Battelle
. . . Putting Technology To Work

Chemistry Support Services for the NTP
NIH Contract No.: N01-ES-05456
Battelle Project No.: G004110-BMX
NTP ChemTask No.: CHEM07027
CAS No.: 84-65-1

BIOLOGICAL SAMPLE METHOD DEVELOPMENT REPORT

ANTHRAQUINONE

7-064-BSMD-47

December 17, 2003

Prepared By:

[redacted]

Task Leader

Approved By:

[redacted]

Principal Investigator

Submitted to:

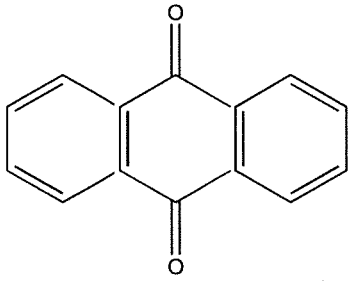
[redacted]

National Institute of Environmental Health Sciences
Mail Drop: EC-06
4401 Commons Building, Suite 100
Research Triangle Park, NC 27709

BIOLOGICAL SAMPLE METHOD DEVELOPMENT REPORT

ANTHRAQUINONE

CAS No.: 84-65-1	Lot Nos.: 1-Hydroxyanthraquinone – 254-2B (ChemService) 2-Hydroxyanthraquinone – 33-217-H (Narchem Corporation)
Battelle Chemical ID Code: 064	Samples Received: Not applicable
Battelle Task No.: 7-064-BSMD-47	Sample Receipt Date: Not applicable
NTP ChemTask No.: CHEM07027	Sample Submitter: Not applicable
Program Supported: CAR	Sample Collection Date: Not applicable
Analysis Dates: 7/17-9/20/03	Receipt Condition: Not applicable
Interim Results Date: Not applicable	Shipping Container: Not applicable
	Storage Condition (@ Battelle): Not applicable

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

EXECUTIVE SUMMARY

A chromatographic method that had been developed and validated by Battelle for separation and analysis of various metabolites of anthraquinone was used for this work. A liquid-liquid extraction method based on the solubilities of the analytes was developed. The resulting method was developed and validated over the concentration ranges of 2.4 to 15 µg/mL for 1-hydroxyanthraquinone (1-HANQ) and 30 to 200 µg/mL for 2-hydroxyanthraquinone (2-HANQ). These concentrations were based on a preliminary analysis of samples from the rodent metabolism study.

The method was evaluated using three runs and found to be suitable based on the following.

The standard curves had acceptable regression parameters for both analytes.

The specificity of the method was acceptable with no significant chromatographic interference for the test articles or internal standard and the y-intercepts were statistically equal to zero in three of the six runs and slightly outside the acceptance range in the other three with no clear trend to either negative or positive bias.

The accuracy of the method met the acceptance criteria of having within and between day average relative errors within 20% of nominal at all concentrations of the standards and QCs.

The precision of the method met the acceptance criteria of having between and within day relative standard deviations (RSD) for the quality control (QC) samples of 20% or less for all runs.

The sensitivity of the method was acceptable with an experimental limit of quantitation of 2.4 μg/mL for 1-HANQ and 30 μg/mL for 2-HANQ using a 2-mL sample. The calculated limits of detection were 0.2055 and 0.9292 μg/mL for 1- and 2-HANQ, respectively. The calculated limits of quantitation were 0.6851 and 3.097 μg/mL for 1 and 2-HANQ, respectively.

The recoveries of the analytes from the urine were 93.4 ± 5.1 and 97.5 ± 4.3 for the 1- and 2-HANQ, respectively. The recovery of the IS was 97.6 ± 3.8%

Analysis of the QCs indicated that analytes in urine were stable for at least 60 days when stored at ≤-20°C and through 3 freeze/thaw cycles. The extracts were found to be stable for 2 days at room temperature.

Two analysts conducting the analysis on separate chromatographic systems obtained equivalent data indicating the method is sufficiently rugged.

TABLE OF CONTENTS

Page

1 INTRODUCTION 1
2 TEST ARTICLES 1
3 METHOD DEVELOPMENT 1
4 METHOD VALIDATION 2
4.1 Experimental Design 2
4.2 Method 3
4.2.1 Preparation and Storage of Standards 3
4.2.1.1 Stock Standards 3
4.2.1.2 Spiking Standards 3
4.2.1.3 Fischer 344 Rat Urine Standards 4
4.2.1.4 Preparation of Solvent Recovery Standards 4
4.2.2 Preparation of Blank Fischer 344 Rat Urine 4
4.2.3 Preparation of Fischer 344 Rat Urine Quality Control (QC) Samples 4
4.2.3.1 Stock Solution 4
4.2.3.2 Spiking Solutions 4
4.2.3.3 Fischer 344 Rat Urine QCs 5
4.2.3.4 Fischer 344 Rat Urine ELOQ QCs 5
4.2.4 Preparation of Specificity Samples 5
4.2.5 Preparation of Fischer 344 Rat Urine Standards, Blanks, QCs, and Specificity Samples for Analysis 5
4.3 Analysis 5
4.4 Calculations 7
4.5 Results 8
4.5.1 Linearity 8
4.5.2 Specificity 9
4.5.3 Accuracy – Standards 10
4.5.4 Precision and Accuracy – QC Samples 13
4.5.5 Precision – Standards 15
4.5.6 Limit of Quantitation QCs 16
4.5.7 Dilution QC 17
4.5.8 Recovery 18
4.6 Discussion and Conclusions 19
4.6.1 Linearity 19

TABLE OF CONTENTS (CONTINUED)

Page

4.6.2 Specificity 19
4.6.3 Accuracy 19
4.6.4 Precision 20
4.6.5 Sensitivity – ELOQ QCs 20
4.6.6 Dilution QC 20
4.6.7 Recovery 20
4.6.8 Ruggedness 20
4.7 Stability 20
4.7.1 Study Designs 20
4.7.2 Results 20
4.7.2.1 Freeze/Thaw 20
4.7.2.2 Storage 21
4.7.2.3 Extract Stability 22
4.7.3 Discussion and Conclusions 22
5 ACKNOWLEDGEMENTS 22

LIST OF TABLES

Table 1. Validation Design Summary 2
Table 2. Preparation of Spiking Standards 3
Table 3. Preparation of QC Spiking Solutions 4
Table 4. HPLC System 6
Table 5. Regression Curve Parameters – 1-HANQ 8
Table 6. Regression Curve Parameters – 2-HANQ 8
Table 7. Run 1 Fischer 344 Rat Urine Standards (1-HANQ) 10
Table 8. Run 1 Fischer 344 Rat Urine Standards (2-HANQ) 11
Table 9. Run 2 Fischer 344 Rat Urine Standards (1-HANQ) 11
Table 10. Run 2 Fischer 344 Rat Urine Standards (2-HANQ) 11
Table 11. Run 3 Fischer 344 Rat Urine Standards (1-HANQ) 12
Table 12. Run 3 Fischer 344 Rat Urine Standards (2-HANQ) 12
Table 13. Standard Accuracy (% Relative Error) All Runs – 1-HANQ 12
Table 14. Standard Accuracy (% Relative Error) All Runs – 2-HANQ 13
Table 15. Run 1 Fischer 344 Rat Urine QC Results (1-HANQ) 13
Table 16. Run 1 Fischer 344 Rat Urine QC Results (2-HANQ) 13
Table 17. Run 2 Fischer 344 Rat Urine QC Results (1-HANQ) 14

LIST OF TABLES (CONTINUED)

Page

Table 18. Run 2 Fischer 344 Rat Urine QC Results (2-HANQ) 14
Table 19. Run 3 Fischer 344 Rat Urine QC Results (1-HANQ) 14
Table 20. Run 3 Fischer 344 Rat Urine QC Results (2-HANQ) 15
Table 21. Summary of QC Results (1-HANQ) 15
Table 22. Summary of QC Results (2-HANQ) 15
Table 23. Summary of Fischer 344 Rat Urine Standards (1-HANQ – Between Days) 16
Table 24. Summary of Fischer 344 Rat Urine Standards (2-HANQ – Between Days) 16
Table 25. ELOQ Fischer 344 Rat Urine QC Results (1-HANQ) 17
Table 26. ELOQ Fischer 344 Rat Urine QC Results (2-HANQ) 17
Table 27. Fischer 344 Rat Urine Dilution QC Results (1-HANQ) 18
Table 28. Fischer 344 Rat Urine Dilution QC Results (2-HANQ) 18
Table 29. Recovery Results (1-HANQ) 18
Table 30. Recovery Results (2-HANQ) 19
Table 31. Freeze/Thaw Results (1-HANQ) 21
Table 32. Freeze/Thaw Results (2-HANQ) 21
Table 33. Storage Results (1-HANQ) 22
Table 34. Storage Results (2-HANQ) 22

LIST OF FIGURES

Figure 1. Representative Overlaid Chromatograms of a High and Low Fischer 344 Rat Urine Standard, Blank with IS and Blank (Full Scale – Top and Reduced Scale – Bottom) 7
Figure 2. Regression Curves (Run 1) Fischer 344 Rat Urine 9
Figure 3. Overlaid Chromatograms of a Representative Low Urine Standard and Fischer 344 Rat Urine Specificity Samples 10

APPENDIX A – REPORT DATA SUMMARY

BIOLOGICAL SAMPLE ANALYSIS METHOD DEVELOPMENT

Method Development A-1
Method Validation A-1

1 INTRODUCTION

The scope of this work was to develop and validate analysis methods for 1- and 2-hydroxyanthraquinone, metabolites of anthraquinone, in Fischer 344 rat urine.

This report presents:

- a description of method development activities;
- a description of a method to analyze Fischer 344 rat urine for 1- and 2-hydroxyanthraquinone;
- data from the validation of the analysis method for Fischer 344 rat urine;
- our conclusions; and
- an appendix.

This task was conducted at Battelle, 505 King Avenue, Columbus, OH 43201 in support of CAR studies.

2 TEST ARTICLES

Ten (10) g of 1-hydroxyanthraquinone (1-HANQ), Lot No. 254-2B, was received from ChemService on October 6, 2000 in twenty amber glass vials. The chemical was received and stored at room temperature until chemical handling was performed. The net weight received was determined to be 11.09 g. The weight of chemical remaining after chemical handling (homogenization and sampling) was 9.65 g. The remaining chemical was stored at room temperature.

