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Development of a method for measuring lysine and methionine bioavailability in rumen-protected products for cattle

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ABSTRACT

The objective of the experiment was to find a quantitative test to determine the lysine or methionine bioavailability of rumen-protected amino acids. For these purposes we elaborated a blood test including an *in vivo* calibration phase and we used this test on two commercial products whose bioavailability is known (SmartamineM™ and SmartamineML™). Three ruminally and duodenally fistulated low-yielding (10 kg/d) Holstein cows were used. The calibration phase consisted of relating blood plasma methionine and lysine concentrations to duodenal infusion of graded amounts of methionine and lysine. The blood plasma responses of methionine and lysine to graded amounts infused duodenally were linear. The methionine response was the same for all cows, but the lysine response varied with the cows. Quantification of the bioavailability of commercial products is achieved by relating the blood level variations, as induced by product supplementation, to those obtained by duodenal infusion during the calibration phase. Bioavailability of methionine supplied by SmartamineM™ and SmartamineML™ was 75 and 84%, respectively. The reliability of the test is related to the amount of amino acid provided by the product supplementation: providing a low quantity of amino acids resulted in an overestimation of the bioavailability. This tendency is particularly clear for lysine bioavailability estimation. The proposed blood test is valuable for the determination of the bioavailability of methionine, but not lysine, of rumen-protected products.

KEY WORDS: methionine, lysine, bioavailability, blood test, dairy cattle

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INTRODUCTION

Methionine and lysine are the most limiting amino acids of milk protein synthesis in dairy cows, as demonstrated by post-ruminal infusions of increasing doses of lysine (Rulquin et al., 1990; King et al., 1991; Schwab et al., 1992; Guinard and Rulquin, 1994) and methionine (Socha et al., 1994a,b,c; Guinard and Rulquin, 1995; Pisulewski et al., 1996). In practice, those amino acids must be given in a form that ensures their protection against degradation in the rumen and absorption in the small intestine. This is possible with raw materials like gluten meal or fish meal, for example. Bioavailability (the amount of amino acid useable by animals), however, varies greatly according to the technological treatment undergone by feedstuffs. Moreover, only small amounts of methionine or lysine can be supplemented this way. As an example, more than one kg of fish meal supplement is necessary to provide approximately 12 g of absorbable methionine. Therefore, a significant effort has been deployed and dedicated to the development of protected forms of pure amino acids, using different techniques.

Many tests have been developed to assess the effectiveness of this protection :

- *in vitro* laboratory tests (Robert, 1992)
- *in sacco* tests (Overton et al., 1996; Robert et al., 1997; Bach and Stern, 2000)
- *in vivo* tests measuring digestive flux (Robert and Williams, 1997; Koenig et al., 1999; 2002)
- tests based on variations of plasma concentrations of the protected amino acid consumed (Overton et al., 1996; Bach and Stern, 2000).

These various techniques all have advantages and disadvantages: laboratory tests *in vitro* are remote from *in vivo* reality. The methods proposed can only test pH-sensitive forms. Tests *in sacco* do not take the mechanical aspects of digestion into account (mastication, rumination, transit rate in the rumen). The *in sacco* method cannot be used with small-sized or soluble products, the tested substrate can be contaminated by rumen bacteria, giving false or biased results, particle loss is also a risk if the grain size of the tested products is close to the mesh of the bags.

In vitro and *in sacco* tests are semi-quantitative tests and so give numerical but overestimated values because they do not take a number of digestive phenomena into account; they will help in segregating good products from the really bad ones as a first step, but their discriminating power is too low to precisely rate the bioavailability of the various protected amino acids. Tests that measure the duodenal digestive fluxes of free amino acids originating from protected forms are especially cumbersome and costly. They require using cannula-fitted animals and infusing flux and digestibility markers. To be quantitative, the measurement has to be performed according to equally cumbersome experimental designs (Latin square, double inversion etc.) with imprecise flux results, even in well-conducted experiments. Tests based on blood level variations have the advantage of being global and of reflecting the bioavailability of the protected product, but they are not quantitative.

