Published in final edited form as:
JPEN J Parenter Enteral Nutr. 2019 August ; 43(6): 726–733. doi:10.1002/jpen.1462.

The citrulline generation test: what does it measure?

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Abstract

Background: The citrulline generation test (CGT) has been proposed as a tool to determine gut function. However, the increase in plasma citrulline concentration that follows a bolus dose of alanyl-glutamine may also result from a reduction in citrulline clearance due to competition with glutamine for transport.

Materials and Methods: A swine model was developed and stable isotope tracers were used to determine the mechanism behind the increase in plasma citrulline that follows a bolus dose of alanyl-glutamine. Plasma concentrations and enrichments were determined and a non-steady state model used to calculate rates of appearance, disappearance and conversion.

Results: The pig model recapitulated the increase in plasma citrulline observed in humans after a dose of alanyl-glutamine. The dipeptide was rapidly hydrolyzed to its constative amino acids. Both citrulline plasma concentration and citrulline rate of appearance increased up to ~45% after the bolus dose of alanyl-glutamine. The conversion of citrulline to arginine and the rate of arginine also increased. Glutamine contributed up to 25±2% of the rate of appearance of citrulline. No changes in the rate of disappearance of citrulline were observed.

Conclusion: Our results indicate that a single bolus dose of alanyl-glutamine increases plasma citrulline concentration by increasing citrulline production without any effect on citrulline disposal. Our findings strongly indicate that the CGT assesses the metabolic response of the gut and that CGT can become a useful tool to evaluate gut mass and function.

Keywords

arginine; citrulline; glutamine; gut function; test

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Conflict of interest: None declared.

Introduction

In a landmark study, Windmueller and Spaeth[1] demonstrated that the small intestine utilizes glutamine and releases citrulline. Since then, the central role of the gut as the main site for citrulline production has been firmly established.[2–4] Because citrulline is produced in the gut, is usually absent in the diet and escapes hepatic metabolism appearing in the peripheral circulation, plasma citrulline concentration has been proposed as a marker for gut mass and function.[5, 6] However, a single fasting citrulline concentration value may not reflect a reduction in gut function;[7] for this reason a dynamic test (Citrulline Generation Test, CGT) has been proposed.[8, 9] The CGT consists in the administration of a large bolus dose of glutamine, as the dipeptide alanyl-glutamine, and in measuring the subsequent increase in plasma citrulline concentration.

The physiological basis for the test is based on the observation that both enteral and parenteral glutamine administration increases plasma citrulline[10–12] and the belief that glutamine is the main precursor for citrulline synthesis.[13, 14] However, we have shown that this apparent contribution of glutamine to citrulline synthesis was an artifact created by the choice of tracer and mathematical models used (for a detail discussion see[15]). Utilizing tracers that follow the carbon skeleton of glutamine, we have demonstrated in multiple species, including humans, that the contribution of glutamine to citrulline synthesis is rather modest.[16–19]

Although the increase in plasma citrulline after glutamine supplementation is indisputable, the mechanism behind it has not been fully explored. An increase in plasma concentration can reflect an increase in production, a reduction in clearance, or both. In fact, glutamine and citrulline compete for transport in many cell types;[20–23] for this reason, the supra-physiological plasma glutamine concentration achieved by the CGT may reduce citrulline clearance and result in an increase in the plasma concentration of citrulline independently of any change in its production. If this were true, the usefulness of the CTG to monitor gut mass and function would be limited.

The present study was conducted to determine if the pig is an appropriate model for humans, increasing plasma citrulline concentration after the administration of a bolus dose of alanyl-glutamine and to elucidate the mechanism behind this increase in plasma citrulline concentration.

Methods

General

Domestic conventionally-reared crossbred pigs were purchased from a local commercial swine farm. All animal procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Experiment 1 Preliminary study

To determine if the pig is an appropriate model for humans replicating the plasma citrulline increase after a bolus dose of alanyl-glutamine, 4 male pigs underwent surgery at 9 days of

age to implant an indwelling catheter in the jugular vein. After 5 days of recovery and a 8 h of feed deprivation, pigs (4.1±0.4 kg) received an intravenous single bolus dose of alanyl-glutamine (0.5 g/kg). Blood samples were collected from the catheter before (−20 and 0 min) and after (every 20 min for 200 min) the bolus dose of alanyl-glutamine, transferred to an EDTA-coated tube, placed on ice and centrifuged immediately at 3000 × g at 4 °C. Plasma was then transferred to an Eppendorf tube and stored at −80 °C until analysis.

Experiment 2 Tracer Study

To determine the mechanism involved in the increase of plasma citrulline after a bolus dose of alanyl-glutamine, a pregnant sow was brought to the animal facility 7 d before the scheduled birth. Piglets were born naturally and remained with the sow until they were 11 d old, when jugular and carotid catheters were surgically implanted. Piglets (4.5±0.5 kg; 3 males, 4 females) were then single housed and fed a milk replacement diet (Soweena Litter Life, Merrick's Inc., Middleton WI) until the day of the tracer study at 14 d of age.

Infusion schedule and blood sampling

On the day of the study, and after 8 h of feed deprivation, piglets were primed-continuously infused for 6.5 h using the jugular catheter with (ureido)[^{15}N] citrulline (prime: 2.5 μmol/kg; continuous: 2.5 μmol•kg^{-1}•h^{-1}) and [$^{15}N_4$] arginine (prime: 4.75 μmol/kg; continuous: 4.75 μmol•kg^{-1}•h^{-1}) to determine the rate of appearance of these amino acids. After 3 h of infusion (steady state period; Figure 1), pigs received a bolus dose of alanyl-glutamine (0.45 g/kg; 18% solution w/v) intravenously over ~5 minutes. In addition, the bolus administration contained alanine (0.21 mmol/kg; 0.74% w/v) and 2,3,3,4,4 [$^{2}H_5$] glutamine (0.21 mmol/kg; 1.25% w/v) to achieve a 10% enrichment in the glutamine infused and determine the utilization of glutamine for citrulline synthesis (Figure 1). With the addition of these free amino acids the bolus dose delivered the same amount of alanine and glutamine as in Experiment 1.

