Chemistry Support Services for the NTP

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NTP ChemTask No.: CHEM07521

CAS No.: 84-65-1

BIOLOGICAL SAMPLE ANALYSIS REPORT

ANALYSIS OF URINE SAMPLES FOR 1- AND 2-HYDROXYANTHRAQUINONE

8-064-BSA-82

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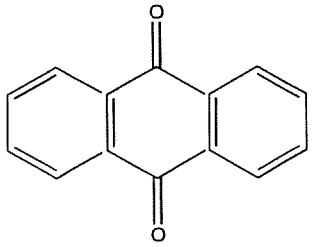
BIOLOGICAL SAMPLE ANALYSIS REPORT

ANALYSIS OF URINE SAMPLES FOR 1- AND 2-HYDROXYANTHRAQUINONE

CAS No.: 84-65-1	Lot Nos: 5893 (Zeneca Fine Chemical), 2Y011 (Kawasaki Kasei Chemicals LTD), 64005 (Environmental Biocontrol Intl.), GSTU 2517770 (Environmental Biocontrol Intl.)
Battelle Chemical ID Code: 064	Samples Received: Urine
Battelle Task No.: 8-064-BSA-82	Sample Receipt Date: 7/8/03
NTP ChemTask No.: CHEM07521	Submitter: [redacted]
Program Supported: CAR	Study Lab: Battelle-Columbus
Analysis Dates: 8/6-8/7/03	Sample Collection Date: 6/25/03
Interim Results Date: 9/4/03	Receipt Condition: Not applicable
	Shipping Container: Not applicable
	Storage Conditions (@ Battelle): ≤-70°C

STRUCTURE	Mol. Wt.	Mol. Formula
	208.2 g/mol	$C_{14}H_8O_2$

EXECUTIVE SUMMARY

Urine samples from F344 male rats dosed with anthraquinone in irradiated NTP-2000 feed for 7 days were analyzed for 1- and 2-hydroxyanthraquinone, metabolites of anthraquinone. The samples were analyzed by a high performance liquid chromatography (HPLC) method with ultraviolet detection following extraction from the urine. Measurable levels of both metabolites were found.

QUALITY ASSURANCE STATEMENT

BIOLOGICAL SAMPLE ANALYSIS REPORT

ANALYSIS OF URINE SAMPLES FOR 1- AND 2-HYDROXYANTHRAQUINONE

NTP ChemTask No.: CHEM07521
Battelle Study No.: G004110-BUT
Battelle Task No.: 8-064-BSA-82

Listed below are the phases and/or procedures performed by Battelle that were reviewed by the Quality Assurance Unit during performance of the task described in this report. Adverse findings, if any, were reported to the study director at the time of review.

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Audit study file	10/22/03	10/22/03
Audit analytical report	10/22/03	10/22/03

This report reflects the procedures and raw data generated in this study.

In addition to the study-specific audits/inspections cited above, routine inspections of the general facilities and equipment were performed by the QAU and reports were submitted to management as follows:

Facility/Equipment	Date Inspected	Date of Report to Management
Chemistry Technical Center inspection	4/23/00	4/24/00
	12/8-12/12/00	12/13/00
	10/17/01	10/19/01
	9/6/02	9/6/02

[redacted] 10-24-03
Date

Battelle

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1 INTRODUCTION

The purpose of this work was to analyze urine samples from F344 male rats dosed with anthraquinone for 1- and 2-hydroxyanthraquinone from a metabolism study supporting the chronic carcinogenicity study. This study was conducted at Battelle-Columbus. This task was performed at Battelle, 505 King Avenue, Columbus, OH 43201.

2 TEST ARTICLE

Four lots of anthraquinone (CAS No. 84-65-1) were used to dose the animals whose urine was analyzed. The suppliers were Zeneca Fine Chemicals (Wilmington, DE), Kawasaki Kasei Chemicals LTD (Kawasaki City, Kanagawa, Japan), and Environmental Biocontrol Intl./Arkion Life Sciences LLC (Wilmington, DE). Procurement of anthraquinone from Zeneca Fine Chemicals occurred on a separate contract (Toxicology Branch, Contract No. N01-ES-25337), whereas the procurement from Kawasaki Kasei Chemicals and Environmental Biocontrol Intl. occurred via a separate task on the Chemistry Support Services contract (NTP ChemTask No. CHEM07209; Battelle Study No. G004110-BPP). Environmental Biocontrol Intl. provided two different samples as described below. The data related to procurement are maintained with the records of the relevant contract and task. The information in Table 1 summarizes the test article information.

