1 - Methyl - 3 - n - butyl - 3 - cyanopyrrolidine (XX).—Similarly, 8.1 Gm. (48.5%) of XX, b.p. 136° at 25 mm., was obtained from 18.4 Gm. (0.1 mole) of *n*-butyl iodide; picrate, m.p. 158–160°.

Anal.—Calcd. for $C_{16}H_{21}N_5O_7$: N, 17.71. Found: 17.78, 17.62.

1 - Methyl - 3 - cyclopentyl - 3 - cyanopyrrolidine (XXI).—Similarly, 17.9 Gm. (0.12 mole) of cyclopentyl bromide yielded 3.3 Gm. (18.7%) of XXI, b.p. 106–108° at 5 mm.; picrate, m.p. 204–206°.

Anal.—Calcd. for $C_{17}H_{21}N_5O_7$: N, 16.78. Found: 17.10, 17.21.

1 - Methyl - 3 - benzyl - 3 - cyanopyrrolidine (XXII).—Similarly, 11.5 Gm. (57.6%) of XXII, b.p. 134–136° at 2 mm., was obtained from 12.7 Gm. (0.1 mole) of benzyl chloride; picrate, m.p. 176–177°.

Anal.—Calcd. for $C_{19}H_{19}N_5O_7$: N, 16.31. Found: 16.12, 16.38.

1 - Methyl - 3 - (1 - methyl - 3 - pyrrolidinyl)-3 - cyanopyrrolidine (XXIII).—Similarly, 19.7 Gm. (0.12 mole) of freshly prepared VI yielded 2.7 Gm. (13.8%) of XXIII, b.p. 120–126° at 8 mm.; dipicrate, m.p. 232–233° dec.

Anal.—Calcd. for $C_{23}H_{25}N_9O_{14}$: N, 19.35. Found: 19.60, 19.52.

1 - Methyl - 3 - (2 - dimethylaminoethyl) - 3-cyanopyrrolidine (XXIV).—Similarly, with double the amount of sodium amide used for the preceding compounds, 28.0 Gm. (0.12 mole) of 2-dimethylaminoethylbromide hydrobromide yielded 2.9 Gm. (16.2%) of XXIV, b.p. 110–112° at 6 mm.; dipicrate, m.p. 148–150° (opaque liquid), clear at 155–160°.

Anal.—Calcd. for $C_{22}H_{25}N_9O_{14}$: N, 19.71. Found: 19.60.

REFERENCES

(1) Lunsford, C. D., Ward, J. W., Pallota, A. J., Tusing, T. W., Rose, E. K., and Murphey, R. W., *J. Med. Pharm. Chem.*, **1**, 73(1959).
(2) Lunsford, C. D., U. S. pat. 2,838,521(1958).
(3) Franko, B. V., and Lunsford, C. D., *J. Med. Pharm. Chem.*, **2**, 523(1960).
(4) Parke, Davis and Co., Brit. pat. 831,934(1960).
(5) Biel, J. H., U. S. pat. 3,091,570(1963).
(6) Lunsford, C. D., U. S. pat. 2,878,264(1959).
(7) Winder, C. V., Wax, J., Serrano, B., Scott, L., Stackhouse, S. P., and Wheelock, R. H., *J. Pharmacol. Exptl. Therap.*, **133**, 117(1961).
(8) Parke, Davis and Co., Brit. pat. 907,424(1962).
(9) Wu, Y.-H., Feldkamp, R. F., Corrigan, J., and Rhodes, H. J., *J. Org. Chem.*, **26**, 1524(1961).
(10) Wu, Y.-H., and Feldkamp, R. F., *ibid.*, **26**, 1529(1961).
(11) Schuler, W. A., U. S. pat. 2,784,185(1957).
(12) Scarborough, H. C., U. S. pat. 3,073,826(1963).
(13) Lunsford, C. D., Cale, A. D., Jr., Ward, J. W., Franko, B. V., and Jenkens, H., *J. Med. Chem.*, **7**, 302 (1964).
(14) Smilely, R. A., and Arnold, C., *J. Org. Chem.*, **25**, 257(1960).
(15) Ames, D. E., *J. Chem. Soc.*, **1960**, 2780.
(16) Gould, F. E., Johnson, G. S., and Ferris, A. F., *J. Org. Chem.*, **25**, 1658(1960).
(17) Scarborough, H. C., Minelli, J. L., Lawes, B. C., Lobek, W. C., Jr., Corrigan, J. R., and Wu, Y.-H., *ibid.*, **26**, 3955(1961).
(18) Basu, N. K., *J. Proc. Inst. Chemists*, **29**, 73(1958); through *Chem. Abstr.*, **52**, 1183*i*(1958).
(19) Cavalla, J. F., *J. Chem. Soc.*, **1959**, 851.
(20) Cope, A. C., Holmes, H. L., and House, H. O., "Organic Reactions," vol. 9, Adams, R., Blatt, A. H., Cope, A. C., Curtin, D. Y., McGrew, F. C., and Nieman, C., eds., John Wiley & Sons, Inc., New York, N. Y., 1957, pp. 107–331.
(21) Reppe, W., *et al.*, *Ann.*, **596**, 141(1955).
(22) Abruzov, Yu. A., and Ovchinikov, Yu., *Dokl. Akad. Nauk SSSR*, **117**, 813(1957).
(23) Greenlee, K. W., and Henne, A. L., "Inorganic Synthesis," vol. 2, Fernelius, W. S., ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1946, p. 128.

