Determination of Sulfadimethoxine in Animal Tissues

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A method is described for the determination of residue levels of sulfadimethoxine in animal tissues. The sulfonamide is extracted from the tissues with chloroform, partitioned into dilute ammonia, and reacted in the Bratton-Marshall procedure. The dye formed is extracted into *n*-butanol prior to colorimetry. As little as 0.1 p.p.m. of sulfadimethoxine was accurately determined in this fashion on a 10-gram tissue sample. Average recovery for all tissues ranged from 70 to 90%. Nonspecific Bratton-Marshall color of untreated control tissues is kept below 0.05 p.p.m. of apparent sulfonamide. As little as 1.0 μg. of sulfadimethoxine can be measured accurately.

Sulfadimethoxine (4-sulfanilamido-2,6-dimethoxypyrimidine, SDM) has found application as an antimicrobial agent in animal therapy (Fish, 1960). In addition to its use in the treatment of infectious diseases of cattle (Stowe and Sisodia, 1963), SDM has been shown to be an efficacious agent for the prophylaxis and therapy of avian coccidiosis (Mitrovic and Bauernfeind, 1967) as well as for the treatment of microbial diseases of poultry (Mitrovic, 1967). Application of SDM to food-producing animals created the need for a highly sensitive tissue residue procedure, capable of accurately determining the disappearance of the drug from the animal organism. The Bratton-Marshall reaction (Bratton and Marshall, 1939), which depends on the diazotization and coupling of an aromatic amine, has long been used to measure sulfonamides in tissues and body fluids. It is suitable for the determination of residue levels of SDM, provided a sufficiently large tissue sample can be used. However, since even sulfonamide-free (control) tissues contain enough diazotizable aromatic amines to produce high levels of nonspecific Bratton-Marshall color, the use of large tissue samples is possible only in conjunction with a rigorous extraction and clean-up procedure to concentrate and purify the sulfonamide prior to color development. Recovery of drug must be as high and uniform as possible, while keeping the procedure sufficiently simple to allow the processing of large numbers of samples. A cation exchange resin was used by Mooney and Pasarela (1964) to purify the sulfonamides prior to Bratton-Marshall reaction. However, the method gave low recoveries when applied by us to SDM. Another method, published after completion of the work reported here, utilizes a solvent mixture to extract the sulfonamides from the tissues with good recoveries and low control values (Tishler *et al.*, 1968). The required 50-gram tissue samples might however become limiting when dealing with poultry tissues such as kidney or liver.

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The method described here consists of the following steps: extraction of SDM from the tissues by homogenization with chloroform; extraction of SDM from the chloroform by dilute ammonia; acidification and Bratton-Marshall reaction; and concentration of the Bratton-Marshall color by extraction into *n*-butanol prior to spectrophotometric determination. Using this procedure, it was possible to determine as little as 0.1 p.p.m. of SDM in 10 grams of calf, chicken, turkey, or swine tissues, while limiting the nonspecific Bratton-Marshall color of control tissue to less than 0.05 p.p.m. of apparent sulfonamide. Average recovery ranged from 70 to 90%. A minimum of 1 μg. of SDM could be measured accurately by the present method.

EXPERIMENTAL

Reagents. Aqueous ammonium hydroxide, 1% (v./v.), containing sodium chloride, 2.5% (w./v.). Sodium nitrite, 0.1%; ammonium sulfamate, 0.5%; and *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 0.1%, prepared fresh daily. Chloroform, reagent grade, redistilled fresh daily.

Preparation of Standards and Fortified Samples. To prepare the stock standard, weigh exactly 100 mg. of sulfadimethoxine into a 100-ml. flask, suspend in 10 ml. of water, dissolve with the aid of 3 drops of concentrated ammonium hydroxide, and bring to 100 ml. This stock standard (1 mg. of SDM per ml.) is stable for at least one week at room temperature. The working standards containing 1, 2, 4, 8, and 16 μg. of SDM per ml. are prepared daily from the stock standard by two successive dilutions.