Table 1 – Test Article Information

Supplier	Manufacturing Process	Lot No.	ChemTask No.[a]	Study No.[a]
Zeneca Fine Chemical	Nitric Acid Oxidation	5893	--[b]	SC940122
Kawasaki Kasei Chemicals LTD	Diels-Alder	2Y011	CHEM07319	G004110-BRS
Environmental Biocontrol Intl.	Diels-Alder	64005	CHEM07321	G004110-BRU
Environmental Biocontrol Intl.	Friedel-Crafts	GSTU 2517770	CHEM07320	G004110-BRT

a. Bulk Chemical Limited Analysis Task.
b. Procured under an NTP Toxicology Branch contract.

3 STANDARDS

1-Hydroxyanthraquinone, Lot No. 254-2B, was received from ChemService on October 6, 2000. The chemical was homogenized by stirring with a spatula for approximately five minutes. The net weight of chemical remaining after sampling was determined to be 9.65 g. The chemical was stored at room temperature.

The standards and QCs for this study were prepared using this lot.

2-Hydroxyanthraquinone, Lot No. 33-217-H, was received from Narchem Corporation on September 4, 2001. The chemical was homogenized by stirring with a spatula for approximately five minutes. The net weight of chemical remaining after sampling was determined to be 21.0 g. The chemical was stored at room temperature.

The standards and QCs for this study were prepared using this lot.

The identity of these lots will be confirmed and the purity determined and reported by Battelle under NTP ChemTask No. CHEM05679, Battelle Study No. G004110-AUE.

4 SAMPLES

The urine samples from all male F344 rats shown in Table 2 were received on July 8, 2003. The volumes from the ten individual animals within a dose group were pooled prior to submission for analysis. The sample collection times are the actual collection times. CON was the control group. NO-Z is the Zeneca product produced by nitric acid oxidation. DA-K is the Kawasaki Kasei product produced by the Diels-Alder method. DA-E is the Environmental Biocontrol product produced by the Diels-Alder method. FC-E is the Environmental Biocontrol product produced by the Friedel-Crafts method.

Table 2 – Male F344 Rat Urine Samples (7500 ppm Dosage)

Animal IDs					Urine Start Time (6/24/03 am)	Urine End Time (6/25/03 am)	Dosage (ppm)	Treatment Group
1	2	3	4	5	8:40	8:28	0	CON
6	7	8	9	10				
11	12	13	14	15	8:50	8:39	7500	NO-Z
16	17	18	19	20				
21	22	23	24	25	9:01	8:46		DA-K
26	27	28	29	30				
31	32	33	34	35	9:08	8:54		DA-E
36	37	38	39	40				
41	42	43	44	45	9:15	9:01		FC-E
46	47	48	49	50				

5 SAMPLE ANALYSIS

The analysis method was developed and validated as part of the biological sample method development task, NTP ChemTask No. CHEM07027, Battelle Study No. G004110-BMX. The samples were analyzed in one run.

5.1 Preparation of Stock, Spiking, and Urine Standards

5.1.1 Stock Standards

The amounts of 1- and 2-hydroxyanthraquinone shown in Table 3 were weighed into individual 100-mL volumetric flasks. The chemicals were dissolved in acetone using sonication as needed. The flasks were diluted to volume with acetone, sealed and mixed well. These stock standards were stored at 2-8°C when not in use.

Table 3 – Preparation of Stock Standards

Standard ID	Target Conc (µg/mL)		Target Weight (mg)	
	1 HANQ	2 HANQ	1 HANQ	2 HANQ
A	150	2000	15 ± 0.3	200 ± 3
B	120	1500	12 ± 0.3	150 ± 3

5.1.2 Spiking Standards

Spiking standards were prepared by dilution of the stock standards with acetone as shown in Table 4. A single spiking standard was prepared at each concentration.

