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Different elemental infant formulas show equivalent phosphorus and calcium bioavailability in healthy volunteers

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ABSTRACT

Retrospective chart reviews have reported hypophosphatemia associated with elemental formula use in infants and children with systemic disease involving multiple diagnoses. The present study aims to evaluate the bioavailability of phosphorus from 2 commercial elemental formulas and to test our hypothesis of bioequivalence of the 2 products in healthy volunteers receiving gastric acid-suppressive medication. A single-center, double-blind, randomized, cross-over study was conducted in healthy volunteers with esomeprazole-induced hypochlorhydria. After a standardized low phosphorus meal followed by overnight fasting, subjects consumed 1 gram of phosphorus in a single oral dose of 1217 kcal of Product A (Neocate) or Product B (Elecare). The alternate product was given following a 1-week washout period. Blood and urine were collected at baseline and different time-points for up to 6 hours after product consumption. Area-under-the-curve (AUC) and peak values (C_{peak}) for serum phosphate and calcium and urinary creatinine-corrected phosphate and calcium were assessed for bioequivalence of Products A and B. Results show that the geometric mean ratio (GMR) and 90% CI for serum phosphate were 1.041 (0.998–1.086) and 1.020 (0.963–1.080) for AUC_{0-360} and C_{peak} , respectively, meeting the predetermined criteria for bioequivalence. Urinary creatinine-corrected phosphate followed a similar pattern after intake of Product A and B, but did not reach bioequivalence criteria (GMR: $AUC_{0-370} = 1.105$ (0.918–1.330); $C_{peak} = 1.182$ (1.040–1.343)). Serum calcium concentrations (GMR: $AUC_{0-360} = 1.002$ (0.996–1.009); $C_{peak} = 0.991$ (0.983–0.999)) and urinary creatinine-corrected calcium excretion (GMR: $AUC_{0-370} = 1.117$ (1.023–1.219); $C_{peak} = 1.157$ (1.073–1.247)) demonstrated bioequivalence of

Abbreviations: AE, adverse event; ALP, alkaline phosphatase; AUC, area-under-the-curve; C_{peak} , peak concentrations; Ca, calcium; CMA, cow's milk allergy; GMR, geometric mean ratio; IgE, immunoglobulin E; iPTH, intact parathyroid hormone; P, phosphate; T_{peak} , time of peak concentration; TRP, tubular reabsorption of phosphate.

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the products. In conclusion, both elemental infant formulas showed equivalent serum phosphorus and calcium bioavailability in healthy volunteers even if combined with treatment with acid-suppressive medication.

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1. Introduction

Preserving mineral status within healthy ranges is important, particularly for developing infants and children. Calcium and phosphorus serve many different functions in human physiology, including bone mineralization, energy production and cellular signaling [1]. Most of the body's calcium (99%) and phosphorus (85%) are stored in bone. Plasma levels of both minerals are tightly regulated balancing uptake from the diet, redistribution towards and from different body compartments, and renal excretion [2,3].

Mineral absorption from food is influenced by many different factors including, but not limited to, food matrix and viscosity, food processing, mineral composition (chemical source of salt and concentration), co-ingestion of other food items, route of administration (e.g., oral intake vs postpyloric tube feeding), as well as physiological factors like mineral status, functional state of the gastrointestinal tract, age and use of medication (e.g., proton-pump inhibitors) [4].

In contrast to standard infant milk formulas, where a considerable portion of minerals comes with the milk proteins, in elemental amino acid-based formulas all minerals need to be added separately as salts and need to be carefully selected to ensure optimal stability, palatability, and bioavailability. Elemental formulas are used for the dietary management of severe or complex immunoglobulin E (IgE)- and non-IgE-mediated cow's milk allergy (CMA) in formula-fed infants [5]. Dietary management of CMA with an elemental formula has been proven to be well-tolerated, safe and effective in reducing symptoms of CMA. Normal growth in infants on elemental formula has been widely reported [5–12]. Additionally, it has been shown that infants aged 0 to 8 months diagnosed with CMA who received an elemental formula for 16 weeks had blood (trace) mineral concentrations (calcium, phosphorus, chloride, sodium, potassium, magnesium, and ferritin) within the age-specific reference ranges [13,14]. Despite this, recent retrospective chart reviews [15–20] have reported cases of hypophosphatemia associated with elemental formula use in infants and children with complex medical conditions and extensive medical histories.

Hypophosphatemia may be caused by three main mechanisms: (1) decreased intake and intestinal absorption of phosphorus e.g., due to low dietary intake, malnutrition, prematurity, diarrhea, vomiting, gastrointestinal symptoms, intestinal mucosal hypoplasia, short gut, postpyloric feeding, and total parenteral nutrition, low plasma levels of vitamin D, phosphate absorption-inhibitors such as phosphate binders, and acid-suppressive medication), (2) increased urinary loss (e.g., due to lack of renal conservation, metabolic acidosis or (diuretic) drug use, secondary hyperparathyroidism, chronic kidney disease or kidney diseases such as Fanconi syndrome),

or (3) internal redistribution of phosphate from serum to cells (e.g., due to recovery from malnutrition, refeeding syndrome, recovery from diabetic ketoacidosis, respiratory alkalosis, rapid cell proliferation/uptake (e.g., hungry bone syndrome, acute leukemia, Burkitt lymphoma), and hormonal and other agents (e.g., insulin, glucagon, epinephrine, dopamine, beta-2 agonists, steroids, xanthine derivatives) [21,22]. Many of the infants and children described in the case series had at least one of the above-mentioned risk factors for hypophosphatemia. In the vast majority of patients described with hypophosphatemia on elemental formulas, gastric acidification was bypassed either by postpyloric feeding and/or acid-suppressive medication (90%–100% of described cases). Previous *in vitro* studies have indicated that bypassing gastric acidification can affect the bio-accessibility (i.e., the fraction of minerals that is released from the product matrix into the gastro-intestinal tract and becomes available for intestinal absorption) of mineral salts from different amino acid-based medical nutrition products [4]. Furthermore, it has been reported that the use of acid-suppressive medication like proton-pump inhibitors can compromise mineral bioavailability [23] and has been related to increased fracture risk in adults [24,25]. Solubility of minerals, which can be pH dependent, is one of the factors influencing mineral bio-accessibility, and bio-accessibility is a primary requisite for mineral bioavailability (i.e., the fraction of minerals that is absorbed from the diet and used for normal body functions).

The sources of calcium and phosphorus in the Neocate powdered infant and junior product range have been reviewed with the aim to further optimize the solubility of the mineral salts. Venema et al. showed that the bio-accessibility of phosphorus in the updated Neocate was high even under conditions bypassing stomach acidification [4]. The present study aims to evaluate the bioavailability of phosphorus and calcium of Neocate Infant DHA/ARA (with dipotassium phosphate, dicalcium phosphate, tricalcium phosphate, calcium carbonate, tricalcium citrate, and calcium glycerophosphate) and to test the hypothesis that the bioavailability of phosphorus and calcium of Neocate is bioequivalent to an alternate commercially available elemental formula under hypochlorhydric conditions mimicking the complex settings described in the case series.

