THE INFLUENCE OF LIPID ON THE PAPER CHROMATOGRAPHIC BEHAVIOUR OF SYMPATHOMIMETIC CATECHOLAMINES IN PLASMA EXTRACTS

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Inconsistent results have been obtained during the separation and estimation of catecholamines in extracts of rabbit plasma and the cause traced to displacements of R_F values by lipid. An extraction technique which overcomes this is described. The possibility of misinterpretation of results after chromatographic separation on paper of the catecholamines from biological extracts is discussed.

USING a modified version (Vogt, 1952) of the recommendations of Crawford and Outschoorn (1951) for the quantitative separation of catecholamines from biological extracts and tissue fluids by paper chromatography, inconsistent recoveries, high blank activities and assay results diverging from parallelism were encountered in this laboratory when estimating concentrations of noradrenaline, adrenaline and isoprenaline in rabbit plasma. The inconsistent results were obtained with the eluates from the paper chromatograms of all three amines. It seemed that, with the phenol-hydrochloric acid solvent system used, neither the formation of lactyl-noradrenaline or lactyl-adrenaline (R_F values 0·57 and 0·80 respectively; Lockett, 1954), nor the interference by 5-hydroxytryptamine (5-HT) or dopamine (R_F values 0·51 and 0·37; Vogt, 1959) were major causes of the irregularities. As the extraction techniques convert all the amines to hydrochloride salts and the developing solvent contained hydrochloric acid, possible multiple spot formation (West, 1959; Beckett, Beaven and Robinson, 1960) was eliminated since the use of the same acid in salt and solvent system resulted in compact spots in the chromatography of divers bases (Munier, 1952). The R_F values of these catecholamines have been shown to be dependent on many factors (Roberts, 1963) it was decided, therefore, to investigate the effects of plasma as a source of explanation of the results obtained.

METHODS

Preparation of the Extracts for Chromatography

Blood was collected from the femoral arteries of heparinised (500 u./kg.) rabbits under urethane anaesthesia (6·0 ml./kg. of a 20 per cent w/v solution in 0·8 per cent w/v NaCl injected intravenously via a marginal ear vein) and was then centrifuged at 3000 r.p.m. for 10 min. The plasma was removed and aliquots mixed with 0·1 ml. of a solution of noradrenaline adrenaline and isoprenaline in distilled water (500 μg. each amine per ml.), before preparation of an extract for chromatography by the methods of Vogt (1952), Peacock (1960) or that now described.

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The plasma was shaken in a large conical flask with six times its volume of ice-cold acid-ethanol (1·0 ml. N HCl/litre absolute ethanol) and left for 1 hr. at −20° to complete precipitation of proteins. The mixture was centrifuged (3000 r.p.m. for 3 min.), the precipitate washed with more acid-ethanol (5–10 ml.), re-centrifuged and the combined supernatants transferred to a suitable R.B. Quickfit flask. The solution was then distilled at 0·5–1·0 mm. Hg (bath temperature 30–35°). The moist residue was shaken vigorously with 4·0 ml. ether : benzene mixture (5 : 2) plus 4·0 ml. 0·01N hydrochloric acid and the resultant mixture transferred to a centrifuge tube. The flask was washed with a further 2·0 ml. 0·01N hydrochloric acid and the total 10·0 ml. centrifuged at 3000 r.p.m. for 5 min. The "milky" upper layer and any interfacial "cream" was removed and the clear aqueous layer remaining was mixed with an equal volume of absolute ethanol, saturated with sodium chloride, and distilled as before. The residue was washed thoroughly with acetone : ethanol (1 : 1) to a total volume of 1·0 ml. and the resultant solution centrifuged (3000 r.p.m., 5 min.), and the supernatant chromatographed on paper.

In some experiments the residues were taken up in distilled water (0·5 ml.) and applied to the paper as an aqueous solution.

Chromatography

Chromatograms were developed by the ascending technique using phenol containing 15 per cent v/v 0·01N hydrochloric acid and apparatus and conditions previously described (Roberts, 1963). The amines were located by spraying the papers with a solution of potassium ferricyanide (0·44 g.) in sodium hydroxide (100 ml., 0·05N). Because of the dependence of the R_F values of the catecholamines on factors such as temperature and distance of solvent flow (Roberts, 1963) extracts were compared with each other on a single sheet of paper and a quantitative relationship between the chromatograms was not sought.

For the recovery experiments, the papers were sprayed with an aqueous solution of ascorbic acid (Crawford and Outschoorn, 1951) before use, and the air inside the chromatography tanks was displaced by carbon dioxide (Vogt, 1952). After development the papers were washed thoroughly with benzene to remove the phenolic solvent and the amines eluted overnight using techniques essentially similar to those described by Crawford and Outschoorn (1951). Distilled water only was used as the eluant since I have found solutions of sodium dihydrogen phosphate to give rise to "blank" activity.

Biological Assay of Eluates

In addition to using the rat uterus, rat colon and rat blood pressure, after urethane or pentobarbitone anaesthesia, for the biological estimation of the eluates containing sympathomimetic catecholamines, extensive use was made of the blood pressure of pithed rats both before and after the potentiation by cocaine (2·5 mg./kg.) administered intravenously.

Results and Discussion

Displacement caused by Plasma

Of 10 experiments, in each of which 2·5, 5 and 10 ml. samples of plasma containing the reference amines were extracted using the method of Vogt (1952) and chromatographed as spots, 6 showed displacements in R_F values as shown in Fig. 1, three resulted in a concentration of the amines around the adrenaline R_F value and one produced an upward migration, greater than shown in the figures, for all three amines. Similar experiments in which the extracts were applied as strips 5 cm. long again resulted in definite, but less marked, shifts in R_F values proportional to the volume of plasma used.

