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A NEW METHOD FOR THE QUANTITATIVE EVALUATION OF THIN-LAYER CHROMATOGRAMS

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SUMMARY

A new method is described for the quantitative elution of material in spots separated by thin-layer chromatography. By using recently devised apparatus*, the substances can be quantitatively eluted without scraping the layer off the plate. In comparison with conventional methods of elution, this technique usually affords higher recovery and better reproducibility. The advantages and possible applications of the new device are illustrated by examples drawn from the analysis of pharmaceuticals.

INTRODUCTION

Two methods are commonly used for the quantitative evaluation of substances separated by thin-layer chromatography (TLC), *viz.*

(a) direct evaluation of the TLC plates with a densitometer, or

(b) elution, in which the layer containing the spot is scraped from the plate, and the substance is extracted from the adsorbent by elution and is then determined in the eluate.

Many descriptions of the application of these methods have been published, and summaries of their uses have been made by several authors[1–7].

With the object of eliminating certain stages from the elution process, an apparatus was devised by means of which, after the substances have been separated by TLC, a circular elution chamber is applied to each spot, and the desired component is then eluted separately with a suitable solvent. The principal advantages of the apparatus in comparison with the conventional elution procedure are:

(1) The number of plates per analysis is reduced, with corresponding saving in time and expenditure.

(2) Only small amounts of material and small volumes of solvent are required.

(3) It is not necessary to scrape the adsorbent from the plate, so that there is no necessity for centrifugation or filtration after elution.

* This apparatus is manufactured under licence by Camag (Muttenz, Switzerland) and is sold under the name of Eluchrom.

(4) Once the elution chamber has been fitted, the rest of the elution procedure is automatic.

(5) It is subsequently possible to verify that all the material that was in the spot has been eluted, since the adsorbent remains unchanged in the eluted area.

DESCRIPTION AND OPERATION OF THE APPARATUS

Fig. 1 shows how the apparatus (which consists of six elution units, so that it is possible simultaneously to elute material from six spots) is operated. Each elution unit comprises a piston pump driven by a motor and delivering the necessary volume of solvent, a four-way cock and an elution cylinder, the various parts being connected by PTFE tubing. A single electric motor drives all six pumps. Each elution cylinder is fashioned from a block of PTFE, with the elution chamber as a flat round recess in the lower surface, which is surrounded by a sealing ring. The solvent is delivered to the elution chamber by way of a passage bored through the cylinder and flows out at the other side through a similar passage. Each unit may have a separate supply of solvent, or, if desired, all can be supplied from a common reservoir.

Before the elution cylinder can be fitted, the adsorbent must be scraped off in a ring round the area to be eluted; this is done by means of a special ring scraper, which is applied by hand. The elution cylinder is then applied with the seal fitting exactly into this cleared ring, and is clamped down until the elution chamber is her-

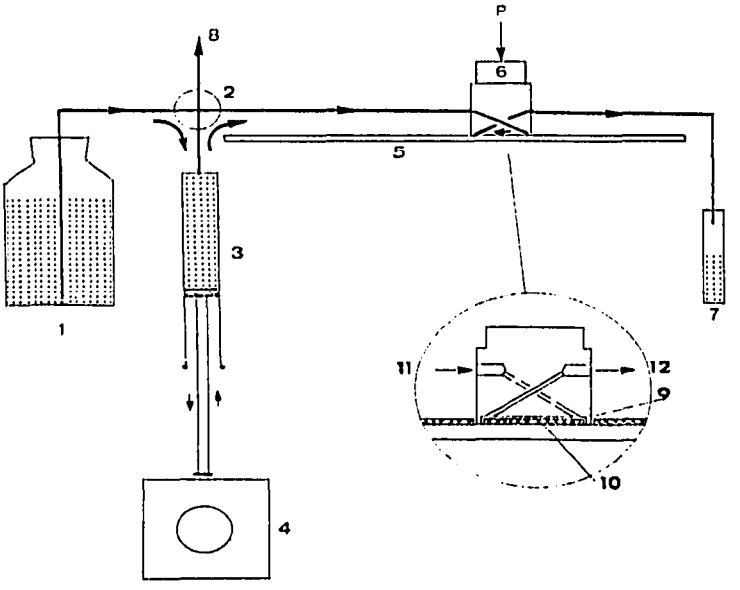

Fig. 1. Diagram showing the operating principle of the apparatus. 1 = Solvent reservoir; 2 = four-way cock; 3 = piston pump; 4 = electric motor; 5 = TLC plate; 6 = elution cylinder; 7 = cell; 8 = air supply for drying the elution chamber after elution; 9 = sealing ring; 10 = layer including the spot; 11 = inlet; 12 = outlet; P = direction of pressure applied to clamp the elution cylinder to the plate.

metically sealed to the underlying plate by the compression of the sealing ring. As soon as the electric motor is switched on, it starts to drive the piston pumps and produces a continuous flow of solvent through the elution chambers. Elution ceases automatically as soon as the pre-determined volume of solution has accumulated.