2. Methods and materials

2.1. Study design

This was a single-center, double-blind, randomized, 2-period, cross-over trial conducted at the Nutrition Clinical Research

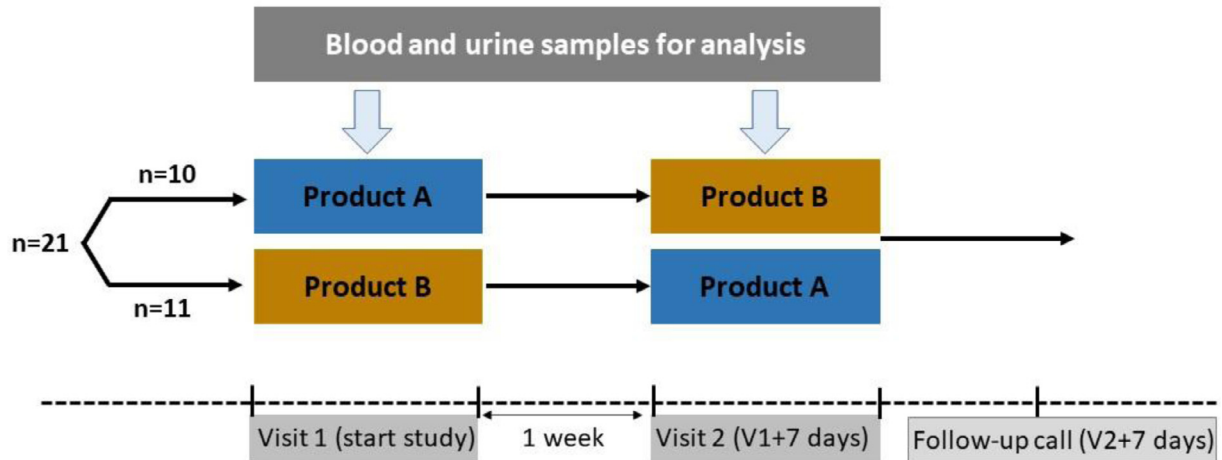


Fig. 1 – Schematic diagram of study design.

Unit, Utrecht, the Netherlands between October 2018 and March 2019. The study (registered at www.trialregister.nl as NTR7575/Trial NL7367) was approved by the Medical Review and Ethics Committee of Foundation BEBO (Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands) and conducted in accordance with the principles of the Declaration of Helsinki [26] and Good Clinical Practice [27]. The study consisted of four phases: (1) screening for eligibility (day –28 to –6), (2) a first visit that included blood and urine sampling for pharmacokinetic analyses (Visit 1), (3) a 1-week wash-out period, and (4) a second visit that included blood and urine sampling for pharmacokinetic analyses (Visit 2; Fig. 1). For the entire duration of the study, i.e., from screening to Visit 2, subjects took 10 µg vitamin D3 daily by mouth. On the 4 days prior to Visit 1 and Visit 2, subjects self-administered 40 mg esomeprazole twice daily by mouth to induce hypochlorhydria. Additionally, participants were instructed not to eat or drink any phosphorus rich foods or beverages (Supplemental Table S1) for 24 hours prior to the study visits. On the evening prior to each study visit, the participants came to the study site after being fasted for 4 hours and took the esomeprazole at the site. At least 30 minutes after intake of the esomeprazole, participants received a standardized low phosphorus meal (containing 280–415 mg phosphorus, dependent on gender, age, body weight, and height) and went home. The subsequent morning, participants came to the study site for Visit 1 or Visit 2 after fasting since the standardized dinner the evening before. Participants were allowed to drink water until 2 hours before the study visits. A nasogastric tube was placed and an additional dose (40 mg) of esomeprazole was given by mouth. Subjects were block-randomized in the order they entered the study using a random number generator for the order in which they would receive product A (Neocate Infant DHA/ARA, Nutricia, Liverpool, UK with dipotassium phosphate, dicalcium phosphate, tricalcium phosphate, calcium carbonate, tricalcium citrate, and calcium glycerophosphate) or Product B (Elecare Infant, Abbott Nutritionals, Columbus, OH, USA with calcium phosphate, potassium phosphate, and calcium carbonate). Both products were isocaloric with an energetic

value of 1217 kcal per serving to provide 1 g of phosphorus. Details on the ingredients and nutritional composition of both formulas are presented in Table 1. The products were consumed from a blinded cup within 10 minutes in one concentrated serving of approximately 475 mL (~256 kcal/100 mL: 3.8 times more concentrated than the standard dilution of 68 kcal/100 mL for normal use in infants). Ten to 20 minutes after product intake, hypochlorhydria (stomach pH ≥ 5.5) was confirmed by a 5 mL (~1% of the bolus provided) gastric aspirate pH measurement. Blood samples were collected before study product intake ($T = -20$ and $T = -5$ minutes) and for up to 6 hours after product consumption at $T = 20, 40, 60, 80, 100, 120, 140, 160, 180, 210, 240, 300,$ and 360 minutes. Timed urine collection occurred at the following time points after product consumption: $T = 70, 130, 190, 250, 310,$ and 370 minutes. Throughout the study visits, subjects were not allowed to eat, but were requested to drink 150 mL of water every hour after study product intake (starting at $T = 125$). In addition, subjects were asked to refrain from any physical exercise other than incidental walking during the study visits.

2.2. Subjects

Due to the ethical considerations and the inability of infants to provide the large number of blood samples required for this study, the present study was conducted in healthy male and female (nonpregnant, nonlactating) volunteers aged 18 to 40 years. Eligible participants had a body mass index (BMI) between 18.5 and 25 kg/m². Due to ethnic differences in treatment response to proton-pump inhibitors [28], only non-Asian participants were eligible for inclusion. Subjects were required not to have any medical condition interfering with digestion and/or gastrointestinal function, nor to have any known renal or hepatic failure, diabetes mellitus type I or type II, or food allergy and/or food intolerance. Other exclusion criteria were: any ongoing cancer or cancer treatment; any known bleeding disorder; hemoglobin at screening <7.5 mmol/L in men and <7.0 mmol/L in women, 25(OH)D <50 nmol/L at screening; known hypersensitivity to esomeprazole, fructose intolerance, glucose-galactose malabsorption, or sucrase-isomaltase

Table 1 – Macro- and micronutrient composition of the study products per 100 kcal and as consumed (per 1217 kcal) by the healthy volunteers (n = 21) and list of ingredients.

