contact with the movable bar. Readjust the front plate of the carrier so that the syringes protrude about 1/2 in.

Moisten the blotter paper in the side panels with eluting solvent and put them in place. Insert the syringe racks into the appropriate slots and adjust so that the tips of the syringes are in firm contact with the centers of all strips. Attach the lid and the motor assemblies.

The motors are started after about 10 minutes to permit the partial saturation of the air in the elutor with the solvent. The time of elution can be varied by using different gears on the motor assembly. A short elution period is desirable with highly volatile, nonpolar solvents. The elution process can be automatically stopped when the plungers are near the bottom of the syringe barrel by powering the motors through a timer.

After allowing the solvent to drain from the strips into the cups for 10 minutes, remove the lid and the motor assemblies of the elutor. Adjust the syringe rack so the ends of the syringes will clear the spacer bar; remove the racks and the side panels. Evaporate the more volatile solvents by connecting the variable heater in the base of the elutor. Detach the needle rack from the cup holder and remove the spacer bar with attached paper and needle rack. Retain this assembly for additional studies which are indicated. Complete the drying of the cups by loosely wrapping the cup holder in aluminum foil and placing it in a vacuum oven. After drying, cool the cup holder to room temperature in a desiccating cabinet and reweigh the cups. Treat the results as indicated in the section on *Theory*.

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Gravimetric Evaluation of Paper Chromatograms II

Studies on Ouabain, Other Glycosides, and Prednisone

By ALBERT E. H. HOUK

Results are reported on the analyses of milligram quantities of some pure and crude steroid preparations. Samples are chromatographed on paper, and the entire sheet is rapidly eluted by a previously described technique. Components, in concentrations of 0.2 per cent or less, can theoretically be separated and detected. This technique may be found of equal benefit in the study of other compounds which can be separated by paper chromatography.

ANALYSES of steroid preparations by paper chromatography have been impeded by the difficulty in locating, rapidly eluting, and quantitating all components separated. This led to the development in this laboratory of new instrumentation and procedures reported previously (1). Results of some applications of this technique to steroid analyses are now given.

EXPERIMENTAL

Solvents

Neutral, readily volatile solvents were used. All solvents were percolated rapidly through a column of anion-cation exchange resin.[1] The first effluent through the column was discarded; the remainder was collected and redistilled. Formamide was redistilled under vacuum and stored in a desiccator over sulfuric acid until free of ammonia. Constant boiling fractions were returned to their original glass containers. Teflon[2] liners were placed in each bottle cap.

Chromatographic Paper

Whatman No. 3-mm. chromatographic paper in 8 × 8-in. sheets was ordinarily used. When required, 8 × 11 1/4-in. sheets were cut from a 23-cm. roll. Guidelines for streaking and development of paper were marked on all sheets; the paper was washed, dried, and stored (1).

Equipment

(*a*) Thomas-Mitchell chromatographic tank assembly with glass rods, troughs, and continuous technique accessories[3] was used with 8 × 8-in. sheets. The slotted cover and clips were coated with Teflon (1). (*b*) Thomas-Kolb chromatographic jar[3] was used with 8 × 11 1/4-in. sheets. A double-slotted cover, similar to the above accessory, was made for the jar and was coated with Teflon. (*c*) Two Pyrex 3-quart utility baking dishes,[4] each 9 1/4 × 14

Received November 6, 1962, from the Division of Pharmaceutical Chemistry, Bureau of Biological and Physical Sciences, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington 25, D. C.
Accepted for publication December 6, 1962.
The author extends his thanks to Dr. F. H. Wiley for his constant encouragement and guidance and to Mr. P. R. Litz for the illustrations.

[1] Rohm and Haas Amberlite MB-1, analytical grade, indicator-free was satisfactory.

[2] Marketed by E. I. du Pont de Nemours & Co., Inc. Wilmington, Del.
[3] Arthur H. Thomas Co., Philadelphia, Pa.
[4] Most department stores.

