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A New Phe-Free Protein Substitute Engineered to Allow a Physiological Absorption of Free Amino Acids for Phenylketonuria

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Abstract

An innovative technology (Physiomimic Technology) has been applied to amino acids (AAs) formulated for patients with phenylketonuria, with the objective of masking AA taste and odor and prolonging AA release in the gut, allowing a physiological absorption. This technology entails that the AAs are processed with functional additives that are able to modify their release and their organoleptic features. Two prototypes, obtained using sodium alginate + ethylcellulose (engP-1) or sodium alginate + ethylcellulose + glyceryl dibehenate (engP-2), have been tested for AA prolonged release versus the same AAs (n-engP) without the application of the Physiomimic Technology. In vitro tests indicated that the technology is able to prolong the release of the engineered AAs versus the free compounds. A crossover in vivo kinetic study in pigs showed reduced peak concentrations (C_{max}) and, as expected, similar areas under the concentration/time curve (up to 5 hours) for the engineered products versus the free AAs. Significantly lower C_{max} values ($P < .01$) were attained for essential AAs, large neutral AAs, and branched-chain AAs, indicating that the technology is able to reduce the typical absorption peak of free AAs. Taste and odor masking has been obtained as a consequence of the AA coating. The Physiomimic Technology, applied to free AAs, provided AA mixes with improved organoleptic features and with modified AA kinetics sustaining a more physiological AA absorption.

Keywords

prolonged release, amino acids, phenylketonuria, physiological AA absorption, protein substitute, odor masking, taste masking, dietary proteins

Background and Aim

Phenylketonuria (PKU)[1] is a rare inborn error of metabolism causing, in uncontrolled newborn and young children, phenylalanine (Phe) accumulation in blood and brain,[2,3] where Phe neurotoxic effects lead to progressive, irreversible intellectual impairment and other important consequences including seizures and motor deficits.[4]

Nowadays, the majority of patients, thanks to the adoption of newborn screening programs, are identified at birth and are counseled to follow long-term or lifetime dietary protein restrictions in order to maintain Phe blood levels within the recommended ranges. Phe-free protein substitutes, compensating a reduced protein intake from natural sources, represent a fundamental part of the diet.[5] Maintenance of Phe levels within recommended range highly contributes to prevention of the major severe deficits associated with Phe accumulation. However, some impairments, in insufficiently controlled patients,[6-8] including adults,[9] as well as subtle disabilities in patients with controlled or almost controlled levels of blood Phe,[9-11] have been reported.

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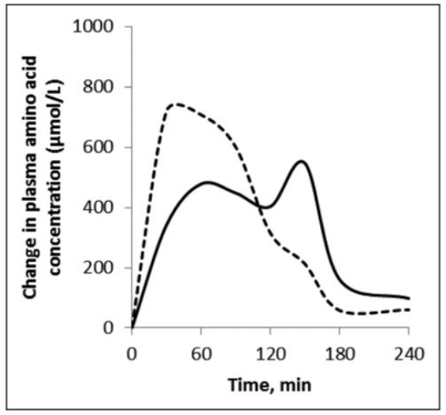


Figure 1. Total plasma AAs after administering food proteins (cottage cheese, solid line) or an equivalent mixture of free AAs with an identical amino acid composition (broken line) in fasting healthy volunteers (n = 10).[13] [Adapted from Gropper et al, 1991].

Deviations from the recommended diet, which are also related to the palatability of protein substitutes, are thought to play a role, causing effects ranging from metabolic consequences to psychological distress.[12]

Moreover, free AAs typically contained in protein substitutes show absorption profiles dissimilar to those of intact proteins, with higher plasma concentrations, faster absorption peaks, and steeper blood concentration reductions (Figure 1).[13] Indications that the fast absorption kinetics of free AAs might affect body physiology raise the question about the potential benefits of a physiological absorption of protein substitutes for patients with PKU. It has been shown that free AAs given alone cause more physiological and metabolic unbalances than balanced isocaloric diets, suggesting the need to find nutritional strategies for patients with PKU as close as possible to a natural state.[14,15]

Mönch et al[16] reported that the administration of an AA bolus increases the amount of nitrogen excreted in urine when the rapid increase in blood AAs exceeds the capacity of anabolic processes to incorporate them into nascent proteins. Similarly, when young healthy subjects are fed "slow" proteins (eg, casein [cas]), protein retention is greater than in subjects fed "fast" proteins (eg, whey), where the rapid uptake is associated with a rapid increase and higher oxidation rates.[17,18]

