GRAS Notice (GRN) No. 635
http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm

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ORIGINAL SUBMISSION

THORNE - EXHIBITE EX1017

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ORIGINAL

Spherix.
CHROMADEX CONSULTING

GRN 000635

March 8, 2016

Office of Food Additive Safety
HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

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FOOD ADDITIVE SAFETY

To Whom It May Concern:

Enclosed please find three copies of the dossier entitled "Generally Recognized as Safe (GRAS) Determination for Niagen™ (Nicotinamide Riboside Chloride)" and the GRAS Expert Panel Consensus Statement. This GRAS determination has been prepared by Spherix Consulting, Inc., on behalf of its parent company, ChromaDex, Inc.

The data and information that serve as the basis for this GRAS determination is available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., President, Spherix Consulting, Inc., 11900 Parklawn Drive, Suite 200, Rockville, MD 20852, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: clairek@chromadex.com, or will be sent to FDA upon request.

Should you have any questions or concerns, please contact me at the number listed above.

Sincerely,
(b) (6)

Claire L, Kruger, Ph.D., D.A.B.T.
President

Enclosures:

Three copies of the GRAS Panel Consensus Statement for the above-referenced GRAS Notification

Three copies of the dossier entitled "Generally Recognized as Safe (GRAS) Determination for Niagen™ (Nicotinamide Riboside Chloride)"

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GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR NIAGEN™ (NICOTINAMIDE RIBOSIDE CHLORIDE)

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of the use of Niagen™ (nicotinamide riboside chloride or NR) as an ingredient in vitamin waters, protein shakes, nutrition bars, gum and chews, based upon scientific procedures as described under 21 CFR §170.30(b). The intake of Niagen from the intended uses specified above has been shown to be safe and GRAS based on generally available and accepted information, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s).

To demonstrate that Niagen is safe under its intended conditions of use, the safety of the intake of Niagen resulting from its consumption in foods and beverages at the intended level of use has been established. The safety determination is based on the following:

- Product is manufactured in a facility that complies with cGMP for foods.
- Product specifications are set to ensure a food-grade product; stability over shelf life has been documented.
- Finished product batches reproducibly meet compositional standards and comply with limits on contaminants appropriate for food-grade ingredients.
- All processing aids used in the production are determined GRAS for their use and/or comply with regulations set forth in 21 CFR for use in food.
- Vitamin B_3 is defined as the dietary precursor to nicotinamide adenine dinucleotide (NAD+) other than the amino acid tryptophan (Erdman et al. 2012). The two major forms of vitamin B_3, nicotinic acid and nicotinamide, are together commonly known as niacin, and constitute the most well-known NAD+ precursors. NR is a recently discovered form of vitamin B_3 (Erdman et al. 2012).
- Derivation of a tolerable upper intake level (UL)[1] for Niagen is based on:
    - A single dose clinical pharmacokinetic study demonstrated that Niagen is metabolized similarly to nicotinamide in healthy humans and can be utilized as a form of Vitamin B_3. No clinically adverse effects on hematology, clinical

[1] "the highest daily level of chronic nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population"

chemistry, urinalysis or liver or kidney function parameters were noted in this study of single doses of 100, 300 and 1000 mg.

- Niagen was evaluated in published genotoxicology assays including an Ames assay, *in vitro* chromosome aberration assay, and *in vivo* micronucleus assay; results demonstrated that Niagen is not genotoxic under the conditions of these assays (Conze et al. 2015).
- A published 90-day toxicology study evaluated rats gavaged daily with control vehicle (water), 300, 1000, or 3000 mg/kg Niagen or a positive control of nicotinamide at 1260 mg/kg/day dose level (equivalent to 3000 mg/kg/day dose of Niagen on equimolar basis) (Conze et al. 2015). NR is a form of Vitamin B_3 that is metabolized similarly to nicotinamide and other NAD+ precursors. Thus, a positive control group treated with nicotinamide was included.
  - Adverse effects at 3000 mg/kg body weight/day of NR included treatment-related adverse effects in liver, kidneys, testes, epididymides and ovaries. Importantly, these effects also occurred in the nicotinamide groups with a similar magnitude.
  - Niagen administration at 1000 mg/kg/day resulted in treatment related increases in liver and kidney relative weights and increases in neutrophils, leukocytes, ALT and triglycerides which were statistically significant in female rats only. Changes in neutrophils and leukocytes were not associated with significant inflammatory changes in all organs examined. The significant increase in ALT was noted only in one gender, occurred in conjunction with an increase in triglycerides but was below the 2-fold increase typically used to establish a biologically significant signal of liver damage in the absence of histological change (Hall et al. 2012). The increased kidney weight at this dose occurred in the absence of corresponding histopathology. Therefore, the effects in liver at 1000 mg/kg/day are considered to be treatment-related but mild and potentially adaptive in nature. Based on the minimal changes at 1000 mg/kg/day, this dose was considered the Low-Observed-Adverse-Effect-Level (LOAEL).
  - Administration of Niagen at 300 mg/kg/day did not result in treatment related adverse effects in any of the parameters monitored and is considered to be the No-Observed-Adverse-Effect-Level (NOAEL).
  - The NOAEL from the 90-day rodent study is 300 mg/kg/day and effects noted at the LOAEL of 1000 mg/kg/day are considered to be mild and potentially

adaptive in nature due to prolonged exposure to this form of niacin. Therefore, application of a 100-fold safety factor applied to the NOAEL is considered appropriately conservative to derive a UL.

- Application of these safety factors to the NOAEL results in a UL of 3 mg/kg/day or 180 mg/day for a 60 kg individual.
- This UL is conservative and below the UL for nicotinamide identified by the European Commission of 900 mg/day (EC SFC, 2002) and 500 mg/day identified by the UK Expert Working Group (2003). The derivation of the UL for nicotinamide by the EU SCF (2002) considered major long-term studies in patients with Type 1 diabetes mellitus, at dosages of 2-3 g of nicotinamide per day. The UK Expert Group (2003) calculated the UL for nicotinamide based on human studies of large doses (up to 3000 mg/day for periods of up to 3 years).
  - Because animal toxicology studies on nicotinic acid and nicotinamide are limited and do not comply with current standardized testing protocols, such as OECD (Unna, 1939; Handler and Dann, 1942; Chen et al., 1938; OECD SIDS, 2002), authoritative bodies have used the results of clinical studies in which high doses of nicotinic acid and nicotinamide have been administered to derive the ULs.
  - Although nicotinamide does not appear to be associated with flushing, the IOM established a UL of 35 mg/day for adults 19 years and older for both nicotinic acid and nicotinamide based on flushing because it is considered to be protective against potential adverse effects. The European Commission Scientific Committee on Food (SCF) established a UL for nicotinic acid of 10 mg /day based on flushing (EC SFC, 2002).
  - NR may not cause flushing because just like nicotinamide, it does not bind GPR109a, the high affinity G-protein coupled receptor that is believed to play an important role in mediating the vasodilatory effects of nicotinic acid (Canto et al. 2012).

- Over 10% of the total U.S. population of 2+ years were identified as consumers of Niagen from the proposed food uses. The mean intakes of Niagen by all users aged 2+ years from all proposed food uses were estimated to be 51 mg/person/day or 0.8 mg/kg body weight/day. The heavy consumer (90th percentile all-user) intake of Niagen from all proposed food-uses in persons aged 2+ years was estimated to be 145 mg/person/day
- The Estimated Daily Intake (EDI) is below the established UL.

Determination of the GRAS status of Niagen under the intended conditions of use has been made through the deliberations of Drs. Roger Clemens, Thomas Sox and John Thomas. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of Niagen and the potential human exposure resulting from its intended use as an ingredient in foods and beverages and have concluded:

> *There is no evidence in the information reviewed on Niagen that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when this product is used at levels that might reasonably be expected from the proposed applications. Niagen is GRAS for use in foods and beverages as proposed by ChromaDex, Inc.*

Therefore, Niagen is safe and GRAS as the proposed levels of ingestion, and is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

(b) (6)

Roger Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
School of Pharmacy
University of Southern California

Signature: [redacted]

Date: December 21, 2015

(b) (6)

Thomas E. Sox, PhD, JD
GRAS Expert Panel Member
Senior Consultant
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Signature: [redacted]

Date: December 21, 2015

(b) (6)

John A. Thomas, PhD, DATS, FACT
GRAS Expert Panel Member
Indiana University School of Medicine

Signature [redacted]

Date: December 21, 2015

#635

Generally Recognized as Safe (GRAS) Determination for Niagen™ (Nicotinamide Riboside Chloride)

Prepared for:

ChromaDex, Inc.
10005 Muirlands Boulevard., Suite G
Irvine, CA 92618

Prepared by:

Spherix Consulting, Inc.
11900 Parklawn Drive, Suite 200
Rockville, MD 20852

December 21, 2015

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I. EXECUTIVE SUMMARY

A. NAME AND ADDRESS OF SPONSOR

ChromaDex, Inc.
10005 Muirlands Boulevard, Suite G
Irvine, CA 92618

Contact:
Troy Rhonemus, Chief Operating Officer
Tel: 949-600-9734
Fax: 949-356-1634
TroyR@chromadex.com

B. COMMON AND USUAL NAME OF GRAS SUBSTANCE

The substance of this Generally Recognized As Safe (GRAS) determination is Nicotinamide Riboside Chloride (NR), a form of vitamin B_3. NR is a single chemical moiety containing nicotinamide and ribose (Chi and Sauve, 2013). NR is sold by ChromaDex under the name Niagen™.

C. INTENDED USE

ChromaDex, Inc. proposes to add Niagen to selected foods and beverages to provide a source of vitamin B_3 (vitamin waters, protein shakes, nutrition bars, gum and chews). The intended maximum use level is 0.027% by weight and will be in powdered beverages designed to be reconstituted with water or milk. The intended maximum use level in all other foods will be 0.0057% by weight.

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of Niagen as an ingredient in vitamin waters, protein shakes, nutrition bars, gum and chews, is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of Niagen from the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that Niagen is safe, and GRAS, under the intended conditions of use, the safety of the intake of this product has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts

qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

To demonstrate that Niagen is safe under its intended conditions of use, the safety of the intake of Niagen resulting from its consumption in foods and beverages at the intended level of use has been established. The safety determination is based on the following:

- Product is manufactured in a facility that complies with cGMP for foods.
- Product specifications are set to ensure a food-grade product; stability over shelf life has been documented.
- Finished product batches reproducibly meet compositional standards and comply with limits on contaminants appropriate for food-grade ingredients.
- All processing aids used in the production are determined GRAS for their use and/or comply with regulations set forth in 21 CFR for use in food.
- Vitamin B_3 is defined as the dietary precursor to nicotinamide adenine dinucleotide (NAD+) other than the amino acid tryptophan (Erdman et al. 2012). The two major forms of vitamin B_3, nicotinic acid and nicotinamide, are together commonly known as niacin, and constitute the most well-known NAD+ precursors. NR is a recently discovered form of vitamin B_3 (Erdman et al. 2012).
- Derivation of a tolerable upper intake level (UL)[1] for Niagen is based on:
  - A single dose clinical pharmacokinetic study demonstrated that Niagen is metabolized similarly to nicotinamide in healthy humans and can be utilized as a form of Vitamin B_3. No clinically adverse effects on hematology, clinical chemistry, urinalysis or liver or kidney function parameters were noted in this study of single doses of 100, 300 and 1000 mg.
  - Niagen was evaluated in published genotoxicology assays including an Ames assay, *in vitro* chromosome aberration assay, and *in vivo* micronucleus assay; results demonstrated that Niagen is not genotoxic under the conditions of these assays (Conze et al. 2015).

[1] "the highest daily level of chronic nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population"

- A published 90-day toxicology study evaluated rats gavaged daily with control vehicle (water), 300, 1000, or 3000 mg/kg Niagen or a positive control of nicotinamide at 1260 mg/kg/day dose level (equivalent to 3000 mg/kg/day dose of Niagen on equimolar basis) (Conze et al. 2015). NR is a form of Vitamin B_3 that is metabolized similarly to nicotinamide and other NAD+ precursors. Thus, a positive control group treated with nicotinamide was included.
  - Adverse effects at 3000 mg/kg body weight/day of NR included treatment-related adverse effects in liver, kidneys, testes, epididymides and ovaries. Importantly, these effects also occurred in the nicotinamide groups with a similar magnitude.
  - Niagen administration at 1000 mg/kg/day resulted in treatment related increases in liver and kidney relative weights and increases in neutrophils, leukocytes, ALT and triglycerides which were statistically significant in female rats only. Changes in neutrophils and leukocytes were not associated with significant inflammatory changes in all organs examined. The significant increase in ALT was noted only in one gender, occurred in conjunction with an increase in triglycerides but was below the 2-fold increase typically used to establish a biologically significant signal of liver damage in the absence of histological change (Hall et al. 2012). The increased kidney weight at this dose occurred in the absence of corresponding histopathology. Therefore, the effects in liver at 1000 mg/kg/day are considered to be treatment-related but mild and potentially adaptive in nature. Based on the minimal changes at 1000 mg/kg/day, this dose was considered the Low-Observed-Adverse-Effect-Level (LOAEL).
  - Administration of Niagen at 300 mg/kg/day did not result in treatment related adverse effects in any of the parameters monitored and is considered to be the No-Observed-Adverse-Effect-Level (NOAEL).
  - The NOAEL from the 90-day rodent study is 300 mg/kg/day and effects noted at the LOAEL of 1000 mg/kg/day are considered to be mild and potentially adaptive in nature due to prolonged exposure to this form of niacin. Therefore, application of a 100-fold safety factor applied to the NOAEL is considered appropriately conservative to derive a UL.
- Application of these safety factors to the NOAEL results in a UL of 3 mg/kg/day or 180 mg/day for a 60 kg individual.

- This UL is conservative and below the UL for nicotinamide identified by the European Commission of 900 mg/day (EC SFC, 2002) and 500 mg/day identified by the UK Expert Working Group (2003). The derivation of the UL for nicotinamide by the EU SCF (2002) considered major long-term studies in patients with Type 1 diabetes mellitus, at dosages of 2-3 g of nicotinamide per day. The UK Expert Group (2003) calculated the UL for nicotinamide based on human studies of large doses (up to 3000 mg/day for periods of up to 3 years).
  - Because animal toxicology studies on nicotinic acid and nicotinamide are limited and do not comply with current standardized testing protocols, such as OECD (Unna, 1939; Handler and Dann, 1942; Chen et al., 1938; OECD SIDS, 2002), authoritative bodies have used the results of clinical studies in which high doses of nicotinic acid and nicotinamide have been administered to derive the ULs.
  - Although nicotinamide does not appear to be associated with flushing, the IOM established a UL of 35 mg/day for adults 19 years and older for both nicotinic acid and nicotinamide based on flushing because it is considered to be protective against potential adverse effects. The European Commission Scientific Committee on Food (SCF) established a UL for nicotinic acid of 10 mg /day based on flushing (EC SFC, 2002).
  - NR may not cause flushing because just like nicotinamide, it does not bind GPR109a, the high affinity G-protein coupled receptor that is believed to play an important role in mediating the vasodilatory effects of nicotinic acid (Canto et al. 2012).

- Over 10% of the total U.S. population of 2+ years were identified as consumers of Niagen from the proposed food uses. The mean intakes of Niagen by all users aged 2+ years from all proposed food uses were estimated to be 51 mg/person/day or 0.8 mg/kg body weight/day. The heavy consumer (90th percentile all-user) intake of Niagen from all proposed food-uses in persons aged 2+ years was estimated to be 145 mg/person/day

- The Estimated Daily Intake (EDI) is below the established UL.

E. DETERMINATION OF GRAS

Determination of the GRAS status of Niagen under the intended conditions of use has been made through the deliberations of Drs. Roger Clemens, Thomas Sox, and John Thomas. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of Niagen and the potential human exposure resulting from its intended use as an ingredient in foods and beverages and have concluded:

> There is no evidence in the information reviewed on Niagen that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when this product is used at levels that might reasonably be expected from the proposed applications. Niagen is GRAS for use in foods and beverages as proposed by ChromaDex. Niagen is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

F. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., President, Spherix Consulting, Inc., 11900 Parklawn Drive, Suite 200, Rockville, MD 20852, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ClaireK@chromadex.com; or be sent to FDA upon request.

G. SIGNATURE

Pursuant to the criteria provided in proposed 21 CFR 170.36, ChromaDex, Inc. hereby notifies the Food and Drug Administration that there is no evidence from authoritative opinion or in the available literature on Niagen that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when Niagen are used under the intended conditions of use. Therefore, Niagen is GRAS for use in food products as proposed by ChromaDex, Inc. and is therefore excluded from the definition of a food additive and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

(b) (6)

Troy Rhonemus, COO
ChromaDex, Inc.

December 21, 2015
Date

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II. DESCRIPTION OF SUBSTANCE

A. IDENTITY

1. Chemical Name and Identity

The subject of this GRAS determination is Nicotinamide Riboside Chloride (NR), a form of vitamin B_3. NR is a single chemical moiety containing nicotinamide and ribose (Chi and Sauve, 2013). NR is sold by ChromaDex under the name Niagen.

Vitamin B_3 is defined as the dietary precursor to nicotinamide adenine dinucleotide (NAD+) other than the amino acid tryptophan (Erdman et al. 2012). The two major forms of vitamin B_3, nicotinic acid and nicotinamide, are together commonly known as niacin, and constitute the most well-known NAD+ precursors. NR is a recently discovered form of vitamin B_3 (Erdman et al. 2012).

2. Common or Trade Name

Nicotinamide Riboside Chloride (3-(Aminocarbonyl)-1-β-D-ribofuranosyl-pyridinium chloride (1:1)) is sold by ChromaDex, Inc., under the name Niagen.

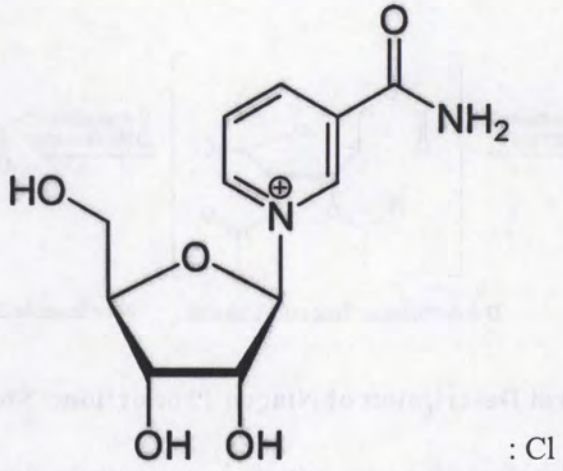
3. CAS Registry Number

Nicotinamide Riboside Chloride- 23111-00-4

4. Empirical and Structural Formula

$C_{11}H_{15}N_2O_5.Cl$

Figure 1. Structural Formula of Niagen - Nicotinamide Riboside Chloride



B. PHYSICAL AND CHEMICAL PROPERTIES

The physical and chemical properties of Niagen are presented in Table 1. Niagen is an off-white powder, soluble in water.

Table 1. Niagen-Physical and Chemical Properties

Property	Value
Molecular Weight	290.70
Appearance	Off white powder
Melting Point	115 -125°C
Solubility in water	446.5 mg/ml

C. PRODUCTION PROCESS

1. Process Description

Niagen is manufactured in accordance with current good manufacturing practice (cGMP) under 21 CFR § 110, in a two-step process. A brief description of the manufacturing steps is included below (Figures 2 and 3) and a flow diagram depicting the production process for Niagen is presented in Figures 4-6.

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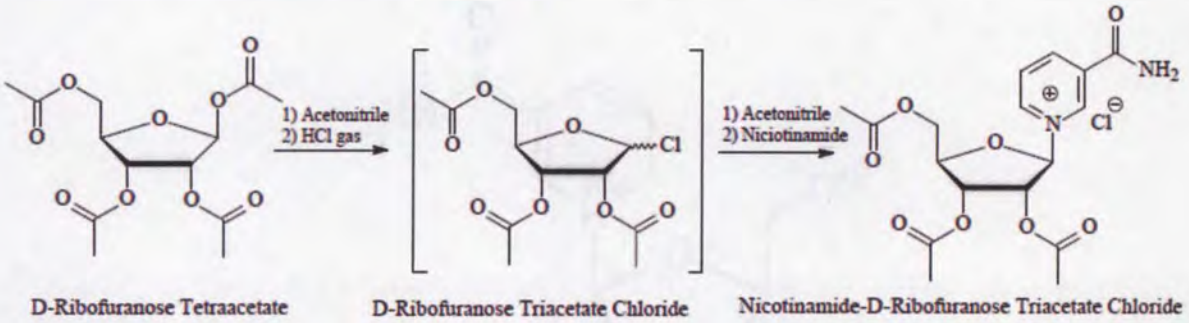
Figure 2. Niagen Production: Step 1

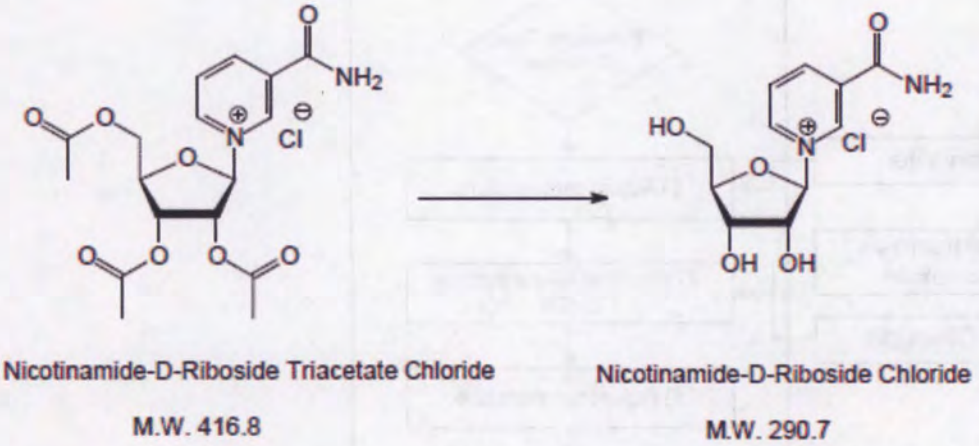


General Description of Niagen Production: Step 1

To a solution of D-Ribofuranose tetra-acetate in acetonitrile, gaseous hydrogen chloride is slowly charged. After completion of the charge, the reaction solution is sampled for reaction completion to the intermediate D-ribofuranose triacetate chloride via NMR. With the reaction confirmed complete, a slurry of nicotinamide in acetonitrile is charged and stirred until the reaction to Nicotinamide-D-Riboside Chloride is complete.

Since the reaction uses HCl and nicotinamide as reagents to do the coupling of the acetoxy ribose, residual nicotinamide gets converted to nicotinamide. HCl salt. With the coupling complete, the product slurry is cooled down and a molar excess of tributylamine (relative to excess nicotinamide) added to break the nicotinamide. HCl salt in order to facilitate it being washed off with acetonitrile and other solvents during the subsequent steps. The slurry is charged, stirred, isolated via centrifugation and washed with acetonitrile. The filter-cake is analyzed and held for use in Step 2 of the reaction. Isolated yield of Nicotinamide-D- Riboside triacetate is expected to be 48–50%.