	Product A (Neocate Infant DHA/ARA)		Product B (Elecare Infant)	
	Per 100 kcal	Per 1217 kcal	Per 100 kcal	Per 1217 kcal
Macronutrients				
Protein equivalent, g	2.8	34.0	3.1	37.1
Fat, g	5.1	61.7	4.8	58.9
Carbohydrates, g	10.8	131.0	10.7	130.7
Minerals				
Sodium, mg	39.1	476	45.3	551
Chloride, mg	79.9	973	60.0	730
Potassium, mg	109	1323	150	1832
Calcium, mg	116	1414	116	1409
Phosphorus, mg	82.2	1000	84.2	1025
Magnesium, mg	10.6	129	8.4	103
Iron, mg	1.5	18.4	1.8	21.5
Zinc, mg	1.1	13.4	1.2	14.1
Manganese, µg	41.4	504	84.2	1025
Copper, µg	84.9	1033	126	1535
Iodine, µg	20.7	252	8.9	108
Selenium, µg	2.3	28.5	2.6	32.3
Chromium, µg	2.2	26.7	2.3	28.2
Molybdenum, µg	3.0	36.5	2.5	30.7
Vitamins				
Vitamin A, µg RE	84.1	1023	82.1	999
Vitamin C, mg	10.7	130	9.1	110
Vitamin D, µg	1.8	22.2	1.5	18.2
Vitamin E, IU	1.4	17.1	2.1	25.6
Vitamin K, µg	8.8	107	12.6	154
Vitamin B1, µg	143	1739	211	2562
Vitamin B2, µg	112	1361	105	1281
Vitamin B5, mg	0.60	7.31	0.42	5.12
Vitamin B6, µg	112	1361	84.2	1025
Vitamin B12, µg	0.27	3.3	0.42	5.1
Niacin, mg	1.01	12.3	1.68	20.5
Folic acid, µg	13.3	161	29.5	359
Biotin, µg	3.9	47.9	4.2	51.2
Choline, µg	19.8	240	14.9	182
Inositol, µg	22.4	272	5.1	61.5
Ingredients				
	Corn Syrup Solids, Refined Vegetable Oil (Medium Chain Triglycerides (Palm kernel and/or coconut oil), High Oleic Sunflower Oil, Sunflower Oil, Canola Oil), L-Arginine L-Aspartate, L-Leucine, L-Lysine Acetate, L-Glutamine, L-Proline, CAEM (an emulsifier), L-Valine, Glycine, L-Isoleucine, Dipotassium Phosphate, L-Threonine, L-Phenylalanine, L-Tyrosine, L-Serine, Calcium Phosphate Dibasic, L-Histidine, L-Alanine, Sodium Chloride, Tripotassium Citrate, Tricalcium Phosphate, L-Cystine, Magnesium Chloride, L-Tryptophan, Calcium Carbonate, Tricalcium Citrate, Calcium Glycerophosphate, Choline Bitartrate, L-Methionine, M. Alpina Oil, Magnesium L-Aspartate, C. Cohnii Oil, M-Inositol, L-Ascorbic Acid, Taurine, Ferrous Sulfate, Zinc Sulfate, L-Carnitine, Uridine-5-monophosphate disodium salt, Cytidine-5-monophosphate, Niacinamide, Inosine-5-monophosphate disodium salt, Adenosine-5-monophosphate, Calcium D-Pantothenate, Guanosine-5-monophosphate disodium salt, DL-alpha Tocopheryl Acetate, Ascorbyl Palmitate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Manganese Sulfate, Riboflavin, Vitamin A Acetate, DL-alpha Tocopherol, Potassium Iodide, Mixed Tocopherols, Folic Acid, Chromium Chloride, Phylloquinone, Sodium Selenite, Sodium Molybdate, D-Biotin, Vitamin D3, Cyanocobalamin		Corn Syrup Solids, High Oleic Safflower Oil, Medium-Chain Triglycerides, Soy Oil, L-Glutamine, C. Cohnii Oil, M. Alpina Oil, L-Asparagine, L-Leucine, L-Lysine Acetate, DATEM, Calcium Phosphate, L-Valine, Potassium Phosphate, L-Isoleucine, L-Arginine, L-Phenylalanine, L-Tyrosine, L-Threonine, Potassium Citrate, Sodium Citrate, L-Proline, L-Serine, L-Alanine, Glycine, L-Histidine, L-Methionine, Ascorbic Acid, Magnesium Chloride, Calcium Carbonate, L-Cystine Dihydrochloride, L-Tryptophan, Salt, Choline Chloride, Inositol, Ferrous Sulfate, Taurine, Ascorbyl Palmitate, Zinc Sulfate, dl-Alpha Tocopheryl Acetate, L-Carnitine, Niacinamide, Calcium Pantothenate, Thiamine Hydrochloride, Copper Sulfate, Manganese Sulfate, Vitamin A Palmitate, Riboflavin, Pyridoxine Hydrochloride, Folic Acid, Beta-Carotene, Biotin, Phylloquinone, Chromium Chloride, Potassium Iodide, Sodium Selenate, Sodium Molybdate, Vitamin D3, Vitamin B12	

insufficiency; use of any medication within 1 week of the study visits (except for oral contraceptives and incidental use of paracetamol, nonsteroidal anti-inflammatory drugs and/or common cold relievers); use of nutritional supplements (other than vitamin D) within 2 weeks of the study visits; adherence to a weight loss program or severe involuntary weight loss; smoking (current or stopped <3 months prior to screening visit); alcohol use >21 glasses/week for men and >14 glasses/week for women or any other patient characteristic (e.g., not able or not willing to consume the standardized meal or study product) or (medical) history (e.g., blood donation within 2 months prior to study entry) that could interfere with the conduct or assessments of the study. All participants were requested to refrain from taking any medication (except for oral contraceptives and incidental use of paracetamol, nonsteroidal anti-inflammatory drugs, or common cold relievers) and nutritional supplements (except for vitamin D to ensure sufficient intake during the course of the study) for the duration of the study, and to refrain from the consumption of alcohol 24 hours prior to the study visits. Additionally, they were asked to maintain their normal dietary habits, habitual activity level and habitual day and sleep pattern and refrain from smoking, taking alcohol and donating blood for the duration of the study. All participants were informed of the experimental procedure with its aims and risks and gave their written consent before entering the study.

2.3. Study outcomes

The primary outcome of this study was serum phosphate (P) area-under-the-curve (AUC_{0-360}) of Product A and Product B. Secondary outcome parameters were peak concentrations (C_{peak}) and time of peak concentration (T_{peak}) of Product A and B for serum P, and AUC, C_{peak} , and T_{peak} for urinary creatinine-corrected P, serum calcium and urinary creatinine-corrected calcium (Ca). From the serum and urine phosphorus and creatinine concentrations, the tubular reabsorption of phosphate (TRP) was determined as follows [29]:

$$TRP = 1 - \frac{(\text{urine P} \times \text{serum Cr})}{(\text{serum P} \times \text{urine Cr})}$$

As we hypothesized that the glucose load and insulin rise following study product intake would cause phosphate to redistribute from serum to the intracellular component [22], exploratory outcome parameters defined in this study were AUC_{0-360} , C_{peak} , and T_{peak} for serum glucose, serum insulin, serum P/serum glucose and serum P/serum insulin. Additionally, AUC_{0-360} , C_{peak} , and T_{peak} for serum alkaline phosphatase (ALP) were assessed and serum intact parathyroid hormone (iPTH) was measured at $T = 60$ and 360 minutes.

Serum insulin and serum iPTH were measured by chemiluminescent immunometric assays (for serum insulin: Immulite 2000 XPI, Siemens Healthcare GmbH, Erlangen, Germany; for serum iPTH: Abbott Architect i2000, Abbott Laboratories, Lake Bluff, IL, USA). All other laboratory parameters were measured on an automated analyzer, Cobas 8000 (c702, Roche Diagnostics, Basel, Switzerland) using photometric (for P and Ca in serum and urine), enzymatic (for glucose in serum and creati-

nine in urine) and colorimetric (for ALP in serum) methodologies. All analyses were performed blinded to treatment group.