These working standards are used to prepare the fortified samples and the aqueous standard curve. To prepare the fortified samples, 1 ml. of the appropriate SDM working

standard is added to 10 grams of ground control tissue or 10 ml. of whole blood or plasma in the homogenizer cup and mixed in thoroughly prior to the addition of chloroform. The aqueous standard curve is prepared by adding the appropriate working standard to 30 ml. of the sodium chloride–ammonia solution in a 100-ml. glass-stoppered graduated cylinder and proceeding as indicated under "Bratton-Marshall Color Development. Acidify the solution. . . ."

Bratton-Marshall Color Development. Acidify the solution in the 100-ml. graduated cylinder by adding 2 ml. of $6N$ HCl. The solution, which may be slightly turbid, is now ready for the Bratton-Marshall color development. To the acidified solution, add 0.5 ml. of the 0.1% sodium nitrite solution. Mix well and let stand for 6 minutes. Add 0.5 ml. of the 0.5% ammonium sulfamate solution. Mix well and let stand for 2 minutes. Add 0.5 ml. of the Bratton-Marshall reagent. Mix well and let stand in the dark for 10 minutes while color develops. Add 10 grams of sodium chloride and 5.0 ml. of *n*-butanol. Shake vigorously for 15 seconds and allow layers to separate for 5 minutes. Some undissolved sodium chloride may collect at the bottom of the lower, aqueous phase. Transfer all of the butanol phase along with some of the aqueous phase to a 15-ml. centrifuge tube. Centrifuge at moderate speed for 5 minutes. Withdraw the upper, butanol layer, which must be absolutely clear, by means of a syringe with a long, blunt needle, and transfer it to a Beckman DU spectrophotometer cell of 1-cm. path length. Care must be taken not to shake the centrifuge tube or to penetrate the aqueous layer with the needle so as not to create any turbidity in the butanol. Determine absorbance at 545 mμ against pure *n*-butanol. Calculate sulfadimethoxine level from aqueous standard curve.

Sample Preparation and Extraction (Muscle, Liver, Kidney, Skin, and Fat). Cut the entire sample into small pieces and pass through a meat-grinder. Weigh 10 $\pm$ 0.1 grams of ground sample into a 250-ml. Virtis homogenizer cup. Add 100 ml. of chloroform and homogenize (Virtis-45 high speed homogenizer) for approximately one minute at a medium to high speed setting. With the aid of several small portions of chloroform, quantitatively transfer the homogenate to a 250-ml. centrifuge tube and centrifuge for 5 minutes at 2000 r.p.m. Place 15 ml. of the ammonia–sodium chloride solution into a 250-ml. separatory funnel. Decant the chloroform from the centrifuge tube into the separatory funnel through a fluted Whatman No. 2V filter paper into which 1 gram of dry Celite analytical filter aid (Johns-Manville) had been placed, while retaining the packed tissue with a glass rod. Rinse centrifuge tube and packed tissue with a small portion of chloroform. Shake gently by rocking the separatory funnel back and forth manually at about 1 cycle per second for 1 minute. Avoid more vigorous shaking so as not to form an undesirable emulsion. Allow layers to separate for approximately 10 minutes. The chloroform phase may remain somewhat turbid while the aqueous phase is virtually clear. The chloroform phase is drained off into another 250-ml. separatory funnel. Material which may have collected at the interface is retained with the aqueous phase. If separation of the phases is difficult because of emulsion, it can be speeded up by a brief application (10 seconds) of the spark from a high frequency induction coil (vacuum leak detector) to the circumference of the separatory funnel, followed by a 5-minute rest period to allow layers to separate. Application of the spark may be repeated after most of the chloroform has been drained off, to obtain a clean interface. Drain the aqueous phase from the separatory funnel through a small plug of glass wool into a 100-ml. glass-stoppered graduated cylinder. Rinse the separatory funnel with 3 to 5 ml. of water which are added to the graduated cylinder. Extract the chloroform with another 15-ml. portion of the ammonia–sodium chloride solution. Slightly more vigorous shaking may now be employed. Allow layers to separate and discard chloroform. Transfer the aqueous phase to the 100-ml. graduated cylinder, filtering through the same plug of glass wool. Rinse the separatory funnel and the glass wool with 3 to 5 ml. of water which are added to the graduated cylinder. Continue as under "Bratton-Marshall Color Development. Acidify the solution. . . ."

