Analysis of Selected Pharmaceuticals by Quantitative Thin-Layer Chromatography

By J. C. MORRISON* and J. M. ORR

Thin-layer chromatography was employed to separate the components of 14 selected commercial pharmaceutical mixtures in tablet and capsule form. The components included the amphetamines, certain barbiturates, and several related compounds. A recording photoelectric densitometer with an electronic integrator was utilized to scan and estimate quantitatively the various constituents which were rendered visible by specific reagents. Silica Gel G was the adsorbent used and a dioxane–benzene–25 per cent ammonia mixture (40:40:10 v/v) was found to be a suitable developing solvent for separating the mixtures examined. Most of the drugs studied could be estimated with an experimental error of approximately 5 per cent when applied in concentrations of 25 to 100 mcg.

MULTICOMPONENT tablets and capsules provide two of the most convenient forms for the oral administration of drugs since they are easily mass produced and provide a compact accurate dose in a convenient form. For this reason they constitute the method of administration for over 75% of all drugs prescribed.

The widespread use of these dosage forms has given rise to problems in pharmaceutical analysis. In many cases the concentration of an incorporated drug is extremely small—a milligram or less—and rapid techniques of semimicro analysis must be devised for its assay. As the formulation of the preparation becomes more complex the greater are the assay problems introduced since the components must be separated and estimated individually. For the purpose of quality control, three or more components may have to be isolated and assayed in the product.

Since its introduction by Stahl in 1956 (1) the technique of thin-layer chromatography has been used extensively in many fields. In the past 3 years, however, it has been applied increasingly to pharmaceutical analysis because of its rapidity and the high degree of resolution achieved. Under specified conditions the technique lends itself to quantitative interpretation and can be used for analytical control and toxicological investigations. Direct elution techniques from the adsorbent may be used provided no extracted contaminants interfere with the assay, although colorimetric reagents can be employed to ensure that only specific chemicals are estimated (2). Since there is a correlation between the amount of drug applied to a plate and the area of the developed spot, drugs have been estimated by careful measurement of spot area (3). By this method Morrison and Chatten estimated antihistamines in drug mixtures and measured the spot areas involved manually (4).

The present work was undertaken to develop the technique of densitometric measurement, a method in which spot area is calculated electronically. This method of area measurement is simple to operate and compares favorably with gravimetric or spectrophotometric techniques. Furthermore, in using a photoelectric device, the process of measurement is rapid and convenient. Such an instrument allows analytical procedures to become automated when their reliability has been established and these procedures can then be carried out by semiskilled operators. By employing specific reagents to render the drug or drugs visible on the chromatoplate, the technique nullifies the effect of any impurities present in the adsorbent and ensures that only the drug is estimated. The need for elution or extraction of the drug is thus eliminated (5–7).

The authors report the application of such a technique to pharmaceutical preparations available in Canada and suggest possible sources of error in the method.

EXPERIMENTAL

Materials and Apparatus.—Glass plates (200 × 200 mm.) in glass developing tanks lined with solvent saturated filter paper.

Preparation of the Plates.—The plates were coated with a layer of adsorbent 250 mμ thick according to the method of Stahl (1). The slurry was prepared by mixing 30 Gm. of Silica Gel G with 60 ml. of 25% 1,2-dimethoxyethane in distilled water. This resulted in a smooth even film of adsorbent which was not liable to flake or crack.

Chemicals.—All chemicals and reagents used were analytical reagent grade. The chemical purity of the standards was checked by observing their melting points and comparing them with the literature. As further proof of purity, each standard produced only one spot on chromatographic examination. The drugs used in this investigation were acetylsalicylic acid, salicylic acid, phenacetin, amphetamine sulfate,

Received March 28, 1966, from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.
Accepted for publication June 8, 1966.
* Present address: School of Pharmacy, College of Technology, Portsmouth, Hampshire, England.

