β-blocking drugs were evaluated, the carbohydrate fructose, four organic acids, and the hydrocarbon n-$C_{22}H_{46}$ utilized by Shanfield *et al.*[4] as a reference substance.

Where fluorescence was observed by all three methods (which was usually the case), the estimated intensity was similar. Quantitative comparisons were not made in this set of experiments because of the unavailability of a chromatogram spectrophotometer. Diazepam responded sensitively with all methods at the 40-ng level, as did oxprenolol at the 200-ng level. Fructose showed fluorescence with the thermal and $ZrCl_4$ methods, but not noticeably with the gas discharge method, as previously reported by Shanfield *et al.* Benzoic and hippuric acids failed to give fluorescence with the $ZrCl_4$ method; instead, UV quenching (365-nm lamp) was observed. Lactic and oxalic acids did not respond by any of the methods. Finally, pamatolol responded weakly to the $ZrCl_4$ method.

At the present time, one can merely speculate on the reasons why such procedures convert varied organic structures to fluorescing materials on a silica gel substrate. (A ground glass surface, for example, does not result in fluorescence.) All three methods supply energy to adsorbed molecules, and molecular rearrangement and/or decomposition probably occurs. On the silica gel substrate, however, these new structures are held more or less rigidly on the surface. Thus, metastable products can remain so for extended time periods, as Shanfield *et al.*'s work[4] showed. Metastable substances are also susceptible to further reaction, which occurs with the thermal and

TABLE I

COMPARISON OF THERMAL, $ZrCL_4$ DIP AND GASEOUS DISCHARGE METHODS (HPTLC CHROMATOPLATES)

Compound	*Amount (ng)*	*Estimated fluorescent intensity**		
		Thermal	*$ZrCl_4$ Dip*	*Gaseous discharge***
Diazepam	40	5	5	5
Fructose	40	2	2	§
Benzoic acid	100	3	§	3
Hippuric acid	100	3	§	3
Lactic acid	100	***	***	***
Oxalic acid	100	***	***	***
Alprenolol	200	4	4	4
Atenolol	200	3	3	3
Oxprenolol	200	10	10	10
Pamatolol	200	4	1–2	4
Practolol	200	4	4	4
Prenalterol	200	4	4	4
Propanolol	200	4	4	4
Timolol	200	4	4	4
n-$C_{22}H_{46}$	1000	3	3	3

* 1 = weak, 10 = strong, observed with 365-nm UV lamp.
** NH_4HCO_3 in chamber during discharge.
*** No fluorescence observed.
§ Quenching observed.

gas discharge procedures, since exposure to additional reactants (*e.g.*, NH_3, H_2O, CO_2) is now provided.

The striking similarity between resultant fluorescing materials has been noted previously, and it is tempting to speculate that a parent structure is responsible. The surface characteristics of silica gel at the molecular level may indeed, promote common structures. Work on fluorescence has shown that fluorescence of substances is greatly enhanced either by building "rigidity" into the molecule, or by adsorption on solid substrates[7]. This is illustrated by phenolphthalein which does not fluoresce in solution *versus* fluorescein which does so strongly. The difference between the two structures lies solely in an oxygen bridging two phenyl groups in fluorescein, conferring rigidity on the molecule. Presumably, this tends to keep excitation energy localized so it cannot dissipate throughout the molecule.

Fluorescence is, of course, known to occur in a large number of aromatic compounds. Generalizations have been found respecting molecular features which contribute to fluorescence (*e.g.*, large polynuclear aromatic structures which are geometrically rigid). It is known that groups like methoxyl, hydroxyl, cyano and amino are effective in shifting the fluorescence spectrum toward longer wavelengths, and fluorescence is intensified as well[8]. Cyano and amino groups, at least, seem plausible under the conditions used in the thermal and gas discharge methods, followed by exposure to vapors of ammonium compounds. It is directions of this kind which we intend to pursue in future work. This choice is partly prompted by the observation that compounds on chromatoplates which were induced to fluoresce by gas discharge, suffer no loss of fluorescence whatever when treated with bromine

water or bromine–CCl_4 solution. This would appear to eliminate simple, non-aromatic, conjugated double bond systems.

Shanfield *et al.*[4] showed that argon, an inert gas, is capable of activating organic compounds in 30 sec in the electrical discharge chamber, and that they would subsequently fluoresce when exposed to vapors of NH_4HCO_3 in a few minutes. About 4 h are required to accomplish the same result at 130°C with vapors from aqueous NH_4HCO_3 (Segura's thermal method). This suggests that the two procedures differ mainly in the level and rate of energy deposition, and may not otherwise differ greatly in principle. It could account for the null observation on fructose by gaseous discharge, for which the thermal method showed weak fluorescence. Evidently drastic decomposition occurs under discharge conditions.

Results for acid vapor-induced fluorescence

Table II lists the results obtained with acid vapor-induced fluorescence on HPTLC chromatoplates. Included for comparison are diazepam and fructose. The latter compound gave only modest fluorescence in the thermal and $ZrCl_4$ dip processes (see Table I).

TABLE II

ACID VAPOR-INDUCED FLUORESCENCE (HPTLC CHROMATOPLATES)

Compound	*Amount (ng)*	*Estimated fluorescent intensity**		
		*HCl****	*HBr*****	*HNO_3*[§]
Diazepam	20	10	10	10
Testosterone	200	7	6	6
β-D-Fructose	200	8	6	7
Dextrose	200	8	6	7
Ephedrine	200	1	0	1
1,1'-Carbonyl diimidazole	200	1	8	1
Acetyl acetonates				
Beryllium	200	1	–	1
Aluminium	200	1	–	1
Vanadium	200	3	–	3
Iron(II)	200	0	–	0
Cerium	200	10	–	10
Manganese	200	8	–	8

* 1 = weak, 10 = strong, observed with 365-nm UV lamp.
** 37% acid.
*** 40% acid.
[§] 70% acid.