Twenty five (25) g of 2-hydroxyanthraquinone (2-HANQ), Lot No. 33-217-H, was received from Narchem Corporation on September 4, 2001 in one amber glass bottle. The chemical was received and stored at room temperature until chemical handling was performed. The net weight received was determined to be 25.9 g. The weight of chemical remaining after chemical handling (homogenization and sampling) was 21.0 g. The remaining chemical was stored at room temperature.

A bulk chemical limited analysis is being conducted to confirm the identity and evaluate the purity of both of these standards (Battelle Study No. G004110-AUE, NTP ChemTask No. CHEM05679).

These materials were used to prepare the standards and quality control samples (QC) for this work.

3 METHOD DEVELOPMENT

The instrumental analysis methods selected for initial evaluation were based on previous chromatographic work that had been done for the analysis of anthraquinone. This work included developing chromatographic systems capable of resolving the hydroxyanthraquinones and nitro anthracenes from each other and anthraquinone.

High performance liquid chromatographic systems with ultraviolet and fluorescence detection and a gas chromatographic system with flame ionization detection were evaluated for sensitivity and specificity. The high performance liquid chromatographic system was found to have the best sensitivity and specificity.

Sample preparation was done using liquid-liquid extraction with a variety of solvents. Ethyl acetate was found to be the best solvent based on its ease of use, ability to extract the anthraquinones and cleanliness of the extract.

An evaluation was done of the resulting method. This evaluation indicated that the method could quantify 1-hydroxyanthraquinone down to approximately 10 ng/mL and 2-hydroxyanthraquinone down to approximately 25 ng/mL in a 2-mL sample.

The method was subsequently modified prior to validation for a higher concentration range based on the analysis of the Fischer 344 rat urine samples from the metabolism study that showed the actual sample concentrations would be significantly higher than the original curve range.

4 METHOD VALIDATION

This section describes the validation design, the analytical method, and the results and conclusions from the validation.

4.1 Experimental Design

The validation was designed to be conducted in three separate runs. It also included the use of at least two analysts, two different instruments, and two different LC columns from the same supplier. The acceptance criteria were based on the SOP for conducting BSMD tasks. The design is summarized in Table 1.

Table 1 – Validation Design Summary

Element	Design	Acceptance Criteria
Linearity/Curve Fit	Analyze duplicate aliquots of Fischer 344 rat urine standards for each run. Analyze a single Fischer 344 rat urine blank and a single Fischer 344 rat urine blank with IS.	The correlation coefficient for linear models will be ≥0.99. The coefficient of determination for quadratic models will be ≥0.98. Determine and report regression parameters for most acceptable model and weighting.
Specificity	In the validation run, analyze at least six Fischer 344 rat urine samples from different sources and analyze a single specificity blank + IS.	Any peak with retention time of 1-HANQ or 2-HANQ or the IS will have an average response <30% of the lowest standard. Evaluate calculated y-intercept from standard curves.
Precision	Calculate relative standard deviations for QCs.	Values ≤20%.
Accuracy	Calculate individual and average relative errors for standards and QCs.	Average values within 20% of nominal.
Sensitivity (ELOQ)	Evaluate using six replicates of the lowest vehicle standard during one run.	Average relative error within 20% of nominal and relative standard deviations of ≤20%.
Recovery	Compare responses of extracted urine standards to solvent standards.	Recovery of ≥85 ± 15%.
Storage Stability	Compare data from the initial analysis of both levels of QCs to data from stored QCs.	None; determine and report.
Freeze/Thaw Stability	Compare data from the initial analysis of both levels of QCs to data from QCs which have undergone three freeze/thaw cycles.	None; determine and report.

Table 1 – Validation Design Summary (Continued)

Element	Design	Acceptance Criteria
Processed Sample Stability	Compare Fischer 344 rat urine standard and QC values following storage of the LC vials at room temperature in the light for at least 48 hours after initial analysis to values of unstored Fischer 344 rat urine standards and QCs.	All original acceptance criteria should be met.
Sample Dilution	Dilute a rat urine QC into the range of the standard curve and analyze. If needed this will be performed during analysis of the actual study samples.	None; determine and report.

4.2 Method

4.2.1 Preparation and Storage of Standards

4.2.1.1 Stock Standards

Two stock standards were prepared at target concentrations of 150 (A) and 120 (B) µg/mL of 1-HANQ and 2000 (A) and 1500 (B) µg/mL of 2-HANQ by dissolving 15 ± 0.3 and 12 ± 0.3 mg of 1-HANQ and 200 ± 3 and 150 ± 3 mg of 2-HANQ, respectively, in acetone, diluting to a final volume of 100 mL with acetone and mixing well. These solutions were used throughout the validation. They were stored at 2-8°C when not in use. They were also used as the two most concentrated spiking standards.

4.2.1.2 Spiking Standards

Spiking standards were prepared by dilution of the stock standards, A and B, with acetone as shown in Table 2. A single spiking standard was prepared at each concentration for each validation run.

Table 2 – Preparation of Spiking Standards

Standard	1 HANQ Target Conc (µg/mL)	2 HANQ Target Conc (µg/mL)	Source	Source Vol (mL)	Final Vol (mL)
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

4.2.1.3 Fischer 344 Rat Urine Standards

Five hundred (500) µL of each spiking standard was pipetted into individual 5-mL volumetric flasks containing approximately 2.5 mL of blank Fischer 344 rat urine. The flasks were diluted to volume with blank rat urine, sealed, and shaken. This produced urine standards with target concentrations of 15, 12, 9, 7.2, 4.5 and 2.4 µg/mL for 1-HANQ and 200, 150, 120, 90, 60 and 30 µg/mL for 2-HANQ. A single urine standard at each concentration was prepared for each run and used on the day of preparation. Duplicate 2-mL aliquots of each standard were pipetted into individual extraction tubes.

4.2.1.4 Preparation of Solvent Recovery Standards

Solvent recovery 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]. Milli-Q water has a resistivity = 18 megohm/cm. 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 recovery standard was made from each spiking standard. An aliquot of each solvent recovery standard was transferred to an autoinjector vial and the vials were sealed.

4.2.2 Preparation of Blank Fischer 344 Rat Urine

Blank Fischer 344 rat urine was prepared by diluting 0.5 mL of acetone to 5 mL with blank Fischer 344 rat urine. 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.

4.2.3 Preparation of Fischer 344 Rat Urine Quality Control (QC) Samples

4.2.3.1 Stock Solution

One stock solution (C) was prepared at a target concentration of 250 µg/mL of 1-HANQ and 3000 µg/mL of 2-HANQ by dissolving 12.5 ± 0.2 mg of 1-HANQ and 150 ± 5 mg of 2-HANQ, respectively, in acetone, diluting to a final volume of 50 mL with acetone, and mixing well.

4.2.3.2 Spiking Solutions

Spiking standards were prepared by dilution of stock solution C with acetone as shown in Table 3.

Table 3 – Preparation of QC Spiking Solutions

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

4.2.3.3 Fischer 344 Rat Urine QCs

Five (5) mL of stock or spiking solution was pipetted into individual 50-mL volumetric flasks containing approximately 25 mL of blank rat urine. The flasks were diluted to volume with blank rat urine, sealed, and shaken. This produced urine QCs with target concentrations of 25 (dilution QC), 10, and 5 µg/mL for 1-HANQ and 300 (dilution QC), 120, and 60 µg/mL of 2-HANQ.

Two (2)-mL aliquots of QC were pipetted into individual extraction tubes. The tubes were sealed and stored at -20°C until used during an analysis. Four replicates of the dilution QC were analyzed with one run. Four replicates of each of the other QCs were analyzed with each run.

4.2.3.4 Fischer 344 Rat Urine ELOQ QCs

Two and one-half (2.5) mL of spiking standard S6 was pipetted into a 25-mL volumetric flask approximately half full with blank Fischer 344 rat urine. The flask was diluted to volume with blank rat urine and sealed. The contents were mixed well. Two (2)-mL aliquots were pipetted into six extraction tubes.

4.2.4 Preparation of Specificity Samples

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

4.2.5 Preparation of Fischer 344 Rat Urine Standards, Blanks, QCs, and Specificity Samples for Analysis

Fischer 344 rat urine standards, blanks, QCs, and specificity samples were prepared for analysis by adding 200 µL of blank Fischer 344 rat urine 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 the internal standard [an approximately 320 µg/mL solution of 9-anthracenemethanol in acetone:Milli-Q water (80:120 v/v)] was added to each tube except for the blanks without internal standard to which 200 µL of acetone was added instead. Two (2) mL of ethyl acetate was added to each tube and the tubes were rotated for approximately 5 minutes and centrifuged at a setting of approximately 2000 rpm for approximately 5 minutes. The top (ethyl acetate) layer was transferred to another tube. The extraction was repeated twice using the same procedure with the extracts being combined in the second tube. The combined extract was evaporated to dryness using nitrogen and a water bath set at approximately 50°C. The residue was reconstituted in 10 mL of HPLC mobile phase B. An aliquot was transferred to an autoinjector vial and the vial was sealed.

4.3 Analysis

Single injections were made from each vial using the HPLC system shown in Table 4. Representative overlaid chromatograms of a high and low standard, blank with IS, and blank from one validation run are shown in full (top)

and reduced (bottom) scale in Figure 1. The chromatograms for the ELOQ QCs were virtually identical to those of the lowest standard.

