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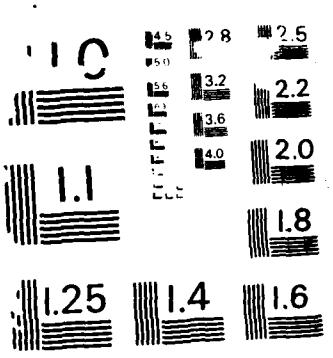

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Quantitation of nitroguanidine in rodent feed was accomplished by extraction with water and analysis by high pressure liquid chromatography (HPLC). Using methylnitroguanidine as an internal standard the assay was used to quantitate nitroguanidine at concentrations ranging from 1 to 50 mg/g feed.

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QUANTITATION OF NITROGUANIDINE IN RODENT CHOW

INTRODUCTION

The assay described in this report was developed to verify the concentrations of nitroguanidine (NGu) in feed mixtures used in GLP animal studies. The target concentrations of NGu in the animal diets ranged from approximately 1 to 22 mg NGu/g feed. These diets were prepared using a mixture of feed (premix) with a much higher concentration of NGu (approximately 50 mg NGu/g feed). The assay employs HPLC and is suitable for determining NGu over this entire range of concentrations.

Using methylnitroguanidine (MNGu) as an internal standard, weighed amounts of the animal diets were diluted, filtered and analyzed by HPLC. The peak area ratios were then used to quantitate the concentration of NGu in the final dilution. Sample weights and dilution factors permit the calculation of the NGu concentration in the original feed mixture.

MATERIALS

Chemicals

NGu was obtained from the Sunflower Army Ammunition Plant, Desoto, Kansas (lot #SOW85F011-028). MNGu was synthesized as described under METHODS using 1-methyl-3-nitro-1-nitrosoguanidine, 97% (MNNG, lot #02308AT, Aldrich Chemical Co., Milwaukee, Wisconsin) and methylamine (40% wt % in water, lot #0719AL, Aldrich Chemical Co.). HPLC grade water was prepared from distilled water with the removal of all organic impurities by ultraviolet (UV) irradiation using a Barnstead ORGANICpure® water purifier (Sybron/Barnstead, Boston, MA). Methanol (OmniSolv® grade) was obtained from EM Science, Cherry Hill, NJ.

Feed

Certified rodent chow #5002 (lot #JULY21871CMEAL) was obtained from Ralston-Purina, St. Louis, MO.

Equipment

The melting point of methylnitroguanidine was uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian XL-300 NMR (Varian Instruments, Palo Alto, CA) using tetramethylsilane as a reference. Infrared spectra (IR) were obtained using a Perkin-Elmer 983G IR spectrophotometer (Perkin-Elmer, Norwalk, NJ), and ultraviolet (UV) spectra were obtained with a Hitachi 110-A spectrophotometer (Hitachi Instruments, Inc., Mountain View, CA).

Chromatographic analysis was performed with an HPLC system comprised of a Kratos Spectroflow 400 HPLC pump (Kratos Analytical Instruments, Ramsey, NJ), a Waters model 712 WISP automated sample injector (Waters, Inc., Milford, MA), a Brownlee RP-18 spheri-5 column (4.6 x 250 mm, Brownlee Labs, Inc., Santa Clara, CA), a Kratos Spectroflow 783 absorbance detector and a Shimadzu C-R3A integrator (Shimadzu, Kyoto, Japan).

METHODS

Synthesis of Methylnitroguanidine

MNGu was prepared according to the method of McKay [1]. MNNG (10 g, 68 mmoles) was added to a 125-ml flask containing water (8 ml), ethanol (20 ml), and a magnetic stirring bar. An aqueous solution of methylamine (40 wt %, 7.32 ml, 85 mmoles) was slowly added over a period of 10 min. The reaction mixture was then allowed to stand for 20 min before the white crystalline precipitate was collected by suction filtration. After recrystallization from ethanol a yield of 3.55 g was obtained [2]. The filtrate was saved for further recovery of MNGu. MP 160-162° C [2] (lit.[1] 159-161° C); IR (KBr): 3414, 3251 (broad), 1641, 1600, 1409, 1368, 1293, 1168, 1140, 1071, 781, 680, 589 cm^{-1} [3]. NMR (DMSO): ∂ 3.36 (s, 3 H, CH_3), 7.80, 8.30 (broad s, NH) [4]. Two peaks were observed by UV spectrometry (peak maxima, molar extinction coefficient): 215 nm, 5400 and 268 nm, 14,210 [5]

Chromatographic analysis

HPLC analysis was performed under the following conditions: solvent, 10% methanol/90% water; injection volume, 10 µl; flow rate, 0.7 ml/min; wavelength monitored, 265 nm. Under these conditions the retention time was 5.1 min for NGu and 6.2 min for methylnitroguanidine.