TABLE I.—SPRAY REAGENTS USED FOR DRUG DETECTION

Spray Reagent	Constituents	Color, Spot	Color, Background
50% aqueous sulfuric acid	...	Black–brown	White
Furfural spray	20% furfural in *o*-phosphoric acid	Blue–black	White
Mercury-dithizone	(*a*) Suspend 5 Gm. mercuric oxide in 100 ml. of water and add 20 ml. of conc. H_2SO_4. Cool and dilute to 250 ml. with water. (*b*) 10% diphenylthiocarbazone in chloroform. Spray with (*a*) and then (*b*).	Reddish–brown	Gray
Sodium molybdate	0.1% sodium molybdate in conc. sulfuric acid.	Brown	White
Ferric chloride	(*a*) 10% aqueous ferric chloride, 2 parts. (*b*) 5% aqueous potassium ferricyanide, 1 part. (*c*) Distilled water, 8 parts. Mix and spray immediately.	Blue–mauve	Pale green
Dragendorff's reagent (modified)	Bismuth subnitrate, 3.4 Gm. Glacial acetic acid, 20.0 ml. Potassium iodide, 10.0 Gm. Distilled water, 60.0 ml. Dilute 1 ml. of above with 3 ml. of glacial acetic acid and 6 ml. of distilled water.	Orange–red	Gray
Bratton-Marshall reagent	(*a*) 1 *N* hydrochloric acid. (*b*) 5% sodium nitrite. (*c*) 0.1% solution of *N*-(1-naphthyl) ethylene diamine dihydrochloride. Spray with (*a*) and (*b*). Heat and spray with (*c*).	Red–purple	Gray

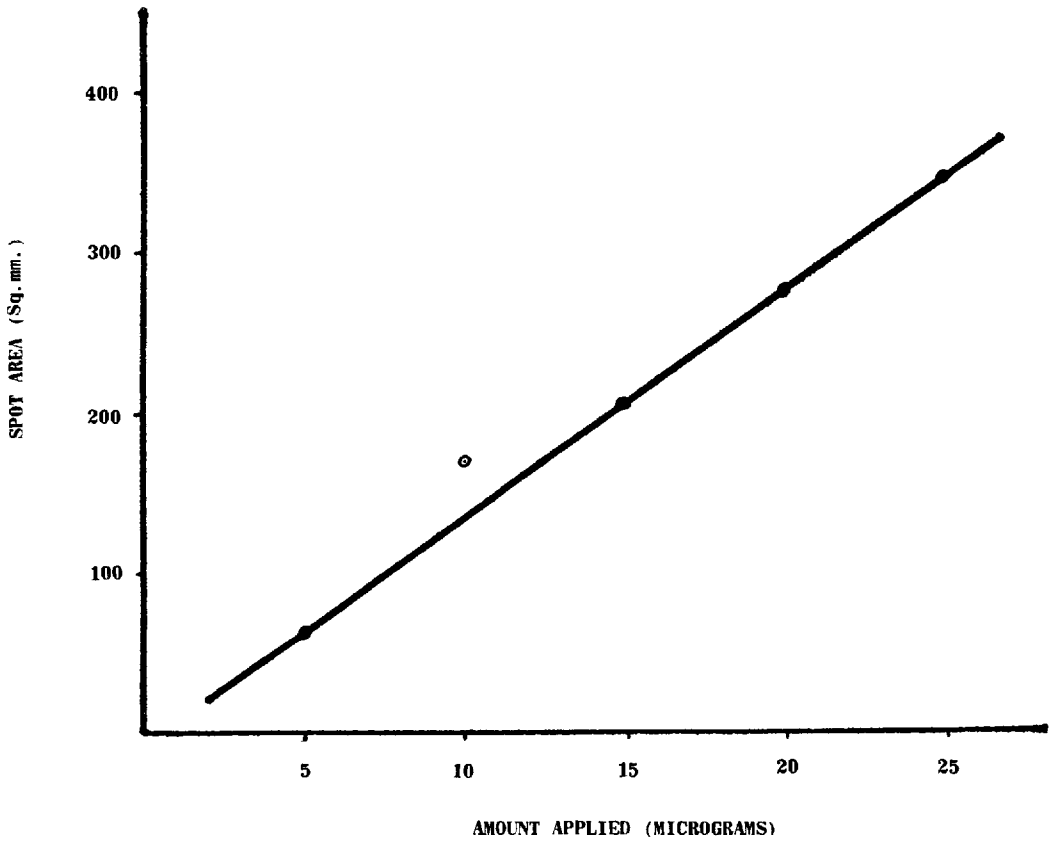

Fig. 1.—Relationship between spot area and spot weight for amphetamine sulfate.

methamphetamine hydrochloride, theophylline, caffeine, ephedrine, phenobarbital, amobarbital, butabarbital, pentobarbital, carbromal, meprobamate, and prochlorperazine maleate.

