Determination of Urea in Animal Tissue by Gas Chromatography

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A new procedure has been developed that determines urea in biological materials by gas chromatography as trifluoroacetyl urea. The samples are extracted with water, cleaned up by zinc hydroxide precipitation, and then followed by separation by ether fractionation. An aliquot of the ether solution is then treated with trifluoroacetic anhydride to give trifluoroacetylurea, which is gas chromatographed on a 4-ft × 2-mm 3% neopentyl glycol sebacate–2% phosphoric acid column with electron capture detection. The average recovery from selected biological materials was 91%.

The extensive use of urea as a nonprotein nitrogen source for ruminant animals has created an interest in the assay of various animal tissues for urea content. Several assay methods have been used for urea; a common one involves hydrolysis of urea to ammonia by urease, with subsequent measurement of the ammonia (Hawk, 1967). There has also been an increased use of reactions involving urea itself, such as colorimetric methods employing *p*-dimethylaminobenzaldehyde or α-diketones (Briggs, 1967).

A new approach, described in this paper, is the gas chromatography of the trifluoroacetylurea derivative using electron capture detection.

EXPERIMENTAL

Apparatus. A Barber-Colman Model 10 gas chromatograph equipped with a Sr^{90} ionization detector was used in the electron capture mode for this study. A Lourdes Model MM-1 Multi-mixer equipped to use 4-oz French square bottles was used to blend the samples.

Reagents. A trifluoroacetylurea analytical reagent material was synthesized and is available from the Sampling Coordinator, Agricultural Department, The Dow Chemical Co., Midland, Mich. The solutions of this material should be made fresh each week. All other materials were reagent grade.

ANALYTICAL PROCEDURES

The trifluoroacetylurea was chromatographed on a 4-ft × 2-mm 3% neoglycol sebacate–2% phosphoric acid on 80/100 mesh Chromosorb WAW support operated with a column temperature of 120° C, injector block temperature of 145° C, and a detector temperature of 180° C. The nitrogen carrier gas flow rate was 80 cm^3/min with the detector operating voltage set at 13 V.

Fluid samples were stored frozen at 0° C while other tissues were passed through a meat chopper and then stored frozen at 0° C.

Weigh 10 g of sample into a 12-dram vial, add 50% $ZnSO_4 \cdot 7\ H_2O$ solution, 20% sodium hydroxide solution, and water, in the quantities shown in Table I. Cap and shake the mixture for 5 min on a Burrell wrist-action shaker and centrifuge at maximum speed for a clinical centrifuge. Evaporate an aliquot equivalent to 5 g of tissue to dryness with air and moderate heat on a steam bath. Add two drops of water and 4 ml of ammonium hydroxide in methanol, and warm on a steam bath followed by loosening the material from the beaker with a spatula.

Prepare a 1-cm Hyflo Super-Cel filter in a 12-mm × 17-cm chromatographic tube with a coarse sintered glass disk. Wash this filter with 5 ml of 5% ammonium hydroxide in methanol. Add 1 ml of ether to the methanol solution above, extract, and decant this ether–methanol solution onto the filter. To the residue in the beaker add 2.5 ml of ammonium hydroxide in methanol, warm, and add 0.5 ml of ether and decant this solution onto the filter. Repeat this step until 10 ml of filtrate are collected.

Take a 0.1- to 0.5-ml aliquot from the filtrate and evaporate it to dryness with air in a 13-mm × 10-cm screw cap test tube. Add 0.2 ml of benzene and 0.05 ml of trifluoroacetic anhydride. Cap and shake 10 min. Add 4.95 ml of benzene, mix well, and immediately add a 2-ml aliquot to a column made up with a freshly prepared mixture of 0.5 g Hyflo Super-Cel filter aid and 0.2 ml of 30% aqueous sodium acetate packed in a 12-mm × 17-cm chromatographic tube. Elute the column with 1:1 (v/v) ether:benzene solution, collecting the effluent in a 10-ml volumetric flask. Collect exactly 10 ml and then dilute 1:10 with benzene. Chromatograph 2 μl of the diluted solution and determine its urea concentration by referring to the standard curve prepared by plotting peak heights *vs.* the corresponding trifluoroacetyl urea concentration obtained over the range of 0.025 to 0.1 μg/ml. The urea being acetylated is effectively diluted to 260 ml for injection into the gas chromatograph, giving a dilution factor of 100 as 1 μg of urea is equivalent to 2.6 μg of trifluoroacetylurea.

