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Determination of Methylglyoxal in Ruminal Fluid by High-Performance Liquid Chromatography Using Fluorometric Detection

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There is no reported method for the quantification of methylglyoxal in ruminal fluid. The method reported here is based on the conversion of methylglyoxal to 6-methylpterin, followed by quantification of the resulting pteridinic compound by fluormetric detection using liquid chromatography. Ruminal fluid was collected and preserved with 1 M HCl at −20 °C. Cation exchange prior to derivatization was used to eliminate possible interfering peaks. The detection limit of 0.125 μg/mL was calculated. The recoveries were >80%, and the coefficients of variation were <15%. This method has proven to be rugged and accurate for the detection of methylglyoxal concentration in ruminal fluid collected from cows fed diets deficient in degradable intake protein as a marker. Methylglyoxal is produced by ruminal bacteria in response to low nitrogen levels in the rumen. The ruminal methylglyoxal concentration has the potential to be a useful marker to assess ruminal nitrogen status to aid in more accurate diet formulation.

KEYWORDS: Methylglyoxal; HPLC; ruminants; nitrogen, carbohydrate

INTRODUCTION

When ruminants are consuming dormant forage or diets that are high in cereal grains but low in forage, the ruminal microbes may experience growth conditions where carbohydrate is in excess of microbial requirements and nitrogen is limiting. To overcome these inadequacies, cattle are supplemented with protein supplements formulated to meet their nutrient requirements (*1*). These protein supplements may contain ruminally degradable protein, undegradable protein, or combinations of both. However, prediction of the effectiveness of the supplementation program, in regards to the degradable protein requirement of the microbial population, is based on the ammonia (NH_3) concentration in ruminal contents. Ammonia is used by the ruminal bacteria to synthesize microbial protein and thereby supply the ruminant with amino acids for absorption (*2*). Ammonia in the rumen is a pool of several inputs and outputs. Ammonia is derived from the degradation of dietary protein and dietary NPN, from the hydrolysis of urea recycled to the rumen, and from the degradation of microbial crude protein (*1*). Ammonia disappears from the rumen pool due to uptake by the microbes, absorption by the microbes, absorption through the rumen wall, and flushing to the omasum. Alterations of nitrogen input or output will alter the NH_3 concentration in the rumen (*3*). Thus, the NH_3 concentration alone is not a good indicator of the nitrogen status of the ruminal environment.

Methylglyoxal (MG) is a highly reactive and toxic compound that disrupts DNA, inhibits protein synthesis, and kills bacteria (*4*). In mammals, MG is synthesized by the host animal or microorganisms in the digestive tract. MG-producing bacteria use a variety of pathways such as glycolytic bypass, glycerol degradation, and amino acid catabolism to produce MG. Aerobes such as *Escherichia coli* can convert D-lactate to pyruvate via a flavoprotein-linked dehydrogenase, but this reaction is not active under anaerobic conditions. The microorganisms that predominate in the rumen are saccharolytic. Carbohydrates, such as cellulose and other polysaccharides, make up most of the ruminant diet and constitute the main substrate available for fermentation. *Prevotella ruminicola* B_14 accounts for as much as 19% of the cultivable bacteria from the rumen (*6*). When there is a loss of balance between nitrogen and carbon metabolism in the rumen bacterium, *P. ruminicola* B_14 produces MG (*6*). Therefore, it has been hypothesized that MG production by ruminal bacteria would indicate a loss of balance between carbon and nitrogen metabolism in the rumen.

Currently, there is not an analytical method available for the quantification of MG in ruminal fluid. High-performance liquid chromatography (HPLC)-based methods using fluorescence detection have been developed for tissue, blood, and urine (*8*, *9*). MG does not exhibit native fluorescence; therefore, derivatization procedures involving the conversion of MG with *o*-phenylenediamine (OPD) or its derivatives such as dichloro-1,2-phenylenediamine and 1,2-diamine-4,5-dimethoxybenzene were used. Using these protocols, the actual compound detected is the quinoxaline derivative.