Table 4 – Preparation of Spiking Standards

Standard ID	Target Conc (µg/mL)		Source	Source Vol (mL)	Final Vol (mL)
	1 HANQ	2 HANQ			
S1	150	2000	A	NA	NA
S2	120	1500	B	NA	NA
S3	90	1200	A	3	5
S4	72	900	B	3	5
S5	45	600	A	3	10
S6	24	300	B	1	5

5.1.3 Urine Standards

Urine standards were prepared by pipetting 0.5 mL of each spiking standard into individual 5-mL volumetric flasks half-full with blank F344 rat urine. The flasks were diluted to volume with blank F344 rat urine, sealed and mixed well. A single urine standard was made from each spiking standard. This produced urine standards with target concentrations of 1-HANQ at 15, 12, 9, 7.2, 4.5, and 2.4 µg/mL and 2-HANQ at 200, 150, 120, 90, 60, and 30 µg/mL.

Duplicate aliquots of each standard were analyzed.

5.2 Preparation of QC Samples

5.2.1 Stock Standard

The amounts of 1- and 2-hydroxyanthraquinone shown in Table 5 were weighed into a 50-mL volumetric flask. The contents were dissolved in acetone using sonication as needed. The flasks were diluted to

volume with acetone, sealed and mixed well. This produced a solution with target concentrations of 1-HANQ at 250 µg/mL and 2-HANQ at 3000 µg/mL.

Table 5 – Preparation of QC Stock Standard

Standard ID	Target Conc (µg/mL)		Target Weight (mg)	
	1 HANQ	2 HANQ	1 HANQ	2 HANQ
C	250	3000	12.5 ± 0.2	150 ± 5

5.2.2 Spiking Standards

Spiking standards were prepared by dilution of the stock standard with acetone as shown in Table 6. The flasks were sealed and contents mixed well.

Table 6 – Preparation of Spiking Standards

Standard ID	Target Conc (µg/mL)		Source Vol (mL)	Final Vol (mL)
	1 HANQ	2 HANQ		
S7	100	1200	4	10
S8	50	600	2	10

5.2.3 Quality Control Samples

Five thousand (5000) µL of each spiking standard was pipetted into individual 50-mL volumetric flasks containing approximately 25 mL of blank F344 rat urine. The flasks were diluted to volume with blank F344 rat urine, sealed and mixed well. This produced a high QC with target concentrations of 1-HANQ at 10 µg/mL and 2-HANQ at 120 µg/mL and a low QC with target concentrations of 1-HANQ at 5 µg/mL and 2-HANQ at 60 µg/mL.

Two (2) mL of the QCs were aliquoted into extraction tubes. The tubes were sealed and stored at -20°C until used. Four QCs at each concentration were analyzed.

5.3 Preparation of Blanks

Five hundred (500) µL of acetone was pipetted into a 5-mL volumetric flask approximately half-full with blank F344 rat urine. The flasks were diluted to volume with blank F344 rat urine, sealed and mixed well. Two (2) mL of this preparation was pipeted into two extraction tubes to serve as a single blank and a single blank with internal standard. A single blank and blank with internal standard were analyzed.

5.4 Dilution of Samples

Any urine sample with an expected concentration greater than 15 µg/mL of 1-HANQ or 200 µg/mL of 2-HANQ was diluted with Milli-Q water (which has a resistivity =18 megohm/cm) prior to analysis. A minimum of 500 µL of the original urine sample was diluted in each case.

5.5 Preparation of Urine Standards, QCs, Blanks, and Samples for Analysis

Two (2) mL of each standard, QC, blank, or sample was pipetted into an extraction tube. Two hundred (200) µL of blank rat urine was added to each except the samples, to which 200 µL of acetone was added. All tubes were vortexed to mix. Two hundred (200) µL of internal standard (9-anthracenemethanol, in acetone: Milli-Q water (80:120 v/v), at a concentration of approximately 320 µg/mL) was added to each tube except the blank without internal standard, to which 200 µL of acetone was added. Two (2) mL of ethyl acetate was added to each tube and the tubes were rotated for approximately 5 minutes. The tubes were centrifuged at a setting of approximately 2000 rpm for approximately 5 minutes. The upper (organic) layer was transferred to an evaporation tube. The ethyl acetate extraction was carried out twice more and the extracts were combined. The solvent was evaporated to dryness using nitrogen and an approximately 50°C water bath. The residue was reconstituted in 10 mL of mobile phase B [75:25 (v:v) ACN:Milli-Q water] and vortexed to dissolve the residue. An aliquot was transferred to an autoinjector vial and sealed.

