STEROIDS

CCXI. CENTRIFUGALLY ACCELERATED PAPER PARTITION CHROMATOGRAPHY OF STEROIDS*

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The analysis of steroids by paper partition chromatography[2] has made possible the study of a variety of problems related to their biosynthesis and metabolism. This has been true not only in biochemical studies but also in organic synthesis[3]. Normally the development of a chromatogram in which the solvent travels 30 cm from the line of sample application takes 2 h or more. In reaction rate studies which require many consecutive analyses the time factor can be important. Recently, the advent of centrifugally accelerated paper chromatography[4] presented a possibility for making this technique even more useful by reducing the time for analysis from hours to minutes. The following is a study of the variables in the centrifugally accelerated partition chromatography of steroids on paper.

EXPERIMENTAL AND RESULTS

Apparatus

The apparatus (Fig. 1) consists of two 10 in. aluminium pie plates, B and G, between which is held the impregnated chromatographic paper C. The lower plate, G, is

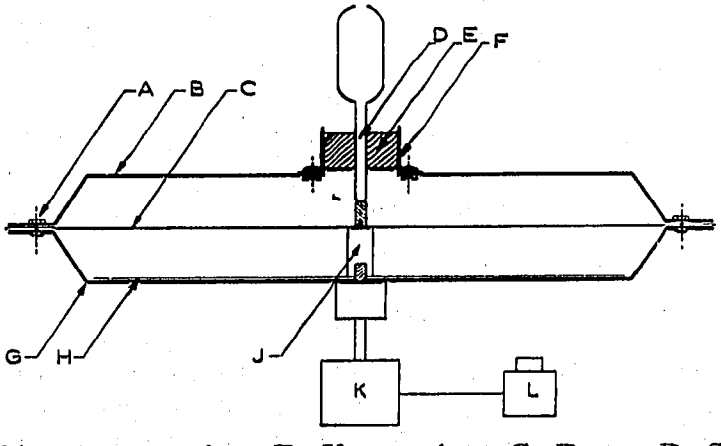

Fig. 1. A: Screw to hold covers together. B: Upper plate. C: Paper. D: Solvent applicator. E: Cork liner to hold applicator. F: Flange to hold cork liner. G: Lower plate. H: Paper liner. J: Brass rod support. K: Motor and shaft assembly. L: Variac to control motor velocity.

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attached to the motor shaft and centered by means of a threaded ½ in. brass rod J, which supports the center of the paper. This support has a raised point which pierces, the center of the paper disc. A Whatman No. 3 MM liner, covers the inside bottom of the lower plate and is held in place by J. The liner is wet with the mobile phase before the start of a run. The upper plate, B, has a 1 in. hole in the center. A flange, F, 1.25 in. high is centered over the hole, and has a cork, E, with a hole for the solvent applicator, D. The whole assembly is held together by means of four screws, A, evenly spaced around the perimeter of the plates. The speed of the motor is controlled by variac, L.

The volatile solvents used in steroid chromatography require that a closed system be used. In addition, paper wet with the stationary phase is not strong enough to be supported only at the center while it is being accelerated centrifugally. Even if supported with pins[5] it does not suspend at the velocity used in this work and therefore it must be held around the edge. The apparatus described meets both of these requirements.

Solvent application

The solvent is applied to the center of the paper disc by means of a paper wick inside a 6 mm internal diameter glass tube 9 cm high with a 4 cm high × 13 mm internal diameter reservoir at the top. An indented ridge about an inch above the lower end prevents the wick from receding completely into the glass tube. The tube is long enough that the wick can be centered on the point of support J before the upper lid is lowered so that the solvent is applied exactly to the center of the paper disc. The wick is made by tightly rolling a one inch width of washed Whatman No. 1 paper around a wooden cotton applicator stick. The stick is recessed to allow for the point on support J.

The velocity of the solvent, passing through the wick is regulated by the amount of paper that is used for the wick. Flow rates of 1 ml/min were used in this work. The wick is calibrated by placing 6 ml of hexane saturated with formamide in the applicator and measuring the time required for a given volume to pass through. Five to six ml of solvent was applied to the paper disc. Due to the effect of centrifugal velocity most determinations required 10 to 15 min to apply this amount.

This method of solvent application was adopted because of the difficulties encountered in using capillaries. After each run, the wick is cleaned of stationary phase by applying vacuum at the upper end of the tube and placing the wick in some methanol, then drying by pulling the wick out of the solvent and allowing air to pass through with vacuum applied. Once a wick is prepared it is good for a large number of determinations.