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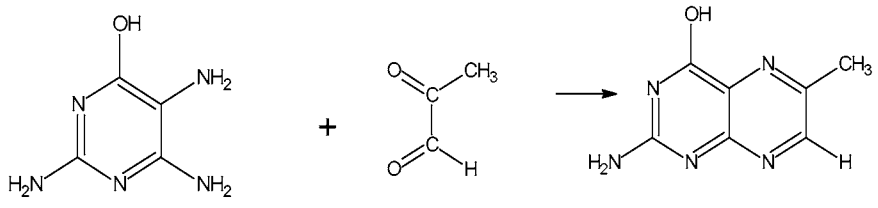

6-hydroxy-2,4,5-triaminopyrimidine methylglyoxal 6-methylpterin

Figure 1. Derivization of MG with TRI to form 6-meth.

Alternatively, a reversed phase HPLC and precolumn derivatization of MG with 6-hydroxy-2,4,5-triaminopyrimidine (TRI) to form a 6-methylpterin (6-meth) (**Figure 1**) protocol has been developed for human urine and mouse corneas (*9*). The resulting compound is fluorescent, and quantification with HPLC results in high recoveries and adequate sensitivity. Derivatization procedures using OPD or TRI yield similar detection limits. Espinosa-Mansilla (*9*) reported a detection limit of 11 pmol for MG from urine samples. Chaplen et al. (*8*) derivatized MG OPD and reported a detection limit of 7 pmol. The objective of this study was to develop an HPLC methodology for the detection of MG-derivatized TRI in ruminal fluid.

MATERIALS AND METHODS

Chemicals and Solutions. All chemicals were HPLC grade. MG, 6-meth, and TRI were purchased from Sigma (St. Louis, MO). Sodium acetate and glacial acetic acid were purchased from Fisher (Chicago, IL).

Sample Collection. The New Mexico State University Institutional Animal Care and Use Committee approved all procedures involving animals. The animals were previously cannulated. The ruminal fluid was collected via ruminal cannulae from four English-crossbred cows (BW = 544 kg) and composited. The ruminal fluid was strained through four layers of cheesecloth into an insulated thermal container, returned to the laboratory, and centrifuged (10000*g*, 10 min, 10 °C) to remove bacterial cells. The supernant was removed and centrifuged (10000*g*, 10 min, 10 °C).

Sample Preparation. The resulting clarified ruminal fluid (CRF) was decanted, and six aliquots of 2 mL each were placed in 2.5 mL screw cap centrifuge tubes (Fisher). Half of the samples were acidified with 200 μL of 1 M HCl, spiked with known quantities of MG (5.0, 1.0, and 0.25 μg/mL final concentration), and frozen at (−20 °C) until analyzed. The pH of the acidified samples was adjusted to a pH of 4.0 with 1 M NaOH prior to freezing. The remaining three aliquots of CRF were not acidified but were spiked with the same concentration of MG as the acidified samples and frozen at (−20 °C) until analyzed. An additional sample was acidified but not spiked to serve as a CRF blank to test for background MG levels. The CRF used for method validation had no detectable MG (data not shown).

Quantities of MG ranging from 25 to 0.125 μg were used to evaluate sensitivity and linearity of the HPLC assay. Each level of MG equivalent detected was prepared as three separate samples injected in duplicate. Cation exchange (CE) columns (SCX Extract-Clean, 500 mg; Alltech, Deerfield, IL) were used for sample preparation with a vacuum manifold (Alltech). The CE column was rinsed with 5 mL of methanol followed by 5 mL of methanol:0.1 M HCl (1:1, w/v) and finally with 0.1 M HCl. Care was used between each of the column preparation steps to avoid drying the column bed. Samples (900 μL; pH 4.0) were applied to the column. The solid phase extraction cartridge was then rinsed with 700 μL of double distilled water (ddH_2O) followed by 900 μL of 1 M ammonium hydroxide (Sigma). The eluants from the application of the sample, ddH_2O, and ammonium hydroxide were collected individually to determine which fraction resulted in the greatest recovery.