Figure 3. Niagen Production: Step 2



General Description of Niagen Production: Step 2

Nicotinamide-beta-riboside triacetate chloride is slurried into methanol and chilled. While maintaining the solution chilled, ammonium hydroxide is slowly added, and the reaction solution mixed until de-acetylation is confirmed as complete by HPLC. The solution is placed under vacuum to strip the excess ammonia then methyl t-butyl ether is charged to precipitate the product. The chilled slurry is isolated via centrifugation. The methanol wet filter-cake is transferred back to the reactor and re-slurried in a cold methanol /water mix. The slurry is re-filtered via centrifugation and the methanol wet filter-cake re-slurred in an acetone/water mix. The slurry is checked for residual methanol in the filter-cake. If the methanol is not completely removed, the slurry is isolated via centrifugation and washed with acetone/water. The product is off-loaded and vacuum dried until residual solvent specifications are met. The material is then milled, sieved, and passed through the magnet to yield final product Nicotinamide Riboside Chloride.

Figure 4. Flow Diagram Showing the Production of Niagen (Step 1)

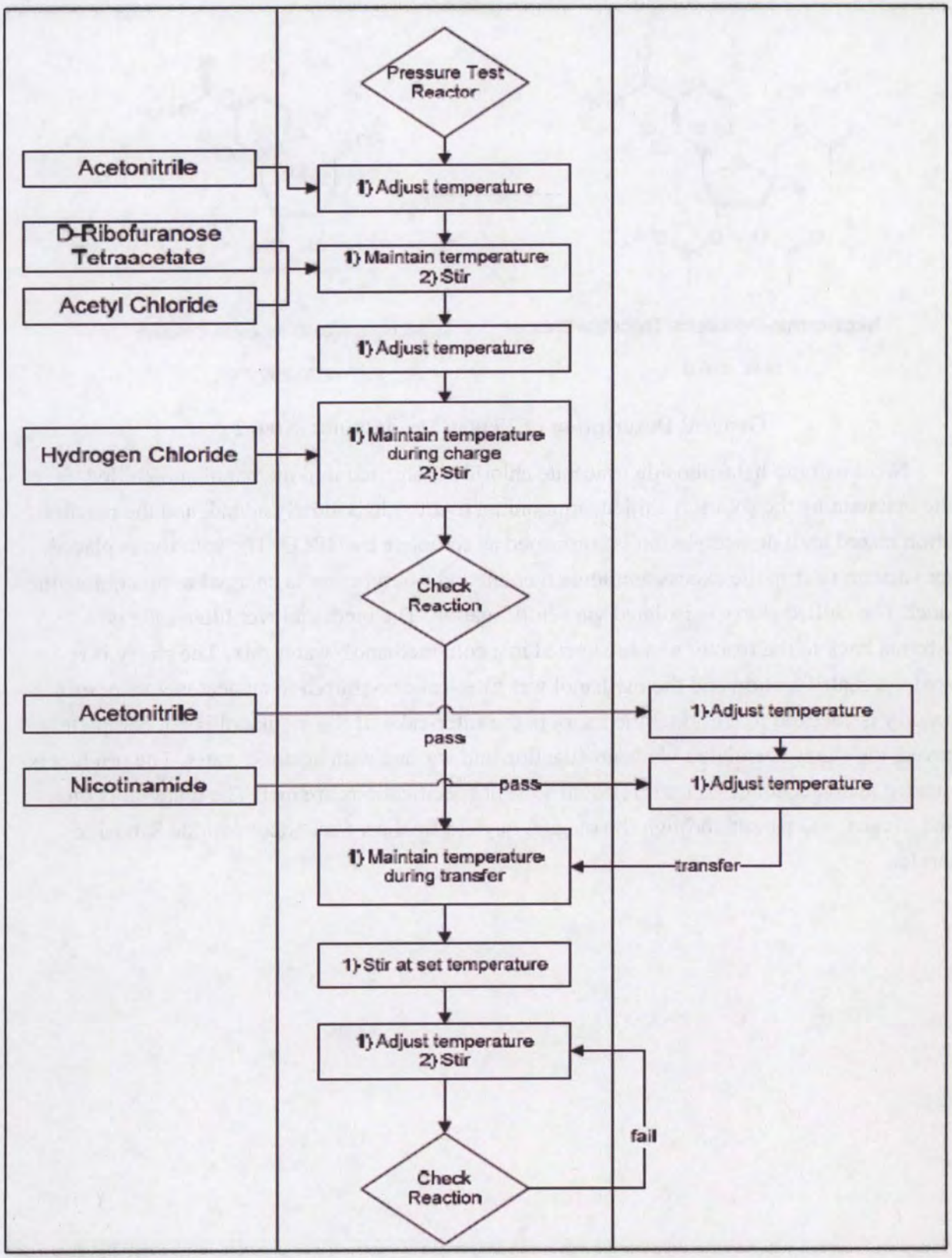


Figure 5. Flow Diagram Showing the Production of Niagen (Step 1) (continued)

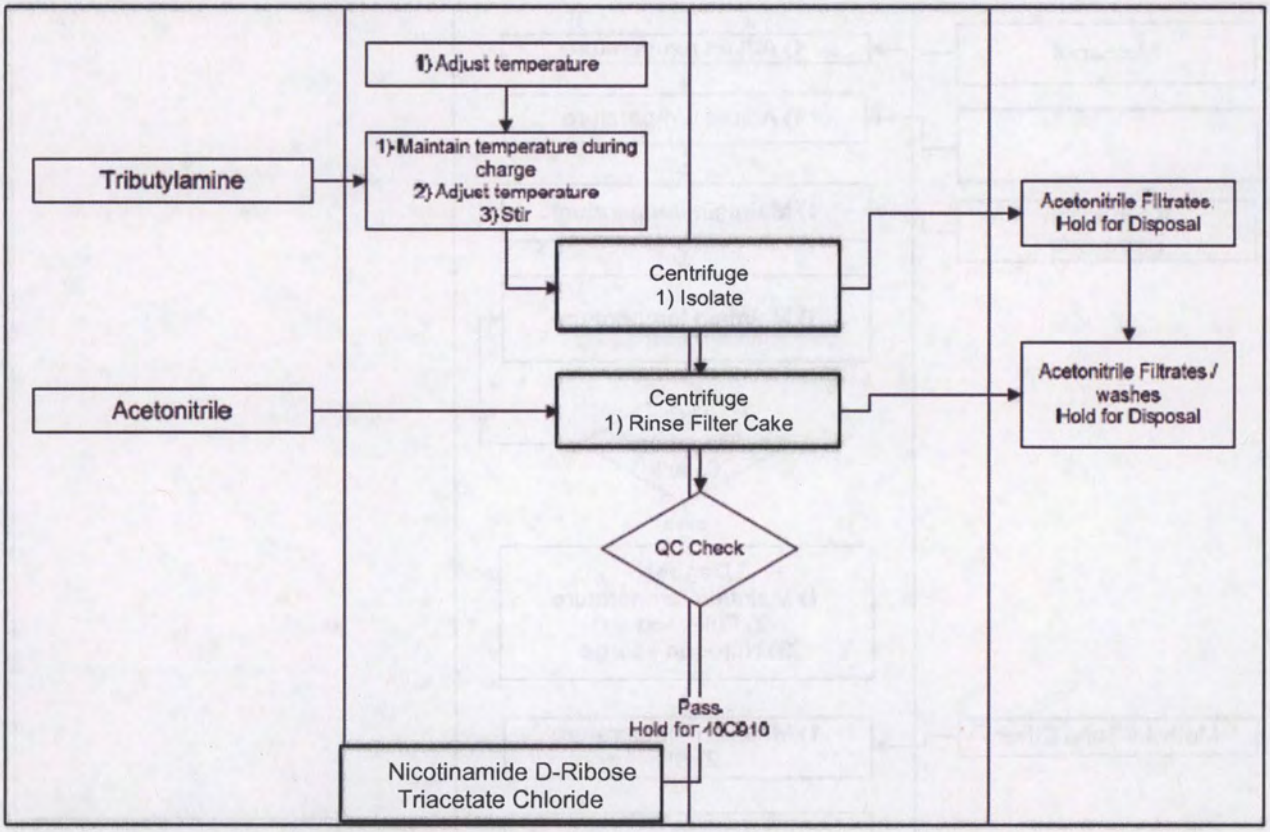


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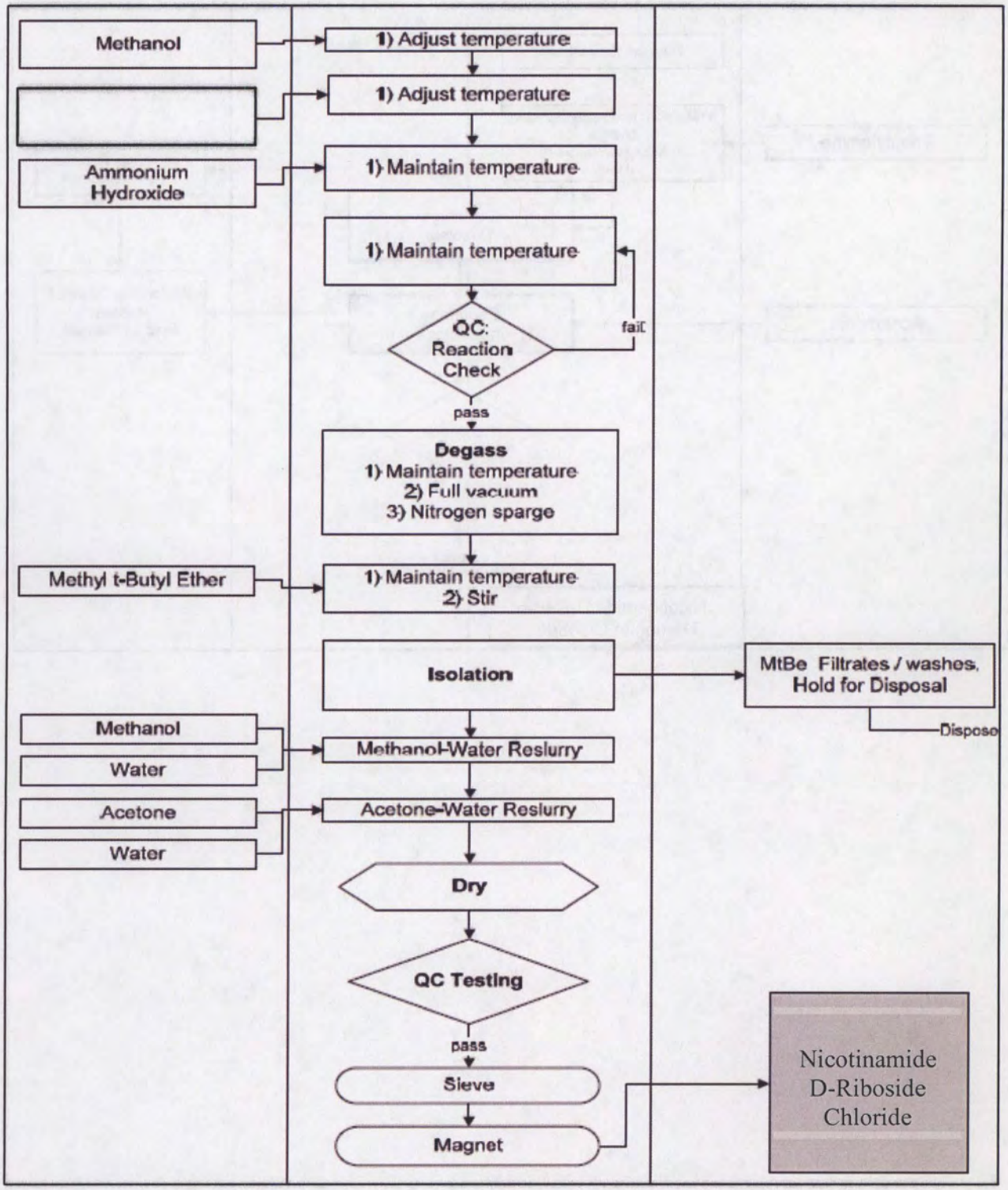
Figure 6. Flow Diagram Showing the Production of Niagen (Step 2)



2. Raw Materials and Chemicals

Certificates of Analysis (CoAs) for D-ribofuranose tetraacetate and nicotinamide document appropriate specifications; specifications for nicotinamide comply with USP 2015.

3. Processing Aids

Specifications are set for the final product to comply with appropriate controls on residual solvents and other processing aids for food. The processing aids used in the production of Niagen are listed below:

a. Solvents

Acetone: Approved as a secondary direct food additive under 21 CFR § 173.210 and is a Class 3 solvent [2] for pharmaceutical products with permitted daily exposure of 50 mg/day and a concentration limit of 5000 ppm (ICH Q3C (R5) 2011; MAPP 2014).

A residual amount of acetone in finished product of ≤ 3000 ppm is determined to be GRAS in GRN 491 for rooster comb extract. The calculated EDIs for rooster comb and Niagen are similar, therefore the limit of ≤ 3000 ppm determined in GRAS Notification 491 serves as precedent for a specification of ≤ 3000 ppm for acetone for Niagen.

Methyl t-butyl ether: Categorized as a Class 3 Solvent for Pharmaceutical products with permitted daily exposure of 50 mg/day and a concentration limit of 5000 ppm (ICH Q3C (R5) 2011; MAPP 2014). The solvent is safe for use as a processing aid for Niagen under the conditions that the specification for residue is not detected at the LOD (4ppm).

Acetonitrile: A residual amount of acetonitrile in finished product of ≤ 40 ppm is determined to be GRAS in GRN 202 for the use of polyoxyethanyl-alpha-tocopheryl sebacate (PTS) as a solubilizer for the dietary ingredient coenzyme Q10. The specification for residue is not detected at the LOD (6 ppm).

Methanol: A residual amount of methanol in finished product of ≤ 740 ppm is determined to be GRAS based on the methanol specification of 200 ppm in GRNs 448, 329, 323, 304, 303, 275, 253 for steviol glycosides. GRN 275 may be used for calculation purposes to

[2] Solvents in Class 3 may be regarded as less toxic and of lower risk to human health. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the solvents in Class 3. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies. It is considered that amounts of these residual solvents of 50 mg per day or less (corresponding to 5,000 ppm or 0.5 percent under Option 1) would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and good manufacturing practice (GMP).

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derive a specification for methanol for Niagen. The ADI for steviol equivalents is 4 mg/kg. In GRN 275, steviol glycoside is 36% steviol equivalents by weight, therefore the ADI of final product is up to 667 mg/day for a 60 kg person. At a specification of 200 ppm for methanol, this results in an exposure to methanol of 0.13 mg/day. At an ADI for Niagen of 180 mg/day for a 60 kg person, a specification of 740 ppm for methanol results in an exposure to methanol of 0.13 mg/day.

b. Other Processing Aids

***Sodium Carbonate*:** The affirmation of this ingredient as GRAS as a direct human food ingredient is under 21 CFR §184.1742. Ingredient must meet FCC specifications. In accordance with 21 CFR §184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice.

Acetyl Chloride: The affirmation of this ingredient as GRAS as a direct human food ingredient is under 21 CFR §184.1005. In accordance with 21 CFR §184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice.

Acetyl chloride is used in the first step as a moisture mopping agent to clean up any residual moisture present in the starting material (D-Ribofuranose tetraacetate) and reactor system. It is highly reactive with water (Lide et al., 1999; Budavari et al., 1996). The preparation of the D-Ribofuranose triacetate chloride intermediate using hydrogen chloride gas occurs efficiently when the reaction system is moisture free. Acetyl chloride is used in less than 0.05 molar equivalent levels relative to ribofuranose tetraacetate. Acetyl chloride has high solubility in organic solvents such as acetic acid, petroleum ether, acetone etc. This is expected to be cleared during the washing stage (with acetonitrile) in the first processing step. In the unlikely event that there was any residual acetyl chloride remains after the processing of step 1, it will get quenched in the second step during the addition of methanol (into methyl acetate) or with any residual moisture/water in the second step into acetic acid. Both methyl acetate and acetic acid are relatively innocuous chemicals. Based on the processing conditions for producing the Nicotinamide Beta-Riboside Chloride product, the possibility of any residual acetyl chloride remaining in the material is remote and therefore no specification was set.

***Hydrochloric acid (gas)*:** The affirmation of this ingredient as GRAS when used as a buffer and neutralizing agent is under 21 CFR § 182.1057; it is used in accordance with good manufacturing practice.

Tributyl amine (tri-n-butyl amine): is an amine base used in the first step of the NR production process to quench the reaction once completed. Tributyl amine is highly soluble in

acetone, ethanol, ethyl ether and most organic solvents. Solubility in water is relatively limited at about 142mg/L at 25°C.

Tributyl amine (tri-n-butyl amine): It is an amine base used in the first step of the Niagen production process to quench the reaction once completed. Tributyl amine is highly soluble in acetone, ethanol, ethyl ether and most organic solvents (Lide et al., 2000; Lewis et al., 1997). Solubility in water is relatively limited at about 142mg/L at 25°C (Riddick et al., 1985). The preparation and processing of Nicotinamide Beta Riboside Triacetate chloride (Step 1 product) involves rinsing the material with excess volumes of acetonitrile. The material is re-slurried and rinsed with additional volumes of acetonitrile. Any residual tributyl amine will be washed away during the slurry and rinsing process. In addition, the processing conditions during the subsequent second step preparation of Nicotinamide Beta-Riboside Chloride (Step 2 product) involves rinsing the material with large volumes of methanol, methyl-t-butyl ether and acetone.

In the unlikely event that there was any residual tributyl amine remaining after the processing of step 1, the rinsing and washing with organic solvents during step 2 of the process will remove any remaining residual. Based on the processing conditions for producing the Nicotinamide Beta-Riboside Chloride product, the possibility of any residual tributyl amine remaining in the material is extremely remote, therefore no specification was set.

Ammonia hydroxide: The affirmation of this ingredient as GRAS is under 21 CFR § 184.1139 with no limitation other than current good manufacturing practice. Ammonium hydroxide (NH_4OH, CAS Reg. No. 1336-21-6) is produced by passing ammonia gas into water.

Sulfuric Acid: The affirmation of this ingredient as GRAS as a direct human food ingredient is under 21 CFR §184.1095. Ingredient must meet FCC specifications. In accordance with 21 CFR §184.1(b)(1), the ingredient is used in food with no limitation other than current good manufacturing practice.

D. PRODUCT SPECIFICATIONS AND BATCH ANALYSES

1. Specifications for Niagen are Presented in Table 2

2. Batch Analysis Results

Batch analysis for three commercial batches of Niagen are shown in Table 2 and document that each batch of Niagen complies with the specifications established by ChromaDex, Inc. for this product. Accordingly, the batch data demonstrate that the production process for Niagen can consistently yield a product suitable for consumption.

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3. Impurities, Residuals, and Contaminants of Concern

Potential contaminants of Niagen include microbial contamination, heavy metals, residual solvents, processing aids and by-products. The specifications set for Niagen control these impurities to assure acceptable final product. Batch data for three different lots document control of final product to meet these specifications. Specifications and batch data are presented in Table 2.

a. Potential Reaction By-Products

Methyl acetate: It is a potential byproduct in the manufacture of Nicotinamide Riboside Chloride, but undetected in the final product. It is used as an extraction solvent in the production of some foods. In EU directive 88/344/EEC the European Union established a limit when used in the production of sugar from molasses of 1 mg/kg in the sugar. A limit of 20 mg/kg is permitted when used in decaffeination of coffee and tea. It is classified as a Class 3 Solvent for Pharmaceutical products with permitted daily exposure of 50 mg/day and a concentration limit of 5000 ppm (ICH Q3C (R5) 2011; MAPP 2014). A specification for Niagen is set at below the LOD/BLOQ (limit of detection/below limit of quantitation; 5/15 ppm).

Acetamide: Acetamide is a byproduct that could be formed during the preparation of the Nicotinamide Riboside Chloride as a result of the deacetylation step with ammonium hydroxide (Step 2). Acetamide is undetectable in the final product; it is removed based on its high solubility in solvents (alcohol, water) used to wash the product (Maryadele et al., 2006; Yalkowsky et al., 1992). The preparation and processing of Nicotinamide Riboside Chloride (Step 2 product) involves removing the volatiles under vacuum. Subsequently, the slurry is rinsed with large volumes of methyl-t-butyl ether, methanol, methanol/water and acetone /water. The use of these large volumes of organic solvents such as methanol, acetone and also volumes of water is expected to remove any residual acetamide that could remain in the product. The solubility in alcohol is high at about 0.5g/mL. Solubility in water is extremely high at >2g/mL at 25°C (Maryadele et al., 2006; Yalkowsky et al., 1992). A specification for Niagen is set at below the LOD/BLOQ (limit of detection/below limit of quantitation; 10/25 ppm)

Acetic Acid: Approved as a direct food substance affirmed as GRAS under 21 CFR §184.1005 at levels not to exceed current good manufacturing practice. A specification for Niagen is set at ≤ 5000 ppm.

Table 2. Specifications and Batch Analyses for Commercial Batches of Niagen

Parameter	Specifications	Method***	Batch 14207	Batch 14209	Batch 15201
Color	White to light brown	Visual	White to Off-white	White to Off-white	White to light brown
Form	Powder	Visual	Powder	Powder	Powder
Purity	95 – 102 (wt%) by HPLC	99.1-CD-3.0-000591	99.2	99.9	97.8
Identification	Conforms to structure by NMR	99.1-CD-1.0-000122	Conforms	Conforms	Conforms
Water Content	≤ 0 1%	99.1-CD-6.0-000094	0.1%	0.1%	0.1%
Residual Solvents					
Acetone	≤ 3000 ppm	99.1-CD-7.0-000115	1140	1576	1776
Methanol	≤ 740 ppm	99.1-CD-7.0-000115	172	263	631
Acetonitrile	ND*	99.1-CD-7.0-000115	ND	ND	ND
Methyl *t*-Butyl Ether	ND*	99.1-CD-7.0-000115	ND	ND	ND
Reaction by-products					
Methyl acetate	ND/BLOQ*	99.1-CD-7.0-000115	ND	ND/BLOQ**	ND
Acetamide	ND/BLOQ*	99.1-CD-1.0-000616	ND	ND	ND/BLOQ**
Acetic Acid	≤ 5000 ppm	99.1-CD-7.0-000115	1020	745	965
Microbiological limits					
Total Plate Count	≤1,000 CFU/g	AOAC 990.12; CMMEF, APHA CHP 7	<10 CFU/g	<10 CFU/g	<10 CFU/g
Yeast and Mold	≤100 CFU/g	AOAC 997.02; CMMEF APHA CHP 20	<10 CFU/g	<10 CFU/g	<10 CFU/g
E.coli	Absent/10g	USP <62>; USP <2022>	Absent/ 10g	Absent/ 10g	Absent/ 10g
Heavy Metals					
Arsenic	≤1 ppm	AOAC 993.14	<0.5 ppm	<0.5 ppm	<0.5 ppm
Mercury	≤ 1 ppm	AOAC 993.14	<0.1 ppm	<0.1 ppm	<0.1 ppm
Cadmium	≤ 1 ppm	AOAC 993.14	<0.25 ppm	<0.25 ppm	<0.25 ppm
Lead	≤ 0.5 ppm	AOAC 993.14	<0.05 ppm	<0.05 ppm	<0.05 ppm

**ND- Not Detected (For methyl acetate LOD is 5 ppm; for acetamide LOD is 10 ppm; for acetonitrile the LOD is 6 ppm; for Methyl t-Butyl Ether the LOD is 4 ppm)*
***BLOQ- Below Limit of Quantitation (For methyl acetate LOQ is 15 ppm; for acetamide LOQ is 25 ppm)*
****In House validated analytical methods*

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4. Stability of Niagen

Stability data of Niagen powder: Stability studies under controlled conditions are currently underway for Niagen in powder form. Results from ambient conditions (25° C and 60% relative humidity) and accelerated conditions (40° C. and 75% relative humidity) over 5 years at designated intervals will be used to establish product stability. Stability is established for 11 months under ambient conditions and 9 months under accelerated conditions.

Results from the first three months are presented in Tables 3 and 4 for ambient and accelerated conditions, respectively.

