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COMPUTER-CONTROLLED EVALUATION IN THIN-LAYER CHROMATOGRAPHY

RESULTS AND POSSIBILITIES

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SUMMARY

The results of quantitative optical *in situ* scanning with a computer-controlled double-beam densitometer for thin-layer chromatography are given. Each spot is positioned in an optimum way with respect to the light beam so that deviations in sample application and in the chromatographic process no longer cause errors. The measurement of peak height in connection with the internal standard method gives very good results. As there is no time-consuming step in the quantitation, this system is suitable for routine analysis in pharmaceutical quality control (content uniformity test). Furthermore, peak areas can be determined by digital integration or by a special peak approximation. The detection limit is lowered, as signal averaging or integrating analogue to digital conversion gives better signal-to-noise ratios than in the usual scanning methods.

INTRODUCTION

Quantitative determinations of different classes of substances on thin-layer plates by optical scanning have been carried out for about 15 years. During the past 10 years, chromatogram photometers for reflectance, transmission and fluorescence measurements have been commercially available. There are highly sophisticated instruments for avoiding errors due to the irregularities of the sorbents, solvents and the chromatographic process itself, which operate in a double-beam or double-wavelength mode or by simultaneous measurement of the reflectance and transmission. Quantitative *in situ* thin-layer chromatography (TLC) is a very flexible analytical method and the separations of different compounds from each other and from the interfering matrix are effected in a single procedure directly followed by the quantitation.

EXPERIMENTAL

TLC-photometer

A Schoeffel SD 3000 was used with a modified stage, driven by stepper motors controlled by a Hewlett-Packard 9830 or 9810 desk calculator (*cf.*, Fig. 3).

Reference substances

Reference substances were of pharmaceutical quality, reagent grade.

Solvents

Solvents were reagent grade materials obtained from E. Merck (Darmstadt, G.F.R.).

TLC and high-performance TLC-plates

Pre-coated Kieselgel 60 plates were obtained from Merck.

Sample application

Microcaps (0.5 μl) were used (Drummond, Broomall, Pa., U.S.A.).

AUTOMATIC SCANNING UNITS

The time-consuming step in the quantitation of the separated spots is the positioning of each track in the centre of the light beam. The commonly used TLC densitometers are sensitive to mispositioning and the resulting errors can easily exceed 1–2%. We have described two methods for constructing electronically controlled automatic scanning units for TLC: one system with precision analogue time bases[1] and the other with digital electronics[2]. Small errors in spotting and deviations in developing the chromatogram can cause large errors. Therefore, another system has been developed commercially (Zeiss, Zürich, Switzerland) that avoids this error by a special scanning mode, as shown in Fig. 1. Each track of the plate can be scanned,

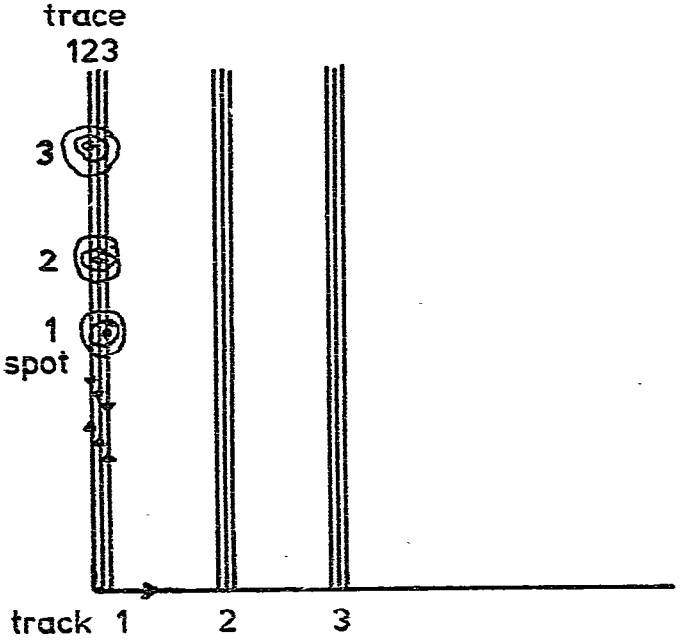

Fig. 1. Principle of the automatic scanning unit for TLC (Zeiss, Zürich, Switzerland).

examining close traces different distances apart (0.1, 0.2, ... mm). Scanning with and against the direction of development with averaging of the two integration counts for each peak leads to better results and to a higher reproducibility[3]. The system is coupled to a computer and from each scan all peak areas are compared to give the most probable value for the estimation of the content of the spots to be determined (Fig. 2).

