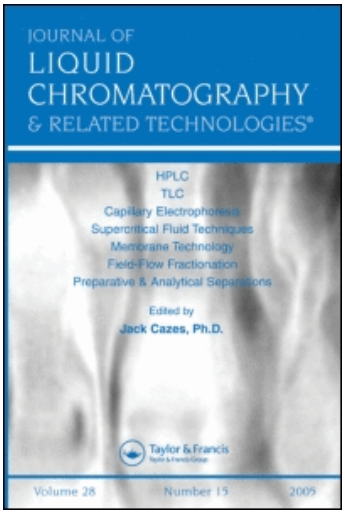

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TWO-DIMENSIONAL TLC AND FLUORESCENCE ANALYSIS WITH CCD VIDEO CAMERA USED TO DETERMINE THE DISSOCIATION OF DIPHENYLHEXATRIENE INCLUDED IN ß-CYCLODEXTRIN

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ABSTRACT

Two-dimensional TLC coupled to fluorescence analysis by CCD video camera is a sensitive, precise, and rapid method for the study of complexes which contain at least one fluorescent component. The method applied to the diphenylhexatriene·ß-cyclodextrin complex in water/acetonitrile system enables the separation of free and bound DPH and an accurate estimation of their respective quantities on the plate. Free DPH eluted by the hydrocarbon media was evaluated by the emitted fluorescence intensity by comparison with the linear 0 to 50 picomoles calibration scale. DPH bound to BCD was dissociated with a brief polar migration followed by elution with a non polar solvent mixture and evaluated as above. The balance of the free and/or dissociated fluorophore is maintained equal in every section of the TLC.

INTRODUCTION

The solubility of 1,6-diphenyl 1,3,5-hexatriene (DPH) and many other low polar molecules is increased in an essentially aqueous media when complexed with cyclodextrin (1,2). However, the inclusion compound is formed with a significant amount of the free form of DPH remaining in the preparation media. The advantages of studying DPH-BCD complexes with TLC are the possibilities to separate DPH into its free and complexed forms and to rapidly film the fluorescent emission data before the unbound form degradates (3). It has been shown that DPH's fluorescence quantum yield is sensitive to the polarity, polarisability and viscosity of the media (4). It has also been demonstrated that the fluorescence of polycyclic aromatics significantly increases in the presence of BCD (5). DPH included in BCD can be dissociated with a two-dimensional elution with the second migration perpendicular to the first one. This elution scheme places dissociated DPH at the same level as free DPH and ensures that their fluorescent quantum yield are identical. Both these DPH species can therefore be evaluated in the same conditions and be compared.

MATERIALS

Chemicals and supplies

Roquette ß-cyclodextrin crystals were recrystallized from water. The purity of Lancaster DPH was controlled by TLC, by the measure of its molar extinction coefficient with UV-absorption spectroscopy and by its fluorescence emission spectra in hexane and in acetonitrile. Diethyl ether, acetonitrile, acetic acid,

and 2-methyl propanol of analytical grade were used without further purification. Water and hexane were bidistilled.

The 0.2 mm thick TLC plates without fluorescent indicator were supplied by Merck (Art.5553).

Apparatus

The spots were deposited on TLC with a NICHIRYO capillary micropipette (0.05 μl precision).

Image recording of DPH's fluorescence on TLC was performed in a Camag illuminator cabinet under excitation at 366 nm.

A SONY CCD-TR705E video camera sensitive to 2 lux with two superimposed 370 nm (Corning 3-75) and 420 nm (Corning 3-73) cut-off filters and Hi-8 Metal-E magnetic bands were used to film the fluorescence emitted on TLC. The analogic images were numerized with a VITEC VideoMaker card on a 486-66 MHz compatible PC. The capture rate was 25 s^{-1} and the spatial definition equal to 768x576 pixels in 16 million colours.

