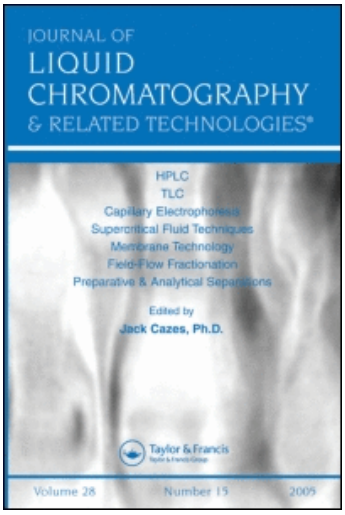

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SIMPLE THIN LAYER CHROMATOGRAPHY METHOD WITH FIBRE OPTIC REMOTE SENSOR FOR FLUORIMETRIC QUANTIFICATION OF TRYPTOPHAN AND RELATED METABOLITES

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ABSTRACT

Tryptophan (TP), 5-hydroxytryptophan (5-HTP), 3-indoleacetic acid (IAA) and serotonin (5-HT) were separated by TLC, with chloroform-methanol-ammonia (12-7-1) (v-v-v) as eluent and cellulose as stationary phase. A fibre optic-based fluorescence instrument for in situ scanning was used for quantitative measurements. The compounds were determined over the range 10-100 ng, with relative standard deviations between 1.70-6.52% and detection limits over the range 16.39-22.50 ng.

INTRODUCTION

Thin layer chromatography (TLC) offers significant advantages for the separation and identification of compounds of analytical interest.[1] In the quantification of analytes separated by TLC, densitometry has proved most useful.[2-4] Recently, as an alternative, the use of fibre optic sensors has been suggested since it

allows the measurement of fluorescence emitted by fluorophors at some distance from the source of excitation and the detector.[5-8]

The possibility of transporting light from one place to another, by means of optical fibres, facilitates taking readings of TLC plates. Thus it is possible to transmit useful spectral information for qualitative and quantitative analysis with minimal loss of precision and resolution.

Tryptophan (TP), 5-hydroxytryptophan (5-HTP), 3-indoleacetic acid (IAA) and serotonin (5-hydroxytryptamine) (5-HT) are highly important biological compounds that actively participate in biosynthetic routes in both the animal and vegetable worlds.[9-11] Among other aspects of interest, the analysis of some of these derivatives in brain tissue or cerebrospinal liquid is used in the diagnosis of mental disease and nervous disorders.[12]

Owing to their natural fluorescence, 5-HT,[13-15] TP[16] and IAA[17] have been determined spectrofluorimetricaly. Other analytical techniques, such as voltametry[18] and phosphorescence[19] have also been proposed for the quantification of these substances. However, in the analysis of real samples, these determinations lack selectivity, which makes previous separation imperative. Thus, the most important techniques of determination are HPLC, in the normal or inverse phase modes, and electrochemical,[20] UV[21] or fluorescence detection, with chemical derivatization,[22] or without it.[23]

In this study we set out to quantify TP, 5-HTP, IAA and 5-HT with conventional spectrofluorimetry, after separation by TLC, by means of in situ reading of the analytes. Excitation and emission radiations were transmitted by optical fibres.

EXPERIMENTAL

Apparatus

Fluorescence measurements and spectra were made with a Perkin-Elmer LS-50 luminescence spectrometer equipped with a Perkin-Elmer fluorescence plate-reader accessory. A bifurcated fibre optic was used to transfer excitation and emission energy between the plate and the spectrometer. The spectrometer was connected via an RS232C interface to an Epson PCAX2e, containing Fluorescence Data Manager Software (FLDM) that controls the instrument.

Reagents

3-Indoleacetic acid (IAA), 5-hydroxytryptophan (5-HTP) and serotonin-creatinine sulphate (5-HT) were obtained from Aldrich, D-tryptophan (TP) was from Sigma and solvents were purchased from Merck.

Solutions of the indolic compounds were prepared in methanol-water (60-40) (v-v) at a concentration of 1 mg mL^{-1} and diluted as required.

