Journal of Chromatography A, 655 (1993) 31–38
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 998

Image analysis of photochemically derivatized and charge-coupled device-detected phenothiazines separated by thin-layer chromatography

F. García Sánchez*, A. Navas Díaz and M.R. Fernández Correa

Department of Analytical Chemistry, Faculty of Sciences, University of Málaga, 29071 Málaga (Spain)

ABSTRACT

Phenothiazines previously separated by thin-layer chromatography were detected on the plates by photochemical derivatization and gave different R_F values in six solvent systems. The methanol–acetic acid (95:5, v/v) system gave the best separation and greatest chromatic and fluorescence intensities. Qualitative and quantitative information about propiomazine, acetopromazine and chlorpromazine was obtained by image analysis. Images were obtained with a charge-coupled device camera (CCD) under different conditions using both pseudocolour red–green–blue (RGB) and grey-scale tones and then filtering the light emitted when the derivative spots were excited by UV light. Calibration graphs for acetopromazine were linear from 3.94 to 22.00 m*M*, for chlorpromazine from 2.69 to 13.00 m*M* and for propiomazine from 3.31 to 15.00 m*M*.

INTRODUCTION

In contrast to liquid chromatography, thin-layer chromatography (TLC) suffers from a lack of automation. Image analysis can be useful in attempts to automate the analysis of TLC plates because it minimizes human intervention. Image analysis facilitates the extraction of quantitative characteristics from images and objective interpretation. Several articles have recently appeared in the literature describing the capabilities of multichannel imaging devices such as charge-coupled devices as detectors in TLC [1–4]. These systems involve the capture of the image; this image can then be stored in digital form on a host computer and operated on at any future time [5,6].

Computer-controlled scanning densitometers are the state of the art in commercial instruments to obtain quantitative information from TLC plates. The performances of video densitometers and scanning densitometers have previously been compared [1]. The mechanical scanners tend to be slow and expensive, and the results can be user dependent, however better spatial resolution is achieved than with video densitometers.

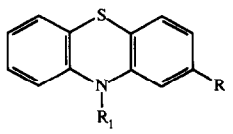
In situ photoxidation is particularly suitable for obtaining labelled compounds because the derivative is formed without dipping or spraying of the plate. Post-chromatographic derivatization in TLC allows the reaction of all standards and samples simultaneously under the same conditions, and the separation properties of the solutes are not changed by the reaction.

The phenothiazines (Fig. 1) are a group of well-known psychopharmaceuticals. These compounds contain a reduced sulphur that can be photoxidized to a sulphoxide [7]. Both chemical and photochemical oxidations of phenothiazines to fluorescent sulphoxide derivatives have been applied and significantly improve the detectability of these analytes [8–10]. However, this method has not been exploited for detecting phenothiazines separated by TLC.

In this work we determined the optimal sepa-

* Corresponding author.

0021-9673/93/$06.00



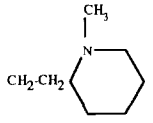
	R_1	R
Trimeprazine	CH_2-CH(CH_3)-CH_2-CH_2-$N(CH_3)_2$	H
Promethazine	CH_2-CH(CH_3)-$N(CH_3)_2$	H
Promazine	CH_2-CH_2-CH_2-$N(CH_3)_2$	H
Propiomazine	CH_2-CH(CH_3)-$N(CH_3)_2$	CO-CH_3
Thioridazine		S-CH_3
Ethopropazine	CH_2-CH(CH_3)-$N(C_2H_5)_2$	H
Chlorpromazine	CH_2-CH_2-CH_2-$N(CH_3)_2$	Cl
Acetopromazine	CH_2-CH_2-CH_2-$N(CH_3)_2$	CO-CH_3

Fig. 1. Structure of phenothiazines.

ration conditions for post-chromatographic photochemical detection on TLC plates. The plate images were acquired by using a charge-coupled device camera and then processed to extract the maximum information about the TLC plate.

