Journal of Chromatography, 204 (1981) 51–58
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,352

FIRST AND SECOND DERIVATIVE RECORDING OF THIN-LAYER CHROMATOGRAMS

APPLICATION TO THE ASSAY OF UNRESOLVED COMPOUNDS

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SUMMARY

The practical advantages of direct first or second derivative recording of thin-layer chromatographic (TLC) or high-performance TLC separations to expand the qualitative and quantitative scope of TLC are considered. By coupling TLC scanning photodensitometry with a derivative recording technique, trace analysis and detailed studies of unresolved compounds can be significatively simplified without introducing any additional experimental burden.

INTRODUCTION

In parallel with liquid chromatography, thin-layer chromatography (TLC) has recently experienced a significant optimization of instrumental and operational parameters, resulting in what has been considered to be the second generation of TLC generally referred to as high-performance TLC (HPTLC)[1]. Thus, the new technology in coating materials, developing chambers and dosage methods has led to a considerable improvement in separation power and analytical capacity, all with relatively short running times. This, coupled with the performance of modern photometric scanning instruments, has facilitated direct quantitative evaluations of HPTLC plates in a number of applications in various fields[2–5].

Speed and simplicity are of utmost importance in the development of analytical methods, especially for product quality control purposes, and in this regard the advantages of HPTLC are well proven. However, speed and simplicity usually necessitate operating conditions that do not favour selectivity, resolution or sensitivity.

In our recent experience with the application of derivative spectroscopic techniques to different complex mixtures[5,6] we came to the conclusion that the coupling of HPTLC with first or second derivative spectrophotodensitometric detection of the components separated on the plates could enhance the resolving power and detecta-

0021-9673/81/0000–0000/$02.50

bility in standard HPTLC separations. This is demonstrated here with a few examples of interest in the field of pharmaceutical sciences.

To our knowledge, derivative techniques have not been systematicaly applied to chromatographic systems, despite the fact that they could improve detectability and resolution without the need to resort to extensive modifications of the operating parameters. The paucity of reports in this field is perhaps a consequence of the technical difficulties associated with the generation of derivative curves by either electronic or computer methods.

However, the derivative signals generated by a computer from a given TLC spot have been used to define its centre (R_F) and width[1] and recently derivative spectroscopy has been used to enhance the discriminatory capibility of size exclusion chromatography[7]. Second derivative has also been succesfully coupled with an HPLC fluorimetric system for the detection of porphyrins[8]. Although the electronically generated first and second derivative signals were obtained in both instances from circuits designed and built in the authors' laboratories, it is now possible to simplify the whole operation by using standard derivative instrumental units designed for UV spectroscopy.

EXPERIMENTAL

Samples

The active ingredients of different pharmaceutical formulations were assayed by TLC in order to establish (a) the residual content of *p*-chloroacetanilide in commercial batches of the phenacetin used in various pharmaceutical applications; (b) the content of xanthinol nicotinate and hydrochlorothiazide in sugar-coated tablets (Lacer, Barcelona, Spain); and (c) the content of reserpine and rescinamine in commercial samples of *Rauwolfia* alkaloids (Giulini Pharma, Hannover, G.F.R.). The samples were diluted to the appropriate concentration prior to their application to the TLC plates.

Methods

The HPTLC and TLC separations were performed on Merck (Darmstadt, G.F.R.) 60 F254 silica gel plates (10 × 20 cm or 20 × 20 cm, layer thickness 0.25 mm), except for reserpine–rescinamine mixtures, for which the plates used had a concentrating zone (Merck, K 60 F254).

Samples were spotted with an Evachrom sample applicator and the plates were developed by ascending elution in closed glass chambers, using the following solvent compositions (by volume) and running conditions: (a) acetone–benzene–light petroleum (1:1:1), solvent front migration distance 15 cm, time 90 min; (b) methanol–25% ammonia solution (100:0.25), solvent front migration 4 cm, time 10 min; (c) methanol–methyl ethyl ketone–*n*-heptane (84:33.6:58), solvent front migration 4 cm, time 10 min. The separations were carried out in the dark.