5.6 Analysis

Single injections were made from each vial using the chromatographic conditions provided in Table 7.

Table 7 – Chromatographic System

LC System	Waters Model 2695 (Milford, MA)
Column	Phenomenex, Intersil ODS-2, 150 mm x 3.00 mm (ID), 5µ (Torrance, CA)
Mobile Phase	Mobile Phase A- 25:75 (v:v) ACN:Milli-Q Water Mobile Phase B- 75:25 (v:v) ACN:Milli-Q Water
Mobile Phase Gradient	Time (minutes) / % A / % B 0 / 100 / 0 5 / 0 / 100 25 / 0 / 100 26 / 100 / 0 35 / 100 / 0
Flow rate	0.8 mL/minute
Injection Volume	20 µL
Detector Type	UV
Detector Wavelength	260 nm
Run Time	~35 minutes

5.7 Calculations

The integration of the 1-HANQ, 2-HANQ and internal standard peaks by the data system was evaluated to assure it was correct in all chromatograms and manually reintegrated, if necessary, to achieve consistent integration. Linear regression equations weighted 1/x were calculated relating the response ratios of 1-HANQ and 2-HANQ divided by the internal standard (y) to the concentration (x) of the urine standards. The concentration of each urine standard, QC, and sample was calculated using its individual response, the regression equation, and any dilution factor. The concentration values for the standards and QCs were used to calculate the individual and average concentrations and relative errors, standard deviation and percent relative standard deviation as appropriate for the standards and the QCs.

5.8 Acceptance Criteria

The following acceptance criteria were established prior to the analysis of samples based on the method validation results.

5.8.1 Linearity

The regression line will have a correlation coefficient of 0.99 or greater. Up to 25% of the standards may be excluded from a run, if necessary. The individual relative errors of the remaining standards must be between -30 and 30%. The average relative errors of the remaining standards must be between -20 and 20%.

5.8.2 Selectivity

Acceptable selectivity is defined as having no co-eluting peaks in the blank that have areas greater than 20% of the average peak area of the analyte or internal standard in the lowest standard.

5.8.3 Sensitivity

The LOQ will be the lowest standard that meets all acceptance criteria for a run.

5.8.4 Accuracy

The average percent relative errors for each QC level will be between -20 and 20% and the individual relative errors for at least 66% (and a minimum of 2 at each concentration) of the individual QCs will be between -20 and 20%.

5.8.5 Precision

The percent relative standard deviation for the QCs will be =20%.

5.8.6 Acceptance of Partial Runs

Should problems occur during analysis, the results of study samples that are bracketed by acceptable QCs may be considered acceptable. Also, a partial acceptable range of the curve may be used, if the resulting curve uses four or more standard concentrations.

5.8.7 Repeat Analysis of Study Samples

For samples that are repeated for technical reasons, only one replicate needs to be run. For samples that are repeated for other reasons, at least duplicate aliquots must be analyzed, if sufficient sample volume exists. When multiple samples are analyzed, the average of all acceptable values will be reported, unless a value can be statistically excluded.

5.9 Results

The samples were analyzed in one run.

5.9.1 Linearity

The standard curves for the analyses met acceptance criteria for the correlation coefficient. The average relative errors for the standards met acceptance criteria for the run.

The standard curves are shown in Figures 1 and 2.