The eluate can be collected in the cell in which the extinction or fluorescence is to be measured (perhaps after the eluted substance has undergone some reaction designed to develop colour or fluorescence); it is not necessary to filter or to centrifuge the solution.

Up to six spots can be eluted simultaneously from a TLC plate or foil measuring 20 × 20 cm. This is possible provided that the clear space between the spots is never less than 2.5 mm wide and that none of the spots is more than about 20 mm wide after development of the chromatogram.

The layer of absorbent under the elution cylinder is not damaged by the elution process; it is therefore possible to verify that the material in the spot has been completely eluted by removing the elution cylinder and spraying the plate with a reagent or, if plates with a fluorescent indicator are used, by examination under ultraviolet (UV) radiation.

ELUTION PROCEDURE

The proposed method makes it possible to work with very small amounts of material and very small volumes of solvent. The amount of material depends principally on the extinction coefficient and on the type of cell to be used for measurement, since only a very small quantity of solvent is required for complete elution of the material in the spot. This is illustrated in Fig. 2 which shows the successive phases in the elution of a dye. It can be seen from Fig. 2 that, with the first filling of the chamber, *i.e.*,

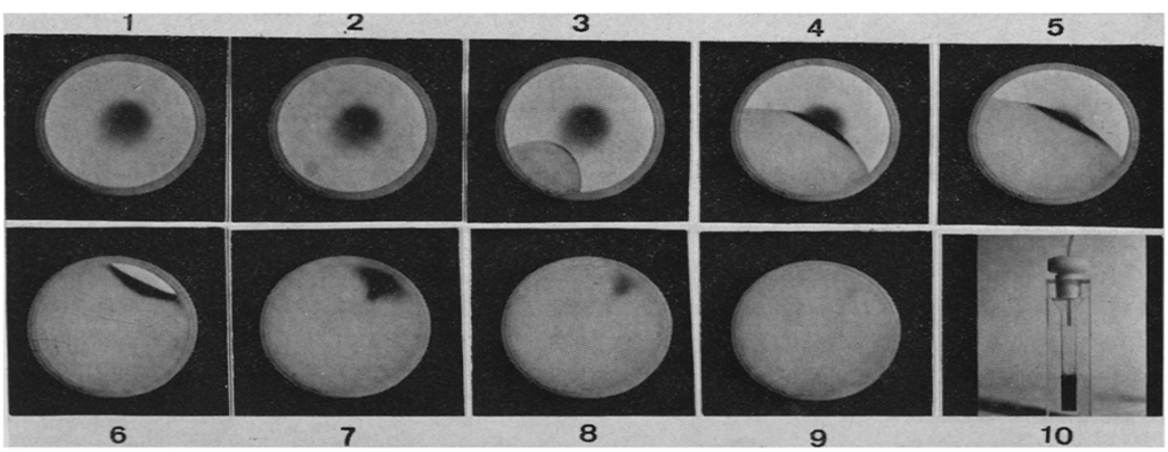

Fig. 2. Elution of indophenol blue (viewed from below through the glass of the TLC plate). 1 = The spot with the elution cylinder fitted into a ring from which the adsorbent has been removed; 2 = solvent starts to flow into the chamber; 3 = beginning of the elution process; 4–6 = further stages of elution; 7, 8 = the substance is washed out of the elution chamber through the outlet; 9 = elution is complete; 10 = cell containing the eluted substance. Plate pre-coated with silica gel 60 (Merck); ascending development for 15 cm with dichloromethane; elution with 0.4 ml of methanol at 0.04 ml/min.

with less than 0.25 ml of solvent, the substance is virtually completely extracted from the layer and washed out of the chamber. This favourable elution characteristic of the apparatus also holds for the elution of other substances, and can be demonstrated by collecting the eluate in separate fractions and analysing each fraction separately. Table I summarises results obtained with caffeine, benzocaine and phenacetin.

TABLE I

EFFICIENCY OF RECOVERY

Substance	*Plate**	*Amount applied (μg)*	*Solvent**	*Percentage of active substance in the eluate***	
				1st fraction (0.5 ml)	*2nd fraction (0.25 ml)*
Caffeine	1	36	2	100.6	<0.25
Benzocaine	2	13.5	3	100.0	<0.25
Phenacetin	3	18.0	12	99.2	<0.25

* See notes on Table II.

