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BIOLOGICAL SAMPLE ANALYSIS REPORT

ANALYSIS OF URINE SAMPLES FOR 1- AND 2-HYDROXYANTHRAQUINONE

8-064-BSA-96

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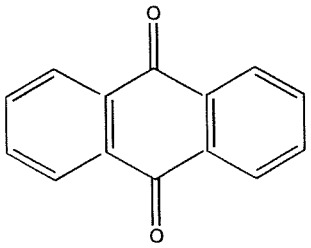
BIOLOGICAL SAMPLE ANALYSIS REPORT

ANALYSIS OF URINE SAMPLES FOR 1- AND 2-HYDROXYANTHRAQUINONE

CAS No.: 84-65-1	Lot No.s: 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-96	Sample Receipt Date: 10/3/03
NTP ChemTask No.: CHEM07624	Submitter: [redacted]
Program Supported: CAR	Study Lab: Battelle-Columbus
Analysis Dates: 9/24-10/7/03	Sample Collection Date: 10/3/03
Interim Results Date: 10/20/03	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

A method for the analysis of 1- and 2-hydroxyanthraquinone in urine was originally validated (Battelle Study No. G004110-BMX, NTP ChemTask No. CHEM07027) over the ranges of 2.5 to 15 µg/mL for 1-hydroxyanthraquinone (1-HANQ) and 30 to 200 µg/mL for 2-hydroxyanthraquinone (2-HANQ). Due to the use of lower dose concentrations than a previous metabolism study, a modification to the previously used urine analysis method was required. This consisted primarily of lowering the concentrations for the lowest calibrations standards. A limited revalidation was done for the modified method over the extended ranges of 0.12 to 15 µg/mL for 1-HANQ and 1.5 to 200 µg/mL for 2-HANQ. The limited validation consisted of a single analytical run containing: vehicle standards (duplicates at each of ten concentrations), solvent standards (a single replicate at each vehicle standard concentration), a vehicle blank with and without internal standard, six specificity blanks, quadruplicate quality control samples at each of three concentrations, and six replicates of quality control samples at the experimental limit

of quantitation (the concentration of the lowest vehicle standard). The validation met all acceptance criteria for accuracy and precision.

Urine samples from F344 male rats dosed with anthraquinone in irradiated NTP-2000 feed for 9 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.: CHEM07624
Battelle Study No.: G004110-BXH
Battelle Task No.: 8-064-BSA-96

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	10/28/03	10/28/03
Audit analytical report	10/28/03	10/28/03

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	10/17/01	10/19/01
	9/6/02	9/6/02
	10/13/03	10/13/03

[redacted]

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.

This task was a follow on to a previous metabolism study, which was identical in design, but utilized a higher dosed feed concentration (Battelle Study No. G004110-BUH, NTP ChemTask No. CHEM07479).

2 TEST ARTICLE

Four lots of anthraquinone (CAS No. 84-65-1) were used to prepare the formulations 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 (Battelle Study No. G004110-BPP, NTP ChemTask No. CHEM07209). 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 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 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 Battelle Study No. G004110-AUE, NTP ChemTask No. CHEM05679.

4 LIMITED VALIDATION OF EXTENDED CURVE

A method for the analysis of 1- and 2-hydroxyanthraquinone in urine was originally validated (Battelle Study G004110-BMX, NTP ChemTask No. CHEM07027) over the ranges of 2.5 to 15 µg/mL for 1-hydroxyanthraquinone (1-HANQ) and 30 to 200 µg/mL for 2-hydroxyanthraquinone (2-HANQ). Due to lower dose concentrations for this study compared to the original metabolism study, a modification to the original method was required. A limited revalidation of the modified method was performed over the extended standard ranges of 0.12 to 15 µg/mL for 1-HANQ and 1.5 to 200 µg/mL for 2-HANQ. The limited validation consisted of a single analytical run containing: vehicle standards (duplicates at each of ten concentrations), solvent standards (a single replicate at each vehicle standard concentration), a vehicle blank with and without internal standard, six specificity blanks, quadruplicate quality control samples at each of three concentrations, and six replicates of quality control samples at the experimental limit of quantitation (the concentration of the lowest vehicle standard).

4.1 Preparation of Stock, Spiking, Urine, and Solvent Standards

4.1.1 Stock Standards

The amounts of 1- and 2-hydroxyanthraquinone shown in Table 2 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 the contents mixed well. These stock standards were stored at 2-8°C when not in use.

Table 2 – Preparation of Stock Standards (Limited Validation)

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

4.1.2 Spiking Standards

Spiking standards were prepared by dilution of the stock standards with acetone as shown in Table 3. A single spiking standard was prepared at each concentration.

Table 3 – Preparation of Spiking Standards (Limited Validation)

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
S7	12	160	A	2	25
S8	7.2	90	B	3	50
S9	3.0	40	A	0.5	25
S10	1.2	15	B	0.5	50

4.1.3 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, 2.4, 1.2, 0.72, 0.3, and 0.12 µg/mL and 2-HANQ at 200, 150, 120, 90, 60, 30, 16, 9, 4, and 1.5 µg/mL.

Duplicate aliquots of each standard was analyzed.

4.1.4 Solvent Standards

Solvent standards were prepared by pipetting 1 mL of each spiking standard into individual 10-mL volumetric flasks and diluting to volume with mobile phase B [75:25 (v:v) ACN:Milli-Q water]. A 2-mL aliquot of each diluted spiking standard was then pipetted into individual 10-mL volumetric flasks and diluted to volume with dilute internal standard (approximately 8 µg/mL 9-anthracenemethanol in mobile phase B). A single solvent standard was made from each spiking standard. This produced solvent standards with the same target concentrations as the vehicle standards after extraction assuming 100% recovery. A single aliquot of each standard was analyzed.