Separation and Quantitative Determination of Adrenaline Using Thin-Layer Chromatography

By N. H. CHOULIS

The separation of adrenaline from noradrenaline and dopamine and its quantitative determination using thin-layer chromatography have been studied. A complete spot separation of the above amines can be achieved by the use of cellulose thin layers and a phenol–water solvent system. For the quantitative determination of the separated adrenaline two methods were used, namely (*a*) a method studying the weight/area relationship of the spots and (*b*) a method employing elution of the spots and measurement of the ultraviolet absorption of the eluents.

PAPER CHROMATOGRAPHIC methods for the separation of catecholamines and related compounds using various solvent systems have been reported (1, 2). These methods require 5–20 hr. for a satisfactory development of the chromatograms, and as a result of the prolonged exposure, oxidation of the catecholamines to the corresponding red aminochromes is usually observed on the paper.

Received April 25, 1966, from the School of Pharmacy, Texas Southern University, Houston 77004.
Accepted for publication October 19, 1966.
Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Dallas meeting, April 1966.

Thin-layer chromatographic methods have often been used for the separation of various components of a mixture. The importance of this method and the ease with which it is carried out have been generally recognized since Stahl (3–5) introduced it as an analytical tool.

A complication in the interpretation of chromatograms of catecholamines (on paper or thin-layer chromatography) may result from the presence of multiple spots which are sometimes produced during chromatography of pure substances

(6–11). Multiple spots are formed when catecholamines are chromatographed in the presence of acids (*i.e.*, dichloro- or trichloroacetic, picric, etc.) and also when pure samples of salts of catecholamines (tartrate, hydrochloride) are chromatographed in *n*-butanol–HCl or *n*-butanol–acetic acid–water solvent systems (2, 12).

Adrenaline and noradrenaline have been previously chromatographed on buffered silica gel plates, using a 70% solution of ethanol as solvent system (13). Waldi (14) has also achieved the separation of adrenaline, noradrenaline, and serotonin and other catecholamines on Silica Gel G plates, but after conversion of the amines to their acetyl derivatives.

For quantitative thin-layer chromatography a number of methods have been used. Seher (15) has suggested that in thin-layer chromatography the weight of the material and the spot are proportional. Purdy and Truter (16) found that quantitation is based on a linear relationship existing between the square root of the area of a component after chromatographing, and the logarithm of the weight of the applied sample; according to them Seher's data as well as that of Stahl (17) and Breuner and Niederwieser (18) fit the relationship for loads of 1–80 mcg. per spot. Pelka and Metcalf (19) examining long-chain tertiary amines, found the method applicable for loads of approximately 200 mcg.

Recently Morrison and Orr (20), in an analysis of selected pharmaceutical mixtures in tablet and capsule forms, by using thin-layer chromatography, obtained quantitative results from the developed chromatograms. A linear relationship between the spot area, in sq. mm., and the weight of the spot was observed.

The method was applicable for concentrations up to 250 mcg. However, this method requires a satisfactory spray reagent, otherwise its use for quantitative work is doubtful.

Ganshirt *et al.* (21) used the thin-layer chromatography for the quantitative assay of a number of bile acids, and Zollner *et al.* (22) found the method applicable for the quantitative estimation of cholesterol esters.

Millett *et al.* (23) described other techniques for quantitative thin-layer chromatography using furoic and hydroxymethylfuroic acids as model compounds. An application of thin-layer chromatography for quantitative assay of tryptamine was suggested by Eble and Brooker (24).

