Microdetermination of the Drug Ingredients, 2-Chloro-4-nitrobenzamide, 4′-[(p-Nitrophenyl)sulfamoyl]-acetanilide and 4-Aminobenzenearsonic Acid in Medicated Finished Feeds

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Although more than one method is available for determination of the individual drug ingredients, 2-chloro-4-nitrobenzamide (aklomide), 4′-[(*p*-nitrophenyl)sulfamoyl]acetanilide (sulfanitran), and 4-aminobenzenearsonic acid (*p*-arsanilic acid), no suitable procedure is reported for their determination when present as a mixture in medicated finished feeds. In this report the estimation of microamounts of aklomide, sulfanitran, and *p*-arsanilic acid is described. Using a thin-layer alumina chromatoplate, aklomide and sulfanitran were separated from *p*-arsanilic acid and individually estimated colorimetrically using the Bratton-Marshall reagent. By repeating the determination after alkaline digestion of a separate sample of the same feed, the total of sulfanitran and *p*-arsanilic acid was obtained, from which *p*-arsanilic acid was calculated by difference. The recovery of aklomide and sulfanitran was more than 96% and that of *p*-arsanilic acid was about 90%.

In many animal and poultry feeds, aklomide, sulfanitran, and *p*-arsanilic acid are incorporated along with other medicaments. Aklomide and sulfanitran alone or in combination are used in several oral veterinary preparations to protect against severe infections of *Eimeria (E.) tenella, E. necatrix, E. acervulina, E. brunetti,* and *E. maxima.* Because of its low toxicity *p*-arsanilic acid has been widely incorporated as a feed additive since 1950. Several reports are available regarding the effect of concentrations of these drugs on pigmentation, growth, selenium-poisoning symptoms in pigs (Whalstrom and Olson, 1959), symptoms of thiamine deficiency (Abbott *et al.*, 1954), egg production, and hatchability (Moore and coworkers, 1954). Also, it has been reported that *p*-arsanilic acid and its metabolites are retained to some extent in certain edible parts of meat (Calesnick *et al.*, 1966; Overby and Frederickson, 1965; Overby *et al.*, 1965). Because the concentration of these drugs is critical in producing beneficial or adverse effects, the regulatory process demands a careful control which, in turn, warrants sound analytical procedures for the determination of these drugs in medicated feeds.

Many of the coccidiostats and growth-promoting substances which are incorporated in animal and poultry feeds are free or derivatized organic amines and organonitro compounds. During extraction several organic compounds similar to these drugs are brought into solution from the medicated feed. Although several methods are available for the determination of an individual drug, they cannot be applied when many drugs are concurrently present in finished feeds. The functional groups are converted to free amines and estimated as a soluble colored dye through diazotization and coupling (Bratton and Marshall, 1939).

It has, therefore, become necessary to separate these drugs cleanly from feed extracts. Several mixtures of drugs have been separated using thin-layer chromatography (Antkowiak and Spatorico, 1967) with a view to identifying them. A convenient method was not available for the separation and estimation of aklomide, sulfanitran, and *p*-arsanilic acid, but now these medicaments have been successfully separated using alumina thin-layer chromatography. Aklomide is estimated by the quantitative reduction of the nitro to the amino compound (Knecht and Hibbert, 1925). Sulfanitran is determined by the method of Cavett (1956, 1963) and *p*-arsanilic acid by modifying the procedure described by Merwin (1954).

APPARATUS

The filter funnel is fabricated of a medium-walled borosilicate glass tube (30 × 150 mm.) and provided with a 25-mm. coarse fritted disk and a ₸ 10/30 outer joint. The suction adapter is made of the same glass and provided with a ₸ 10/30 inner joint and a ₸ 24/40 outer joint.

REAGENTS

All solvents are glass-distilled. Benzene and chloroform are dried over activated alumina (Merck). Water is

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doubly distilled using borosilicate glassware. One per cent concentrated hydrochloric acid in methanol is prepared when required. One per cent aqueous sodium nitrite solution, 5% aqueous ammonium sulfamate solution, and 0.1% aqueous solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride are freshly prepared each week and kept cold in a refrigerator.

