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The Separation of Choline Esters by Paper Chromatography

KLAS-BERTIL AUGUSTINSSON and MARGARETA GRAHN

Institute of Organic Chemistry and Biochemistry, University, Stockholm, Sweden

In connection with work on the identification of various choline esters in tissue extracts, a method was worked out in this laboratory for the separation of such compounds by means of filter-paper chromatography.

Only a few papers have been published on the application of paper chromatography to the separation of choline esters. In the course of our experiments, Whittaker and Wijesundera [1] reported a method employing *n*-propanol- and *n*-butanol-water mixtures. Special accounts have been given by these authors [2] of the separation of the hydrolysis products of succinyldicholine. At the same time, Malyoth and Stein [3] published their work on the separation of choline esters, sugars, and aneurine. These authors used mixtures of acetic ester and pyridine with certain amounts of water. The method described in the present communication gives good separation of the choline esters. In addition the quantitative determination of acetylcholine based on the elution technique with paper chromatograms is reported.

METHODS

Chromatographic technique. Munktell No. OB filter paper was used in all the experiments, unless otherwise stated. Whatman No. 4 was used in some cases, but it showed no appreciable advantage over the Munktell paper. The Whatman paper possesses the properties of slower ascension and gives the same R_F values as the Swedish paper. Aqueous solutions 0.2—1 % of the compounds to be chromatographed were placed, with micropipettes, at points marked on a pencil line. The volume of solution used was about 5 μl, corresponding to 10—50 μg of the compound; the diameter of the spot was about 10 mm. The spots were then air-dried. The papers were placed in an air-tight glass chamber in a room, the temperature of which was maintained at 20.0 ± 0.5° C. Both ascending and descending techniques were used; the former was found to be more advantageous. When the solvent had run a convenient distance (about 40 cm in 16 hours in the upward and in 9 hours in the downward irrigation), the paper was dried and developed.

Solvents. A great many solvents have been tried which will be discussed below. The following is the composition of the mixture which we have found most useful: *n*-butanol-ethanol-acetic acid-water (8 : 2 : 1 : 3).

Colour developing reagent. The compounds were made visible on the paper by spraying with a solution of dipicrylamine (hexanitro diphenylamine, $Mg[N(C_6H_2(NO_2)_3)_2I_2]$ containing 0.2 g in 50 ml acetone and 50 ml distilled water. On spraying dried sheet with this

solution, choline and its esters at once produce dark yellow spots on a light yellow ground. The colour is strongest immediately after spraying and fades on standing for a week. Dipicrylamine also produces colour with other compounds which are described below.

Other developing reagents have been tried, *e.g.*, the iodine method (immersion in an alcoholic solution of iodine), spraying with a solution of bromphenol blue in 1 *M* K_2HPO_4, and the carboxylic reagent (hydroxylamine-ferric chloride) applied by Hestrin [4] to the quantitative estimation of acetylcholine. All three methods have been found to be less convenient than that described above.

The modification of Hestrin's method recently described by Whittaker and Wijesundera [1], but published after the above method had been worked out, has been tried in this laboratory. Non-aqueous reagents are used in this modification in contradistinction to Hestrin's reagents. This method seems to have definite advantages over the older one; it produces distinct spots (with acetylcholine) which are visible for several weeks. It is applicable only for detection of esters on chromatograms, not for the quantitative assay in solutions or extracts.

Quantitative estimation of acetylcholine. The quantitative estimation of acetylcholine on chromatograms was carried out by using the elution technique of spots in combination with the original method of Hestrin [4]. A test strip was cut out and developed. This strip with the coloured spots was placed alongside the rest of the chromatogram, the corresponding spots marked with a pencil, and the areas cut out and eluted with 2.0 ml of 0.0001 *M* hydrochloric acid (acetylcholine has maximum stability at pH 4). The acetylcholine present was estimated by mixing 1.0 ml of this eluate with 2.0 ml of a mixture of equal volumes of 3.5 *M* sodium hydroxide and 2 *M*-hydroxylamine hydrochloride. After adjusting to pH 1.4 ± 0.2 with hydrochloric acid, 1.0 ml 0.37 *M* ferric chloride ($FeCl_3 \cdot 6H_2O$) in 0.1 *M* HCl was added and the red colour promptly determined colorimetrically. The extinction coefficient of the solution was measured at 5 400 Å with an EEL colorimeter (Evans Electroselenium LTD, Harlow, Essex).