The acid vapor procedure appears to offer the gratifying combination of simplicity with sensitivity comparable to the other methods described. In particular, fructose responded strongly to this technique.

To the eye, the fluorescent spots appear to be similar, in many cases, to that obtained with the other procedures. In the absence of a spectrophotometer, it was not possible to examine this question in detail.

The mechanism by which the transformation to fluorescent compounds occurs is completely unknown. The chemical simplicity of an acid like HCl reduces the investigative complexity compared, for example, with what must be occurring in the thermal or gaseous discharge methods. Accordingly, we plan to attempt some characterization of the fluorescent materials produced by the acid vapor technique.

Finally, it should be noted that one added advantage for HNO_3 vapor is that the chromatoplate background was significantly reduced compared with HCl or HBr. This provides extra sensitivity because of the enhanced contrast.

Comparison of sensitivity of UV vs. gaseous discharge fluorescence

A quantitative comparison between UV absorption and fluorescence by gaseous discharge was made with several β-blocking drugs. The results are shown in Fig. 1. It is clear that much higher sensitivity is achieved with oxprenolol, in which fluorescence is induced, than with UV absorption. It is estimated that for this drug, the minimum level of detectability by the gaseous discharge method is about 1–2 ng, *i.e.*, about one order of magnitude more sensitive than UV absorption.

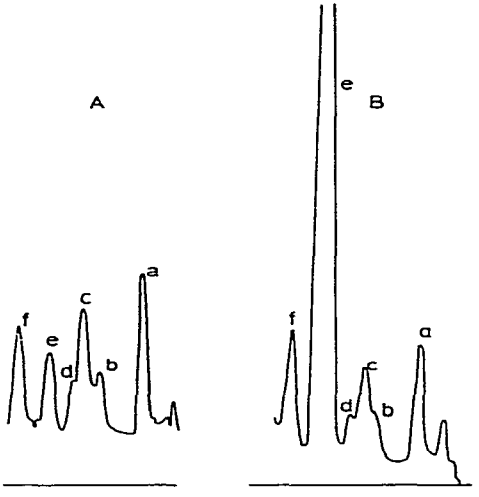

Fig. 1. HPTLC chromatograms of some β-blocking drugs. A, UV absorption; B, fluorescence viewed at 365 nm. a = Practolol and atenolol; b = pamatolol; c = timolol; d = metoprolol; e = oxpranolol; f = propranolol. Solvent system: dichloromethane–ether–formic acid (20:20:4).

HPTLC chromatoplate response to gaseous discharge species

Table III lists the results obtained when HPTLC chromatoplates are exposed to the gaseous discharge chamber filled at 5 Torr with nitrogen and methane, in the presence of what are termed "activating compounds". This procedure combines the two steps normally used in the gaseous discharge procedure, into a single one. Whatever energetic species arise, do so from the chamber gas plus whatever is contributed due to the vapor pressure of the activating compounds.

Ammonium acetate, ammonium benzoate and maleic anhydride all produced fluorescence which was particularly strong after the plates were heated. In the case of maleic anhydride, strong fluorescence was observed without heating. Methane tends to produce stronger fluorescence on the whole compared with nitrogen, but maleic

TABLE III

EFFECT OF N_2 AND CH_4 AS DISCHARGE CHAMBER GASES ON HPTLC CHROMATOPLATES WITH VARIOUS ACTIVATING SUBSTANCES PRESENT

Activating compound	*Estimated fluorescent intensity**			
	N_2		CH_4	
	Before heating	*After heating*	*Before heating*	*After heating*
None present	None	None	None	None
$ZrCl_4$	None	None	None	1
Phthalic anhydride	None	None	None	1
Ammonium formate	–	–	None	1
$NaHCO_3$	–	–	None	1
NH_4HCO_3	None	None	1	2
Ammonium acetate	1	5	8	10
Ammonium benzoate	1	8	1	10
Maleic anhydride	10	10	10	10

* 1 = weak, 10 = strong; observed with 365-nm UV lamp.

anhydride results were indifferent to the gas used, or to heating. In spite of the low volatility of compounds like $ZrCl_4$ and $NaHCO_3$, some fluorescence could be observed. However, the presence of impurities cannot be ruled out.

One other gas was tested by itself in the chamber, namely methyl amine. This gave a fluorescent intensity of 1 before heating, and 10 after heating. This stands in contrast to CH_4, which alone gave no fluorescence. However, the C–N bond strength in methyl amine is much lower than the C–H bond strength in methane, so that free radicals should form with greater ease in CH_3NH_2 during the electrical discharge.

CONCLUSIONS

Based on the work done to date, we have concluded the following:

(1) A variety of fluorescence-inducing procedures can be employed successfully for HPTLC, which adds enhanced sensitivity to its other benefits.

(2) The acid vapor technique for inducing fluorescence in compounds appears to offer the greatest simplicity together with significant gains in sensitivity.

(3) Fluorescent compounds produced by the acid vapor technique obviate, to some extent, the complexities introduced by the other fluorescence-inducing methods described.

(4) The success of widely differing techniques for inducing fluorescence in compounds on silica gel chromatoplates is probably a reflection of the strong catalytic properties of silica gel surfaces.

(5) Fluorescent spot intensity remains stable for a period of at least two weeks, when the plates are stored in a desiccator.

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