Table 4 – HPLC System

Instrument	Waters Module 1-Plus and Model 2690 (Milford, MA); Agilent 1100 Series (Palo Alto, CA)
Column	Phenomenex (Torrance, CA) Inertsil ODS-2, 150 mm x 3.00 mm (ID), 5μ (or equivalent)
Guard Column	Phenomenex Inertsil ODS-2 Guard Cartridge
Mobile Phase Components	A: 25:75 (v:v) of Acetonitrile:Milli-Q Water B: 75:25 (v:v) of Acetonitrile:Milli-Q Water
Mobile Phase Gradient	Time (minutes) %A %B All changes are linear with time. 0 100 0 5 0 100 25 0 100 26 100 0 35 100 0
Column Flow Rate	0.8 mL/minute
Injection Volume	20 μL in first run, 10 μL in all other runs
Detector Type	UV
Detector Wavelength	260 nm

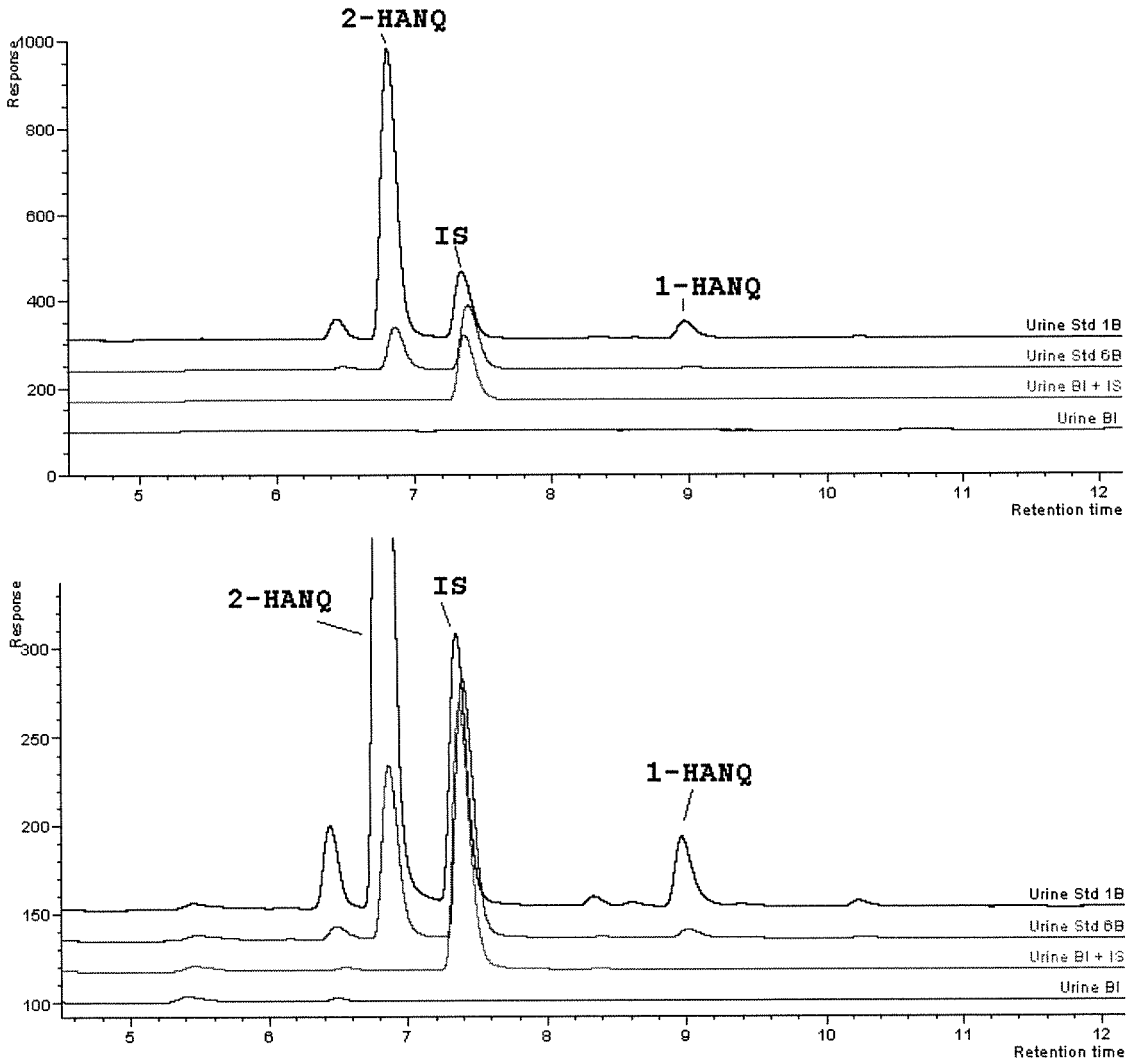


Figure 1 – Representative Overlaid Chromatograms of a High and Low Fischer 344 Rat Urine Standard, Blank with IS, and Blank (Full Scale – Top and Reduced Scale – Bottom)

4.4 Calculations

The 1- and 2-HANQ and the IS peaks were integrated by the data system and manually reintegrated, if necessary, to assure proper integration.

A 1/x weighted linear regression equation was calculated for each analyte relating the response ratio of the analyte/IS (y) to its concentration (x) in the urine standards. The use of weighting was found to be necessary to achieve acceptable accuracy at the low concentration of the curves. The analyte concentration of each standard was

calculated using its individual response and the regression equation. These values were used to calculate the individual and average concentrations and relative errors, standard deviation and percent relative standard deviation as appropriate at each concentration. The response ratios of the QCs were used to calculate their individual concentrations. These values were used to calculate the individual and average concentrations and relative errors, standard deviation and percent relative standard deviation as appropriate at each concentration.

Recovery was calculated at each standard concentration by dividing the response of the analyte in the urine standard by the response of the analyte in the solvent standard. The average recovery and standard deviation were calculated from the individual values.

4.5 Results

4.5.1 Linearity

The critical regression parameters are shown in Tables 5 (1-HANQ) and 6 (2-HANQ). The regression curves from the first validation run are shown in Figure 2. The curves from the other runs were similar.

Table 5 – Regression Curve Parameters – 1-HANQ

Run	Slope	y Intercept	Std Error	Correlation Coefficient
1	0.0165	-0.0025	0.0041	0.9986
2	0.0206	-0.0027	0.0019	0.9998
3	0.0158	-0.0063	0.0047	0.9981

Table 6 – Regression Curve Parameters – 2-HANQ

Run	Slope	y Intercept	Std Error	Correlation Coefficient
1	0.0194	0.0293	0.0343	0.9996
2	0.0228	-0.0039	0.0272	0.9998
3	0.0186	0.0263	0.0222	0.9998

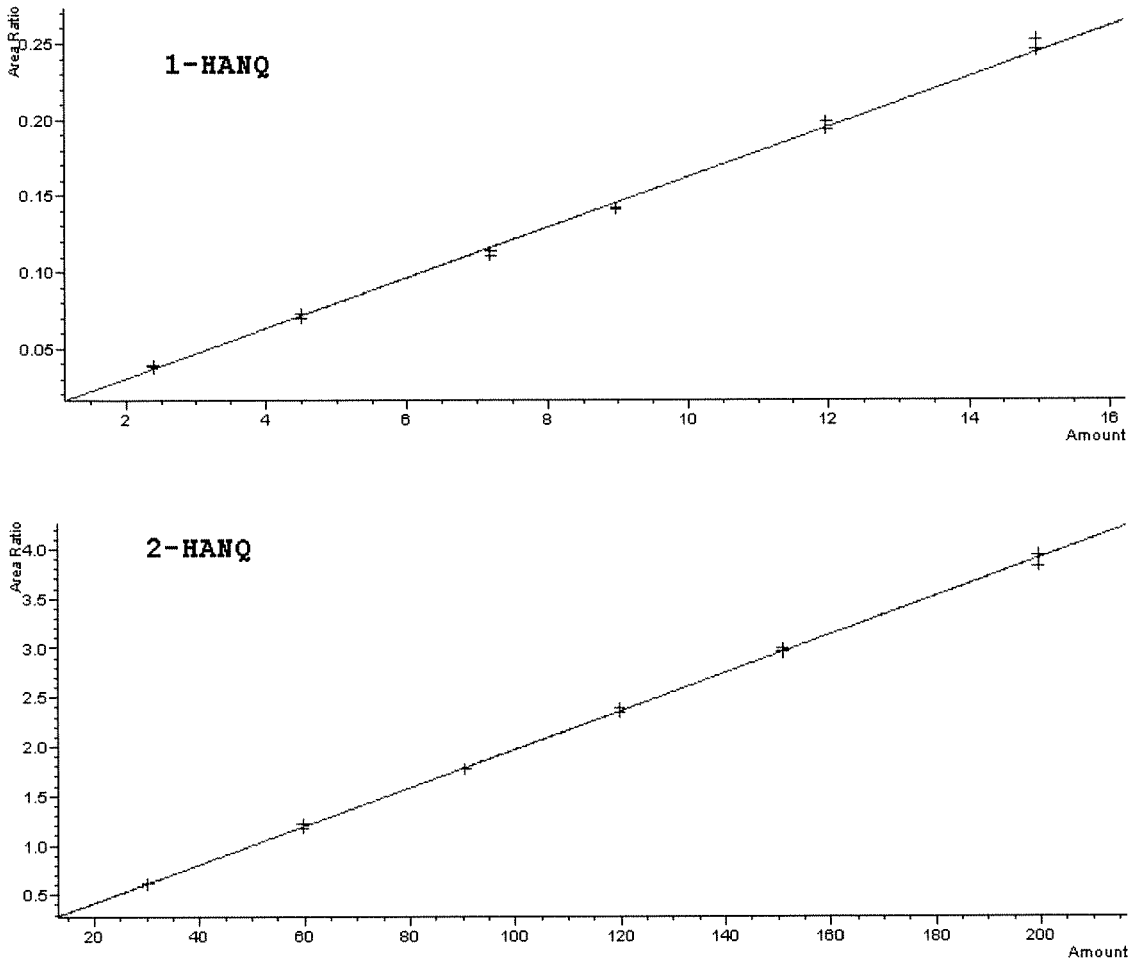


Figure 2 – Regression Curves (Run 1) Fischer 344 Rat Urine

4.5.2 Specificity

The chromatograms from the multiple specificity samples did not have any peaks which interfered with either analyte or the IS. Chromatograms of a representative low standard and the specificity samples are presented in Figure 3.

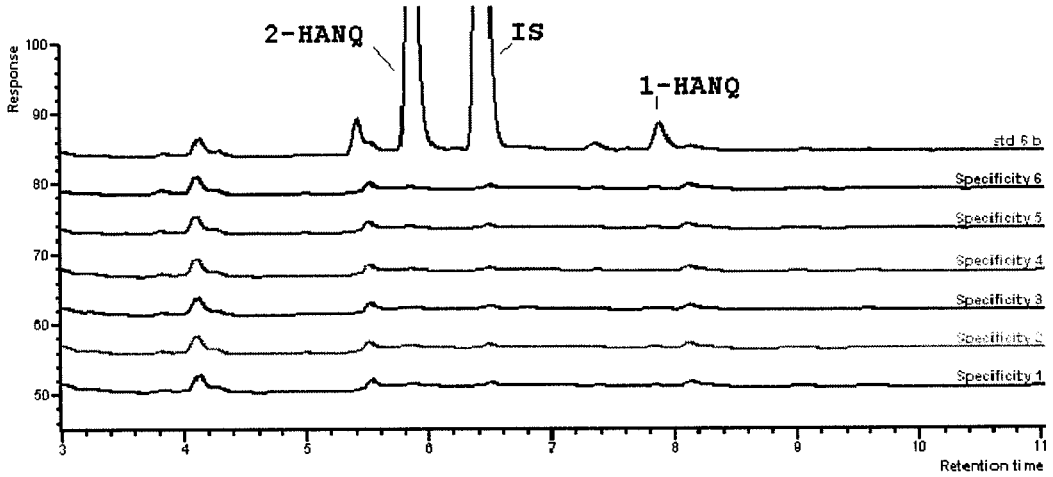


Figure 3 – Overlaid Chromatograms of a Representative Low Urine Standard and Fischer 344 Rat Urine Specificity Samples

4.5.3 Accuracy – Standards

The results for the Fischer 344 rat urine standards from the individual runs are shown in Tables 7 through 12. The average accuracy data for all runs are shown in Tables 13 and 14.

