(32) P. Rivaille and D. Raulis, *C. R. Acad. Sci. (Paris)*, **269**, 1121(1969).
(33) S. Ghosal, *Planta Med.*, **21**, 200(1972).

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PHARMACEUTICAL ANALYSIS

Quantitative TLC of Salicylazosulfapyridine

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Abstract □ Nine lots of raw material salicylazosulfapyridine from six suppliers were analyzed by an early spectrophotometric method. The lower limit of purity set by this method is 88%. Using a highly purified reference standard, results of the spectrophotometric method were compared to a salicylazosulfapyridine-specific, quantitative TLC method. Results confirmed the nonspecificity of the spectrophotometric method. In an extreme case, material with a 94% spectrophotometric purity value was shown to be only 85% by quantitative TLC. As many as eight extraneous spots in addition to salicylazosulfapyridine were found in some lots of raw material when developed chromatographs were sprayed with an azo-indicating stain. The minimum visible detection level of the impurities was between 0.01 and 0.1% of the total salicylazosulfapyridine spotted. Isolated impurities showed spectrophotometric absorbance at the wavelength of the spectrophotometric method, resulting in erroneous purity values. Drug recovery by the quantitative TLC method was 98% with an assay standard deviation of less than ±0.5%. These results led to commercial upgrading of synthesis and purification, so that raw material salicylazosulfapyridine at the 96% level by quantitative TLC was subsequently available for drug production.

Keyphrases □ Salicylazosulfapyridine—quantitative TLC analysis, compared to literature spectrophotometric method □ TLC—analysis, salicylazosulfapyridine, compared to literature spectrophotometric method

Salicylazosulfapyridine (I), indicated for use in the treatment of ulcerative colitis, has been marketed in the United States for about 20 years. The first monograph for this drug appeared in 1953 (1). Chemical specifications for I found in that reference are as current as can be found and call for a purity of not less than 88%.

During the development of I dosage forms in this laboratory, it was necessary to explore analytical procedures for assurance of raw material purity as well as chemical stability. Three methods have been described: a titanium trichloride titration used in 1925 (2), a spectrophotometric procedure (2), and the more recent polarographic technique (3). Both the spectrophotometric and the azo titration procedures were included in Ref. 1. These methods were compared by Berggren and Hansen (2), who theorized that the similarity of potency values obtained for I raw material was due to the specificity of both assays for the azo linkage (—N=N—). More recent studies (3) showed that normal production lots of I contain azo impurities which have been proven to be spectrally active. Thus, neither method can be considered specific or meaningful for I potency unless azo impurities are first removed.

Consideration has been given to polarography (3) in an attempt to overcome the impurity disturbances of the spectral method; however, in the final analysis of this work, an average difference of only 0.6% between the two methods is found. This slight difference indicates that polarography is not a significantly more specific assay than spectrophotometry for production lots of I. It was further stated that the spectrophotometric method was unquestionably more sensitive and rapid. Therefore, it was evident from the literature reviewed that a specific, quantitative assay for I did not exist.

Kiger and Kiger (4) reported qualitative TLC techniques for I in their work on sulfonamide differentiation. Two distinct spots for this drug were reported, and one was designated as an impurity. After repeating the qualitative TLC procedure in these laboratories, it was confirmed that indeed two spots were visible with the naked eye at the reported R_f

Table I—Physical–Chemical Properties of Salicylazosulfapyridine Reference Standard

	Found	Reported	Theory
Melting range	255–258°	240–245°[a]	—
Absorptivity	668[b]	658[c]	—
TLC	One spot	Two spots[d]	
Elemental analysis, %	C 54.83	55.19[c]	54.23
	H 3.52	3.75	3.54
	N 14.18	14.00	14.06
	S —	7.88	8.05

[a] Reference 6. [b] Calculated from Beer's law by least-squares method, n = 14. [c] Reference 2. [d] Reference 4.

values. However, further investigation using UV lamps and azo-indicating stains revealed as many as 10–12 additional spots in some raw material lots of I. By using TLC techniques, therefore, it was the objective to develop a quantitative isolating procedure for I and incorporate the highly sensitive spectrophotometric method for the purity determination.

