Improved Procedure for Determination of Microamounts of Sulfamethazine and Procaine Penicillin Admixed with Chlortetracycline and *p*-Arsanilic Acid in Medicated Feeds

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Abstract □ An improved technique is presented for the separation and determination of both sulfamethazine and procaine penicillin in medicated feeds. This method utilizes diazotization of the free amino compounds with nitrite and coupling with the Bratton-Marshall reagent to produce a soluble red dye. The absorbance of this dye at 545 mμ is measured. Results on six laboratory-mixed medicated feeds are reported.

Keyphrases □ Sulfamethazine—analysis, medicated feeds □ Procaine penicillin—analysis, medicated feeds □ TLC—separation □ Bratton-Marshall reaction—colorimetric analysis □ UV spectrophotometry—analysis

Sulfamethazine and procaine penicillin in combination with other drugs, such as chlortetracycline, tylosine, and *p*-arsanilic acid, are admixed in swine and cattle feeds for the prevention of infection associated with hemorrhagic septicemia, swine dysentery, bacterial scours in calves (1) and coccidiosis in chickens (2). Gerriets (3) observed that chickens fed with sulfamethazine-incorporated, riboflavin-deficient fodder developed typical clinical riboflavin avitaminosis, and he concluded that in sulfamethazine therapy both riboflavin and vitamin K_3 should be supplemented in the feed. It has been shown that the excretion of sulfamethazine is not complete and the concentration of the sulfa drug in bovine milk (4, 5) and plasma proteins (6) can be relatively high. Borsnk (7) noted that injection of sulfamethazine in hen eggs affected embryonic development, resulting in poor hatchability, several growth disturbances, and prenatal malformations. Because of these and other harmful side effects recorded in the literature, it has become necessary for the regulatory agencies to develop reliable analytical methods for estimating these drugs in feeds.

Several reports have appeared in the literature concerning the separation and determination of sulfamethazine and procaine penicillin in combination with other drugs, and these methods have been summarized in a previous communication (8). The present paper describes an improvement over the procedure outlined in the earlier method (8) for the estimation of sulfamethazine and procaine penicillin, and their extraction from the adsorbent alumina is accomplished with considerable saving of time. By this procedure, six laboratory-prepared samples containing varying amounts of *p*-arsanilic acid, chlortetracycline, sulfamethazine, and procaine penicillin have been extracted and the components separated cleanly from one another on thin-layer alumina plates and estimated spectrophotometrically.

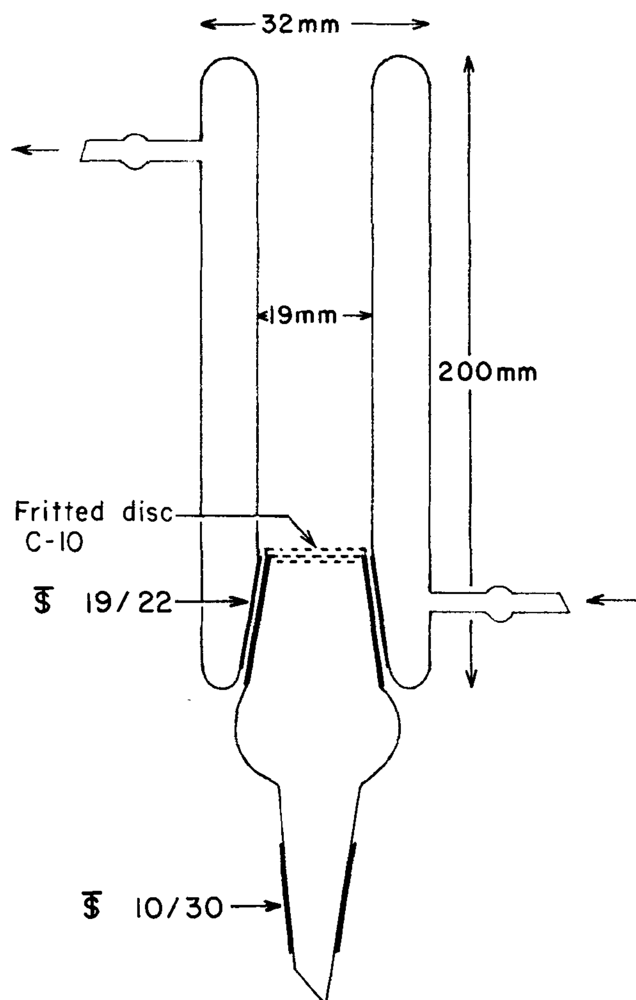

Figure 1—*Extraction apparatus.*

EXPERIMENTAL

Apparatus—The extraction apparatus for eluting the drugs from thin-layer alumina is illustrated in Fig. 1. The alumina chromatoplates were prepared as previously described (9).