Table 7 – Run 1 Fischer 344 Rat Urine Standards (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
14.97	15.09	15.52	15.31	0.8	3.7	2.2
11.97	11.89	12.20	12.05	-0.7	1.9	0.6
8.982	8.727	8.764	8.746	-2.8	-2.4	-2.6
7.182	6.885	7.015	6.950	-4.1	-2.3	-3.2
4.491	4.544	4.392	4.468	1.2	-2.2	-0.5
2.394	2.514	2.444	2.479	5.0	2.1	3.6

Table 8 – Run 1 Fischer 344 Rat Urine Standards (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
199.5	195.4	201.9	198.7	-2.0	1.2	-0.4
150.9	150.6	152.4	151.5	-0.2	1.0	0.4
119.7	119.3	121.2	120.3	-0.3	1.3	0.5
90.55	89.90	89.98	89.94	-0.7	-0.6	-0.7
59.84	61.54	58.99	60.26	2.8	-1.4	0.7
30.18	30.08	29.98	30.03	-0.3	-0.7	-0.5

Table 9 – Run 2 Fischer 344 Rat Urine Standards (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
14.97	14.88	15.18	15.03	-0.6	1.4	0.4
11.97	11.95	11.95	11.95	-0.2	-0.2	-0.2
8.982	8.882	9.098	8.990	-1.1	1.3	0.1
7.182	7.108	7.186	7.147	-1.0	0.1	-0.5
4.491	4.457	4.482	4.469	-0.8	-0.2	-0.5
2.394	2.413	2.409	2.411	0.8	0.6	0.7

Table 10 – Run 2 Fischer 344 Rat Urine Standards (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
199.5	200.0	200.0	200.0	0.3	0.3	0.3
150.9	152.3	148.4	150.3	0.9	-1.7	-0.4
119.7	120.7	119.6	120.2	0.9	-0.1	0.4
90.55	91.35	89.28	90.31	0.9	-1.4	-0.3
59.84	60.31	58.66	59.49	0.8	-2.0	-0.6
30.18	30.66	30.05	30.36	1.6	-0.5	0.6

Table 11 – Run 3 Fischer 344 Rat Urine Standards (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
14.97	15.09	15.67	15.38	0.8	4.7	2.7
11.97	NV	11.80	11.80	NV	-1.4	-1.4
8.982	8.595	9.004	8.800	-4.3	0.2	-2.0
7.182	6.882	7.092	6.987	-4.2	-1.3	-2.7
4.491	4.393	4.535	4.464	-2.2	1.0	-0.6
2.394	2.452	2.494	2.473	2.4	4.2	3.3

NV = No value; wrong standard appears to have been used for this aliquot.

Table 12 – Run 3 Fischer 344 Rat Urine Standards (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
199.5	200.0	199.7	199.8	0.2	0.1	0.2
150.9	NV	147.9	147.9	NV	-2.0	-2.0
119.7	120.4	119.5	119.9	0.6	-0.2	0.2
90.55	90.77	90.92	90.85	0.3	0.4	0.3
59.84	60.80	61.03	60.91	1.6	2.0	1.8
30.18	30.02	29.44	29.73	-0.6	-2.5	-1.5

NV = No value; wrong standard appears to have been used for this aliquot.

Table 13 – Standard Accuracy (% Relative Error) All Runs – 1-HANQ

Nominal Conc (µg/mL)	Avg Daily RE (%)			Grand Avg RE (%)
14.97	2.2	0.4	2.7	1.8
11.97	0.6	-0.2	-1.4	-0.1
8.982	-2.6	0.1	-2.0	-1.5
7.182	-3.2	-0.5	-2.7	-2.1
4.491	-0.5	-0.5	-0.6	-0.5
2.394	3.6	0.7	3.3	2.5

Table 14 – Standard Accuracy (% Relative Error) All Runs – 2-HANQ

Nominal Conc (µg/mL)	Avg Daily RE (%)			Grand Avg RE (%)
199.5	-0.4	0.3	0.2	0.0
150.9	0.4	-0.4	-2.0	-0.4
119.7	0.5	0.4	0.2	0.3
90.55	-0.7	-0.3	0.3	-0.2
59.84	0.7	-0.6	1.8	0.6
30.18	-0.5	0.6	-1.5	-0.5

4.5.4 Precision and Accuracy – QC Samples

The results from the Fischer 344 rat urine QCs from the three validation runs are shown in Tables 15 through 20. Tables 21 and 22 summarize the day-to-day QC results.

Table 15 – Run 1 Fischer 344 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 (%)
4.940	4.976	4.960	0.062	1.3	0.7	0.4
	5.003				1.3	
	4.993				1.1	
	4.868				-1.5	
9.880	10.07	10.03	0.15	1.5	1.9	1.5
	9.967				0.9	
	10.21				3.3	
	9.864				-0.2	

Table 16 – Run 1 Fischer 344 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 (%)
58.48	60.09	59.92	0.48	0.8	2.7	2.5
	60.50				3.5	
	59.66				2.0	
	59.42				1.6	
117.0	119.8	119.6	1.3	1.1	2.4	2.2
	119.5				2.1	
	121.1				3.5	
	118.0				0.9	

Table 17 – Run 2 Fischer 344 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 (%)
4.940	4.952	4.967	0.048	1.0	0.2	0.6
	4.958				0.4	
	4.924				-0.3	
	5.036				1.9	
9.880	9.848	9.897	0.078	0.8	-0.3	0.2
	9.860				-0.2	
	10.01				1.4	
	9.865				-0.1	

Table 18 – Run 2 Fischer 344 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 (%)
58.48	58.96	58.99	0.49	0.8	0.8	0.9
	58.89				0.7	
	58.48				0.0	
	59.65				2.0	
117.0	116.1	116.4	1.0	0.8	-0.7	-0.5
	116.0				-0.9	
	117.8				0.7	
	115.7				-1.1	

Table 19 – Run 3 Fischer 344 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 (%)
4.940	4.885	4.921	0.045	0.9	-1.1	-0.4
	4.885				-1.1	
	4.935				-0.1	
	4.979				0.8	
9.880	10.47	10.40	0.25	2.4	6.0	5.2
	10.07				1.9	
	10.38				5.1	
	10.66				7.9	

Table 20 – Run 3 Fischer 344 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 (%)
58.48	61.29	61.35	0.23	0.4	4.8	4.9
	61.31				4.8	
	61.68				5.5	
	61.15				4.6	
117.0	124.7	124.3	8.3	6.6	6.6	6.2
	113.7				-2.8	
	124.8				6.7	
	133.9				14.5	

Table 21 – Summary of QC Results (1-HANQ)

QC	Run	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
4.940	1	4.960	4.949	0.025	0.5	0.4	0.2
	2	4.967				0.5	
	3	4.921				-0.4	
9.880	1	10.03	10.11	0.26	2.6	1.5	2.3
	2	9.897				0.2	
	3	10.40				5.3	

Table 22 – Summary of QC Results (2-HANQ)

QC	Run	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
58.48	1	59.92	60.09	1.19	2.0	2.5	2.8
	2	58.99				0.9	
	3	61.35				4.9	
117.0	1	119.6	120.1	4.0	3.3	2.2	2.6
	2	116.4				-0.5	
	3	124.3				6.2	

4.5.5 Precision – Standards

The results from the analysis of the standards from all validation runs are shown in Tables 23 and 24.

Table 23 – Summary of Fischer 344 Rat Urine Standards (1-HANQ - Between Days)

Run	Avg Det'd Conc (µg/mL)					
1	15.31	12.05	8.746	6.950	4.468	2.479
2	15.03	11.95	8.990	7.147	4.469	2.411
3	15.38	11.80	8.800	6.987	4.464	2.473
Avg Conc*	15.24	11.96	8.845	7.028	4.467	2.454
s*	0.30	0.15	0.186	0.124	0.066	0.042
RSD*	2.0	1.2	2.1	1.8	1.5	1.7

* Calculated using determined concentrations.

Table 24 – Summary of Fischer 344 Rat Urine Standards (2-HANQ - Between Days)

Run	Avg Det'd Conc (µg/mL)					
1	198.7	151.5	120.3	89.94	60.26	30.03
2	200.0	150.3	120.2	90.31	59.49	30.36
3	199.8	147.9	119.9	90.85	60.91	29.73
Avg Conc*	199.5	150.3	120.1	90.37	60.22	30.04
s*	2.1	2.1	0.8	0.77	1.16	0.39
RSD*	1.1	1.4	0.6	0.9	1.9	1.3

* Calculated using determined concentrations.

4.5.6 Limit of Quantitation QCs

The results from the Fischer 344 rat urine ELOQ QCs are shown in Tables 25 (1-HANQ) and 26 (2-HANQ). The limit of detection, defined as three times the standard deviation of the ELOQ QC, was 0.2055 for 1-HANQ and 0.9292 µg/mL for 2-HANQ. The limit of quantitation, defined as ten times the standard deviation of the ELOQ QC, was 0.6851 for 1-HANQ and 3.097 µg/mL for 2-HANQ.

Table 25 – ELOQ Fischer 344 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 (%)
	2.611				9.1	
	2.438				1.8	
2.394	2.422	2.477	0.069	2.8	1.2	3.4
	2.478				3.5	
	2.456				2.6	
	2.454				2.5	

Table 26 – ELOQ Fischer 344 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 (%)
	30.77				1.9	
	30.03				-0.5	
30.18	30.17	30.36	0.31	1.0	0.0	0.6
	30.72				1.8	
	30.31				0.4	
	30.17				0.0	

4.5.7 Dilution QC

The results from the various experiments of diluting a Fischer 344 rat urine QC into the standard curve range are shown in Tables 27 (1-HANQ) and 28 (2-HANQ).

