Metabolism and Hemoglobin Adduct Formation of Acrylamide in Humans

Timothy R. Fennell[1], Susan C.J. Sumner[2], Rodney W. Snyder[1], Jason Burgess[1], Rebecca Spicer[3], William E. Bridson[3], and Marvin A. Friedman[4]

[1]Research Triangle Institute, Research Triangle Park, NC.

[2]Paradigm Genetics, Inc., Research Triangle Park, NC.

[3]Covance Clinical Research Unit, Inc, Madison WI

[4]UMDNJ, Newark, NJ.

Address Correspondence to
Timothy R. Fennell
Research Triangle Institute
P.O. Box 12190
3040 Cornwallis Road,
Research Triangle Park
NC 27709
(919) 485-2781
Fennell@RTI.org

Running Title: Metabolism and Adducts of Acrylamide in Humans

Abstract

Acrylamide, used in the manufacture of polyacrylamide and grouting agents, is produced during the cooking of foods. Workplace exposure to acrylamide can occur through the dermal and inhalation routes. The objectives of this study were to evaluate the metabolism of acrylamide in humans following oral administration, to compare hemoglobin adduct formation on oral and dermal administration, to measure hormone levels, and to monitor the safety of acrylamide in people exposed under controlled conditions. Prior to conducting exposures in humans, a low-dose study was conducted in rats administered 3 mg/kg 1,2,3-$^{13}C_3$ acrylamide by gavage. The study protocol was reviewed and approved by Institute Review Boards both at RTI which performed the sample analysis, and the clinical research center conducting the study. 1,2,3-$^{13}C_3$ Acrylamide (AM) was administered in an aqueous solution orally (single dose of 0.5, 1.0, or 3.0 mg/kg) or dermally (3 daily doses of 3.0 mg/kg) to sterile male volunteers. Urine samples (3 mg/kg oral dose) were analyzed for AM metabolites using ^{13}C NMR spectroscopy. Approximately 86 % of the urinary metabolites were derived from GSH conjugation, and excreted as *N*-acetyl-*S*-(3-amino-3-oxopropyl)cysteine and its *S*-oxide. Glycidamide, glyceramide, and low levels of *N*-acetyl-*S*-(3-amino-2-hydroxy-3-oxopropyl)cysteine were detected in urine. On oral administration, a linear dose response was observed for *N*-(2-carbamoylethyl)valine (AAVal) and *N*-(2-carbamoyl-2-hydroxyethyl)valine (GAVal) in hemoglobin. Dermal administration resulted in lower levels of AAVal and GAVal. This study indicated that humans metabolize acrylamide via glycidamide to a lesser extent than rodents, and dermal uptake was approximately 5% of that observed with oral uptake.

Acrylamide is used in the manufacture of water- soluble polymers (European Union, 2002). These polymers are then used for wastewater and sludge treatment, paper manufacture, soil stabilization, mining and many other uses (European Union, 2002). Acrylamide is also a chemical intermediate in the manufacture of other monomeric chemicals and used for grouting and preparation of laboratory gels for electrophoresis. Human exposure through these applications is very small (European Union, 2002). Previously, it has been postulated that dermal absorption was the major route of human exposure to acrylamide (European Union, 2002). The magnitude of this dermal absorption is highly relevant as one of the uses of acrylamide based polymers is in the formulation of skin creams (European Union, 2002). Estimates of dermal absorption based on in vitro and rodent studies have ranged from 3% to 100 (European Union, 2002). Recently, exposure to acrylamide in a variety of cooked foods has been described (Rosen and Hellenas, 2002; Tareke *et al.*, 2002). Human exposure via this route is substantial, with estimated exposures as high as 70 µg per day proposed (Tareke *et al.*, 2002).

Acrylamide is metabolized by two main pathways: glutathione conjugation (Dixit *et al.*, 1982; Edwards, 1975; Hashimoto and Aldridge, 1970; Miller *et al.*, 1982; Sumner *et al.*, 1992), and oxidation to glycidamide (Calleman *et al.*, 1990; Sumner *et al.*, 1992). The metabolism of acrylamide in vivo results in the formation of a number of metabolites. These metabolism of acrylamide in vivo has been investigated by administration of 1,2,3-$^{13}C_3$ acrylamide to rodents, with the detection and quantitation of metabolites by ^{13}C NMR spectroscopy (Sumner *et al.*, 1999; Sumner *et al.*, 1992; Sumner *et al.*, 2003). The

oxidation reaction to glycidamide is catalyzed by cytochrome P450 2E1 in rodents (Sumner *et al.*, 1999). Both acrylamide and glycidamide react with hemoglobin producing a stable adduct which can be measured as an indicator of exposure. Correlations have been made with hemoglobin adducts and neurotoxicity, but there has been no systematic standardization of hemoglobin adducts with dose. Glycidamide is weakly mutagenic in the Salmonella test (Hashimoto and Tanii, 1985). It can react with DNA in vitro to produce a guanine derivative N7-(2-carbamoyl-2-hydroxyethyl)guanine (Gamboa da Costa *et al.*, 2003; Segerback *et al.*, 1995). In vivo, administration of acrylamide to rats and mice produces low levels of N7-(2-carbamoyl-2-hydroxyethyl)guanine (Gamboa da Costa *et al.*, 2003; Segerback *et al.*, 1995).

Acrylamide induces a characteristic peripheral neurotoxicity in animals and man (Spencer and Schaumburg, 1974a, b, 1975). This toxicity manifests itself as a distal to proximal loss of nerve function and dying back of cells. Acrylamide also effects rodent reproduction, namely smaller litter size. At elevated acrylamide doses other reproductive effects are seen, likely as a consequence of the neurotoxicity.

Acrylamide is carcinogenic in drinking water studies in laboratory rats (Friedman *et al.*, 1995; Johnson *et al.*, 1986). In male rats, it induces tumors of the tunica vaginalis testes and the thyroid, while in females, it induces mammary fibroadenomas and thyroid tumors (Friedman *et al.*, 1995). The mechanism for this tumorigenicity is unclear, although interaction with the dopamine receptor has been postulated as well as genotoxicity (Tyl and Friedman, 2003). If the mechanism were genotoxicity, then conversion of

acrylamide to glycidamide is directly proportional to carcinogenic activity. Understanding the mechanism of tumorigenicity is important, since conventional risk assessment techniques place the order of magnitude of the risk at approximately 10^{-3} with exposures of 70 μg/ day.

The relative contributions of acrylamide and glycidamide in the mode of action of acrylamide are the subject of debate and current research. Understanding the conversion of acrylamide to glycidamide and differences that may occur between species, exposure route, and dose are important considerations in assessing the risk of the possible effects of acrylamide exposures in the diet, in consumer products, and in the workplace.

The primary objectives of this study were to evaluate the conversion of acrylamide to glycidamide in people exposed to acrylamide, and to evaluate the extent of uptake following dermal administration. This was conducted by administering a low dose of ^{13}C labeled acrylamide to volunteers orally or dermally, and by measuring urinary metabolites or hemoglobin adducts derived from the glycidamide pathway and comparing them to metabolites and hemoglobin adducts derived from acrylamide directly. More specifically, we intended to evaluate urinary metabolites and hemoglobin adducts, and to measure hormone levels after exposure to a known dose of acrylamide. As a secondary and no less important objective, we intended to monitor the safety of acrylamide in people exposed under controlled conditions.

MATERIALS AND METHODS

Chemicals

1,2,3-$^{13}C_3$ Acrylamide (CLM-813, lot number 11085) was obtained from Cambridge Isotopes Limited. Identity and purity were confirmed by ^{1}H and ^{13}C NMR spectroscopy. Glycidamide was synthesized by H_2O_2 oxidation of acrylonitrile (Payne and Williams, 1961), and stored at –20 ^{o}C. *N*-(2-Carbamoylethyl)valine (AAVal), *N*-(2-carbamoylethyl)valine -$^{13}C_5$ (AAVal-$^{13}C_5$), *N*-(2-Carbamoyl-2-hydroxyethyl)valine (GAVal) and *N*-(2-carbamoyl-2-hydroxyethyl)valine -$^{13}C_5$ (GAVal-$^{13}C_5$) were synthesized and purified as described previously by Fennell et al. (2003). The AAVal phenylthiohydantoin derivative (AAVal PTH), the corresponding ^{13}C labeled standard AAVal-$^{13}C_5$, GAVal PTH and ^{13}C GAVal PTH standards were prepared as described by Fennell et al. (2003). AAVal-leu-anilide was obtained from Bachem Bioscience Inc. (King of Prussia, PA).

Human Study

Institutional Review Board Approval.

This study was conducted in accordance with the CFRs governing Protection of Human Subjects (21 CFR 50), IRB (21 CFR 56), retention of data (21 CFR 312) as applicable and consistent with the Declaration of Helsinki. The administration of ^{13}C acrylamide to the study subjects was conducted at Covance Clinical Laboratories. Institutional Review Board approval of the protocol and the consent form was obtained at Covance Clinical

Laboratories. Institutional Review Board approval was also obtained at RTI, where the analysis of the samples was conducted.

Acrylamide Exposure

Twenty-four volunteers participated in this study. They were all male Caucasians (with the exception of one Native American) weighing between 71 and 101 kg and between 26 and 68 years of age. All volunteers were aspermic and had not used tobacco products for the past 6 months. They passed a drug screen and had not taken prescription drugs or caffeinated products over the previous 3 days. Each experimental group consisted of 6 individuals of which 1 was a placebo. There were 2 phases to this study: an oral phase and a dermal phase.

A comprehensive physical exam was conducted on each individual upon check-in to the clinic, at 24 hours after compound administration, and 7 days after check out. This exam included medical history, demographic data, neurological examination, 12-lead ECG, vital signs (including oral temperature, respiratory rate and automated seated pulse and blood pressure), clinical laboratory evaluation (including clinical chemistry, hematology, and complete urinalysis). Each individual also had screens for HIV, hepatitis, and selected drugs of abuse, and provided a semen sample to confirm aspermia.

In the oral phase, three groups of six people were administered 0.5, 1.0 or 3.0 mg/kg $^{13}C_3$ acrylamide. Individuals were presented with test substance at approximately 9:00 in the morning to initiate the study. Urine was collected at 0-2, 2-4, 4-8, 8-16 and 16-24 hours. Blood was collected immediately prior to compound administration and 24 hours later.

Hormone blood samples (testosterone, LH, and prolactin levels) were drawn immediately prior to compound administration, 24 hours later and on the follow up visit on day 8.

In the dermal phase, a 50% solution of $^{13}C_3$ acrylamide was applied directly on the skin to a clean, dry, marked off, 24 cm^2 (3 cm x 8 cm) area on the volar forearm. After applying the appropriate amount of material, the liquid was evaporated to dryness using a commercial hair dryer and covered with a sterile gauze pad. Dermal applications alternated between left and right arms, starting with the subject's dominant arm. Removal of dermal application consisted of washing with 1000 mL of water and an appropriate number of additional pieces of gauze. Blood was collected immediately prior to compound administration and 24, 48, 72 and 96 hours later (immediately prior to administration of the second and third doses, after gauze removal and prior to leaving the clinic). Hormone blood samples were drawn immediately prior to compound administration, after 24 hours and on day 5 when the volunteers left the clinic.

