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The influence of hydrochloric acid on the chromatographic behaviour of sympathomimetic catecholamines

D. J. ROBERTS

The behaviour of three catecholamines on paper chromatograms is shown to be markedly influenced by hydrochloric acid. Double spot formation exhibited by noradrenaline acid tartrate, for example, results from the use of hydrochloric acid in the developing solvent, but the formation of the second spot is prevented when the amine is applied to the paper from solution in dilute hydrochloric acid. Retention of amine at the application area and additional multiple spot phenomena are exhibited by all of the three amines investigated when chromatographed from concentrated hydrochloric acid. The significance of these and related observations is discussed in relation to the use of hydrochloric acid during the separation of catecholamines from biological tissues and fluids.

IN 1952 Shepherd & West demonstrated that adrenaline can form multiple spots when chromatographed on paper in the presence of trichloroacetic and certain other acids and they attributed their results to the formation of a complex between the phenolic amines and the acids as described by Kendall (1916). Tissue amines other than catecholamines can in certain circumstances also produce more than one spot (West, 1959). The phenomenon was further investigated and elucidated by Beckett, Beaven & Robinson (1960a,b) who warned against the use of trichloroacetic acid as a protein precipitant during the preparation of biological extracts for paper chromatography.

Double spot formation by noradrenaline acid tartrate in the presence of hydrochloric acid has also been reported (Roberts, 1963a) and because hydrochloric acid is used during both the extraction and the paper chromatographic separation of sympathomimetic catecholamines (Vogt, 1952; Lockett, 1954; Roberts, 1963b) it was decided to investigate the ways in which this acid might influence the chromatographic behaviour of these amines.

Methods

The apparatus, materials and techniques used have been previously described (Roberts, 1963a). Noradrenaline, adrenaline and isoprenaline were chromatographed on Whatman No. 1 papers (washed or sprayed with 0·01 N hydrochloric acid or untreated) from distilled water or hydrochloric acid (0·01, 0·1, 1·0 and 10 N) solution. The developing solvent was phenol containing 15% v/v 0·1 N hydrochloric acid and the amines were located by spraying the developed chromatograms with potassium ferricyanide (0·44 g) in sodium hydroxide solution (100 ml, 0·5 N).

From the Department of Physiology and Pharmacology, Chelsea College of Science and Technology, Manresa Road, London, S.W.3.
Present address: The Department of Pharmacology, School of Pharmacy, Brighton College of Technology, Lewes Road, Moulsecoomb, Brighton 7.

Two dimensional chromatograms were first developed as in the single dimensional experiments and the papers were washed free of the phenolic solvent with benzene (without locating the amines). When dry the papers were refashioned into new cylinders at right angles to the original direction of flow, and development was continued in this second direction. The positions of the amines after the first development were arrived at by comparison with single dimensional chromatograms developed simultaneously.

Drugs. (−)-Noradrenaline acid tartrate (L. Light & Co. Ltd.), (−)-adrenaline acid tartrate (Burroughs Wellcome & Co.) and (±)-isoprenaline sulphate (Burroughs Wellcome & Co.) were obtained commercially. The doses quoted in the text refer to the quantity of amine calculated as its salt.

Results

Each amine (25μg) chromatographed from 0·01 ml distilled water or hydrochloric acid (0·01, 0·1, 1·0 and 10 N) behaved typically as shown in Fig. 1. When chromatographed from distilled water, isoprenaline and

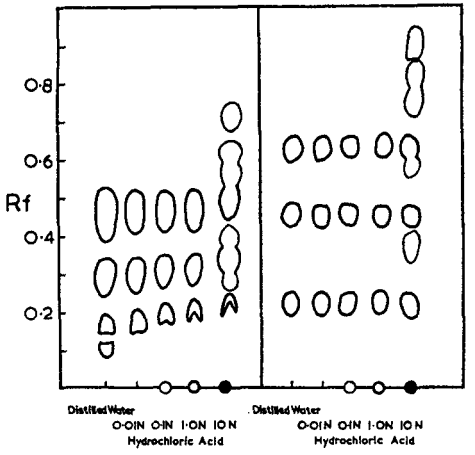

FIG. 1. The influence of application from hydrochloric acid on the chromatographic behaviour of noradrenaline (lower spots), adrenaline (middle spots) and isoprenaline (upper spots) on untreated (left hand side) and acid-treated (right hand side) papers. Circles on the base line represent retention of catecholamine. Developing solvent, phenol containing 15% v/v 0·1 N HCl.

