Final Report

ETHYL TERTIARY BUTYL ETHER ORAL ABSORPTION IN MALE AND FEMALE RATS

SUBMITTED TO:

Section 211(b) Research Group
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RTI Protocol:	RTI-932
RTI Study Code:	Rt05-932
RTI Report No.	RTI/0209408.007 and 0212607.000

February 12, 2015

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Study Director

**RTI International is a trade name of Research Triangle Institute.*

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SUMMARY

Male and female Fischer 344 rats were administered a single gavage dose of ethyl tertiary butyl ether (ETBE) in aqueous solution (50 and 10 mg/kg). Prior to dosing, the rats were implanted with jugular vein cannulas for the serial sampling of blood. Blood samples were withdrawn at approximately 15, 30 min, 1, 2, 4, 8, and 24 hr. Samples were immediately placed in tared headspace vials and crimp sealed. The samples were weighed, and after addition of internal standard (MTBE), were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) equipped with a headspace autosampler. No significant levels of ETBE were detected in the predosing samples collected from each animal. ETBE levels reached maximal values at the first timepoint (0.25 hr) and declined rapidly thereafter, falling to less than the limit of quantitation by 8 hr at the high dose and 2-4 hr at the low dose. Non-compartmental pharmacokinetic analysis was conducted for each animal using WinNonlin. No statistically significant differences between gender were observed in Tmax, apparent Cmax, half life, AUCall, AUCINF(observed), or lamda z. At the high dose, elimination half lives of 1.061 ± 0.326 hr for males and 1.105 ± 0.340 hr for females were observed. At the low dose, elimination half lives of 0.508 ± 0.191 hr for males and 0.573 ± 0.176 hr for females were observed. Differences between dose levels in half life and AUCINF(observed)/Dose may be consistent with saturation of metabolism of ETBE. The study demonstrated that ETBE is taken up following oral administration, and is rapidly eliminated from blood.

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Quality Assurance Statement

Study Title: **Ethyl Tertiary Butyl Ether Oral Absorption in Male and Female Rats**

Sponsor: **Section 211(b) Research Group, American Petroleum Institute**

Protocol Number: **RTI-932**

Study Code: **Rt05-932**

This study was audited by the Regulatory, Quality and Records Management – Quality Assurance Unit and the results of the inspections and audits were reported to the Study Director and management as identified below.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Study Director and Management
Protocol Audit	August 3, 9, 14, 2006	August 14, 2006
Protocol Audit – Revised Protocol	February 15, 2007	February 15, 2007
Protocol Amendment	June 8, 2007	June 8, 2007
Blood Collection Inspection	June 21, 2007	June 21, 2007
Data and Report Audit	October 30-November 8, 2012	November 8, 2012
Report Review, revised draft report	January 12, 2015	January 13, 2015

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Date

Laboratory GLP Compliance Statement

This study was carried out in compliance with the EPA Good Laboratory Practices (GLP) Standards for Inhalation Exposure Health Effects Testing, 40 CFR part 79, subpart F § 79.60.

The method of synthesis of the test chemical ethyl tertiary butyl ether or its location was not available at the time of conduct of the study.

There were no significant protocol deviations (Appendix A) that would affect the integrity or quality of the study or the interpretation of the results. Raw data, and the final report generated as a result of this study are archived by the Sponsor at EPL Archives, Sterling, Virginia.

Timothy R. Fennell, Ph.D. Date

Study Director

Sponsor's Representative Date

Table of Contents

Page

List of Tables

Page

List of Figures

Page

List of Appendices

1.0 INTRODUCTION

Ethyl *tertiary* butyl ether (ETBE) is an oxygenate used in the manufacture of blended gasolines. A number of studies have investigated the metabolism and pharmacokinetics of ETBE following inhalation exposure in rodents and humans (Amberg et al., 2000; Bernauer *et al*., 1998; Dekant *et al*., 2001; Johanson et al., 1995; Nihlen *et al*., 1998a, 1998b). Metabolism of ETBE occurs via oxidation and subsequent conjugation or further oxidation (Bernauer *et al.* 1998; Dekant *et al.* 2001). Physiologically based pharmacokinetic models have been developed to describe the behavior of ETBE in rodents and humans. Despite intensive investigations of the behavior of ETBE following inhalation exposure, the kinetics of uptake and elimination following oral administration in rats had not been investigated previously.

2.0 OBJECTIVES AND PROTOCOL

The objectives of this study on ETBE were to:

Develop a method for the analysis of ETBE in blood by headspace gas chromatography – mass spectrometry (GC-MS).

Conduct an evaluation of oral absorption by measuring ETBE in blood in male and female Fischer 344 rats administered ETBE by gavage at one of two dose levels, 10 and 50 mg/kg.

Copies of the approved protocol and protocol amendments and protocol deviations for these studies are included in Appendix A.

3.0 MATERIALS AND METHODS

3.1 Test Substance

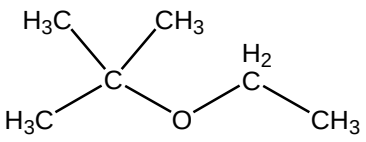
NAME: Ethyl *tertiary* Butyl Ether (ETBE; *tertiary* butyl ethyl ether, 2-ethoxy-2-methylpropane)

CAS No.: 637-92-3

MOLECULAR FORMULA: $C_6H_{14}O$

MOLECULAR WEIGHT: 102.17

STRUCTURE:



SOURCE OF TEST SUBSTANCE: ETBE was purchased from Sigma-Aldrich, Milwaukee, WI (Catalog number 253898, specified purity 99%). A certificate of analysis was obtained from the vendor, which indicated a purity of 99.3%.

LOT NUMBER(S): 04608LD

IDENTITY AND PURITY: The identity of the unlabeled ETBE was confirmed at RTI by ^{1}H and ^{13}C NMR, and by mass spectrometry. Details of the analysis conducted are contained in Appendix B. The purity of the test chemical was determined by GC with flame ionization detection (FID) also contained in Appendix B.

STORAGE CONDITIONS: ETBE was stored in the dark at room temperature.

STABILITY: Upon receipt, the identity and purity of the test substance ETBE was confirmed as described above. Periodically, and at the end of the experimental portion of the study, ETBE was reanalyzed by ^{1}H NMR and by GC-MS to verify stability. Initial analysis in March 2006 indicated a purity of 99.66 $\pm$ 0.04%, with purity following the completion of the study of 99.26 $\pm$ 0.17%. Details of the analysis are included in Appendix B.

3.2 Reference Substances

Methyl tertiary butyl ether (MTBE) was obtained from Sigma Aldrich (Milwaukee, Wi, Catalog number 443808, specified purity 99%). This material was assigned a chemical receipt number of BOC-B-0456.

3.3 Test System

Source: Male Fischer 344 rats for methods development were purchased from Charles River (Kingston, NY). Male and female Fischer 344 rats with jugular vein cannulae were obtained from Harlan Sprague Dawley (Indianapolis, In).

Diet: Animals were fed Certified Purina Rodent Chow #5002 and were furnished tap water *ad libitum*. The analysis of each feed batch for nutrient levels and possible contaminants was performed by the supplier, examined by the Study Director, and maintained in the study records. The feed was stored at approximately 60–70 °F, and the period of use did not exceed six months from the milling date. The source of the water was the City of Durham, NC. Approximately once a year, the City of Durham provides analyses of the drinking water for potential contaminants. Documentation of these analyses were inspected by the Study Director and maintained in the study records. In addition, samples of water were collected during the study for analysis of tertiary butyl alcohol, tertiary amyl methyl ether, ethyl tertiary butyl ether, diisopropyl ether, and methyl tertiary butyl ether. The samples were sent to Kiff Analytical (Davis, CA 95616) for analysis.

Identification: Individual ear tags were used to uniquely identify animals used.

Housing: Rats were housed (maximum of three per cage) in polycarbonate cages with stainless steel bar lids accommodating a water bottle until they were used in an experiment. Cage sizes are 19" x 10.5" x 8" high (143 sq. in. floor space). Contact bedding was Sani-Chips hardwood chips (P. J. Murphy Forest Products Co.; Montville, NJ). Cannulated rats were housed individually in polycarbonate cages with dimensions of 9 ¼" x 8 ¼" x 8" (ca. 76.3 sq. in. floor space), and were used within 1-3 days of arrival at RTI.

Following dosing, the cannulated animals were housed individually in polycarbonate cages with dimensions of 9 ¼" x 8 ¼" x 8" (ca. 76.3 sq. in. floor space), and were placed in a hood.

Quarantine: Rats without cannulas that were used as blood donors were quarantined for a minimum of one week. Rats with implanted jugular vein cannulas received a truncated quarantine period of 1 day. A veterinarian or qualified designee examined the animals prior to their release from quarantine.

Randomization: The ear tag numbers of animals were assigned in numerical order to sequential values using a series of computer generated numbers as described in SOP DPK-HUS-001 Assignment of Animals into Groups, using the procedure for Assignment of Animals within a Single Group or Multiple Groups without Regard to Weight Mean and Range. Four groups were prepared: Study A (n=9), Study B (n=9), Study C (n=9), and Study D (n=9). Animals were ordered specifically for each study, with Studies C and D performed 1 week later than Studies A and B. Of the animals in each group, the first four animals were administered ETBE and the remaining animals were held as extras in the event of dosing accidents or inability to obtain blood samples.

Environmental: Temperature and relative humidity in RTI animal rooms were continuously monitored, controlled, and recorded using an automated system (Siebe/Barber-Colman Network 8000 System with Revision 4.4.1 for Signal® software [Siebe Environmental Controls (SEC)/Barber-Colman Company; Loves Park, IL]). The target environmental ranges were 64–79 °F (18 °C - 26 °C) for temperature and 30–70% relative humidity, with a 12-h light cycle per day.

Euthanasia: At the end of the in-life phase, the rats were euthanized by overexposure to carbon dioxide.

3.4 Test Chemical Preparation and Analysis

ETBE was prepared for gavage dosing as a solution in water. For dosing of animals, the dose solution was prepared within 24 hours of dosing, and maintained in a refrigerator. The nominal concentration was calculated from the weight of ETBE and the weight of water added to the dose preparation. Dose solutions were prepared at two concentrations, the first at approximately 5 mg/ml and the second at 1 mg/ml, to ensure that the volume of dose administered per kg body weight is similar in the high and the low dose groups. Prior to dosing, a method was developed for the quantitative analysis of ETBE in water, by dissolving a weighed aliquot of the ETBE/water solution in methanol (see Appendix C). Aliquots of this solution were analyzed directly by gas chromatography (GC) using a standard curve

for the quantitation of ETBE. The stability of ETBE in water was determined by analysis of aliquots of a mock dose solution of ETBE kept at room temperature for three days (Appendix E).

3.5 Gavage Dosing

Each rat was weighed prior to dosing to determine the amount of dose to be administered. A single gavage dose was administered using a syringe fitted with a blunt tipped gavage dosing needle. The dose administered to each animal was determined from the weight of the full syringe minus that of the empty syringe. The dose time was recorded. Dosing of animals was spaced apart to allow blood collection at the appropriate times (see section 3.6.1 below). The groups were designated according to the dose administered and sex of the rats: Group A male 50 mg/kg, Group B female 50 mg/kg, Group C male 10 mg/kg, and Group D female 10 mg/kg. At least four cannulated rats per group were dosed with ETBE. Group and animal numbers are indicated in Table 1.

3.6 Collection and Analysis of Blood Samples

3.6.1 Blood Sample Collection

Blood samples were collected prior to dosing from each rat (time = 0), from 4 rats of each sex, at each dose at approximately 15 and 30 min, and at approximately 1, 2, 4, 8, and 24 hr after dosing. Jugular vein cannulated rats were maintained with the heparin lock solution supplied by the vendor untouched until the day of dosing. After removing the heparin lock solution, and verification of patency, the initial blood sample (time = 0) was collected. After this and subsequent samples, the cannula was filled with sterile isotonic saline (Baxter Healthcare Corporation), containing 20 units sodium heparin/mL (prepared with sodium heparin, 1000 units/mL, Baxter Healthcare Corporation), until the next sample. Prior to collection of the blood sample, the heparin/saline was removed from the cannula with a syringe and blunt needle until blood filled the cannula. The syringe contents were discarded. A fresh syringe and needle was then used to collect the blood sample.

Blood samples (approximately 100 µl) were drawn from the jugular vein cannula (except those indicated in the protocol deviation list) into a heparinized 1 ml syringe, and the sample was immediately placed in a preweighed headspace vial. The vial was immediately crimped and weighed, and the weight of the blood aliquot was determined. After the last sample had been collected from each animal at 24 hr, the rats were euthanized by exposure to CO_2. When cannulas failed during the collection of samples, several samples were collected at 15 and 30 min, and at 8 hr, via the tail vein, and at 24 hr, blood was collected by cardiac puncture under CO_2 anesthesia at sacrifice. The blood samples were maintained on ice, until placed in the headspace autosampler for analysis. All blood samples were analyzed by GC-MS within 24 hours of collection.

Blood samples from unexposed rats were used for method development. Blood samples were collected by cardiac puncture, under CO_2 anesthesia, and the rats were euthanized.

3.6.2 Blood Sample Analysis

A GC-MS method was developed for quantitating ETBE using blood from control male Fischer 344 rats. The methods involved the development of a standard curve for analysis of ETBE in the headspace of airtight vials containing a known volume of control blood and a known concentration of ETBE. The stability of ETBE was evaluated under storage conditions that were used in this study. This included analysis of ETBE immediately after spiking in control blood, after storage at room temperature (8 hr), and at approximately 4°C (24 hr). The concentration of ETBE in the headspace of vials containing a known volume of blood from rats administered ETBE by gavage was determined by comparison to the standard curve.

3.7 Data Collection and Reporting

Study data was collected and reported in the Debra™ system version 5.5.10.75 (Lablogic Systems Limited, Sheffield, England). This includes data for dosing, dose times, sample collection times, and pot weights and sample weights. The data collected and calculations in Debra™ are described in Appendix F, and were reported with the Debra™ system.

3.7 Pharmacokinetic Analysis

Individual and mean blood concentration-time data for each dose and sex were analyzed by noncompartmental (model-independent) methods using the least-squares fitting program WinNonlin™ (Statistical Consulting Inc., Cary, NC). Pharmacokinetic parameters were extracted from the WinNonlin generated outputs. These include:

Pharmacokinetic Parameters		
WinNonlin Output	**Table/Report**	
Tmax	T_{max}	Time of maximum observed concentration.
Cmax	C_{max}	Maximum observed concentration, occurring at T_{max}
lambda_z	λ_z	Terminal elimination rate constant
t1/2_lamda_z	$T_{1/2\lambda z}$	Terminal elimination half-life
AUCall	AUCall	Area under the plasma concentration-time curve from time zero to the last measured time point
AUCINF(observed)	AUCINF(observed)	Area under the plasma concentration-time curve from time zero extrapolated to infinity
AUCINF(observed)/D	AUCINF(observed)/ Dose	Area under the plasma concentration-time curve from time zero extrapolated to infinity/Dose
Vz(observed)/F*	Vz(observed)/F	Volume of Distribution at steady state
Cl(observed)/F*	Clearance(observed)/F	Systemic clearance
MRTlast	MRTlast	Mean residence time based upon values from the time of dosing up to the last measured concentration

*For extravascular models the fraction of dose absorbed cannot be estimated, therefore Volume of Distribution and Clearance for these models are actually Volume/F or Clearance/F where F is the fraction of dose absorbed.

Statistical comparison of the parameters Tmax, Cmax, t1/2 Lambda z, Lambda z, AUCall, AUCobserved and AUCobserved/Dose were conducted using the Student's t-test available in SAS® Version 9 (SAS Institute Inc., 2004, 2005, 2006a,b). The males were compared to the females within dose group for each parameter. Additionally, for the parameter AUCobserved/Dose the low dose was compared to the high dose within each sex. Grubbs test for outliers was conducted to determine whether individual values were outliers (Grubbs, 1969).

4.0 RESULTS

4.1 Analysis of Test Chemical Identity and Purity

Information on the identity, purity, and stability of the test chemical is included in Appendix B. Prior to study initiation, the test chemical purity was determined by GC to be 99.66 ± 0.04 %. Following completion of the study, test chemical purity was determined to be 99.26 ± 0.17 %, indicating that the test chemical was stable under the conditions of storage.

4.2 Method Validation for Analysis of ETBE in Blood

A method for the determination of ETBE in blood using headspace GC-MS was developed and validated prior to the study conduct. Details of the method, the method validation plan, and the validation report are contained in Appendix C. The analytical method titled Project Specific Method for Analysis of Ethyl tertiary Butyl Ether in Blood Samples (AM-0209408.007) was validated in preparation for analyses of blood samples to determine concentrations of ethyl tertiary butyl ether (ETBE) present in blood in this study. This validation established a Limit of Quantitation (LOQ), accuracy, and precision of the method. The method consists of GC/MS analysis of ETBE with injection of headspace from sample vials.

The validation procedure established a calibration range of 0.110 μg/ml to 250 μg/ml for analysis of ETBE in the headspace of blood samples using two calibration curves. The low-concentration curve was defined by a linear regression (y=bx+a) of slope 0.08065, intercept -0.0006558, and linear correlation coefficient of 0.9998. The high-concentration curve was defined by the linear regression (y=bx+a) of slope 0.09673, intercept -0.1292, and linear correlation coefficient of 0.9994. Using the regression equation, calculated concentrations for all calibration standards were within the acceptance criteria of ± 15% (20% for the LOQ) of the nominal concentrations. The limit of quantitation was established as 0.11 μg/ml. Mean calculated concentrations for the replicate concentration points assayed at 0.540 μg/ml, 5.06 μg/ml, and 101 μg/ml for determination of precision and accuracy ranged from 96.2% to 99.3% of nominal concentration. Precision around the mean for each of these replicate concentration points was calculated to be well within the acceptance criteria of 15% CV. Additionally, the mean values for each

concentration were well within the acceptance criteria of 15% deviation from nominal concentration. Storage stability of ETBE in blood was verified at room temperature for 8 hr, and at approximately 4°C for 24 hr. Storage stability of standard solutions was verified at 7 days at approximately -20°C.

4.3 Animal Dosing

Animals were administered a single gavage dose of ETBE in water. Analysis of the ETBE dose solutions was conducted prior to dosing by GC with FID detection (see Table 2). The concentration of ETBE determined analytically was used to calculate the amount of ETBE dose solution to administer. The amount of dose solution administered was calculated by the difference between the weight of the charged syringe and needle with dose, and the discharged syringe and needle after dosing. Information on the doses administered is reported in Table 3. The doses administered were within ± 10% of the target dose. Doses of ETBE were administered to 4 animals in each group AM, BF, and DF. Five animals were dosed in group CM, but one of these (CM-04) was dosed with less than the expected dose per kg body weight due to a dosing error (16.6 % less than expected). The error was discovered during the review of the dosing data immediately following dosing. One of the extra animals (the next animal in the series that had a patent cannula, CM-08) was dosed, and the samples that had been obtained from rat CM-04 were not analyzed with the exception of the predose sample, which was in the process of analysis.

4.4 Sample Analysis

Sample analysis was conducted on the day of collection, with a target of completing sample analysis within 16 hr of sample collection. Calibration curves prepared with rat blood with known concentrations of ETBE added were constructed, at the same time as sample analysis. Linear regression equations were derived for two standard curves: a low concentration curve, with concentrations ranging from 0.1 μg/ml to 5.2 μg/ml; and a high concentration curve, with concentrations ranging from 5.2 to 250 μg/ml. Details of the calibration curves and method performance are provided in Appendix F. Blood concentration data for each dose group are reported in Tables 4-7.

With gavage administration of 50 mg ETBE/kg, ETBE reached a maximum in blood at the first timepoint (0.25 h), and declined rapidly, falling to <LOQ at 8 hr (Tables 4 and 5). Similarly, with gavage administration of 10 mg ETBE/kg, ETBE reached a maximum in blood at the first timepoint (0.25 h), and declined rapidly, falling to <LOQ by 2-4 hr in males and 2-4 hr in females (Tables 6 and 7).

4.5 Pharmacokinetic Analysis

Pharmacokinetic analysis was conducted using a non-compartmental model for extravascular input with uniform weighting. The WinNonlin output reports for individual animals are included in Appendix D. An example semilog plot of ETBE concentration vs. time for rat BF01 is shown in Figure 5. Semilog plots for each of the animals are shown in Appendix D. For animal DF05, the pharmacokinetic

behavior was considered different from the other animals in the group, and this animal was treated as a pharmacokinetic outlier as per the study protocol, section 9.0 Pharmacokinetic Analysis, item 4. A Grubbs test for outlier was not significant for the concentration of ETBE at 0.25 h. Data including and excluding this animal are presented in Tables 8 and 9. With DF05 included, the calculated half life for the low dose female group (DF) was 1.558 ± 1.976 hr (mean ± standard deviation). However, a Grubbs test for outlier indicated that the value of t1/2_lambda_z of 4.513 h for DF05 was an outlier when compared with the mean value of 1.558 and standard deviation of 1.976 h for the DF group ($p<0.05$). Exclusion of DF05 reduced the mean half life by a factor of approximately three to 0.573 ± 0.176 hr (Table 9).

The mean Tmax was achieved at the first time point following dosing in all dose groups (Table 9). This precluded accurate determination of Cmax and Tmax, since the absorption phase was not captured in the data obtained. The apparent Cmax values measured for males and females in the high dose groups, (AM and BF) were similar at 6.514 ± 0.727, and 5.837 ± 1.586 μg/ml. In the low dose groups, the Cmax values measured for males and females (CM and DF) were similar at 1.057 ± 0.174, and 1.103 ± 0.133 μg/ml. The half lives in males and females at the high dose were similar (1.061 ± 0.326 hr for males and 1.105 ± 0.340 hr for females). While there were no significant gender differences, the half lives in males and females at the low dose were approximately half of those observed at the high dose (0.508 ± 0.191 h for males and 0.573 ± 0.176 h for females). There was little difference in AUCall and AUCINF(observed) between genders, at both the high and low doses. However, when AUCINF(observed) was normalized by dose (AUCINF(observed)/D), the values calculated for the high dose (0.123 ± 0.014 μg.h/ml/mg/kg for AM, and 0.122 ± 0.004 for BF compared with 0.066 ± 0.004 for CM and 0.078 ± 0.016 for DF) were significantly different from the low dose.

5.0 DISCUSSION

This investigation indicated that the uptake of ETBE was rapid following gavage administration, with the highest concentrations observed at the first timepoint, and therefore the apparent Cmax was achieved at the first timepoint. No information on the uptake rate was determined from pharmacokinetic modeling, because the uptake phase was not captured in the sampling schedule. Since the absorption phase of the kinetic curve was not captured, the maximal concentrations of ETBE achieved may have occurred at a time earlier than the first sample, and may have been higher than that observed. ETBE was rapidly eliminated, and fell below the LOQ by 8 hr at the high dose, and by 4 hr in the low dose males, and 2-4 hr in the low dose females.

No statistically significant differences between males and females were observed in the pharmacokinetic parameters measured. There were however differences between the high and the low dose groups in both males and females. The longer half lives and increased AUCINF(observed)/D at the high dose are consistent with saturation of metabolism, which may be expected based on the requirement

for oxidative metabolism as one of the mechanisms for elimination of ETBE. However, because the concentration of ETBE rapidly reached LOQ at the low dose, the ability to determine saturation is limited.

6.0 REFERENCES

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7.0 RECORDS AND REPORTS

The following will be maintained in the record:

a) Protocol and any amendments
b) Animal receipt records
c) Quarantine records
d) Temperature and humidity records for the treatment rooms
e) Animal research facility room logs
f) Feed and water analysis for contaminants

g) Randomization records
h) Test chemical receipt, storage, and use records
i) Balance calibration log references
j) Correspondences
k) All other raw data and documentation

Upon acceptance of the audited draft report by the Sponsor, a final report will be issued.

8.0 STORAGE OF RECORDS AND BIOLOGICAL SAMPLES

A copy of the final report and the records for this study, including all raw data, will be retained by the Sponsor, for the length of time specified in the appropriate regulations.

The test substance ETBE is an ether that can generate explosive peroxides on storage. Therefore, an archived sample of test chemical will not be retained. The samples of blood collected will not afford analysis beyond the duration of the study. Wet specimens of blood will be disposed of after quality assurance verification (after the QAU assures that discarding the samples does not negatively impact the integrity of the study).

9.0 REGULATORY COMPLIANCE

These studies were performed in compliance with the EPA Good Laboratory Practices (GLP) Standards for Inhalation Exposure Health Effects Testing, 40 CFR part 79, subpart F § 79.60. Deviations from the approved protocol are documented in Appendix A.

10.0 STUDY PARTICIPANTS

RTI International	
Study Director:	Timothy R. Fennell, Ph.D.
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	Christine Myers
Secretary:	Kathleen. G. Ancheta

Table 1. Male and Female Fischer 344 Rats Administered ETBE

Group	Subject	Species	Strain	Gender	Body Weight [a] (g)
Group A	AM01	Rat	Fischer 344	Male	150.57
Group A	AM02	Rat	Fischer 344	Male	161.91
Group A	AM03	Rat	Fischer 344	Male	158.09
Group A	AM04	Rat	Fischer 344	Male	146.05
Group B	BF01	Rat	Fischer 344	Female	135.82
Group B	BF02	Rat	Fischer 344	Female	132.95
Group B	BF03	Rat	Fischer 344	Female	138.13
Group B	BF04	Rat	Fischer 344	Female	131.39
Group C	CM01	Rat	Fischer 344	Male	139.26
Group C	CM02	Rat	Fischer 344	Male	141.44
Group C	CM03	Rat	Fischer 344	Male	135.27
Group C	CM04	Rat	Fischer 344	Male	180.64
Group C	CM08	Rat	Fischer 344	Male	150.54
Group D	DF01	Rat	Fischer 344	Female	136.36
Group D	DF02	Rat	Fischer 344	Female	129.57
Group D	DF03	Rat	Fischer 344	Female	140.44
Group D	DF04	Rat	Fischer 344	Female	137.40
Group D	DF05	Rat	Fischer 344	Female	130.57

[a]Body weight on the day of dosing

Table 2. Dose Formulation Analysis.

Dose Formulation	Nominal Concentration (mg/ml)	Actual Concentration (mg/ml)	
		Mean	SD
ETBE 5 mg/mL Group A	5	5.2388	0.0960
ETBE 5 mg/ml Group B	5	5.2859	0.0707
ETBE 1 mg/mL Group C	1	1.1486	0.0058
ETBE 1 mg/mL Group D	1	0.9902	0.0081

Table 3. Dose of ETBE Administered by Gavage

Group	Subject	Treatment	Full Syringe Weight (g)	Empty Syringe Weight (g)	Dose Solution Admin. (g)	ETBE Admin. (mg)	Actual Dose (mg/kg)	Dose error (%)[a]
A	AM01	ETBE 5 mg/mL Group A	7.7369	6.3137	1.4232	7.4559	49.518	-0.96 %
A	AM02	ETBE 5 mg/mL Group A	7.8845	6.3096	1.5749	8.2506	51.958	1.92 %
A	AM03	ETBE 5 mg/mL Group A	7.8140	6.3036	1.5104	7.9127	50.052	0.10 %
A	AM04	ETBE 5 mg/mL Group A	7.6482	6.3065	1.3417	7.0289	48.127	-3.75 %
B	BF01	ETBE 5 mg/ml Group B	7.2363	5.9442	1.2921	6.8299	50.286	0.57 %
B	BF02	ETBE 5 mg/ml Group B	7.2166	5.9473	1.2693	6.7094	50.466	0.93 %
B	BF03	ETBE 5 mg/ml Group B	7.2905	5.9456	1.3449	7.1090	51.466	2.93 %
B	BF04	ETBE 5 mg/ml Group B	7.2328	5.9409	1.2919	6.8289	51.974	3.95 %
C	CM01	ETBE 1 mg/mL Group C	7.5584	6.3083	1.2501	1.4359	10.311	3.11 %
C	CM02	ETBE 1 mg/mL Group C	7.6016	6.3055	1.2961	1.4887	10.525	5.25 %
C	CM03	ETBE 1 mg/mL Group C	7.4832	6.2976	1.1856	1.3618	10.067	0.67 %
C	CM04[b]	ETBE 1 mg/mL Group C	7.6203	6.3091	1.3112	1.5060	8.337	-16.63 %
C	CM08	ETBE 1 mg/mL Group C	7.6597	6.3382	1.3215	1.5179	10.083	0.83 %
D	DF01	ETBE 1 mg/mL Group D	7.3260	5.9315	1.3945	1.3808	10.126	1.26 %
D	DF02	ETBE 1 mg/mL Group D	7.2674	5.9193	1.3481	1.3349	10.302	3.02 %
D	DF03[c]	ETBE 1 mg/mL Group D	7.3515	5.9046	1.4469	1.4327	10.202	2.02 %
D	DF04	ETBE 1 mg/mL Group D	7.3276	5.9231	1.4045	1.3907	10.122	1.22 %
D	DF05	ETBE 1 mg/mL Group D	7.2577	5.9076	1.3501	1.3369	10.239	2.39 %

[a] Dose error calculated as 100*((Actual dose – target dose)/target dose)
[b] Rat CM04 was removed from the study because of a dosing error.
[c] Rat DF03 was removed from the study because of loss of cannula patency.

Table 4. Individual and Mean Blood Concentrations of ETBE in Male Rats Following Gavage Administration of 50 mg/kg ETBE.

Time (h)	ETBE Concentration (μg/ml)					
	AM01	AM02	AM03	AM04	Mean	SD
0	<LOQ[a]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
0.25	5.91	5.95	7.44	6.76	6.51	0.727
0.5	3.59	3.04	4.99	2.97	3.65	0.938
1	1.73	1.88	2.36	1.38	1.84	0.405
2	0.566	0.630	0.690	0.673	0.640	0.0553
4	0.311	0.269	0.261	0.336	0.294	0.0356
8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

[a] Less than the limit of quantitation, 0.11 μg/ml

Table 5. Individual and Mean Blood Concentrations of ETBE in Female Rats Following Gavage Administration of 50 mg/kg ETBE.

Time (h)	ETBE Concentration (μg/ml)					
	BF01	BF02	BF03	BF04	Mean	SD
0	<LOQ[a]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
0.25	5.16	4.15	6.16	7.87	5.84	1.59
0.5	4.12	2.92	3.33	4.05	3.60	0.578
1	1.95	2.21	1.50	1.98	1.91	0.298
2	1.10	0.923	0.697	0.627	0.836	0.215
4	0.197	0.306	0.347	0.193	0.261	0.0779
8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

[a] Less than the limit of quantitation, 0.11 μg/ml

Table 6. Individual and Mean Bloods Concentration of ETBE in Male Rats Following Gavage Administration of 10 mg/kg ETBE.

Time (h)	ETBE Concentration (μg/ml)					
	CM01	CM02	CM03	CM08	Mean	SD
0	<LOQ[a]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
0.25	1.02	1.24	1.14	0.834	1.06	0.17
0.5	0.423	0.503	0.547	0.436	0.48	0.05
1	0.219	0.221	0.270	0.190	0.225	0.033
2	0.0958	<LOQ	<LOQ	0.0800	0.0879	0.0122
4	0.029	<LOQ	<LOQ	<LOQ	0.029	0.000
8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

[a] Less than the limit of quantitation, 0.11 μg/ml

Table 7. Individual and Mean Blood Concentrations of ETBE in Female Rats Following Gavage Administration of 10 mg/kg ETBE.

Time (h)	ETBE Concentration (μg/ml)					
	DF01	DF02	DF04	DF05	Mean	SD
0	<LOQ[a]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
0.25	1.26	1.05	1.01	0.282	0.898	0.425
0.5	0.653	0.554	0.460	0.308	0.494	0.147
1	0.320	0.259	0.245	0.269	0.273	0.033
2	0.120	0.095	0.101	<LOQ	0.110	0.014
4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

[a] Less than the limit of quantitation, 0.11 μg/ml

Table 8. Pharmacokinetic Analysis of ETBE Following a Single Gavage Dose of ETBE.

Parameter (units)	AM[a] Male Rats 50 mg/kg		BF[a] Female Rats 50 mg/kg		CM[a] Male Rats 10 mg/kg		DF[b] Female Rats 10 mg/kg	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tmax (h)	0.243	0.012	0.235	0.037	0.247	0.018	0.316	0.118
Cmax (μg/ml)	6.514	0.727	5.837	1.586	1.057	0.174	0.904	0.412
t1/2_Lambda_z (h)	1.061	0.326	1.105	0.340	0.508	0.191	1.558	1.976
Lambda_z (1/h)	0.694	0.181	0.667	0.174	1.525	0.581	1.018	0.691
AUCall (μg.h/ml)	5.641	0.762	5.795	0.401	0.571	0.035	0.560	0.268
AUCINF(observed) (μg.h/ml)	6.102	0.670	6.237	0.247	0.675	0.043	1.085	0.600
AUCINF(observed)/D (μg.h/ml/mg/kg)	0.123	0.014	0.122	0.004	0.066[b]	0.004	0.106[c]	0.058

[a] Values represent mean and standard deviation, n = 4.

[b] Significantly different from AM, 50 mg/kg, $p<0.05$

[c] Significantly different from BF, 50 mg/kg, $p<0.05$

Table 9. Pharmacokinetic Analysis of ETBE Following a Single Gavage Dose of ETBE (excluding DF05).

Parameter	AM[a] Male Rats 50 mg/kg		BF[a] Female Rats 50 mg/kg		CM[a] Male Rats 10 mg/kg		DF[b] Female Rats 10 mg/kg	
(units)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Tmax (h)	0.243	0.012	0.235	0.037	0.247	0.018	0.257	0.023
Cmax (μg/ml)	6.514	0.727	5.837	1.586	1.057	0.174	1.103	0.133
t1/2_Lambda_z (h)	1.061	0.326	1.105	0.340	0.508	0.191	0.573	0.176
Lambda_z (1/h)	0.694	0.181	0.667	0.174	1.525	0.581	1.306	0.468
AUCall (μg.h/ml)	5.641	0.762	5.795	0.401	0.571	0.035	0.674	0.170
AUCINF(observed) (μg.h/ml)	6.102	0.670	6.237	0.247	0.675	0.043	0.792	0.156
AUCINF(observed)/D (μg.h/ml/mg/kg)	0.123	0.014	0.122	0.004	0.066[c]	0.004	0.078[d]	0.016

[a] Values represent mean and standard deviation, n = 4.

[b] Values represent mean and standard deviation, n = 3.

[c] Significantly different from AM, 50 mg/kg, $p<0.05$

[d] Significantly different from BF, 50 mg/kg, $p<0.05$

Figure 1. Headspace GC-MS Analysis of ETBE in control blood with no added internal standard.
Upper panel: selected ion monitoring at m/z 87 (ETBE), and lower panel, selected ion monitoring at m/z 73 (MTBE, internal standard).

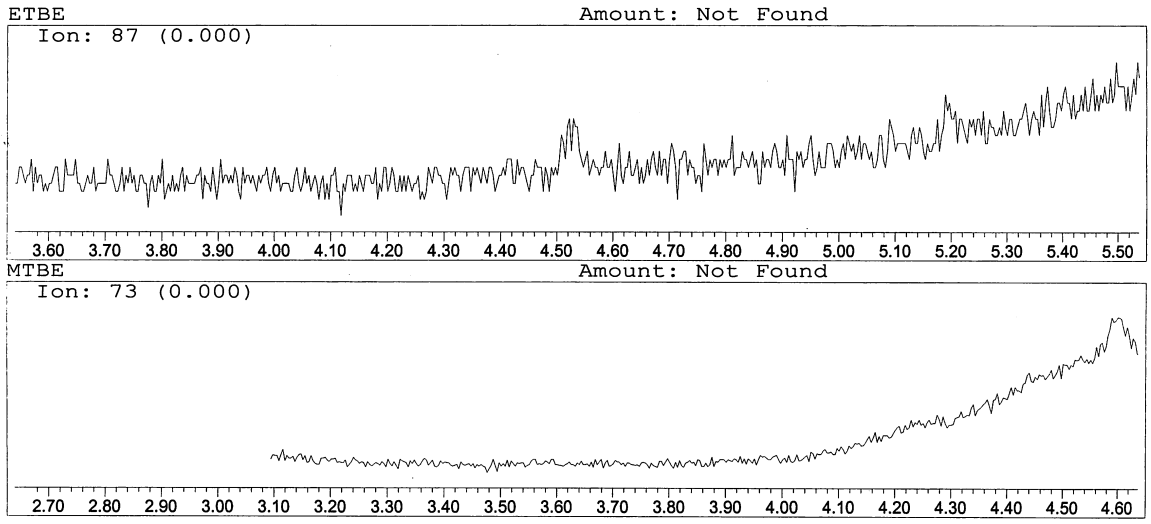


Figure 2. Headspace GC-MS Analysis of ETBE in control blood with internal standard.
Upper panel: selected ion monitoring at m/z 87 (ETBE), and lower panel, selected ion monitoring at m/z 73 (MTBE, internal standard).