Developing Solvent.—The developing solvent used in all cases was dioxane–benzene–25% ammonia (40:50:10 v/v).

Spray Reagents.—The compositions of the seven spray reagents used in this investigation are listed in Table I.

Application of Drugs and Development of Chromatogram.—The samples for analysis were applied in ethanolic solution approximately 1 in. from the edge of the plate. Self-filling lambda pipets cali-

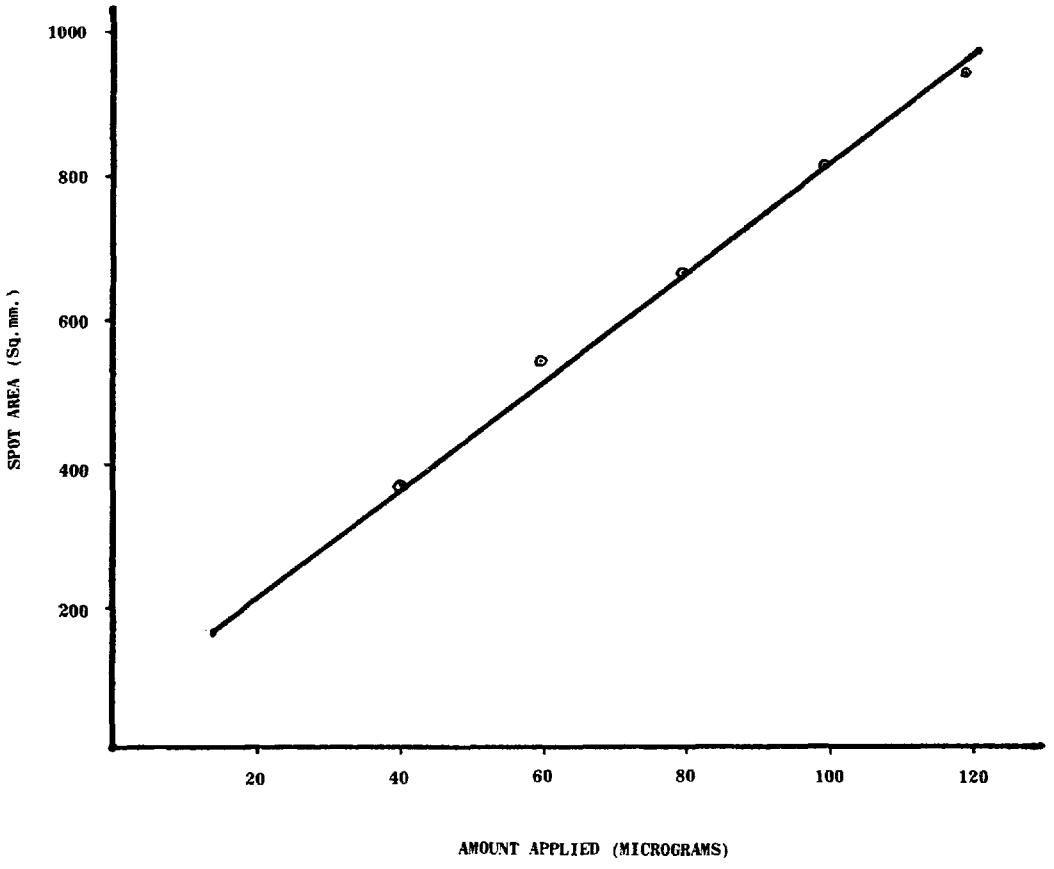

Fig. 2.—Relationship between spot area and spot weight for phenobarbital.

brated to deliver accurately known volumes were used for application, and the spot area was 6–8 mm. in diameter. The plates were developed through a distance of 15 cm., dried at room temperature for 15 min., oven dried for 20 min. at 110°, and sprayed with the appropriate reagent. Reference standards for each drug mixture were applied on the same plate and in concentrations commensurate with the drugs in the sample.