A spectrofluorimeter assembled in the laboratory was used to record the emission spectra of DPH on TLC. The apparatus consists of a polychromatic light source (Xenon XBO 150 W) and two scanning Jobin Yvon (H 25) grating monochromators. The excitation monochromator has a series of diaphragms which permits the localization of the light beam on the spot in such a way as to reduce the background noise due to the TLC's silica gel. The signals were detected with a Hamamatsu R928 photomultiplier linked to a Keithley 610C electrometer.

Softwares

Imager^{TM} was used to capture the filmed images on computer, Photostyler^{TM} to transform the images, and a

program written in the laboratory to determine the density of the spots.

METHODS

Preparation of BCD-DPH complexes

0.08 g BCD was dissolved in 3 ml water/acetonitrile 62/38 (w/w) to obtain a final concentration of 0.02 $mol.l^{-1}$. 10 mg excess DPH was added to this mixture which was then sealed, protected from light and agitated for 48 hours in a thermally controlled shaker at 60°C. The non solubilized DPH was filtered without cooling with an Ederol n°11 filter paper. The filtrate containing solubilized DPH was then diluted with water/acetonitrile 62/38 (w/w) to obtain a 0.01 $mol.l^{-1}$ solution of BCD with DPH.

Two-dimensional TLC

The following steps in the procedure were carried out in a darkroom.

A 10x7 cm TLC plate was divided into four sections with two perpendicular lines which also indicated the final solvent front of each of the two developments. 1 μl of the 10 mM BCD/DPH system was spotted in each of the four sections labeled A, B, C and D. The different sections were eluted in the directions shown in figure 1.

Section A was not eluted by either eluent. In the first dimension, Sections B and C were eluted with hexane/ether 97/3 (v/v). Spots from section C, and section D were eluted in a dimension perpendicular to the first one. Two migration protocols were applied to the second dimension. The calibration scale consisting of 10, 20, 40 and 50 picomoles DPH in pure acetonitrile could be placed in section B or D.

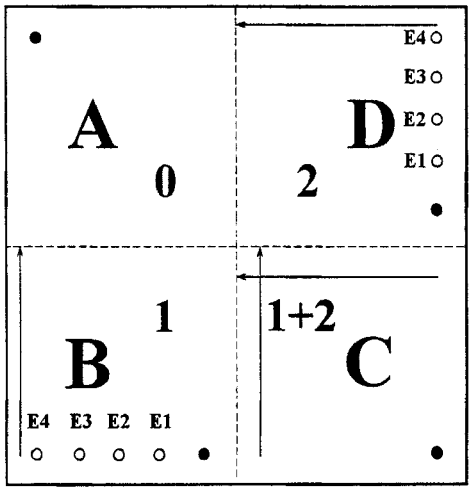

FIGURE 1. TLC spotting scheme. A: 0 dimension (no elution). B: elution in dimension 1 only. C: elution in 1+2 dimensions. D: elution in dimension 2 only. DPH calibration standards E1 to E4 can be placed in section B or C.

In the first method, the second migration was done in one step with the water/2-methyl propanol/acetic acid 5/12/3 (v/v/v) mixture. The calibration scale was placed in section D and eluted in the second dimension.

In the second protocol, the second migration was performed in two steps. The polar eluent, water/2-methyl propanol/acetic acid 5/12/3 (v/v/v) was used for an initial development over 3 mm. The plate was dried for one minute at 110°C and the migration was continued in the same direction with hexane/ether 97/3 (v/v). In this case, the DPH standards were placed in section B and eluted in the first dimension only.

Analysis of emission fluorescence data

1. Analysis by CCD camera.

The video camera was placed in automatic mode and the plate was immediately filmed upon excitation at 366 nm

for several seconds. The time lapse allowed the cell to adapt itself to the intensity of the emitted fluorescence. The images were numerized in 24 bits per pixel and transformed twice. The first transformation converted the images into 8 bits per pixel with a 256-grey scale. The images were then transformed a second time into 1-bit images with the Floyd-Steinberg algorithm (6,7). The last conversion increased the resolution of the image while completely conserving the light intensity data.