Table 3. Ambient Conditions: Batch # 14207 (Manufacture Date: August 12 2014)

Analyte	Units	Spec.	8 months	11 months
Water Content	%	< 1.0	< 0.2	< 0.2
Aerobic Plate Count	CFU/g	NMT 1×10^3	Pass (<10)	Pass (<10)
Yeast and Mold	CFU/g	NMT 1×10^2	Pass (<10)	Pass (<10)
Escherichia coli	/10g	Absent	Pass (ND)	Pass (ND)
Color	NA	White to light brown	Yellow-White D	Yellow-White D
Appearance	NA	Powder	Powder	Powder
NR Chloride	wt%	≥ 95%	99.2	98.9
Nicotinamide	wt%	≤ 5%	0.803	0.773

**Method Detection Limit*
***BRL- Below Reporting Limit*

Table 4. Accelerated Conditions: batch # 14207 (Manufacturing Date: August 12 2014)

Analyte	Units	Spec.	8 months	9 months	11 months
Water Content	%	< 1.0	BRL < 0.2	0.23	0.28
Aerobic Plate Count	CFU/g	NMT 1×10^3	Pass (<10)	Pass (<10)	Pass (<10)
Yeast and Mold	CFU/g	NMT 1×10^2	Pass (<10)	Pass (<10)	Pass (<10)
Escherichia coli	/10g	Absent	Pass (ND)	Pass (ND)	Pass (ND)
Color	NA	White to light brown	Yellow-White D	Yellow-White D	Yellow-White D
Appearance	NA	Powder	Powder	Powder	Powder
NR Chloride	wt%	≥ 95%	98.9	95.4	92.1
Nicotinamide	wt%	≤ 5%	0.773	2.17	3.93

**Method Detection Limit*
***BRL- Below Reporting Limit*

Stability data in solution: Stability studies under controlled conditions are currently underway for Niagen at a concentration of 1 mg/mL, using a range of acidic pH solutions (pH 2.5, 3.5, 4.7) and three different temperatures (4° C, 25° C and 40° C) (Tables 5-7). Levels of NR and nicotinamide are monitored at designated interval.

These preliminary studies indicate that Niagen is stable at 4 ° C for up to 83 days (95% NR). Ambient temperatures or higher (25° C and 40° C) resulted in considerable degradation of NR into nicotinamide within a day (82.4% NR), with further reductions to 38.9% by day 73. During incubation at higher temperature (40° C), Niagen undergoes accelerated degradation leading to almost complete degradation by three weeks at 40° C. The data also show that the acidic pH conditions tested (pH 2.5, 3.5, 4.7) have minimal impact on Niagen stability. Microbiological testing of Niagen in solution is currently ongoing.

Thus, Niagen is unstable in acidic solutions as a function of temperature. Beverages containing Niagen will be stored under refrigerated conditions for up to 83 days.

Table 5. Stability of Niagen (batch #14207) in a 1mg/mL solution- 4° C

Analyte	Units	pH	Day 0	Day 16	Day 55	Day 83
NR Chloride	wt%	2.5	100	98.7	97.2	95.6
Nicotinamide	wt%		0.00	0.780	1.77	3.03
NR Chloride	wt%	3.5	99.7	98.3	96.6	92.0
Nicotinamide	wt%		0.00	0.834	1.80	3.04
NR Chloride	wt%	4.7	100	98.6	97.2	94.3
Nicotinamide	wt%		0.00	0.795	1.82	2.91

Table 6. Stability of Niagen (batch #14207) in a 1mg/mL solution- 25° C

Analyte	Units	pH	Day 0	Day 14	Day 20	Day 35	Day 55	Day 73
NR Chloride	wt%	2.5	100	82.4	77.1	64.6	50.0	38.9
Nicotinamide	wt%		0.00	7.68	10.1	15.6	21.8	26.4
NR Chloride	wt%	3.5	99.7	81.7	76.7	63.7	52.6	38.5
Nicotinamide	wt%		0.00	7.78	10.2	15.8	23.5	26.4
NR Chloride	wt%	4.7	100	82.1	76.7	64.3	49.5	38.8
Nicotinamide	wt%		0.00	7.43	9.68	14.8	20.4	24.6

Table 7. Stability of Niagen (batch #14207) in a 1mg/mL solution- 40° C

Analyte	Units	pH	Day 0	Day 1	Day 3	Day 7	Day 14	Day 20
NR Chloride	wt%	2.5	100	80.6	53.1	21.5	4.74	1.38
Nicotinamide	wt%		0.00	8.44	19.9	32.8	40.3	42.0
NR Chloride	wt%	3.5	99.7	80.3	53.3	21.6	4.69	1.36
Nicotinamide	wt%		0.00	8.55	20.0	32.8	40.1	42.2
NR Chloride	wt%	4.7	100	79.6	52.7	21.6	4.60	1.33
Nicotinamide	wt%		0.00	8.57	20.1	33.5	40.4	43.5

5. Analytical Methods

ChromaDex is using a validated method to establish identity and purity of Nicotinamide Riboside Chloride by HPLC. Structural confirmation support comes from NMR and mass spectral analysis.

6. Adverse Events/Serious Adverse Events

ChromaDex has a Standard Operating Procedure in place for receiving, documenting, and reporting Adverse Health Effects.

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III. INTENDED TECHNICAL EFFECT

A. INTENDED TECHNICAL EFFECT

Niagen will be added to foods and beverages as another source of vitamin B_3.

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IV. HISTORY OF USE AND INTENDED USE

A. HISTORY OF USE: NATURAL OCCURRENCE OF NICOTINAMIDE RIBOSIDE (NR) IN FOODS AND DAIRY PRODUCTS

Humans are exposed to NR via dietary sources such as milk. It is thought that NR in milk is derived from catabolism of NAD+. Unpublished studies indicate that fat-free non-organic and organic cow's milk contain 3.1 μM and 1.9 μM of NR, respectively (Table 8, Trammel and Brenner, personal communication), indicating that NR is the second most abundant NAD precursor in milk after nicotinamide. Moreover, NR levels in milk do not change significantly when milk is stored at room temperature for 24 hrs (Brenner, unpublished). Thus, the estimated amount of NR ingested by humans from the equivalent of 710 ml/day (3 cups) of cow's milk is ~545 μg/day.

Table 8. Levels of NR and Related Metabolites in Fat-Free Bovine Milk (μM ± SEM)

Metabolite	Organic milk (n = 4)	Commercial milk (n = 4)
Nicotinamide	5.6 ± 1.2	5.2 ± 1.7
Nicotinamide Riboside	1.9 ± 0.49	3.1 ± 0.80
Nicotinic acid Riboside	<0.4	<0.4
Nicotinic acid	<1.0	<1.0
Nicotinamide Mononucleotide	<0.4	<0.4
NADH	<0.08	<0.08
NAD	<0.08	<0.08
NaMN	<0.03	<0.03
NADP	<0.02	<0.02
NaAD	<0.008	<0.008

Method=LC-MS/MS *(Trammell and Brenner, 2013)*

B. INTENDED USE

1. Introduction

ChromaDex intends to add Niagen (nicotinamide riboside) to the following foods and beverages in the U.S. food supply: vitamin waters, protein shakes, nutrition bars and gums or chews. The intended maximum use level is 0.027% by weight and will be in powdered beverages designed to be reconstituted with water or milk. The intended maximum use level in all other foods will be 0.0057% by weight.

Estimates for the intake of Niagen were based on the proposed food uses and maximum use levels in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) 2009-2010 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2006; USDA, 2012; Bodner-Montville et al, 2006). Calculations for the mean and 90th percentile intakes were performed for all proposed food uses of Niagen combined. The intakes were reported for the following population groups:

- infants, ages 0 to 1 year,
- toddlers, ages 1 to 2 years,
- children, ages 2 to 5 years,
- children, ages 6 to 12 years,
- teenagers, ages 13 to 19 years,
- adults, ages 20 years and up,
- total population (age groups from 2 and up combined, excluding infants of 0-1 year and toddlers of 1-2 years)

2. Food Consumption Survey Data

a. Survey Description

The National Health and Nutrition Examination Surveys (NHANES) for the years 2009-2010 are available for public use. NHANES are conducted as continuous, annual surveys, and are released in 2-year cycles. In 2009-2010, approximately 10,000 people across the U.S. completed the health examination component of the survey. Any combination of consecutive years of data collection is a nationally representative sample of the U.S. population. It is well

established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Gregory et al, 1995). Because two 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) are available from the NHANES 2009-2010 survey, these data were used to generate estimates for the current intake analysis.

The NHANES provide the most appropriate data for evaluating food-use and food-consumption patterns in the United States, containing 2 years of data on individuals selected via stratified multistage probability sample of civilian non-institutionalized population of the U.S. NHANES 2009-2010 survey data were collected from individuals and households via 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Day 1 data were collected in-person in the Mobile Examination Center (MEC), and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed. Fifteen PSUs are visited each year. For the 2009-2010 NHANES, there were 13,272 persons selected; of these 10,253 were considered respondents to the MEC examination and data collection. 9754 of the MEC respondents provided complete dietary intakes for Day 1 and of those providing the Day 1 data, 8,405 provided complete dietary intakes for Day 2.

In addition to collecting information on the types and quantities of foods being consumed, NHANES 2009-2010 collected socioeconomic, physiological, and demographic information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. Among those who completed food intake survey on both Day 1 and Day 2, 8301 respondents also provided physiological information including age, sex and weight; of these 7738 were 2 years and older.

Sample weights were incorporated with NHANES 2009-2010 to compensate for the potential under-representation of intakes from specific population groups as a result of sample

variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (CDC, 2006; USDA, 2012).[3]

b. Statistical Methods

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of Niagen by the U.S. population. The statistical programming language R (https://www.r-project.org/) was used for ETL (Extract-Transform-Load) operations and Matlab (Natick, MA, http://www.mathworks.com/products/matlab/) for collating and calculations on data gathered using R. Estimates for the daily intake of Niagen represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2009-2010 data; these average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated incorporating sample weights in order to provide representative intakes for the entire U.S. population. "All-person" intake refers to the estimated intake of Niagen averaged over all individuals surveyed, regardless of whether they consumed food products that will contain Niagen, and therefore includes "zero" consumers (those who reported no intake of food products containing Niagen during the 2 survey days). "All-user" intake refers to the estimated intake of Niagen by those individuals consuming food products that will contain Niagen, hence the "all-user" designation. Individuals were considered users if they consumed 1 or more food products that will contain Niagen on either Day 1 or Day 2 of the survey.

3. Food Usage Data

Food codes representative of each proposed use were chosen from the Food and Nutrition Database for Dietary Studies (FNDDS). In FNDDS, the primary (usually generic) description of a given food is assigned a unique 8-digit food code (CDC, 2006; USDA, 2012).

Niagen will be incorporated into foods from 4 food categories and 47 food codes. The amount of Niagen to be added to each food code is determined to be 0.027% (w/w) for the powdered beverages designed to be reconstituted with water. This concentration is designed to yield an average concentration of 0.0057% (w/w) once water is added to the drinks. The intended maximum use level in all other foods is also 0.0057% by weight.

[3] A sample weight is assigned to each sample person. It is a measure of the number of people in the population represented by that sample person in NHANES, reflecting the unequal probability of selection, non-response adjustment, and adjustment to independent population controls.

4. Food Survey Results

The estimated "all-user" daily intakes of Niagen from proposed foods supplemented with 0.0057% Niagen is summarized in Table 9 below.

Table 9. Estimated "All-user" Daily Intake (EDI) of Niagen in Foods Supplemented with 0.0057% Niagen (wt/wt) by Population Group (2009-2010 NHANES Data)

Population Group	N users	N population	% Users	Mean mass (kg)	Mean EDI (mg)	90th % EDI (mg)	Mean EDI (mg/kg)	90th % EDI (mg/kg)
ages 0-1	7	408	1.72	7.9	31.06	38.88	3.93	4.92
ages 1-2	12	235	5.11	11.6	53.93	97.19	4.64	8.36
ages 2-5	83	764	10.86	15.7	47.85	84.23	3.04	5.35
ages 6-12	205	1388	14.77	33.6	50.42	107.99	1.50	3.21
ages 13-19	139	1124	12.37	65.4	57.61	138.23	0.88	2.11
ages 20 and up	505	5812	8.69	81.6	49.86	150.00	0.61	1.84
ages 2 and up	932	9088	10.26	66.6	50.71	144.71	0.76	2.17

5. Conclusions

In summary, over 10% of the total U.S. population of 2+ years were identified as consumers of Niagen from the proposed food uses. The mean intakes of Niagen by all Niagen consumers aged 2+ years ("all-user") from all proposed food uses were estimated to be 51 mg/person/day or 0.8 mg/kg body weight/day. The heavy consumer (90th percentile all-user) intake of Niagen from all proposed food-uses in persons aged 2+ years was estimated to be 145 mg/person/day or 2.2 mg/kg body weight/day.

V. SAFETY OF NICOTINAMIDE RIBOSIDE

The two major forms of vitamin B_3, nicotinic acid and nicotinamide, are together commonly known as niacin, and constitute the best well-known NAD+ precursors. Nicotinamide riboside (NR) is a single chemical moiety containing nicotinamide and ribose and, because it is a precursor of nicotinamide adenine dinucleotide (NAD+), is considered to be a form of vitamin B_3 (reviewed in Chi and Sauve 2013; Penberthy and Kirkland, 2012; Bogan and Brenner, 2008).

Intake of nicotinic acid at doses of 50 mg/day and higher consistently results in undesirable side effects, including vasodilation effects (flushing) and pruritus (itching), which can occur within 30 minutes following oral administration (Guyton and Bays, 2007), whereas NR and nicotinamide are not associated with these adverse effects. NR may not cause flushing because just like nicotinamide, it does not bind GPR109a, the high affinity G-protein coupled receptor that is believed to play an important role in mediating the vasodilatory effects of nicotinic acid (Canto et al. 2012).

A. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME) OF NICOTINAMIDE RIBOSIDE

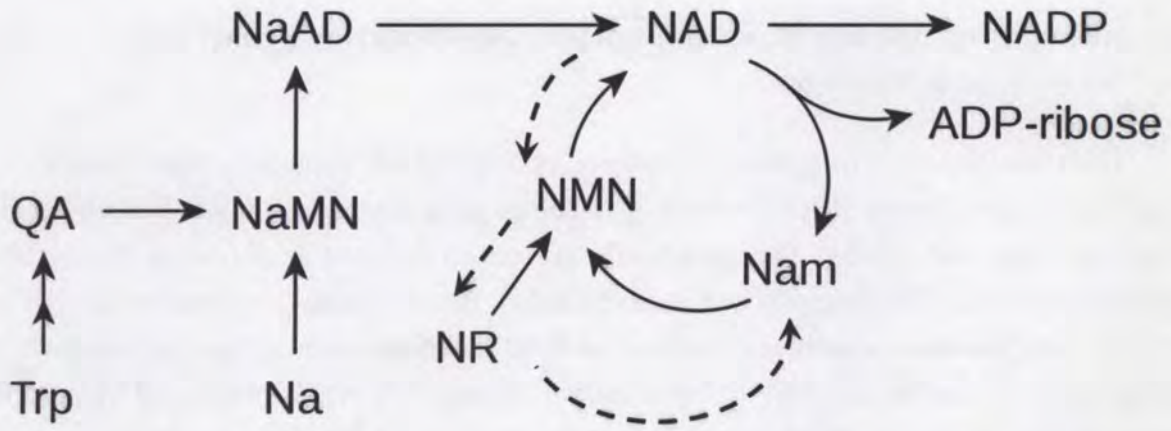
Even though cells are capable of synthesizing NAD+ from tryptophan *de novo*, it is thought that a major source of NAD+ production comes from salvage pathways (Belenky et al., 2007a; Rongvaux et al., 2003). The main NAD+ precursors that feed the salvage pathways are nicotinamide and NR (Bieganowski and Brenner, 2004). Recent studies have shown that NR is used in a conserved salvage pathway that leads to NAD+ synthesis through the formation of nicotinamide mononucleotide (NMN). Upon entry in the cell, NR is phosphorylated by the NR kinases (NRKs), generating NMN, which is then converted to NAD+ by nicotinamide mononucleotide adenylyltransferase (NMNAT) (Bieganowski and Brenner, 2004). Because NMN is the only metabolite that can be converted to NAD+ in mitochondria, nicotinamide and NR are the two candidate NAD+ precursors that can replenish NAD+ and thus improve mitochondrial fuel oxidation (Figure 7).

Studies indicate that nicotinamide is the predominantly absorbed form of niacin when the dietary source is NAD+ (Kimura et al., 2006; Sadoogh-Abasian and Evered, 1980). It has also been reported that NAD+ is metabolized by pyrophosphatases to NMN and hydrolyzed to NR, which was found in the lumen of the upper small intestine in rats (Baum et al., 1982; Gross and Henderson, 1983). According to Gross and Henderson (1983), up to 80% of NAD+ is converted to NR after 15 minutes in the rat intestine lumen. Subsequently, a fraction of NR in the intestine is hydrolyzed to nicotinamide by enzymatic action of mucosal cells. The absorption rate of NR in

the intestine is far slower than the conversion rate of NR to nicotinamide, which is readily absorbed. Therefore, it is plausible that NR is incorporated into the bioavailable NAD+ pool in two ways: via the action of the Nrk pathway (Bieganowski and Brenner, 2004) or via the nicotinamide salvage pathway after conversion to nicotinamide by phosphorolysis in the gut (Gross and Henderson 1983).

Figure 7. Biosynthetic and Salvage Pathways in NAD Metabolism

De novo biosynthesis of NAD+ begins with the conversion of tryptophan (Trp) to NAD in a series of enzymatic steps. NAD+-consuming enzymes, such as sirtuins, break the bond between the nicotinamide (Nam) and ADP-ribosyl moieties of NAD, producing ADP-Ribose and Nam. Nam, which is also a dietary component, is salvaged to form NMN, which is adenylylated to form NAD+. NR coming from dietary sources is salvaged by nicotinamide riboside kinases (Nrk1 and Nrk2) and converted to nicotinamide mononucleotide (NMN). In the gastrointestinal tract, dietary NAD can be hydrolyzed to NR, which in turn is further converted to Nam and absorbed by the intestine (dashed arrows). Because NMN is the only metabolite that can be converted to NAD in mitochondria, nicotinamide and NR are the only NAD+ precursors that can replenish mitochondrial NAD levels.



Legend: Trp: tryptophan, **QA**: quinolinic acid, **Na**: nicotinic acid, **NaMN**: nicotinic acid mononucleotide, **NaAD**: nicotinic acid adenine dinucleotide, **NAD**: nicotinamide adenine dinucleotide, **NADP**: nicotinamide adenine dinucleotide phosphate, **NR**: nicotinamide riboside, **NMN**: nicotinamide mononucleotide, **Nam**: nicotinamide, **ADP-ribose**: adenosine diphosphate ribose.

B. PHYSIOLOGIC EFFECTS:

Several studies found that NR supplementation resulted in increased NAD+ levels in yeast and cultured human cells (Belenky et al., 2007a; Yang et al., 2007, Canto et al., 2012). NR also extended the lifespan of yeast cells in a Sir2-dependent manner, suggesting that NAD+-mediated sirtuin activation is the potential mechanism of action (Belenky et al., 2007b).

More recently, several studies (summarized in Table 10), evaluated the physiologic effects of prolonged NR-supplementation in rodents (Canto et al., 2012; Khan et al., 2014, Brown 2014, Tummala et al., 2014, Scheibye-Knudsen et al., 2014). Canto et al. (2012) fed wild-type mice a high fat diet (35% fat, 26% carbohydrate, and 26% protein) with and without NR for 12 weeks. The mice receiving NR gained weight at a slower rate, had improved insulin sensitivity, increased endurance, enhanced mitochondrial oxidative capacity in brown adipose tissue, were more able to maintain body temperature during cold exposure, and increased muscle and liver NAD+ concentrations compared to the mice receiving the high fat diet alone. Khan et al. (2014) fed wild-type (control) and Deletor mice, which are prone to developing mitochondrial myopathy, a normal diet (11% fat, 65% carbohydrate, and 24% protein) supplemented with and without NR for 16 weeks. Although NR supplementation did not significantly increase skeletal muscle NAD+ concentrations in either wild-type or Deletor mice, the NR-containing diet increased mitochondrial biogenesis in skeletal muscle and enhanced lipid oxidation in brown adipose tissue of both wild-type and Deletor mice. More recently, in a study reported by Tummala et al., (2014) NR supplemented diet significantly increased pancreatic NAD+ levels after 12 weeks in a murine cancer model.

The effects of NR supplementation has also been assessed in several animal models of neurodegeneration (Gong et al., 2013; Shindler et al., 2007, Brown et al., 2014). Gong et al. (2013) fed Tg2576 transgenic mice that develop symptoms that resemble Alzheimer's disease (AD) NR for 4 months and found that NR supplementation attenuated the effects of beta-amyloid toxicity at the behavioral and cellular levels (Gong et al., 2013). Shindler et al. (2007) injected NR intraocularly to mice with Experimental Autoimmune Encephalomyelitis (EAE). Compared to mice injected with PBS, injections of NR delayed retinal ganglion cell (RGC) death. Sasaki et al. (2006) treated mouse dorsal root ganglion neurons with NAD+ precursors including NR, nicotinamide and nicotinic acid, and found that all three increased activity of the NAD+ pathway and delayed axonal degeneration. Brown et al. (2014) showed that following acoustic trauma caused by high decibel noise exposure, cochlear NAD+ levels were decreased in mice, and that injection of NR was able to rescue the effects of acoustic trauma which normally results in noise-induced hearing loss. Another study by Scheibye-Knudsen et al., (2014) showed that NR injections in mouse model of Cockayne Syndrome can partially restore reduced cerebellum NAD+ levels. Taken together, these results suggest that increased NAD+ availability resulting from NR supplementation may delay neuronal degeneration.

In some of these studies it was demonstrated that NR is as effective as other NAD+ precursors (nicotinamide and nicotinic acid) in increasing NAD+ levels (Chi and Sauve, 2013). Thus, Chi and Sauve (2013) proposed that NR is a third form of vitamin B_3 and plays a role in ameliorating metabolic and age-related deficiencies of mitochondrial function.