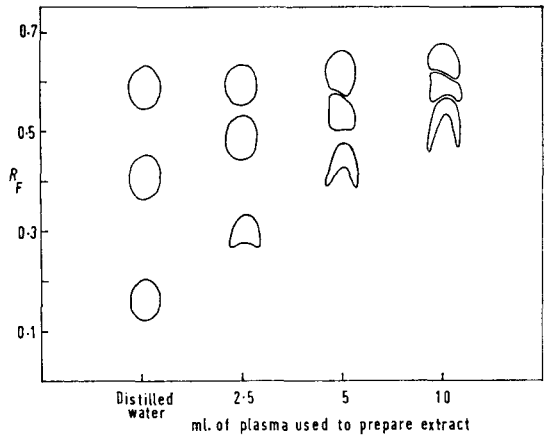

FIG. 1. Displacement of the R_F values of noradrenaline (lower spots), adrenaline (middle spots) and isoprenaline (upper spots) caused by lipid when chromatographed from plasma extracts (Vogt, 1952) compared with values obtained from aqueous solution. Developing solvent, phenol containing 15 per cent v/v 0·1N hydrochloric acid.

The figure indicates that the use of aqueous solutions of catecholamines for reference chromatograms for plasma extracts would give misleading results in that much noradrenaline and adrenaline, being displaced, would not be taken up in their eluates, their biological activity then appearing as "blank" activity at the surrounding R_F value levels. The use of "adjusted" aqueous R_F values (Crawford and Outschoorn, 1951) would be equally misleading. Usually, reference chromatograms are developed from plasma extracts to compensate for any alteration in R_F values, but often any suitable volume of plasma is used, and even when the volumes of the control and test plasma samples are the same, the practice of applying the reference extract as a spot (i.e. a greater local concentration of the interfering substance) and the test extract as a strip, invalidates the control.

Applying a reference extract as a strip uses valuable space on the chromatography paper but an attempt to locate the catecholamines

present in a plasma extract applied as a series of 10 spots, by using a reference extract prepared from 1/10th of the volume of the test sample and applied as a single spot, was unsuccessful.

Protein Precipitation

The course of protein precipitation by acid-ethanol over 24 hr. was followed by treating aliquots of the supernatant with an equal volume of 10 per cent aqueous trichloroacetic acid and comparing the turbidities produced. It was impossible to obtain complete removal of proteins by this method but the conditions described on page 580 gave optimal precipitation.

Lipid Extraction

Crawford and Outschoorn (1951) have described their final extracts as "faintly yellow" and "lipid-like" and I have found this to apply to extracts prepared by the method of Vogt (1952). Papers held to the light showed well-defined grease-spots at the application areas; this indicated that lipid rather than residual protein was responsible for the displacement of the R_F values and that some form of lipid extraction before application to the paper might stabilise the chromatographic behaviour of the catecholamines. An extraction of this kind is included in the method of Peacock (1960) where the supernatant, after acid-ethanol protein precipitation, is evaporated to 5–10 ml. and the lipids removed with ether. Unfortunately when this method was tried, the residual solution after evaporation was miscible with ether presumably as a result of azeotropic mixture formation. When benzene was added to the mixture the acid-ethanol was displaced from solution in the ether. This is in agreement with the results of Lockett (personal communication) who found that a mixture of benzene and ether was the most satisfactory solvent for extracting lipid from human plasma with minimum loss of catecholamine.

The extraction method I have described results in constant R_F values for the catecholamines when they are chromatographed as spots from extracts prepared from 1 to 10 ml. volumes of plasma. These values were higher than those from aqueous solution, but when the amines were chromatographed from 0·9 per cent sodium chloride the R_F values were similarly elevated and therefore saline solutions were used to produce reference chromatograms. By applying the extracts as strips 10 cm. long it was possible to use extracts from plasma volumes of up to 100 ml. without significant change in R_F values. Furthermore, blank activity between the spots was largely eliminated, biological assays were parallel and the grease-spots and "clogging" of the paper at the application areas were no longer evident. The recoveries of 5μg. of each amine were seldom greater than 60 per cent but were relatively constant for extracts prepared from a wide range of plasma volumes.

The Influence of Lipid

The influence of lipid was confirmed by adding varying amounts of lipid, extracted from rabbit plasma, to acetone-ethanol solutions of the

catecholamines when the R_F values were again displaced in proportion to the amount of lipid added. When lipid-free extracts were prepared from volumes of plasma in excess of 10 ml. and applied as spots, displacement of the amines was again observed even when the final residues were applied to the paper from aqueous solution. It was also found that chromatography, using distilled water, of extracts of plasma (1–25 ml.) prepared by the method of Vogt (1952), resulted in constant R_F values up to 10 ml. of plasma after which increased migration, particularly of noradrenaline, was observed. Much larger shifts occurred as the plasma volumes were increased. The interfering substance(s) must be soluble in both distilled water and acetone-ethanol but the phenomenon has not been investigated further.

The unsatisfactory results initially obtained may be explained by the inadequacy of the reference chromatograms, because the variable displacements of the amines could result in variable recoveries and leave unknown amounts of amines to appear as blank activity. When displacement is severe, the contamination of one amine with another will give rise to lack of parallelism in biological assays. There also exists the possibility of new active substances and metabolites (Roberts and Lockett, 1961) being contaminated or falsely "identified" because of the difference between the R_F values obtained on the reference and test chromatograms of the naturally occurring catecholamines. Similar results were obtained with cat plasma.

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