Preparation of standards

Stock solutions of NGu (1 mg/ml water) and MNGu (1 mg/ml water) were prepared in separate volumetric flasks and stored at 4° C. Standards were prepared on the day of analysis by transferring varying amounts of the stock solutions to 25 ml volumetric flasks and diluting to volume (see TABLE 1).

TABLE 1. Preparation of standard solutions (concentrations are in mg/ml, total dilution volume is 25 ml).

	Final Concentration		Ml Stock Solution	
Standard #	NGu	MNGu	NGu	MNGu
1	0.01	0.04	0.25	1.0
2	0.02	0.04	0.50	1.0
3	0.04	0.04	1.00	1.0
4	0.06	0.04	1.50	1.0
5	0.08	0.04	2.00	1.0
6	0.10	0.04	2.50	1.0
7	0.12	0.04	3.00	1.0

Extraction of standards from feed

Volumes of standards added to samples of feed and final dilution volumes were chosen to duplicate the concentrations employed in GLP studies. Typically the concentrations of NGu range from 1 to 22 g/kg feed for the final diets and approximately 50 g/kg feed for the premix. The feed mixtures that were prepared for assay verification are shown in TABLE 2.

TABLE 2. Preparation of feed mixtures for assay validation.

Grams of Rat Chow	Amount of NGu per g Feed	Amount of Stock Solution Added		Final Dilution
		NGu	MNGu	
1.00	1.0 mg	1.0 ml	1.0 ml	25 ml
1.00	5.0 mg	5.0 ml	4.0 ml	100 ml
1.00	10.0 mg	10.0 ml	10.0 ml	250 ml
1.00	15.0 mg	15.0 ml	10.0 ml	250 ml
1.00	20.0 mg	20.0 ml	20.0 ml	500 ml
1.00	25.0 mg	25.0 ml	20.0 ml	500 ml
0.25	50.0 mg	12.5 ml	10.0 ml	250 ml

Extracts were prepared by adding the respective amounts of feed and stock solutions to volumetric flasks. A stir bar was added and the contents stirred for at least 30 min. An aliquot was removed from each flask and centrifuged to sediment the feed. The supernatant was filtered through a 0.2-mm filter and analyzed.

The feed mixtures were prepared on 5 different days and analyzed to determine interday variability. On the first day of analysis multiple injections of the 0.01 and 0.12 mg NGu/ml standard solutions were performed to determine the precision of the HPLC analysis. On the last day the 1 and 25 mg NGu/g feed samples were each prepared in quintuplicate and analyzed to determine intraday variability.

CALCULATIONS

The standard curve was prepared by plotting NGu concentration against either peak height or area ratios and determining the equation of the line by linear least squares regression analysis. This equation was used to calculate the concentration of NGu in the feed samples. Multiplication by the dilution volume provided the concentration of NGu in the feed.

RESULTS

Repeated injections showed a high degree of reproducibility for both peak area and height ratios (TABLE 3). In this report the results were calculated using peak area ratios. The results for the analysis of the feed samples are presented in TABLE 4.

TABLE 3. Reproducibility of peak height and area ratios during repetitive analysis.

Date of Analysis	Conc of NGu in Standard	Injection #	Peak Area Ratio	Peak Height Ratio
23 Oct 87 [6]	0.01 mg/kg feed	1	0.326	0.396
		2	0.344	0.419
		3	0.334	0.411
		4	0.326	0.400
		5	0.325	0.398
23 Oct 87 [6]	0.12 mg/kg feed	1	3.465	4.264
		2	3.472	4.285
		3	3.467	4.255
		4	3.465	4.260
		5	3.468	4.282

TABLE 4. Concentration of NGu in feed as determined by analysis using peak area ratios.