Urine and blood samples were obtained from sterile male volunteers who were exposed to 0.5 (low), 1.0 (mid) or 3.0 mg/kg (high) 1,2,3-$^{13}C_3$ acrylamide by a single oral dose, or 3 x 3.0 mg/kg 1,2,3-$^{13}C_3$ acrylamide administered dermally. Each exposure group contained 6 volunteers. Of the 6 volunteers in each group, 5 received the designated amount of acrylamide, and one received no acrylamide. For the groups administered acrylamide orally, a blood sample was collected immediately before acrylamide administration, and a second sample was collected at 24 h following the administration. For the group administered acrylamide dermally, blood samples were collected immediately before administration. Acrylamide was applied to the skin for 24 hours on

one forearm, and a blood sample was collected at 24 hours following the first administration. This was repeated on the following two days, with acrylamide applied on alternating arms, for a total of three dermal doses of acrylamide at 24 h intervals. A total of 5 blood samples was collected from each volunteer administered acrylamide dermally, on day 1 (prior to the first dose), day 2, day 3, day 4, and day 5. The sample obtained on day 5 was at 24 hours following removal of the occlusion at the site of application.

Blood samples were processed for storage and shipment at the Covance Clinical Research Unit as follows: the blood samples were centrifuged and plasma was removed, an equal volume of isotonic saline was added to the red cell pellets, the remaining washed red blood cells were washed by centrifugation with isotonic saline. The washing procedure was repeated a total of three times. The samples were stored frozen until shipped to RTI.

Urine samples were collected at intervals of 0-2, 2-4, 4-8, 8-16 and 16-24 hr following administration of acrylamide. The volume of urine in each sample was recorded, and sample aliquots were transferred to sample vials for storage.

Samples of urine and washed red blood cells were shipped to RTI from Covance Clinical Laboratories on dry ice, and were stored at –20 ^{o}C until processed for analysis.

Acrylamide Analysis

The dose solutions, dermal dam solutions, and wash solutions provided by the Covance Clinical Research Unit were analyzed for the concentration of acrylamide using a

reversed phase HPLC method. A calibration curve was prepared with acrylamide over a concentration range of 5–200 µg/mL. Analysis was conducted with a Waters HPLC system consisting of two 515 pumps, a 717 autosampler, and an Applied Biosystems 759A UV detector. Data was recorded with a Waters Millennium data system. Chromatography was conducted on a Beckman Ultrasphere ODS column (4.5 mm x 25 cm) eluted at a flow rate of 1 mL/min with 100% water. Elution was monitored by measuring UV absorbance at 195 nm. The measurements of acrylamide concentration in dose solutions were used to confirm the amount of acrylamide administered in oral dose solutions, and in the dose solution used for dermal applications. The amount of acrylamide recovered from dermal application in both the dermal dam, which was used to outline the skin area for application, and the skin washings following removal of the gauze covering the application site were also measured. The total amount of acrylamide recovered following dermal application was used to calculate the maximum amount of acrylamide that was available for absorption (total dose applied – amount recovered in the dermal dam and washing solutions).

Urinary Metabolite Analysis

Metabolites of 1,2,3-$^{13}C_3$ acrylamide in urine were analyzed from the group of volunteers exposed to 3 mg/kg AM orally by ^{13}C NMR spectroscopy, essentially as described by Sumner et al. (1991). No analyses were conducted on the samples from the volunteers administered 0.5, or 1.0 mg/kg orally , or those administered acrylamide dermally, due to the sensitivity of the methodology. Samples were prepared for all urine samples from subject 016 (3 mg/kg). Composite urine samples were prepared for analysis of each

subject administered 3.0 mg/kg orally. Aliquots of each sample were combined in the appropriate proportion to make a total of 10 mL. Samples were concentrated by mixing 5.0 mL of urine with 10 mL of methanol, centrifuging at 5000 g for 10 min. The supernatant was reduced in volume under a stream of nitrogen in a preweighed tube to approximately 300 – 600 µL. The weight of the concentrated urine was recorded. Water was added to make a total volume of 600 µL, and a solution of dioxane in D2O was added (200 µL).

NMR Analysis of Urinary Metabolites

Initial analysis of urine samples was conducted on a Bruker 500 MHz NMR spectrometer operating a 125 MHz for ^{13}C. Quantitative analysis of metabolites was conducted on a Varian 500 MHz NMR spectrometer operating at 125 MHz. Samples were prepared by adding D_2O, or D_2O containing dioxane at a known concentration (200 µl) to an aliquot of a urine sample, a composite urine sample, or a concentrated composite urine sample (800 µl). Carbon-Carbon connectivity was established using two-dimensional incredible natural abundance double quantum transfer spectra (INADEQUATE) using the Varian pulse sequence.

LC-MS/MS Analysis of Hemoglobin Adducts

Initial LC-MS/MS analyses of hemoglobin adducts were conducted using an API-3000 LC/MS/MS system with a heated nebuliser source coupled to an Agilent 1100 HPLC system. Data was processed using Analyst version 1.1 software. The HPLC system was composed of a binary HPLC pump, a refrigerated vial/96 well plate autosampler, a photodiodearray detector and a column heater. Direct infusion experiments were

conducted with an infusion rate of 10 µl/min, source temperature of 100°C, an ionspray voltage of 5000 V, curtain gas flow rate of 8, nebuliser gas flow rate of 5, and collision gas flow rate of 4. Nitrogen was used as the curtain gas, nebuliser gas, and collision gas.

Final quantitative analysis of all of the human globin samples was conducted using an API-4000 LC/MS/MS system with a heated nebuliser source coupled to an Agilent 1100 HPLC system. Data was processed using Analyst version 1.3 software. The HPLC system was composed of a binary HPLC pump, a refrigerated vial/96 well plate autosampler, a photodiodearray detector and a column heater.

Hemoglobin Adduct Analysis

N-(2-carbamoylethyl)valine (AAVal) and *N*-(2-carbamoyl-2-hydroxyethyl)valine (GAVal), formed by reaction of acrylamide and glycidamide, respectively, with the N-terminal valine residue in hemoglobin, were measured by an LC-MS/MS method. Globin was isolated from washed red cells (Mowrer et al., 1986). Samples were derivatized with phenylisothiocyanate in formamide to form adduct phenylthiohydantoin derivatives in a manner analogous to the modified Edman degradation (Tornqvist et al., 1986; Bergmark, 1997; Perez et al., 1999). Internal standards, AAValPTH-$^{13}C_5$ and GAVal PTH-$^{13}C_5$ were added, and the samples were extracted using a Waters Oasis HLB 3 cc (60 mg) extraction cartridge (Milford, MA). The samples were eluted with methanol, dried, and reconstituted in 100µl of 50:50 MeOH:H2O (containing 0.1% formic acid). Analysis was conducted using an HP 1100 HPLC system interfaced to a PE Sciex API 4000 LC-MS with a Turboionspray interface. Chromatography was conducted on a Phenomenex Luna Phenyl-Hexyl Column (50 mm × 2 mm, 3 µm) eluted with 0.1% acetic acid in

water and methanol at a flow rate of 350 µl/min, with a gradient of 45-55% methanol in 2.1 min. The elution of adducts was monitored by Multiple Reaction Monitoring (MRM) in the negative ion mode for the following ions:

Natural Abundance Analytes

AAVal-PTH: m/z 304 → 233 ($M-H^- \rightarrow M-H^- - CH_2-CH_2-CONH_2$)
GAVal-PTH: m/z 320 → 233 ($M-H^- \rightarrow M-H^- - CH_2-CHOH-CONH_2$)

Adducts Derived from 1,2,3-^{13}C Acrylamide

$^{13}C_3$-AAVal-PTH: m/z 307 → 233 ($M-H^- \rightarrow M-H^- - {}^{13}CH_2-{}^{13}CH_2-{}^{13}CONH_2$)
$^{13}C_3$-GAVal-PTH: m/z 323 → 233 ($M-H^- \rightarrow M-H^- - {}^{13}CH_2-{}^{13}CHOH-{}^{13}CONH_2$)

Internal Standards

AAVal-PTH-$^{13}C_5$: m/z 309 → 238 ($M-H^- \rightarrow M-H^- - CH_2-CH_2-CONH_2$)
GAVal-PTH-$^{13}C_5$: m/z 325 → 238 ($M-H^- \rightarrow M-H^- - CH_2-CHOH-CONH_2$)

Quantitation of AAVal was conducted using the ratio of analyte to internal standard, with a calibration curve generated using AAVal-leu-anilide. Quantitation of GAVal was conducted using the ratio of analyte to internal standard.

Rat Study

Prior to conducting the study in humans, male Fischer 344 rats were administered [1,2,3-^{13}C] acrylamide at a dose of 3 mg/kg by gavage in distilled water. This component of the study was conducted in parallel with administration of 50 mg [1,2,3-^{13}C] AM/kg to rats by gavage described in detail previously (Sumner *et al.*, 2003). The rats were placed in metabolism cages for 24 hours following dosing for collection of urine. After 24 hours, the rats were euthanized, and blood samples were collected by cardiac puncture. Washed

red blood cells were prepared by centrifugation, and globin was isolated by the method of Mowrer et al. (1986). Globin samples were analyzed by LC-MS/MS for AAVal, $^{13}C_3$-AAVal, GAVal and $^{13}C_3$-GAVal as described in (Sumner *et al.*, 2003). Urine samples were analyzed by ^{13}C NMR spectroscopy for metabolites of acrylamide (Sumner *et al.*, 2003). For quantitation of metabolites, samples were concentrated by addition of methanol to a 3 mL sample of urine, centrifugation, and evaporation under a stream of nitrogen. D_2O and dioxane were added.

In Vitro Reaction Rate Constants

Washed red cells from male F-344 rats, or from human blood were lysed with an equal volume of distilled water, and incubated with acrylamide or glycidamide at a concentration of 100 mM. Samples were removed at 0, 2, 5, 10, 15, 30 and 60 minutes, and the reactions were terminated by placing the tubes on ice and immediately adding 3 ml of 50 mM HCl in isopropanol. Globin was then isolated as described by Mowrer et al. (1986).

RESULTS

Rat Study

Analysis of Urinary Metabolites

Prior to conducting the administration of acrylamide in humans, the ability of the methods used to detect urinary metabolites and hemoglobin adducts was evaluated with a single dose of 3 mg/kg $^{13}C_3$ acrylamide administered by gavage in rats. The analysis of urinary metabolites was conducted as described previously (Sumner *et al.*, 2003). The

metabolites could be detected in urine samples. However, for quantitation of urinary metabolites, concentration of urine samples was required. The results of the quantitative analysis are shown in Table 1. Conjugation with GSH to form metabolite 1 accounted for 59% of the total metabolites. Metabolites 2,2' and 3,3', produced by GSH conjugation of glycidamide accounted for 25 and 16% of the urinary metabolites, respectively. Glycidamide was detected in urine samples from 2 of the rats prior to concentration, but was below the limit of quantitation in the concentrated samples. Metabolism via oxidation to glycidamide (2,2'-5, Table 1) accounted for approximately 41% of the urinary metabolites.