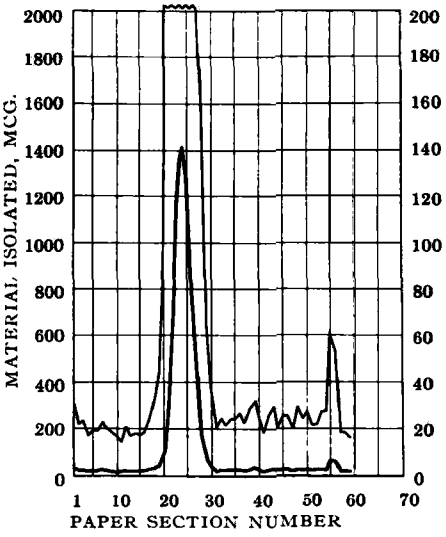

Fig. 1.—Weight-distribution curve of commercial ouabain (Sample A).

× 2½-in. and covered with a glass plate, were used to hold the immobile solvent during impregnation of the paper. (*d*) A drying rack similar to the commercial item[3] but 17 in. high; (*e*) desiccating cabinet[3] with glass trays and phosphorus pentoxide desiccant; (*f*) disposable plastic gloves[3] for handling the paper; (*g*) capillary melting point tubes,[5] each 1.2 mm. × 8 cm.-size and approximately 114 mg. weight. A 2-cm. section was broken off each and discarded; the remainder was fire polished. (*h*) Aluminum rod ⅛ × 10 in. was used to handle the cut chromatogram. (*i*) Thin-walled glass tube ⅛ × 6 in. was used to attach ends of paper strips to needle rack. (*j*) Sample applicator box, paper chromatogram cutting equipment, Teflon weighing cups, microbalance, and elutor apparatus (1).

General Procedure

Conclusions drawn from data obtained by this procedure are usually based on weights of small magnitude. Consequently, it is essential that precautions be taken to avoid nonvolatile contaminants. Glassware should be washed with chromic acid cleaning solution, thoroughly rinsed, drained, and stored in a dust-free cabinet. When in use, the equipment should be protected as far as possible from such contaminants as dust, fingerprints, chemical fumes, etc. The use of polyethylene gloves has been found helpful.

For initial quantitative analyses, three approximately equal aliquots of a sample were weighed in Teflon microcups. The first aliquot was used for gravimetric paper chromatography as previously described (1); the second aliquot was used to prepare a standard reference solution; the third aliquot was used as a control. To it and also to an empty, preweighed Teflon cup were added immobile, mobile, and eluting solvents equivalent approximately to that from an eluted paper strip. These cups were dried later with the cups from the elution apparatus and reweighed to determine nonvolatile components in solvents used, efficiency of drying, and weight changes of sample during processing. Chemical studies were conducted on solutions (equivalent to the reference solution) made from the residues in these cups and from the residues of the eluted fractions and the corresponding blank chromatogram. Thus both the recovery by weight and the recovery by chemical analysis of the fractions isolated were determined. Weighable residues remaining in the cup and capillary tube (after transfer of the sample to the paper for chromatography) were determined chemically also. Once a procedure for a sample was established, unnecessary control work was eliminated. Often the average of the blank areas of a sample chromatogram, eliminating the solvent front, was used to calculate the weight of the "fractions" above the "background weight."

Dry weight of the sample placed on the paper for fractionation normally agrees with summation of the dry weights of fractions isolated. If results indicate incomplete elution, the same chromatographic paper may be re-extracted, with the same or different solvent, into the same cups. Increases in weight of residue over increased blank confirm initial incomplete elution. There is no limit to the number of times the paper may be re-extracted, provided the solvent is evaporated between each operation. Further indication of the completeness of the extraction can be obtained by drying and dipping or spraying the eluted chromatogram with a developing reagent. Any fluorescence or color on the paper, characteristic of the sample and usually near the bottom of the strips, indicates incomplete elution.

The Teflon cups used to collect the fractions are chemically inert to most chemicals. Spot tests often were done directly in each cup. The cups were placed also in separate test tubes and color reactions completed there without transfer of residue.