Amino acid levels may also impact body metabolism with effects on insulin release, glycemic control, and endocrine responses or the transport of AAs across the blood–brain barrier (BBB). Insulinogenic AAs, mainly branched-chain amino acids (BCAAs), and among them leucine (LEU), induce an anabolic response[19] that is also sustained by the concomitant stimulation of incretins (glucagon-like peptide-1 [GLP-1] and gastric inhibitory polypeptide [GIP]) with similar anabolic effects.[20-22]

Large neutral AAs (LNAAs) also play an important role as they represent the precursors of important neurotransmitters; in PKU, the presence of unusually high blood Phe levels determines a higher influx across the BBB,[23] with potential consequences on neurotransmitter synthesis.[24,25]

Abnormal blood Phe fluctuations in patients with PKU, showing opposite patterns when compared to the healthy population, can be associated with poorer outcomes, inconsistent adherence to dietary treatments, and excessive catabolic processes such as nocturnal catabolism.[26-28]

The purpose of this research was to obtain kinetic data on Phe-free AA mixes engineered to modify their release and absorption in order to provide products mimicking the physiological absorption of dietary proteins. A pharmaceutical process applied to AAs, patented as the Physiomimic Technology, allows the production of small coated granules processed to gradually release the AAs in the gut, modifying the release of the AAs. Moreover, the Physiomimic Technology remarkably masks the taste and the odor of the AAs, with positive consequences on the typical unpleasant aftertaste of traditional formulations.

The consumption of products engineered with this technology is expected to contribute to the control of those metabolic aspects affected by the use of free AAs and to provide new options with organoleptic features possibly meeting the needs of patients.

Materials and Methods

Tested Products

Two Phe-free engineered mixtures of AAs were tested: engP-1 and engP-2, both containing 17 AAs (alanine [ALA], arginine [ARG], aspartic acid [ASP], cystine [C-C], glutamine [GLN], glycine [GLY], histidine [HIS], isoleucine [ILE], leucine [LEU], lysine [LYS], methionine [MET], proline [PRO], serine [SER], threonine [THR], tryptophan [TRP], tyrosine [TYR], and valine [VAL]). Phe is obviously not present in the mix, as well as asparagine and glutamic acid represented by aspartic acid and glutamine, respectively. The formulation also contains carnitine and taurine.

Functional additives (sodium alginate + ethylcellulose in engP-1 and sodium alginate, + ethylcellulose + glyceryl dibehenate in engP-2) were used to modify the AA release and consequently the absorption kinetics. Sodium alginate is used as a granulating agent, while ethylcellulose and glyceryl dibehenate are coating agents specifically chosen, among those allowed for medical foods, for their performance in prolonging the release of the AAs up to the desired extent. The 2 engineered mixes were compared with the same AA mix consisting of free AAs without the application of the Physiomimic Technology (n-engP). The relative content in AA of the engineered and nonengineered products was accurately equivalent. Absorption kinetics of cas, a known slow-release protein, were also tested for reference.

Experiments

In vitro ponderal dissolution tests and an in vivo kinetic study in female pigs (*Sus scrofa domesticus*) were performed on the 2 engineered products and on the corresponding free AA mixture in order to obtain preliminary data on the release profile determined by the Physiomimic Technology and to select the most appropriate engineered product for further development.

In Vitro Studies

The ponderal dissolution profiles of the 2 study products—engP-1 and engP-2—were carried out using USP dissolution apparatus (apparatus 2, Paddle Apparatus, USP <711>, Electrolab, Mubai, India; 50 rpm) according to the following analytical conditions: dissolution medium: pH 1.2 ± 0.1 (0.1 N hydrochloric acid: 8.3 mL/L); temperature: 37°C ± 0.5°C; volume medium: 500 mL; sample: product amount corresponding to 2.0 g of AA base form; gentle mix at start; sampling time: 30-60-120-180 minutes.

At each sampling time, samples were filtered; the remaining powder and filter were dried for about 4 hours in vacuum oven at 50°C until constant weight. Samples were weighed (net of the insoluble additives, ie, ethylcellulose and glyceryl dibehenate) and the undissolved AA percentage was calculated.