4.2 Preparation of QC Samples

4.2.1 Stock Standard

The amounts of 1- and 2-hydroxyanthraquinone shown in Table 4 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 the contents mixed well. This produced a solution with target concentrations of 1-HANQ at 250 µg/mL and 2-HANQ at 3000 µg/mL.

Table 4 – 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

4.2.2 Spiking Standards

Spiking standards were prepared by dilution of stock C with acetone as shown in Table 5. The flasks were sealed and contents mixed well.

Table 5 – Preparation of QC Spiking Standards

Standard ID	Target Conc (µg/mL)		Source Vol (mL)	Final Vol (mL)
	1 HANQ	2 HANQ		
QC1	100	1200	4	10
QC2	25	300	1	10
QC3	5	60	1	50

4.2.3 Low, Mid, and High 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 QCs with target concentrations of 1-HANQ at 10, 2.5, and 0.5 µg/mL and 2-HANQ at 120, 30, and 6 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.

4.2.4 ELOQ Quality Control Samples

An aliquot (2.5 mL) of spiking standard S10 was pipetted into a 25-mL volumetric flask half-full of blank F344 rat urine. The flask was diluted to volume with blank F344 rat urine, sealed, and mixed well. This produced an ELOQ QC with target concentrations of 1-HANQ at 0.12 µg/mL and 2-HANQ at 1.5 µg/mL. Six 2-mL aliquots were pipetted into individual extraction tubes and analyzed.

4.3 Preparation of Blanks

Five hundred (500) µL of acetone was pipetted into a 5-mL volumetric flask half-full with blank F344 rat urine. The flasks were diluted to volume with blank F344 rat urine, sealed and the mixed well. Two (2) mL of this preparation was pipetted 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.

4.4 Preparation of Specificity Samples

An aliquot (2 mL) of each specificity sample was pipetted into individual extraction tubes.

4.5 Preparation of Urine Standards, QCs, Blanks, and Specificity Samples for Analysis

Two (2) mL of each standard, QC, and blank was pipetted into individual extraction tubes. Two hundred (200) µL of blank F344 rat urine was added to each except the specificity samples, to which 200 µL of acetone was added. All tubes were vortexed to mix the contents. 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 in the same manner and the extracts were combined. The solvent was evaporated to dryness using nitrogen at approximately 50°C. The residue was reconstituted in 10 mL of mobile phase B and vortexed to dissolve the residue. An aliquot was transferred to an autoinjector vial and sealed.

4.6 Analysis

Single injections were made from each vial using the chromatographic conditions provided in Table 6. Representative overlaid chromatograms for a high and low urine standard, blank with IS, and blank are shown in full (top) and reduced (bottom) scale in Figure 1. The chromatograms for the QCs were virtually identical to those of the standards.

Table 6 – Chromatographic System (Limited Validation)

LC System	Waters Model 2690 (Milford, MA)
Column	Metachem ODS-2, 150 mm x 3.00 mm (ID), 5μ (Lake Forest, 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	10 μL
Detector Type	UV
Detector Wavelength	260 nm
Run Time	~35 minutes

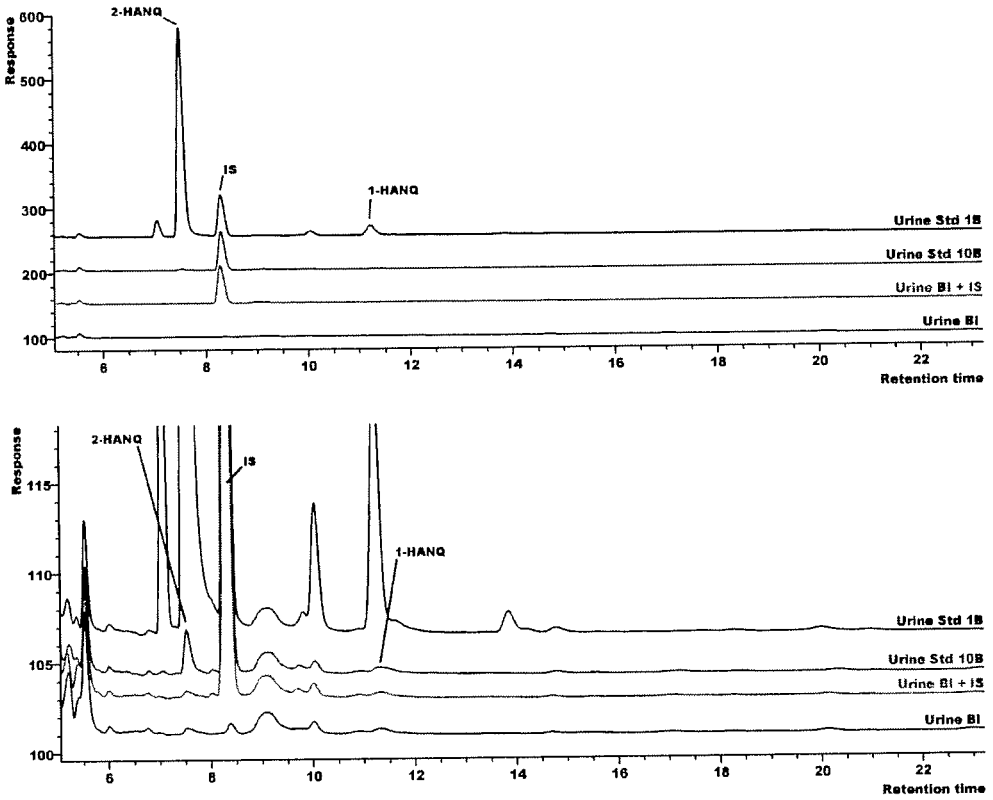

Figure 1 – Representative Overlaid Chromatograms of a High and Low F344 Rat Urine Standard, Blank with IS, and Blank (Full Scale - Top and Reduced Scale - Bottom)

4.7 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 standard and QC 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.

