Improved Method for Determination of Sulfonamides in Milk and Tissues

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Utilizing the amphoteric nature of most sulfonamides, a simple extraction method followed by the colorimetric development, based on the diazotization-coupling reaction with *N*-(1-naphthyl) ethylenediamine dihydrochloride, is described for the determination of sulfonamides alone and in combination with procaine penicillin G in milk. Recoveries for the ten sulfonamides studied ranged from 81 to 100% recovery at the 100-p.p.b. level. With slight modifications in the initial extraction and evaporation, the procedure has been adapted to swine, poultry, and calf tissues (kidney, liver, muscle, and fat) with a recovery ranging from 76 to 100% at the 100-p.p.b. level. The average milk controls gave an apparent sulfonamide concentration of 8 p.p.b. while the highest average control from tissue was 39 p.p.b.

One of the major requirements in obtaining approval for new veterinary formulations for cows, swine, and chickens is the presentation of methods for determining residues in edible products such as tissues, milk, and eggs. For sulfonamides, the method used for tissues and milk must be sensitive to 0.1 p.p.m.

The most widely used method for the colorimetric determination of sulfonamides is the Bratton-Marshall reaction (Bratton and Marshall, 1939). The procedure described below utilizes the Bratton-Marshall reaction after isolation of the sulfonamide from tissues and milk.

Selzer and Banes (1963) first developed a column chromatographic method suitable for determining sulfonamides in milk at the 0.1-p.p.m. level with a 60 to 91% recovery; however, the procedure is time-consuming, and limits the number of samples a chemist can analyze during a day.

Mooney and Pasarela (1964), also utilizing the Bratton-Marshall reaction, used a strong cationic exchange resin for absorbing the sulfonamide after extraction from tissue and milk. The sulfonamide was then eluted with concentrated ammonia. In addition to being time-consuming, many of the sulfonamides used in our investigation did not yield suitable recoveries when tested using their method.

The procedure described below is faster, offers a sensitivity of at least 0.1 p.p.m. with a minimum of 75% recovery at this level, is applicable when certain other aromatic amine compounds, such as procaine penicillin G, are present, requires no controls, and gives much lower blank values than the methods previously described.

EXPERIMENTAL

Sulfonamide Standard Solution. Solution A: Accurately weigh 100 mg. of the sulfonamide and dissolve in 100 ml. of acetone. Solution B: Dilute 10 ml. of Solution A to 100 ml. with acetone. Solution C: Further dilute 5 ml. of Solution B to 100 ml. with acetone. Solution D: Finally, dilute 25 ml. of Solution C to 100 ml. with acetone. Final concentration: 4 ml. = 5 μg.

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Table I. Recovery of Various Sulfonamides from Milk

Sulfonamide	P.P.B. Added	P.P.B. Found[a]	% Recovery
Sulfachloropyridazine[b]	100	84	84
	100	88	88
	100	88	88
	100[c]	81	81
	200	178	89
	200	176	88
	200	173	87
	200[c]	170	85
Sulfachloropyrazine	100	90	90
	100	92	92
	100	90	90
	100[c]	89	89
	200	180	90
	200	184	92
	200[c]	178	89
N^1-(3-Methyl-1-phenyl-5-pyrazolyl)-sulfanilamide[d]	100	100	100
	100	94	94
Sulfisomidine[e]	100	92	92
	100	88	88
Sulfathiazole	100	94	94
	100	90	90
Sulfaquinoxaline	100	96	96
	100	89	89
Sulfadiazine	100	100	100
	100	94	94
Sulfamerazine	100	92	92
	100	91	91
Sulfamethazine	100	96	96
	100	92	92
Sulfanilamide	100	92	92
	100	88	88
Control milk[f]	0	8 ± 3	...

[a] All recoveries corrected for their respective controls.
[b] CIBA's trade name, Vetisulid.
[c] Samples also spiked with procaine penicillin G, 22 p.p.m.
[d] CIBA's trade name, Vesulong.
[e] CIBA's trade name, Elkosin.
[f] Average result of ten controls with standard deviation.

