Thin-Layer Chromatography in Drug Analysis I

Identification Procedure for Various Sulfonamides in Pharmaceutical Combinations

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A thin-layer chromatographic procedure is described for identifying various sulfonamides when present in pharmaceutical combinations. Successful identification has been accomplished for sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, sulfacetamide, sulfanilamide, sulfaquinoxaline, and sodium sulfabromomethazine. The critical effect of water in the solvent system and the use of various detection methods, including fluorescent plates and Ehrlich's and Bratton-Marshall's reagent sprays are described. The procedure can be completed within 2 hours.

SINCE the introduction of thin-layer chromatography (TLC) into pharmaceutical literature by Stahl (1), numerous articles have been published pertaining to drug analysis by use of this technique (2–9). The advantage of TLC over paper chromatography is its speed and ability to withstand corrosive substances for use as spraying agents, such as sulfuric acid for the determination of meprobamate (2). A disadvantage is the difficulty in obtaining reproducible R_f values. This, however, can be overcome by use of standards along with the unknown samples.

The U.S.P. XVI (10) uses a precipitation procedure for its identification of a trisulfapyrimidine combination of sulfadiazine, sulfamerazine, and sulfamethazine. The N.F. XI (11) uses a paper chromatographic separation for the identification of sulfadiazine and sulfamerazine in the presence or absence of sulfacetamide. These are time consuming and tedious to perform. The TLC procedure described in this paper can be done within 2 hours. Wollish, Schmall, and Hawrylyshyn (8) reported a TLC method and approximate R_f values for sulfanilic acid, sulfadiazine, N[1]-(3,4-dimethyl-5-isoxazoyl) sulfanilamide and its acetyl derivative,[1] and 2,4 - dimethoxy - 6 - sulfanilamido - 1,3 - diazine.[2]

The purpose of this paper is to provide, by means of TLC, a relatively rapid systematic separation of commercially available sulfonamide combinations. Using a modification of the solvent system described by Wollish, *et al.* (8), separation and identification of various sulfonamides was accomplished. The influence of the amount of water on the successful separation has also been studied.

EXPERIMENTAL

Primary Preparation of Plates.—The Stahl apparatus[3] and method were used for the preparation of the chromatoplates. Silica gel G,[4] 30 Gm., was placed in a dry 8-oz. mortar.

Preparation of Neutral Plates.—Distilled water, 40 ml., was added while stirring slowly. When the entire mass was of a uniform consistency, an additional 20 ml. of distilled water was added, and the mixture was stirred until uniform.

Preparation of Fluorescent Plates.—Fluorescein, 20 mg., was dissolved in 1.2 ml. of 0.1 *N* sodium hydroxide. Sufficient distilled water was added to make 60 ml. The preparation was then the same as described for the neutral plates, using the fluorescein solution instead of water.

The total stirring time should not exceed 1.5 minutes. The suspension was poured into the applicator and spread immediately over five plates (200 × 200 mm.) or 20 strips (200 × 50 mm.). After air drying for approximately 5 minutes to allow evaporation of excess water, the plates were stored in a desiccator over silica gel. Before use, they were activated at 105° for 30 minutes and set aside to cool. Starting points were marked along an imaginary line 15 mm. from one edge, and a finishing line was drawn 150 mm. from the start.

Standards.—A 10-mg. quantity of each sulfonamide was dissolved in separate 50-ml. portions of acetone. Sulfonamides used were: sulfadiazine (I), sulfamerazine (II), sulfamethazine (III), sulfathiazole (IV), sulfacetamide (V), sulfanilamide (VI), sulfaquinoxaline (VII), and sodium sulfabromomethazine (VIII).

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[1] Marketed as Gantrisin and Gantrisin acetyl by Hoffmann-LaRoche.

[2] Marketed as Madribon by Hoffmann-LaRoche.

[3] Obtainable from C. Desaga, Heidelberg, Germany, Importer, Brinkmann Instruments Inc., Great Neck, L. I., N. Y.

[4] A standardized mixture of silicic acid and calcium sulfate obtainable from E. Merck, Darmstadt, West Germany; Importers, Brinkmann Instruments Inc., Great Neck, L. I., N. Y.; Terra Chemicals Inc., 500 Fifth Ave., New York 36, N. Y.

Tablet Samples.—A quantity containing approximately 10 mg. of the individual sulfonamide in the largest concentration was added to a 125-ml. glass-stoppered Erlenmeyer flask and extracted with 50 ml. of acetone.

Suspension Samples.—The extraction procedure was the same as that for the tablets except where the products contained interfering ingredients. To these latter samples, 1 ml. of distilled water was added before shaking with the acetone. Cloudy solutions were centrifuged before applying a sample to the plate.