Spectrophotodensitometric detection

The plates (air-dried in an oven for 5 min) were read on a Zeiss KM 3 chromatogram spectrophotodensitometer (Carl Zeiss, Oberkochen, G.F.R.) operated in the reflectance mode for cases (a) and (b) and in the fluorescence mode for case (c) with slits of (a) 8 × 0.5 mm, (b) and (c) 3.5 × 0.5 mm. The scanning speeds were (a) and (b) 100 mm/min and (c) 50 mm/min. The plates were read in the direction of solvent flow.

Derivative TLC or HPTLC

The derivative curves from the spectrophotodensitometric zero-order signals were directly recorded by coupling to the recorder unit (Perkin-Elmer Hitachi Model 200) a Perkin-Elmer Model 200-0628 derivative spectrum attachment.

RESULTS AND DISCUSSION

Trace analysis, which is always a difficult task, becomes impossible in cases such as that illustrated in Fig. 1. In this particular instance it was necessary to determine the residual content of *p*-chloroacetanilide (PCA) in commercial batches of phenacetin so as to verify compliance with the accepted upper limit of 0.03 or 0.05% set by the US and European Pharmacopoeias, respectively[9,10]. These two compounds can be separated by TLC with the R_F values indicated in Fig. 1, except when the relative amount of phenacetin in the samples is such that it completely masks the peak of PCA. Accordingly, the samples would either have to be re-run on another solvent system, not always easy to prepare without resorting to an empirical trial-and-error approach, or alternatively the PCA would have to be isolated by usually tedious

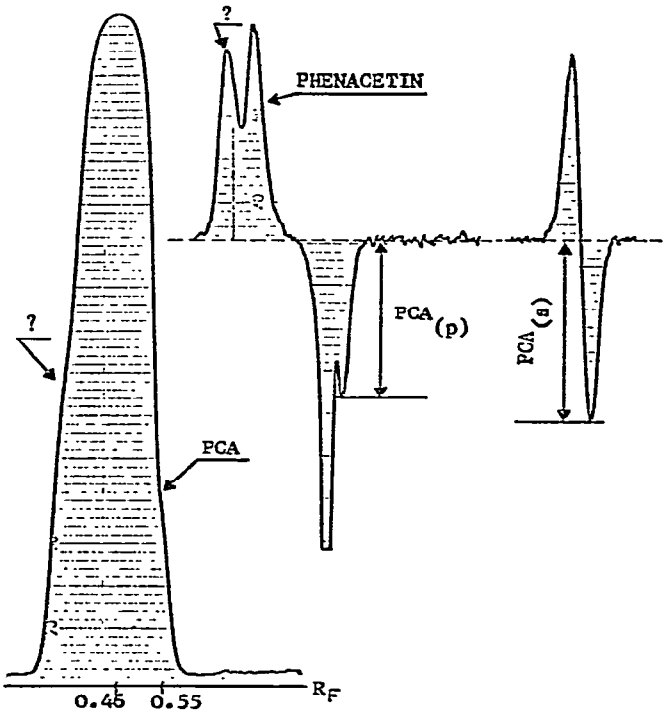

Fig. 1. TLC assay of traces of *p*-chloroacetanilide (PCA) (R_F = 0.55) in a commercial batch of phenacetin (R_F = 0.46). The photodensitometric response shown corresponds to a sample containing microgram amounts of phenacetin applied on the origin of the plate. $PCA_{(s)}$ indicates the measurement taken on the second half of the first derivative curve generated from a standard. $PCA_{(p)}$ indicates the corresponding measurement on the first derivative curve of the HPTLC spot at R_F = 0.44–0.55. The unknown product was not quantitated.

extraction procedures. However, this problem can be solved in a much simpler and rapid way by the application of first derivative techniques to detect the small slope change on the back edge of the large TLC peak, thus resolving it into its two components.

