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CHROMATOGRAHIC MULTISPOT PHENOMENA AS A POSSIBLE SOURCE OF HYPOTENSIVE SUBSTANCES IN TRICHLOROACETIC ACID EXTRACTS OF RABBIT HEART

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Abstract—The influence of trichloracetic and hydrochloric acids on the paper chromatographic behaviour of adrenaline and noradrenaline is reported, with special reference to the use of these acids in a published method of preparing extracts of tissues containing catecholamines, for chromatography. In the presence of trichloracetic acid two spots, or one spot with a modified R_f value, were obtained from each amine. By contrast, the extraction technique yielded multiple spots which were unrelated to the use of trichloracetic acid as a protein precipitant; they were, however, related to the use of hydrochloric acid to elute the amines from alumina. The significance of the phenomena is discussed.

IT HAS been suggested that isoprenaline-like substances found in extracts of biological tissues and fluids could have resulted from multiple spot formation by adrenaline in the presence of the hydrochloric acid used during certain extraction procedures.[1] Experimental evidence supporting this suggestion was presented. Hypotensive (rat blood pressure) and positive inotropic (rat auricles) activity has recently been reported in eluates prepared from strips cut at the isoprenaline R_f value from paper chromatograms of rabbit and guinea pig heart extracted with trichloracetic acid.[2] A variety of sympathomimetic and other naturally occurring amines have been shown to produce more than one spot when chromatographed on paper in the presence of trichloracetic acid,[3–6] but these multispots were of different origin to those demonstrated following the use of hydrochloric acid.[1,7] Prior to chromatography the catecholamines in the trichloracetic acid extracts of Jacob and Maitre[2] were adsorbed onto alumina and eluted therefrom with hydrochloric or sulphuric acids.

We were interested, therefore, in further investigating the influence of trichloracetic, hydrochloric and sulphuric acids on the paper chromatographic behaviour of catecholamines and in examining the possibility that under the extraction and chromatographic conditions described by Jacob and Maitre,[2] adrenaline or noradrenaline could give rise to an artifact spot at the isoprenaline R_f value.

EXPERIMENTAL

The materials and techniques used for chromatography were the same as described previously.[1,7,8] Potassium ferricyanide (0·5 g) in sodium hydroxide solution (100 ml, 0·5 N) was used to locate catecholamines and multispots; trichloracetic acid was

detected by spraying dried chromatograms with an aqueous solution of bromophenol blue (0·04% w/v) containing 5% w/v ethanol followed by potassium iodide (1% w/v) followed by potassium iodate (1% w/v) starch solution.[6]

RESULTS AND DISCUSSION

50, 100 and 200 μg quantities of adrenaline base were chromatographed from solution (2·5 mg/ml) in trichloracetic acid (0·1–10% w/v) in parallel with adrenaline acid tartrate and isoprenaline sulphate from solution in distilled water as references (Table 1). From solution in 0·1% trichloracetic acid all concentrations of adrenaline produced

TABLE 1. THE INFLUENCE OF TRICHLORACETIC ACID ON THE CHROMATOGRAPHIC BEHAVIOUR OF ADRENALINE

	Concentration of trichloracetic acid % w/v					
R_f values	0·1	0·25	0·5	1	5	10
Trichloracetic acid	Not detectable	Not detectable	Not detectable	0·60	0·73	0·73
Adrenaline Range	(a) 0·39–0·46 (b) 0·56–0·66	0·55–0·67	0·54–0·63	0·57–0·70	0·70–0·81	0·71–0·80
Adrenaline Mean	(a) 0·43 (b) 0·62	0·61	0·60	0·64	0·75	0·76

Developing solvent, phenol containing 15% v/v 0·1 N HCl. Mean figures calculated from at least six observations. In the same solvent system R_f values from solution in distilled water were for adrenaline 0·35–0·44 (mean 0·38) and for isoprenaline 0·52–0·64 (mean 0·58).