MG Derivatization. Purified fractions were derivatized with differing concentrations of TRI in 2 mL (12 mm × 32 mm) silanized HPLC sampling vials (Alltech). Samples (200 μL) were derivatized with 800 μL of TRI, and 900 μL of 0.02 M sodium acetate (pH 4.05) was added to bring the reaction volume to 1.9 mL. Three concentrations of TRI and three samples sizes were evaluated as follows: The concentrations of TRI tested were 7.02, 10.53, and 14.04 mM and were equivalent to ratios of 4:1, 6:1, and 8:1 MG to TRI, respectively. Sample sizes of 100, 200, and 500 were evaluated with the three levels of TRI. Derivatization was completed by incubation in a 60 °C water bath for 45 min. After the incubation period, the samples were passed through a 0.45 μm syringe filter (Waters Corp., Milford, MA) into 2 mL (12 mm × 32 mm) silanized HPLC sampling vials (Alltech). The recovery was calculated as the ratio of the slope on a line describing the change in 6-meth as increasing amounts of MG were added to the samples and the slope of a theoretical line generated by assuming 100% recovery of the added MG.

Detection of Pterdinic Derivative. The pterdinic derivative of MG, 6 methylpterin, was detected using an HPLC system equipped with a fluorescence detector (Varian 9075, Varian Inc., Walnut Creek, CA), 96 vial autosampler fitted with a size appropriate sampling loop (Alltech 570), and an in-line solvent degasser (Alltech). A reversed phase Novapak C_{18} analytical column (150 mm × 3.9 mm; Waters Corp.) was maintained at 30 °C with a column heater (Waters Corp.). Data capture and analysis were performed by the Star Chromatography Workstation software package (Varian Inc.). The mobile phase consisted of 0.02 M sodium acetate (pH 4.05) filtered through a 0.2 μm nylon filter (Fisher). The analysis conditions were as follows: The fluorescence was measured at 352 nm excitation and 447 nm emission, the mobile phase flow was controlled by a isocratic pump at a (Rainin model SD200, Mettler-Toledo, Inc., Columbus, OH) rate of 1.5 mL/min; typical injection size, 50 μL; and column temperature, 30 °C. Duplicate injections of each sample were made. Quantification was based on peak area. The average retention time of 6-meth was 4.0 min.

The detection limit was calculated as the smallest concentration of derviatized MG that gave a measurable response based on two times the signal-to-noise ratio. The limit of quantitation was defined as the smallest concentration of MG that gave a response that could be accurately quantified (RSD > 3.0%). Accuracy was tested by spiking known concentrations of MG and 6-meth into CRF. The concentrations were 25.0, 20.0, 15.0, 10.0, 5.0, 1.0, 0.5, 0.25, and 0.125 μg/mL final concentration. This procedure was replicated three times using separately prepared samples. Each sample was injected in duplicate and compared to the known amount added for each spike. These data were used to calculate a calibration curve. The precision was tested in two ways. First, the reliability of the injection was evaluated by preparing a standard solution of derivatized MG (1.0 μg) in a matrix of ruminal fluid and injected 10 times. The standard deviation (SD) for the run was ≤5%. Second, injecting five standard solutions of derivatized MG three times assessed the intraassay variation. The SD of ≤2% was achieved.

Statistical Analysis. The stability of MG in acidified ruminal fluid was analyzed as a completely random design using the GLM procedure of SAS 8.2 (SAS Inst. Inc., Cary, NC). The model included terms for acidification, MG equivalent, and recovery. When significant, F statistics were noted and means were separated using the least significant difference procedure. The sample size and TRI concentration means for MG and recovery were analyzed with preplanned contrasts of sample size vs TRI concentration. The F statistics of $P < 0.05$ were considered significant.

Table 1. Effect of Acidification with 1 M HCl (pH 4.0) vs No Acidification on the Stability of MG in Ruminal Fluid Spiked with 1 μg/mL and Stored Frozen (–20 °C)[a] for 2 Months

sample[b]	MG equivalent detected (μg/mL)	recovery[c] (%)
	unacidified	
1	0.70	70
2	0.78	78
3	0.77	77
average		75
	acidified	
1	0.99	99
2	0.96	96
3	0.91	91
average		95

[a] HPLC detection conditions: elution buffer, sodium acetate (20 mM, pH 4.05); flow rate, 1.5 mL/min; sample injection size, 50 μL; fluorescence excitation/emission, 352/447 nm. [b] Samples were injected in triplicate and averaged. [c] $P < 0.001$ unacidified vs acidified.