Blood samples were collected from the carotid catheter to determine background isotopic enrichment (−210, −195 and −180 min), at plateau enrichment during steady state to determine baseline citrulline production (−30, −15 and 0 min), and during the unsteady state phase following alanyl-glutamine administration (every 20 min for 200 min; Figure 1). Blood samples were collected, processed and stored as indicated above.

Sample analysis

Amino acid enrichments and concentrations were determined as their dansyl derivatives by LC-MS/MS as described previously.[2, 24] The appropriate corrections were performed to account for the semi-labile nature of the C_2 deuterium of the glutamine tracer as described previously.[19]

Calculations

The use of the classical rate of appearance equation assumes steady state conditions [Eq. 1]; however these conditions were only maintained during the first part of the study, before the bolus dose of alanyl-glutamine. To account for the non-steady state condition, the correction proposed by Steele was used [Eq. 2].[25]

$$RaCitSS = {i_{rate}}/{\text{TTR}} \quad \text{[Eq. 1]}$$

$$RaCitSteele = i_{rate} - V \bullet \left[(C_1 + C_2)/2\right] \bullet \left[(TTR_2 - TTR_1)/(t_2 - t_1)\right] \Big/ (TTR_1 + TTR_2)/2$$

[Eq. 2]

were *RaCitSS* and *RaCitSteele* are the rates of appearance of citrulline (μmol•kg^{-1}•h^{-1}) using the steady state or Steele equations, respectively; i_{rate} is the continuous infusion rate of ureido [^{15}N] citrulline (μmol•kg^{-1}•h^{-1}); C1 and C2 are the plasma citrulline concentration (μmol/L) and TTR_1 and TTR_2 are the tracer-tracee ratio of ureido [^{15}N] citrulline (unitless) at two consecutive time points; ($t_2 - t_1$) is the time between two consecutive blood samples (i.e., 1/3 h), and V an assumed volume of distribution where citrulline enters initially and is mixed instantaneously. For this calculation V was assumed to be equal to the extracellular space (0.23 L/kg body weight).

To calculate rate of disappearance (*Rd*, μmol•kg^{-1}•h^{-1}) the following equation was used

$$Rd = RaCitSteele - V \bullet (c_2 - c_1)/(t_2 - t_1) \quad \text{[Eq. 3]}$$

where the variables are as defined above. Identical calculations were performed to determine the rate of appearance and disappearance of arginine.

The fractional contribution of citrulline to arginine ($F_{Cit\ to\ Arg}$) was calculated as shown below (%)

$$F_{Cit\ to\ Arg} = TTR_{Arg}/TTR_{Cit} \bullet 100 \quad \text{[Eq. 4]}$$

where TTR_{Arg} is the tracer-tracee ratio of the product (i.e., [$^{15}N_1$] arginine) due to the infusion of the precursor (i.e., (ureido) [^{15}N] citrulline) and TTR_{Cit} is the tracer-tracee ratio of (ureido) [^{15}N] citrulline. The absolute rate of conversion of citrulline to arginine (*RcCittoArg*; μmol•kg^{-1}•h^{-1}) can then be obtained by multiplying this fractional contribution by the rate of appearance of arginine [Eq. 5].

$$RcCit\ to\ Arg = RaArg \bullet F_{Cit\ to\ Arg}/100 \quad \text{[Eq. 5]}$$

where the variables are as defined above. Similar calculation were performed to determine the contribution of glutamine to citrulline production. The underlying assumption during the non-steady state period of the study is that this conversion is very fast (i.e., no lag time).

To account for the likely lag time in the transport of the labeled precursor and the synthesis and release of the product back in the circulation, we calculated the average increase in plasma concentration and rate of appearance over the 200 min following the bolus dose of alanyl-glutamine. The increase (Δ) was calculated as an absolute value (μmol/L or μmol•kg $^{-1}$•h^{-1}) or as a fraction of the baseline.

$$\Delta_{absolute} = \Sigma_{i=0}^{200}(C_{it} - C_{base})/n \quad \text{[Eq. 6]}$$

where C_{ti} is the concentration (or rate of appearance) at time i from 0 to 200 min, C_{base} is the concentration (or rate of appearance) at baseline (during the steady state period), and n is the number of time points between 0 and 200 min.

By dividing the absolute increase by the baseline values the fractional increase ($\Delta_{\%}$) was obtained as follows,

$$\Delta_{\%} = \Delta(absolute) \bullet 100/C_{base} \quad \text{[Eq. 7]}$$

Statistical Methods

Data (plasma concentrations and kinetic parameters) were analyzed using the PROC MIXED procedure of SAS (version 9.2; SAS Institute, Cary, NC) with pig as the random effect of the model. Return to baseline was determined by pairwise comparison between time points after the alanyl-glutamine bolus with baseline values. All data are reported as means±SEM and differences were considered significant at $P < 0.05$.

Results

Preliminary study

The bolus dose of alanyl-glutamine resulted in a rapid increase in the plasma concentration of this dipeptide reaching a maximal concentration of 4.3±0.6 mmol/L at 20 min and returning to baseline by 75±5 min (Figure 2A). The concentration of plasma glutamine and alanine increased manifold, reaching ~2.2±0.2 and 1.8±0.2 mmol/L at 20 min, respectively. Alanine concentration returned to baseline values at 130±6 min; glutamine concentration returned to baseline in only two pigs, out of the four studied, by the end of the study (200 min; Figure 2A).

The plasma concentration of citrulline increased 49±6% of the baseline value at 40 min post bolus (Figure 2B); arginine, also reached the maximal plasma concentration at 40 min and the increase accounted for 73±13% of the arginine baseline concentration. The plasma concentration of both amino acids then declined slowly reaching their baseline values at 133±32 and 140±22 min, respectively (Figure 2B).

Plasma amino acid concentrations

In the second experiment, the bolus dose of alanyl-glutamine also resulted in a rapid increase in the plasma concentration of the dipeptide. The maximal concentration of 1.4±0.1 mmol/L was achieved at 20 min and became negligible (< 3 μmol/L) by ~80 min (Figure 3A). The concentration of plasma glutamine and alanine increased rapidly reaching 2±0.1 and 1.9±0.1 mmol/L at 20 min, respectively, and returned to baseline values by 140 min (Figure 3A).

