A randomized pilot trial to evaluate the bioavailability of natural versus synthetic vitamin B complexes in healthy humans as reflected in homocysteine, oxidative stress and antioxidant levels

Nonmedical study director
PD Mag. Dr. Willibald Wonisch
Institut für Physiologische Chemie
A-8010 Graz, Harrachgasse 21/2
Mail: willibald.wonisch@medunigraz.at
Tel: +43 664 – 42 33 177

Medical study director with responsibility for the study center
Prim. Dr. Meinrad Lindschinger
Institut für Ernährung und Stoffwechselerkrankungen
A-8301 Laßnitzhöhe, Hauptstraße 24/Top21
Mail: office@lindschinger.at
Tel: +43 3133 30 660

Responsible for biostatistics and randomization
Dr. Wolfgang Schimetta
Arbeitsgruppe zur Systemoptimierung klinischer Forschungsprojekte ASOKLIF
Abteilung für Angewandte Systemforschung und Statistik
Johannes Kepler Universität
A-4040 Linz, Altenbergerstraße 69
Mail: wolfgang.schimetta@asoklif.at
Tel: +43 0664 4433446

Design:
Monocentric double blind crossover study

Study period:
2017

Inhaltsverzeichnis

1 Scientific background

The vitamin B complex comprises 8 vitamins: thiamin (B1), riboflavin (B2), niacin (B3) panthothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9), and cobalamin (B12). B vitamins are water soluble and essential. A B vitamin deficiency can lead to neurological disorders (amnesia, funicular myelosis), heart failure, coagulopathies, diminished hormone formation and digestive disorders (food intolerances, allergies). Literature searches gave no clear indication as to whether natural or synthetic B vitamins have better bioavailability. Numerous animal studies on guinea pigs, rats and mice, however, suggest that natural vitamins have clear advantages with respect to bioavailability and effectiveness, as well as sustainability.

2 Aim

A 23-week randomized, double-blind crossover study will examine the bioavailability of natural B vitamins in comparison to synthetic B vitamins in humans. The primary working hypothesis of the study is: Natural B vitamins have better bioavailability and are more sustainable than synthetic B vitamins.

The secondary working hypothesis is: Supplementation with a vitamin B complex will either reduce homocysteine or increase the antioxidative potential, with a reduction in oxidative stress.

This study constitutes basic research.

The results of the study are intended to form the basis for generating hypotheses for further studies.

3 Participants

Participants will be 18-65 years old, gender neutral, age correlated BMI >19 and <29. Written informed consent will be required.

The medical director of the study will choose the participants.

The participants should feel “healthy and fit.” Serious illnesses, pregnancy and other medical risks are grounds for exclusion from the study.

Detailed exclusion criteria:

- Total cholesterol >240 mg/dl
- Participation in a study in the last 2 months
- Pregnancy and nursing
- Chronic infections, inflammatory bowel and joint diseases
- Consumption of vitamins, trace elements or fatty acid supplements within the three months prior to the beginning of the study.
- Serious liver or kidney disease
- Diagnosed CHD (myocardial infarction > 1 Jahr previously), tumors, malignant diseases
- Consuming diseases
- Psychiatric diseases
- Lipid lowering medication
- Diabetes mellitus
- Autoimmune diseases

- Maldigestion and malabsorption disorders
- Beer consumption > 1 beer (0.5l/5 vol%) / day
- Vegan nutrition

4 Study variables

4.1 Vitamins

The following serum vitamin concentrations will be determined every time blood is drawn (BA1 to BA12):

- Thiamine (Vitamin B1) [µg/l]
- Riboflavin (Vitamin B2) [µg/l]
- Pyridoxin (Vitamin B6) [µg/l]
- Folic acid (Vitamin B9) [ng/ml]
- Cobalamin (Vitamin B12) [pg/ml]

4.2 Antioxidants and oxidative stress biomarkers

The following serum concentrations or serum activities will be determined every time blood is drawn (BA1 to BA12

- Homocysteine → [µmol/l)
- Peroxidase activity [mU/l]
- Antioxidant status [mmol/l]
- Peroxides [µmol/l]
- Polyphenols [mmol/l]

4.3 Demographic data

The following will be documented at BA1:

- Age [years]
- Gender [m/w]
- Height [cm]
- Weight [kg]

5 Test products

5.1 Natural vitamin B complex (verum product)

Production:

The verum product is available as a food ingredient with the trade name PANMOL® B-COMPLEX and also marketed as a nutritional supplement (e.g. PanMol®-B-Complex GPH capsules) and is made by the vis vitalis gmbh, Salzburg, Austria