Quantity of Sulfonamide Applied.—A 1-mcg. quantity of sulfonamide was applied to the chromatoplates.

Primary Solvent System.—Chloroform-ethanol-heptane (1:1:1 plus an amount of water depending upon the sulfonamide combination). It was prepared by mixing 66 ml. each of reagent grade chloroform, anhydrous ethanol, and heptane. Anhydrous sodium sulfate was added and the mixture shaken. Before using, sufficient water was added to the decanted solvent to obtain the optimum water percentage. After the addition of water, the level was checked using Karl Fisher reagent.

Concentration of Water.—Table I shows the amounts of water used for various mixtures. Slight variances from the values shown altered the chromatoplates sufficiently to reduce the sensitivity of the procedure.

TABLE I.—AMOUNT OF WATER ADDED TO THE PRIMARY SOLVENT

	Suspected Sulfonamides	Water,[a] %
A	I, II, III, VII, VIII	1.0–1.8 (1.5 optimum)
B	IV	1.2
C	V	1.5–1.8
D	VI	1.5

[a] Per cent water for sulfonamides in A was used when no others were suspected. B, C, and D were used when these sulfonamides appeared in addition to any of those in A.

Spray Reagents.—Aqueous hydrochloric acid, 1*N*, 5% aqueous sodium nitrite, 0.1% alcoholic N-(1-naphthyl)ethylenediamine dihydrochloride (Bratton-Marshall reagent) were used as spray reagents.

Distance Developed.—The chromatoplates were developed over a distance of 150 mm.

Method for Neutral Plates (A).—Samples (5 μl.) and standard solutions (5 μl.) representing each sulfonamide theoretically present in the samples were applied to the plates and dried by a current of air. The developing tanks were allowed to equilibrate at least 3 hours before inserting the chromatoplate. Up to 18 individual samples were applied per plate. When development of 150 mm. was completed, the plates were removed, air dried, and sprayed with 1 *N* hydrochloric acid followed by 5% sodium nitrite.

Any yellow spots representing sulfathiazole or sulfaquinoxaline were marked. The plates were then dried at 100° (usually for less than 5 minutes) until free from excess nitrous acid (when no yellow areas other than those marked could be seen). Coupling with 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride produced reddish-purple spots which were marked and the R_f values calculated. Measurements were made from markings of the leading edge of the spots. The samples were identified by comparison of their R_f values to those of the standards. Development of 200 mm. was also tried on a 200 × 230 mm. plate using a larger developing tank.

Method for Fluorescent Plates (B).—The application of standards and samples, development, and air drying were identical to the procedure described for the neutral plates. Instead of the spray reagents, a short wave U.V. lamp (257 mμ) was used to observe the separation of the components, and the dark nonfluorescent spots were then marked. A summary of R_f ranges for the various sulfonamides tested is shown in Table II.

Figures 1 and 2 show typical, developed neutral chromatoplates of commercial products and standards using 1.2 and 1.5% water in the solvent system.

TABLE II.—APPROXIMATE R_f VALUES AND RANGES OBTAINED FOR VARIOUS SULFONAMIDES[a]

Sulfonamide	Av. R_f	Std. Deviation	Exptl. Ranges
Sulfacetamide	0.42	0.03	0.36–0.47
Sulfadiazine	0.47	0.03	0.43–0.53
Sulfathiazole	0.50	0.03	0.47–0.60
Sulfanilamide	0.53	0.02	0.48–0.57
Sulfamerazine	0.57	0.03	0.53–0.64
Sulfamethazine	0.64	0.04	0.59–0.72
Sulfaquinoxaline	0.66	0.05	0.62–0.75
Sodium sulfabromomethazine	0.77	0.04	0.70–0.83

[a] Data accumulated from 12 or more plates, using 1.5% water in the solvent system. In 1.2% water, the values for all except sulfacetamide, sulfathiazole, and sulfanilamide are reduced by at least 0.05.

RESULTS AND DISCUSSION

Procedure A.—Using the proper concentration of water, the sulfonamides, except VII, were sufficiently separated to allow identification by observation of the developed spot. Though the R_f value for VII closely approximated that for III, it was readily differentiated from the other by its yellow diazotization product. The identification of IV, if overlapped, was also possible due to its yellow diazotization product. Close observation of freshly sprayed spots showed IV to have a brown tint, V a red tint, and the others to be reddish-purple. On standing the colors faded.

The development of 200 mm. gave more distinct separation but did not justify the increased development time. Table III shows the various types of pharmaceutical combinations tested. The other components present did not interfere with the procedure.