two spots having R_f values (0·43 and 0·62) similar to those of adrenaline (0·38) and isoprenaline (0·58). Only one spot was obtained from the other solutions but with increase in the concentration of trichloracetic acid the R_f value of this spot increased from 0·61–0·76. Rationale was given to these observations when it was found that the location of trichloracetic acid on the paper varied with the concentration in a similar manner (Table 1). Similar results were obtained with noradrenaline (R_f 0·17) and the R_f values of the two spots obtained in the presence of the dilute trichloracetic acid (0·18 and 0·57) confirms the association of the upper spot with the acid. Beckett, Beavan and Robinson[5,6] have previously investigated the formation of multiple spots by sympathomimetic amines in the presence of trichloracetic acid and although our use of the phenol/HCl solvent system gave us qualitatively different results, their explanations of the phenomena appear to fit our findings. Thus, since the p*Ka* of trichloracetic acid (0·65) is much less than that of phenol (9·89), and the R_f value of trichloracetic acid (0·6–0·7) is greater than that of adrenaline (0·35–0·44) or noradrenaline (0·16–0·22), double spot formation may be anticipated. The fact that we were only able to demonstrate the phenomenon from dilute trichloracetic acid indicates that under our conditions the formation of the second spot is much more sensitive to changes in the relative proportion of the base to the acid than under the conditions described by Beckett *et al.*[5,6] 2·5 mg adrenaline in 1 ml 0·1% trichloracetic acid represents a slight molar excess of base, and the faster of the two spots seen when this solution is chromatographed (R_f 0·62) is associated with the trichloracetic acid (R_f 0·60) while the slower spot (R_f 0·43) consists of the free amine in equilibrium with the

solvent. The same amount of adrenaline dissolved in 1 ml 0·25% v/v trichloracetic acid however represents a two-fold molar excess of acid, and in this case all of the base is associated with the acid giving one spot at the higher R_f value. In the experiments described by Beckett *et al.*,[5,6] however, adrenaline produced two spots even in the presence of a 20-fold molar excess of trichloracetic acid. The increase in the R_f value of our faster running spot to 0·76 when 10% w/v trichloracetic acid (R_f 0·73) was used to prepare the adrenaline solution is additional evidence of its association with the acid.

When trichloracetic acid is used as a protein precipitant during the preparation of a biological extract containing amines for paper chromatography therefore, some or the whole of the amine (depending on the degree of removal of the acid prior to chromatography) could appear on the paper at the acid R_f value instead of the base R_f value. In the phenol HCl (this paper) and the butanol:acetic acid: water[6] solvent systems trichloracetic acid has an R_f value similar to that of isoprenaline and therefore the use of this acid with either of these two solvent systems could give rise to biologically active material at the isoprenaline R_f value on chromatograms of tissue extracts containing adrenaline or noradrenaline. It should also be noted that this phenomenon is not unique for sympathomimetic amines since it has already been shown that acetylcholine can complex with trichloracetic acid[9] and that in the water saturated butanol solvent system it has an R_f value of 0·5–0·9 when chromatographed in the presence of this acid instead of its usual R_f of 0·05–0·15 when chromatographed from solution in water.[10] This may have already resulted in confusion concerning the nature of the substance responsible for acetylcholine-like activity in brain extracts.[10]

Our investigations were continued by subjecting 200 μg quantities of adrenaline base (sufficient to allow the detection of a 1 per cent conversion to other ferricyanide sensitive substances) to the extraction processes of Jacob and Maitre[2] as if they were in fact whole hearts; in some of these experiments trichloracetic acid was omitted. In 12 out of 15 experiments in which hydrochloric acid was used to elute the adrenaline from the alumina, multiple spots were obtained when the 'extracts' were chromatographed on acid washed papers, although tailing marred some of the experiments. The number and R_f values of the multispots were inconsistent from experiment to experiment, but there appeared to be no qualitative difference between the chromatograms of trichloracetic acid 'extracts' and those of distilled water 'extracts' (Fig. 1). By contrast their R_f values corresponded well with those of some of the multispots previously demonstrated with adrenaline and hydrochloric acid.[7,11] Our suspicions were confirmed when similar multispots were obtained on chromatograms of solutions of adrenaline base (200 μg) in 10 ml 0·25 N HCl (the amount used to elute the adrenaline from the alumina) which had been evaporated to small volume under reduced pressure.

When sulphuric acid (0·25 N) was substituted for hydrochloric acid in all of the above experiments, the resultant concentrated extracts dissolved the paper and chromatography was impossible. It has however been shown, using thin layer chromatography on silica gel plates, that in contrast to hydrochloric acid multiple spots are not evident when adrenaline is chromatographed from concentrated sulphuric acid (Broadley and Roberts, unpublished results).