4.8 Results

4.8.1 Linearity

The critical regression parameters are shown in Tables 7 (1-HANQ) and 8 (2-HANQ). The regression curves are shown in Figure 2.

Table 7 – Regression Curve Parameters (1-HANQ)

Curve	Slope	y Intercept	Std Error	Correlation Coefficient
Vehicle	0.0216	0.0066	0.0040	0.9994
Solvent	0.0236	0.0067	0.0008	1.0000

Table 8 – Regression Curve Parameters (2-HANQ)

Vehicle	0.0243	0.0070	0.0363	0.9997
Solvent	0.0244	0.0052	0.0083	1.0000

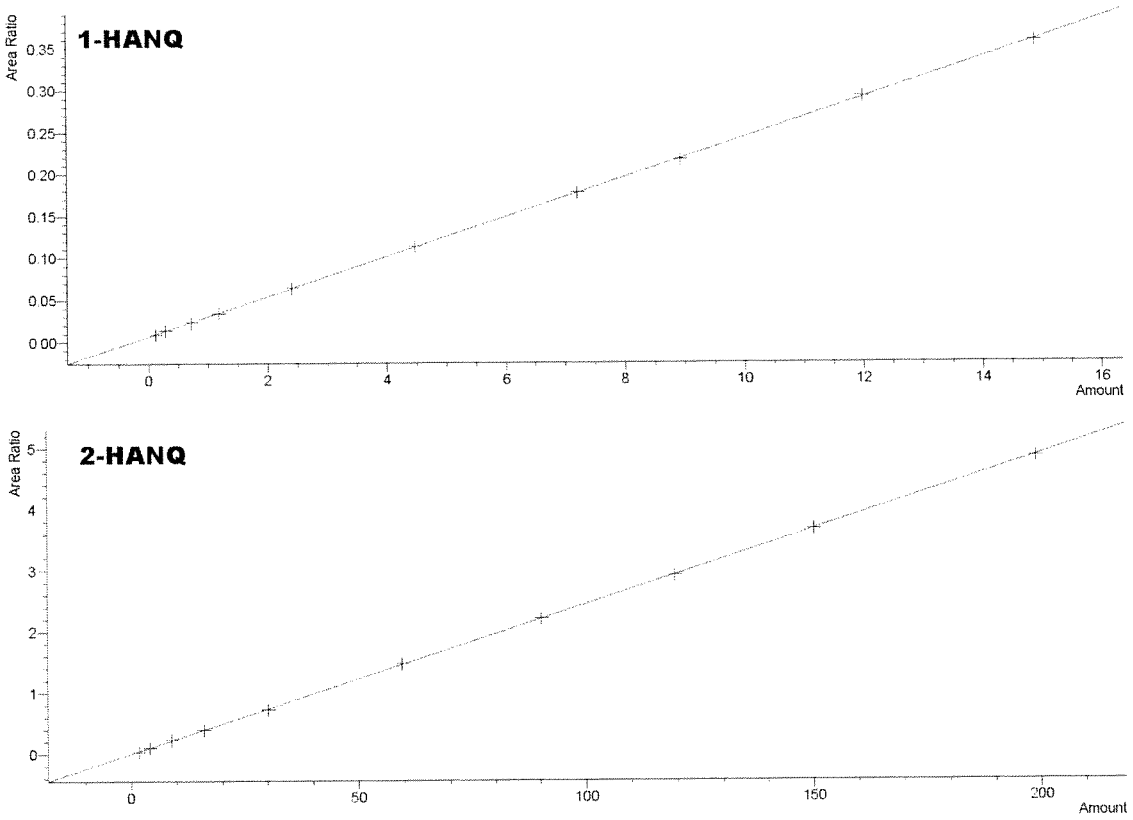

Figure 2 – Regression Curves - F344 Rat Urine Standards

4.8.2 Specificity

The multiple specificity samples had peaks that interfered with 1-HANQ, 2-HANQ, and the IS. The interferences averaged 85% the response of 1-HANQ, 21% the response of 2-HANQ, and 1.2% the response of IS in the lowest concentration vehicle standard. Despite this interference, the precision and accuracy of the method was acceptable at all concentrations. Overlaid chromatograms of the specificity samples and representative low urine and solvent standards are presented in Figure 3.