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Table 10. Physiological and Safety Observations from Supplementation with Nicotinamide Riboside -Animal Studies

Reference	Study Design	NR Dose and Route Of Administration	Duration	Physiology Endpoints	Safety or Toxicity Observations
Canto et al., 2012	Mammalian cell lines HEK293T	range 0-1000uM in culture media	24 hrs	Increased intracellular NAD+ levels No activation of overexpressed GPR109A	No cell death reported
	Mice raised on a high fat diet	400 mg/kg/day in chow	16 wks	Reduced weight gain, increased energy metabolism. Significantly increased NAD+ levels in muscle. Improved energy metabolism in mitochondria	No adverse effects reported
Khan et al., 2014	Wild-type and Deletor mice, which are prone to developing mitochondrial myopathy (MM)	400 mg/kg/day in chow	16 wks	NR treatment delayed early- and late-stage disease progression in muscle and increased mitochondrial biogenesis. No statistically significant increases in NAD+ levels in muscle and liver after 16 wks of NR administration	No adverse effects reported
Gong et al., 2013	Tg2576 mice Transgenic model for Alzheimer's Disease 7-8 months old	250 mg/kg/day in drinking water	3 months	Improved cognitive function in behavioral assays, coinciding with increased NAD+ levels in the cerebral cortex Attenuated Aβ toxicity through activation of PGC-1alpha transcription	No adverse effect reported in vivo
	Hippocampal brain slices from Tg2576 mice	10uM in bath	4 hrs	Improves synaptic function in hippocampal slices in culture No difference in Long Term Potentiation (LTP)	No toxicity reported
Sasaki et al.,2006	Mouse dorsal root ganglion neurons ex vivo	1mM in culture media	24 hrs before axon transection	Delayed axonal degeneration, possibly due to Sirt1 activation	No toxicity reported
Shindler et al., 2007	Mice treated with M tuberculosis-EAE model	Intraocular injection of 16.67 mM or 66.67 mM	up to 14 days	Prevented retinal degeneration of RGC neurons, possibly due to Sirt1 activation	No change in RGC survival in control mice

Table 10. Physiological and Safety Observations from Supplementation with Nicotinamide Riboside -Animal Studies

Reference	Study Design	NR Dose and Route Of Administration	Duration	Physiology Endpoints	Safety or Toxicity Observations
Brown et al. 2014	Mouse model (*WldS* mutation) resistant to noise-induced hearing loss. WT mice	Twice a day injection of 1,000 mg/kg of NR.	5 days prior to acoustic trauma, up to 14 days following acoustic trauma induction.	The *WldS* allele expresses a gene fusion between the NAD+ biosynthetic enzyme *Nicotinamide mononucleotide adenylyl transferase 1* and *Ube4a*. Following noise exposure, cochlear NAD+ levels were decreased in WT but not in WldS mice. NR prevented noise-induced hearing loss (NIHL) and spiral ganglia neurite degeneration.	No toxicity reported.
Tummala et al. 2014	Genetically engineered mouse model for hepatocyte-specific carcinoma induction	500 mg NR/kg/day in diet	48 wks	Nicotinamide Riboside (NR) restored NAD+ pools, and seemed to prevent DNA damage and tumor formation. NR supplementation significantly increased pancreatic NAD+ levels after 12 weeks of NR supplementation in both normal and mutant Ela-1-myc mice.	No toxicity reported.
Scheibye-Knudsen et al., 2014	Csb m/m Mouse model for Cockayne Syndrome	Injection of 500 mg NR /kg/d	1 wk	Older Csb m/m mice had reduced cerebellum NAD+ levels compared to younger Csb m/m mice. After 1 week of saline injections, NR significantly increased NAD+ levels in both WT and Csb m/m mice.	No toxicity reported.

C. TOXICOLOGY STUDIES

1. Summary

Pivotal published genotoxicity and toxicology studies demonstrate the safety of ingestion of Niagen at proposed intake levels (Conze et al. 2015). Niagen was evaluated in an Ames assay, *in vitro* chromosome aberration assay, and *in vivo* micronucleus assay; results demonstrated that Niagen is not genotoxic under the conditions of these assays

Niagen is not acutely toxic in rats, with no mortality occurring at the limit dose of 5000 mg/kg. Results from a 14-day range finding study were used to determine the doses in a 90-day toxicology study in rats. In the 90-day toxicology study, administration of Niagen at 0, 300, 1000 or 3000 mg/kg/day and nicotinamide (positive control) at 1260 mg/kg/day by oral gavage to Sprague Dawley rats for 90 consecutive days resulted in treatment-related adverse findings at 1000 and 3000 mg/kg/day (Niagen) and 1260 mg/kg/day (nicotinamide). Administration of Niagen at 300 mg/kg/day did not result in treatment-related adverse effects in any of the parameters monitored.

Adverse effects at 3000 mg/kg/day Niagen and nicotinamide included: treatment-related organ weight changes in liver, kidneys, testes, epididymides and ovaries; decrease in body weight, food consumption; increases in clinical chemistry parameters related to hepatocyte damage correlated with liver weight increase, centrilobular hepatocellular hypertrophy and single cell necrosis. In addition, thyroid follicular cell hypertrophy and increased kidney weight with exacerbation of chronic progressive nephropathy were observed. Importantly, similar types and magnitude of adverse effects were noted in the nicotinamide positive control group.

Niagen administration at 1000 mg/kg/day dose level resulted in treatment related organ weight changes in liver and kidney and increases in neutrophils, ALT and triglycerides which were statistically significant in female rats only. These changes were considered adverse based on dose response. The significant increases in ALT at this dose were noted only in one gender, occurred in conjunction with an increase in triglycerides but were below the 2-fold increase typically used to establish a biologically significant signal of liver damage in the absence of histological change (Hall et al. 2012). The kidney weight increases at this dose occurred in the absence of corresponding histopathology. Therefore, the effects in liver at 1000 mg/kg/day are considered to be treatment-related but mild and potentially adaptive in nature due to prolonged exposure to this form of niacin. Based on the minimal changes at 1000 mg/kg/day, this dose was considered the LOAEL.

Administration of Niagen at 300 mg/kg/day did not result in any treatment related adverse effects in the parameters monitored. A slight decrease (8%) in overall body weight (Day 90) was considered adaptive and therefore, not adverse.

Under the conditions of the 90-day study, the LOAEL of Niagen is 1000 mg/kg/day and the NOAEL of Niagen is 300 mg/kg/day in males and females Sprague Dawley rats when administered test article by oral gavage for 90 days.

D. GENOTOXICITY AND MUTAGENICITY

1. Bacterial Reverse Mutagenicity (Ames Assay)

a. Methods

Bacterial reverse mutation assays were performed in compliance with the Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practices (GLP) and Guideline No. 471. Niagen (>99% nicotinamide riboside chloride; CAS No. 23111-00-4) was supplied by ChromaDex, Inc. 2-aminoanthracene, 2-nitrofluorene, sodium azide, 9-aminoacridine, and 4-nitroquinoline-*N*-oxide were obtained from Sigma Aldrich Chemical Co., Inc. Aroclor 1254-induced rat liver S9 homogenate was obtained from Xenometrix AG. *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 were obtained from the National Collection of Type Cultures. *Escherichia coli* WP2 uvrA (pKM101) was obtained from Xenometrix.

The mutagenicity of Niagen was determined using the plate incorporation and pre-incubation methods. In the plate incorporation method, 50, 159, 501, 1582, and 5000 µg Niagen was mixed with selective top agar containing 0.6-0.8% agar, 0.5% NaCl, the tester strains *S. typhimurium* TA98, TA100, TA1535 and TA1537 or *Escherichia coli* WP2 *uvr*A pKM101, histidine and biotin or tryptophan (depending on the type stain used), with and without of a metabolic activation system (S9 mix; 5-30% of the Aroclor 1254-induced rat liver S9 homogenate, NADP, glucose-6-phosphate, magnesium chloride, and potassium chloride) at 45-50°C. The mixture was overlaid onto solidified Vogel-Bonner minimal E basal agar (Vogel and Bonner, 1956), and after the selective top agar solidified, the plates were incubated at 37°C for 67 hr. The plates were examined for the presence of a background lawn and precipitate, and the number of revertant colonies were counted manually. In a confirmatory assay, the tester strains *S. typhimurium* TA98, TA100, TA1535 and TA1537 or *Escherichia coli* WP2 *uvr*A pKM101, and 99, 265, 699, 1869, or 5000 µg Niagen were preincubated at 37°C for 30 min in the presence or absence of the S9 mix. Molten selective top agar containing 0.6-0.8% agar, 0.5% NaCl, and histidine and biotin or tryptophan (depending on the type stain used) was then added

and the resulting mixture was plated and incubated at 37°C for 67 hr. The plates were examined for the presence of a background lawn and precipitate, and the number of revertant colonies were counted manually.

Importantly, all experiments were performed in triplicate with vehicle and strain-specific positive controls. In the presence of the S9 mix, the positive controls for all strains was 2-aminoanthracene. In the absence of the S9 mix, the positive controls for strains TA98, TA100 and TA1535, TA1537, and WP2 *uvr*A pKM101 were 2-nitrofluorene, sodium azide, 9-aminoacridine, and 4-nitroquinoline-*N*-oxide, respectively. Niagen was considered cytotoxic if there was a 50% reduction in the mean number of revertants per plate compared to the mean vehicle control and/or at least a moderate reduction in the background lawn. Niagen was considered mutagenic if there was a concentration-related increase in the number of revertants per plate in at least one tester strain over a minimum of two increasing concentrations of Niagen. In the case of the strains TA98, TA100, and WP2 *uvr*A pKM101 the result was considered positive if the mean number of revertants was equal to or greater than two times the number of revertants obtained with the negative control. In the case of the strains TA1535 and TA1537 the result was considered positive if the mean number of revertants was equal to or greater than three times the number of revertants obtained with the negative control.

b. Results

Niagen was not cytotoxic at any of the doses used in this study (data not shown), and compared to the vehicle control, did not increase the number of revertant colonies in any of the frameshift or base-pair tester strains either when incubated in the presence or absence of the S9 mix, or using the plate incorporation or preincubation methods (Table 11). In contrast, all positive controls (2-Aminoanthracene 2-Nitrofluorene, 9-Aminoacridine, Sodium Azide, 4-Nitroquinoline-*N*-oxide) significantly increases in the number of revertant colonies ($p<0.05$), demonstrating both the sensitivity and validity of the assay. Therefore, Niagen was not mutagenic under the conditions used in the studies.

Table 11. Ames Assay Results

	Test Item	Test Concentrations (µg/plate)	Mean Colonies/Plate				
			Frameshift types		Base-pair types		Frameshift and base-pair types
			TA98	TA1537	TA1535	WP2uvrA pKM101	TA100
Plate Incorporation Method							
With S9 Mix	Water	-	22 ± 3	8 ± 2	10 ± 3	149 ± 3	101 ± 1
	Niagen	50	27 ± 6	8 ± 2	12 ± 3	153 ± 3	108 ± 6
		159	23 ± 4	11 ± 3	8 ± 2	152 ± 3	103 ± 3
		501	22 ± 5	8 ± 3	10 ± 2	142 ± 4	104 ± 3
		1582	26 ± 1	9 ± 3	12 ± 2	144 ± 8	103 ± 1
		5000	24 ± 4	10 ± 8	11 ± 6	144 ± 6	104 ± 2
	2-Aminoanthracene	4	597 ± 14	83 ± 3	108 ± 4	-	883 ± 12
	2-Aminoanthracene	30	-	-	-	736 ± 6	-
Without S9 Mix	Water	-	21 ± 4	8 ± 4	12 ± 4	119 ± 6	104 ± 2
	Niagen	50	27 ± 4	9 ± 2	14 ± 5	121 ± 16	106 ± 3
		159	23 ± 6	9 ± 5	14 ± 3	116 ± 6	108 ± 5
		501	27 ± 4	7 ± 2	17 ± 5	111 ± 10	111 ± 6
		1582	26 ± 3	7 ± 4	18 ± 7	122 ± 14	121 ± 15
		5000	27 ± 4	8 ± 3	18 ± 4	122 ± 17	121 ± 3
	2-Nitrofluorene	2	228 ± 10	-	-	-	-
	9-Aminoacridine	4	-	82 ± 3	-	-	-
	Sodium Azide	1	-	-	124 ± 3	-	526 ± 7
	4-Nitroquinoline-*N*-oxide	4	-	-	-	621 ± 3	-

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Table 11. Ames Assay Results

	Test Item	Test Concentrations (µg/plate)	Mean Colonies/Plate				
			Frameshift types		Base-pair types		Frameshift and base-pair types
			TA98	TA1537	TA1535	WP2uvrA pKM101	TA100
Preincubation method							
With S9 Mix	Water	-	22 ± 6	8 ± 1	10 ± 4	105 ± 6	108 ± 5
	Niagen	99	21 ± 5	6 ± 1	9 ± 1	96 ± 11	104 ± 6
		265	22 ± 4	6 ± 1	8 ± 1	107 ± 16	103 ± 6
		699	26 ± 5	9 ± 2	14 ± 1	86 ± 17	117 ± 4
		1869	22 ± 3	8 ± 2	15 ± 4	110 ± 10	105 ± 13
		5000	26 ± 6	5 ± 1	9 ± 1	99 ± 2	112 ± 4
	2-Aminoanthracene	4	571 ± 6	90 ± 7	101 ± 5	-	919 ± 8
	2-Aminoanthracene	30	-	-	-	562 ± 18	-
Without S9 Mix	Water	-	20 ± 8	9 ± 1	14 ± 2	96 ± 4	104 ± 6
	Niagen	99	16 ± 1	9 ± 4	12 ± 2	98 ± 7	103 ± 9
		265	18 ± 5	8 ± 1	11 ± 2	95 ± 4	109 ± 3
		699	19 ± 4	8 ± 4	11 ± 3	91 ± 4	103 ± 8
		1869	17 ± 3	6 ± 4	9 ± 1	92 ± 7	113 ± 7
		5000	22 ± 2	6 ± 3	9 ± 1	94 ± 7	120 ± 4
	2-Nitrofluorene	2	216 ± 4	-	-	-	-
	9-Aminoacridine	4	-	92 ± 4	-	-	-
	Sodium Azide	1	-	-	126 ± 7	-	419 ± 9
	4-Nitroquinoline-*N*-oxide	4	-	-	-	505 ± 16	-

"-" denotes not tested
Values are mean +/- standard deviation

2. *In vitro* Chromosomal Aberration Assay

a. Methods

In vitro chromosomal aberration assays were performed in compliance with the OECD Principles of GLP and Guideline No. 473. Niagen (>99% nicotinamide riboside chloride) was supplied by ChromaDex, Inc. Cyclophosphamide monohydrate and ethyl methanesulphonate were obtained from Sigma Aldrich Chemical Co., Inc. Aroclor 1254-induced rat liver S9 homogenate was obtained from Xenometrix AG. Human peripheral blood lymphocytes (PBLs) were obtained from whole blood harvested from a healthy donor who was approximately 35 years-old, had no history of smoking or alcoholism, and had not received medication one month prior to the blood draw. The whole blood was cultured in RPMI 1640 medium containing 10% fetal bovine serum, heparin, EDTA, amphotericin, penicillin, streptomycin, and phytohaemagglutinin (PHA) at 37°C and 5% CO_2 for 3 days per OECD Guideline 473 and International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH)-harmonized guidances on genotoxicity testing of pharmaceuticals.

To determine the clastogenic activity of Niagen, the PHA-stimulated whole blood cultures were centrifuged and the resulting PBLs were resuspended in RPMI 1640 containing 10% fetal bovine serum, amphotericin, penicillin, streptomycin, either vehicle (water), 1.25, 2.5, or 5 mg/ml of Niagen, or the appropriate positive control (cyclophosphamide monohydrate and ethyl methanesulphonate), supplemented with either phosphate buffered saline or the metabolic activation system (S9 mix; 10% of the Aroclor 1254-induced rat liver S9 homogenate, 4mM NADP, 5 mM glucose-6-phosphate, 8 mM magnesium chloride, and 33 mM potassium chloride). The mixtures were then incubated at 37°C and 5% CO_2 for 3 and 19 hours, at which point colchicine was added to the cultures to a final concentration of 2 μg/ml. Three hours later, the cells were harvested by centrifugation at 800 to 1000 rpm for approximately 10 minutes, resuspended in 0.56 % pre-warmed potassium chloride, and incubated at room temperature for 25 to 30 minutes. The cell suspension was then centrifuged 800 to 1000 rpm for approximately 10 minutes, the resulting supernatant was discarded, and the cellular pellet was resuspended and incubated in cold fixative (acetic acid: methanol (1:3)) at room temperature for 10 to 15 minutes. This process was repeated 3 additional times with one incubation at 4 °C for a minimum of 1 hour followed by two incubations at room temperature for 10 to 15 minutes. After the final incubation, the cells suspension was dropped onto clean, cold slides, which were then gently dried over a flame, stained with 5% Giemsa, and scored for the presence of metaphase cells and the presence of aberrations. To determine the mitotic index, which was used as an indicator of cytotoxicity, a minimum of 1000 cells were scored for each group and the total number of metaphases was divided by the number of cells counted. The quotient was then multiplied by

100. To determine the types of aberrations (chromatid gaps, chromosomal gaps, chromosomal breaks, chromatid breaks, deletions, and fragments) a minimum of 300 metaphases containing 46 +/- 2 centromere regions were counted and the number of cells containing one or more different types of aberrations were recorded. The data was the subjected to a one-tailed Fisher Exact test. Niagen was considered cytotoxic if there was a 45+/- 5% reduction in the mitotic index compared to the vehicle control. Niagen was considered mutagenic if there was a concentration-related and statistically significant increase ($p<0.05$) in the number of chromosome aberrations.

b. Results

Niagen was not cytotoxic to ex vivo human peripheral blood lymphocytes at any of the concentrations used in the study as determined by the mitotic index (data not shown), and, compared to the vehicle control, did not increase the number of aberrant metaphases when incubated with or without S9 mix for 6 hours (Table 12). Moreover, the types of aberrations (chromatid gaps, chromosomal gaps, chromosomal breaks, chromatid breaks) detected in the vehicle- and Niagen-treated cells were similar. In contrast, the positive controls, cyclophosphamide and ethyl methansulphonate, significantly increased the number of aberrant metaphases ($p<0.05$), characterized as chromatid gaps, chromosomal gaps, chromosomal breaks, chromatid breaks, deletions, and fragments, thus confirming the sensitivity and validity of the assay. Similar results were also found when the lymphocytes were incubated with increasing amounts of Niagen for 22 hours in the absence of the S9 mix (data not shown). Niagen was therefore not clastogenic under the conditions used in the study.

Table 12. Summary of Results – *In vitro* Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes

	Dose Level per mL of Test Medium				
	Vehicle Control[a]	**Niagen 1.25 mg**	**Niagen 2.5 mg**	**Niagen 5 mg**	**Positive Control[b]**
Without S9					
Total number of metaphases read	350	300	300	300	300
Number of aberrant metaphases					
Including gaps	5	3	1	2	**30***
Excluding gaps	4	1	0	1	**28***
With S9					
Total number of metaphases read	300	300	300	300	300
Number of aberrant metaphases					
Including gaps	1	3	3	5	**25***
Excluding gaps	0	2	1	2	**25***

[a]: Vehicle control= 150 µl sterile water

[b]: Positive controls= 650 µl ethyl methanesulphonate (EMS) in the absence of metabolic activation (without S9); or 55 µg cyclophosphamide in the presence of metabolic activation (with S9).

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3. *In vivo* Micronucleus Assay

a. Methods

The in vivo micronucleus assay performed in compliance with the OECD Principles of GLP, OECD Guideline No. 474, and the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCESA), Government of India. The rats were obtained from Harlan Laboratories. Nutrilab Rodent Pellet feed was obtained from Provimi Animal Nutrition. Niagen (>99% nicotinamide riboside chloride; CAS No. 23111-00-4) was supplied by ChromaDex, Inc. Niagen (>99% nicotinamide riboside chloride) was supplied by ChromaDex, Inc. Cyclophosphamide was obtained from Sigma Aldrich Chemical Co., Inc.

All rats were housed at three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and, except for the overnight fast prior to euthanasia on day 91, provided feed and water *ad libitum* throughout the study. Prior to dosing the rats were randomized by body weight to two groups (n=6/sex/group). At dosing, a single dose of vehicle (water), 500, 1000, and 2000 mg/kg of Niagen or 40 mg/kg cyclophosphamide was administered by gavage at a rate of 10 ml/kg body weight. Twenty-four hours after dosing the vehicle, 500 mg/kg Niagen-, 1000 mg/kg Niagen-, 2000 mg/kg Niagen-, and 40 mg/kg cyclophosphamide-treated groups were euthanized by carbon dioxide asphyxiation. Forty-eight hours after dosing, an additional vehicle and 2000 mg/kg Niagen-treated group was also euthanized by carbon dioxide asphyxiation. The animals were observed for mortality at 1 and 2 hours and then twice daily after dosing for 2 days. Clinical signs were monitored 1 and 2 hours after dosing and then once daily for 2 days.

Immediately after euthanisation, bone marrow was harvested from the femurs of each animal, and centrifuged. The centrifuged cell suspension was smeared on 2 slides, which were then air-dried, fixed in methanol, and stained using a May-Gruenwald and Giemsa solution. The test item was considered toxic if polychromatic erythrocyte/total erythrocyte ratio less than that in vehicle control group. Niagen were be considered mutagenic if, at least one of the treatment groups exhibited a statistically significant ($p<0.05$) increase in the frequency of micronucleated immature erythrocytes when compared with the concurrent vehicle control.

b. Results

No mortalities or clinical signs of toxicity were observed in any of the rats receiving Niagen. In addition, bone marrow analyses showed that compared to the negative control, the administration of 500, 1000, and 2000 mg/kg of did not result in cytotoxicity or increase the percentage of polychromatic erythrocytes at either 24 or 48 hours of administration (Table 13). In contrast, the positive control cyclophosphamide induced a statistically significant ($p < 0.05$) increase in the percentage of polychromatic erythrocytes at 24 hours demonstrating both the sensitivity and validity of the assay. Therefore, Niagen was not genotoxic under the conditions used in this study.

Table 13. Summary of Results - *In vivo* Micronucleus test

Parameters	Dose (mg/kg bw)					
	Vehicle Control 0	**Niagen 500**	**Niagen 1000**	**Niagen 2000 (a)**	**Niagen 2000 (b)**	**Positive Control 40**
Males						
Sampling time (hours)	24	24	24	24	48	24
PCE's with micronuclei (%)	0.00	0.00	0.00	0.00	0.00	**0.28***
Range	0-1	0	0-1	0	0	**8-15**
Mean P/E ratio	0.34	0.37	0.39	0.38	0.39	0.40
Females						
Sampling time (hours)	24	24	24	24	48	24
PCE's with micronuclei (%)	0.00	0.00	0.00	0.00	0.00	**0.27***
Range	0	0	0	0	0	**7-13**
Mean P/E ratio	0.40	0.43	0.44	0.44	0.41	0.40

mg/kg b. wt.: milligram/kilogram body weight, Positive Control: Cyclophosphamide,
% - per cent, PCE – Polychromatic erythrocytes,
P/E: Polychromatic erythrocytes/ Total Erythrocytes
* = P value Significant

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E. ANIMAL TOXICOLOGY STUDIES

1. Test Material Used in Acute Toxicity, Dose Range Finder, and Subchronic Toxicity Safety Studies

The batch of Niagen used as test article used for the animal toxicity studies described below -batch lot #13202 was produced using manufacturing conditions slightly different from those described in the manufacturing section for production of commercial material (batch analysis data in Table 14). Between preliminary batch Lot #13202 and subsequent commercial batches, the washing steps of the production process were further modified and optimized. Mainly, the changes involved altering the rinse procedures such as replacing the acetone slurry in the first step; reversing addition of solvents, introducing an extended methanol water slurry; a reduced acetone water slurry and updated drying condition in the second the second step of the process. This led to an overall improvement in the levels of solvent residues remaining in the final commercial ingredient product. The batch used in the toxicology studies had slightly higher acetone residue than specifications for commercial product, however, all other specifications were met. This did not compromise the use of this batch for testing purposes because it represents a worst case from the perspective of acetone residue. Commercial batches comply with all specifications.