EXPERIMENTAL

Chemicals and Equipment—Unless otherwise stated, all chemicals used in this study were analytical reagent grade quality. Precoated silica gel TLC plates (20 × 20 cm) were used. For the quantitative assay, silica gel with fluorescent indicator[1] was used; for the qualitative assay, additional types of TLC plates[2,3] were also employed. Other equipment included capillary pipets[4] (5 μl), a spectrophotometer[5], a mechanical shaker, a forced-air blower, a chromatographic sprayer, and short and long UV lamps.

Samples—Nine samples of raw material from salicylazosulfapyridine (I) production lots of six manufacturers were procured and dried at 105° for 90 min. Samples were then stored in a desiccator until required. The reference standard of I[6] was dried and stored in the same manner and was characterized by determining chromatographic purity, absorptivity, melting range, and carbon, hydrogen, and nitrogen content.

Spectrophotometric Assay—The spectrophotometric assay for I (1) was carried out on each sample in comparison to the reference standard. In this procedure, solutions of sample and standard were prepared by dissolving about 150 mg of I, accurately weighed, in enough 0.1 N sodium hydroxide solution to make 100 ml of solution. A 5-ml aliquot was transferred to a 1000-ml volumetric flask containing about 750 ml of water and 20 ml of 0.1 N acetic acid solution. Enough additional water was added to make 1000 ml. The absorbance of these solutions was read on a spectrophotometer at 360 nm against water, and the purity percentage of the sample was calculated. A Beer's plot of the reference standard was prepared according to the spectrophotometric procedure for the determination of the absorptivity value.

Quantitative TLC Assay—Standard and sample solutions were prepared by dissolving about 250 mg of I, accurately weighed, in 10 ml of dimethylformamide and diluting this solution to 25 ml with methanol. The chromatographic bath was prepared in a glass chamber (28 × 25 × 9 cm) using 60 ml chloroform and 15 ml each of acetone, n-butanol, and 88% formic acid. The chamber was lined on one side with filter paper, which was in contact with the mobile phase. The chamber was tightly sealed and allowed to equilibrate overnight for use on the next day only. Chromatographic plates were prerinsed with anhydrous methanol and thoroughly dried. Five microliters of sample and 5 μl of standard solutions were spotted alternately in triplicate on the TLC plates. To prevent thermal expansion, capillary pipets were held with a flag made of tape and the outer surface of the delivery end was coated with a thin film of silicone grease to prevent creep back of the solution. Pipet filling was done by capillary action using the nongreased end. Following each application, the pipet was rinsed with 5 μl of methanol, which was applied to its respective spot to ensure complete transfer. This step was then followed by rinsing the pipet with the next solution to be spotted. The same pipet was used for each six-spot series.

After drying the applied spots thoroughly, chromatographic development was started and continued until the solvent front was 10–12 cm above the origin. The plates were removed from the chamber and dried in a stream of forced warm air. The yellow drug spots visible at R_f 0.6–0.7 were outlined under long UV light to ensure total removal. These spots were individually cut from the plastic-backed plates. The three sample spots and the three standard spots were combined in separate 50-ml glass elution vessels. The drug was eluted from the silica gel with 18 ml of 0.016 N sodium hydroxide (one part 0.1 N sodium hydroxide plus five parts water) by mechanically agitating the tightly sealed vessels for 15 min. The mixtures were then transferred to a syringe-mounted, Teflon filter[7]. Six-milliliter aliquots of the filtrates were separately combined with 4 ml of 0.1 N acetic acid solution, and the absorbances were compared at the 360-nm maximum using water as the blank.

Qualitative TLC—For qualitative assessment of the raw material purity of I, developed chromatographs[8] were first viewed under short and long UV light followed by spraying with a solu-

Table II—Comparative Assays of Salicylazosulfapyridine Raw Material Samples

Sample Code	Purity[a], % Quantitative TLC Method	Purity[a], % Spectrophotometric Method (1)	Impurities[b], %	Melting Range[c]
A-1	97.7	98.7	2.4	256–258°
A-2	96.2	98.1	1.9	256–258°
A-3	92.1	94.4	3.8	249–251°
B-1	91.4	97.9	5.7	250–252°
C-1	90.2	93.7	5.2	248–250°
D-1	89.9	96.8	5.8	246–248°
B-2	85.6	94.1	10.2	247–249°
E-1	81.9	89.8	8.8	243–245°
F-1	77.4	88.9	12.5	237–239°

[a] All results based on reference standard. [b] Impurities isolated by TLC that show absorbance at 360 nm. [c] USP procedure 1a.