** The eluate is diluted to 3.0 ml and the extinction is determined at the absorption maximum. The value for 100% is determined from the extinction of a standard solution (containing the same amount of active substance in the same volume) that has not been submitted to TLC.

These results show that the elution process consists principally of displacement of the substance from the adsorbent as the solvent front flows through it; the high polarity of methanol, which was used as the solvent, may well be of decisive importance here. Methanol can be used as the solvent in virtually every instance. Apart from the advantage of its high polarity, most of the substances that have so far been determined are sufficiently soluble in it, and it is also possible to carry out normal UV spectrophotometric determination directly on the eluate over the whole range of wavelengths that is conventionally used.

The rate of elution must be adapted to the quality of the plates that have been used. Plates with firm layers (that is, most of the usual commercial plates) must be eluted more slowly than the less-compact layers prepared in the laboratory. By selecting a suitable speed of elution and a suitable quality of plate, it is usually possible to achieve almost quantitative elution.

The precision with which elution can be carried out is, within wide limits, independent of the amount of material in the area to be eluted. This is illustrated in Table II by reference to paracetamol, taking 60 or 6 μg, and to caffeine, taking 100, 10 or 5 μg.

It is clear from the results in Table II that, in the range of concentrations examined, there are no analytically significant differences in either the recovery rate or the standard deviation.

EXAMPLES OF THE USE OF THE METHOD

The results obtained with the proposed method are summarised in Table II. The substances were dissolved in a suitable solvent (usually methanol), either singly or as mixtures, and, generally, 10 μl of the solution were spotted on to the plate from

TABLE II

PRACTICAL EXAMPLES INCLUDING RECOVERY AND STANDARD DEVIATION

In all instances, spots on the plates were detected in UV radiation (254 nm) and eluted with methanol.

Substance	*Plate*[*]	*Solvent*[**]	*Amount applied (µg)*	*Elution*		*Method of determination*	*Recovery (%) (average of 12 determinations)*[***]	*Standard deviation for each single determination (%)*[§] *(calculated from 12 determinations)*
				ml/min	*total volume (ml)*			
Single substances								
Paracetamol	1	1	60	0.08	1.0	Extinction measured at 248 nm after dilution to 10 ml with methanol	98.0	0.4
Paracetamol	1	1	6	0.08	1.0	Extinction measured at 248 nm	98.9	0.2
Caffeine	1	2	100	0.08	1.0	Extinction measured at 272 nm after dilution to 10 ml with methanol	98.9	0.9
Caffeine	1	2	10	0.08	1.0	Extinction measured at 272 nm	100.3	0.7
Caffeine	1	2	5	0.08	1.0	Extinction measured at 272 nm	100.0	0.5
Benzocaine	2	3	10	0.2	2.0	Extinction measured at 292 nm	99.9	0.8
Leucinocaine	3	4	30	0.2	2.5	Extinction measured at 293 nm	98.3	0.8
Thioridazine	2	5	15	0.2	2.0	Extinction measured at 262 nm	100.7	0.6
Ergotamine tartrate	2	6	60	0.2	1.0	Van Urk reaction; extinction measured at 545 nm	98.7	0.8

(Continued on p. 284)

TABLE II *(continued)*

Substance	*Plate**	*Solvent***	*Amount applied (µg)*	*Elution*		*Method of determination*	*Recovery (%) (average of 12 determinations)****	*Standard deviation for each single determination (%)* § *(calculated from 12 determinations)*
				ml/min	*total volume (ml)*			
Mixtures								
1 Acetylsalicylic acid, phenacetin and caffeine								
Acetylsalicylic acid	1	7	200	0.08	2.0	Extinction measured at 276 nm	100.2	0.5
Phenacetin	1	7	75	0.08	2.0	Extinction measured at 248 nm after dilution to 10 ml with methanol	97.5	0.5
Caffeine	1	7	25	0.08	2.0	Extinction measured at 272 nm	97.5	0.5
2 Allyl isobutyl barbitone, phenobarbitone and barbitone								
Allyl isobutyl barbitone	1	8	20	0.08	2.0	Extinction measured at 238 nm after addition of 1 ml of 0.1 N NaOH	100.1	0.8
Phenobarbitone	1	8	12	0.08	2.0	As for allyl isobutyl barbitone	100.1	1.0
Barbitone	1	8	36	0.08	2.0		100.1	1.2
3 Allyl isobutyl barbitone and aminophenazone								
Allyl isobutyl barbitone	1	9	30	0.08	1.0	As for mixture 2	101.4	0.8
Aminophenazone	1	9	35	0.08	1.0	Extinction measured at 270 nm after the addition of 1.0 ml of 0.1 N hydrochloric acid	99.9	1.3