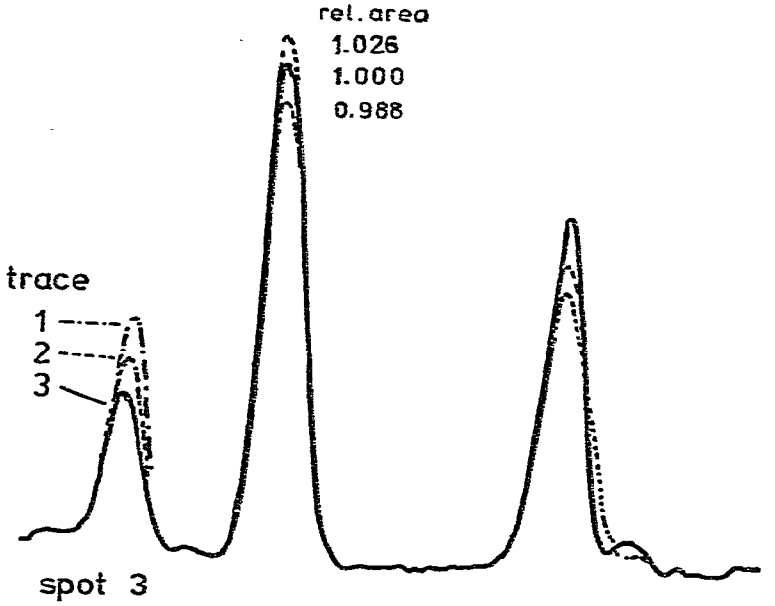

Fig. 2. Chromatogram recorded using the method of scanning shown in Fig. 1 with the results of the electronic integration.

COMPUTER-CONTROLLED TLC DENSITOMETER

We recently described a computer-controlled TLC densitometer[4–6]. In this system, the signal of the densitometer is fed by an analogue–digital converter or by a

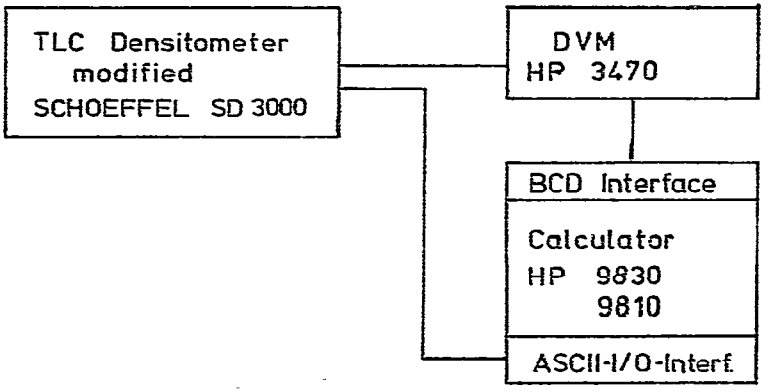

Fig. 3. Schematic diagram of the computer-controlled TLC densitometer.

digital voltmeter with BCD output via an interface to a programmable desk calculator (Fig. 3). The calculator works like a process computer and is able to control the stage of the TLC densitometer. Therefore, a Schoeffel SD 3000 double-beam instrument was equipped with stepper motors for the x- and y-directions of the stage. This system allows the position of each spot to be controlled by the computer optimally with respect to the light beam, as shown in Fig. 4. The stage is moved in discrete steps in the y-direction (the direction of development) until the first peak is found by four successive pieces of increasing data. With a lower resolution defined by the software, the maximum peak height is searched first in the y- and then in the x-direction. In order to avoid errors due to irregular spots, this procedure is repeated a second time using backscan for a defined distance. The resolutions A_y, a_y, a_x and the distance of the backscan, D_y can be changed, in order to give optimal values for different problems or in order to scan the commonly used TLC plates as well as the new high-performance TLC plates. The resolution is related to the stepper motors and the gear, and it is possible to position any spot to within 0.05–0.1 mm. This permits a reproducibility (expressed as relative standard deviation), measured on 20 spots using 25 scans of the same plate, of less then 0.4%.