The first derivative curve illustrated in Fig. 1 clearly shows a peak profile sufficiently resolved to allow quantitation of PCA relative to the response of a standard. The enhanced qualitative possibilities of direct derivative TLC are also demonstrated by the detection of an unknown interference on the upward slope of the main peak (labelled by a question mark on Fig. 1), clearly resolved on the first derivative curve. By this procedure the estimated level of PCA in this particular sample could be calculated as 0.43% and the practical limit of detection of PCA was established as 0.05%. Thus, this method effectively competes with the more elaborate procedure described in the pharmacopoeias.

Another interesting application of derivative TLC, in this instance HPTLC, is the quantitative evaluation of hydrochlorothiazide in the presence of nicotinic acid. The commercial product contained xanthinol nicotinate and hydrochlorothiazide, the first of which decomposes on the TLC plates to xanthinol and nicotinic acid. In the solvent system used the nicotinic acid spot (N at $R_F = 0.94$) overlaps the spot of hydrochlorothiazide (HCT at $R_F = 0.90$), as shown in Fig. 2. The xanthinol by-product is well separated with $R_F = 0.49$. The UV absorption spectra taken at 10-nm intervals on the leading and trailing edges of the large unresolved spot are shown at either side of the spectrophotodensitometric trace. The scan of the upward slope of the main peak produces a UV spectral pattern essentially consistent with that of authentic HCT, with a major maximum at 270 nm and a secondary maximum at 320 nm. The spectrum of the downward slope, corresponding mainly to nicotinic acid, is characterized by a simpler envelope from 240 to 290 nm, with a maximum at 270 nm. However, for quantitative purposes significative cross-contributions to both of these UV spectra cannot be ruled out. For this reason, the first and second derivative signals from the zero-order spectrophotodensitometric TLC scan were obtained, as shown in the two lower traces in Fig. 2.

In this case, the two R_F values are so close that the shape of first derivative curve does not indicate the presence of two components in the HPTLC spot. Nevertheless, as shown in Table I, HCT could be quantitated by measuring the distance OB in the first half of the derivative curve. The presence of two components in this spot is more clearly indicated on the second derivative curve. As shown by the second derivative profile, the minimum in this curve is resolved into two adjacent minima, unequivocally pointing to a second component of almost coincidental R_F within the peak of HCT, in this instance nicotinic acid.

Table I gives the values obtained from three different samples of a commercial formulation. In the second derivative mode the OA measure is the more precise relative to the real values, as it corresponds to the part of the curve least affected by the unresolved nicotinic acid (the beginning of the upward slope of the HCT + N peak).

Finally, Fig. 3 illustrates a case of special relevance such as the quantitation of reserpine and rescinamine, two alkaloids of the *Rauwolfia* which, owing to structural similarities, are difficult to resolve. In fact, there has been only one reported separation[11], and we have not been able to reproduce it. After various attempts to