The plasma concentration of citrulline increased up to 43±6% of the baseline value at 60 min post bolus (Figure 3B); arginine, however, reached the maximal plasma concentration earlier at 40 min and the increase accounted also for 43±4% of the arginine baseline concentration. Note that the absolute increase in the plasma concentration of these amino acids was 61±7 and 37±5 μmol/L for citrulline and arginine, respectively. Plasma citrulline and arginine concentrations returned to baseline values by 169±13 and 106±18 min (Figure 3B). The average plasma concentration increase over the baseline for citrulline and arginine were 20±3% and 18±3%, respectively.

Isotopic plasma enrichments and rate of appearances

Plasma [^{15}N] citrulline, [$^{15}N_4$] arginine, and [$^{15}N_1$] arginine isotopic plateau enrichments were achieved during the steady state phase of the infusion before the administration of the bolus dose of alanyl-glutamine (Figure 4A). Following the bolus of the dipeptide there was a sudden decrease in the enrichment of citrulline and arginine which returned to pre-bolus values by the end of the experiment (Figure 4A). The calculated rate of appearance using the steady state and Steele equations showed a rapid increase in the production of citrulline from a baseline value of 60±3 μmol•kg^{-1}•h^{-1} to a maximal value of 82±6 and 88±5 μmol•kg^{-1}•h^{-1} at 60 and 40 min, respectively (Figure 5). For the Steele equation this represented a 46±4% increase; this increase was transient and returned to baseline values by 137±19 min (steady state) or 93±17 min (Steele). The average entry rate increase for the rate of appearance of citrulline during the period studied were 11.0±1.4 and 10.9±1.4 μmol•kg^{-1}•h^{-1}, representing an average increase of 18.6±2.1 and 19.9±3.3% over the baseline concentration for the steady state and Steele equations, respectively.

The baseline rate of appearance of arginine was 189±9 μmol•kg^{-1}•h^{-1} and after the bolus dose of alanyl-glutamine increased slightly to 216±9 and 217±7 μmol•kg^{-1}•h^{-1} reaching its maximal value at 70 and 60 min for the steady state and Steel equations, respectively, and returning to baseline values soon after (Figure 6).

The maximal enrichment value of 5.5±0.3 mpe for 2,3,3,4,4 [$^{2}H_5$] glutamine was observed at the first time point (20 min after the bolus dose of alanyl-glutamine; Figure 4B). Glutamine enrichment then declined over time but it was still substantial (0.86±0.05 mpe) by the end of the experiment. The resulting labeling of citrulline by the [$^{2}H_5$] label followed a similar pattern (Figure 4B).

Rates of conversion

The contribution of citrulline to arginine synthesis at baseline was 6.4±0.2% of the rate of appearance of arginine and after the bolus dose of alanyl-glutamine increased to 9.2±0.5 % when it peaked at 60 min. The average contribution of citrulline to circulating arginine was 8.4±0.4 % during the post alanyl-glutamine period, representing a 31±6 % increase over the baseline values.

The contribution of glutamine to citrulline synthesis peaked at 25±2.4% 60 min after the bolus dose of alanyl-glutamine. The average contribution of glutamine to citrulline production was 20.1±1.2% of the rate of appearance of citrulline. The [$^2H_5$] recovered as plasma citrulline over the period studied was 2.23±0.1 μmol, equivalent to ~1% of the [$^2H_5$] glutamine tracer administered.

Rates of disappearance

The rate of disappearance of citrulline remained unchanged (P = 0.75) during the study (Figure 7). However, there was a small fluctuation in the rate of disappearance of arginine (Figure 7).

Discussion

The interorgan nature of the metabolism of citrulline, together with its negligible extraction by the liver, implies that citrulline produced by the gut enters the peripheral circulation. For this reason citrulline has been proposed as a marker for gut mass and function.[6] Recently, this concept has been further supported by a meta-analysis demonstrating that plasma citrulline concentration is negatively correlated with different acute and chronical enteral conditions.[26] However, plasma citrulline concentration also depends on its clearance, mainly by the kidney. For this reason plasma citrulline has also been proposed as a marker for renal disease.[27] In fact, plasma citrulline is elevated in renal patients[28] and in rodent models plasma citrulline concentration increases immediately after kidney ligation[29] or as a result of partial nephrectomy.[29, 30]

Accordingly, a single fasting citrulline concentration value may not reflect the citrulline produced by the gut and thus it may be of a limited value in assessing gut function in clinical practice.[7] Because a dynamic functional test measures changes within a subject, the CGT has the potential to circumvent the limitations of a single isolated plasma sample. However, the nature of the citrulline increase remains to be solved before the CGT can be used more widely. To answer this question we developed a swine model and used stable isotope labeled tracers to determine if the increase in plasma citrulline concentration following a bolus dose of alanyl-glutamine is due to an increased citrulline production, decreased citrulline disposal or a combination of both processes.

Pigs recapitulate the citrulline response following a bolus dose of alanyl-glutamine

To develop a swine model of CGT we used the same alanyl-glutamine dose (0.5 g/kg) used in humans. However, there are some differences in the metabolism of glutamine between these two species. Although pigs have lower plasma glutamine concentrations than humans,

[31] the rate of appearance of this amino acid is similar when compared to a pediatric healthy population.[32] In this study, the dose of glutamine was the equivalent to 3 times the hourly rate of appearance of this amino acid.[19] Alanyl-glutamine disappeared rapidly from circulation and resulted in the increase in the plasma concentration of its two constitutive amino acids.

Pigs responded to a bolus dose of alanyl-glutamine in a similar fashion to humans, reaching a similar maximal plasma citrulline concentration (pigs 43–49%, humans 44%).[8] Pigs have higher plasma citrulline concentration than humans[9, 31] and the maximal absolute increase was ~40 μmol/L compared to ~20 μmol/L in adult control subjects.[9] The increase in plasma citrulline was evident within 20 min after the bolus dose of alanyl-glutamine, peaked at 40–60 min and returned to baseline values by 160–180 min. Despite these quantitative differences, the swine model developed recapitulated the increase in plasma citrulline observed in humans after a dose of alanyl-glutamine.

