

SOME FACTORS AFFECTING THE R_F VALUES OF SYMPATHOMIMETIC CATECHOLAMINES

BY D. J. ROBERTS*

From the Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.3

Received March 25, 1963

The behaviour of three catecholamines on paper chromatograms is reported as an investigation of the effects of variables on R_F values. The use of these values as a means of locating and identifying catecholamines on paper is misleading as they are dependent on too many different factors.

PAPER chromatography has been extensively used as a means of separating catecholamines in biological tissues and fluids, but the use of R_F values as an aid to identification is incompatible with the differing values reported for individual catecholamines. The chromatographic behaviour of some catecholamines has been examined, using a single solvent, to seek an explanation of the differences in values.

METHODS

Whatman No. 1 papers were washed with hydrochloric acid (0.01N) by continuous descending flow for 20 hr., dried and cut. Circles of 1 cm. diameter, placed 3 cm. apart, were marked out 5 cm. from the bottom edge of the papers which were then fashioned into cylindrical form (Wolfson, Cohn and Devaney, 1949), and solutions of usually 25 μ g. of each amine in 0.01 ml. distilled water were applied within the marked areas. The papers were placed into tanks (55 cm. high, 14 cm. diameter) containing 180 ml. of phenol containing 15 per cent v/v 0.01N hydrochloric acid, equilibrated with the internal atmosphere for not less than 24 hr. at room temperature. Chromatography was at 22–29° by the ascending technique for either 16 to 22 or 40 to 46 hr., the solvent fronts advancing 20 to 30 cm. and 35 to 44 cm. respectively. After removing the solvent by washing with benzene, and drying, the spots were located by spraying the papers with a solution of potassium ferricyanide (0.44 g.) in sodium hydroxide (100 ml. 0.05N). The R_F values were measured from the centre of each spot.

Drugs. (–)-Noradrenaline acid tartrate (L. Light & Co. Ltd.), (–)-adrenaline acid tartrate (Burroughs Wellcome & Co.) and (±)-isoprenaline sulphate (Burroughs Wellcome & Co.) were obtained commercially.

RESULTS

As expected R_F values were found to depend on the grade of paper (Table I) and temperature (Table II). 2.5 μ g. of adrenaline or noradrenaline, and 1.0 μ g. of isoprenaline were the minimum amounts required to

* Present address: School of Pharmacy, Brighton College of Technology, Moulsecoomb, Brighton, 7.

R_F VALUES OF CATECHOLAMINES

obtain visible spots on oxidation. Although the use of more than 200 $\mu\text{g.}$ of each amine caused streaking and tailing, the volume applied was without effect as shown by the identical R_F values obtained from seven different dilutions each containing 25 $\mu\text{g.}$ of the amines (0.005 ml. to 0.5 ml.).

TABLE I

R_F VALUES OF REFERENCE AMINES COMPARED SIMULTANEOUSLY ON TWO DIFFERENT MEDIUM FLOW GRADES OF PAPER

Solvent, phenol containing 15 per cent v/v 0.1N HCl. Temperature 22 to 25°. Solvent front 25 to 30 cm. above point of application

Paper No.		Catecholamine R_F values		
		Noradrenaline	Adrenaline	Isoprenaline
Whatman No. 1	Range over 13 observations	0.09-0.14	0.34-0.43	0.54-0.64
	Mean with standard error	0.12 (± 0.006)	0.38 (± 0.007)	0.58 (± 0.007)
Whatman No. 40	Range over 13 observations	0.055-0.084	0.23-0.32	0.44-0.55
	Mean with standard error	0.068 (± 0.002)	0.29 (± 0.012)	0.51 (± 0.01)

With papers previously washed with hydrochloric acid (0.01N) compact spots were obtained from all three amines, but when the acid washing process was omitted, tailing of the isoprenaline and, to a lesser extent, the adrenaline spots was observed, while noradrenaline showed complete separation into two spots. That the additional spot was derived from noradrenaline was proved by chromatographing the amines individually.

