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HMX: THE DETERMINATION OF FREE NITRITE IN MOUSE STOMACH 1/1

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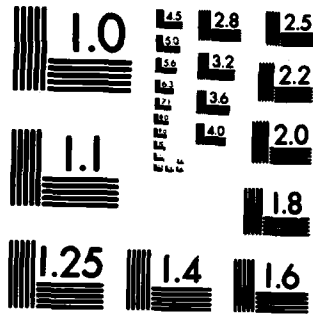
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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
	AD-A171 599	
4. TITLE (and Subtitle) HMX: The Determination of Free Nitrite in Mouse Stomach Contents	5. TYPE OF REPORT & PERIOD COVERED FINAL April 1981-April 1982	
	6. PERFORMING ORG. REPORT NUMBER 416877/2342	
7. AUTHOR(s) B. Craig, J.N. Done	8. CONTRACT OR GRANT NUMBER(s)	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Inveresk Research International Limited Musselburgh, EH21 7UB, Scotland	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62720A.3E162720A835.00.104	
11. CONTROLLING OFFICE NAME AND ADDRESS Jesse J. Barkley, Jr., US Army Medical Research and Development Command, Fort Detrick, Maryland, U.S.A.	12. REPORT DATE 17 September 1985	
	13. NUMBER OF PAGES 23	
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report) NON-CLASSIFIED	
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
16. DISTRIBUTION STATEMENT (of this Report) <div style="border: 1px solid black; padding: 5px; text-align: center;">DISTRIBUTION STATEMENT A Approved for public release Distribution Unlimited</div>		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES Principal Investigator: A.B. Wilson		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) HMX Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazoline, Explosives, Mouse, Nitrite, Stomach Contents, Toxicology		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A sensitive method for the analysis of nitrite in the stomach contents of mice was developed. The stomach contents of mice from a 90 day toxicity study on HMX were analysed at termination of the study. Only 0.1 to 1.1 ppm was found. The content was not related to the HMX content of the diet and was consistent with what might have been expected from the laboratory animal diet.		

AD-A171 599

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IRI Report No. 2342

HMX: The Determination of Free Nitrite in Mouse Stomach Contents

Final Report by:

B. Craig
J.N. Done

September, 1985

Supported by:

U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland, 21701

Contract No. DAMD-17-80-C-0053
IRI Project 416877

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The findings in this report are not to be construed as an official Department of the Army position unless to designated by other authorised documents.

FOREWORD

"I, the undersigned, hereby declare that this work was performed under my supervision, according to the procedures herein described and that this report represents a true and accurate record of the results obtained."

A. B. Wilson

A.B. Wilson, B.V.Sc., M.R.C.V.S.,
D.A.B.T.
Principal Investigator



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Project No. 416877

Report No. 2342

QUALITY ASSURANCE AUTHENTICATION

The execution of this type of short-term study is not individually inspected. The processes involved are inspected at intervals according to a pre-determined schedule.

This report has been audited by IRI Quality Assurance Personnel according to the appropriate Standard Operating Procedure and is considered to describe the methods and procedures used in the study. The reported results accurately reflect the original data of the study.

IRI Project No. 416877

Report No. 2342

Signed: Andrew Waddell
(Quality Assurance Manager)

Date: 14th January 1986

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SUMMARY

A sensitive method for the analysis of nitrite in the stomach contents of mice fed on diet mixed with HMX has been developed, based on that used for the estimation of nitrite in water and waste waters. The nitrite is diazotised to produce a coloured azo dye, the concentration of which is proportional to the amount of nitrite originally present.

The samples were analysed by extraction with nitrite-free distilled water, reaction with suphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride and measurement, at 543 nm, of the absorbance of the coloured dye produced. The analyses were quantified using standard solutions of nitrite.

The results of the analysis showed that small amounts of nitrite were present in the stomach contents. The mean values found for each group were between 0.1 and 1.1 ppm, but there was no relationship between the quantities found and the concentration of HMX fed to the animals.

Laboratory diet used at Inveresk Research International limited usually contains between 1 and 3 ppm nitrite and hence the levels found during this work are consistent with being derived from the diet and not the HMX.

LOCATION OF EXPERIMENTAL PROGRAMME

The analytical experiments described in this report were performed at the Inveresk Gate Laboratories of Inveresk Research International Limited, Musselburgh, Scotland during February and March 1982.

The toxicity study (IRI Project No. 416877/Report No. 2195) and the necropsies were undertaken at the Elphinstone Research Centre. The necropsies were carried out between 21 and 23 April 1981.

