CHROM. 14,229

APPLICATION OF ELECTRONICALLY DIFFERENTIATED HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC DENSITOGRAMS TO THE ASSAY OF SOME PRESERVATIVES USED IN PHARMACEUTICAL FORMULATIONS*

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(Received July 21st, 1981)

SUMMARY

The results of an extended study on the feasibility and scope of derivative high-performance thin-layer chromatography (HPTLC) for the assay of unresolved components are presented. It is shown that as the resolution decreases the accuracy of quantitative digital electronic integration, area and peak height measurements diminishe to a point where the differentiated signal becomes the only valid approach to the direct quantitation of unresolved HPTLC spots. Using the first derivative, the accuracy of these assays can be maintained within 2–3 %, independently of the resolution, and the derivative techniques provide a very useful alternative to direct HPTLC analysis of small amounts of a component masked by major peaks. In practice, this simplifies the optimization of experimental parameters in HPTLC, and results in shorter analysis times.

The HPTLC spots are estimated in a spectrophotodensitometer, the signal of which is electronically differentiated by means of an analog circuit, capable of recording the first or second derivative spectrum. Instrumental possibilities for optimizing and enhancing the differential signal are discussed.

INTRODUCTION

It has already been shown[1] that the use of electronically differentiated first and second order signals in thin-layer chromatography (TLC) improves the quantitation of poorly resolved components. According to our experience, this technique could save a lot of time and effort while enhancing the scope of standard TLC or high-performance TLC (HPTLC) techniques, especially where overlapping of the ana-

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lytes precludes accurate measurement of the necessary parameters usually derived from a graphical evaluation of peak shape.

As a further check of the potential of derivative HPTLC, we have studied the performance of this procedure in the assay of a group of preservatives found in a wide range of pharmaceutical products and foods[2]. Relative to ultraviolet spectroscopic or gas chromatographic methods, quicker analysis of these products by TLC, is said to be possible if confirmation only is desired[3] and that for speed and quantitation high-performance liquid chromatography (HPLC) is more appropriate. Although this may generally be true, we demonstrate that the application of electronic differentiation of spectrophotodensitometric HPTLC signals is also very rapid and specific, sometimes obviating the need for tedious empirical adjustments of the mobile phases in order to quantitate poorly resolved compounds.

EXPERIMENTAL

Samples

The *p*-hydroxybenzoic esters used in this work were purchased from Lemmel S.A. (Barcelona, Spain). All are commercially available pharmaceutical preservatives.

Methods

The HPTLC separations were performed on E. Merck (Darmstadt, G.F.R.) reversed-phase nanoplates Sil-C_{18} 50 % and RP-18 F_{254} (10 × 10 cm). TLC runs were carried out on silica gel G-60 F_{254} plates (regular or silanized for reversed-phase TLC), also from E. Merck.

Samples were spotted with an Evachrom sample applicator and the plates were developed by the ascending technique in closed non-saturated glass chambers at 18–20 C for 6–7 min. The reversed-phase plates were developed with methanol–water solutions of various compositions, depending on the degree of separation desired. The solvent front was allowed to rise 4 cm from the sample spots. The methanol was purchased from E. Merck.

Resolution was calculated from the expression $R = 2 (x_2 - x_1) : (b_2 + b_1)$ where x_1 and x_2 are the distances from the starting point to the peak maximum for compounds 1 and 2, and b_1, b_2 are the corresponding peak widths at half-height. In cases where overlapping of peaks 1 and 2 did not allow the graphical measurement of these two parameters, the values were extrapolated from those of standards spotted alongside the mixture.

Spectrophotodensitometric detection

The plates (air dried in an oven for 5 min) were read on a Zeiss KM3 chromatography spectrophotometer (Carl Zeiss, Oberkochen, G.F.R.) operated in the reflectance mode at $\lambda = 270$ nm and variable slits of 3.5 × 2–0.2 mm, as discussed below. The scanning speed was usually set at 100 mm/min. The plates were read in the direction of solvent flow.