Ouabain (Sample A).—Sample—7.556 mg. commercial ouabain (20.2% water) dissolved in water-methanol (1 + 2). Paper size—8 × 11¼ in. Immobile solvent—*n*-butanol-saturated water in meth-

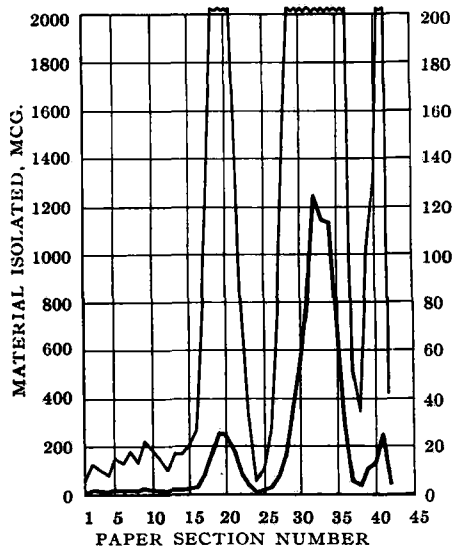

Fig. 2.—Weight-distribution curve of commercial ouabain (Sample B).

[5] Micro-Ware, Inc., Vineland, N. J.

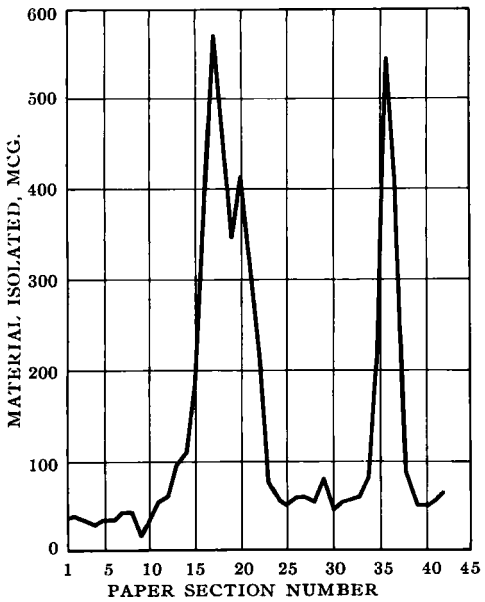

Fig. 3.—Weight-distribution curve of a mixture of ouabain, K-strophanthoside, and strophanthidin.

anol (3 + 1). Mobile solvent—water-saturated *n*-butanol. Development—20 hours at 28° by continuous ascending slit technique. Chromatogram—vacuum dried at 105° for 1 hour and cut into 59 strips. Elution—50% ethanol for 80 minutes. Drying—eluting solvent evaporated at 28° overnight (2), and residues vacuum dried in the presence of P_2O_5, at 130° for 5 hours.

Ouabain (Sample B).—Sample—9.999 mg. commercial ouabain (19.3% water) dissolved in water-methanol (1 + 2). Paper size—8 × 8 in. Immobile solvent—*n*-butanol-saturated water in acetone (1 + 1). Other techniques were as given under *Ouabain (Sample A)*, except the chromatogram was cut into 42 strips.

Mixture of Glycosides.—Sample—approximately 2 mg. each of ouabain (20.2% water), K-strophanthoside (strophosid; 4% water), and strophanthidin dissolved in methanol. Paper size—8 × 8 in. Immobile solvent—*n*-butanol-saturated water (paper was air dried until it appeared damp). Mobile solvent—water-saturated *n*-butanol. Development—to finish line by ascending technique in a sealed tank (approximately 4 hours at 28°). Chromatogram—vacuum dried at 105° for 1 hour and cut into 42 strips. Other techniques were as given under *Ouabain (Sample A)*.

Strophanthin K.—Sample—approximately 10 mg. commercial strophanthin K dissolved in water-methanol (1 + 2). Other techniques were as given under procedure for *Mixture of Glycosides*.

Digitoxin.—Sample—approximately 5 mg. commercial digitoxin dissolved in methanol-chloroform (1 + 3). Paper size—8 × 8 in. Immobile solvent—35% formamide in acetone. Mobile solvent—benzene-chloroform (1 + 1). Development—to finish line by ascending technique in a sealed tank (approximately 1 hour at 28°). Chromatogram—dried in a stream of air at 105° for 45 minutes and cut into 42 strips. Elution—50% aqueous pyridine for 60 minutes. Drying—solvent evaporated and residue dried in vacuum at 130° for 2 hours.