Table 27 – Fischer 344 Rat Urine Dilution QC Results (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
	25.74				4.2	
24.70	24.18	24.86	0.66	2.6	-2.1	0.6
	24.91				0.9	
	24.61				-0.4	

Table 28 – Fischer 344 Rat Urine Dilution QC Results (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
	282.5				-3.4	
292.4	279.5	281.0	1.2	0.4	-4.4	-3.9
	280.8				-4.0	
	281.4				-3.8	

4.5.8 Recovery

The results of the recovery analyses are shown in Tables 29 and 30.

The recoveries of 1-HANQ are shown in Table 29.

Table 29 – Recovery Results (1-HANQ)

Target Conc (µg/mL)	Avg Recovery (%)	Grand Avg Recovery (%)	s
15	103.1		
12	94.4		
9	92.4	93.4	5.1
7.2	91.3		
4.5	88.8		
2.4	90.4		

The recoveries of 2-HANQ are shown in Table 30. The recovery of the IS was 97.6 ± 3.8%.

Table 30 – Recovery Results (2-HANQ)

Target Conc (µg/mL)	Avg Recovery (%)	Grand Avg Recovery (%)	s
200	106.0		
150	96.9		
120	95.5	97.5	4.3
90	95.9		
60	94.0		
30	96.4		

4.6 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.

4.6.1 Linearity

The standard curves for both analytes had correlation coefficient values greater than 0.998 (Tables 5 and 6). This indicates the model has an appropriate fit over the selected concentration range.

4.6.2 Specificity

The method met acceptance criteria. There were no peaks that interfered with either analyte or the internal standard. The y-intercept values were slightly negative for 4 of the 6 curves and statistically equal to zero for 3 of the 6 curves.

4.6.3 Accuracy

The within day average relative errors met all acceptance criteria. The maximum relative error for an individual standard on any day was 5.0% for 1-HANQ in run 1 (Table 7) and 2.8 for 2-HANQ in run 1 (Table 8).

The maximum average relative error for any standard over all days was 3.6% for 1-HANQ (Table 7) and -2.0 for 2-HANQ (Table 12).

The within day and overall average relative errors for the Fischer 344 rat urine QCs met acceptance criteria in all days. The maximum within day relative error for 1-HANQ was 5.3% for the high QC on Day 3 (Table 21) and the maximum overall average relative error for 1-HANQ was 2.3% for the high QC (Table 21). The maximum within day relative error for 2-HANQ was 6.2% for the high QC on Day 3 (Table 22) and the maximum overall average relative error for 2-HANQ was 2.8% for the low QC (Table 22).

4.6.4 Precision

The overall RSDs for all standards met acceptance criteria with a maximum value of 2.1% for 1-HANQ (Table 23) and 1.9% for 2-HANQ (Table 24).

The within day RSDs for the QCs met acceptance criteria with a maximum value for 1-HANQ of 2.4% for the high QC in run 3 (Table 19) and for 2-HANQ of 6.6% for the high QC in run 3 (Table 20).

The between day RSDs met acceptance criteria with a maximum value of 2.6% for 1-HANQ (Table 21) and 3.3% for 2-HANQ (Table 22).

4.6.5 Sensitivity – ELOQ QCs

The ELOQ QCs met all acceptance criteria for the lowest standard evaluated (2.394 µg/mL for 1-HANQ and 30.18 µg/mL for 2-HANQ) (Tables 25 and 26).

4.6.6 Dilution QC

The results of the urine dilution experiments indicated that QCs with concentrations greater than the highest standard can be diluted (Tables 27 and 28).

4.6.7 Recovery

The recoveries of both analytes and the IS were acceptable (Tables 29 and 30).

4.6.8 Ruggedness

The data from runs conducted by different analysts, using multiple instruments and multiple LC columns from the same supplier were essentially equivalent. This indicates that trained analysts can produce acceptable data and that the method can utilize equivalent columns.

4.7 Stability

4.7.1 Study Designs

The stability studies were designed to evaluate the stability of the analyte under those conditions which would apply to typical samples (storage, freeze/thaw cycles and extract storage).

The rat stability study was conducted using the QCs from the validation.

4.7.2 Results

4.7.2.1 Freeze/Thaw

The determined concentrations of the QCs that had undergone 3 freeze/thaw cycles are shown in Tables 31 and 32. These values agreed very well with the QCs from the same run (Run 3), which had not been subjected to multiple freeze/thaw cycles.

Table 31 – Freeze/Thaw Results (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	Avg RE (%)
4.940	4.916 4.920 4.904 4.987	4.932	-0.2
9.880	10.32 10.36 10.76 10.78	10.56	6.8

Table 32 – Freeze/Thaw Results (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	Avg RE (%)
58.48	61.02 61.71 61.71 61.24	61.42	5.0
117.0	124.1 123.8 124.3 124.4	124.2	6.1

4.7.2.2 Storage

The determined concentrations of the QCs that had been stored for 61 days at -20°C are shown in Tables 33 and 34.

Table 33 – Storage Results (1-HANQ)

Avg Det'd Conc from Validation (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	% Difference
4.949	4.278 4.256 4.275 4.164	4.243	-14.3
10.11	8.805 8.837 8.794 8.859	8.824	-12.7

Table 34 – Storage Results (2-HANQ)

Avg Det'd Conc from Validation (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	% Difference
60.09	54.36 54.22 54.46 54.32	54.34	-9.6
120.1	110.2 111.6 111.8 111.2	111.2	-7.4

4.7.2.3 Extract Stability

Extracts, which had been stored for at least 48 hours did not show any significant differences to the values from their original analyses.

4.7.3 Discussion and Conclusions

The analytes were stable under all the conditions that were evaluated.

5 ACKNOWLEDGMENTS

Tudor Fernando and Darren Brown conducted the analytical work. Steven Graves wrote this report. Melissa Cloud reviewed the data and report for completeness and accuracy. Assessment of the overall quality of the data and report was performed by Kathleen Reed.

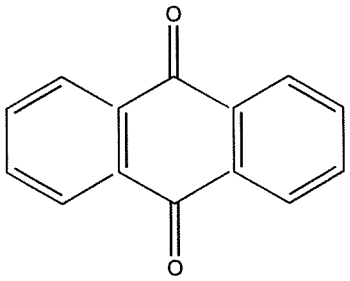
APPENDIX A – REPORT DATA SUMMARY

BIOLOGICAL SAMPLE ANALYSIS METHOD DEVELOPMENT

Name: Anthraquinone
Molecular Formula: $C_{14}H_8O_2$
Molecular Weight: 208.2 g/mol
Sources: 1-Hydroxyanthraquinone – ChemService
2-Hydroxyanthraquinone – Narchem Corporation

Lot Nos.: 1-Hydroxyanthraquinone – 254-2B
2-Hydroxyanthraquinone – 33-217-H

Structure:



METHOD DEVELOPMENT

The instrumental analysis methods selected for initial evaluation were based on previous chromatographic work that had been done for the analysis of anthraquinone. This work included developing chromatographic systems capable of resolving the hydroxyanthraquinone and nitro anthracenes from each other and anthraquinone.

High performance liquid chromatographic systems with ultraviolet and fluorescence detection and a gas chromatographic system with flame ionization detection were evaluated for sensitivity and specificity. The high performance liquid chromatographic system was found to have the best sensitivity and specificity.

Sample preparation was done using liquid-liquid extraction with a variety of solvents. Ethyl acetate was found to be the best solvent based on its ease of use, ability to extract the anthraquinones and cleanliness of the extract.

An evaluation was done of the resulting method. This evaluation indicated that the method could quantify 1-hydroxyanthraquinone down to approximately 10 ng/mL and 2-hydroxyanthraquinone down to approximately 25 ng/mL in a 2-mL sample.

The method was subsequently modified prior to validation for a higher concentration range based on the analysis of the Fischer 344 rat urine samples from the metabolism study that showed the actual sample concentrations would be significantly higher than the original curve range.

METHOD VALIDATION

This section describes the validation design, the analytical method, and the results and conclusions from the validation.

Experimental Design

The validation was designed to be conducted in three separate runs. It also included the use of at least two analysts, two different instruments and two different LC columns from the same supplier. The acceptance criteria were based on the SOP for conducting BSMD tasks. The design is summarized in Table 1.

Table 1 – Validation Design Summary

Element	Design	Acceptance Criteria
Linearity/Curve Fit	Analyze duplicate aliquots of Fischer 344 rat urine standards for each run. Analyze a single Fischer 344 rat urine blank and a single Fischer 344 rat urine blank with IS.	The correlation coefficient for linear models will be ≥0.99. The coefficient of determination for quadratic models will be ≥0.98. Determine and report regression parameters for most acceptable model and weighting.
Specificity	In the validation run, analyze at least six Fischer 344 rat urine samples from different sources and analyze a single specificity blank + IS.	Any peak with retention time of 1-HANQ or 2-HANQ or the IS will have an average response <30% of the lowest standard. Evaluate calculated y-intercept from standard curves.
Precision	Calculate relative standard deviations for QCs.	Values ≤20%.
Accuracy	Calculate individual and average relative errors for standards and QCs.	Average values within 20% of nominal.
Sensitivity (ELOQ)	Evaluate using six replicates of the lowest vehicle standard during one run.	Average relative error within 20% of nominal and relative standard deviations of ≤20%.
Recovery	Compare responses of extracted urine standards to solvent standards.	Recovery of ≥85 ± 15%.
Storage Stability	Compare data from the initial analysis of both levels of QCs to data from stored QCs.	None; determine and report.
Freeze/Thaw Stability	Compare data from the initial analysis of both levels of QCs to data from QCs which have undergone three freeze/thaw cycles.	None; determine and report.
Processed Sample Stability	Compare Fischer 344 rat urine standard and QC values following storage of the LC vials at room temperature in the light for at least 48 hours after initial analysis to values of unstored Fischer 344 rat urine standards and QCs.	All original acceptance criteria should be met.
Sample Dilution	Dilute a rat urine QC into the range of the standard curve and analyze. If needed this will be performed during analysis of the actual study samples.	None; determine and report

Method

Preparation and Storage of Standards

Two stock standards were prepared at target concentrations of 150 (A) and 120 (B) µg/mL of 1-HANQ and 2000 (A) and 1500 (B) µg/mL of 2-HANQ by dissolving 15 ± 0.3 and 12 ± 0.3 mg of 1-HANQ and 200 ± 3 and 150 ± 3 mg of 2-HANQ, respectively, in acetone, diluting to a final volume of 100 mL with acetone and mixing well. These solutions were used throughout the validation. They were stored at 2-8°C when not in use. They were also used as the two most concentrated spiking standards.