Derivative 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

The components of a mixture of methyl (MHB), ethyl (EHB), propyl (PHB), butyl (BHB) and benzyl (BzHB) p-hydroxybenzoates cannot be resolved on standard silica gel HPTLC plates as shown in Fig. 1A. The only TLC approach yielding the complete separation of these five compounds implies the use of double development on properly activated and silanized silica G-60 plates, as illustrated in Fig. 2. This approach was described by Rangone and Ambrosio[2] who, for quantitative purposes, removed the bands and extracted the compounds from the silica gel with methanol. However, according to our experience, this procedure is limited in practice by the relatively short life of the silanized TLC surface, which tends to suffer serious disruption and cracking when wetted by the aqueous solvent front. Thus, reproducibility is rather poor for quantitative purposes.

A different approach is based on the use of modern reversed-phase systems using a relatively high water content in the mobile phase. In this context, two recent communications have described the use of circular HPTLC for the rapid separation of PHB, EHB and MHB[4] and the HPTLC quantitation of MHB and PHB[5] on reversed-phase layers. However, both of them do not deal with butyl and benzyl p-hydroxybenzoates which cannot be resolved on reversed-phase layers, even when the water content of the mobile phase approaches the maximum allowable by the hydrophobicity of these layers.

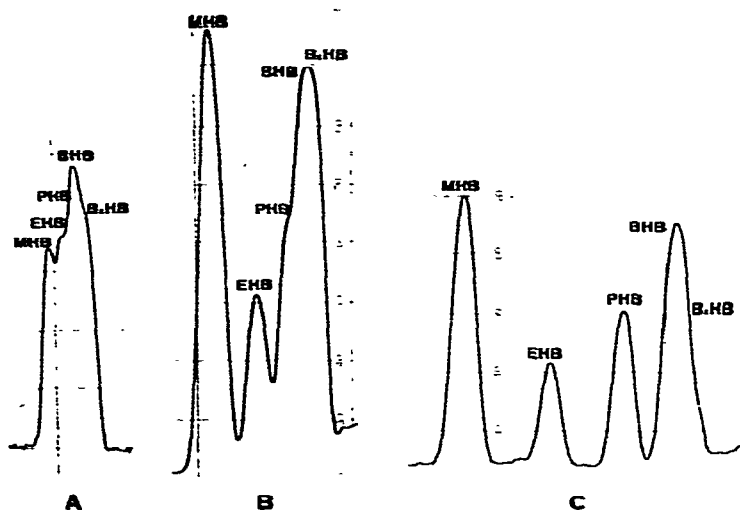The first advice one would get from a TLC expert would be to "change or modify

Fig. 1. TLC and HPTLC separations of different p-hydroxybenzoates (from low to high R_F): BzHB = benzoyl p-hydroxybenzoate (80 ng); BHB = butyl p-hydroxybenzoate (400 ng); PHB = propyl p-hydroxybenzoate (160 ng); EHB = ethyl p-hydroxybenzoate (80 ng) and MHB = methyl p-hydroxybenzoate (320 ng). The amounts shown in parentheses were dissolved in the 0.2 μl methanol deposited on the plates. A. Sorbent silica gel. G-60 F_{254}. 10 × 10 cm; solvent system pentane–acetic acid (22:3). B. Sorbent Sil-C_{18} 50%, 10 × 10 cm; solvent system methanol–water (40:60). C, Sorbent RP-18 F_{254}. 10 × 10 cm; solvent system methanol–water (85:15), triple development. Slit widths: A and B, 0.5 mm; C, 0.35 mm. Linear scanning at 100 mm/min. Recorder speeds: A and B. 120 mm/min; C. 240 mm min.
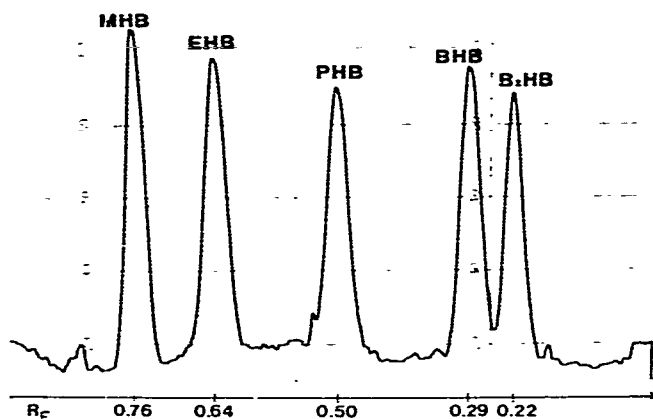
Fig. 2. Separation of the same mixture as in Fig. 1 on activated and silanized silica G-60 plates. Solvent system: borate buffer (pH 11)-dioxane (9:1). Double development. Spectrophotodensitometer conditions as in Fig. 1A and B. Volumes of 0.2 μl containing 240 ng of each compound were spotted.

