Postruminal Delivery System for Amino Acids and Proteins in Cattle

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Received February 12, 2007
Accepted October 2, 2007

Abstract

Sýkora T., M. Rabišková, J. Třináctý, D. Vetchý, A. Häring, P. Dvořák: Postruminal Delivery System for Amino Acids and Proteins in Cattle. Acta Vet. Brno 2007, 76: 547-552.

The purpose of this experiment was to develop an effective postruminal transport system (PTS) with a high content of suitable vegetable proteins and amino acids. PTS serves for nutrient delivery to the abomasum and small intestine of dairy cows in order to increase the milk yield. Direct addition of proteins and amino acids to the diet is not useful as the ruminal microbes will utilize active substances before they reach absorption sites in the small intestine. PTS has several advantages, e.g. a possibility of the direct application in a food, low cost, and nutritional and therapeutical improvement. PTS consists of a core (pellets, small tablets) and a coating, which protects the core against the environment of rumen and enables to release the core content in the environment of abomasum and small intestine.

Lenticular tablets - cores of PTS were prepared by wet granulation method and compression. Qualitative indicators of tablets (average weight, weight uniformity, hardness, friability, disintegration time) were determined according to valid Czech and European Pharmacopoeias. Cores were subsequently coated with several types of coating - ethylcellulose, stearic acid and pH sensitive polymer poly-(2-vinylpyridine-co-styren), alone or in combination of various rates.

Nine samples of coated protein tablets exhibiting appropriate characteristics *in vitro* were prepared. The presence of the pH sensitive polymer at least in 10% concentration of the coating and the coating amount of 9.0 to 12.6% per tablet were necessary to ensure the requested PTS properties.

Rumen-stable system, pH sensitive, coating, dairy cow, protein, nutrition

Balanced intake of amino acids and proteins is very important for high milk and meat production (Pell et al. 2000). To remedy the amino acids deficiency, farmers offer their animals high protein feed or preparations containing specific amino acids, e.g. lysine and methionine (Wu et al. 1981). Methionine is the first limiting amino acid for protein synthesis in growing ruminants, lactating dairy cows and wool producing sheep (Südekum et al. 2004). Metabolic disorders induced by long-term deficiency of these amino acids are often associated with a reduced productivity of livestock. Highly productive dairy cows are the basis of successful dairy industry and their nutrition is of paramount importance.

The rumen constitutes a transport obstacle for safe drug delivery to the absorption site in the small intestine, methionine and lysine had to be supplied in PTS stable in the environment of rumen, leading thus to the increase of milk protein and milk fat content (Rossi et al. 2003). Commercial methionine products with various types of protection are being offered by different companies in the U.S.A. - Meprone M85® (Degussa Corporation), Met-Plus® (Nisso America, Inc.), and Smartamine M® and ML® (Aventis Animal Nutrition), containing from 65 to 85% of D,L-methionine and/or D,L - lysine (Südekum et al. 2004).

PTS protects the active substances from rumination and degradation processes caused by microorganisms in the rumen and it enables their release in the abomasum, their absorption

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and transport to blood circulation in the small intestine (Wu and Papas 1997). The effective coating of PTS ensures to maintain the rumen environment without any damage for 24 h. However, in the acid abomasum environment (pH 1 - 2) it is rapidly dissolved (30 min, maximum up to 60 min), thus releasing proteins and amino acids (Rabišková et al. 2004). The core of PTS should have high active ingredient content, optimal size (up to 6 mm) and shape (sphere or short cylinder), smooth surface, appropriate specific gravity (1.2 - 1.7 g/cm^3) and good mechanical properties to withstand the following coating process. These core characteristics have high influence on the particle movement within the rumen (Desborders and Welch 1984; Firkins 1997; Třináctý et al. 2002).