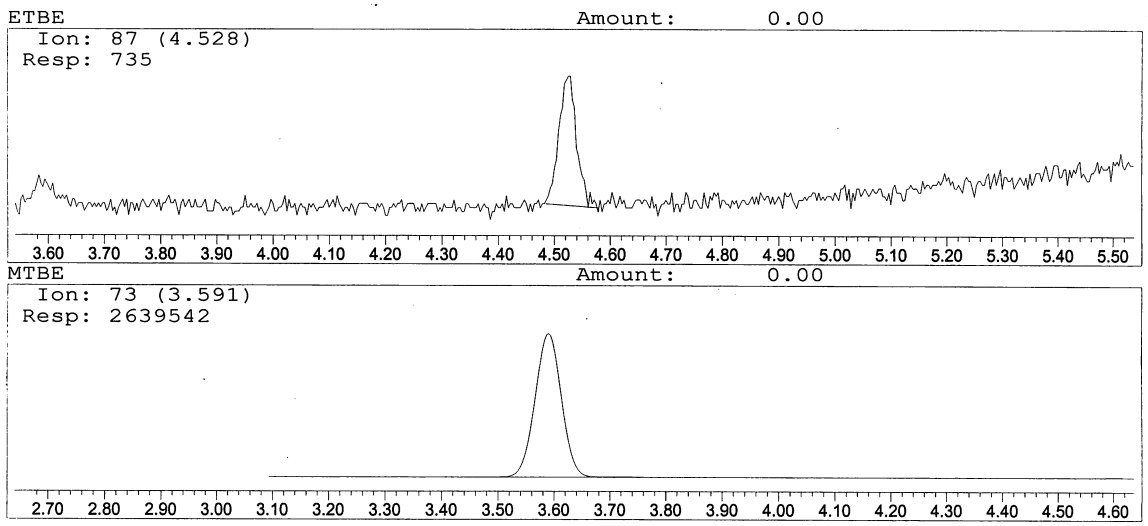


Figure 3. Headspace GC-MS Analysis of ETBE in predose blood with internal standard.

Upper panel: selected ion monitoring at m/z 87 (ETBE), and lower panel, selected ion monitoring at m/z 73 (MTBE, internal standard).

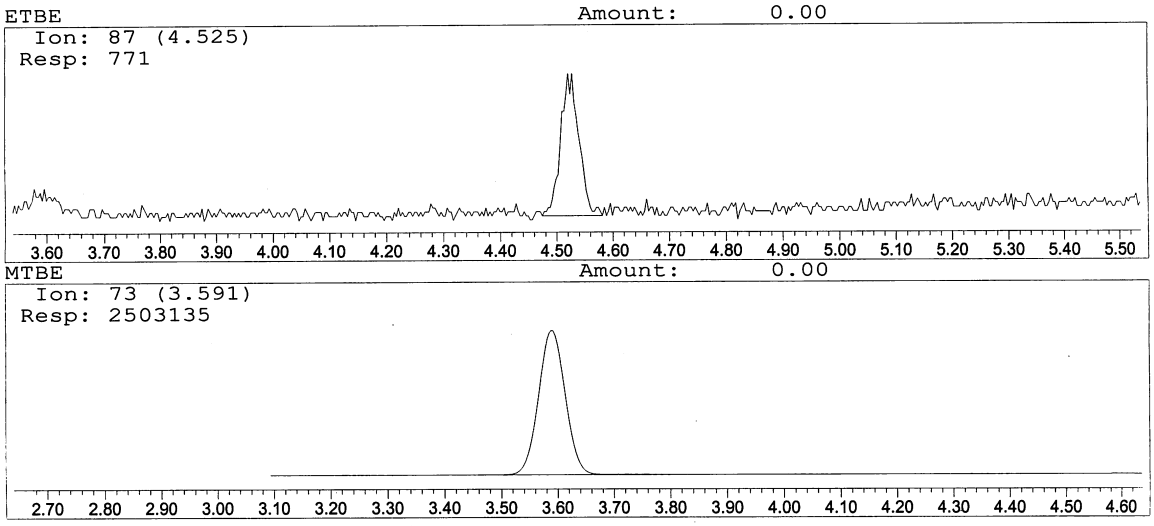


Figure 4. Headspace GC-MS Analysis of ETBE in rat blood following dose administration.

Upper panel: selected ion monitoring at m/z 87 (ETBE), and lower panel, selected ion monitoring at m/z 73 (MTBE, internal standard).

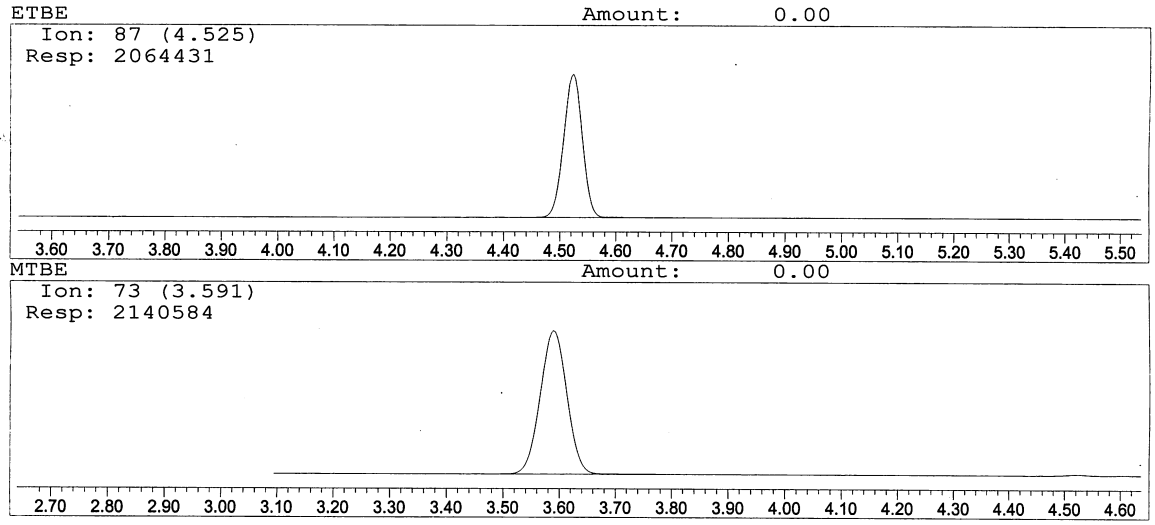
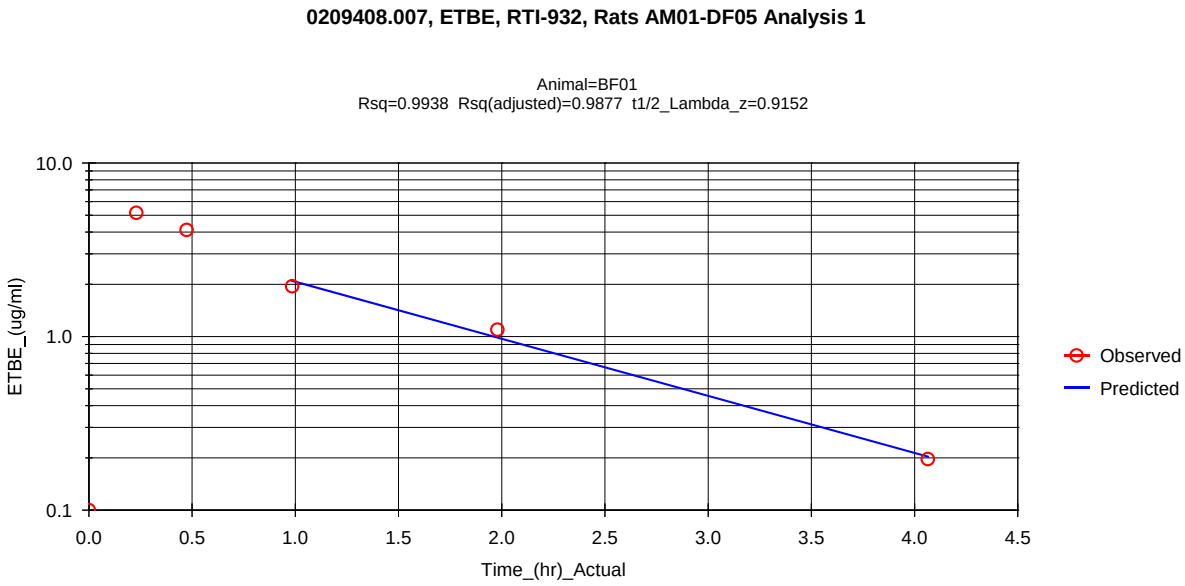


Figure 5. Example pharmacokinetic analysis for blood concentration of ETBE for Animal BF01, administered 50 mg/kg ETBE by gavage.



Appendix A

Approved Study Protocol, Amendments, and Deviations

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Page 1 of 16

TITLE: ETHYL TERTIARY BUTYL ETHER ORAL ABSORPTION IN MALE AND FEMALE RATS

SPONSOR: Section 211(b) Research Group
American Petroleum Institute
1220 L Street NW
Washington, DC 20005

TESTING FACILITY: RTI International*
Science and Engineering
3040 Cornwallis Road
Post Office Box 12194
Research Triangle Park, NC 27709

RTI PROJECT NO.: 0209408.007

RTI Study Code: Rt05-932

RTI STUDY DIRECTOR: Timothy R. Fennell

PROPOSED EXPERIMENTAL START DATE: March 1st, 2007

PROPOSED EXPERIMENTAL TERMINATION DATE: May 31st, 2007

AMENDMENTS:

No.	Date	Section	Pages
1			
2			
3			
4			

Thomas M. Gray, MS, DABT
Sponsor's Representative
2/22/07
Date

Timothy R. Fennell, Ph.D.
Study Director
RTI International
2/23/07
Date

RTI International is a trade name of Research Triangle Institute.

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Page 2 of 16

Table of Contents

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Page 3 of 16

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Page 4 of 16

1.0 OBJECTIVES

The objectives of this study on ethyl *tertiary* butyl ether (ETBE) are to:

Develop a method for the analysis of ETBE in blood by headspace gas chromatography – mass spectrometry (GC-MS).

Conduct an evaluation of oral absorption by measuring ETBE in blood in male and female Fischer 344 rats administered ETBE by gavage at one of two dose levels, 10 and 50 mg/kg.

2.0 PERSONNEL

- Timothy R. Fennell, Ph.D. , Study Director
- Susan Sumner, Ph.D. – Chemist
- Norman Gaudette, B.S. – Research Chemist
- Rodney Snyder, M.S. – Research Chemist
- Yan Hong, M.S – Research Chemist
- Jem Scott-Emuakpor, DVM – Veterinarian
- Melody Gower – Biologist

Other personnel will be used as required. A full list of study participants will be included in the study report.

3.0 STUDY DESIGN

For the pharmacokinetic (PK) analysis of ETBE, a GC-MS method will be developed for the quantitation of ETBE in blood. To provide rat blood for development and validation of the method, up to ten male rats will be sacrificed for the collection of control blood. The rats will be euthanized under CO_2 as needed, to provide blood, and exsanguinated by cardiac puncture.

For the analysis of blood concentrations, all time-point blood samples from ETBE exposures will be placed in glass crimp seal vials and sealed. Control blood samples will be used to develop a GC-MS method for analyzing the concentration of ETBE in blood. Once the method is verified, blood samples will be used to prepare a standard curve. Freshly prepared standards, consisting of rat blood to which a solution of ETBE is added, will be used for definitive pharmacokinetic studies. Details of the method will be recorded in the raw data.

ETBE will be administered in water at a dose of 10 or 50 mg/kg. For each dose level, six male rats and six female rats will be cannulated with jugular vein cannulas up to 4 days prior to dosing and the cannulas will be kept patent. Two additional male rats and two additional female rats will be cannulated and kept on hand in the event that a cannula fails. One additional male rat and one additional female rat will be available in the event of an accidental death prior to cannulation. ETBE will be administered by gavage in water. The syringe, needle and contents will be weighed prior to dosing, and after dosing the weight of the empty syringe and needle will be recorded. The weight of the dose administered will be calculated.

The only biological samples collected for analysis in this study will be blood.

It is anticipated that the study will be conducted with male rats administered 50 mg/kg ETBE on day 1, female rats administered 50 mg/kg on day 2. Analysis of the samples from this part of the study will be conducted prior to the start of the second portion of the study approximately 1 week later in which male rats will be dosed with 10 mg/kg ETBE on day 8 and female rats will be dosed with 10 mg/kg ETBE on day 9. Male and female rats will be ordered specifically for each dose group, and thus will not be randomly assigned to a treatment group.

4.0 JUSTIFICATIONS

4.1 Animal Species

The present studies are designed to evaluate pharmacokinetics of ETBE to provide information that will be used for safety assessments to humans. No *in vitro* techniques are available that allow for adequate determination of uptake, distribution, and excretion of chemicals by mammals. Fischer 344 rats are an established animal species and strain for toxicological testing and PK studies.

4.2 Numbers of Animals

The numbers of animals used in this study are considered acceptable to develop the analytical procedures, and to conduct an evaluation of the pharmacokinetics of ETBE in male and female rats at two doses.

4.3 Routes of Administration and Dose Levels

The route of administration is one of the expected potential exposure routes in humans (through ground water). Oral administration is commonly used in toxicity or safety assessment studies. The dose vehicle, water, corresponds to a potential human exposure scenario. The doses are 10 and 50 mg/kg, and are expected to be without significant toxicity. The high dose was selected based on the concentration of ETBE that can be reasonably achieved in a dose volume of 10 ml/kg. The reported solubility of ETBE in water is 1.2%, which is expected to enable preparation of a solution of 5 mg/ml for gavage dosing.

5.0 REGULATORY COMPLIANCE

This study will be carried out in compliance with 40 CFR part 79, subpart F § 79.60 Good Laboratory Practices (GLP) Standards for Inhalation Exposure Health Effects Testing. The Quality Assurance Unit at the testing facility will prepare and sign a QA Statement to be included in the final report. It will specify the phases of the study that were inspected, the dates on which inspections were made, and the dates on which results of the inspections were reported to the Study Director and the Study Director's management.

The final report will include a Compliance Statement signed by the Study Director that the final report accurately reflects the raw data obtained during the performance of the study and that there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study. If deviations are encountered that will affect the quality or integrity of the study, each deviation will be described in detail.

6.0 TEST SUBSTANCE

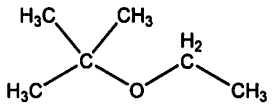
NAME: Ethyl *tertiary* Butyl Ether (ETBE; *tertiary* butyl ethyl ether, 2-ethoxy-2-methylpropane)

CAS No.: 637-92-3

MOLECULAR FORMULA: $C_6H_{14}O$

MOLECULAR WEIGHT: 102.17

STRUCTURE:



SOURCE OF TEST SUBSTANCE: ETBE will be purchased from Sigma-Aldrich, Milwaukee, WI (Catalog number 253898, specified purity 99%). A certificate of analysis will be obtained from the vendor.

LOT NUMBER(S): To be listed in the final report.

IDENTITY AND PURITY: The identity of the unlabeled ETBE will be confirmed at RTI by ^{1}H and ^{13}C NMR, and by mass spectrometry. The purity of the test chemical will be determined by GC-MS.

STORAGE CONDITIONS: ETBE will be stored in the dark at room temperature.

STABILITY: Upon receipt, the identity and purity of the test substance ETBE will be confirmed as described above. Periodically, and at the end of the experimental portion of the study, ETBE will be reanalyzed by ^{1}H NMR and by GC-MS to verify stability.

7.0 ANIMALS

1. Species and Strains: Fischer 344 rats
2. Approximate Age: 8-9 weeks old at time of dosing. (Male rats that are used as blood donors for analytical methods development may be significantly older than the 8-9 weeks of age, and this will not affect the study).
3. Approximate Weight: 200 g
4. Number/Sex: Up to 28 male rats and 18 female rats.
5. Sources: Charles River Laboratories Inc. (Kingston, NY) will be the primary source of animals. In the event that suitable animals cannot be provided from the primary source,

acceptable alternate sources are Charles River Laboratories, Inc. (Portage, MI), and Harlan (Indianapolis, IN). The source(s) of all animals will be documented in the raw data, and included in the Final Report.

7.1 Husbandry

Research Triangle Institute is accredited by AAALAC International. Animal procedures detailed in this protocol are in accordance with the Animal Welfare Act, "Guide for the Care and Use of Laboratory Animals" (NRC, 1996), and the Office of Laboratory Animal Welfare (NIH). All animal procedures will be reviewed by RTI's Institutional Animal Care and Use Committee (IACUC) before initiation of the studies. In the opinion of the Sponsor and Study Director, the study does not unnecessarily duplicate any previous work.

7.1.1 Identification

Rats will be identified by individual metal eartags. All individual animal data will be referenced to either eartag number or to treatment group and animal number or to both. Cages will be individually coded by color and number that are related to dose and treatment group.

7.1.2 Quarantine

Uncannulated animals will be quarantined for a minimum of seven days before use on a study. Animals will be examined by a veterinarian or their representative prior to their release from quarantine, and only animals determined to be in good health as indicated by the absence of clinical signs will be used. During the quarantine period and prior to initiation of the experiments detailed in Section 8.0, rats will be housed (maximum of 3 per cage) in polycarbonate cages with stainless steel bar lids accommodating a water bottle. Cage sizes are approximately 19" x 10 1/2" x 8" high (ca.143 sq. in. floor space). Contact bedding will be Sani-Chips® (P.J. Murphy Forest Products Corp, Montville, NJ). Cannulated animals will be housed individually and will be used within 1-2 days of arrival at RTI.

7.1.3 Feed and Water

Animals will be provided Certified Purina Rodent Chow (5002) *ad libitum*. Water will be provided *ad libitum*. The source of the water is the City of Durham, NC. The analysis of water and analysis of the rodent chow for chemical composition and possible chemical contamination will be provided by the suppliers and maintained in the study records. Approximately once a year, the City of Durham provides analyses of the drinking water for potential contaminants. Documentation of these analyses will be inspected by the Study Director and maintained in the study records. In addition, approximately once per year, RTI conducts an analysis of drinking water contaminants using an outside laboratory. Samples of water will be collected before and after the study for analysis for tertiary butyl alcohol, tertiary amyl methyl ether, ethyl tertiary butyl ether, diisopropyl ether, and methyl tertiary butyl ether. The samples will be sent

to Kiff Analytical (Davis, CA 95616) for analysis. Documentation of these analyses will also be inspected by the Study Director and maintained in the study records. It is anticipated that contaminant levels will be below those permitted in the certified feed, and will not affect the design, conduct, or conclusions of this study. It is anticipated that contaminant levels measured in the water will not affect the design, conduct or conclusions of this study.

7.1.4 Environmental

Air circulation will be 100% fresh air. Room temperature will be maintained at 64–79°F and relative humidity at 30–70% and monitored at least once a day. Light/darkness will be cycled at 12-hr intervals. Any deviations from these conditions shall be included in the study records. Environmental parameters will be recorded automatically using a computerized HVAC Monitoring and Control System.

7.1.5 Acclimation and Housing during Studies

Animals will be acclimated for a minimum of 7 days prior to use on studies. Animals will be housed in polycarbonate cages with stainless steel lids. Following administration of ETBE, the cages will be placed in a hood for the duration of the post dosing period.

7.2 Randomization and Assignment of Animals to Treatment Groups

Animals designated as blood donors for method development will not be randomized. Animals designated for dosing with ETBE will be specifically ordered to be at the appropriate age and body weight at the time of dosing. To accommodate the dosing schedule, separate groups of 9 male and female rats will be purchased for each dose group. For each dose group, a total of 9 cannulated rats will be available prior to dosing. Animals within each treatment group will be assigned in numerical order a number from 1-9, using a series of computer generated numbers as described in SOP DPK-HUS-001 *Assignment of Animals into Groups*, using the procedure for Assignment of Animals within a Single Group or Multiple Groups without Regard to Weight Mean and Range. Animals assigned numbers 1–4 in the treatment groups will be designated as "core" members of the group; animals assigned higher numbers will be designated as "extra" animals (see Section 11.0 for an explanation of the use of the "extra" animals).

7.3 Body Weights

Individual body weights will be measured during the quarantine period, the day of exposure, and at sacrifice.

7.4 Found Dead/Moribund Animals

The Study Director or the veterinarian with the approval of the Study Director will authorize euthanasia of animals with life-threatening clinical signs that indicate that they are unlikely to survive until

the next scheduled observation. The time of death will be estimated as precisely as possible and recorded.

7.5 Euthanasia

Rats will be euthanized with CO_2 exposure.

8.0 STUDY PROCEDURES

8.1 Test Chemical Preparation and Analysis

ETBE will be prepared for gavage dosing as a solution in water. For dosing of animals, the dose solution will be prepared within 24 hours of dosing, and maintained at room temperature. The nominal concentration will be calculated from the weight of ETBE and the weight of water added to the dose preparation. Dose solutions will be prepared at two concentrations, the first at approximately 5 mg/ml and the second at 1 mg/ml, to ensure that the volume of dose administered per kg body weight is similar in the high and the low dose groups. Prior to dosing, a method will be developed for the quantitative analysis of ETBE in water, by dissolving a weighed aliquot of the ETBE/water solution in a suitable solvent such as methanol. Aliquots of this solution will be analyzed directly by gas chromatography (GC) using a standard curve for the quantitation of ETBE. The stability of ETBE in water will be determined by analysis of aliquots of a mock dose solution of ETBE kept at room temperature for three days.

When appropriate methods have been developed for the detection of ETBE in blood, and for the quantitative analysis of ETBE in dosing solutions, and the stability of ETBE in blood and dose solution have been established, the animal study will be conducted.

8.2 Cannulation of Rats for Blood Collection

Rats with indwelling jugular vein cannullae will be purchased from Charles River Laboratories Inc., Kingston NY. Animals will be cannulated by the vendor, and shipped to RTI the day after surgery. The rats will be administered ETBE within 1 to 2 days after arrival at RTI. Cannula patency will be maintained with daily flushings with heparinized saline (sterile saline containing 20 IU/mL of sodium heparin).

8.3 Dosing

Each rat will be weighed prior to dosing to determine the amount of dose to be administered. A single gavage dose will be administered using a syringe fitted with a blunt tipped gavage dosing needle. The dose administered to each animal will be determined from the weight of the full syringe minus that of the empty syringe. The dose time will be recorded. Dosing of animals will be spaced apart to allow blood collection at the appropriate times (see section 8.5.1 below). The groups will be designated according to the dose administered and sex of the rats: Group A male 50 mg/kg, Group B female 50 mg/kg, Group C

male 10 mg/kg, and Group D female 10 mg/kg. At least four cannulated rats per group will be dosed with ETBE. Group and animal numbers are indicated in Table 1.

8.4 Ante mortem Observations and Functional Assessments

Animals will be observed twice per day for mortality, morbidity, signs of toxicity, and for any acute distress that might be related to the test procedure or test substances. Animals exhibiting adverse reactions will be closely monitored. All signs of poor health or abnormal behavior will be recorded. Moribund animals will be euthanized by CO_2 exposure following authorization by the Study Director or the veterinarian with the approval of the Study Director. Blood samples collected from dead or moribund animals will be included in the analysis if the animal was not moribund/dead at the time of collection. The Sponsor will be notified as soon as possible if it is anticipated that the sacrifice may affect the integrity of the study. If possible, an extra animal will be substituted for the animal removed from the study (see 11.0 Data from "Extra" Animals).

8.5 Collection and Storage of Biological Samples

8.5.1 Blood

Blood samples will be collected prior to dosing from each rat (time = 0), from 4 rats of each sex, at each dose at approximately 15 and 30 min, and at approximately 1, 2, 4, 8, and 24 hr after dosing.

Blood samples will be drawn from the jugular vein cannula into a heparinized 1 ml syringe, and the sample will be immediately placed in a preweighed headspace vial. The amount of blood will be approximately 100 µl. The vial will be immediately crimped and weighed, and the weight of the blood aliquot will be determined. After the last sample has been collected from each animal at 24 hr, the rats will be euthanized by exposure to CO_2. Should cannulas fail between the 8 and 24 hr blood samples, blood will be collected by cardiac puncture under CO_2 anesthesia at sacrifice at 24 hr. The blood samples will be maintained at room temperature until analyzed. It is anticipated that blood samples will be analyzed by GC-MS within 24 hours of collection.

Blood samples from unexposed rats will be used for method development. Blood samples will be collected by cardiac puncture, under CO_2 anesthesia, and the rats will be euthanized.

8.6 Analysis of Blood Samples

A GC-MS method will be developed for quantitating ETBE using blood from control male Fischer 344 rats. The methods will involve the development of a standard curve for analysis of ETBE in the headspace of airtight vials containing a known volume of control blood and a known concentration of ETBE. The stability of ETBE will be evaluated under storage conditions that may be used in this study

during this method development. This will include analysis of ETBE immediately after spiking in control blood, after storage at room temperature (8 hr), or at approximately 4°C (24 hr). The concentration of ETBE in the headspace of vials containing a known volume of blood from rats administered ETBE by gavage will be determined by comparison to the standard curve.

9.0 PHARMACOKINETIC ANALYSIS

Individual and mean blood concentration-time data for each dose and sex will be analyzed, as appropriate, by noncompartmental (model-independent) methods using the least-squares fitting program WinNonlin™ (Statistical Consulting Inc., Cary, NC). The following PK parameters will be determined as appropriate: terminal elimination rate constant, terminal elimination half-life ($T_{1/2}$), area under the blood concentration-time curve extrapolated from time zero to infinity (AUC), maximum concentration achieved (C_{max}), and time to maximum concentration (T_{max}).

Occasionally, a data point (i.e., a concentration) that cannot be predicted by a PK method of analysis may be encountered. The following procedure will be used to evaluate such data points as outliers.

1. Identify suspected outliers by visual inspection of the data. Types of data points that should be considered as suspected outliers include non-zero concentrations prior to dosing, an individual concentration that is much different from that predicted by the PK method of analysis (such as lone high concentration preceded and followed by much lower concentrations).
2. Rule out physiological or other processes which may explain the suspected outlier. Certain processes, such as enterohepatic circulation or absorption from more than one site in the gastrointestinal tract, may result in unusual C-T profiles, which a PK method of analysis would be unable to approximate. Furthermore, non-zero concentrations at time zero may be possible if the analyte is present endogenously, or if some endogenous material interferes with the assay for the target analyte. If a concentration is deemed to be an outlier solely because it cannot be explained by PK methods of analysis, the possibility of some process which might explain it such as sample analysis should be considered.
3. Once a concentration that is suspected as a PK outlier cannot be explained by PK methods of analysis or physiologic processes, the Grubbs method (Grubbs 1969) will be used to test whether it is an outlier.
4. If a concentration cannot be explained by PK methods of analysis and is substantially different from that seen in the other animals at the same time (based on the above

criteria), it is considered a PK outlier and will not be included in the calculation of PK parameters or mean concentrations. Reasons for exclusion will be documented in the raw data and in the final report.

10.0 STATISTICAL ANALYSIS

All blood concentration data will be reported in tables as the mean ± standard deviation (SD). PK data will be presented as mean ± SD. The methods to be used for statistical analysis will be added by amendment.

11.0 DATA FROM "EXTRA" ANIMALS

To better ensure that complete data from the required number of animals in each treatment group will be obtained, one or more additional animals, predesignated as "extras", will start the study as part of each treatment group. If a "core" study animal fails to complete the study due to loss of samples, misdosing, morbidity, etc., it will be replaced with a designated "extra" animal and data will cease to be obtained from the "core" animal. The "extra" animal will then become part of the "core" group and the original animal will be removed from the "core" group. All such substitutions will be documented as to reason and approved in writing by the Study Director. An "extra" animal may also be substituted for a "core" animal as described in Section 8.4. Terminal blood samples or blood samples obtained via the tail vein may be included in the analysis in the event that cannulas are no longer patent. Except for animals that become part of a "core" group, samples from "extra" animals will not normally be analyzed. However, all data obtained in the study will be reported. Data used to construct group means and to obtain PK parameters will consist of the data obtained from the "core" study animals (not the "extras"), except in cases where an "extra" has taken the place of a "core" animal. In that case, the data from the "extra" animal will be used instead, and data from the "core" animal that was eliminated from the study will not be used in these calculations. Data from the "core" animal that was eliminated from the study will be used in these calculations, if the data are from samples collected prior to elimination.

12.0 RECORDS AND REPORT

The following will be maintained in the record:

a. Protocol and any amendments
b. Animal receipt records
c. Quarantine records
d. Temperature and humidity records for the treatment rooms
e. Animal research facility room logs
f. Feed and water analysis for contaminants

g. Randomization records
h. Test chemical receipt, storage and use records
i. Balance calibration log references
j. Correspondences; and
k. All other raw data and documentation.

Results of the studies will be described in an audited draft report, which will be submitted to the Sponsor for approval. This report will include but not be limited to:

a. Name and address of the facility performing the study, dates of study initiation and completion, and RTI study number.
b. A copy of the signed, dated and approved protocol and all deviations and authorized amendments to the original protocol.
c. A detailed description of all methods used, including the randomization method.
d. The lot number(s) of the test substances and details of the formulation of doses.
e. Animal information to include: supply source, species, strain or substrain, sex, individual animal weights (at randomization through sacrifice), approximate age at initiation of dosing, and procedure used for individual animal identification and assignment to the treatment group.
f. Tabulated individual results for blood concentration.
g. Tabulated mean results for blood concentration.
h. Pharmacokinetic and statistical analysis of the data.
i. A QA statement prepared and signed by QAU specifying that audits/inspections were reported to management and the study director.
j. A compliance statement signed by the Study Director.

Upon acceptance of the audited draft report by the Sponsor, a final report will be issued. Two bound and one unbound copies of the audited draft report and two bound and one unbound copies of the final report will be shipped to:

Derek Swick
Regulatory Analysis and Scientific
Affairs
American Petroleum Institute
1220 L Street NW
Washington, DC 20005
p: (202) 682-8341
f: (202) 682-8031

13.0 MAINTENANCE OF RECORDS AND RAW DATA

Records will be maintained in the laboratories of the study personnel while the studies are being conducted. Raw data generated while conducting the study and any transformations, calculations or operations performed on the data will be recorded in the study records. All original study records, protocols, amendments, and the final report will be stored in the RTI International archives, under the control of the RTI Quality Assurance Unit in accordance with EPA GLP regulations 40 CFR part 79, subpart F § 79.60 Good Laboratory Practices (GLP) Standards for Inhalation Exposure Health Effects Testing. Documentation and raw data will be maintained in the archives for a period of ten years following issuance of the final report. The test substance ETBE is an ether that can generate explosive peroxides on storage. Therefore, an archived sample of test chemical will not be retained. The samples of blood collected will not afford analysis beyond the duration of the study. Wet specimens of blood will be disposed of after quality assurance verification (after the QAU assures that discarding the samples does not negatively impact the integrity of the study).

Materials will be maintained in the RTI Archive for a period of one year after the signature of the final report as part of the initial study cost. At that point, the Sponsor will be contacted to determine the final disposition of these materials. The Sponsor may continue to store these materials in the RTI Archive, have RTI ship them to the Sponsor or an alternative archive facility, or have RTI dispose of them. The Sponsor will be responsible for all costs associated with the storage of these materials beyond 1 year from the issuance of the final report, and for any costs associated with the shipment of these materials to the Sponsor or to any other facility designated by the Sponsor.

14.0 SAFETY PRECAUTIONS

ETBE is a flammable liquid, with the potential to form explosive peroxides. The container should be tightly closed when not in use. Storage under a nitrogen atmosphere is recommended to avoid the generation of peroxides. Use personal protective equipment, including safety glasses, lab coat, and chemical resistant gloves.

15.0 PROTOCOL AMENDMENTS AND DEVIATIONS

This protocol may be amended by the Study Director with agreement of the Sponsor as the study progresses. Normally, a formal amendment will be prepared and signed by the Study Director and the Sponsor's Representative prior to the change. If instances arise where a change is urgent, the change may become effective upon approval by the Study Director. A notification of the urgent change will be sent to the Sponsor's Representative (email, facsimile, or telephone) as soon as feasible (no more than

24 h after the Study Director's approval). Subsequently, a formal protocol amendment will be prepared for approval by the Study Director and the Sponsor's Representative.

Any deviations from the protocol that occur in the course of the conduct of the study will be documented. The cause for the deviation and its effect if any on the outcome of the study will be explained and the Study Director will sign the document.

16.0 REFERENCES

Grubbs FE (1969). Procedure for detecting outlying observations in samples. Technometrics, 11:1–21.

National Research Council (1996). Guide for the Care and Use of Laboratory Animals. National Academy Press: Washington, DC.

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Page 16 of 16

TABLE 1. ANIMAL NUMBER ASSIGNMENT

	Animal Group ID			
Initial Designation	Group A Male rats 50 mg/kg ETBE	Group B Female rats 50 mg/kg ETBE	Group C Male rats 10 mg/kg ETBE	Group D Female rats 10 mg/kg ETBE
Core	AM01	BF01	CM01	DF01
Core	AM02	BF02	CM02	DF02
Core	AM03	BF03	CM03	DF03
Core	AM04	BF04	CM04	DF04
Extra	AM05	BF05	CM05	DF05
Extra	AM06	BF06	CM06	DF06
Extra	AM07	BF07	CM07	DF07
Extra	AM08	BF08	CM08	DF08
Extra	AM09	BF09	CM09	DF09

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Amendment 1 Page 1 of 4

AMENDMENT 1

TITLE: ETHYL TERTIARY BUTYL ETHER ORAL ABSORPTION IN MALE AND FEMALE RATS

SPONSOR: Section 211(b) Research Group
American Petroleum Institute
1220 L Street NW
Washington, DC 20005

TESTING FACILITY: RTI International*
Science and Engineering
3040 Cornwallis Road
Post Office Box 12194
Research Triangle Park, NC 27709

RTI PROJECT NO.:	0209408.007
RTI Study Code:	Rt05-932
RTI STUDY DIRECTOR:	Timothy R. Fennell
PROPOSED EXPERIMENTAL START DATE:	June 13th, 2007
PROPOSED EXPERIMENTAL TERMINATION DATE:	June 22nd, 2007

Thomas M. Gray, MS, DABT — 6/08/07 (Date)
Sponsor's Representative
American Petroleum Institute

Timothy R. Fennell, Ph.D. — 6-11-07 (Date)
Study Director
RTI International

**RTI International is a trade name of Research Triangle Institute.*

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Amendment 1 Page 2 of 4

Protocol Change No.: 1

Change (Title Page, Page 1):

PROPOSED EXPERIMENTAL START DATE: March 1st, 2007

PROPOSED EXPERIMENTAL TERMINATION DATE: May 31st, 2007

To:

PROPOSED EXPERIMENTAL START DATE: June 13th, 2007

PROPOSED EXPERIMENTAL TERMINATION DATE: June 22nd, 2007

Reason for change:

To include the experimental start date and experimental termination date as required by GLP regulations.

Protocol Change No.: 2

Change (2.0 Personnel, Page 2):

- Jem Scott-Emuakpor, DVM – Veterinarian

To:

- Christopher Johnson, DVM – Veterinarian
- Serena Young, DVM – Veterinarian

Reason for change:

Dr. Scott-Emuakpor is no longer employed by RTI.

Protocol Change No.: 3

Change (7.1.2 Quarantine, Page 7):

Cannulated animals will be housed individually and will be used within 1-2 days of arrival at RTI.

To:

Cannulated animals will be housed individually in cages with dimensions of 9 ¼" x 8 ¼" x 8" (ca. 76.3 sq. in. floor space), and will be used within 1-2 days of arrival at RTI.

Reason for change:

To describe the cages that will be used for individually housing animals.

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Amendment 1 Page 3 of 4

Protocol Change No.: 4

Change (7.1.5 Acclimation and Housing during Studies, Page 8):

Animals will be acclimated for a minimum of 7 days prior to use on studies.

To:

Animals will be acclimated for a minimum of 7 days prior to use on studies. Cannulated animals will be acclimated for approximately 1-2 days before use on studies.

Reason for change:

To clarify the acclimation for cannulated animals.