Spiking standards were prepared by dilution of the stock standards, A and B, with acetone as shown in Table 2. A single spiking standard was prepared at each concentration for each validation run.

Table 2 – Preparation of Spiking Standards

Standard	1 HANQ Target Conc (µg/mL)	2 HANQ Target Conc (µg/mL)	Source	Source Vol (mL)	Final Vol (mL)
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

Five hundred (500) µL of each spiking standard was pipetted into individual 5-mL volumetric flasks containing approximately 2.5 mL of blank Fischer 344 rat urine. The flasks were diluted to volume with blank rat urine, sealed, and shaken. This produced urine standards with target concentrations of 15, 12, 9, 7.2, 4.5, and 2.4 µg/mL for 1-HANQ and 200, 150, 120, 90, 60, and 30 µg/mL of 2-HANQ. A single urine standard at each concentration was prepared for each run and used on the day of preparation. Duplicate 2-mL aliquots of each standard were pipetted into individual extraction tubes.

Solvent recovery 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]. Milli-Q water has a resistivity =18 megohm/cm. 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 recovery standard was made from each spiking standard. An aliquot of each solvent recovery standard was transferred to an autoinjector vial and the vials were sealed.

Preparation of Blank Fischer 344 Rat Urine

Blank Fischer 344 rat urine was prepared by diluting 0.5 mL of acetone to 5 mL with blank Fischer 344 rat urine. 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.

Preparation of Fischer 344 Rat Urine Quality Control (QC) Sample

One stock solution (C) was prepared at a target concentration of 250 µg/mL of 1-HANQ and 3000 µg/mL of 2-HANQ by dissolving 12.5 ± 0.2 of 1-HANQ and 150 ± 5 mg of 2-HANQ, respectively, in acetone, diluting to a final volume of 50 mL with acetone and mixing well.

Spiking standards were prepared by dilution of the stock solution C with acetone as shown in Table 3.

Table 3 – Preparation of QC Spiking Solutions

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

Five (5) mL of stock or spiking solution was pipetted into individual 50-mL volumetric flasks containing approximately 25 mL of blank rat urine. The flasks were diluted to volume with blank rat urine, sealed, and shaken. This produced urine QCs with target concentrations of 25 (dilution QC), 10, and 5 µg/mL for 1-HANQ and 300 (dilution QC), 120, and 60 µg/mL of 2-HANQ.

Two (2)-mL aliquots of QC were pipetted into individual extraction tubes. The tubes were sealed and stored at -20°C until used during an analysis. Four replicates of the dilution QC were analyzed with one run. Four replicates of each of the other QCs were analyzed with each run.

Two and one-half (2.5) mL of spiking standard S6 was pipetted into a 25-mL volumetric flask approximately half full with blank Fischer 344 rat urine. The flask was diluted to volume with blank rat urine and sealed. The contents were mixed well. Two (2)-mL aliquots were pipetted into six extraction tubes.

Preparation of Specificity Samples

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

Preparation of Fischer 344 Rat Urine Standards, Blanks, QCs, and Specificity Samples for Analysis

Fischer 344 rat urine standards, blanks, QCs, and specificity samples were prepared for analysis by adding 200 µL of blank Fischer 344 rat urine 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 the internal standard [an approximately 320 µg/mL solution of 9-anthracenemethanol in acetone:Milli-Q water (80:120 v/v)] was added to each tube except for the blanks without internal standard to which 200 µL of acetone was added instead. Two (2) mL of ethyl acetate was added to each tube and the tubes were rotated for approximately 5 minutes and centrifuged at a setting of approximately 2000 rpm for approximately 5 minutes. The top (ethyl acetate) layer was transferred to another tube. The extraction was repeated twice using the same procedure with the extracts being combined in the second tube. The combined extract was evaporated to dryness using nitrogen and a water bath set at approximately 50°C. The residue was reconstituted in 10 mL of HPLC mobile phase B. An aliquot was transferred to an autoinjector vial and the vial was sealed.

Analysis

Single injections were made from each vial using the HPLC system shown in Table 4. Representative overlaid chromatograms of a high and low standard, blank with IS, and blank from one validation run are shown in full (top) and reduced (bottom) scale in Figure 1. The chromatograms for the ELOQ QCs were virtually identical to those of the lowest standard.

Table 4 – HPLC System

Instrument	Waters Module 1-Plus and Model 2690 (Milford, MA); Agilent 1100 Series (Palo Alto, CA)
Column	Phenomenex (Torrance, CA) Inertsil ODS-2, 150 mm x 3.00 mm (ID), 5μ (or equivalent)
Guard Column	Phenomenex Inertsil ODS-2 Guard Cartridge
Mobile Phase Components	A: 25:75 (v:v) of Acetonitrile:Milli-Q Water B: 75:25 (v:v) of Acetonitrile:Milli-Q Water
Mobile Phase Gradient	Time (minutes) %A %B All changes are linear with time. 0 100 0 5 0 100 25 0 100 26 100 0 35 100 0
Column Flow Rate	0.8 mL/minute
Injection Volume	20 μL in first run, 10 μL in all other runs
Detector Type	UV
Detector Wavelength	260 nm

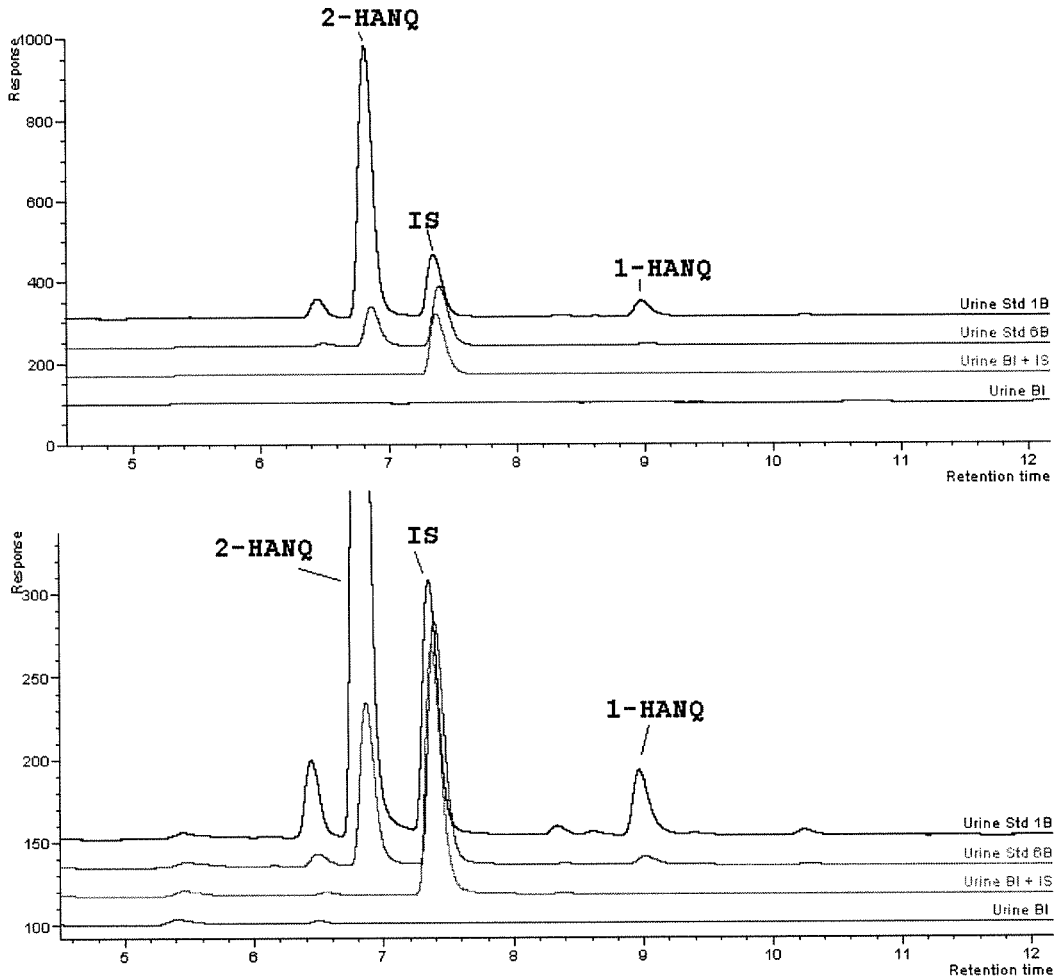


Figure 1 – Representative Overlaid Chromatograms of a High and Low Fischer 344 Rat Urine Standard, Blank with IS, and Blank (Full Scale – Top and Reduced Scale – Bottom)

Calculations

The 1- and 2-HANQ and the IS peaks were integrated by the data system and manually reintegrated, if necessary, to assure proper integration.

A 1/x weighted linear regression equation was calculated for each analyte relating the response ratio of the analyte/IS (y) to its concentration (x) in the urine standards. The use of weighting was found to be necessary to achieve acceptable accuracy at the low concentration of the curves. The analyte concentration of each standard was calculated using its individual response and the regression equation. These values were used to calculate the individual and average concentrations and relative errors, standard deviation and percent relative standard deviation as appropriate at each concentration. The response ratios of the QCs were used to calculate their individual concentrations. These values were used to calculate the individual and average concentrations and relative errors, standard deviation and percent relative standard deviation as appropriate at each concentration.

Recovery was calculated at each standard concentration by dividing the response of the analyte in the urine standard by the response of the analyte in the solvent standard. The average recovery and standard deviation were calculated from the individual values.

Results

Linearity

The critical regression parameters are shown in Tables 5 (1-HANQ) and 6 (2-HANQ). The regression curves from the first validation run are shown in Figure 2. The curves from the other runs were similar.