In Vivo Study

Eight healthy female pigs, aged 70 days and with a body weight range between 26.65 to 30.75 kg at enrollment, were selected. Animals underwent a surgical procedure to implant a central venous catheter in the jugular vein. Following appropriate recovery, the animals were given a single dose of each of the 4 products (engP-1, engP-2, n-engP, or cas), according to a crossover design. A washout period of at least 48 hours was observed between consecutive administrations.

The administered dose of each product was calculated based on body weight (0.8 g AA base form/kg body weight and 0.8 g cas/kg body weight). Each product dose was mixed with 300 mL of water and administered orally by gavage to ensure fast consumption (≤ 5 minutes). The animals were fasted for 13.5 hours before each dosing, and access to water was suspended 1 hour before and 1 hour after the treatment.

Blood samples were obtained at time 0.75, 0.50, and 0.25 hours before and at 0.25, 0.50, 0.75, 1, 1.25, 1.50, 2, 2.50, 3, 4, and 5 hours after each product dose. The plasma concentrations of the AAs were assayed using a validated method to monitor the content of ALA, ARG, ASP, GLN, GLY, HIS, ILE, LEU, LYS, MET, PRO, SER, THR, TRP, TYR, and VAL in pig plasma. The method, based on Phenomenex EZ: FAAST kit (Aschaffenburg, Germany), consisted of a solid-phase extraction step performed via a sorbent packed tip followed by a derivatization and a liquid/liquid extraction. The derivatized samples were analyzed by liquid chromatography/mass spectrometry-mass spectrometry (LC/MS-MS Column Phenomenex EZ: FAAST [4 µm AAA-MS 250 × 2.0 mm], mobile phase: ammonium formate 10 mM in H_2O and ammonium formate 10 mM in CH_3OH, flow rate 0.25 mL/min using a gradient program, mass spectrometry positive electrospray mode in multiple reaction monitoring).

The method to evaluate C–C has not been validated, while MET and ASP were excluded from the analysis due to stability issues. Tyrosine release was anomalous, probably linked to some type of interference with the coating; TYR data were not included in the statistical analysis.

The data of the 13 AAs included in the statistical analysis (ALA, ARG, GLN, GLY, HIS, ILE, LEU, LYS, PRO, SER, THR, TRP, and VAL) fell into the respective validated range. In eng-P1 and eng-P2, this pool of AAs accounted for the 78.8% of the whole AA administered dose.

The following kinetic parameters were elaborated by noncompartmental analysis for all AAs and for clinically relevant AA subgroups, namely essential AAs (EAAs; ARG, HIS, ILE, LEU, LYS, THR, TRP, and VAL), LNAAs (HIS, ILE, LEU, THR, TRP, and VAL), and BCAAs (ILE, LEU, and VAL):

i. time to peak concentration (T_{max}),
ii. area under the concentration/time curve ($AUC_{0\text{-}last}$),
iii. peak concentration (C_{max}), and
iv. last measured concentration at 5 hours (C_{last}).

The AUC and concentration data were analyzed by 1-way repeated-measures analysis of variance (ANOVA) with post hoc analysis with Bonferroni adjustment. The $AUC_{0\text{-}last}$, C_{max}, and C_{last} parameters were log-transformed before statistical analysis. Time to peak concentration results were analyzed by Friedman test followed by multiple sign test for pairwise comparison. The analyses were performed using IBM SPSS Statistics (version 23).

Ethical Statement

The study was conducted in full compliance with the principles of Good Laboratory Practices. The procedures involving animals were authorized by the Animal Welfare Body of Vetspin Srl and by Ministry of Health (Authorization n° 543/2016-PR), and animals were managed according to the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes, enforced by the Italian Legislative Decree n° 26 of March 14, 2014. The least number of animals was used in compliance with current regulations and scientific integrity. Animals were monitored by veterinary surgeons and handled by qualified personnel at Vetspin Srl, Villanova di Castenaso (BO), Italy.

Results

Ponderal Dissolution Tests

The dissolution profiles of the 2 study products—engP-1 and engP-2—manufactured with the application of the Physiomimic Technology, together with that of the same AA mixture (n-

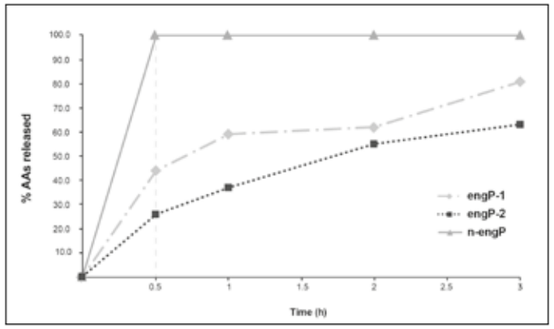


Figure 2. In vitro ponderal dissolution profile of engP-1, engP-2, and n-engP.