Compounds tested. Choline and choline esters were used as the chlorides with the exception of succinyldicholine iodide * and the M compounds **.

(M 111, the iodide of dicholine adipic acid ester; M 114, the bromide of the ethyl derivative of M 111; M 116, the iodide of the dicholine sebacic acid ester.) Phosphorylcholine chloride was used as the calcium salt, and acetylaneurine as the chloride hydrochloride.

RESULTS

The R_F values of the compounds tested for the solvent *n*-butanol-ethanol-acetic acid-water are presented in Table 1. Both descending and ascending techniques were employed, but the latter were found to be more convenient. The values obtained are the same whether one or many compounds are run in a mixture; this is illustrated with some compounds in Fig. 1.

Several solvent mixtures have proved unsatisfactory. Alkaline solvents, for instance those containing ammonia, cannot be used due to hydrolysis of the esters. Phenol is not useful; buffering with sodium citrate, KH_2PO_4, and ascorbic acid gives no separation. *Iso*-butanol-acetic acid gives small spots and high R_F-values. We have found *n*-butanol-acetic acid to be more satisfactory, giving lower R_F values. Good resolving power has been exhibited by *n*-butanol-ethanol, but in some cases duplicate spots are obtained with one ester. The resolving power of ethanol-acetic acid is unsatisfactory. However, the addition of certain amounts of acetic acid to the *n*-butanol-ethanol-water

* Prepared and kindly supplied by assistant L.-E. Tammelin, Research Institute of National Defence, Sweden.

** These compounds were kindly placed at our disposal by Messrs. Österreichische Stickstoffwerke, Austria.

Table 1. The R_F values (mean values) in n-*butanol-ethanol-acetic acid-water (8 : 2 : 1 : 3) at 20° C. The chlorides were used, unless otherwise stated.*

Substance	R_F	
	Descending	Ascending
Choline	0.37	0.38
Acetylcholine	0.47	0.46
Acetylthiocholine iodide	—	0.58
Acetyl-β-methyl-choline (mecholyl)	0.55	0.55
Propionylcholine	0.57	0.57
Butyrylcholine	0.67	0.66
Benzoylcholine	0.70	0.71
Carbaminoylcholine	0.30	0.30
Succinyldicholine iodide	0.18	0.12
M 111	0.22	—
M 114	0.25	—
M 116	0.54	—
Salicylcholine	0.64	0.64
Acetylsalicylcholine	0.65	0.67
Phosphorylcholine	0.19	0.21
Acetylaneurine	0.31	0.33
Aneurine	0.25	0.26
Betaine	0.47	0.46
D-Tubocurarine	0.45	0.45
Physostigmine salicylate	0.72	0.75
Prostigmine bromide	0.63	0.63
Histamine	—	0.21

mixture gives the best results. Formic acid may replace acetic acid but gives somewhat higher R_F-values; hydrochloric acid can also be used, but the paper must be neutralized before development with dipicrylamine. With propionic acid or *iso*-butyric acid the spots are more elongated and the R_F values low.

The solvent mixture, ethylene chlorohydrin-*n*-butanol-acetic acid-water, in the proportions 2 : 10 : 1 : 3, has been found useful. The R_F values produced are about the same as those obtained with the *n*-butanol mixture used generally in these studies. Distinct spots are obtained, but duplicate spots are usually produced by acetylcholine. The solvent mixture may be found satisfactory in a second run for twodimensional chromatograms.

Small variations in the composition of solvent produce great changes in the results. The proportion of acetic acid to water has been found to be of especially great importance. The most suitable composition of the solvent mixture used, *n*-butanol-ethanol-acetic acid-water, is 8 : 2 : 1 : 3. This solvent produces good resolving power with most of the choline esters studied. The following esters are easily separated in one-dimensional chromatogram: acetyl-, acetyl-β-methyl- or propionyl-, butyryl- or benzoyl, phosphoryl-, and carbaminoylcholine, and also choline. The dicholine esters of succinic acid and adipic acid (M 111) respectively, produce "tailing" and are not separated satisfactorily. The diester of sebacic acid (M 116), however, is easily separated from the other dicholine esters studied; aqueous solutions of M 116 kept for 3 hours or more give two distinct spots. Salicylcholine and especially phosphorylcholine give