Table 5 – Regression Curve Parameters – 1-HANQ

Run	Slope	y Intercept	Std Error	Correlation Coefficient
1	0.0165	-0.0025	0.0041	0.9986
2	0.0206	-0.0027	0.0019	0.9998
3	0.0158	-0.0063	0.0047	0.9981

Table 6 – Regression Curve Parameters – 2-HANQ

Run	Slope	y Intercept	Std Error	Correlation Coefficient
1	0.0194	0.0293	0.0343	0.9996
2	0.0228	-0.0039	0.0272	0.9998
3	0.0186	0.0263	0.0222	0.9998

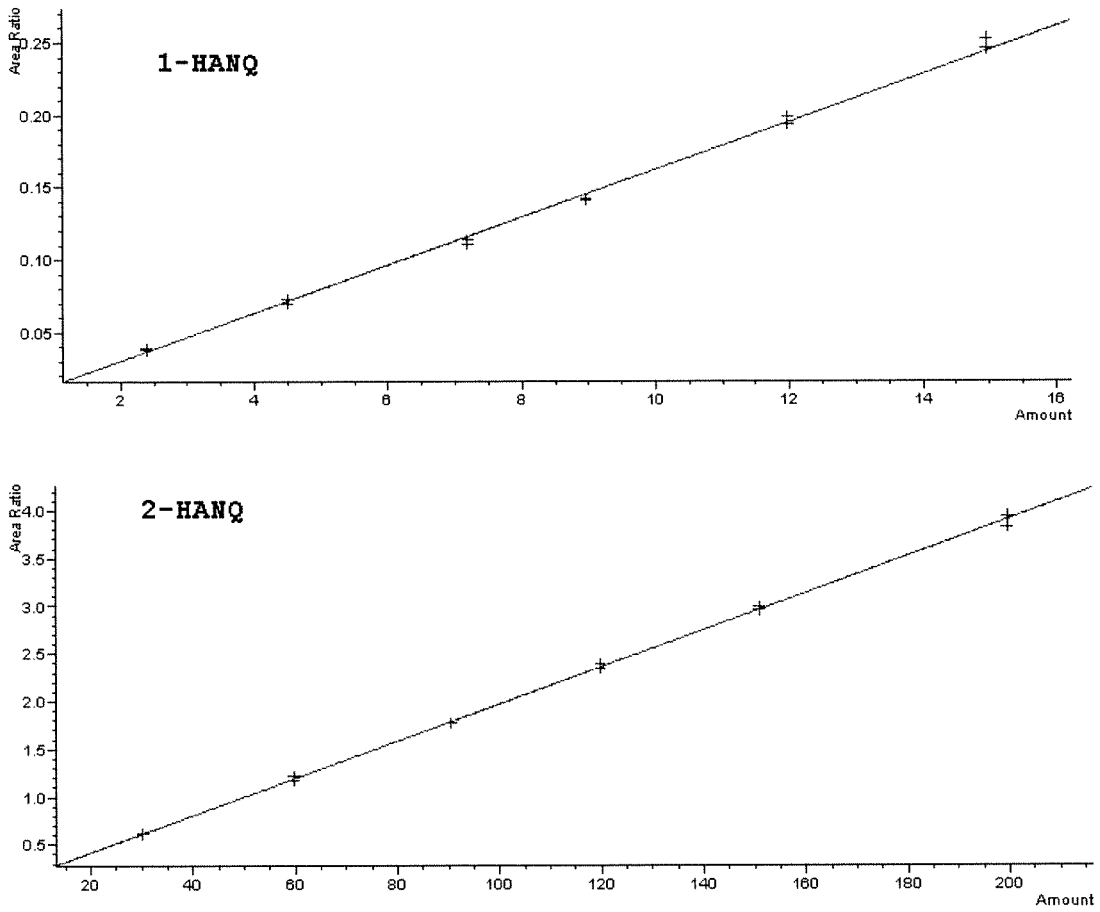


Figure 2 – Regression Curves (Run 1) Fischer 344 Rat Urine

Specificity

The chromatograms from the multiple specificity samples did not have any peaks with interfered with either analyte or the IS. Chromatograms of a representative low standard and the specificity samples are presented in Figure 3.

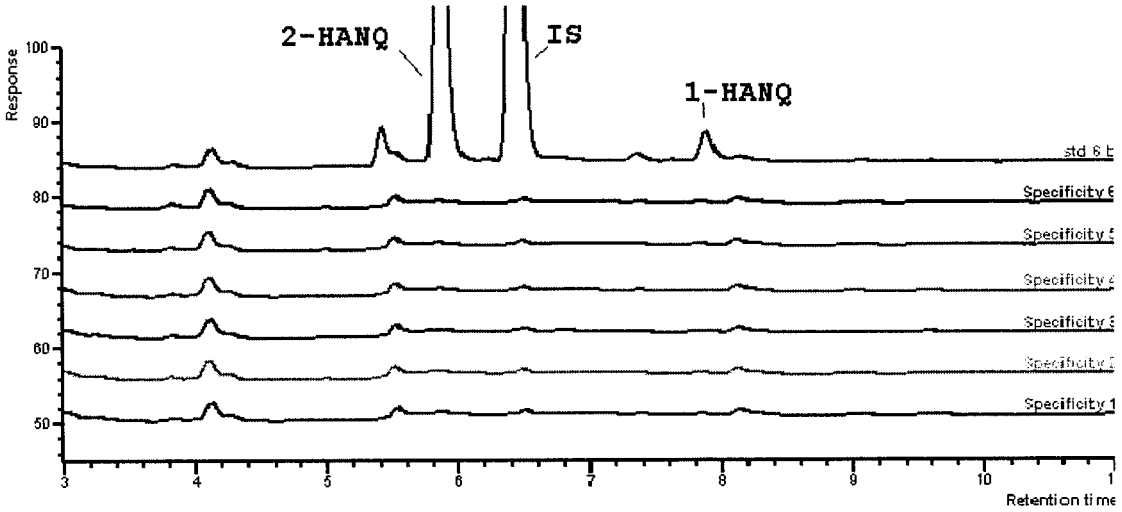


Figure 3 – Overlaid Chromatograms of a Representative Low Urine Standard and Fischer 344 Rat Urine Specificity Samples

Accuracy - Standards

The results for the Fischer 344 rat urine standards from the individual runs are shown in Tables 7 through 12. The average accuracy data for all runs are shown in Tables 13 and 14.

Table 7 – Run 1 Fischer 344 Rat Urine Standards (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
14.97	15.09	15.52	15.31	0.8	3.7	2.2
11.97	11.89	12.20	12.05	-0.7	1.9	0.6
8.982	8.727	8.764	8.746	-2.8	-2.4	-2.6
7.182	6.885	7.015	6.950	-4.1	-2.3	-3.2
4.491	4.544	4.392	4.468	1.2	-2.2	-0.5
2.394	2.514	2.444	2.479	5.0	2.1	3.6

Table 8 – Run 1 Fischer 344 Rat Urine Standards (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
199.5	195.4	201.9	198.7	-2.0	1.2	-0.4
150.9	150.6	152.4	151.5	-0.2	1.0	0.4
119.7	119.3	121.2	120.3	-0.3	1.3	0.5
90.55	89.90	89.98	89.94	-0.7	-0.6	-0.7
59.84	61.54	58.99	60.26	2.8	-1.4	0.7
30.18	30.08	29.98	30.03	-0.3	-0.7	-0.5

Table 9 – Run 2 Fischer 344 Rat Urine Standards (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
14.97	14.88	15.18	15.03	-0.6	1.4	0.4
11.97	11.95	11.95	11.95	-0.2	-0.2	-0.2
8.982	8.882	9.098	8.990	-1.1	1.3	0.1
7.182	7.108	7.186	7.147	-1.0	0.1	-0.5
4.491	4.457	4.482	4.469	-0.8	-0.2	-0.5
2.394	2.413	2.409	2.411	0.8	0.6	0.7

Table 10 – Run 2 Fischer 344 Rat Urine Standards (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
199.5	200.0	200.0	200.0	0.3	0.3	0.3
150.9	152.3	148.4	150.3	0.9	-1.7	-0.4
119.7	120.7	119.6	120.2	0.9	-0.1	0.4
90.55	91.35	89.28	90.31	0.9	-1.4	-0.3
59.84	60.31	58.66	59.49	0.8	-2.0	-0.6
30.18	30.66	30.05	30.36	1.6	-0.5	0.6

Table 11 – Run 3 Fischer 344 Rat Urine Standards (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
14.97	15.09	15.67	15.38	0.8	4.7	2.7
11.97	NV	11.80	11.80	NV	-1.4	-1.4
8.982	8.595	9.004	8.800	-4.3	0.2	-2.0
7.182	6.882	7.092	6.987	-4.2	-1.3	-2.7
4.491	4.393	4.535	4.464	-2.2	1.0	-0.6
2.394	2.452	2.494	2.473	2.4	4.2	3.3

NV = No value; wrong standard appears to have been used for this aliquot.

Table 12 – Run 3 Fischer 344 Rat Urine Standards (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)		Avg Det'd Conc (µg/mL)	RE (%)		Avg RE (%)
199.5	200.0	199.7	199.8	0.2	0.1	0.2
150.9	NV	147.9	147.9	NV	-2.0	-2.0
119.7	120.4	119.5	119.9	0.6	-0.2	0.2
90.55	90.77	90.92	90.85	0.3	0.4	0.3
59.84	60.80	61.03	60.91	1.6	2.0	1.8
30.18	30.02	29.44	29.73	-0.6	-2.5	-1.5

NV = No value; wrong standard appears to have been used for this aliquot.

Table 13 – Standard Accuracy (% Relative Error) All Runs – 1-HANQ

Nominal Conc (µg/mL)	Avg Daily RE (%)			Grand Avg RE (%)
14.97	2.2	0.4	2.7	1.8
11.97	0.6	-0.2	-1.4	-0.1
8.982	-2.6	0.1	-2.0	-1.5
7.182	-3.2	-0.5	-2.7	-2.1
4.491	-0.5	-0.5	-0.6	-0.5
2.394	3.6	0.7	3.3	2.5

Table 14 – Standard Accuracy (% Relative Error) All Runs – 2-HANQ

Nominal Conc (µg/mL)	Avg Daily RE (%)			Grand Avg RE (%)
199.5	-0.4	0.3	0.2	0.0
150.9	0.4	-0.4	-2.0	-0.4
119.7	0.5	0.4	0.2	0.3
90.55	-0.7	-0.3	0.3	-0.2
59.84	0.7	-0.6	1.8	0.6
30.18	-0.5	0.6	-1.5	-0.5

Precision and Accuracy – QC Samples

The results from the Fischer 344 rat urine QCs from the three validation runs are shown in Tables 15 through 20. Tables 21 and 22 summarize the day-to-day QC results.