Aklomide (from Salsbury Laboratories, Ltd., Charles City, Iowa) was purified by passing a warm solution, in benzene, through a column containing 10% Darco in Celite 545 and eluting with benzene. After evaporation of the solvent, the crude solid was crystallized from a mixture of hot benzene-redistilled Skellysolve B (b.p. 66–68° C.) to give pale yellow crystals. This was recrystallized twice from the same solvent mixture to give pale yellow crystals (m.p. 172.0–72.5° C.; reported 172° C.).

Sulfanitran (from Salsbury Laboratories, Ltd., Charles City, Iowa). The sample for standard use (m.p. 258–58.5° C.) was used without further purification.

***p*-Arsanilic acid** (from Eastman Kodak Co.) (no definite melting point) was crystallized from a Darco-treated boiling solution of *p*-arsanilic acid in 95% ethanol-water (1 to 1) to give a white crystalline solid. This was twice recrystallized from the same solvent mixture and white crystals were obtained. After drying at 100° C. and 14-mm. pressure, the solid did not melt but decomposed above 300° C.

Celite 545 was treated with warm 6*N* aqueous hydrochloric acid, washed successively with hot water, warm methanol, and pentane, and finally air-dried at 105° C.

PROCEDURE

Iron-free Alumina (pH 7.2 to 7.4) for Thin-Layer Chromatography. FROM MERCK ALUMINUM OXIDE G (according to Stahl). About 100 grams of aluminum oxide G (E. Merck Co., Darmstadt, for thin-layer chromatography, 50 to 200 p.p.m. Fe, pH 7.5) was treated with 400 ml. of warm aqueous hydrochloric acid (1 to 7), washed free of acid, and dried for 6 hours (pH 4.4). The dry alumina was then suspended in 400 ml. of aqueous sodium hydroxide (50 ml. of 0.5*N* alkali solution in 350 ml. of water), vigorously stirred for about 30 minutes, washed with water and methanol, and air-dried at 105° C. for 2 hours (pH 7.2 to 7.4). The dry alumina (ca. 88 grams) was thoroughly mixed with 12.5 grams of calcium sulfate (Mallinckrodt analytical reagent) using a Fisher-Kendall mixer and activated at 175–180° C. for 12 to 16 hours.

FROM CAMAG ALUMINA DS-O (pH 9.5 to 10.0). About 100 grams of Camag alumina DS-O in 400 ml. of water containing 6.6 to 6.8 ml. of 1*N* HCl was magnetically stirred and, after 90 minutes, washed successively with 3 × 100-ml. portions of warm distilled water and 100 ml. of methanol. After drying at 105° C., the alumina (ca. 88 grams, pH 7.2 to 7.4) was admixed with 12.0 grams of calcium sulfate (Mallinckrodt analytical reagent), thoroughly mixed with the binder on a Fisher-Kendall mixer, and activated at 175–80° C., for 12 to 16 hours. A slurry of 43 grams of this alumina in 55 ml. of distilled water was applied onto five 200 × 200 mm. glass plates to get a uniform layer, 0.25 to 0.3 mm. thick. The plates were activated at 145° C. for about 12 hours and stored in stainless steel cabinets over anhydrous calcium chloride.

Preparation of Medicated Feed Samples. Approximately 100 grams of previously analyzed unmedicated feed was carefully weighed into a stainless steel container of a Sorvall Omni-Mixer. To this were added known weights of aklomide, sulfanitran, and *p*-arsanilic acid, and the mixtures were blended for about 5 minutes with intermittent shaking and external cooling. The feed sample was then spread on a glazed paper and manually quartered until a homogeneous mixture was obtained. Thus, seven samples of the medicated feed containing varying amounts of each drug ranging from 13.9 to 125 mg. per 100 grams were prepared.