2.4. Sample size

The sample size was calculated using PROC POWER procedure (SAS version 9.4, SAS Institute Inc., Cary, NC, USA) to evaluate 2-sided hypotheses regarding bioequivalence of the 2 study products with the following assumptions based on a previous study (not published): a significance level (α) of 0.05, a within-subject correlation of 0.3, a coefficient variation of 0.2, a geometric mean ratio (GMR) of 0.95, and a power of 90%. An estimated sample size of at least 18 paired observations (i.e., complete cases) was considered to be sufficient to detect bioequivalence for the AUC of P between the 2 study products. An interim analysis was performed after $n = 8$ subjects completed the study to re-estimate the sample size. Based on the interim analysis, it was recommended to continue the study with the planned sample size of at least 18 subjects that completed the study.

2.5. Statistical analyses

The trapezoidal method was used to calculate the AUCs for serum and urine outcome parameters. Means of the 2 prebaseline measurements ($T = -20$ and $T = -5$) were taken as baseline levels ($T = 0$). The assessment of bioequivalence is based upon 90% CI for the ratio of the population geometric means. The AUC and C_{peak} values were transformed prior to analysis using a logarithmic transformation (natural log). The CI for the difference between formulations on the log-transformed scale was back transformed to obtain the desired CI for the ratio on the original scale. In line with FDA and EMA guidance, bioequivalence between the 2 products is demonstrated if the 90% CIs fall entirely within the prespecified equivalence margins of 0.8 and 1.25 [30–32]. The AUC and C_{peak} were compared by using a general linear model with subject, period, and study product as fixed factors for outcomes with complete crossover data, whereas a mixed model analysis of variance with fixed effects for period and study product and compound symmetry covariance matrix with the Kenward-Roger option for the degrees of freedom was used for outcomes with incomplete crossover data.

Differences between the two products for T_{peak} were evaluated using the median difference and its 95% CI using the methodology of Hodges-Lehmann as recommended by Senn [33]. The derivation of the Hodges-Lehmann estimate and the distribution-free 95% CI for the difference between Product A and Product B was calculated for T_{peak} as described by Han et al. [34].

Equivalence of the 2 study products for iPTH (measured at $T = 0$, $T = 60$, and $T = 360$ minutes) was determined through a linear mixed model including logarithm of iPTH (at $T = 60$ and $T = 360$) in the outcome vector and adjusting for logarithm of baseline ($T = 0$), study product, time, and the interaction of study product by time as fixed factors. Mean differences in serum P, serum Ca, urinary creatinine-corrected P, and urinary creatinine-corrected Ca between baseline and the mean time point that the peak concentration is observed were studied using a paired t test.

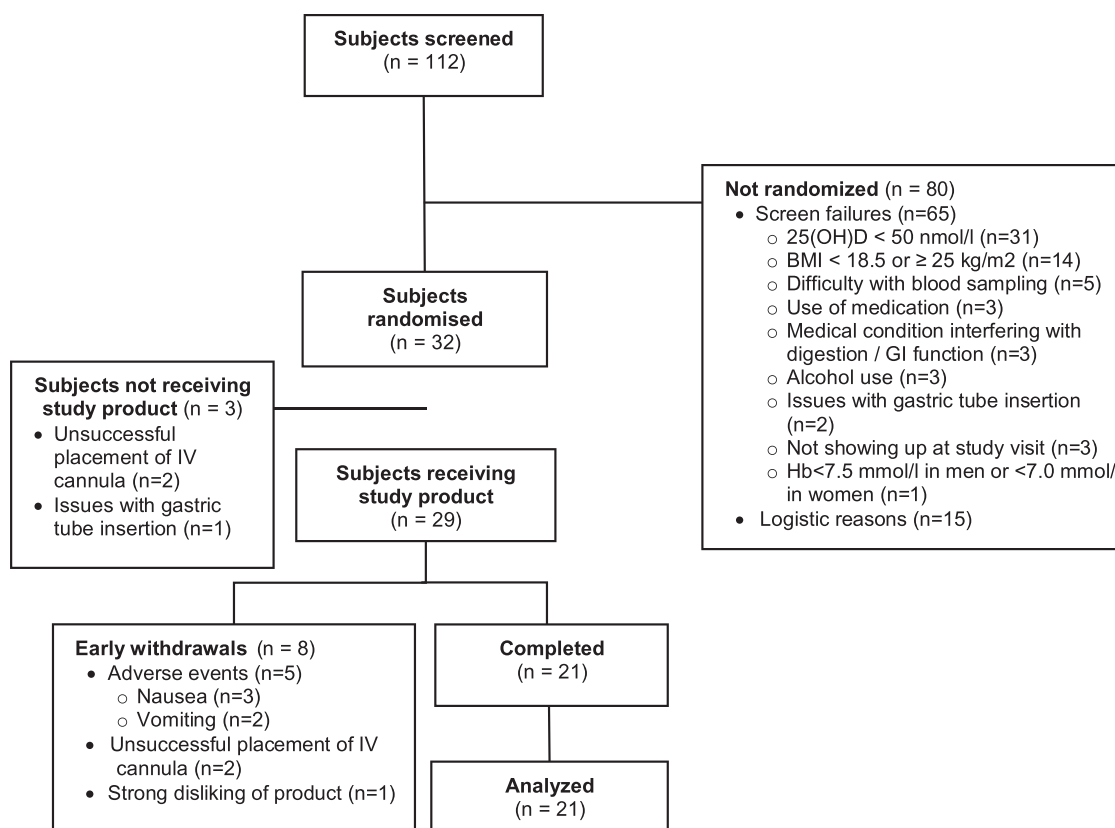


Fig. 2 – Study flow chart.

Correlation plots as a measure of the strength and direction of a linear relationship were presented for serum glucose AUC₀₋₃₆₀ and serum P AUC₀₋₃₆₀, and for serum insulin AUC₀₋₃₆₀ and serum P AUC₀₋₃₆₀, reporting the Pearson correlation coefficient and significance test for correlation (PROC CORR). SAS system (SAS version 9.4, SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

3. Results

3.1. Subjects

A total of 112 potentially eligible subjects were screened of whom 32 (29%) were randomized, 15 subjects (13%) were not randomized due to logistic constraints and 65 (58%) subjects failed to meet inclusion criteria. Main reasons for not meeting the inclusion criteria were a vitamin D level < 50 nmol/L (n = 31) and BMI < 18.5 or ≥ 25 kg/m² (n = 14; Fig. 2). A total of 29 subjects received at least 1 sip of study product. Of these 29 subjects, 21 (72%) completed the study and 8 (28%) subjects were discontinued from the study, due to adverse events (n = 5), unsuccessful placement of the IV cannula (n = 2) and strong dislike of the taste and smell of the product (n = 1). The mean age of the study population was 25.7 ± 5.1 years (ranging from 20 to 37 years) with a mean BMI of 22.3 ± 1.8

and female subjects constituting almost two-thirds of the study population. All subjects had a stomach pH ≥ 5.5 ten to 20 minutes after study product intake.