adrenaline showed tailing, and noradrenaline complete separation into two spots, on untreated paper; on acid-washed or acid-sprayed papers all three amines gave compact spots with higher Rf values. On untreated papers increasing the concentration of acid was without effect on the Rf values of isoprenaline and adrenaline and did not prevent the tailing phenomenon; noradrenaline produced a variety of results ranging from double spot formation (distilled water), through a single spot with the higher Rf value (0·01 N HCl), to a crescent-shaped spot showing even

greater movement up the paper (0·1 and 1·0 N HCl). On acid-treated papers compact spots were obtained from all three amines and although the Rf values were higher than on the untreated papers, application from hydrochloric acid was without effect on these values (Fig. 1). When the conditions were such that noradrenaline applied from hydrochloric acid had similar Rf values to those of adrenaline applied from distilled water (untreated paper, less than 25 cm solvent development), the adrenaline and isoprenaline Rf values obtained from the acid solutions were also increased.

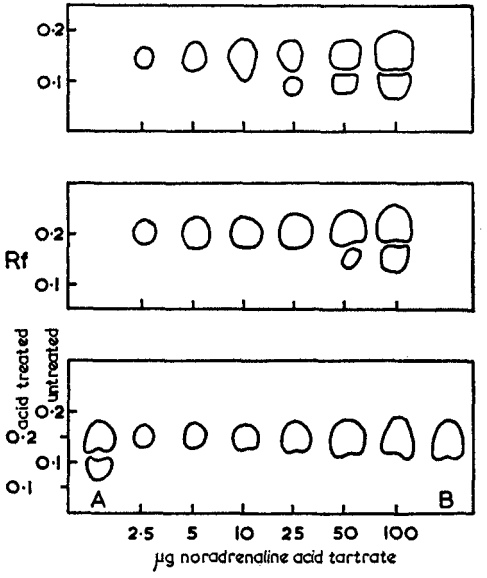

FIG. 2. The influence of hydrochloric acid, in the developing solvent (upper diagram, untreated paper), on the paper (middle diagram, acid-treated paper) and as a solvent for the amine (lower diagram, untreated or acid-treated paper), on the chromatographic behaviour of noradrenaline acid tartrate. Developing solvent, phenol containing 15% v/v 0.1 N HCl.

The double spot formation exhibited by noradrenaline was further examined by chromatographing 2·5, 5, 10, 25, 50 and 100 μg of the amine (1·0 mg/ml) from distilled water and hydrochloric acid (0·01 N). Typical results are shown in Fig. 2. Application from distilled water on untreated papers resulted in single spots of the higher Rf value when 10 μg or less were chromatographed; double spot formation was evident when 25 μg or more were used (Fig. 2, upper diagram). When the amines were applied from hydrochloric acid, however, the faster running spot only was obtained from all concentrations chromatographed (Fig. 2, lower diagram) On acid treated papers, 2·5 to 25 μg gave single spots of high Rf value, 50 μg gave a similar spot plus a smaller one of lower Rf value and 100 μg gave two equally sized spots, when applied from distilled water (Fig. 2, middle diagram). Application from hydrochloric acid again resulted

in compact spots of the higher Rf value for all concentrations of noradrenaline applied. However, when 100 μg amounts of noradrenaline were chromatographed from more concentrated solutions (10 mg/ml, distilled water or hydrochloric acid), double spot formation was obtained from distilled water and hydrochloric acid (0·01 N), but from hydrochloric acid (0·1 N) only a single spot of the higher Rf value was obtained (Fig. 2, lower diagram A and B). These results were obtained on both untreated and acid-treated papers.