Recovery Determinations. Milk normally contains urea, so that it was necessary to pretreat milk with 0.1 g of jackbean urease to 200 ml of milk and allow this material to stand overnight to get a control for urea-free milk to be used for fortification studies. The urea-free milk was then fortified at levels of 10 to 300 ppm and analyzed by the

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Table I. Sample Preparation

Sample type	Sample wt, g	$ZnSO_4$ soln, ml	NaOH, ml	Water, ml	Centrifuge, min	Represents (ml = g)
Milk	10	1.0	0.6	2.4	15	7 = 5
Rumen fluid	10	1.0	0.6	2.4	15	7 = 5
Blood	10	1.0	0.6	20.4	15	15 = 5
Urine	1	0.5	0.3	8.2	5	5 = 0.5
Muscle	10	2.0	1.0	21	15	15 = 5
Liver and kidney	10	2.0	1.0	20	30	15 = 5
Fat	10	1.0	0.5	18.5	5	10 = 5

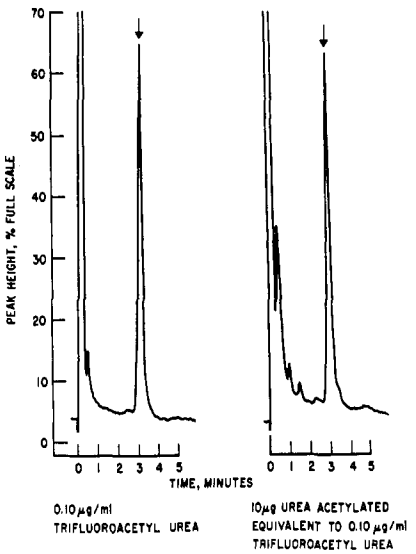

Figure 1. Chromatograms: 10 μg urea acetylated compared to equivalent amount of trifluoroacetylurea standard

method employed for treated samples. Rumen fluid was also fortified with 100-ppm urea and analyzed in the same manner.

RESULTS AND CONCLUSIONS

This analytical procedure for urea gave recoveries in milk and rumen fluid of 92 and 90%, respectively. These data were corrected for control values and are shown in Table II. The conversion of urea to trifluoroacetylurea is quantitative. Nine trials on acetylating 10 μg of urea gave a range of 87 to 100% conversion, with an average of 98%. Solutions of trifluoroacetylurea are relatively stable, but stability data (Table III) suggest that it would be prudent to make up dilute standard solutions each week. The chromatograms of acetylated urea are equivalent to the same amount of trifluoroacetylurea standard, as shown in Figure 1.

Bovine liver, kidney, muscle, milk, rumen fluid, blood, fat, and urine were analyzed by this procedure and the values obtained are tabulated in Table IV. These values agree quite well with those found in the literature. Chromatograms for urea determination from these various tissues are shown in Figure 2.

The removal of trifluoroacetic anhydride and acid from the acetylation mixture by liquid chromatography on the sodium acetate column makes this method for urea possible. Both the trifluoroacetic anhydride and acid are very good electron-capturing molecules with retention times that cover the

Table II. Summary of Recovery of Urea from Control Samples

ppm of Urea added	ppm of Urea found: Gross	ppm of Urea found: Net	% Recovery
	Milk		
0	2.2		
0	2.2		
10		9.6	96
10		8.2	82
25		21.4	86
25		24.5	98
100		85.8	86
100		92	92
300		288	96
300		292	97
			Average 92
	Rumen fluid		
0	0.5		
0	<0.5		
100		88	88
100		99	99
100		84	84
			Average 90

Table III. Stability of the 0.1 μg/ml Trifluoroacetylurea Standard[a]

Standard no.	Time, days: 1	2	3	4	5
1			100	100	99
2	98	100	94	93	
3	88	68	65		
4	98	96			91
5	100			99	96
6			98	92	92
7	95	95	98	100	
8	100	98	96		
9	100	98			
10	100				

[a] The values expressed in this table are in percent of freshly prepared standard solution.

