CHROM. 14,014

USE OF METAL SALTS AS FLUORESCENCE-INDUCING REAGENTS IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

In the procedure described for the detection and quantitation of organic compounds in thin-layer chromatography, the formation of fluorescent derivatives is induced by thermal treatment of the chromatographic plates in the presence of a metal salt or another inorganic compound. Silicon tetrachloride and tin(IV) chloride can be applied in gaseous form, whereas zirconyl chloride and zirconyl sulphate have to be applied in aqueous solution. Zirconium derivatives have several advantages over other fluorogenic reagents, namely convenience of application, higher sensitivity of detection (allowing the detection of nanogram or picogram amounts of some organic compounds), speed of reaction (requiring only a few seconds or minutes of treatment), great versatility and reasonable stability of the fluorophores obtained (several weeks).

INTRODUCTION

Although thin-layer chromatography (TLC) has several well known advantages, it suffers from two important limitations: lack of automation and generally poor quantitative results. The ideal procedure for quantitative TLC would be one that offers the highest sensitivity, namely the production of fluorescent derivatives, one that assures an even distribution of the reagent in the layer and, at the same time, gives a low background (in order to achieve accurate and reproducible results) and one that produces a derivative stable enough for adequate measurement. It is obvious that, in most instances, these goals are not achieved.

Segura and Gotto[1] introduced the use of ammonium hydrogen carbonate as a reagent that satisfied some of the aspects mentioned above; the thermal treatment of the plates in the presence of the above reagent induces the formation of fluorescent derivatives with many types of organic compounds (and gives a rather low background), produces fluorophores that are stable for long periods of time and allows the quantitation of submicrogram amounts of substances. However, some limitations of this procedure, namely the need for a long period of heating (as much as 6–8 h at 150°C for lipids) and its limited sensitivity, promted us to investigate the usefulness of other compounds (ammonium halides, metal halides, etc.) as fluorogenic reagents.

We found that heating the plates in the presence of silicon tetrachloride produces very strong fluorescent derivatives with different types of organic compounds, especially steroids and carbohydrates; in addition, the fluorophores can be obtained in a very short time (about 30 min). The fact that silicon tetrachloride is a very volatile, corrosive and irritating compound led us to look for other types of reagents, both volatile and non-volatile. We have found that the zirconium derivatives are the reagents that give the best fluorogenic response of all of those that we have tested.

EXPERIMENTAL

Compounds and reagents

More than 50 organic compounds were studied. The substances were dissolved in suitable solvents and kept refrigerated. All solvents used in the chromatographic separation were of analytical or spectroscopic quality.

Chromatographic plates

Most of the experiments were performed on pre-coated silica gel 60 plates, without fluorescent indicator, with a layer thickness of 0.25 mm (E. Merck, Darmstadt, G.F.R.). In some instances pre-coated silica gel 60 plates for high-performance thin-layer chromatography (HPTLC), without fluorescent indicator, were used.

In addition, the following adsorbents (TLC grade) were tested: silica gel (Woelm Pharma, Eschwege, G.F.R.), silica gel H, type 60 (Merck), acidic, neutral and basic alumina (Woelm Pharma), Florisil (Schuchardt, Munich, G.F.R.), cellulose powder MN 300 (Macherey, Nagel & Co., Düren, G.F.R.) and polyamide powder (Woelm Pharma).

The plates were coated with the adsorbent, suspended in ethanol, by means of a Desaga (Heidelberg, G.F.R.) spreader at a thickness of 0.25 mm. After air drying, the plates were purified by double development with a mixture of methanol and concentrated ammonia (1:1). Prior to use, the plates were activated at 120°C for 30 min. The same procedure was followed for the purification of pre-coated plates.

Application of the samples

All compounds studied in a particular series were applied in the same volume (usually in 5-μl samples) by means of a 25-μl Hamilton (Reno, NV, U.S.A.) microsyringe equipped with a repeating dispenser. Each sample was applied as a band of three closely spaced spots, 5 mm wide, perpendicular to the direction of the chromatographic development.

With certain groups of compounds the different components were separated on the plate with a suitable development system. The different plasma lipid classes were separated on silica gel by development first with ethanol, containing 1% of formic acid, up to 1 cm from the origin, and, after drying, with 1,2-dichloroethane. Estrogens were separated on silica gel with benzene–ethanol (9:1). Bile acids were separated on silica gel by development with a mixture of 1,2-dichloroethane–ethanol–formic acid (80:17:3).