Rumen stability of PTS can be evaluated using *in vitro* buffer incubation, *in situ* fermentation and/or *in vivo* methods. *In vitro* incubation test is based on a buffer system simulating rumen pH and temperature. Typical test measures stability in a phosphate buffer of pH 5.5 - 7.4 or rumen liquid collected from dairy cattle at 40 °C for 24 h. Mechanical stirring may be included to simulate agitation of the rumen contents. If such a system fails this test, the results are predictive of poor protection. Passing the test, however, does not predict the resistance of a system to the microbial fermentation in an active rumen environment. In addition, this method does not evaluate the release of the protected compound for absorption (Wu and Papas 1997; Welch 1990). *In situ* nylon bag, nylon capsule and mobile bag methods have been used to determine ruminal and intestinal digestibility of nutrients. Nylon bags with the delivery system, placed either directly in the rumen (*in situ*) or *in vitro* rumen system containing active rumen microbes and simulating rumen function, allow free inflow of the rumen fluid and exit of solubilized active ingredients. The loss of active ingredients in samples is determined (Třináctý et al. 1999, 2003).

Buffers and enzymes or actual digesta collected via cannula are used for the estimation of abomasum release and drug availability for absorption. Buffers simulating the abomasum environment are particularly useful for evaluating the delivery system based on pH sensitive coatings (Wu and Papas 1997). *In vitro* Tilley-Terry methods (Tilley and Terry 1963) and methods designed by Calsamiglia (Calsamiglia and Stern 1995) simulate digestion in the abomasum. Both methods bring incubation of the tested material in a pepsin solution of pH 2.0 for 1 - 2.5 h at 39 °C (Rhône Poulenc, Smartamine™M, Smartamine™ML 1994).

In blood response method *in vivo* the tested material is either given orally or placed in the rumen via cannula. Blood levels of metabolites released from transported tablets are monitored over the time. An increase of the contents of proteins and amino acids in blood indicates that the delivery system is at least partially stable in the rumen and releases the active ingredient postruminally (Papas et al. 1984a, 1984b).

Materials and Methods

Vegetable protein concentrate HP 300 was chosen as the main active ingredient; the second adjuvant active ingredient was methionine (both Hamlet Protein a.s., DK). Since it is impossible to compress proteins directly and without any excipients, wet granulation method and five binding agents were used to prepare the granulate. Sucrose (Jan Kulich, CZ), gelatine (Ardea Pharma, CZ), and wheat starch (Tamda, CZ) were selected as inexpensive binders of natural origin and used at a minimal concentration in order to keep the protein content as high as possible. Povidon (Kollidon® 30, BASF, D) as a highly efficient synthetic binder and ethylcellulose (Sigma Aldrich, USA) as a semisynthetic binder, insoluble in water, were selected to compare the granulate and tablet core characteristics. Ferric oxide (Pancreac Quimica SA, E) was used as an adjuvant increasing the core specific gravity. For granulation (Stephan UMC 5, D), sucrose was used as 64% solution in water, wheat starch as 5% water solution, gelatine and povidone as 3% water solutions, and ethylcellulose as 3% solution in ethanol 95%. Wet granulate was sieved through the sieve with 0.8 mm apertures (Retsch®, D) and dried at 40 °C for 2 - 4 h in a ventilated oven (Horo 048B, D). The dry granulate was sieved through the sieve with 1.0 mm apertures to remove secondary agglomerates, and mixed with colloidal silicon dioxide Aerosil® 200 (Degussa, D) in order to improve the flow properties of granulates as well as the hardness and friability of tablet cores. Five samples of tablet cores of a lenticular shape and the size of 5 mm in diameter were compressed using an eccentric tablet machine (Korsch EK 0, D) and compression force of 18.5 - 19.0 kN.

Important core characteristics: hardness (Tablet Hardness Tester C50, Engineering Systems, GB), friability (Friabilator Roche, type Tar 10, Erweka GmbH, D), and disintegration (Disintegration Tester, Erweka type ZT 501, Erweka GmbH, D) were determined according to PhB and PhEur methods. The tablet specific gravity was measured in a glass pycnometer using a liquid not dissolving the samples (ether for the uncoated tablets, water for the coated tablets).