Status:

Nutritional supplement

Dosage:

Vitamin	Total amount that will be taken during the study	NRV	UL

B1 (thiamine)	2.60 mg	1.1 mg	no UL established
B2 (riboflavin)	5.11 mg	1.4 mg	no UL established
B3 (niacin)	30.84 mg	16 mg	UL: Nicotinic acid: 10 mg/d UL: Nicotinamide: 900 mg/d or 12.5 mg/kg KG
B5 (pantothenic acid)	12.53 mg	6 mg	no UL established
B6 (pyridoxine)	3.57 mg	1.4 mg	25 mg/d
B7 (biotin)	0.106 mg	0.05 mg	no UL established
B9 (folic acid)	0.8 mg	0.20 mg	no UL established, but consumption of not more than 1 mg/d recommended
B12 (cobalamin)	8.7 µg	2.5 µg	no UL established

Abbreviations:
NRV (Nutrient referent value) = Reference amount for daily consumption
UL (tolerable upper intake level) = tolerable maximal amount for regular consumption

Composition:

Filled in hydroxypropylmethylcellulose capsules (size 0, white)

3 capsules (one daily dose) contain 2100 mg pulverized quinoa sprouts with biologically active vitamins:

- Vitamin B1 (as thiamine) ……..2.60 mg
- Vitamin B2 (as riboflavin) ……5.11 mg
- Vitamin B3 (as niacin) …….30.84 mg
- Vitamin B5 (as pantothenic acid) …...12.53 mg
- Vitamin B6 (as pyridoxine) ……3.57 mg
- Vitamin B7 (as biotin) ……..0.106 mg
- Vitamin B9 (as folic acid) ……0.80 mg
- Vitamin B12 (as cobalamin) …….8.7 µg

These are minimum amounts of vitamins. Due to natural variations during the sprouting of quinoa seeds, the contents of the individual vitamins can be as much as 50% higher than as given in the table.

5.2 Synthetic vitamin B complex (control product)

Production:

The control product will be made by the vis vitalis gmbh, Salzburg

Status:

Nutritional supplement

Dosage:

Vitamin	Total amount that will be taken during the study	NRV	UL
B1 (thiamine)	2.60 mg	1.1 mg	no UL established
B2 (riboflavin)	5.11 mg	1.4 mg	no UL established
B3 (niacin)	30.84 mg	16 mg	UL: Nicotinic acid: 10 mg/d UL: Nicotinamide: 900 mg/d or 12.5 mg/kg KG
B5 (pantothenic acid)	12.53 mg	6 mg	no UL established
B6 (pyridoxine)	3.57 mg	1.4 mg	25 mg/d
B7 (biotin)	0.106 mg	0.05 mg	no UL established
B9 (folic acid)	0.8 mg	0.20 mg	no UL established, but consumption of not more than 1 mg/d recommended
B12 (cobalamin)	8.7 µg	2.5 µg	no UL established

Abbreviations:
NRV (Nutrient referent value) = Reference amount for daily consumption
UL (tolerable upper intake level) = tolerable maximal amount for regular consumption

Composition:

Filled in hydroxypropylmethylcellulose capsules (size 0, white)

3 capsules (one daily dose) contain 2100 mg pulverized quinoa sprouts with biologically active vitamins:

- Vitamin B1 (as thiamine) ……..2.60 mg
- Vitamin B2 (as riboflavin) ……5.11 mg
- Vitamin B3 (as niacin) …….30.84 mg
- Vitamin B5 (as pantothenic acid) …...12.53 mg
- Vitamin B6 (as pyridoxine) ……3.57 mg
- Vitamin B7 (as biotin) ……..0.106 mg
- Vitamin B9 (as folic acid) ……0.80 mg
- Vitamin B12 (as cobalamin) …….8.7 µg

The vitamin contents of the control product are identical to those of the verum product and will be adjusted to those of the verum product when the final vitamin contents of the verum product are available.

5.3 Consumption

Three capsules per day.

To be taken by mouth: Three capsules in the morning before breakfast with sufficient water (approx. ¼ liter).

5.4. Blinding/randomization

The person responsible for producing the test product and blinding it provides for identical appearance of the verum and control products. The test product packages are prepared on the basis of the list of group assignments provided by

the person responsible for randomization (in the following “randomizer”) (1 test product package consists of 126 capsules per phase and participant). Each test product package will be labeled with the appropriate participant’s number (1,2,3…) and the phase (phase I, phase II). Only these two labels distinguish the test product packages from one another.