Date of Preparation	Target Concentration of NGu in Feed (g NGu/kg feed)	Concentration of NGu Determined by Analysis (g NGu/kg feed)	% Target
22 Oct 87	1.0	1.03	103.0
[6]	5.0	5.10	102.0
	10.0	10.18	101.8
	15.0	15.36	102.4
	20.0	20.36	101.8
	25.0	25.54	102.2
	50.0	50.70	101.4
26 Oct 87	1.0	0.99	99.1
[7]	5.0	4.98	99.3
	10.0	9.99	99.8
	15.00	15.12	100.8
	20.0	20.05	100.3
	25.0	25.19	100.8
	50.0	50.66	101.3
10 Nov 87	1.0	1.02	102.5
[8]	5.0	5.06	101.2
	10.0	10.07	100.7
	15.0	14.92	99.5
	20.0	19.95	99.7
	25.0	25.49	102.0
	40.0*	40.19	100.5
11 Nov 87	1.0	0.98	98.3
[9]	5.0	5.05	101.0
	10.0	10.24	102.4
	15.0	15.33	102.2
	20.0	20.20	101.0
	25.0	25.43	101.7
	50.0	50.84	101.7
12 Nov 87	1.0	1.01	101.0
[10]	1.0	1.01	100.6
	1.0	1.00	100.0
	1.0	1.00	100.1
	1.0	1.01	100.7
	5.0	5.05	100.7
	10.0	10.12	101.2
	15.0	15.07	100.4
	20.0	20.06	100.4
	25.0	25.12	100.3
	25.0	25.30	101.2
	25.0	25.35	101.5
	25.0	25.12	100.4
	25.0	25.17	100.7
	50.0	50.50	101.0

*10 mg of NGu was added to 0.25 g feed

Equations for the standard curves and correlation coefficients were as follows:

22 Oct 87 $Y = 28.86x + 0.052$, $r^2 > 0.999$

26 Oct 87 $Y = 29.48x + 0.056$, $r^2 > 0.999$

10 Nov 87 $Y = 30.1xx + 0.03$, $r^2 > 0.999$

11 Nov 87 $Y = 29.84x + 0.05$, $r^2 > 0.999$

12 Nov 87 $Y = 29.58x + 0.04$, $r^2 > 0.999$

DISCUSSION

MNGu is a suitable internal standard because it is a close analog of NGu with a different retention time (see FIGURES 1 and 2). Extraction of feed to which nothing had been added showed no components with the same retention times as NGu and MNGu [11].

Repeated injections of the standard solutions show high reproducibility in terms of peak area and height ratios. The maximum deviation from the average peak area ratio was 3.9% and 0.13%, respectively, for 0.01 and 0.12-mg NGu/ml standard solutions.

The standard curve is linear from 0.01 to 0.12 mg NGu/ml. The equations for the standard curve obtained at the beginning or end of an analysis or on different days were almost identical.

The maximum deviation of analytically determined values from target concentrations was three percent with most deviations falling significantly below this. Assay variability is presented in TABLE 5. The interday variability (CV) ranged from 0.28 to 2.07%. Intraday variability was less than 0.6% for concentrations of 1 and 25 mg NGu/g feed.

TABLE 5. Assay variability.

Concentration (mg NGu/g feed)			
Target	Observed (average)	SD	CV (%)
Interday (n=5)			
1.0	1.01	0.02	2.07
5.0	5.05	0.04	0.85
10.0	10.12	0.10	0.96
15.0	15.16	0.18	1.22
20.0	20.12	0.16	0.79
25.0	25.37	0.16	0.64
50.0*	50.68	0.14	0.28
Intraday (n=5)			
1.0	1.01	0.02	0.54
25.0	25.20	0.12	0.46

*n=4

NOTES

1. MNNG is highly mutagenic and must be handled very carefully.

2. Adjustable pipettes (Gilson Pipetman®, Rainin Instrument Co., Woburn, MA) were used in this study. They were calibrated periodically to check both accuracy and pipetting technique. This should be done for any type or style of pipette used.

3. Standards should be analyzed both before and after the feed extracts. This is especially important when a large number of samples are analyzed, because the last feed extract may be analyzed hours after the standards.

REFERENCES

[1] McKay AF, inventor; Honorary Advisory Council for Scientific and Industrial Research, Ottawa, Canada, assignee. 1-Substituted-3-nitro-guanidines. Can. patent 519,488. 13 Dec 1955. In: Chemical Abstracts 1956;50:12107.