Preparation of Standard Curve for *p*-Arsanilic Acid. Ten aliquots of a methanolic solution of *p*-arsanilic acid ranging in concentration from 15.0 to 124.2 μg. were placed in 50-ml. volumetric flasks. After 2.0 ml. of 6*N* HCl and 10 ml. of water had been added to each flask, the contents were heated on a water bath (70° to 80° C., Caution!) to evaporate the methanol with a gentle stream of nitrogen until no odor of methanol persisted. The contents were diluted with another 10 ml. of water and cooled in an ice bath for about 10 minutes. Three milliliters of 1% ice-cold sodium nitrite solution was added to each flask, which was shaken well and allowed to stand for 10 minutes. To this diazotized solution was added, with careful intermittent shaking, 1.5 ml. of 5% ammonium sulfamate to decompose the excess nitrite. After 15 minutes, 5 ml. of 0.1% ice-cold *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution was added and the mixture shaken vigorously and allowed to stand for the next 30 minutes at room temperature to complete the coupling reaction. The solutions were made to volume and the absorbance measured at 545 mμ using 1-cm. quartz cells and a Beckman Model DU spectrophotometer. The plot of concentration *vs.* absorbance obeyed Beer-Lambert's law over the concentration range studied.

Extraction of Medicated Feed and Separation of Drug Ingredients. Exactly 1 gram of feed sample was extracted by refluxing with 75 ml. of methanol for 7 to 8 hours. After the methanolic extract had been filtered through a 1-inch bed of Celite 545 in the sintered glass filter funnel (30 × 150 mm.), the residue was warmed with 3 × 10-ml. portions of methanol and transferred onto the Celite bed. The filtrate (about 150 ml.) was evaporated under reduced pressure to about 10 to 15 ml. (bath temperature below 35° C.). The concentrate was heated with 25 ml. of acetone on a steam bath to coagulate most of the proteins (5 minutes). After 30 minutes at 20° C., the suspension was filtered off through the same filter funnel along with 3 × 5-ml. portions of methanolic rinsings into the same receiver. The combined filtrate was concentrated as before to about 10 ml. and an equal volume of absolute ethanol was added. The solution was re-evaporated to 3 to 5 ml. and quantitatively transferred with repeated washings to a 10-ml. volumetric flask and made to volume. (Caution. A good portion of the aklomide will be lost if proper care is not exercised at this stage.)

On the processed alumina thin-layer plate, a thick line was drawn to divide the alumina layer into 6-cm. and 14-cm. portions. In the broader region, exactly 4 × 250 μl. of the above extract of the medicated feed was spotted on the chromatoplate in the form of a streak 10 to 12 cm.

long and 0.5 cm. wide, leaving a 1-cm. margin at the edges of the strip. Exactly 20 μl. of a methanolic solution of a mixture of known weights (20 to 50 μg.) of authentic samples of the drugs was spotted in the narrow strip of the plate. The plate was first developed in an ascending manner in a benzene-chloroform mixture (92 to 8) (60 to 75 minutes). After air-drying for 3 minutes, the plate was redeveloped in a second solvent mixture, acetonitrile-benzene-methanol (60:32:8) (45 to 50 minutes). The plate was then air-dried and the regions corresponding to the drugs were located using a short-wave ultraviolet lamp and marked. In our preliminary work, the compounds were located using the Bratton-Marshall reagent (Figure 1). (3,5-Dinitrobenzamide and zoalene, if present in the medicated feed, will interfere with the estimation of 2-chloro,4-nitrobenzamide, since these two amides have similar R_f's under these TLC conditions.)

Extraction of Aklomide and Sulfanitran from Chromatoplate. The region corresponding to the standard aklomide was carefully scraped off the plate and the adhering alumina was swept with a camel's hair brush onto a glazed paper. After the powder had been transferred to a 50-ml. Erlenmeyer flask, the scraped-off region was carefully rinsed with methanol into the flask. Similarly, all the regions corresponding to the two drugs in the standard and feed portions were collected in separate flasks.