Detection

When using non-volatile reagents (see Table I), the plates were usually sprayed with an excess of a 20% solution of the reagent; alternatively, the plate may be

dipped into the reagent solution for 1 min or, with some types of separations, the reagent may be incorporated into the layer prior to the development; a mixture of estrone, 17β-estradiol and estriol can be fractionated with a similar degree of resolution both on silica gel or on silica gel impregnated with zirconyl sulphate or zirconyl chloride (the plates are dipped into a 5% aqueous solution of the salt and dried at 60°C for 60 min prior to use).

The plates treated according to one of the procedures just described were placed inside an oven, or on a hot plate, and heated at 150–180°C for a variable period of time (5–60 min) according to the type of adsorbent and the nature of the compound to be determined. The different plasma lipid fractions separated on silica gel were heated at 160°C for 30 min when using zirconyl sulphate or zirconyl chloride as the fluorogenic reagents. Estrogens can be visualized on silica gel plates impregnated with zirconyl sulphate after less than 5 min of thermal treatment at 160°C.

When volatile reagents were used (see Table I), the plates were placed in a sealed tank of 4 gallon capacity, containing 5 ml of the volatile reagent. The tank thus prepared was placed inside an oven and heated at 160°C for 30–60 min, according to the type of compound studied. The different plasma lipid fractions separated on silica gel are rendered fluorescent after 60 min of heating at 160°C in the presence of silicon tetrachloride.

Fluorescence measurements

The fluorescent derivatives were measured *in situ* with a Zeiss (Oberkochen, G.F.R.) Model KM 3 chromatogram spectrometer equipped with a mercury source. For the evaluation of the excitation wavelength the radiation is dispersed by the monochromator and a particular wavelength radiation, which falls vertically on the sample, is selected; the light emitted is collected at a 45° angle through a barrier filter. To evaluate the emission maxima the illumination geometry is inverted; the radiation, selected by a barrier filter, impinges at a 45° angle on the chromatographic plate; the emitted and reflected light are collected vertically, dispersed in the monochromator and the selected radiation is directed to the detector.

The plates are scanned in the same direction as that of development. The areas of the peaks corresponding to each type of compound are integrated by triangulation or, more usually, by means of an electronic integrator (Autolab Minigrator; Spectra-Physics, Santa Clara, CA, U.S.A.).

RESULTS

Heating in the presence of different halides or of several metal salts induces the formation of fluorescent derivatives of a large number of organic compounds separated on thin-layer plates. Of the reagents tested (Table I), zirconyl chloride ($ZrOCl_2$) and zirconyl sulphate ($ZrOSO_4$) among the non-volatile compounds and silicon tetrachloride ($SiCl_4$) and tin(IV) chloride ($SnCl_4$) among the volatile compounds were the most efficient.

The fluorescent derivatives formed by heating in the presence of these reagents are readily detected by viewing the treated plate under UV light, where they show, in most instances, a yellow-greenish or blueish fluorescence. After spraying with an aqueous solution of zirconyl chloride or sulphate, the fluorescent derivatives are formed in a very short time, which varies according to the temperature of heating

TABLE I

COMPOUNDS TESTED AS POTENTIAL FLUOROGENIC REAGENTS IN THIN-LAYER CHRO-
MATOGRAPHY

Type	Compounds	
Volatile	HCl	
Non-volatile	$SbCl_3$	$SnCl_4$
	$SiCl_4$	$TiCl_4$
	$AlCl_3$	$TiCl_3$
	$BaCl_2$	Zr (metal)
	$CaCl_2$	$ZrCl_4$
	$CrCl_3 \cdot 6H_2O$	$ZrOCl_2 \cdot 4H_2O$
	$FeCl_3$	ZrF_4
	$MgCl_2 \cdot 6H_2O$	$ZrO(NO_3)_2 \cdot H_2O$
	$MnCl_2 \cdot 4H_2O$	ZrP_2O_7
	$SnCl_2$	$ZrO(SO_4)H_2SO_4 \cdot 3H_2O$

and the nature of the original compound. Steroid derivatives, such as cholesterol, cholesteryl esters, testosterone and bile acids, are rendered fluorescent in a few seconds at 200°C. Under the same conditions, sugars, estrogens, non-esterified fatty acids, etc., react also very rapidly (in a few minutes). On the other hand, amino acids and purine and pyrimidine derivatives require prolonged heating.