Prednisone.—Sample—approximately 3 mg. commercial prednisone dissolved in chloroform. Paper size—8 × 8 in. Immobile solvent—40% formamide in methanol. Mobile solvent—petroleum ether-chloroform (1 + 2). Development—2 hours at 28° by continuous ascending slit technique. Chromatogram—vacuum dried at 105° for 1 hour and cut into 42 strips. Elution—95% ethanol for 60 minutes. Drying—solvent evaporated and residue dried at 105° for 5 hours.

RESULTS

Figures 1 and 2 show the quantitative weight-distribution curves of two samples of ouabain. These are the purest and the most contaminated of eight samples examined for selecting reference standard material and evaluating the chemical assay procedures in current use. The left ordinate of each graph is for the lower curve, the right ordinate for the ten-fold expanded upper curve. The latter shows small weight variations in the base line.

On a dry weight basis, ouabain (Sample A) was recovered 100 ± 1% in the main fraction. Its "weight background" or base line averaged 22 mcg. per cup with extreme variations of ±8 mcg. The material recovered from the solvent front, where evaporation took place for 20 hours during development, weighed 81 mcg. A blank chromatogram gave an equivalent baseline and solvent front. On the basis of alkaline picrate color reaction (3), 99.5 ± 0.5% of the active component was recovered in the main fraction. The solvent front contained no chromogenic material. The extracted dried chromatogram sprayed with zinc chloride reagent (3) was negative.

With Sample B, 81.5 ± 1% of the starting material was recovered on a dry weight basis from the main fraction. When the alkaline picrate color reaction was applied to the unchromatographed material, the results indicated the presence of 87.4 ± 0.5% of ouabain. However, impurities less polar than ouabain and equal to 5.0% of the sample subjected to chromatography were found superimposed on the solvent front. This fraction gave a strong re-

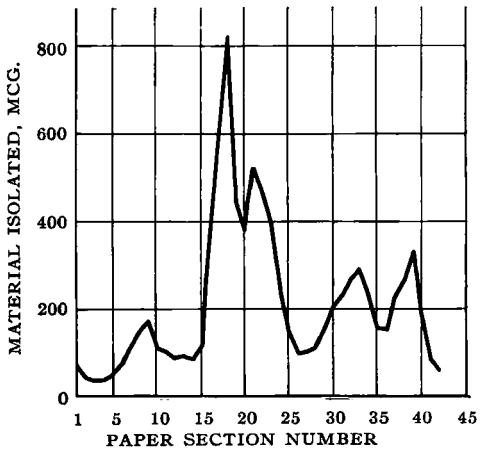

Fig. 4.—Weight-distribution curve of commercial strophanthin K.

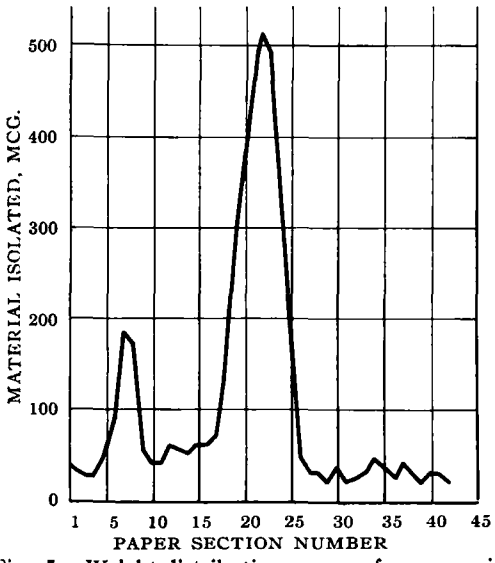

Fig. 5.—Weight-distribution curve of commercial digitoxin.

action with the alkaline picrate reagent and thus was included in the colorimetric assay of the unchromatographed sample. In addition to the above, impurities more polar than ouabain accounted for 13.4% of the dry weight of sample and gave little alkaline picrate color but had a strong anthrone color reactivity. We have occasionally observed the type of asymmetry seen at the apex of the main fraction. It usually disappears in subsequent weight-determination curves and may be associated with the physical characteristics of the zone and the finite width of the paper strips. The two examples cited above are typical of the quantitative recoveries which can be achieved by this technique.