Protocol Change No.: 5

Change (8.2 Cannulation of Rats for Blood Collection, Page 9):

Rats with indwelling jugular vein cannullae will be purchased from Charles River Laboratories Inc., Kingston NY. Animals will be cannulated by the vendor, and shipped to RTI the day after surgery. The rats will be administered ETBE within 1 to 2 days after arrival at RTI. Cannula patency will be maintained with daily flushings with heparinized saline (sterile saline containing 20 IU/mL of sodium heparin).

To:

Rats with indwelling jugular vein cannulae will be purchased from a commercial vendor described in 7.0 Animals. Animals will be cannulated by the vendor, and shipped to RTI as soon as practical after surgery. The rats will be administered ETBE within 1 to 2 days after arrival at RTI. Cannula patency following dosing will be maintained with flushings with heparinized saline (sterile saline containing 20 IU/mL of sodium heparin).

Reason for change:

Clarification of the source and shipment of animals. Clarification of when flushing cannulae with heparinized saline should start.

Protocol Change No.: 6

Add (8.7 Data Collection, Page 11):

8.7 Data Collection

The Debra™ laboratory information management system will be used for collection of body weights, animal observations, dose administered, dosing time, sample collection times and sample weights. Therefore, the raw data for these measurements will be the electronic data collected in Debra unless otherwise noted in the study records. The Debra system will be used to calculate and report dose

PROTOCOL	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI- 932 Amendment 1 Page 4 of 4

administered in each animal. The Debra system will be used to calculate and report data for time between dosing and sample collection, and for weight of sample collected.

Temperature and humidity data will be collected using a computerized HVAC Monitoring and Control System. All other data, such as animal receipt and quarantine records, will be manually recorded unless noted otherwise in the study record.

Reason for change:

To clarify the systems used for collection of data.

RTI Protocol No. RTI-935 (RTI Project No. 020908.007)

Deviations from the Approved Protocol

The deviations listed below did not affect the results of the study.

Deviation
The protocol states (section 8.5.1) that: “The blood samples will be maintained at room temperature until analyzed.” They were maintained on ice until placed in the headspace autosampler for analysis.

Reason for Deviation
Oversight. The storage conditions to be investigated and found to maintain ETBE concentrations were noted in section 8.6: "This will include analysis of ETBE immediately after spiking in control blood, after storage at room temperature (8 hr), or at approximately 4ºC (24 hr)." ETBE in blood under all conditions tested was found to be stable.

Deviation
The protocol states (section 7.1.5) that:
“Following administration of ETBE, the animals will be placed in the hood for the duration of the post-dosing period.” Group C animals dosed the previous night were transferred out of the hood in the morning on 6/21/07 prior to collection of the 24 hr blood collection in order to allow placement of Group D Animals in the hood following dosing of the animals.

Reason for Deviation
Sample analysis results indicate that Group C animals are no longer exhaling ETBE in amounts (above the assay limit of quantitation) which may cross-contaminate other study samples or expose study personnel to ETBE. This also affords dosing of Group D animals earlier in the morning.

Deviation
The protocol states (section 10.0) that:
“The statistical analysis to be used will be added by amendment” The following staticial analysis will be performed. Statistical comparison of the parameters Tmax, Cmax, t1/2 Lambda z, Lambda z, AUCall, AUCobserved and AUCobserved/Dose will be conducted using the Student’s t-test available in SAS® Version 9 (SAS Institute Inc., 2004, 2005, 2006a,b). The males will be compared to the females within dose group for each parameter. Additionally, for the parameter AUCobserved/Dose the low dose will be compared to the high dose within each sex.
SAS Institute Inc. (2004). SAS/STAT® User’s Guide, Version 9.1, Cary, NC: 5136 pp.
SAS Institute Inc. (2005). SAS® Language Reference: Concepts, Version 9.1.3, Cary, NC: 664 pp.
SAS Institute Inc. (2006a). SAS® Language Reference: Dictionary, Version 9.1.3, Cary, NC: 1908 pp.
SAS Institute Inc. (2006b). SAS® Procedures Guide, Version 9.1.3, Cary, NC: 1934 pp.

Reason for Deviation
To expedite statistical analysis, the proposed statistical analysis was agreed with the sponsor.

Deviation
At the following time points for the respective animals, blood was collected from the tail vein instead of the cannula.

8h (AM01, BF03, DF05),

24h (AM01, AM02, AM03, AM04, BF03, BF04, DF02)

15m (DF05), 30m (DF05), 4h (BF03)

Reason for Deviation
Cannulae failed in all cases. For the earlier timepoints, a sufficient number of extra animals were not available to complete the study by dosing additional animals. For 8-24h timepoints, tail vein bleeds were considered an appropriate method of blood collection in the absence of a patent cannula, and a complete time course to 24h was desired.

Deviation
Periodic stability analyses of the ETBE test substance were conducted via Gas Chromatography with Flame Ionization Detection (GC-FID) instead of GC-MS, and Proton-NMR was not used.

Reason for Deviation
The GC-MS was in constant use during method validations and study sample analyses during this study. GC-FID was considered an appropriate alternative and provided a quantitative method for assessing purity.

Deviation
Group A animals were released from quarantine on the same day of receipt.

Reason for Deviation
Animals were not available from the vendor on a prior day to allow for one day between receipt and and release from quarantine. The animals were administered ETBE the day following release.

Deviation
Dose formulations were stored in a refrigerator at ca. five degrees Celcius overnight between preparation and analysis. The protocol stated room temperature.

Reason for Deviation
The test substance is very volatile, and a lower temperature was considered prudent to provide additional assurance that ETBE remained in the formulation between preparation and dosing. Both room temperature and refrigerator temperature had been evaluated and both were acceptable.

Appendix B

Test Chemical Analysis Report

Test Chemical Analysis Report

Ethyl Tertiary Butyl Ether

RTI Reference 12307-11

SUBMITTED TO:

Section 211(b) Research Group
American Petroleum Institute
1220 L Street NW
Washington, DC 20005

TESTING FACILITY:

RTI International*
3040 Cornwallis Road
P.O. Box 12194
Research Triangle Park, NC 27709-2194

Timothy R. Fennell, Ph.D. 03-04-2013 Date

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

Two x 5g of tert-Butyl ethyl ether (ETBE) was purchased from Sigma Aldrich. The material received on March, 9, 2006 was 99% tert-Butyl ethyl ether, Product Number 253898, Lot Number 04608LD, Formula Weight 102.17. Purity from the Vendor Certificate of Analysis was 99.3% by GLC. No expiration date was indicated by the vendor.

RTI assigned this material a Test Article Number of 12307-11. RTI confirmed the identity of the material using nuclear magnetic resonance spectroscopy, and mass spectrometry. RTI confirmed the purity of the material by gas chromatography.

Nuclear Magnetic Resonance Spectroscopy.

All NMR data were acquired on a 300 MHz Bruker spectrometer. The ^{1}H NMR spectra were acquired with a relaxation delay of 30 sec, a 6173 Hz sweep width, and an 8 µsec pulse. The sample was prepared in $CDCl_3$ (deuterochloroform). The ^{1}H-decoupled ^{13}C NMR spectrum was acquired with a relaxation delay of 2 sec, a sweep width of 23810 Hz, and a 5.5 µsec pulse. The NMR analysis was conducted on 03/17/06.

The ^{1}H NMR spectrum of the sample contained an overlapping singlet and triplet at approximately 1.2 ppm, and a quartet at 3.4 ppm (Figure 1). The singlet at 1.2 ppm is attributed to the CH_3 groups of tertiary butyl portion of the molecule. The overlapping triplet at 1.2 ppm is attributed to the ethyl CH_3 group. The quartet at 3.4 ppm is attributed to the ethyl CH_2 group. The ratio of integrals (12.485:2.000) is consistent with the expected ratio for 12 methyl protons, and 2 methylene protons.

The ^{13}C NMR spectrum of the sample contained singlets at approximately 72.9, 57.2, 28.0 ppm and 16.7 ppm (Figure 2). The triplet at approximately 77.0 ppm is assigned to $CDCl_3$. The large signal at 22.8 ppm is consistent with the tertiary butyl CH_3 groups, and the small singlet at 72.9 ppm is consistent with the quaternary carbon of the tertiary butyl group. The signal at 57.2 ppm is consistent with the ethyl CH_2 group, and the singlet at 16.7 ppm is consistent with the ethyl CH_3 group.

The ^{1}H and ^{13}C NMR data for the sample are consistent with the structure of ETBE.

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

GC Analysis

Analysis of the supplied material was conducted as described below on March 10 and May 16, 2006.

Equipment: Agilent 6890 gas chromatograph equipped with a split-splitless injector and a flame ionization detector.
Agilent 6890 autoinjector with controller
Millenium data system.

Column DB-1, J&W Scientific 30m x 0.53 mm i.d., 3 µm film thickness (J&W, Agilent Technologies, Wilmington, DE)

Injection port	split/splitless
Temperature	200 ºC
Split ratio	100:1
Carrier gas	Helium
Flow rate	1 ml/min
Injection volume	1 µl
Initial temperature	35 ºC
Initial time	1 min
Temperature rate	5 ºC/min
Final temperature	220 ºC
Final time	1 min

From August 22, 2006, analyses were conducted as described above with the following exceptions:

Split ratio	50:1
Flow rate	4 ml/min
Temperature rate	10 ºC/min
Final time	2 min

Empower 2 has replaced Waters Millenium 32 Version 4.0 as the chromatography data system used for the analyses since March 7, 2007.

Purity of the material was determined by injection of 3 1-µl samples onto the GC column with the exception that injection of 3 3-µl samples for analyses on June 7, 2007. The initial purity determined was 99.66 %, with a standard deviation of 0.04 %. Figure 3 shows a typical chromatogram. The purity of the material measured on subsequent dates is presented in Table 1.

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

GC-MS analysis

Analysis of the supplied material was conducted as described below on March 10, 2006.

Equipment: Agilent 6890 gas chromatograph equipped with a split-splitless injector and a flame ionization detector.
Agilent 5973 Mass Selective Detector.

Column DB-624 30m x 0.32 mm i.d., 1.8 μm film thickness
(J&W, Agilent Technologies, Wilmington, DE)

Injection port	split/splitless
Temperature	150 ºC
Split ratio	5:1
Carrier gas	Helium
Flow rate	1.7 ml/min
Injection volume	1 µl
Initial temperature	30 ºC
Initial time	3 min
Temperature rate	5 ºC/min
Final temperature	80 ºC
Final time	0
Ramp	100 ºC/min
Final temperature	200 ºC
Final time	1 min
5973 MSD	
Mode	EI mode
Scan	10-150 amu
Source temperature	230 ºC
Quad temperature	150 ºC
Transfer line	250 ºC
Tune	Atune.u
Solvent delay	2.75 min

Identity was verified by GC-MS analysis. A sample of 10 µl ETBE was dissolved in 20 ml of methanol, and 1 µl was injected.

The total ion chromatogram showed a single peak at approximately 4.6 min (Figure 4, upper panel). The mass spectrum of this peak (Figure 4, lower panel) showed a base peak at *m/z* 87 (consistent with M-CH_3), and major fragment ions

at 59, 57, and 45 (consistent with CH_3-$CH{=}OH^+$). A library search indicated a match with the spectrum of tert-butyl ethyl ether.

Conclusion

The NMR and mass spectral data of the supplied material are consistent with the structure of ETBE. The initial purity of the material measured by GC with FID on March 10, 2006 was 99.66 ± 0.04 %. Measurement of purity between March 2006 and June 2007 (99.26 ± 0.17 %) indicated that the material is stable under the conditions of storage at RTI.

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

Table 1. Purity of ETBE determined by gas chromatography with flame ionization detection.

Date of Analysis	Purity (Mean ± SD, 3 determinations)
March 10, 2006	99.66 ± 0.04
May 16, 2006	99.88 ± 0.07
August 22, 2006	99.14 ± 0.06
November 29, 2006	99.11 ± 0.04
March 7, 2007	99.17 ± 0.02
June 7, 2007	99.31± 0.05
June 21, 2007	99.26 ± 0.17

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

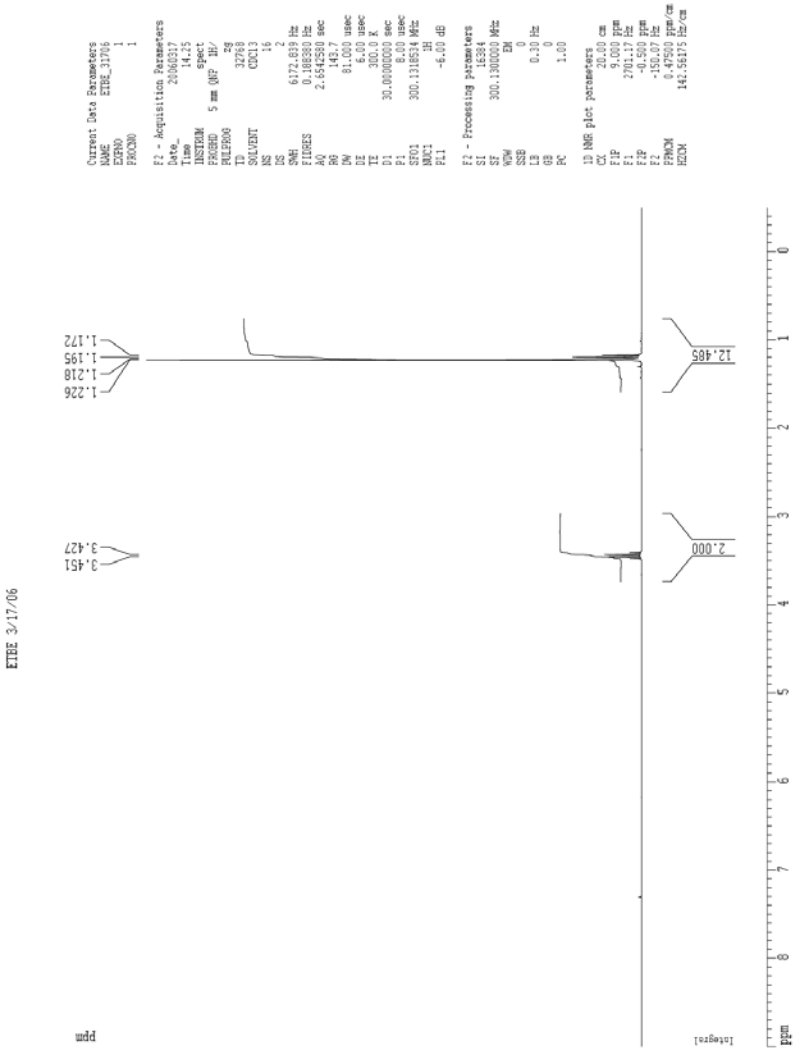


Figure 1. 300 MHz ^{1}H NMR of Vendor Supplied ETBE, analyzed on 3/17/06 by J. Burgess

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

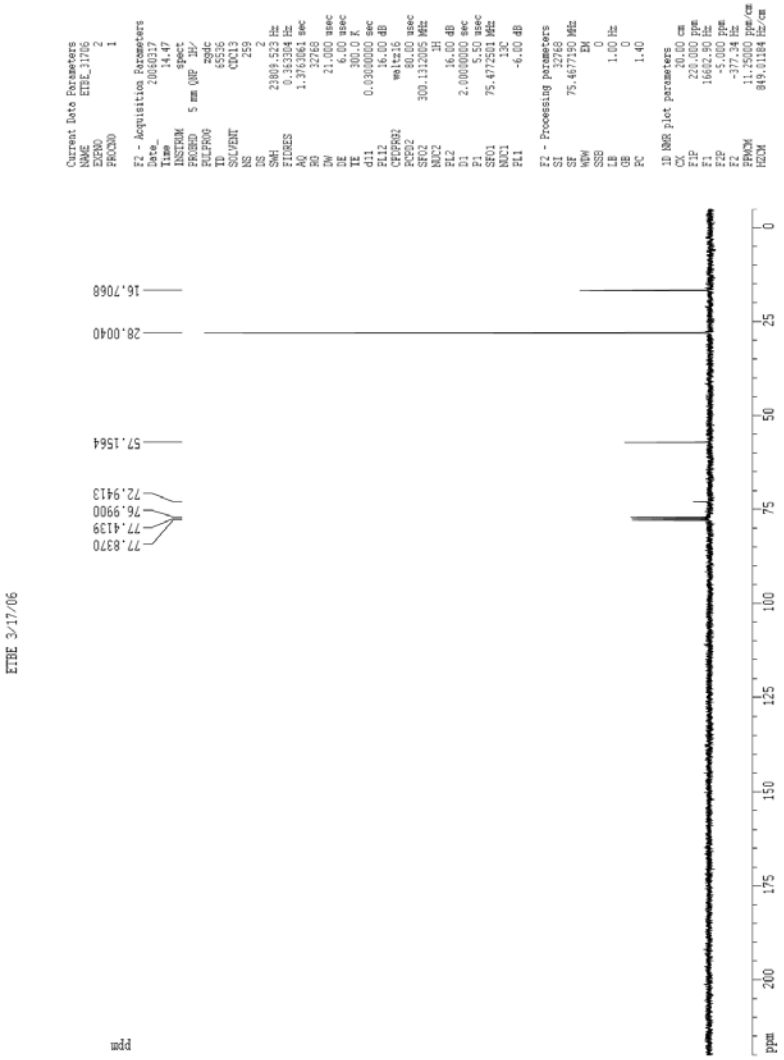


Figure 2. 75 MHz ^{13}C NMR of Vendor Supplied ETBE, analyzed on 3/17/06 by J. Burgess

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

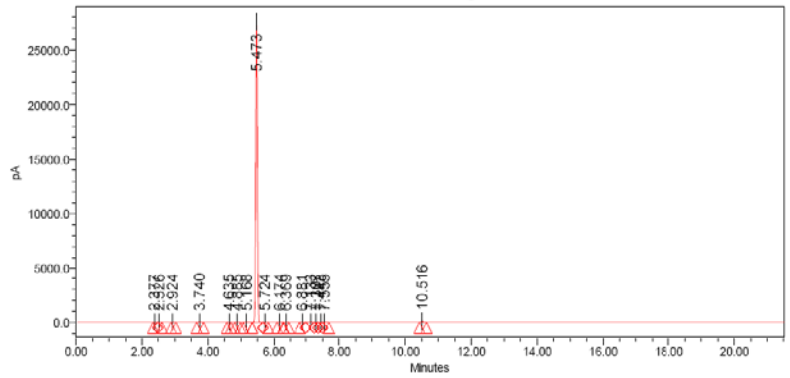
Individual Report Report

Reported by User: Yan Hong (HongY) Project Name: API

SAMPLE INFORMATION

Sample Name:	ETBE 12307-17	Acquired By:	HongY
Sample Type:	Unknown	Date Acquired:	3/7/2007 9:48:37 AM EST
Vial:	1	Acq. Method Set:	Purity
Injection #:	1	Date Processed:	3/7/2007 10:59:50 AM EST
Injection Volume:	1.00 ul	Processing Method:	Purity
Run Time:	21.5 Minutes	Channel Name:	HP6890 Ch1
Sample Set Name:	030707 ETBE Purity	Proc. Chnl. Descr.:	FID

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	% Area
1		2.377	5	2	0.00
2		2.526	9	4	0.01
3		2.924	8	3	0.01
4		3.740	133	45	0.14
5		4.635	6	2	0.01
6		4.885	7	2	0.01
7		5.168	31	10	0.03
8		5.473	95179	27545	99.19
9		5.724	31	9	0.03
10		6.174	5	2	0.01
11		6.369	20	6	0.02

	Name	RT	Area	Height	% Area
12		6.881	44	7	0.05
13		7.133	82	6	0.09
14		7.307	31	6	0.03
15		7.443	24	4	0.02
16		7.555	30	4	0.03
17		10.516	310	98	0.32

Report Method: Individual Report Printed 11:00:06 AM8/7/2007 Page: 1 of 1

Figure 3. GC-FID Chromatogram of ETBE, analyzed on March, 7, 2007 by Y. Hong.

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

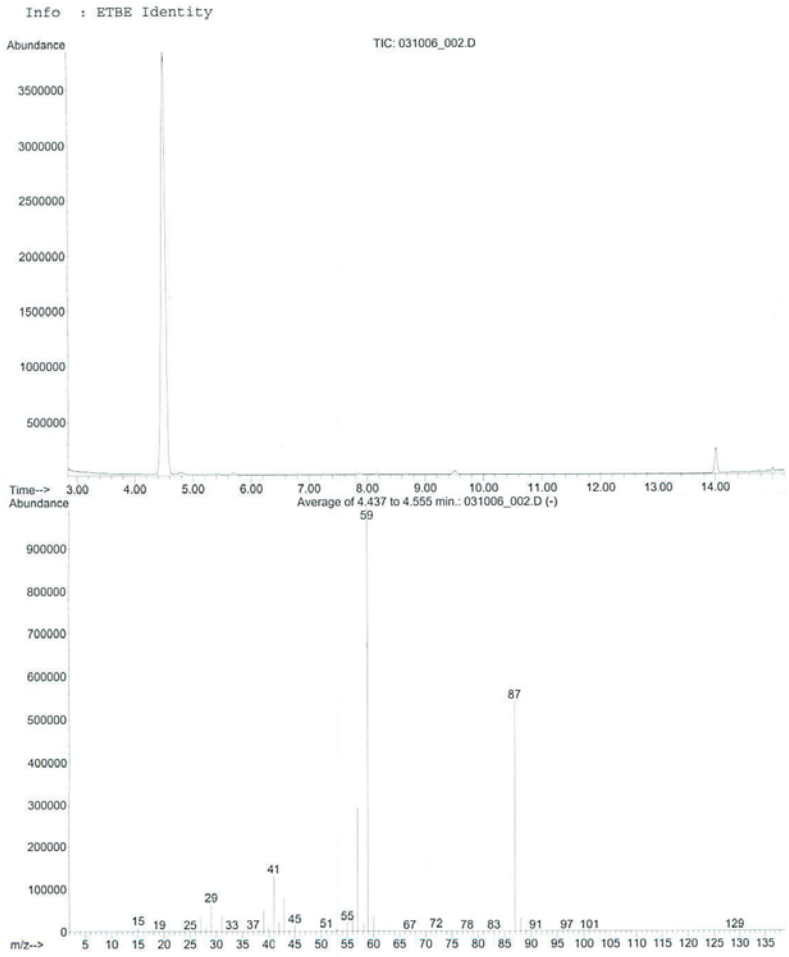


Figure 4. Total Ion Chromatogram (top) and Mass Spectrum (bottom) of vendor supplied ETBE, analyzed on March, 10, 2006 by Y. Hong.

Test Chemical Analysis Report **tert- Butyl Ethyl Ether**
RTI Reference 12307-11

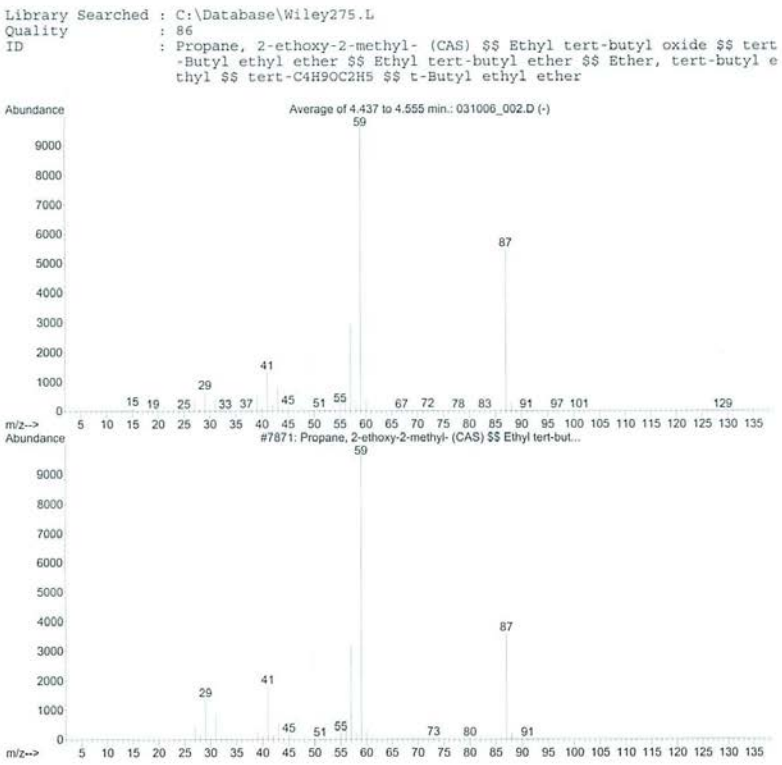


Figure 5. EI Mass Spectrum of ETBE (top) and Library Search (bottom)

Appendix C

Method Validation Plan

Analytical Method

Validation Report

Method Validation Report

Analysis of Ethyl Tertiary Butyl Ether in Blood Samples

SPONSOR: Section 211(b) Research Group
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Washington, DC 20005

TEST SITE: RTI International*
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Research Triangle Park, NC 27709

RTI PROTOCOL NO: RTI-932

RTI PROJECT NO.: 0209408.007

PRINCIPAL INVESTIGATOR: Timothy R. Fennell

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Norman F. Gaudette, Jr. Date

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Approval:

Timothy R. Fennell 1/12/2015
Timothy R. Fennell, Ph.D Date

Principal Investigator, RTI

RTI International is a trade name of Research Triangle Institute.

SUMMARY

The analytical method titled Project Specific Method for Analysis of Ethyl tertiary Butyl Ether in Blood Samples (AM-0209408.007) was validated in preparation for analyses of blood samples in order to determine concentrations of Ethyl tertiary butyl ether (ETBE) present in the matrix following an oral exposure study. This validation established a Limit of Quantitation (LOQ), accuracy, and precision of the method. The method consists of GC/MS analysis of ETBE using mass-selective detector with injection of headspace from sample vials.

The validation procedure established a calibration range of 0.110 μg/ml to 250 μg/ml for analysis of ETBE in the headspace of blood samples using two calibration curves. The low-concentration curve was defined by a linear regression (y=bx+a) of slope 0.08065, intercept -0.0006558, and linear correlation coefficient of 0.9998. The high-concentration curve was defined by the linear regression (y=bx+a) of slope 0.96736, intercept -0.1292, and linear correlation coefficient of 0.9994. Using the regression equation, calculated concentrations for all calibration standards were within the acceptance criteria of ± 15% (20% for the LOQ) of the nominal concentrations. Mean calculated concentrations for the replicate concentration points assayed at 0.540 μg/ml, 5.06 μg/ml, and 101 μg/ml for determination of precision and accuracy ranged from 96.2% to 99.3% of nominal concentration. Precision around the mean for each of these replicate concentration points was calculated to be well within the acceptance criteria of 15% CV. Additionally, the mean values for each concentration were well within the acceptance criteria of 15% deviation from nominal concentration.

Table of Contents

Page

1.0 INTRODUCTION

The analytical method titled Project Specific Method for Analysis of Ethyl tertiary Butyl Ether in Blood Samples (AM-0209408.007) was validated in preparation for analyses of blood samples in order to determine concentrations of Ethyl tertiary butyl ether (ETBE) present in the matrix following an oral exposure study (Ethyl Tertiary Butyl Ether Oral Absorption in Male and Female Rats, Study Protocol, RTI Protocol RTI 932). This validation established a Limit of Quantitation (LOQ), accuracy, and precision for the method and its calibration range. The method utilizes GC/MS analysis of headspace from vials containing the blood samples with mass-selective detection. Procedures conducted in the validation included preparation of calibration standards, analysis of standards and samples, regression analysis, comparison to acceptance criteria, and sample concentration calculation. This validation was conducted with a Validation Plan (Ethyl Tertiary Butyl Ether Bioanalytical Method Validation, RTI Validation Plan). Criteria for acceptance of the validation (US Food and Drug Administration, 2001) were specified in the Validation Plan, and they are summarized in Table 1 below.

Table 1: Method Validation Acceptance Criteria

Item	Acceptance Criteria
Accuracy	Acceptance Criteria
Limit of Quantitation (LLOQ)	± 20% of Nominal
Low Conc. (above the LLOQ).	± 15% of Nominal
Middle Conc.	± 15% of Nominal
High Conc.	± 15% of Nominal
Precision	
Limit of Quantitation (LLOQ)	± 20%
Low Conc (above LLOQ)	± 15 % CV
Middle Conc:	± 15 % CV
High Conc.	± 15 % CV
Calibration Curve	
Linear Correlation Coefficient (r)	≥ 0.990

2.0 ETHYL TERTIARY BUTYL ETHER TEST SUBSTANCE (ETBE)

The ETBE test substance was utilized as the analytical standard for preparation of calibration standards.

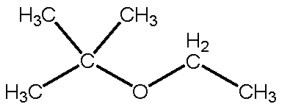
NAME: Ethyl *tertiary* Butyl Ether (ETBE; *tertiary* butyl ethyl ether, 2-ethoxy-2-methylpropane)

CAS No.: 637-92-3

MOLECULAR FORMULA: $C_6H_{14}O$

MOLECULAR WEIGHT: 102.17

STRUCTURE:



SOURCE OF TEST SUBSTANCE: ETBE was purchased from Sigma-Aldrich, Milwaukee, WI (Catalog number 253898, specified purity 99%). A certificate of analysis was obtained from the vendor.

LOT NUMBER(S): 04608LD *IDENTITY AND PURITY:* The identity of the unlabeled ETBE was confirmed at RTI by ^{1}H and ^{13}C NMR, and by mass spectrometry. The purity of the test chemical was determined by GC. Results of purity and identity determinations were previously reported to the sponsor in a separate report prior to initiation of the validation.

STORAGE CONDITIONS: ETBE was stored in the dark at room temperature.

3.0 REAGENTS AND CHEMICALS

Ethyl tertiary Butyl Ether (ETBE): 04608LD 99% purity, Sigma-Aldrich, St. Louis, MO. This test substance lot was used for preparation of all standards and QC samples. See Section 2.0 for a detailed description of the ETBE test article.

Methyl tertiary Butyl Ether (MTBE): Batch 02047CC, 99.9%, HPLC, Sigma-Aldrich, St. Louis, MO. This compound serves as the internal standard.

N, N-Dimethylformamide (DMF): Batch 01045AD, 99.8%, A.C.S reagent, Sigma-Aldrich, St. Louis, MO

Distilled/Deionized (D/I) water

Helium: 99.996% purity, obtained from National Welders, Durham NC.

Blank Rat Blood: Obtained from male Fischer 344 rats via cardiac puncture using syringes coated with sodium heparin.

4.0 ETBE STOCK SOLUTIONS

Two ETBE stock solutions were prepared at 25110 µg/ml (Stock A) and 25260 µg/ml (Stock B) in dimethylformamide. Use of two stock solutions to prepare separate calibration points was intended to provide additional evidence of the method's accuracy. Alternating the use of these stocks for preparation of adjacent calibration standards demonstrated accuracy through quality of the linear regression. The stock solutions were stored at approximately -20 °C.

The ETBE stock solutions were prepared by weighing DMF and ETBE in a 10 ml volumetric flask containing dimethylformamide. Just prior to addition of ETBE, a volume of DMF equivalent to the volume of ETBE about to be added was removed from the flask. ETBE was then added to the flask recording the mass added to the flask. Flask contents were then mixed by hand and immediately cooled in ice.

5.0 INTERNAL STANDARD SOLUTION

An internal standard solution of 50 µg/ml MTBE was prepared by dilution from a 1250 µg/ml stock solution. The stock solution was prepared by weighing 16 µl of MTBE into a 10-ml volumetric flask filled with distilled/deionized water. Prior to adding MTBE, an equivalent volume of distilled/deionized water was removed from the flask. Aliquots of the internal standard solution were sealed in separate vials ensuring a fresh unopened aliquot was used for preparing each set of standards or samples. The internal standard solution aliquots were stored at approximately -20 °C.

6.0 PREPARATION OF CALIBRATION STANDARDS AND SAMPLES FOR ANALYSIS

6.1 Calibration Spiking Solutions

Eleven calibration spiking solutions encompassing the concentration range of 1 and 2500 µg/ml were prepared for use in producing calibration standards (Table 2). Adjacent spiking standard concentrations were prepared using different stock solutions. Weights of water added to the solution and total solution weight were recorded. Dilution factors for each spiking standard solution were calculated by dividing the weight of added spiking standard solution by the total weight of solution. The density of DMF was used to calculate the volume of stock solution added, which is divided into the total weight of solution for dilution factor. Aliquots of each calibration spiking solution were removed and stored at approximately -20 °C for use in preparation of separate sets of calibration standards and samples.

Table 2

Calibration Spiking Solution Preparation

Spiking Standard Solution	Water (g)	Stock or Spiking Solution Used	Total Solution Weight (g)	Dilution Factor	Solution Concentration (µg/ml)
Spk. Std. A6	3.3037	Stock A	3.6459	0.0994	2497
Spk. Std. B5	3.5381	Stock B	3.6763	0.0398	1006
Spk. Std. A5 (HQC)	2.9509	Spk. Std. A6	3.6925	0.201	501
Spk. Std. B4	2.9357	Spk. Std. B5	3.9102	0.249	251
Spk. Std. A4	2.8996	Spk. Std. A5	3.6459	0.205	103
Spk. Std. B3 (MQC)	2.8896	Spk. Std. B4	3.6203	0.202	50.6
Spk. Std. A3	2.8800	Spk. Std. A4	3.8641	0.255	26.1
Spk. Std. B2	2.8852	Spk. Std. B3	3.6215	0.203	10.3
Spk. Std. A2 (LQC)	2.8214	Spk. Std. A3	3.5566	0.207	5.40
Spk. Std. B1	2.9262	Spk. Std. B2	3.9091	0.251	2.59
Spk. Std. A1 (LLQC)	2.8896	Spk. Std. A2	3.6250	0.203	1.10

6.2 Calibration Standards

Calibration standards were prepared in headspace vials at eleven ETBE concentrations using the calibration spiking solutions, blank Fischer 344 rat blood, and the 50 µg/ml MTBE internal standard solution. Three replicates were prepared at each concentration level for generation of the standard curves. In addition to the eleven ETBE standards concentrations, three blank blood samples (no internal standard), and six blank blood samples with internal standard were prepared. Table 3 details the composition of all blanks and standard concentrations.

Table 3.

Calibration Standard and QC Sample Components

Standard or QC Sample ID	ETBE Concentration (µg/ml)	Blank Blood (µl)	Spiking Solution Used	Spiking Solution Aliquot Volume (µl)	Volume Internal Standard Solution (µl)
Blood Blank	0	100	n/a	0	0
ISTD Blank	0	100	n/a	0	10
Std A1 (LLQC)	0.110	90	A1	10	10
Std B1	0.259	90	B1	10	10
Std A2 (LQC)	0.540	90	A2	10	10
Std B2	1.03	90	B2	10	10
Std A3	2.61	90	A3	10	10
Std B3 (MQC)	5.06	90	B3	10	10
Std A4	10.3	90	A4	10	10
Std B4	25.1	90	B4	10	10
Std A5	50.1	90	A5	10	10
Std B5 (HQC)	101	90	B5	10	10
Std A6	250	90	A6	10	10

6.3 LOQ, Accuracy, and Precision

Three additional replicate samples were prepared at each of the 0.540 µg/ml (LQC), 5.06 µg/ml (MQC) and 101 µg/ml (HQC) concentrations for use in accuracy and precision determinations in addition to the three samples prepared for use as calibration standards. Three additional replicate samples were

also prepared at the 0.110 µg/ml (LLQC)) concentration for use in establishing the Lower Limit of Quantitation (LLOQ) also referred to as the LOQ.

7.0 STABILITY STUDIES

7.1 Stability in Solution

Concentration stability of ETBE spiking solutions was investigated over a seven day period at -20 °C. The two spiking solutions used to prepare the HQC and LQC calibration standards were prepared and stored at approximately -20 °C for seven days prior to analysis. At the analysis timepoint, three aliquots of the two spiking solutions were transferred to separate headspace autosampler vials. Aliquots (10 µl) of the 50 µg/ml of the internal standard solution were then added to each vial prior to analysis.

7.2 Stability in Blood

The stability of ETBE in blood samples was tested under sample storage conditions expected to be utilized during the animal studies. During the studies, samples are expected to be analyzed within 24 h following collection. Four sets of blank blood samples were prepared. Each set consisted of three samples at two blood concentrations: 0.540 µg/ml (LQC) and HQC 101 µg/ml (HQC). One set was analyzed immediately following preparation. The second set was stored for 8 hours at room temperature prior to analysis. The third and fourth sets were stored in refrigerator temperatures at approximately 4 °C for 16 h and 24 h, respectively.

8.0 STANDARD/SAMPLE LIST

The following table (Table 4) details the list of samples/standards prepared and analyzed during conduct of the validation.