In the analysis of plasma lipids, consisting of various compounds, most of the components show a good response after heating for 5–10 min at 170°C when using an aliquot of extract corresponding to 0.1–0.2 μl of plasma. Triglycerides show a response and require heating for 30 min or more. Fig. 1 shows a typical ... gram, corresponding to the separation of an isopropanol extract of normal plasma (equivalent to 0.2 μl of plasma), heated at 160°C for 30 min in the presence of zirconyl sulphate and scanned at 460 nm, with the lower setting of the chromatogram spectrometer.

Relative efficiency of the fluorogenic reagents

Quantitative evaluation of the same amount of compound (the different lipid fractions contained in an extract equivalent to 0.2 μl of normal plasma) treated with the five best fluorogenic reagents showed that zirconyl sulphate is the reagent of choice (Fig. 2); note the difference in the responses between zirconyl chloride and sulphate in relation to the triglyceride fraction. On the other hand, zirconyl chloride appears to be the reagent of choice for the detection and quantitation of estrogens (Fig. 3 and Table II).

TABLE II

RELATIVE RESPONSES OF ESTROGENS TREATED WITH FLUORESCENT REAGENTS

Results are peak-area ratio $\cdot 10^{-3}$ per microgram of compound. Conditions as in Fig. 3.

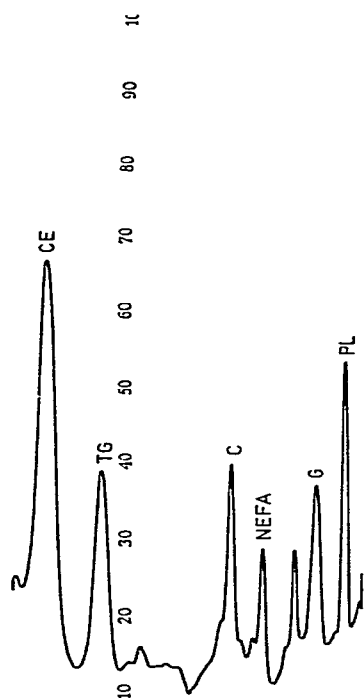| Compound	$ZrOSO_4$	$ZrOCl_2$	$ZrO(NO_3)_2$
Estriol	488	690	353
Estradiol	1269	1440	962
Estrone	1017	1922	788

Fig. 1. Chromatogram showing the different lipid fractions contained in an isopropanol extract corresponding to 0.2 μl of normal plasma. The compounds have been rendered fluorescent by treatment at 160°C for 30 min in the presence of zirconyl sulphate. Scanning conditions: excitation, 365 nm; emission, 460 nm. PL = phospholipids; G = glucose; NEFA = non-esterified fatty acids; C = cholesterol; TG = triglycerides; CE = cholesteryl esters.
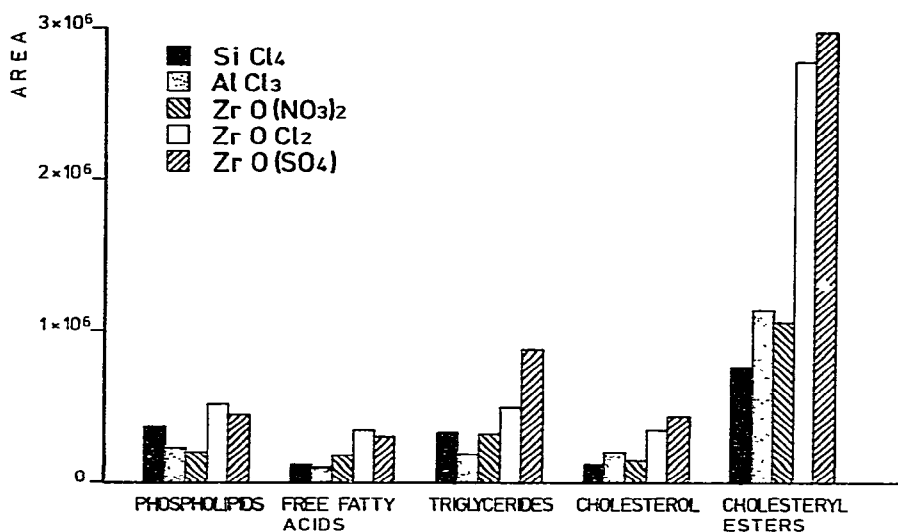

Fig. 2. Relative responses of different plasma lipid fractions treated at 160°C with different fluorogenic reagents. All samples were measured under identical spectrofluorimetric settings: excitation, 365 nm; emission, 460 nm.