EXPERIMENTAL

Thin-layer chromatography

The phenothiazine compounds were obtained from Sigma and the solvents used were purchased from Merck. All samples were prepared in ethanol and were sonicated for at least 10 min. The stock solutions of trimeprazine 8.13 m*M*, promethazine 4.58 m*M*, promazine 4.30 m*M*, propiomazine 18.51 m*M*, thioridazine 5.31 m*M*, ethopropazine 6.30 m*M*, chlorpromazine 15.14 m*M* and acetopromazine 25.23 m*M* were stored in the dark.

Phenothiazine mixtures were chromatographed on HPTLC silica gel 60 plates (Merck) following the procedure described previously [11] using methanol–acetic acid (95:5, v/v) as mobile phase.

Samples were applied by the spray-on technique using a Camag Linomat IV microprocessor-controlled device. Spots of 10-μl aliquots from the samples were placed on the plates at 10.0-mm intervals.

Phenothiazines were detected on the TLC plates by photolysis and analysing the images acquired by using a charge-coupled device camera and suitable software.

Photochemical–fluorimetric procedures

After chromatographic separation the plates were exposed for a fixed time to light from a photochemical reactor equipped with a 125-W mercury vapour lamp and water cooling [12].

For the photochemical–fluorimetric measurements in liquid solution, 100 ml of phenothiazine solution were placed in the photochemical reactor and irradiated for a fixed time. The fluorescence intensity of this solutions was then measured in a Perkin-Elmer LS-50 spectrofluorimeter.

Image acquisition

Images of the plate were captured with a Sony AVC-D7CE video camera with an 8-mm F/1.3 lens. Digitization of the video images was performed with an S-151 AT/VME digitizing tablet. The images were transferred in binary format to a host computer, which controls the working of the system through Visilog version 3.6 software.

To obtain absorbance measures, a light box with back illumination of the TLC plate was used. The geometrical arrangement of two tubular lamps (30 W each) in the light box ensured uniform lighting of the entire area of the plate. It should be noted that these are not true absorbance data since no logarithmic calculation was performed. To obtain fluorescence measures a 6-W Mineralight UV lamp Model, UVGL-58, in front the plate, at 45°, is used to excite the sample and a 365-nm filter was attached to the

camera lens to reduce extraneous excitation light.

The developed plates were placed on the light box surrounded by a non-transmitting background and the boundaries for the scanning area were set. To obtain complete information about the plate, two images were acquired with boundaries defined by the user. Orientation of the plate was fixed in such way that the "starting wells" were situated horizontally, and the direction of development was vertical, from the bottom to top of the image.

Images obtained were stored in a digital format in the computer, and they could be both displayed and manipulated. Digitization consisted of the transformation of the image into a matrix; each element of this matrix corresponds to a picture element or pixel. Each pixel can be considered as a miniature cuvette, and its intensity is a transmission, reflection or fluorescence value. From a monochrome image, each pixel is characterized by its grey-level value, coded by an integer number. Polychrome images are coded in the same way, but with three matrices corresponding to each colour channel: red, green and blue (RGB).

Monochrome and polychrome images were obtained in each mode —"absorbance" and fluorescence.

Image analysis

The digital image was used for quantitative as well as for qualitative work. A previous image processing is necessary to enhance and improve the image. Thus, the noise can be reduced. The noise results in pixels having abnormal grey levels that differ from those of their closest neighbours. To reduce noise, both random and systematic, we applied median filtering. In this case the neighbours of a given pixel were sorted according to their grey level. The pixel to be processed was replaced by the median grey level.

Once obtained, enhanced images were processed in order to extract a number of quantitative features. The steps result in the separation of objects from the background. Each separated spot can then be characterized by a number of numerical features. Thus, several morphological parameters were obtained which could be used to discriminate between the different detected spots according to shape or size or a combination thereof.