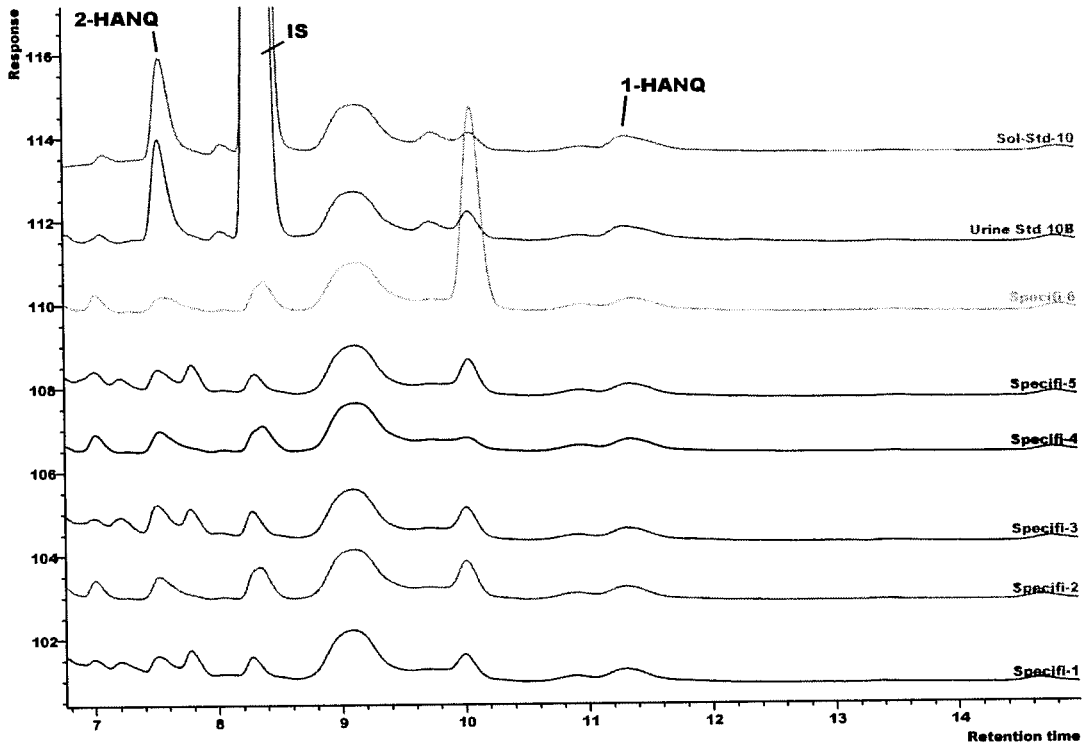

Figure 3 – Representative Overlaid Low Solvent and Urine Standards, and F344 Rat Urine Specificity Sample Chromatograms

4.8.3 Accuracy

The results for the F344 rat urine standards are shown in Tables 9 and 10.

Table 9 – F344 Rat Urine Standards (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
14.88	14.76	14.87	14.81	-0.8	-0.1	-0.4
12.00	12.38	12.39	12.39	3.2	3.2	3.2
8.928	8.588	8.674	8.631	-3.8	-2.9	-3.3
7.200	7.314	7.462	7.388	1.6	3.6	2.6
4.464	4.399	4.475	4.437	-1.5	0.2	-0.6
2.400	2.281	2.354	2.317	-5.0	-1.9	-3.4
1.190	1.081	1.111	1.096	-9.2	-6.7	-8.0
0.7200	0.6845	0.6915	0.6880	-4.9	-4.0	-4.4
0.2976	0.3009	0.3236	0.3122	1.1	8.7	4.9
0.1200	0.1277	0.1350	0.1314	6.4	12.5	9.5

Table 10 – F344 Rat Urine Standards (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
199.0	202.1	201.0	201.6	1.6	1.0	1.3
150.3	150.0	149.7	149.9	-0.2	-0.4	-0.3
119.4	116.3	115.9	116.1	-2.6	-2.9	-2.7
90.17	91.82	91.25	91.53	1.8	1.2	1.5
59.69	59.75	59.75	59.75	0.1	0.1	0.1
30.06	29.86	30.44	30.15	-0.6	1.3	0.3
15.92	15.58	15.76	15.67	-2.1	-1.0	-1.5
9.017	8.803	8.966	8.884	-2.4	-0.6	-1.5
3.979	3.925	4.070	3.997	-1.4	2.3	0.5
1.503	1.482	1.597	1.539	-1.4	6.3	2.4

4.8.4 Precision and Accuracy

The results from the F344 rat urine QCs are shown in Tables 11 and 12.

Table 11 – F344 Rat Urine QC Results (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
0.4948	0.4820	0.4825	0.0069	1.4	-2.6	-2.5
	0.4816				-2.7	
	0.4916				-0.7	
	0.4747				-4.1	
2.474	2.417	2.392	0.043	1.8	-2.3	-3.3
	2.396				-3.1	
	2.424				-2.0	
	2.330				-5.8	
9.896	10.26	10.19	0.10	0.9	3.7	3.0
	10.13				2.3	
	10.29				4.0	
	10.10				2.1	

Table 12 – F344 Rat Urine QC Results (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
5.906	6.030	6.048	0.128	2.1	2.1	2.4
	6.211				5.2	
	6.053				2.5	
	5.899				-0.1	
29.53	29.89	29.88	0.05	0.2	1.2	1.2
	29.94				1.4	
	29.88				1.2	
	29.81				1.0	
118.1	121.2	120.8	0.5	0.4	2.6	2.3
	120.2				1.8	
	121.2				2.6	
	120.5				2.0	

4.8.5 Limit of Quantitation QCs

The results from the F344 rat urine ELOQ QCs are shown in Tables 13 (1-HANQ) and 14 (2-HANQ). The limit of detection, defined as three times the standard deviation of the ELOQ QC, was 0.02764 for 1-HANQ and 0.1342 µg/mL for 2-HANQ. The limit of quantitation, defined as ten times the standard deviation of the ELOQ QC, was 0.09214 for 1-HANQ and 0.4475 µg/mL for 2-HANQ.