Measurement of Spot Area.—A photovolt densicord (model 542) was used to obtain quantitative results from the developed chromatograms which were positioned directly under the scanning head at a height of approximately 6 mm. The response control selected for the instrument varied with the chemical reagent used to render the drugs visible. The resulting graph plotted by the instrument was rendered quantitative by the densicord electronic integrator which calculated the area under the curve. Results were calculated with reference to the appropriate standard.

Preparation of Standard Curves.—To establish the relationship between spot area and concentration, amphetamine, phenobarbital, and acetylsalicylic acid were spotted in various concentrations in their respective detectable ranges. Following development, the chromatograms were scanned and the integrated areas found for each concentration.

Assay of Simulated Drug Mixtures.—To check the reliability of the method ethanolic solutions of several drugs in concentrations approximating those of various commercial preparations were spotted on plates and developed. The chromatograms were then scanned with the densitometer and compared with relative standards.

Assay of Commercial Preparations.—Each of the preparations was treated individually due to differences in the concentrations of the constituents. The amount of drug applied to the chromatogram varied from one product to another, but this was necessary to ensure that the spot would contain the minimum assayable quantity of the drug. The general procedure for tablets consisted in crushing several weighed tablets in a mortar and dissolving a weighed portion in approximately 15 ml. of ethanol. After shaking for 20 min., the solution was filtered, rinsed with two portions of ethanol, and made up to volume. Assay limits were established by running a series of chromatograms containing several concentrations of the drugs. The appropriate standards were applied to the plate to ensure a total of 10 assays for each constituent. Standards and samples were spotted alternately on the plate to facilitate assay and nullify any variation in the silica gel layer. After development, the plates were sprayed with the appropriate reagent to de-

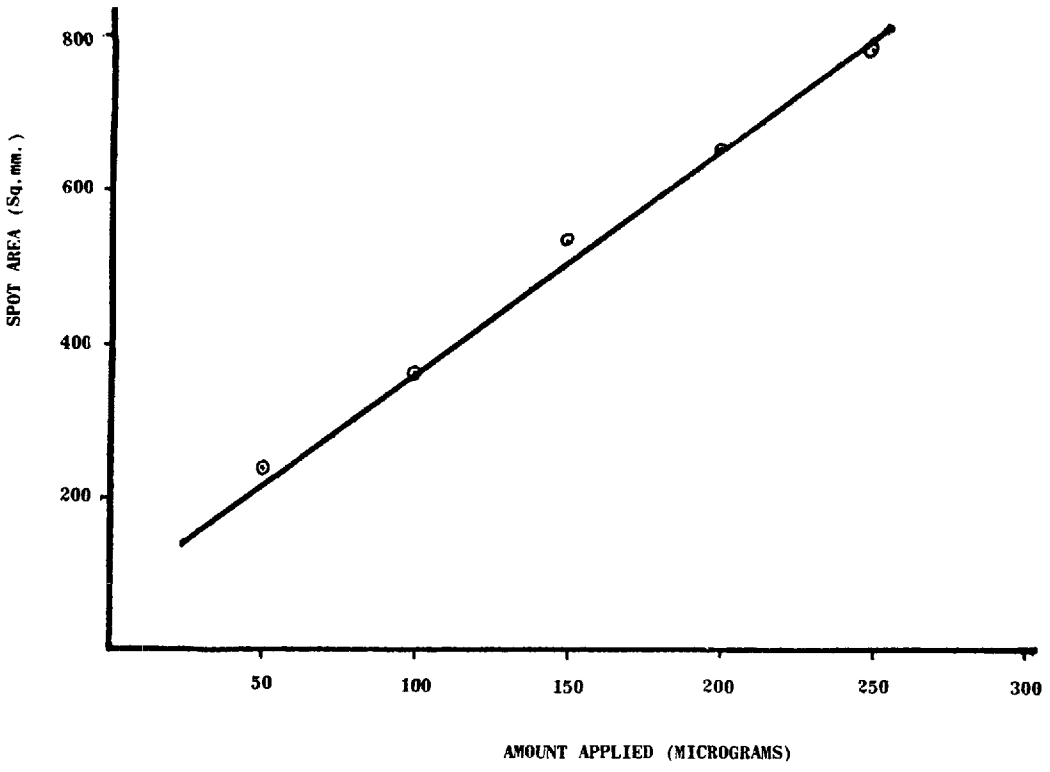

Fig. 3.—Relationship between spot area and spot weight for acetylsalicylic acid.