Extraction from Blood and Plasma. WHOLE BLOOD. Place 10 ml. of blood into a 250-ml. centrifuge tube. Add 90 ml. of water and 1 ml. of $0.1N$ hydrochloric acid and mix well. Add 100 ml. of a 1-to-1 mixture of chloroform and ethyl acetate. Stopper the tube and place on mechanical shaker for 15 minutes. Centrifuge for 20 minutes at 2000 r.p.m. Remove the upper (aqueous) phase by aspiration, taking care not to touch the semisolid interface. Pour the organic phase into a 250-ml. separatory funnel through a fluted Whatman No. 2V filter paper. The filter will retain the material from the interface. Rinse centrifuge tube and filter with three portions of 5 ml. each of chloroform which are added to the separatory funnel. Add 15 ml. of the ammonia–sodium chloride solution. Continue above: "Shake gently. . . ."

PLASMA. Place 10 ml. of plasma into a 250-ml. centrifuge tube and add 1 ml. of $0.1N$ hydrochloric acid. Add 100 ml. of chloroform. Stopper the tube and place on mechanical shaker for 15 minutes. Transfer to 250-ml. separatory funnel. Allow phases to separate for 5 to 10 minutes. Drain chloroform phase into a 250-ml. separatory funnel through a fluted Whatman No. 2V filter paper into which 1 gram of dry Celite analytical filter aid (Johns-Manville) has been placed. Any residual emulsion may be broken by swirling with an additional small quantity of chloroform prior to filtration. Add 15 ml. of the ammonia–sodium chloride solution. Continue above: "Shake gently. . . ."

RESULTS AND DISCUSSION

The use of a water-immiscible solvent, chloroform, to extract SDM from the tissues presents several advantages including good recovery, clean separation of the phases, and limited contamination by nonspecific Bratton-Marshall positive materials. When extracting whole blood, chloroform is replaced by a 1-to-1 mixture of chloroform and ethyl acetate to get good separation of the layers. Removal of the nonspecific Bratton-Marshall positive materials is accomplished in the next step, extraction of SDM from chloroform into dilute ammonia. While SDM could be extracted from chloroform by either dilute acid or dilute ammonia, use of the latter resulted in a much better separation from the Bratton-Marshall positive

Table I. Recovery of Sulfadimethoxine from Fortified Calf, Chicken, and Turkey Tissues

