portion of the DMCTC standard solution treated in the same manner.

The corrected sample absorbance will give a direct measure of the DMTC when used with the standard curve.

Calculation—(*a*) mg. of antibiotic (DMTC and DMCTC) in sample weight:

$$\frac{\text{sample abs. at 368 m}\mu}{\text{standard abs. at 368 m}\mu} + 10.0 \times \frac{100}{1000} \times \frac{500}{5} = \text{mg.}$$

(*b*) corrected sample absorbance value to be used with concentration curve: $\frac{100}{\text{mg.}} \times$ abs. at 425 mμ (sample) = corrected value

SUMMARY

With the use of nitric acid, an assay for DMTC in DMCTC has been developed. DMCTC is unstable to nitric acid while DMTC is sufficiently stable for purpose of assay. The data presented suggest that DMTC could also be measured in the presence of CTC, TC, and OTC by this procedure.

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Keyphrases

6-Demethyltetracycline in 6-demethylchlortetracycline—analysis

6-Demethylchlortetracycline—nitric acid transformation

Spectrophotometry—analysis

Quantitative Thin-Layer Chromatography of Sympathomimetic Amines

By N. H. CHOULIS* and C. E. CAREY

Thin-layer chromatography for quantitative estimation of sympathomimetic amines has been used. Two methods were performed: the weight/area relationship and the elution of the spots and spectrophotometric determination in the ultraviolet. For the first method, solutions of the amines, in increasing concentration, were applied on cellulose thin layer, and a solution of unknown concentration of the same amines was run parallel to the standards. The spot was compared with the standards using a recording photoelectric densitometer. For the second method the standard spots were eluted from the cellulose plates and their absorption was measured. The absorptivity of a similarly eluted spot of unknown concentration, but parallel running, was measured and compared with the standards. The accuracy of the methods appeared to be between 95–98 percent.

THE APPLICATION of thin-layer chromatography to pharmaceutical analysis has been used increasingly during the past years. A number of methods for quantitative analysis have been employed since the technique was first introduced by Stahl (1) in 1956.

Seher (2) has suggested that in thin-layer chromatography the weight of the material and the spot area are proportional. Purdy and Truter (3) 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 (4) and Breuner and Niederwieser (5), fit the relationship for loads of 1 to 80 mcg./spot. Pelka and Metcalf (6) examining long-chain tertiary amines, found the method applicable for loads of approximately 200 mcg.

Morrison and Orr (7), in an analysis of selected pharmaceutical mixtures in tablet and capsule forms and 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 spots 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.* (8) used thin-layer chromatography for the quantitative assay of a number of bile acids, and Zollner *et al.* (9) found the method applicable for the quantitative estimation of cholesterol esters.

Millett *et al.* (10) described other techniques for

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quantitative thin-layer chromatography using furoic acid and hydroxymethylfuroic acids as model compounds. Eble and Brooker (11) suggested an application of thin-layer chromatography for quantitative assay of tryptamine.

Choulis (12) recently used thin-layer chromatography for quantitative estimation of adrenaline.

The purpose of the present paper is to examine the applicability of the technique as a general method for quantitation for sympathomimetic amines in pharmaceutical formulations.

EXPERIMENTAL

Materials—Amphetamine hydrochloride, noradrenaline hydrochloride, normetanephrine hydrochloride, metanephrine hydrochloride, dopamine, ephedrine hydrochloride, isopropylarterenol hydrochloride, nordefrin hydrochloride, dopa, and phenylephrine hydrochloride were used.

Developing System—*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).

Detection—Amphetamine was detected by the *p*-nitroaniline diazo reagent described by Wickstrom and Salversen (13). The reagent was prepared by dissolving 0.25 Gm. *p*-nitroaniline in 25 ml. of 1 *N* hydrochloric acid and diluted to 50 ml. with ethanol. The solution was cooled in an ice bath and 0.1 Gm. sodium nitrate was added before use; the amphetamine was detected by the appearance of a pink spot.