Table 14. Specifications and Batch Analysis for Niagen Batch # 13202

Parameter	Specifications	Method	Batch 13202
Color	White to light brown	Visual	Off-white
Form	Powder	Visual	Powder
Purity	95 – 102 (wt%) by HPLC	99.1-CD-3.0-000591	99.6 (wt%)
Identification	Conforms to structure by NMR	99.1-CD-1.0-000122	Conforms to structure
Water Content	NMT 1%	99.1-CD-6.0-000094	0.14%
Residual Solvents			
Acetone	≤ 3000 ppm*	99.1-CD-7.0-000115	5784
Methanol	≤ 740 ppm*	99.1-CD-7.0-000115	717
Acetonitrile	ND*	99.1-CD-7.0-000115	ND
Methyl *t*-Butyl Ether	ND*	99.1-CD-7.0-000115	288
Reaction By-products			
Methyl acetate	ND /BLOQ*	99.1-CD-7.0-000115	ND/BLOQ**
Acetamide	ND/BLOQ*	99.1-CD-1.0-000616	ND/BLOQ**
Acetic Acid	≤ 5000 ppm*	99.1-CD-7.0-000115	587
Microbiological Limits			
Total Plate Count	≤1,000 CFU/g	USP <2023(h)>	<1000 CFU/g
Yeast and Mold	≤1000 CFU/g	USP <2023(h)>	<100 CFU/g
E.coli	Absent/10g	USP <2023(h)>	Absent/ 10g
Heavy Metals			
Arsenic	≤1 ppm	AOAC 993.14	<0.5 ppm
Mercury	≤1 ppm	AOAC 993.14	<0.1 ppm
Cadmium	≤1 ppm	AOAC 993.14	<0.25 ppm
Lead	≤ 0.5 ppm	AOAC 993.14	<0.05 ppm

ND- Not Detected (For methyl acetate LOD is 5 ppm; for acetamide LOD is 10 ppm; for acetonitrile the LOD is 6 ppm; for Methyl t-Butyl Ether the LOD is 4 ppm)

BLOQ- Below Limit of Quantitation (For methyl acetate LOQ is 15 ppm; for acetamide LOQ is 25 ppm)

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2. Acute Toxicity

a. Methods

The acute toxicity study was performed in female Sprague Dawley SD rats in compliance with the OECD Principles of GLP, the Guidance for Industry, Single Dose Acute Toxicity Testing for Pharmaceuticals from the United States Food and Drug Administration, and the recommendations of the AAALAC and CPCESA, Government of India. The rats were obtained from Harlan Laboratories. Nutrilab Rodent Pellet feed was obtained from Provimi Animal Nutrition. Niagen (>99% nicotinamide riboside chloride) was supplied by ChromaDex, Inc.

All rats were housed two to three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and, except for the overnight fast prior to dosing, provided feed and water *ad libitum* throughout the study. Prior to dosing the rats were randomized by body weight to two groups (n=5/sex/group). At dosing, a single dose of vehicle (water) or 5000 mg/kg of Niagen was administered by gavage at a rate of 10 ml/kg body weight. Over the course of the following 14 days all rats were monitored for morbidity, mortality, and visible clinical signs. Detailed clinical examinations were conducted on day 1, 8 and 15 and included evaluations of the skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity, changes in gait, posture, response to handling, and presence of clonic or tonic movements, stereotypes, or bizarre behaviors. Body weights were recorded prior to treatment on day 1 and then on day 8 and 15. Food consumption for each cage was measured on day 8 and 15 and food consumption per rat was calculated by dividing the total food consumption during the interval per cage by the number of rats multiplied by the number of days. On day 15, all animals were euthanized and examined for gross pathological changes. Analyses were conducted using two-tailed tests for a minimum significance level of 5% comparing the Niagen and vehicle-treated group for each sex. All quantitative variables, like body weight, body weight gain, and food consumption were subjected to Student's t-test. Males and females were considered separately for each analyses and a p value of <0.05 was considered statistically significant.

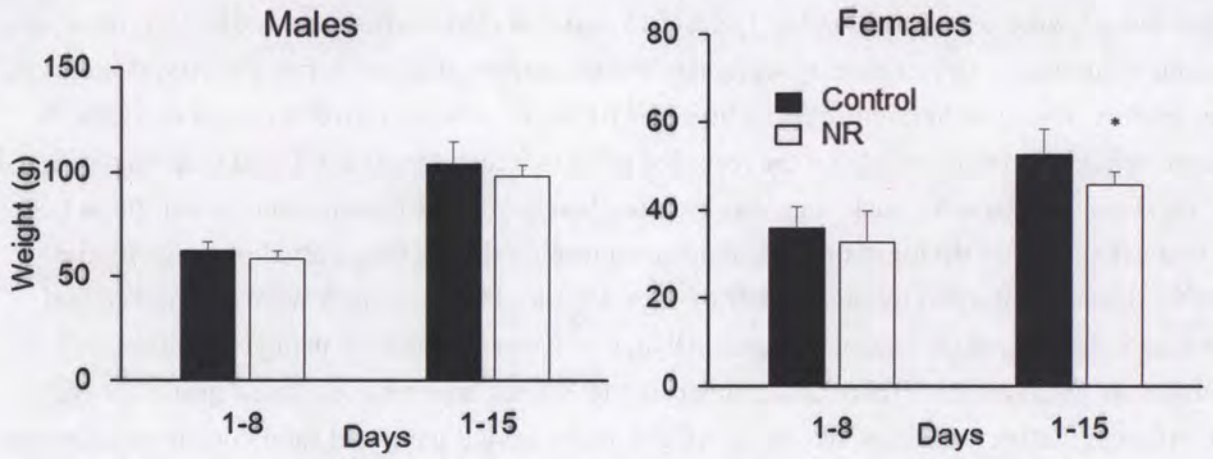
b. Results

There was no mortality observed in males or females at the limit dose of 5000 mg/kg.

No clinical signs were observed in male and female rats. No statistically significant changes were observed in body weight in both sexes. Cumulative body weight gain (days 1-15) was significantly lower in female rats as compared to control group. Since change in day 15 body weight was minimal (-3%), this was considered as treatment related but non-adverse.

There were no statistically significant changes in food consumption in either sex. There were no gross pathological lesions observed in male and female rats (Figure 8).

Figure 8. Effects of Acute Oral Dose of NR on Weight in Sprague-Dawley Rats



After a single oral dose (5000 mg/kg bw) of Niagen, rats were monitored for clinical signs of toxicity including body weights, for 15 days after Niagen administration. A small but significant reduction in body weight was observed in female rats at day 15 ($p < 0.05$).

3. 14-Day Study Dose Range Finder

a. Methods

The objectives of the study were to evaluate the potential systemic toxicity of Niagen when administered by oral gavage for 14 days in Sprague Dawley rats and to select doses for subchronic toxicity study. A 14 day repeat-dose study was conducted in Sprague Dawley rats in compliance with the OECD Guideline 407, but for 14 days instead of 28 days, and the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCESA), Government of India. The rats were obtained from Harlan Laboratories. Nutrilab Rodent Pellet feed was obtained from Provimi Animal Nutrition. Niagen (>99% nicotinamide riboside chloride) was supplied by ChromaDex, Inc.

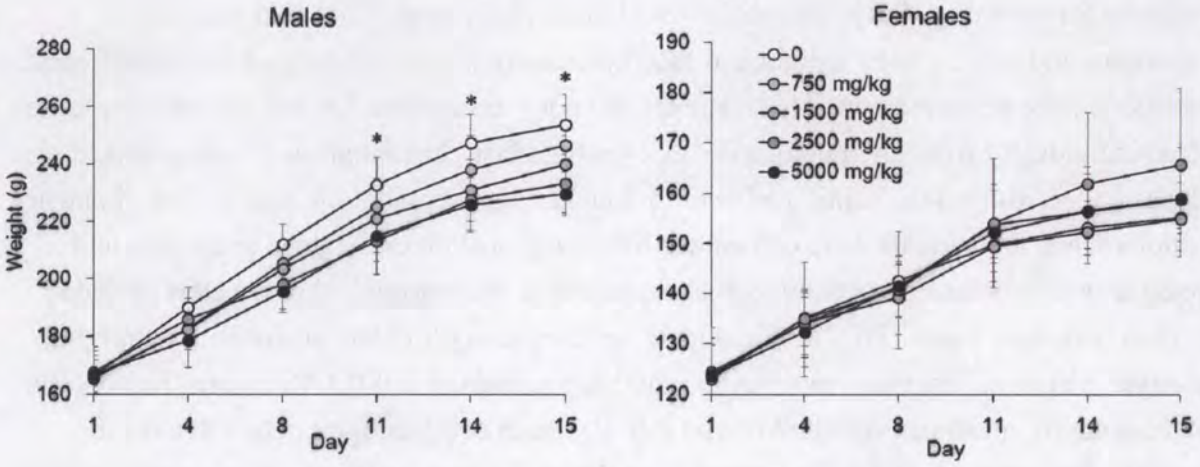
All rats were housed two to three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and provided feed and water *ad libitum* throughout the study. Prior to dosing the rats were randomized to five groups (n=5/sex/group) according to body weight. During the 14-day treatment period, each group was gavaged daily with either vehicle (water) or 750, 1500, 2500, or 5000 mg/kg/day of Niagen at a rate of 10 mL/kg body weight. Dose formulation

analyses showed that Niagen was completely soluble in water and the dose formulations were homogeneous and contained the targeted concentrations of nicotinamide riboside. Stability analyses showed that when Niagen was dissolved in water, nicotinamide riboside was stable up to 6 hours at room temperature and 7 days at 2-8 °C. During the 14 day-treatment period all rats were monitored for morbidity, mortality, and visible clinical signs. Detailed clinical examinations were conducted on day 1, 8 and 15 and included evaluations of the skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity, changes in gait, posture, response to handling, and presence of clonic or tonic movements, stereotypes, or bizarre behaviors. Body weights were recorded prior to treatment on day 1 and then on day 8 and 15. Food consumption for each cage was measured on day 8 and 15 and food consumption per rat was calculated by dividing the total food consumption during the interval per cage by the number of rats multiplied by the number of days. On day 15, all animals were euthanized and examined for gross pathological changes. All analyses were conducted using two-tailed tests for a minimum significance level of 5% comparing the Niagen and vehicle-treated group for each sex. All quantitative variables, like body weight, body weight gain, and food consumption were tested for normality and homogeneity of variances within the group before performing a one-factor ANOVA by treatment. When the data were found to be non-optimal, the data were log transformed prior to performing the ANOVA. Comparisons of the means differences between the Niagen- and vehicle-treated groups was performed using Dunnett's post-hoc test. When normality/homogeneity was significant, even after transformation, the data was subjected to the Kruskal-Wallis test followed by Dunn's post-hoc test. Males and females were considered separately for each analyses and a p value of <0.05 was considered statistically significant.

b. Results

Minimal reduction in mean body weight compared to the vehicle-treated group was observed in male rats at 2500 mg/kg/day (7-8% reduction) and 5000 mg/kg/day (8-9% reduction) on Days 8, 11, 14 and 15 (Figure 9). A test article related decrease in overall (Day 1-14) feed consumption was observed at 5000 mg/kg/day (8%) in male rats. No test item-related changes were observed in body weight and feed consumption in female rats. No gross pathological lesions were observed in male and female rats. Based on a combination of both body weight reduction of approximately 10% correlated with a feed intake reduction at 5000 mg/kg/day in males, this dose level was considered to be too high for the 90-day study. Based on the body weight reduction of 7-8% at 2500 mg/kg/day in the 14-day study, the doses of 300, 1000 and 3000 mg/kg/day were chosen for the 90-day subchronic toxicity study in rats.

Figure 9. Effects of 14 Day Administration of Niagen on Body Weight in Rats



4. Subchronic Toxicity

a. Methods

A 90-day oral subchronic toxicity study in Sprague Dawley rats was conducted in compliance with the OECD Principles of GLP, the OECD Guideline 408 for testing of chemicals, and the recommendations of the AAALAC and CPCESA, Government of India. The rats were obtained from Harlan Laboratories. Nutrilab Rodent Pellet feed was obtained from Provimi Animal Nutrition. Niagen (>99% nicotinamide riboside chloride) was supplied by ChromaDex, Inc. Nicotinamide (≥ 99.5%; CAS No. 98-92-0) was obtained from Sigma Aldrich.

All rats were housed two to three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and, except for the overnight fast prior to euthanasia on day 91, provided feed and water *ad libitum* throughout the study. Prior to dosing the rats were randomized by body weight to 5 groups (n=10/sex/group). During the 90-day treatment period, each group was gavaged daily with either vehicle (water), 300, 1000, 3000 mg/kg of Niagen or 1260 mg/kg/day of nicotinamide, which is equivalent to 3000 mg/kg/day of Niagen on a molar basis. Importantly, studies have shown that NR is a form of Vitamin B_3 that is metabolized similarly to nicotinamide and other NAD+ precursors. Thus, a positive control group treated with nicotinamide was also included.

Dose formulation analyses showed that both Niagen and nicotinamide were completely soluble in water and the dose formulations contained the targeted concentrations of nicotinamide riboside or nicotinamide. Stability analyses showed that when Niagen and nicotinamide were

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dissolved in water, both nicotinamide riboside and nicotinamide were stable up to 6 hours at room temperature and 7 days at 2-8 °C. The parameters evaluated during the study were twice daily checks for mortality, daily evaluations for clinical signs, weekly detailed clinical examinations, and weekly body weight and food consumption measurements. Ophthalmological examinations were performed prior to treatment and prior to sacrifice. On day 91, after urine was collected individually from all animals after an overnight fast, the animals were anesthetized, and blood was collected from the sublingual vein for hematology, coagulation, and clinical chemistry evaluations. Then the animals were euthanized by exsanguination under deep anesthesia and subjected to necropsy and gross pathological examination. Hematological parameters included differential leukocyte count (DLC), reticulocyte, leukocyte, erythrocyte, eosinophil, neutrophil, lymphocyte platelets, basophils, monocytes, and large unstained cell (LUC) counts, hemoglobin (Hgb), hematocrit, mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean hemoglobin concentration (MCHC), prothrombin time (PT), and activated partial thromboplastin time (APTT). Plasma clinical chemistry parameters included total protein, albumin, bile acids, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGTP), globulin, alkaline phosphatase (ALP), total cholesterol (TC), triglycerides, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorous (Pi), calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), and chloride (Cl) levels. The organs that were collected, weighed, and preserved included the adrenals, aorta, bone marrow smear, brain including medulla/pons, cerebellum and cerebrum, caecum, colon, duodenum, epididymides, esophagus, eyes with optic nerve, biceps femoris muscles, femur bone with joint gross lesions, heart, ileum with Peyer's patches, jejunum, kidneys, liver, lungs with main bronchi and bronchioles, mandibular lymph nodes, mesenteric lymph nodes, mammary gland, ovaries, oviducts, pancreas, pituitary, prostate, seminal vesicles and coagulating glands, rectum, salivary glands (mandibular, parotid and sublingual), sciatic nerve, skin (inguinal region), spinal cord at 3 levels - cervical, mid-thoracic and lumbar, spleen, sternum with marrow, stomach, testes, thymus, thyroid and parathyroid, tongue, trachea, urinary bladder, uterus with cervix, and vagina. The preserved tissues were processed and embedded in paraffin, sectioned and stained with Haematoxylin and Eosin.

All samples were fixed in 10% neutral buffer formalin and stained with hematoxylin-eosin. The eye, optic nerve, and Harderian gland were pre-fixed in a 2.5% glutaraldehyde solution, and the nasal cavity, testis, and epididymis were pre-fixed in Bouin's solution.

All quantitative variables such as body weight, body weight gain, food consumption, hematology, clinical chemistry, urinalysis, organ weights and organ weight ratios were tested for normality and homogeneity of variance within the group before performing a one-factor

ANOVA. In cases, wherein, the data was found to be non-optimal (non-normal or heteroschedastic), ANOVA was done using a log transformation. Comparison of means between treatment groups and control group was done using a Dunnett's test. Even after transformation, when normality/homogeneity tests were significant, data was subjected to a Kruskal-Wallis test followed by a Dunn's test. All analyses and comparisons were evaluated at the 5% level.

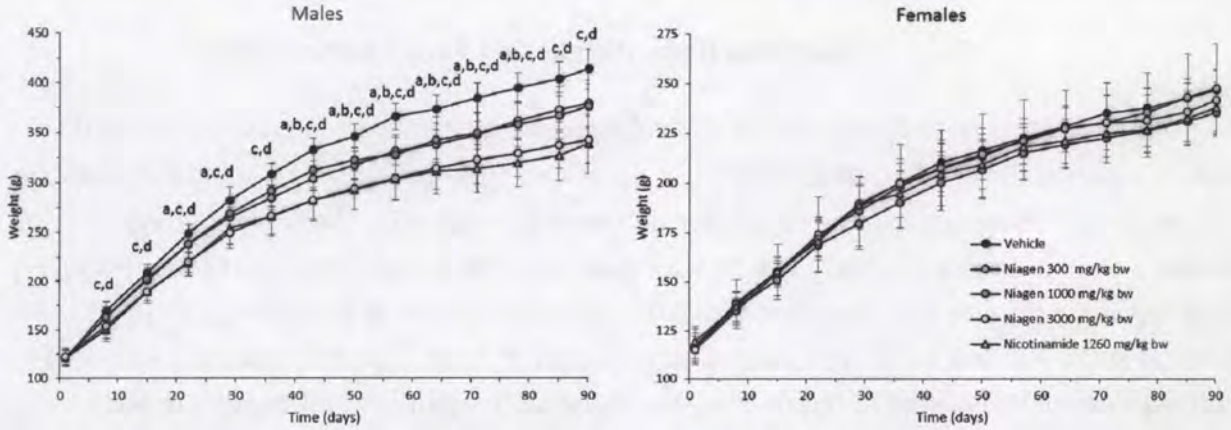
b. Results

i. Mortality, Body Weight and Feed Consumption

No treatment related mortality or clinical signs were observed at any dose level in this study. Compared to vehicle-treated controls, a significant ($p<0.05$) treatment-related decrease in body weight (17% reduction) was noted in male rats at the high dose (3000 mg/kg/day) of Niagen; a similar decrease in body weight was observed at an equimolar dose (1260 mg/kg/day) of nicotinamide (Figure 10). Significant ($p<0.05$) 8-9% reductions in body weight (<10%) were observed at the 300 and 1000 mg/kg/day dose of Niagen in male rats. This decrease was <10% and therefore not considered to be adverse. No statistically significant differences in body weights were seen in Niagen- or nicotinamide-treated females. In male rats, decreases in feed consumption were noted at 3000 mg/kg/day of Niagen (9-14%) and 1260 mg/kg/day of nicotinamide (9-17%) throughout the treatment period. Decreases in feed consumption also occurred at 300 mg/kg/day on days 57-64 and at 1000 mg/kg/day on days 50-57. In female rats, decreases in feed consumption occurred at days 1-8 in the nicotinamide treated group and at days 15-22 in the 3000 mg/kg/day Niagen group.

Figure 10. Body Weights of Male and Female Rats Treated with Vehicle Control, Nicotinamide Riboside or Nicotinamide For 90-Days

"a", "b", "c", and "d" denote significant (p<0.05) differences between rats treated with 300, 1000, and 3000 mg/kg/day of Niagen, or 1260 mg/kg/day of nicotinamide, respectively, and rats treated with the vehicle control.



ii. Hematological, Clinical Chemistry and Urinalysis Tests

Similar treatment-related changes in hematology parameters were observed at the high dose Niagen- and nicotinamide-treated groups (Table 15). Statistically significant ($p < 0.05$) treatment related increases in white blood cells (WBC) and neutrophils occurred in both males and females. Statistically significant ($p<0.05$) increases in monocytes were also noted in females treated with 3000 mg/kg/day of Niagen and 1260 mg/kg/day of nicotinamide. At 1000 mg/kg/day of Niagen, significant increases in WBC and neutrophils were observed in males and females, respectively. There were no significant changes in hematological parameters at male or female rats treated with 300 mg/kg/d of Niagen. Importantly, the significant effects were not associated with any inflammatory changes in any of the organs examined. All other changes in hematology parameters, including those determined to be statistically significant, were considered to be due to normal biological variation, and not due to the administration of the test item.

Niagen produced statistically significant ($p < 0.05$) increases at 3000 mg/kg/day in ALT, ALP, GGT, triglycerides and bile acids; the effects on ALT and triglycerides were significant at 1000 mg/kg/day of Niagen in females (Table 16). Comparable effects on clinical chemistries were seen in the nicotinamide-treated group. A minimal but statistically significant decrease in sodium in females and chloride in males and females was noted at 3000 mg/kg/day of Niagen. Similar results were seen in the nicotinamide-treated group. Significant reductions ($p<0.05$) in

sodium were also observed in males and females treated with 1000 mg/kg/day. All other changes in clinical chemistry parameters were considered incidental as the magnitude of change minimal and were considered to be due to normal biological variation.

Urinalysis showed increased urine volume in both nicotinamide and high dose Niagen-treated males (data not shown). This effect was considered a treatment-related effect, which may have been correlated with microscopic changes in the adrenals. Urine pH was decreased in males (7.6 ± 0.64 in controls vs. 6.11 ± 0.16 in high dose) and females (7.2 ± 0.53 in controls vs. 6.3 ±6.3 ± 0.35 in high dose) at 3000 mg/kg/day of Niagen but not the males and females treated with nicotinamide. The slightly acidic pH may have been due to the excretion of test item and an acidic metabolite.

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Table 15. Summary of Significant Changes-Hematological Parameters Day 91					
	Doses (mg/kg/day)				
Parameters	**Vehicle 0**	**Niagen 300**	**Niagen 1000**	**Niagen 3000**	**Nicotinamide 1260^**
Males					
White Blood Cells	8.47±1.97	9.16±1.54	**11.11 ±1.77***	**13.51± 2.64***	**14.11±1.66***
Total Neutrophils	2.12±0.7	2.30±1.67	2.86±1.20	**5.76± 1.47***	**6.02±0.7***
Total Monocytes	0.28 ±0.09	0.33±0.30	0.40±0.19	0.42±0.09	0.45±0.12
Females					
White Blood Cells	6.25±1.20	6.23 ±1.84	7.40±1.01	**10.38± 1.51***	**9.99±2.64***
Total Neutrophils	0.93±0.31	1.11±0.57	**1.63±0.29***	**3.36± 1.19***	**4.13±1.56***
Total Monocytes	0.16 ±0.07	0.15±0.05	0.15±0.04	**0.32±0.08***	**0.33±0.21***

Values represented as Mean ± SD

*Significantly higher than the vehicle control group at $p < 0.05$.

^: equimolar ratio to Niagen 3000 mg/kg/d

Table 16. Summary of Significant Changes-Clinical Chemistries Day 91

	Doses (mg/kg/day)				
Parameters	**Vehicle 0**	**Niagen 300**	**Niagen 1000**	**Niagen 3000**	**Nicotinamide 1260^**
Males					
ALT (U/L)	85.23±26.11	75.94±12.20	106.17±45.34	159.35 ±27.56*	152.11± 25.50*
AST (U/L)	122.18±27.34	116.10±13.94	138.23±49.03	132.93±23.63	139.47±26.99
ALP (U/L)	99.85±22.69	120.94±16.68	110.96±18.67	131.63 ±19.73*	139.48± 24.88*
GGT (U/L)	3.09±0.90	4.13±1.22	3.47±1.20	3.82±1.22	3.81±1.16
Trig (mg/dL)	49.84±18.96	59.30±29.48	69.65±29.91	128.34± 47.88*	98.57 ±34.72*
Sodium (mmol/L)	139.15 ±3.37	141.03 ±6.05	137.99 ±3.52	137.1 ±4.7	135.88 ±1.9
Chloride (mmol/L)	101.55 ±2.66	102.13 ±4.41	99.21 ±1.3	97.34 ±3.24*	97.52 ±1.25*
Females					
ALT (U/L)	56.09 ±15.54	56.34 ±10.47	76.50 ±20.42*	121.81 ±22.35*	125.22 ±30.29*
AST (U/L)	101.02 ±18.67	106.1 ±18.52	126.69 ±35.51	126.27 ±33.09	126.31 ±16.24*
ALP (U/L)	60.79 ±5.16	69.66 ±11.23	85.96 ±22.06	105.10 ±26.99*	114.41 ±30.91*
GGT (U/L)	3.15 ±1.17	3.9 ±0.92	3.83 ±1.42	5.01 ±1.36*	5.46 ±1.75*
Trig (mg/dL)	28.38 ±6.9	28.24 ±7.92	46.69 ±12.97*	61.85 ±23.31*	87.00 ±38.48*
Sodium (mmol/L)	137.71 ±1.28	137.86 ±1.25	136.68 ±1.46*	134.42 ±1.22*	135.83 ±1.17*
Chloride (mmol/L)	102.12 ±0.96	101.43 ±1.13	100.69 ±1.52	98.12 ±1.63*	98.93 ±1.33*

Values represented as Mean ± SD

*Significantly higher than the vehicle control group at $p < 0.05$.