The contents of the Erlenmeyer flask containing standard aklomide were extracted with 12 × 10-ml. portions of hot methanol, decanting each time into the sintered glass funnel containing a bed of prewashed Celite 545 (2 cm. high) and filtered into a 300-ml. round-bottomed flask. (Avoid bumping.) After evaporation, as before, the concentrate was quantitatively transferred to a 50-ml. volumetric flask with several rinsings of hot methanol, 5 ml. of 0.1*N* sodium hydroxide, and hot water. In the same manner the flask contents containing alumina coated with aklomide from the feed were extracted into a separate 50-ml. volumetric flask. The volumetric flasks were heated in a water bath (70° C.) and the methanol was evaporated by blowing a gentle stream of nitrogen into the flask. After the odor of methanol had disappeared, 2.0 ml. of 0.5*N* hydrochloric acid was added with intermittent shaking and the flask was allowed to stand for a 10-minute period. Then 1.0 ml. of 10*N* aqueous sodium hydroxide was added to basify the contents of the flask, followed by the addition of 0.4 ml. of 4% titanous chloride solution in 2*N* hydrochloric acid. The flask was shaken well until a white gelatinous precipitate persisted. The solution was acidified with 2 ml. of concentrated hydrochloric acid and allowed to stand for an hour.

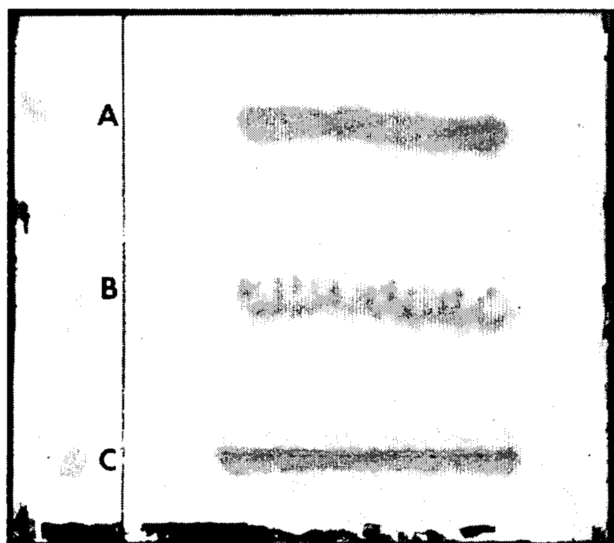

Figure 1. Chromatogram of a mixture of aklomide (*A*), sulfanitran (*B*), and *p*-arsanilic acid (*C*) after Bratton-Marshall reaction

The suspensions of alumina containing sulfanitran were extracted with 10 × 10-ml. portions of hot 1% methanolic hydrochloric acid. The filtration and concentration were carried out in the same manner as in the case of aklomide and the concentrate (5 ml.) was quantitatively transferred to a 50-ml. volumetric flask with several rinsings of hot methanol, 10 ml. of hot 0.5*N* aqueous hydrochloric acid, and 3 ml. of water. The flasks were then immersed in a water bath (70° C.) for about an hour, during which time sulfanitran was deacetylated and the methanol evaporated with a gentle stream of nitrogen.

Combined Extraction of *p*-Arsanilic Acid and Sulfanitran. Exactly 1.0 gram of the medicated feed was extracted with 5 ml. of 0.5*N* aqueous sodium hydroxide and 45 ml. of water on a steam bath for 15 to 20 minutes and allowed to stand at room temperature for 12 to 16 hours. Five milliliters of 6*N* aqueous hydrochloric acid was added to the digest, which was further heated on the steam bath for 30 minutes. The contents and the 3 × 5-ml. portions of hot water rinsings of the flask were transferred to a 100-ml. borosilicate glass centrifuge tube along with a pinch of Celite 545. The mixture was shaken thoroughly and centrifuged at 2500 r.p.m. for 15 minutes. The clear supernatant liquid was filtered through a Celite bed in a sintered glass funnel and the filtrate collected under suction into a 300-ml. round-bottomed flask. The sediment in the centrifuge tube was then shaken with 2 × 10-ml. portions of hot water, centrifuged for 10 minutes each time, and filtered. During the final washing, the precipitate was transferred to the Celite bed and rinsed with another 10-ml. portion of distilled water.