4 Caffeine, paracetamol, quinine gluconate and codeine hydrochloride

Caffeine	1	10	25	0.08	2.0	Extinction measured at 272 nm	99.0	0.5
Paracetamol	1	10	40	0.08	1.5	Extinction measured at 248 nm after dilution with 3.5 ml of 0.5 N sodium hydroxide	98.2	0.5
Quinine gluconate	1	10	120	0.08	2.0	Extinction measured at 325 nm	96.3	0.9
Codeine hydrochloride	3	11	25	0.08	1.0	Extinction measured at 450 nm after reaction with dimethylaminobenzaldehyde and H_2SO_4	99.8	1.0

* Plates used: 1 = silica gel $60F_{254}$, pre-coated, (Merck No. 5715); 2 = silica gel GF_{254} basic (Merck No. 7730) 0.25-mm layer; 3 = silica gel GF_{254} (Merck No. 7730) 0.25-mm layer.

** Solvent composition (v/v): 1 = Chloroform–methanol–conc. aq. ammonia ($d = 0.91$) (90:10:2); 2 = Chloroform–absolute ethanol (90:10); 3 = Chloroform–methanol (95:5); 4 = Chloroform–benzene–ether–conc. aq. ammonia ($d = 0.91$) (60:25:15:0.75); 5 = Benzene–acetone–conc. aq. ammonia ($d = 0.91$) (75:15:7.5); 6 = Chloroform–absolute ethanol (100:10); 7 = Benzene–ether–glacial acetic acid–methanol (60:30:9:0.5); 8 = Ether–methanol–conc. aq. ammonia ($d = 0.91$) (90:10:4); 9 = Acetone–cyclohexane (40:50); 10 = Chloroform–methanol–diethylamine (86:10:4); 11 = Chloroform–diethylamine (90:10); 12 = Chloroform–methanol–conc. aq. ammonia ($d = 0.91$) (95:5:0.15).

*** Determined by comparison with a standard solution (containing the stated amount of material in the stated volume of solvent) that had not been submitted to TLC.

§ Standard deviation (%) $= \frac{100}{\bar{X}} \sqrt{\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{n-1}}$, where n (the number of determinations) is 12.

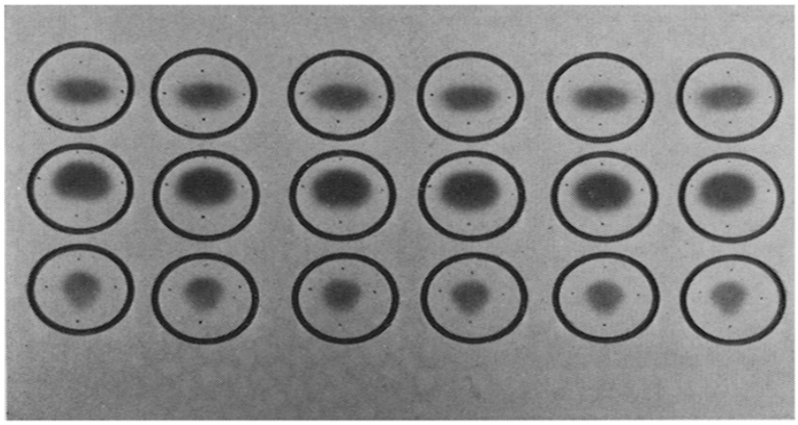

Fig. 3. Developed chromatogram before elution (detection at 254 nm); see text for details.

a Hamilton syringe. The recovery rate and the standard deviation were determined from twelve separate runs on two plates. By way of illustration of the working technique, Fig. 3 shows a photograph (taken under UV radiation at 254 nm) of a plate on which a mixture of acetylsalicylic acid, phenacetin and caffeine has been separated (acetylsalicylic acid, R_F approx. 0.5; phenacetin, R_F approx. 0.4; caffeine, R_F approx. 0.25). The spots were examined under UV radiation and each was marked with four dots; these dots served as reference points for the ring scraper. Fig. 4 shows the plate after elution; it can be seen that, for each spot, elution has been practically quantitative.

