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ULTRAMICRODETERMINATION OF CHOLESTEROL, TESTOSTERONE AND LSD BY FLUORESCENCE MEASUREMENTS OF TLC SPOTS

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SUMMARY

Fluorescent derivatives of many organic materials adsorbed on t.l.c. plates are obtained by reaction with ammonia liberated by heating ammonium hydrogencarbonate. Spots whose fluorescence can be measured quantitatively with high sensitivity over wide ranges of concentrations can be obtained with testosterone (1—250 ng), LSD (2—150 ng) and cholesterol (4—150 ng).

Segura and Gotto [1] were the first to report the appearance of stable, highly fluorescent spots in t.l.c. after heating the plates in an atmosphere of ammonia liberated from ammonium hydrogencarbonate; many biological materials, including cholesterol and testosterone, were examined. After the chromatograms had been developed the fluorescent spots were scanned; a linear relation between the square root of the peak area of the spot and the concentration was obtained. The limit of determination was in the range 1—0.1 μg. The present work extends the sensitivity of the method to the nanogram range for the determination of cholesterol, testosterone, and LSD. This high sensitivity was obtained by direct measurement of the fluorescence of homogeneous, well-defined spots without any kind of scanning. This method can be used after separation of the materials by liquid chromatography or h.p.l.c. [2]. One of the most important advantages of this reaction is that the reagent is gaseous; an identical quantity of reagent is applied therefore to each spot, hence improving the reproducibility of the method. However, forming uniform t.l.c. layers (which is critical in this method) is not easy; the standard deviation of the results is caused mainly by the deviation of the layer thickness on the plates. How to overcome this difficulty is described under Experimental.

On heating, alumina and silica gel dehydrate molecules containing hydroxyl groups, thereby producing double bonds which increase the wavelength of excitation of the molecule and make those molecules available for fluorescence measurements. The ammonia in the system increases the fluorescence intensity by forming products in which the nitrogen atom is conjugated to the double bonds and its two free electrons participate in the π-electron

system. The reaction takes place with many organic materials, hence difficulties arise in getting a non-fluorescent background. To overcome this problem, the alumina and silica gel should be ultrapure. The level of the fluorescent background was minimized by heating the alumina and the silica (before making the suspension) and "washing", and by using pure solvents as described below.

As the wavelengths of excitation and emission differ only slightly from compound to compound, the same wavelengths have been used for all compounds. The wavelength of excitation was 405 nm (mercury source); emission was at 470 nm.

EXPERIMENTAL

Reagents

All chemicals were reagent grade. All the solvents were distilled before use. Alcoholic stock solutions (400 ppm) of the materials to be determined were diluted to the appropriate concentrations.

Preparation of the t.l.c. plates

Plastic strips were unsuccessful; their elastic property makes it difficult to keep spots in a constant position on the strip before reading the fluorescence. Small glass plates (7.5 × 2.5 cm) were optimal for the method. From stocks of glass plates (Chance Propper Ltd., Spon Lane, Smethwick, Warley, England) plates of 0.92 ± 0.01 mm thickness were selected with a micrometer. These small plates were put on moistened large plates (20 × 20 cm) which were placed on the plate holder; the small plates stick to the large plates and give a stable system. This makes the process of spreading easy and homogeneous plates are obtained.

A mixture of (1 + 2, w/w) of alumina gel (neutral, Merck) and silica gel (Merck) was prepared. The mixture was placed in a porcelain crucible and in an oven at 700°C for 4 h, and then cooled to room temperature. To 30 g of mixture, 70 ml of water, triply distilled, was added and mixed well for 10 min. The suspension was poured into a clean spreader (Desaga), adjusted to 0.25-mm thickness, and spread with constant rate of advance of the spreader. The plates were left to dry at room temperature for 6 h. The plates were removed carefully and "washed" 4 times by development with absolute methanol. After the final drying, a strip, 1 cm wide, from both edges of the layer was scraped off to allow the t.l.c. plates to enter the fluorimeter holder smoothly.