ANALYTICAL PROCEDURE

Milk (Procaine Penicillin G Not Present). Pipet 50-ml. aliquots of the milk sample into separate 500-ml. separatory funnels. Add 200 ml. of chloroform-acetone mixture (2 to 1). Extract by inverting the separatory funnel very gently for 2 minutes. Try to eliminate as much emulsion as possible. Allow the layers to separate, then run the organic phase through Whatman No. 12 filter paper into a 1000-ml. round-bottomed flask. Do not allow any of the small amount of emulsion that may form to run onto the filter. Repeat the extraction two more times with 200-ml. portions of chloroform-acetone mixture, combining the extracts in the 1000-ml. round-bottomed flask.

Evaporate the combined extracts on a rotary evaporator in a water bath at 50° C. until only a small amount of oily residue remains. Transfer the residue to a 250-ml. centrifuge bottle with four 25-ml. portions of hexane, two 3-ml. portions of acetone, and, finally, two more 25-ml. portions of hexane. Add 10 ml. of 0.5*N* HCl, cap with a rubber stopper covered with Saran Wrap, and shake vigorously for 2 minutes. Centrifuge for 5 minutes at 1500 r.p.m. Draw off the acid layer with the aid of a 10-ml. syringe equipped with a 14-gage round-nosed 15-cm. needle, and filter through Whatman No. 42 paper into a 50-ml. graduated cylinder. Repeat the acid extraction with three 5-ml. portions of 0.5*N* HCl, combining the extracts in the 50-ml. graduated cylinder. Dilute to 30 ml. with 0.5*N* HCl, and mix well.

Evaporate a 4-ml. aliquot of Standard Solution D to dryness in a 50-ml. beaker with the aid of a hair dryer. Transfer the standard residue to a 50-ml. graduated cylinder with 0.5*N* HCl, and dilute to 30 ml. with 0.5*N* HCl.

Color Development. To the sample and standard, add 2 ml. of 0.1% aqueous sodium nitrite, mix well, and let stand for 3 minutes. Add 2 ml. of 0.5 aqueous ammonium sulfamate, mix well, and let stand for 2 minutes. Pass the solutions through a fine sintered glass filter. Pipet 15-ml. aliquots of the solutions into separate 50-ml. Erlenmeyer flasks. To one sample and one standard, add 1 ml. of 0.1% aqueous *N*-(1-naphthyl) ethylenediamine dihydrochloride, mix well, and let stand for 15 minutes. To the remaining sample and standard, add 1 ml. of distilled water (blanks). Determine the absorbance of the sample and standard solutions against their respective blanks at 545 mμ in a 5-cm. cell.

Table II. Recovery of Sulfonamides from Various Tissues

Sulfonamide—Tissue	P.P.B. Added	P.P.B. Found[a]	% Recovery
Sulfachloropyridazine			
Calf			
Fat[b]	100	83	83
	100	91	91
	100	91	91
	200	174	87
	200	168	84
Control[c]	0	27[c] ± 4	...
Kidney[b]	100	78	78
	100	79	79
	100	85	85
	200	154	77
	200	162	81
Control[c]	0	39 ± 8	...
Muscle[d]	100	93	93
	100	90	90
	200	178	89
	200	180	90
Control[c]	0	31 ± 7	...
Liver[e]	100	76	76
	100	76	76
	100	79	79
	100	82	82
	200	160	80
	200	162	81
Control[c]	0	37 ± 8	...
Swine			
Fat[b]	100	88	88
	100	85	85
Control[c]	0	14 ± 5	...
Kidney[b]	100	99	99
	100	93	93
Control[c]	0	33 ± 7	...
Muscle[d]	100	92	92
	100	87	87
Control[c]	0	28 ± 6	...
Liver[d]	100	85	85
	100	79	79
Control[c]	0	23 ± 5	...
Sulfachloropyrazine			
Chicken			
Fat[b]	100	85	85
	100	80	80
	200	200	100
	200	190	95
Control[c]	0	27 ± 4	...
Sulfachloropyrazine			
Chicken			
Kidney[e]	100	97	97
	100	90	90
	200	186	93
	200	186	93
Control[c]	0	13 ± 3	...
Muscle[e]	100	83	83
	100	80	80
	100	83	83
	200	188	94
	200	180	90
Control[c]	0	5 ± 2	...
Liver[e]	100	83	83
	100	81	81
	200	180	90
	200	180	90
	200	174	87
	200	160	80
Control[c]	0	29 ± 6	...