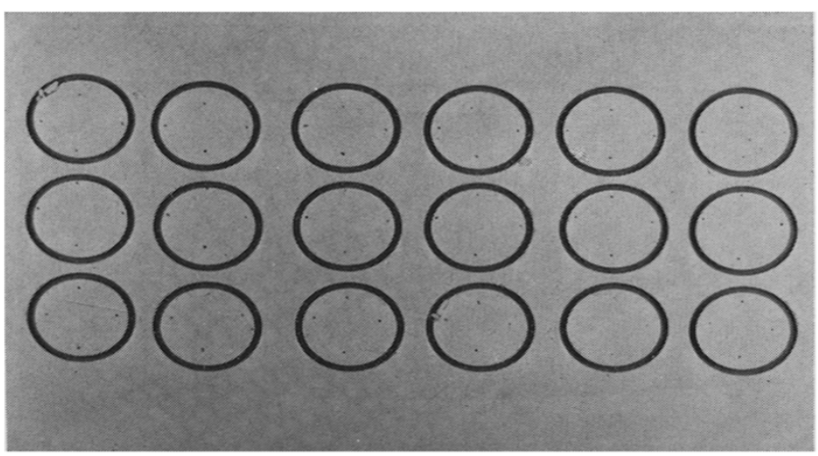

Fig. 4. Developed chromatogram after elution (detection at 254 nm); see text for details.

DISCUSSION

The results obtained show that the proposed method usually yields a higher recovery rate and better reproducibility than do conventional elution techniques. A particular advantage is the considerable saving in time and material in comparison

with the conventional method; this arises principally from the fact that six determinations can be carried out simultaneously on a single plate, so that the number of plates required per analysis is reduced.

Since, within wide limits, the accuracy of the proposed method is only slightly dependent on the amount of material to be determined, the method can be applied with considerable advantage to the analysis of pharmaceutical specialities that contain several drugs in widely differing quantities.

One limitation on the use of the method is that the substance that has been separated on the chromatogram must be detected in some way that does not interfere with the subsequent determination in the eluate. Plates containing a fluorescent indicator and on which spots can be detected by quenching of the fluorescence are particularly suitable, or the substance may be spontaneously fluorescent or it may undergo a colour reaction with iodine vapour.

Another limitation on the use of the method is that the separation of the spots must be sufficient to provide space for the fitting of the elution cylinder. If there is difficulty in obtaining sufficient separation, direct evaluation of the spots with a densitometer is to be preferred.

The time required for the determination of one substance per analysis by the proposed method is about the same as with a densitometer. With the densitometer, however, attainment of a similar degree of accuracy requires at least two determinations per analysis, which means that each plate (20 × 20 cm) will serve for up to three analyses. Where several components are to be examined per analysis, direct evaluation on the plates is quicker. Very expensive equipment is needed to achieve this saving in time with a densitometer. The output from the densitometer can be fed directly into a computer[8–12]; however, with the proposed apparatus, no computer is necessary.

On the other hand, use of the proposed apparatus has the advantage that it is hardly affected by certain disturbing effects that are liable to occur during evaluation *in situ*, especially deformation of the spot (which might arise, for example, from the presence in the chromatogram of large quantities of excipients derived from pharmaceutical preparations, or by peripheral effects associated with the use of complicated mixtures of solvents or by changes in layer thickness).

Likewise, the decomposition of unstable substances on the plate that sometimes takes place while they are undergoing direct evaluation can be avoided to a great extent by using the proposed method. The examples quoted, and the experience gained with them, show that the method constitutes a valuable addition to the technique of quantitative evaluation in TLC.

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REFERENCES

1 H. Gänshirt, in E. Stahl (Editor), *Dünnschichtchromatographie*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1967, p. 33.
2 W. Kinze, *Pharm. Zentralh.*, 104 (1965) 705.

3 H. Jork, *J. Chromatogr.*, 33 (1968) 297.
4 D. Waldi, *Cosmo-Pharma*, 1 (1966) 1.
5 R. W. Frei, in A. Niederwieser and G. Pataki (Editors), *Progress in Thin-Layer Chromatography and Related Methods*, Ann Arbor Sci. Publ., Ann Arbor, Mich., Vol. II, 1971, p. 1.
6 N. Seiler and H. Möller, *Chromatographia*, 2 (1969) 273, 319, 470.
7 E. J. Shellard (Editor), *Quantitative Paper and Thin-Layer Chromatography*, Academic Press, New York, London, 1968.
8 R. R. Goodall, *J. Chromatogr.*, 78 (1973) 153.
9 V. Pollak, *J. Chromatogr.*, 63 (1971) 145.
10 G. Kynast, *Z. Anal. Chem.*, 256 (1971) 20.
11 G. Kynast, *Chromatographia*, 3 (1970) 425.
12 A. A. Boulton and V. Pollak, *J. Chromatogr.*, 63 (1971) 75.