Alanyl-glutamine increases the rate of appearance of citrulline

Estimating rates of appearance in a non-steady state system is difficult, but the Steele correction offers a good approximation. However, the calculation depends on a good estimation of V, the volume of distribution. Here, we used a value for V equivalent to the extracellular compartment (230 mL/kg).[25] This seems to be an appropriate value, since compartmental kinetics in pigs of different ages yielded a similar volume of distribution (Marini, unpublished).

The rate of appearance of citrulline during the steady state phase of the study was 60 μmol•kg^{-1}•h^{-1}, about six-fold greater than in humans.[19, 33] After the bolus dose of alanyl-glutamine, the rate of appearance of citrulline increased almost 50% reaching 88 μmol•kg^{-1}•h^{-1}. To the best of our knowledge no comparable data in humans are available.

Contribution of glutamine to citrulline synthesis

Our previous estimations of the contribution of plasma glutamine to the synthesis of citrulline in pigs were rather modest (< 8.5%).[18, 19] Likewise, we have also determined a small contribution of glutamine for citrulline production in rodents and humans.[16, 19, 34] Here we have determined a more substantial (up to 25%) contribution; however, it is likely that the supra-physiological glutamine concentration increased the contribution of this amino acid to the synthesis of citrulline. Regardless, only a small fraction of the glutamine administered (~1%) was converted into citrulline.

Alanyl-glutamine increases the rate of appearance of arginine

The rate of appearance of arginine, together with an increase in the rate of conversion of citrulline to arginine, increased after the bolus dose of alanyl-glutamine suggesting that more citrulline was available for *de novo* arginine synthesis.

Alanyl-glutamine does not affect the disposal of citrulline

Glutamine and citrulline compete for transport into cells, since they share a common transporter system.[20–23] However, the rate of disappearance of citrulline remained

unchanged after the bolus dose of alanyl-glutamine despite the large increase in plasma glutamine concentration. This seems to indicate that the competition for transport between glutamine and citrulline determined in *ex vivo* and *in vitro* studies[20–23] is not present *in vivo*.

In conclusion, our results indicate that a single bolus dose of alanyl-glutamine increases plasma citrulline concentration by increasing citrulline production without any effect on citrulline disposal. Our findings strongly indicate that the CGT assesses the metabolic response of the gut. This offers a sound physiological basis for a test that can become a useful tool to evaluate gut mass and function as previously proposed by others.

Acknowledgments

This work was supported by federal funds from the U.S. Department of Agriculture, Agricultural Research Service under Cooperative Agreement Number 58-6250-6-001, and the National Institutes of Health (R01 GM108940).

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Clinical Relevance Statement

The citrulline generation test has been proposed as a tool to determine the function of the gut. However, the increase in plasma citrulline concentration that follows a bolus dose of alanyl-glutamine may also result from a reduction in citrulline clearance due to competition with glutamine for transport. This study shows that the citrulline generation test results in an increase in citrulline production with no changes in the rate of citrulline disposal. For this reason, the citrulline generation test is an appropriate tool to evaluate gut function

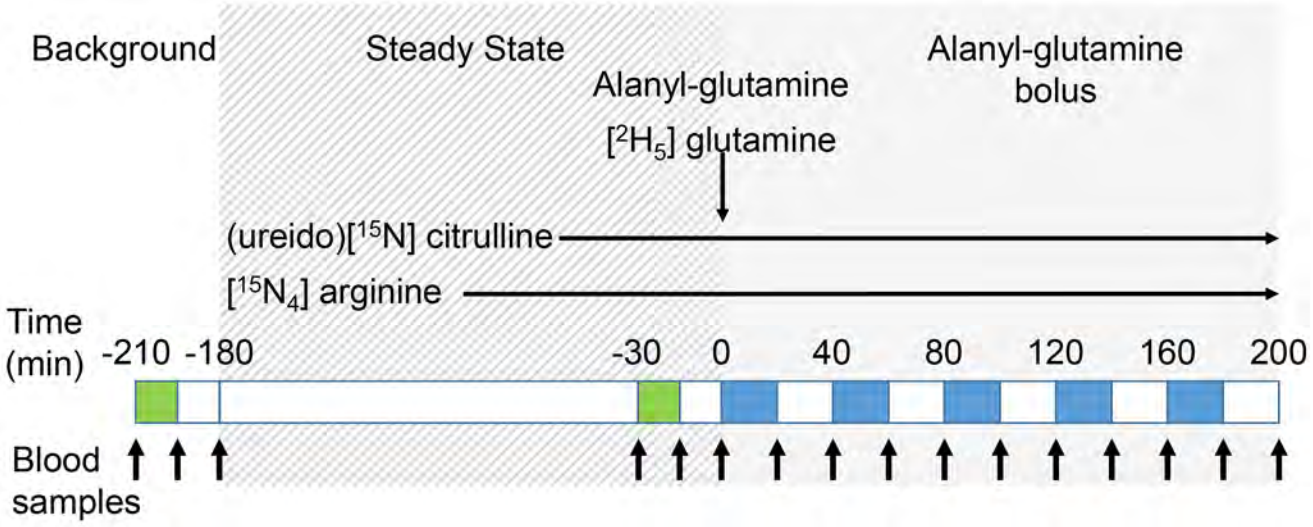

Figure 1.

Study schedule. A bolus of alanyl-glutamine (plus 2,3,3,4,4 [$^{2}H_{5}$] glutamine) was administered at time 0 min. At time −180 min a primed continuous infusion of [^{15}N] citrulline and [$^{15}N_{4}$] arginine started for the remainder of the study. Multiple blood samples were taking at background and during the steady state and the alanyl-glutamine phase of the study (indicated by arrows).