Analysis of the hemoglobin adducts from rats administered 3 mg/kg $^{13}C_3$ acrylamide is shown in Table 2. $^{13}C_3$-AAVal and $^{13}C_3$-GAVal were both increased by administration of acrylamide. The ratio of $^{13}C_3$-GAVal: $^{13}C_3$-AAVal was 0.84 ± 0.07.

Human Study

Clinical Findings

No adverse events were reported in the oral phase of the study. With the dermal administration, one individual appeared to have a mild contact dermatitis which is a known response to acrylamide and was part of the informed consent. This individual was seen by a dermatologist who performed a skin biopsy which was consistent with a delayed hypersensitivity reaction. The skin reaction resolved 39 days after the first application of acrylamide and 23 days after the reaction was manifested. Thus, the acrylamide caused a delayed hypersensitivity reaction when placed on the skin, a reaction

that took more than three weeks to resolve completely. An increase in the liver enzyme alanine aminotransferase (ALT) was observed above the upper limit of the reference range (normal) in 4 of the 5 individuals who received acrylamide by dermal application, one of whom had a preexisting elevation of this enzyme prior to receiving the dose (not shown). One individual who received dermal acrylamide also had an elevation in serum aspartate transaminase (not shown). The elevated liver function tests returned to within or near the reference range at subsequent determinations and were judged to be not clinically significant. When administered to the skin, acrylamide may cause a moderate increase in ALT levels. Serum prolactin, testosterone, and luteinizing hormone did not differ between subjects who received acrylamide and those who received placebo (not shown). All blood parameters and hormone levels were within the normal range. There were no neurological or cardiovascular findings among these individuals.

Dose Administered

For oral administration, acrylamide was administered in a constant volume of 200 mL to give the appropriate dose of 0.5, 1.0, or 3.0 mg/kg. The actual amount of acrylamide administered was verified by analysis of aliquots of the dose solution with calculation of the amount of acrylamide in 200 mL for each subject and the amount of acrylamide administered per kg body weight. One individual from each dose group did not receive acrylamide and this was verified by analysis of the administered dose. The mean doses calculated were 0.43 ± 0.01, 0.89 ± 0.01, and 2.75 ± 0.03 mg/kg, and were 86, 89, and 92% of the nominal dose at 0.5, 1.0, and 3.0 mg/kg, respectively.

With dermal administration, the appropriate volume of a 50% solution of acrylamide was applied to the skin. The dose applied each day based on HPLC analysis was calculated as 2.48 mg/kg, and was 83% of the nominal dose.

After drying the acrylamide solution, the tape which had been used to demark the area of application was removed and placed in a vial containing 20 mL of water. The water (dermal dam solution) was analyzed for acrylamide by HPLC and was found to contain between 9 and 54 mg of acrylamide (Table 3). The site of application was covered with gauze for 24 h at which time the gauze was removed and the area was washed with 1000 mL of water. The recovered wash water was analyzed by HPLC for acrylamide. The amount of acrylamide recovered ranged between 62 and 154 mg. The total acrylamide per day recovered in dermal dam and wash solutions ranged between 85 and 190 mg and accounted for 36–86% of the applied acrylamide. While considerable variability was observed in the recovery of acrylamide, the mean recovery in dam and washes ranged between 65 and 71% of the total acrylamide applied. The acrylamide recovered in the dermal dam and wash solutions would not be available systemically. The amount of dose that could have been taken up was calculated as:

$$\text{Absorbed dose} = \frac{\text{Total Dose Administered} - (\text{Dermal Dam} + \text{Wash})}{\text{Body Weight}} \qquad (1)$$

The mean absorbed dose ranged from 0.73 to 0.86 mg/kg/day (Table 3). The cumulative absorbed dose (over the three days of administration) was 2.35 ± 0.50 mg/kg (Table 3). This value includes material that could be retained at the site of application, and probably represents the maximum that could be absorbed rather than the actual amount absorbed.

Analysis of Urinary Metabolites

Urine samples from a single individual administered 3 mg/kg orally were evaluated qualitatively by ^{13}C NMR spectroscopy prior to quantitative analysis. A sample of each time point collection was analyzed from subject 15. The majority of the metabolite signals were found in the 2–4, 4–8, and 8–16 h samples. No signals indicative of the presence of $^{13}C_3$ acrylamide or its metabolites were found in the predose urine.

To achieve the necessary signal to noise ratio for quantitative analysis, and to collect data that were similar to those obtained from studies in rodents, aliquots of the urine samples from individuals were combined in the appropriate proportions to make a 24-h composite sample, which was then concentrated. Dioxane was added as an internal standard for quantitation of the metabolites.

The ^{13}C NMR spectrum of a composite urine sample obtained from a volunteer who did not receive acrylamide is shown in Figure 2. A number of signals from endogenous metabolites, including urea at 162.5 ppm can be seen. In the samples of urine from volunteers administered 3.0 mg/kg 1,2,3-$^{13}C_3$ acrylamide, there are additional signals arising from acrylamide and its metabolites (Figure 3). These signals show characteristic multiplets arising from coupling with adjacent labeled carbon atoms. Many but not all of the signals present in urine samples from rats and mice administered 1,2,3-$^{13}C_3$ acrylamide were present in the human urine samples. Some additional signals that had not been previously observed were also present. These signals are presented in Table 4. The nomenclature used previously (Sumner *et al.*, 1992; Sumner *et al.*, 1997; Sumner *et al.*, 2003) is also used to describe the metabolites in this report. The major metabolites present in all of the composite urine samples were derived from direct glutathione

conjugation of acrylamide (Sumner *et al.*, 1992; Sumner *et al.*, 1997; Sumner *et al.*, 2003).

The major metabolites, 1 and 1', corresponding to *N*-acetyl-*S*-(3-amino-3-oxopropyl)cysteine and *S*-(3-amino-3-oxopropyl)cysteine, showed signals at 27 ppm (doublet), 34.7 ppm (2 doublets of doublets, Figure 3), and at 177 ppm (2 doublets, Figure 3). An additional set of signals has been tentatively assigned as 1'' at 27.8 ppm. Signals associated with glycidamide, metabolite 4, were observed at 46.9 ppm (4a, Figure 3), and at 48.5 ppm (4b). Signals from the hydrolysis product of glycidamide (2,3-dihydroxypropionamide) were observed at 62.99 ppm (5a, doublet), at 72 ppm (5,5'b, doublet of doublets, Figure 3), and at 175.5 ppm (5,5'c, doublet). Low intensity signals that may be due to metabolite 2 (*N*-acetyl-*S*-(3-amino-2-hydroxy-3-oxopropyl)cysteine) were observed at 35.8 ppm and at 70.2 ppm. Signals that are associated with metabolite 3 (*N*-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)cysteine) were not observed. A metabolite that had not been previously observed in rats and mice gave a signal at 46.47 ppm (doublet, J = 37 Hz). An INADEQUATE spectrum was used to establish the carbon-carbon connectivity of the main signals of metabolite 1, glycdamide and glyceramide. In addition, the connectivity of the doublet at 46.47 ppm was established with a complex doublet of doublets at 27.60 ppm, and a doublet at 175.4 ppm. These signals have been assigned to the labeled carbon atoms of *N*-acetyl-*S*-(3-amino-3-oxopropyl)cysteine-S-oxide, based on the comparison with a synthesized standard (to be reported in an additional publication).

Quantitation of the metabolites present was conducted using the integral of the metabolite signals and dioxane added as internal standard. Spectra for quantitation were acquired

with decoupling only during acquisition to ensure that nuclear Overhauser enhancement was minimized so that accurate quantitation could be conducted. This resulted in the decrease in intensity of some of the low intensity signals to the point were quantitation was not readily possible, e.g. with metabolite 2. Although signals for acrylamide were readily apparent, these were not quantitated because of the long relaxation time for the signals of acrylamide. A summary of the quantitative information for urinary metabolites is presented in Table 5. Approximately 34% of the administered dose of acrylamide was recovered in the total urinary metabolites within 24 h of administration. Metabolite 1 accounted for approximately 72% of the metabolites excreted. The sulfoxide derived from metabolite 1 accounted for approximately 14% of the metabolites measured. Metabolites that are known to be derived from glycidamide (4 and 5) represented approximately 14% of the metabolites.

Analysis of Hemoglobin Adducts

Analysis of AAVal and GAVal was set up and validated as described previously (Fennell et al., 2003). A standard curve was developed with AAVal-leu-anilide standard and $^{13}C_5$-AAVal PTH. GAVal was measured based on the ratio of analyte to added $^{13}C_5$ GAVal PTH. In each of the samples obtained prior to administration of 1,2,3-$^{13}C_3$ acrylamide, measureable adduct backgrounds for AAVal and GAVal were detected.

Typical chromatograms for AAVal and GAVal from a volunteer administered $^{13}C_3$ acrylamide orally (0.5 mg/kg) are shown in Figure 4 (before administration) and Figure 5 (following administration). The administration of 1,2,3-^{13}C acrylamide resulted in an

increase in the peak height and area for $^{13}C_3$-AAVal and $^{13}C_3$-GAVal in the Figure 5 B and E. The mean values for hemoglobin adduct levels from the various groups administered acrylamide orally are presented in Table 6. AAVal, GAVal, $^{13}C_3$-AAVal and $^{13}C_3$-GAVal were measured for each individual prior to exposure to $^{13}C_3$ acrylamide, and at 24 hours following administration. The majority of the individual values (not shown) for AAVal prior to exposure were in the range of 40 – 200 fmol/mg globin. Most of the values for AAVal measured before and after exposure were similar. One exception to this was noted. One volunteer had high levels of AAVal in the first sample (986 fmol/mg), which dropped in the second sample (43 fmol/mg). The reason for this discrepancy can not be explained, and the value for the pre exposure sample has been excluded from calculations of statistical parameters. Prior to administration of acrylamide, the levels of GAVal were in the range of 16 – 67 fmol/mg globin and peaks associated with $^{13}C_3$-AAVal and $^{13}C_3$-GAVal were not detected. Following administration of acrylamide, $^{13}C_3$-AAVal and $^{13}C_3$-GAVal adduct levels increased in 5 out of the 6 members of each group. The subjects who received no 1,2,3-$^{13}C_3$ acrylamide were readily identified by a lack of detectable $^{13}C_3$-AAVal and $^{13}C_3$-GAVal.

On oral administration of 1,2,3-$^{13}C_3$ acrylamide, levels of $^{13}C_3$-AAVal increased in a dose-dependent manner (Table 6). A plot of $^{13}C_3$-AAVal vs. the nominal dose administered was linear (Figure 6). Similarly, on oral administration of 1,2,3-$^{13}C_3$ acrylamide, levels of $^{13}C_3$-GAVal increased in a dose-dependent manner (Table 6). A plot of $^{13}C_3$-GAVal vs. the nominal dose administered was also linear (Figure 6). The levels of $^{13}C_3$-GAVal formed were considerably lower than those of $^{13}C_3$-AAVal, with a ratio of $^{13}C_3$-GAVal: $^{13}C_3$-AAVal of ranging from 0.36 – 0.44 (Table 6).