The randomizer and all nonblinded personnel involved in the production and blinding of the test product are bound to confidentiality toward third parties. The randomizer prepares both a sealed envelope containing the randomization procedure used and the list with group classifications (master list) that may only be opened after the study had been evaluated, and a sealed envelope for each study participant with the respective individual decoding that can be opened in an emergency.

Group classification of the study participants is based on a 1:1 ratio of group A to group B (group A: verum product in phase I and control product in phase II; group B: control product in phase I and verum product in phase II).

Only those study participants will be randomized for whom prior to the beginning of phase I no dropout criteria were identified (for other premature termination of participation in the study, see section “Dropout criteria”). Participants accepted for the run-in phase receive a specific study participant number (R1, R2, R3…). When they are accepted for the randomized part of the study (from phase I on), this number is then replaced in ascending order with the final study participant number (1,2,3…), which is also on the respective test product package.

6 Methodology

6.1 Blood sampling

Per sample, a maximum of 20 ml of venous blood is drawn from the seated patient. Side effects may be pain from the venipuncture or circulatory problems.

6.2 Blood analyses

Dr. Meinrad Lindschinger, an internist at the Institute for Nutrition and Metabolic Diseases in Lassnitzhöhe, is responsible for blood sampling. Blood analyses are done at Med. u. Chem. Labordiagnostik Lorenz & Petek GmbH (8010 Graz) under the direction of Dr. Petek, and at the Medical University of Graz under the direction of Dr. Wonisch.

6.3 Procedure

3 weeks: Run-in phase without consumption of test product
- Baseline exam (morning, fasting) (BA1)
- Nutrition instruction (see attachment)

- Randomization into groups A and B at end of the run-in phase
- 6 weeks: Test product consumption (Phase I):
  - Group A – daily consumption of verum product
  - Group B – daily consumption of control product

- Blood sampling on Phase I day 1 (morning, fasting, hours as from consumption of the first test product tablet: 0h (BA2), 1.5h (BA3), 4h (BA4), 7h (BA5))
- Blood sampling at the end of the 6 weeks (fasting, morning of the day after the last test product was taken on Phase I day 42, BA6)

2 weeks: Test product is not taken (washout phase)

6 weeks: Test product consumption (Phase II):

- First day: Group A – consumption of control product
- Group B – consumption of verum product
- Blood sampling on Phase II day 1 (morning, fasting, hours as from consumption of the first test product tablet: 0h (BA7), 1.5h (BA8), 4h (BA9), 7h (BA10))
- Blood sampling after 6 weeks (fasting, morning of the day after the last test product was taken on Phase I day 42) BA11 and final physical exam

6 weeks (± 3 days): No consumption of product (follow-up phase):

- Blood sampling 6 weeks (± 3 days) after final physical exam (morning, fasting): BA12 (follow-up physical exam)

7 Dropout criteria

A case is classified as a dropout when at least one of the following criteria is met, with loss of status as a valid per protocol analysis case:

- Withdrawal of consent to participate prior to the final physical exam
- Inavailability of vitamin parameters and antioxidant and oxidative stress biomarkers (exception: follow-up study)
- Consumption of less than 80% of the test products per phase (I and II)
- Nonconsumption of the test product on the first and last day of the respective phase (I and II)
- Consumption of vitamin B complex or single vitamin B preparations between the baseline and final physical exam
- Change in the vitamin B serum concentration at BA7 vis a vis BA2 by more than 35%
- Relevant noncompliance with nutritional guidelines before the final physical exam (as classified by the trial director before unblinding)
- Unblinding before the final physical exam

If a dropout criterion is met before the end of the washout phase, the individual's participation is terminated prior to entry into Phase I.

8 Study parameters

8.1 Primary outcome measures

Apply to vitamins B1, B2, B6, B9 und B12 (specific vitamin units):

- Vitamin Bx serum concentrations at BA3 and BA8
- Increase of Vitamin Bx serum concentrations at BA3 (xBA3 minus xBA2) and BA8 (xBA8 minus xBA7)

- Vitamin Bx serum concentrations at BA4 and BA9
- Increase in Vitamin Bx serum concentration at BA4 (xBA4 minus xBA2) bzw. BA9 (xBA9 minus xBA7)
- Vitamin Bx serum concentration at BA5 and. BA10
- Increase in Vitamin Bx serum concentration at BA5 (xBA5 minus xBA2) and BA10 (xBA10 minus xBA7)
- Vitamin Bx serum concentration at BA6 and BA11
- Increase in Vitamin Bx serum concentration at BA6 (xBA6 minus xBA2) and BA11 (xBA11 minus xBA7)
- Baseline-adjusted (xBAy minus xBA2 and minus xBA7) Area Under the Curve (AUC) of vitamin Bx serum concentrations at BA2, BA3, BA4 and BA5 and at BA7, BA8, BA9 und BA10 per unit time (divided by 7) corresponding to the average increase in the vitamin Bx serum concentrations between BA2 and BA5 and BA7 und BA10