INTRODUCTION

Inveresk Research International Limited have recently undertaken a range of toxicity tests on Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) for the US Army (DAMD 17-80-C-0053). As part of this programme it was decided to analyse selected mouse stomach contents for the nitrite ion after the termination of the 90 day mouse toxicity study. This report describes the procedures used and the results obtained.

The method of analysis was based on that used for the estimate of nitrite in water and waste water⁽¹⁾ and involved the photometric determination of the reddish purple azo dye produced by the coupling of N-(1-naphthyl)ethylenediamine dihydrochloride with diazotised sulphanilic acid. At the pH of the reaction, the sulphanilic acid is diazotised by the nitrite and the amount of dye produced is proportional to the original amount of nitrite present.

All data generated and recorded during this study will be stored in the Scientific Archives of Inveresk Research International Limited for 5 years after issue of the final report.

MATERIALS AND METHODS

Materials

The following reagents were used:

Sulphanilamide (Sigma) 1% w/v solution in 1.2N hydrochloric acid

N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma) 0.1 w/v in water

Sodium nitrite 97% (Aldrich)

Sodium oxalate 99+% A.C.S. reagent (Aldrich)

Potassium permanganate AnalaR (BDH)

Hydrochloric acid AnalaR (Koch-Light)

Sulphuric acid AnalaR (BDH)

All solutions and dilutions were made using glass distilled water which was shown to be free of nitrite.

Method

The working standard solution of sodium nitrite (1 ml = 1.643 µg nitrite) was prepared by serial dilution of a stock solution made by dissolving sodium nitrite (0.616 g) in distilled water (500 ml). This stock solution was standardised by reacting 25 ml with excess standardised 0.05N potassium permanganate solution and titrating the excess with standardised 0.05N sodium oxalate.

Instrumentation

After development of the coloured solution the intensity of the absorption at 543 nm was measured using a Beckmann Model 25 spectrophotometer. Matched 1 cm quartz cells were used and the absorption was recorded on a chart recorder with the sensitivity setting at 0.1 absorbance units full scale (0.1 AUFS).

Samples

Stomach contents were removed from the randomly selected animals used for pharmacokinetic blood sampling at the end of the 90 day mouse toxicity study (IRI Project No. 416877/Report No. 2195). The animals had been fed ad libitum on powdered laboratory rodent diet (Appendix 1) up until the time of sacrifice. Six animals from each group had been designated for this purpose but no group 6♀ samples were available since all animals in this group were premature decedents. Samples were stored frozen at -20°C until they were analysed.

Analytical Method

The method was based on that described in "Standard Methods for the Examination of Water and Waste Water". The levels found were low and some difficulty was experienced in measuring the very low absorbances. This was overcome by appropriate use of reference samples for each sample analysed.

The contents of each stomach were weighed into a 30 ml centrifuge tube and 5 ml of distilled water added. The tubes were then shaken for 15 min and centrifuged at 2500 rpm for 15 min. The supernatant was then removed and filtered through a 0.45 μm millipore filter and the extraction repeated with a further 3 ml of distilled water. The filtrates were combined with a 1 ml washing of the filter and placed in a 10 ml graduated flask which was made up to the mark with distilled water.

The analysis was carried out by adding 100 μl of the 1% sulphanilamide solution to 5 ml of the sample extract and shaking for 2 min. Immediately afterwards 100 μl of the N-(1-naphthyl)-ethylenediamine dihydrochloride solution was added and the visible absorption at 543 nm measured after 10 min. There are strict requirements for the timing of these operations such that the second reagent must be added no later than 10 min after the addition of the first and the absorbance must be measured within 2 h of the colour development. The analysis was completed by comparison with standards prepared by appropriate dilution of the standard nitrite solution. A calibration line covering nitrite concentrations from 0 to 65.7 $\mu\text{g.l}^{-1}$ was prepared and a further diluted standard was used as a quality control solution to check the analytical procedure. The standards and quality control samples were prepared using 50 ml solutions of nitrite solution and 1 ml of each reagent.

Accurate measurement of the absorbances of the diazotised samples was facilitated by using the untreated portions as the reference, after addition of 200 μl of distilled water to compensate for the volume of reagents added to the samples.

It should be noted that in the above procedure no checks are made on the efficiency of recovery of nitrite from the stomach contents. Data quoted for nitrite content are therefore based on the assumption of complete recovery.