Following dermal administration of acrylamide (days 1, 2, and 3), both AAVal and GAVal increased in a linear manner after each dose, on days 2, 3, and 4 (Table 6). Little change was noted between days 4 and 5 when no administration took place. A graph of $^{13}C_3$-AAVal and $^{13}C_3$-GAVal vs. the cumulative nominal dose administered produced a straight line (Figure 7). However, the initial point for both $^{13}C_3$-AAVal and $^{13}C_3$-GAVal were below the line in each case. The ratio of $^{13}C_3$-GAVal:$^{13}C_3$-AAVal ranged from 0.48 – 0.68.

To enable the comparison of dose groups, the data for hemoglobin adducts have been normalized by the nominal dose of acrylamide administered (Table 7), and by the actual dose estimated from analysis of acrylamide administered and recovered (Table 8). Comparison of the hemoglobin adducts formed in humans on oral administration with those formed in rats on gavage administration is presented in Table 9. Adduct levels normalized for dose are approximately 3-fold higher in humans for AAVal and 1.7 fold higher for GAVal in humans. The ratio of GAVal:AAVal in humans on oral administration was similar to that previously reported (Fennell *et al.*, 2003) for rats administered acrylamide by gavage at 50 mg/kg (Table 9). On dermal administration in humans, the ratio of GAVal:AAVal was slightly increased compared with oral administration (0.57 vs 0.39), the magnitude of the increase was much less than that observed in rats (1.7 vs 0.38).

Hemoglobin adducts can be used to calculate the internal dose or area under the curve in blood, using the reaction rate constant measured in vitro (Osterman-Golkar *et al.*, 1976), using the relationship:

$$\text{AUC} = \text{Adduct concentration/reaction rate constant} \qquad (2)$$

where the second order reaction rate constant is expressed in units of l/g globin/hr and adduct concentration is the amount of adduct per g globin. In Table 9, the rate constants measured in this laboratory are presented. These are similar in magnitude to those reported previously by Bergmark et al. (1993). For the human AAVal rate constant, our value of 4.24×10^{-6} agrees well with the previously reported value of 4.4×10^{-6}. However, our values for glycidamide reaction with rat and human hemoglobin are approximately half of those reported previously. Calculated values for AAVal and GAVal normalized for administered or absorbed dose are shown in Table 10. These values converted to AUC normalized for administered or absorbed dose are shown in Table 11. Compared with the AUC calculated for acrylamide in rats administered 3 mg/kg, the AUC in humans ranged from 2.75 to 3.7 fold higher. In contrast, the AUC for glycidamide in humans was similar to that in the rat, ranging from approximately 1.2 – 1.4 times that of rat.

DISCUSSION

The administration of a low dose (3 mg/kg) of acrylamide by gavage to rats resulted in a greater amount of metabolism via glycidamide (41 % of the urinary metabolites) compared with a higher dose of 59 mg/kg (28% of the urinary metabolites, (Sumner *et*

al., 2003)). The fate of glycidamide was primarily conjugation with GSH, resulting in the excretion of two mercapturic acids (metabolites 2 and 3).

The ratio of GAVal:AAVal has been observed to differ with route of exposure, and reflects the relative AUCs for acrylamide and glycidamide in blood. At a dose of 3 mg/kg in rats, GAVal:AAVal was lower (1.2) than that observed at 50 mg/kg (2.6, (Sumner *et al.*, 2003)). Both this observation and the larger percentage of metabolites in urine derived from glycidamide at the lower dose are consistent with saturation of the oxidation of acrylamide at the higher dose administered (Calleman *et al.*, 1992).

The urinary metabolites of acrylamide in humans showed similarities and differences with data obtained previously in the rat and mouse. The main pathway of metabolism in humans was via direct glutathione conjugation, forming *N*-acetyl-*S*-(3-amino-3-oxopropyl)cysteine, as observed in the rat and mouse, and its *S*-oxide, which has not been reported previously. Oxidation to glycidamide was the other important pathway, with glyceramide formed as a major metabolite in humans. Glycidamide was detected in low amounts. The glutathione conjugation of glycidamide, which is a major pathway in rodents, appeared to occur at very low levels in humans, with metabolite 2 detected, but not quantitated, and metabolite 3 not detected. Metabolism via glycidamide (derived from glycidamide and glyceramide) in humans was approximately 12% of the total urinary metabolites. This is considerably lower than the amount of glycidamide derived metabolites reported for oral administration of acrylamide in rats (28% at 50 mg/kg, (Sumner *et al.*, 2003) and in mice (59% at 50 mg/kg, (Sumner *et al.*, 1992)).

This study has provided data on the amount of hemoglobin adducts derived from acrylamide and glycidamide following administration of a defined dose of acrylamide to people. Both AAVal and GAVal increased linearly with increasing dose of acrylamide administered orally, suggesting that over the range of 0.5–3.0 mg/kg, there is no saturation of metabolism of acrylamide to glycidamide. The ratio of GAVal:AAVal produced by administration of acrylamide was similar to the ratio of the background adducts prior to exposure. Compared with the equivalent oral administration in rats (3 mg/kg), the ratio of $^{13}C_3$-GAVal: $^{13}C_3$-AAVal in humans was lower (0.44 ± 0.06) than in rats (0.84 ± 0.07), and the absolute amount of $^{13}C_3$-AAVal formed in humans was approximately 2.7 fold higher than in the rat. The absolute amount of $^{13}C_3$-GAVal was approximately 1.4 fold higher than that formed in the rat.

The extrapolation of dose between species is generally conducted using a scaling factor of body weight$^{3/4}$. For extrapolation from rats to humans, a scaling factor of 4.5 would be used. For effects that are mediated via the action of acrylamide, based on the AAVal comparison between rats and humans, a factor of 2.7 would appear appropriate, whereas for effects mediated by glycidamide, a factor of 1.4 would appear appropriate.

Dermal administration of acrylamide resulted in much lower levels of AAVal and GAVal formed compared with an equivalent dose by the oral route. Comparing AAVal after dermal and oral administration indicated approximately 5.6% of the dermally administered dose was taken up, assuming 100% oral absorption. Dermal administration also resulted in much lower formation of GAVal (8.6% of that formed on oral administration). Approximately 66% of the administered dose of acrylamide was recovered in dam solutions and the wash solutions, and thus was not systemically

absorbed on dermal administration. This suggests that only approximately 33% of the dermally applied dose could have been absorbed. If AAVal formed on oral and dermal administration is normalized with the absorbed dose, 16.4% of the absorbed dose (Equation 1) would have been systemically available (Table 8, 172 fmol/mg globin/mg acrylamide/kg for dermal vs. 1047 fmol/mg globin/mg acrylamide/kg for oral). This may indicate that 80% of the acrylamide that penetrated the skin was not available systemically. GAVal on dermal administration normalized for the actual dose in a similar manner was 24.4% of that formed on oral administration (Table 8, 99 fmol/mg globin/mg acrylamide/kg for dermal vs. 405 fmol/mg globin/mg acrylamide/kg for oral). An alternative explanation is that the acrylamide and glycidamide may be more rapidly metabolized on dermal exposure, resulting in a lower AUC and lower adduct formation per mg/kg.

The normalized formation of AAVal per unit dose, averaged for all of the oral dose groups, was calculated as 924 fmol/mg globin/mg acrylamide/kg. Using this value, the amount of acrylamide exposure that would be expected in a human from the diet can be calculated. In this study, the average pre-exposure AAVal level was 79 ± 49 fmol/mg globin (excluding subject 13). Hagmar *et al.* (2001) reported a range of 0.02–0.07 nmol AAVal/g globin (equivalent to 20–70 fmol AAVal/mg globin) in unexposed individuals. The steady state adduct level from continuous exposure is calculated to be $a.t_{er}/2$, where a is the daily adduct increment, and t_{er} is the erythrocyte lifespan, which in humans is approximately 120 days. The amount of adduct formed per day, assuming similar exposure per day over the erythrocyte lifespan, would be $1/60^{th}$ of the daily adduct increment. For the average level of 80 fmol/mg:

Daily adduct increment = 2*80 fmol/mg globin/120 days = 1.33 fmol/mg globin/day

The amount of acrylamide taken in can be estimated by:

$$\text{Acrylamide intake} = \frac{1.33 \text{ fmol/mg globin/day}}{924 \text{ fmol/mg globin/mg acrylamide/kg body wt.}} \quad (3)$$

$$= 1.44 \text{ μg/kg/day.}$$

Similarly for the lower level of 20 fmol/mg, the daily adduct increment is 0.33 fmol/mg globin/day, and acrylamide intake is 0.36 μg/kg/day.

These estimates suggest a daily intake in the range of 25 – 101 μg for an average 70-kg person, and can be compared with a variety of estimates that have been produced for the average daily intake of acrylamide. The World Health Organization consultation (2002) estimated a daily intake of 0.8 μg acrylamide/kg/day for the average consumer, based on acrylamide levels in foods. Similarly, Svennson *et al.* (2003) estimated a mean daily intake of 31 μg/day based on food consumption data, which corresponds to 0.44 μg/kg/day for a 70-kg person. Törnqvist *et al.* (1998) have estimated a daily intake of approximately 100 μg per person based on hemoglobin adduct measurements of 30 fmol/mg globin, reported in Tareke et al. (2002). These calculations are based on a complex equation that relates the estimated dose to adduct level accumulated over the erythrocyte lifespan, rate of elimination (k), the reaction rate constant for adduct formation, the volume of distribution, and the erythrocyte lifespan (t_{er}).

Uptake (g/kg/day) =

$$\frac{\text{Adduct level } (30 \times 10^{-12} \text{ mol/g globin}) \times k\ (0.15\ \text{hr}^{-1}) \times 71\ \text{g/mol} \times \text{VD } (1\ \text{L/kg})}{[\text{Erythrocyte lifespan} \times 0.5\ (63\ \text{days})] \times \text{reaction rate constant } (4.4 \times 10^{-6}\ \text{L/g globin/hr})} \quad (4)$$

$$= 1.1\ \mu\text{g/kg/day}.$$

Of the parameters in this equation, two are estimated: the elimination rate constant in humans, and the volume of distribution (Calleman, 1996). Using the data obtained in this study for a single exposure in a modified equation enables the estimation of possible values for the elimination rate constant and volume of distribution.

Dose Administered (1 mg/kg) =

$$\frac{\text{Adduct level } (924 \times 10^{-12} \text{ mol/g globin}) \times k\ (0.15\ \text{hr}^{-1}) \times 71\ \text{g/mol} \times \text{VD (L/kg)}}{\text{Reaction rate contant } (4.24 \times 10^{-6}\ \text{L/g globin/hr})} \quad (5)$$

Rearranging and solving for VD yields a value of 0.44 L/kg, or distribution into 31 liters in a 70 kg person. This is approximately 73% of the estimated 42 liters of total body water. Using the value for VD of 0.44 L/kg and an adduct level of 30 pmol adduct/g globin, an uptake of acrylamide of 0.46 µg/kg/day is calculated, or 32 µg /day for a 70 kg person. This estimated uptake is more in line with the estimates of acrylamide exposure based on food consumption.