The tablets of suitable mechanical properties were coated in a fluid bed coater Wurster (type M-100, Medipo, CZ). Several substances individually or as mixtures were tested for the coatings protecting the cores: Surelease® - aqueous dispersion of ethylcellulose (Colorcon, GB); ethylcellulose, pH sensitive polymer poly-(2-vinylpyridine-co-styren) (both Sigma Aldrich, USA) and stearic acid (Jan Kulich, CZ) in different ratios. Surelease® was diluted with purified water in the ratio 3 : 2 (sample 1); ethylcellulose and stearic acid were dissolved in ethanol 96% (samples 2 - 4); poly-(2-vinylpyridine-co-styren), stearic acid and ethylcellulose were used as solutions in the mixture of ethanol 96% and 1,2-dichlorethane (Pliva-Lachema, CZ) in the ratio 1 : 1 (samples 5 - 14).

The weight of 20 uncoated and 20 coated tablets of each sample was determined three times; a weight increase of the coated tablets (practical weight increase) was calculated and expressed in %. The effectiveness of the tablet coating was proved *in vitro*, using the dissolution apparatus (Erweka type DT - D6, Erweka GmbH, D) and phosphate buffer of pH 6.4 at 39 °C, simulating the rumen, reticulum and omasum environment. The test was performed with 20 coated tablets for more than 24 h (Murphy et al. 1989). Each tablet coating was carefully studied under the microscope.

The specific gravity of PTS was measured in the interval of 3 days by glass pycnometer, using purified water as a liquid. The coated tablets were also evaluated in buffer of pH 2.0 at 39 °C, simulating the abomasum environment. 20 coated tablets were inserted into the buffer in the dissolution apparatus, the buffer with tablets were constantly mixed with a rotating paddle and the particles' dissolution/disintegration time (dissolution of the coating, disintegration of the core) was determined (Welch 1990; Murphy et al. 1989). The composition of uncoated tablets or their coatings is in Tables 1 and 3, respectively. The results are summarized in Tables 2 and 4 as average values from five measurements ± SD.

Results and Discussion

Firstly, the characteristics of protein cores with different binders (Table 1) were studied. Gelatine as a binder (sample II) was considered to give the best tablet cores of the lowest friability value, the fastest disintegration time, sufficient hardness, and specific gravity close to 1.2 g/cm^3 (Table 2). Cores containing sucrose (sample I) as a binder contained only 74.9% of HP 300, had the lowest specific gravity and the slowest disintegration time. Sample III containing wheat starch had the highest friability. Samples IV and V using synthetic and semisynthetic binders had comparable physical properties to sample II, however, the sample containing a natural protein binder was preferred.

Table 1. Composition of uncoated tablets

Substance [%]	Sample					
	I	II	III	IV	V	VI
HP 300	74.9	97.0	96.2	97.0	97.1	54.4
Methionine	-	-	-	-	-	22.8
Fe_2O_3	-	-	-	-	-	19.8
Sucrose	23.1	-	-	-	-	-
Gelatine	-	1.0	-	-	-	1.0
Wheat starch	-	-	1.8	-	-	-
Povidone (Kollidon® 30)	-	-	-	1.0	-	-
Ethylcellulose	-	-	-	-	0.9	-
Aerosil®	2.0	2.0	2.0	2.0	2.0	2.0

Secondly, in order to improve the nutrient value methionine was added, and to increase the specific gravity ferric oxide was included into the formulation (Table 1, sample VI). The optimum amount of ferric oxide was previously studied and reported (Sýkora et al. 2003). Core characteristics are given in Table 2. This change in formulation resulted in higher core hardness and the requested specific gravity (1.38 g/cm^3). The disintegration