SDM, P.P.M. Added	Calf			
	Liver		Kidney	
	P.P.M. found[a]	% recovery	P.P.M. found	% recovery
0	0.043	...	0.029	...
0.1	0.069(±0.024)	69	0.090(±0.014)	90
0.2	0.155(±0.043)	78	0.176(±0.016)	88
0.4	0.313(±0.027)	78	0.350(±0.016)	88
0.8	0.624(±0.036)	78	0.680(±0.042)	85
1.6	1.24 (±0.041)	78	1.34 (±0.034)	84
	Muscle		Fat	
0	0.026	...	0.043	...
0.1	0.079(±0.002)	79	0.074(±0.022)	74
0.2	0.160(±0.002)	80	0.166(±0.015)	83
0.4	0.340(±0.004)	88	0.313(±0.039)	78
0.8	0.650(±0.007)	81	0.616(±0.023)	77
1.6	1.30 (±0.014)	81	1.27 (±0.056)	80
	Blood		Plasma	
0	0.022	...	0.017	...
0.1	0.089(±0.007)	89	0.070(±0.008)	70
0.2	0.150(±0.005)	75	0.132(±0.008)	66
0.4	0.290(±0.009)	73	0.270(±0.013)	68
0.8	0.590(±0.015)	74	0.620(±0.025)	78
1.6	1.26 (±0.028)	79	1.25 (±0.025)	78
	Chicken			
	Liver		Kidney	
0	0.026	...	0.031	...
0.1	0.082(±0.015)	82	0.079(±0.010)	79
0.2	0.168(±0.018)	84	0.160(±0.014)	80
0.4	0.335(±0.027)	84	0.327(±0.008)	82
0.8	0.638(±0.051)	80	0.025(±0.027)	78
1.6	1.22 (±0.044)	76	1.25 (±0.026)	78
	Muscle		Fat	
0	0.017	...	0.036	...
0.1	0.074(±0.010)	74	0.079(±0.012)	79
0.2	0.160(±0.015)	80	0.160(±0.016)	80
0.4	0.321(±0.012)	80	0.332(±0.026)	83
0.8	0.652(±0.035)	82	0.652(±0.048)	82
1.6	1.34 (±0.094)	84	1.29 (±0.028)	81
	Turkey			
	Liver		Kidney	
0	0.033	...	0.035	...
0.1	0.064(±0.003)	64	0.100(±0.003)	100
0.2	0.142(±0.014)	71	0.168(±0.018)	84
0.4	0.316(±0.012)	79	0.370(±0.020)	93
0.8	0.614(±0.018)	77	0.646(±0.018)	81
1.6	1.21 (±0.034)	76	1.33 (±0.029)	83
	Muscle		Fat	
0	0.017	...	0.043	...
0.1	0.082(±0.008)	82	0.082(±0.007)	82
0.2	0.163(±0.011)	81	0.158(±0.009)	79
0.4	0.337(±0.010)	84	0.348(±0.031)	87
0.8	0.665(±0.058)	83	0.687(±0.028)	86
1.6	1.28 (±0.039)	80	1.33 (±0.040)	83

[a] Values corrected for controls. Standard deviations calculated from 10 replicate samples.

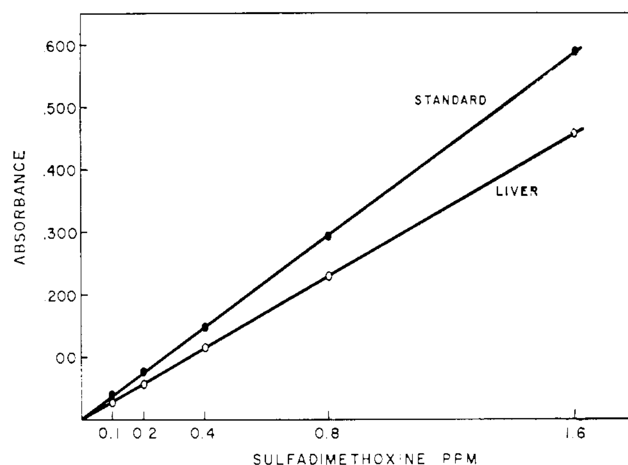

Figure 1. Sulfadimethoxine aqueous standard curve and recovery curve from calf liver (10-gram samples)

aromatic amines responsible for the high control values. The addition of sodium chloride to the aqueous ammonia solution aids in reducing the formation of emulsions and promotes a better separation of the phases. Advantage is taken of the extreme solubility of the Bratton-Marshall dye in *n*-butanol to concentrate the dilute solution after color development, so as to be able to take instrument readings in a desirable range of absorbancies. In addition, this extraction step eliminates the occasional turbidity encountered in the final aqueous solution with some tissue samples. Near-saturation of the aqueous phase with sodium chloride promotes a very sharp separation of the butanol.

The results of a series of recovery experiments with 10-gram samples of fortified control tissues from calf, chicken and turkey are shown in Table I. With all tissues, average recovery of SDM ranged from 70 to 90% for the concentrations studied (0.1 to 1.6 p.p.m.). Replication was good, as evidenced by the standard deviations calculated from 10 replicate assays. The good linearity of the results is illustrated in Figure 1. Control values for unmedicated tissues were maintained at 0.02 to 0.04 p.p.m. of apparent sulfonamide. Based on these data, the requirements outlined in the introduction have been fulfilled by the present method.

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