The purpose of the present paper is to provide, by means of thin-layer chromatography, a rapid method of separation and quantitative determination of catecholamines.

EXPERIMENTAL

Materials.—Adrenaline, dopamine, and noradrenaline were used. Adrenaline and noradrenaline were liberated from the hydrochloride and bitartrate monohydrate salts, respectively, by the addition of a dilute ammonium hydroxide solution containing a trace of sodium metabisulfite. The precipitates were filtered off, washed with water, methanol, ether, and dried at low temperature. Thin-layer chromatography, melting point determinations, and infrared spectra analysis demonstrated that each compound had a high degree of purity.

Solvent Systems.—The following solvent systems were used: (*1*) *n*-butanol–acetic acid–water, 4:1:5 v/v (the liquids were shaken together and set aside overnight; the organic layer was separated and used as the running solvent). (*2*) *n*-Amyl alcohol–acetic acid–water, 4:1:5 v/v. (*3*) Methanol–acetone–triethylamine, 50:50:1 v/v. (*4*) Dimethylformamide. (*5*) Phenol–water, 8:2 w/w.

Detection.—The spots were visualized by spraying the plates with a solution of 0.6 Gm. of potassium ferrocyanide and 0.5 Gm. of sodium hydroxide in 100 ml. of water (25).

An ultraviolet lamp was used also to locate the spots in cases where spraying reagents had to be avoided.

Thin-Layer Plates.—All plates used were 20 × 20 cm.

Using MN 300G cellulose powder (Machery, Nagel & Co.) Silica Gel G (Merck), and aluminum oxide G (Merck), plates were prepared according to Stahl (3). The plates were kept in a desiccator using calcium chloride and reactivated at 100° for 5 min. before use.

Methods

Separation of Amines.—Two per cent solutions of the amines were prepared using a 5% acetic acid solution in water. One-tenth microliter quantities were applied 1.5 cm. from the bottom edge of the plates. A distance of 10 cm. from the origin was marked off, and ascending chromatograms were run to this mark (at room temperature).

Quantitative Determination of Adrenaline.—*Weight/Area Relationship.*—A solution of adrenaline was prepared by dissolving 300 mg. of pure compound in 10 ml. of a 2% solution of acetic acid in water, in a 10-ml. volumetric flask. Using a micropipet, quantities of 1 to 5 μl. were applied to the plates 1.5 cm. from the bottom edges. Ascending chromatograms were run (at room temperature).

When the solvent front reached the 10 cm. mark (60–70 min.), the plates were removed and air dried, sprayed, and the spot areas were measured by means of a photovolt Densicord (model 52-C); the graph plotted by the instrument was rendered quantitative by an electronic integrator (Integraph model-49) adapted to the Densicord, and which calculated the spot area which then was expressed in the equivalent square centimeters by the use of a Gelman spot calculator. The area of the spots representing the various concentrations was taken and plotted, using the appropriate relationship.

Elution of the Spots.—A solution of adrenaline was prepared by dissolving 100 mg. of the amine in 3 ml. of a 2% solution of acetic acid in water (master solution). Five different concentrations

of the amine were prepared by diluting quantities of 1 to 5 μl. of the master solution into 10-ml. volumetric flasks and bringing up to the volume using the same solvent. The ultraviolet absorption of each solution was taken at 280 mμ, and from the values obtained, a standard curve was plotted.

Quantities of 1 to 5 μl. of the adrenaline solution (master solution) were applied to thin-layer plates of either silica gel or alumina, and the chromatograms were run in the usual manner.

After drying the plates, the spots were located using an ultraviolet lamp and were marked off. Each spot was then scraped off into small conical flasks. The adrenaline was eluted from the coating material by adding about 7 ml. of the solvent. Each flask was shaken well for about 10 min., centrifuged, and filtered into 10-ml. volumetric flasks. Solvent was added to volume.

No adrenaline residues were found on the filter, testing with the detection reagent (25).

A similar elution process was followed for a blank sample of the coating material alone.

Ultraviolet absorption of each solution was taken at the same wavelength as above (280 mμ), and the values obtained after reducing the absorption due to the blank were recorded next to the standard curve and compared.

To all solvent systems used, small quantities of sodium metabisulfite were added as antioxidant. All chromatograms were carried out in chromatographic chambers enriched in nitrogen atmosphere.

RESULTS AND DISCUSSION

All experiments were performed in duplicate.