Procedure B.—Though the total time consumed was reduced by the elimination of the spraying procedure, the sensitivity was also reduced. This was overcome by increasing the amount of sample applied to the plate. To increase the visibility of the samples, the diazotization and coupling can be carried out on the fluorescent plate. The reddish spots are then clearly visible on the yellow background.

Experimental plates were developed in solvent

TABLE III.—COMPONENTS OF PHARMACEUTICAL COMBINATIONS TESTED

Samples[a]	Sulfonamides I	II	III	IV	V	Others[b]
A and B	x	x	x			
C	x	x	x			Pink dye
D	x	x	x			Penicillin
E-G	x	x	x			
H	x	x		x		Green dye
I	x	x		x		
J	x	x			x	Yellow dye
K	x	x			x	Hyoscyamus
L and M	x	x				
N-P	x	x	x			
R-T	x	x	x			
U and W	x	x		x		
X	x	x			x	Sulfanilamide[c]
Z	x	x	x		x	Sulfanilamide[c]

[a] A to K, inclusive, represent tablets; L to Z represent suspensions. [b] Suspensions contained preservatives and suspending and flavoring agents. [c] Not stated by the formula.

systems containing various amounts of water. The water concentration was critical in the separation of sulfathiazole and sulfanilamide from the others. When these two were not present, an optimum range existed whereby the spots were sufficiently separated to allow easy identification.

A water concentration of 1.2% was found to be optimum for the separation of sulfathiazole from sulfadiazine, and 1.5% water was found to be optimum for the separation of sulfanilamide from sulfamerazine. Higher concentrations (1.8%) caused an overlapping of sulfanilamide and sulfadiazine; lower concentrations (1.2%) caused sulfanilamide to overlap the sulfamerazine. Sulfacetamide showed the best separation at a water level between 1.5 and 1.8%, though no overlapping occurred at the other levels tested.

Greater concentrations of water gave further movements for certain compounds but decreased the separation areas. A weakening of the adsorptive property of the silica gel G on some of the compounds by the water caused the increased movements. An anhydrous solvent system gave less separation, overlapping, and more tailing.

For the preliminary investigation of an unknown mixture, it is recommended that duplicate plates be prepared. One plate should be developed in the