the solvent", although occasionally this is not effective as illustrated in Fig. 1B and C. These HPTLC profiles show examples of the kind of results obtained in two of the multiple solvent systems tested. Changing the mobile phase did not result in a separation of the five compounds comparable to that of Fig. 2, but, as expected, it did affect the overall resolution so that we can merge or separate any of these components at will, except for BzHB and BHB which always overlap. Thus, in line with our recent experience on the application of derivative spectroscopic techniques to overlapped HPTLC spots, this particular system appeared to be an ideal model for the further study of the performance of the derivative technique.

One of the first aspects examined was the relative merits of conventional area and peak height measurements *vs.* first derivative determinations as a function of peak resolution. For this purpose, HPTLC of the ethyl and propyl esters was selected as a suitable working model since both compounds can be resolved by appropriate modifications of the methanol–water ratio in the mobile phase. The data in Fig. 3 summarize our experience of different laboratory applications over the past 2 years. That is, electronically differentiated signals consistently yield a higher precision and accuracy of the quantitative measurements at low resolutions. As shown in Fig. 3, for baseline-resolved peaks ($R > 1$), quantitation by peak area, peak height or first derivative provides in all cases a satisfactory degree of accuracy, irrespective of the method used. However, as resolution decreases, the accuracy of the peak height or peak area measurements also decreases to a point where quantitation of the merged peaks is no longer possible. Nevertheless, even when resolution is very poor or practically nil, the components can in many cases still be quantitated by application of derivative techniques, as illustrated in Fig. 4. In this example, the quantitation of EHB and PHB, even at a resolution of only 0.28, can readily be achieved by measuring the distances OA and OB in the first derivative curve of the unresolved HPTLC spot, relative to the corresponding standards.

Consequently, whenever peak area measurements cannot be used and peak height values are significantly affected by a lack of resolution, the electronically differentiated signals obtained directly from the overlapped peaks may provide the only
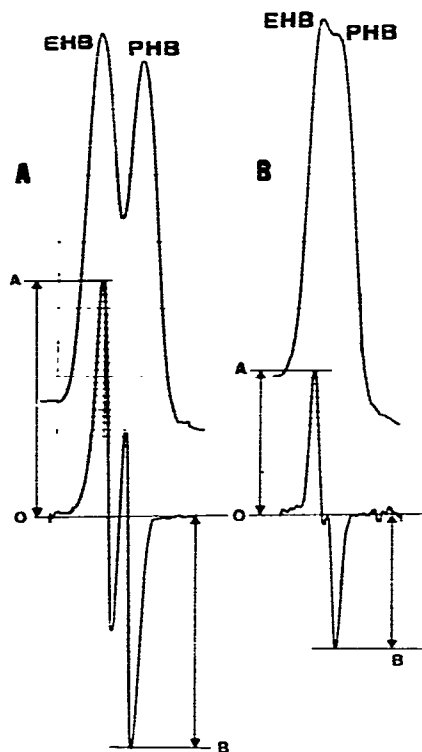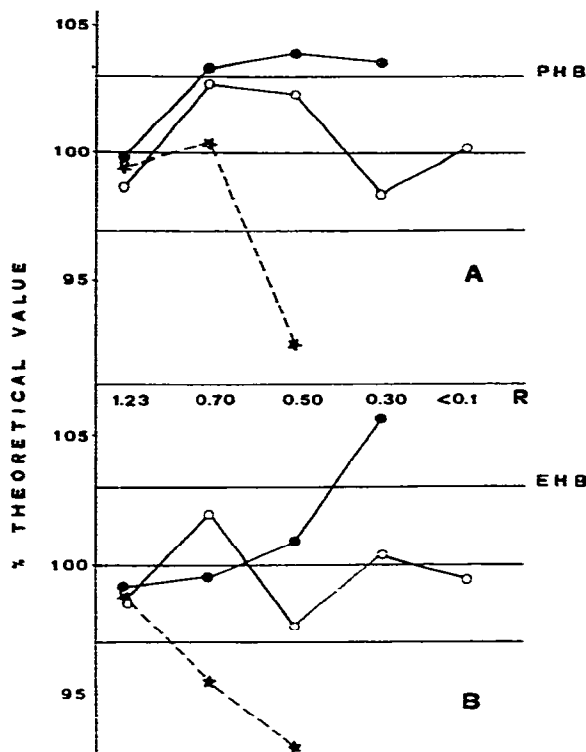
Fig. 3. Accuracy of peak height and first derivative procedures for the quantitation of unresolved HPTLC spots. The chromatographic resolution R, of PHB and EHB is dependent on the methanol–water ratio (in parentheses below). as follows: R = 1.23 (8:4); 0.70 (7:1); 0.50 (10:1); 0.30 (20:1); <0.1 (50:1). The data were obtained on RP-18 F_{254} plates with a slit width of 0.5 mm and a scanning speed of 100 mm/min. Mode 6. except when R = 0.30 and <0.1. where the modes used were 4 and 5. respectively. The 100 % line represents the theoretical value corresponding to the actual content of propyl (A) and ethyl p-hydroxybenzoates (B). ★. Data points calculated from electronically integrated peak areas; ●. peak height values; O. first derivative measurements of electronically differentiated signals. All data points represent the averages of four determinations.