RESULTS AND DISCUSSION

Table 1 shows the data for the calibration lines generated on each analytical occasion. The values found for the quality control samples are also given. The results show that a satisfactory analytical procedure was being used. The mean values for the groups are collected in Table 2. The individual values are given in Appendix 2. Some of the samples were not taken because of difficulty in removal during the necropsy and those samples which weighed less than 25 mg are not included in the tables because the small sample size would lead to a large error in the final result. The values found for the nitrite concentrations are all very small and are comparable with the values often found in the laboratory diets used at Inveresk Research International Limited. There does not appear to be any real differences between the treated and untreated groups although the Group 2♀ values are higher than the others.

REFERENCE

- (1) **Standard Methods for the Examination of Water and Waste Water, Fourteenth Edition, American Public Health Association, Washington, U.S.A., 1976.**

TABLE 1

Calibration and Quality Control Data for the Analysis of Nitrite in Mouse Contents*

Analytical Sample Numbers	Coefficient of Correlation	Value Determined for Quality Control Sample (Theoretical Value 32.5 $\mu\text{g.l}^{-1}$)
1-10	0.9999	30.0
11-19	0.9993	30.0
20-33	0.9994	36.6
34-43	0.9988	30.9
44-54	0.9997	30.2

* = One calibration curve was recorded with each batch of stomach contents analysed. Unknown concentrations were obtained by interpolation

TABLE 2

HMX: The Determination of Free Nitrite in
 Mouse Stomach Contents
 Mean Values for the Concentration Found

Group No.	Males		Females	
	Dose (mg.kg ⁻¹ .day ⁻¹)	Mean Concentration ppm	Dose (mg.kg ⁻¹ .day ⁻¹)	Mean Concentration ppm
1	0	0.2	0	0.3
2	5	0.2	10	1.1
3	12	N.D.	30	0.3
4	30	<0.1	90	<0.1
5	75	0.1	250	<0.1
6	200	0.2	750	-

N.D. = Not detected

APPENDIX 1HMX: The Determination of Free Nitrite in
Mouse Stomach Contents

Diet Analysis

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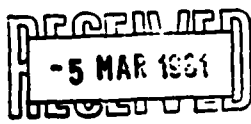
PRODUCT: RAT & HOUSE NO.1 (MODIFIED) EXPANDED FINE GROUND

BATCH NO: 1081

PREMIX BATCH NO: P121/128

DATE OF MANUFACTURE: 27TH JANUARY 1981

Nutrient	Found Analysis		Contaminant	Found Analysis		Limit of Detection
Moisture	8.6	%	Fluorine	8.2	mg/kg	1.0 mg/kg
Crude Fat	3.6	%	Nitrate as NaNO ₃	7.0	mg/kg	1.0 mg/kg
Crude Protein	15.3	%	Nitrite as NaNO ₂	2.5	mg/kg	1.0 mg/kg
Crude Fibre	3.5	%	Lead	1.5	mg/kg	0.25 mg/kg
Ash	5.3	%	Arsenic	0.11	mg/kg	0.2 mg/kg
Calcium	0.74	%	Cadmium	0.21	mg/kg	0.05 mg/kg
Phosphorus	0.65	%	Mercury	<0.01	mg/kg	0.01 mg/kg
Titanium	0.16	%	Selenium	0.08	mg/kg	0.02 mg/kg
Chlorine	0.51	%				
Potassium	0.83	%				
Magnesium	0.18	%				
Iron	212	mg/kg	Total Aflatoxins	NONE DETECTED	mg/kg	1 mg/kg each of B1, B2, G1, G2
Copper	14	mg/kg				
Manganese	63	mg/kg	Total P.C.B.	NONE DETECTED	mg/kg	0.001 mg/kg
Zinc	46	mg/kg	Total D.D.T.	0.011	mg/kg	0.001 mg/kg
			Dieldrin	0.001	mg/kg	0.001 mg/kg
			Lindane	0.004	mg/kg	0.001 mg/kg
			Heptachlor	0.001	mg/kg	0.001 mg/kg
			Malathion	0.012	mg/kg	0.02 mg/kg
Vitamin A	4750	Iu/kg	Total Viable Organisms	3.25×10^3	per gram	1000/g
Vitamin E	85	mg/kg	Mesophilic Spores	15.0×10^2	per gram	100/g
Vitamin C		mg/kg	Salmonellae Species	NONE DETECTED	per gram	Absent in 20 gm
			Presumptive E. coli	NONE DETECTED	per gram	Absent in 10 gm
			E. coli Type 1	NONE DETECTED	per gram	Absent in 10 gm
			Fungal Units	NONE DETECTED	per gram	Absent in 10 gm
			Antibiotic Activity			10 gm