^: equimolar ratio to Niagen 3000 mg/kg/day

Significantly higher / lower than the vehicle control group at $p < 0.05$.

% Change: % change from vehicle control

^: equimolar ratio to Niagen 3000 mg/kg/d

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iii. Gross Pathology

Bilateral small-size testes observed in 3000 mg/kg/day Niagen- and nicotinamide-treated males was considered to be treatment-related and associated with degeneration/atrophy of the seminiferous tubules.

One single incidence of auxiliary region subcutaneous nodule microscopically associated with adenocarcinoma of mammary gland was observed in one Niagen high dose male. This neoplastic change was considered an incidental tumor of spontaneous origin as it is reported to occur naturally in young Sprague Dawley male rats (Ikezaki et al., 2011).

All other single or low incidences of gross pathologic findings observed in different groups were considered incidental and not related to test item as they were randomly distributed in different groups and were not dose-dependent.

iv. Organ Weights

At 3000 mg/kg/day, there were statistically significant reductions in absolute organ weights of brain, spleen, testes, epididymides, prostate, thyroid/parathyroid, pituitary and heart in males; brain and pituitary absolute organ weights were reduced and liver and kidney absolute organ weights were increased in females. In the nicotinamide-treated group, there were statistically significant reductions in absolute organ weights of brain, spleen, testes, epididymis, prostate, thyroid/parathyroid, pituitary, thymus and heart in males; brain and pituitary absolute organ weights were reduced and liver and ovary weights were increased in females. At 1000 mg/kg/day, there were statistically significant reductions in absolute organ weights of thyroid/parathyroid, pituitary and heart in males; no effects on absolute organ weights were seen in females. At 300 mg/kg/day, there were statistically significant reductions in absolute organ weights of brain and heart in males; no effects on absolute organ weights were seen in females. Relative organ weight changes in the 3000 mg/kg/day Niagen-treated rats were similar to those of animals ingesting an equimolar dose of nicotinamide (Table 17). Treatment-related organ weight changes were observed in liver, kidneys, testes, epididymides and ovaries in 3000 mg/kg/day Niagen- and nicotinamide-treated groups. At 1000 mg/kg/day of Niagen, increases in liver and kidney weights were statistically significant. All other relative to body weight organ weight changes which reached statistical significance were likely secondary to decrease in terminal body weight and/or random biological variation and not considered treatment related.

Relative to brain weight, at 3000 mg/kg/day, there were statistically significant reductions in heart, epididymides, prostate and thyroid/parathyroid in males; liver, heart, ovaries and kidney were increased in females. In the nicotinamide-treated group, there were statistically significant reductions in weights of spleen, epididymides, testes and heart in males; liver weight was increased. At 1000 mg/kg/day there were no changes in organ weights relative to brain weight in males and a statistically significant increase in liver weight in females. No changes in organ weights relative to brain weights were seen in either males or females treated with 300 mg/kg/day of Niagen.

Table 17. Summary of Significant Changes-Organ Weight Ratio Relative to Body Weight At Day 91

	Dose (mg/kg/day)				
Parameters	**Vehicle 0**	**Niagen 300**	**Niagen 1000**	**Niagen 3000**	**Nicotinamide 1260^**
Males					
Terminal Body Wt.	395.7 ±18.36	363.56 ±23.22 *	354.23 ±21.85 *	317.21±25.8 *	312.87±26.74 *
Organ/body weight					
Liver	2.958 ±0.143	3.013 ±0.163	3.200 ±0.18 *	3.600±0.272 *	3.703 ±0.116 *
Kidneys	0.715 ±0.047	0.701 ±0.033	0.777 ±0.02 *	0.876±0.063 *	0.833 ±0.086 *
Brain	0.507 ±0.027	0.526 ±0.038	0.550 ±0.024 *	0.572 ±0.042	0.583 ±0.043 *
Heart	0.368 ±0.019	0.356 ±0.018	0.373 ±0.015	0.384 ±0.02	0.387 ±0.015
Thymus	0.068 ±0.011	0.076 ±0.015	0.086 ±0.014 *	0.07 ±0.009	0.068 ±0.008
Adrenals	0.012 ±0.001	0.013 ±0.001	0.014 ±0.001 *	0.014 ±0.001 *	0.015 ±0.001 *
Females					
Terminal Body Wt.	232.29 ±8.1	234.43 ±23.28	219.51 ±9.92	216.19 ±14.75	215.61 ±11.16 *
Organ/body weight					
Liver	2.902 ±0.191	3.003 ±0.327	3.295 ±0.181*	4.046 ±0.174 *	4.465 ±0.239 *
Kidneys	0.676 ±0.053	0.645 ±0.06	0.678 ±0.058	0.822 ±0.044 *	0.766 * ±0.019
Brain	0.78 ±0.034	0.788 ±0.055	0.801 ±0.047	0.798 ±0.041	0.781 ±0.031
Heart	0.392 ±0.016	0.393 ±0.015	0.398 ±0.022	0.433 ±0.032 *	0.424 * ±0.029
Thymus	0.09 ±0.011	0.097 ±0.024	0.093 ±0.013	0.087 ±0.014	0.084 ±0.017
Adrenals	0.027 ±0.003	0.028 ±0.003	0.027 ±0.002	0.029 ±0.004	0.027 ±0.003
Ovaries	0.036 ±0.005	0.037 ±0.005	0.036 ±0.006	0.045 ±0.008 *	0.049 ±0.007 *

Values represented as Mean ± SD

*: Significantly higher / lower than the vehicle control group at $p < 0.05$.

^: equimolar ratio to Niagen 3000 mg/kg/day

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v. Histopathological Findings

Treatment-related histopathological changes were observed in liver, thyroid, kidneys, testes, epididymides, ovaries and adrenals in both the 3000 mg/kg/day Niagen-treated and nicotinamide-treated groups. All other single or low incidences of microscopic findings observed were considered incidental and not related to test item as they were randomly distributed among groups. Importantly, the treatment-related histopathological changes noted in Niagen at the high dose were similar to the findings observed in the equimolar nicotinamide group.

In the livers of 3000 mg/kg/day Niagen-treated and nicotinamide-treated males and females, centrilobular hepatocellular hypertrophy was reported. This was characterized by enlarged hepatocytes containing granular eosinophilic cytoplasm, follicular cell hypertrophy, characterized by enlarged follicular epithelium which contained pale eosinophilic cytoplasm and small clear vacuoles, and hepatocyte single cell necrosis, which was considered a treatment-related adverse change. In the kidneys, chronic progressive nephropathy characterized by presence of foci or areas of basophilic tubules, with or without simple tubular hyperplasia, hyaline casts, atrophic tubules, dilated tubule, focal glomerular sclerosis/atrophy and mononuclear cell infiltration was seen.

In male rats, both 3000 mg/kg/day Niagen-treated and nicotinamide-treated rats exhibited degeneration/atrophy of seminiferous tubules characterized by the presence of some tubules containing degenerating germ cells. Some tubules were also depleted of all germ cells and lined only by Sertoli cells and while others were partially depleted of germ cells. Degenerating tubules contained multinucleated germ cells, spermatid head retention, Sertoli cell cytoplasmic vacuolation and disorganization of germ cells. Reduced sperm and cell debris in epididymal lumen in nicotinamide and Niagen high dose males were considered treatment-related effects.

In female rats of the 3000 mg/kg/day Niagen-treated and nicotinamide-treated groups, hypertrophy of corpora lutea was seen. The affected ovaries contained large sized corpora lutea and lightly eosinophilic cytoplasm of the enlarged luteal cells.

In male and female rats of the 3000 mg/kg/day Niagen-treated and nicotinamide-treated groups, hypertrophy of the zona glomerulosa of the adrenal cortex was considered a treatment-related non-adverse change. Hypertrophy of zona glomerulosa was characterized by increased thickness of zona glomerulosa layer and cytoplasm of hypertophic cells was lightly eosinophilic.

c. Discussion of Animal Toxicology and Genotoxicity

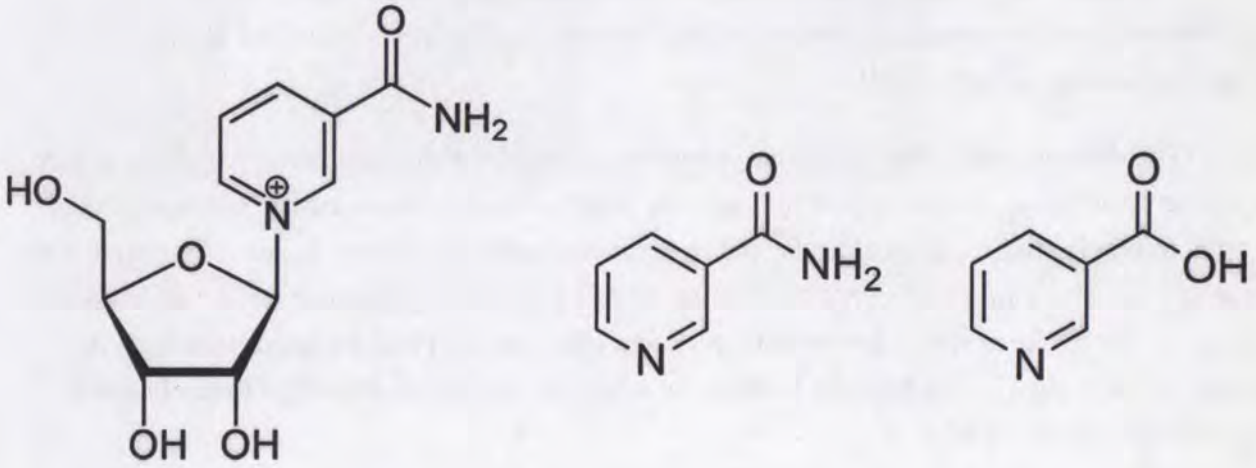
The safety of a synthetic form of NR was evaluated in an Ames assay, in vitro chromosome aberration assay, in vivo micronucleus assay and acute, 14-day and 90-day rat toxicology studies to derive a UL.

The determination of a UL for any vitamin is based on well-established principles of risk assessment and relies on data concerning adverse health effects from excessive nutrient intakes in epidemiologic studies, clinical trials, and experimental studies. Several factors associated with these various data sources influence the derivation of a UL. Among the most important of these factors are the intake at which adverse effects occur (the LOAEL) and the maximum level of intake, which is always less than the LOAEL, at which no adverse health effects are observed (the NOAEL) (IOM, 1998).

There are no animal toxicology studies on nicotinic acid and nicotinamide that comply with current standardized testing protocols from which to derive a UL. Therefore, authoritative bodies have used the results of clinical studies in which high doses of nicotinic acid and nicotinamide have been administered. A UL of 35 mg/day for adults was established for both nicotinic acid and nicotinamide by the IOM (1998) based on the flushing in humans as the critical adverse effect. However, nicotinamide does not induce flushing when either given as an intravenous injection or orally at high-doses to patients with diabetes (EC SCF, 2002), and therefore, it is currently thought that the flushing effects are related to the presence of the carboxyl group on the pyridine nucleus of nicotinic acid (Figure 11) (EC SFC, 2002). NR also lacks this carboxyl group and thus may not produce the flushing response that is associated with high nicotinic acid intakes (Figure 11). Furthermore, in vitro studies have shown that NR does not induce the GPR109A-mediated calcium flux, which is believed to be required for nicotinic acid-induced flushing (Benyo et al., 2005; Canto et al., 2012). It is therefore appropriate to derive the ULs for nicotinamide and NR based on endpoints other than flushing.

Consistent with this, the European Commission and UK Expert Group on Vitamins and Minerals derived independent ULs for nicotinamide and nicotinic acid. The EC SCF (2002) set the UL for nicotinamide of 900 mg/day based on long-term studies in patients with Type 1 diabetes mellitus, at dosages of 2-3 g of nicotinamide per day whereas the UK Expert Group on Vitamins and Minerals set that UL at 500 mg/day (UK Expert Group, 2003) based on human studies where large doses (up to 3000 mg/day for periods of up to 3 years) appeared to be well tolerated.

Figure 11. Structure of Nicotinamide Riboside (Left), Nicotinamide (Middle), And Nicotinic Acid (Right)



NR is not genotoxic. In addition, NR is thought to exhibit the same toxicity profile as nicotinamide because evidence from a single dose pharmacokinetic study in humans suggests that it is metabolized in a manner similar to nicotinamide (unpublished results). Because there are no publicly available 90-day studies on either nicotinamide or NR, a toxicology study was completed where 300, 1000, or 3000 mg/kg body weight/day of NR, or 1260 mg/kg body weight/day of nicotinamide, which is equivalent to 3000 mg/kg/day dose of NR on a molar basis, was administered to rats over the course of 90 days. Adverse effects at 3000 mg/kg body weight/day of NR included treatment-related adverse effects in liver, kidneys, testes, epididymides and ovaries. These effects included increases in clinical chemistry parameters related to hepatocyte damage (ALT, ALP, and GGT) and a corresponding increase in liver weight, centrilobular hepatocellular hypertrophy and single cell necrosis. In addition, thyroid follicular cell hypertrophy and increased kidney weight with exacerbation of chronic progressive nephropathy were observed. Statistically significant but minor reductions in sodium and chloride were seen and microscopically associated with hypertrophy of zona glomerulosa in adrenals at 3000 mg/kg. Importantly, these effects also occurred in the nicotinamide groups with a similar magnitude.

NR administration at 1000 mg/kg/day dose level resulted in treatment-related organ weight changes in liver and kidney and increases in neutrophils, ALT and triglycerides, which were statistically significant in female rats only. Although these changes were considered adverse, based on their dose-dependent responsiveness, the increases in ALT and triglycerides occurred only in one gender and were below the 2-fold increase that is typically used as the cut-off for a biologically significant effect in the absence of histological results (Hall et al., 2012). The kidney weight increases at this dose also occurred in the absence of corresponding

histopathology. Therefore, the liver and kidney effects at 1000 mg/kg/day were considered to be treatment-related, but mild and potentially adaptive in nature due to prolonged exposure to this form of niacin. There were no treatment related adverse effects noted at 300 mg/kg/day, although there was a slight decrease (8%) in overall body weight (Day 90) at 300 mg/kg/day, which was considered adaptive. The NOAEL and LOAEL for NR were determined to 300 and 1000 mg/kg body weight/day, respectively.

A UL for human exposure to NR is derived by application of a 100-fold safety factor to the NOAEL determined from this 90-day study; the UL for nicotinamide riboside is 3 mg/kg/day or 180 mg/day, assuming a body weight of 60 kg. Importantly, because the UL for NR falls below the ULs for nicotinamide and the adverse effects associated with ingestion of NR were similar those found with equimolar amounts of nicotinamide, this level of intake provides an adequate margin of safety to protect consumers from the adverse effects associated with NR ingestion.

F. CLINICAL STUDY (STUDY NUMBER 14NBHC)

1. Summary

The pharmacokinetics and safety of single administration of three dosages of Niagen was studied in healthy human subjects in a randomized, double-blind three-arm crossover trial. Results indicate that Niagen is metabolized similarly to nicotinamide in healthy humans and can be utilized as a form of Vitamin B_3. No clinically adverse effects on hematology, clinical chemistry, urinalysis or liver or kidney function parameters were noted.

2. Test Article Used in Study 14NBHC

The test article used in the clinical trial came from preliminary batch lot #13201, produced using manufacturing conditions slightly different from those described in the manufacturing section (batch analysis data in Table 18). Between preliminary batch Lot #13201 and subsequent commercial batches, the washing steps of the production process were further modified and optimized. Mainly, the changes involved altering the rinse procedures such as replacing the acetone slurry in the first step; reversing addition of solvents, introducing an extended methanol water slurry; a reduced acetone water slurry and updated drying condition in the second the second step of the process. This led to an overall improvement in the levels of solvent residues remaining in the final ingredient product. The batch used in the clinical study had slightly higher lead residue than specifications for commercial product allow however, all other specifications were met. This did not compromise the use of this batch for testing purposes because it represents a worst case from the perspective of lead contamination. Commercial batches all comply with specifications.

Table 18. Specifications and Batch Analysis for Niagen Batch # 13201

Parameter	Specifications	Method	Batch 13201
Color	White to light brown	Visual	Off-white
Form	Powder	Visual	Powder
Purity	95 – 102 (wt%) by HPLC	99.1-CD-3.0-000591	99.9 (wt%)
Identification	Conforms to structure by NMR	99.1-CD-1.0-000122	Conforms to structure
Water Content	NMT 1%	99.1-CD-6.0-000094	0.25%
Residual Solvents			
Acetone	≤ 3000 ppm	99.1-CD-7.0-000115	4818
Methanol	≤ 740 ppm	99.1-CD-7.0-000115	378
Acetonitrile	≤ 40 ppm	99.1-CD-7.0-000115	ND
Methyl *t*-Butyl Ether	ND*	99.1-CD-7.0-000115	ND
Reaction By-products			
Methyl acetate	ND/BLOQ**	99.1-CD-7.0-000115	ND
Acetamide	ND/BLOQ**	99.1-CD-1.0-000616	ND
Acetic Acid	≤ 5000 ppm*	99.1-CD-7.0-000115	87
Microbiological Limits			
Total Plate Count	≤1,000 CFU/g	USP <2023(h)>	<1000 CFU/g
Yeast and Mold	≤1000 CFU/g	USP <2023(h)>	<1000 CFU/g
E.coli	Absent/10g	USP <2023(h)>	Absent/ 10g
Heavy Metals			
Arsenic	≤1 ppm	AOAC 993.14	<0.5 ppm
Mercury	≤ 1 ppm	AOAC 993.14	<0.1 ppm
Cadmium	≤ 1 ppm	AOAC 993.14	<0.25 ppm
Lead	≤ 0.5 ppm	AOAC 993.14	0.054 ppm

ND- Not Detected (For methyl acetate LOD is 5 ppm; for acetamide LOD is 10 ppm; for acetonitrile the LOD is 6 ppm; for Methyl t-Butyl Ether the LOD is 4 ppm)

BLOQ- Below Limit of Quantitation (For methyl acetate LOQ is 15 ppm; for acetamide LOQ is 25 ppm)

3. Study Design

The clinical study is a randomized, double-blind, single-dose, three-arm crossover, 24 hr pharmacokinetic study. Twelve healthy participants (6 males and 6 females) were enrolled in the study and randomized to a three treatment sequence after screening and passing eligibility criteria, with all 12 subjects receiving each dose (100mg, 300mg and 1000mg) of Niagen on separate study days with a 7 day wash-out period in between study days. This study was

conducted in subjects of any ethnicity. The period from screening to study completion was approximately six weeks.

Standardized meals devoid of whey protein, milk and dairy products were provided. Breakfast were provided after the 1 h sampling, lunch after the 4h sampling and supper between the 6h and 12h sampling. Subjects were counseled to refrain from consuming vitamins, nutritional yeast, milk and other whey proteins, energy drinks and dairy products between the 12h and 24h sampling. Adverse events including vasodilation (flushing), were assessed at each study visit.

Fasting blood sample was collected for hematology parameters (hemoglobin, hematocrit, WBC, RBC, MCV, MCH, MCHC, RDW, platelets, neutrophils, lymphocytes, monocytes), clinical chemistries (electrolytes, AST, ALT, GGT and bilirubin) as well as for measuring Niagen and Niagen metabolite levels at 24hours post dose time point. Urine samples from 12-24hours interval were also collected. Vitals were taken for 24 hours post dose time point.

Niagen (NR) and NR metabolites in plasma were analyzed at pre-dose, 7.5min, 15min, 30min, 1h, 2h, 3h, 4h, 5h, 6h, 8h, 12h, and 24 h post-dose. Twelve participants completed the study. Participants completing the study had a mean age of 45.8 years with an average BMI of 25.85 ± 2.49.

4. Results

a. Clinical Measures

Evaluation of vital sign measures showed that there were no differences between 100mg, 300mg or 1000mg dosages of Niagen in SBP, DBP or HR. All values for blood pressure and heart rate were at a normal and acceptable range for healthy adults. Although there some significant within group differences during the course of the 24 hour post-dose period with respect to blood pressure and heart rate these values remained in the normal and acceptable range for healthy adults.

b. Hematology and Blood Chemistry

There were no clinically relevant, treatment related adverse findings in the hematology or blood chemistry. There were no between group differences in hemoglobin, hematocrit, WBC, RBC, MCV, MCH, MCHC, RDW, platelets, lymphocytes, monocytes in 100mg, 300mg and the 1000mg dosages of Niagen, except for neutrophils. Participants administered 300mg of Niagen showed an increase while participants administered with 1000mg of Niagen showed a decrease

in neutrophils resulting in a significant difference between these two groups (p=0.04). However, this difference is most likely due to one individual with a low level of neutrophils in the Niagen 1000mg dosage group that was not considered clinically significant.

c. Liver and Kidney Function Tests

AST, ALT, GGT and Total bilirubin, kidney function tests: creatinine and eGFR and the electrolytes: sodium, potassium and chloride were not different at 24h post-dose between the 100mg 300mg or the 100mg dosage groups of Niagen. The 24h post-dose urate levels were similar between all tested doses. The 24h post-dose urate levels were similar between the 100mg, 300mg or the 1000mg dosage groups of Niagen.

Within groups, 24h post-dose urate levels decreased (p=0.002) and potassium levels increased (p=0.005) when participants were on the 100mg Niagen dose. At the 300mg dose group of Niagen, urate levels decreased (p=0.007) and potassium increased (p=0.032) 24h post-dose. There was an increase in sodium (p=0.040), potassium (p=0.008) and chloride (p=0.001) at 24h post-dose at the 1000mg Niagen dose. These within group differences were not deemed clinically significant.

No adverse clinically significant treatment related effects were seen in urinalysis parameters.

d. Adverse Events (AEs)

A total of 18 adverse events were reported by 10 participants during the study. All AEs ranged in severity from mild to moderate. A total of 5 AE's were reported by 5 participants receiving the 100mg dose of Niagen. Two of these were categorized as General Disorders and Administration Site Conditions (feeling of warmth and tiredness). As these were not dose-dependent, both AE's were interpreted by the CRO as unlikely related to the 100 mg dose of Niagen. One AE was categorized as Skin and Subcutaneous Disorder and another as Nervous System Disorder. Both were considered to be "unlikely" related to the 100mg dose of Niagen. One AE categorized as Investigations (decrease in hemoglobin) was classified as "possibly" related to the study product and the participants were withdrawn from the study.

Seven participants receiving the 300mg dose of Niagen reported 9 AEs. Four of these AEs were categorized as Nervous System Disorders (headache) and were classified as "unlikely" related to the study product. Two AEs were categorized as General Disorders and Administration Site Conditions (flushing and feeling warm) and were categorized as "unlikely" related to the study product. Two AEs were classified as Gastrointestinal disorders (soft/loose stools) and were

considered "unlikely" related to the study product. One AE was categorized as Investigations (decrease in WBC count) and was considered "possibly" related to test item administration.