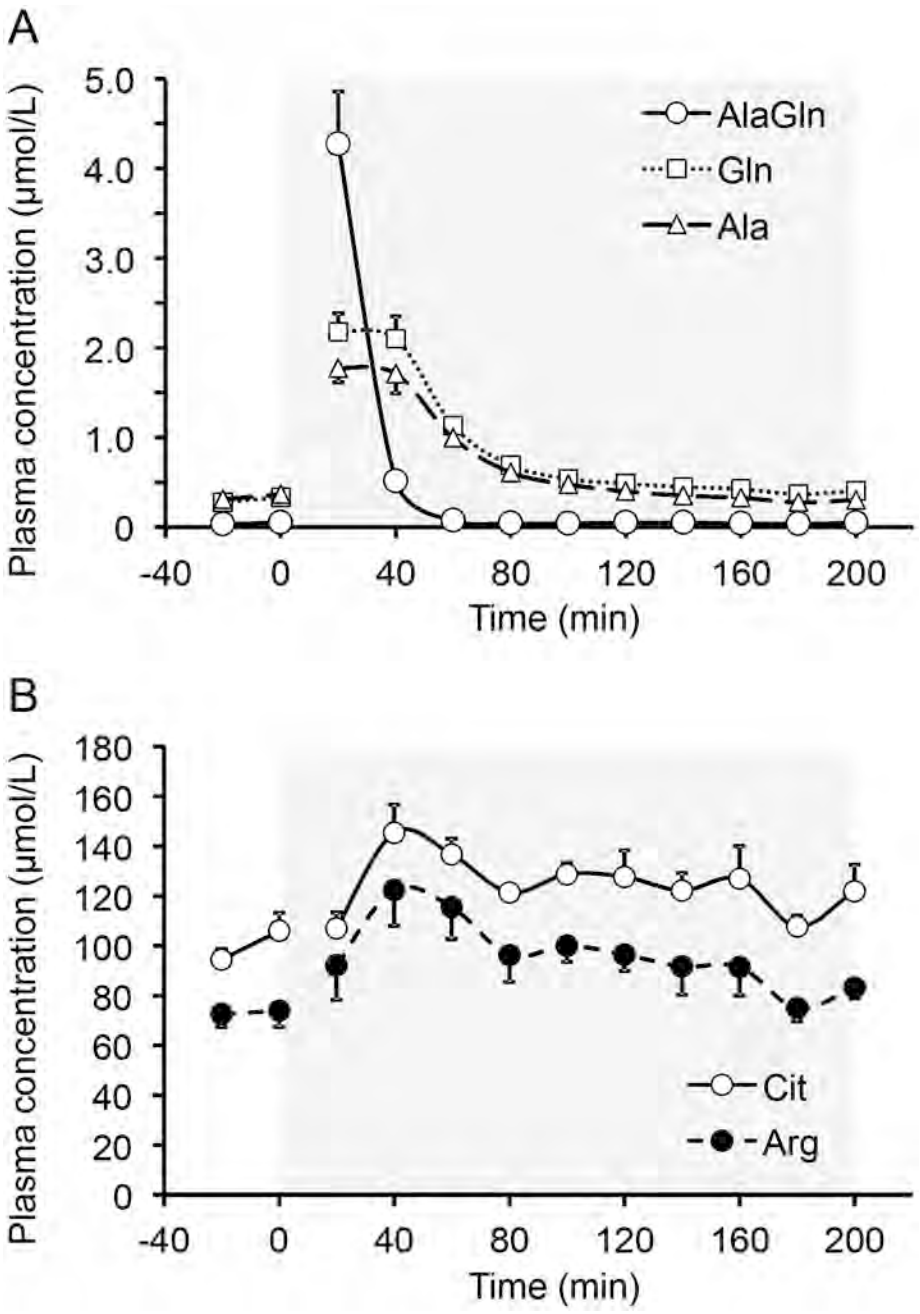

Figure 2.

Plasma alanyl-glutamine, glutamine and alanine (A), and citrulline and arginine (B) concentration in pigs before and after a bolus dose of alanyl-glutamine. Alanyl-glutamine (0.5 g/kg) was administered at time 0. Symbols are means±SEM, n = 4. Ala, alanine; AlaGln, alanyl-glutamine, Arg, arginine; Cit, citrulline; Gln, glutamine.