Figures 3–6 show the type of general information that may be obtained from rapid qualitative weight-distribution measurements. Figure 3 shows the weight-distribution curve of a synthetic mixture of ouabain with two other glycosides, K-strophanthoside and strophanthidin. This figure illustrates the curve characteristics under two extreme conditions: (*a*) when a component is at or near the solvent front, and (*b*) when two components overlap. Strophanthidin (very close to the solvent front) gave a typical sharp inflection, indicating a buildup behind the solvent line. K-strophanthoside (slightly ahead of ouabain) easily showed its presence by a very sharp inflection above the ouabain background. A similar chromatogram when dried, sprayed with zinc chloride reagent, and viewed under ultraviolet light, showed only one fluorescent band for the ouabain-K-strophanthoside fraction. Where required, the fraction containing such an overlapping mixture could be rechromatographed, using a solvent system which would give a complete separation of the components.

Figure 4 shows a more complex type of weight-distribution curve obtained from strophanthin K. This sample was obtained as a possible pure reference standard. A chromatogram when dried, sprayed with zinc chloride reagent, and viewed under ultraviolet light, showed a series of overlapping fluorescent bands of varying intensity from the starting to the finishing line. It was difficult to estimate the quality of the product. However, the weight curve clearly showed it was unsuitable for a standard. It contained five major and five minor components; three were colored pigments with peaks near paper section numbers 8, 22, and 37. It was a mixture of glycosides similar to strophanthin N. F. X.

Figures 5 and 6 show the weight-distribution curves for commercial digitoxin and prednisone samples in which high boiling point formamide was used successfully as an immobile solvent. When the paper was dried before elution, it was difficult to remove completely the more nonpolar steroids because of adsorption by the paper. However, complete removal was readily obtained by cutting and eluting the wet paper with alcohol. The alcohol then was evaporated from the cups and the formamide removed under vacuum at 90°. The weight curve for digitoxin shows gitoxin near the starting line and traces of two components near paper sections numbers 12 and 15 more polar than digitoxin. The two minor components had not been observed previously by conventional paper chromatography but were confirmed by high concentration chromatography and use of slit technique. The weight curve for prednisone indicates absence of marked impurities. It does show a broad solvent front due to evaporation of the highly volatile mobile solvents within the developing chamber and below the slit of its lid. Multiple solvent fronts, due to different evaporation points from the paper by mixed mobile solvents, were no problem in these systems.

DISCUSSION

In conventional chromatography, it is becoming more important to control the purity of the paper and solvents used. It is critical in the gravimetric procedure herein described, since the presence of nonvolatile, soluble contaminants influences the accuracy of quantitative studies and may obscure the presence of minor components of the sample. Washing with acetone removed 2.8 mg. of soluble material from one sheet of Whatman No. 1 filter

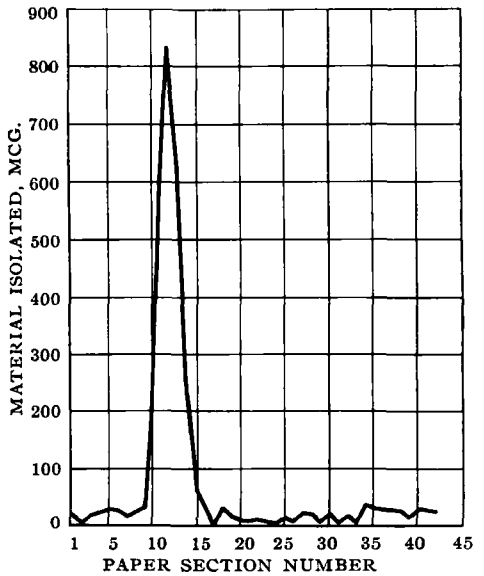

Fig. 6.—Weight-distribution curve of prednisone.

paper.[6] Total and acetone-soluble materials were even higher in Whatman No. 3 paper. These substances are particularly troublesome when aqueous solvents are used as eluents.