In summary, this study has examined the metabolism of acrylamide in people, and compared the internal dose of acrylamide and glycidamide in people with that observed in rats. The data reported are consistent with slower elimination of acrylamide in humans, and slower metabolism of acrylamide to glycidamide in humans. The hemoglobin adduct measurements obtained will provide a calibration for estimates of

exposure from diet, lifestyle, and the workplace.

ACKNOWLEDGEMENTS

The authors would like to acknowledge CIIT Centers for Health Research, Research Triangle Park, NC, where the administration of acrylamide to rats and analyses of rat urine and globin were conducted. This study was funded by SNF SA.

REFERENCES

Bergmark, E., Calleman, C. J., He, F., and Costa, L. G. (1993). Determination of hemoglobin adducts in humans occupationally exposed to acrylamide. *Toxicol. Appl. Pharmacol.* **120**, 45-54.

Calleman, C. J. (1996). The metabolism and pharmacokinetics of acrylamide: implications for mechanisms of toxicity and human risk estimation. *Drug Metab. Rev.* **28**, 527-590.

Calleman, C. J., Bergmark, E., and Costa, L. G. (1990). Acrylamide is metabolized to glycidamide in the rat: evidence from hemoglobin adduct formation. *Chem. Res. Toxicol.* **3**, 406-412.

Calleman, C. J., Stern, L. G., Bergmark, E., and Costa, L. G. (1992). Linear versus nonlinear models for hemoglobin adduct formation by acrylamide and its metabolite glycidamide: implications for risk estimation. *Cancer Epidemiol. Biomarkers Prev.* **1**, 361-368.

Dixit, R., Seth, P. K., and Mukjtar, H. (1982). Metabolism of acrylamide into urinary mercapturic acid and cysteine conjugates in rats. *Drug Metab Dispos* **10**, 196-197.

Edwards, P. M. (1975). The distribution and metabolism of acrylamide and its neurotoxic analogues in rats. *Biochem. Pharmacol.* **24**, 1277-1282.

European Union (2002). European Union Risk Assessment Report

Acrylamide, p. 210 pp, Luxembourg.

Fennell, T. R., Snyder, R. W., Krol, W. L., and Sumner, S. C. (2003). Comparison of the hemoglobin adducts formed by administration of N-methylolacrylamide and acrylamide to rats. *Toxicol Sci* **71**, 164-175.

Friedman, M. A., Dulak, L. H., and Stedham, M. A. (1995). A lifetime oncogenicity study in rats with acrylamide. *Fundam. Appl. Toxicol.* **27**, 95-105.

Gamboa da Costa, G., Churchwell, M. I., Hamilton, L. P., Von Tungeln, L. S., Beland, F. A., Marques, M. M., and Doerge, D. R. (2003). DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice. *Chem Res Toxicol* **16**, 1328-1337.

Hagmar, L., Törnqvist, M., Nordander, C., Rosen, I., Bruze, M., Kautiainen, A., Magnusson, A. L., Malmberg, B., Aprea, P., Granath, F., and Axmon, A. (2001). Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. *Scand. J. Work Environ. Health* **27**, 219-226.

Hashimoto, K., and Aldridge, W. N. (1970). Biochemical studies on acrylamide, a neurotoxic agent. *Biochem. Pharmacol.* **19**, 2591-2604.

Hashimoto, K., and Tanii, H. (1985). Mutagenicity of acrylamide and its analogues in Salmonella typhimurium. *Mutat. Res.* **158**, 129-133.

Johnson, K. A., Gorzinski, S. J., Bodner, K. M., Campbell, R. A., Wolf, C. H., Friedman, M. A., and Mast, R. W. (1986). Chronic toxicity and oncogenicity study on acrylamide incorporated in the drinking water of Fischer 344 rats. *Toxicol. Appl. Pharmacol.* **85**, 154-168.

Miller, M. J., Carter, D. E., and Sipes, I. G. (1982). Pharmacokinetics of acrylamide in Fisher-344 rats. *Toxicol. Appl. Pharmacol.* **63**, 36-44.

Mowrer, J., Törnqvist, M., Jensen, S., and Ehrenberg, L. (1986). Modified Edman Degradation applied to hemoglobin for monitoring occupational exposure to alkylating agents. *Toxicol. Env. Chem.* **11**, 215-231.

Osterman-Golkar, S., Ehrenberg, L., Segerbäck, D., and Hallstrom, I. (1976). Evaluation of genetic risks of alkylating agents. II. Haemoglobin as a dose monitor. *Mutat. Res.* **34**, 1-10.

Payne, G. B., and Williams, P. H. (1961). Reactions of Hydrogen Peroxide. VI. Alkaline epoxidation of acrylonitrile. *J. Org. Chem.* **26**, 651-659.

Rosen, J., and Hellenas, K. E. (2002). Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. *Analyst* **127**, 880-882.

Segerback, D., Calleman, C. J., Schroeder, J. L., Costa, L. G., and Faustman, E. M. (1995). Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [14C]acrylamide. *Carcinogenesis* **16**, 1161-1165.

Spencer, P. S., and Schaumburg, H. H. (1974a). A review of acrylamide neurotoxicity. Part I. Properties, uses and human exposure. *Can J Neurol Sci* **1**, 143-150.

Spencer, P. S., and Schaumburg, H. H. (1974b). A review of acrylamide neurotoxicity. Part II. Experimental animal neurotoxicity and pathologic mechanisms. *Can J Neurol Sci* **1**, 152-169.

Spencer, P. S., and Schaumburg, H. H. (1975). Nervous system degeneration produced by acrylamide monomer. *Environ Health Perspect* **11**, 129-133.

Sumner, S. C., Fennell, T. R., Moore, T. A., Chanas, B., Gonzalez, F., and Ghanayem, B. I. (1999). Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. *Chem. Res. Toxicol.* **12**, 1110-1116.

Sumner, S. C., MacNeela, J. P., and Fennell, T. R. (1992). Characterization and quantitation of urinary metabolites of [1,2,3-^{13}C]acrylamide in rats and mice using ^{13}C nuclear magnetic resonance spectroscopy. *Chem. Res. Toxicol.* **5**, 81-89.

Sumner, S. C., Selvaraj, L., Nauhaus, S. K., and Fennell, T. R. (1997). Urinary metabolites from F344 rats and B6C3F1 mice coadministered acrylamide and acrylonitrile for 1 or 5 days. *Chem. Res. Toxicol.* **10**, 1152-1160.

Sumner, S. C., Williams, C. C., Snyder, R. W., Krol, W. L., Asgharian, B., and Fennell, T. R. (2003). Acrylamide: a comparison of metabolism and hemoglobin adducts in rodents following dermal, intraperitoneal, oral, or inhalation exposure. *Toxicol Sci* **75**, 260-270.

Svensson, K., Abramsson, L., Becker, W., Glynn, A., Hellenas, K. E., Lind, Y., and Rosen, J. (2003). Dietary intake of acrylamide in Sweden. *Food Chem Toxicol* **41**, 1581-1586.

Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., and Tornqvist, M. (2002). Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J Agric Food Chem* **50**, 4998-5006.

Törnqvist, M., Bergmark, E., Ehrenberg, L., and Granath, F. (1998). Risk Assessment of Acrylamide. Swedish Chemicals Inspectorate, Solna, Sweden.

Tyl, R. W., and Friedman, M. A. (2003). Effects of acrylamide on rodent reproductive performance. *Reprod Toxicol* **17**, 1-13.

WHO (2002). Joint FAO/WHO Consultation on Health Implication of Acrylamide in Food., p. 33 pp. World Health Organization, Geneva, Switzerland.

Table 1. Quantitation of urinary metabolites by NMR spectroscopy in rats administered 3 mg 1,2,3-^{13}C acrylamide/kg body wt.

	3 mg/kg [^{13}C]Acrylamide (8.7 ± 0.46 µmol)[a]				
Metabolite	% of Total	mM	µmol	µmol/kg bw	% of Dose
1,1'	59 ± 1.5	0.31 ± 0.061	2.6 ± 0.31	12 ± 1.6	29 ± 4.5
2,2'	25 ± 0.19	0.14 ± 0.028	1.1 ± 0.15	5.3 ± 0.73	13 ± 2.1
3,3'	16 ± 1.7	0.085 ± 0.026	0.69 ± 0.17	3.3 ± 0.82	8.0 ± 2.1
4	BDG[b]	BDG	BDG	BDG	BDG
5,5'	BD[c]	BD	BD	BD	BD
2,2'-5	41 ± 1.5	0.22 ± 0.053	1.8 ± 0.32	8.6 ± 1.5	21 ± 4.2
Total	100 ± 0	0.53 ± 0.11	4.3 ± 0.63	21 ± 3.0	50 ± 8.6

[a] N = 4, (Average ± Standard Deviation of dose in µmols). Urine samples were concentrated.

[b] BDG = Below detection limit in concentrated urine samples. Metabolite 4 (Glycidamide) constitutes 0.76-3.5 % of total measured excreted metabolites in 2 rats prior to concentration of urine. Following concentration, Glycidamide signals are not observed.

[c] BD = Below detection limit.

Table 2. Mean AAVal and GAVal levels following administration of 3 mg/kg 1,2,3-$^{13}C_3$ acrylamide to male rats by gavage.

	AAVal (fmol/mg globin)	GAVal (fmol/mg globin)	$^{13}C_3$-AAVal (fmol/mg globin)	$^{13}C_3$-GAVal (fmol/mg globin)
Control	156 ± 9	117 ± 16	19 ± 2	ND
3 mg/kg gavage	224 ± 100	111 ± 19	907 ± 176	752 ± 106

Table 3. Dermal dose delivered.

Subject	Body Wt (kg)	Dose Administered (mg)	Dermal Dam Total Acrylamide (mg)	Total Acrylamide in Wash (mg)	Total Acrylamide Recovered (mg)	Total Dose - (Dam + Wash) (mg)	Actual Dose (mg/kg)
Day 1	85.8 ± 9.1	212.4 ± 21.7	38.8 ± 11.5	100.2 ± 23.0	139.0 ± 18.4	73.4 ± 15.4	0.86 ± 0.14
Day 2	85.8 ± 9.1	212.4 ± 21.7	36.5 ± 19.2	113.3 ± 19.0	149.8 ± 23.6	62.6 ± 19.1	0.73 ± 0.2
Day 3	85.8 ± 9.1	212.4 ± 21.7	29.5 ± 9.0	115.2 ± 35.7	144.6 ± 37.8	67.7 ± 47.7	0.77 ± 0.48
Total	85.8 ± 9.1	637.1 ± 65.1	104.7 ± 22.1	328.7 ± 30.5	433.4 ± 42.4	203.7 ± 58.8	2.35 ± 0.5

Table 4. Chemical shift and coupling constants of metabolites in human urine derived from 1,2,3-$^{13}C_3$ acrylamide

Metabolite Identification[a]	Chemical Shift (ppm)[b]	Carbon-carbon Coupling (J_{cc}, Hz)	Observed in This Study
1a	26.97	35	Yes
1b	34.72	35, 48	Yes
1'b	34.68	35, 48	Yes
1c	177.14	48	Yes
1'c	177.08	48	Yes
2a	35.96	38	Yes
2'a	35.76	38	Yes
2b	70.22	38,52	Yes
2'b	70.09	38,52	Yes
2c	177.61	52	No
3a	61.45	37	No
3'a	61.48	37	No
3b	49.69	38,50	No
3'b	49.63	38,50	No
3c	175.54	50	No
4a	46.81	26	Yes
4b	48.45	26,63	Yes
4c	173.84	63	No
5a	62.99	40	Yes
5b	72.00	40,50	Yes
5c	175.46	50	Yes
6b	27.60	37,51	Yes
6a	46.47	37	Yes
6c	175.4	51	Yes

[a] Signals for metabolites are labeled according to the letter designating the carbon derived from acrylamide ($_aCH_2 =_bCH-_cCONH_2$). Derivation of metabolites given in Sumner et al. (1992, 1997, 2003).

