Determination of Ormetoprim in Animal Tissues

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A method is described for the determination of low levels of the sulfonamide potentiator ormetoprim [2,4-diamino-5-(4,5-dimethoxy-2-methylbenzyl)pyrimidine] in animal tissues. The compound is extracted from tissues with methylene chloride–ethyl acetate 1:9, cleaned up by partition between organic and aqueous phases, oxidized with permanganate to 4,5-dimethoxy-*o*-toluic acid, and determined by spectrofluorometry. As little as 0.1 ppm of ormetoprim was accurately measured on a 10-g tissue sample. Average recovery from all tissues ranged from 72 to 94%. Control values for unfortified tissues ranged from 0 to 0.02 ppm of apparent ormetoprim.

Rofenaid, a safe and efficacious coccidiostat and antibacterial agent for poultry (Mitrovic *et al.*, 1969a,b, 1970a,b; Marusich *et al.*, 1969, 1970) consists of two drugs, sulfadimethoxine (4-sulfanilamido-2,6-dimethoxypyrimidine) and ormetoprim [2,4-diamino-5-(4,5-dimethoxy-2-methylbenzyl)pyrimidine] in a ratio of 5:3. Application of Rofenaid to food-producing animals has resulted in the need for highly sensitive tissue residue procedures, capable of accurately measuring the disappearance of both drugs from the animal organism. A method for the determination of sulfadimethoxine has already been described (Fellig and Westheimer, 1968); the determination of ormetoprim forms the subject of the present report. Schwartz *et al.* (1969) have described the determination in body fluids of the sulfonamide potentiator trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] by permanganate oxidation to 3,4,5-trimethoxybenzoic acid.

EXPERIMENTAL

The method consists of the following steps: extraction of ormetoprim from the tissues by homogenization with methylene chloride–ethyl acetate 1:9; purification by partitioning between aqueous and organic phases; permanganate oxidation of ometoprim to 4,5-dimethoxy-*o*-toluic acid; and fluorometric determination of the latter compound.

Preparation of Standards and Fortified Samples. To prepare the stock standard, weigh exactly 10 mg of ormetoprim into a 100-ml flask, dissolve in 10 ml of 1 *N* hydrochloric acid, and bring to 100 ml. This stock standard is stable for at least 1 week at room temperature. The working standards, containing 1, 2, 4, and 8 μg of ormetoprim per ml, are prepared daily from the stock standard by the appropriate dilutions. They are used to prepare the fortified samples and the aqueous standard curve. To prepare the fortified samples, 1 ml of the appropriate ormetoprim working standard is added to 10 g 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 solvents. To prepare the aqueous standard curve, place 1 ml of the appropriate working standard into a 50-ml glass-stoppered centrifuge tube, add 1 ml of 0.1 *N* sodium hydroxide, and proceed as indicated under permanganate oxidation ". . . followed by 2 ml of alkaline permanganate."

Permanganate Oxidation. Dissolve the residue from the evaporation of the chloroform in 1 ml of 0.1 *N* hydrochloric acid, add 2 ml of 0.1 *N* sodium hydroxide, followed by 2 ml of alkaline permanganate (0.1 *M* potassium permanganate in 0.1 *N* sodium hydroxide). Mix well and place in a heating block maintained at 80° C for a period of 10 min. Remove from the heating block, add 1 ml of 10% w/v sodium nitrite (prepared fresh daily), followed immediately by 4 ml of 1 *N* sulfuric acid. Mix well, and let stand for 5 min with occasional vigorous shaking. Add 2 ml of 10% w/v ammonium sulfamate (prepared fresh daily), mix well, and let stand with occasional vigorous shaking until there is no further evolution of nitrogen (5 min). Add 10 ml of chloroform (reagent grade, glass distilled), stopper and mix thoroughly for 30 sec on a Vortex mixer. Allow phases to separate. Transfer the lower, chloroform layer to a Farrand spectrofluorometer cell of 1-cm path length by means of a syringe with a long blunt needle. Measure fluorescence in μA at an activation wavelength of 305 nm and an emission wavelength of 345 nm. Calculate concentration of ormetoprim from aqueous standard curve.