[a] All recoveries corrected for their respective controls.
[b] Method B.
[c] Average result of five controls with standard deviation.
[d] Method C.
[e] Method A.

Calculation.

$$\frac{As}{Ast} \times 100 = \text{p.p.b. sulfonamide}$$

As = absorbance of sample
Ast = absorbance of standard

Milk (Procaine Penicillin G and Other Aromatic Amines Present). Extract milk and hexane solutions exactly as above, only combine 0.5*N* HCl extractions in a 125-ml. separatory funnel. Add 3 ml. of 10*N* sodium hydroxide to the acid extracts. Wash the basic solution with two 25-ml. portions of chloroform, discarding the chloroform layer after each washing. Make the solutions acidic (about pH 1) with concentrated hydrochloric acid. Filter the solutions through fine sintered funnels into a 50-ml. graduated cylinder, and dilute to 30 ml. with 1*N* HCl. Evaporate a 4-ml. aliquot of Standard Solution D, and dilute to 30 ml. with 1*N* HCl in a 50-ml. graduated cylinder. Develop color as described under Color Development, eliminating the filtration step.

Tissue (Procaine Penicillin G Not Present). METHOD A. Cut a portion of the tissue, in excess of 50 grams, into small pieces, and weigh 50 grams of sample tissue into a Waring Blendor. Add 90 to 100 ml. of acetone-chloroform mixture (1 to 1) and homogenize for at least 1 minute at low speed. Transfer as much as possible of the homogenized mixture to a Whatman No. 2V filter and collect the extract in a 1000-ml. round-bottomed flask. Repeat the extraction of the material remaining in the blender with two additional 90- to 100-ml. portions of acetone-chloroform, collecting all extracts in the 1000-ml. round-bottomed flask. Wash the filter paper and contents with several portions of the acetone-chloroform mixture with the aid of a plastic wash bottle (about 25 ml. total). Evaporate the acetone-chloroform mixture on a rotating evaporator at a temperature of about 90° C., and quantitatively transfer the residue to a 250-ml. centrifuge bottle, using in order four 25-ml. portions of hexane, two 3-ml. portions of acetone, and two 25-ml. portions of hexane.

Add 10 ml. of 1*N* HCl, shake gently for 2 minutes, and centrifuge. Draw off the acid phase with the aid of a syringe equipped with a round-nosed needle and filter through a Whatman No. 42 filter into a 50-ml. graduated cylinder. Repeat the extraction with three 5-ml. portions of 1*N* HCl, drawing off the acid phase and filtering each time. Wash the filter with 3 ml. of 1*N* HCl, dilute to 30 ml. with 1*N* HCl, and mix well.

Evaporate a 4-ml. aliquot of Standard Solution D to dryness in a 50-ml. beaker with the aid of a hair dryer. Transfer the standard residue to a 50-ml. graduated cylinder using small portions of 1*N* HCl. Finally, dilute to 30 ml. with 1*N* HCl, and mix well.

METHOD B. Proceed with extraction as above, only add 15 ml. of 1*N* HCl before evaporation (a residue of HCl and oils will remain in the flask; approximately 20 to 25 ml. of solution remain; evaporation should not exceed 45 minutes). Quantitatively transfer the residue to a 250-ml. centrifuge bottle, using in order four 25-ml. portions of hexane, two 3-ml. portions of acetone, and two 25-ml. portions of hexane. Shake gently for 2 minutes, centrifuge, and draw off the acid phase as above. Repeat the extraction with three 5-ml. portions of 1*N* HCl, drawing off the acid phase and filtering each time. Wash the filter with 3 ml. of 1*N* HCl, dilute to 30 ml. with 1*N* HCl, and mix well.

Prepare standard as above.