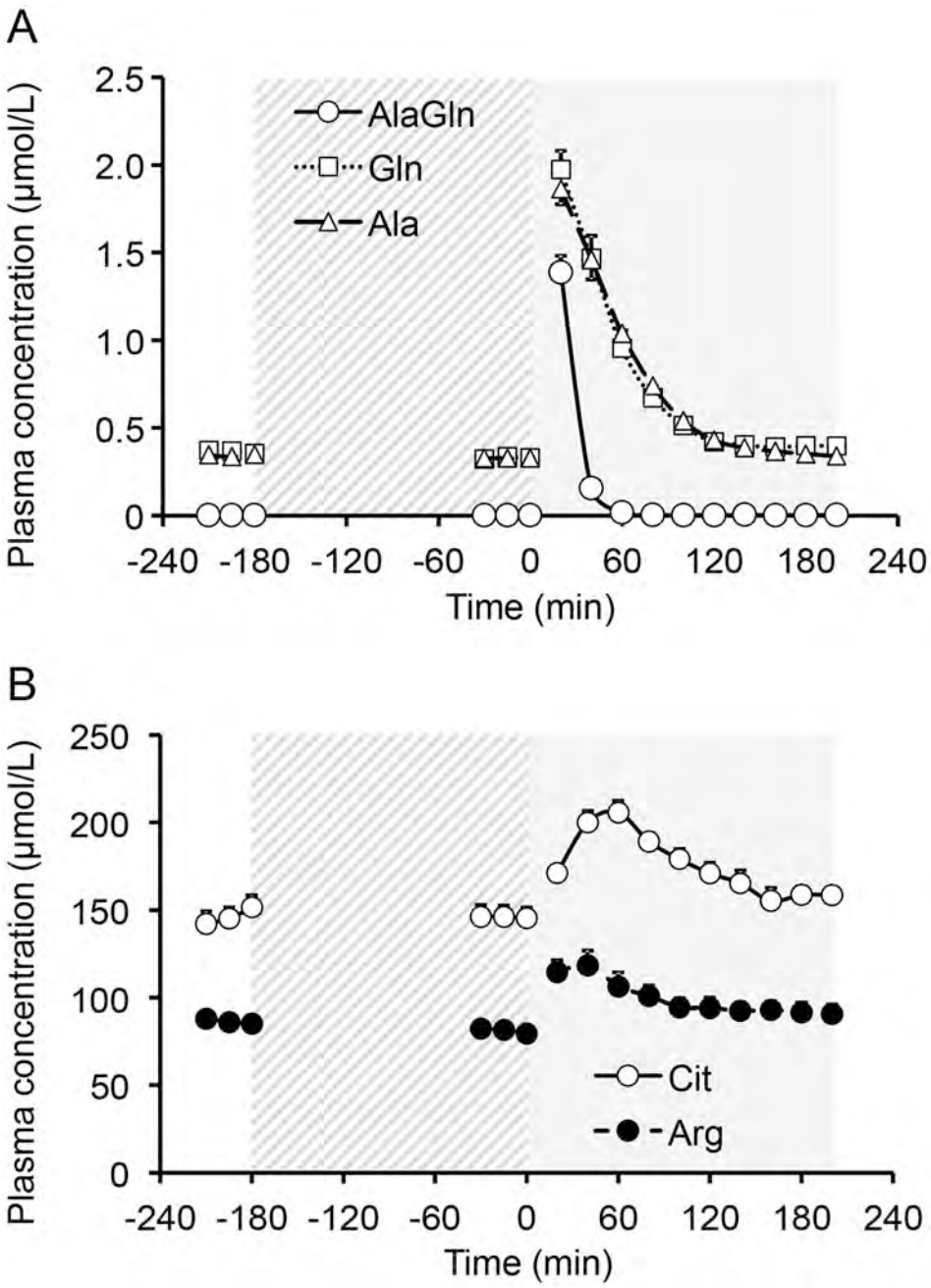

Figure 3.

Plasma alanyl-glutamine, glutamine and alanine (A), and citrulline and arginine (B) concentration in pigs before and after a bolus dose of alanyl-glutamine. Alanyl-glutamine (0.5 g/kg) was administered at time 0. The experiment consisted of three periods: baseline (no shading), steady state (fasting; hatched area) and post alanyl-glutamine bolus (greyed area). Symbols are means±SEM, n = 4. Ala, alanine; AlaGln, alanyl-glutamine, Arg, arginine; Cit, citrulline; Gln, glutamine.

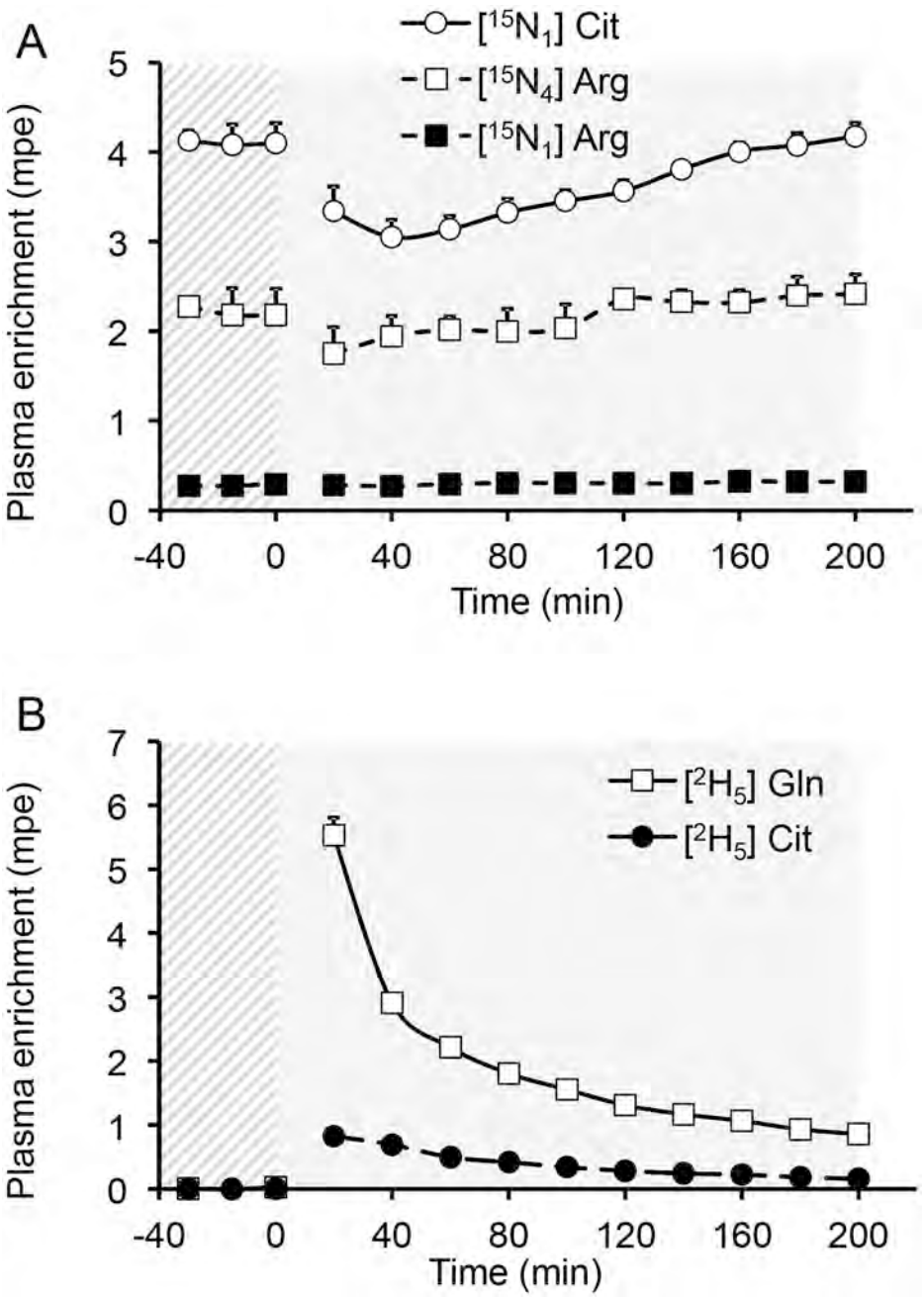

Figure 4.