The combined filtrate was evaporated under reduced pressure to a volume of about 20 ml., when a slight turbidity appeared. This solution was carefully transferred to a 50-ml. volumetric flask and made to volume with several rinsings of the evaporation flask. After centrifuging the solution as before, two 5.0-ml. aliquots were pipetted into two 50-ml. volumetric flasks (A and B). To each flask were added 2.0 ml. of 6*N* aqueous hydrochloric acid and 10 ml. of distilled water.

Estimation of Drugs. The volumetric flasks containing reduced aklomide, deacetylated sulfanitran, and the combined *p*-arsanilic acid and deacetylated sulfanitran flask A were diazotized and coupled with *N*-(1-naphthyl)-ethylenediamine dihydrochloride, as previously described. Exactly 5 ml. of *N*-(1-naphthyl)-ethylenediamine dihydrochloride reagent was added to flask B and this was used as a blank for flask A. The absorbance was measured at 545 mμ using 1-cm. cells and a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

The separation of aklomide, sulfanitran, and *p*-arsanilic acid from one another is highly dependent on the pH of the alumina used for thin-layer chromatography. By judicious selection of the solvent systems the adsorbent with a pH of 7.2 to 7.4 was found appropriate for their separation. When the concentration of iron in the adsorbent was higher than 50 to 70 p.p.m., sulfanitran trailed considerably and resulted in poor recoveries. The iron content of the alumina was reduced to 50 to 70 p.p.m. range by leaching with 1.5N aqueous hydrochloric acid and washing repeatedly with distilled water. The pH of the alumina was restored to 7.2 to 7.4 by treating with 400 ml. of 0.063N aqueous alkali. By using this alumina, a known volume of the extract was spotted and developed first in a benzene-chloroform mixture (92 to 8) to elute the polyenes, carotenes, and other interfering substances which, if not separated from the drugs, could move along with aklomide in the second solvent system. After drying in air, the plate was then developed in the second solvent system, acetonitrile-benzene-methanol (60:32:8). By this method aklomide and sulfanitran were cleanly separated from *p*-arsanilic acid (R_f's 0.62 to 0.65, 0.26 to 0.29, and 0.00, respectively).

A preliminary investigation of the unmedicated feed was undertaken, to verify the presence of these medicaments. Four poultry rations of the type laying mash, pullet developer, growing mash, and complete chick grower were analyzed, as described above, to determine the drug content of the feeds. None of the four samples contained any aklomide or sulfanitran, but they did contain some procaine penicillin (R_f 0.70 to 0.75) which did not interfere with the estimation of aklomide (R_f 0.60 to 0.65).

Determination of *p*-arsanilic acid by extraction of the feed in alkaline medium indicated that some contaminant which gave a colored dye with the Bratton-Marshall reagent was also extracted. This dye also had an absorbance

Table I. Concentration of "Contaminant" in Unmedicated Samples

Sample	Reg. No.	Apparent Arsanilic Acid Concn., µg./G. of Feed[a]
Laying mash	6424	34.0
Pullet developer	2385	45.0
Growing mash	2510	34.0
Complete chick grower	2538	32.0

[a] Average of two analyses.