The results for the separation of the amines, using alumina, cellulose, or silica gel plates and the various solvent systems, indicate that although there was a considerable movement of the amine spots on alumina plates, when No. *1* and *2* solvent systems were used, the mixture of the three amines could not be separated completely, since the observed R_f values were very close together. A complete separation, however, between dopamine and noradrenaline can be achieved, when the No. *1* solvent system is used.

Similarly on silica gel plates, separation between two amines can be observed, but not complete separation of the three amines.

On cellulose thin layer, solvents No. *1* and *2* showed a partial separation between the amine spots, but when phenol–water (solvent No. *5*) was used, the separation between the three amines was complete (Fig. 1). This indicates that among the layers studied, only that of cellulose, along with the phenol–water solvent system, can give a complete separation when even large quantities of the amines are used.

However, for very small loads (1–30 mcg.) combinations of the above-mentioned solvent systems and alumina, cellulose, or silica gel plates would give complete separation of the amines with the exception of alumina plate and the No. *3* solvent system. Furthermore, in no case was separation between adrenaline and noradrenaline observed when alumina and the No. *4* solvent system or silica gel and the No. *3* or *4* solvent systems were used, but a complete separation of these amines from dopamine always occurred.

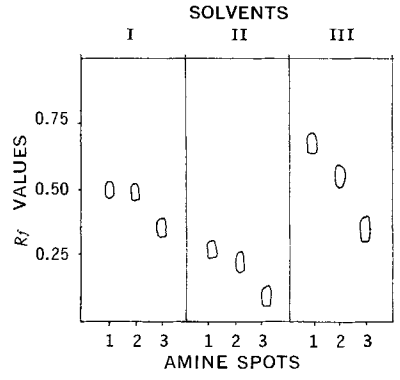

Fig. 1.—Thin-layer chromatography of adrenaline [1], dopamine [2], and noradrenaline [3] on cellulose layers using various solvent systems. Solvents: I, *n*-butanol–acetic acid–water, 4:1:5 v/v; II, *n*-amyl alcohol–acetic acid–water, 4:1:5 v/v; III, phenol–water, 8:2 w/w.

The results obtained for the quantitative estimation of adrenaline by applying the weight/area relationship method when *n*-butanol–acetic acid–water or phenol–water solvent systems were used are shown in Figs. 2 and 3, respectively. In Fig. 4 results obtained from the elution method are depicted.

For the weight/area method, a linear relationship between the spot area and the weight of the compound has been found when silica gel or alumina plates and either solvent systems No. *1* or *5* were used. This relationship shows the dependence of the area of the spot upon the amount of the sample used, and although the size of the spot varied with the thickness of the layers, the temperature of the experiment and the purity of the solvent used, the observed results were reproducible within ±5%. However, it is advisable ro run on the same plate both the standards and the sample.

Although the best separation of adrenaline from the other amines was achieved on cellulose plates,

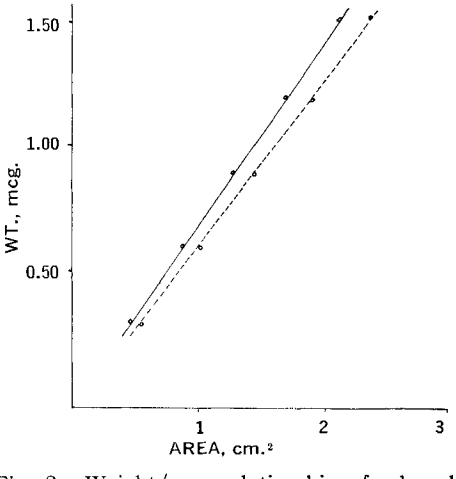

Fig. 2.—Weight/area relationship of adrenaline spots when *n*-butanol–acetic acid–water, 4:1:5 v/v solvent system, was used. Key: - - - - - -, silica gel plates; ——, alumina plates.

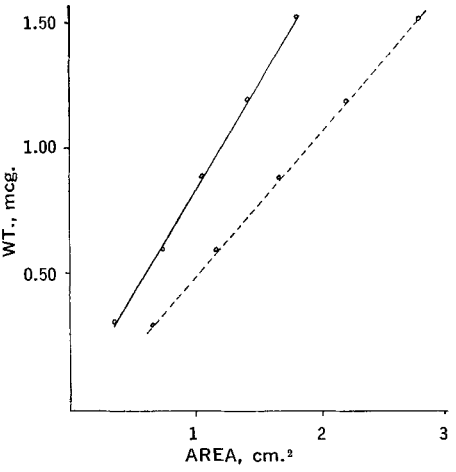

Fig. 3.—Weight/area relationship of adrenaline spots when phenol–water, 8:2 w/w solvent system, was used. Key: - - - - - -, silica gel plates; ——, alumina plates.