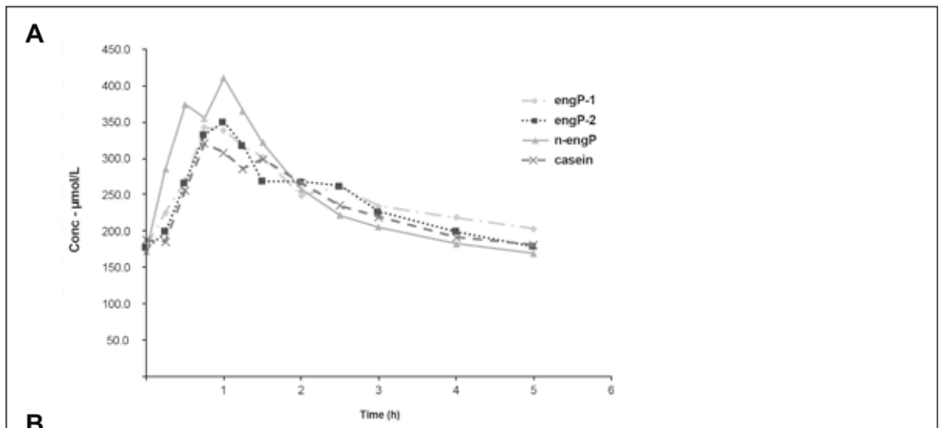
engP; no technology applied), are shown in Figure 2. The free AA mixture is characterized by a fast and complete release of the entire AA dose in less than 30 minutes. On the contrary, both engP-1 and engP-2 show a slower AA release, with less than 50% of the AAs released after 30 minutes and a gradual and sustained release of the dose in the following 3 hours.

In Vivo Study

The plasma concentrations of the AAs included in the analysis were used to calculate the kinetic parameters for each of the AA groups (Figures 3-6). With respect to n-engP, both engineered products—engP-1 and engP-2—showed a lower plasmatic peak of AA (C_{max}), a similar AUC, a retarded plasmatic peak of AA (T_{max}), and a higher plasmatic AA concentration at 5 hours (C_{last}) in all the analyzed AA groups.

As expected, $AUC_{0\text{-}last}$ of engP-1 and engP-2 was not statistically different from the $AUC_{0\text{-}last}$ of n-engP. Area under the concentration results indicate that the amount of absorbed AAs in engP-1 and engP-2 is comparable to that of n-engP, although complete AA absorption should be observed over >5 hours.

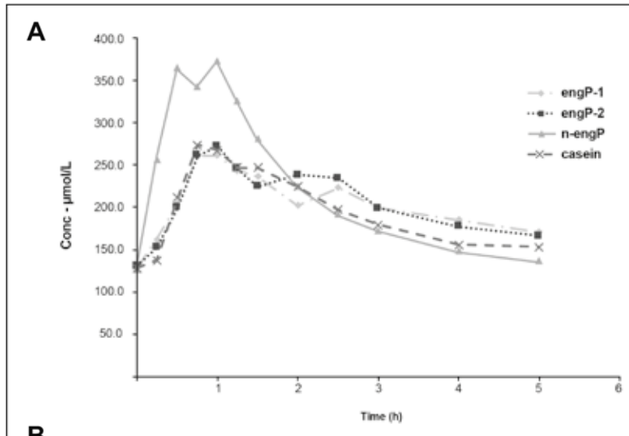
In the EAA, LNAA, and BCAA groups (Figures 4-6), the C_{max} values of the engineered products were consistently statistically lower than C_{max} of n-engP, while the C_{max} reduction



B

All amino acids§	$AUC_{0\text{-}last}$ (µmol/l *h)			C_{max} (µmol/l)			C_{last} (µmol/l)			T_{max} (h)		
	Mean	±	SD	Mean	±	SD	Mean	±	SD	Median	/	Mean
engP-1	1259.8	±	204.1	361.4	±	76.6	203.0	±	32.1	1.00		1.03
engP-2	1213.5	±	168.9	363.9	±	93.3	179.0	±	15.5	1.00		1.22
n-engP	1250.7	±	201.1	439.3	±	72.2	169.4	±	27.4	0.88		0.88
Casein	1171.6	±	262.5	335.7	±	108.3	181.3	±	36.6	1.13		1.25

Figure 3. Mean plasma concentration (µmol/L) versus time (hours after dosing; A) and calculated kinetic parameters (B) of all AAs included in the statistical analysis.
Mean = arithmetic mean; SD = standard deviation.
§ALA, ARG, GLN, GLY, HIS, ILE, LEU, LYS, PRO, SER, THR, TRP, VAL.