Table 13 – ELOQ F344 Rat Urine QC Results (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
0.1200	0.1063	0.1201	0.0092	7.7	-11.4	0.1
	0.1121				-6.6	
	0.1229				2.4	
	0.1254				4.5	
	0.1315				9.6	
	0.1223				2.0	

Table 14 – ELOQ F344 Rat Urine QC Results (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
	1.475				-1.8	
	1.595				6.1	
1.503	1.586	1.549	0.045	2.9	5.5	3.1
	1.545				2.8	
	1.568				4.4	
	1.522				1.3	

4.8.6 Recovery

The recoveries of 1-HANQ and 2-HANQ are shown in Tables 15 and 16. The average recovery of the IS was 93.6 ± 2.8%.

Table 15 – Recovery Results (1-HANQ)

Target Conc (µg/mL)	Avg Recovery (%)	Grand Avg Recovery (%)	s
15	87.1		
12	90.4		
9	82.3		
7.2	88.9		
4.5	85.7	86.7	2.9
2.4	83.8		
1.2	82.9		
0.72	88.6		
0.3	87.5		
0.12	89.6		

Table 16 – Recovery Results (2-HANQ)

Target Conc (µg/mL)	Avg Recovery (%)	Grand Avg Recovery (%)	s
200	96.2		
150	95.5		
120	89.0		
90	95.2		
60	94.2	94.0	2.5
30	94.3		
16	91.5		
9	95.4		
4	91.9		
1.5	97.2		

4.9 Discussion and Conclusions

The acceptability of the method to meet the pre-established acceptance criteria for the various design elements is discussed in the following sections. The method was found to meet all criteria except specificity.

4.9.1 Linearity

The standard curves for both analytes had a correlation coefficient greater than 0.999 (Tables 7 and 8). This indicates the model has an appropriate fit over the selected concentration range. There was no significant difference between the vehicle and solvent standard curves.

4.9.2 Specificity

There were interferences observed in the specificity samples with average responses of 85% the response of 1-HANQ, 21% the response of 2-HANQ, and 1.2% the response of IS in the lowest concentration vehicle standard. The solvent and urine standards show nearly identical profiles and response (see Figure 3), indicating a source of the background peaks other than the urine. The method met all acceptance criteria for accuracy and precision despite the high background.

4.9.3 Accuracy

The maximum relative error for an individual standard was 12.5% for 1-HANQ (Table 9) and 6.3% for 2-HANQ (Table 10). The maximum average relative error for any standard was 9.5% for 1-HANQ (Table 9) and -2.7% for 2-HANQ (Table 10).

The average relative errors for the F344 rat urine QCs met acceptance criteria for all analytes. The maximum average relative errors for 1-HANQ and 2-HANQ were -3.3% and 2.4%, respectively (Tables 11 and 12).

4.9.4 Precision

The RSDs for the QCs met acceptance criteria with a maximum value for 1-HANQ and 2-HANQ of 1.8% and 2.1%, respectively (Tables 11 and 12).

4.9.5 Sensitivity – ELOQ QCs

The ELOQ QCs met all acceptance criteria for the lowest standard evaluated (0.12 µg/mL for 1-HANQ and 1.5 µg/mL for 2-HANQ) (Tables 13 and 14).

4.9.6 Recovery

The recoveries of both analytes and the IS were acceptable (Tables 15 and 16).

4.10 Stability

The QCs analyzed with study samples were stored longer than the samples, so no additional stability testing was required.

5 SAMPLES

The urine samples from all male F344 rats shown in Table 17 were received on October 3, 2003. The volumes from the individual animals within a dose group were pooled prior to submission for analysis. The samples were collected on October 3, 2003. 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 17 – Male F344 Rat Urine Samples

Animal IDs	Dosage (ppm)	Treatment Group
1 2 3 4 5 6 7 8 9 10	0	CON
11 12 13 14 15 16 17 18 19 20	938	NO-Z
21 22 23 24 25 26 27 28 29 30	3750	NO-Z
31 32 33 34 35 36 37 38 39 40	938	DA-K
41 42 43 44 45 46 47 48 49 50	3750	DA-K
51 52 53 54 55 56 57 58 59 60	938	DA-E
61 62 63 64 65 66 67 68 69 70	3750	DA-E
71 72 73 74 75 76 77 78 79 80	938	FC-E
81 82 83 84 85 86 87 88 89 90	3750	FC-E

6 SAMPLE ANALYSIS

The analysis method was originally developed and validated as part of the biological sample method development task, Battelle Study No. G004110-BMX, NTP ChemTask No. CHEM07027. A limited validation of the extended curve range used for this task was performed and was presented in Section 4 of this report. The samples were analyzed in one run.

6.1 Preparation of Stock, Spiking, and Urine Standards

6.1.1 Stock Standards

The amounts of 1- and 2-hydroxyanthraquinone shown in Table 18 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 the contents mixed well. These stock standards were stored at 2-8°C when not in use.

Table 18 – Preparation of Stock Standards (Sample Analysis)

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

6.1.2 Spiking Standards

Spiking standards were prepared by dilution of the stock standards with acetone as shown in Table 19. A single spiking standard was prepared at each concentration.