[2] Wheeler CR. Nitrocellulose-nitroguanidine projects. Laboratory Notebook # 85-01-006, p 65. Letterman Army Institute of Research, Presidio of San Francisco, CA.

[3] *Ibid.* p. 67.

[4] *Ibid.* pp. 68-69.

[5] *Ibid.* p. 70.

[6] Wheeler CR. Nitrocellulose-nitroguanidine projects. Laboratory Notebook # 85-12-022, pp 42-43. Letterman Army Institute of Research, Presidio of San Francisco, CA

[7] *Ibid.* pp 46-48.

[8] *Ibid.* pp. 49-50.

[9] *Ibid.* pp. 51-52.

[10] *Ibid.* pp. 53-55.

[11] *Ibid.* pp. 56-57.

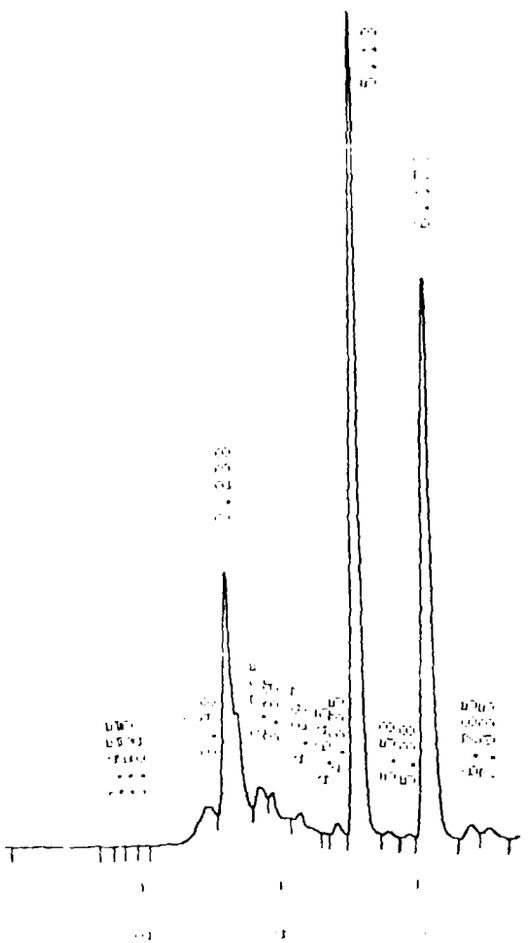

FIGURE 1. Chromatogram of extracted rat feed. The concentration of NGu was 1 mg/g feed and the dilution volume was 25 ml

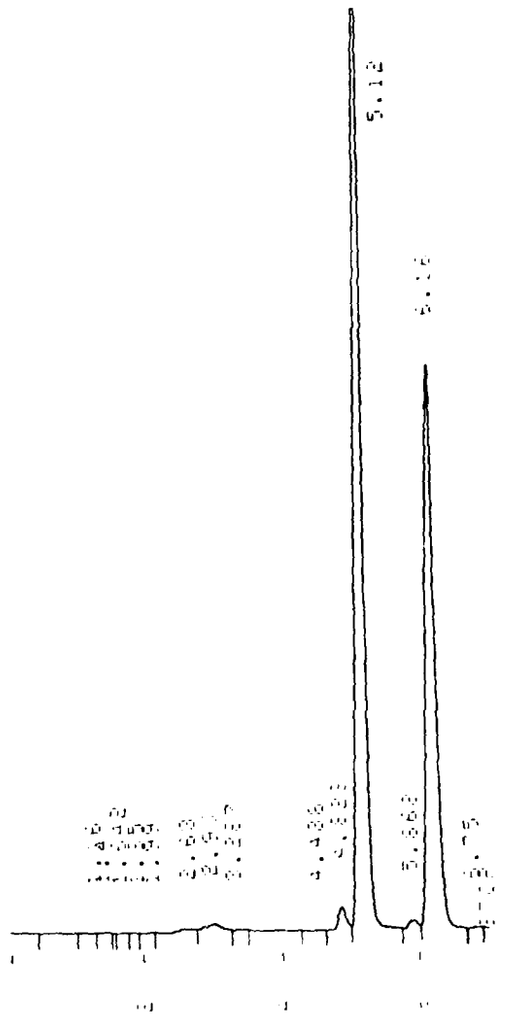

FIGURE 2. Chromatogram of extracted rat feed. The concentration of NGu was 25 mg/g feed. The dilution volume was 500 ml.

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