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In vitro Evaluation of Three Sources of non Protein Nitrogen for Prolonged Release in the Rumen

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Abstract: The objective of this study was the development and the evaluation of press-coated tablets for the modified release of nonprotein nitrogen in the rumen based on: urea, ammonium phosphate monobasic and ammonium phosphate dibasic, in order to be used as a protein supplement in ruminants. The release profiles, showed that tablets with urea had a better control of release although were faster than the other sources. The 80% of total urea was released in 31 h whereas, ammonium phosphate monobasic and dibasic released only 27% at the same time in the dissolutor with distilled water. Testing the press-coated tablets with ruminal fluid presented similar performance, with a half time of release of 26.45 h urea, 41.25 h ammonium phosphate monobasic and 25.02 ammonium phosphate dibasic. The press-coated tablets are potential technology to provide nonprotein nitrogen for prolonged time to ruminal microbes and the ruminant.

Key words: Non protein nitrogen sources, urea, ammonium phosphate monobasic, ammonium phosphate dibasic, press-coated tablets, modified release, ruminants

INTRODUCTION

The nonprotein nitrogen (NPN) compounds have been used satisfactorily as a protein substitute in the feeding of ruminants; however, they have the characteristic to be rapidly degraded in the rumen (Ørskov, 1988). Therefore reducing the rate of ammonia release would be an alternative to improve the efficiency of N utilization in the rumen (Galo *et al*., 2003). The most common NPN used is the urea, but there are other compounds such as diammonic phosphate and ammonia polyphosphate, which also could provide phosphorous as a nutrient to the animal (Kolb, 1971).

Forage diets are usually deficient in N, besides, the ruminal pH and NH_3 pKa, allows fast ammonia absorption under those dietary conditions (Haresign and Cole, 1988), therefore, the use of sustained release of N could be an alternative to improve the use of N in the rumen in forage diets.

A press-coated tablets could be an alterative to develop N of sources for slow release in the rumen, therefore the objective of this experiment was to design a inert matrix with ethilcellulose (Ethocel® std 100 premium FP) as a polymer with different sources of NPN (urea, ammonium phosphate monobasic and ammonium phosphate dibasic) and to evaluate the ammonia release *in vitro*, with and without ruminal microbes.

MATERIALS AND METHODS

Elaboration of press-coated tablets: The press-coated tablets were made in two steps; first, the cores were manually elaborated with barium sulphate (94%), carnauba wax (5%) and magnesium stearate (1%) using U.S.A. standard sieves No 30. Then, were weighed in a precision balance (Mettler Toledo model AL204) and mixed manually by 10 min.

The cores were elaborated by direct compression in a rotary tablet instrument (Riva piccola B/10 model), resulting a sinker with a mean weight of 703 mg, 7 mm of diameter and densities above 3 g mL^{-1}.

In the second step, the matrix systems were elaborated with Ethocel® std 100 premium FP, as inert polymer to form the porous network where the NPN sources were dispersed; designed to be evaluated in the rumen.

In order to select the particle size of the 3 NPN sources was necessary to grind the powder in a helix

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crusher machine (Krups Tipo 203) then, the powder was passed through of normalized sieves ASTM E-11 with several screens (meshes 60, 70, 80, 100, 120, 140) with an electric sieve (Rotap RX-29) with continuous vibration during 15 min. Obtaining a granular fraction between 125 and 177 μm for the ammonium phosphates and lower than 180 μm for the urea. The Ethocel® std 100 premium FP was used directly from the supplier Colorcon de México, S de R.L. de C.V. with a particle size of 53-63 μm. Later the powder was weighted in precision balance (Mettler Toledo mod. AL204) to obtain the binary mixtures with different NPN sources/polymer in a proportion of 30/70%; the ingredients were mixed during 15 min in a "v" mixer with capacity of 1 L.

The press-coated tablets were elaborated by direct compression in a hydraulic press (Carver, model C), placing the cores in the center of the matrix powders, obtaining cylindrical and flat tablets, with a mean of 13 mm of diameter and 2.23 g of weight. One tablet was elaborated each time, for which was required the individual weight of each powder mixture corresponding to a tablet. During its compression, the maximum forces applied and accepted for each powder mixtures, were 1500 psi for urea and 1250 psi for both ammonium phosphates.

Quality controls of tablets

Resistance to the fracture: To verify the resistance to pressure, 6 final tablets from each batch, were tested and subjected with a diametrical force with a tablet hardness tester (model vankel 2000), which determines the force in kp required to produce the rupture of tablets.