3.2. Serum phosphate and serum calcium

Fig. 3 shows the means (±SD) for serum P (Fig. 3A) and Ca (Fig. 3B) concentrations as a function of time after oral intake of either Product A or Product B. The pharmacokinetic parameters and bioequivalence assessments are shown in Table 2. Intake of both products initially led to a significant decrease in serum P levels when compared to baseline (Product A: 1.03 ± 0.14 mmol/L [T = 0 minutes], 0.93 ± 0.14 mmol/L [T = 60 minutes], P < .001; Product B: 1.01 ± 0.14 mmol/L [T = 0 minutes], 0.90 ± 0.14 mmol/L [T = 60 minutes], P < .001), followed by a gradual increase in P until the end of sample collection (Fig. 3A). The geometric means AUC₀₋₃₆₀ for serum P were 378 mmol/L*min for Product A and 363 mmol/L*min for Product B, with a GMR of 1.041. The geometric means C_{peak} were 0.89 mmol/L for Product A and 0.87 mmol/L for Product B, with a GMR of 1.020. For both products, a negative peak of serum P was observed 40 to 60 minutes after product intake. The 90% CI for the GMR (Product A/Product B) of AUC₀₋₃₆₀ (0.998–1.086) and C_{peak} (0.963–1.080) were within the acceptance range for bioequivalence [30]. Product A and B displayed

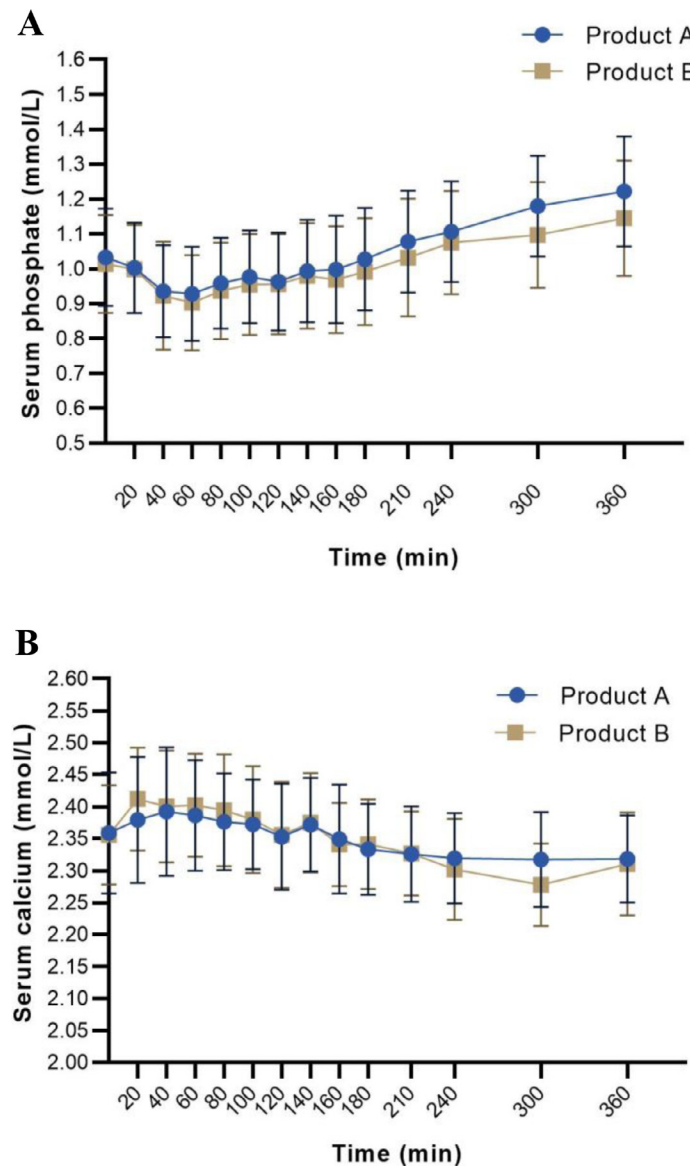


Fig. 3 – Serum phosphate concentration (mmol/L) (A) and serum calcium concentration (mmol/L) (B) after oral intake of Product A (●) or Product B (■) in healthy volunteers (n = 21). Values are means $\pm$ SD. Intake of both products led to a significant decrease in serum P ($P < .001$) and a significant increase in serum calcium ($P < .001$).

comparable medians and ranges for T_{peak} (Supplemental Table S2).

For serum Ca, the geometric means AUC_{0-360} were 844 mmol/L*min for Product A and 842 mmol/L*min for Product B, with a GMR and 90% CI of 1.002 (0.996–1.009). For both products, the means C_{peak} were reached after about 20 to 40 minutes leading to a significant increase compared to baseline (Product A: 2.36 ± 0.09 mmol/L [$T = 0$ minutes], 2.39 ± 0.10 mmol/L [$T = 40$ minutes], $P < .001$; Product B: 2.36 ± 0.08 mmol/L [$T = 0$ minutes], 2.41 ± 0.08 mmol/L [$T = 20$ minutes], $P < .001$) with a subsequent gradual decline in concentration toward baseline levels near the end of sample collection (Fig. 3B). The geometric means C_{peak} were 2.43 mmol/L for Product A and 2.45 mmol/L for Product B, and the GMR and 90% CI of Product A/Product B was 0.991 (0.983–0.999).

The 90% CI for the GMRs of AUC_{0-360} and C_{peak} were within the acceptance range for bioequivalence [30]. Medians and ranges of T_{peak} were comparable for Product A and Product B (Supplemental Table S2).

3.3. Urinary phosphate and urinary calcium

The amount of creatinine-corrected P excreted in urine remained stable for the first three hours after product intake and thereafter significantly increased throughout the remainder of the study reaching more than 2.5 times baseline levels for both products by the end of the sample collection at $T = 370$ (Product A: 0.64 ± 0.42 mmol/L [$T = 70$ minutes], 1.78 ± 0.80 mmol/L [$T = 370$ minutes], $P < .001$; Product B: 0.53 ± 0.32 mmol/L [$T = 70$ minutes], 1.54 ± 0.87 mmol/L

Table 2 – Comparing area-under-the-curves (AUCs) and peak concentrations (C_{peak}) for serum phosphorus (P) and calcium (Ca) and for urinary creatinine (Cr)-corrected P and Ca of Product A and Product B.

	Product A		Product B		Product A/Product B	
	n	Geometric Mean	n	Geometric Mean	Ratio of means	90% CI
Serum P						
AUC ₀₋₃₆₀ (mmol/L*min)	21	378	21	363	1.041 ^b	0.998-1.086 ^b
C_{peak} (mmol/L) ^d	21	0.89	21	0.87	1.020 ^b	0.963-1.080 ^b
Urinary P/Cr ratio						
AUC ₇₀₋₃₇₀ (mmol/L*min)	19	238.6	18	216.0	1.105 ^c	0.918-1.330 ^{c,a}
C_{peak} (mmol/L) ^d	19	1.66	18	1.40	1.182 ^c	1.040-1.343 ^{c,a}
Serum Ca						
AUC ₀₋₃₆₀ (mmol/L*min)	21	844	21	842	1.002 ^b	0.996-1.009 ^b
C_{peak} (mmol/L) ^d	21	2.43	21	2.45	0.991 ^b	0.983-0.999 ^b
Urinary Ca/Cr ratio						
AUC ₇₀₋₃₇₀ (mmol/L*min)	19	130.0	17	116.4	1.117 ^c	1.023-1.219 ^c
C_{peak} (mmol/L) ^d	19	0.71	17	0.61	1.157 ^c	1.073-1.247 ^c

^a Not fulfilling the 80%-125% bioequivalence criteria.