Table III indicates the fluorescence response, per microgram of compound, obtained with five different types of fluorogenic reagents; again, zircony sulphate is the reagent that produces the highest yield, especially with triglycerides. It should be emphasized that in all of the experiments the spectrofluorimeter was operating at its lower range of sensitivity.

TABLE III

RELATIVE RESPONSES OF PLASMA LIPIDS TREATED WITH DIFFERENT FLUORESCENT REAGENTS

Results are peak-area ratio · 10^{-3} per microgram of compound. conditions as in Fig. 1.

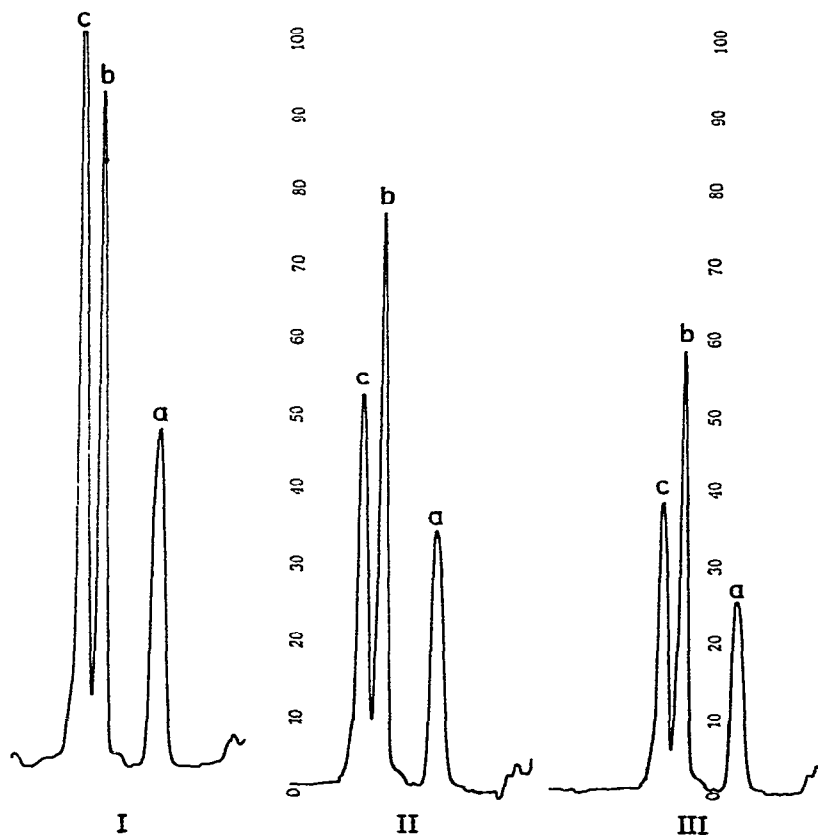| Compounds	$SiCl_4$	$AlCl_3$	$ZrO(NO_3)_2$	$ZrOCl_2$	$ZrOSO_4$
Phospholipids	364	235	199	497	440
Cholesterol	809	1404	1013	2238	2885
Triglycerides	442	257	425	665	1155
Cholesteryl esters	1415	2112	1979	4755	5482

Fig. 3. Relative responses of identical amounts of estrogens separated on silica gel and rendered fluorescent by thermal treatment (15 min at 160°C) in the presence of different fluorogenic reagents. Spectrofluorimeter settings: excitation, 365 nm; emission, 460 nm. I = Zirconyl chloride; II = zirconyl sulphate; III = zirconyl nitrate. a, Estriol; b, 17β-estradiol; c, estrone.

Spectral characteristics of the fluorophores

Regardless of the nature of the original product, all organic compounds show very similar fluorescence characteristics. All show excitation maxima at the 365 nm line of the mercury source, and the emission maxima for a many compounds show a remarkable similarity (Table IV).