Fig. 4. First derivative measurements of unresolved PHB and EHB at resolutions of 0.70: (A) and 0.30 (B). Conditions as in Fig. 3. The response shown corresponds to 320 ng of each compound.

practical means to perform rapidly the desired assay, thus avoiding the sometimes cumbersome and tedious process of finding another suitable mobile phase system. Also. as already demonstrated by us[1], the derivative approach in many cases provides valid quantitative information even when peaks are totally overlapped (R < 0.1 in Fig. 3).

In all of the present cases (R = 1.23 to <0.1), the values calculated from the first derivative curves approach the true theoretical value to within $\pm$ 3 %, an accuracy which cannot be achieved by peak area or peak height measurements at resolutions lower than 0.71 (see Fig. 3). The corresponding precision values expressed as the coefficient of variation of replicate determinations at three different resolutions are given in Table I. These data clearly show that seriously overlapped peaks (R < 0.1) can be quantitated by the derivative technique with a relatively high degree of ac-

TABLE I

PRECISION OF DIFFERENT MEASUREMENTS AT DIFFERENT RESOLUTIONS

PHB = Propyl ester of p-hydroxybenzoic acid; EHB = ethyl ester of p-hydroxybenzoic acid. The values represent the coefficients of variation (%) corresponding to the points shown in Fig. 3. In all cases there were four replicate determinations per plate. The data were obtained by application of the data pair procedure[*]. The corresponding calibration curves and linear regression analysis are given in Table II.

	0.70		0.50		0.30		<0.1	
	PHB	EHB	PHB	EHB	PHB	EHB	PHB	EHB
Peak area (electronic integrator)	2.48	4.60	3.55	3.40	–	–	–	–
Peak height	0.24	0.48	0.73	0.75	1.94	2.44	–	–
First derivative	0.49	0.44	0.59	0.62	3.88	1.35	1.23	1.05

curacy and precision, whereas electronic peak area and peak height measurements become inoperative. It is also evident that the derivative technique shows its advantages in extreme situations where all other conventional quantitative approaches fail.

The calibration curves obtained for instance for PHB by application of three different types of regression formulae to the first derivative measurements confirm the higher quality of fit of the log, log and reciprocal $1/y$ vs. $1/x$ regression[6] according to Table II. The concentration of PHB spotted on the HPTLC plate ranged from 16 to 320 ng. As a test of the discriminating potential of the derivative technique, a series of parallel assays were carried out in the presence of a direct and constant interference of a fixed amount of EHB (320 ng added to all of the samples of PHB). These assays were carried out at a resolution of the order of 0.3. As shown in Table II, the accuracy of the calibration data obtained with and without EHB is maintained since the first derivative determination of PHB is not affected by the relatively high excess of unresolved EHB. In other words, the derivative spectra effectively discriminate against the overlapping effect of EHB.