Also, the intensity values for each pixel were obtained for describing the distribution of light intensities. The graphic output can be considered a chromatogram, with intensity values on the y-axis and pixel on the x-axis. Because distortions of spots compromise resolution and introduce reading errors, readings were taken in four different positions of each spot. The fluorescence intensity was recorded as peaks of light intensity. In the absorbance mode the graphic output was blank subtracted because the intensity values are transmitted light.

The imaging detectors are wide-band, and filters or monochromators are necessary for extracting information in smaller bands. A cut-off 365-nm filter was used to filter excitation light in fluorescence mode. Additionally, absorbance mode images were also obtained with a 470-nm cut-off filter attached to the lens.

RESULTS AND DISCUSSION

Photolysis in ethanolic solutions

In order to obtain data about time of irradiation and chromogenic and fluorogenic behaviour subsequent to the photoexcitation of the reagents under study, the photolysis was studied in ethanolic solution, the medium used to solubilize the phenothiazine drugs. Table I shows the spectral characteristics before and 2 h after irradiation. Irradiation of phenothiazine drugs, which have weak native fluorescence, results in relevant changes in its fluorescence and colour characteristics. Up to a wavelength (λ) of 113 nm blue-shift in excitation (λ_{ex}) and up to 130 nm red-shift in emission (λ_{em}) maxima is observed. As an exception a 15-nm blue-shift emission maxima is observed for promethazine. A five-fold increase in relative fluorescence intensity at the emission maximum for promazine and ethopropazine was observed.

Photoxidation takes place immediately for all the compounds studied except for chlorpromazine, and is complete 2 h later. Propiomazine needs 6 h for complete photoxidation.

TABLE I

FLUORESCENCE OF PHENOTHIAZINES IN 100% ETHANOL

25°C; $slit_{ex}$ = 2.5 nm; $slit_{em}$ = 5 nm

Compound	Before irradiation			2 h after irradiation		
	λ_{ex}	λ_{em}	RFI[a]	λe_x	λ_{em}	RFI[a]
Trimeprazine, 1.02 m*M*	305	440	419	370	485	142
Promethazine, 0.60 m*M*	277, 330	440	227	390	455	150, 370
Promazine, 0.12 m*M*	300	440	211	320	365	1000
Propiomazine, 0.10 m*M*	300	530	114	360[b]	400[b]	143[b]
Thioridazine, 0.39 m*M*	300, 330	460	154	360	375	396
Ethopropazine, 0.11 m*M*	300	440	151	330	365	687
Chlorpromazine, 0.41 m*M*	300	440	145	370	455	180
Acetopromazine, 0.42 m*M* [c]	340, 395	560	70	390	485	190

[a] Relative fluorescence intensity.
[b] 6h after irradiation.
[c] $Slit_{ex}$ = 2.5 nm; $slit_{em}$ = 10 nm.

These data were used for the detection of the spots in the plates.

Chromatographic conditions

To optimize the mobile phase, a series of binary, ternary and quaternary solvent mixtures were prepared. In binary mixtures a fixed solvent was used and the other solvent was varied. Because the nature of photoproducts to be detected can be affected by the medium [1], the influence of acidity was considered by using as second solvent 25% ammonia, acetic acid and buffer of pH 9 and pH 3. Other solvent mixtures were also used. In Table II the different solvent compositions and the R_F values of each compound in the solvents studied are shown. The best separation was achieved by using the binary mixtures methanol–25% ammonia (95:5, v/v) and methanol–acetic acid (95:5, v/v) as mobile phases. In contrast, mobile phases methanol–buffer pH 9 (40:20, v/v) and methanol–buffer pH 3 (40:20, v/v) with similar acidity characteristics did not give good separation. The other systems gave intermediate results.

Before separation and irradiation all the spots were slightly coloured and fluorescent. After 5 min of plate irradiation the spots colour changes. The colour and fluorescence of spots changed with the mobile phase used. With methanol–25% ammonia (95:5, v/v) and methanol–acetic acid (95:5, v/v) all the compounds showed fluorescence and colour. With the methanol–acetic acid (95:5, v/v) system the values were greater.