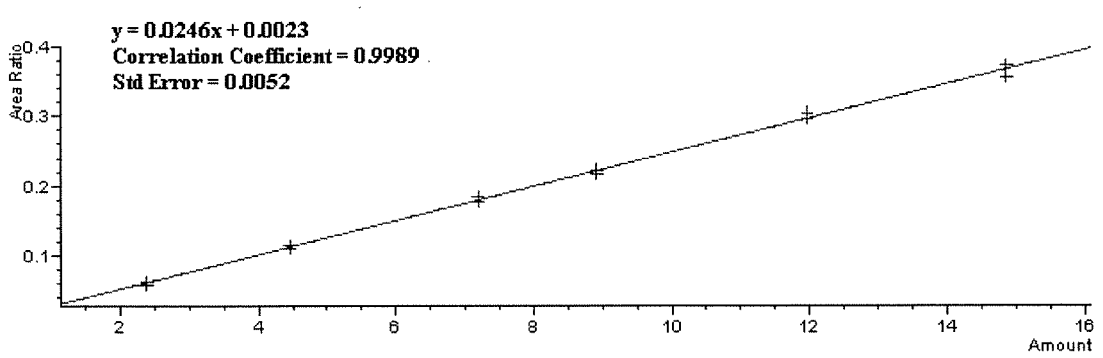

Figure 1 – Standard Curve, 1-HANQ

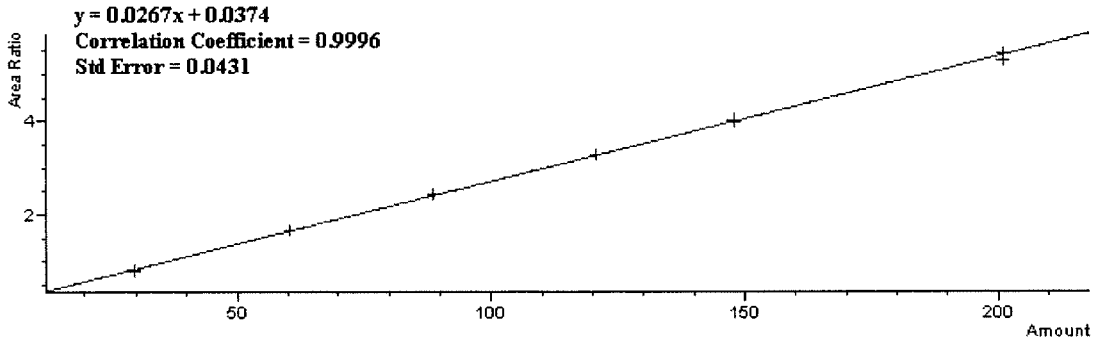

Figure 2 – Standard Curve, 2-HANQ

5.9.2 Selectivity

Acceptance criteria for the selectivity was met for the run.

5.9.3 Accuracy and Precision

Acceptance criteria for accuracy and precision were met for the run.

The results for the QCs are shown in Tables 8 and 9.

Table 8 – 1-HANQ Low and High QC Results

Replicate	Nominal Conc. (µg/mL)	Det'd Conc. (µg/mL)	RSD	%RE	Avg %RE
Low-1	4.940	4.428	1.5	-10.4	-11.1
Low-2		4.299		-13.0	
Low-3		4.451		-9.9	
Low-4		4.398		-11.0	
High-1	9.880	8.682	0.8	-12.1	-12.2
High-2		8.732		-11.6	
High-3		8.580		-13.2	
High-4		8.712		-11.8	

Table 9 – 2-HANQ Low and High QC Results

Replicate	Nominal Conc. (µg/mL)	Det'd Conc. (µg/mL)	RSD	%RE	Avg %RE
Low-1	58.48	54.37	1.2	-7.0	-7.7
Low-2		53.05		-9.3	
Low-3		54.35		-7.1	
Low-4		54.23		-7.3	
High-1	117.0	105.9	0.8	-9.5	-9.0
High-2		107.2		-8.3	
High-3		105.5		-9.8	
High-4		107.2		-8.3	

The results showing the average relative errors for the standards are shown in Tables 10 and 11.