TABLE II.—DATA SHOWING CORRELATION BETWEEN SPOT WEIGHT AND SPOT AREA

Drug	Amt. Applied, mcg.	Area, Sq. mm.
Amphetamine sulfate	5	60
	10	168
	15	204
	20	276
	25	348
Phenobarbital	40	372
	60	540
	80	660
	100	804
	120	936
Acetylsalicylic acid	50	240
	100	360
	150	528
	200	648
	250	780

velop visible spots. R_f values were recorded and the chromatograms scanned by the densitometer.

RESULTS

Relationship Between Spot Area and Concentration.—By preparing a series of known standards it was possible to demonstrate a linear relationship between spot area and spot content and to reproduce this correlation within certain parameters (Figs. 1–3). This relationship was established for amphetamine sulfate in concentration ranges between 5 and 25 mcg. and for phenobarbital and acetylsalicylic acid in concentrations between 40 and 120 mcg. and between 50 and 250 mcg., respectively. The slight variation in the slopes of the lines can be attributed to several factors such as the intensity of stray light

TABLE III.—QUANTITATIVE RESULTS FOR SIMULATED DRUG MIXTURES

Drug	Amt./Tablet, mcg.	Mean % Recovery	R_f Value	Spray Reagent
Simulated preparation *8*				
d-Amphetamine sulfate	12.0	96.4 ± 2.3	0.44	50% sulfuric acid
Phenobarbital	48.0	97.0 ± 2.4	0.62	
Simulated preparation *1*				
d-Amphetamine sulfate	5.0	99.7 ± 3.7	0.21	50% sulfuric acid
Meprobamate	400.0	98.3 ± 2.0	0.51	Furfural
Simulated preparation *12*				
Amobarbital	32.0	95.4 ± 1.3	0.79	Mercury-dithizone
Phenacetin	160.0	98.7 ± 3.6	0.48	Ferric chloride