8.2 Secondary outcome measures

Apply to all antioxidant and oxidative stress biomarkers (specific biomarker units)

- Biomarker x serum concentration or activity at BA3 and BA8
- Increase in biomarker x serum concentration or activity at BA3 (xBA3 minus xBA2) and BA8 (xBA8 minus xBA7)
- Biomarker x serum concentration or activity at BA4 and BA9
- Increase in biomarker x serum concentration or activity at BA4 (xBA4 minus xBA2) and BA9 (xBA9 minus xBA7)
- Biomarker x serum concentration or activity at BA5 and BA10
- Increase in biomarker x serum concentration or activity at BA5 (xBA5 minus xBA2) and BA10 (xBA10 minus xBA7)
- Biomarker x serum concentration or activity at BA6 and BA11
- Increase in biomarker x serum concentration or activity at BA6 (xBA6 minus xBA2) and BA11 (xBA11 minus xBA7)
- Baseline-adjusted (xBAy minus xBA2 and minus xBA7) Area Under the Curve (AUC) of the biomarker x serum concentrations and activities at BA2, BA3, BA4 und BA5 and at BA7, BA8, BA9 und BA10 Per unit time (divided by 7) corresponding to the average increase in the vitamin Bx serum concentrations between BA2 and BA5 and between BA7 und BA10

8.3 Further parameters

Demographic data at BA1:

- Age [years]
- Gender [m/w]
- Height [cm]
- Weight [kg]
- Body Mass Index (BMI) [kg/m²]

For vitamins B1, B2, B6, B9 und B12:

- Vitamin Bx serum concentrations at BA1
- Vitamin Bx serum concentrations at A2 and BA7
- Vitamin Bx serum concentration at BA12

For all antioxidant and oxidative stress biomarkers:

- Biomarker x serum concentration or activity at BA1
- Biomarker x serum concentration or activity at BA2 and BA7
- Biomarker x serum concentration or activity at BA12

Compliance parameters:

- Number of vitamin B complex capsules taken in Phase I and Phase II [-]
- Adherence to nutritional guidelines in the run-in phase, Phase I, the washout phase, and Phase II [yes/no]

9 Biometric study design and evaluation

9.1 Study design

This interventional crossover comparison will use descriptive and exploratory statistics.

9.2 Power calculation

The case number of 30 for this crossover study with a postulated dropout rate of 20% should provide sufficient preliminary information to determine whether the bioavailability and sustainability of natural B vitamins are superior to those of synthetic B vitamins.

9.3 Collective for evaluation

The following collectives will be evaluated:

- Intent-To-Treat-Analysis:
  All cases (valid cases and dropouts) who at least began to take the test product. Only random descriptive and safety parameters will be analyzed.
- Per-Protocol-Analysis:
  All cases that did not involve a dropout criterion (all valid cases). All parameters under consideration will be analyzed. The per-protocol analysis is the principal evaluation concept.
- Full Analysis Set:
  All patients included (participants in the intent-to-treat collective plus participants whose participation in the study was terminated before the beginning of Phase I, i.e. before taking the first test product capsule. For all the participants who did not belong to the intent-to-treat collective, all parameters will be analyzed with descriptive statistics, as long as their number exceeds 5; otherwise there will be a narrative description of these cases.

9.4 Implausible values and missing values

- Implausible values:
  Implausible values will be identified by the study directors and included among the missing values

- Missing values:
  Missing values will not be replaced

9.5 Statistical evaluation

- Crossover comparisons (comparison of the two interventions)
  A parametric crossover approach will be used. If the data deviate strongly from normal distribution (tested with the Kolmogorov-Smirnov test with Lilliefors significances, alpha=10%), a nonparametric rather than a parametric test will be used. To test the carryover or phase effects, an alpha of 10% applies.
- Group comparisons:
  The usual parametric and nonparametric tests will be used.
- Estimating the true size of the effects
  A two-sided 95% confidence interval (95% CI) is calculated for the main outcome measures and the chosen random descriptive parameters.
- Alpha error level:
  There is no adjustment of the alpha error level for multiple tests, so that all statistical results are purely descriptive.

9.6 Presentation of results

All documented data will be presented in tables with the numbers of observed and missing values.