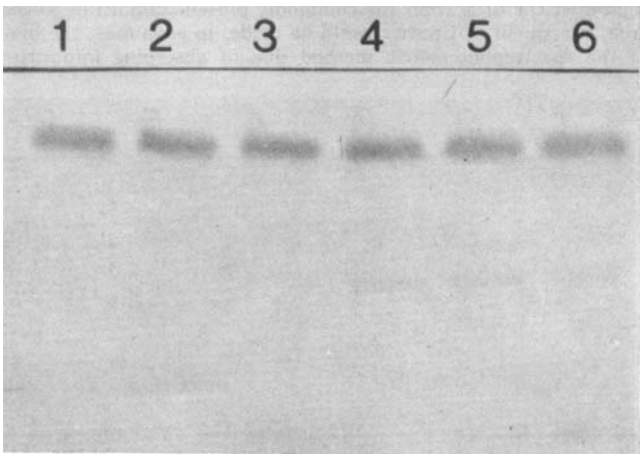

Figure 1—*Qualitative TLC of salicylazosulfapyridine (silica gel F254, fast running). Key: 1, reference standard; 2, A-2, 3, A-3; 4, D-1; 5, E-1; and 6, F-1. See Table II for purity values.*

[1] Eastman Chromagram (13181), Eastman Kodak Corp., Rochester, N.Y.
[2] Silica gel F254 (fast running), Brinkmann Instruments, Westbury, NY 11590
[3] MN silica gel N-HR/UV254, Brinkmann Instruments, Westbury, NY 11590
[4] Drummond Microcaps (55-mm length), Bolab, Inc., Derry, N.H.
[5] Beckman DB, Beckman Instrument Co., Fullerton, Calif.
[6] A gift of Orgamol Corp., Switzerland, through J. H. DeLamar, Inc., Chicago, Ill.
[7] Millipore Corp., Bedford, Mass.
[8] Spotted with a 10-μl applicator, Cordis Laboratories, Miami, Fla.

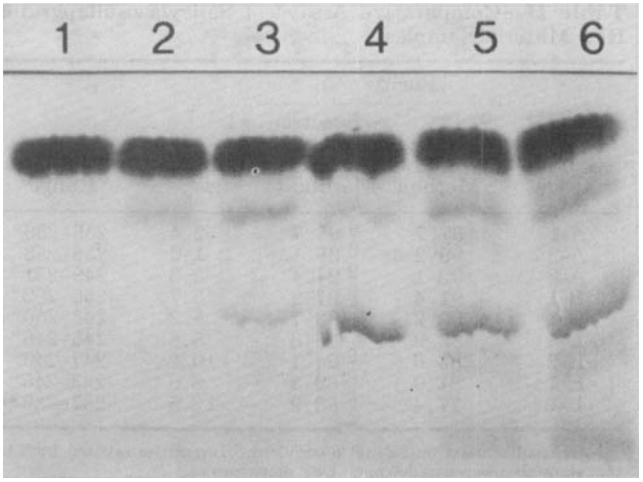

Figure 2—*Qualitative TLC of salicylazosulfapyridine (silica gel 13181). Key: same as Fig. 1. See Table II for purity values.*

tion prepared just prior to use by combining 20 parts of 4-dimethylaminocinnamaldehyde (II) solution (0.1% in a 50:50 mixture of glacial acetic acid and water) with one part of titanium trichloride solution (20% technical grade) (5).

RESULTS AND DISCUSSION

The reference standard of I used in this study was found to be higher in purity than any previously reported material, as shown by the comparative physical-chemical properties listed in Table I. The absorptivity reported by Berggren and Hansen (2) represents a calculated value based on their 98% standard. Our findings indicate that their standard was really 96.5% of I and that the use of the 658 absorptivity value in the spectrophotometric method results in a 1.5% positive bias. This indicates that the lower limit of purity by this currently accepted assay method is not 88% as stated but actually 86.5%.

The qualitative TLC system, capable of visualizing as little as 5 ng with respect to I, revealed that the reference standard was essentially homogeneous, with one trace band just above the drug band. The trace band was not consistently visualized and was considered to be insignificant.