Table 19 – Preparation of Spiking Standards (Sample Analysis)

Standard ID	Target Conc (µg/mL)		Source	Source Vol (mL)	Final Vol (mL)
	1 HANQ	2 HANQ			
S1	90	1200	A	3	5
S2	72	900	B	3	5
S3	45	600	A	3	10
S4	24	300	B	1	5
S5	12	160	A	2	25
S6	7.2	90	B	3	50
S7	3	40	A	0.5	25
S8	1.2	15	B	0.5	50

6.1.3 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 the contents mixed well. A single urine standard was made from each spiking standard. This produced urine standards with target concentrations of 1-HANQ at 9, 7.2, 4.5, 2.4, 1.2, 0.72, 0.3, and 0.12 µg/mL and 2-HANQ at 120, 90, 60, 30, 16, 9, 4, and 1.5 µg/mL.

Duplicate aliquots of each standard were analyzed.

6.2 Preparation of QC Samples

The low (0.5 µg/mL 1-HANQ and 6 µg/mL 2-HANQ) and mid (2.5 µg/mL 1-HANQ and 30 µg/mL 2-HANQ) quality control samples prepared for the validation (see section 4.2) were used as the low and high QCs for this analysis.

6.3 Preparation of Blanks

Five hundred (500) µL of acetone was pipetted into a 5-mL volumetric flask half-full with blank F344 rat urine. The flask was 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.

6.4 Dilution of Samples

Any urine sample with an expected concentration greater than 9 µg/mL of 1-HANQ or 120 µg/mL of 2-HANQ was diluted into the range of the calibration curve with Milli-Q water (which has a resistivity = 18 megohm/cm) prior to analysis.

6.5 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 F344 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 over nitrogen at approximately 50°C. 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.

6.6 Analysis

Single injections were made from each vial using the chromatographic conditions provided in Table 20.

Table 20 – Chromatographic System (Sample Analysis)

LC System	Waters Model 2695 (Milford, MA)
Column	Metachem Intersil ODS-2, 150 mm x 3.00 mm (ID), 5µ (Lake Forrest, 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	10 µL
Detector Type	UV
Detector Wavelength	260 nm
Run Time	~35 minutes

6.7 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.

6.8 Acceptance Criteria

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

6.8.1 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%.

6.8.2 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.

6.8.3 Sensitivity

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

6.8.4 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 two at each concentration) of the individual QCs will be between -20 and 20%.

6.8.5 Precision

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

6.8.6 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.

6.8.7 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.

6.9 Results

The samples were analyzed in one run.

6.9.1 Linearity

The standard curves for the analysis 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 4 and 5.

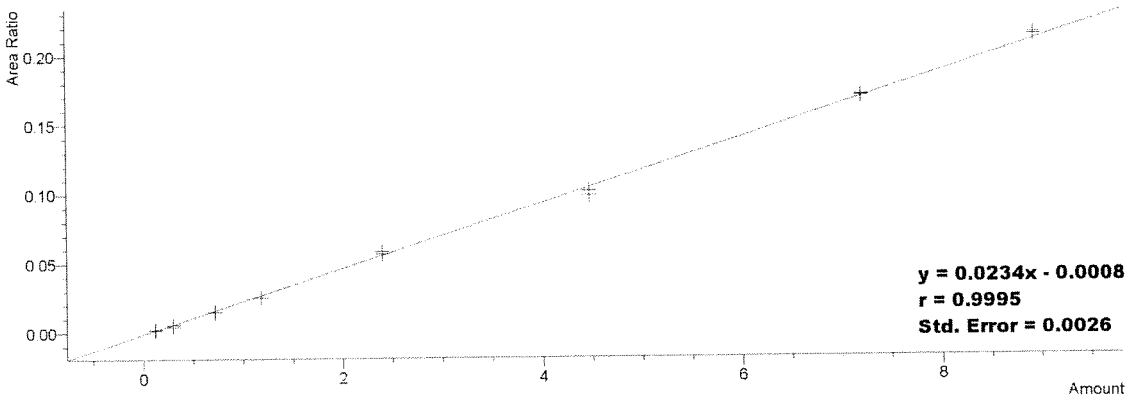

Figure 4 – Standard Curve, 1-HANQ

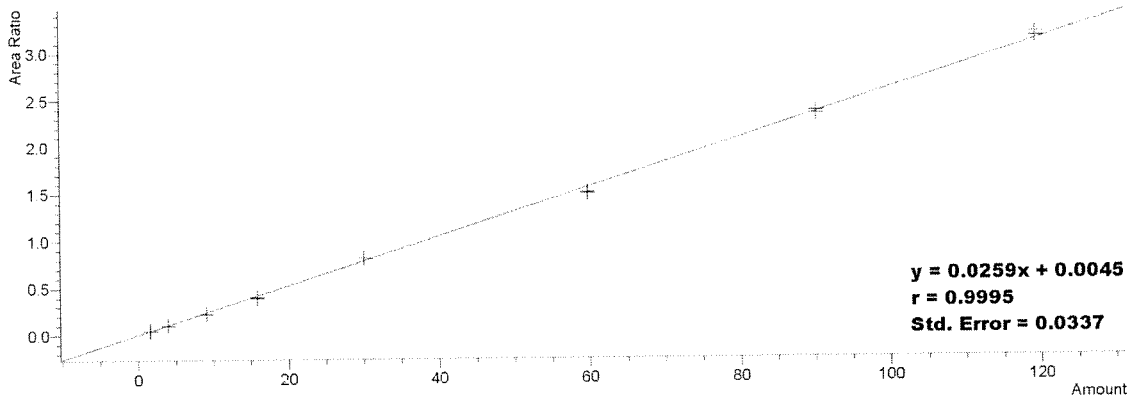

Figure 5 – Standard Curve, 2-HANQ

6.9.2 Selectivity

Acceptance criteria for selectivity was not met for the run. As seen in the validation, there were background peaks in the blanks that were above the acceptable range. The background peaks did not significantly affect the accuracy and precision of the analysis.