B

Essential amino acids§	$AUC_{0\text{-}last}$ (µmol/l *h)			C_{max} (µmol/l)			C_{last} (µmol/l)			T_{max} (h)		
	Mean	±	SD	Mean	±	SD	Mean	±	SD	Median	/	Mean
engP-1	1020.5	±	180.9	277.0*	±	68.5	170.7**	±	27.7	1.25		1.50
engP-2	1029.0	±	114.7	292.9*	±	63.0	166.2***	±	17.5	1.00		1.19
n-engP	1098.5	±	170.7	409.0	±	72.7	138.2	±	14.8	0.75		0.88
Casein	969.9	±	154.5	299.1	±	79.2	153.0	±	21.0	1.00		1.16

Figure 4. Mean plasma concentration (µmol/L) versus time (hours after dosing; A) and calculated kinetic parameters (B) of EAAs.
Mean = arithmetic mean; SD = standard deviation.
§ARG, HIS, ILE, LEU, LYS, THR, TRP, VAL.
eng-P vs. n-engP=*p<0.01; **p=0.029; ***p=0.017 [one-way ANOVA; post-hoc Bonferroni adjustment]

of the whole set of AAs (Figure 3) showed a reduction of 17% and 18% compared with the n-engP, respectively. The C_{last} values of eng-P1 and eng-P2, consistently higher in EAA, LNAA, and BCAA groups than in n-engP corresponding groups, corroborate the indication of a prolonged release with more AAs potentially available for release over the 5 hours.

Casein kinetics were also calculated for reference, due to the known kinetic characteristics of "slow protein" reported for this nutrient. Although statistical analysis was not performed due to the different relative AA content of cas with respect to the tested AA mixes, the kinetic profiles of both engP-1 and engP-2 resemble quite closely that of cas for all the considered groups of AAs.

Discussion

In patients with PKU, a diet ensuring appropriate energetic and nutritional requirements, guaranteeing normal growth in children and maintenance of an overall good nutritional status in adults, is fundamental. Health-care professionals, caregivers, and patients, despite clinical manageability of PKU, still have daily challenges to overcome. As reported by Brown and Lichter-Konecki, more than half (51.7%) respondents of a survey have difficulty in managing their PKU, reporting issues ranging from the desire to increase intake of natural protein to the wish of improving their mental health (ie, by reducing depression and anxiety). In addition, an unresolved aspect of compliance to the diet and to the protein substitutes is still present despite the numerous advances of the last decade.[29]

Based on these considerations, a new option has been conceived with the aim of delivering AAs in a physiological way. The patented Physiomimic Technology has been developed to provide prolonged AA release and odor and taste masking features, with the aim of reducing some of the unpleasant and unwanted characteristics of the free AAs.

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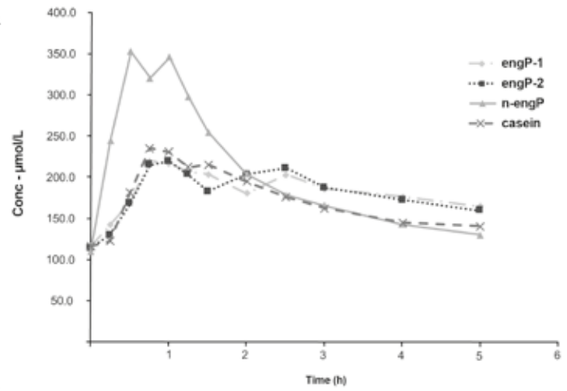

