THE USE OF REPEATED DEVELOPMENT IN SPREAD-LAYER CHROMATOGRAPHY

L. STÁRKA AND R. HAMPL

Research Institute of Endocrinology, Prague (Czechoslovakia)

(Received March 25th, 1963)

In paper partition chromatography, repeated one-dimensional development of the chromatogram with the same or different solvents is a current technique, and has frequently been utilized[1-4], e.g., in the separation of steroids. To calculate the resulting R_F values after the second development, PRUSÍKOVÁ[3] derived a general relationship, which was verified practically[3-5]. Recently, thin-layer chromatography, i.e. chromatography on a thin non-adhering layer (thin-layer chromatography without binder, spread-layer chromatography), employing the technique of MOTTIER AND POTTERAT[6], has been successfully applied to the chromatographic separation of steroids[7-11]. The present study was undertaken to determine the value of the repeated development in this technique and to establish the factors which influence the mobility of the substances.

METHODS

Chromatographic procedure

Approximately 25 g alumina (activity II–III; 250 mesh) are spread on a glass plate (12 × 27 cm) and a layer 10 cm wide is smoothed by means of a glass rod which is sleeved at each end with a 2 cm length of polyethylene tubing. The thickness of the tubing is 0.6–0.8 mm and this determines the depth of the adsorption layer of alumina on the glass plate. Steroid samples or azodyes in chloroform are spotted approx. 2 cm from the narrower edge of the prepared glass plate.

The glass plate with the adsorption layer is placed in a glass tank (20 × 30 × 20 cm) at a slope of 15–25° from the horizontal and 50 ml of the mobile phase is poured into the bottom of the tank. The chromatogram is developed by the ascending technique until the solvent reaches the other side of the glass plate (approx. 30–45 min).

Detection

17-Ketosteroids were detected by spraying with the Zimmermann reagent (2 parts 1 % ethanolic m-dinitrobenzene, 1 part 5 N aqueous KOH) on the alumina surface when dry. The steroids appear as violet spots without heating after 2–3 min. Care must be taken not to disturb the alumina layer when spraying.

RESULTS AND DISCUSSION

Factors influencing the R_F values in a single run

The flow rate of the solvent front, i.e. the relation between the time and the distance travelled by the front from the start is parabolic, the first 5 cm being run within

5–10 min, depending on the nature of the mobile phase and other conditions, whereas 20 cm requires 30–60 min. The flow rate decreases with increasing slope of the glass plate and with decreasing grain size of alumina.

TABLE I

THE INFLUENCE OF GRAIN SIZE OF ALUMINA ON
THE R_F VALUES OF 17-KETOSTEROIDS

Steroids	R_F	
	grain size	
	< 0.06 mm	> 0.07 mm
Androstane-3,17-dione	0.83	0.58
4-Androstene-3,17-dione	0.73	0.48
5-Androsten-3β-ol-17-one	0.41	0.26
Etiocholan-3α-ol-11,17-dione	0.28	0.18
5-Androsten-3β-ol-7,17-dione	0.23	0.17
5-Androstene-3β,7α-diol-17-one	0.12	0.07
Flow rate of solvent front	22 cm/61 min	22 cm/23 min

Therefore, the R_F values, in the early stages, depend to a considerable degree on the distance the solvent has run and only become constant after a run of more than 10–12 cm. The slope of the glass plate and the distance of the starting line from the level of the solvent are of practically no importance. The R_F values increase with decreasing grain size of the adsorbent (Table I) and are influenced by the thickness of the adsorbent layer (Table II).

TABLE II

THE INFLUENCE OF THE THICKNESS OF THE ALUMINA LAYER ON
THE R_F VALUES OF SOME 17-KETOSTEROIDS

Steroids	R_F	
	Thickness of Al_2O_3-layer	
	0.9 mm	2.7 mm
Androstane-3,17-dione	0.98	0.90
5-Androsten-3β-ol-17-one	0.58	0.46
Androstan-3α-ol-17-one	0.53	0.43
Androstan-3α-ol-11,17-dione	0.23	0.17
Etiocholan-3α-ol-11,17-dione	0.14	0.12
Etiocholane-3α,11β-diol-17-one	0.06	0.05

Repeated development using different solvents

For the repeated development using different solvents with different distances for the respective solvent fronts, PRUSÍKOVÁ[3] derived a general equation, which slightly modified[4] is as follows:

$$``R_F" = a/b \cdot R_1 (1 - R_2) + R_2 .$$

TABLE III

THE R_F VALUES OF SOME 17-KETOSTEROIDS AFTER REPEATED DEVELOPMENT WITH DIFFERENT SOLVENTS FOR THE SAME DISTANCE.