6.9.3 Accuracy and Precision

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

The results for the QCs are shown in Tables 21 and 22.

Table 21 – Low and High QC Results (1-HANQ)

Replicate	Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	RSD	RE (%)	Avg RE (%)
1	0.4948	0.4358	4.6	-11.9	-11.0
2		0.4203		-15.1	
3		0.4690		-5.2	
4		0.4370		-11.7	
1	2.474	2.290	0.4	-7.4	-7.5
2		2.286		-7.6	
3		2.297		-7.1	
4		2.277		-8.0	

Table 22 – Low and High QC Results (2-HANQ)

Replicate	Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	RSD	RE (%)	Avg RE (%)
1	5.906	5.719	1.3	-3.2	-4.6
2		5.629		-4.7	
3		5.541		-6.2	
4		5.656		-4.2	
1	29.53	28.72	0.2	-2.7	-3.0
2		28.56		-3.3	
3		28.66		-2.9	
4		28.59		-3.2	

The results showing the average relative errors for the standards are shown in Tables 23 and 24.

Table 23 – Standard Results (1-HANQ)

Average % Relative Error for Standards							
0.1200 µg/mL	0.2976 µg/mL	0.7200 µg/mL	1.190 µg/mL	2.400 µg/mL	4.464 µg/mL	7.200 µg/mL	8.928 µg/mL
16.5	-7.9	-4.8	-5.4	3.3	-4.2	0.6	1.9

Table 24 – Standard Results (2-HANQ)

Average % Relative Error for Standards							
1.503 µg/mL	3.979 µg/mL	9.017 µg/mL	15.92 µg/mL	30.06 µg/mL	59.69 µg/mL	90.17 µg/mL	119.4 µg/mL
9.4	-1.7	-5.1	-4.5	3.7	-3.5	0.0	1.8

6.9.4 Chromatography

Representative overlaid chromatograms in full (upper) and reduced (lower) scale from a high standard, low standard, blank with IS, and blank are shown in Figure 6. Representative overlaid chromatograms from the QCs are shown in Figure 7. Representative overlaid chromatograms from a standard, one of the samples, and a control animal are shown in Figure 8. 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.

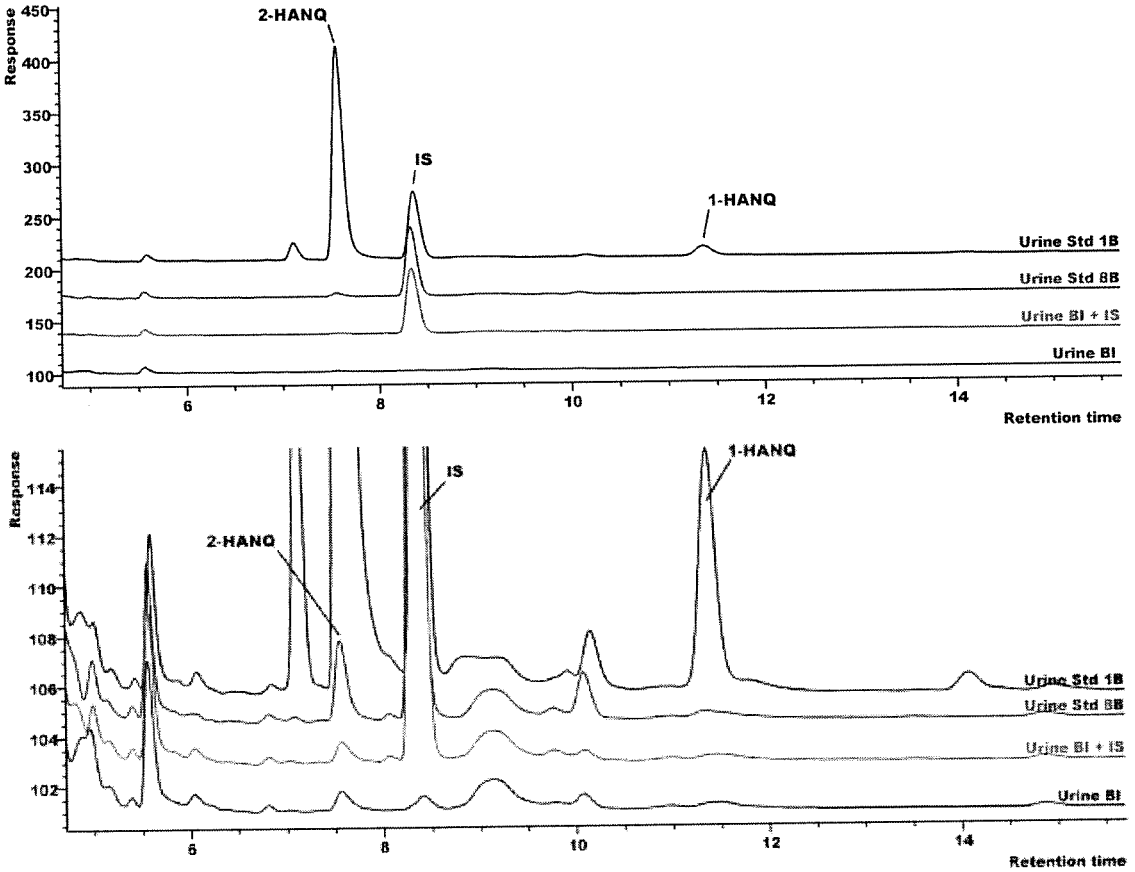

Figure 6 – Representative Overlaid Chromatograms for the High and Low Standard, Blank with IS, and Blank (Full Scale - Top and Reduced Scale - Bottom)