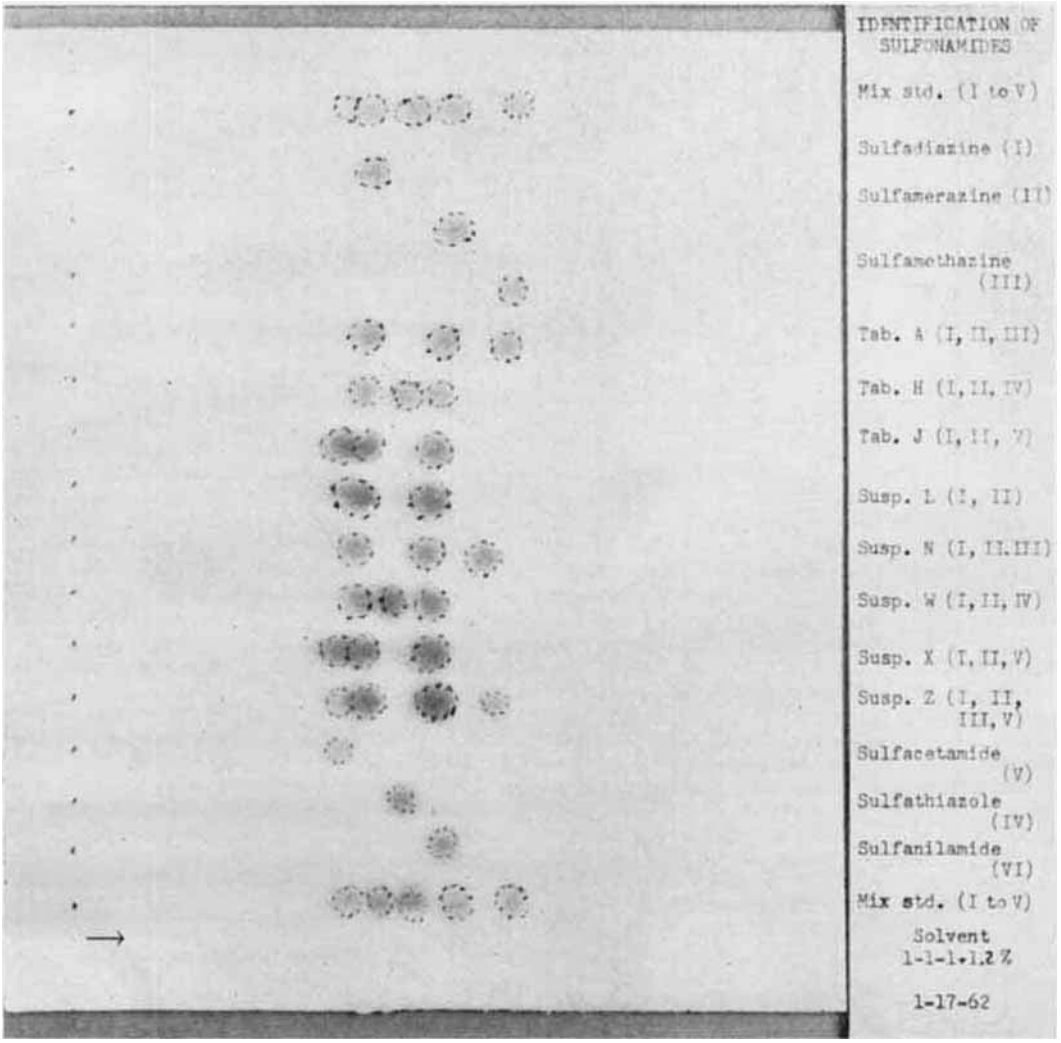

Fig. 1.—Thin-layer chromatographic separation of pharmaceutical combinations. Solvent: chloroform-heptane-ethanol (1:1:1 + 1.2% water).