Images of acetopromazine, chlorpromazine, propiomazine and mixtures at several concentrations were obtained. The results of the morphological measurements are given in Table III. The measured parameters are: area, perimeter and two form factors to account for specific shape characteristics. The first form factor (eccentricity) measures the elongation of the object in the chromatographic development direction. Its value is zero for round particles. The second form factor (compactness) is based on the ratio of perimeter squared to area and is minimal and equal to 1 for round particles. An elongated set has a high shape factor. It also measures an object's roughness, so a cranked disc has a high shape factor even though it is globally circular.

The results in Table III indicate that the area and perimeter spots increase with concentration of phenothiazine. From the near-zero eccentricity values it is deduced that the elongation of chlorpromazine and propiomazine spots is minimal, *i.e.* they are round spots. Greater elongation is observed for acetopromazine spots. The

TABLE II

R_F VALUES FOR PHENOTHIAZINES USING DIFFERENT MOBILE PHASES

Mobile phase composition (v/v)	Compound							
	Trimeprazine	Promethazine	Promazine	Propiomazine	Thioridazine	Ethopropazine	Chlorpromazine	Acetopromazine
Methanol–chloroform (1:9)	0.32	0.31	0.21	0.29	0.27	six spots	0.18	0.18
Methanol–25% ammonia (95:5)	0.66	0.61	0.53	0.65	0.56	three spots	0.59	0.56
Methanol–acetic acid (95:5)	0.42	three spots	0.34	0.50	0.46	–	0.40	0.31
Methanol–buffer borax hydrochloride pH 9 (40:20)	0.05	–	0.05	0.18	0.03	–	0.05	0.04
Methanol–buffer phthalate hydrochloride pH 3 (40:20)	0.31	–	0.28	0.31	0.24	–	0.28	0.23
Methanol–acetic acid–30% hydrogen peroxide (94:5:1)	0.58	–	0.57	0.60	0.54	–	0.60	0.57
Methanol–acetic acid–chloroform (45:15:30)	0.67	–	0.60	0.66	0.67	–	0.64	0.53
Acetone–isopropanol–25% ammonia (27:21:12)	0.73	0.72	0.78	0.73	0.73	–	0.92	0.71
Isopropanol–chloroform–water–25% ammonia (35:30:4:1)	0.64	0.55	0.44	0.63	0.53	four spots	0.53	0.47

TABLE III

MORPHOLOGICAL PARAMETERS OF PHENOTHIAZINE SPOTS SEPARATED BY TLC

	Quantity spotted (μg)	Area (mm^2)	Perimeter (mm)	Eccentricity	Compactness
Acetopromazine	17.4	5.00	15.50	2.72	3.82
	44.2	16.00	26.91	1.10	3.60
	97.3	33.00	40.26	0.31	3.91
Chlorpromazine	9.5	42.00	45.80	0.03	3.97
	25.3	63.00	52.37	0.02	3.46
	46.2	58.00	60.14	0.54	4.96
Propiomazine	15.1	41.00	43.02	0.00	3.59
	37.2	56.00	58.45	0.49	4.85
	68.5	83.00	75.41	0.84	5.45

second form factor values are high and similar for all spots. A combination of both form factors indicates that chlorpromazine and propiomazine spots are round with no elongation and roughness. Acetopromazine spots can be deduced to be highly elongated.