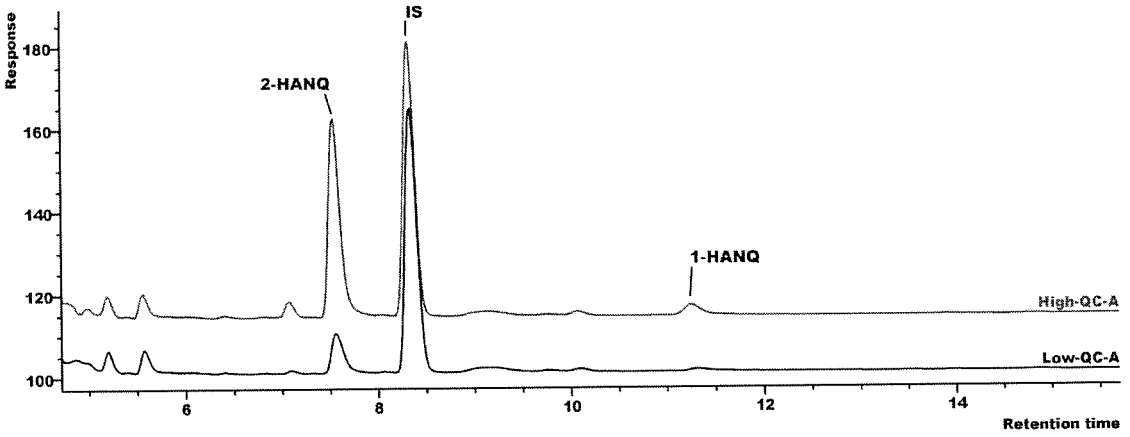

Figure 7 – Representative Overlaid Chromatograms for the High and Low QCs

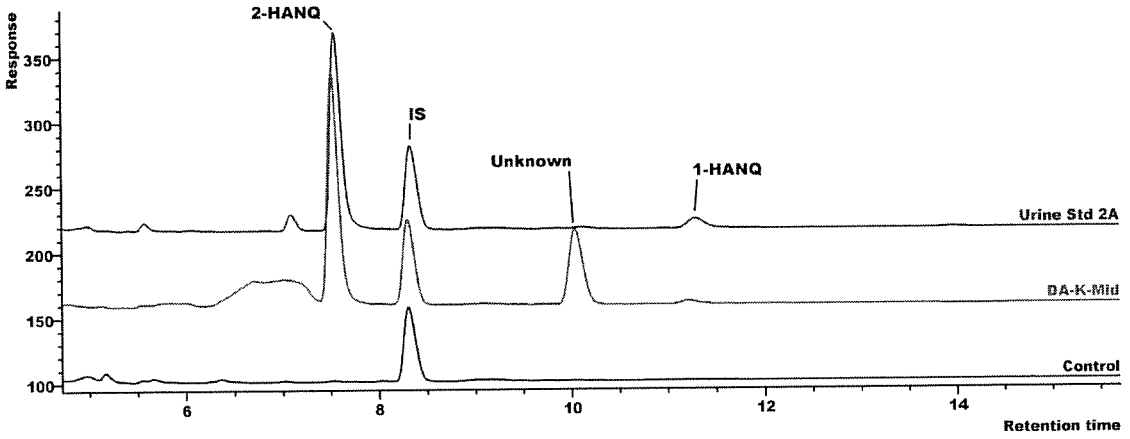

Figure 8 – Representative Overlaid Chromatograms of a Standard, Sample, and Control Animal

6.9.5 Sample Results

The concentrations of 1-HANQ and 2-HANQ in the samples are shown in Tables 25 and 26. The limits of quantitation are 0.1398 and 1.645 μg/mL of 1-HANQ and 2-HANQ, respectively.

Table 25 – Sample Results (1-HANQ)

Sample ID	Dosage (ppm)	Det'd Conc (µg/mL)	Dilution Factor	Conc (µg/mL)
CON	0	Not Detected	None	BLOQ
NO-Z	938	0.1958	None	0.1958
	3750	1.867	None	1.867
DA-K	938	0.4211	None	0.4211
	3750	2.369	None	2.369
DA-E	938	0.3851	None	0.3851
	3750	1.610	2	3.219
FC-E	938	0.5569	None	0.5569
	3750	1.776	2	3.552

Table 26 – Sample Results (2-HANQ)

Sample ID	Dosage (ppm)	Det'd Conc (µg/mL)	Dilution Factor	Conc (µg/mL)
CON	0	0.2362	None	BLOQ
NO-Z	938	13.43	None	13.43
	3750	90.80	None	90.80
DA-K	938	30.52	None	30.52
	3750	99.47	None	99.47
DA-E	938	28.12	None	28.12
	3750	74.51	2	149.0
FC-E	938	39.41	None	39.41
	3750	78.89	2	157.8

6.10 Conclusions

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

7 ACKNOWLEDGMENTS

The analyses were conducted by [redacted]. The report was prepared by [redacted] and [redacted] reviewed the data for completeness and accuracy; [redacted] conducted the review of the report.