Friability: Resistance was also tested with a friabilator (Elecsa mod FE30), constituted by a plastic disc carried in its interior, in which three tablets with a weight equivalent of 6 g were placed and they were put under rotation by 4 min at 25 rpm. At the end of the assays, the weight loss in tablets was recorded (Vila-Jato, 2001).

***In vitro* evaluation of press coated tablets**

Release assays: The release assays of the tablets were conducted *in vitro* in a dissolutor (Erweka), using the apparatus 2 of dissolution (FEUM, 2000) during 31 h at 50 rpm in 700 mL of distilled water at 39±2°C. Each assay was realized by triplicate.

Quantification of drugs: The ammonium phosphate was determined with a conductivimeter (Orion 150) connected to a computer, recording conductivity data and temperature every 5 min. Urea was measured by the technique of Chaney and Marbach (1962); which is based on the hydrolysis with urease producing ammonia and CO_2. Immediately the ammonia reacts with phenol-hypochlorite using sodium nitroprussiate like catalyst. The blue color formed (indophenols) was quantified in a spectrophotometer Cary 100, at 560 nm.

Profiles and mechanism of release: Release profiles were obtained plotting the amount nitrogen released versus time.

In order to define the transport mechanisms and type of release, three kinetic models were applied in which the data corresponding to initial period of adjustment and the time of exhaustion were not considered (<5 % and >70%) (Melgoza *et al*., 2007):

$$\text{Zero order model } Q = kt + a$$
$$\text{Higuchi Model (1963): } Q = kt^{1/2} + a$$
$$\text{Korsmeyer Model (1983): } Q = kt^{n} + a$$

Evaluation of press-coated tablets with ruminal microbes

Press-coated tablets *in vitro* ammonia N release: Ammonia N release with ruminal microbes, was evaluated after the incubation of the tablets with the technique from Tilley and Terry (1963), using the artificial McDougall (1948) at pH of 6.8 and inoculum source ruminal fluid collected from a ruminally cannulated sheep fed with a alfalfa: concentrate diet (50:50).

In nalgene tubes by triplicate, 50 mL of the mixed saliva-inoculum (relation of 1:4 v/v) were added to a 1 tablet of NPN sources, with blanks without tablets. Fermentation was stopped at 12, 24, 36, 48, 60 and 72 h.

The technique of McCullough (1967) was used to quantify the N-NH_3 release; 2 mL of fluid were centrifuged to 3000 g ×10 min; the metaphosphoric acid was added (ratio 1: 4) to precipitate proteins, after 4 h in refrigeration, sample was centrifuged again (3000 g ×25 min). A 20 μL from the supernatant were placed in tubes adding 1 ml of phenol and 1 mL of sodium hypochlorite for the reaction, using a spectrophotometer Cary 100 at 630 nm. The results were analyzed by regression according to a first order kinetic model, estimating the half time and the proportional rate of release of the NNP source (Rojo *et al*., 2005).

RESULTS AND DISCUSSION

Quality controls of tablets: The friability results were satisfactory because the weight loss was lower than the recommended value of 0.8% in all the tablets, with a mean of 0.24%. Regarding to the fracture, the obtained values of 50 kp, indicated that the tablets will not suffer deterioration in their structure and may stay intact to diverse manipulation conditions and movements inside the rumen without damage.

Profiles and mechanism of release: The solubility is an inherent physicochemical propriety to each source tested, that influences its rate of release and this is reflected in the profiles observed in Fig. 1. The urea is released more quickly from the matrix tablets, releasing more than 50% during the first 12 h in contrast to ammonium phosphate monobasic and dibasic, which at the same time did not reach the 20%. At the end of the experiment (31 h) the urea reached 80% and the phosphates only the 27%.

The results obtained from the models, are presented in Table 1. The model of zero order did not show a good fit with a low r^2. The Higuchi model (squared root of time) shows a better adjustment in the three types of sources, indicating, that the mechanism of release is diffusional.

The analysis with Korsmeyer model confirms that mechanism of transport of urea from matrix tablets is fickian (n = 0.5), reason why it is observed a fast initial release, followed by a reduction with the time. The mechanism of transport for the ammonium salts assumes a condition release known as super case II (n<0.5), which involves an initial fast release but slower than the fickian release, which also is reduced with the time (Fig. 1) (Bernad *et al*., 2003).