With the exception of three subjects who were withdrawn from the study due to low hemoglobin (two subjects) and low WBC (one subject), there were no clinically significant changes in hematology and blood chemistry values in this study. It is possible that the decrease in hemoglobin observed in two of the subjects that were removed was due to the repeated blood draws conducted over a prolonged period of time throughout the study.

e. Pharmacokinetics of a Single Dose of Niagen

The pharmacokinetics analysis was conducted on all randomized participants (n=12) that completed the study. Graphs showing the mean concentrations of urine, plasma, and white blood cell NR and NR metabolites over time were provided for each dosage. Graphs showing corrected values to pre-dose were done by subtracting the pre-dose values. Descriptive statistics, including means and standard deviations were calculated for each dosage.

f. NR Bioavailability

In this study there was very low detection of NR in blood or urine. Subsequent analytical work by ChromaDex has indicated that the method of blood collection was not appropriate for the detection of NR in the blood (unpublished results).

g. Urine and Blood Plasma Levels of NR metabolites:

Urine and plasma concentrations of NR metabolites Nam, MeNam, Py_4, and Py_2, exhibited a dose dependent relationship for each collection time (0-6h, 6-12h, and 12-24h) following treatment with 100mg, 300mg, or 1000mg Niagen (data shown in Figures 12-19). Between group changes among the time-points 6-12h and 12-24h were also found for Nam, MeNam, Py_4, Py_2, and NaR.

Despite the lack of detection of NR, several metabolites that are hallmark of niacin status are increased upon ingestion of NR in a dose dependent manner. Increased levels of Nam, meNam, Py2 and Py4 are indicative of underlying increases in cellular NAD+ concentrations. Increases in NAD+ were observed in some subjects, however the intrinsic between-subject variability in the baseline values precluded detection of statistically significant changes in plasma or WBC NAD+ levels.

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The pharmacokinetics analysis from the first in human clinical study for Niagen (14NBHC) show that MeNam, 4 Py and 2 Py are the predominant NAD+ related metabolites

that are detectable after ingestion of a single dose of Niagen. Dose-related increase in these metabolites are seen in plasma and urine. Importantly, normal metabolism of nicotinamide and NAD+ in humans leads to the production of these metabolites (Shibata et al., 1989).

Thus, it is evident that single doses of Niagen are metabolized in a similar fashion to nicotinamide in humans, based on the identity of the metabolites produced. This strongly supports that Nicotinamide Riboside is a form of Vitamin B_3 that can function as a NAD+ precursor.

In conclusion, these data corroborate that Niagen is metabolized as nicotinamide in humans and can be utilized as a form of Vitamin B_3.

Figure 12. Nicotinamide: Mean (±SEM) relative urine concentration and (B) mean (±SEM) change from pre-dose mean relative urine concentration of Nam standardized by creatinine for the pre-dose, 0-6 Hour, 6-12 Hour, and 12-24 Hour urine collection periods following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)

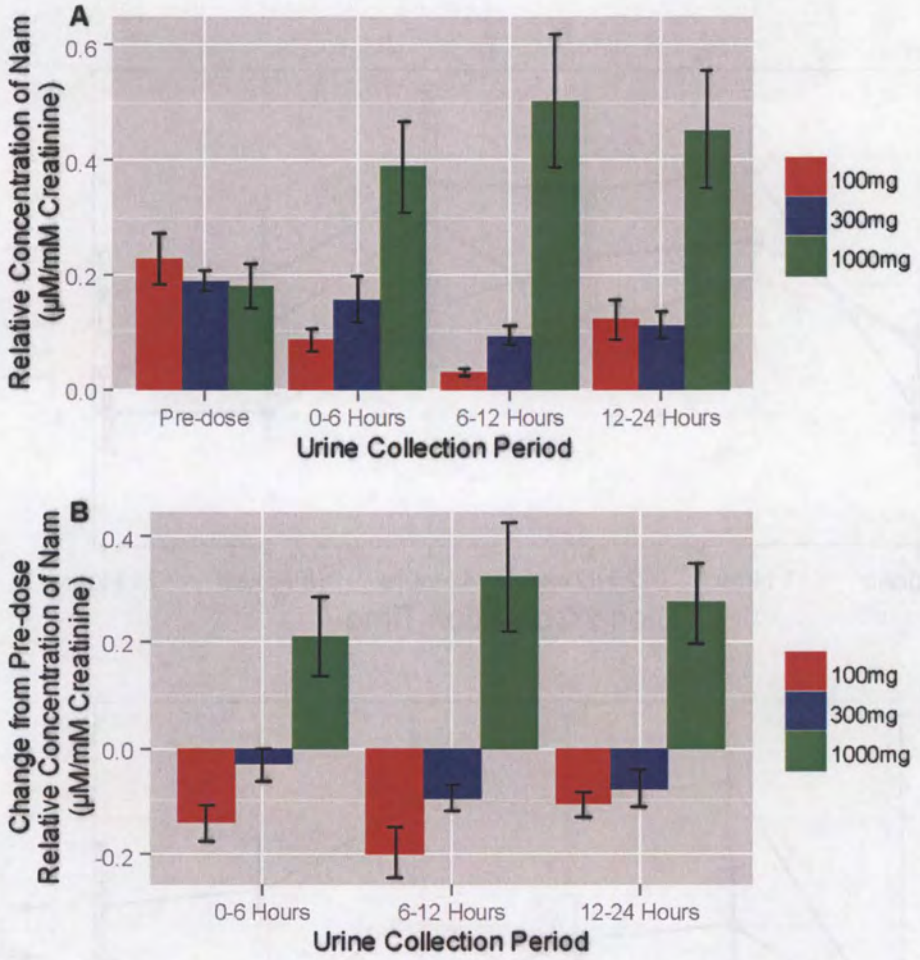

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Figure 13. Nicotinamide: Mean (±SEM) blood plasma concentration and (B) mean (±SEM) change from pre-dose blood plasma concentration of Nam for the pre-dose, 1h, 2h, 4h, 8h, and 24h blood collection times following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)

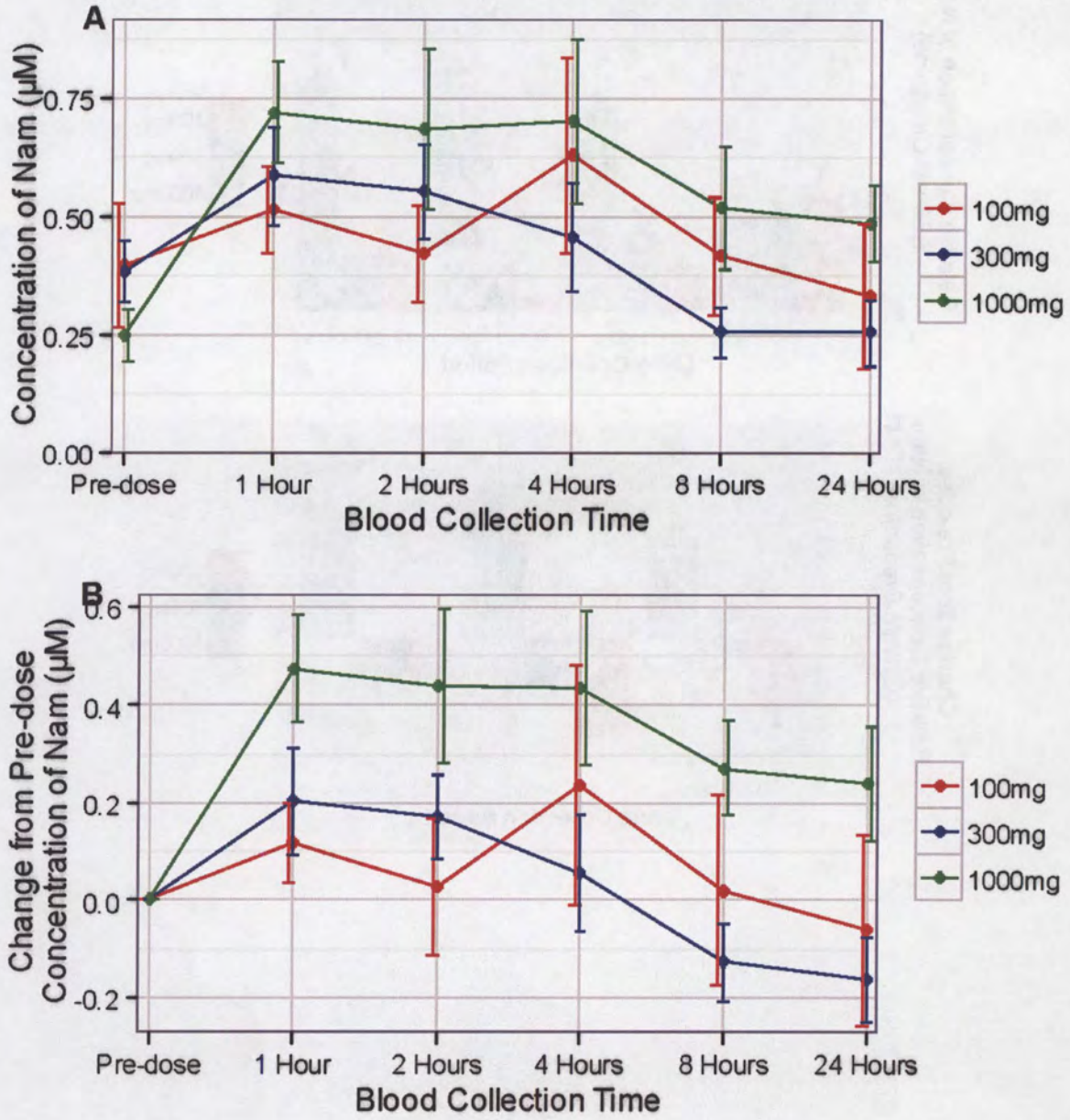

Figure 14. Methyl-nicotinamide: Mean (±SEM) relative urine concentration and (B) mean (±SEM) change from pre-dose mean relative urine concentration of MeNam standardized by creatinine for the pre-dose, 0-6 Hour, 6-12 Hour, and 12-24 Hour urine collection periods following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)

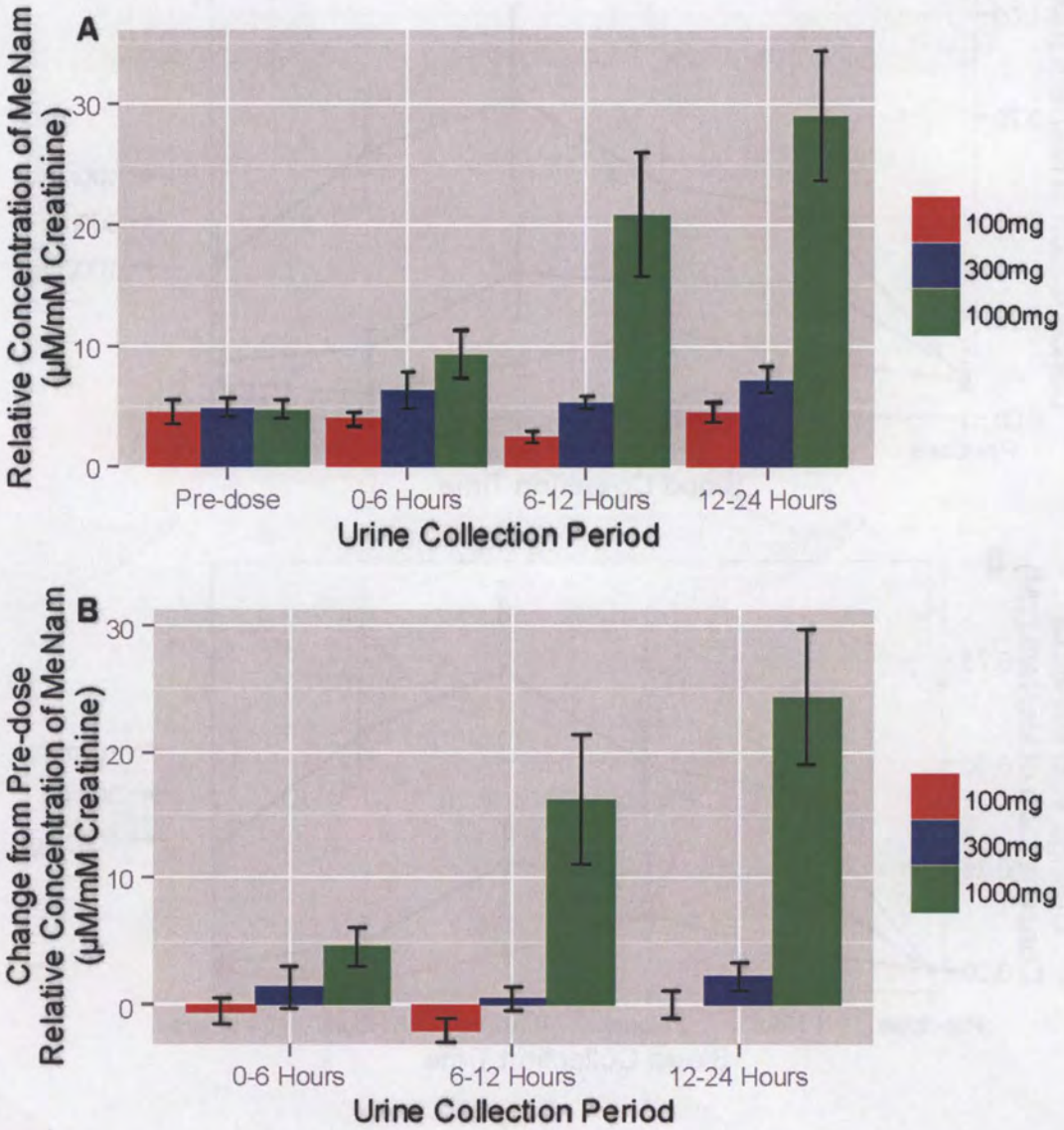

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Figure 15. Methyl-nicotinamide: Mean (±SEM) blood plasma concentration and (B) mean (±SEM) change from pre-dose blood plasma concentration of MeNam for the pre-dose, 1h, 2h, 4h, 8h, and 24h blood collection times following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)

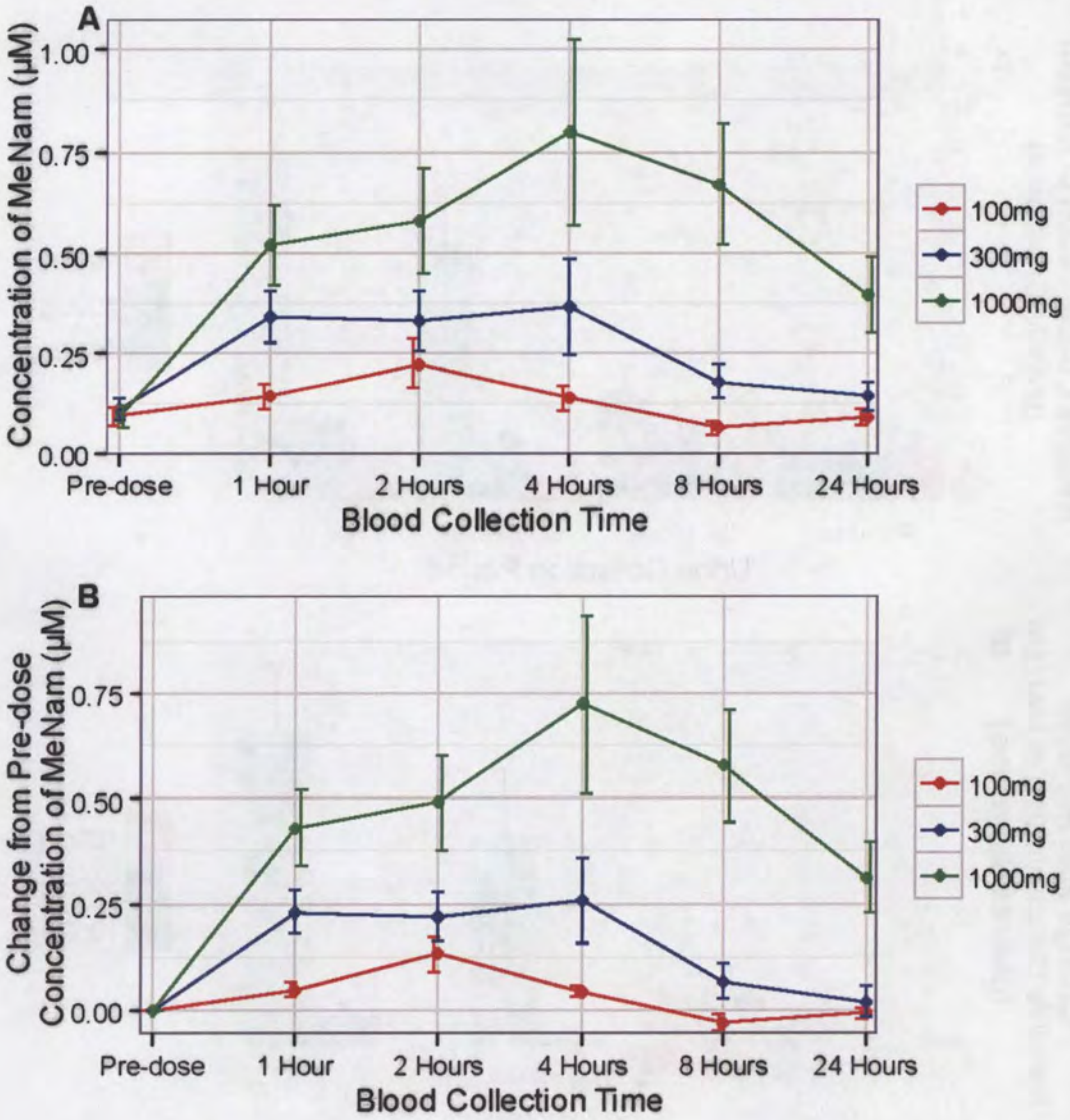

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Figure 16. Mean (±SEM) relative urine concentration and (B) mean (±SEM) change from pre-dose mean relative urine concentration of Py 4 standardized by creatinine for the pre-dose, 0-6 Hour, 6-12 Hour, and 12-24 Hour urine collection periods following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)

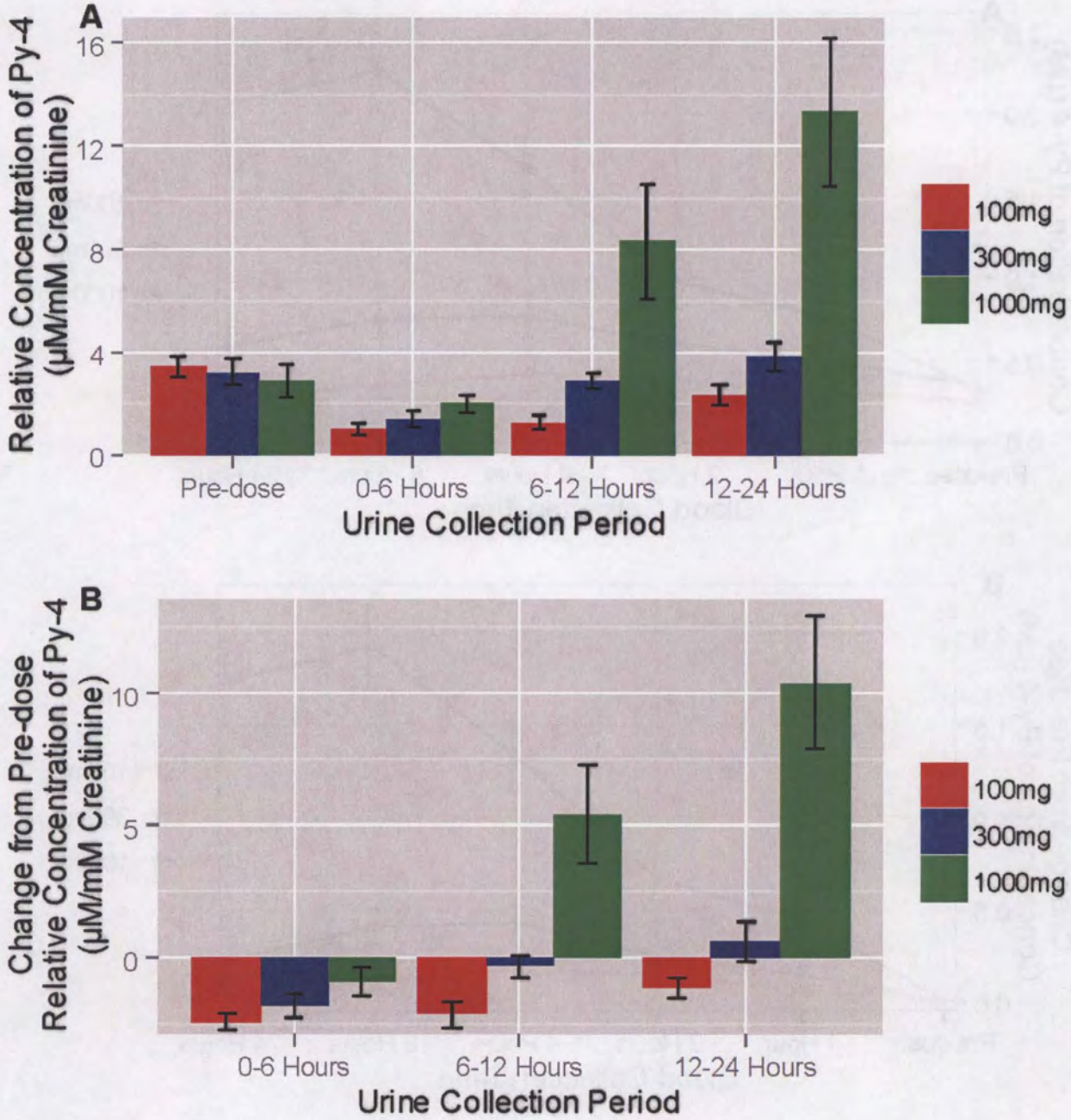

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Figure 17. Mean (±SEM) blood plasma concentration and (B) mean (±SEM) change from pre-dose blood plasma concentration of Py_4 for the pre-dose, 1h, 2h, 4h, 8h, and 24h blood collection times following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)