Comparative raw material purity values for I samples from six sources are presented in Table II. The TLC system effectively separated the drug from the commonly present impurities so the true assessment of I purity could be made. In each case, the bias of the spectrophotometric method due to absorbing impurities

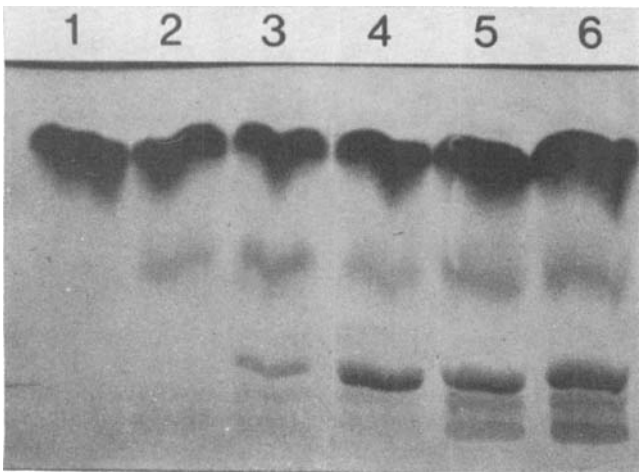

Figure 3—*Qualitative TLC of salicylazosulfapyridine (MN silica gel N-HR/UV254). Key: same as Fig. 1. See Table II for purity values.*

Table III—Statistical Evaluation of the Quantitative TLC Method

Sample Code	Purity[a], %	Mean Purity, %	Relative Standard Deviation, %
A-3	92.3[b]	92.1	±0.15
	92.0		
	92.0[c]		
	92.0		
D-1	89.9[b]	90.4	±0.39
	90.7[c]		
	90.5		
	90.6		
A-2	95.6[b]	95.8	±0.47
	96.2		
	95.3		
	96.2[c]		
			Average = ±0.34

[a] Individual assays based on reference standard. [b] Assayed on Day 1. [c] Assayed on Day 2.

was eliminated, resulting in significantly lower purity values by the quantitative TLC procedure. Impurity absorbance at 360 nm was confirmed by collective elution of all bands except I. Estimates for the total amount of impurities were calculated assuming the absorptivity to be similar to I. These values approximate the difference in percent purity found by the two methods.

All samples tested were within or above the currently accepted 220–240° melting range. As shown in Table II, melting ranges tended to indicate a rank-order correlation with I purity. The purity values obtained from the quantitative TLC method correlated better with melting temperature than did the purity values from the spectrophotometric analysis.

The methods of qualitative examination used for developed TLC plates revealed numerous impurities in most I samples studied. Figures 1–3 show six chromatographed samples of I ranked according to their quantitative TLC purity. A rank-order correlation was demonstrated between the quantitative TLC assay values and the number and intensity of impurity bands. Initially (Fig. 1), only one distinct impurity band could be seen at R_f 0.35 with the drug band at R_f 0.6–0.7. Spraying the chromatographs with the azo-reducing (titanium trichloride), amine-indicating solution of II allowed further visualization of this and other impurities (Figs. 2 and 3). The silica gel medium of the plate represented in Fig. 3 allowed for the greatest resolution of I from impurities. Although the R_f of the drug remained at about 0.7, the R_f 0.35 impurity was suppressed to about R_f 0.2.

As many as eight azo bands were located in the sample at position 6 (F-1). This material represented the lowest purity of I tested and, although it passed current specifications, it was found to contain only 77% of I by quantitative TLC. Chromatographs sprayed with the II solution alone showed only one free amine to be present in Sample F-1, which was subsequently confirmed as sulfapyridine.

Sample A-2 (position 2) and A-1 (not shown) represent production lots of I synthesized and purified by improved methods. This type of material is of the highest purity commercially available with essentially all azo impurities eliminated.

Some chromatographs viewed under short UV light contained a fluorescent band at a slightly greater R_f than the drug. This impurity was confirmed as being salicylic acid. No other short UV fluorescence was seen. Long UV fluorescence, common in all lots except A-1 and A-2, was observed at R_f 0.55, 0.2, and 0. These additional impurities were not the same as those responding to the azo spray and may account for the remaining impurities not seen by the spectrophotometric method.