- Nominally scaled data are given in tables with absolute and relative frequencies
- Ranked data are given in tables with absolute and relative frequencies and/or median, quartiles, minimum and maximum
- For quantitative data, the following values are given for their distribution:
  - Minimum
  - Median
  - Quartiles
  - Maximum
  - Average
  - Standard deviation

Graphics (box plots, bar diagrams) will be created as needed.

9.7 Intermediate evaluation

An intermediate evaluation is not foreseen

9.8 Post-hoc analyses

Post-hoc analyses can be made when the planned analyses reveal constellations that suggest that they would be useful. The resultant statistics, however, are understood to be purely descriptive.

10 Data security

The participants will be pseudonymized. The data will be encoded, saved in an Excel table and then analyzed.

Only authorized persons will have access to the original data.

11 Risk-benefit evaluation

The participants have no direct benefit from the study.

Side effects of blood sampling can be pain from the venipuncture or circulatory problems

No side effects are known for the vitamins in the test products in the given dosage.

12 References

Human plasma and tissue α-tocopherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E.
Burton G.W., Traber M.G., Acuff R.V., Walters D.N., Kayden H., Hughes L., Ingold K.U.
Am J Clin Nutr 1998;67:669–84

Effect of vitamin E source, natural versus synthetic, and quantity on serum and tissue α-tocopherol concentrations in finishing swine.
Yang H., Mahan D.C., Hill D.A., Shipp T.E., Radke T.R., Cecava M.J.
J. Anim. Sci. 2009. 87:4057–4063

Relative bioavailabilities of natural and synthetic vitamin E formulations containing mixed tocopherols in human subjects.
Chopra R.K., Bhagavan H.N.
Int J Vitam Nutr Res 1999; 69(2):92-95

Synthetic or food-derived vitamin C – are they equally bioavailable?
Carr A.C., Vissers M.C.M.
Nutrients 2013; 5:4282-4304

Natural vitamins may be superior to synthetic ones.
Thiel R.J.
Med Hypotheses 2000; 55(6):461-469

Fortification of yogurts with different antioxidant preservatives: a comparative study between natural and synthetic additives.
Caleja C., Barros L., Antonio A.L., Carocho M., Oliveira M.B.P.P., Ferreira I.C.F.R.
Food Chemistry 2016; 210:262-268

Resveratrol and propolis as necrosis or apoptosis inducers in human prostate carcinoma cells.
Scifo C., Cardile V., Russo A., Consoli R., Vancheri C., Capasso F., Vanella A., Renis M.
Oncol Res. 2004; 14(9):415-426

Quantitative combination of natural anti-oxidants prevents metabolic syndrome by reducing oxidative stress.
Gao M., Zhao Z., Lu P., Li YF. Gao J., Zhang M., Zhao B.
Redox Biology 2015; 6:206-217

Antioxidative responses during germination in quinoa grown in vitamin B-rich medium.
Pitzschke A., Fraundorfer A., Guggemos M., Fuchs N.
Food Science & Nutrition 2015; 3(3): 242–251

Effects of folate treatment and homocysteine lowering on resistance vessel reactivity in atherosclerotic subjects.
Stanger O., Semmelrock H.J., Wonisch W., Bös U., Pabst E., Wascher T.C.
JPET 2002; 303:158-162

Enzymatic and non-enzymatic antioxidative effects of folic acid and ist reduced derivates.
Stanger O. & Wonisch W.
Subcell Biochem 2012: 56:131-161

Dual method for the determination of peroxidase activity and total peroxides-iodide leads to a significant increase of peroxidase activity in human sera.
Tatzber F., Griebenow S., Wonisch W., Winkler R.
Anal Biochem. 2003 May 15;316(2):147-53.

Antioxidant supplementation attenuates oxidative stress in patients undergoing coronary artery bypass graft surgery.
Stanger O, Aigner I, Schimetta W, Wonisch W.
Tohoku J Exp Med. 2014;232(2):145-54.

Vitamine
Physiologie, Pathophysiologie, Therapie - ff.67-131
Eds Biesalski H.K., Schrezenmeir J., Weber P., Weiß H.
Georg Thieme Verlag – Stuttgart – New York 1997

Tolerable Upper Intake Levels for Vitamins and Minerals.
European Food Safety Authority. 2006; Download von
http://europa.eu.int/comm/food/fc/sc/scf/index_en.html am 01.03.2017.

Ernährung des Menschen.
Elmadfa, I. & Leitzmann, C. 2004; 4. Auflage, Ulmer Verlag, Stuttgart.

13 Appendix

13.1 Nutrition instruction

The study is partially supported by the vis vitalis gmbh (Salzburg) in the form of an unrestricted grant and the blinded test products.