TABLE IV.—QUANTITATIVE RESULTS FOR COMMERCIAL PHARMACEUTICALS

Prepn.	Labeled Strength/ Tablet	Mean % Recovery	Min. Detectable Quantity, mcg.	Min. Assayable Quantity, mcg.	Max. Assayable Quantity, mcg.	Spray Reagent	R_f Value
1 (tablets)							
d-Amphetamine sulfate	5.0	99.4 ± 3.4	10	10	20	50% sulfuric acid	0.22
Meprobamate	400.0	98.0 ± 3.3	80	80	1600	Furfural	0.49
2 (tablets)							
d-Amphetamine sulfate	5.0	94.5 ± 2.7	5	10	200	50% sulfuric acid	0.21
Prochlorperazine maleate	2.5	92.6 ± 3.5	5	10	150		0.38
3 (tablets)							
d-Amphetamine sulfate	5.0	94.6 ± 4.8	5	10	200	50% sulfuric acid	0.29
Amobarbital	32.0	93.7 ± 2.6	40	50	300	Mercury-dithizone	0.85
4 (tablets)							
d-Amphetamine sulfate	5.0	97.1 ± 5.2	5	10	200	Sodium molybdate	0.27
Sodium butabarbital	32.0	94.1 ± 1.7	40	50	300	Mercury-dithizone	0.55
5 (tablets)							
d-Amphetamine sulfate	15.0	95.8 ± 5.6	5	10	200	Sodium molybdate	0.31
Amobarbital	100.0	95.9 ± 2.2	40	50	300		0.86
6 (tablets)							
d-Amphetamine phosphate	5.0	96.3 ± 3.9	5	10	200	50% sulfuric acid	0.44
Pentobarbital	32.0	94.2 ± 2.6	40	50	300	Sodium molybdate	0.73
7 (tablets)							
Methamphetamine hydrochloride	15.0	94.0 ± 2.9	5	10	200	50% sulfuric acid	0.20
Phenobarbital	64.8	95.2 ± 5.0	40	50	350		0.85
8 (tablets)							
d-Amphetamine sulfate	12.0	97.9 ± 4.9	5	10	200	50% sulfuric acid	0.46
Phenobarbital	48.0	96.2 ± 3.9	40	50	350		0.60
9 (capsules)							
Carbromal	250.0	96.4 ± 4.3	85	100	300	Furfural	0.73
Pentobarbital	100.0	95.6 ± 3.4	40	50	300	Sodium molybdate	0.59
10 (tablets)							
Acetylsalicylic acid	160.0	98.9 ± 5.8	50	50	350	Ferric chloride	0.00
Phenacetin	160.0	98.5 ± 6.5	50	50	350	Ferric chloride	0.47
dl-Amphetamine	2.5	99.4 ± 3.8	10	10	350	50% sulfuric acid	0.18
11 (tablets)							
Phenobarbital	25.0	97.3 ± 2.7	40	50	300	50% sulfuric acid	0.40
Ephedrine hydrochloride	48.0	91.8 ± 3.3	10	10	200	Dragendorff's	0.18
Theophylline	180.0	No recovery	...	...	...	...	0.00
12 (tablets)							
Amobarbital	32.0	99.1 ± 3.5	40	50	300	Mercury-dithizone	0.72
d-Amphetamine sulfate	5.0	100.6 ± 2.9	5	10	300	50% sulfuric acid	0.23
Acetylsalicylic acid	160.0	96.5 ± 3.3	50	80	400	Ferric chloride	0.00
Phenacetin	160.0	96.4 ± 3.7	50	80	400	Ferric chloride	0.51
13 (tablets)							
Acetylsalicylic acid	200.0	93.8 ± 3.0	50	80	300	Dragendorff's	0.00
Phenacetin	150.0	93.8 ± 3.4	50	80	300	Ferric chloride	0.63
Caffeine citrate	30.0	96.6 ± 2.5	10	30	300	Ferric chloride	0.53
Meprobamate	200.0	96.9 ± 3.5	80	80	1600	Furfural	0.45
Triple-sulfas (tablets)							
Sulfamethazine	167.0	96.6 ± 4.9	0.2	0.5	2.5	Bratton-Marshall	0.75
Sulfadiazine	167.0	94.5 ± 2.7	0.2	0.5	2.5	reagent	0.63
Sulfamerazine	167.0	96.3 ± 3.8	0.2	0.5	2.5		0.71

on the photocell, the detection spray used, and variations in layer thickness (8). The results are shown in Table II.

Results for Simulated Drug Mixtures and Commercial Preparations.—The percentage recoveries obtained from the drug mixtures simulating existing commercial preparations are shown in Table III, and the recoveries from the commercial preparations are shown in Table IV which includes the labeled strength of each preparation and the mean recovery from 10 assays on each preparation. The R_f values and the minimum and maximum assayable quantities are included for each preparation together with the staining reagent used to render the drugs visible.

DISCUSSION

From the results obtained in this study it is suggested that the technique of densitometric measurement is sufficiently accurate for routine pharmaceutical analysis and can be applied to pharmaceutical dosage forms. The average error involved was found to be about 5%, a figure which is similar to that reported in related fields (9–11).

Several experimental factors were found to be critical in obtaining consistent and accurate results. Since the area of the spot can increase during development from 25 to 100%, the size of the initial spot application should be as small and as uniform as possible. An applied spot of 6–8 mm. was found to be satisfactory. Spots which are not uniformly

applied may occupy different areas, and this difference with its consequent error will be registered by the photocell. Similarly, variations in the layer thickness of the silica gel can cause alterations in the light reflected by the background, and this error can be recorded by the densitometer since it is designed to produce results which are based on the difference in photoelectric intensity between the visible spot and the background of the plate. For this reason it is essential to use spray reagents which produce stains specific for individual drugs and which do not color the background to any appreciable extent, otherwise the contrast between spot and background will be diminished. If a satisfactory spray reagent cannot be developed, it is doubtful whether the method could be used for quantitative work.

In using thin-layer densitometry it was essential that the drugs being assayed separated sharply and distinctly as any degree of spot overlap renders area measurement techniques liable to error. To some extent it was found possible to counteract the effects of overlap by spraying the plate with a stain which rendered only a particular drug visible for purposes of assay. Alternatively, two or more plates could be used, one of which was treated to allow estimation of one fraction of the drug mixture while the other plate was developed to estimate any additional components.