Table 2. Effect of Sample Size and TRI Concentration on MG Recovery in Ruminal Fluid as Quantified with HPLC Fluorescence Detection

sample size[a] (μL)	TRI (mM)	MG added (μg/mL)	MG equivalent detected (μg/mL)	recovery (%)
100	7.02	0	ND[b]	
		0.25	0.19	76.0
		0.5	0.43	86.0
		1.0	0.83	83.0
	10.53	0	ND	
		0.25	0.13	52.0
		0.5	0.42	84.0
		1.0	0.82	82.0
	14.04	0	ND	
		0.25	0.13	52.0
		0.5	0.41	82.0
		1.0	0.75	75.0
200	7.02	0	ND	
		0.25	0.24	96.0
		0.5	0.48	96.0
		1.0	0.99	99.0
	10.53	0	ND	
		0.25	0.22	88.0
		0.5	0.47	94.0
		1.0	0.94	94.0
	14.04	0	ND	
		0.25	0.22	88.0
		0.5	0.45	90.0
		1.0	0.95	95.0
500	7.02	0	ND	
		0.25	0.17	68.0
		0.5	0.30	60.0
		1.0	0.65	65.0
	10.53	0	ND	
		0.24	0.10	42.0
		0.5	0.39	78.0
		1.0	0.63	63.0
	14.04	0	ND	
		0.25	0.15	60.0
		0.5	0.35	70.0
		1.0	0.66	66.0

[a] Mean value for four independent samples. Comparison of sample size (μL) 200 vs 100 and 500 ($P = 0.02$). Comparison of TRI (mM) vs sample size ($P = 0.87$). [b] ND = not detectable.

RESULTS AND DISCUSSION

An essential aspect of obtaining accurate measurements of MG in ruminal fluid is the development of collection and preservation techniques. The stability of MG in ruminal fluid was unknown and required investigation. The recovery of MG from acidified samples was higher (95%) as compared to nonacidified samples (75%; **Table 1**). On the basis of these findings, all ruminal samples were acidified with 1 M HCl for the remaining experiments. MG is a highly reactive electrophile at physiological pH, and under acidic conditions (pH $\leq$ 4), MG is stable (*10*).

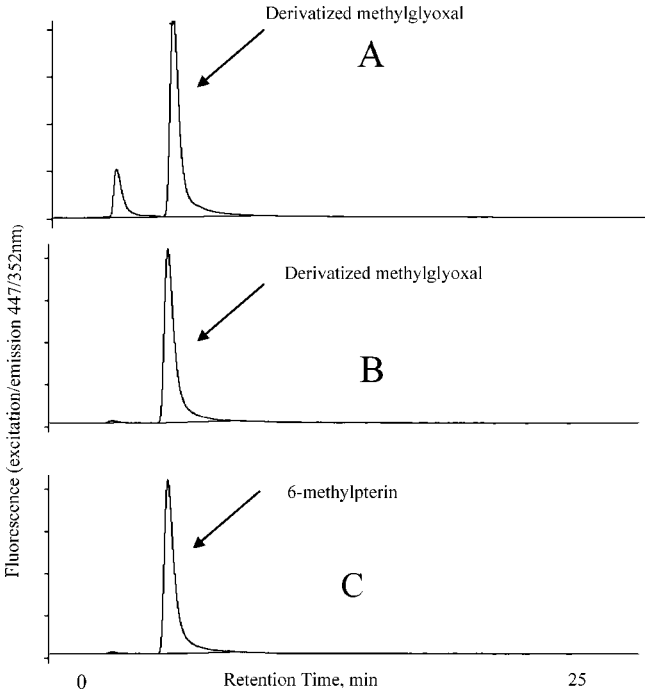

Figure 2. Comparison of derivatized samples of ruminal fluid with (**A**) no prederivatization purification step or (**B**) purified with a prederivatization CE step; (**C**) chromatograph of 6-meth.