^b Results are obtained from general linear model with subject, period, and study product as fixed factors

^c Results are obtained from mixed model analysis of variance on log-transformed values with fixed effects for period and study product and compound symmetry covariance matrix, the Kenward-Roger option for degrees of freedom

^d C_{peak} either shows a positive peak (for urinary P/Cr ratio, serum Ca, and urinary Ca/Cr ratio) or a negative peak (for serum P).

[T = 370 minutes], $P < .001$). Although urinary creatinine-corrected P levels followed a similar pattern after intake of Product A and Product B, predefined bioequivalence criteria were not reached. The total excreted amount tended to be slightly higher for product A, with a GMR and 90% CI of 1.105 (0.918-1.330) and 1.182 (1.040-1.343) for AUC₇₀₋₃₇₀ and C_{peak} , respectively (Fig. 4A, Table 2).

Urinary creatinine-corrected Ca levels increased significantly until 130 minutes after product intake (Product A: 0.33 ± 0.17 mmol/L [T = 70 minutes], 0.75 ± 0.28 mmol/L [T = 130 minutes], $P < .001$; Product B: 0.38 ± 0.20 mmol/L [T = 70 minutes], 0.68 ± 0.31 mmol/L [T = 130 minutes], $P < .001$), and then decreased back to baseline levels (Fig. 4B). Urinary creatinine-corrected Ca excretion demonstrated bioequivalence of the two products (Table 2).

Equivalence was also reached for AUC₆₀₋₃₆₀ of TRP with a GMR and 90% CI of 0.993 (0.984-1.002).

3.4. Serum glucose and serum insulin and its association with serum phosphate

Serum glucose (Fig. 5A) and glucose-induced serum insulin (Fig. 5B) levels increased rapidly after product intake and only returned to basal values near the end of the study. For serum insulin, peak values were observed around 120 to 160 minutes after product intake, whereas for serum glucose no clear time of peak effect was observed. For serum glucose and serum insulin, bioequivalence was established between Product A and Product B as the GMRs of AUC and C_{peak} were both within the 80% to 125% range. Mean serum P/serum glucose remained fairly stable at around 0.18 mmol/L from product intake until 240 minutes and thereafter sharply increased to 0.27 mmol/L at the end of the study (Fig. 6A). Serum P AUC₀₋₃₆₀ was weakly (negatively) correlated with serum glucose AUC₀₋₃₆₀ (Fig. 6C, $r = -0.277$; $P = .076$). Serum P/serum insulin rapidly decreased after product intake from about 0.4 mmol/L at baseline to zero between 20 and 240 minutes

and thereafter increased to about 0.20 mmol/L at 360 minutes (Fig. 6B). Serum P AUC₀₋₃₆₀ appears to have a negative association with serum insulin AUC₀₋₃₆₀ (Fig. 6D, $r = -0.343$; $P = .026$).

3.5. Serum alkaline phosphatase and serum intact parathyroid hormone

Average serum alkaline phosphatase levels remained very stable throughout the study, at around 63 U/L. Bioequivalence was established between Product A and Product B as the GMRs of AUC (GMR and 90% CI: 0.992 (0.969-1.015)) and C_{peak} (GMR and 90% CI: 0.998 (0.971-1.025)) were both within the 80% to 125% range. Serum iPTH at both measured time points was higher in Product B (geometric means: 2.17 pmol/L at T = 60 and 3.86 pmol/L at T = 360 minutes) compared to Product A (geometric means: 1.99 pmol/L at T = 60 and 3.24 pmol/L at T = 360 minutes). Equivalence between the two products was reached at T = 60 min (GMR and 90% CI: 0.915 (0.837-1.000)), whereas equivalence of serum iPTH was not demonstrated at T = 360 min (GMR and 90% CI: 0.838 (0.767-0.916)).

3.6. Adverse events

In the study, a total of 56 adverse events (AEs) were reported in 24 subjects (82.8% of all subjects receiving study product): 31 AEs in 18 subjects after intake of Product A and 25 AEs in 11 subjects after intake of Product B. The number of AEs per subject ranged from 0 to 5. There were 29 AEs reported as related to the study procedures, frequently reported types of AEs were unpleasantness/discomfort related to the blood withdrawal or fasting. Most cases of AEs were of a mild to moderate intensity (71.4% and 26.8%, respectively). A total of 26 reported AEs were assessed to be related to the intake of the energy dense bolus of the study product. The most frequently reported types of AEs were gastro-intestinal disorders (e.g., nausea, vomiting, abdominal distension) and

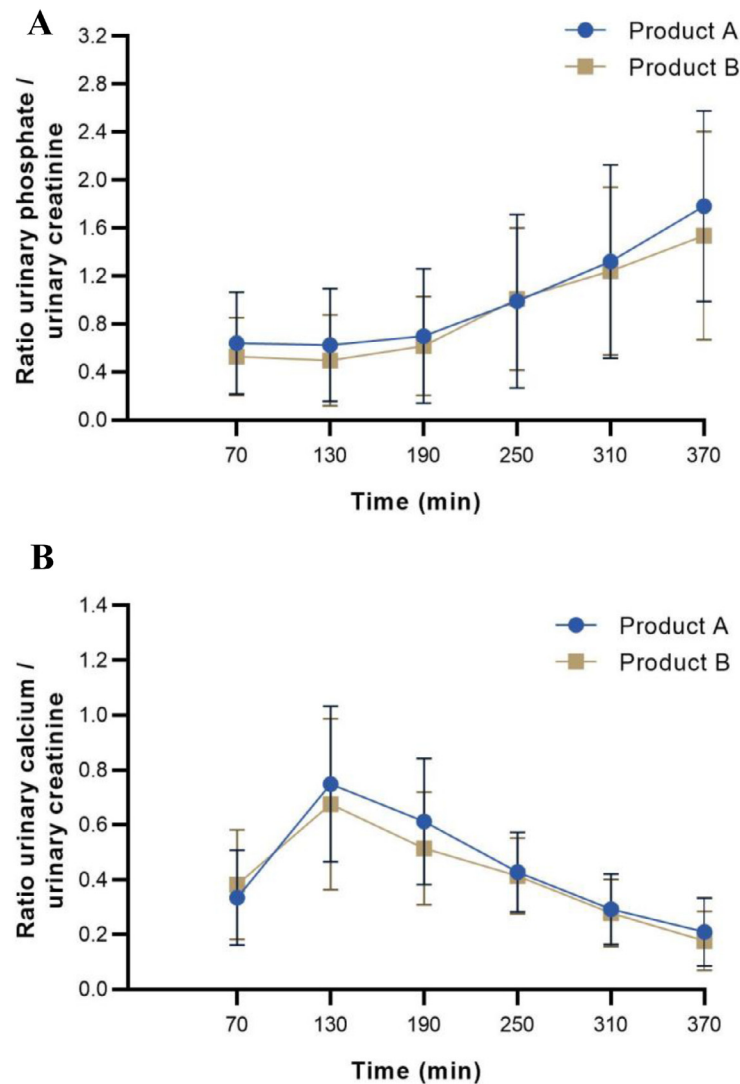


Fig. 4 – Urinary creatinine-corrected phosphate (mmol/L) (A) and urinary creatinine-corrected calcium (mmol/L) (B) after oral intake of Product A (●) or Product B (■) in healthy volunteers (n = 21). Values are means $\pm$ SD. Intake of both products led to significant increases in urinary creatinine-corrected phosphate ($P < .001$) and urinary creatinine-corrected calcium ($P < .001$).

nervous system disorders (e.g., dizziness, headache). One AE (diarrhea), reported to be related to Product A, was classified as severe. The subject recovered within the same day and did not require any medication or further follow-up. There were no serious AEs reported in the study.