The minimum quantity of a drug necessary for detection on a thin-layer plate appeared to depend on the specificity of the spray reagent used to produce the necessary color, and it was observed that these chemical stains varied in their relative sensitivities of detection. For example, with the exception of phenobarbital, the barbiturates did not appear distinctly after charring with sulfuric acid, but when a 2% mercuric sulfate spray was used followed by 0.1% sodium molybdate in concentrated sulfuric acid both amobarbital and butabarbital appeared as distinct brown spots on a gray background and could be assayed satisfactorily. Consequently, the minimum assayable quantity for these drugs using the acid-molybdate spray was 40 mcg. as opposed to 50 mcg. with the sulfuric acid spray. Thus, it was found necessary to vary the amount of solution applied to a plate in such a way that the amount of drug being estimated contained the minimum detectable quantity. For example, in preparation *I* the tablets have a ratio of amphetamine to meprobamate of 1–80 and the amounts of solution applied to the plate in the assay of this preparation must be varied to allow the accurate estimation of each drug.

Due to the very low solubility of prochlorperazine maleate in the extracting solvents the agitation time during its extraction was increased from 30 to 60 min. to ensure its complete removal from the tablet. Solubility was also a factor in the assay of preparation *11* when it was found that the theophylline in the tablets has such a low solubility and was so slowly soluble that quantitative estimation by this method proved unreliable.

Triple-sulfa tablets U.S.P. were included in this study to demonstrate the application of the densitometric technique to systems where distinct stains exist or can easily be found. Using the method reported by Wehrli (12) for the qualitative separation of these sulfonamides, it was found that quantitative evaluation of the tablets could be readily carried out by this method.

It is suggested that if a sensitive spray reagent exists and the correct experimental conditions are observed regarding the parameters of the assay, the densitometric method could be applied in various fields of pharmaceutical analysis to yield quantitative results.

SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent has been devised for the separation and analysis of 14 selected pharmaceuticals which included amphetamines, barbiturates, and related compounds. It was demonstrated that a linear relationship exists between spot area and spot content. Quantitative evaluation was achieved without elution from the adsorbent by using a photoelectric densitometer coupled to an electronic integrator which computed the spot areas. The experimental error was found to be approximately 5%. The technique gave quantitative results when the drugs were applied to the plates within certain concentrations. These concentrations varied with individual drugs but were in the range of 25 to 100 mcg. generally. Dioxane–benzene–25% ammonia (40:50:10 v/v) was found satisfactory for resolving the selected pharmaceuticals into their respective components. Results were obtained more rapidly and with greater convenience than by planimetry or visual area measurement. Several experimental factors which influence quantitative recovery are discussed. The technique could be applied routinely in microanalysis and has specific application to pharmaceuticals.

REFERENCES

(1) Stahl, E., *Pharmazie*, **11**, 633(1956).
(2) Morrison, J. C., and Chatten, L. G., *J. Pharm. Pharmacol.*, **17**, 655(1965).
(3) Purdy, S. J., and Truter, E. V., *Analyst*, **87**, 802 (1962).
(4) Morrison, J. C., and Chatten, L. G., *J. Pharm. Sci.*, **53**, 1205(1964).
(5) Privett, O. S., and Blank, M. L., *J. Lipid Res.*, **2**, 37 (1961).
(6) Attaway, J. A., and Edwards, G. J., *Anal. Chem.*, **37**, 1(1965).
(7) Privett, O. S., Blank, M. L., and Lundberg, W. O., *J. Am. Oil Chemists' Soc.*, **38**, 312(1961).
(8) Semenuk, G., and Beher, W. T., *J. Chromatog.*, **21**, 27(1966).
(9) Randerath, K., "Thin-Layer Chromatography," Academic Press Inc., New York, N. Y., 1964, p. 61.
(10) Genest, K., *J. Chromatog.*, **19**, 531(1965).
(11) Stahl, E., "Thin-Layer Chromatography," Academic Press Inc., New York, N. Y., 1965, p. 49.
(12) Wehrli, A., *Can. Pharm. J.*, **97**, 208(1964).