The same concepts were next applied to three-component HPTLC spots with the aim of verifying the potential use of the derivative technique in more complex

TABLE II

FIRST DERIVATIVE CALIBRATION OF PHB BY THREE LINEAR REGRESSION METHODS COMPARED TO THE DATA OBTAINED FOR PHB IN THE PRESENCE OF EHB AT A RESOLUTION OF 0.30

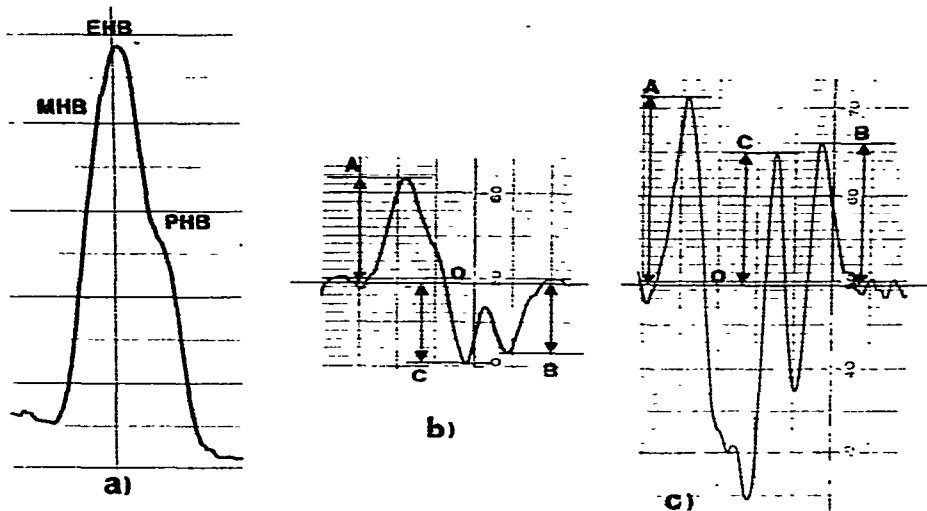|		A	B	r	$S_{y \cdot x}$
$y = Ax + B$					
	PHB	0.018	0.89	0.9817	0.22
	PHB + EHB	0.023	1.56	0.9796	0.23
$ln\ y = A\ ln\ x + B$					
	PHB	0.6518	1.57	0.9971	0.09
	PHB + EHB	0.630	1.44	0.9823	0.09
$1/y = A \cdot 1/x + B$					
	PHB	25.51	0.07	0.9997	0.03
	PHB + EHB	11.90	0.09	0.9989	0.05

Fig. 5. A. Spectrophotodensitometric profile of an unresolved HPTLC spot containing MHB. EHB and PHB. Sorbent: RP-18 F_{254}. Solvent: methanol–water (9:0.7). Slit width: 0.2 mm. Scanning at 100 mm/min. B. First derivative scan of profile A. C. Second derivative scan of profile A. Derivative conditions: scan speed 240 mm/min; mode 5: absorbance scale 0.2.

situations. An example of the kind of results obtained for a peak representing a mixture of MHB. EHB and PHB is illustrated in Fig. 5. Whereas the components hidden behind the leading (PHB) and trailing edges (MHB) of the main peak (EHB) can readily be quantitated by measuring the distances OA and OB in the first derivative curves, as for the binary mixtures (see Fig. 4), the ascending and descending slopes of the first or second derivative curves corresponding to the middle component are significantly affected by the shape of those of the other two components. In other words, the middle component (EHB) would have to be quantitated using for example the distance OC in the derivative traces, a measurement clearly subject to significant deviations from the true values, as shown graphically in Fig. 6. Thus, while the MHB and PHB in this example can still be quantitated to within $\pm 10\%$ by first or second derivative measurements this is not so for EHB. However, as shown by the higher deviations of the other points, this accuracy level applies only to the optimum 0.5-mm slit width of the spectrophotodensitometer. This leads to a consideration of the need to optimize the instrumental parameters.