4. Discussion

The present study showed that the bioavailability of phosphorus and calcium from Product A is equivalent to Product B in healthy volunteers with esomeprazole-induced hypochlorhydria, thereby supporting our hypothesis. Bioequivalence of both products was demonstrated for serum P, as well as for serum and urinary creatinine-corrected Ca. Urinary creatinine-corrected P followed a similar pattern after intake of both products, but predefined bioequivalence criteria

were not reached. Additionally, equivalence was established between products for serum ALP, TRP, and for iPTH at T = 60 minutes.

Interestingly, serum P levels initially declined after intake of both products, followed by a gradual increase in P until the end of sample collection. Since urine P excretion only started rising toward the end of the observational period, the initial decline of serum P may be due to insulin-mediated P uptake into peripheral tissues as a result of the accompanying glucose or fructose challenge with study product intake, which has been described before [35–39]. In line with this hypothesis, the peak increase in postprandial glucose and insulin at 20–40 minutes preceded the nadir decrease in serum P at 60 minutes. Additionally, the transient reduction in serum P levels could be due to the secretion of gastrin following the high bolus ingestion of the products causing stimulation of calcitonin. Calcitonin inhibits osteoclasts and thereby reduces the release

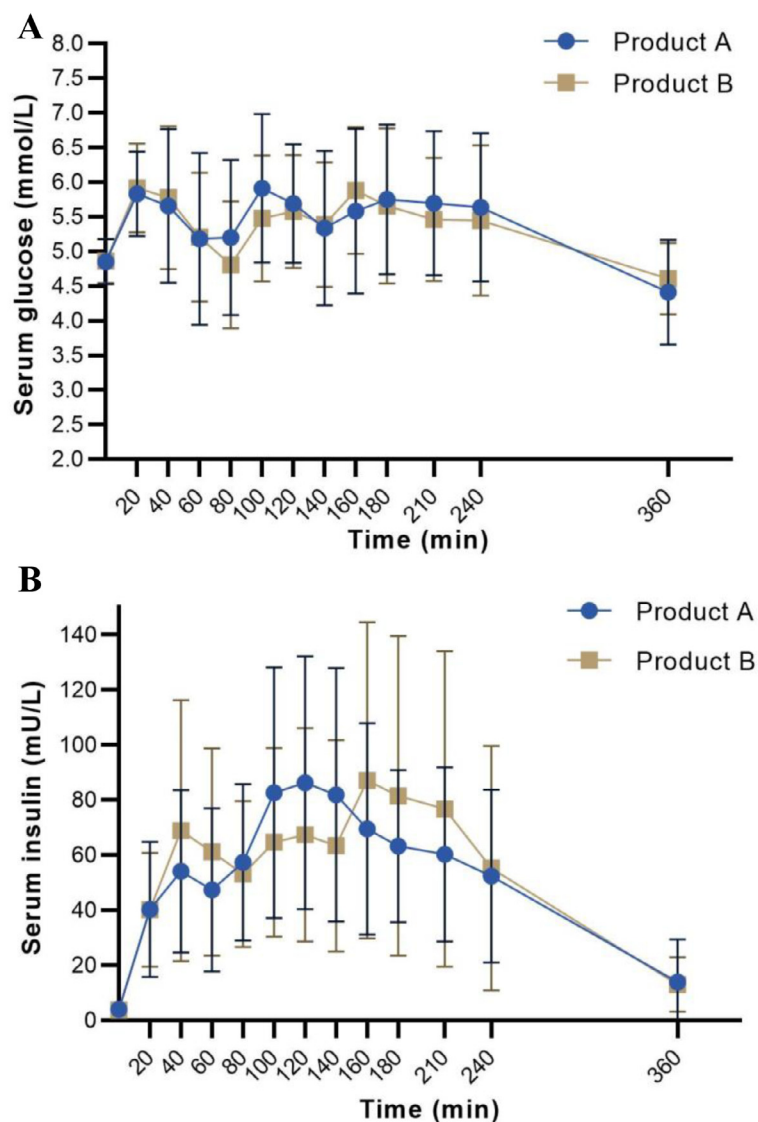


Fig. 5 – Serum glucose (mmol/L) (A) and serum insulin (mU/L) (B) after oral intake of Product A (●) or Product B (■) in healthy volunteers (n = 21). Values are means $\pm$ SD. Intake of both products led to significant increases in serum glucose and serum insulin.

of calcium and phosphorus from bone matrix into the blood [35,40]. Similarly, Trautvetter et al. [35] found that the single or 3-weeks repeated consumption of bread enriched with pentacalcium hydroxy-triphosphate providing 0.5 g phosphorus and 1 g calcium per day significantly decreased P concentrations after 30, 60, 120, and 180 minutes compared to baseline.

Following the early decrease, we observed a delayed increase of serum P which was accompanied by an increase in urine P excretion which may be due to release of P from intracellular stores after postprandial redistribution of P [22]. However, diurnal variation in fasting serum P may be a second explanation as it has been shown that P levels reach a nadir in early morning and increase to a plateau by 4 PM (~420 minutes after baseline) [41,42].

Different from serum P, serum Ca levels appeared homeostatically more tightly regulated, which after increasing on average 2% to 3% (+0.05–0.07 mmol/L) from baseline to

20 to 40 minutes after product intake had returned back to baseline by the 120 minutes time point. This was likely due to a steep increase in urinary Ca excretion in the first 120 minutes following product intake and suggests that different from serum P, Ca is removed from the bloodstream by renal excretion to compensate for increased oral Ca intake [43]. Similarly, Hanzlik et al. [43] showed that oral administration of 1200 mg calcium resulted in a 5% increase in serum Ca levels after 60 minutes, although the changes in serum Ca were partially attributed to normal diurnal variations by the authors [43]. In addition, the calcium source, the matrix in which the calcium is provided (i.e., tablet or food) and the stomach pH may influence the bioavailability of Ca [44–46].