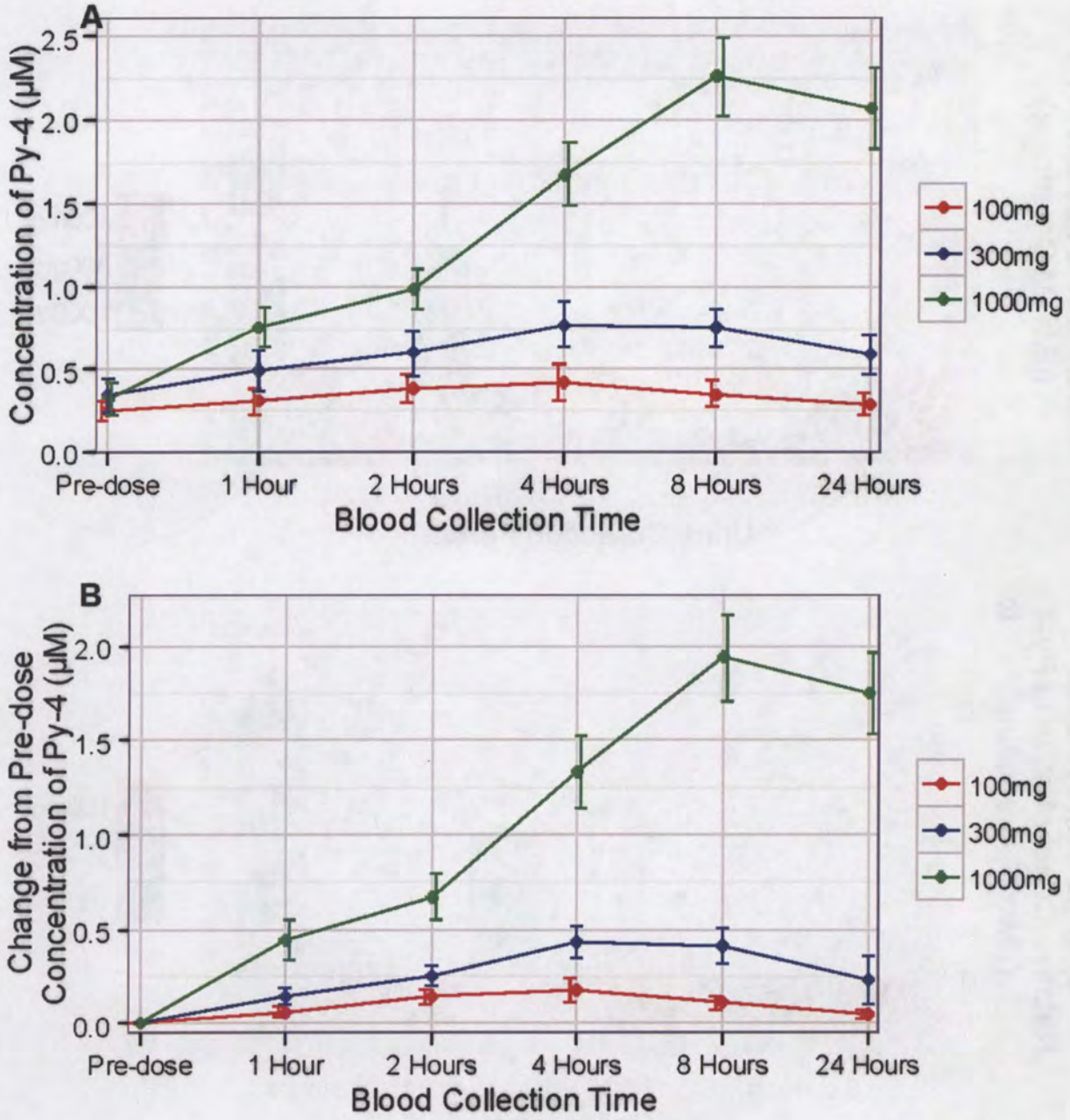

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Figure 18. Mean (±SEM) relative urine concentration and (B) mean (±SEM) change from pre-dose mean relative urine concentration of Py 2 standardized by creatinine for the pre-dose, 0-6 Hour, 6-12 Hour, and 12-24 Hour urine collection periods following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)

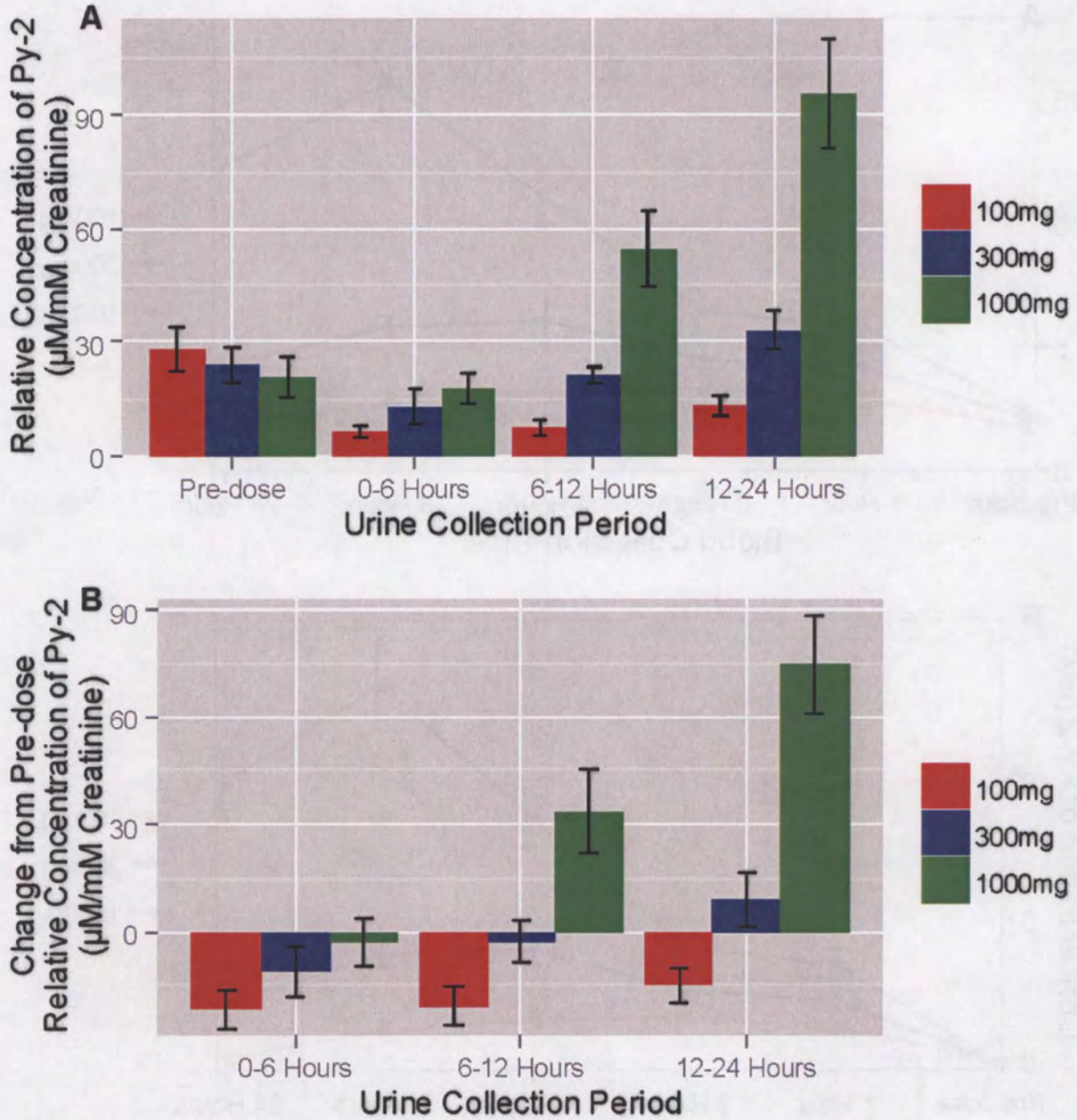

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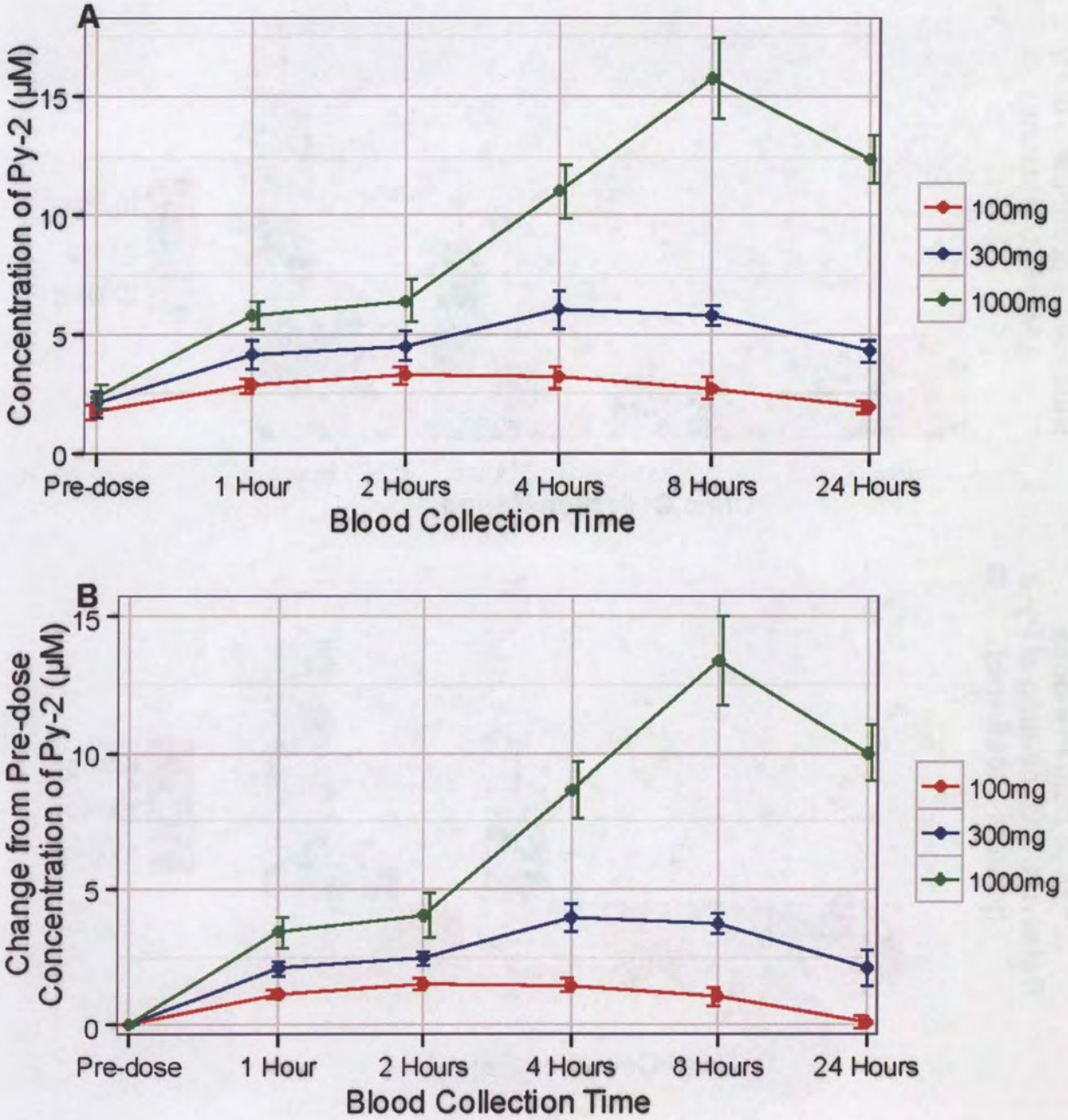
Figure 19. Mean (±SEM) blood plasma concentration and (B) mean (±SEM) change from pre-dose blood plasma concentration of Py_6 for the pre-dose, 1h, 2h, 4h, 8h, and 24h blood collection times following administration of 100mg, 300mg or 1000mg of Niagen to Healthy Adults (N=12)



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G. CORROBORATIVE SAFETY DATA ON NICOTINAMIDE

1. Introduction

Because NR is a form of Vitamin B_3 (niacin), understanding the safety profile of nicotinamide is relevant when assessing the safety of Niagen (NR chloride) in human subjects. NR might also be converted partly into nicotinamide *in vivo*, further highlighting the relevance of nicotinamide metabolism for Niagen.

Animal and human studies indicate that oral administration of nicotinamide is well tolerated (OECD SIDS, 2002) (Cosmetic Ingredient Review Expert, 2005). Genotoxicity tests on nicotinamide have shown that it is not mutagenic in bacterial strains and does not induce clastogenic effects in micronucleus testing. The oral LD_{50} for nicotinamide has been reported to be 3000-7000 mg/kg bw in rodents. A 4-week rat feeding study with nicotinamide showed increased liver weight with extramedullary haematopoiesis in the spleen (in females) at 1000 mg/kg bw/day, which were considered by the authors to be minor and due to adaptation to repeated nicotinamide administration (OECD SIDS, 2002). Importantly, the study established a NOAEL of 215 mg/kg bw/day, but because only 215 and 1000 mg/kg bw/day were tested, the true NOAEL for nicotinamide may be higher than 215 mg/kg/day. Handler and Dann et al. (1942) administered nicotinamide to young laboratory animals at 1 or 2 percent of the diet (equivalent to 1000 to 2000 mg/kg bw/day) and found that nicotinamide depressed growth at both doses.

In lifetime carcinogenicity studies, there was no reported increase in the incidence of tumors in mice receiving 1 % nicotinamide in the drinking water, (approximately 3 mg/kg bw/day) starting at six weeks of age (Toth, 1983).

2. Clinical Studies on Nicotinamide

Because animal toxicology studies on nicotinic acid and nicotinamide are limited and do not comply with current standardized testing protocols, such as OECD (Unna, 1939; Handler and Dann, 1942; Chen et al., 1938; OECD SIDS, 2002), authoritative bodies have used the results of clinical studies in which high doses of nicotinic acid and nicotinamide have been administered to derive the ULs. The most severe forms of toxicity after nicotinic acid ingestion are hepatotoxicity and glucose intolerance, and occur at doses greater than 500 mg/day. Flushing has also been reported, and generally occurs at doses greater than 50 mg/day. As a result, it is considered to be the most sensitive endpoint of nicotinic acid effects. In contrast, after ingestion of supplemental nicotinamide, no cases of flushing or glucose intolerance have been reported. No significant adverse effects have been reported in clinical trials which have used doses up to

the equivalent of 3000 mg/day for up to 3 years to evaluate the possible benefits of nicotinamide administration to patients with or at risk of developing Type 1 diabetes (Lampeter et al., 1998; Pozzilli et al., 1995). In addition, doses of 25 and 42 mg/kg bw/day had no effect on a variety of biochemical parameters, such as those that assessed liver and kidney function. Only one case of hepatotoxicity was reported following the ingestion of greater than 3 g/day for several days (Winter and Boyer, 1973).

Although nicotinamide does not appear to be associated with flushing, the IOM established a UL of 35 mg/day for adults 19 years and older for both nicotinic acid and nicotinamide based on flushing because it is considered to be protective against potential adverse effects. The EC SCF established a UL of 10 mg acid/day for nicotinic acid based on flushing (EC SFC, 2002).

The SCF established a separate UL of 900 mg/day for nicotinamide based on hepatic function in diabetic subjects given nicotinamide and did not use data on flushing after nicotinic acid as the default for the UL for nicotinamide as was used by IOM (EC SFC, 2002). This value represents the lowest dose that did not produce an adverse health effect from trials considered by the European Commission to be of high quality, used sensitive markers of hepatic function and glucose homeostasis, and included a range of age groups, with some subjects treated with up to 50 mg/kg bw/day. Although no adverse effects were identified, an uncertainty factor of 2 has been used to allow for the fact that many of the subjects in these studies were children, data for children may not reflect the full extent of intersubject variability that could occur in an older population, and because adults may eliminate nicotinamide more slowly. This represents a guidance value of 12.5 mg/kg bw/day for nicotinamide, which is equivalent to 900 mg/day of nicotinamide in a 70 kg adult, for supplementation purposes only.

More recent clinical trials (Crino et al., 2005; Cabrera-Rode et al., 2006) have used high levels of nicotinamide for extended periods of time, up to 5 years. These studies indicated that subjects were monitored for adverse events including liver enzymes and other signs of toxicity throughout the study, however, data was not reported. Even though these safety outcomes are not published for these studies, there were no reports of subjects discontinuing the long term use of nicotinamide due to hepatotoxicity and other adverse events related to treatment.

Only one case of hepatotoxicity has been reported in a subject receiving a high dose of nicotinamide (3000 mg/day followed by 9000 mg/day for several days) (Winter and Boyer, 1973). However, the relevance of nicotinamide in inducing these effects is unclear because subject also received high doses of nicotinic acid for an extended period of time.

3. Relationship Between Metabolic Fate of Niagen and Nicotinamide

The pharmacokinetic analysis from the first in human clinical study (14NBHC) shows that Methyl-nicotinamide (MeNam), Py 4 and Py 2 are predominant NAD+ related metabolites that appear within hours after ingestion of a single dose of Niagen. The generation of these NR metabolites is consistent with nicotinamide being an intermediate in the metabolism of Niagen in humans.

Methyl-nicotinamide is a biomarker of niacin status and correlates with NAD+ levels in red blood cells in humans. (Jacob et al., 1989; Fu et al., 1989). Moreover MeNam, Py 4 and Py 2 concentrations in urine correlate with the levels of niacin intake and NAD+ levels in humans. (Jacob et al., 1989; Fu et al., 1989; Shibata et al., 1989).

Thus, the pharmacokinetic data strongly suggest that NR is metabolized in a similar fashion to nicotinamide in humans. This supports that NR is another form of Vitamin B_3 that can function as a NAD+ precursor in cellular energy metabolism.

H. Derivation of a Tolerable Upper Intake Level (UL) for NR

The definition of a UL is "the maximum level of daily nutrient intake that is likely to pose no risk of adverse effects" (IOM 2000). The model that was developed and used to determine ULs is based on well-established principles of risk assessment and relies on data concerning adverse health effects from excessive nutrient intakes in epidemiologic studies, clinical trials, and experimental studies. Several factors associated with these various data sources influence the derivation of a UL. Among the most important of these factors are the intake at which adverse effects are documented (the LOAEL) and the maximum level of intake, which is always less than the LOAEL, at which no adverse health effects are observed (the NOAEL).

The method mirrors guidance provided by FDA in the Redbook approach to the determination of an Acceptable Daily Intake (ADI) for safety evaluation of food additives. Toxicologists exercise their best scientific judgement in determining what toxicity studies are needed to adequately assess the safety of an ingredient, taking into account what is already known about the properties of a compound, its intended conditions of use, and current standards for toxicity testing. From this data, the NOAEL is determined, an appropriate safety factor is selected, and the ADI is calculated. The use of safety factors is based on the observation that toxic substances usually have thresholds below which toxic effects cannot be detected. The safety factor attempts to account for differences between animals and humans and differences in sensitivity among humans. Use of the safety factor is intended to provide an adequate margin of

safety for consumers. For non-cancer endpoints, the NOAEL is divided by a safety factor to obtain an estimate of the maximum acceptable daily intake (ADI) of the additive for humans. The selection of a safety factor is based on the biological significance of the endpoint, uncertainties inherent in extrapolating information about adverse effects from toxicity studies in animals to human populations, and other judgmental factors. The food additive procedural regulations (21 CFR 170.22) state that a safety factor of 100 will be used as a general rule in applying animal test data to man.

The two major forms of vitamin B_3, nicotinic acid and nicotinamide, are together commonly known as niacin, and constitute the most well-known NAD+ precursors. NR is a recently discovered form of vitamin B_3 (Erdman et al. 2012). Niagen is metabolized by the body similarly to nicotinamide as demonstrated in a single-dose human clinical trial. No clinically adverse effects on hematology, clinical chemistry, urinalysis or liver or kidney function parameters were noted in the clinical trial. An Ames assay determined that Niagen is not genotoxic under the conditions of the assay.

In a pivotal 90-day toxicology study of Niagen, adverse effects identified at 3000 mg/kg/day Niagen included: treatment-related organ weight changes in liver, kidneys, testes, epididymides and ovaries; decrease in body weight, food consumption; increases in clinical chemistry parameters related to hepatocyte damage correlated with liver weight increase, centrilobular hepatocellular hypertrophy and single cell necrosis. Treatment related decreases in leukocytes, neutrophils and monocytes were noted. In addition, thyroid follicular cell hypertrophy and increased kidney weight with exacerbation of chronic progressive nephropathy were observed. Importantly, similar types and magnitude of adverse effects were noted in the nicotinamide positive control group.

Niagen administration at 1000 mg/kg/day dose level resulted in treatment related organ weight changes in liver and kidney and increases in neutrophils and leukocytes, ALT and triglycerides which were statistically significant in female rats only. Changes in neutrophils and leukocytes were not associated with significant inflammatory changes in all organs examined. The significant increases in ALT at this dose were noted only in one gender, occurred in conjunction with an increase in triglycerides but were below the 2-fold increase typically used to establish a biologically significant signal of liver damage in the absence of histological change (Hall et al. 2012). The kidney weight increases at this dose occurred in the absence of corresponding histopathology. Therefore, the effects in liver at 1000 mg/kg/day are considered to be treatment-related but mild and potentially adaptive in nature. Based on the minimal changes at 1000 mg/kg/day, this dose was considered the LOAEL.

Administration of Niagen at 300 mg/kg/day did not result in any adverse effects in any of the parameters monitored and is considered to be the NOAEL. A slight decrease (8%) in overall body weight (Day 90) was considered adaptive and therefore, not adverse.

The NOAEL from the 90-day rodent study is 300 mg/kg/day and effects noted at the LOAEL of 1000 mg/kg/day are considered to be mild and potentially adaptive in nature. Therefore, application of a 10-fold safety factor to account for extrapolation from rodents to humans and a 10-fold safety factor to account for intra-individual variability applied to the NOAEL is considered appropriately conservative to derive a UL. Application of these safety factors results in a UL of 3 mg/kg/day or 180 mg/day for a 60 kg individual.

Importantly, Niagen is another form of Vitamin B_3 and the toxicity of Niagen and nicotinamide was demonstrated to be similar in the 90-day rodent study. Therefore, the derivation of the UL for Niagen is conservative and below the UL identified by the European Commission of 900 mg/day for nicotinamide (EC SFC, 2002) as well as the UL identified by the UK Expert Group on Vitamins and Minerals (May 2003) of 500 mg/day.

The UK Expert Group (2003) calculated the UL for nicotinamide based on human studies of large doses (up to 3000 mg/day for periods of up to 3 years) that appear to be well tolerated. Two of the best conducted studies are those by Pozzilli et al. (1995) and Lampeter et al. (1998) and were used for guidance purposes. In these studies, doses of 25 and 42 mg/kg bw/day did not affect a range of biochemical parameters, including liver and kidney function tests in small groups of Type 1 diabetics (or those at high risk of developing the condition). Although no adverse effects were detected, the nature of the study population and the small numbers involved mean that these findings may not be applicable to the whole population. Although no adverse effects were identified, the Expert Group applied a UF of 3 to account for inter-individual variability because of the nature of the study population. Thus, 25/3 results in a guidance value, for supplementation only, of 8.3 mg/kg bw/day for nicotinamide. This is equivalent to 500 mg/day supplemental nicotinamide in a 60 kg adult.

The derivation of the UL for nicotinamide by the SCF EU (2002) considered major long-term studies in patients with Type 1 diabetes mellitus, at dosages of 2-3 g of nicotinamide per day, (ENDIT - see Reimers et al., 1993; IMDIAB III - see Pozzilli et al., 1995; DENIS - see Lampeter et al., 1998). The IMDIAB III study involved a double-blind trial in which 28 newly diagnosed patients with Type 1 diabetes mellitus were given 25 mg/kg bw of nicotinamide daily for 12 months, and a similar number treated with placebo; no adverse effects were reported and biochemical parameters including liver and kidney function were normal during follow-up (the publication describes the measurement of bilirubin). The DENIS trial (Deutsche Nicotinamide

Intervention Study) was a study in young children (average age 3 years) at high risk of developing Type 1 diabetes mellitus in which 25 subjects were randomised to receive nicotinamide (1.2 g per m^2 per day), and 30 to receive placebo; the trial continued for 3 years and during this period all biochemical markers (including alanine aminotransferase, aspartate aminotransferase and bilirubin) were in the normal range. No significant adverse effects have been reported in trials on the possible benefits of nicotinamide in patients with or at risk of diabetes, which have used doses up to the equivalent of 3 g per day, for periods up to 3 years. The NOAEL from these studies is approximately 25 mg/kg bw/day. This value represents the lowest reported dose in a number of recent trials of high quality, many of which used sensitive markers of hepatic function and glucose homeostasis, and included a range of age groups, with some subjects treated with up to 50 mg/kg bw/day. An uncertainty factor of 2 was used to allow for the fact that adults may eliminate nicotinamide more slowly than the study groups, many of which were children, and that data for children would not reflect the full extent of intersubject variability that could occur in an older population. The upper level for nicotinamide is established at 12.5 mg/kg bw/day or approximately 900 mg/day for adults.

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