Quantitative analysis

Several phenothiazine mixtures were spotted and chromatographed. Figs. 2 and 3 represent graphic output of the blue component of the plates image in absorbance mode using a 470-nm filter. In the fluorescence mode the graphic

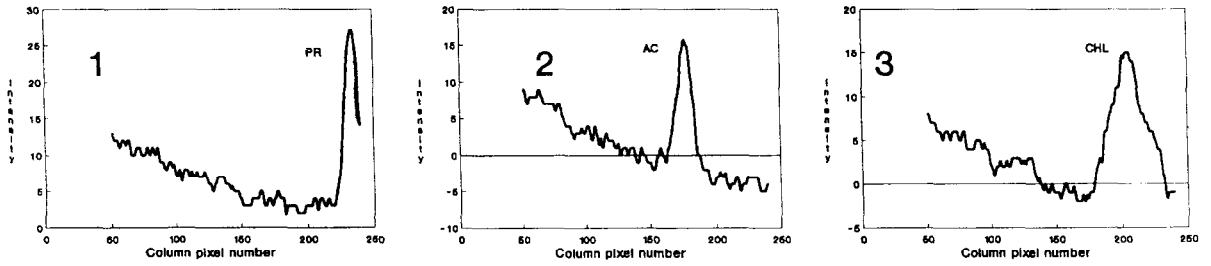


Fig. 2. Graphic output for the blue component in the absorbance mode and using a 470-nm filter. AC = Acetopromazine standard; CHI = chlorpromazine standard; PR = propiomazine standard.

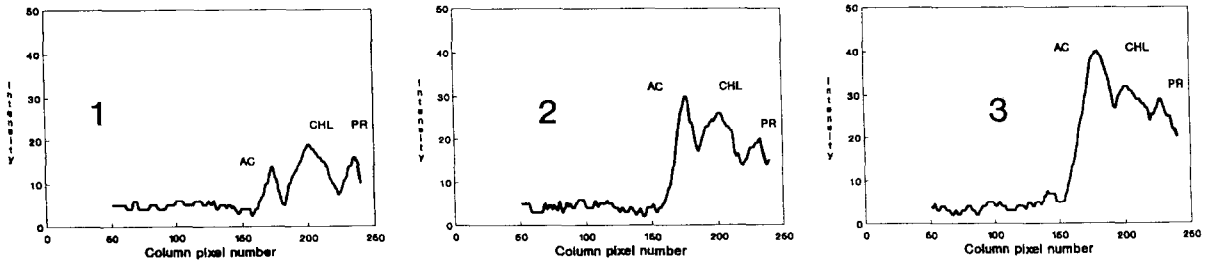


Fig. 3. Graphic output for the blue component in the absorbance mode and using a 470-nm filter. 1 = Mixture of 3.94 m*M* AC, 2.69 m*M* CHL and 3.31 m*M* PR; 2 = mixture of 10 m*M* AC, 7.14 m*M* CHL and 8.15 m*M* PR; 3 = 22 m*M* AC, 13 m*M* CHL and 15 m*M* PR. AC = Acepromazine; CHL = chlorpromazine; PR = propiomazine.

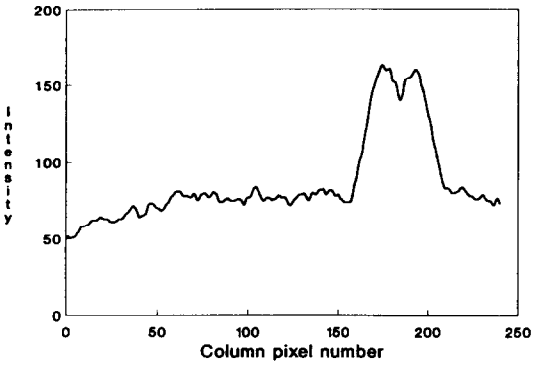


Fig. 4. Graphic output for the blue component in the fluorescence mode. Mixture of 10 m*M* acepromazine, 7.14 m*M* chlorpromazine and 8.15 m*M* propiomazine.

output shows wide peaks with low resolution (Fig. 4). The best-resolved peaks are obtained in the absorbance mode. The red colour gives greater noise than green and blue. Green gives data with lower noise but little signal intensity. Blue colour provides high intense signal and low noise. This illustrates the utility of pseudocolour selectivity.