Sample Preparation and Extraction (Muscle, Kidney, Skin and Fat). The entire sample is passed through a meat grinder. Weigh 10 ± 0.1 g of ground tissue into a 250-ml Virtis homogenizer cup. Add 1 ml of 10% ammonia solution, 10 ml of methylene chloride (reagent grade), and 90 ml of ethyl acetate (reagent grade). Homogenize (Virtis-45 High Speed Homogenizer) for approximately 1 min at a medium to high-speed setting. Pour contents of cup through a Whatman No. 2V 25-cm fluted filter paper into a 250-ml separatory funnel. Rinse cup and residue with 20 ml of ethyl acetate. Add 35 ml of 0.1 *N* hydrochloric acid to the separatory funnel and extract by shaking for 1 min. Allow phases to separate and drain the aqueous phase into a 125-ml separatory funnel. Reextract the organic phase with another 35-ml portion of 0.1 *N* hydrochloric acid and combine the aqueous phases. If emulsions form, they can be broken by a brief application of the spark from a high-frequency induction coil (vacuum leak detector) to the circumference of the separatory funnel. Make the combined aqueous phase alkaline by the addition of 4 ml of 8 *N* sodium hydroxide. Add 15 ml of chloroform and extract by shaking gently for 1 min. Allow phases to separate and drain the chloroform into another 125-ml separatory funnel through a small plug of cotton. Reextract the aqueous phase with two additional 15-ml portions of chloroform and combine the chloroform phases. Extract the chloroform with two 12-ml portions of 0.1 *N* hydrochloric acid, allow the phases to separate, and combine the acid extracts in a 125-ml separatory funnel. Add 1 ml of 8 *N* sodium hydroxide. Extract the alkaline solution with two 10-ml portions of chloroform, allow the phases to separate, and combine the chloroform layers in a

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Table I. Recovery of Ormetoprim from Fortified Chicken and Turkey Tissues

Ormetoprim ppm added	Muscle (chicken) ppm found[a]	% recovery	Kidney (chicken) ppm found	% recovery
0	0.010	...	0	...
0.1	0.092 (±0.005)	92	0.086 (±0.006)	86
0.2	0.156 (±0.008)	78	0.185 (±0.003)	92
0.4	0.342 (±0.011)	86	0.325 (±0.010)	81
0.8	0.700 (±0.027)	88	0.612 (±0.005)	77
	Skin and fat (chicken)		**Blood (chicken)**	
0	0.017	...	0	...
0.1	0.073 (±0.003)	73	0.085 (±0.002)	85
0.2	0.174 (±0.006)	87	0.184 (±0.008)	92
0.4	0.349 (±0.011)	87	0.374 (±0.004)	94
0.8	0.707 (±0.028)	88	0.698 (±0.000)	87
	Liver (chicken)		**Muscle (turkey)**	
0	0	...	0.010	...
0.1	0.085 (±0.005)	85	0.075 (±0.004)	75
0.2	0.183 (±0.008)	92	0.180 (±0.011)	90
0.4	0.304 (±0.009)	76	0.312 (±0.013)	78
0.8	0.726 (±0.013)	91	0.655 (±0.020)	82
	Kidney (turkey)		**Skin and fat (turkey)**	
0	0	...	0.020	...
0.1	0.085 (±0.007)	85	0.085 (±0.007)	85
0.2	0.155 (±0.026)	78	0.177 (±0.016)	88
0.4	0.310 (±0.030)	78	0.315 (±0.009)	78
0.8	0.641 (±0.033)	80	0.711 (±0.030)	89
	Blood (turkey)		**Liver (turkey)**	
0	0	...	0.010	...
0.1	0.072 (±0.012)	72	0.072 (±0.009)	72
0.2	0.145 (±0.010)	73	0.165 (±0.013)	81
0.4	0.326 (±0.023)	81	0.302 (±0.025)	73
0.8	0.645 (±0.039)	81	0.596 (±0.010)	75

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

round-bottomed, glass-stoppered 50-ml centrifuge tube. Place the tube in a heating block maintained at 80° C and evaporate the chloroform in a stream of high-purity nitrogen filtered through a silica gel or molecular sieve filter-dryer.

Liver. The entire sample is passed through a meat grinder. Weigh 10 ± 0.1 g of ground tissue into a 250-ml Virtis homogenizer cup. Add 10 ml of methylene chloride and 90 ml of ethyl acetate. Homogenize at medium speed for 15 sec. Add 1 g of dibasic potassium phosphate and 15 g of anhydrous sodium sulfate. Homogenize for 1 min at medium to high speed. Pour contents of cup through a Whatman No. 2V 25-cm fluted filter paper into a 250-ml separatory funnel. Rinse cup and residue with an additional 20 ml of ethyl acetate. Continue as above: "Add 35 ml of 0.1 *N* hydrochloric acid...."