[b] Chemical shift for center of multiplet pattern.

Table 5. Quantitation of urinary metabolites by NMR spectroscopy in humans administered 3 mg 1,2,3-^{13}C acrylamide/kg body wt orally.

		3 mg/kg [^{13}C]Acrylamide			
Metabolite		% of Total	mM	µmol	% of Dose
1,1'		72 ± 6.5	0.33 ± 0.09	679 ± 162	22 ± 5.3
6		14.1 ± 3.9	0.06 ± 0.01	130 ± 35	4.2 ± 1.1
2,2’		BD	BD	BD	BD
3,3'		BD	BD	BD	BD
4		2.6 ± 0.57	0.01± 0.00	25 ± 7.9	0.79 ± 0.24
5,5'		11.0 ± 3.7	0.05 ± 0.02	102 ± 40	3.3 ± 1.1
2,2'-5		13.5 ± 3.9		126 ± 44	4.1 ± 1.2
Total		100 ± 0		1056 ± 183	34 ± 5.7

Table 6. Mean AAVal and GAVal levels prior to and following administration of 1,2,3-$^{13}C_3$ acrylamide orally and dermally.

	AAVal (fmol/mg globin)	GAVal (fmol/mg globin)	$^{13}C_3$-AAVal (fmol/mg globin)	$^{13}C_3$-GAVal (fmol/mg globin)	$^{13}C_3$-GAVal/ $^{13}C_3$-AAVal
Pre-Dose Oral Group A1 (0.5 mg/kg)	89 ± 52	32 ± 17	ND [a]	ND	-
Pre-Dose Oral Group A2 (1 mg/kg)	63 ± 29	26 ± 5	ND	ND	-
Pre-Dose Oral Group A3 (3 mg/kg)	271 ± 354 [b]	288 ± 5	ND	ND	-
Oral Group A1 (0.5 mg/kg)	81 ± 41	34 ± 12	514 ± 49	186 ± 47	0.36 ± 0.06
Oral Group A2 (1 mg/kg)	66 ± 21	26 ± 6	914 ± 125	344 ± 52	0.38 ± 0.03
Oral Group A3 (3 mg/kg)	64 ± 26	35 ± 7	2479 ± 685	1076 ± 237	0.44 ± 0.06
Pre-Dose Dermal Group B (3 mg/kg)	167 ± 307 [c]	24 ± 6	ND	ND	-
Dermal Day 2	45 ± 11	27 ± 8	116 ± 15	55 ± 12	0.48 ± 0.09
Dermal Day 3	43 ± 9	28 ± 5	292 ± 78	167 ± 55	0.57 ± 0.11
Dermal Day 4	41 ± 5	27 ± 6	440 ± 101	292 ± 95	0.66 ± 0.11
Dermal Day 5	48 ± 14	30 ± 6	464 ± 62	316 ± 67	0.68 ± 0.11

[a] ND = not detected
[b] Recalculation with 13 removed: 128 ± 56
[c] Recalculation with 19 removed: 42.0 ± 12.1

Table 7. Hemoglobin adducts in humans normalized by nominal dose of 1,2,3-13C3 acrylamide (mg/kg).

Dose (Route) (mg/kg)	$^{13}C_3$-AAVal [a] (fmol/mg globin/ mg acrylamide/kg)	$^{13}C_3$-GAVal (fmol/mg globin/ mg acrylamide/kg)
0.5 (Oral)	1027 ± 98	372 ± 95
1 (Oral)	914 ± 125	344 ± 53
3 (Oral)	829 ± 228	359 ± 99
Combined (Oral)	924 ± 171	358 ± 73
1 x 3 (Dermal)	39 ± 5	18 ± 4
2 x 3 (Dermal)	49 ± 13	28 ± 9
3 x 3 (Dermal)	49 ± 11	32 ± 11
Combined (Dermal)	45 ± 11	26 ± 10

[a] Values represent mean ± SD for 5 individuals.

Table 8. Hemoglobin adducts in humans normalized by actual dose of 1,2,3-$^{13}C_3$ acrylamide (mg/kg).

Dose (Route) (mg/kg)	Actual Dose[a] (mg/kg)	$^{13}C_3$-AAVal [b] (fmol/mg globin/ mg acrylamide/kg)	$^{13}C_3$-GAVal (fmol/mg globin/ mg acrylamide/kg)
0.5 (Oral)	0.43 ± 0.01	1206 ± 105	436 ± 107
1 (Oral)	0.89 ± 0.01	1032 ± 139	389 ± 60
3 (Oral)	2.75 ± 0.03	903 ± 250	391 ± 87
Combined (Oral)		1047 ± 207	405 ± 84
1 x 3 (Dermal)	0.86 ± 0.14	140 ± 34	66 ± 20
2 x 3 (Dermal)	1.58 ± 0.12	183 ± 35	104 ± 27
3 x 3 (Dermal)	2.35 ± 0.50	194 ± 56	128 ± 41
Combined (Dermal)		172 ± 47	99 ± 39

[a] Dose calculated from analysis of dose solutions (oral) or absorbed dose (dermal).

[b] Values represent mean ± SD for 5 individuals.

Table 9. Rate constant for reaction of acrylamide and glycidamide with the *N*-terminal valine residue of hemoglobin.

	AAVal l/g/h	GAVal l/g/h	AAVal/GAVal
Rat	3.82×10^{-6}	4.96×10^{-6}	0.77
Human	4.27×10^{-6}	6.72×10^{-6}	0.64
Rat/human	0.89	0.73	

Table 10. Comparison of hemoglobin adducts normalized by administered or absorbed dose (mmol/kg) in rats and humans.

Dose (Route) (mg/kg)	Actual Dose (mg/kg)	$^{13}C_3$AAVal [a] (nmol/g globin/ mmol acrylamide/kg)	$^{13}C_3$ GAVal (nmol/g globin/ mmol acrylamide/kg)	GAVal: AAVal
Human				
0.5 (Oral)	0.43 ± 0.01	89.2 ± 7.8	33.2 ± 7.9	0.36 ± 0.06
1 (Oral)	0.89 ± 0.01	76.4 ± 10.2	28.8 ± 4.4	0.38 ± 0.03
3 (Oral)	2.75 ± 0.03	66.9 ± 18.5	28.9 ± 6.5	0.44 ± 0.06
Combined		77.4 ± 15.3	30.0 ± 6.2	0.39 ± 0.06
1 x 3 (Dermal)	0.86 ± 0.14	10.3 ± 2.5	4.9 ± 1.5	0.48 ± 0.09
2 x 3 (Dermal)	1.58 ± 0.12	13.5 ± 2.6	7.7 ± 2.0	0.57 ± 0.11
3 x 3 (Dermal)	2.35 ± 0.50	14.4 ± 4.1	9.5 ± 3.0	0.66 ± 0.11
Combined		12.7 ± 3.4	7.3 ± 2.8	0.57 ± 0.12
Rat [b]				
Oral	3	21.8 ± 4.1	18.1 ± 2.7	0.84 ± 0.07
Oral	50	26.4 ± 4.9	9.9 ± 1.6	0.38 ± 0.07
i.p.	50	20.6 ± 1.4	14.4 ± 1.5	0.71 ± 0.03
Dermal (1)	150	0.75 ± 0.3	1.2 ± 0.3	1.7 ± 0.42
Dermal (2)	150	2.8 ± 0.3	4.4 ± 1.4	1.7 ± 0.27
Dermal (2) [c]	150	12.8 ± 3.1	20.8 ± 4.3	

[a] Values represent mean ± SD for 5 individuals.
[b] Values for the rat are from this study, or are published in (Fennell *et al.*, 2003; Sumner *et al.*, 2003).
[c] Calculated based on dose recovered in tissue, excreta, and carcass.

Table 11. Comparison of AUC normalized by actual dose (mmol/kg) in rats and humans.

Dose (Route) (mg/kg)	Actual Dose (mg/kg)	$^{13}C_3$Acrylamide [a] (mM.h/ mmol acrylamide/kg)	$^{13}C_3$ Glycidamide (mM.h/ mmol acrylamide/kg)
Human			
0.5 (Oral)	0.43 ± 0.01	20.9 ± 1.8	4.9 ± 1.8
1 (Oral)	0.89 ± 0.01	17.9 ± 2.4	4.3 ± 0.6
3 (Oral)	2.75 ± 0.03	15.7 ± 1.5	4.3 ± 0.9
Combined		18.1 ± 4.5	4.5 ± 0.9
1 x 3 (Dermal)	0.86 ± 0.14	2.4 ± 0.6	0.73 ± 0.22
2 x 3 (Dermal)	1.58 ± 0.12	3.2 ± 0.6	1.1 ± 0.3
3 x 3 (Dermal)	2.35 ± 0.50	3.4 ± 1.0	1.4 ± 0.45
Combined		3.0 ± 0.8	1.1 ± 0.4
Rat [b]			
Oral	3	5.7 ± 1.1	3.7 ± 0.5
Oral	50	6.9 ± 1.3	2.0 ± 0.3
i.p.	50	5.4 ± 0.4	2.9 ± 0.3
Dermal (1)	150	0.20 ± 0.08	0.24 ± 0.06
Dermal (2)	150	0.73 ± 0.08	0.89 ± 0.28
Dermal (2) [c]	150	3.4 ± 0.8	4.2 ± 0.9

[a] Values represent mean ± SD for 5 individuals.
[b] Values for the rat are from this study, or published previously (Fennell *et al.*, 2003; Sumner *et al.*, 2003).
[c] Calculated based on dose recovered in tissue, excreta, and carcass.

Legends to Figures

Figure 1. Metabolism of acrylamide.

Figure 2. **^{13}C** NMR spectrum of human urine from an unexposed volunteer.

Figure 3. ^{13}C NMR spectrum of a 0-24 h composite human urine sample from a volunteer administered 3 mg/kg 1,2,3-^{13}C acrylamide orally.

Figure 4. LC-MS/MS analysis of AAVal and GAVal. Adducts were monitored by MRM for (A) AAVal; (B) $^{13}C_3$-AAVal; (C) $^{13}C_5$-AAVal; (D) GAVal; (E) $^{13}C_3$-GAVal; and (F) $^{13}C_5$-GAVal. Globin was obtained from a volunteer prior to administration of acrylamide.