Signed *C. R. Popplestone*Dated *23rd Feb 1981*C. R. POPPLESTONE M.Sc., Ph.D., C.Chem., M.R.S.C.
Quality Control ManagerB.P. Nutrition (U.K.) Limited
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APPENDIX 2

HMX: Analytical Results for the Determination of Free Nitrite
In Mouse Stomach Contents

Group 1

Group	Analytical Sample Number	Animal Number	Weight of Stomach Contents (mg)	Absorbance	Nitrite Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Nitrite Concentration (ppm)	Average Value (ppm)
18	1	6			Insufficient Sample		
	2	12	160.0	0.024	4.7×10^{-3}	0.3	0.2
	3	14	63.7	0.018	0	0	
	4	18	73.7	0.022	3.0×10^{-3}	0.4	
	5	19			Insufficient Sample		
19	6	128	100.5	0.019	5.1×10^{-4}	0.05	
	7	130	79.1	0.018	0	0	
	8	135	56.5	0.023	3.9×10^{-3}	0.7	
	9	140	272.5	0.019	5.1×10^{-4}	0.02	
	10	122	79.1	0.024	4.7×10^{-3}	0.6	

APPENDIX 2 (continued)

Group 2

Group	Analytical Sample Number	Animal Number	Weight of Stomach Contents (mg)	Absorbance	Nitrite Concentration ($\mu\text{g}\cdot\text{m}^{-1}$)	Nitrite Concentration (ppm)	Average Value (ppm)
28	11	146			Insufficient Sample		
	12	149	115.7	0.042	2.2×10^{-2}	1.9	
	13	151	58.2	0.026	8.2×10^{-3}	1.4	1.1
	14	153			Insufficient Sample		
	15	159	42.7	0.016	0	0	
28	16	25	107.2	0.020	3.0×10^{-3}	0.3	
	17	26	32.6	0.017	3.9×10^{-4}	0.1	0.2
	18	37	72.2	0.017	3.9×10^{-4}	0.05	
	19	38	55.0	0.018	1.3×10^{-3}	0.2	

APPENDIX 2 (continued)

Group 3/4

Group	Analytical Sample Number	Animal Number	Weight of Stomach Contents (mg)	Absorbance	Nitrite Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Nitrite Concentration (ppm)	Average Value (ppm)
39	20	170	120.5	0.011	0	0	
	21	173			No Sample		
	22	175	136.4	0.017	1.03×10^{-3}	0.08	0.3 ϕ
	23	177	33.5	0.019	3.0×10^{-3}	0.9	
	24	180	53.9	0.020	4.0×10^{-3}	0.7	
38	25	41			Insufficient Sample		
	26	43			Insufficient Sample		
	27	51			Insufficient Sample		
	28	60	51.1	0.013	0	0	Not Detected
48	29	182	37.9	0.013	0	0	
	30	185			Insufficient Sample		
	31	192	99.0	0.012	0	0	
	32	195	302.4	0.017	1.03×10^{-3}	0.03	<0.1
	33	199	85.5	0.014	0	0	

 ϕ = Includes analytical sample 34 result

APPENDIX 2 (continued)

Group 4/5

Group	Analytical Sample Number	Animal Number	Weight of Stomach Contents (mg)	Absorbance	Nitrite Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Nitrite Concentration (ppm)	Average Value (ppm)
3♀	34	164	33.3	0.016	0	0	0
	35	205	132.0	0.017	2.4×10^{-4}	0.02	
5♀	36	212	67.6	0.014	0	0	<0.1
	37	214	187.3	0.016	0	0	
	38	220	90.0	0.017	2.4×10^{-4}	0.03	
4♂	39	65	49.9	0.018	1.1×10^{-3}	0.22	
	40	61	70.0	0.017	2.4×10^{-4}	0.03	
	41	66	48.1	0.014	0	0	<0.1
	42	78			No Sample		
	43	80	40.6	0.016	0	0	

APPENDIX 2 (continued)

Group 5/6

Group	Analytical Sample Number	Animal Number	Weight of Stomach Contents (mg)	Absorbance	Nitrite Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	Nitrite Concentration (ppm)	Average Value (ppm)
58	44	83	126.8	0.015	0	0	
	45	86		Insufficient Sample			
	46	90	91.3	0.020	2.2×10^{-3}	0.2	0.1
	47	91	58.4	0.019	1.3×10^{-3}	0.2	
	48	95		Insufficient Sample			
	49	98		Insufficient Sample			
68	50	101		Insufficient Sample			
	51	103	54.1	0.015	0	0	
	52	105	258.5	0.017	0	0	0.2
	53	109		Insufficient Sample			
	54	112	76.6	0.023	4.6×10^{-3}	0.6	

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