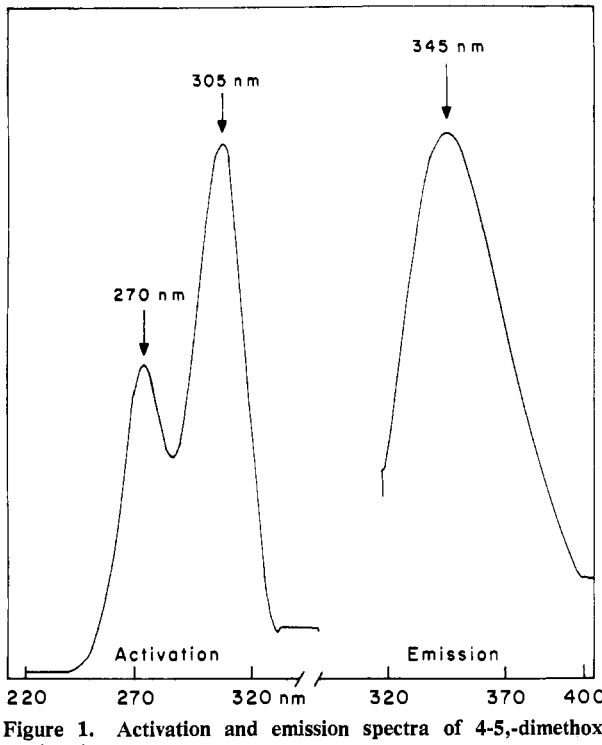

Figure 1. Activation and emission spectra of 4-5,-dimethoxy-*o*-toluic acid

Blood and Plasma. Place a 10-ml sample into a 250-ml Virtis homogenizing cup. Add 1 ml of 10% ammonia solution, mix, and add 10 ml of methylene chloride and 90 ml of ethyl acetate, followed by 3 g of Celite analytical filter aid. Homogenize at medium to high speed for 1 min. Pour contents of cup through a Whatman No. 2V 25-cm fluted filter paper, into which 1 g of Celite had been placed, into a 250-ml separatory funnel. Rinse cup and residue with an additional 20 ml of ethyl acetate. Continue as above: "Add 35 ml of 0.1 *N* hydrochloric acid...."

RESULTS AND DISCUSSION

Methylene chloride–ethyl acetate 1:9, the water-immiscible solvent system selected to extract ormetoprim from the tissues, presents the advantages of good recovery and minimal formation of emulsions. In the case of liver, recovery was better when the tissue was partially dehydrated by addition of anhydrous sodium sulfate, and aqueous ammonia replaced by dibasic potassium phosphate. Advantage is taken of the basic nature of ormetoprim to accomplish a further cleanup by extracting the drug from the organic phase into dilute hydrochloric acid and, after neutralization of the aqueous solution, back into chloroform. These steps are needed to achieve low control values with unfortified tissues. By

calibration of the spectrofluorometer with authentic 4,5-dimethoxy-*o*-toluic acid, the yield of the permanganate oxidation was found to average 65% of theory under the optimal conditions specified by the method. Other oxidants were unsatisfactory. The characteristic activation and emission spectra of 4,5-dimethoxy-*o*-toluic acid are shown in Figure 1. Sodium nitrite was chosen to destroy the excess permanganate in preference to formaldehyde or sodium bisulfite, because it was difficult to reduce manganese dioxide completely with the former and very thorough elimination of sulfur dioxide was necessary with the latter because SO_2 was found to quench the fluorescence.

Table I shows the results of a series of recovery experiments with 10-g samples of chicken and turkey control tissue fortified with ormetoprim. Recovery from all tissues ranged from 72 to 94% (average 83%) over the range of concentrations studied (0.1 to 0.8 ppm), with good linearity. Replication was good, as shown by the standard deviations calculated from five replicate assays. Control values for unfortified tissues ranged from 0 to 0.02 ppm of apparent ormetoprim. It is concluded that the present method is capable of accurately measuring as little as 1 μg of ormetoprim in a 10-g tissue sample for a sensitivity of 0.1 ppm.

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