To demonstrate the remarkable effect of the slit width on the accuracy of the derivative data, in Fig. 7A and B the first derivative values measured for PHB and EHB at different resolutions and with a slit of 0.5 mm (Fig. 3A and B) are compared to the corresponding values obtained at different slit widths. As shown, smaller or larger slits than 0.5 mm produce a remarkable decrease in accuracy. Thus, in derivative recording the slit width of the spectrophotometer is a critical parameter which must be adjusted in accordance with the peculiarities of each problem and instrument in order to optimize resolution.

Other instrumental parameters which may influence the accuracy of derivative measurements are the scan speed and the mode (differentiator time constant) selected.
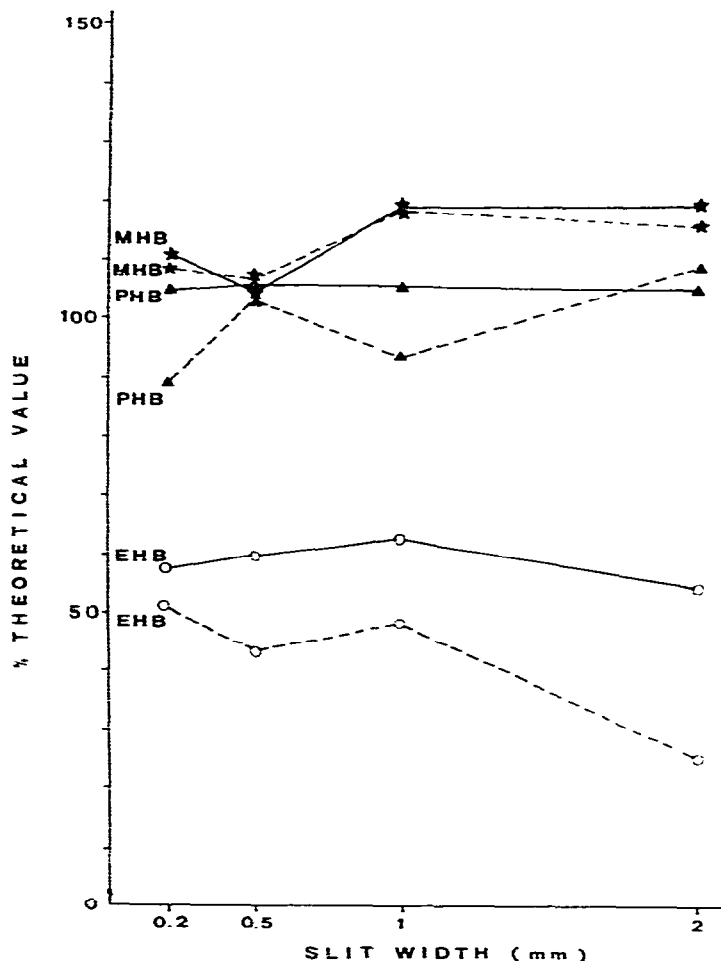
Fig. 6. Assay of an unresolved three-component peak (see Fig. 5) by first (———) and second ($-$ $-$ $-$) derivative measurements at different slit widths. PHB (▲) was measured using the distance OB (Fig. 5). MHB (★) using the distance OA and EHB (O) using distance OC.