Table 10 – 1-HANQ Standard Results

Average % Relative Error for Standards					
2.394 µg/mL	4.461 µg/mL	7.182 µg/mL	8.922 µg/mL	11.97 µg/mL	14.87 µg/mL
-0.8	0.2	1.1	-0.6	1.1	-1.0

Table 11 – 2-HANQ Standard Results

Average % Relative Error for Standards					
29.60 µg/mL	60.34 µg/mL	88.81 µg/mL	120.7 µg/mL	148.0 µg/mL	201.1 µg/mL
-1.7	0.9	1.1	0.1	0.6	-1.0

5.9.4 Chromatography

Representative overlaid chromatograms in full (upper) and reduced (lower) scale from a high standard, low standard, blank, and blank with IS are shown in Figure 3. Representative overlaid chromatograms from the QCs are shown in Figure 4. Overlaid chromatograms from a standard, one of the samples, and a control animal are shown in Figure 5. The sample chromatogram has a large unknown peak that is absent in the control urine, blanks, standards and QCs indicating that it is probably either anthraquinone or another anthraquinone metabolite. This unknown peak was seen in all the sample chromatograms.

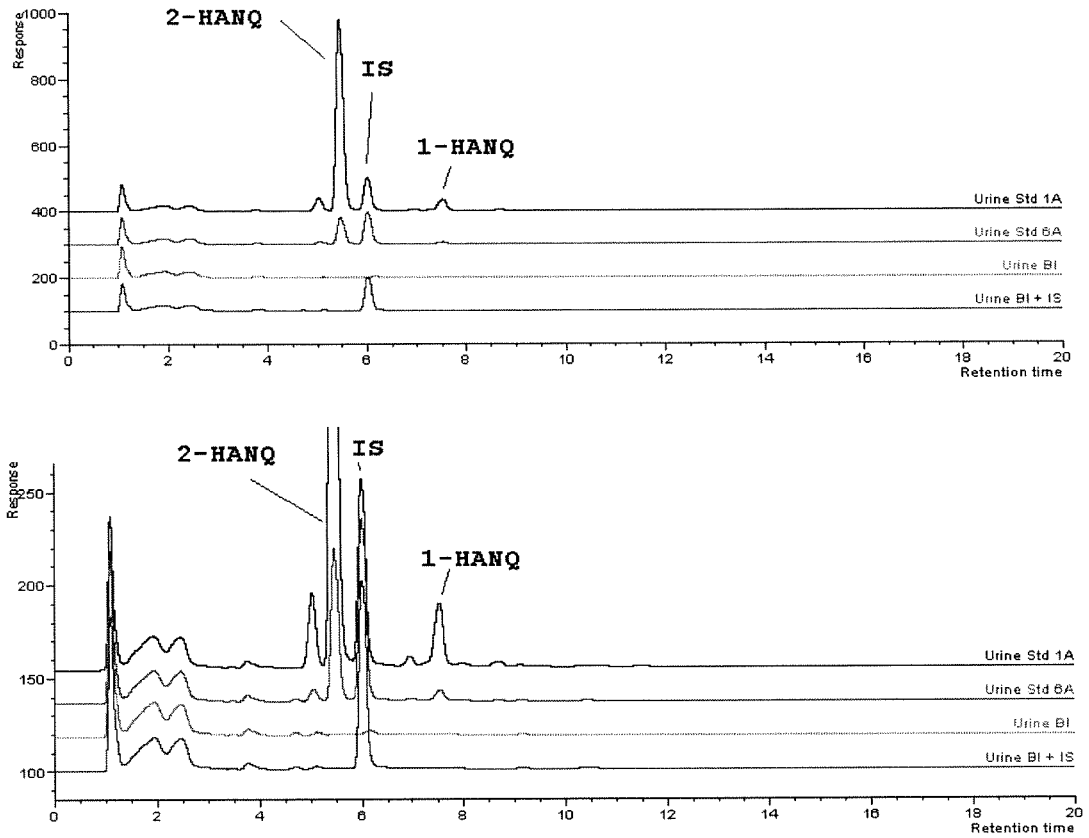

Figure 3 – Representative Overlaid Chromatograms for the High and Low Standard, Blank, and Blank with IS