	Mobile phase					
	Chloroform: Diethylether		1. Chloroform 2. Diethylether		1. Diethyl ether 2. Chloroform	
	R_1	R_2	"R_F"		"R_F"	
			Found	Calculated	Found	Calculated
5-Androstene-3β-ol-17-one	(0.49)	0.84	0.86	0.92	0.97	0.92
Androstane-3α-ol-11,17-dione	(0.35)	0.39	0.60	0.62	0.49	0.62
Etiocholane-3α-ol-11,17-dione	(0.33)	0.22	0.45	0.48	0.36	0.48
Etiocholane-3α,11β-diol-17-one	(0.20)	0.32	0.48	0.46	0.29	0.46

where "R_F" is the resulting location of the spot with respect to the second front, R_1 and R_2 are the R_F values in the first and second system, respectively, and a and b are the distances of the front after the first and second development, respectively. This relationship was shown to be valid for the separation of steroids using spread-layer chromatography by the agreement between the experimental results and the theoretical calculation according to the above equation, providing that the first solvent did not substantially alter the activity of the adsorbent, i.e., by the use of less polar solvents. The addition of alcohols, the more polar ethers, or esters to the mobile phase during the first development has a definite influence on the results. The use of two different solvents is justified especially if the solvents differ in their behaviour; sometimes it is advantageous to use a combination of two solvents having electron donor and electron acceptor properties, respectively. Some results are shown in Table III.
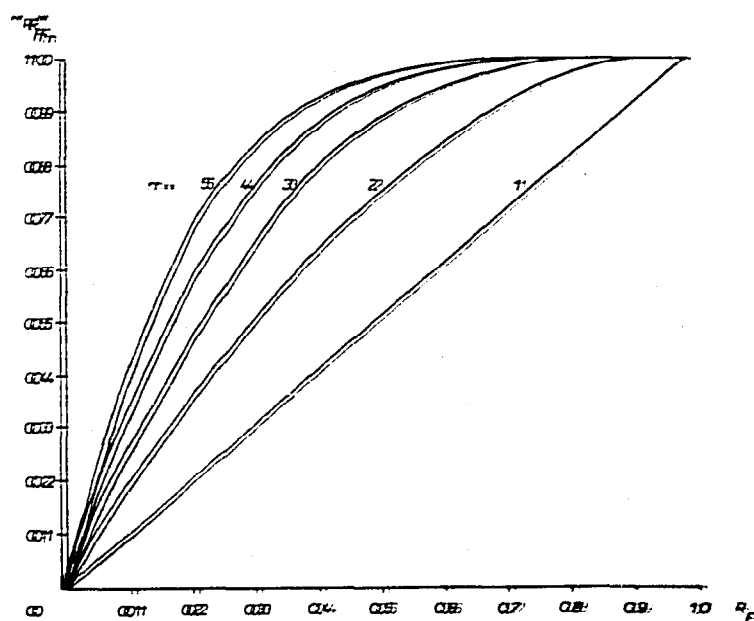

Fig. 1. The apparent "R_F" value after the n-th development in the same system to the identical front distance for any original R_F.

Multiple repeated development in the same system

Multiple repeated development with only one solvent system is now widely employed and has proved successful in many practical cases. If the chromatogram is developed with the same solvent always to the same distance, the equation for the second development is simplified to:

$$"R_F"_2 = 2R - R^2$$

and, for the n-th development it is:

$$"R_F"_n = R(1 - R_{n-1}) + R_{n-1},$$

where $"R_F"_n$ is the apparent R_F after the n-th development, R is the R_F value after the first and R_{n-1} the value after the $(n-1)$-th development. The graphic representation of the resulting mobility related always to the constant solvent front—$"R_F"$—for the first to fifth development and any arbitrary original R_F is given in Fig. 1.

TABLE IV

THE R_F VALUES AFTER THE n-th TIME OF REPEATED DEVELOPMENT WITH THE SAME SOLVENT FOR THE SAME DISTANCE

	$n=$	1	2	3	4
Mobile phase: benzene–ethanol (98:2)					
5-Androsten-3β-ol-17-one	found	0.14	0.24	0.32	0.40
	calc.	—	0.26	0.36	0.45
4-Androstene-3,17-dione	found	0.33	0.50	0.62	0.73
	calc.	—	0.55	0.70	0.79
Mobile phase: tetrachloromethane					
Sudan red	found	0.15	0.30	0.42	0.51
	calc.	—	0.28	0.41	0.50
Sudan yellow	found	0.32	0.53	0.68	0.80
	calc.	—	0.54	0.68	0.78
Azobenzene	found	0.66	0.90	0.95	front
	calc.	—	0.88	0.97	0.99

The validity of this relationship was verified by a repeated development of several 17-ketosteroids in benzene–ethanol (98:2) and in tetrachloromethane and is shown in Table IV. It is also valid in those cases where the activity of alumina may be slightly affected by the mobile phase employed.

SUMMARY

The relationship for $"R_F"$ of some steroids after repeated development in different systems was found to be valid in spread-layer chromatography and a relationship for the mobility of some steroids developed several times in the same system was also established.

REFERENCES

1 R. Neher and A. Wettstein, *Helv. Chim. Acta*, 35 (1952) 276.
2 J. Barrollier and J. Heilmann, *Z. Physiol. Chem.*, 309 (1957) 221.
3 M. Prusíková, *Experientia*, 15 (1959) 460.
4 L. Stárka, *J. Chromatog.*, 4 (1960) 334.
5 P. Lisboa, *Acta Endocrinol.*, in the press.
6 M. Mottier and M. Potterat, *Anal. Chim. Acta*, 13 (1955) 46.
7 V. Černý, J. Joska and L. Lábler, *Collection Czech. Chem. Commun.*, 26 (1961) 1658.
8 L. Stárka and J. Malíková, *J. Endocrinol.*, 22 (1961) 215.
9 S. Heřmánek, V. Schwarz and Z. Čekan, *Collection Czech. Chem. Commun.*, 26 (1961) 1669.
10 V. Schwarz and K. Syhora, *Collection Czech. Chem. Commun.*, 28 (1963) 101.
11 O. Adamec, J. Matis and M. Galvánek, *Lancet*, I (1961) 81.