Plasma (ureido)[^{15}N] citrulline, U-[$^{15}N_4$] arginine, and [$^{15}N_1$] arginine isotopic enrichments in pigs before and after a bolus dose of alanyl-glutamine (A), and plasma [$^{2}H_5$] citrulline and [$^{2}H_5$] glutamine after the bolus dose of alanyl-glutamine (B). Alanyl-glutamine was administered at time 0. The experiment consisted of two periods: steady state (fasting; hatched area) and post alanyl-glutamine bolus (greyed area). Symbols are means±SEM, n = 7. [$^{15}N_1$] Arg, [$^{15}N_1$] arginine; [$^{15}N_4$] Arg, [$^{15}N_4$] arginine; [$^{15}N_1$] Cit, [$^{15}N_1$] citrulline; [$^{2}H_5$] Cit, [$^{2}H_5$] citrulline; [$^{2}H_5$] Gln, [$^{2}H_5$] glutamine; mpe, mole percent excess.

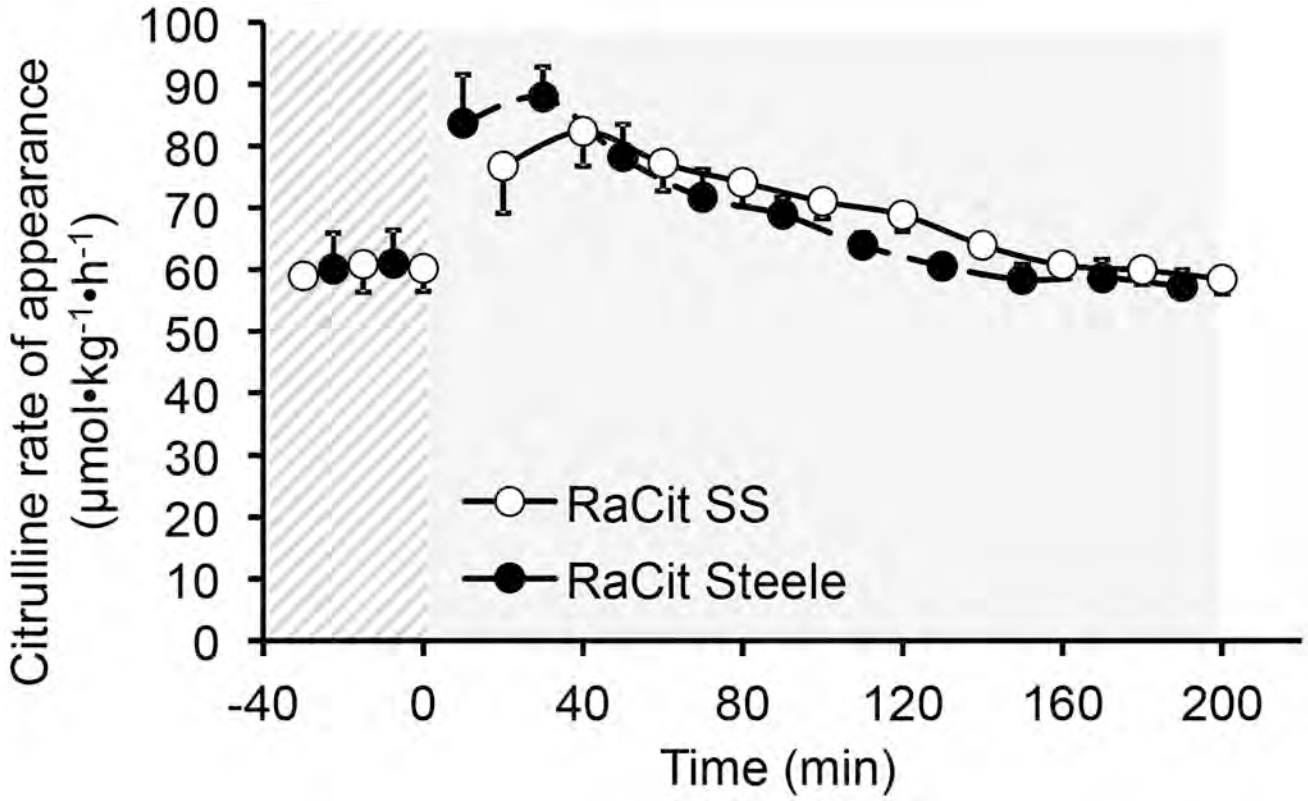

Figure 5.

Rate of appearance of citrulline calculated with the steady state equation and Steele correction for non-steady state conditions. Alanyl-glutamine was administered at time 0. The experiment consisted of two periods: steady state (fasting; hatched area) and post alanyl-glutamine bolus (greyed area). Symbols are means±SEM, n = 7. RaCit SS, rate of appearance of citrulline calculated with the steady state equation; RaCit Steele, rate of appearance of citrulline calculated with Steele's equation.