Table II. Determination of Aklomide, Sulfanitran, and *p*-Arsanilic Acid in Medicated Feeds (Micrograms per Gram)

Sample	Aklomide		Sulfanitran		*p*-Arsanilic Acid	
	Added	Found	Added	Found	Added	Found
A	200.6	209	287.2	312	720.7	616
		204		287		636
		187		289		672
		195		316		656
		...		...		662
		...		...		678
Av.		199		301		653
S.D.		±8.4		±13.0		±21.3
B	207	183	211	201	529	490
		182		189		498
		203		216		470
		202		244		474
		...		...		446
		...		...		467
Av.		193		213		474
S.D.		±10.0		±20.5		±16.7
C	139	158	154	147	574	526
		141		133		522
		151		138		481
		135		164		490
		144		165		...
		133		165		...
Av.		144		152		505
S.D.		±8.7		±13.4		±19.5
D	503	495	498	508	742	659
		467		505		648
		505		484		636
		484		484		616
		540		515		...
		496		523		...
Av.		498		503		640
S.D.		±22.3		±14.6		±16.0
E	386	345	269	312	637	642
		374		267		654
		375		304		620
		364		240		624
		386		311		602
		351		317		...
Av.		366		292		628
S.D.		±14.2		±28.5		±17.5
F	550	534	556	575	1242	1140
		560		588		1129
		499		591		1050
		556		588		1188
		526		553		1152
Av.		535		579		1132
S.D.		±22.1		±14.1		±45.4
G	291	262	373	367	848	701
		274		344		694
		278		405		760
		269		368		792
		262		370		724
Av.		269		371		735
S.D.		±6.4		±20.1		±37.0

maximum at 545 mμ. Merwin (1954) reported that extraction of feeds with acid or alkali gave a higher concentration for *p*-arsanilic acid than expected. The collaborators in this work claimed that the higher yield was probably due to tryptophan, released from the proteins by this extraction procedure. In fact, tryptophan does not interfere with the estimation of *p*-arsanilic acid (Merwin, 1954). The contaminant in these rations was probably procaine penicillin (which would react with the Bratton-Marshall reagent to give a colored dye having an absorbance at the same wavelength) or *p*-arsanilic acid itself, either of which might have resulted from cross-contamination.

Table I shows the concentrations of the contaminant present in our four samples. The amount of contaminant seems to be fairly consistent with an average of 36.2 μg. per gram of feed. This value, although insignificant at higher concentrations of *p*-arsanilic acid present in the feed (Merwin, 1954), would make a significant contribution to the drug content at levels of 100 μg. per gram. Hence, the estimated amount of the contaminant has been included in the total concentration of *p*-arsanilic acid in the present study.

The analyses for aklomide in the seven spiked samples indicate that the drug was almost quantitatively extracted, both from the feed and from the alumina plate (Table II). The average loss was approximately 4%, with a standard deviation (S.D.) ranging from ±6.4 to 22.0 for a drug content of 140 to 550 μg. per gram. When known amounts of authentic sample of aklomide were spotted on the alumina plate, the average recovery of the drug was between 96 and 98%.

Again, in the case of sulfanitran, the extraction of the sulfa drug from the feed and from the alumina plate was almost quantitative; more than 97% was recovered. The average loss was about 3.5% except for sample E. The standard deviation ranged from ±13.0 to 28 for a drug content of 150 to 560 μg. per gram.

In the combined extract of sulfanitran and *p*-arsanilic acid in alkaline medium, all the sulfa drug was brought into solution. The absorbance of *p*-arsanilic acid could be calculated by subtracting the absorbance of the sulfa drug from the total absorbance of the combined extract. By this procedure, the recovery of *p*-arsanilic acid was only 90%. The loss may be partly due to the irreversible bonding of the drug to the protein, as observed by Winkler (1962), and partly due to its adsorption on the Celite. However, the loss seemed to be consistent and ranged from 8.9 to 13.8% when the concentration of the drug varied from 520 to 1240 μg. per gram of the feed.

CONCLUSION

This method is valid for the determination of aklomide and sulfanitran in medicated feeds. Considering the variety of organic compounds present in the feed, 10% loss of *p*-arsanilic acid is not surprising. By employing a suitable correction factor to the calculation of *p*-arsanilic acid, this method is applicable in the estimations of the three drugs.

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