Table 4: Standard and Sample Analysis List [1]

Standard/Sample	Replicate Number	ETBE Concentration (µg/ml)	Purpose[2]
Blank Blood	1	-	Blank
Blank Blood	2	-	Blank
Blank Blood	3	-	Blank
Blank Blood + IS	1	-	Blank
Blank Blood + IS	2	-	Blank
Blank Blood + IS	3	-	Blank
Blank Blood + IS	4	-	Blank
Blank Blood + IS	5	-	Blank
Blank Blood + IS	6	-	Blank
Blank Blood + IS	7	-	Blank
Std A1	1	0.110	Calib Std, LLOQ
Std A1	2	0.110	Calib Std, LLOQ
Std A1	3	0.110	Calib Std, LLOQ
Std A1	4	0.110	Calib Std, LLOQ
Std A1	5	0.110	Calib Std, LLOQ
Std A1	6	0.110	Calib Std, LLOQ
Std B1	1	0.259	Calib Std
Std B1	2	0.259	Calib Std
Std B1	3	0.259	Calib Std
Std A2	1	0.540	Calib Std, P + A
Std A2	2	0.540	Calib Std, P + A
Std A2	3	0.540	Calib Std, P + A
Std A2	4	0.540	Calib Std, P + A
Std A2	5	0.540	Calib Std, P + A
Std A2	6	0.540	Calib Std, P + A
Std B2	1	1.03	Calib Std
Std B2	2	1.03	Calib Std
Std B2	3	1.03	Calib Std
Std A3	1	2.61	Calib Std
Std A3	2	2.61	Calib Std
Std A3	3	2.61	Calib Std
Std B3	1	5.06	Calib Std, P + A
Std B3	2	5.06	Calib Std, P + A
Std B3	3	5.06	Calib Std, P + A
Std B3	4	5.06	Calib Std, P + A
Std B3	5	5.06	Calib Std, P + A
Std B3	6	5.06	Calib Std, P + A.

[1] Note: Order in the table does not indicate order of analysis in the sample set.
[2] P + A = Precision and Accuracy, Calib Std = Calibration Standard

Table 4 (Continued): Sample/Standard Analysis List [1]

Standard/Sample	Replicate Number	ETBE Concentration (µg/ml)	Purpose[2]
Std A4	1	10.3	Calib Std
Std A4	2	10.3	Calib Std
Std A4	3	10.3	Calib Std
Std B4	1	25.1	Calib Std
Std B4	2	25.1	Calib Std
Std B4	3	25.1	Calib Std
Std A4	1	10.3	Calib Std
Std A5	1	50.1	Calib Std
Std A5	2	50.1	Calib Std
Std A5	3	50.1	Calib Std
Std B5	1	101	Calib Std, P + A
Std B5	2	101	Calib Std, P + A
Std B5	3	101	Calib Std, P + A
Std B5	4	101	Calib Std, P + A
Std B5	5	101	Calib Std, P + A
Std B5	6	101	Calib Std, P + A
Std A6	1	250	Calib Std
Std A6	2	250	Calib Std
Std A6	3	250	Calib Std
LQC RT 0 h	1	0.540	Stab in Blood
LQC RT 0 h	2	0.540	Stab in Blood
LQC RT 0 h	3	0.540	Stab in Blood
HQC RT 0 h	1	101	Stab in Blood
HQC RT 0 h	2	101	Stab in Blood
HQC RT 0 h	3	101	Stab in Blood
LQC RT 8 h	1	0.540	Stab in Blood
LQC RT 8 h	2	0.540	Stab in Blood
LQC RT 8 h	3	0.540	Stab in Blood
HQC RT 8 h	1	101	Stab in Blood
HQC RT 8 h	2	101	Stab in Blood
HQC RT 8 h	3	101	Stab in Blood
LQC 4 °C 16 h	1	0.540	Stab in Blood
LQC 4 °C 16 h	2	0.540	Stab in Blood
LQC 4 °C 16 h	3	0.540	Stab in Blood
HQC 4 °C 16 h	1	101	Stab in Blood
HQC 4 °C 16 h	2	101	Stab in Blood
HQC 4 °C 16 h	3	101	Stab in Blood

[1] Note: Order in the table does not indicate order of analysis in the sample set.
[2] P + A = Precision and Accuracy, Calib Std = Calibration Standard, Stab in Blood = Stability in Blood

Table 4 (Continued): Sample/Standard Analysis List [1]

Standard/Sample	Replicate Number	ETBE Concentration (µg/ml)	Purpose[2]
LQC 4 °C 24 h	1	0.540	Stab in Blood
LQC 4 °C 24 h	2	0.540	Stab in Blood
LQC 4 °C 24 h	3	0.540	Stab in Blood
HQC 4 °C 24 h	1	101	Stab in Blood
HQC 4 °C 24 h	2	101	Stab in Blood
HQC 4 °C 24 h	3	101	Stab in Blood
Spk LQC (0 days)	1	5.40	Spike Sol Stab
Spk LQC (0 days)	2	5.40	Spike Sol Stab
Spk LQC (0 days)	3	5.40	Spike Sol Stab
Spk MQC (0 days)	1	50.6	Spike Sol Stab
Spk MQC (0 days)	2	50.6	Spike Sol Stab
Spk MQC (0 days)	3	50.6	Spike Sol Stab
Spk LQC (7 days)	1	5.40	Spike Sol Stab
Spk LQC (7 days)	2	5.40	Spike Sol Stab
Spk LQC (7 days)	3	5.40	Spike Sol Stab
Spk MQC (7 days)	1	50.6	Spike Sol Stab
Spk MQC (7 days)	2	50.6	Spike Sol Stab
Spk MQC (7 days)	3	50.6	Spike Sol Stab

[1] Note: Order in the table does not indicate order of analysis in the sample set.
[2] Stab in Blood = Stability in Blood, Spike Sol Stab = Spike Solution Stability.

9.0 GC/MS ANALYSIS OF SAMPLE AND STANDARDS

Samples and standards were assayed using an Agilent Model 6890 gas chromatograph fitted with an Agilent Model 5973 Mass Selective Detector. Samples of air from the headspace of sample vials were introduced into the gas chromatograph using an Agilent Model G1888A headspace autosampler. Data acquisition and instrument control was performed by Agilent MSD Security Chemstation Software Version A.02.01 (Agilent technologies, Wilmington, DE). Instrumental operating parameters and other analysis parameters are detailed in Table 5. A representative chromatogram, shown in Figure 1, contain selected ion chromatograms for m/z 87 (ETBE) and m/z 73 (MTBE).

Table 5: Instrument Parameters

6890 GC	
Injection method	split/splitless
Temperature	150 ºC
Split ratio	15:1 (calculated by adding flow rate of HSS)
Carrier gas	Helium
Flow rate	1.7 ml/min
Column	DB-624 30m x 0.32 mm i.d. 1.8 µm film thickness
	(J&W, Agilent technologies, Wilmington, DE)
GC oven program	
Initial temperature	40 ºC
Initial time	2 min
Temperature program rate	5 ºC/min
Final temperature	50 ºC
Final time	0 min
Temperature rate A	50 ºC/min
Final temperature A	150 ºC
Final time A	0 min
Run time	6 min
G1888A headspace sampler	
Loop size	1 ml
Vial Pressure	15 psig
Carrier Pressure	2.8 psig
Headspace oven	65 ºC
loop temperature	90 ºC
Transfer line temperature	110 ºC
Equilibration time	10 min
GC cycle time	10 min
Pressurization	0.2 min
Loop Fill	0.2 min
Loop Equilibration	0.05 min
Inject	0.5 min
Shake	low
5973 MSD	
Scan	EI mode
SIM	ETBE (m/z) 87; MTBE (m/z 73)
Source temperature	230 ºC
Quad temperature	150 ºC
Transfer line	230 ºC
Tune	Atune.u
Solvent delay	3.0 min
Timed detector off	6.0 min

10.0 CALCULATIONS

Peak areas for ETBE and the MTBE internal standard were used to calculate the internal standard ratio. For each chromatographic run, the internal standard ratio was computed using the following equation:

$$\text{Internal Standard Ratio} = \frac{\text{ETBE Peak Area}}{\text{Internal Standard Peak Area}}$$

Resulting concentration and internal standard ratios for all calibration standards were input into the curve fitting software TableCurve 2D for Windows (Version 2.04, Systat Software Inc., Richmond Ca) for calculation of the linear regression coefficients. Regression coefficients of slope and intercept were then used to quantitate ETBE in all standards and samples.

11.0 VALIDATION RESULTS AND DISCUSSION

11.1 Calibration Curve

Two calibration curves were constructed to encompass the entire standard concentration range. The low concentration curve included standards from 0.110 μg/ml to 5.06 μg/ml ETBE (Table 6 and Figure 2). The high concentration curve encompassed standard concentrations from 5.06 μg/ml to 101 μg/ml ETBE (Table 7 and Figure 2). Both curves were weighted by a factor of 1/x in order to provide accuracy at the lower end of each calibration range. Calculation of linear (least squares) regression generated a slope of 0.08065 and intercept value of -0.0006558 for the low curve. The linear correlation coefficient (r) for the low concentration curve was 0.9998. Slope and intercept values were 0.09673 and -0.1292, respectively for the high concentration curve. The linear correlation coefficient (r) was 0.9994 for the high curve. Correlation coefficients resulting from both curves exceeded the minimum acceptance criterion of 0.990.

Concentrations of the individual calibration standards were then calculated using the respective regression equations. Resulting concentrations (Table 6) were within ±15% of the nominal values. The method validation plan included this limit for acceptance of the calibration curves. Therefore, the standard curves constructed by the analytical method met both criteria stated for acceptance in the validation plan.

11.2 Limit of Quantitation

Results of six analyses of standards at the 0.110 μg/ml ETBE concentration yielded a mean concentration of 0.115 μg/ml ETBE. The mean value was within ± 20% of nominal concentration stated in the validation plan for acceptance. The individual concentration determinations for each replicate varied

around the mean by 1.54% (%CV). The precision around the mean was also within the ± 20% CV criterion specified in the validation plan. Therefore, this standard concentration met criteria in the validation plan for establishing it as the limit of quantitation.

11.3 Accuracy and Precision

Determination of precision and accuracy utilized results obtained from analyses of six standards at each of three ETBE concentrations: 0.540 μg/ml, 5.06 μg/ml, and 101 μg/ml. Table 8 details assay results and precision and accuracy calculation results. The accuracy of the analytical method was defined as the closeness of test results obtained by the method to the nominal values of each standard. The acceptance criterion for accuracy of the method was a mean concentration determination within ± 15% of the nominal concentration at each of the three standard concentrations. Precision was measured by calculating the Percent Coefficient of Variation (%CV) around the mean for each set of determinations per concentration. The acceptance limit for precision was ± 15% for each set of determinations per concentration.

Mean calculated concentrations for the three ETBE standard concentrations were 0.520 μg/ml, 5.03 μg/ml, and 98.5 μg/ml, respectively. These mean concentration results were well within the acceptance criterion of ± 15% of nominal concentration, and correspond to accuracy values of 96.2%, 99.3%, and 101% of the nominal concentrations. Therefore, accuracy of the method was established within the calibration range. An additional measure of accuracy in this validation was provided by linearity of the standard curve. Adjacent calibration concentrations in the curve were prepared from different stock solutions. Therefore, attainment of acceptable calibration curves provided this additional measure.

11.4 Stability in Blood

Mean values resulting from analyses of blood samples of 0.540 μg/ml and 101 μg/ml ETBE concentrations stored at room temperature for 8 h were determined to be 101% and 102% of the initial concentration prior to storage (Table 9). Analysis of blood samples (also 0.540 μg/ml and 101 μg/ml ETBE) following storage at 4 °C for 16 h yielded mean concentrations that were 93.2% and 91.6% of the initial concentration respectively (Table 10). Following storage for 24 h at 4 °C, ETBE concentration was determined to be 87.6% and 90.4% for the two concentrations, respectively. In all cases, measured concentrations were within the ± 15% of initial concentration acceptance criterion following storage for 8 h at room temperature or at 4 °C for 16 h or 24 h.

11.5 Stability in Solutions

Mean concentrations obtained from analyses of two ETBE spiking solutions stored at -20 °C for seven days were within the acceptance criterion of ± 15% of original measured concentrations at preparation. Analysis results are reported in Table 11. Mean analysis results for each of the two solutions at preparation were 5.18 μg/ml and 50.0 μg/ml ETBE, respectively. Following storage for seven

days, mean analysis results were 5.20 μg/ml and 48.3 μg/ml, respectively. These concentrations were 100% and 96.6% of the initial concentrations prior to storage for the 5.18 μg/ml and 50.0 μg/ml solutions, respectively.

12.0 REFERENCES

Ethyl Tertiary Butyl Ether Oral Absorption in Male and Female Rats, Study Protocol, RTI Protocol RTI-932, February 23, 2007.

Ethyl Tertiary Butyl Ether Bioanalytical Method Validation, RTI Validation Plan, April 2, 2007.

Project Specific Analytical Method for Analysis of Ethyl Tertiary Butyl Ether in Blood Samples, Analytical Method, AM-0209408.007, April 2, 2007.

US Food and Drug Administration (2001). Guidance for Industry: Bioanalytical Method Validation.

Table 6

Standard Concentration Results (Low Concentration Curve)

Standard	Internal Standard Ratio	Nominal Conc. (µg/ml)	Calculated Conc. (µg/ml)	Accuracy (%)	Mean Calc. Conc. (µg/ml)
Std A1 (LLOQ)	0.00867	0.110	0.116	105	0.116
Std A1 (LLOQ)	0.00869	0.110	0.116	105	
Std A1 (LLOQ)	0.00879	0.110	0.117	106	
Std B1	0.0203	0.259	0.260	100	0.257
Std B1	0.0196	0.259	0.251	96.9	
Std B1	0.0203	0.259	0.260	100	
Std A2	0.0419	0.540	0.528	97.8	0.524
Std A2	0.0416	0.540	0.523	96.9	
Std A2	0.0414	0.540	0.522	96.7	
Std B2	0.0795	1.03	0.994	96.5	1.00
Std B2	0.0801	1.03	1.00	97.2	
Std B2	0.0811	1.03	1.01	98.4	
Std A3	0.205	2.61	2.55	97.7	2.60
Std A3	0.212	2.61	2.63	101	
Std A3	0.211	2.61	2.62	100	
Std B3	0.412	5.06	5.12	101	5.11
Std B3	0.410	5.06	5.09	101	
Std A3	0.411	5.06	5.11	101	

Table 7

Standard Concentration Results (High Concentration Curve)

Standard	Internal Standard Ratio	Nominal Conc. (µg/ml)	Calculated Conc. (µg/ml)	Accuracy (%)	Mean Calc. Conc. (µg/ml)
Std B3	0.412	5.06	5.60	111	5.59
Std B3	0.410	5.06	5.57	110	
Std B3	0.411	5.06	5.59	110	
Std A4	0.849	10.3	10.1	98.2	9.77
Std A4	0.876	10.3	10.4	101	
Std A4	0.721	10.3	8.79	85.4	
Std B4	2.21	25.1	24.2	96.2	24.3
Std B4	2.26	25.1	24.7	98.6	
Std B4	2.20	25.1	24.1	96.0	
Std B2	4.68	50.1	49.8	99.3	49.4
Std B2	4.64	50.1	49.3	98.4	
Std B2	4.62	50.1	49.1	98.0	
Std A3	9.26	101	97.1	96.5	98.9
Std A3	9.74	101	102	101	
Std A3	9.32	101	97.6	97.1	
Std B3	24.9	250	259	104	254
Std B3	24.6	250	256	102	
Std A3	23.7	250	247	98.8	

Table 8

Precision and Accuracy Results

Standard	Internal Standard Ratio	Nominal Conc. (µg/ml)	Calculated Conc. (µg/ml)	S.D. (%)	% RSD	Mean Calc. Conc. (µg/ml)	Mean Accuracy (%)
Std A1 (LLOQ)	0.00867	0.110	0.116	0.00177	1.54	0.115	104
Std A1 (LLOQ)	0.00869	0.110	0.116				
Std A1 (LLOQ)	0.00879	0.110	0.117				
LLOQ	0.00844	0.110	0.113				
LLOQ	0.00861	0.110	0.115				
LLOQ	0.00844	0.110	0.113				
Std A2	0.0419	0.540	0.528	0.00725	1.40	0.520	96.2
Std A2	0.0416	0.540	0.523				
Std A2	0.0414	0.540	0.522				
LQC2	0.0402	0.540	0.507				
LQC	0.0410	0.540	0.517				
LQC	0.0413	0.540	0.520				
Std B3	0.412	5.06	5.12	0.220	4.38	5.03	99.3
Std B3	0.410	5.06	5.09				
Std B3	0.411	5.06	5.11				
MQC	0.415	5.06	5.15				
MQC	0.412	5.06	5.12				
MQC	0.369	5.06	4.58				
Std B5	9.26	101	97.1	2.10	2.14	98.5	97.9
Std B5	9.74	101	102				
Std B5	9.32	101	97.6				
HQC	9.45	101	99.0				
HQC	9.46	101	99.1				
HQC	9.15	101	95.9				

Table 9

Concentration of ETBE in Blood Samples Stored at Room Temperature

Analysis Timepoint	Nominal Conc. (µg/mL)	Measured Conc. (µg/mL)	Mean Measured Conc. (µg/mL)	SD	%CV	Percent of Time 0 Conc. (%)
Time 0	0.540	0.482	0.485	0.0400	0.825	n/a
		0.484				
		0.490				
	101	93.1	94.1	0.923	0.981	n/a
		94.4				
		94.9				
8 h	0.540	0.480	0.492	0.0119	2.43	101
		0.504				
		0.492				
	101	97.2	96.4	0.954	0.99	102
		96.7				
		95.3				

Table 10

Concentration of ETBE in Blood Samples Stored at Approximately 4 °C

Analysis Timepoint	Nominal Conc. (µg/mL)	Measured Conc. (µg/mL)	Mean Measured Conc. (µg/mL)	SD	%CV	Percent of Time 0 Conc. (%)
Time 0	0.540	0.482 0.484 0.490	0.485	0.0400	0.825	n/a
	101	93.1 94.4 94.9	94.1	0.923	0.981	n/a
16	0.540	0.461 0.452 0.444	0.452	0.00873	1.93	93.2
	101	84.6 87.2 87.0	86.3	1.45	1.68	91.6
24	0.540	0.426 0.429 0.419	0.425	0.00507	1.19	87.6
	101	85.1 86.7 83.5	85.1	1.57	1.85	90.4

Table 11

Concentration of ETBE Calibration Spiking Solutions Stored at Approximately -20 °C

Analysis Timepoint	Nominal Conc. (µg/mL)	Measured Conc. (µg/mL)	Mean Measured Conc. (µg/mL)	SD	%CV	Percent of Day 0 Conc. (%)
Day 0	5.40	5.24 5.17 5.12	5.18	0.0611	1.18	n/a
	50.6	50.8 50.2 49.0	50.0	0.917	1.83	n/a
7 Days	540	5.29 5.11 5.19	5.20	0.0918	1.77	100
	50.6	49.3 48.1 47.6	48.3	0.891	1.84	96.6

Figure 1: Representative Total Ion Chromatogram and Single-Ion Chromatograms Obtained During Analysis of an ETBE Standard

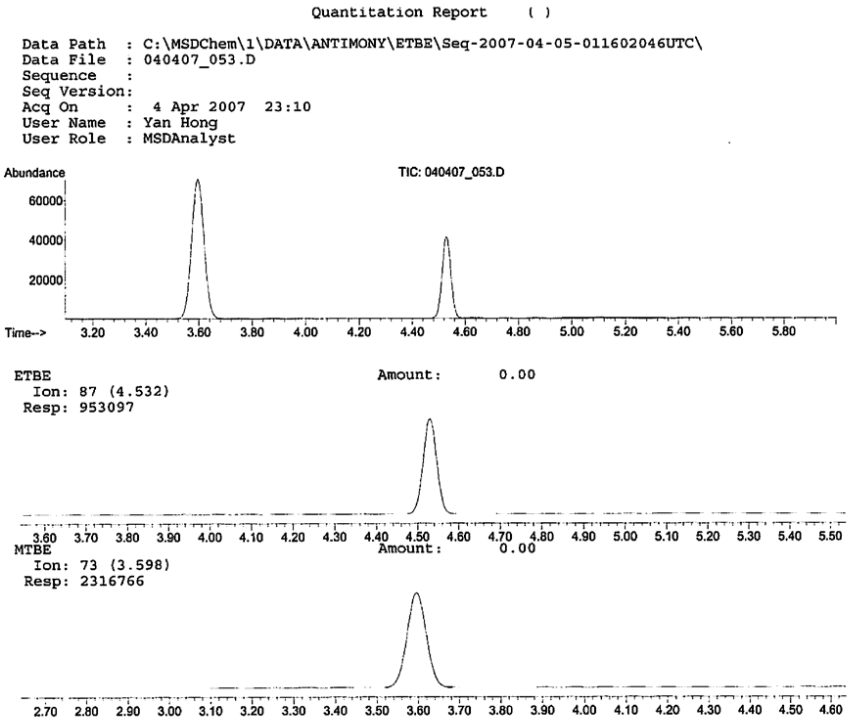
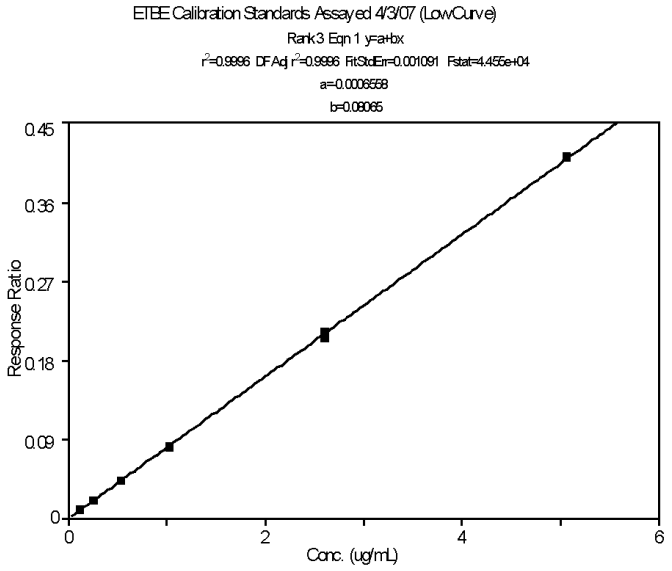
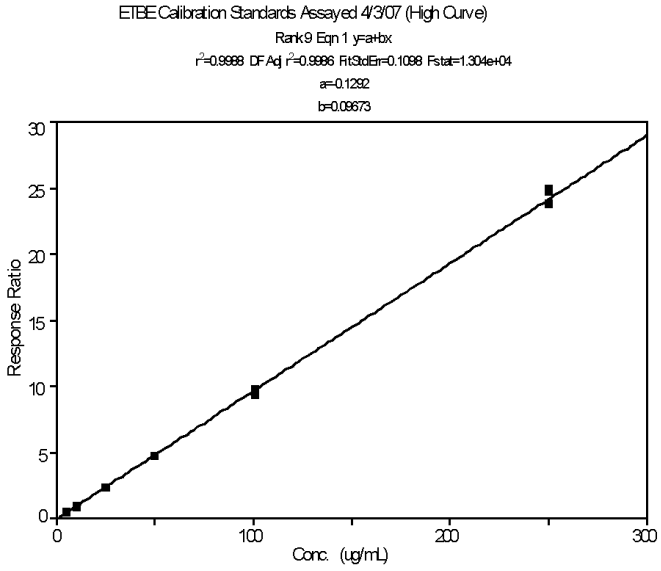


Figure 2: ETBE Standard Calibration Curves





Appendix 1

Method Validation Plan

VALIDATION PLAN	RTI INTERNATIONAL* POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	Page 1 of 6

TITLE: **Ethyl tertiary Butyl Ether Bioanalytical Method Validation**

SPONSOR: Section 211(b) Research Group
American Petroleum Institute
1220 L Street NW
Washington, DC 20005

TESTING FACILITY: Science and Engineering
RTI International*
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Research Triangle Park, NC 27709

RTI PROJECT NO.: 0209408.007

RTI STUDY DIRECTOR: Timothy R. Fennell

APPROVAL SIGNATURES:

Timothy R. Fennell, Ph.D.
Study Director, RTI

04-02-07
Date

*RTI International is a tradename of Research Triangle Institute.

VALIDATION PLAN	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	Page 2 of 6

Table of Contents

VALIDATION PLAN	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	Page 3 of 6

1.0 PRINCIPLES AND CRITERIA FOR VALIDATION OF ANALYTICAL METHOD

This document details components and acceptance criteria for validating a method designed to determine the concentration of Ethyl tert-Butyl Ether (ETBE) in rat blood. Analysis of samples, standards, and quality control samples will be performed using gas chromatography-mass spectrometry (GC/MS) coupled with headspace sample introduction. The analysis system has been previously validated with respect to the accuracy and reliability of generated data and computed results.

1.1 Project Specific Analytical Method (PSAM)

- A detailed project specific analytical method (PSAM) describing the analysis method will be written prior to the start of validation.
- Included in the PSAM will be a statement of the principle of the analytical method, a listing of necessary reagents, test solutions and mixtures with directions for their preparation and specified storage conditions. It will also contain a listing of the required instrumentation, instrumental parameters and a step-by-step description of the entire analytical procedure.

1.2 LOQ

- **Definition:** The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable accuracy and precision under the stated experimental conditions. The response at the LOQ is at least 5 times the response compared to blank response.
- **Raw data required:** The LOQ will be established by analyzing at least five replicate samples from rat blood matrix, which is also LLQC, at the lowest concentration in the range to be quantitated. These replicate samples will consist of three calibration standards used to generate the lowest curve concentration and an additional three replicate samples.
- **Acceptance specifications:** Two criteria must be met in order to establish this concentration as the LOQ. The mean value must be within ±20% of the nominal value. The precision around the mean value must not exceed 20% CV.

1.3 Quality Control Standards

- **Definition:** A spiked sample used to monitor the performance of a bioanalytical method to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

VALIDATION PLAN	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	Page 4 of 6

- **Raw data required:** A Quality control standards (QCSs) will be prepared in a rat blood matrix, which include a minimum number of three concentrations (LQC, MQC and HQC) representing the entire range of a standard curve required by the study. LQC will be at a concentration within 3 x the LLOC, MQC will be near the center and HQC will be near the upper limit of the standard curve. The QC samples in duplicate at each concentration must be analyzed along with a sample batch.
- **Acceptance specifications:** At least 4 out of 6 of QCSs must fall within 15% of the nominal value. The two QC samples that may fail the criteria must not be at the same concentration.

1.4 Accuracy and Precision

- **Definition:** The accuracy of an analytical method is the closeness of test results obtained by that method to the nominal or true value. The Precision is a measure of the degree of reproducibility of the analytical method under normal operating circumstances.
- **Raw data required:** Determination of accuracy and precision for concentrations of analyte through the range of standard curve, will be accomplished by analyzing replicate sets of known ETBE concentration in rat blood. The accuracy and precision will be determined using six replicate samples for each sets concentrations. Accuracy and precision for the lowest point, the LLQC, will be determined as described in section 1.2.
- **Acceptance specifications:** The mean value for the each set must be within ±15% of the nominal value. The precision around the mean value must not exceed 15% coefficient of variation (CV).

1.5 Standard Curve

- **Definition:** A standard curve details the relationship between instrument response ratio (analyte to internal standard) and known concentration of the analyte. Acceptance of the standard curve is also a reflection of accuracy of the method when adjacent calibration points are prepared from different stock solutions.
- **Raw data required:**

1. A minimum number of six standards per curve will define the relationship between concentration and response.
2. A standard curve will be prepared by spiking the rat blood with known concentration of ETBE and a consistent amount of MTBE as internal standard.

VALIDATION PLAN	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	Page 5 of 6

3. Standard concentration and curve range will be determined based on the requirement of the study.
4. A standard curve must consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and minimum six non-zero samples covering the expected range, including LOQ.
5. Adjacent concentration points on the curve will be prepared from different stock solutions. Two stock solutions will be used.

- **Acceptance specifications:** A regression equation describing the concentration and instrument response relationship will be determined. A minimum of six standards, when back-calculated using the regression equation, must fall within 15% of the nominal value, except for LOQ (20%). The correlation coefficient (r) must be greater than 0.990.

1.6 Stability

- **Definition:** The chemical stability of an analyte in a given matrix under specific conditions for specified time intervals. Stability procedures will evaluate the stability of the analyte during sample handling and short-term storage. Each step in the procedure will be assessed to determine the extent to which experimental and matrix variables may affect the quantitation of the analyte in the matrix.
- **Raw data required for stability:** To demonstrate storage stability of ETBE, a minimum of three determinations at two concentrations (LQC and HQC) is examined.
- **Determination of Stability of ETBE Concentration in blood samples:** Samples used for determining concentration stability in blood will be prepared by placing blank aliquots of blood matrix, ETBE, and the internal standard in headspace vials. These blood matrix samples will be maintained at room temperature until analysis. It is anticipated that blood samples will be analyzed by GC/MS within 24 hours of collection in the animal studies.

  ETBE stability will be determined by preparing four sample sets (a sample set will consist of three replicates at two concentrations). One set of samples will be analyzed immediately after preparation. The second set will be stored at room temperature for at least 8 hours prior to analysis. The third set will be stored at approximately 4° C in a refrigerator for 16 hours before analysis. The fourth set will be stored at approximately 4° C in a refrigerator for 24 hours before analysis.

VALIDATION PLAN	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	Page 6 of 6

- **Spiking solution stability:** Concentration of ETBE in spiking solutions will be determined following storage at approximately -20 ° C for 7 days. A minimum of two spiking solutions at low (LQC) and medium (MQC) concentrations will analyzed in three replicates by using the calibration curve in triplicate of the freshly prepared spiking solutions by relationship of the response ratio of ETBE to internal standard of MTBE versus the spiking concentration. 10 µl of spiking solution will be added into the headspace vial, and the vial crimp sealed; 10 µl of internal standard solution will be added into the vial through septum using 10-µl syringe for headspace GC/MS analysis.
- **Acceptance specifications:** The mean value determined for each concentration of ETBE level must be within ±15% of its initial analyzed concentration. The precision around the mean value must not exceed 15% CV.

1.7 Analysis Preparation for Validation

1 Prepare three sets of calibration standards including one set of QC samples including LLQC, LQC, MQC and HQC before analysis, individually.

2 Prepare four sets of the LQC and HQC in triplicate, which will be analyzed immediately, after storage at room temperature for 8 hours and after storage at approximately 4° C for 16 hours and for 24 hours.

4 The analysis order for validation is listed below:
The prepared calibration curves are stored in refrigerator when waiting for analysis. The stability samples are stored under the required conditions.

1) Calibration curve set 1, including one set of QC samples.
2) Stability samples
3) Calibration curve set 2, including one set of QC samples.
4) Stability samples.
5) Calibration curve set 3, including one set of QC samples.

1.8 Instrument Validation

- Analysis of samples, standards and quality control samples will be performed on Headspace GC-MS system that has been validated with respect to the accuracy and reliability of generated data and computed results. Complete documentation of the system validation procedure will be available for inspection.

Appendix 2

Analytical Method

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 1 of 13

TITLE: Project Specific Analytical Method for Analysis of Ethyl tertiary Butyl Ether in Blood Samples

SOURCE: Science and Engineering

AUTHOR: Signed Yan Hong
Yan Hong
Date 04-02-2007

APPROVED BY: Signed Timothy R. Fennell
Timothy R. Fennell
Date 04-02-07

EFFECTIVE DATE: 04-02-07
(This version)

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 2 of 13

1.0 INTRODUCTION

The methodology described in this document details procedures necessary for the quantitation of ethyl tert-butyl ether (ETBE) in rat blood. In this method, samples are analyzed using Gas Chromatography coupled with Mass Spectrometry detection (GC/MS). Samples are introduced into the instrument using a headspace autosampler and methyl tert-butyl ether (MTBE) serves as the internal standard. Procedures include preparation of calibration standards, analysis of standards and samples, regression analysis, acceptance criteria, and sample concentration calculations. This method is intended to be utilized in analyzes of ETBE in rat blood samples collected during studies designed to determine absorption and disposition of ETBE following oral administration. Blood samples (ca. 100 µL) collected from indwelling jugular cannulae from rats during the study will be used for analysis.

2.0 REAGENTS AND CHEMICALS

Ethyl tertiary Butyl Ether (ETBE): 99% purity, Sigma-Aldrich, St. Louis, MO. This test article lot approved by the study sponsor will be used for preparation of all standards and QC samples.

Methyl tertiary Butyl Ether (MTBE): 99.9%, HPLC, Sigma-Aldrich, St. Louis, MO. This compound serves as the internal standard.

N, N-Dimethylformamide (DMF): 99.8%, A.C.S reagent, Sigma-Aldrich, St. Louis, MO

Distilled/Deionized (D/I) water: Obtain this reagent from the Corning Still in Hermann 210.

Helium: 99.996% purity, obtained from National Welders, Durham NC.

3.0 EQUIPMENT

Equipment necessary for completion of procedures in this method are detailed in the table below.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 3 of 13

Table 1

Equipment

Item	Description
Electronic Balance	Four-place electronic balance, Mettler AG 204, or equivalent
Gas Chromatograph/Mass Spectrometer (GC/MS)	Agilent Model 6890 gas chromatograph equipped with an Agilent Model 5973 Mass Selective Detector (MSD), or equivalent
Headspace Autosampler interfaced to the GC/MS	Agilent G1888A headspace autosampler, or equivalent
Electronic Pipet (100 µL)	Rainin Model LTS-100, or equivalent
Electronic Pipet (1000 µL)	Rainin Model LTS-1000, or equivalent
Electronic Pipet (10 µL)	Rainin Model LTS-10, or equivalent
Microsyringe (10 µL)	Hamilton, Gastight #1801, or equivalent
Headspace Vial	Agilent Technologies, 10 ml Flat botton,or equivalent, silylated with hexamethyldisilazane using a vacuum oven at 225 °C
Headspace Crimp Cap	Agilent Technologies, PTFE/Si sep, 20mm, or equivalent
Electronic Crimper	Agilent Technologies, 20mm, or equivalent

4.0 SOLUTION PREPARATION (STOCK, INTERNAL STANDARD, AND SPIKING)

4.1 Stock Solutions

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 4 of 13

4.1.1 ETBE Stock Solution

Prepare two stock solutions (Stocks A and B) at a target concentration of 25000 µg/ml for subsequent dilution in preparation of calibration standards. Concentration of stock solution should be calculated in units of micrograms ETBE per milliliter (mL) of solution. Prepare the stock solution according to the follow procedure. Store the ETBE stock solutions at approximately -20 °C.

1) Fill a 10-ml volumetric flask with to the 10-mL volume mark.
2) Remove 328 µl of DMF from the flask using a pipet.
3) With the flask on an analytical balance, tare/zero the balance.
4) Add 340 µl of ETBE to the flask using a syringe. This volume of ETBE fills the flask, up to the 10-mL mark.
5) Record the mass (balance reading) of ETBE added to the flask.
6) Immediately cool the flask contents in wet ice. Mix the stock solution by hand.
7) Calculation the actual concentration of stock solutions.

4.1.2 MTBE Stock Solution

Prepare the MTBE stock solutions as described below.

1) Fill a 10-mL volumetric flask with distilled/deionized water to the volume mark.
2) With the flask on the analytical balance, remove 12 µl of water using pipet.
3) Tare/zero the analytical balance with the volumetric flask on the balance.
4) Add 16 µl of MTBE to the flask using a syringe. This volume will fill the solution up to the 10-mL volume mark.
5) Record the mass (balance reading) of MTBE added to the flask
6) Immediately cool the flask contents in wet ice. Mix the stock solution by hand.
7) Calculation the actual concentration of the stock solution.
8) Store MTBE stock solution at approximately -20 °C.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 5 of 13

4.2 Internal Standard (MTBE) Working Solution

The target internal standard solution concentration is 50 µg/ml. Store the internal standard solution at approximately -20 °C.

1) Calculate the actual amount of MTBE stock solution required to prepare a 10 mL volume of a 50 µg/mL MTBE solution.
2) Transfer the appropriate amount of the MTBE stock solution to a 10-mL volumetric flask.
3) Add water to the flask completing the 10-mL dilution. Mix the flask contents by hand.
4) Aliquot and store the internal standard working solution at approximately -20 °C.

4.3 Preparation of ETBE Spiking Solutions

Prepare a set of eleven calibration spiking solutions encompassing the concentration range of 1 and 2500 µg/mL. Adjacent spiking standard concentrations are prepared using different stock solutions. Prepare spiking solutions in wet ice. Ensure water used to prepare solutions is cooled in wet ice prior to use. Prepare the spiking solutions as described below. Amount of stock solution, distilled/deionized water and the target final volume required for preparation of calibration spiking solution are shown in the table 2. Collect the weight of added distilled/deionized water and total solution volumes where noted.