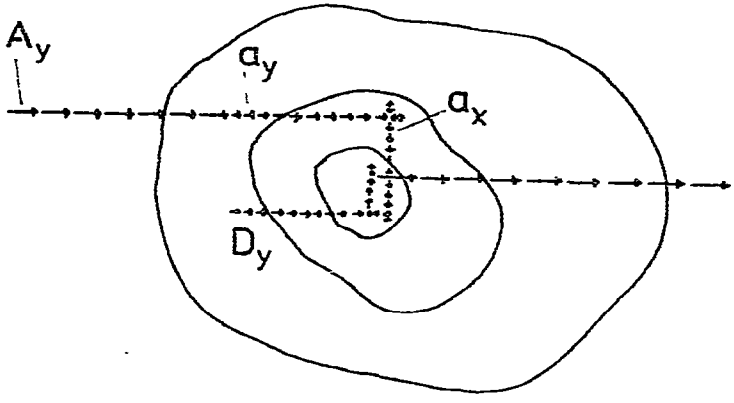

Fig. 4. Method of positioning the spot in the light beam of the TLC densitometer. A_y and a_y, resolution in direction of development; a_x, resolution at right-angles to solvent flow; D_y, backscan before the second positioning.

EVALUATION USING PEAK HEIGHT AND INTERNAL STANDARD

In a recent paper, we compared the estimation of the same spots by the integration and peak height methods[7]. In routine pharmaceutical analysis, the peak height method gives very good results only if there is a limited range of concentration[8] or at low concentrations[9,10]. Using the internal standard method[11–13], the error caused by spotting the solution on the plate can be eliminated and a larger number of analyses can be carried out on the same plate. Using a double-beam densitometer, many effects of the sorbents (gradients in thickness or particle size) and the solvents (UV absorption near the front) have smaller influence on the results in comparison with single-beam instruments. A large number of routine analyses have been carried out with this computer-controlled TLC densitometer in the past 2 years and some results

are given in Tables I–III. All of these results are based on peak height measurements with an internal standard. The time that is needed to scan one plate depends on the numbers of tracks, spots and the distance from the first to the last spot. A mixture of three components plus an internal standard, with 8 tracks (32 singular spots), is processed in about 25 min using the equipment shown in Fig. 3 or in about 18 min using a fast analog-to-digital converter instead of the digital voltmeter. The method described can be used, for instance, in the content uniformity test.

TABLE I

DETERMINATION OF DIMETHINDEN IN VIBROCIL WITH PHENAZON AS INTERNAL STANDARD[14]

Dimethinden: 100% ≈ 1.88 μg per spot ≈ 2.5 mg per 10 ml. Solvent: acetonitrile–ethanol–25% NH_3 (30:5:3). λ = 254 nm; slit width ≈ 7 × 0.5 mm.

Stock No.	*Results*			
	Mean (%)	*Standard deviation (%)*	*Number of results*	*Mode**
2240	97.6	1.12	20	a
2250	98.5	1.74	20	
2200	102.9	1.53	16	
2240	98.8	1.09	30	b
2250	98.0	2.43	29	
2200	102.5	2.51	16	

* a, 4 analyses/4 references/plate; b, 6 analyses/2 references/plate.

TABLE II

DETERMINATION OF CAFFEINE AND ASPIRIN IN CAFASPIN TABLETS (CONTENT UNIFORMITY TEST) WITH ACETOPHENETIDIN AS INTERNAL STANDARD[14]

Caffeine: 100% ≈ 0.25 μg per spot ≈ 50 mg per tablet. Aspirin: 100% ≈ 2.5 μg per spot ≈ 500 mg per tablet. Solvent: *n*-hexane–dioxan–formic acid (45:40:2). λ = 275 nm; slit width ≈ 6 × 0.3 mm.