B

Large neutral amino acids§	$AUC_{0\text{-}last}$ (µmol/l *h) Mean ± SD	C_{max} (µmol/l) Mean ± SD	C_{last} (µmol/l) Mean ± SD	T_{max} (h) Median / Mean
engP-1	925.0 ± 172.5	244.3* ± 54.2	166.9** ± 31.3	2.25° / 2.16
engP-2	921.8 ± 91.6	240.0* ± 44.9	161.8* ± 16.7	1.00 / 1.22
n-engP	1028.3 ± 142.7	383.9 ± 50.0	132.5 ± 15.3	0.75 / 0.69
Casein	868.9 ± 130.2	256.6 ± 63.4	143.0 ± 19.0	1.00 / 1.16

Figure 5. Mean plasma concentration (µmol/L) versus time (hours after dosing; A) and calculated kinetic parameters (B) of LNAAs.
Mean = arithmetic mean; SD = standard deviation.
§HIS, ILE, LEU, THR, TRP, VAL.
eng-P vs. n-engP = *p<0.01; **p=0.031 [one-way ANOVA; post-hoc Bonferroni adjustment]
eng-P vs. n-engP = °p<0.01 [Friedman test and multiple sign test for pairwise comparison]

The addition of functional additives to the AAs is compliant with the requirements of food ingredients to be safely used in medical foods. In particular, ethylcellulose, the functional additive present in higher proportion, not being metabolized by humans, is considered an indigestible dietary fiber passing through the gastrointestinal tract essentially unchanged after its oral ingestion. Lifelong consumption of ethylcellulose, at the maximum estimated daily doses, contained in this new protein substitute, can be considered safe.[30-32]

The initial in vitro ponderal dissolution tests corroborated the hypothesis that the coating could modify the release of the AAs in a way compatible with the sought kinetics. Compared to the free AAs, the engineered products showed a substantial reduction of the peak, followed by a gradual release of the remaining AAs over 3 hours, suggesting a further potential for AA release over longer periods of time.

In vivo, the prolonged release of the engineered AAs, measured over 5 hours, showed prolonged absorption kinetics in clinically interesting groups of AAs such as EAAs, LNAAs, and BCAAs. Interestingly, absorption kinetics of the engineered AA mixes were similar to those of cas, a known slow-release protein.

Casein has been shown to sustain a net postprandial protein deposition in contrast to whey proteins. Whey proteins, despite a transient initial stimulation of protein synthesis, promote a lower net protein deposition, due to higher rates of nitrogen loss,[33] oxidation, and a less potent anabolic stimulation.[15,34-36]

The kinetic profile of a product prepared with the Physiomimic Technology could therefore sustain metabolic processes similar to slow-release protein, rebalancing a dietary regimen necessarily contemplating the assumption of fast absorbed free AAs, often concentrated in 2 or 3 daily doses.

Moreover, in patients with PKU, some studies revealed high fluctuations of Phe and confirmed its inverse diurnal variation with levels highest in the morning.[37,38] Another study correlated the stabilization of Phe fluctuations, when protein

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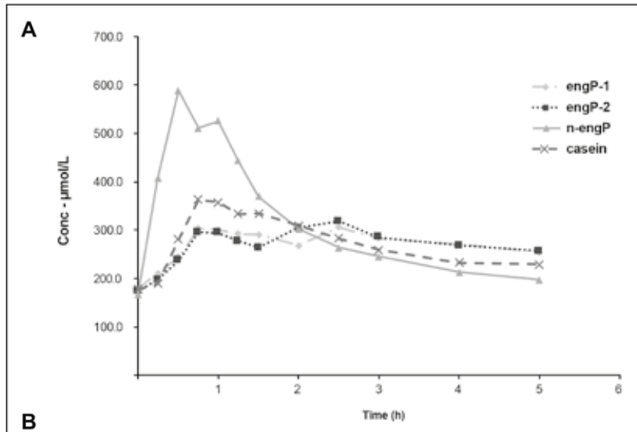

B

Branched-chain amino acids§	$AUC_{0\text{-}last}$ (µmol/l *h) Mean ± SD	C_{max} (µmol/l) Mean ± SD	C_{last} (µmol/l) Mean ± SD	T_{max} (h) Median / Mean
engP-1	1365.8 ± 260.4	350.9* ± 65.0	253.7** ± 43.8	2.50° / 2.41
engP-2	1367.3 ± 93.9	335.8* ± 39.0	256.2* ± 32.4	2.00°° / 1.72
n-engP	1556.1 ± 166.3	628.3 ± 67.4	197.0 ± 21.1	0.75 / 0.63
Casein	1365.3 ± 186.1	401.1 ± 82.6	228.7 ± 29.3	1.00 / 1.16