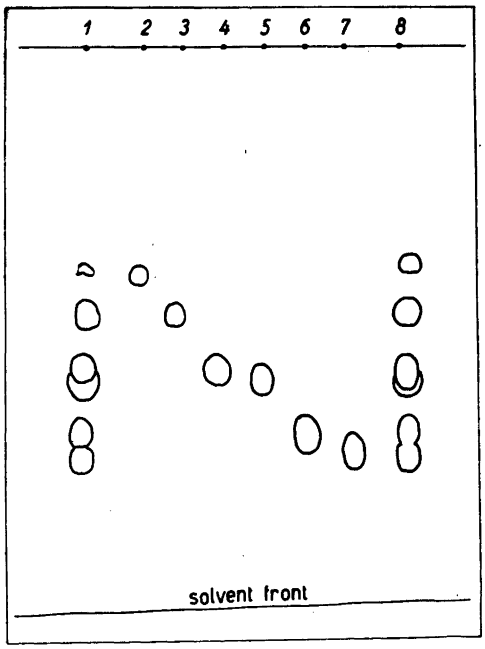

Fig. 1. Chromatogram of choline and various choline esters. Solvent: n-*butanol-ethanol-acetic acid-water (8 : 2 : 1 : 3). Upward irrigation at 20° C. Run of solvent front 42 cm in 16 hours. 1 and 8: mixtures of all compounds 2—7; 2: choline; 3: acetylcholine; 4: acetyl-β-methylcholine; 5: propionylcholine; 6: butyrylcholine; 7: benzoylcholine.*

spots which are more or less elongated. The spots produced by acetylsalicylcholine, on the other hand, show no "tailing".

In preliminary experiments some non-choline esters which may be of interest in connection with acetylcholine, and its occurrence and metabolism in tissues were tested. A mixture of physostigmine and prostigmine can be resolved satisfactorily; the "anticholinesterases" show R_F values which differ greatly from those of choline and acetylcholine. Tubocurarine produces "tailing". Acetylaneurine separates from acetylcholine and aneurine. The method may also be useful for the separation of histamine from acetylcholine and other choline esters. Betaine gives the same R_F value as acetylcholine but decolourises the dipicrylamine.

The dipicrylamine reagent is most useful for the development of colour on the chromatogram of choline esters. It was first used by Ackermann and Mauer [5] as a sensitive agent for the detection (precipitation) of acetylcholine and later used as developing reagent in paper chromatography [3]. Dipicrylamine gives yellow spots on a light yellow ground for choline and its esters. Sometimes choline appears as a light purple spot and may then be distinguished from the esters by this colour difference. Succinylcholine gives a colour which is darker than that produced by the other choline esters. The esters and choline itself may be detected in concentrations of about five micrograms or more. Phosphorylcholine must be used in higher concentrations in order to be visible after development. All spots must be marked soon after development.

The dipicrylamine reagent is not specific for choline and its esters. Among compounds which give positive reaction, acetylaneurine produces a yellow-red colour. Most other compounds, not mentioned in Table 1, appear in case of positive reaction as yellow spots, *e.g.* adrenaline and nor-adrenaline, creatine, hordenine, spermine, pilocarpine. The amino acids with the exception of arginine and tryptophane are not detected with this reagent. Negative results are also obtained with nucleic acids and their degradation products. Betaine produces decolourization.

Elution of spots of acetylcholine. The amount of acetylcholine (or any carboxylic acid ester of choline) on chromatograms can be estimated quantitatively with great accuracy by using the elution procedure. Acetylcholine can be eluted quantitatively with 10^{-4} *M* hydrochloric acid from a spot containing about 0.20 μmoles (38 μg acetylcholine chloride). The results obtained with 0.30 μmole of acetylcholine chromatographed together with the same amount of choline, are recorded in Fig. 2. The chromatogram is shown in the lower half of the figure. The numbered areas (one cm wide) were extracted with hydrochloric acid after cutting in small pieces and the amount of acetylcholine in each area was determined by the hydroxylamine-ferric chloride test (described by Hestrin [4]). It will be seen that good recovery and localization were obtained. It is unnecessary to mince the paper.

The extracts of the spots of acetylcholine have also been used for pharmacological assay. The guinea-pig ileum was used as test object in the usual way. It has thus been possible to estimate acetylcholine in parallel experiments both chemically and pharmacologically. These investigations are still in progress.