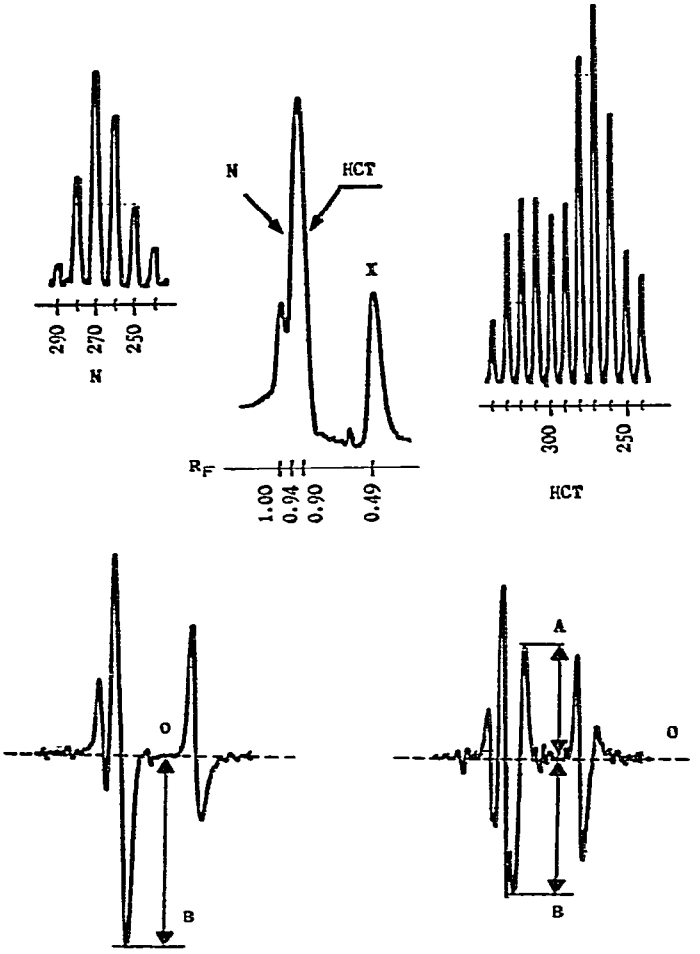

Fig. 2. HPTLC separation of a sample containing xanthinol nicotinate and hydrochlorothiazide ($R_F = 0.90$). Decomposition of the former yields xanthinol ($R_F = 0.49$) and nicotinic acid ($R_F = 0.94$). On the right- and left-hand sides of the chromatogram are shown the responses obtained by scanning at selected wavelength intervals the upward and downwards slopes of the main peaks. Lower left, first derivative; lower right, second derivative.

TABLE I

HPTLC ASSAY OF THE CONTENT OF HYDROCHLOROTHIAZIDE IN A COMMERCIAL FORMULATION

The actual values read at $\lambda = 340$ nm (no interference from the spectrum of nicotinic acid at this λ) were 103, 107, 105, respectively. Values represent percentages of the theoretical content (100%); r = correlation coefficient of the calibration curves used.

Derivative	*Sample*			*r*
	A	*B*	*C*	
Second derivative —				
A B	97	91	90	0.9549
O A	103	94	98	0.9916
First derivative —				
O B	102	100	102	0.9945