Quantitative data were obtained using peak heights of standards, and calibration curves were constructed by plotting peak heights *versus* concentration. Table IV shows the standard curves for acetopromazine, chlorpromazine and propiomazine obtained in the absorbance mode. Propiomazine only showed a detectable signal in the fluorescence mode and by analysing the blue component in the absorbance mode. Calibration graphs were linear for acetopromazine in the range 3.94–22.00 m*M*, for chlorpromazine in the range 2.69–13.00 m*M* and for propiomazine in the range 3.31–15.00 m*M*.

CONCLUSIONS

The results obtained show that reliable man-independent techniques for qualitative and quantitative thin-layer chromatography can be achieved by computer-assisted image analysis.

Phenothiazines separated by TLC can be detected by photochemical derivatization and give rise to coloured or fluorescent spots. Images of the plates can be obtained by using a charge-coupled device, and subsequent analysis methods are easily performed given the right software.

The obtained morphological parameters of the spots gave quantitative information about their shape or size, and could be useful in future experiments as a measure of the separation quality. Image analysis under different conditions using both pseudocolour red–green–blue (RGB) and grey-scale tones, and then filtering

TABLE IV

STANDARD CURVES FOR ACETOPROMAZINE, CHLORPROMAZINE AND PROPIOMAZINE

y = concentration; x = intensity.

Compound	Image component analysed	Calibration curve ($n = 5$)	Correlation coefficient
Acetopromazine	Grey	$y = 1.1x - 24.7$	0.99
	Blue	$y = 0.3x - 15.1$	0.94
	Blue filter	$y = 0.7x - 6.6$	0.95
	Red	$y = 4.4x - 140.4$	0.99
	Red filter	$y = 1.4x - 48.2$	0.99
Chlorpromazine	Grey	$y = 0.4x - 11.7$	0.98
	Blue	$y = 0.3x - 14.6$	0.99
	Blue filter	$y = 0.8x - 12.5$	0.96
	Red	$y = 0.5x - 23.4$	0.98
	Red filter	$y = 0.4x - 19.0$	0.97
Propiomazine	Blue filter	$y = 0.8x - 10.2$	0.96

the light emitted when the derivative spots were excited by UV light, illustrates the utility of pseudocolour selectivity.

In future, the sensitivity and resolution of separation may be improved by better plate illumination and wavelength selection, respectively. Also, the acquisition of successive images during the chromatographic process could improve the separation in a similar manner to diode-array detection in liquid chromatography.

REFERENCES

1 P.B. Oldham, *Anal. Instrumentation*, 19 (1990) 49.
2 D.H. Burns, J.B. Callis and G.D. Christian, *Trends Anal. Chem.*, 5 (1986) 50.
3 P.K. Aldrigde, J.B. Callis and H. Burns, *J. Liq. Chromatogr.*, 13 (1990) 2829.
4 S.H. Wettlaufer, L.H. Weinstein, *J. Chromatogr.*, 441 (1988) 361.
5 D. Betrand, M.-F. Devaux and P. Robert, *Trends Anal. Chem.*, 10 (1991) 237.
6 P. Geladi, E. Bengtsson, K. Esbensen and H. Grahn, *Trends Anal. Chem.*, 11 (1992) 41.
7 J.W. Birks, *Chemiluminescence and Photochemical Reaction Detection in Chromatography*, VCH, New York, 1989, p. 151.
8 U. Breyer, *Biochem. Pharmacol.*, 18 (1969) 777.
9 A.H.M.T. Scholten, U.A.Th. Brinkman and R. Frei, *Anal. Chim. Acta*, 114 (1980) 137.
10 D. Chen, A. Ríos, M.D. Luque de Castro and M. Valcárcel, *Talanta*, 38 (1991) 1227.
11 A. Navas Díaz, *Anal. Chim. Acta*, 255 (1991) 297.
12 J.J. Aaron, J. Fidanza and M.D. Gaye, *Talanta*, 30 (1983) 649.