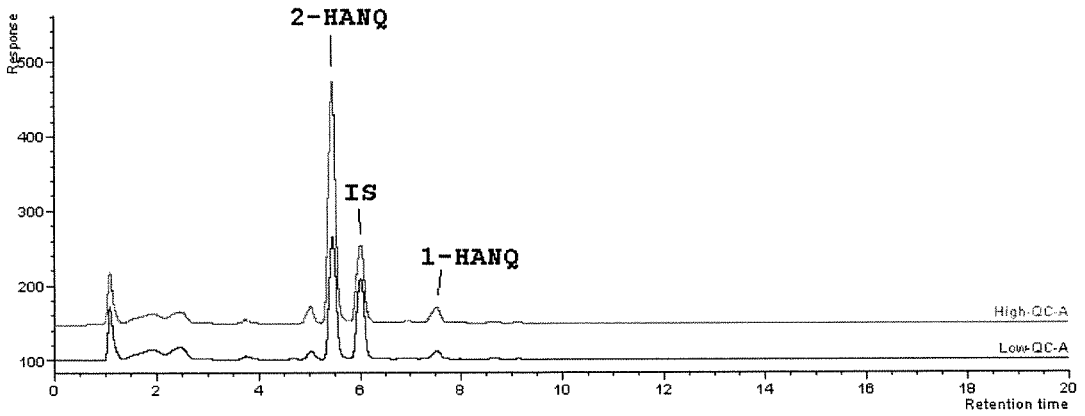

Figure 4 – Representative Overlaid Chromatograms for the High and Low QCs

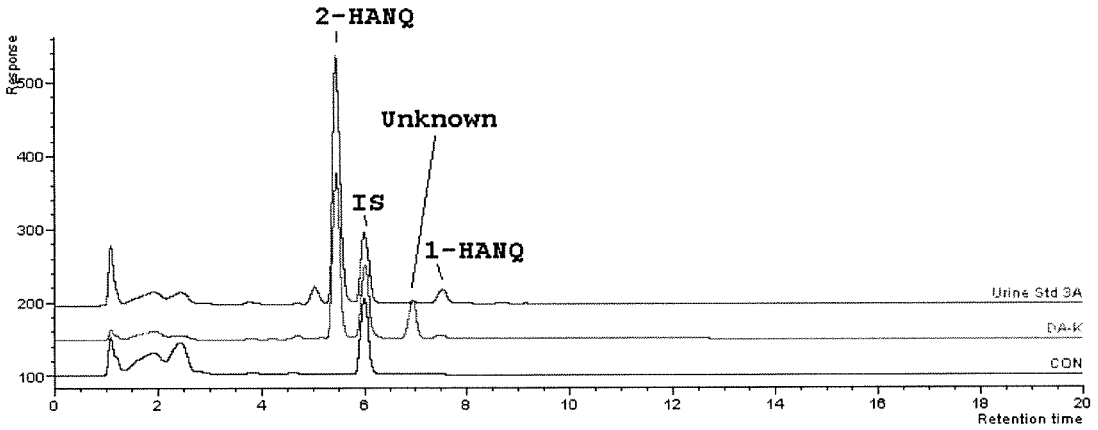

Figure 5 – Overlaid Chromatograms of a Standard, Sample, and Control Animal

5.9.5 Sample Results

The concentrations of 1-HANQ and 2-HANQ in the samples are shown in Tables 12 and 13.

Table 12 – 1-HANQ in Male F344 Rats (7500 ppm Dosage)

Sample ID	Det'd Conc (µg/mL)	Dilution Factor	Conc (µg/mL)
CON	Not Detected	None	Not Detected
NO-C	3.688	None	3.688
DA-K	5.933	None	5.933
DA-E	8.305	None	8.305
FC-E	5.789	None	5.789

Table 13 – 2-HANQ in Male F344 Rats (7500 ppm Dosage)

Sample ID	Det'd Conc (µg/mL)	Dilution Factor	Conc (µg/mL)
CON	Not Detected	None	Not Detected
NO-C	163.8	None	163.8
DA-K	77.77	4	311.1
DA-E	69.53	4	278.1
FC-E	73.40	4	293.6

5.10 Conclusions

The analytical method performed well. The accuracy of the standards and the precision and accuracy of the QCs were acceptable.

6 ACKNOWLEDGMENTS

The analysis was conducted by [redacted] The report was prepared by [redacted] and [redacted] The data and report were reviewed for completeness and accuracy by [redacted]