Ammonia N concentration *in vitro* with ruminal microbes: In Table 2 are shown the results of ammonia N release, showing a higher degradation in the urea tablets in all incubation times, similar performance to that observed in the dissolution (Fig. 1). Regarding to the ammonium phosphates, presented similar ammonia release in most of the incubation times. Even when there were numerically and biologically differences in half time and rate of ammonia release, differences were not statistically detected among sources.

Studies whit a commercial coated urea product has demonstrated that can be reduced the ammonia levels in the rumen (García-González *et al*., 2007) but in fermenters did not affect ammonia concentration (Harrison *et al*., 2007a). Evaluation with producing animal are scarce; administration in dairy cattle of a slowly NPN source did not improve the milk production (Galo *et al*., 2003).

One evaluation with dissolutor indicated that the N from Optigen was released in 100% at 6 h (Melgoza *et al*., 2007) and that may be the reason why some studies with ruminal microbes only reports ammonia N up to 8 h incubations (Garcia-Gonzalez *et al*., 2007). The tablets designed here have the potential to sustain ammonia release for longer period of time than Optigen, however only *in vivo* studies will show if the polymer coating may resist damage of during feed mixing or transport (Galo *et al*., 2003) or the rumen movements.

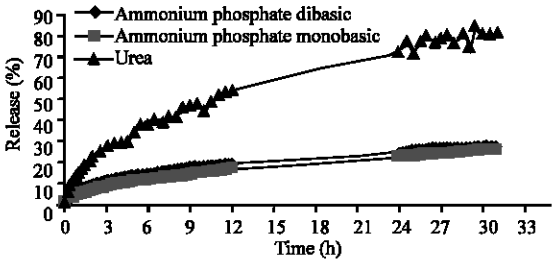

Fig. 1: Percentage of nitrogen release from tablets with 30% of drug

Table 1: Models kinetics of release

NNP sources	Zero orden model		Higuchi model		Korsmeyer model		
30%	K_0 (%t^{-1})	r^2	b (%$t^{-1/2}$)	r^2	K (%t^n)	N	r^2
Urea 30%	0.0503	0.909	1.940	0.993	2.081	0.490	0.993
$NH_4H_2PO_4$ 30%	0.01	0.975	0.571	0.999	0.771	0.465	0.999
$(NH_4)_2HPO_4$ 30%	0.0098	0.951	0.554	0.995	1.416	0.393	0.998

t = minutes

Table 2: Ammonia N release *in vitro* (mg/dl) with different nitrogen sources in the tablets, rate of release and half time according to a first order kinetic model

Time h	Urea	$(NH_4)_2HPO_4$	$NH_4H_2PO_4$	C.V.
12	54.32^a	19.03^b	20.03^b	22.77
24	152.79^a	40.72^b	31.75^b	12.64
36	226.98^a	52.31^b	38.76^b	11.43
48	241.46^a	65.63^b	40.683^c	16.10
60	304.36^a	82.05^b	49.37^b	19.14
72	321.09^a	122.61^b	62.23^c	16.88
Kd %/h	2.62	2.77	1.68	
$T_{1/2}$ h	26.45	25.02	41.25	

^{a,b,c} Means within column with the same literal are different (p<0.05). C.V. Coefficient of variation, Kd Rate of degradation. $T_{1/2}$ Half time life

The comparison of Optigen with urea by Garcia-Gonzalez *et al*. (2007) with ruminal microbes presents only ammonia N results at 2 and 4 h, which do not allow the evaluation for longer times with ruminal microbes. Other studies have been oriented to the protein substitution with Optigen (Harrison *et al*., 2007a; Wahrmund and Hersom, 2007) and there is lack of information on ruminal ammonia concentration to arrive a conclusion on its sustainable release in the rumen. Nevertheless, there is an agreement, that Optigen may substitute up to 9% of the proteins without negative effects in rumen fermentation or microbial protein synthesis (Harrison *et al*., 2007b).

CONCLUSION

The results of friability and resistance to the fracture indicate that the tablets may resist the conditions of movements in the rumen to their action. The ammonia

in vitro releases show that the design of matrix tablets was able to release slowly the nitrogen source, releasing the active principle during more than 30 h.

Besides the urea, this study presents the alternative of inclusion of ammonium phosphate monobasic and dibasic, both the sources of NPN and phosphorous, witch present a reduced risk of toxicity, however the first is economically more feasible and could be preferred to develop new sources of NPN for ruminant production.

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