The aim of the tests described in this paper was to propose quantitative assessment methods to determine in circulating blood the additional amount of the free amino acid originated by the protected product (added amino acid bioavailability). Quantification is achieved by relating the blood level variations, as induced by product supplementation, to those obtained by duodenal infusion of known doses of pure amino acids. After verifying the linearity of blood level response according to the doses infused, that technique was applied to two commercial protected amino acid products.

MATERIAL AND METHODS

Treatments

Animal response calibration was performed by infusing water or 15 g DL-Met and 26 g L-Lys·HCl or 20 g DL-Met and 38 g L-Lys·HCl in the duodenum. The infusions were performed according to a 3 x 3 Latin square design with 4 d periods. At the end of the trial two calibration periods were added: 3 g DL-Met and 13 g L-Lys·HCl and 30 g DL-Met and 51 g L-Lys·HCl. Additional treatment consisted of supplementing 20 g DL-Met and 38 g L-Lys·HCl by continuous infusion into the rumen or by introduction into the rumen cannula twice a day, 15 min after the distribution of feeds.

Between these two calibration periods, the bioavailability (amount absorbed/amount supplemented) of rumen-protected amino acids was determined with several doses. The test doses were 30 and 40 g SmartamineM™ (SmM), then 88, 132 and 50 g SmartamineML™ (SmML), respectively. The analytical specifications of these products, protected by a pH-sensitive coating, were as follows, % DM: for SmartamineM™ and SmartamineML™: 99.6 and 96.7; N: 6.53 and 9.71; Met: 78.1 and 16.8; Lys: 0 and 39.4.

The protected amino acids were introduced during 4 days in the rumen cannula twice a day, 15 min after the distribution of feeds. Pure amino acids were dissolved in 5 kg water and infused continuously with a peristaltic pump (Ismatec, Bioblock, Illkirch, France).

Animals

Three multiparous Holstein cows fitted with rumen and duodenal fistulae were used. They were fitted with a catheter inserted in the jugular vein. The cows' body weight was 776 ± 55 kg and they were all in late lactation (350 ± 8 days), producing 10 ± 1.3 kg milk.

Diet and feeding

The animals were fed a standard diet composed of (%): maize silage, 94; tanned meal, 4.1 (80% soyabean and 20% rapeseed); urea, 0.7; mineral vitamin supplement, 1.3. That diet was fed to the amount of 14.9 kg DM/d in two equal parts at 6.30 and 18.30, and covered 127% of energy requirements and 115% of protein requirements.

Sampling and analysis

On the last day of each experimental period, a series of blood samples (10 ml) was collected from the jugular vein with a heparinized syringe (Monovette^{R} Starsted) every hour from 06.00 to 17.00. The plasma collected by centrifugation of blood at 3000 rpm for 10 min at + 4°C was protein-depleted with sulphosalicylic acid 6% (1 volume of plasma to 1 volume of acid). The mixture was then centrifuged at 3000 rpm for 10 min. A supernatant aliquot was used to make up a sample per cow representative of the average day and the remainder was stored to study variations later in the day. The amino acid composition of those samples was determined by ion exchange chromatography with an amino acid analyser (LC 3000, Biotronik) according to Pisulewski et al. (1996).

Statistical analysis

Statistical analyses were performed according to the SAS procedure. ANOVA was used to test the difference in bioavailability of methionine from the SmM product administered in doses of 30 and 40 g/day and to estimate the residual standard deviation of the blood test technique.

The GLM procedure was used to compare the slopes and ordinates at the origin of calibration lines. It was also used, based on linear and quadratical orthogonal contrasts, to verify the linearity of the bioavalability results of the SmML product amino acids. The equilibrium hypothesis was tested by using dose/time interactions by the GLM repeated time procedure.

RESULTS AND DISCUSSION

Control selection

Various possible controls were tested comparatively. No significant difference was found between treatments, type of infusion (water in the duodenum vs amino acids in the rumen) or in the infusion mode (continuous or intermittent) for Met and

Lys (Table 1). However, highly significant differences in blood Lys concentration were noted between cows. The above results showed that methionine and lysine were metabolized in the rumen, and are consistent with Patterson's results (1988).