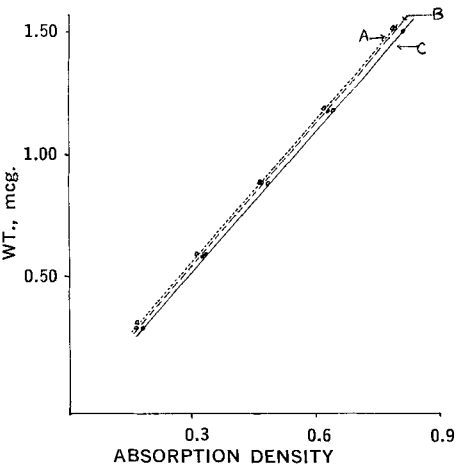

Fig. 4.—Adrenaline recovery after elution of the spots from thin-layer chromatograms of alumina (A) and silica gel (B). The straight line (C) indicates the blank. *n*-Butanol–acetic acid–water, 4:1:5 v/v solvent system, was used.

using the phenol–water solvent system, it was found that for the quantitative estimation of the separated amine, alumina or silica gel plates and the No. *1* solvent system gave the most accurately reproducible results (Fig. 4).

The recovery of the compounds, after the elution of the spots, was 93 and 97%, respectively, for thin layers of alumina or silica gel. The lower recovery on the alumina thin layer can be attributed to the strong adsorption forces by which the substance is held on the alumina (11). A recovery of below 85% was observed when cellulose layers were used. Here the adsorption and partition forces influence the recovery (11).

However, for quantitative determination of adrenaline, the above-mentioned methods may be regarded as simple and accurate, though the second method has been found more reliable, since factors which might have influenced the spot area in the first method are involved only to a certain extent in the elution method.

The use of these methods can also be extended for quantitative determination of other sympathomimetic amines and other compounds.

REFERENCES

(1) McGreer, E. G., and Clark, W. G., *J. Chromatog.*, **14**, 107(1964).
(2) Mattok, G. L., *ibid.*, **16**, 254(1964).
(3) Stahl, E., *Pharmazie*, **11**, 633(1956).
(4) Stahl, E., *Chemiker-Ztg.*, **82**, 323(1958).
(5) Stahl, E., *Pharmazeutische Rundschau I*, Spec. Edit. No. 2, 1959.
(6) Shepherd, D. M., and West, G. B., *Nature*, **169**, 797 (1952).
(7) West, G. B., *J. Pharm. Pharmacol.*, **11**, 595(1959).
(8) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *Nature*, **186**, 775(1960).
(9) Beckett, A. H., Beaven, M. A., and Robinson, A. E., *J. Pharm. Pharmacol., Suppl.*, **12**, 203(1960).
(10) Beckett, A. H., and Choulis, N. H., *ibid.*, **15**, 236 (1963).
(11) Beckett, A. H., and Choulis, N. H., *XIII Intern. Kongr. Pharm. Wiss.*, Münster, Germany, 1963.
(12) Roberts, J., *J. Pharm. Pharmacol.*, **15**, 532(1963).
(13) Teicher, K., Mutschler, E., and Rochelmeyer, H., *Deut. Apotheker-Ztg.*, **100**, 283(1960).
(14) Waldi, D., *Arch. Pharm.*, **295**, 125(1962).
(15) Seher, A., *Mikrochim. Acta*, **1961**, 308.
(16) Purdy, S., and Truter, E. R., *Chem. Ind.*, **1962**, 506.
(17) Stahl, E., *Z. Anal. Chem.*, **181**, 303(1961).
(18) Breuner, M., and Niederwieser, A., *Experientia*, **16**, 378(1960).
(19) Pelka, J. R., and Metcalf, L. D., *Anal. Chem.*, **37**, 603(1965).
(20) Morrison, J. C., and Orr, J. M., *J. Pharm. Sci.*, **55**, 936(1966).
(21) Ganshirt, H., Koss, F. W., and Morianz, K., *Arzneimittel-Forsch.*, **10**, 943(1960).
(22) Zollner, N., Wolfram, G., and Amin, G., *Klin. Wochschr.*, **40**, 273(1962).
(23) Millett, M. A., Moore, W. E., and Seaman, J. F., *Anal. Chem.*, **36**, 491(1964).
(24) Eble, J. N., and Brooker, R. M., *Experientia*, **18**, 524 (1962).
(25) James, G., *Nature*, **161**, 551(1948).