Reagents—An analytical sample of sulfamethazine (m.p. 198–198.5°) was obtained by crystallizing a technical sample[1] twice from a mixture of methanol–petroleum ether (b.p. 66–68°) followed by a final recrystallization from benzene–petroleum ether (b.p. 66–68°) mixture. The sample was shown to be identical to an authentic sample by determination of mixed melting point, elemental analysis, R_f values, and IR and NMR spectra. From the UV absorption spectrum, the sample was found to be 99.8% pure.

Procaine Penicillin[2]—Analytical standard was used without further purification. All organic solvents were glass-distilled. The alumina[3] for thin-layer chromatography was processed according to the method of Malaiyandi *et al.* (9) with plates activated at 120°

[1] American Cyanamid Co.
[2] Chas. Pfizer Co.
[3] Camag alumina supplied by Mondray Co. Ltd., Montreal, Quebec, Canada.

instead of 145°. Aqueous hydrochloric acid, 6 *N*; aqueous sodium nitrite solution, 1.0%; aqueous ammonium sulfamate solution, 5.0%; Bratton-Marshall reagent (aqueous *N*-(1-naphthyl)ethylenediamine dihydrochloride), 0.1%. The last three reagents were all freshly prepared each week and stored in a refrigerator (5°).

PROCEDURE

Standard and Recovery Curves for Procaine Penicillin and Sulfamethazine—*Standard Curve*—Ten aliquots (in duplicate) containing known amounts of different concentrations of procaine penicillin and sulfamethazine in ethanol were transferred to separate 50-ml. volumetric flasks. To these flasks was added 3.0 ml. of 6 *N* HCl followed by 15 ml. of distilled water. The flasks were heated on a water bath at 70–80° for 15 min. and the ethanol was evaporated off with a gentle stream of nitrogen (about 45 min.). The solutions were diluted with 10 ml. water and estimated by the procedure described for the preparation of standard curve for *p*-arsanilic acid (9).

Recovery Curve—A concentrated ethanolic solution containing known amounts of a mixture of procaine penicillin and sulfamethazine was prepared. Eight aliquots (in duplicate) containing amounts varying from 10 to 200 mcg. of drugs were applied to thin-layer alumina plates (0.25–0.30 mm. thick) along with a clearly visible reference spot at one side of the plate. The plates were developed as described in the earlier method (8).[4]

The areas[5] containing the drugs were separately scraped off the plate, and the adhering alumina was swept with a camel hair brush onto glazed paper. The alumina was transferred to the hot water-circulated extraction apparatus over a bed of prewashed methanol-moistened diatomaceous earth (Celite 545, about 1.5–2.0 cm. high). The drug was eluted with 175 ml. of hot 2.0% methanolic hydrochloric acid. The eluate was evaporated using a rotary vacuum evaporator (bath temperature below 37°) to a volume of about 15 ml. The concentrate was quantitatively transferred to a 50-ml. volumetric flask with three rinsings of about 5-ml. portions of aqueous methanol. The flask was heated on a water bath at 70–80° and the methanol evaporated with a gentle stream of nitrogen. When the evaporation of methanol was complete, the contents of the flask were cooled, diazotized, and coupled with the Bratton-Marshall reagent as described previously. After 30 min. the solutions were diluted to volume, shaken thoroughly, and centrifuged. The absorbance was then measured and plotted to obtain the recovery curve.

Preparation of Laboratory Premixes—Six laboratory-blended samples of medicated feed containing *p*-arsanilic acid, chlortetracycline, procaine penicillin, and sulfamethazine at levels ranging from 120 to 1250 mcg./g. of feed were prepared by grinding 50-g. portions of unmedicated feed with varying amounts of the four drugs in a Sorvall-Omni mixer. After mixing for about 5 min., the mixture was tumbled on a Fisher-Kendall mixer for about 15 min. These operations were repeated twice and the sample was carefully transferred to a container which was then tumbled for 1 hr. to achieve thorough mixing.