Whatman-41 filter paper, ion exchange chromatography paper (P81 and DE81, Whatman), and TLC plates of silica gel (Merck), cellulose (Merck) and KC18 reverse phase (Whatman) were used as solid surfaces.

All chemicals used were of analytical reagent grade and used without further purification.

Thin Layer Chromatography and Analytical Method

Sample application was by the spray-on technique using a microprocessor-controlled Camag Linomat IV device. Sample volumes of 1-10 μL (containing from 10 to 100 ng of each one of the analytes) were applied to the plates at a rate of 15 $sec \cdot \mu L^{-1}$.

Indolic compounds were chromatographied on 10x10 cellulose TLC plastic sheets, layer thickness 0.1 mm (Merck). The TLC plates were without fluorescence indicator and activated before use. The thin layer plate was developed in chloroform-methanol-ammonia (12-7-1) (v-v-v), light protected, until the solvent migrated a distance of 8 cm up to the plate.

Once the spot corresponding to the analyte had been located, in situ quantitative scans were done at λ_{exc}= 280 nm and λ_{em}= 347 nm, using slits of 5 nm for excitation and emission. As a blank to correct fluorescence intensity measurements, we used the signal corresponding to the dry stationary phase, after elution with the above-mentioned mobile phase.

Procedure for the Determination of 3-Indoleacetic Acid, Tryptophan, 5-Hydroxytryptophan and Serotonin in Serum

A 1 mL aliquot of the serum was deproteinized with 100 μL of perchloric acid

Table 1

Spectrofluorimetric Characteristics of the Indolic Derivatives on Different Solid Surfaces. C_a= 40 ng/μL, Slits 5 nm.

Surfaces	5-HT		TP		IAA		5-HTP	
	$\lambda_{ex}/\lambda_{em}$	I^*_f	$\lambda_{ex}/\lambda_{em}$	I^*_f	$\lambda_{ex}/\lambda_{em}$	I^*_f	$\lambda_{ex}/\lambda_{em}$	I^*_f
Whatman-41	277/337	392	278/346	334	278/361	20	278/337	464
Cellulose	276/347	10	278/350	21	280/358	15	278/337	41
Silica gel	278/336	46	278/338	15	275/348[b]	10	278/337	30
P-81	278/336	210	279/344	210	278/364	8	277/338	264
DE-81	278/336	212	279/344	252	279/335	23	277/337	172
C-18	277/334	118	279/334	212	277/335	8	276/335	127

[b] C_a = 400 ng/μL, Slits 10 nm.

(70%) and centrifuged (speed: 3000 rpm, 5 min.). An aliquot of 400 μL was taken from the supernatant, spiked with 32 μg of each indole and dilute 1:2 with methanol-water (60-40) (v-v). The sample was analyzed in accordance with the analytical method previously described.

RESULTS AND DISCUSSION

The fluorescence emitted by a fluorophor largely depends on the nature of the medium in which it is found. In TLC, the solid constituting the stationary phase is, at the same time, the medium in which solutes are retained after separation occurs. Consequently, selection of the stationary phase must be made bearing in mind its chromatographic properties and its possible influence on the spectrofluorimetric characteristics of the analytes.

The combination of a fibre optic sensor and conventional spectrofluorimeter enables one to obtain spectra of excitation and emission of solutes trapped on a solid surface. Figure 1 shows representative examples of emission spectra of the indolic derivatives considered in this study deposited on a cellulose plate. The main characteristics of 5-HT, TP, IAA and 5-HTP, adsorbed on different solid surfaces, are summarized in Table 1. In general, the excitation wavelengths are similar for all the derivatives. The greatest differences appear in the wavelengths of maximum emission, especially in the case of IAA, although with respect to the spectral band widths these differences are not too significant from an analytical point of view.