Derivatization conditions were evaluated based on rumen fluid aliquot size and the amount of TRI added. Three sample sizes and three concentrations of TRI were evaluated. **Table 2** shows a comparison between sample size and TRI concentration. Sample sizes of 200 and 800 μL of TRI (7.02×10^{-3} M) placed in a 2.0 mL silanized HPLC sampling vial with the remaining volume composed of 900 μL of sodium acetate (0.02 M, pH 4.05) were optimal as these variables gave the highest and most reliable MG recovery values. Additionally, the 200 μL sample size and TRI concentration of 7.02×10^{-3} M are in agreement with methods reported in the literature (*9*, *11*). Smaller sample sizes resulted in too much background interference from unknown components in the ruminal fluid. Larger sample sizes resulted in poor recovery of MG probably due to incomplete derivatization of MG in the sample (**Table 2**). The concentration of TRI did not seem to affect recoveries as dramatically as sample size.

A prederivatization CE step was added to reduce interferences. **Figure 2a** shows a chromatograph of a derivatized CRF sample prepared without the CE step; **Figure 2b** shows a derivatized CRF sample prepared with the CE step. The CE step reduced or eliminated the earlier eluting peak. The peak that eluted prior to MG was not stable in its retention and tended to float around our peak of interest causing interference and inaccurate integration. Additionally, CE reduced but did not eliminate tailing by the MG peak resulting in more accurate integration and lower standard errors. Prior to CE, standard errors were >10.0% for samples spiked with 1.0 μg/mL MG injected 10 times. The CE reduced the standard error to <2.0% in samples spiked with 1.0 μg/mL MG.

Table 3. Accuracy Evaluation of the HPLC Method for Detection of MG in Ruminal Fluid

MG added (μg/mL)	MG equivalent detected (μg/mL)[a]	CV[b] (%)
25.0	23.13	3.0
20.0	19.87	2.4
15.0	13.02	1.8
10.0	9.81	4.7
5.0	4.87	2.1
1.0	0.88	2.9
0.5	0.42	0.9
0.25	0.22	4.7
0.125	0.11	2.1
0	ND	

[a] Mean of three independent samples injected in duplicate. [b] CV = coefficient of variation.

Derivatized MG and 6-meth (injected directly) were compared, and results indicated that derivatized MG was converted to 6-meth by TRI (**Figure 2c**). The overall recovery of derivatized MG was 87% and agreed well with the value of 85% reported by Shamsi et al. (*11*) but was lower than the 98% reported by Espinosa-Mansilla et al. (*9*). Part of this difference may be due to losses in the CE step. When MG was not subjected to the CE step, the recovery of derivatized MG was 56% (SD = 25%, $n = 15$).

The detection of MG by derivatization to 6-meth has proven to be accurate and precise. **Table 3** shows the accuracy of the method. The reliability of the injection was evaluated by preparing a standard solution spiked with 1.0 μg/mL of MG. The sample was derivatized and quantified by multiple injections (10) on the same sample (RSD = 4.7%). The resulting curve was linear ($R^2 = 0.99$). The calculated detection limit for this assay was 0.125 μg/mL.

This is the first documented protocol for the quantification of MG in ruminal fluid. The derivatization process is simple, and a short time (45 min) is necessary to complete the derivatization process. A recovery of 87% was accomplished, and there was an acceptable chromatographic resolution. During the initial validation experiments used, a run time of 25 min ensured that there was no carry over of interfering peaks to subsequent runs. The run time was shortened after validation to 10 min. The detection limit of 0.125 μg/mL should be sensitive enough to detect MG levels in ruminal fluid based on the in vitro data collected by Russell (*6*). However, there are no reported data on the concentration of MG in ruminal contents. *P. ruminicola* B_14 produced approximately 2.5 μg/mL of MG in response to nitrogen limitation and excess carbohydrate in vitro (*6*). *Prevotella* species are one of the most numerous groups of ruminal bacteria and are found in rumens of cattle (or sheep) fed a variety of diets (*7*). Therefore, it is plausible to hypothesize that MG concentrations in ruminal contents could be correlated to the nutrient environment of the ruminal bacteria.

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