Dopa, dopamine, and ephedrine hydrochloride were located by spraying with a solution of ninhydrin in *n*-butanol or acetone (0.2% w/v) and heating the plates at 100° for 4 min. (14).

All other amines were detected by using an aqueous solution containing 0.6% w/v potassium ferricyanide and 0.5% w/v sodium hydroxide (15).

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

Preparation of the Plates—The plates (20 × 20 cm.) were coated with cellulose layer, 250 mμ thick, according to the method of Stahl (1), also (12).

General Procedure—Solutions of the amines were prepared by dissolving 200 mg. of the pure compounds in 10 ml. of a 5% solution of acetic acid in water, in 10-ml. volumetric flasks (master solutions).

Using accurately calibrated microliter pipets, samples of the solutions were applied to the plates, 1.5 cm. from the bottom edges, and ascending chromatograms were run at room temperature (about 23°). The solvent front was allowed to travel 10 cm.; the plates were removed, air dried, and sprayed with the appropriate reagent.

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.

Methods—(*a*) Spot area/concentration relationship—The relationship between spot area and concentration was established by spotting various concentrations of the amine solutions on thin layers. The chromatograms were developed and the spots were detected using various spray reagents. The detected chromatograms were positioned on the stage of a Photovolt Densitometer, model 52-C, and the spots were scanned perpendicularly to the direction of chromatographic development. Using a 1- × 10-mm. slit which covered the spots completely, the machine was adjusted to read zero absorbance at a point a few millimeters from the spot; scanning was begun and the peak area was traced on a Varicord recorder with a 6.5-in. paper. The area under the peak was integrated with the photovolt electronic integrator, and also calculated with the use of Gelman high-precision planimeter. Standard curves were constructed from plots of the area *versus* concentration of various amines.

(*b*) Ultraviolet absorption/concentration relationship—The relationship between ultraviolet absorption and concentration of the amines was established by spotting again various concentrations of the amine solutions on thin layers. The chromatograms were developed and the spots were detected using an ultraviolet lamp and marked. The marked areas, which corresponded to the spots, were carefully scraped off and the cellulose was quantitatively transferred to 20-ml. conical flasks. The amines were eluted from the coating material by adding about 7 ml. of the solvent; the flasks were shaken well for about 10 min., centrifuged, and filtered into 10-ml. volumetric flasks. Solvent was added to the volume. No amine residues were found on the filters testing with the appropriate amine reagent. A similar elution process was followed for a blank sample of the coating material alone.

Spectrophotometric measurements were carried out at λ maxima of each individual amine, using a Beckman DU spectrophotometer with 1-cm. cells, and standard curves of absorptivity *versus* concentration of the various amines (after reducing the absorbance due to the blank) were constructed.

RESULTS

Relationship Between Spot Area and Concentration—The quantitation of the amine spots, after spraying the chromatograms with the appropriate reagent, was calculated from plots of the peak areas *versus* concentrations with the standard deviation on the plate, and tabulated as indicated in Table I where the relationships for amphetamine hydrochloride and phenylephrine hydrochloride in concentration ranges between 1–15 mcg. were established. The average deviation from the theoretical value was calculated and expressed in percentages.

TABLE I—RECOVERY VALUES AND PERCENTAGE DEVIATION OF AMPHETAMINE HCl AND PHENYLEPHRINE HCl USING PHOTOELECTRIC DENSITOMETER

Compounds	Quantities Spotted on Plates, mcg.	Theoretical Values of Integrated Area Units, Pips	Received Mean-Values of Integrated Area Units, Pips	Av. Deviation from 100%
Amphetamine hydrochloride	1	8	8	
	3	16	16	
	5	32	31	−5
	7	48	46	
	9	54	58	
Phenylephrine hydrochloride	3	25	24	
	6	50	50	
	9	75	72	−3
	12	100	96	
	15	125	121	

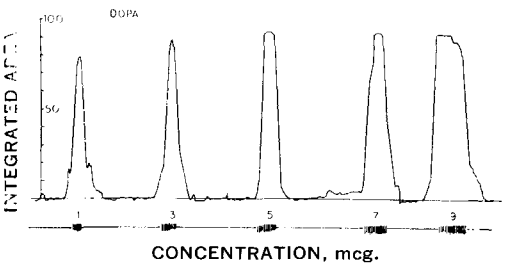

Fig. 1—Typical Varicord traced areas for dopa.