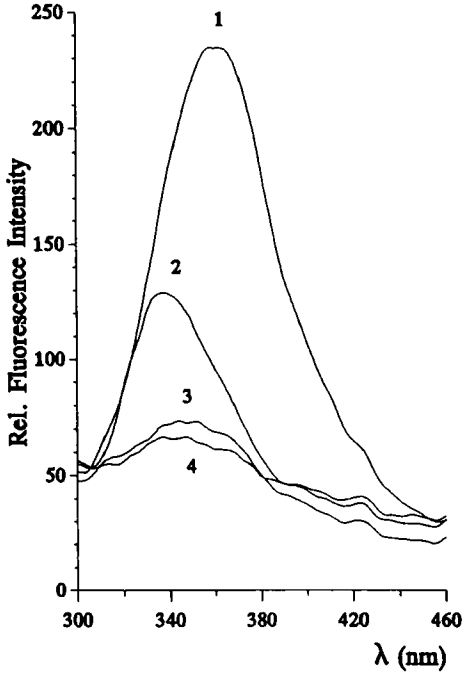

Figure 1. In situ emission spectra of 500 ng IAA (1), 60 ng 5-HTP (2), 60 ng TP (3) and 60 ng 5-HT (4) adsorbed onto cellulose obtained with the fibre optic sensor. Spectrofluorimetric conditions as described in Table 1.

The intensities of fluorescence emitted by TP, 5-HT, IAA and 5-HTP largely depend on the nature of the solid surface used. The corrected values(I^*_f) for this parameter, taking into account the signal emitted by the respective surfaces, are grouped in Table 1. In general terms, the low values of I^*_f, corresponding to IAA, stand out. With regard to the nature of the surfaces, the different types of paper and silica gel provide the greatest intensities of fluorescence.

When different solid surfaces were used as stationary phases for the chromatographic separation of indolic derivatives, the best resolutions were obtained using cellulose plates and silica gel. With the latter, the analytic sensitivity of IAA is far less than that of the other analytes. For this reason cellulose was selected as the stationary phase for the separation and quantification of TP, 5-HTP, 5-HT and IAA.

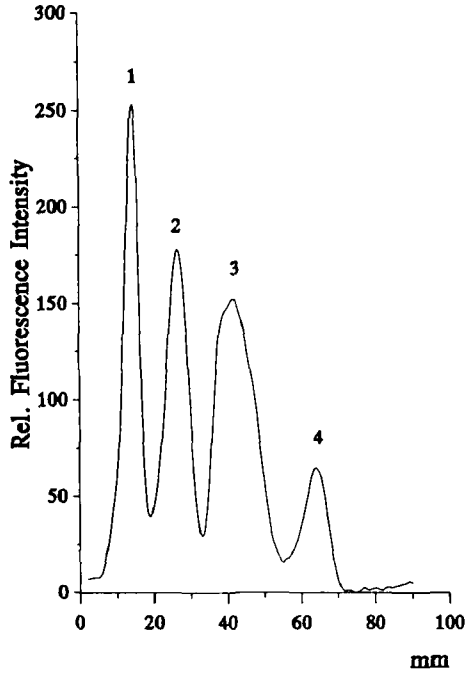

Figure 2. TLC chromatogram of a mixture of 5-HTP (1), TP (2), IAA (3) and 5-HT (4) obtained with the fibre optic sensor. Chromatographic conditions as described in the text. C_a= 100 ng, Slits 5 nm, λ_{ex}=280 nm, λ_{em}=347 nm.

Chromatographic Separation

Once the stationary phase had been chosen, we tested the behavior of different mobile phases used in the separation of similar substances.[24] One of them was the mixture of chloroform-methanol-ammonia (12-7-1) (v-v-v), which provided the best results.

Table 2 shows the most representative chromatographic properties of the indolic derivatives. For the statistical evaluation of the R_f values, we performed nine elutions of each solute in the stationary phase of cellulose, with the selected mobile phase. For a probability of 95%, uncertainty in R_f values ranged between ±0.001 and ±0.028. Coefficients of Variation (CV) showed that the dispersion of results corresponding to serotonin was considerably lower than those of the other analytes.

Table 2

Chromatographic Characteristics of the Indolic Derivatives
C_a= 40 ng/μL, Slits 5 nm, λ_{ex}=280 nm, λ_{em}=347 nm.