Apparatus

The fluorescence measurements were made with a Perkin-Elmer Fluorescence Spectrophotometer model 203, into which a holder for the t.l.c. plates was fitted as described previously [2].

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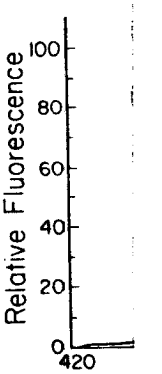

Fig. 1. Fluo
source).

Preparation of the spot on t.l.c. plates

The solutions were delivered from a motorized microsyringe (which can also be used manually) as described previously [2, 3]. The needle of the microsyringe permits delivery of 5-μl drops of the solutions onto the t.l.c. plates which are placed in a holder slightly modified from that described previously [2]. The size of the holder was adjusted to the size of the plates, 7.5 × 2.5 cm. Two spots are placed on every plate, one on each side, one as a blank and the other of the appropriate concentration. After drying, the t.l.c. plates are inserted into a tank of 2.5 l capacity to which 2.5 g of ammonium hydrogencarbonate are added. The sealed tank is put in an oven at 120°C for 3 h. The plates are removed carefully, inserted into the holder, and fluorescent readings are then taken. Two filters are used; one before the sample cuts out all radiation over 470 nm and the other, placed after the sample, passes all radiation over 480 nm. The fluorescence is viewed at 45°.

RESULTS AND DISCUSSION

The optimum fluorescence reaction takes place on heating at 120°C for 3 h. With longer heating the background becomes significant from the reaction of residual impurities with the ammonia.

Figure 1 shows the fluorescence spectrum of the testosterone derivative (after subtraction of the blank). The shape of the spectrum is not symmetrical because of the two filters used. The optimal emission wavelength for quantitative measurements was 470 nm. A linear dependence over a wide range was found between the concentration and the intensity of fluorescence (Fig. 2).

A theoretical treatment of fluorescent densitometry of thin layer chromatograms has been given by Goldman [4]. His mathematical treatment is based on the model and assumptions of the Kubelka—Munk theory. In this

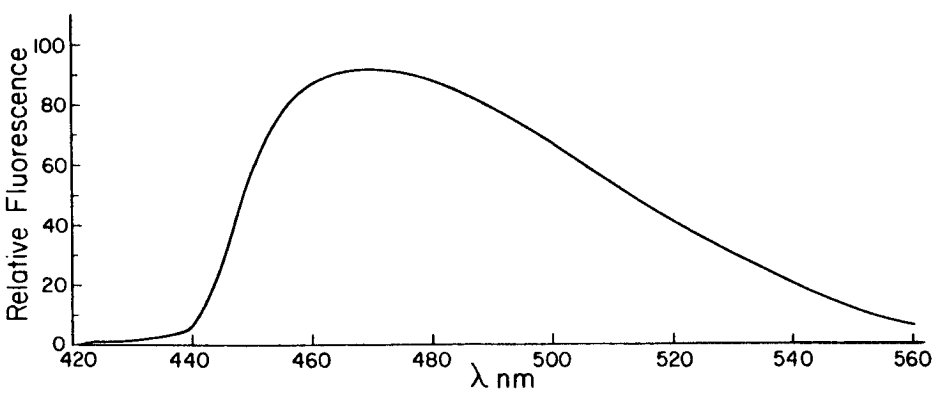

Fig. 1. Fluorescence spectrum of testosterone derivative excited at 405 nm (mercury source).

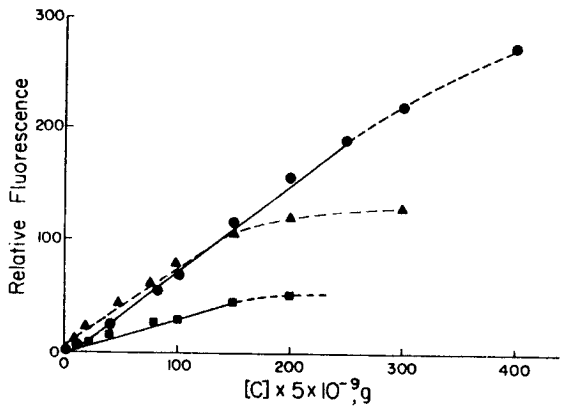

Fig. 2. Calibration curves for the testosterone (●), LSD (▲) and cholesterol (■) derivatives. Each point is the average for 4 spots after deduction of the blank.