Figure 1 illustrates typical Varicord-traced areas for dopa, in concentration ranges between 1 and 9 mcg. Similar results were observed for all other examined amines using concentration areas between 2 and 40 mcg.

Relationship Between Ultraviolet Absorption and Concentration—The quantitation of the amine spots was determined from plots of absorbance *versus* concentration and tabulated, using the mean values from a number of measurements, as indicated in Table II, where the relationships of amphetamine hydrochloride and phenylephrine hydrochloride in concentration ranges between 0.1 and 5 mcg. were established.

Here also the average deviation from the theoretical value was calculated and expressed in percentages.

DISCUSSION

In the present investigation two methods for quantitative determination of sympathomimetic amines are employed, namely the spot area/concentration and the ultraviolet absorption/concentration.

All experiments were carried out in triplicate and the mean values were recorded.

For the first method, linear relationships between the spot areas and the weight of the compounds used were found using densitometric measurements. This relationship shows the dependence of the area of the spot upon the amount of the amine used. From the calculated mean values, a deviation of ±5% was observed.

Although the method is considered sufficiently accurate for routine pharmaceutical analysis, some limitations may be imposed. For example, a tendency to overload the capacity of the plates with an excess of spots, or even to overload the spots must be avoided. The best results were obtained with 25-mcg. or less quantities/spot, although higher concentrations can be determined accurately provided that the size of the initial spot application area should be as small as possible, due to the fact that the spot area is increased during the development of the chromatogram, and also that the photocell of the densitometer cannot detect areas outside its optical field.

Moreover, the temperature of the experiments (room temperature) must be constant, and the purity of the solvents used must be examined so that R_f values will be more reproducible.

The greater difficulty, however, is in the preparation of the appropriate plates, because variations in the thickness of the cellulose layer alter the light reflected by the background. Spray reagents which color the background will also lead to incorrect results, since the densitometer records the difference in intensity between the detected spot and the noncolored background.

Table II—Recovery Values and Percentage Deviation of Amphetamine HCl and Phenylephrine HCl Using Ultraviolet Spectrophotometer

Compounds	Quantities Spotted on Plates, mcg.	Theoretical Absorptivity Values	Absorptivity after Elution, Mean Value	Deviation from 100%
Amphetamine hydrochloride	0.3	0.06	0.06	
	0.6	0.12	0.11	
	0.9	0.18	0.19	−2
	1.2	0.24	0.24	
	1.5	0.30	0.28	
Phenylephrine hydrochloride	1	0.10	0.10	
	2	0.20	0.195	
	3	0.30	0.29	−4
	4	0.40	0.38	
	5	0.50	0.47	

Using the ultraviolet absorption/concentration method, linear relationships between the absorption density of the eluates and the weight of the compounds used were observed; the recovery of the compounds after elution of the spots was approximately 95% (±3%).

Previous observations (12) of a low recovery of the amines from cellulose layers had not recurred. Presumably the higher concentration of the acid solution used (5% instead of 2%) overpowered the influence of the absorption and partition forces, which otherwise operate exclusively on cellulose layers (16).

For quantitative estimation of sympathomimetic amines, the above-mentioned methods may be regarded as simple and accurate, and the use of these two techniques in routine microanalysis is suggested

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Keyphrases

Sympathomimetic amines—analysis
TLC—separation
Densitometry—spot area/concentration relationship
UV spectrophotometry—analysis