Compound	Rf ± I	CV(%)	R
5-HTP	$0.015 \pm 7 \cdot 10^{-4}$	5.52	
			1.85
TP	0.193 ± 0.015	9.53	
			1.5
IAA	0.370 ± 0.028	8.16	
			2.3
5-HT	0.846 ± 0.013	1.90	

Figure 2 shows a representative chromatogram of the separation of the four indolic derivatives. For each pair of analytes eluted consecutively, the spatial resolving power of the chromatographic system was calculated by means of the following expression:

$$R = \frac{P_2 - P_1}{(W_2 + W_1)/2} \qquad (1)$$

in which P_2 and P_1 represent the position of the adjacent peaks in mm, while W_2 and W_1 are the peak width at half-height. R values as ranged from 1.5 to 2.3.

Analytical Characteristics

Analytical determinations on solid surfaces are strongly conditioned by background signal and reproducibility of the measurements.

Radiation reaching the detector consists of background signal superimposed on fluorescence emitted by the components of the sample. Thus the ability of the system to detect and measure the sample emission is limited by the magnitude of the background signal and noise.

The background signal is generally elevated by the high amount of diffusely

Table 3

S/N Ratios for the Detection of 40 ng of Each Compound Spotted on Silica Gel After Elution

Compound	E_{bk}	E_S	σ_s	σ_{bk}	S/N
5-HTP	50.33	30.76	2.43	1.78	10.2
TP	50.99	15.61	2.89	0.72	5.2
IAA	47.82	23.19	2.08	1.55	8.9
5-HT	48.37	18.06	1.19	2.37	6.8

Table 4

Representative Statistical Parameters of the Analytical Methods

Compound	LDR(ng)	LOD(ng)	S_M	CV(%)	E(%)
5-HT	10 - 100	19.56	0.40	1.70	2.90
TP	10 - 100	19.32	0.95	4.00	3.27
IAA	10 - 100	22.50	0.76	2.84	2.50
5-HTP	10 - 100	16.39	0.86	6.52	5.80

scattered radiation. Moreover, the use of a fibre optic to transport the radiation allows a certain amount of light from the surroundings to be added to the fluorescence emitted by the analyte, thus increasing the background signal. Table 3 shows the values obtained for the signal/noise ratios for each of the compounds studied, using the expression:[25]

$$S/N = E_s / (\sigma_s^2 + \sigma_{bk}^2)^{1/2} \qquad (2)$$

in which E_s is the analytical signal, obtained by subtracting the blank signal from the total, while σ_s and σ_{bk} express the standard deviations of the analytical and blank signals respectively. A minimum of nine determinations were performed for each of the parameters included in the expression (2).

The quantification of the solutes separated by TLC was seen to be notably

Table 5

Recoveries of Tryptophan, Serotonin, 3-Indoleacetic Acid and 5-Hydroxytryptophan in Enriched Samples of Deproteinized Rat Serum.

Compounds	Added (ng)	Found (ng)	R(%)
5-HT	50	48.50	97.20
	100	107.2	107.21
TP	40	39.60	99.00
	80	78.79	98.49
IAA	40	42.31	105.78
	80	75.44	94.30
5-HTP	40	38.44	96.10
	80	78.44	98.05

attained in the sample application and in the measurements and efficacy provided by the chromatographic system.

For the four indolic derivatives, fluorescence intensities, registered as peak heights, showed a lineal function for concentrations below 100 ng. In all cases the correlation coefficients were higher than 0.996. Table 4 shows the most representative statistical parameters of the analytical methods established, as well as the detection limits.

Coefficients of Variation were relatively small, with the greatest dispersion of results corresponding to TP and 5-HTP determinations. Detection limits[26] oscillated between 16.39 and 22.50 ng, being higher than those found in the literature where HPLC was used.[11]

However, the detection limit obtained in this study for IAA was approximately half of that described by others after derivatization with o-phthalaldehyde (OPA) by TLC.[27]

By means of the elution of synthetic samples containing variable quantities of the four analytes studied, from 40 to 80 ng, we obtained mean recoveries ranging from 87.2% to 119.67%.

Determinations in Rat Serum

In order to test the applicability of the methods established for the determination of TP, 5-HT, IAA and 5-HTP in complex matrices, we used samples of deproteinized rat serum,[28] enriched by the addition of dissolutions containing different amounts of each of the four analytes. The results obtained, Table 5, show recoveries whose values range from 94.3% to 107.2%.

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