TABLE II

R_F VALUES OF REFERENCE AMINES CHROMATOGRAPHED AT DIFFERENT TEMPERATURES

Solvent, phenol containing 15 per cent v/v 0.1N HCl. Solvent front 18 to 22 cm. above point of application

Catecholamine	Temperature °C		
	11	23	40
Isoprenaline	0.55	0.60	0.72
	0.53	0.61	0.73
Adrenaline	0.36	0.41	0.52
	0.35	0.41	0.53
Noradrenaline	0.13	0.14	0.25
	0.12	0.15	0.25

In addition, the R_F values of each of the amines were markedly reduced on papers not washed with acid. The effects of washing with acid were reproduced when the papers were sprayed with hydrochloric acid (0.01N), but not when they were sprayed with distilled water. In all instances the right half of the paper only was sprayed, the remainder serving as control. On chromatographing the amines (25 $\mu\text{g.}$) from hydrochloric acid (0.01N) solution (0.025 ml.) on papers half-sprayed with acid as above, noradrenaline produced only a single spot on both the untreated and acid-treated paper but the tailing of the isoprenaline and adrenaline spots and the lower R_F values on the unsprayed half were still evident.

When the amines were applied at 5 cm. intervals from the bottom edge of the paper the results indicated that R_F values increased with the distance of the solvent front above the application point or decreased with the distance of this point from the solvent surface (Fig. 1). Further experiments in which the distance of the solvent front above a fixed application point was varied showed that R_F values were lower the further the solvent front advanced. Fig. 1 also shows that on one occasion this relationship was not true for the application point nearest the solvent surface.

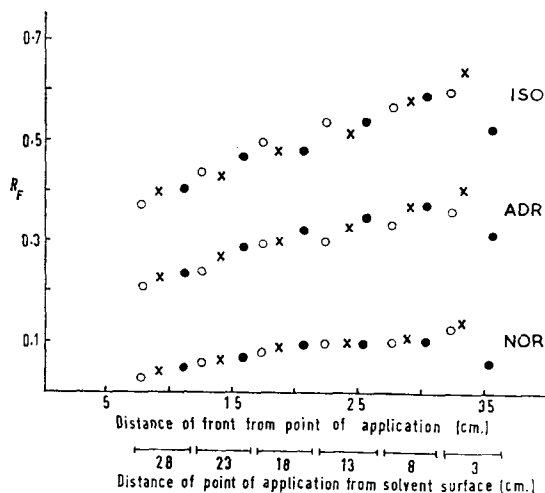


FIG. 1. R_F values of noradrenaline (NOR), adrenaline (ADR) and isoprenaline (ISO) developed in phenol containing 15 per cent v/v 0.1 N HCl when applied from distilled water at varying distances from the solvent surface. Open circles, crosses and closed circles represent three different experiments.

Successive running of chromatograms in any one tank resulted in a fall in R_F values, the effect appeared to be dependent on the number of times the solvent was used and was intensified by omission of the equilibration process or by gassing the tanks with carbon dioxide before use. In the absence of equilibration, prespraying the papers with distilled water and allowing them to dry at room temperature resulted in significant (but smaller than after acid treatment) increases in R_F values an effect that was completely inhibited by drying the papers at 40°. The R_F values on acid-sprayed papers remained high after drying at 40°. A return of R_F values to levels slightly higher than originally obtained was observed after beakers of distilled water or hydrochloric acid (250 ml. 0.01N) had been placed in the centre of the solvent and the internal atmosphere of the tank allowed to equilibrate for 12 hr. When some of the previous experiments were repeated in the presence of this excess of aqueous phase in a tank of fresh solvent, the results were qualitatively the same as before, but the R_F values were higher and showed good stability with time. The R_F values of the amines when applied close to the solvent

R_F VALUES OF CATECHOLAMINES

surface, however, were always lower than expected when curves similar to Fig. 1 were plotted.

Aqueous solutions of the amines were also chromatographed using distilled water as the developing solvent. The papers were rapidly dried at 40° before location of the spots to prevent excessive diffusion, and although no separation was evident the amines appeared as streaks in the bulk of the paper.

DISCUSSION

It is now accepted that simple partition between a stationary aqueous phase supported by the paper and a mobile organic phase is not the sole phenomenon involved in the separation of substances by paper chromatography. The results obtained using distilled water as the developing solvent and the demonstration that R_F values are dependent upon the grade of paper used indicate that adsorptive forces are important and that the "water cellulose complex" (Haynes and Isherwood, 1949; Martin, 1950), and hence the distribution isotherm, varies from paper to paper.

In general, R_F values fall with decreasing water content when miscible pairs of liquids are used as chromatographic solvents. In the phenol-hydrochloric acid system used for this study, preferential absorption of water results in the solvent becoming more "phenolic" as it flows up the paper. This phenomenon of "frontal analysis", previously noted in the collidine-water system by Kowkabany and Cassidy (1952), together with the change in the water content of the solvent as each successive chromatogram is taken out of the tank, offers an explanation of why the R_F values obtained vary inversely with the distance the solvent travels and with the number of times the solvent is used.