Substance	*Results*		
	Mean (%)	*Standard deviation (%)*	*Number of results*
Caffeine	100.5	1.16	29
Aspirin	99.4	1.64	29

TABLE III

DETERMINATION OF AN ANALGESIC MIXTURE IN DRAGEES (CONTENT UNIFORMITY TEST) WITH CORNECAIN AS INTERNAL STANDARD[14]

Dichloralphenazon: 100% ≈ 10 μg per spot ≈ 100.0 mg per dragée. Caffeine: 100% ≈ 2 μg per spot ≈ 20.0 mg per dragée. Papaverin: 100% ≈ 2 μg per spot ≈ 20.0 mg per dragée. Solvent: acetonitrile–25% NH_3 (50:4). λ = 280 nm; slit width ≈ 6 × 0.5 mm.

Substance	*Results*		
	Mean (%)	*Standard deviation (%)*	*Number of results*
Dichloralphenazon	98.2	2.69	24
Caffeine	101.0	3.42	24
Papaverin	99.1	3.39	24

QUANTITATION OF HIGH-PERFORMANCE TLC PLATES

As mentioned above, all steps in the *x*- and *y*-directions are controlled by the software and can easily be changed. Using the HP 9830 desk calculator, a dialogue is possible between the user of the system and the computer. Therefore, there is no limitation to the estimation of spots on high-performance TLC plates. A typical result is given in Table IV. Some lipophilic dyestuffs used by Ripphahn and Halpaap[15] for controlling the separation properties of the thin layer were also quantified by peak height measurement and the internal standard method. Only a measurement at one wavelength (ca. 420 nm with a relative large bandwidth) was made, and therefore some peaks are very small and others very large. In some instances the ratios of the peak heights exceed 1:10, as shown in Fig. 5.

TABLE IV

DETERMINATION OF 30 ng OF DYESTUFFS (*cf.*, FIG. 5) ON HIGH-PERFORMANCE TLC PLATES WITH COMPOUND 4 AS INTERNAL STANDARD[14]

Mode: alternating tracks with reference and analysis.

No.	*Substance*	*Results*		
		Mean (ng)	*Standard deviation (%)*	*Number of results*
1	Ceresviolett BRN	29.8	0.49	8
2	Ceresschwarz G	29.8*	1.68	8
3	Accompanying substance with 2	29.9*	2.81	8
4	Fettgelb (internal standard)	—	—	—
5	Bleu VI F Organrot	29.7	4.69	8
6	Ceresrot G	29.3	2.57	8
7	Ceresbraun BRN	30.1	3.19	8

* Relative results.

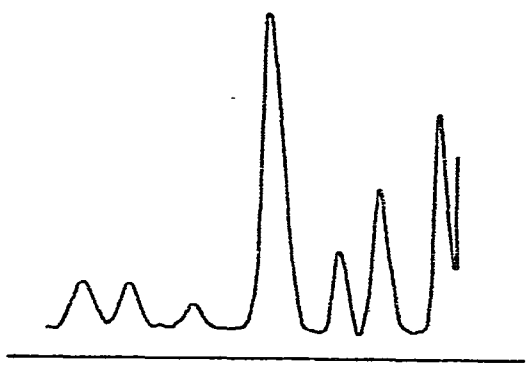

Fig. 5. Mixture of seven lipophilic dyestuffs[15] measured at about 420 nm and relatively large monochromator exit slit.

PEAK AREA DETERMINATION

In some instances, the determination of only the peak height is erroneous. For instance, in the examination of biological fluids or tissue extracts, there are many interferences by the matrix in the analysis. Therefore, the determination of the integral peak area is more useful. After positioning the spot in the centre of the light beam, the stage is re-scanned for a defined length and the peak area is found by scanning and numerical integration. The method of determining peak areas by numerical integration is used in many commercial data systems (Hewlett-Packard, Siemens, IBM, etc.), and some special problems with regard to TLC have been described in the literature[15,16]. Another method is the determination of peak areas by a modified Gauss approximation[17].