Figure 6. Mean plasma concentration (µmol/L) versus time (hours after dosing; A) and calculated kinetic parameters (B) of BCAAs.
Mean = arithmetic mean; SD = standard deviation.
§ILE, LEU, VAL.
eng-P vs. n-engP = *p<0.01; **p<0.045 [one-way ANOVA; post-hoc Bonferroni adjustment]
eng-P vs. n-engP = °p<0.01; °°p=0.031 [Friedman test and multiple sign test for pairwise comparison]

substitutes were administered more frequently throughout the 24 hours, if compared with fewer doses concentrated in daily hours, while it did not correlate the observed variations to Phe intake from the diet.[26] Again, a physiological AA absorption might contribute to control blood Phe fluctuations by providing AAs gradually over time.

Protein substitutes typically contain a good proportion of LNAAs, exerting competition with other AAs for the L-type amino acid transporter (LAT-1) AA transporter on the BBB. In the presence of a gradual AA absorption, the sustained availability of LNAAs competing with Phe for transport across the BBB could reduce the influx of Phe in the brain,[39] a mechanism thought to relate to some of the neurocognitive symptoms in PKU patients, including those in control.[40-42]

Another goal of the Physiomimic Technology was to offer products that could remarkably cover the unpleasant odor and taste caused by some AAs in free form, often also echoed by an unfriendly aftertaste, so as to potentially facilitate social management of the disorder, especially in school-age children and young adults.

These initial data have some limitations, planned to be clarified by the conduction of additional studies to better characterize the kinetic and clinical profile of the product. In this preliminary study, the kinetics have been calculated on 13 out of 17 AAs constituting the tested mixes. Tyrosine, a conditionally essential AA for patient with PKU, was excluded from the analysis due to an anomalously low release. The reason of its low solubility is thought to be dependent on the functional coating. For this reason, a formula optimization, consisting in the addition of only 1 functional additive to TYR has been studied in order to overcome this issue. Verifications by in vitro dissolution tests have confirmed a kinetic release profile of TYR in the same range of the other AAs; a final confirmation of sufficient TYR release and absorption is expected by the results of a human kinetic study currently ongoing.

In the pig study, blood samples were collected from the jugular vein rather than the portal vein, not allowing to discern the adsorbed AAs from those deriving from the metabolism. Branched-chain AAs, unlike other AAs, are not subject to first-pass hepatic metabolism; their absorption could reflect more closely the intestinal absorption following the dosing, and therefore, they could be considered representative of the prolonged release deriving from the Physiomimic Technology (Figure 6).

The comparison of the absorption kinetics among the engineered and the free AA mixes is, at this stage, indicative of a prolonged release. Differently from EEA, LNAA, and BCAA groups, all showing prolonged release kinetics (statistically inferior C_{max} and statistically not different $AUC_{0\text{-last}}$), a statistical difference has not been reached for whole set of 13 AAs. Since EAAs as well as LNAAs and BCAAs are known for their roles in modulating the metabolism, this should be a sufficient reason to recommend an engineered protein substitute delivering AAs gradually. Nevertheless, the administration of a protein substitute with the whole set of AAs presenting a prolonged release would be ideal. To verify this aspect, the results of a formal kinetic study in healthy human volunteers are awaited.

Moreover, the kinetic profiles of engP-1 and engP-2 show kinetic parameters not dissimilar to cas. This gives reassurance on the overall kinetic behavior of the engineered formulations, mimicking a slow release protein despite their different relative AA composition.

Given the similar results of the 2 engineered products, engP-1 has been selected for product scale-up, favoring the use of the minimal number of functional additives (sodium alginate + ethylcellulose). The AAs engineered with the Physiomimic Technology are complemented with the required set of micronutrients and trace elements, as recommended by nutritionists for PKU patients in different age groups. These components are available based on their natural kinetic profiles.

In conclusion, an innovative patented technology has been developed with the objective of providing a new option to potentially ameliorate the management of PKU thanks to the provision of AAs absorbed in a physiological way, resembling natural proteins, and that present a remarkable masking of AA odor and taste. Additional benefits of protein substitutes obtained with the Physiomimic Technology, potentially influencing some aspects of AA metabolism and the overall well-being of patients, will have to be verified in appropriate studies.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: NG, VP, AF, AC are employees of APR Applied Pharma Research sa. GR is founder and stakeholder of APR Applied Pharma Research sa. GG is an employee of Vetspin.

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