1) Add the cold distilled/deionized water into a tared vial, and record the weight of water added.
2) Transfer the appropriate amount of stock or spiking solution into the vial. Record the weight after spiking standard solution added. Total amount of solution = stock wt + water wt.
3) Mix the spiking standard solution by hand.
4) Calculate dilution factors for each spiking standard solution by dividing the weight of spiking standard solution by the total weight of solution except for the stock solution. The density of DMF is used to calculate the volume of stock solution added, which is divided to the total weight of solution for dilution factor.
5) Aliquot and store the spiking solutions at approximately -20 °C when not in use.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 6 of 13

Table 2

Preparation of ETBE Spiking Solutions

Spiking Standard Solution	Water Required (mL)	Stock or Spiking Solution required	Stock or Spiking Standard Aliquot Volume (mL)	Nominal Dilution Factor	Target Nominal Concentration (µg/mL)
Spk. Std. A6	X	Stock A	0.375	1:10	2500
Spk. Std. B5	Y	Stock B	0.150	1:25	1000
Spk. Std. A5 (HQC)	3	Spk. Std. A6	0.75	1:5	500
Spk. Std. B4	3	Spk. Std. B5	1	1:4	250
Spk. Std. A4	3	Spk. Std. A5	0.75	1:5	100
Spk. Std. B3 (MQC)	3	Spk. Std. B4	0.75	1:5	50
Spk. Std. A3	3	Spk. Std. A4	1	1:4	25
Spk. Std. B2	3	Spk. Std. B3	0.75	1:5	10
Spk. Std. A2 (LQC)	3	Spk. Std. A3	0.75	1:5	5.0
Spk. Std. B1	3	Spk. Std. B2	1	1:4	2.5
Spk. Std. A1 (LLQC)	3	Spk. Std. A2	0.75	1:5	1.0

X = [(Conc. Stock A x 0.375 mL)/(2500 µg/mL)]-0.375mL

Y = [(Conc. Stock B x 0.15 mL)/1000 µg/mL]-0.150mL

Note: Dilution factors shown in the table are targets for using pipets to perform the dilution. Weights of added water and total dilution weights must be collected for calculation of dilution factors by weight.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 7 of 13

5.0 PREPARATION OF CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES

Prepare blank samples (without internal standard), zero samples (containing internal standard), calibration standards, and quality control (QC) samples prior to analysis according to the procedure listed below and Table 3.

1) Aliquot 90 µl of blank blood into a 10-ml headspace vial.
2) Transfer 10 µl of ETBE spiking solution to the vial.
3) Immediately seal the vial with a crimp-top.
4) Add 10 µl of internal standard working solution to the vial through septum using a 10-µl microsyringe, except for the blank sample.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 8 of 13

Table 3

Preparation of ETBE Calibration Standards QC Samples in Blood

Standard ID	Spiking Solution ID	Volume of Rat Blood (µl)	Volume of Spiking Solution (µl)	Final Volume (µl)	Nominal final concentration (µg/ml)	Volume of ISTD added (µl)
Blood Blank	n/a	100	0	100	0	0
ISTD Blank	n/a	100	0	100	0	10
Std A1 (LLQC)	A1	90	10	100	0.1	10
Std B1	B1	90	10	100	0.25	10
Std A2 (LQC)	A2	90	10	100	0.50	10
Std B2	B2	90	10	100	1.00	10
Std A3	A3	90	10	100	2.50	10
Std B3 (MQC)	B3	90	10	100	5.00	10
Std A4	A4	90	10	100	10.0	10
Std B4	B4	90	10	100	25.0	10
Std A5	A5	90	10	100	50.0	10
Std B5 (HQC)	B5	90	10	100	100	10
Std A6	A6	90	10	100	250	10

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 9 of 13

6.0 PREPARATION OF BLOOD SAMPLES FOR ANALYSIS

Follow the procedure listed below for preparation of blood samples obtained during study for analysis.

1) Immediately upon collection of blood, place the blood sample in a pre-weighed 10-ml headspace vial. Seal the vial with a crimp-style cap and reweigh the filled vial to determine the weight of the whole blood sample.
2) Add 10 µl of an aqueous internal standard working solution of MTBE through the septum using 10-µl microsyringe.
3) Analyze samples as soon as possible. Storage limits are as follows: Room temperature for no longer than 8 hours, or refrigeration at approximately 4 ºC for no longer than 24 hours prior to loading these samples into the headspace autosampler.
4) Analyze QC samples throughout the blood samples.

7.0 ANALYSIS

5.1 Instrument Method

Assay samples and standards according to GC/MS instrument parameters detailed in Table 4.

5.2 Analysis Sequence

Analysis of standards and samples should proceed in the order detailed below.

1) Daily Tune of the MS spectrometer (prior to sample analysis)
2) Chromatography system verification sample (A spiking solution used for checking peak shape of analytes)
3) Calibration standard (Set 1)
4) Rat blood samples
5) Calibration standards (Set 2)
6) Rat blood samples
7) Calibration standards (Set 3)

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 10 of 13

Table 4 Instrument Conditions

6890 GC	
Injection method	split/splitless
Temperature	150 °C
Split ratio	15:1 (calculated by adding flow rate of HSS)
Carrier gas	Helium
Flow rate	1.7 ml/min
Column	DB-624 30m x 0.32 mm i.d. 1.8 µm film thickness (J&W, Agilent technologies, Wilmington, DE)
GC oven program	
Initial temperature	40 °C
Initial time	2 min
Temperature program rate	5 °C/min
Final temperature	50 °C
Final time	0 min
Temperature rate A	50 °C/min
Final temperature A	150 °C
Final time A	0 min
Run time	6 min
G1888A headspace sampler	
Loop size	1 ml
Vial Pressure	15 psig
Carrier Pressure	2.8 psig
Headspace oven	65 °C
loop temperature	90 °C
Transfer line temperature	110 °C
Equilibration time	10 min
GC cycle time	10 min
Pressurization	0.2 min
Loop Fill	0.2 min
Loop Equilibration	0.05 min
Inject	0.5 min
Shake	low
5973 MSD	
Scan	EI mode
SIM	ETBE (m/z) 87; MTBE (m/z 73)
Source temperature	230 °C
Quad temperature	150 °C
Transfer line	230 °C
Tune	Atune.u
Solvent delay	3.0 min
Timed detector off	6.0 min

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 11 of 13

8.0 CALCULATION OF RESULTS

8.1 Calibration Standards

Perform the following calculations using calibration standard assay results. When possible, use Microsoft Excel spreadsheets for the calculation.

1) Integrate the ETBE and MTBE peaks in all samples and standards to generate peak areas.

2) Generate two plots of peak area (internal standard) ratio of ETBE/MTBE versus the nominal standard concentration containing the following standard ranges: 1) ca. 0.1 µg/mL to ca. 5.0 µg/mL, and 2) ca. 5.0 µg/mL to ca. 250 µg/mL. Include all three replicate standards at each concentration in each plot.

3) Using the program TableCurve (Systat Software Inc., Richmond Ca), calculate the weighted (1/x) linear regression equation (Y=mx+b) for each plotted standard range.

4) Determine the correlation coefficient (r) for each linear regression.

5) Compute the concentration of all calibration standards using the regression equations and their coefficients of slope and intercept.

6) Compute the accuracy (%) for each standard by dividing the calculated concentration by the nominal standard concentration. Multiply the result by 100 to complete the calculation.

7) For the method validation results, compute concentration, mean concentration, standard deviation, and relative standard deviation for the six replicates prepared at three concentrations (three calibration standards plus the three additional standards prepared per concentration). Also, calculate the mean accuracy value for each of the three concentrations. These results are used to establish accuracy and precision of the method during validation.

8.2 Quality Control (QC) Samples

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 12 of 13

1) Use the linear regression equation to calculate concentration of ETBE in the QC samples.

2) Divide the calculated concentration by the nominal concentration and multiply the result by 100 to obtain the Accuracy % value.

8.3 Blood Samples

If the calibration curves meet acceptance criteria stated in Section 9.1, use the linear regression equations to calculate sample concentrations. Use Microsoft Excel spreadsheets to perform these calculations. Use the weight of sample collected to correct for differences between the actual sample weight and the volume of blood used for generation of standards.

9.0 ACCEPTANCE CRITERIA

9.1 Linear Regression and Calibration Curve

Acceptance criteria for the calibration curve are as follows:

1) Correlation coefficient (*r*) greater than or equal to 0.990

2) Measure concentrations of individual calibration standards must be within ± 15 % of the nominal value except the lowest concentration point (LLOQ) which must be ± 20 %.

9.2 QC Samples

If QC samples (LLQC excluded) are distributed throughout the sample set, measure concentrations of at least 4 out of 6 of QC samples must fall within 15% of the nominal value for acceptance of results for samples bracketed in between them.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-0209408.007.0 Page 13 of 13

REVIEW/ REVISION LOG

Rev. #	Rev. Date	Description
0	NA	Original version.

Appendix D

Pharmacokinetic Analysis

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=AM01

Start Time: 12:33:41 07-15-2007
End Time:   12:33:42 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 49.518
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:41

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2600	*	5.914	3.887	2.028	.7689	.1999	1.000
.5122	*	3.588	3.217	.3711	1.967	.6256	1.000
.9850	*	1.732	2.257	-.5248	3.225	1.464	1.000
2.053	*	.5660	1.014	-.4482	4.452	2.994	1.000
4.054	*	.3114	.2264	.8503E-01	5.330	5.420	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.8849
Rsq(adjusted)	.8465
Corr(x:y)	-.9407
Tlag	.0000
Tmax	.2600
Cmax	5.9143
No._points_Lambda_z	5
Tlast	4.0536
Clast	.3114
AUClast	5.3297
Lambda_z	.7495
Lambda_z_lower	.2600
Lambda_z_upper	4.0536
t1/2_Lambda_z	.9249
AUCall	5.3297
AUCINF(observed)	5.7452
AUCINF(observed)/D	.1160
AUC_%Extrap(obs.)	7.2320
Vz(observed)/F	11.5003
Cl(observed)/F	8.6190
AUCINF(predicted)	5.6318
AUCINF(predicted)/D	.1137
AUC_%Extrap(pred.)	5.3631
Vz(predicted)/F	11.7319
Cl(predicted)/F	8.7926
AUMClast	5.4198
AUMCINF(observed)	7.6584
AUMC_%Extrap(obs.)	29.2310
AUMCINF(predicted)	7.0471
AUMC_%Extrap(pred.)	23.0923
MRTlast	1.0169
MRTINF(observed)	1.3330

```
MRTINF(predicted)                1.2513

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=AM02

Start Time: 12:33:42 07-15-2007
End Time:   12:33:43 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 50.958
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:42

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2386		5.949			.7098	.1694	
.4903	*	3.038	2.540	.4976	1.841	.5354	1.000
.9828	*	1.876	1.810	.6587E-01	3.051	1.356	1.000
1.984	*	.6302	.9094	-.2792	4.305	2.904	1.000
3.974	*	.2692	.2312	.3799E-01	5.200	5.214	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9465
Rsq(adjusted)	.9197
Corr(x:y)	-.9729
Tlag	.0000
Tmax	.2386
Cmax	5.9491
No._points_Lambda_z	4
Tlast	3.9744
Clast	.2692
AUClast	5.2001
Lambda_z	.6879
Lambda_z_lower	.4903
Lambda_z_upper	3.9744
t1/2_Lambda_z	1.0076
AUCall	5.2001
AUCINF(observed)	5.5914
AUCINF(observed)/D	.1097
AUC_%Extrap(obs.)	6.9985
Vz(observed)/F	13.2485
Cl(observed)/F	9.1136
AUCINF(predicted)	5.5362
AUCINF(predicted)/D	.1086
AUC_%Extrap(pred.)	6.0707
Vz(predicted)/F	13.3807
Cl(predicted)/F	9.2046
AUMClast	5.2136
AUMCINF(observed)	7.3377
AUMC_%Extrap(obs.)	28.9478
AUMCINF(predicted)	7.0379
AUMC_%Extrap(pred.)	25.9212
MRTlast	1.0026
MRTINF(observed)	1.3123

```
MRTINF(predicted)               1.2713

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=AM03

Start Time: 12:33:43 07-15-2007
End Time:   12:33:44 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 50.052
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:43

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2358	*	7.435	5.513	1.922	.8767	.2068	1.000
.4950	*	4.991	4.380	.6103	2.487	.7541	1.000
.9939	*	2.358	2.814	-.4557	4.320	1.955	1.000
1.986	*	.6902	1.167	-.4764	5.833	3.798	1.000
3.983	*	.2605	.1985	.6204E-01	6.782	6.203	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9373
Rsq(adjusted)	.9164
Corr(x:y)	-.9682
Tlag	.0000
Tmax	.2358
Cmax	7.4352
No._points_Lambda_z	5
Tlast	3.9828
Clast	.2605
AUClast	6.7819
Lambda_z	.8871
Lambda_z_lower	.2358
Lambda_z_upper	3.9828
t1/2_Lambda_z	.7813
AUCall	6.7819
AUCINF(observed)	7.0756
AUCINF(observed)/D	.1414
AUC_%Extrap(obs.)	4.1508
Vz(observed)/F	7.9740
Cl(observed)/F	7.0739
AUCINF(predicted)	7.0056
AUCINF(predicted)/D	.1400
AUC_%Extrap(pred.)	3.1940
Vz(predicted)/F	8.0536
Cl(predicted)/F	7.1445
AUMClast	6.2028
AUMCINF(observed)	7.7035
AUMC_%Extrap(obs.)	19.4816
AUMCINF(predicted)	7.3462
AUMC_%Extrap(pred.)	15.5650
MRTlast	.9146
MRTINF(observed)	1.0888

```
MRTINF(predicted)                1.0486

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=AM04

Start Time: 12:33:44 07-15-2007
End Time:   12:33:45 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 48.127
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:44

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2364		6.758			.7987	.1888	
.5108		2.970			2.134	.6162	
1.018	*	1.381	1.234	.1463	3.236	1.357	1.000
1.990	*	.6732	.7947	-.1215	4.235	2.691	1.000
4.009	*	.3361	.3185	.1765E-01	5.253	5.403	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9569
Rsq(adjusted)	.9139
Corr(x:y)	-.9782
Tlag	.0000
Tmax	.2364
Cmax	6.7576
No._points_Lambda_z	3
Tlast	4.0086
Clast	.3361
AUClast	5.2534
Lambda_z	.4530
Lambda_z_lower	1.0178
Lambda_z_upper	4.0086
t1/2_Lambda_z	1.5302
AUCall	5.2534
AUCINF(observed)	5.9954
AUCINF(observed)/D	.1246
AUC_%Extrap(obs.)	12.3763
Vz(observed)/F	17.7209
Cl(observed)/F	8.0274
AUCINF(predicted)	5.9564
AUCINF(predicted)/D	.1238
AUC_%Extrap(pred.)	11.8031
Vz(predicted)/F	17.8368
Cl(predicted)/F	8.0799
AUMClast	5.4032
AUMCINF(observed)	10.0156
AUMC_%Extrap(obs.)	46.0523
AUMCINF(predicted)	9.7734
AUMC_%Extrap(pred.)	44.7154
MRTlast	1.0285
MRTINF(observed)	1.6706

```
MRTINF(predicted)                1.6408

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=BF01

Start Time: 12:33:45 07-15-2007
End Time:   12:33:46 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 50.286
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:45

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2300		5.163			.5937	.1366	
.4733		4.118			1.723	.5182	
.9847	*	1.951	2.096	-.1453	3.275	1.508	1.000
1.979	*	1.098	.9875	.1105	4.790	3.542	1.000
4.064	*	.1966	.2035	-.6847E-02	6.140	6.641	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9938
Rsq(adjusted)	.9877
Corr(x:y)	-.9969
Tlag	.0000
Tmax	.2300
Cmax	5.1629
No._points_Lambda_z	3
Tlast	4.0644
Clast	.1966
AUClast	6.1398
Lambda_z	.7573
Lambda_z_lower	.9847
Lambda_z_upper	4.0644
t1/2_Lambda_z	.9152
AUCall	6.1398
AUCINF(observed)	6.3994
AUCINF(observed)/D	.1273
AUC_%Extrap(obs.)	4.0566
Vz(observed)/F	10.3757
Cl(observed)/F	7.8580
AUCINF(predicted)	6.4084
AUCINF(predicted)/D	.1274
AUC_%Extrap(pred.)	4.1919
Vz(predicted)/F	10.3610
Cl(predicted)/F	7.8469
AUMClast	6.6410
AUMCINF(observed)	8.0389
AUMC_%Extrap(obs.)	17.3890
AUMCINF(predicted)	8.0876
AUMC_%Extrap(pred.)	17.8863
MRTlast	1.0816
MRTINF(observed)	1.2562

```
MRTINF(predicted)                1.2620

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=BF02

Start Time: 12:33:46 07-15-2007
End Time:   12:33:47 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 50.466
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:46

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2867		4.150			.5948	.1705	
.4678	*	2.919	2.846	.7336E-01	1.235	.4019	1.000
.9792	*	2.215	2.053	.1621	2.548	1.306	1.000
1.984	*	.9231	1.080	-.1572	4.125	3.316	1.000
4.046	*	.3061	.2895	.1660E-01	5.392	6.480	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9889
Rsq(adjusted)	.9834
Corr(x:y)	-.9945
Tlag	.0000
Tmax	.2867
Cmax	4.1497
No._points_Lambda_z	4
Tlast	4.0456
Clast	.3061
AUClast	5.3916
Lambda_z	.6388
Lambda_z_lower	.4678
Lambda_z_upper	4.0456
t1/2_Lambda_z	1.0851
AUCall	5.3916
AUCINF(observed)	5.8708
AUCINF(observed)/D	.1163
AUC_%Extrap(obs.)	8.1621
Vz(observed)/F	13.4566
Cl(observed)/F	8.5962
AUCINF(predicted)	5.8448
AUCINF(predicted)/D	.1158
AUC_%Extrap(pred.)	7.7537
Vz(predicted)/F	13.5164
Cl(predicted)/F	8.6344
AUMClast	6.4801
AUMCINF(observed)	9.1687
AUMC_%Extrap(obs.)	29.3242
AUMCINF(predicted)	9.0229
AUMC_%Extrap(pred.)	28.1819
MRTlast	1.2019
MRTINF(observed)	1.5618

```
MRTINF(predicted)                1.5438

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=BF03

Start Time: 12:33:47 07-15-2007
End Time:   12:33:48 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 51.466
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:47

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2011		6.164			.6198	.1247	
.4869		3.333			1.977	.5338	
1.014	*	1.502	1.317	.1846	3.252	1.363	1.000
2.032	*	.6970	.8449	-.1479	4.371	2.859	1.000
4.211	*	.3473	.3266	.2064E-01	5.509	5.996	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9460
Rsq(adjusted)	.8919
Corr(x:y)	-.9726
Tlag	.0000
Tmax	.2011
Cmax	6.1641
No._points_Lambda_z	3
Tlast	4.2111
Clast	.3473
AUClast	5.5086
Lambda_z	.4362
Lambda_z_lower	1.0142
Lambda_z_upper	4.2111
t1/2_Lambda_z	1.5892
AUCall	5.5086
AUCINF(observed)	6.3048
AUCINF(observed)/D	.1225
AUC_%Extrap(obs.)	12.6278
Vz(observed)/F	18.7150
Cl(observed)/F	8.1630
AUCINF(predicted)	6.2575
AUCINF(predicted)/D	.1216
AUC_%Extrap(pred.)	11.9670
Vz(predicted)/F	18.8566
Cl(predicted)/F	8.2247
AUMClast	5.9957
AUMCINF(observed)	11.1737
AUMC_%Extrap(obs.)	46.3412
AUMCINF(predicted)	10.8659
AUMC_%Extrap(pred.)	44.8213
MRTlast	1.0884
MRTINF(observed)	1.7723

```
MRTINF(predicted)                1.7365

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=BF04

Start Time: 12:33:48 07-15-2007
End Time:   12:33:49 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 51.974
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 5
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:48

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2217		7.872			.8725	.1934	
.4969	*	4.048	3.147	.9014	2.513	.7104	1.000
.9892	*	1.984	2.085	-.1012	3.998	1.688	1.000
1.992	*	.6272	.9016	-.2744	5.307	3.299	1.000
4.025	*	.1935	.1647	.2872E-01	6.141	5.360	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9582
Rsq(adjusted)	.9372
Corr(x:y)	-.9789
Tlag	.0000
Tmax	.2217
Cmax	7.8718
No._points_Lambda_z	4
Tlast	4.0250
Clast	.1935
AUClast	6.1410
Lambda_z	.8361
Lambda_z_lower	.4969
Lambda_z_upper	4.0250
t1/2_Lambda_z	.8290
AUCall	6.1410
AUCINF(observed)	6.3724
AUCINF(observed)/D	.1226
AUC_%Extrap(obs.)	3.6311
Vz(observed)/F	9.7553
Cl(observed)/F	8.1562
AUCINF(predicted)	6.3380
AUCINF(predicted)/D	.1219
AUC_%Extrap(pred.)	3.1088
Vz(predicted)/F	9.8082
Cl(predicted)/F	8.2004
AUMClast	5.3604
AUMCINF(observed)	6.5684
AUMC_%Extrap(obs.)	18.3921
AUMCINF(predicted)	6.3891
AUMC_%Extrap(pred.)	16.1014
MRTlast	.8729
MRTINF(observed)	1.0308

```
MRTINF(predicted)                1.0081

NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=CM01

Start Time: 12:33:49 07-15-2007
End Time:   12:33:50 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.311
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 4
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:49

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2367		1.019			.1206	.2853E-01	
.4958	*	.4226	.3939	.2869E-01	.3074	.8693E-01	1.000
.9917	*	.2188	.2431	-.2432E-01	.4664	.1927	1.000
1.985	*	.9580E-01	.9250E-01	.3304E-02	.6226	.3949	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9844
Rsq(adjusted)	.9687
Corr(x:y)	-.9922
Tlag	.0000
Tmax	.2367
Cmax	1.0189
No._points_Lambda_z	3
Tlast	1.9850
Clast	.0958
AUClast	.6226
Lambda_z	.9730
Lambda_z_lower	.4958
Lambda_z_upper	1.9850
t1/2_Lambda_z	.7124
AUCall	.6226
AUCINF(observed)	.7211
AUCINF(observed)/D	.0699
AUC_%Extrap(obs.)	13.6549
Vz(observed)/F	14.6965
Cl(observed)/F	14.2990
AUCINF(predicted)	.7177
AUCINF(predicted)/D	.0696
AUC_%Extrap(pred.)	13.2464
Vz(predicted)/F	14.7661
Cl(predicted)/F	14.3667
AUMClast	.3949
AUMCINF(observed)	.6916
AUMC_%Extrap(obs.)	42.8969
AUMCINF(predicted)	.6813
AUMC_%Extrap(pred.)	42.0394
MRTlast	.6342
MRTINF(observed)	.9590
MRTINF(predicted)	.9493

```
 NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=CM02

Start Time: 12:33:50 07-15-2007
End Time:   12:33:51 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.525
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 3
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:50

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2383	*	1.239	1.077	.1612	.1476	.3518E-01	1.000
.4906	*	.5035	.6206	-.1171	.3673	.1036	1.000
.9953	*	.2206	.2058	.1485E-01	.5500	.2213	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9543
Rsq(adjusted)	.9087
Corr(x:y)	-.9769
Tlag	.0000
Tmax	.2383
Cmax	1.2386
No._points_Lambda_z	3
Tlast	.9953
Clast	.2206
AUClast	.5500
Lambda_z	2.1872
Lambda_z_lower	.2383
Lambda_z_upper	.9953
t1/2_Lambda_z	.3169
AUCall	.5500
AUCINF(observed)	.6509
AUCINF(observed)/D	.0618
AUC_%Extrap(obs.)	15.4957
Vz(observed)/F	7.3931
Cl(observed)/F	16.1705
AUCINF(predicted)	.6441
AUCINF(predicted)/D	.0612
AUC_%Extrap(pred.)	14.6051
Vz(predicted)/F	7.4710
Cl(predicted)/F	16.3409
AUMClast	.2213
AUMCINF(observed)	.3678
AUMC_%Extrap(obs.)	39.8316
AUMCINF(predicted)	.3579
AUMC_%Extrap(pred.)	38.1742
MRTlast	.4023
MRTINF(observed)	.5651
MRTINF(predicted)	.5557

```
NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=CM03

Start Time: 12:33:51 07-15-2007
End Time:   12:33:52 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.067
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 3
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:51

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2381	*	1.135	1.022	.1129	.1351	.3217E-01	1.000
.4931	*	.5471	.6408	-.9375E-01	.3496	.1010	1.000
.9933	*	.2704	.2563	.1406E-01	.5541	.2357	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9623
Rsq(adjusted)	.9245
Corr(x:y)	-.9810
Tlag	.0000
Tmax	.2381
Cmax	1.1353
No._points_Lambda_z	3
Tlast	.9933
Clast	.2704
AUClast	.5541
Lambda_z	1.8318
Lambda_z_lower	.2381
Lambda_z_upper	.9933
t1/2_Lambda_z	.3784
AUCall	.5541
AUCINF(observed)	.7017
AUCINF(observed)/D	.0697
AUC_%Extrap(obs.)	21.0340
Vz(observed)/F	7.8318
Cl(observed)/F	14.3462
AUCINF(predicted)	.6940
AUCINF(predicted)/D	.0689
AUC_%Extrap(pred.)	20.1608
Vz(predicted)/F	7.9185
Cl(predicted)/F	14.5049
AUMClast	.2357
AUMCINF(observed)	.4629
AUMC_%Extrap(obs.)	49.0838
AUMCINF(predicted)	.4511
AUMC_%Extrap(pred.)	47.7502
MRTlast	.4253
MRTINF(observed)	.6596
MRTINF(predicted)	.6499

```
NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=CM08

Start Time: 12:33:52 07-15-2007
End Time:   12:33:53 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.083
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 4
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:52

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2733		.8336			.1139	.3114E-01	
.5261	*	.4362	.3840	.5216E-01	.2744	.8894E-01	1.000
.9919	*	.1903	.2293	-.3899E-01	.4203	.1863	1.000
1.996	*	.7999E-01	.7540E-01	.4589E-02	.5560	.3613	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9621
Rsq(adjusted)	.9243
Corr(x:y)	-.9809
Tlag	.0000
Tmax	.2733
Cmax	.8336
No._points_Lambda_z	3
Tlast	1.9961
Clast	.0800
AUClast	.5560
Lambda_z	1.1074
Lambda_z_lower	.5261
Lambda_z_upper	1.9961
t1/2_Lambda_z	.6259
AUCall	.5560
AUCINF(observed)	.6282
AUCINF(observed)/D	.0623
AUC_%Extrap(obs.)	11.4983
Vz(observed)/F	14.4933
Cl(observed)/F	16.0494
AUCINF(predicted)	.6241
AUCINF(predicted)/D	.0619
AUC_%Extrap(pred.)	10.9106
Vz(predicted)/F	14.5896
Cl(predicted)/F	16.1560
AUMClast	.3613
AUMCINF(observed)	.5707
AUMC_%Extrap(obs.)	36.6964
AUMCINF(predicted)	.5587
AUMC_%Extrap(pred.)	35.3351
MRTlast	.6498
MRTINF(observed)	.9084
MRTINF(predicted)	.8952

```
 NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=DF01

Start Time: 12:33:53 07-15-2007
End Time:   12:33:54 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.126
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 4
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:53

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2436		1.255			.1529	.3725E-01	
.5014	*	.6526	.6075	.4507E-01	.3989	.1188	1.000
.9822	*	.3202	.3559	-.3572E-01	.6327	.2731	1.000
1.989	*	.1202	.1162	.4040E-02	.8545	.5519	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9879
Rsq(adjusted)	.9758
Corr(x:y)	-.9939
Tlag	.0000
Tmax	.2436
Cmax	1.2555
No._points_Lambda_z	3
Tlast	1.9892
Clast	.1202
AUClast	.8545
Lambda_z	1.1118
Lambda_z_lower	.5014
Lambda_z_upper	1.9892
t1/2_Lambda_z	.6234
AUCall	.8545
AUCINF(observed)	.9626
AUCINF(observed)/D	.0951
AUC_%Extrap(obs.)	11.2341
Vz(observed)/F	9.4613
Cl(observed)/F	10.5191
AUCINF(predicted)	.9590
AUCINF(predicted)/D	.0947
AUC_%Extrap(pred.)	10.8978
Vz(predicted)/F	9.4972
Cl(predicted)/F	10.5589
AUMClast	.5519
AUMCINF(observed)	.8643
AUMC_%Extrap(obs.)	36.1437
AUMCINF(predicted)	.8538
AUMC_%Extrap(pred.)	35.3587
MRTlast	.6459
MRTINF(observed)	.8978
MRTINF(predicted)	.8903

```
NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=DF02

Start Time: 12:33:54 07-15-2007
End Time:   12:33:55 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.302
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 3
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:54

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2442	*	1.046	.9611	.8478E-01	.1277	.3118E-01	1.000
.4761	*	.5544	.6273	-.7292E-01	.3133	.9140E-01	1.000
.9783	*	.2589	.2490	.9914E-02	.5175	.2213	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9755
Rsq(adjusted)	.9510
Corr(x:y)	-.9877
Tlag	.0000
Tmax	.2442
Cmax	1.0459
No._points_Lambda_z	3
Tlast	.9783
Clast	.2589
AUClast	.5175
Lambda_z	1.8395
Lambda_z_lower	.2442
Lambda_z_upper	.9783
t1/2_Lambda_z	.3768
AUCall	.5175
AUCINF(observed)	.6583
AUCINF(observed)/D	.0639
AUC_%Extrap(obs.)	21.3847
Vz(observed)/F	8.5079
Cl(observed)/F	15.6503
AUCINF(predicted)	.6529
AUCINF(predicted)/D	.0634
AUC_%Extrap(pred.)	20.7357
Vz(predicted)/F	8.5781
Cl(predicted)/F	15.7795
AUMClast	.2213
AUMCINF(observed)	.4355
AUMC_%Extrap(obs.)	49.1905
AUMCINF(predicted)	.4273
AUMC_%Extrap(pred.)	48.2151
MRTlast	.4276
MRTINF(observed)	.6616
MRTINF(predicted)	.6545

```
NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=DF04

Start Time: 12:33:55 07-15-2007
End Time:   12:33:56 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.122
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 4
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:55

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.2839		1.008			.1430	.4061E-01	
.4981	*	.4603	.4292	.3110E-01	.3002	.9579E-01	1.000
.9722	*	.2453	.2714	-.2614E-01	.4675	.2067	1.000
2.032	*	.1006	.9753E-01	.3102E-02	.6508	.4413	1.000

*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

Dosing_time	.0000
Rsq	.9862
Rsq(adjusted)	.9724
Corr(x:y)	-.9931
Tlag	.0000
Tmax	.2839
Cmax	1.0077
No._points_Lambda_z	3
Tlast	2.0317
Clast	.1006
AUClast	.6508
Lambda_z	.9662
Lambda_z_lower	.4981
Lambda_z_upper	2.0317
t1/2_Lambda_z	.7174
AUCall	.6508
AUCINF(observed)	.7549
AUCINF(observed)/D	.0746
AUC_%Extrap(obs.)	13.7965
Vz(observed)/F	13.8773
Cl(observed)/F	13.4080
AUCINF(predicted)	.7517
AUCINF(predicted)/D	.0743
AUC_%Extrap(pred.)	13.4283
Vz(predicted)/F	13.9366
Cl(predicted)/F	13.4653
AUMClast	.4413
AUMCINF(observed)	.7607
AUMC_%Extrap(obs.)	41.9869
AUMCINF(predicted)	.7509
AUMC_%Extrap(pred.)	41.2262
MRTlast	.6781
MRTINF(observed)	1.0077
MRTINF(predicted)	.9989

```
NORMAL ENDING
```

```
Input File: Data - [C:\WINNONLN\USERCOMP\ETBE\ETBEDATA.WDO]
Animal=DF05

Start Time: 12:33:57 07-15-2007
End Time:   12:33:57 07-15-2007


          WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM (V01.5A)
                        Core Version 29Oct97

 Listing of input commands

TITLE 1
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1
MODEL 200
DATE
TIME
NVARIABLES 5
NPOINTS 100
XNUMBER 2
YNUMBER 3
DTIME 0
NCONSTANTS 1
CONSTANTS 10.239
METHOD 2  'Linear trapezoidal
MISSING 'Missing'
NOBSERVATIONS 3
DATA 'WINNLIN.DAT'
BEGIN
```

Date: 07/15/2007
Time: 12:33:56

0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1

WINNONLIN NONCOMPARTMENTAL ANALYSIS PROGRAM

Noncompartmental Analysis for Extravascular Administration

Linear Trapezoidal Rule Used to Compute AUC, AUMC

X		Y	Pred.	Res.	AUC	AUMC	WEIGHT
.0000		.0000			.0000	.0000	
.3975	*	.2818	.2952	-.1344E-01	.5600E-01	.2226E-01	1.000
.4908	*	.3078	.2910	.1678E-01	.8351E-01	.3454E-01	1.000
.9517	*	.2686	.2711	-.2547E-02	.2163	.1282	1.000

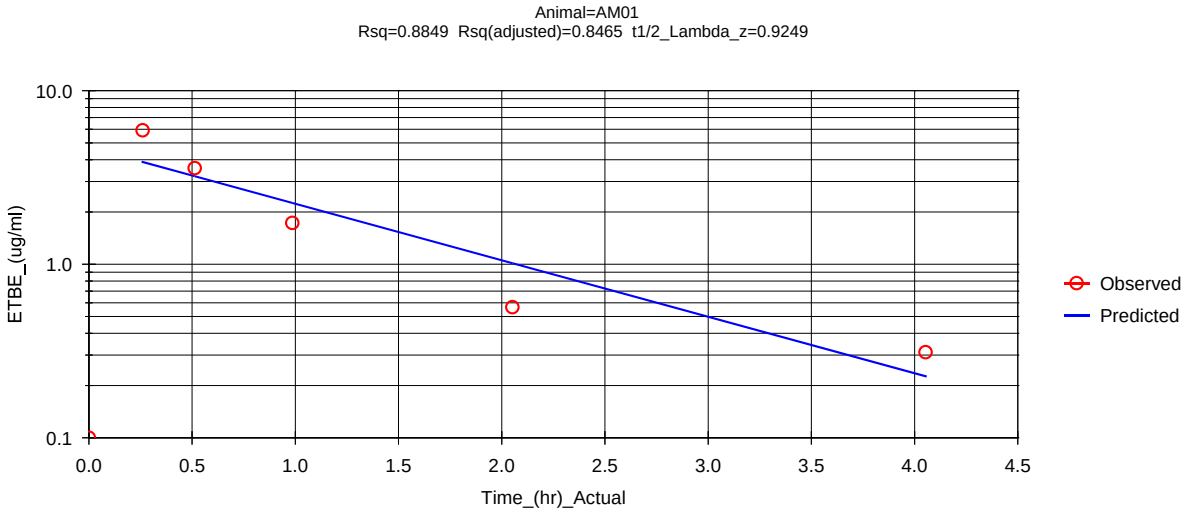
*) Starred values were included in estimation of Lambda_z.