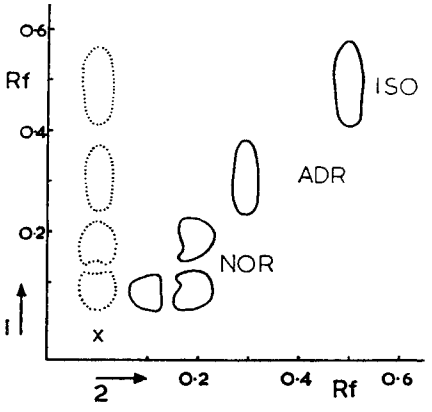

FIG. 3. Combined two-dimensional chromatograms of noradrenaline acid tartrate (NOR), adrenaline acid tartrate (ADR) and isoprenaline sulphate (ISO) developed in phenol containing 15% v/v 0·1 N HCl on untreated papers. Continuous outline indicates the positions of the spots after development in the second dimension. Dotted outline indicates the positions of the spots after the first development.

Further information was obtained by developing individual two dimensional chromatograms of noradrenaline, adrenaline and isoprenaline (100 μg) applied from distilled water to untreated papers. The single dimensional chromatogram of noradrenaline showed two spots but on re-chromatographing in the second direction the lower spot again separated into two while the upper spot persisted (Fig. 3). The shape and relative location of the two new spots were characteristic of the shape and location of the two spots obtained in the single dimensional chromatogram. One way development of adrenaline and isoprenaline resulted in elongated spots, but on re-chromatographing in the second direction no further tailing was observed and there was no indication of any multiple spot phenomena (Fig. 3).

In contrast to the results described above, chromatography of 25–50 μg of each amine from 0·01 ml hydrochloric acid (10 N) resulted in the formation of many extra spots, but as in the previous experiments higher Rf values were obtained on acid-treated papers (Fig. 1). The pink colour of these additional spots, produced on oxidation with alkaline potassium ferricyanide, was not very intense and the overall impression was one of considerable tailing and streaking between and around the three main spots of noradrenaline, adrenaline and isoprenaline. More definite

boundaries to the additional spots were observed when 100 μg quantities of each amine were chromatographed individually on acid-washed papers. The use of 10 N hydrochloric acid resulted in each amine producing three spots (Fig. 4). The more intense of these spots were obtained at the expected Rf values of the amines chromatographed. The remaining spots had Rf values higher than the corresponding amine from which they were formed, and in each case the colour intensity of the spot nearest the parent catecholamine was greater than that of the spot which had travelled farthest up the paper.

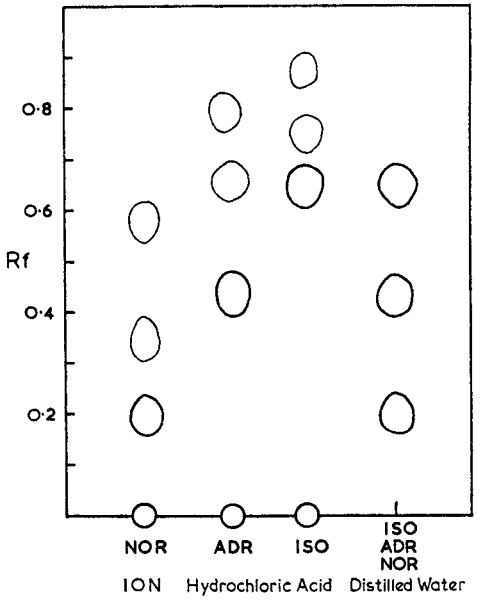

FIG. 4. Multiple spot phenomena exhibited by 100 μg of noradrenaline acid tartrate (NOR), adrenaline acid tartrate (ADR) and isoprenaline sulphate (ISO) when chromatographed from hydrochloric acid (ION) in phenol containing 15% v/v 0.1 N HCl on acid-treated papers. Circles on the base line represent retention of amine. Extreme right, Rf values of the amines obtained from distilled water, for comparison. Some streaking was observed when noradrenaline was chromatographed from 10 N HCl but in the interests of clarity this is not shown.