TABLE IV

FLUORESCENCE MAXIMA (nm) OF ORGANIC COMPOUNDS HEATED AT 160 C IN THE PRESENCE OF ZIRCONYL CHLORIDE AND SULPHATE

Compounds	$ZrOCl_2$	$ZrOSO_4$
Glucose	440	440
Fructose	440	440
Ribose	440	440
Giycine	456	456
Methionine	456	456
Thymidine	465	452
Caffeine	460	460
Melatonin	442	442
Isoprenaline	460	460
Phenylbutazone	470	470
Diazepam	470	470
Cholesterol	472	472
Cholesteryl esters	472	472
Cortisol	450	450
Progesterone	460	505
Testosterone	472	515
Estrone	462	525
Estradiol	465	525
Cholic acid	472	482
Triglycerides	460	460
Phospholipids	460	460
Oleic acid	460	460
Prostaglandins	450	450

Sensitivity of the procedure

Thermal treatment of the plates in the presence of zirconium derivatives allows the detection of very small amounts of compounds. The sensitivity of the procedure is highest for steroids and steroid derivatives; as little as 10 ng of cholesterol can be easily detected on silica gel plates (Fig. 4).

Tabel V shows the limits of detection for five compounds rendered fluorescent after thermal treatment in the presence of zirconyl sulphate, compared with those reported by two other procedures: a fluorometric one (reaction with perchloric acid) and a densitometric one (charring with sulphuric acid). As can be seen, approximately a tenfold increase in sensitivity can be achieved with the use of zirconyl sulphate.

Quantitative determination

For the various compounds studied there is a linear relationship between the logarithm of the amount of compound present in a chromatographic spot or band and the square root of the area of the corresponding peak. This type of relationships extends over a wide range of concentrations. Fig. 5 shows the type of response for

TABLE V

LIMITS OF DETECTION OF VARIOUS ORGANIC COMPOUNDS ACCORDING TO DIFFERENT PROCEDURES OF DERIVATIZATION

Compound	Limit of detection (nmol)		
	3% HClO$_4$ (ref. 9)	10% ZrOSO$_4$	4% H$_2$SO$_4$ (ref. 2)
Triglycerides	0.100	0.056	—
Cholesteryl esters	0.170	0.003	—
Cholesterol	0.110	0.013	0.125
17β-Estradiol	—	0.009	0.179
Testosterone	—	0.008	0.173
Detection	Fluorimetry	Fluorimetry	Densitometry

various amounts of different lipid classes treated with zirconyl chloride; linearity is maintained over almost two orders of magnitude of concentration.

The precision of the method at the high picomole (300–800) levels is quite good with a coefficient of variation of less than 4% (Table VI). Variations within the measuring system contribute significantly to the total coefficient of variation. At a concentration of 0.3 nmol per band, estrone, 17β-estradiol and estriol showed coefficients of variation of 0.52, 0.20 and 0.45%, respectively, when scanned five times in the direction of development.

On the other hand, the coefficients of variation for replicated samples analyzed on different plates are considerably larger than those obtained when samples are both separated and detected on the same plate; this is to be expected when taking into account that the fluorescence response varies somewhat between plates. For this reason, standards corresponding to the different compounds to be separated have to be included on each plate.

The low coefficients of variation of replicated measurements of the same bands and of identical samples on the same plate is an indication of the excellent stability of the fluorophores obtained.

TABLE VI

COEFFICIENTS OF VARIATION OF LIPID DETERMINATIONS ON REPLICATES OF 0.5 μl PLASMA SAMPLES ($n = 10$)

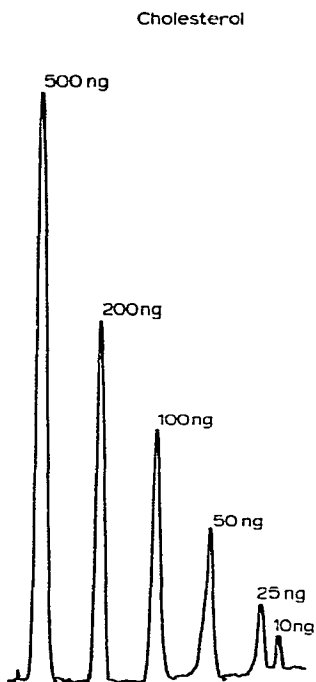| Compounds	Coefficients of variation (%)		
	Scanning (same fraction)	Samples	
		On the same plate	On different plates
Cholesteryl esters (0.835 nmol)	0.37	1.15	4.34
Triglycerides (0.864 nmol)	0.38	3.37	9.49
Cholesterol (0.395 nmol)	0.35	2.41	7.46
Phospholipids (1.306 nmol)	0.28	3.52	10.38