TABLE 1

Effect of the various control treatments on methionine and lysine plasma levels, mg/100 ml (Trial 2)

Control Site Supply	Water Duodenum Infusion	Met and Lys into the rumen		SED	P of effects	
		infusion	twice in 24 h		cows	treatment
Met	0.354	0.348	0.320	0.044	0.32	0.67
Lys	1.095	1.205	1.037	0.083	0.001	0.11

Calibration curves

Plasma methionine or lysine levels varied linearly with the amounts of those amino acids infused in the duodenum. Indeed, the regression R^2 between plasma levels and infusion amounts were between 0.90 and 0.95 for either Met or Lys. Statistically, the ordinates of origin and the slopes did not differ from one another for Met (Figure 1a) but not for Lys (Figure 1b). With Lys the ordinate of origin appeared to be related to the level of protein produced in milk. It is therefore preferable to compute the protection level of a product by using individual calibrations

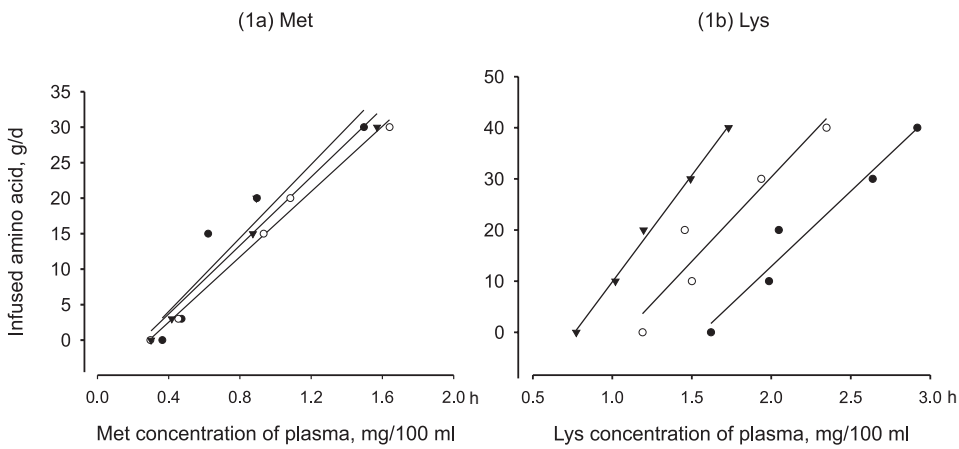

Figure 1. Individual variations of relationships between plasma of Met (1a) or Lys (1b) and amounts infused into the duodenum. Milk protein yield of cows: ● Cow 1 = 189 g/d; ○ Cow 2 = 249 g/d; ▼ Cow 3 = 358 g/d

rather than an average calibration formula. The response being linear over a wide variation range, two calibration points for each cow are sufficient; this will reduce the method application costs.

The response linearity noted in this trial was consistent with the results obtained by Titgemeyer and Merchen (1990), King et al. (1991) and Guinard and Rulquin (1994, 1995). The infused amounts tested (3-30 g methionine and 10-40 g lysine) were similar to those applied by Guinard et al. (1994) and Guinard and Rulquin (1995) who used 32 g methionine and 63 g lysine per cow per day. Conversely, the highest dose of lysine used in this trial was much lower than that of King et al. (1991) - 180 g/cow per day.

In the current trial, the variation range tested was relatively wider with methionine than with lysine and the maximum methionine tested was close to the duodenal flux of dietary methionine, whereas the corresponding lysine maximum only amounted to 25% of the duodenal flux of dietary lysine. That could perhaps explain the lower accuracy of results with lysine as compared with methionine.