An additional phenomenon was observed when the stronger acid solutions were used. In all instances, application from 0·1, 1·0 and 10 N hydrochloric acid resulted in considerable quantities of amine(s) being left at the starting point (Figs. 1 and 4). When individual amines (25 μg) were chromatographed from hydrochloric acid (0·01 ml) it became apparent, using the density of colour produced on oxidation as an index of concentration, that the stronger the acid then the greater was the retention of the amines. However, when 25 μg of any one amine was chromatographed from 0·1 ml of hydrochloric acid (0·01 and 0·1 N) even the dilute acid now caused some amine to be left at the application point. Furthermore, the amount of retention obtained with 0·1 N hydrochloric

acid was equivalent to that obtained previously (i.e. 25 μg of amine from 0·01 ml of acid) with 1·0 N hydrochloric acid. This phenomenon was still evident after continuously washing the papers with acid for three days before use.

Discussion

The multiple spot phenomenon observed with noradrenaline agrees with the arguments presented by Beckett, Beaven & Robinson (1960a, b). On papers untreated with acid the hydrochloric acid present in the developing solvent converts some, but not all, of the 25 μg of noradrenaline acid tartrate applied from distilled water to noradrenaline hydrochloride. This partial conversion results in the formation of two distinct spots, the lower one being noradrenaline base associated with tartaric acid and the upper one being noradrenaline base associated with hydrochloric acid. The results expressed in Fig. 2 (upper diagram) confirm this and show that there is sufficient concentration of acid in the solvent to totally convert 10 μg of noradrenaline acid tartrate to the hydrochloride salt thereby resulting in only a single spot of the higher Rf value. After treatment of the papers with hydrochloric acid (0·01 N), however, the combined concentrations of acid on the paper and in the solvent is sufficient to convert 25 μg of noradrenaline acid tartrate to noradrenaline hydrochloride (Fig. 2, middle diagram). The small size of the lower spot (indicative of incomplete conversion to hydrochloride) obtained when 50 μg of the acid tartrate was chromatographed, suggests that, even when this amount was applied, only a small proportion of the base remained unassociated with hydrochloric acid.

The results obtained when noradrenaline acid tartrate was chromatographed from hydrochloric acid solution confirm that the upper spot is associated with hydrochloric acid rather than tartaric acid and are in agreement with expected results when considered on a molar basis. A solution of 1 mg noradrenaline acid tartrate (i.e. 0·5 mg noradrenaline base) in 1·0 ml hydrochloric acid (0·01 N) represents more than a three-fold molar excess of acid and all of the noradrenaline must be present as the hydrochloride salt. Chromatography of any amount of noradrenaline from this solution is thus expected to result in only a single spot of the upper Rf value and this was found to be so (Fig. 2, lower diagram). A solution of 10 mg noradrenaline acid tartrate (i.e. 5 mg noradrenaline base) in 1·0 ml of hydrochloric acid (0·01 N), however, represents a three-fold molar excess of base and as this will result in a large proportion of noradrenaline remaining associated with tartaric acid, two spots are to be expected and are in fact found (Fig. 2, lower diagram, A). Since this was observed on both untreated and acid-treated papers it is inferred that the combined acid concentration of the developing solvent and that present on the paper is insufficient to complete the conversion of noradrenaline acid tartrate (100 μg) to the hydrochloride salt. Similarly, a concentration of 10 mg noradrenaline acid tartrate (i.e. 5 mg noradrenaline base) in 1·0 ml hydrochloric acid (0·1 N) again represents more than

a threefold molar excess of acid and the upper spot only is observed (Fig. 2, lower diagram, B).