Extraction of Drugs and Their Estimation—Two 1.0-g. portions (a_1 and a_1') and one 5.0-g. portion (a_5) of the medicated feed were extracted with 50–55 ml. of a solvent mixture containing ammonium hydroxide–dimethylformamide–chloroform–ethanol (5:5:20:20). (In the case of a_5, 200 ml. of the same solvent mixture was used.) After refluxing for about 8 hr. the warm extracts were filtered through a bed of diatomaceous earth and concentrated under reduced pressure to about 5 ml. (20 ml. in the case of a_5). The concentrates were then quantitatively transferred to 10-ml. (50 ml. in the case of a_5) volumetric flasks with several rinsings with absolute ethanol and made to volume. Aliquots (4 × 250 μl.) of this extract were applied to a thin-layer alumina plate and developed, and the drugs were extracted and estimated as described in the previous section.

[4] If the humidity of the laboratory is very high, use of a 60:30:8:2 solvent mixture is recommended. When fresh dimethylamine solution is available, a 60:30:5:5 solvent composition is used.

[5] Along with the solvent front, a fluorescent lipid band moved. This lipid band should be excluded to avoid intractable turbidity during centrifugation.

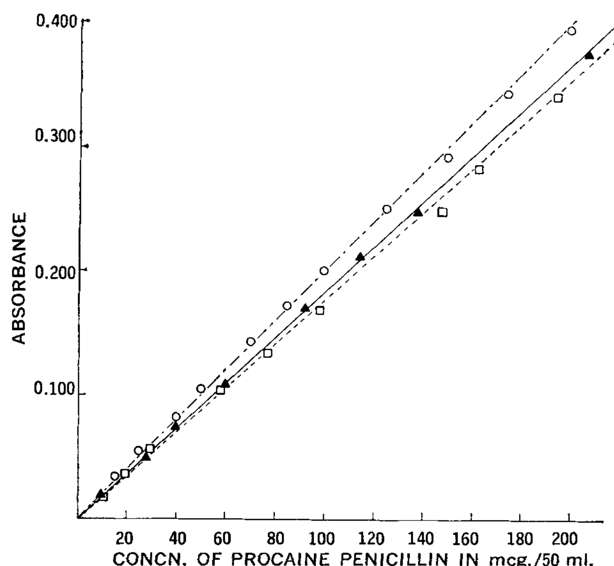

Figure 2—*Standard and recovery curves for procaine penicillin. Key:* ○, *standard curve;* □, *recovery curve by previous method; and* ▲, *recovery curve by present method.*

RESULTS AND DISCUSSION

The need for processed alumina and the choice of range of pH (7.2–7.4) of the adsorbent and the solvent systems to effect a clean separation of procaine penicillin and sulfamethazine from other drugs have been discussed in the earlier report (8). To avoid the laborious procedure of extraction of the drugs from the adsorbent alumina, a convenient technique was developed using a hot water-jacketed filtering funnel. By this expedient the alumina powder containing the drug was directly transferred over the bed of diatomaceous earth in the filtering funnel. The drug was eluted with 2% hot methanolic hydrochloric acid which, by circulating hot water in the jacket, was maintained above 50° during filtration.

It had been previously observed that neither procaine penicillin nor sulfamethazine was completely recovered (88–91%) (8) from adsorbent alumina. Being highly polar compounds, it is not surprising that some of the drugs were firmly adsorbed on the substrate. To evaluate the extent of elution by the current method and compare it with the method described earlier (8), eight aliquots (in duplicate) of a standard mixture containing varying amounts ranging from 10 to 200 mcg. of each drug were applied to thin-

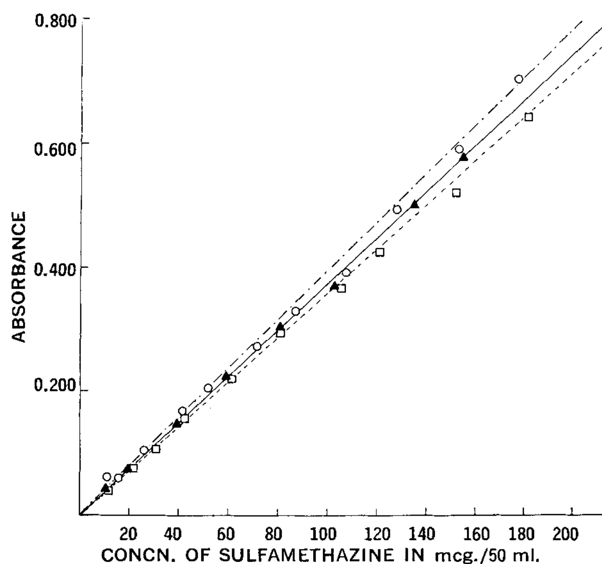

Figure 3—*Standard and recovery curves for sulfamethazine. Key:* ○, *standard curve;* □, *recovery curve by previous method; and* ▲, *recovery curve by present method.*