Cardiac tissue from rabbits and guinea pigs contains more noradrenaline than adrenaline,[2] but noradrenaline also produces multiple spots in the presence of hydrochloric acid[7,11] (Fig. 1). We therefore suggest that the extraction technique under

investigation[2], when used for preparing rabbit and guinea pig hearts for chromatography in phenol HCl would give rise to artifact material from noradrenaline (R_f 0·2) at approximate R_f values of 0, 0·025, 0·4 and 0·6 and to a lesser extent from adrenaline (R_f 0·5) at approximate R_f values of 0, 0·3, 0·65 and 0·8 .Since under normal

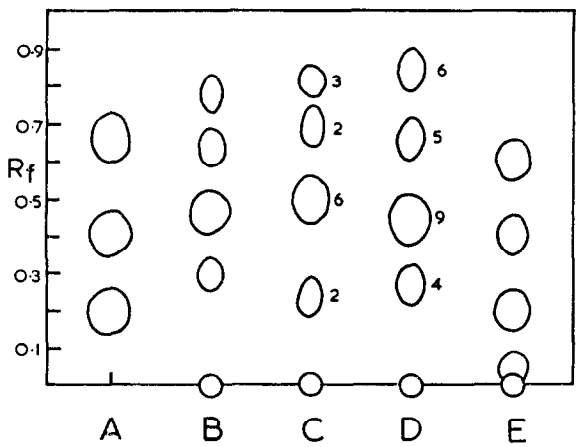

FIG. 1. Mean R_f values of multiple spots obtained from adrenaline and noradrenaline. At A,400 μg of isoprenaline sulphate (upper spot), adrenaline acid tartrate, and noradrenaline acid tartrate (lower spot) chromatographed individually from solution in distilled water; at B,200 μg adrenaline base and at E, 200 μg of noradrenaline base, both chromatographed from solution in 10 N HCl; at C, 200 μg adrenaline base 'extracted' as for rabbit hearts using trichloracetic acid (six experiments and at D,400 μg adrenaline acid tartrate 'extracted' as for rabbit hearts using distilled water instead of trichloracetic acid (nine experiments). Figures next to the spots indicate the number of experiments in which each spot was seen.

circumstances spots on paper chromatograms from tissue extracts extend over about one R_f unit, this amounts to an almost continuous deposition of material from the application point to the solvent front. In the absence of added catecholamine artifact spots are not demonstrable on chromatograms of extracts of rabbit or guinea pig hearts, but with the small amounts of endogenous amine present in these hearts the extra spots may well remain undetected by the alkaline ferricyanide spray reagent; small amounts of artifact substances might nevertheless be present and could have potent biological actions.

Eluates prepared from strips cut at these higher R_f values from chromatograms of 200 μg quantities of amine in hydrochloric acid concentrates show pressor activity on rat blood pressure preparations however, although when smaller amounts of amine are used (1–10 μg) depressor activity can be demonstrated; only small amounts of amine are present in rabbit and guinea pig heart. We further suggest that the intermittent use of sulphuric acid to elute the amines from the alumina could be the reason why hypotensive fractions were absent in some heart extracts.[2]

From the results of previous experiments involving two dimensional chromatography and the use of antioxidants,[12] the substances responsible for the adrenaline multispots described in this paper may be tentatively identified as (working upwards from the base-line in Fig. 1) adrenaline bound to the paper by ether linkages; di-adrenaline ether;[13] adrenaline at its usual R_f value; 5,6-dihydroxy-*N*-methyl indole and

leucoadrenochrome. The reputed absence of any α and β adrenergic activity in either of these latter two compounds[14] is at variance with the biological activity found in our eluates, but other factors such as pH and tonicity can be involved in the production of active eluates;[15] further work will be required to clarify this point. For the moment, however, the results of our preliminary studies with eluates from the higher R_f value artifact spots indicate that while their activity is essentially adrenaline-like, the ratio of α:β[16] adrenergic activity is biased towards the β side. These results would appear to substantiate our proposal that the substances at these R_f values, formed as a result of using and concentrating hydrochloric acid during the extraction techniques, are responsible for isoprenaline-like activity in tissue extracts.

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