The final proof that the double spot phenomenon exhibited by noradrenaline is due to partial conversion of the acid tartrate salt to the hydrochloride salt is obtained from the results of the two dimensional chromatograms. The single dimensional chromatography of 100 μg noradrenaline acid tartrate from distilled water on untreated papers resulted in a fast running spot associated with hydrochloric acid and a slower running spot associated with tartaric acid. If the arguments presented above are correct then re-development of the chromatogram in the second direction, at right angles to the first direction, should result in the formation of three spots. The spot which consists of noradrenaline associated with hydrochloric acid in the first dimension will still run as a discrete spot in the second dimension. The noradrenaline-tartaric acid spot of the first dimension, however, will again separate into two spots in the second dimension as more of the acid tartrate is converted to hydrochloride by the developing solvent; one of these spots, and the single noradrenaline-hydrochloric acid spot, will be equi-distant from the solvent front of the second development. The experimental observations (Fig. 3) comply with these requirements exactly. The fact that adrenaline acid tartrate and isoprenaline sulphate do not exhibit any multiple spot phenomena under similar conditions is interpreted as being the result of the Rf values of these amines differing sufficiently from those of tartaric, hydrochloric and sulphuric acids to be uninfluenced by them. The increases in Rf values of these amines occasionally seen on increasing the concentration of acid used to dissolve them are the result of simple displacement caused by the large upward shift of the noradrenaline spots. The crescent-shaped spots exhibited by noradrenaline when the acid concentration was increased (Fig. 1) are presumably due to concentration of amine at the apex of an elongated acid spot (Beckett, Beaven & Robinson, 1960a, b).

The higher Rf values and absence of tailing by adrenaline and isoprenaline observed on acid treated papers have already been discussed (Roberts, 1963a).

The retention of amine at the application point when applied from strong hydrochloric acid is possibly the same as that noted before with adrenaline by Shepherd & West (1952), although no explanation was offered and drying at an elevated temperature was a prerequisite for this phenomenon in their experiments. In the present case the amount of retention is dependent on the concentration of hydrochloric acid at the application area, for the amount of amine retained from 0·1 ml hydrochloric acid (0·1 N) was the same as that from 0·01 ml hydrochloric acid (1·0 N). A possible cause of the phenomenon is the formation of some insoluble complex between the amines and inorganic ions (not removed from the paper by washing with acid), either with or under the influence of the hydrochloric acid. Alternatively the acid might be causing some change in the molecular and/or ionic structures of the amines causing them to be held at the starting line. Substitution of hydrogen atoms by

chlorine atoms, and the existence of the amines as quaternary ammonium salts in the presence of strong hydrochloric acid, are suggested examples of such changes. The faster moving multiple spots observed when the amines were chromatographed from 10 N hydrochloric acid (Fig. 4) may be due to similar changes in structure giving compounds of differing Rf values.

Double spot formation of the type exhibited by noradrenaline acid tartrate is unlikely to interfere with the chromatography of tissue extracts providing sufficient hydrochloric acid is present in both test and control solutions to ensure that all the catecholamines are in the form of their hydrochloride salts. It is, however, further evidence that the use of different acids in the salt (i.e. during the process of preparing an extract) and solvent system can result in the formation of more than one spot in the chromatography of individual catecholamines. It is significant that when the amines are chromatographed from 10 N hydrochloric acid using the phenol-HCl solvent system, the same acid is being used in the salt and the developing solvent and the multiple spots formed under these conditions must be of different origin to those reported previously in the literature. Furthermore, it may be that the practice of evaporating hydrochloric acid-ethanol extracts to small volume (Vogt, 1952; Lockett, 1954; Roberts, 1963b) is resulting in sufficient concentration of the acid to cause the formation of the substances of higher Rf values discovered in my experiments by the use of 10 N hydrochloric acid. The amounts of these substances formed from the naturally occurring catecholamines present in biological fluid and tissue extracts may be insufficient to show up as spots on the paper, but if they are pharmacologically active they might interfere with the biological assays of the eluates. In this context it is of interest that one of the additional spots formed from adrenaline has an Rf value similar to that of isoprenaline.

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