In the absence of prior saturation or equilibration of the paper, conditions required for "real partition chromatography" (Tscheshe, Grimmer and Seehofer, 1953), the stationary phase is taken either directly from the developing solvent or from its vapour during chromatography. The marked influence on R_F values of decreasing the water content of the vapour by gassing with carbon dioxide and of increasing the water content of the vapour by using beakers of water or acid, or increasing the temperature, infers that the vapour is a major source of the stationary phase; but the aqueous content of the vapour is dependent on the proportion of water in the liquid solvent. The dimensions of the tank, together with the small surface area of the developing solvent, makes it doubtful whether true equilibrium conditions are achieved throughout the vapour phase, and the lowering of R_F values with increased distance of solvent flow could be the result of increased evaporation of water from the higher areas of paper, where the vapour phase is not saturated as it would be near the solvent surface. The hypotheses of a concentration gradient for the water content of the vapour phase inside the tank and of "frontal analysis" are both supported by the fact that the higher the R_F value of the amine the steeper the slope of the linear curve of R_F value versus the distance travelled by the solvent (Fig. 1). Such a relationship might not be expected to hold true for an application point near the solvent

surface, where oversaturation of the paper with aqueous phase could result in interference with partition due to the marked water solubility of the amine salts.

Production of a bound stationary aqueous phase by spraying the paper with distilled water again results in higher R_F values, but the increased migration observed on acid-treated papers and their stability after procedures sufficient to drive off any bound water require further explanation.

The lack of demonstrable differences in the chromatographic behaviour of the amines between acid-washed and acid-sprayed papers means that it is not the removal by washing of traces of metal ions, responsible for retardation and tailing of spots (Haynes and Isherwood, 1949), that gives rise to increased R_F values. Alteration of the physical properties of the paper (increase in capillary size, reduction in adsorption) by the acid, and suppression of the ion-exchange properties of the free carboxylic groups of the carbohydrate network could be responsible for the beneficial effects of pretreating the paper with acid, but probably more than one factor is involved and more work is needed to elucidate the problem.

The phenomenon of multiple spot formation shown by noradrenaline on untreated papers may be explained in terms of the arguments presented by Beckett, Beaven and Robinson (1960) in their report on the effects of acids, other than those present in the developing solvent, on the formation of amine spots. The hydrochloric acid present in the developing solvent converts some of the noradrenaline acid tartrate, when applied from distilled water, to noradrenaline hydrochloride. Two spots are then formed on development; the lower one being noradrenaline associated with tartaric acid, and the upper one noradrenaline associated with hydrochloric acid.

The fact that chromatography of the same amounts of amine, from a concentration of hydrochloric acid sufficient to convert all of the noradrenaline to the hydrochloride salt, results in the formation by noradrenaline of a single spot, of the higher R_F value, lends support to the above suggestions.

The R_F values of adrenaline and isoprenaline must differ sufficiently from those of tartaric, hydrochloric and sulphuric acid to be uninfluenced by them.

Furthermore, since application of the amines from an excess of hydrochloric acid still resulted in lower R_F values on the untreated paper, conversion of the amines to hydrochloride salts is also eliminated as a cause of the increased migration on the acid-treated paper.

The results described are subject to the limiting sensitivity of the spray reagent, but serve to demonstrate the variability of catecholamine R_F values.

Acknowledgement. This work was undertaken while the author was receiving an educational grant from the Pharmaceutical Society of Great Britain.

REFERENCES

- Beckett, A. H., Beaven, M. A. and Robinson, Ann E. (1960). *J. Pharm. Pharmacol.*, **12**, *Suppl.*, 203T-216T.

R_F VALUES OF CATECHOLAMINES

- Haynes, C. S. and Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107-1109.
Kowkabany, G. N. and Cassidy, H. G. (1952). *Analyt. Chem.*, **24**, 643-649.
Martin, A. J. P. (1950). *Ann. Rev. Biochem.*, **19**, 517-542.
Tschesche, R., Grimmer, G. and Seehofer, F. (1953). *Chem. Ber.*, **86**, 1235-1241.
Wolfson, W. Q., Cohn, C. and Devaney, W. A. (1949). *Science*, **109**, 541-543.