This is illustrated in Fig. 8 for the case of the unresolved three-component peak of Fig. 5. The choice of scan speed (Fig. 8A) may be specially critical for the second derivative spectrum since in this case the instrument measures the differential changes of absorbance against time. An increase in scanning speed leads to an increased amplitude of the absorption derivative signal, although the resolution of the spectrum decreases. In this particular unit the mode selector acts as a series of frequency cut-off filters, with higher modes increasing the amplitude of the first and second derivative spectra and reducing the overall high frequency noise, although this is achieved at the expense of resolution. Thus, providing that all other instrumental parameters are fixed, in this case the optimum results would be obtained at mode 4 as shown in Fig. 8B. A plot of the mode setting *vs.* signal/noise ratio for the first and second derivatives of a mixture of PHB and EHB showed that this ratio increases at higher settings. For
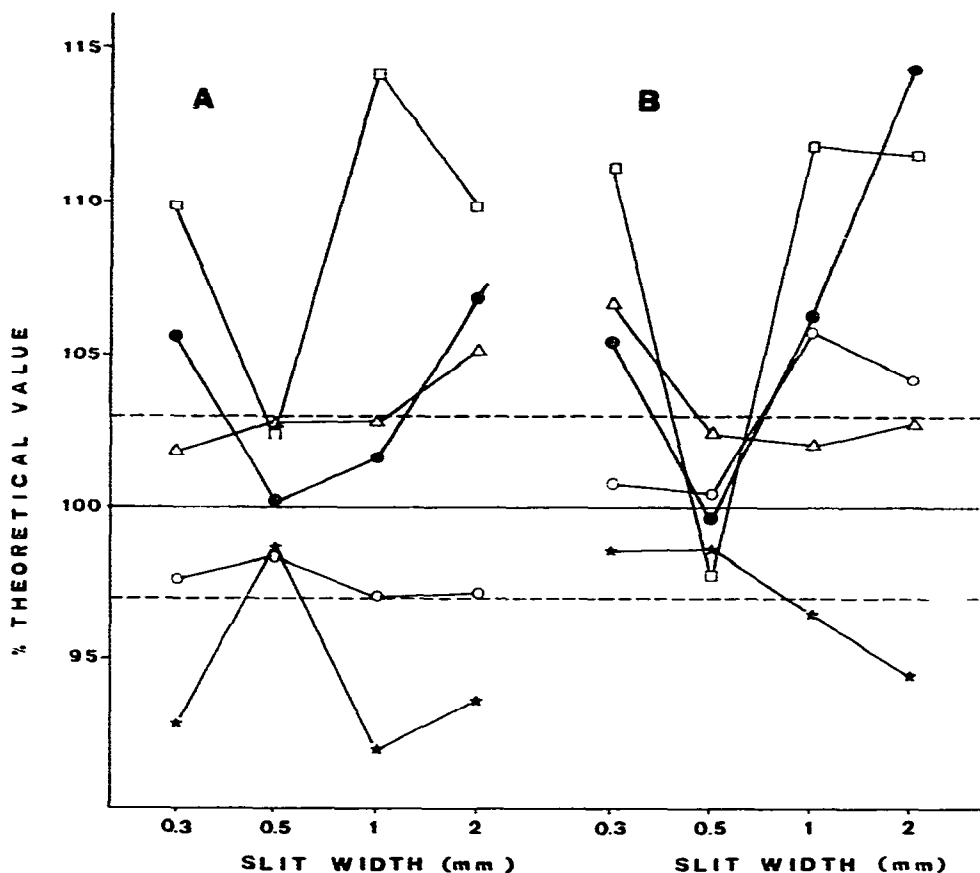
Fig. 7. Accuracy of first derivative determinations of the content of PHB (A) and EHB (B) in a mixture of both prepared in the laboratory, carried out at different resolutions and instrumental slit widths. ★, values obtained at $R = 1.23$; △, values at $R = 0.70$; □, values at $R = 0.50$; O, values at $R = 0.30$; ●, values at $R = <0.1$. Mode 6, scan speed 100 mm/min.