Table 2. Physical characteristics of tablets

Sample	Hardness [N]	Friability [%]	Tablet specific gravity [g/cm^3]	Disintegration time [min]
I	70.5 ± 3.30	0.06 ± 0.04	1.172 ± 0.014	> 60
II	37.4 ± 2.90	0.00	1.194 ± 0.011	7.0 ± 1.5
III	55.7 ± 6.10	0.41 ± 0.17	1.213 ± 0.013	9.5 ± 1.0
IV	56.3 ± 2.90	0.09 ± 0.04	1.228 ± 0.022	8.5 ± 0.0
V	35.0 ± 2.10	0.08 ± 0.07	1.187 ± 0.011	9.0 ± 1.0
VI	66.5 ± 1.47	0.16 ± 0.05	1.380 ± 0.023	28.0 ± 4.4

Results are average values from five measurements ± SD

Table 3. Composition of tablet coatings

Sample	Ethylcellulose [%]	Stearic acid [%]	pH sensitive polymer [%]	Theoretical weight increase [%]	Practical weight increase [%]
1	100.0[a)]	-	-	25.0	23.2
2	11.6	88.4	-	10.0	3.5
3	50.0	50.0	-	15.0	3.5
4	25.0	75.0	-	10.0	11.0
5	6.7	83.3	10.0	15.0	9.9
6	6.7	83.3	10.0	17.0	10.2
7	6.7	83.3	10.0	25.0	19.2
8	6.7	79.3	14.0	15.0	9.0
9	6.7	79.3	14.0	17.0	9.8
10	6.7	76.7	16.7	13.0	9.1
11	6.7	76.7	16.7	17.0	12.6
12	6.7	74.8	18.5	17.0	9.6
13	6.7	73.0	20.3	13.0	9.0
14	6.7	71.3	22.0	17.0	9.8

[a)] ethylcellulose was used as an aqueous dispersion

time was 28 min which is still bellow the limit 30 - 60 min recommended for effective PTS. This composition was used for further coating procedure.

Ten different coating formulations in various amounts were examined; their compositions are given in Table 3. Their dissolution in buffer of pH value 6.4 at 39 °C was observed for more than 24 h, which is considered as the retention time in the rumen (Murphy et al. 1989). The obtained results are presented in Table 4. Insufficient protection of cores was found in sample 1 coated with aqueous dispersion of ethylcellulose and sample 2 with the coating composed of 88.4% stearic acid and 11.6% ethylcellulose. Coated tablets of both samples disintegrated within 1 h. When the ratio of ethylcellulose to stearic acid was changed and the amount of ethylcellulose was increased (samples 3 and 4), the protection of the protein core was improved. Except for samples 1 and 2, all the samples withstood 24 h stay in tested buffer.

As specific gravity is an important indicator of the PTS movement in the gastrointestinal tract of animals, specific gravity observation was performed for 3 days in the buffer of pH 6.4 at 39 °C. All the tested samples (3 - 14, Table 4) showed the appropriate specific gravity (1.282 - 1.456 g/cm^3) keeping approximately the same value within the experiment.

The fast disintegration of the coated tablets in the acidic media, in our experiment simulated with a diluted solution of hydrochloric acid of pH 1.2, is demanded for

Table 4. Disintegration time and specific gravity changes of coated tablets

Sample	Disintegration time at pH 6.4 [hour]	Specific gravity[b] [g/cm^3] 0 day	1 day	2 day	3 day	Disintegration time[c] at pH 1.2 [min]
1	<1	-	-	-	-	-
2	<1	-	-	-	-	-
3	>24	1.334 ± 0.022	1.338 ± 0.051	1.342 ± 0.011	1.325 ± 0.012	insoluble
4	>24	1.436 ± 0.034	1.423 ± 0.009	1.414 ± 0.050	1.380 ± 0.023	insoluble
5	>24	1.442 ± 0.012	1.436 ± 0.008	1.432 ± 0.042	1.424 ± 0.024	27.7 ± 1.51
6	>24	1.423 ± 0.015	1.416 ± 0.051	1.409 ± 0.028	1.398 ± 0.022	36.7 ± 3.97
7	>24	1.328 ± 0.025	1.326 ± 0.007	1.319 ± 0.023	1.314 ± 0.009	insoluble
8	>24	1.362 ± 0.013	1.371 ± 0.053	1.356 ± 0.045	1.354 ± 0.033	22.0 ± 0.62
9	>24	1.414 ± 0.014	1.409 ± 0.006	1.405 ± 0.032	1.401 ± 0.045	31.7 ± 2.78
10	>24	1.415 ± 0.020	1.422 ± 0.008	1.405 ± 0.009	1.403 ± 0.035	27.3 ± 0.60
11	>24	1.450 ± 0.021	1.449 ± 0.009	1.443 ± 0.032	1.445 ± 0.064	31.3 ± 1.15
12	>24	1.315 ± 0.022	1.308 ± 0.023	1.296 ± 0.052	1.282 ± 0.019	25.0 ± 0.35
13	>24	1.451 ± 0.018	1.456 ± 0.034	1.442 ± 0.043	1.437 ± 0.018	23.3 ± 1.25
14	>24	1.453 ± 0.009	1.449 ± 0.031	1.441 ± 0.015	1.379 ± 0.032	15.7 ± 1.18