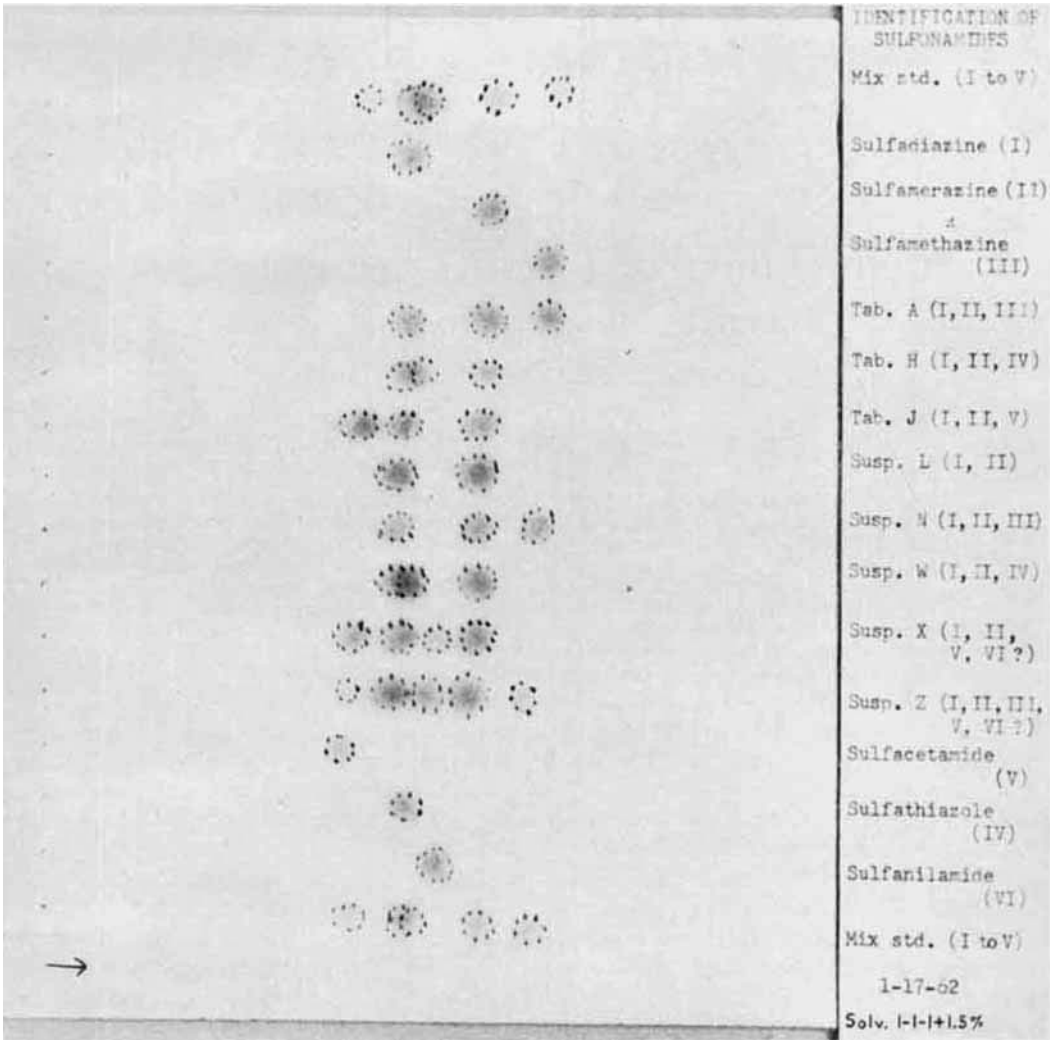

Fig. 2.—Thin-layer chromatographic separation of pharmaceutical combinations. Solvent: chloroform-heptane-ethanol (1:1:1 + 1.5% water).

solvent system containing 1.2% water and the other in the 1.5% water solvent system.

Ehrlich's reagent (*p*-dimethylaminobenzaldehyde, 1% in acidified ethanol) was tried as a replacement for the Bratton-Marshall reagent. Though it was less time consuming, the differentiation of VIII from III and the presence of IV, if overlapped, were not possible. Also, the outlines of the individual spots were not as distinct as those produced by using the Bratton-Marshall reagent.

Due to the uncertainty of reproducing R_f values from one plate to another, it is important to use standards along with the samples.

SUMMARY

1. A thin-layer chromatographic procedure for the identification of various sulfonamides individually and in combination has been developed.

2. Successful separation of mixtures containing the following sulfonamides has been obtained: sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, sulfacetamide, sulfanilamide, sulfaquinoxaline, and sodium sulfabromomethazine.

3. The identification of the following has been made when present in pharmaceutical combinations: sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole, sulfacetamide, and sulfanilamide.

4. The concentration of water is critical for obtaining satisfactory separations of sulfathiazole and sulfanilamide from the other sulfonamides tested.

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Composition of Gum Turpentines from Twenty-Two Species of Pines Grown in New Zealand

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Vapor-phase chromatography, following steam distillation, has provided an effective means of identifying many components of the gum turpentines of oleoresins from 22 *Pinus* species, with the probable exception of the sesquiterpenes and some trace constituents. With the improved injection instruments, it has been possible to analyze samples as small as 0.5 Gm., thus allowing the study of very low-yielding or small trees. This work confirms the specific differences previously reported for the major constituents, with two notable exceptions, *P. muricata* and *P. montezumae*. The results suggest the existence of two varieties of *P. muricata*, distinguishable by a wide difference in turpentine composition.

THE GENUS *Pinus*, as described by botanists, comprises about 100 species, several of which are divided into subspecies or varieties. The chemical composition of the gum turpentine (*i.e.*, the volatile fraction of the oleoresin obtained by wounding the sapwood) has been reported for 92 species and 2 varieties, as summarized by Mirov (1). This work, requiring oleoresin samples of the order of 500 Gm., was done in the main by fractional distillation of the turpentine, followed by determination of the densities, optical rotations, and refractive indexes of the fractions, and finally by the preparation of crystalline derivatives. It was limited by the inadequate methods for isolating and identifying many of the individual compounds which occur only in trace amounts. Physical methods such as vapor-phase chromatography and infrared spectroscopy have simplified the study of turpentine composition, and have the great advantage of being applicable to very small samples. With vapor-phase chromatography alone, however, the comparison of retention volumes depends on the availability of pure reference compounds. Purified samples of terpenes are not readily available and their preparation is often difficult and time-consuming, especially when the compounds occur only as trace quantities. Nevertheless, the value of vapor-phase chromatography in the terpene field has already been recognized and the technique has been used by many workers (1–6).

A satisfactory vapor-phase-chromatography technique for studying the turpentine composition of *P. radiata*, *P. attenuata*, and hybrids between these two species has been developed (7). While extending this work to study individual variation in *P. radiata* (8) we decided to try the technique on a number of other species of pines grown in New Zealand. This would not only test the efficiency of the method for analyzing more complex turpentines, but would also provide a comparison between species grown in New Zealand and elsewhere.

EXPERIMENTAL

Method of Sampling.—All the oleoresin samples except one were collected from single trees growing in or near the New Zealand Forest Service Arboretum at Whakarewarewa, near Rotorua, in February 1961. The remaining sample was collected from a single tree of *P. muricata* on Huckleberry Hill, near Monterey, Calif., in 1960. Holes, 2.5 cm. in diameter, were bored into the sapwood and close-fitting tubes were inserted. With this method the oleoresin had minimum contact with the outside air, and the time required to collect the sample was usually 1 or 2 days, and in no case more than 7 days. The oleoresin samples were tightly

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