METHOD C. Proceed with the extraction as above, only add 5 ml. of concentrated hydrochloric acid before evaporation. (A residue of HCl and oils will remain in the flask. Approximately 15 ml. of solution remain. Evaporation should not take over 45 minutes.) Quantitatively transfer the residue to a 250-ml. centrifuge bottle, using in order four 25-ml. portions of hexane, two 3-ml. portions of acetone, and two 25-ml. portions of hexane. Add 10 ml. of distilled water, shake for 2 minutes, and then centrifuge. Draw off the acid layer as described above, and filter into a 50-ml. graduated cylinder. Repeat the extraction with 10 ml. of distilled water, and dilute the solution to 30 ml. with distilled water. Prepare standard as above, only use 2*N* HCl.

For color development, proceed as directed in Color Development under Milk Determination.

Tissue (Procaine Penicillin G and Other Aromatic Amines Present). Extract tissues and hexane solutions as given in Method A, B, or C, only combine extracts in a 125-ml. separatory funnel and proceed as directed under Milk (Procaine Penicillin G and Other Aromatic Amines Present).

RESULTS AND DISCUSSION

Most of the data presented (Tables I and II) deal with the determination of sulfonamides in milk, since this was the main intent. Some data, however, are presented for the determination of a few sulfonamides in tissue of various animals in order to show that the general method is applicable not only to milk but to tissues with slight modifications.

Table I shows the recovery of 10 sulfonamides from milk at the 100- and 200-p.p.b. level with a minimum recovery of 81%. The average milk blank gave an apparent sulfonamide concentration of 8 p.p.b. (14 p.p.b. highest) which is approximately one fourth of that reported by Selzer and Banes (1963), and approximately one eighth of that reported by Mooney and Pasarela (1964).

Because of the amphoteric nature of sulfonamides, compounds such as procaine penicillin G and other aromatic amines, which interfere in the Bratton-Marshall reaction, can be eliminated with a simple extraction prior to color development. The results of milk samples spiked

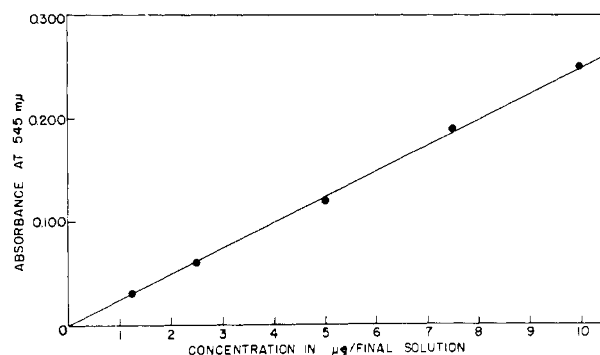

Figure 1. Plot of concentration against absorbance for sulfachloropyridazine

with a sulfonamide and procaine penicillin G are also presented in Table I.

With slight modifications in extraction and evaporation, the procedure described for milk has been adapted to poultry, swine, and calf tissues. Owing to the differences in adsorption of the sulfonamides in the various tissues, slightly different methods of evaporation had to be employed (Methods A, B, and C). Possibly one of the methods would be applicable to all tissues if a different sulfonamide were studied.

Table II summarizes the recovery of two sulfonamides from fat (skin), liver, muscle, and kidney of chickens, calves, and swine. The highest control gave an apparent sulfonamide concentration of 39 p.p.b., which is almost half of the lowest control value reported by Mooney and Pasarela. Although no data are reported in Table II for tissues spiked with procaine penicillin G, the extraction procedure reported above for Milk (Procaine Penicillin G and Other Aromatic Amines Present) should also suffice for tissue samples.

Since all procedures described above state that to divide the sample extract into two portions, one acting as the blank (control), it is not necessary to run tissue or milk controls. The absorbance values obtained from actual controls and from the blanks (controls) as prepared in the outlined procedure showed very little difference between readings. Since controls are not available for regulatory purposes, they have been eliminated in this report; however, they were used in determining recovery data.

The procedures as written above easily can detect 50 p.p.b. of sulfonamide; however, with slight modifications, the procedures can detect 10 p.p.b. Methods are being developed for the determination of sulfonamides at 10 p.p.b., and will be reported at a later date. All of the sulfonamides studied were linear in concentration up to 200 p.p.b. Figure 1 shows the linearity for one of the sulfonamides studied.

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Received for review May 31, 1967. Accepted October 16, 1967.