Cholesterol

Fig. 4. Relative responses of different amounts of cholesterol separated on silica gel and rendered fluorescent by thermal treatment in the presence of zirconyl chloride (30 min at 160 C). All samples were measured with the same spectrofluorimeter settings: excitation, 365 nm; emission, 472 nm.
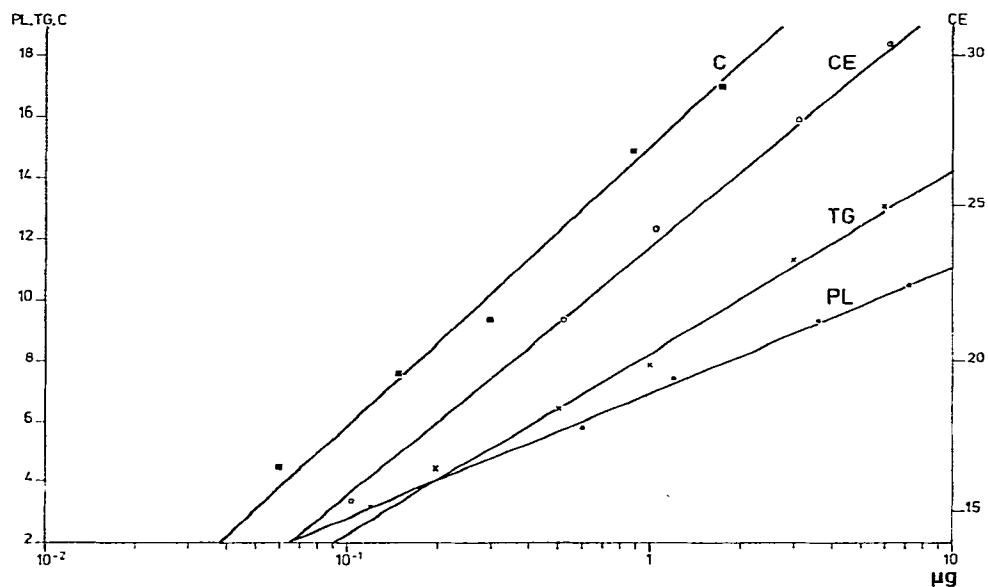

Fig. 5. Calibration graphs for different lipid fractions separated on silica gel and treated with zirconyl chloride for 30 min. Excitation, 365 nm; emission, 460 nm. ■, Cholesterol (C); O, cholesteryl esters (CE); ×, triglycerides (TG); ●, phospholipids (PL).

Relationship between type of adsorbent and fluorescence response

Silica gel appears to be the most suitable adsorbent as a support for the induction of fluorescence with most organic compounds treated with the reagents investigated. The formation of fluorescent derivatives on alumina, in the presence of zirconyl sulphate or chloride, requires longer periods of heating and, with several types of compounds (fatty acids, triglycerides, glucose), is less efficient than formation on silica gel. When operating with organic adsorbents (cellulose, polyamide, etc.) it is difficult to establish the optimal reaction conditions. In any case, these adsorbents show a high background and are unsuitable for quantitative work.

Regardless of the type of adsorbent used, we found it necessary to purify the chromatographic powder or plate prior to the separation and quantitation of the compounds. In our experience, the simplest procedure is double development of the plate with methanol–concentrated ammonia (1:1).

DISCUSSION

TLC, despite its advantages, has commonly been regarded as of limited value for quantitative analysis, but the advent of adsorbents with greater resolving power, more accurate and precise devices for sample application, more sensitive methods of detection and spectrometers equipped with scanning devices for TLC plates has changed the situation.

Ideally, it should be possible to measure all compounds by their emission fluorescence. Fluorescence techniques are more sensitive than photodensitometric techniques owing to their lower background noise[2]. Other spectroscopic techniques (transmission, reflection, fluorescence quenching) are indirect methods in which the sensitivity is determined by the background of the plate. In contrast, fluorescence techniques are direct methods in which only the light emitted by the compound should be measured (the background appearing ideally dark to the instrument) and in which most of the sources of error inherent in the indirect methods (spot deformation, light scattering, ambiguous relationship between sample concentration and light attenuation[3]) should be eliminated.

The main problem with fluorescence detection and quantitation is the necessity in most instances to convert the sample compounds into suitable derivatives. The reagent used for conversion into fluorescent derivatives should be distributed evenly over the entire plate and in the layer, give a low background and produce a derivative stable enough for adequate measurement. We found[1] that heating the plate in the presence of the vapour phase liberated by the thermal decomposition of ammonium hydrogen carbonate ensured a complete uniform reaction over the plate, gave a low background and induced the formation of fluorescent derivatives with most organic compounds. However, the procedure involved heating the plates for a long period (several hours at 160°C) and offered limited sensitivity of detection.