Several precautions may aid in controlling this difficulty. The amount of extraneous material eluted varies with the volume of solvent used. Therefore, the eluting solvent should be kept to the *minimum* required for the complete removal of the components of the sample. The selection of solvent systems which tend to concentrate the fraction to be studied in a narrow band reduces the amount of background material since a smaller area of paper is eluted to obtain the desired fraction.

When it is sufficient to measure the sum of the minor components, the accuracy can be enhanced by using the slit technique and concentrating the less polar materials at the solvent front. Similarly, the more polar components may be concentrated on or near the starting line by the proper choice of solvents.

The limit of sensitivity of the method may be determined by comparing the weight of the sample used and the weight of the background. For example, in ouabain (Sample A), a 10-mg. sample was used and the background weight determined on a blank chromatogram averaged 22 mcg. per cup with extreme variations of ±8 mcg. Since these extremes differ by a maximum of 16 mcg., impurities present in quantities of more than 16 mcg. or 0.16% of a 10-mg. sample and concentrated on a single strip, should produce discernible peaks. The sensitivity is reduced where the impurity is distributed over two or more strips.

If one is interested only in the amount of the main component present, it is not necessary to elute quantitatively the entire chromatogram. The desired result can be obtained by comparing the dry weight of the sample and the dry weight of chief component eluted from the paper.

The elution apparatus described was designed to accommodate an 8 × 8-in. paper cut into 42 strips. Obviously, shorter chromatograms can be used equally well. Long paper may also be used by cutting it into two or more sections and eluting each section separately. In using small samples it may be desirable to use thinner or more narrow paper and with large samples to increase the weight or width of the paper. In the latter case, it will be necessary to increase the height of the elution equipment and the capacity of the cups.

[6] Private communication from L. C. Mitchell of the Food and Drug Administration.

SUMMARY

In a previous publication (1), theory, instrumentation, and procedure were given for the gravimetric determination of the distribution of compounds over an entire paper chromatogram. Some applications of this technique to analysis of steroids are now reported. The method measures components of a drug or other product which are separated by paper chromatography and may be missed by conventional techniques of detection. It eliminates the need for reference standards which for impurities or decomposition products may not be readily available. Precautions in using the method are given. Upon completion of the gravimetric analyses, the isolated fractions were used for additional studies.

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Effects of Some Hypnotic Drugs on Respiration and Oxidative Phosphorylation in Rat Brain

By V. G. ERWIN and HAROLD C. HEIM

Pentobarbital and glutethimide inhibited respiration of rat brain homogenates in the presence of glucose, pyruvate, malate, or glutamate. These effects were not evoked by ethinamate or methyprylon. None of the drugs produced effects on malic dehydrogenase activity, but both pentobarbital and glutethimide inhibited the oxidation of reduced diphosphopyridine nucleotide. Of the four drugs studied only pentobarbital produced significant effects on oxidative phosphorylation by mitochondria.

ALTHOUGH NUMEROUS investigators have studied the effects of narcotics on metabolic processes of the brain *in vitro*, the mechanisms by which these drugs evoke pharmacologic effects are not well understood. It has been demonstrated that barbiturates inhibit oxidation of carbohydrate by brain slices and minces (1–4). Other studies have revealed indications that these drugs interfere with hydrogen transport (5, 6) and with the oxidation of pyruvate (7). It has furthermore been shown that barbiturates in concentrations which more closely approach those present in the brain during anesthesia uncouple oxidation from phosphorylation *in vitro* (8).

Received October 12, 1962, from the School of Pharmacy, University of Colorado, Boulder.
Accepted for publication January 29, 1963.
The authors are most grateful for the generous supplies of ethinamate, methyprylon, and glutethimide furnished by Eli Lilly and Co., Hoffmann LaRoche, Inc., and Ciba Pharmaceutical Products, Inc., respectively.