At this stage, the total emission of each spot was integrated over an area of 52500 pixels. The surrounding background light was also taken into account.

2. Analysis by spectrofluorimetry.

The emission spectra of free DPH and complexed DPH separated on TLC with the hexane/ether 97/3 (v/v) were analyzed with a 45° geometry. 12 µl of the 0.01 mol.l^{-1} BCD/DPH system was spotted on a 1.4x4 cm TLC plate. The resulting spots were excited at 380 nm and the emission spectra recorded from 540 nm to 390 nm.

RESULTS AND DISCUSSION

Spectrofluorimetric characteristics

The two spots on TLC resulting from the separation of the complex into bound and free DPH forms have similar emission spectra. The spectra are also analogous to DPH in hexane, cyclohexane and acetonitrile in solution with the emission maximum at around 430 nm (4). This data confirms that the fluorescent spots observed on the TLC plates are indeed DPH.

Linearity and sensibility precision

Figure 2 shows a chromatogram in which the free form of DPH was separated in the first dimension to R_f 0.5 with the low polar hexane/ether eluent in section B and C. The second dimension was eluted with the polar solvent only (first protocol). In this dimension dissociated and free DPH in section C both migrated to R_f 0.95. The densitometry study of the free samples in sections B and C show that both contain 16 picomoles DPH.

It must be noted that the calibration scale of standards E1 to E4 (10 to 40 pmoles) of DPH in pure acetonitrile does not allow the correct evaluation of all the spots after a polar elution. DPH's very high R_f value of 0.95 resulting from the polar eluent and a short migration distance, renders the differential of the emission intensity across spots E3 and E4 so high that the charge coupled devices saturate. The integration of the spots' density therefore underestimates their emission intensity. With this polar eluent, any calibration scale would be limited to 20 picomoles.

Placing the standards in the section (figure 3) where only the low polar eluent is used lowers the R_f value to 0.5 and avoids the under-evaluation obtained with the polar eluent. In this case, the regression for 0 to 50 picomoles DPH standards in pure acetonitrile is linear and runs through the origin ($R = 0.998$). The precision ranges from 0.4 at 10 picomoles (10 ± 0.4 picomoles) to 0.6 at 20 picomoles and increases to 1.4 at 50 picomoles. The detection limit is of the order of 3 picomoles.

Complex dissociation during the polar migration

To avoid the non linearity observed for the spots containing more than 20 picomoles free DPH, another elution dissociation method must be used.

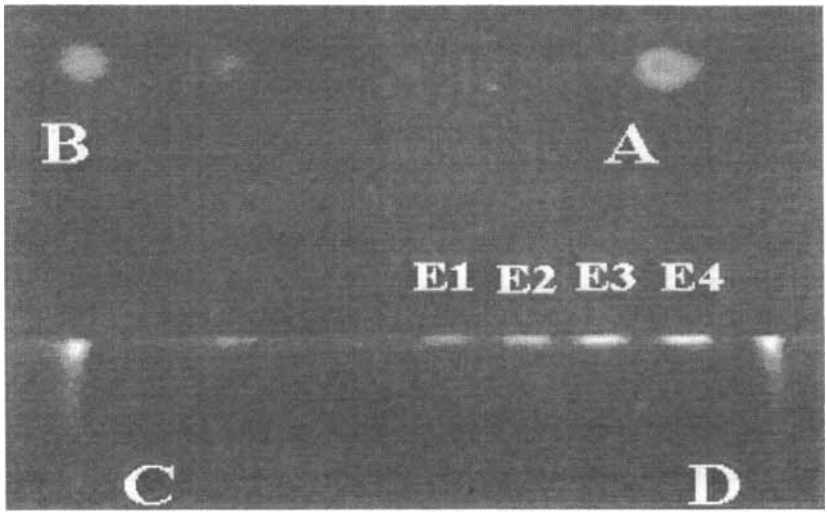

FIGURE 2. A CCD camera view of a two-dimensional TLC plate. The second migration was performed in one step following protocol 1.