Table I—Analysis of Procaine Penicillin and Sulfamethazine in Medicated Feeds

Sample	Procaine Penicillin, mcg./g. Added	Found	$\pm SD$	Sulfamethazine, mcg./g. Added	Found	$\pm SD$
a_1	394	380	19.7	299	290	3.0
a_1'		373	5.0		286	3.5
a_5		386	13.6		285	5.2
b_1	375	402	3.6	437	433	3.6
b_1'		395	5.0		438	3.6
b_5		390	10.0		435	5.0
c_1	161	180	11.6	288	295	1.0
c_1'		174	4.0		306	7.0
c_5		186	8.3		292	5.7
d_1	241	261	7.8	226	237	6.0
d_1'		275	3.6		237	5.0
d_5		258	7.8		231	2.2
e_1	529	541	9.0	1031	999	4.0
e_1'		552	8.0		1009	2.5
e_5		549	17.1		1017	5.7
f_1	1240	1181	12.3	746	744	7.6
f_1'		1207	16.4		749	16.4
f_5		1145	11.2		733	8.3

layer plates, developed, extracted, and estimated. Also, 10 aliquots (in duplicate) of separate standard solutions of procaine penicillin and sulfamethazine were directly determined to obtain the standard curves.

It can be seen from Fig. 2 that by the previous technique, extraction of procaine penicillin at low levels was almost in agreement with the amounts obtained with the standards; whereas at levels above 40 mcg., the recoveries were considerably poorer. This seems to indicate that with the former procedure the alumina was firmly retaining some of the drug. By the current technique, however, it is apparent that the recovery of the drug is very consistent, and better than 93% recoveries are obtained in the range 10–200 mcg.

Examination of Figs. 2 and 3 reveals that the recovery of sulfamethazine by the previous procedure (91%) was better than that of procaine penicillin. It is evident from Fig. 3 that more than 95% recovery of the sulfa drug was obtained by the current technique. By employing the present procedure, two 1-g. and one 5-g. portions of Sample *a* were analyzed and found to be homogeneous.

To test the validity of the method, six laboratory-blended samples were analyzed and the averages of the results of two duplicate analyses of each sample are given in Table I. A close examination of the data reveals that the estimated contents of procaine penicillin are slightly higher than the amount actually added. The standard deviation[6] varies from ±3.0 to 20.0 for the six samples, indicating that although the variation is large it is still acceptable. On the other hand, in the case of sulfamethazine the results are highly reproducible with exceedingly small standard deviations (within ±10.0) and are in close agreement with the amounts actually added.

A comparison of results of the two 1-g. and the 5-g. portions of each sample shows that there is complete agreement between these analyses. For routine work, a 5-g. sample extraction is recommended for the convenience in extraction and makeup of solutions. This extraction procedure can be profitably extended to many other estimations involving thin-layer chromatography.

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[6] The standard deviation was calculated using the formula $SD = \sqrt{\Sigma(x - \bar{x})^2/n}$ where x is the actual value, x the absolute arithme average, and n the number of estimations.

Factors Affecting a Fluorometric Assay of Folic Acid

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Abstract ☐ A fluorometric method of assaying folic acid, based on the oxidation of folic acid by potassium permanganate, has been investigated. Various factors were found to affect the relative fluorescence obtained in the assay. These include the concentration of potassium permanganate, the length of oxidation time, and the pH and temperature of the solution.

Keyphrases ☐ Folic acid—analysis ☐ Potassium permanganate effect—folic acid fluorescence ☐ pH effect—oxidized folic acid fluorescence ☐ Temperature effect—oxidized folic acid fluorescence ☐ Fluorometry—analysis

The oxidative degradation of folic acid by potassium permanganate yields a fluorescent pterine (1). As a result, this oxidation has been used in analytical procedures for the quantitative determination of folic acid (2, 3). The formation of the free pterine derivative provides an approximately 20-fold increase in fluorescent intensity over that of native folic acid fluorescence.

In a comprehensive study, Allfrey *et al.* (2) measured directly the fluorescence of the oxidized mixture as well as that of the chromatographically isolated oxidation

Table I—Final Fluorescent Intensity

Concentration of $KMnO_4$ (moles/l. $\times 10^{-5}$)	Fluorescent Intensity (F_∞)
4.43	80.5
6.33	78.0
9.49	75.0
31.6	80.5