During the last few years we have performed a systematic investigation of different compounds as potential fluorogenic reagents, and have found systems that induce the formation of fluorescent derivatives in a very short period (seconds or minutes compared with hours with the ammonium hydrogen carbonate procedure) and give lower limits of detection (allowing easily the measurement of nanogram amounts of many compounds). We have found that heating the plates in the presence

of the reagents in Table I induces the formation of fluorescent derivatives with various organic compounds and, as discussed above, zirconyl sulphate and chloride appear to be the reagents of choice owing to their convenience and simplicity of application, sensitivity of detection and wide range of compounds detected.

Various types of reagents have previously been used to induce the formation of fluorescent derivatives from a particular group of organic compounds. Touchstone *et al.*[5] reported the appearance of fluorescence with steroids after heating them on silica gel impregnated with ammonium hydrogen sulphate. Truppe and co-workers[6–8] applied the procedure of Touchstone *et al.* to the fluorimetric determination of plasma lipids. More recently, Lykkelund and Damgaard-Pedersen[9] reported the use of perchloric acid for the detection of plasma lipids; this type of reagent had been applied by Faber and co-workers to the fluorimetric detection of nortriptyline, protriptyline[10] and carbamazepine[11].

Treatment with ammonium hydrogen carbonate, introduced by Segura and Gotto in 1974[1], was the first method for the general, non-specific, detection of organic compounds. This procedure has been applied by several workers to the detection and quantitation of different types of compounds separated by TLC (*e.g.*, refs. 12 and 13) and as a detection device for monitoring the effluent from a high-performance liquid chromatograph[14]. Treatment of the plate with hydrogen chloride followed by UV radiation has also been shown to induce fluorescence in a wide variety of compounds[15]. Shanfield and co-workers[16,17] described the use of gaseous electrical discharges to induce fluorescence in organic compounds separated by TLC; although they did not perform a quantitative analysis of the fluorophores, they showed good response with various organic compounds at the microgram level. They concluded that ammonium hydrogen carbonate is the best fluorogenic reagent to be used in conjunction with electrical discharges.

The use of the zirconium derivatives described in this paper permits improvements to some of the main features of fluorescence detection and quantitation in TLC. The reagents can be easily applied either by spraying or dipping the plate into the solution or, alternatively, can be incorporated into the layer prior to development; thus, no special devices or apparatus are needed. The sensitivity of detection is reasonable for most compounds, reaching the lower nanogram or higher picogram levels with some steroid derivatives, estrogens, etc. The fluorophores obtained are stable for days or weeks, so there is no need for an immediate determination of peak area or the establishment of peak area–time characteristics. The time required to complete the reaction is short compared with that required with the ammonium hydrogen carbonate procedure, and is shorter than that required by most of the methods described previously.

The mechanism whereby the different organic compounds are transformed into fluorescent derivatives is unknown. Acid appears to be formed during the reaction; the liberation *in situ* of small quantities of acid could explain, perhaps, the formation of a series of fluorescent derivatives that represent intermediate stages of a more common type of transformation, the charring reaction. Actually, we have observed that heating the plates with fuming sulphuric acid (sulphur trioxide), under carefully controlled conditions, produces fluorescent derivatives with several types of organic compounds. However, these are not as stable as those obtained by heating in the presence of silicon tetrachloride or the zirconium salts, being unsuitable for quantitative work.

Although several indications suggest that the acidic character of the media induced by the fluorogenic reagent is important for the reaction, the role of the corresponding salts cannot be ignored. When the plates are dipped into an aqueous solution of zirconyl sulphate after chromatographic development and, shortly thereafter, are exposed to an atmosphere of concentrated ammonia most organic compounds will still be rendered fluorescent after thermal treatment of the plates; in this case, however, the time needed to complete the reaction is substantially increased. This suggests that, although the liberation of acid is probably an important aspect of the process and is essential in increasing the speed of the reaction, it is not the only determinant in the fluorescent derivatization. Actually, thermal treatment in the presence of hydrogen chloride gas induces fluorescence with cholesterol or cholesterol derivatives but not with other types of organic compounds at the microgram or submicrogram level.

ACKNOWLEDGEMENT

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