Attempts to dissociate spotted samples of the 10 mM BCD/DPH/water/acetonitrile system with simple contact or by dipping the TLC plate in the polar mixture without elution did not work. However, DPH bound to the sugar was dissociated after a brief polar migration. The migration in the second dimension was therefore carried out in two steps (Figure 3).

After the short polar migration, the polyene was eluted with hexane/ether. The R_f value increased from 0.5 (characteristic of the low hexane/ether polar eluent used in the first dimension) to 0.8. This change indicated that either one or several of the polar agents remained on the TLC plate after it was dried.

Table 1 shows the balance of free and dissociated DPH after elution. Section B, eluted only once with the

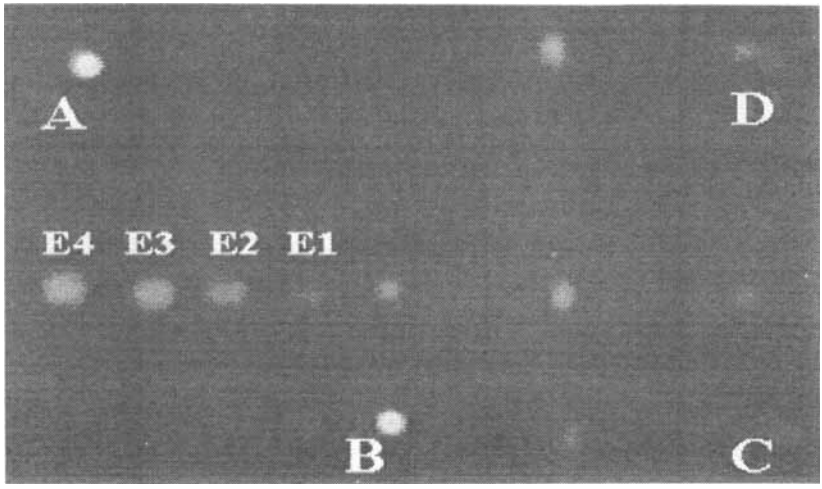

FIGURE 3. A CCD camera view of a two-dimensional TLC plate. The second migration was performed in two steps following protocol 2.

low polar eluent, resulted in 16.5 picomoles free DPH. Section C, eluted in two-dimensions, resulted in 17.2 picomoles free DPH and 12.4 picomoles dissociated DPH. The sum of these two unbound species gives a total of 29.6 picomoles DPH. In section D, eluted only in the second dimension, 28.8 picomoles free plus dissociated DPH were obtained. Despite the different treatments, the total quantities of free and dissociated DPH were conserved.

The good correlation between the value found for free DPH in section B and the value of free DPH in section C indicate that the quantum yield varies very little from one section to another.

It has been previously established (3) using U.V. absorption spectroscopy combined with TLC analysis that the total quantity of DPH bound to BCD (around

TABLE 1

Mass in picomoles of free and/or dissociated DPH separated from BCD in the various elution dimensions (data from Figure 3).

Sect.	elution*	free	dissociated	Total
B	1	16.5±0.6		
C	1+2	17.2±0.6	12.4±0.5	29.6±1.1(calculated)
D	2			28.8±0.9 (measured)

*elution dimension 1: with the low polar eluent, 2: brief polar dissociation followed by low polar elution.

22 picomoles) is greater than the free form. The dissociation is complete but not quantifiable when the elution is only performed with the polar eluent. However, with the double migration in the second dimension, the dissociation is incomplete. A certain quantity of the complex remained at the spotting position C. Supposing the emission quantum yield of this species is the same as that of free or dissociated DPH, then it would correspond to 9.7±0.4 picomoles. Therefore the two step protocol enables approximately a 50% dissociation of the complex.

CONCLUSION

In spite of technical problems associated specifically with the DPH-BCD system, this two-dimensional chromatography technique combined with CCD video analysis appears to be useful in the study of fluorescent cyclodextrin complexes. The method is sensitive, selective, and rapid. It may also be extended to the study of all complexes containing at least one fluorescent component.

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