@) Note - the concentration at time zero (DTIME) was added for extrapolation purposes

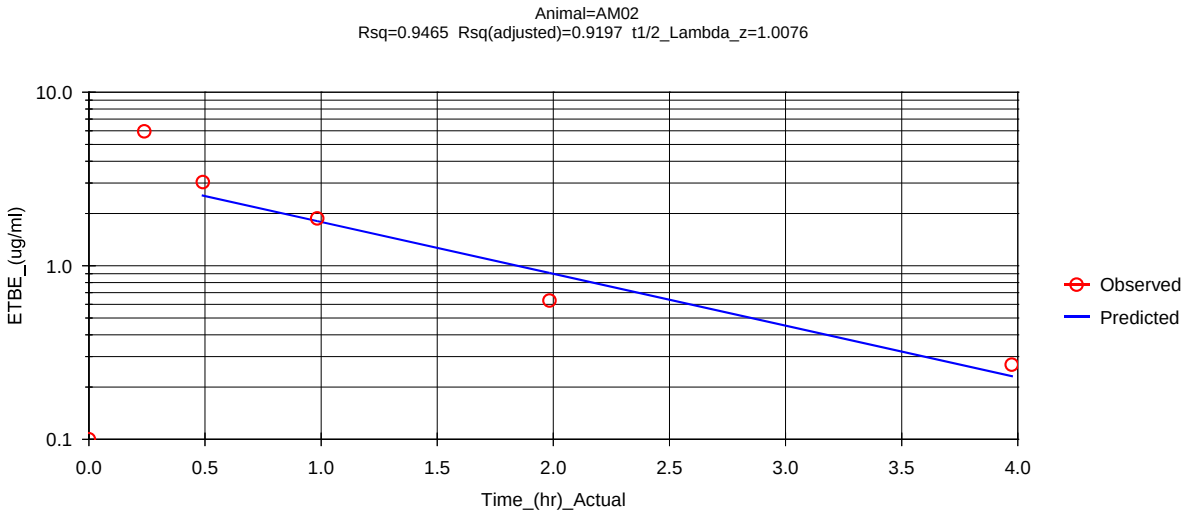
Dosing_time	.0000
Rsq	.4346
Rsq(adjusted)	-.1309
Corr(x:y)	-.6592
Tlag	.0000
Tmax	.4908
Cmax	.3078
No._points_Lambda_z	3
Tlast	.9517
Clast	.2686
AUClast	.2163
Lambda_z	.1536
Lambda_z_lower	.3975
Lambda_z_upper	.9517
t1/2_Lambda_z	4.5134
AUCall	.2163
AUCINF(observed)	1.9651
AUCINF(observed)/D	.1919
AUC_%Extrap(obs.)	88.9924
Vz(observed)/F	33.9275
Cl(observed)/F	5.2104
AUCINF(predicted)	1.9817
AUCINF(predicted)/D	.1935
AUC_%Extrap(pred.)	89.0845
Vz(predicted)/F	33.6436
Cl(predicted)/F	5.1668
AUMClast	.1282
AUMCINF(observed)	13.1797
AUMC_%Extrap(obs.)	99.0270
AUMCINF(predicted)	13.3035
AUMC_%Extrap(pred.)	99.0361
MRTlast	.5928
MRTINF(observed)	6.7069
MRTINF(predicted)	6.7132

```
NORMAL ENDING
```

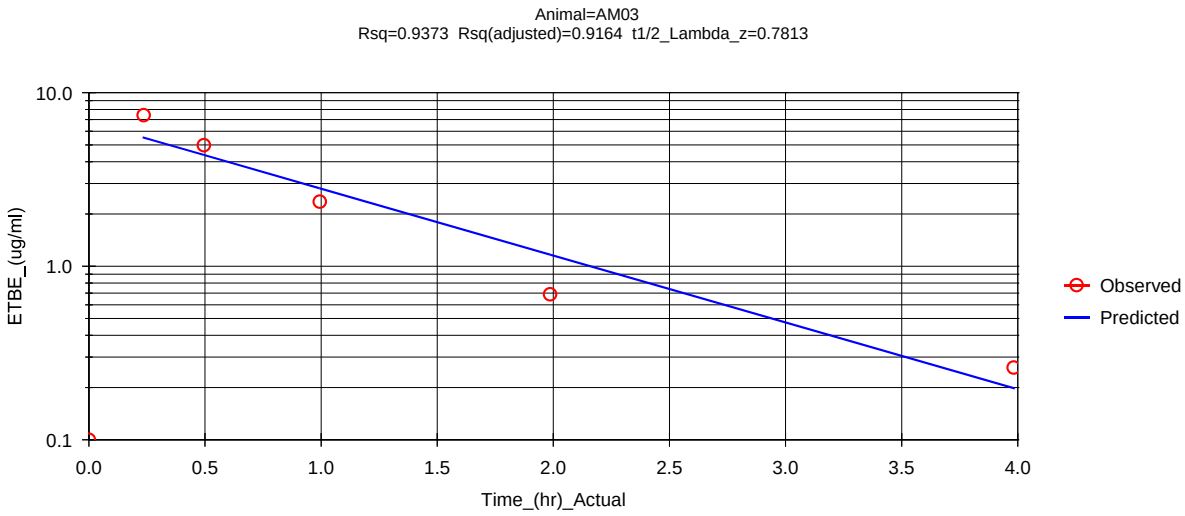
0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1



0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1



0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1



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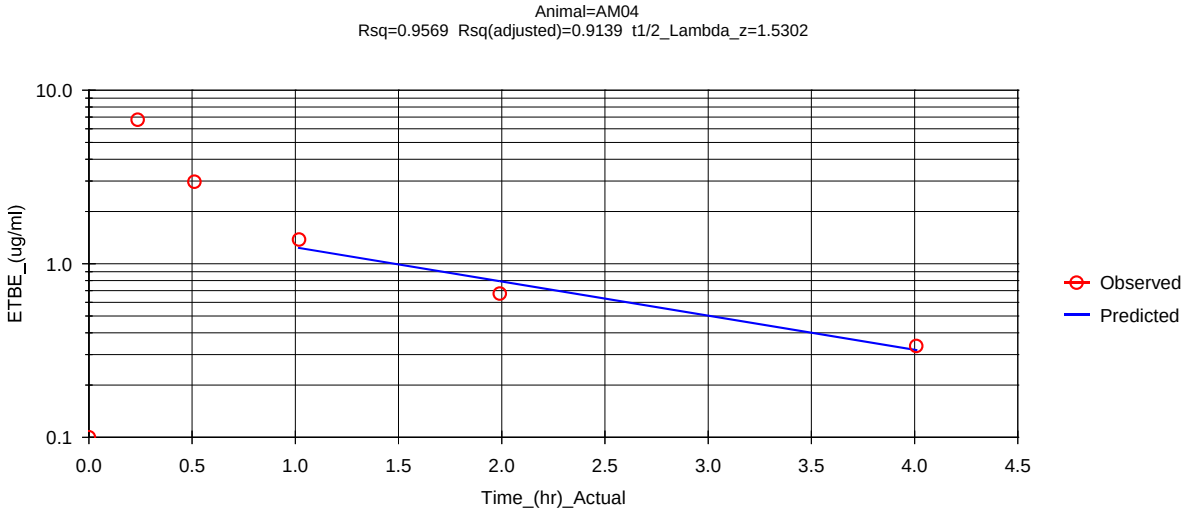

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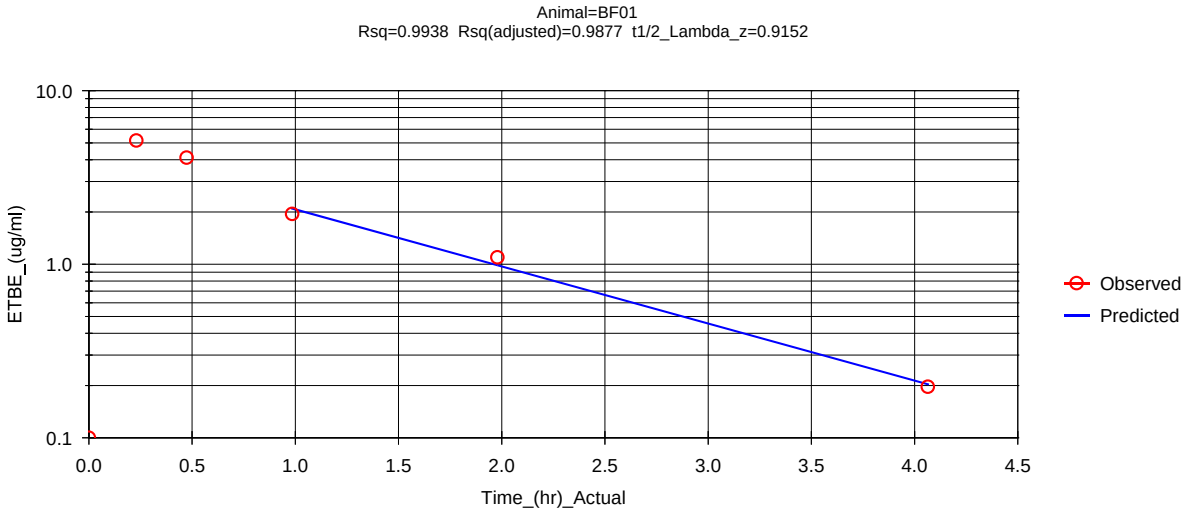

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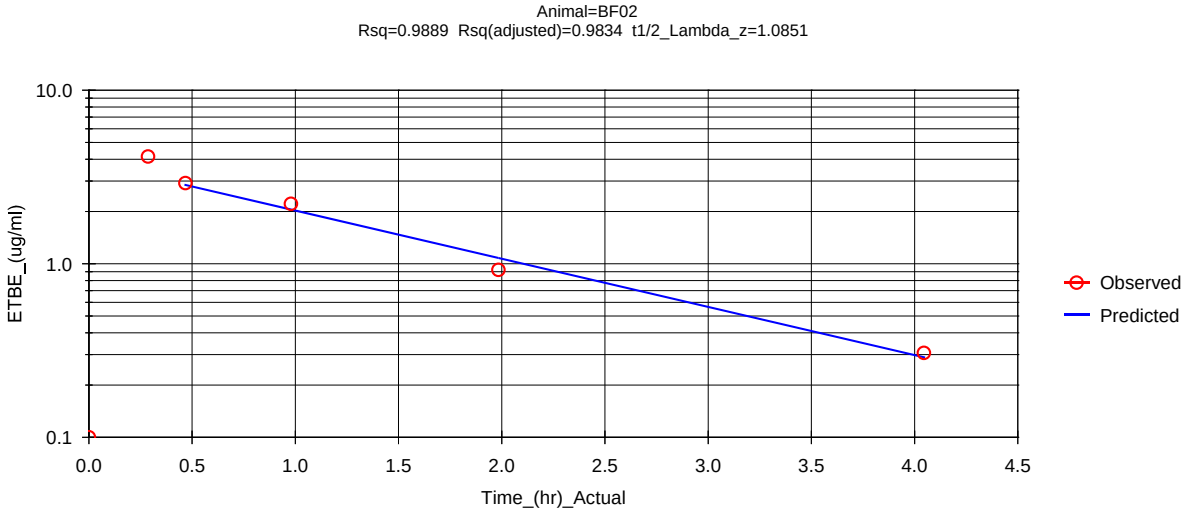

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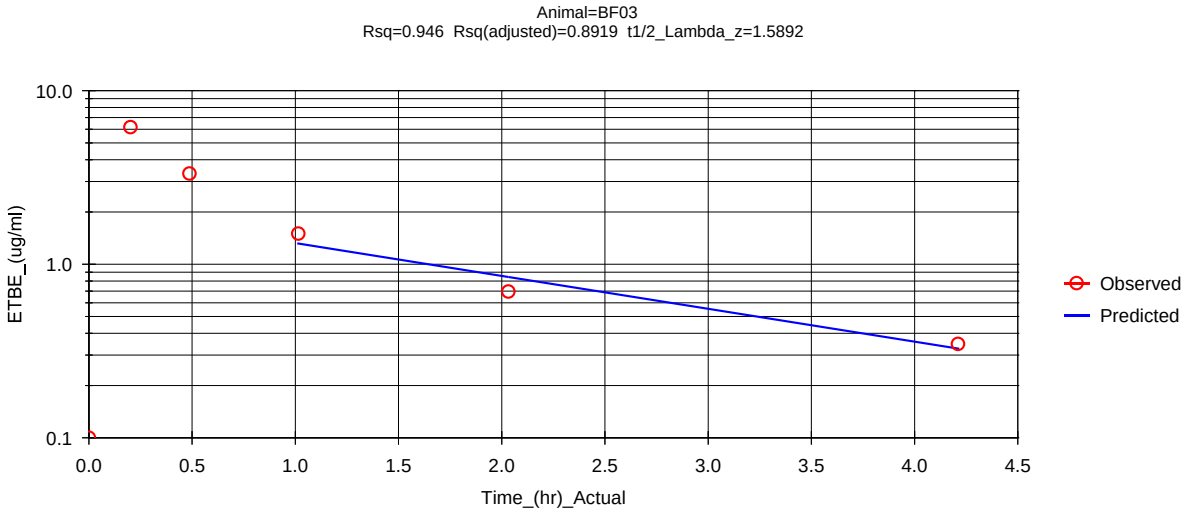

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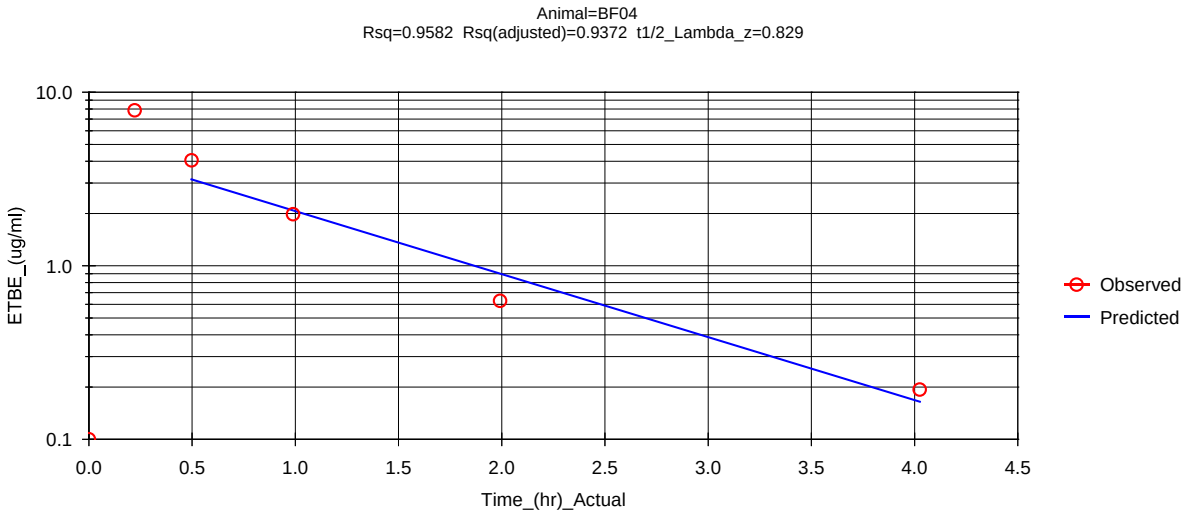

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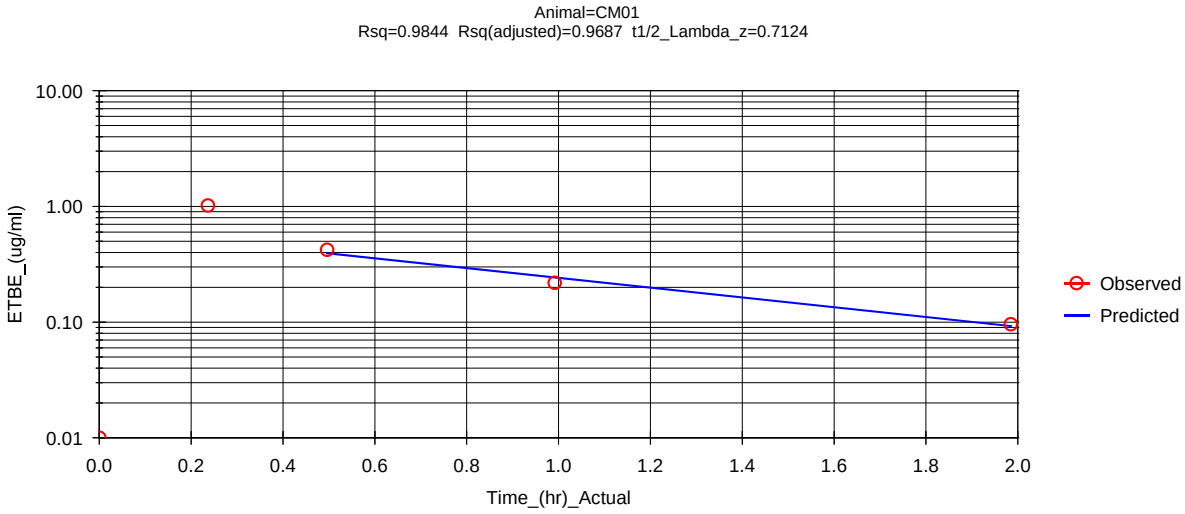

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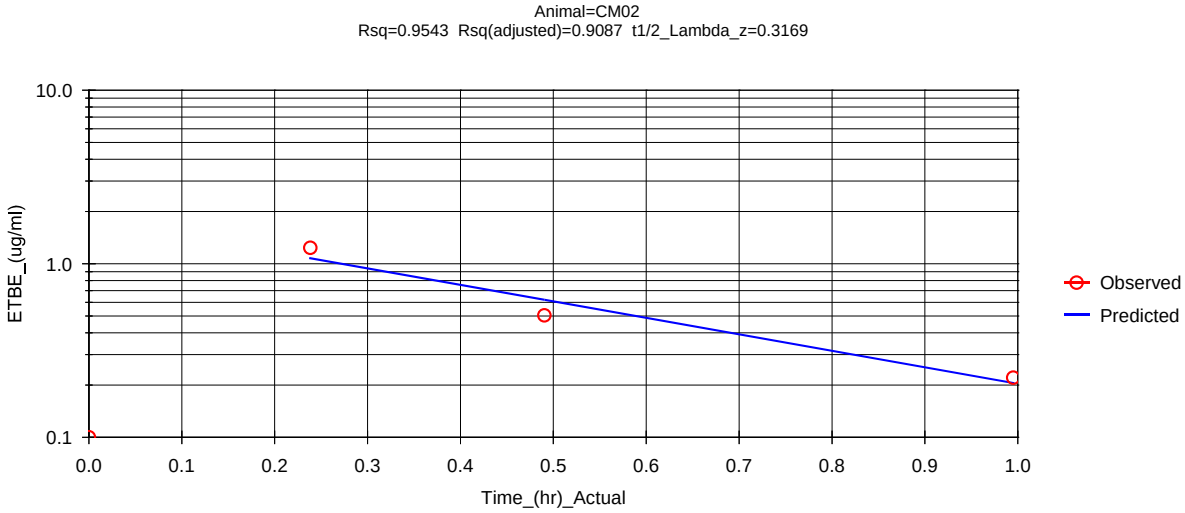
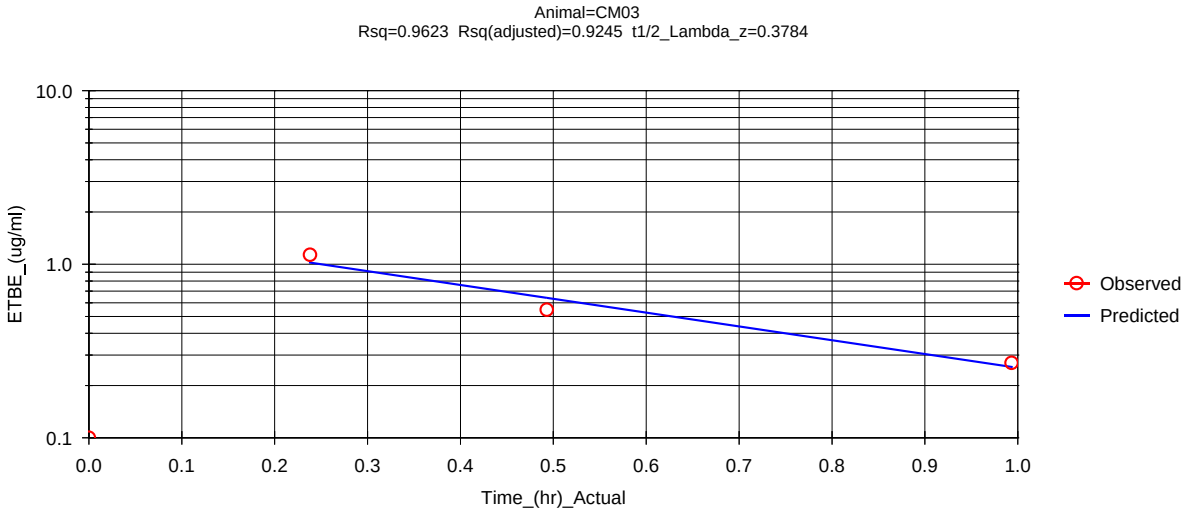


0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1



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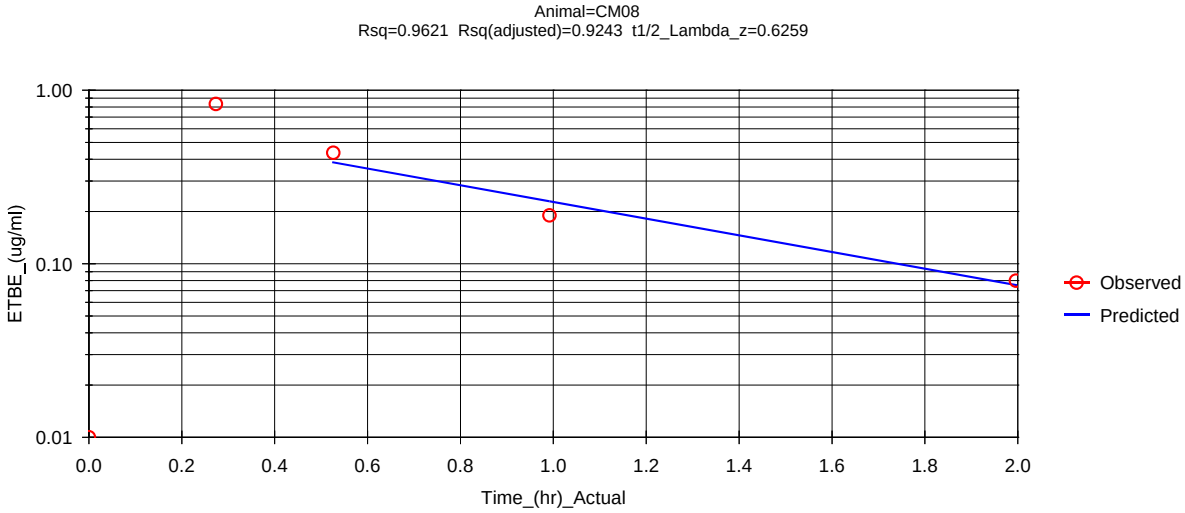

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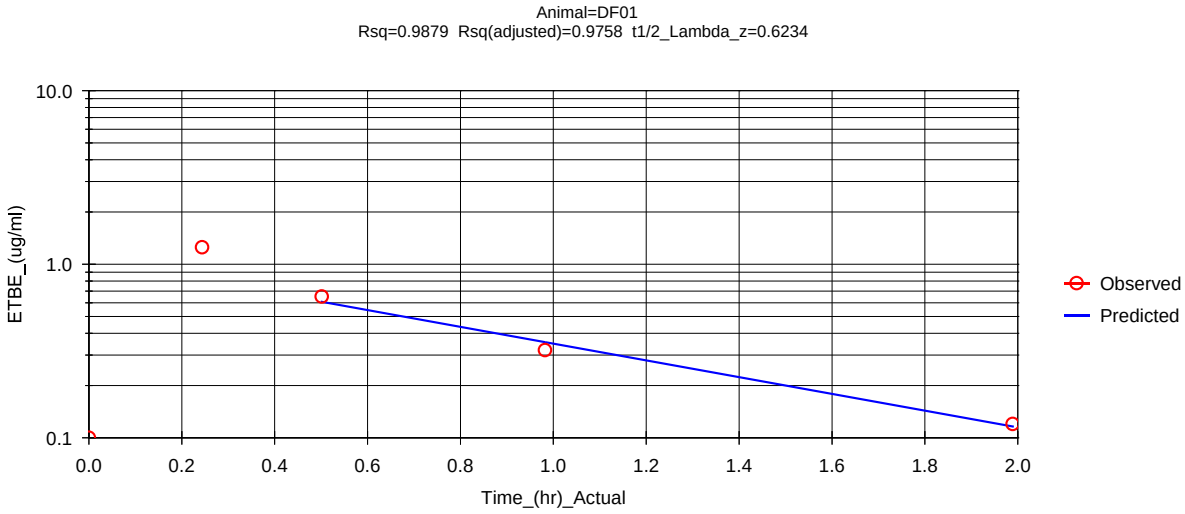
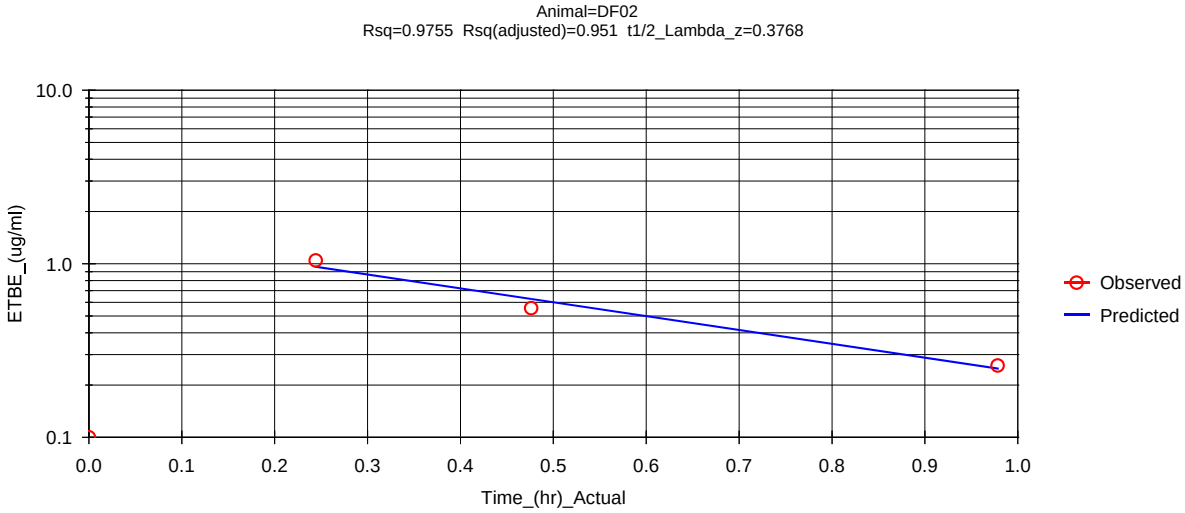


0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1



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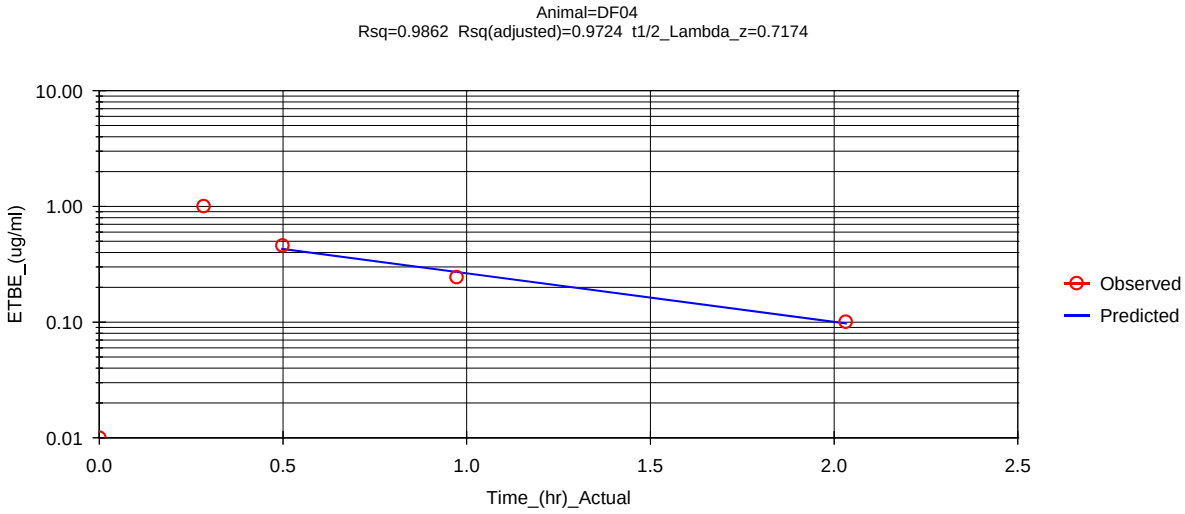
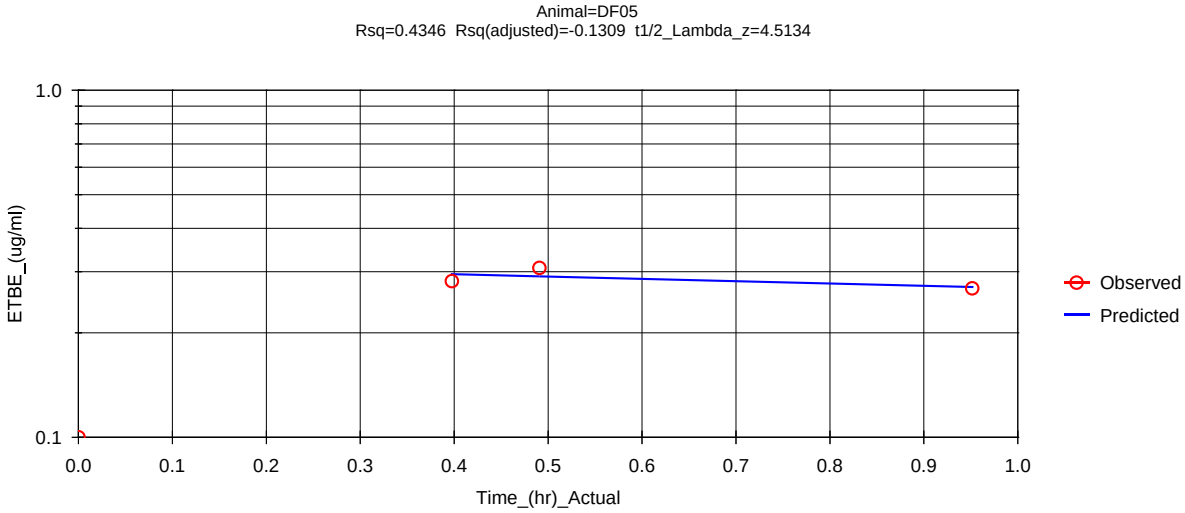


0209408.007, ETBE, RTI-932, Rats AM01-DF05 Analysis 1



Appendix E

Dose Formulation Analysis

Dose formulation analysis was conducted by gas chromatography with flame ionization detection as described in Project Specific Analytical Method for Analysis of Ethyl Tertiary Butyl Ether in Oral Dose Formulations, AM-D0209408.007.0.

Prior to conduct of the in-life portion of the study, the method was used to determine the stability of mock dose formulations at both the low dose (1 mg/ml) and the high dose (5 mg/ml). The dose solutions were found to be stable stored both at room temperature for up to three days and in a refrigerator for 1 day (Table 1).

The dose formulation analysis results are tabulated for each dose solution in Tables 2 – 5. The associated calibration curves and data are shown in Figures 1 – 4.

The Project Specific Analytical Method for Analysis of Ethyl Tertiary Butyl Ether in Oral Dose Formulations, AM-D0209408.007.0 is shown at the end of this Appendix.

Table 1. Dose formulation stability.

Mock Dose.	Storage condition	Concentration of ETBE (μg/ml)	SD	Stability (%)[a]
1.0 mg/mL	0 day	1141	32	-
1.0 mg/mL	Ca. 4°C, 1 day	1156	60	101
1.0 mg/mL	RT[b], 1 day	1166	16	102
1.0 mg/mL	RT, 2 days	1167	14	102
1.0 mg/mL	RT, 3days	1165	24	102
5.0 mg/mL	0 day	5743	533	-
5.0 mg/mL	Ca. 4°C, 1 day	5624	83	97.9
5.0 mg/mL	RT, 1 day	5711	102	99.4
5.0 mg/mL	RT, 2 days	5539	167	96.6
5.0 mg/mL	RT, 3days	5250	106	91.6

[a] Calculated as a percentage of the day 0 value.

[b] RT = Room Temperature

Table 2 Dose formulation analysis for high dose male rats.

	Sample ID	Name	RT	Area	Dilution	Amount	Units	Calibration Id
1	12307-61-1	ETBE	4.052	2196	10.46000	5283.001	µg/mL	5249
2	12307-61-1	ETBE	4.050	2126	10.46000	5111.165	µg/mL	5249
3	12307-61-2	ETBE	4.049	2215	10.42000	5309.806	µg/mL	5249
4	12307-61-2	ETBE	4.050	2213	10.42000	5304.189	µg/mL	5249
5	12307-61-3	ETBE	4.050	2195	10.51000	5304.712	µg/mL	5249
6	12307-61-3	ETBE	4.050	2120	10.51000	5119.573	µg/mL	5249
Mean						5238.8		
Std. Dev.						96.0		
%RSD						1.8		

Table 3. Dose formulation analysis for high dose female rats.

	Sample ID	Name	RT	Area	Dilution	Amount	Units	Calibration Id
1	12307-64-1	ETBE	4.051	2295	10.22000	5366.492	μg/ml	5900
2	12307-64-1	ETBE	4.052	2230	10.22000	5213.326	μg/ml	5900
3	12307-64-2	ETBE	4.052	2143	10.78000	5282.676	μg/ml	5900
4	12307-64-2	ETBE	4.051	2181	10.78000	5377.548	μg/ml	5900
5	12307-64-3	ETBE	4.050	2161	10.58000	5227.800	μg/ml	5900
6	12307-64-3	ETBE	4.052	2169	10.58000	5247.629	μg/ml	5900
Mean						5285.9		
Std. Dev.						70.7		
%RSD						1.3		

Table 4. Dose formulation analysis for low dose female rats.

	Sample ID	Name	RT	Area	Dilution	Amount	Units	Calibration Id
1	12307-81-1	ETBE	4.053	468	9.78000	1152.140	µg/mL	5666
2	12307-81-1	ETBE	4.051	471	918000	1158.194	µg/mL	5666
3	12307-81-2	ETBE	4.052	426	10.56000	1143.368	µg/mL	5666
4	12307-81-2	ETBE	4.051	428	10.56000	1148.662	µg/mL	5666
5	12307-81-3	ETBE	4.049	468	9.72000	1146.016	µg/mL	5666
6	12307-81-3	ETBE	4.050	467	9.72000	1142.978	µg/mL	5666
Mean						1148.6		
Std. Dev.						5.8		
%RSD						0.5		

Table 5. Dose formulation analysis for low dose female rats.

	Sample ID	Name	RT	Area	Dilution	Amount	Units	Calibration Id
1	12307-84-1	ETBE	4.052	443	10.44000	986.780	µg/mL	5830
2	12307-84-1	ETBE	4.053	444	10.44000	989.869	µg/mL	5830
3	12307-84-2	ETBE	4.053	444	10.61000	1005.417	µg/mL	5830
4	12307-84-2	ETBE	4.052	433	10.61000	981.594	µg/mL	5830
5	12307-84-3	ETBE	4.052	423	10.97000	989.709	µg/mL	5830
6	12307-84-3	ETBE	4.053	422	10.97000	987.555	µg/mL	5830
Mean						990.2		
Std. Dev.						8.1		
%RSD						0.8		

Figure 1. Calibration Report 5249 for Analysis of ETBE Dose Formulation.