Serum iPTH levels significantly increased after intake of both products, yet remained within the normal range (1.06–6.89 pmol/L). Serum iPTH was higher after intake of product B

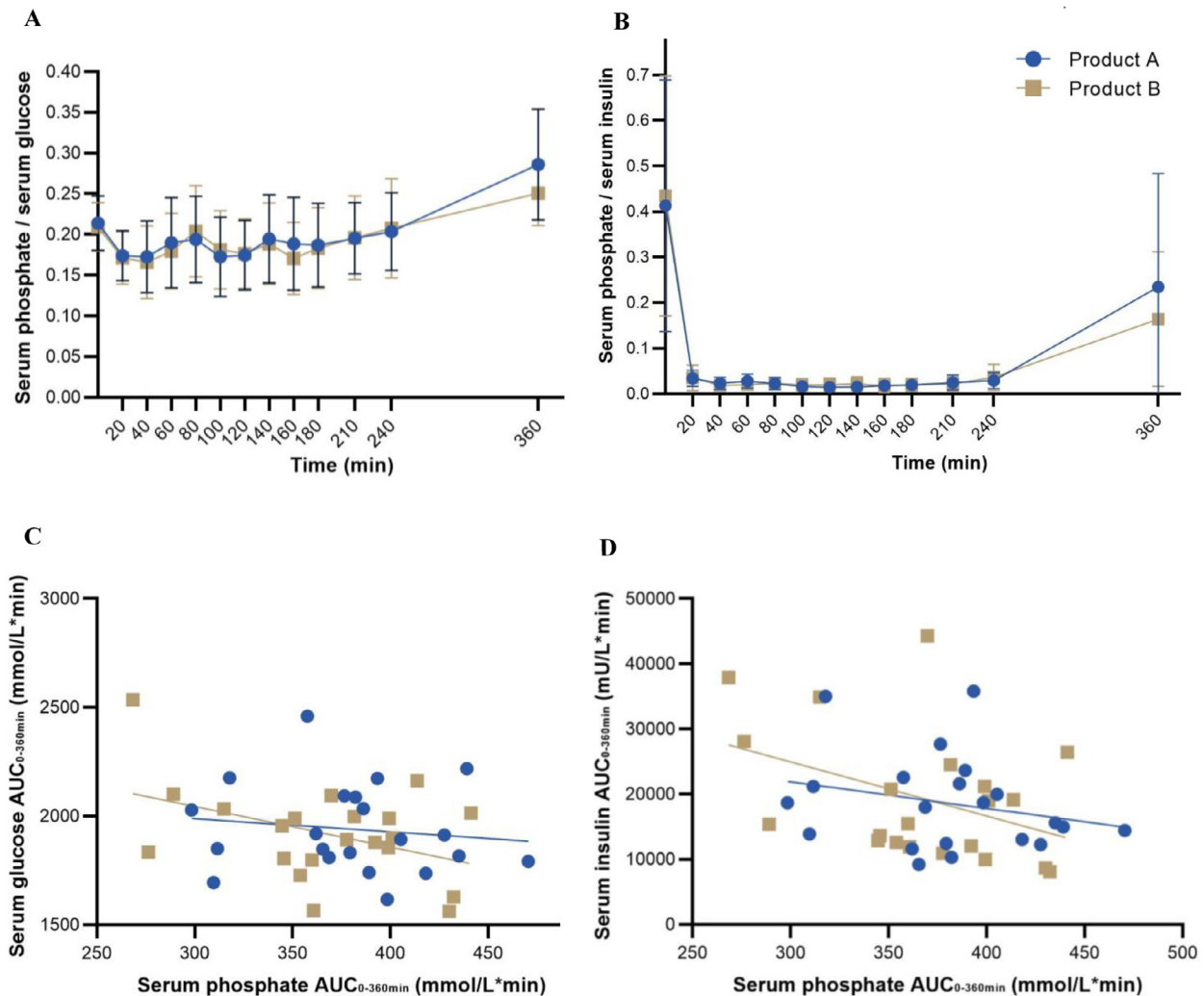


Fig. 6 – Serum phosphate / serum glucose ratio (A) and serum phosphate / serum insulin ratio (B) after oral intake of Product A (●) or Product B (■) in healthy volunteers (n = 21). Correlation plot between serum glucose and serum phosphate (C) and between serum insulin and serum phosphate (D) after oral intake of Product A (●) or Product B (■) in healthy volunteers (n = 21). Values are means $\pm$ SD.

compared to Product A and equivalence of both products was not reached at T = 360 minutes. This could be due to the 0.04 mmol/L lower serum Ca levels achieved by product B at 300 minutes and explain the observed difference of -0.62 pmol/L for iPTH. No significant differences in serum ALP between study products were observed.

Hypochlorhydria induced in healthy adult subjects mimics one risk factor prevalent in many of the recently reported cases of hypophosphatemia associated with elemental infant formula use [15–20]. However, the cases concerned were merely infants and children with multiple additional comorbidities and it is therefore not possible to conclude that the bioavailability of product A and B will be equivalent in the clinical setting. Other limitations of our study include the short duration of the study and single dose of the study products, which limit the relevance of our findings for individuals who require long-term nutrition with amino acid-based formulas. However, we believe these downsides are out-weighted by the

double-blinded crossover design, allowing subjects to serve as their own control, and the frequent and carefully timed collection of serum and urine samples. Furthermore, bias was reduced by using healthy subjects in which hypochlorhydria was induced in a controlled fashion. Additionally, our study is hindered by the relatively high drop-out rate (28%). However, since drop-out occurred equally with both study products and was mainly caused by gastro-intestinal AEs and/or a strong disliking of the product which was, at least partly, caused by the fact that both products were provided highly concentrated, we believe that this does not affect the conclusions.

In conclusion, these data show that serum phosphorus and calcium bioavailability of Neocate powdered formulas with updated mineral salts is bioequivalent to Elecare in healthy volunteers with pharmacologically-induced hypochlorhydria, despite matrix differences between the study products. This observation may be relevant for infants and children with complex medical conditions and histories who receive an

elemental amino acid-based formula via a postpyloric tube and/or are treated with proton-pump inhibitors or other acid-suppressive medication. Up to 60% of infants/children using amino acid-based formula for the nutritional support of acute and chronic complex medical conditions receive the formula via the postpyloric route and/or are prescribed acid-suppressive medication, indicating the importance of our study results [47]. Regular monitoring of mineral status and the ongoing need for acid-suppressive medication remains recommended, especially in complex cases or when multiple risk factors could predispose to altered mineral status.

Supplemental materials for this submission were provided and include: Table S1, List of phosphorus rich foods and beverages that participants were instructed not to eat or drink for 24 hours prior to the study visits and Table S2, Comparing time to peak concentration (T_{peak}) for serum phosphorus (P) and calcium (Ca) and for urinary creatinine (Cr)-corrected P and Ca of Product A and Product B.

Author statement

All authors are responsible for the conception and design on the study. PLHRJ and MV are responsible for the acquisition of the data and SRBME, PLHRJ, MV and AvH are responsible for the initial analysis and interpretation of the data. SRBME and PLHRJ drafted the manuscript and all authors are responsible for revising the work critically for important intellectual content and gave their final approval of this version. All authors are accountable for all aspects of the work and agree to this version of the manuscript.

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Author Declarations

The authors SRBME, PLHRJ, MV, and AvH are employees of Danone Nutricia Research. The authors CB and TOC received research support by Nutricia North America. All authors declare to be responsible for the conception and design of the study. PLHRJ and MV are responsible for the acquisition of the data and SRBME, PLHRJ, MV, and AvH are responsible for the initial analysis and interpretation of the data. SRBME and PLHRJ drafted the manuscript and all authors are responsible for revising the work critically for important intellectual content and gave their final approval of this version. All authors declare to be accountable for all aspects of the work.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.nutres.2020.11.004](https://doi.org/10.1016/j.nutres.2020.11.004).

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Author statement

All authors are responsible for the conception and design on the study. PLHRJ and MV are responsible for the acquisition of the data and SRBME, PLHRJ, MV and AvH are responsible for the initial analysis and interpretation of the data. SRBME and PLHRJ drafted the manuscript and all authors are responsible for revising the work critically for important intellectual content and gave their final approval of this version. All authors are accountable for all aspects of the work and agree to this version of the manuscript.