case the phenomenon is more complicated because of the two kinds of light present in the solid medium: the incident light from outside the medium and fluorescent light produced inside the medium. The incident light undergoes absorption and scattering simultaneously; the fluorescent light is assumed to pass scattering only. For this operation Goldman derives two pairs of Kubelka–Munk differential equations:

$$-\mathrm{d}i/\mathrm{d}x = -(s + k)i + s_j;\ \mathrm{d}j/\mathrm{d}x = -(s + k)j + s_i \quad (1)$$

$$-\mathrm{d}I/\mathrm{d}x = -SI + SJ + (i + j)\alpha k/2;\ \mathrm{d}J/\mathrm{d}x = -SJ + SI + (i + j)\alpha k/2 \quad (2)$$

Equations (1) are for the incident light travelling in the transmission (i) and "reflection" (j) directions. Equations (2) are for the fluorescent light travelling in transmission (I) and reflection (J) directions, where x = thickness of the medium; s = scattering coefficient of the incident light; k = absorption coefficient of the incident light; S = scattering coefficient of the fluorescent light; and $I^+ = I$ at $x = 0$ and $J^+ = J$ at $x = X$, the fluorescence obtained in transmission and reflection, respectively. Solutions for these equations are complicated and a computer is required but, for low concentrations and high scattering power (SX) the solutions become simple:

$$\frac{I^+}{i_0} = \frac{1}{3}\,kX\left(1 - \frac{7}{30}\,sX \cdot kX\right);\ \frac{J^+}{i_0} = \frac{2}{3}\,kX\left(1 - \frac{4}{30}\,sX \cdot kX\right) \quad (3)$$

where i_0 = the initial incident intensity and α = the conversion factor from incident to fluorescent light.

The reflected fluorescence, compared with the transmitted fluorescence, has the important advantages of higher intensity and of being unaffected by scattering power changes in the range 5–20. When the medium has some absorption, a correction for the layer width should be made. The following results help to confirm the theoretical treatment of Goldman [4].

TABLE 1

Equations of the straight lines obtained from linear regression, standard deviations, correlation coefficients and coefficients of variation

	Testosterone	LSD	Cholesterol
Range of conc. (ng)	1—250	2—150	4—150
Intensity of fluorescence	$0.764C - 1.722$	$0.715C + 3.425$	$0.270C + 4.598$
Correlation coefficient	0.99885	0.99795	0.97096
Standard deviation of the regression line (ng)	4.6	3.83	3.88
Coefficient of variation	1.84	2.55	2.59

In this work, simple linear dependence was achieved between the quantity of the fluorescent derivative adsorbed on the layers of the solid phase and the intensity of the fluorescence, when the spots had spherical symmetry and were all irradiated on the same part of the spot. The deviation from linearity in the work of Segura and Gotto [1] may be caused by deviation from spherical symmetry of the spots.

The precision of the method may be seen from 5 readings taken from 5 spots of 500 ng of the testosterone derivative; corrected values of 70, 76, 74, 72 and 75 units were obtained. The relative standard deviation is ca. 3% and is caused mainly by the slight variation of the layer thickness of the solid medium on the glass plate. Calibration curves should be prepared for each determination to minimize deviations arising from the process of preparing the t.l.c. plates. The limit of determination changes from material to material. The testosterone derivative gives the most intense fluorescence and can be determined in the range 5—250 ng; LSD can be determined in the range 25—150 ng; cholesterol gives less intensity but can be determined over the range of concentration, 5—150 ng (Table 1). Under the same conditions, oleic acid, methyl oleate, inolein, β-sitosterin, stigmasterol, lactose and phenylalanine also produce fluorescent products. The fluorescent derivatives are sensitive to sunlight and should be kept in a dark place.

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