DETECTION LIMIT

In some fields (biochemistry, clinical chemistry, naturally occurring compounds), it is of interest to detect substances at very low concentrations. In quantitative *in situ* determinations in TLC, Sudanred is often used to determine the detection limit[18,19]. The detection limit is directly related to the signal-to-noise ratio but also depends on a number of parameters of the chromatographic process[10]. The noise that arises during the recording of a chromatogram arises from the lamp (especially xenon lamps), photomultiplier, amplifier and, to a large extent, small irregularities in the sorbent layer. With a computer-controlled TLC-photometer, the signal-to-noise ratio can be decreased by signal averaging. After having carried out a defined number of

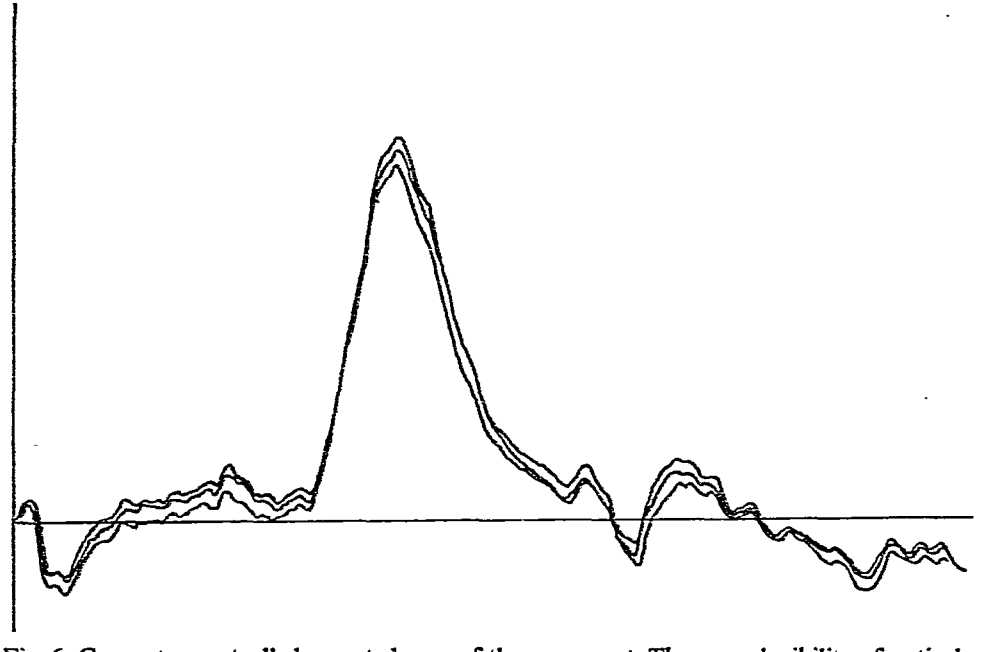

Fig. 6. Computer-controlled repeated scan of the same spot. The reproducibility of optical scanning is evident. Caffeine, 5 ng per 0.5 μl per spot. $\lambda = 275$ nm; slit width $\approx 5 \times 0.3$ mm.

steps in any direction, the stage is stopped and a discrete number of data can be summed and averaged. For instance, a spot containing 10 ng of caffeine (sample application 0.5 μl, spot migration about 5 cm) is detectable with a signal-to-noise ratio of about 4. The detection limit of the computer-controlled system without signal averaging but with integrating analogue–digital conversion defined by the sampling rate of the digital voltmeter (100 ms) is less than 5 ng. Signal averaging, digital smoothing and pseudo-Gaussian approximation[17] can further decrease this detection limit. The real boundary is given by the irregularities of the plate. Fig. 6 shows the computer-controlled scan of the same spot; it can be seen that the deviations in the baseline are not due to statistical noise by the lamp, photomultiplier and amplifier but are real effects from the plate.

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