Paper preparation and sample application

The Whatman No. 1 paper discs were 27 cm in diameter with the center marked and a circle inscribed of 2 cm radius for sample applications. Four holes were punched which corresponded to the screws, A. The discs were impregnated with the stationary phase in the normal manner[2] and the samples applied to the inscribed circle as spots or arcs. It was found that the mode of sample application was very important. If samples were applied dissolved in methanol–chloroform 1:1 there was extensive streaking and tailing in the resulting chromatograms. It was necessary to dissolve the

steroids in chloroform saturated with formamide* if formamide was the stationary phase or benzene if propylene glycol was used. In general 10 to 20 μg of each steroid was applied. No deleterious effect was found in using quantities up to 100 μg.

Centrifugal acceleration

Contrary to what has been found for centrifugally accelerated adsorption chromatography[6] R_F values in accelerated partition chromatography of steroids are not independent of the velocity. At velocities above 200–250 r.p.m. the steroids did not move from the line of application. This is probably because at high velocities the solvent travels too fast to allow for partitioning to occur. The effect of centrifugal acceleration is shown in Table I.

In all subsequent work a velocity of 150 r.p.m. was used.

TABLE I

EFFECT OF CENTRIFUGAL FORCE ON R_F-VALUES OF A MIXTURE OF HYDROCORTISONE, CORTISONE AND 11-DESOXY-17-HYDROXYCORTICOSTERONE[a]

Velocity (r.p.m.)	*Hydrocortisone*	*Cortisone*	*11-Desoxy-17-hydroxycorticosterone*	*Time (min)*
0	0.43	0.81	1.0	40
150	0.47	0.79	0.97	10
300	0	0	0	105

[a] Capillary flow 1 ml/min, Whatman No. 1 impregnated with formamide–methanol, 1:3, chloroform satd. formamide.

Stationary phase content of paper

The amount of stationary phase in the paper affected the appearance of the steroid spots. When formamide–methanol or propylene glycol–methanol 1:1 was used the steroid spots had some tendency to streak and in some cases tailing occurred and no separation was apparent. If too little stationary phase was used (15%) the same effect occurred. Therefore the paper discs were impregnated with methanol solution containing 25% stationary phase. When run under these conditions, the steroid spots elongated laterally but there was no tailing apparent.

R_F values of steroids

To eliminate the possibility of variations in R_F values due to any number of factors, it is best to run standards on the same disc of paper. Although the solvent front assumes an oval shape rather than a circle[6] R_F measurements are still reliable. Thus, when 4 samples of cortisone were run on the same disc, the R_F values obtained were 0.73, 0.74, 0.75 and 0.75 even though the solvent front travelled anywhere from 70 to 77 mm from the line of sample application. In Table II are listed the results of some steroids run separately and in mixtures.

Excess mobile phase can be applied to the paper to give the same effect as in regular descending partition chromatography where the mobile phase is allowed to run off the paper. The advantage in centrifugal chromatography is that the steroids

* Personal communication from Dr. J. DE FLINES, Koninklijke Nederlandsche Gist- en Spiritusfabriek N.V., Delft, The Netherlands, to whom we offer our thanks.

TABLE II

R_F VALUES OF STEROIDS SEPARATELY AND IN MIXTURES

Conditions: 150 r.p.m.; stationary phase applied as 25 % in methanol; wick velocity 1 ml/min; 5 to 6 ml applied.

Stationary phase	*Mobile phase*	*Time of run (min)*	*Mixture*	R_F	
				Separately	*Mixture*
Formamide	Chloroform	10	Estriol	0.10	0.08
			Estradiol	0.80	0.74
			Estrone	0.97	0.95
Formamide	Chloroform	10	Hydrocortisone	0.49	0.47
			Cortisone	0.78	0.79
			11-Desoxy-17-hydroxycorticosterone	0.97	0.97

do not leave the paper because the solvent evaporates where the paper extends beyond the plates and the steroids travel only that far.

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SUMMARY

The apparatus and method for centrifugally accelerated partition chromatography of steroids is described. It is shown that the centrifugal velocity must be relatively low for separations to occur. The other factors studied were the mode of sample application and the stationary phase content of the paper. Using the described procedure, separations occur in 10–15 min. This makes it particularly suited to analyses which require short development times such as in chemical or enzymic kinetic studies or to determine the homogeneity of fractions in column chromatography.

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