Figure 5. LC-MS/MS analysis of AAVal and GAVal. Adducts were monitored by MRM for (A) AAVal; (B) $^{13}C_3$-AAVal; (C) $^{13}C_5$-AAVal; (D) GAVal; (E) $^{13}C_3$-GAVal; and (F) $^{13}C_5$-GAVal. Globin was obtained from a volunteer 24 h following oral administration of [1,2,3-^{13}C] acrylamide (0.5 mg/kg).

Figure 6. Dose response for $^{13}C_3$-AAVal and $^{13}C_3$-GAVal following oral administration of [1,2,3-^{13}C] acrylamide in humans.

Figure 7. Effect of cumulative dermal dose of [1,2,3-^{13}C] acrylamide on formation of $^{13}C_3$-AAVal and $^{13}C_3$-GAVal in humans.

Figure 1

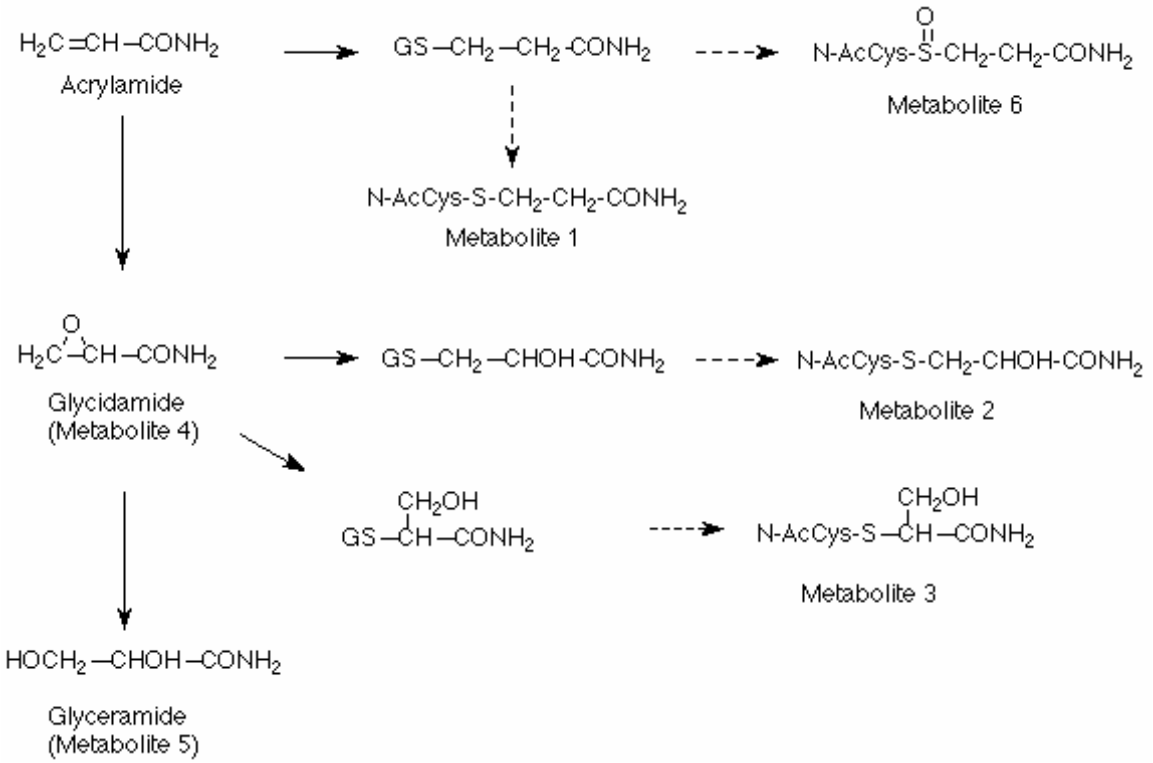

H2C=CH–CONH2
Acrylamide
GS–CH2–CH2-CONH2
N-AcCys-S-CH2-CH2-CONH2
Metabolite 6
N-AcCys-S-CH2-CH2-CONH2
Metabolite 1
H2C–CH–CONH2
Glycidamide
(Metabolite 4)
GS–CH2–CHOH-CONH2
N-AcCys-S-CH2-CHOH-CONH2
Metabolite 2
CH2OH
GS–CH–CONH2
CH2OH
N-AcCys-S–CH–CONH2
Metabolite 3
HOCH2–CHOH–CONH2
Glyceramide
(Metabolite 5)

Figure 2.

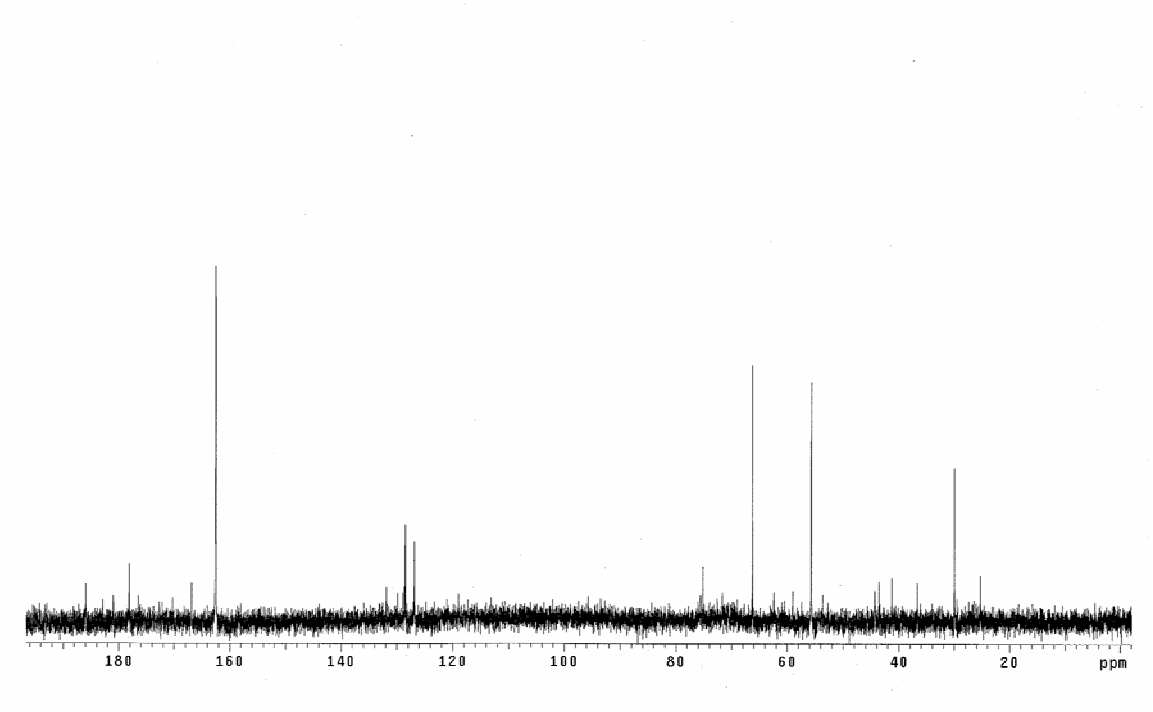


Figure 3.

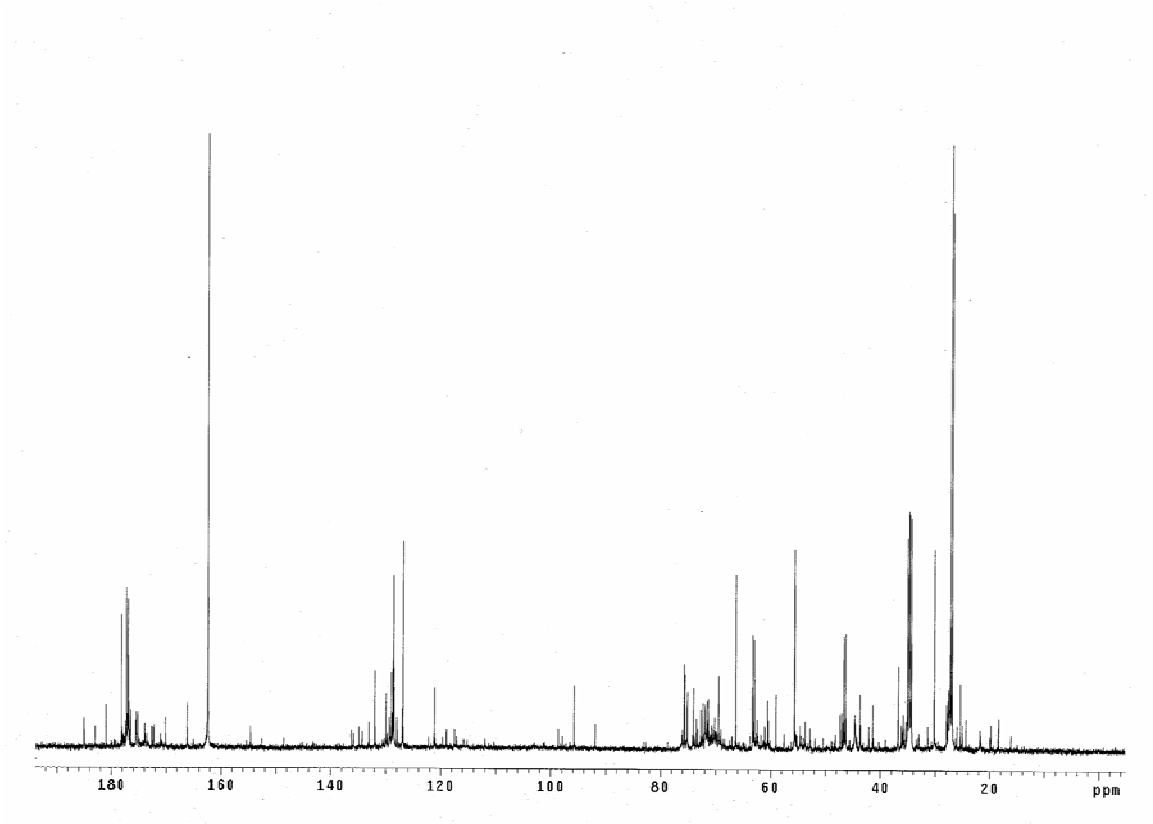


Figure 4.