Table IV. Urea in Several Biological Materials

Tissue	ppm Urea: Values obtained by this method	Values given in literature
Milk	186	100–450[a]
Rumen fluid	<1	Negligible[b]
Blood	380	161–420[b]
		130–580[c]
Muscle	160–400	
Liver	320	
Kidney	380	
Fat	4	
Urine	5000–12000	

[a] Kaplan *et al.* (1960). [b] Briggs (1967). [c] Spector (1956).

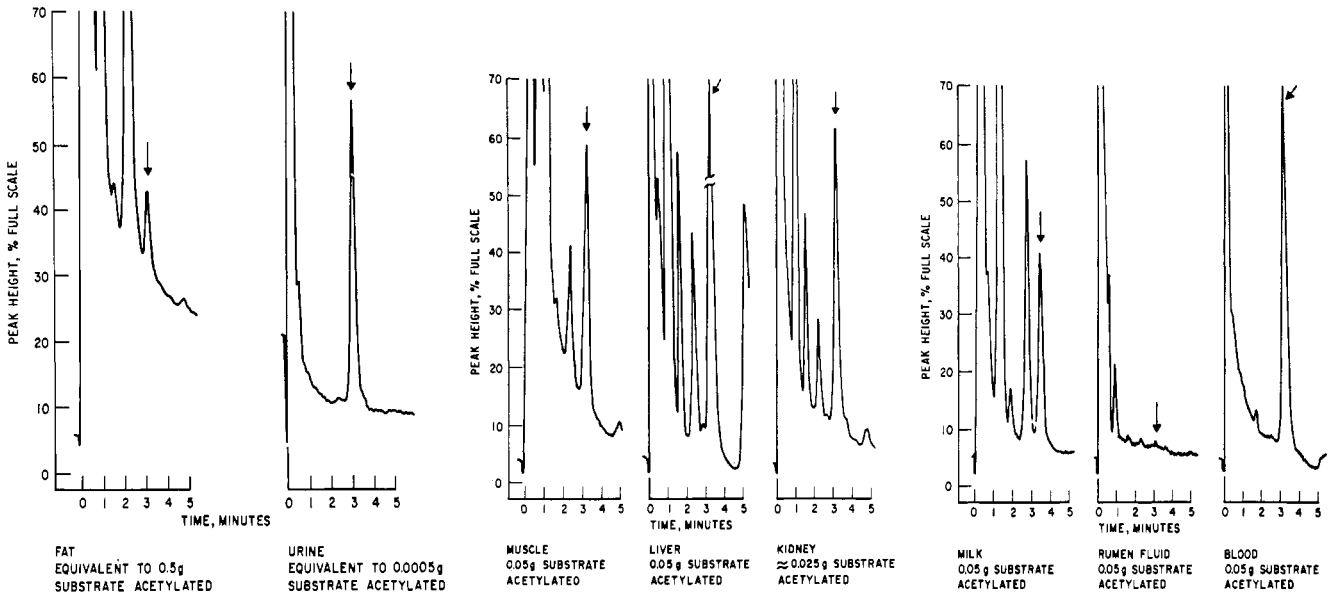

Figure 2. Chromatograms of urea determinations from bovine samples

trifluoroacetylurea peak. The acetate column exchanges the electron capturing trifluoroacetate species with nonelectron capturing acetate which is not detected by the electron capture detector, thereby resolving trifluoroacetylurea.

This method might possibly be used to study low level excretion of urea by the skin or saliva or be used to further study the unrease mechanism.

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