The major impurity, found at R_f 0.35 in all I samples except A-1 and A-2, was isolated from TLC plates and shown to be similar to I in several regards. The UV spectrum and the IR spectrum were essentially the same for both the drug and this impurity. A qualitative examination of solubility showed that, like I, the major impurity was soluble at alkaline pH and could be precipitated by acidic conditions. Melting of this impurity occurred with decomposition over a broad range from about 240 to 260°. Further TLC studies of the isolate showed it to be homogeneous with an R_f of 0.35.

Table IV—Recovery of Salicylazosulfapyridine by the Quantitative TLC Method

Micrograms Recovered[a,b]	Percent Recovered	Micrograms Recovered	Percent Recovered
146.4	97.5	147.7	98.4
146.8	97.8	147.3	98.1
147.7	98.4	147.3	98.1
144.6	96.4	147.3	98.1
148.2	98.7	147.3	98.1
148.2	98.7	146.8	97.8
Mean percent recovered = 98.0 ± 0.65%			

[a] Reference standard, 150.1 μg, was spotted in each of the 12 assays. [b] A separate 5-μl pipet was used for each of the 12 assays.

A statistical evaluation of the quantitative TLC method was conducted on three lots of I. Assays were carried out in a randomized design so that six assays were run on each of 2 days. As shown in Table III, the quantitative TLC method has excellent reproducibility and can be carried out with a relative standard deviation of less than ±0.5%. The potency range of the three lots studied did not show a rank-order correlation with the standard deviation. Day-to-day assay variation did not appear to be a significant factor affecting the results.

Throughout the development of the quantitative TLC method, several techniques had been used. Prior to the final techniques described here, the basic eluate had been buffered to pH 4–5 in the presence of suspended silica gel from the TLC plates. It was subsequently learned that under these conditions erratic adsorption of drug by silica gel as well as various filtration systems resulted in only a 90% recovery with a relative standard deviation of ±1.4–2.5%. The final adjustment in technique, *i.e.*, filtration of the basic eluate for silica gel removal prior to buffering, reduced the assay variation to less than one-fourth that of the previous procedure while increasing the recovery to 98%.

The absorbance values for the reference standard from 12 individual assays were converted to micrograms of the drug recovered. This value was divided by the theoretical amount of standard spotted to obtain the percent recovery (Table IV). Blank assays showed no absorbance at 360 nm. The relative standard deviation of recovery corresponded to that of the delivery precision of the capillary micropipets as determined by Emanuel (7) (n = 12, ±0.64%) and tend to indicate that the TLC technique is highly reproducible.

Since the recovery of I by the quantitative TLC method was sufficiently high and had a low standard deviation, no attempt was made to determine the cause of the 2% drug loss.

SUMMARY AND CONCLUSIONS

A specific method with high precision was developed for the analytical control of salicylazosulfapyridine raw materials as well as dosage forms of this drug. Assay results showed that there are extremely wide purity variations among supplies of this drug. All nine samples tested would be considered acceptable by current spectrophotometric standards, although borderline cases contained as much as 23% of nonsalicylazosulfapyridine materials. The lack of specificity of the spectrophotometric assay for I was confirmed by showing that the isolated impurities possessed strong absorbance at 360 nm, causing assay interference that results in as much as 10–12% positive bias. Most impurities found in commercial grades of I were shown to contain an azo moiety; however, other impurities not responding to the azo test were revealed under UV light. The analytical findings based on the quantitative and qualitative TLC of I led to improvements of methods for synthesis and purification, so material at the 96% purity level was made available for drug production.

REFERENCES

(1) "Tests and Standards for New and Nonofficial Remedies," Lippincott, Philadelphia, Pa., 1953, pp. 256–258.

(2) A. Berggren and S. Hansen, *Farm. Rev.*, **34**, 537(1952).

(3) B. Nygard, J. Olofsson, and M. Sandberg, *Acta Pharm. Suecica*, **3**, 343(1966).

(4) J. L. Kiger and J. G. Kiger, *Ann. Pharm. Fr.*, **24**, 593(1966).

(5) J. P. Moody and R. T. Williams, *Food Cosmet. Toxicol.*, **2**, 698(1964).

(6) "The Merck Index," 8th ed., Merck & Co., Rahway, N.J., 1968, p. 930.

(7) C. F. Emanuel, *Anal. Chem.*, **45**, 1568(1973).

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