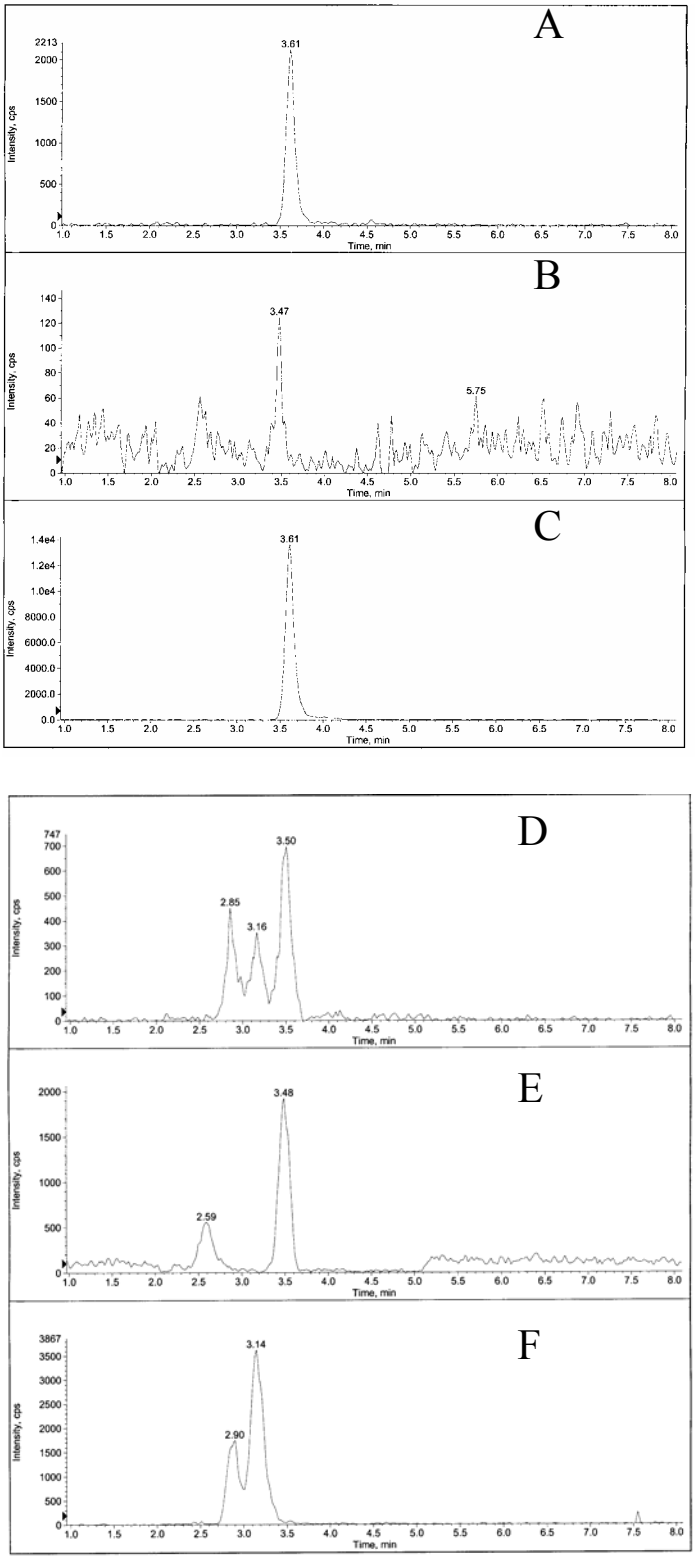

A
3.61
Intensity, cps
Time, min
B
3.47
5.75
Intensity, cps
Time, min
C
3.61
Intensity, cps
Time, min
D
3.50
2.85
3.16
Intensity, cps
Time, min
E
3.48
2.59
Intensity, cps
Time, min
F
3.14
2.90
Intensity, cps
Time, min

Figure 5.

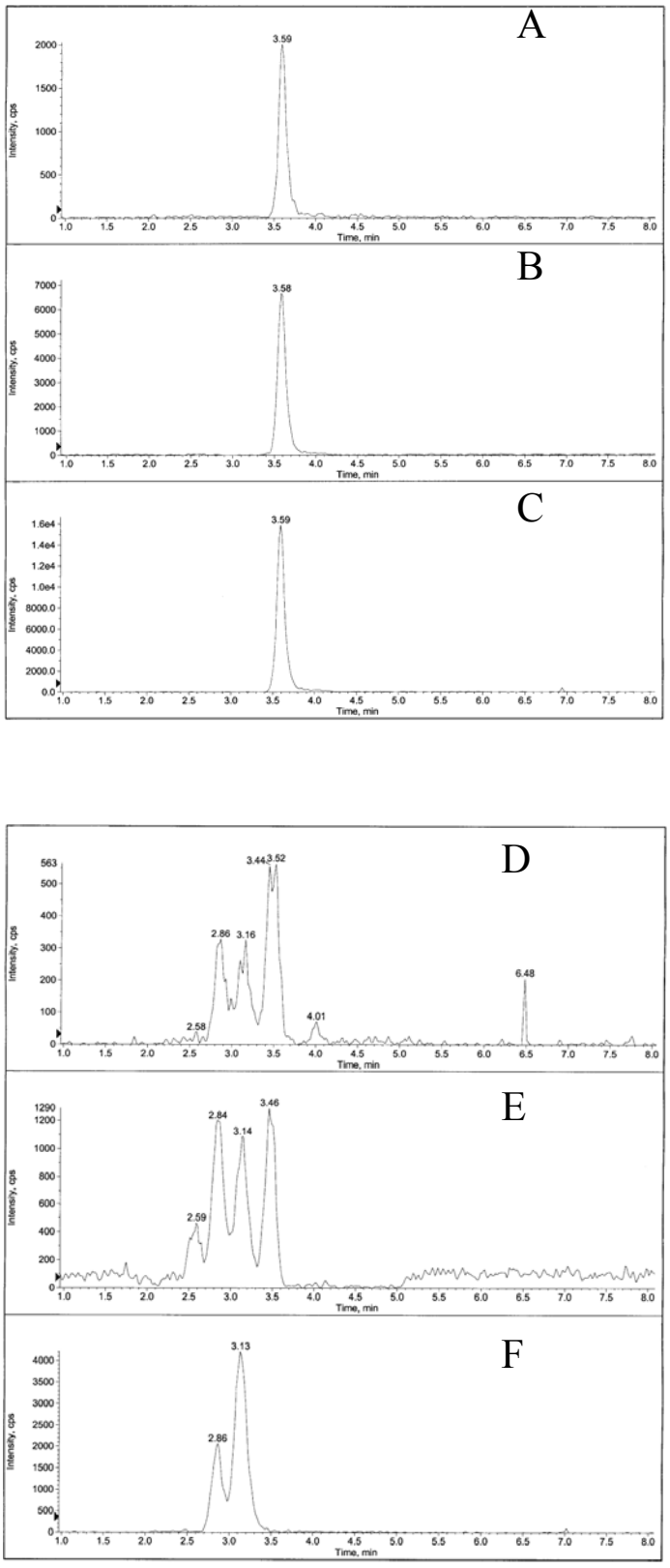

A
3.59
Intensity, cps
Time, min
B
3.58
Intensity, cps
Time, min
C
3.59
Intensity, cps
Time, min
D
3.44
3.52
2.86
3.16
6.48
2.58
4.01
Intensity, cps
Time, min
E
2.84
3.46
3.14
2.59
Intensity, cps
Time, min
F
3.13
2.86
Intensity, cps
Time, min

Figure 6.

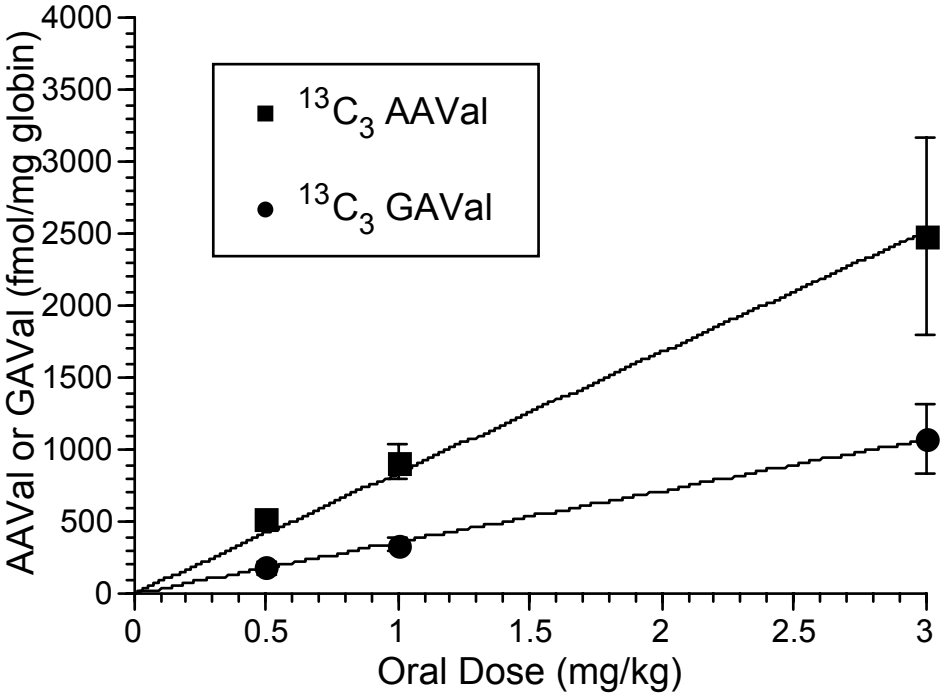
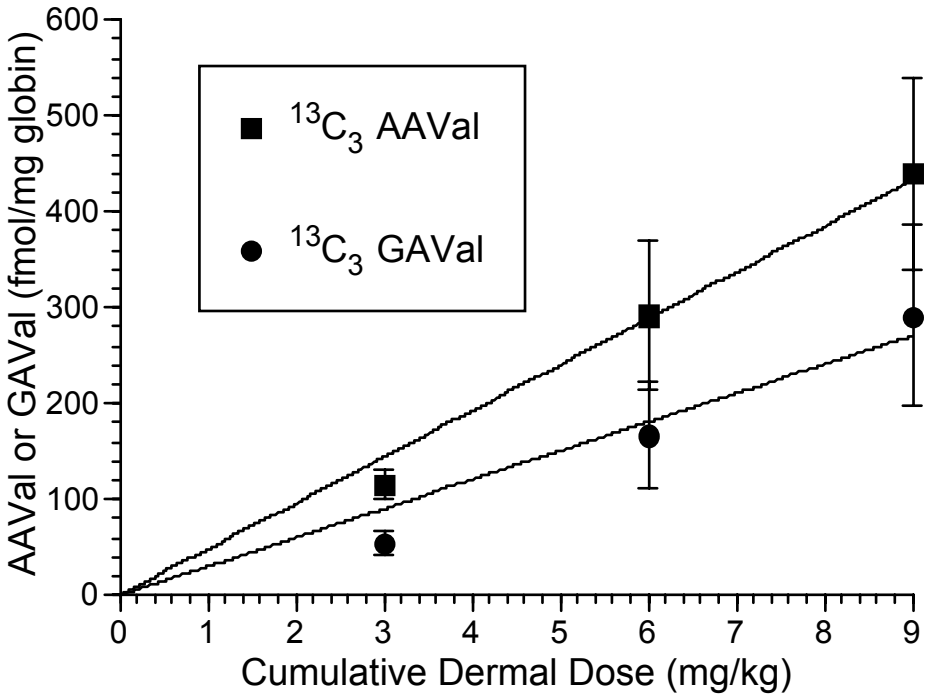

AAVal or GAVal (fmol/mg globin)
4000
3500
3000
2500
2000
1500
1000
500
0
0
0.5
1
1.5
2
2.5
3
Oral Dose (mg/kg)
$^{13}C_3$ AAVal
$^{13}C_3$ GAVal

Figure 7.


AAVal or GAVal (fmol/mg globin)
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
9
Cumulative Dermal Dose (mg/kg)
$^{13}C_3$ AAVal
$^{13}C_3$ GAVal