example, in the first derivative spectra of EHB and PHB, the signal/noise ratio increases from 6 and 4.5, respectively, at mode 3, to 14 and 12.5, respectively, at mode 6. However, the overall resolving power decreases as the mode setting increases; thus, a significant amount of the analytical information contained in the fine detail of these derivative curves is lost. For example, the detail seen in the negative part of the second derivative curve in Fig. 5C, which indicates overlapping of MHB and EHB, would be lost at mode 6 but enhanced at mode 2 or 3. Accordingly, the mode should be adjusted carefully depending on the type of problem. In this particular case, there was no need further to resolve these two second derivative minima by going to a lower mode, since the measurements taken from both minima to the derivative baseline zero (O in Fig. 5C) were not more accurate than those taken on the OA and OC distances, as indicated on this figure. Also, it should be noted that the loss of peak amplitude at the lower mode settings may be a disadvantage in trace analysis[1] or when dealing with very low amounts of sample.
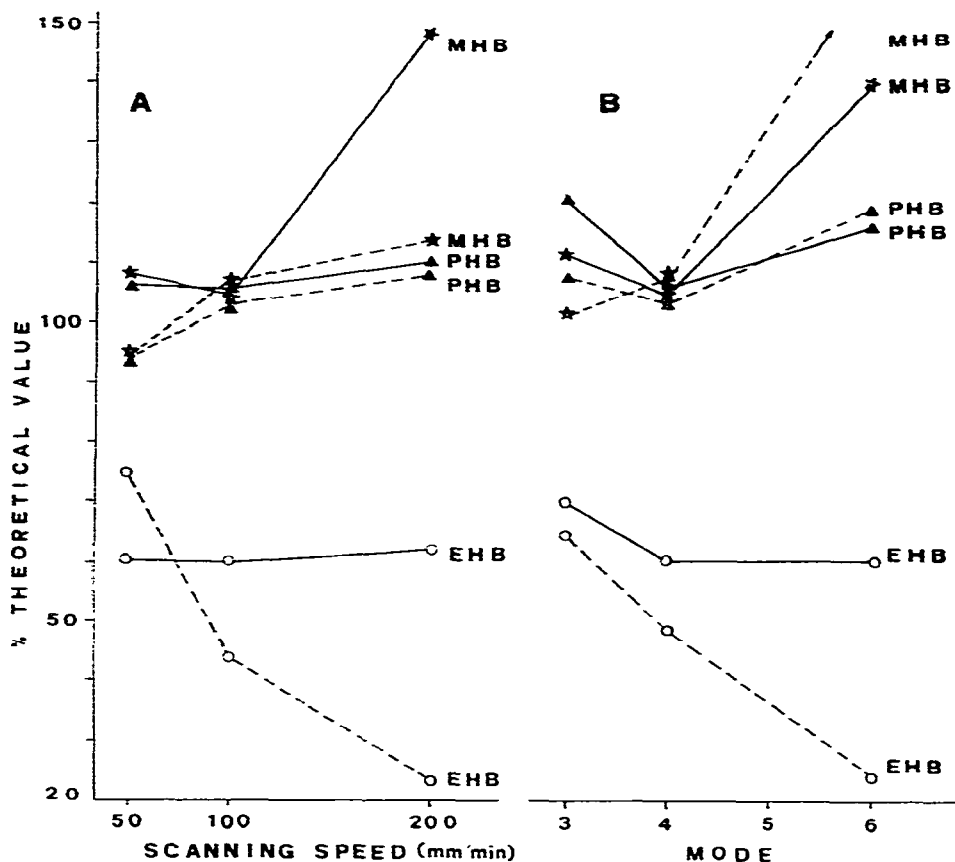
Fig. 8. First (————) and second (— — —) derivative spectrophotodensitometric determination of MHB (★), EHB (O) and PHB (▲) in an unresolved HPTLC spot (see Figs. 5 and 6) at different scan speeds (A) and modes (B). All measurements were carried out at a fixed slit width of 0.5 mm using the distances indicated in the caption of Fig. 6. Mode 6 was used for the readings in A. Data for B were obtained at a scan speed of 100 mm/min.

In this work one has to consider two kinds of resolutions. which should not be confused. On the one hand, the chromatographic resolution, which is fixed by the experimental parameters of plate development and on the other hand, the derivative resolution, which can be used, as shown herein, to improve a given TLC resolution without any further manipulation of the chromatographic parameters. This is why, at very low TLC resolutions, R, the discriminating power of the derivative readings taken on unresolved peaks (*e.g.*, Fig. 4) can be optimized by setting the mode of the differentiating instrument to lower values. For instance, in Fig. 3 the first derivative points corresponding to $R = 0.30$ and $R = <0.1$ were obtained at modes 4 and 5 respectively.

In conclusion, we believe that the application of electronically differentiated signals to the densitometric evaluation of TLC plates, especially for the resolution of seriously overlapping spots, could generate, without any further changes in the chro-

matographic systems, quantitative data of the same accuracy and precision as those obtained by more elaborate and time consuming procedures[8,9].

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