Empower 2 software

Individual_Generic_curve

Processing Method:	ETBE dose	Project Name:	API
Processing Method ID:	4103	System:	GC_08
Channel:	HP6890 Ch1	Calibration ID:	5249
Proc. Chnl. Descr.:	****		
Date Calibrated:	6/13/2007 12:20:57 AM EDT		

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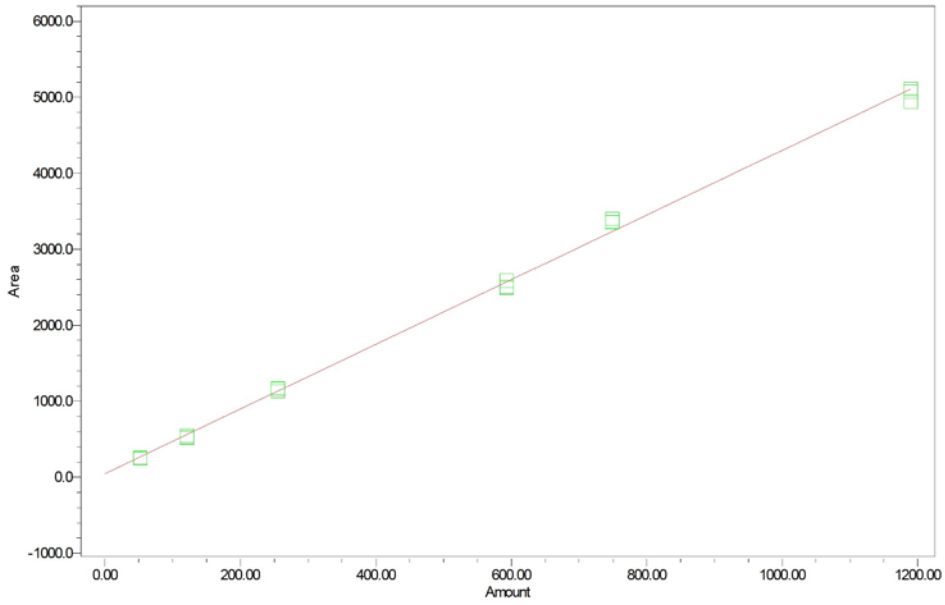
B 4.253309e+000
C 0.000000e+000
D 0.000000e+000
R^2 0.997936

Calibration Plot



ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A1 (12307-60F)	2	ETBE	1	52.400000	248.792461	47.199032	-9.926
2	Std A1 (12307-60F)	13	ETBE	1	52.400000	257.666530	49.285424	-5.944
3	Std A1 (12307-60F)	24	ETBE	1	52.400000	249.398037	47.341409	-9.654
Mean							47.9	
Std. Dev.							1.2	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 1 of 3

Project Name: API
Date Printed:
6/13/2007
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Figure 1 (continued). Calibration Report 5249 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
% RSD							2.4	

ETBE Standard Curve
Peak: ETBELevel: 2

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B1 (12307-60E)	14	ETBE	2	121.000000	547.646558	117.462940	-2.923
2	Std B1 (12307-60E)	3	ETBE	2	121.000000	516.619790	110.168203	-8.952
3	Std B1 (12307-60E)	25	ETBE	2	121.000000	528.237340	112.899618	-6.695
Mean							113.5	
Std. Dev.							3.7	
% RSD							3.2	

ETBE Standard Curve
Peak: ETBELevel: 3

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A2 (12307-60D)	15	ETBE	3	255.000000	1169.445528	263.654764	3.394
2	Std A2 (12307-60D)	4	ETBE	3	255.000000	1130.795409	254.567693	-0.170
3	Std A2 (12307-60D)	26	ETBE	3	255.000000	1162.326639	261.981034	2.738
Mean							260.1	
Std. Dev.							4.8	
% RSD							1.9	

ETBE Standard Curve
Peak: ETBELevel: 4

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B2 (12307-60C)	5	ETBE	4	593.000000	2502.383474	577.043236	-2.691
2	Std B2 (12307-60C)	16	ETBE	4	593.000000	2586.481034	596.815504	0.643
3	Std B2 (12307-60C)	27	ETBE	4	593.000000	2489.833353	574.092564	-3.188
Mean							582.7	
Std. Dev.							12.4	
% RSD							2.1	

ETBE Standard Curve
Peak: ETBELevel: 5

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A3 (12307-60B)	6	ETBE	5	749.000000	3362.793287	779.335112	4.050
2	Std A3 (12307-60B)	17	ETBE	5	749.000000	3355.661034	777.658240	3.826
3	Std A3 (12307-60B)	28	ETBE	5	749.000000	3402.924743	788.770461	5.310
Mean							781.9	
Std. Dev.							6.0	
% RSD							0.8	

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B3 (12307-60A)	18	ETBE	6	1190.000000	5110.802619	1190.311463	0.026
2	Std B3 (12307-60A)	7	ETBE	6	1190.000000	4945.335277	1151.408259	-3.243

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 2 of 3

Project Name: API
Date Printed: 6/13/2007
12:21:41 AM US/Eastern

Figure 1 (continued). Calibration Report 5249 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
3	Std B3 (12307-60A)	29	ETBE	6	1190.000000	5072.070212	1181.205046	-0.739
Mean							1174.3	
Std. Dev.							20.3	
% RSD							1.7	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 3 of 3

Project Name: API
Date Printed:
6/13/2007
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Figure 2. Calibration Report 5900 for Analysis of ETBE Dose Formulation.

Empower 2 software

Individual_Generic_curve

Processing Method:	ETBE dose	Project Name:	API
Processing Method ID:	5608	System:	GC_08
Channel:	HP6890 Ch1	Calibration ID:	5900
Proc. Chnl. Descr.:	****		
Date Calibrated:	7/11/2007 3:51:43 PM EDT		

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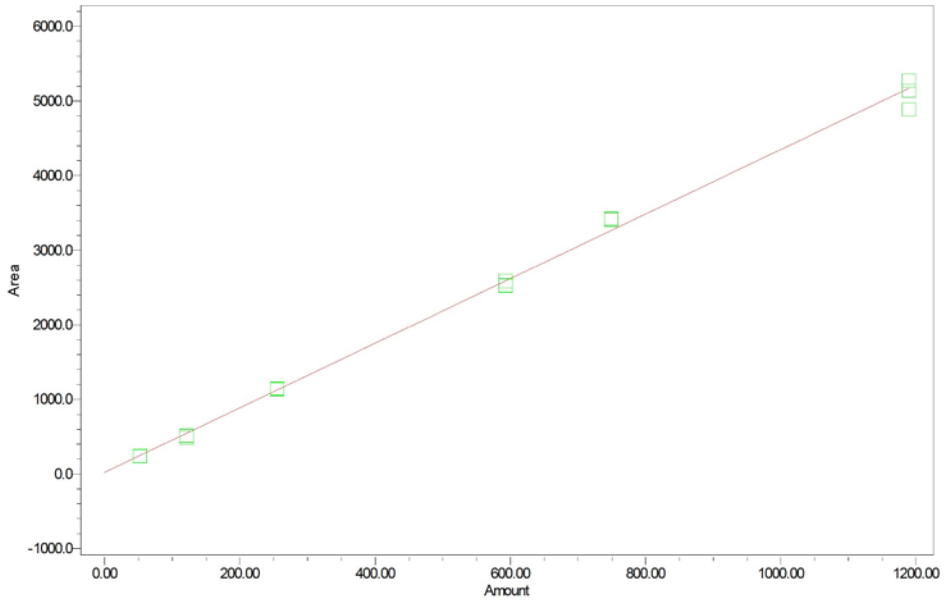
B 4.328341e+000
C 0.000000e+000
D 0.000000e+000
R^2 0.996689

Calibration Plot



ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A1 (12307-60F)	2	ETBE	1	52.400000	236.904352	49.644508	-5.259
2	Std A1 (12307-60F)	13	ETBE	1	52.400000	242.197311	50.867369	-2.925
3	Std A1 (12307-60F)	24	ETBE	1	52.400000	241.171544	50.630381	-3.377
Mean							50.4	
Std. Dev.							0.6	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 1 of 3

Project Name: API
Date Printed:
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Figure 2 (continued). Calibration Report 5900 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
% RSD							1.3	

ETBE Standard Curve
Peak: ETBELevel: 2

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B1 (12307-60E)	14	ETBE	2	121.000000	517.325560	114.431737	-5.428
2	Std B1 (12307-60E)	3	ETBE	2	121.000000	509.245548	112.564968	-6.971
3	Std B1 (12307-60E)	25	ETBE	2	121.000000	483.340189	106.579913	-11.917
Mean							111.2	
Std. Dev.							4.1	
% RSD							3.7	

ETBE Standard Curve
Peak: ETBELevel: 3

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A2 (12307-60D)	4	ETBE	3	255.000000	1141.163054	258.560288	1.396
2	Std A2 (12307-60D)	15	ETBE	3	255.000000	1148.257649	260.199391	2.039
3	Std A2 (12307-60D)	26	ETBE	3	255.000000	1133.675328	256.830358	0.718
Mean							258.5	
Std. Dev.							1.7	
% RSD							0.7	

ETBE Standard Curve
Peak: ETBELevel: 4

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B2 (12307-60C)	16	ETBE	4	593.000000	2586.395727	592.460211	-0.091
2	Std B2 (12307-60C)	5	ETBE	4	593.000000	2521.348095	577.431905	-2.625
3	Std B2 (12307-60C)	27	ETBE	4	593.000000	2529.736508	579.369925	-2.298
Mean							583.1	
Std. Dev.							8.2	
% RSD							1.4	

ETBE Standard Curve
Peak: ETBELevel: 5

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A3 (12307-60B)	6	ETBE	5	749.000000	3426.341318	786.517385	5.009
2	Std A3 (12307-60B)	17	ETBE	5	749.000000	3405.086065	781.606669	4.353
3	Std A3 (12307-60B)	28	ETBE	5	749.000000	3413.845925	783.630506	4.624
Mean							783.9	
Std. Dev.							2.5	
% RSD							0.3	

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B3 (12307-60A)	18	ETBE	6	1190.000000	4886.636104	1123.897189	-5.555
2	Std B3 (12307-60A)	7	ETBE	6	1190.000000	5139.043814	1182.212308	-0.654

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 2 of 3

Project Name: API
Date Printed:
7/11/2007
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Figure 2 (continued). Calibration Report 5900 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
3	Std B3 (12307-60A)	29	ETBE	6	1190.000000	5275.614582	1213.764991	1.997
Mean							1173.3	
Std. Dev.							45.6	
% RSD							3.9	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 3 of 3

Project Name: API
Date Printed:
7/11/2007
4:00:01 PM US/Eastern

Figure 3. Calibration Report 5666 for Analysis of ETBE Dose Formulation.

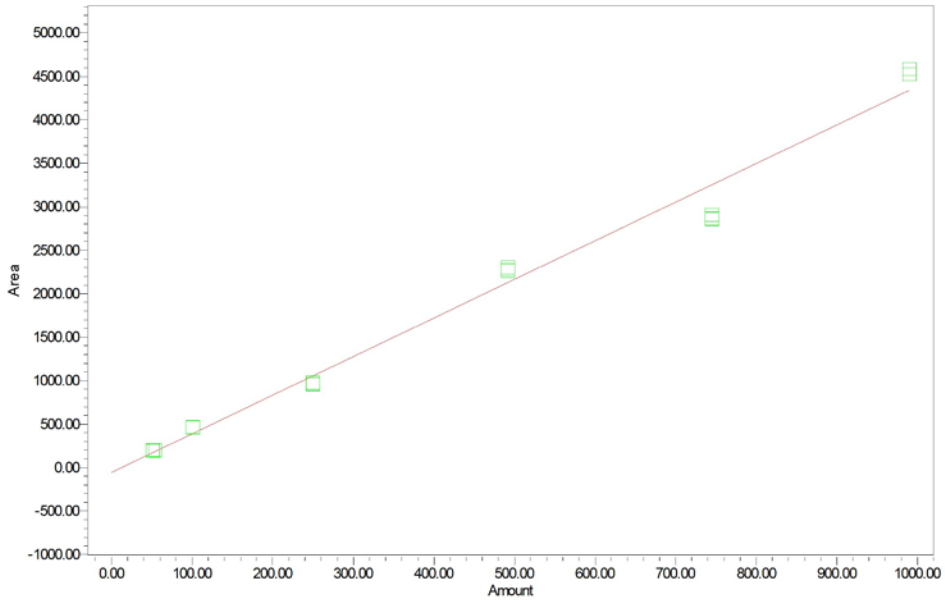
Empower 2 software

Individual_Generic_curve

Processing Method:	ETBE dose	Project Name:	API
Processing Method ID:	5608	System:	GC_08
Channel:	HP6890 Ch1	Calibration ID:	5666
Proc. Chnl. Descr.:	****		
Date Calibrated:	6/19/2007 4:20:17 PM EDT		

A -5.502926e+001
B 4.440321e+000
C 0.000000e+000
D 0.000000e+000
R^2 0.983605

Calibration Plot



ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A1 (12307-80F)	2	ETBE	1	51.400000	193.266755	55.918488	8.791
2	Std A1 (12307-80F)	9	ETBE	1	51.400000	203.592859	58.244019	13.315
3	Std A1 (12307-80F)	23	ETBE	1	54.100000	200.335607	57.510456	6.304
Mean							57.2	
Std. Dev.							1.2	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 1 of 3

Project Name: API
Date Printed:
6/19/2007
4:32:43 PM US/Eastern

Figure 3 (continued). Calibration Report 5666 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
% RSD							2.1	

ETBE Standard Curve
Peak: ETBELevel: 2

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B1 (12307-80E)	10	ETBE	2	101.000000	466.282934	117.404178	16.242
2	Std B1 (12307-80E)	3	ETBE	2	101.000000	461.324653	116.287529	15.136
3	Std B1 (12307-80E)	24	ETBE	2	101.000000	462.827733	116.626036	15.471
Mean							116.8	
Std. Dev.							0.6	
% RSD							0.5	

ETBE Standard Curve
Peak: ETBELevel: 3

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A2 (12307-80D)	4	ETBE	3	250.000000	954.576572	227.372282	-9.051
2	Std A2 (12307-80D)	11	ETBE	3	250.000000	968.370754	230.478856	-7.808
3	Std A2 (12307-80D)	25	ETBE	3	250.000000	978.086279	232.666879	-6.933
Mean							230.2	
Std. Dev.							2.7	
% RSD							1.2	

ETBE Standard Curve
Peak: ETBELevel: 4

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B2 (12307-80C)	12	ETBE	4	492.000000	2274.030993	524.525245	6.611
2	Std B2 (12307-80C)	5	ETBE	4	492.000000	2263.397689	522.130529	6.124
3	Std B2 (12307-80C)	26	ETBE	4	492.000000	2302.573752	530.953330	7.917
Mean							525.9	
Std. Dev.							4.6	
% RSD							0.9	

ETBE Standard Curve
Peak: ETBELevel: 5

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A3 (12307-80B)	6	ETBE	5	745.000000	2906.166222	666.887764	-10.485
2	Std A3 (12307-80B)	13	ETBE	5	745.000000	2864.179030	657.431872	-11.754
3	Std A3 (12307-80B)	27	ETBE	5	745.000000	2855.824799	655.550425	-12.007
Mean							660.0	
Std. Dev.							6.1	
% RSD							0.9	

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B3 (12307-80A)	14	ETBE	6	990.000000	4583.189080	1044.568344	5.512
2	Std B3 (12307-80A)	7	ETBE	6	990.000000	4525.220033	1031.513195	4.193

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 2 of 3

Project Name: API
Date Printed: 6/19/2007
4:32:43 PM US/Eastern

Figure 3 (continued). Calibration Report 5666 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
3	Std B3 (12307-80A)	28	ETBE	6	990.000000	4584.353459	1044.830572	5.538
Mean							1040.3	
Std. Dev.							7.6	
% RSD							0.7	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 3 of 3

Project Name: API
Date Printed:
6/19/2007
4:32:43 PM US/Eastern

Figure 4. Calibration Report 5830 for Analysis of ETBE Dose Formulation.

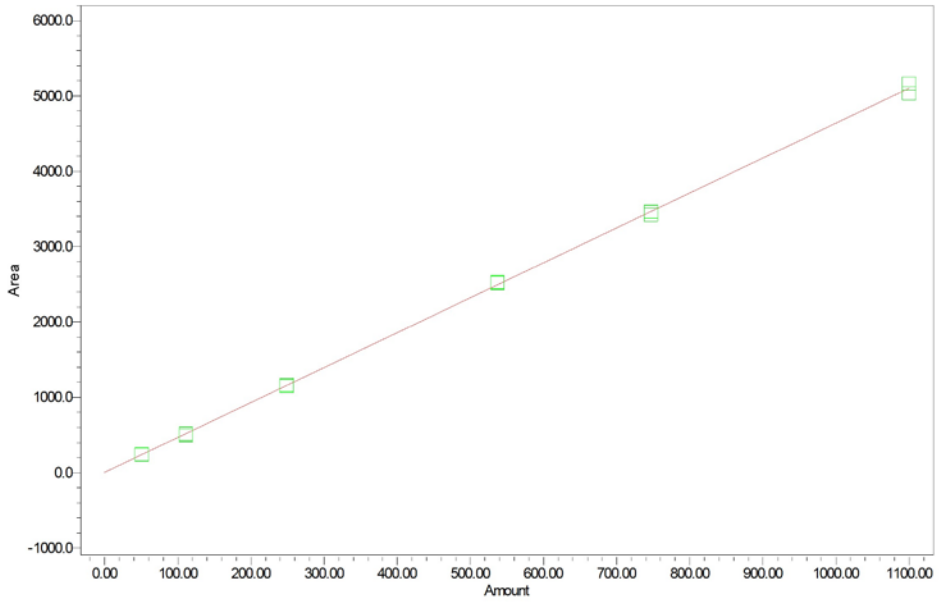
Empower 2 software

Individual_Generic_curve

Processing Method:	ETBE dose	Project Name:	API
Processing Method ID:	5608	System:	GC_08
Channel:	HP6890 Ch1	Calibration ID:	5830
Proc. Chnl. Descr.:	****		
Date Calibrated:	6/20/2007 7:38:11 PM EDT		

A 4.571669e+000
B 4.634501e+000
C 0.000000e+000
D 0.000000e+000
R^2 0.999675

Calibration Plot



ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A1 (12307-83F)	2	ETBE	1	50.600000	239.609321	50.714772	0.227
2	Std A1 (12307-83F)	9	ETBE	1	50.600000	239.441164	50.678488	0.155
3	Std A1 (12307-83F)	23	ETBE	1	50.600000	242.813981	51.406251	1.593
Mean							50.9	
Std. Dev.							0.4	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 1 of 3

Project Name: API
Date Printed:
6/20/2007
7:38:34 PM US/Eastern

Figure 4 (continued) . Calibration Report 5830 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 1

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
% RSD							0.8	

ETBE Standard Curve
Peak: ETBELevel: 2

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B1 (12307-83E)	10	ETBE	2	111.000000	519.929539	111.200298	0.180
2	Std B1 (12307-83E)	3	ETBE	2	111.000000	510.270910	109.116227	-1.697
3	Std B1 (12307-83E)	24	ETBE	2	111.000000	491.623163	105.092547	-5.322
Mean							108.5	
Std. Dev.							3.1	
% RSD							2.9	

ETBE Standard Curve
Peak: ETBELevel: 3

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A2 (12307-83D)	4	ETBE	3	249.000000	1159.481617	249.198348	0.080
2	Std A2 (12307-83D)	11	ETBE	3	249.000000	1145.540838	246.190305	-1.128
3	Std A2 (12307-83D)	25	ETBE	3	249.000000	1165.911059	250.585648	0.637
Mean							248.7	
Std. Dev.							2.2	
% RSD							0.9	

ETBE Standard Curve
Peak: ETBELevel: 4

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B2 (12307-83C)	12	ETBE	4	537.000000	2530.057094	544.931488	1.477
2	Std B2 (12307-83C)	5	ETBE	4	537.000000	2516.418914	541.988738	0.929
3	Std B2 (12307-83C)	26	ETBE	4	537.000000	2524.557833	543.744896	1.256
Mean							543.6	
Std. Dev.							1.5	
% RSD							0.3	

ETBE Standard Curve
Peak: ETBELevel: 5

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std A3 (12307-83B)	6	ETBE	5	747.000000	3461.163773	745.839141	-0.155
2	Std A3 (12307-83B)	13	ETBE	5	747.000000	3423.928419	737.804758	-1.231
3	Std A3 (12307-83B)	27	ETBE	5	747.000000	3465.436370	746.761052	-0.032
Mean							743.5	
Std. Dev.							4.9	
% RSD							0.7	

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
1	Std B3 (12307-83A)	14	ETBE	6	1100.000000	5164.906878	1113.460849	1.224
2	Std B3 (12307-83A)	7	ETBE	6	1100.000000	5033.404520	1085.086195	-1.356

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Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 2 of 3

Project Name: API
Date Printed:
6/20/2007
7:38:34 PM US/Eastern

Figure 4 (continued). Calibration Report 5830 for Analysis of ETBE Dose Formulation.

ETBE Standard Curve
Peak: ETBELevel: 6

	SampleName	Vial	Name	Level	X Value	Response	Calc. Value	% Deviation
Mean							1099.3	
Std. Dev.							20.1	
% RSD							1.8	

Reported by User: Yan Hong (HongY)
Report Method: Individual_Generic_curve
Report Method ID: 4712
Page: 3 of 3

Project Name: API
Date Printed:
6/20/2007
7:38:34 PM US/Eastern

Project Specific Analytical Method for Analysis of Ethyl Tertiary Butyl Ether in Oral Dose Formulations

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-D0209408.007.0 Page 1 of 8

TITLE: Project Specific Analytical Method for Analysis of Ethyl Tertiary Butyl Ether in Oral Dose Formulations

SOURCE: Science and Engineering

AUTHOR: Signed Norman F Gaudette, Jr.

Date 6-5-07

APPROVED BY: Signed Timothy R. Fennell

Date 06-05-07

EFFECTIVE DATE: 6/5/07
(This version)

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-D0209408.007.0 Page 2 of 8

1.0 INTRODUCTION

This document contains procedures for analysis of oral dose formulations containing the compound Ethyl Tertiary Butyl Ether (ETBE). Additionally, procedures for conduct of a stability study establishing the chemical stability of ETBE in aqueous dose formulations at 1 mg/ml and 5 mg/mL are detailed in this document. The methodology described in this document was developed for support of a study conducted under the RTI protocol Pharmacokinetics of Ethyl Tertiary Butyl Ether (ETBE) in Blood following a Single Oral Exposure (RTI Protocol RTI-934).

2.0 REAGENTS AND CHEMICALS

Ethyl tertiary Butyl Ether (ETBE): 99% purity, Sigma-Aldrich, St. Louis, MO. This test article lot approved by the study sponsor will be used for preparation of all standards..

Methanol: HPLC grade or equivalent

Distilled/Deionized (D/I) water: Obtain this reagent from the Corning Still in Hermann 210.

Helium: 99.996% purity, obtained from National Welders, Durham NC.

3.0 EQUIPMENT

Equipment necessary for completion of procedures in this method are detailed in the table below.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-D0209408.007.0 Page 3 of 8

Table 1

Equipment

Item	Description
Electronic Balance	Four-place electronic balance, Mettler AG 204, or equivalent
Gas Chromatograph	Agilent Model 6890 gas chromatograph equipped with an Agilent Model 5973 Mass Selective Detector (MSD), or equivalent
Electronic Pipet (100 µL)	Rainin Model LTS-100, or equivalent
Electronic Pipet (1000 µL)	Rainin Model LTS-1000, or equivalent
Electronic Pipet (20 µL)	Rainin Model LTS-20, or equivalent
Syringe (50 µL)	Hamilton, Gastight#1705, or equivalent

4.0 PROCEDURES

4.1 Preparation of Stock Solutions

Prepare two stock solutions (Stocks A and B) at a target concentration of 1 mg/ml for subsequent dilution in preparation of calibration standards. Concentration of stock solution should be calculated in units of micrograms ETBE per milliliter (mL) of solution. Prepare the stock solutions according to the follow procedure. Store the ETBE stock solutions at approximately -20 °C.

1) Fill a 10-ml volumetric flask with methanol. Remove 17.5 µLof methanol using a pipet and tare the volumetric flask..
2) Using a syringe, add 15 µl of ETBE into the flask and cap the volumetric flask immediately.

3) Record weights of ETBE added to the flask, and place the volumetric flask on ice. Cool and mix the stock solution by hand.
4) Calculate the actual concentration of stock solutions.

4.2 Preparation of Calibration Standard Solutions

Prepare six calibration standards consisting of ETBE in methanol. Perform dilutions according to the table below. Place all solutions in wet ice immediately following preparation. Calculate the actual concentration of each standard solution in units of μg/ml. Transfer an aliquot of each solution to individual autosampler vials in triplicates for analysis. Store the standard solutions at ca. -20 °C.

Table 2: Calibration Standard Preparation

Standard ID	Target ETBE Conc. (μg/ml)	Stock or Standard ID	Stock or Standard Volume (ml)	Volume Methanol Required (ml)	Target Dilution Factor
B3	1000	Stock B	N/A	N/A	N/A
A3	750	Stock A	2.5	X	3:4
B2	500	Standard B3	2	2	1:2
A2	250	Standard A3	1	2	1:3
B1	100	Standard B2	0.75	3	1:5
A1	50	Standard A2	1	3	1:4

X = [(Conc. Stock A x 2.5 mL)/750μg/mL]-2.5 mL

Note: Dilution factors shown in the table are targets for using pipets to perform the dilution. Weights of added methanol and total dilution weights must be collected for calculation of dilution factors by weight.

4.3 Preparation of dose formulation for analysis

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-D0209408.007.0 Page 5 of 8

Dilute the dose formulation with methanol in triplicates using pipets. Record the weight of methanol added to each dilution as well as the total weight of the dilution solution. Calculate a dilution factor according the following equation:

[(Weight of total solution)/Density (methanol)]/ [(Weight of total solution)-(Weight of methanol added)].

Transfer duplicate aliquots of each dilution into inserts in GC vials for analysis.

4.4 Analysis of Standards and Samples

Assay standards and samples using gas chromatography with flame ionization detection. Analysis parameters are detailed below in Table 3.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-D0209408.007.0 Page 6 of 8

Table 3 Instrument Conditions

6890 GC	
Injection method	Splitless
Injector temperature	200 ºC
Carrier gas	Helium
Injector purge flow	15 ml/min
Injector purge start time	0.75 min
Flow rate	7.0 ml/min
Column	DB-1, 30m x 0.530 mm i.d., 3 μm film thickness (J&W, Agilent technologies, Wilmington, DE)
GC oven program	
Initial temperature	40 ºC
Initial time	2 min
Temperature rate	10 ºC/min
Final temperature	60 ºC
Final time	0.10 min
Temperature rate A	100 ºC/min
Final time A	0.5 min
Final temperature A	200 ºC
Run time	6 min
Flame Ionization Detector	
Temperature	250 °C
Hydrogen flow rate	30 ml/min
Helium flow rate	300 ml/min
Makeup gas flow rate	20 ml/min

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-D0209408.007.0 Page 7 of 8

4.5 Calculation of Results

Use the Empower 2 software to calculate the linear regression (standard curve) including the regression's linear correlation coefficient (r). Also, utilize the Empower 2 software to calculate standard concentration, accuracy of each standard solution, and the sample concentration. Calculate mean concentration values, standard deviations, and relative standard deviation (%RSD) for the triplicate standard concentration points and replicate dose formulation concentrations with Empower 2. Use Microsoft Excel spreadsheets to perform the calculation of formulation dilution factors

4.6 Acceptance Criteria

1) Correlation coefficient (r) must be greater than or equal to 0.990.

2) Measured concentration of standards must be within ± 15% of the nominal value for at least 75% of calibration standards

4.7 Formulation Stability Study

Prior to conduct of the animal study, determine the stability of aqueous ETBE dose formulations stored at room temperature for three days. Conduct the study by preparing two mock formulations at 1 mg/ml and 5 mg/ml storing these formulation at room temperature. Analyze triplicate aliquots of the formulation on the day of preparation, the following day, and at termination of the stability study (3 days). Formulation concentration will be considered to be stable under the storage conditions if the concentration is within ± 15 % of the initial concentration measured on the day of preparation.

ANALYTICAL METHOD	RTI INTERNATIONAL POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709-2194	AM-D0209408.007.0 Page 8 of 8

REVIEW/ REVISION LOG

Rev. #	Rev. Date	Description
0	NA	Original version.

Appendix F

Blood Sample Collection and Analysis for ETBE

Sample Naming and Abbreviations

In the tables of raw data values in this appendix, some of the table headings are derived from the conventions of sample naming used in the Debra™ system. These are explained below.

Column Heading	Source	Explanation
Subject		Animal number
Subject wt. (g)	Balance	Animal wt.
Sample		The type of sample, e.g. blood
Time		The time of sample collection
Dose Time	Workstation	The time of dosing
Nominal Time	Input	The nominal sampling time for blood collection
Elapsed Time	Calculated	The time after dosing of actual sample collection
Full Syringe wt (g)	Balance	Weight of full syringe prior to dosing
Empty Syringe wt (g)	Balance	Weight of empty syringe after dosing
Dose Solution Admin. (g)	Calculated	Calculated from Full syringe wt – empty syringe wt
Dose Admin (mg)	Calculated	ETBE Admin calculated from Dose solution administered x concentration of dose solution.
Actual Dose (mg/kg)	Calculated	ETBE admin/Animal wt
Pot wt (g)	Balance	The weight of the empty sample container
Sample wt (g)	Balance	Sample weight, the weight of sample + empty sample container
Corrected Sample wt (g)	Calculated	The corrected sample weight, calculated from Sample wt – Pot wt

Table 1. Blood Sample Collection Times.

Subject	Sample	Dose Time	Nominal Time	Elapsed Time (hh:mm:ss)	Capture Time
AM01	Blood	6/13/2007 10:03	15 m	0:15:36	6/13/2007 10:19
AM02	Blood	6/13/2007 10:10	15 m	0:14:19	6/13/2007 10:24
AM03	Blood	6/13/2007 10:18	15 m	0:14:09	6/13/2007 10:32
AM04	Blood	6/13/2007 10:25	15 m	0:14:11	6/13/2007 10:39
AM01	Blood	6/13/2007 10:03	30 m	0:30:44	6/13/2007 10:34
AM02	Blood	6/13/2007 10:10	30 m	0:29:25	6/13/2007 10:39
AM03	Blood	6/13/2007 10:18	30 m	0:29:42	6/13/2007 10:48
AM04	Blood	6/13/2007 10:25	30 m	0:30:39	6/13/2007 10:56
AM01	Blood	6/13/2007 10:03	1 h	0:59:06	6/13/2007 11:02
AM02	Blood	6/13/2007 10:10	1 h	0:58:58	6/13/2007 11:09
AM03	Blood	6/13/2007 10:18	1 h	0:59:38	6/13/2007 11:18
AM04	Blood	6/13/2007 10:25	1 h	1:01:04	6/13/2007 11:26
AM01	Blood	6/13/2007 10:03	2 h	2:03:09	6/13/2007 12:06
AM02	Blood	6/13/2007 10:10	2 h	1:59:01	6/13/2007 12:09
AM03	Blood	6/13/2007 10:18	2 h	1:59:11	6/13/2007 12:17
AM04	Blood	6/13/2007 10:25	2 h	1:59:24	6/13/2007 12:25
AM01	Blood	6/13/2007 10:03	4 h	4:03:13	6/13/2007 14:06
AM02	Blood	6/13/2007 10:10	4 h	3:58:28	6/13/2007 14:08
AM03	Blood	6/13/2007 10:18	4 h	3:58:58	6/13/2007 14:17
AM04	Blood	6/13/2007 10:25	4 h	4:00:31	6/13/2007 14:26
AM01	Blood	6/13/2007 10:03	8 h	8:07:00	6/13/2007 18:10
AM02	Blood	6/13/2007 10:10	8 h	8:02:06	6/13/2007 18:12
AM03	Blood	6/13/2007 10:18	8 h	7:59:19	6/13/2007 18:17
AM04	Blood	6/13/2007 10:25	8 h	8:00:11	6/13/2007 18:25
AM01	Blood	6/13/2007 10:03	24 h	24:09:52	6/14/2007 10:13
AM02	Blood	6/13/2007 10:10	24 h	24:09:20	6/14/2007 10:19
AM03	Blood	6/13/2007 10:18	24 h	24:06:03	6/14/2007 10:24
AM04	Blood	6/13/2007 10:25	24 h	24:03:00	6/14/2007 10:28
BF01	Blood	6/14/2007 11:13	15 m	0:13:48	6/14/2007 11:27
BF01	Blood	6/14/2007 11:13	30 m	0:28:24	6/14/2007 11:41
BF01	Blood	6/14/2007 11:13	1 h	0:59:05	6/14/2007 12:12
BF02	Blood	6/14/2007 11:18	15 m	0:17:12	6/14/2007 11:35
BF02	Blood	6/14/2007 11:18	30 m	0:28:04	6/14/2007 11:46
BF02	Blood	6/14/2007 11:18	1 h	0:58:45	6/14/2007 12:16
BF03	Blood	6/14/2007 11:27	15 m	0:12:04	6/14/2007 11:40
BF03	Blood	6/14/2007 11:27	30 m	0:29:13	6/14/2007 11:57
BF03	Blood	6/14/2007 11:27	1 h	1:00:51	6/14/2007 12:28

Table 1 (continued). Blood Sample Collection Times.

Subject	Sample	Dose Time	Nominal Time	Elapsed Time (hh:mm:ss)	Capture Time
BF04	Blood	6/14/2007 11:37	15 m	0:13:18	6/14/2007 11:50
BF04	Blood	6/14/2007 11:37	30 m	0:29:49	6/14/2007 12:06
BF04	Blood	6/14/2007 11:37	1 h	0:59:21	6/14/2007 12:36
BF01	Blood	6/14/2007 11:13	2 h	1:58:43	6/14/2007 13:12
BF02	Blood	6/14/2007 11:18	2 h	1:59:03	6/14/2007 13:17
BF03	Blood	6/14/2007 11:27	2 h	2:01:56	6/14/2007 13:29
BF04	Blood	6/14/2007 11:37	2 h	1:59:31	6/14/2007 13:36
BF01	Blood	6/14/2007 11:13	4 h	4:03:52	6/14/2007 15:17
BF02	Blood	6/14/2007 11:18	4 h	4:02:44	6/14/2007 15:20
BF03	Blood	6/14/2007 11:27	4 h	4:12:40	6/14/2007 15:40
BF04	Blood	6/14/2007 11:37	4 h	4:01:30	6/14/2007 15:38
BF01	Blood	6/14/2007 11:13	8 h	8:00:00	6/14/2007 19:13
BF02	Blood	6/14/2007 11:18	8 h	7:59:52	6/14/2007 19:17
BF03	Blood	6/14/2007 11:27	8 h	8:04:35	6/14/2007 19:32
BF04	Blood	6/14/2007 11:37	8 h	8:00:57	6/14/2007 19:38
BF01	Blood	6/14/2007 11:13	24 h	24:02:42	6/15/2007 11:16
BF02	Blood	6/14/2007 11:18	24 h	24:00:12	6/15/2007 11:18
BF03	Blood	6/14/2007 11:27	24 h	24:02:32	6/15/2007 11:30
BF04	Blood	6/14/2007 11:37	24 h	24:00:56	6/15/2007 11:38
CM01	Blood	6/20/2007 9:36	15 m	0:14:12	6/20/2007 9:50
CM02	Blood	6/20/2007 9:40	15 m	0:14:18	6/20/2007 9:55
CM03	Blood	6/20/2007 9:44	15 m	0:14:17	6/20/2007 9:58
CM04	Blood	6/20/2007 9:48	15 m	0:14:34	6/20/2007 10:03
CM01	Blood	6/20/2007 9:36	30 m	0:29:45	6/20/2007 10:06
CM02	Blood	6/20/2007 9:40	30 m	0:29:26	6/20/2007 10:10
CM03	Blood	6/20/2007 9:44	30 m	0:29:35	6/20/2007 10:14
CM04	Blood	6/20/2007 9:48	30 m	0:29:35	6/20/2007 10:18
CM01	Blood	6/20/2007 9:36	1 h	0:59:30	6/20/2007 10:35
CM02	Blood	6/20/2007 9:40	1 h	0:59:43	6/20/2007 10:40
CM03	Blood	6/20/2007 9:44	1 h	0:59:36	6/20/2007 10:44
CM04	Blood	6/20/2007 9:48	1 h	0:59:46	6/20/2007 10:48
CM08	Blood	6/20/2007 10:37	15 m	0:16:24	6/20/2007 10:53
CM08	Blood	6/20/2007 10:37	30 m	0:31:34	6/20/2007 11:08
CM01	Blood	6/20/2007 9:36	2 h	1:59:06	6/20/2007 11:35
CM02	Blood	6/20/2007 9:40	2 h	2:02:08	6/20/2007 11:42
CM03	Blood	6/20/2007 9:44	2 h	2:01:48	6/20/2007 11:46
CM08	Blood	6/20/2007 10:37	2 h	1:59:46	6/20/2007 12:36
CM01	Blood	6/20/2007 9:36	4 h	3:59:41	6/20/2007 13:35
CM02	Blood	6/20/2007 9:40	4 h	4:01:04	6/20/2007 13:41
CM03	Blood	6/20/2007 9:44	4 h	3:59:29	6/20/2007 13:43
CM08	Blood	6/20/2007 10:37	4 h	3:59:39	6/20/2007 14:36

Table 1 (continued). Blood Sample Collection Times.