Blood methionine variations induced by infusion of protected products throughout the day were studied. There was a significant variation ($P<0.05$) of plasma amino acid levels along the day (Figure 2). The variability was not wider with either protected product, SmM or SmML. With SmML there was no significant interaction between blood methionine level and time. That would indicate that a

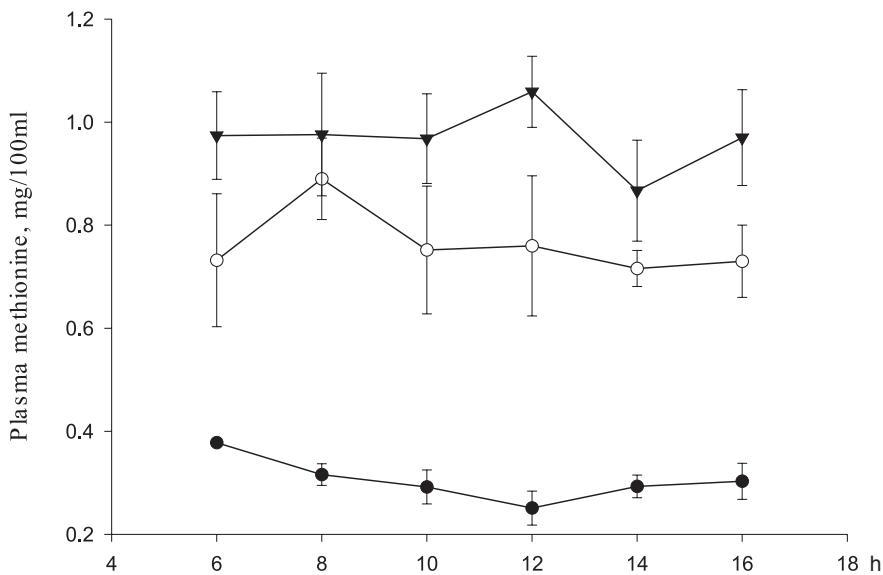

Figure 2. Diurnal variations of plasma methionine concentrations of cows supplied with graded doses of Smartamine ML™: —●— 50 g SmartamineML™, —○— 88 g SmartamineML™, —▼— 132 g Smartamine ML™

steady state was reached after 4 days of treatment; otherwise the kinetics should have been enhanced with higher doses.

These results show, with regard to the application of the technique, that on the one hand it will be necessary to collect several blood samples on the same day, so as to obtain a half-day representative point by pooling samples, and on the other hand, that 4 days of treatment with the product under test will be sufficient to obtain a steady state.

Application to commercial products

The bioavailability level of the Smartamine™ M and Smartamine™ ML amino acids within the same animal were computed using the calibration curve for each cow according to the following formula:

amount "absorbed" = a + b x plasma level

Bioavailability corresponded to the amount absorbed to amount supplemented ratio.

The level of methionine bioavailability was high in both products: 75% in SmM and 84 % in SmML. No significant difference was found with the two doses used with SmM (P=1; Table 2). With SmML, methionine bioavailability tended to decrease with the dose that was used, although nonsignificantly (P=0.11; Table 2).

This may suggest that the technique reached its limitations with very low methionine doses. Bioavailability appeared to be stable between the last two doses, which could mean that at least 15 to 20 g Met are required for the test to be reliable. The fact that bioavailability was the same regardless of the dose used indicates that calibration is possible with these products provided the doses are not too low (below 15 g/d).

The lysine bioavailability of SmML was very high: 100% (Table 2). This result is astonishing because the coating technology is such that there is no reason for lysine bioavailability to be higher than that of methionine, especially in the same product, SmML, where it was 84% (Table 2). This was not due to any aberrant

TABLE 2

Methionine and lysine bioavailability in Smartamine M™ and Smartamine ML™

Indices	Smartamine M™		SED	Smartamine ML™			SED
Product, g/d	30	40		50	88	132	
Met, g/d	23.4	31.2		8.4	14.8	22.2	
Lys, g/d				19.7	34.7	52	
Bioavailability, %							
Met	75.1	75.1	3.43	95.3	79.7	77.7	10.5
Lys				106.3	84.0	109.3	18.2

value because 56 % of the values were above 100%. As with methionine, the lysine bioavailability of SmML was unrelated to the dose used (P= 0.22).