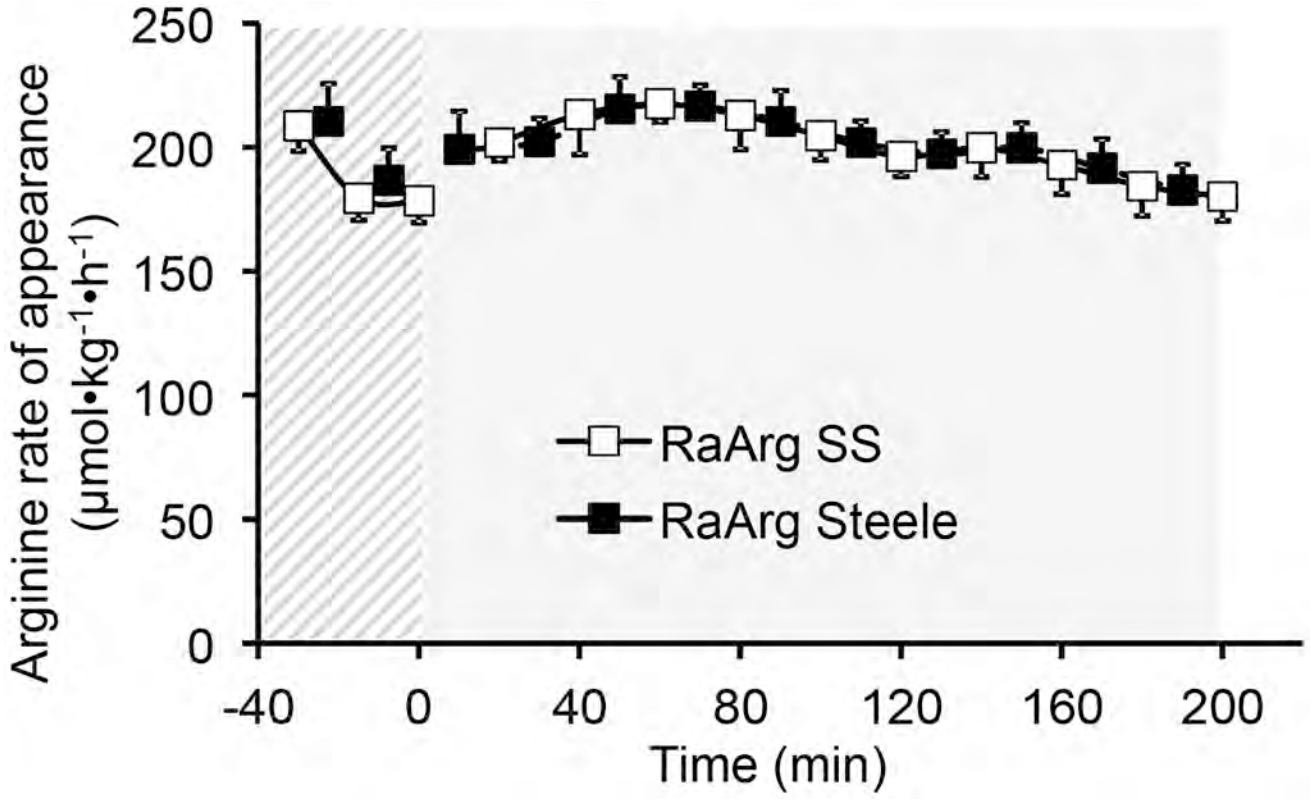

Figure 6.

Rate of appearance of arginine calculated with the steady state equation and Steele correction for non-steady state conditions. Alanyl-glutamine was administered at time 0. The experiment consisted of two periods: steady state (fasting; hatched area) and post alanyl-glutamine bolus (greyed area). Symbols are means±SEM, n = 7. RaArg SS, rate of appearance of arginine calculated with the steady state equation; RaArg Steele, rate of appearance of arginine calculated with Steele's equation.

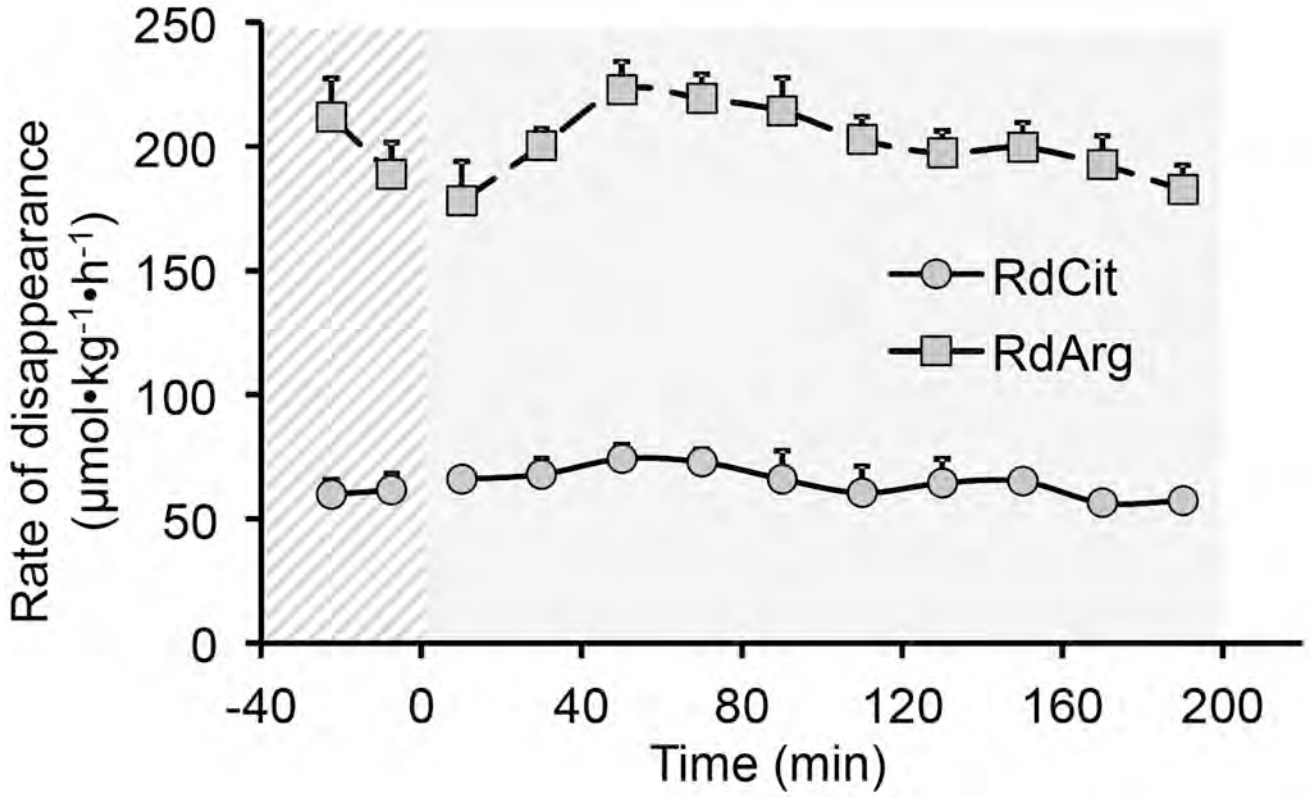

Figure 7.

Rate of disappearance of citrulline before and after a bolus dose of alanyl-glutamine enriched. Alanyl-glutamine was administered at time 0. The experiment consisted of two periods: steady state (fasting; hatched area) and post alanyl-glutamine bolus (greyed area). Symbols are means±SEM, n = 7. RdArg, rate of disappearance of arginine; RdCit, rate of disappearance of citrulline.