Subject	Sample	Dose Time	Nominal Time	Elapsed Time (hh:mm:ss)	Capture Time
CM01	Blood	6/20/2007 9:36	8 h	8:03:04	6/20/2007 17:39
CM02	Blood	6/20/2007 9:40	8 h	8:01:17	6/20/2007 17:42
CM03	Blood	6/20/2007 9:44	8 h	7:59:19	6/20/2007 17:43
CM08	Blood	6/20/2007 10:37	8 h	8:01:21	6/20/2007 18:38
CM01	Blood	6/20/2007 9:36	24 h	23:59:48	6/21/2007 9:36
CM02	Blood	6/20/2007 9:40	24 h	24:03:54	6/21/2007 9:44
CM03	Blood	6/20/2007 9:44	24 h	24:01:51	6/21/2007 9:46
CM08	Blood	6/20/2007 10:37	24 h	24:01:00	6/21/2007 10:38
CM08	Blood	6/20/2007 10:37	1 h	0:59:31	6/20/2007 11:36
DF01	Blood	6/21/2007 10:04	15 m	0:14:37	6/21/2007 10:19
DF02	Blood	6/21/2007 10:11	15 m	0:14:39	6/21/2007 10:25
DF04	Blood	6/21/2007 10:34	15 m	0:17:02	6/21/2007 10:51
DF01	Blood	6/21/2007 10:04	30 m	0:30:05	6/21/2007 10:34
DF02	Blood	6/21/2007 10:11	30 m	0:28:34	6/21/2007 10:39
DF04	Blood	6/21/2007 10:34	30 m	0:29:53	6/21/2007 11:04
DF01	Blood	6/21/2007 10:04	1 h	0:58:56	6/21/2007 11:03
DF02	Blood	6/21/2007 10:11	1 h	0:58:42	6/21/2007 11:09
DF04	Blood	6/21/2007 10:34	1 h	0:58:20	6/21/2007 11:33
DF05	Blood	6/21/2007 11:02	15 m	0:23:51	6/21/2007 11:26
DF05	Blood	6/21/2007 11:02	30 m	0:29:27	6/21/2007 11:31
DF05	Blood	6/21/2007 11:02	1 h	0:57:06	6/21/2007 11:59
DF01	Blood	6/21/2007 10:04	2 h	1:59:21	6/21/2007 12:03
DF02	Blood	6/21/2007 10:11	2 h	2:01:02	6/21/2007 12:12
DF04	Blood	6/21/2007 10:34	2 h	2:01:54	6/21/2007 12:36
DF05	Blood	6/21/2007 11:02	2 h	2:00:50	6/21/2007 13:03
DF01	Blood	6/21/2007 10:04	4 h	3:58:28	6/21/2007 14:03
DF02	Blood	6/21/2007 10:11	4 h	4:00:31	6/21/2007 14:11
DF04	Blood	6/21/2007 10:34	4 h	3:43:14	6/21/2007 14:18
DF05	Blood	6/21/2007 11:02	4 h	4:00:04	6/21/2007 15:02
DF01	Blood	6/21/2007 10:04	8 h	8:00:11	6/21/2007 18:04
DF02	Blood	6/21/2007 10:11	8 h	7:58:18	6/21/2007 18:09
DF04	Blood	6/21/2007 10:34	8 h	7:58:53	6/21/2007 18:33
DF05	Blood	6/21/2007 11:02	8 h	8:08:21	6/21/2007 19:10
DF01	Blood	6/21/2007 10:04	24 h	23:59:27	6/22/2007 10:04
DF02	Blood	6/21/2007 10:11	24 h	24:07:07	6/22/2007 10:18
DF04	Blood	6/21/2007 10:34	24 h	24:00:45	6/22/2007 10:35
DF05	Blood	6/21/2007 11:02	24 h	24:01:44	6/22/2007 11:04

Table 2. Blood Sample Weights from Group A.

Subject	Sample	Time	Pot wt	Sample wt	Corrected Sample Weight
AM01	Blood	Predose	11.4212 g	11.6353 g	0.2141 g
AM01	Blood	15 m	11.1270 g	11.2833 g	0.1563 g
AM01	Blood	30 m	11.1523 g	11.3527 g	0.2004 g
AM01	Blood	1 h	11.1092 g	11.2900 g	0.1808 g
AM01	Blood	2 h	11.1053 g	11.2445 g	0.1392 g
AM01	Blood	4 h	11.4308 g	11.6115 g	0.1807 g
AM01	Blood	8 h	11.4329 g	11.5216 g	0.0887 g
AM01	Blood	24 h	11.1190 g	11.2360 g	0.1170 g
AM02	Blood	Predose	11.4371 g	11.6335 g	0.1964 g
AM02	Blood	15 m	11.3950 g	11.5758 g	0.1808 g
AM02	Blood	30 m	11.4324 g	11.6002 g	0.1678 g
AM02	Blood	1 h	11.1433 g	11.3230 g	0.1797 g
AM02	Blood	2 h	11.1615 g	11.3014 g	0.1399 g
AM02	Blood	4 h	11.4348 g	11.6017 g	0.1669 g
AM02	Blood	8 h	11.4201 g	11.5906 g	0.1705 g
AM02	Blood	24 h	11.2571 g	11.3627 g	0.1056 g
AM03	Blood	Predose	11.1365 g	11.3031 g	0.1666 g
AM03	Blood	15 m	11.1674 g	11.3408 g	0.1734 g
AM03	Blood	30 m	11.1094 g	11.3016 g	0.1922 g
AM03	Blood	1 h	11.4141 g	11.5909 g	0.1768 g
AM03	Blood	2 h	11.4064 g	11.5602 g	0.1538 g
AM03	Blood	4 h	11.1139 g	11.3116 g	0.1977 g
AM03	Blood	8 h	11.4673 g	11.6316 g	0.1643 g
AM03	Blood	24 h	11.1560 g	11.2571 g	0.1011 g
AM04	Blood	Predose	11.4306 g	11.5854 g	0.1548 g
AM04	Blood	15 m	11.1558 g	11.3193 g	0.1635 g
AM04	Blood	30 m	11.4293 g	11.5988 g	0.1695 g
AM04	Blood	1 h	11.4757 g	11.6363 g	0.1606 g
AM04	Blood	2 h	11.0899 g	11.2658 g	0.1759 g
AM04	Blood	4 h	11.1390 g	11.3385 g	0.1995 g
AM04	Blood	8 h	11.1518 g	11.3606 g	0.2088 g
AM04	Blood	24 h	11.3721 g	11.4938 g	0.1217 g

Table 3. Blood Sample Weights from Group B.

Subject	Sample	Time	Pot wt	Sample wt	Corrected Sample Weight
BF01	Blood	Predose	11.1488 g	11.2307 g	0.0819 g
BF01	Blood	15 m	11.2873 g	11.4526 g	0.1653 g
BF01	Blood	30 m	11.0774 g	11.2678 g	0.1904 g
BF01	Blood	1 h	11.0936 g	11.2735 g	0.1799 g
BF01	Blood	2 h	11.3922 g	11.5856 g	0.1934 g
BF01	Blood	4 h	11.2622 g	11.4151 g	0.1529 g
BF01	Blood	8 h	11.0922 g	11.1995 g	0.1073 g
BF01	Blood	24 h	11.1848 g	11.3325 g	0.1477 g
BF02	Blood	Predose	11.1217 g	11.3399 g	0.2182 g
BF02	Blood	15 m	11.3030 g	11.4662 g	0.1632 g
BF02	Blood	30 m	11.2971 g	11.4624 g	0.1653 g
BF02	Blood	1 h	11.2922 g	11.4724 g	0.1802 g
BF02	Blood	2 h	11.3644 g	11.5423 g	0.1779 g
BF02	Blood	4 h	11.0763 g	11.2486 g	0.1723 g
BF02	Blood	8 h	11.3128 g	11.4617 g	0.1489 g
BF02	Blood	24 h	11.0841 g	11.2386 g	0.1545 g
BF03	Blood	Predose	11.3445 g	11.5136 g	0.1691 g
BF03	Blood	15 m	11.1272 g	11.2720 g	0.1448 g
BF03	Blood	30 m	11.0637 g	11.2508 g	0.1871 g
BF03	Blood	1 h	11.1027 g	11.2808 g	0.1781 g
BF03	Blood	2 h	11.1160 g	11.2066 g	0.0906 g
BF03	Blood	4 h	11.2468 g	11.3505 g	0.1037 g
BF03	Blood	8 h	11.0763 g	11.1955 g	0.1192 g
BF03	Blood	24 h	11.1114 g	11.2460 g	0.1346 g
BF04	Blood	Predose	11.1544 g	11.3162 g	0.1618 g
BF04	Blood	15 m	11.0889 g	11.2715 g	0.1826 g
BF04	Blood	30 m	11.3214 g	11.4185 g	0.0971 g
BF04	Blood	1 h	11.0806 g	11.2183 g	0.1377 g
BF04	Blood	2 h	11.1596 g	11.3377 g	0.1781 g
BF04	Blood	4 h	11.3140 g	11.4553 g	0.1413 g
BF04	Blood	8 h	11.2672 g	11.4080 g	0.1408 g
BF04	Blood	24 h	11.1306 g	11.2838 g	0.1532 g

Table 4. Blood Sample Weights from Group C.

Subject	Sample	Time	Pot wt	Sample wt	Corrected Sample Weight
CM01	Blood	Predose	11.3409 g	11.4943 g	0.1534 g
CM01	Blood	15 m	11.1423 g	11.2985 g	0.1562 g
CM01	Blood	30 m	11.3269 g	11.4757 g	0.1488 g
CM01	Blood	1 h	11.2967 g	11.4562 g	0.1595 g
CM01	Blood	2 h	11.4177 g	11.5398 g	0.1221 g
CM01	Blood	4 h	11.3391 g	11.4876 g	0.1485 g
CM01	Blood	8 h	11.3190 g	11.4859 g	0.1669 g
CM01	Blood	24 h	11.1246 g	11.3059 g	0.1813 g
CM02	Blood	Predose	11.4180 g	11.5603 g	0.1423 g
CM02	Blood	15 m	11.2860 g	11.4204 g	0.1344 g
CM02	Blood	30 m	11.3723 g	11.5325 g	0.1602 g
CM02	Blood	1 h	11.2280 g	11.3301 g	0.1021 g
CM02	Blood	2 h	11.3617 g	11.4798 g	0.1181 g
CM02	Blood	4 h	11.1876 g	11.2883 g	0.1007 g
CM02	Blood	8 h	11.2039 g	11.3695 g	0.1656 g
CM02	Blood	24 h	11.3990 g	11.5725 g	0.1735 g
CM03	Blood	Predose	11.2254 g	11.3787 g	0.1533 g
CM03	Blood	15 m	11.2436 g	11.4241 g	0.1805 g
CM03	Blood	30 m	11.3924 g	11.4825 g	0.0901 g
CM03	Blood	1 h	11.2066 g	11.3237 g	0.1171 g
CM03	Blood	2 h	11.2775 g	11.4064 g	0.1289 g
CM03	Blood	4 h	11.3050 g	11.4452 g	0.1402 g
CM03	Blood	8 h	11.2963 g	11.4470 g	0.1507 g
CM03	Blood	24 h	11.3003 g	11.4514 g	0.1511 g
CM08	Blood	Predose	11.4752 g	11.5913 g	0.1161 g
CM08	Blood	15 m	11.4486 g	11.5910 g	0.1424 g
CM08	Blood	30 m	11.1118 g	11.2350 g	0.1232 g
CM08	Blood	1 h	11.1109 g	11.2690 g	0.1581 g
CM08	Blood	2 h	11.4776 g	11.6386 g	0.1610 g
CM08	Blood	4 h	11.4341 g	11.5507 g	0.1166 g
CM08	Blood	8 h	11.4683 g	11.6235 g	0.1552 g
CM08	Blood	24 h	11.4605 g	11.7199 g	0.2594 g

Table 4. Blood Sample Weights from Group D.

Subject	Sample	Time	Pot wt	Sample wt	Corrected Sample Weight
DF01	Blood	Predose	11.4138 g	11.5602 g	0.1464 g
DF01	Blood	15 m	11.2374 g	11.3676 g	0.1302 g
DF01	Blood	30 m	11.1363 g	11.2859 g	0.1496 g
DF01	Blood	1 h	11.3407 g	11.5101 g	0.1694 g
DF01	Blood	2 h	11.1892 g	11.3090 g	0.1198 g
DF01	Blood	4 h	11.1345 g	11.2604 g	0.1259 g
DF01	Blood	8 h	11.4590 g	11.6008 g	0.1418 g
DF01	Blood	24 h	11.2978 g	11.4413 g	0.1435 g
DF02	Blood	Predose	11.3077 g	11.4628 g	0.1551 g
DF02	Blood	15 m	11.3979 g	11.5384 g	0.1405 g
DF02	Blood	30 m	11.3715 g	11.5452 g	0.1737 g
DF02	Blood	1 h	11.1177 g	11.2308 g	0.1131 g
DF02	Blood	2 h	11.3159 g	11.4197 g	0.1038 g
DF02	Blood	4 h	11.4005 g	11.4837 g	0.0832 g
DF02	Blood	8 h	11.1653 g	11.3037 g	0.1384 g
DF02	Blood	24 h	11.2631 g	11.4267 g	0.1636 g
DF04	Blood	Predose	11.1653 g	11.2854 g	0.1201 g
DF04	Blood	15 m	11.2471 g	11.3944 g	0.1473 g
DF04	Blood	30 m	11.1474 g	11.2523 g	0.1049 g
DF04	Blood	1 h	11.3201 g	11.4407 g	0.1206 g
DF04	Blood	2 h	11.1649 g	11.2995 g	0.1346 g
DF04	Blood	4 h	11.1617 g	11.2531 g	0.0914 g
DF04	Blood	8 h	11.1842 g	11.2963 g	0.1121 g
DF04	Blood	24 h	11.1453 g	11.3038 g	0.1585 g
DF05	Blood	Predose	11.4396 g	11.5576 g	0.1180 g
DF05	Blood	15 m	11.3806 g	11.4930 g	0.1124 g
DF05	Blood	30 m	11.1289 g	11.1910 g	0.0621 g
DF05	Blood	1 h	11.1786 g	11.3300 g	0.1514 g
DF05	Blood	2 h	11.3245 g	11.3913 g	0.0668 g
DF05	Blood	4 h	11.4156 g	11.5363 g	0.1207 g
DF05	Blood	8 h	11.3797 g	11.5310 g	0.1513 g
DF05	Blood	24 h	11.1243 g	11.2416 g	0.1173 g

Table 5. Low Concentration Calibration Curve for High Dose Groups A and B.

Sample I.D.	Standard I.D.	Conc. (µg/ml)	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (µg/ml)	Mean (µg/mL)	Accuracy (%)	Error (%)
12307-56A	Blood blank	n/a	0	n/a	n/a	n/a	n/a	-	-
12307-57A	Blood blank	n/a	0	n/a	n/a	n/a	-	-	-
12307-58A	Blood blank	n/a	0	n/a	n/a	n/a	-	-	-
12307-56B	Blood ISTD blank	n/a	0	2641319	n/a	n/a	n/a	-	-
12307-57A	Blood ISTD blank	n/a	0	2154647	n/a	n/a	-	-	-
12307-58A	Blood ISTD blank	n/a	0	2302458	n/a	n/a	-	-	-
12307-56C	Std A1	0.102	22552	2602098	0.00867	0.103	0.104	101%	1.43%
12307-57C	Std A1	0.102	18713	2138062	0.00875	0.104	-	102%	2.45%
12307-58C	Std A1	0.102	19885	2275009	0.00874	0.104	-	102%	2.31%
12307-56D	Std B1	0.252	52855	2554845	0.0207	0.249	0.253	98.8%	-1.25%
12307-57D	Std B1	0.252	44467	2107229	0.0211	0.254	-	101%	0.739%
12307-58D	Std B1	0.252	46168	2172972	0.0212	0.256	-	101%	1.43%
12307-56E	Std A2	0.497	102237	2562139	0.0399	0.481	0.485	96.8%	-3.17%
12307-57E	Std A2	0.497	85024	2102020	0.0404	0.488	-	98.2%	-1.84%
12307-57E	Std A2	0.497	90406	2243675	0.0403	0.486	-	97.8%	-2.22%
12307-56F	Std B2	1.03	209164	2505428	0.0835	1.01	1.03	97.9%	-2.10%
12307-57F	Std B2	1.03	182555	2092192	0.0873	1.05	-	102%	2.33%
12307-58F	Std B2	1.03	188676	2215712	0.0852	1.03	-	99.9%	-0.14%
12307-56G	Std A3	2.43	495521	2476881	0.200	2.42	2.43	99.5%	-0.481%
12307-57G	Std A3	2.43	431537	2116191	0.204	2.47	-	101%	1.44%
12307-57G	Std A3	2.43	445943	2249957	0.198	2.40	-	98.6%	-1.41%
12307-56H	Std B3	5.17	1046174	2490883	0.420	5.08	5.18	98.2%	-1.77%
12307-57H	Std B3	5.17	922001	2129104	0.433	5.24	-	101%	1.28%
12307-58H	Std B3	5.17	952742	2199941	0.433	5.24	-	101%	1.29%

Table 5 (continued). Low Concentration Calibration Curve for High Dose Groups A and B.

Sample I.D.	Standard I.D.	Conc. (µg/ml)	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (µg/ml)	Mean (µg/mL)	Accuracy (%)	Error (%)
12307-65-1	LQC	0.497	101327	2426211	0.0418	0.504	-	101%	1.36%
12307-65-2	LQC	0.497	98466	2404023	0.0410	0.494	-	99.4%	-0.598%
12307-65-1	MQC	5.17	1049759	2348383	0.4470	5.41	-	105%	4.55%
12307-65-2	MQC	5.17	965608	2252523	0.4287	5.18	-	100%	0.260%
	a INTERCEPT	0.0001127							
	b SLOPE	0.08268							
	r	0.9998							

Table 6. High Concentration Calibration Curve for High Dose Groups A and B.

Sample I.D.	Standard I.D.	Conc. (µg/ml)	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (µg/ml)	Mean (µg/mL)	Accuracy (%)	Error (%)
12307-56H	Std B3	5.17	1046174	2490883	0.420	5.45	5.54	105%	5.41%
12307-57H	Std B3	5.17	922001	2129104	0.433	5.58	-	108%	8.01%
12307-58H	Std B3	5.17	952742	2199941	0.433	5.58	-	108%	8.02%
12307-56I	Std A4	9.76	2055076	2477991	0.829	9.66	9.74	99.0%	-1.031%
12307-57I	Std A4	9.76	1801497	2108315	0.854	9.92	-	102%	1.62%
12307-58I	Std A4	9.76	1797962	2175731	0.826	9.63	-	98.7%	-1.34%
12307-56J	Std B4	25.8	5505071	2415755	2.28	24.6	24.9	95.2%	-4.78%
12307-57J	Std B4	25.8	4901307	2117403	2.31	24.9	-	96.6%	-3.35%
12307-58J	Std B4	25.8	5180410	2223813	2.33	25.1	-	97.2%	-2.76%
12307-56K	Std A5	48.9	10630375	2384396	4.46	47.0	47.5	96.1%	-3.93%
12307-57K	Std A5	48.9	9413503	2073300	4.54	47.8	-	97.8%	-2.20%
12307-58K	Std A5	48.9	9961182	2193738	4.54	47.8	-	97.8%	-2.19%
12307-56L	Std B5	104	22891077	2357577	9.71	101	102	96.7%	-3.27%
12307-57L	Std B5	104	20673056	2090026	9.89	103	-	98.5%	-1.48%
12307-58L	Std B5	104	20351677	2078699	9.79	102	-	97.5%	-2.48%
12307-56M	Std A6	249	54977181	2292603	24.0	248	253	99.6%	-0.386%
12307-57M	Std A6	249	50642541	2039543	24.8	256	-	103%	3.13%
12307-58M	Std A6	249	52411517	2118717	24.7	256	-	103%	2.74%
12307-63-1	HQC	104	22085053	2166264	10.2	106	-	102%	1.51%
12307-63-1	HQC	104	24612373	2365010	10.4	108	-	104%	3.60%
	a INTERCEPT	-0.10995							
	b SLOPE	0.09724							
	r	0.9996							

Table 7. ETBE Blood Concentration Analysis for Groups A and B.

Rat.	Time point	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (μg/ml)	Sample weight (g)	Corrected Conc. (μg/ml)
AM 01	Predose	0	2196830	0	< LOQ	0.2141	< LOQ
	15 min	1816910	2302967	0.789	9.24	0.1563	5.91
	30 min	1262099	2141714	0.589	7.19	0.2004	3.59
	1 hr	559988	2161316	0.259	3.13	0.1808	1.73
	2 hr	138439	2121503	0.0653	0.788	0.1392	0.566
	4 hr	94938	2035726	0.0466	0.563	0.1807	0.311
	8 hr	3241	2199731	0.00147	< LOQ	0.0887	< LOQ
	24 hr	0	2525626	0.000	< LOQ	0.1170	< LOQ
AM 02	Predose	0	2222068	0.000	< LOQ	0.1964	< LOQ
	15 min	2067688	2209167	0.936	10.8	0.1808	5.95
	30 min	872119	2068735	0.422	5.10	0.1678	3.04
	1 hr	601965	2158669	0.279	3.37	0.1797	1.88
	2 hr	153565	2103540	0.0730	0.88	0.1399	0.630
	4 hr	78277	2100924	0.0373	0.45	0.1669	0.269
	8hr	14205	2056302	0.00691	< LOQ	0.1705	< LOQ
	24 hr	0	2482458	0.000	< LOQ	0.1056	< LOQ
AM 03	Predose	0	2203725	0.000	< LOQ	0.1666	< LOQ
	15 min	2473802	2162929	1.14	12.9	0.1734	7.44
	30 min	1743477	2118961	0.823	9.59	0.1922	4.99
	1 hr	742683	2153754	0.345	4.17	0.1768	2.36
	2 hr	181407	2064299	0.0879	1.06	0.1538	0.690
	4 hr	87348	2045608	0.0427	0.515	0.1977	0.261
	8 hr	11530	2123129	0.00543	< LOQ	0.1643	< LOQ
	24 hr	0	2413202	0.000	< LOQ	0.1011	< LOQ

Table 7 (continued). ETBE Blood Concentration Analysis for Groups A and B.

Rat.	Time point	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (μg/ml)	Sample weight (g)	Corrected Conc. (μg/ml)
AM 04	Predose	0	2257976	0.000	< LOQ	0.1548	< LOQ
	15 min	2064431	2140584	0.964	11.0	0.1635	6.76
	30 min	901525	2165470	0.416	5.03	0.1695	2.97
	1 hr	395917	2158230	0.183	2.22	0.1606	1.38
	2 hr	206336	2105150	0.0980	1.18	0.1759	0.673
	4 hr	113612	2045057	0.0556	0.671	0.1995	0.336
	8 hr	12628	2095348	0.00603	< LOQ	0.2088	< LOQ
	24 hr	0	2431724	0.000	< LOQ	0.1217	< LOQ
BF 01	Predose	0	2502344	0.000	< LOQ	0.0819	< LOQ
	15 min	1799456	2499526	0.720	8.53	0.1653	5.16
	30 min	1446798	2217557	0.652	7.84	0.1904	4.12
	1 hr	658221	2267553	0.290	3.51	0.1799	1.95
	2 hr	414134	2357315	0.176	2.12	0.1934	1.10
	4 hr	55978	2242089	0.0250	0.301	0.1529	0.197
	8 hr	10761	2474968	0.00435	< LOQ	0.1073	< LOQ
	24 hr	0	2410264	0.000	< LOQ	0.1477	< LOQ
BF 02	Predose	0	2352336	0.000	< LOQ	0.2182	< LOQ
	15 min	1340572	2443639	0.549	6.77	0.1632	4.15
	30 min	903103	2262860	0.399	4.83	0.1653	2.92
	1 hr	744583	2255543	0.330	3.99	0.1802	2.21
	2 hr	315703	2323147	0.136	1.64	0.1779	0.923
	4 hr	96914	2216732	0.0437	0.527	0.1723	0.306
	8 hr	12338	2409563	0.00512	< LOQ	0.1489	< LOQ
	24 hr	0	2367956	0.000	< LOQ	0.1545	< LOQ

Table 7 (continued). ETBE Blood Concentration Analysis for Groups A and B.

Rat.	Time point	Peak Area		Peak Area Ratio	Cal. Conc. (μg/ml)	Sample weight (g)	Corrected Conc. (μg/ml)
		ETBE	MTBE				
BF 03	Predose	0	2390631	0.000	< LOQ	0.1691	< LOQ
	15 min	1825330	2408157	0.758	8.93	0.1448	6.16
	30 min	1107295	2230081	0.497	6.24	0.1871	3.33
	1 hr	521625	2357660	0.221	2.67	0.1781	1.50
	2 hr	122492	2341144	0.0523	0.63	0.0906	0.697
	4 hr	65921	2205701	0.0299	0.36	0.1037	0.347
	8 hr	3110	2387305	0.00130	< LOQ	0.1192	< LOQ
	24 hr	0	2358860	0.000	< LOQ	0.1346	< LOQ
BF 04	Predose	0	2329569	0.000	< LOQ	0.1618	< LOQ
	15 min	3015285	2341472	1.29	14.4	0.1826	7.87
	30 min	749363	2305076	0.325	3.93	0.0971	4.05
	1 hr	515108	2279502	0.226	2.73	0.1377	1.98
	2 hr	210496	2276311	0.0925	1.12	0.1781	0.627
	4 hr	48962	2155643	0.0227	0.273	0.1413	0.193
	8 hr	15377	2358448	0.00652	< LOQ	0.1408	< LOQ
	24 hr	0	2350284	0.000	< LOQ	0.1532	< LOQ

Table 8. Low Concentration Calibration Curve for Low Dose Groups C and D.

Sample I.D.	Standard I.D.	Conc. (μg/ml)	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (μg/ml)	Mean (μg/ml)	Accuracy (%)	Error (%)
12307-76A	Blood blank	n/a	0	n/a	n/a	n/a	n/a	-	-
12307-77A	Blood blank	n/a	0	n/a	n/a	n/a	-	-	-
12307-78A	Blood blank	n/a	0	n/a	n/a	n/a	-	-	-
12307-76B	Blood ISTD blank	n/a	0	2401193	n/a	n/a	n/a	-	-
12307-77A	Blood ISTD blank	n/a	0	2549251	n/a	n/a	-	-	-
12307-78A	Blood ISTD blank	n/a	0	2242567	n/a	n/a	-	-	-
12307-76C	Std A1	0.0987	19679	2385455	0.00825	0.104	0.103	105%	5.12%
12307-77C	Std A1	0.0987	20767	2524119	0.00823	0.103	-	105%	4.86%
12307-78C	Std A1	0.0987	17993	2213878	0.00813	0.102	-	104%	3.67%
12307-76D	Std B1	0.267	52632	2369143	0.0222	0.267	0.267	100%	0.00615%
12307-77D	Std B1	0.267	55885	2491181	0.0224	0.270	-	101%	0.958%
12307-78D	Std B1	0.267	47302	2141561	0.0221	0.266	-	99.4%	-0.554%
12307-76E	Std A2	0.500	95098	2336737	0.0407	0.483	0.481	96.6%	-3.39%
12307-77E	Std A2	0.500	100776	2478539	0.0407	0.483	-	96.5%	-3.47%
12307-78E	Std A2	0.500	86099	2139232	0.0402	0.478	-	95.6%	-4.44%
12307-76F	Std B2	1.08	211638	2347792	0.0901	1.06	1.06	98.3%	-1.75%
12307-77F	Std B2	1.08	222909	2504261	0.0890	1.05	-	97.0%	-2.97%
12307-78F	Std B2	1.08	201097	2192174	0.0917	1.08	-	100%	-0.03%
12307-76G	Std A3	2.51	493023	2339302	0.211	2.47	2.52	98.4%	-1.550%
12307-77G	Std A3	2.51	531189	2456918	0.216	2.53	-	101%	0.99%
12307-78G	Std A3	2.51	471407	2168767	0.217	2.55	-	102%	1.53%
12307-76H	Std B3	5.36	1038513	2281832	0.455	5.33	5.39	99.4%	-0.601%
12307-77H	Std B3	5.36	1131632	2464067	0.459	5.38	-	100%	0.300%
12307-78H	Std B3	5.36	1020627	2189878	0.466	5.46	-	102%	1.79%

Table 8 (continued). Low Concentration Calibration Curve for Low Dose Groups C and D.

Sample I.D.	Standard I.D.	Conc. (μg/ml)	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (μg/ml)	Mean (μg/ml)	Accuracy (%)	Error (%)
12307-85-A	LQC	0.500	101692	2560188	0.0397	0.472	-	94.3%	-5.67%
12307-86-A	LQC	0.500	91467	2379344	0.0384	0.457	-	91.3%	-8.66%
12307-85-B	MQC	5.36	1187024	2584089	0.459	5.38	-	100%	0.323%
12307-86-B	MQC	5.36	1006382	2309839	0.436	5.10	-	95.2%	-4.84%

a	INTERCEPT	-0.00062549
b	SLOPE	0.085542
	r	0.9998

Table 9. High Concentration Calibration Curve for Low Dose Groups C and D.

Sample I.D.	Standard I.D.	Conc. (µg/ml)	Peak Area ETBE	Peak Area MTBE	Peak Area Ratio	Cal. Conc. (µg/ml)	Mean (µg/mL)	Accuracy (%)	Error (%)
12307-76H	Std B3	5.36	1038513	2281832	0.455	5.81	5.86	108%	8.46%
12307-77H	Std B3	5.36	1131632	2464067	0.459	5.85	-	109%	9.22%
12307-78H	Std B3	5.36	1020627	2189878	0.466	5.92	-	110%	10.5%
12307-76I	Std A4	9.91	1966434	2295658	0.857	9.77	10.0	98.6%	-1.394%
12307-77I	Std A4	9.91	2171596	2470927	0.879	10.0	-	101%	0.82%
12307-78I	Std A4	9.91	1940862	2161534	0.898	10.2	-	102.7%	2.72%
12307-76J	Std B4	27.0	5549321	2271644	2.44	25.4	25.6	94.1%	-5.88%
12307-77J	Std B4	27.0	5934902	2435181	2.44	25.4	-	93.9%	-6.09%
12307-78J	Std B4	27.0	5399118	2157220	2.50	26.0	-	96.3%	-3.69%
12307-76K	Std A5	50.2	10647916	2292558	4.64	47.1	47.9	93.9%	-6.13%
12307-77K	Std A5	50.2	11671433	2467353	4.73	48.0	-	95.6%	-4.45%
12307-78K	Std A5	50.2	10276072	2139796	4.80	48.7	-	97.0%	-3.03%
12307-76L	Std B5	109	22795863	2190902	10.4	104	106	95.2%	-4.84%
12307-77L	Std B5	109	25778388	2435123	10.6	106	-	96.8%	-3.20%
12307-78L	Std B5	109	23794507	2191879	10.9	108	-	99.2%	-0.766%
12307-76M	Std A6	254	58000722	2234294	26.0	257	260	101%	1.45%
12307-77M	Std A6	254	63467516	2444398	26.0	257	-	101%	1.47%
12307-78M	Std A6	254	58021039	2173056	26.7	265	-	104%	4.33%
12307-85-C	HQC	109	23890305	2317020	10.3	103	-	94.3%	-5.69%
12307-85-D	HQC	109	24163823	2385436	10.1	101	-	92.7%	-7.32%

a	INTERCEPT	-0.13447
b	SLOPE	0.10142
	r	0.999

Table 10. ETBE Blood Concentration Analysis for Groups C and D.

Sample I.D.	Time point	Peak Area		Peak Area Ratio	Cal. Conc. (μg/ml)	Sample weight (g)	Corrected Conc. (μg/ml)
		ETBE	MTBE				
CM 01	Predose	0	2708273	0	<LOQ	0.1534	<LOQ
	15 min	364965	2693222	0.136	1.59	0.1562	1.02
	30 min	138414	2603538	0.0532	0.629	0.1488	0.423
	1 hr	73836	2526019	0.0292	0.349	0.1595	0.219
	2 hr	24331	2593718	0.00938	0.117	0.1221	0.0958
	4 hr	7766	2587412	0.00300	<LOQ	0.1485	<LOQ
	8 hr	0	2482179	0.000	<LOQ	0.1669	<LOQ
	24 hr	0	2356970	0.000	<LOQ	0.1813	<LOQ
CM 02	Predose	0	2710000	0.000	<LOQ	0.1423	<LOQ
	15 min	378788	2671807	0.142	1.66	0.1344	1.24
	30 min	176491	2581415	0.0684	0.807	0.1602	0.503
	1 hr	47674	2557427	0.0186	0.225	0.1021	0.221
	2 hr	19821	2548846	0.00778	<LOQ	0.1181	<LOQ
	4 hr	6084	2576653	0.00236	<LOQ	0.1007	<LOQ
	8hr	0	2437790	0.000	<LOQ	0.1656	<LOQ
	24 hr	0	2397593	0.000	<LOQ	0.1735	<LOQ
CM 03	Predose	0	2586244	0.000	<LOQ	0.1533	<LOQ
	15 min	454277	2600777	0.175	2.05	0.1805	1.14
	30 min	104522	2516129	0.0415	0.493	0.0901	0.547
	1 hr	66995	2532186	0.0265	0.317	0.1171	0.270
	2 hr	17275	2535485	0.00681	<LOQ	0.1289	<LOQ
	4 hr	8494	2487809	0.00341	<LOQ	0.1402	<LOQ
	8 hr	0	2452134	0.0000	<LOQ	0.1507	<LOQ
	24 hr	0	2457582	0.000	<LOQ	0.1511	<LOQ

Table 10 (continued). ETBE Blood Concentration Analysis for Groups C and D.

Sample I.D.	Time point	Peak Area		Peak Area Ratio	Cal. Conc. (μg/ml)	Sample weight (g)	Corrected Conc. (μg/ml)
		ETBE	MTBE				
CM 08	Predose	0	2678144	0.000	<LOQ	0.1161	<LOQ
	15 min	270768	2683076	0.101	1.19	0.1424	0.834
	30 min	117962	2601598	0.045	0.537	0.1232	0.436
	1 hr	63368	2524024	0.0251	0.301	0.1581	0.190
	2 hr	25761	2479064	0.0104	0.129	0.161	0.0800
	4 hr	4883	2517248	0.0019	<LOQ	0.1166	<LOQ
	8 hr	0	2439774	0.0000	<LOQ	0.1552	<LOQ
	24 hr	0	2327966	0.000	<LOQ	0.2594	<LOQ
DF 01	Predose	0	2589962	0.000	<LOQ	0.1464	<LOQ
	15 min	345332	2480756	0.139	1.63	0.1302	1.26
	30 min	197482	2382581	0.083	0.976	0.1496	0.653
	1 hr	115407	2521028	0.046	0.542	0.1694	0.320
	2 hr	27345	2337992	0.0117	0.144	0.1198	0.120
	4 hr	8001	2453513	0.0033	<LOQ	0.1259	<LOQ
	8 hr	2106	2535169	0.00083	<LOQ	0.1418	<LOQ
	24 hr	0	2367633	0.000	<LOQ	0.1435	<LOQ
DF 02	Predose	0	2510725	0.000	<LOQ	0.1551	<LOQ
	15 min	297826	2381214	0.125	1.47	0.1405	1.05
	30 min	196106	2398964	0.0817	0.963	0.1737	0.554
	1 hr	59294	2427433	0.0244	0.293	0.1131	0.259
	2 hr	19440	2478320	0.00784	<LOQ	0.1038	<LOQ
	4 hr	6952	2464808	0.00282	<LOQ	0.0832	<LOQ
	8 hr	0	2407469	0.00000	<LOQ	0.1384	<LOQ
	24 hr	0	2296209	0.000	<LOQ	0.1636	<LOQ

Table 10 (continued). ETBE Blood Concentration Analysis for Groups C and D.

Sample I.D.	Time point	Peak Area		Peak Area Ratio	Cal. Conc. (μg/ml)	Sample weight (g)	Corrected Conc. (μg/ml)
		ETBE	MTBE				
DF 04	Predose	0	2445110	0.000	<LOQ	0.1201	<LOQ
	15 min	304137	2407123	0.126	1.48	0.1473	1.01
	30 min	97237	2390413	0.0407	0.483	0.1049	0.460
	1 hr	58151	2356125	0.0247	0.296	0.1206	0.245
	2 hr	26052	2376773	0.0110	0.135	0.1346	0.101
	4 hr	11934	2406270	0.00496	<LOQ	0.0914	<LOQ
	8 hr	2192	2434416	0.00090	<LOQ	0.1121	<LOQ
	24 hr	0	2184403	0.000	<LOQ	0.1585	<LOQ
DF 05	Predose	0	2617559	0.000	<LOQ	0.1180	<LOQ
	15 min	65880	2489293	0.0265	0.317	0.1124	0.282
	30 min	39043	2483004	0.0157	0.191	0.0621	0.308
	1 hr	80375	2353090	0.0342	0.407	0.1514	0.269
	2 hr	10540	2351428	0.00448	<LOQ	0.0668	<LOQ
	4 hr	4837	2516128	0.00192	<LOQ	0.1207	<LOQ
	8 hr	0	2450106	0.00000	<LOQ	0.1513	<LOQ
	24 hr	0	2257636	0.000	<LOQ	0.1173	<LOQ