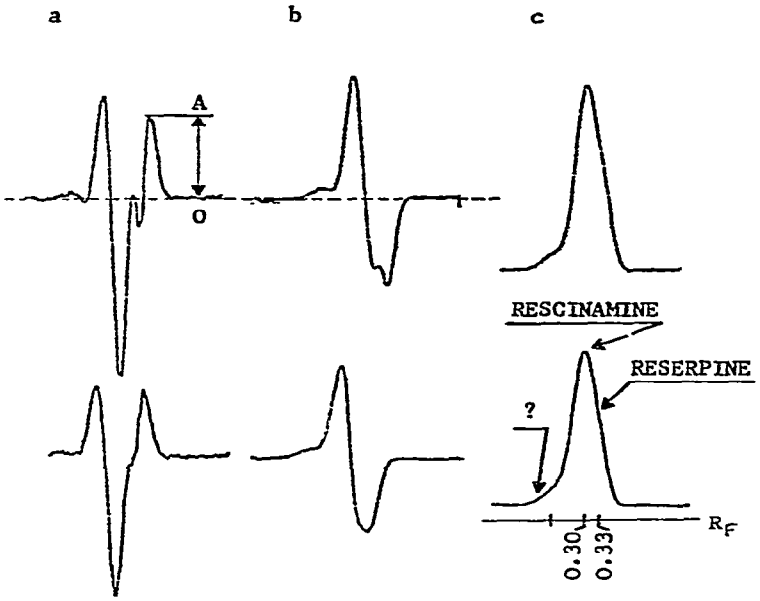

Fig. 3. Spectrophotodensitometric profile (c) of an HPTLC spot (R_F = 0.30) of unresolved reserpine (R_F = 0.33), rescinamine (R_F = 0.30) and an unknown component (?). The corresponding second and first derivatives of this zero-order curve are shown in (a) and (b), respectively. The plate was spotted seven times with the same sample for quantitation according to the data pair procedure[12]. The kind of differences observed in the resolution at various points across the plate is illustrated by the shape of the derivative curves. In contrast, no appreciable difference could be detected by inspection of the chromatographic zero-order profile. Detection was achieved by excitation of the HPTLC spot at 365 nm, reading the fluorescence emission through a Zeiss FL 46 filter.

find a suitable solvent system, we could obtain only a 0.03 difference in R_F values (Fig. 3c), which is not enough to resolve the two compounds. The first and second derivative curves generated from this peak are shown in Fig. 3b and a, respectively. An interesting effect also readily monitored in this fashion is the change in resolving power across an HPTLC plate. For instance, the upper row of zero-order fluorescence and derivative curves in Fig. 3 corresponds to a spot positioned close to the plate border, while those in the lower row correspond to a spot located at the centre of the plate. In this case, the variation of resolution across the TLC plate is of sufficient magnitude to be detected by the first and second derivative curves. There are also indications of an unresolved minor component on the leading edge of the peak (? in Fig. 3c). This component, depending on the prevailing resolution, is occasionally indicated by a second maximum on the last part of the second derivative curve.

The study of the quantitative possibilities of the derivative signals demonstrated that in relation to reserpine, the measure least affected by the overlapping of the rescinamine spot plus the unknown product is the distante AO, corresponding to the first part of the second derivative curve, as illustrated in Fig. 3a.

Regarding the reproducibility in these types of measurements, Table II summarizes the results obtained in replicate determinations of the components in two of the examples discussed here. Considering the difficulties one could foresee when

TABLE II

REPRODUCIBILITY OF THE DERIVATIVE QUANTITATION OF SPECTROPHOTODENSITOMETRIC TLC PROFILES

All determinations were carried out according to the data pair procedure[12].

(A) Determination of traces of PCA in samples of phenacetin. These samples were prepared for assay by addition of 0.2% and 0.05% of PCA to samples of phenacetin.

Parameter	*PCA added (%)*	
	0.2	*0.05*
n	8	8
PCA found ($\bar{x}$) (%)	0.21	0.054
Standard deviation (%)	0.01	0.005
Relative standard deviation (%)	4.5	8.8

(B) Quantitation of reserpine and rescinamine in commercial samples of *Rauwolfia* alkaloids.

Parameter	*Reserpine*	*Rescinamine*
Certified range in commercial samples (%)	7.8–9.6	19.5–24.0
Certified amount in samples analysed (%)	8.7	21.7
Amount found ($\bar{x}$) (%)	8.6	24.5
Standard deviation (%)	0.5	0.6
Relative standard deviation (%)	6.0	2.5
n	16	8

confronted with these totally unresolved TLC profiles (see Figs. 1 and 3), and the speed and simplicity with which the results in Table II can be obtained, a precision of less than 5% and 9% in the determination of 0.2 and 0.05% of PCA, respectively, can be considered as excellent for all practical purposes. The same applies to the example of the two alkaloids, where the precisions can be calculated to be *ca.* 6 and 3% for reserpine and rescinamine, respectively.

In conclusion, derivative TLC provides a rapid, direct, simple and relatively economical means of verifying the homogeneity of any given spot, in many instances enhancing the resolving power of the chromatographic system to a point where the quantitative assay of partially or even totally overlapped components becomes a possibility. Also, the usual changes in resolution across a plate can be readily monitored, resulting in a higher precission of quantitative measurements performed with internal standards applied at different locations on HPTLC or TLC plates.

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