These high lysine bioavailability results could be related to those obtained with methionine supplemented in small doses (i.e. 95% bioavailability with 8 g supplement); in that case, methionine supplementation only represented about 20% of the duodenal flux of dietary origin. Imprecision could also be greater in that case. With lysine the supplementation amounts were between 13 and 33% of the baseline duodenal flux.

This technique of protected amino acid bioavailability assessment appeared to produce good results with methionine. It could in future be simplified by using only two calibration values (0 and 20 or 30 g Met). However, it requires kinetically scheduled blood sampling in order to secure a mean value representative of one half day.

The results of the current trial revealed that methionine bioavailability did not differ between SmM (75%) and Sm ML (84%), and could be defined as 80%. In contrast, the lysine bioavailability of SmML was too high and the reliability of the test for that determination remains to be verified. These results are concordant with those obtained by duodenal assay and faecal digestibility tests, revealing 80% mean bioavailability of methionine in SmartamineM™ (Robert and Williams, 1997). The same tests revealed, however, 87% lysine bioavailability in SmartamineML™, much lower that that obtained with the blood test technique used in the current study.

CONCLUSIONS

In contrast with *in vitro, in sacco* and blood tests without calibration, the blood test with calibration is quantitative. The accuracy of the technique is 3-10%, whereas that of duodenal work-up would be about 20-30% (Robert and Williams, 1997).

The results from the current trial show that the protected amino acid assessment technique produces good results, with methionine in particular. With lysine, the case needs to be confirmed. The method could be simplified by using only two calibration points (0 and 20 or 30 g methionine); it is necessary however, to define blood sampling kinetics to determine a mean value representative of the whole day. The technique is quick and requires few analysis. However, it always requires fistulated animals. In addition, calibration should be performed individually to ensure optimal precision.

The test could be simplified by using calibration with a product whose bioavailability is perfectly known; it would be necessary also that this "calibration product" be absolutely stable over long periods, which remains to be ascertained.

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STRESZCZENIE

Opracowanie metody pomiaru dostępności biologicznej lizyny i metioniny z preparatów chronionych przed rozkładem w żwaczu bydła

Celem badań było opracowanie ilościowego testu do oznaczenia dostępności biologicznej lizyny i metioniny chronionej przed rozkładem w żwaczu (Smartamine M™ i Smartamine ML™). Doświadczenie przeprowadzono na trzech krowach holsztyńskich o niskiej produkcji mleka (10 kg/d), z przetokami do żwacza i dwunastnicy. W fazie kalibracyjnej testu oznaczano stężenie wolnej lizyny i metioniny w krwi żyły jarzmowej i uzyskane wartości odnoszono do stopniowo zwiększanej dozy lizyny i metioniny infundowanej do dwunastnicy: stwierdzono liniową zależność pomiędzy stężeniem tych aminokwasów a ich ilością podawaną. Reakcja krów na podaną metioninę był jednakowa u wszystkich zwierząt, natomiast na lizynę różniła się między zwierzętami.

Dla ilościowej oceny dostępności biologicznej, z handlowych preparatów, aminokwasów chronionych przed rozkładem w żwaczu porównywano zmiany stężenia wolnych aminokwasów w krwi, spowodowane podaniem badanych preparatów, ze zmianami spowodowanymi podawaniem aminokwasów w fazie kalibracyjnej. Dostępność biologiczna metioniny z preparatów Smartamine M™ i Smartamine ML™ wynosiła odpowiednio 75 i 84%. Dokładność testu zależała od ilości aminokwasów w podanym preparacie z zastrzeżeniem, że podanie małej ilości aminokwasu chronionego prowadzi do zawyżenia wartości wyników ich dostępności biologicznej. Ta tendencja była szczególnie wyraźna w przypadku oznaczania dostępności biologicznej lizyny. Opracowany test jest wartościową metodą do oznaczania dostępności biologicznej metioniny, lecz nie lizyny, z preparatów aminokwasów chronionych przed rozkładem w żwaczu.