The enzymatic hydrolysis of dicholine esters. It has been proved previously in this laboratory that succinylcholine is split enzymatically by human plasma, although the rate of hydrolysis as measured with the Warburg technique is comparatively slow. This hydrolysis reaction has also been studied by Löw and Tammelin [6]. Moreover, it has been demonstrated by Whittaker [2] and by Ginzel *et al.*[7] that the monocholine ester of succinic acid is formed as an intermediate during the enzymatic hydrolysis of the dicholine ester.

The chromatographic technique described in the present paper is suitable for the analysis of reaction mixtures, such as those formed in enzymatic hydrolysis processes. Succinyldicholine (1 % solution) was incubated for three hours with horse plasma (the cholinesterase activity of which is about three times higher than that of human plasma). The reaction was carried out under optimum conditions for enzymatic hydrolysis, and the reaction mixture was chromatographed in the manner described above. A new spot with R_F 0.25 was found which is assumed to correspond to succinylmonocholine, and which was separated from choline (R_F 0.38) and the dicholine ester (R_F 0.12).

Similar preliminary studies have been performed with the dicholine esters of adipic acid and sebacic acid respectively. Both these compounds are destroyed comparatively rapidly by horse plasma. New spots are produced on the chromatograms by the reaction mixtures, but a detailed analysis of the compounds produced have not yet been made.

Experiments have also been performed with human erythrocytes in similar enzyme studies. Solutions of the dicholine esters were incubated for two hours

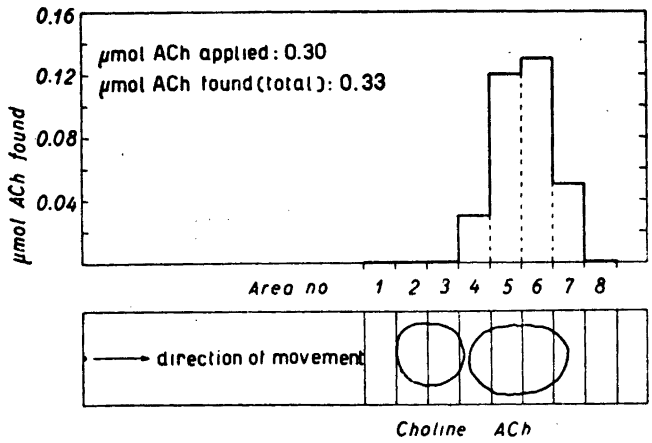

Fig. 2. Quantitative estimation of acetylcholine on a chromatogram by the hydroxylamine-ferric chloride test. Below a duplicate of the paper strip used for the elution of the choline and acetylcholine spots. Areas cut one cm wide.

or more at 25° C with intact washed red cells, in the presence of sodium chloride and magnesium chloride. The cells were then centrifuged and the supernatant liquid analysed by the paper chromatographic method. The original compounds, as found on the chromatograms, were unchanged, and new spots corresponding to degradation products of the esters could not be detected. It is difficult in such experiments to prevent hemolysis.

DISCUSSION

Choline, and most of its esters which have been studied can be separated by paper chromatography using a *n*-butanol-ethanol-acetic acid-water mixture. Dipicrylamine is a valuable reagent for the development of the chromatograms. This method is especially valuable for the separation and identification of mixtures of choline and acetylcholine. Partly purified extracts of various tissues have been examined, but difficulties arise when the method is applied to such material. It has been proved that acetylcholine and choline are in combination with proteins and that these combinations give rise to distorted chromatograms. The method is also valuable for the analysis of reaction mixtures containing acetylcholine and physostigmine or prostigmine. With most general techniques, acetylcholine and physostigmine precipitate together or stay in solution together, but the two compounds can be separated satisfactorally by chromatographic analysis. This technique also separates acetylcholine from histamine.

The method is not suitable for the analysis of choline esters, the acid portion of which is a dicarbonic acid. The method, however, has been used in preliminary experiments with hydrolysis mixtures of dicholine esters, showing that the adipic and sebacic acid esters, and to a lower degree the succinic acid ester, are destroyed by blood plasma and most probably not by erythrocytes.

SUMMARY

The separation of choline esters from each other and from choline by filterpaper chromatography is described. The solvent found to give the best separation is a *n*-butanol-ethanol-acetic acid-water mixture.

The esters on the paper were detected by spraying with a dipicrylamine solution in acetone. Acetylcholine was also estimated quantitatively by the elution technique in conjunction with the hydroxylamine-ferric chloride test.

Preliminary studies have been performed to separate the degradation products from the enzymatic hydrolysis of some dicholine esters (*e.g.* succinyldicholine), by paper chromatography.

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