[b)c)] Results are average values from five measurements ± SD

the fast release of active ingredients in the abomasum and small intestine and their following absorption. Therefore the behaviour of the prepared coated samples was observed in these conditions (Table 4). Samples 3 and 4 that did not contain the pH sensitive polymer were insoluble also in the medium of diluted HCl. Sample 7 with the highest amount of the coating remained also undissolved within the 60 minutes limit. All the other samples disintegrated in 15.7 to 36.7 minutes. From the formulation point of view it can be noticed that with the increasing amount of coating, its dissolution decreases, e.g. samples 5, 6 and 7, samples 8 and 9, samples 10 and 11; and with the increasing concentration of the pH sensitive polymer is their dissolution faster, in samples 11, 12, 13 and 14 (Tables 3 and 4).

Nine samples of coated protein tablets exhibiting requested characteristics *in vitro* were prepared. From the obtained results we can conclude that the presence of the pH sensitive polymer poly-(2-vinylpyridine-co-styren) at least in 10% concentration of the coating, and the coating amount of 9.0 - 12.6% per tablet are necessary to ensure the appropriate PTS properties.

Systém pro postruminální transport aminokyselin a proteinů u skotu

Cílem experimentu bylo vyvinout efektivní postruminální transportní systém (PTS) s vysokým obsahem rostlinných proteinů a aminokyselin. PTS slouží k doručení nutrientů do slezu a tenkého střeva dojnic za účelem zvýšení dojivosti. Přímé přidání proteinů a aminokyselin do potravy není praktické, protože aktivní látky se rozloží v bachoru působením mikroorganismů a nedojde k jejich absorpci v tenkém střevě. Postruminální transportní systém má při aplikaci zvířeti několik výhod. Může se přímo aplikovat s potravou, je levný, zlepšuje výživu a zdravotní stav zvířat. PTS se skládá z jádra (peleta, malá tableta) a obalu, který chrání jádro v prostředí bachoru a umožňuje uvolnění obsahu jádra ve slezu a tenkém střevě.

Čočkovité tablety - jádra se připravila metodou vlhké granulace a lisováním. Jakostní parametry tablet (hmotnostní stejnoměrnost, pevnost, oděr, rozpadavost) se hodnotily lékopisnými metodami, hustota se stanovila s použitím skleněného pyknometru. Jádra se následně obalovala několika typy obalů - ethylcelulosou, kyselinou stearovou a pH

sensitivním polymerem poly-(2-vinylpyridine-co-styren) jednotlivě nebo v kombinaci při různých poměrech.

Vyrobilo se devět vzorků obalených proteinových tablet vhodných vlastností stanovených metodami *in vitro*. Přítomnost pH senzitivního polymeru přinejmenším v 10 % koncentraci v obalu a obal v množství 9,0 - 12,6 % na tabletu byly nutnými podmínkami k zajištění požadovaných vlastností PTS.

Acknowledgement

This experimental study was supported by the Grant Agency of the Czech Republic, project No. 523/02/0164.

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