Table 15 – Run 1 Fischer 344 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 (%)
4.940	4.976	4.960	0.062	1.3	0.7	0.4
	5.003				1.3	
	4.993				1.1	
	4.868				-1.5	
9.880	10.07	10.03	0.15	1.5	1.9	1.5
	9.967				0.9	
	10.21				3.3	
	9.864				-0.2	

Table 16 – Run 1 Fischer 344 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 (%)
58.48	60.09	59.92	0.48	0.8	2.7	2.5
	60.50				3.5	
	59.66				2.0	
	59.42				1.6	
117.0	119.8	119.6	1.3	1.1	2.4	2.2
	119.5				2.1	
	121.1				3.5	
	118.0				0.9	

Table 17 – Run 2 Fischer 344 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 (%)
4.940	4.952	4.967	0.048	1.0	0.2	0.6
	4.958				0.4	
	4.924				-0.3	
	5.036				1.9	
9.880	9.848	9.897	0.078	0.8	-0.3	0.2
	9.860				-0.2	
	10.01				1.4	
	9.865				-0.1	

Table 18 – Run 2 Fischer 344 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 (%)
58.48	58.96	58.99	0.49	0.8	0.8	0.9
	58.89				0.7	
	58.48				0.0	
	59.65				2.0	
117.0	116.1	116.4	1.0	0.8	-0.7	-0.5
	116.0				-0.9	
	117.8				0.7	
	115.7				-1.1	

Table 19 – Run 3 Fischer 344 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 (%)
4.940	4.885	4.921	0.045	0.9	-1.1	-0.4
	4.885				-1.1	
	4.935				-0.1	
	4.979				0.8	
9.880	10.47	10.40	0.25	2.4	6.0	5.2
	10.07				1.9	
	10.38				5.1	
	10.66				7.9	

Table 20 – Run 3 Fischer 344 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 (%)
58.48	61.29	61.35	0.23	0.4	4.8	4.9
	61.31				4.8	
	61.68				5.5	
	61.15				4.6	
117.0	124.7	124.3	8.3	6.6	6.6	6.2
	113.7				-2.8	
	124.8				6.7	
	133.9				14.5	

Table 21 – Summary of QC Results (1-HANQ)

QC	Run	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
4.940	1	4.960	4.949	0.025	0.5	0.4	0.2
	2	4.967				0.5	
	3	4.921				-0.4	
9.880	1	10.03	10.11	0.26	2.6	1.5	2.3
	2	9.897				0.2	
	3	10.40				5.3	

Table 22 – Summary of QC Results (2-HANQ)

QC	Run	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
58.48	1	59.92	60.09	1.19	2.0	2.5	2.8
	2	58.99				0.9	
	3	61.35				4.9	
117.0	1	119.6	120.1	4.0	3.3	2.2	2.6
	2	116.4				-0.5	
	3	124.3				6.2	

Precision - Standards

The results from the analysis of the standards from all validation runs are shown in Tables 23 and 24.

Table 23 – Summary of Fischer 344 Rat Urine Standards (1-HANQ - Between Days)

Run	Avg Det'd Conc (µg/mL)					
1	15.31	12.05	8.746	6.950	4.468	2.479
2	15.03	11.95	8.990	7.147	4.469	2.411
3	15.38	11.80	8.800	6.987	4.464	2.473
Avg Conc*	15.24	11.96	8.845	7.028	4.467	2.454
s*	0.30	0.15	0.186	0.124	0.066	0.042
RSD*	2.0	1.2	2.1	1.8	1.5	1.7

* Calculated using determined concentrations.

Table 24 – Summary of Fischer 344 Rat Urine Standards (2-HANQ - Between Days)

Run	Avg Det'd Conc (µg/mL)					
1	198.7	151.5	120.3	89.94	60.26	30.03
2	200.0	150.3	120.2	90.31	59.49	30.36
3	199.8	147.9	119.9	90.85	60.91	29.73
Avg Conc*	199.5	150.3	120.1	90.37	60.22	30.04
s*	2.1	2.1	0.8	0.77	1.16	0.39
RSD*	1.1	1.4	0.6	0.9	1.9	1.3

* Calculated using determined concentrations.

Limit of Quantitation QCs

The results from the Fischer 344 rat urine ELOQ QCs are shown in Tables 25 (1-HANQ) and 26 (2-HANQ). The limit of detection, defined as three times the standard deviation of the ELOQ QC, was 0.2055 for 1-HANQ and 0.9292 µg/mL for 2-HANQ. The limit of quantitation, defined as ten times the standard deviation of the ELOQ QC, was 0.6851 for 1-HANQ and 3.097 µg/mL for 2-HANQ.

Table 25 – ELOQ Fischer 344 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 (%)
	2.611				9.1	
	2.438				1.8	
	2.422				1.2	
2.394	2.478	2.477	0.069	2.8	3.5	3.4
	2.456				2.6	
	2.454				2.5	

Table 26 – ELOQ Fischer 344 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 (%)
	30.77				1.9	
	30.03				-0.5	
30.18	30.17	30.36	0.31	1.0	0.0	0.6
	30.72				1.8	
	30.31				0.4	
	30.17				0.0	

Dilution QC

The results from the various experiments of diluting a Fischer 344 rat urine QC into the standard curve range are shown in Tables 27 (1-HANQ) and 28 (2-HANQ).

Table 27 – Fischer 344 Rat Urine Dilution QC Results (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
	25.74				4.2	
24.70	24.18	24.86	0.66	2.6	-2.1	0.6
	24.91				0.9	
	24.61				-0.4	

Table 28 – Fischer 344 Rat Urine Dilution QC Results (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s	RSD (%)	RE (%)	Avg RE (%)
	282.5				-3.4	
292.4	279.5	281.0	1.2	0.4	-4.4	-3.9
	280.8				-4.0	
	281.4				-3.8	

Recovery

The results of the recovery analyses are shown in Tables 29 and 30. The recoveries of 1-HANQ are shown in Table 29.

Table 29 – Recovery Results (1-HANQ)

Target Conc (µg/mL)	Avg Recovery (%)	Grand Avg Recovery (%)	s
15	103.1		
12	94.4		
9	92.4	93.4	5.1
7.2	91.3		
4.5	88.8		
2.4	90.4		

The recoveries of 2-HANQ are shown in Table 30. The recovery of the IS was 97.6 ± 3.8%.

Table 30 – Recovery Results (2-HANQ)

Target Conc (µg/mL)	Avg Recovery (%)	Grand Avg Recovery (%)	s
200	106.0		
150	96.9		
120	95.5	97.5	4.3
90	95.9		
60	94.0		
30	96.4		

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.

Linearity

The standard curves for both analytes had correlation coefficient greater than 0.998 (Tables 5 and 6). This indicates the model has an appropriate fit over the selected concentration range.

Specificity

The method met acceptance criteria. There were no peaks that interfered with either analyte or the internal standard. The y-intercept values were slightly negative for 4 of the 6 curves and statistically equal to zero for 3 of the 6 curves.

Accuracy

The within day average relative errors met all acceptance criteria. The maximum relative error for an individual standard on any day was 5.0% for 1-HANQ in run 1 (Table 7) and 2.8 for 2-HANQ in run 1 (Table 8).

The maximum average relative error for any standard over all days was 3.6% for 1-HANQ (Table 7) and -2.0 for 2-HANQ (Table 12).

The within day and overall average relative errors for the Fischer 344 rat urine QCs met acceptance criteria in all days. The maximum within day relative error for 1-HANQ was 5.3% for the high QC on Day 3 (Table 21) and

the maximum overall average relative error for 1-HANQ was 2.3% for the high QC (Table 21). The maximum within day relative error for 2-HANQ was 6.2% for the high QC on Day 3 (Table 22) and the maximum overall average relative error for 2-HANQ was 2.8% for the low QC (Table 22).

Precision

The overall RSDs for all standards met acceptance criteria with a maximum value of 2.1% for 1-HANQ (Table 23) and 1.9% for 2-HANQ (Table 24).

The within day RSDs for the QCs met acceptance criteria with a maximum value for 1-HANQ of 2.4% for the high QC in run 3 (Table 19) and for 2-HANQ of 6.6% for the high QC in run 3 (Table 20).

The between day RSDs met acceptance criteria with a maximum value of 2.6% for 1-HANQ (Table 21) and 3.3% for 2-HANQ (Table 22).

Sensitivity – ELOQ QCs

The ELOQ QCs met all acceptance criteria.for the lowest standard evaluated (2.394 µg/mL for 1-HANQ and 30.18 µg/mL for 2-HANQ) (Tables 25 and 26).

Dilution QC

The results of the urine dilution experiments indicated that QCs with concentrations greater than the highest standard can be diluted (Tables 27 and 28).

Recovery

The recoveries of both analytes and the IS were acceptable (Tables 29 and 30).

Ruggedness

The data from runs conducted by different analysts, using multiple instruments and multiple LC columns from the same supplier were essentially equivalent. This indicates that trained analysts can produce acceptable data and that the method can utilize equivalent columns.

Stability

Study Designs

The stability studies were designed to evaluate the stability of the analyte under those conditions which would apply to typical samples (storage, freeze/thaw cycles and extract storage).

The rat stability study was conducted using the QCs from the validation.

Results

The determined concentrations of the QCs that had undergone 3 freeze/thaw cycles are shown in Tables 31 and 32. These values agreed very well with the QCs from the same run (Run 3), which had not been subjected to multiple freeze/thaw cycles.

Table 31 – Freeze/Thaw Results (1-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	Avg RE (%)
4.940	4.916 4.920 4.904 4.987	4.932	-0.2
9.880	10.32 10.36 10.76 10.78	10.56	6.8

Table 32 – Freeze/Thaw Results (2-HANQ)

Nominal Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	Avg RE (%)
58.48	61.02 61.71 61.71 61.24	61.42	5.0
117.0	124.1 123.8 124.3 124.4	124.2	6.1

The determined concentrations of the QCs that had been stored for 61 days at -20°C are shown in Tables 33 and 34.

Table 33 – Storage Results (1-HANQ)

Avg Det'd Conc from Validation (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	% Difference
4.949	4.278 4.256 4.275 4.164	4.243	-14.3
10.11	8.805 8.837 8.794 8.859	8.824	-12.7

Table 34 – Storage Results (2-HANQ)

Avg Det'd Conc from Validation (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	% Difference
60.09	54.36 54.22 54.46 54.32	54.34	-9.6
120.1	110.2 111.6 111.8 111.2	111.2	-7.4

Extracts, which had been stored for at least 48 hours did not show any significant differences to the values from their original analyses.

Discussion and Conclusions

The analytes were stable under all the conditions that were evaluated.