EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C2 - Management of scientific committees; scientific co-operation and networks

REPORT OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON THE USE OF A HMBI (ISOPROPYL ESTER OF THE HYDROXYLATED ANALOGUE OF METHIONINE)

(Adopted on 25 April 2003)

1. BACKGROUND

A request for authorising isopropyl ester of the hydroxylated analogue of methionine (HMBi) has been submitted on 22 February 2002 by Aventis Animal Nutrition (now ADISSEO) following the procedure of Directive 82/471/EEC on certain products used in animal nutrition. This product is to be used in dairy cows to balance rations deficient in methionine. The estimated daily ration is 25-30g/day.

The request asks for the inclusion of this product in point 4.1 of the Annex of Directive 82/471/EEC.

Name of product group	Name of product.	Designation of nutritive principle or identity of micro-organism	Culture substrate (identifications, if any)	Composition characteristics of the product	Animal species
4.1 Analogue of methionine	4.1.3 Isopropyl ester of the hydroxylated analogue of methionine (HMBi)	CH_3-S-CH_2-C(OH)H-COO-CH-$(CH_3)_2$	-	Monomer esters: 90% minimum	Dairy cows

2. TERMS OF REFERENCE

The Scientific Committee for Animal Nutrition (SCAN) is requested to give an opinion on the following questions:

(1) Does hydroxylated analogue of methionine (HMBi) have nutritional value?

(2) Does the use of hydroxylated analogue of methionine (HMBi) not impair the characteristics of animal products?

(3) Is the use of hydroxylated analogue of methionine (HMBi) safe to dairy cows?

(4) Do the toxicology studies allow to conclude that the proposed use does not present risks for the consumer, for the user, for the workers?

(5) Can the use of hydroxylated analogue of methionine (HMBi) be prejudicial to the environment?

(6) Does the manufacturing process of hydroxylated analogue of methionine (HMBi) have detrimental effect on human or animal health or on the environment?

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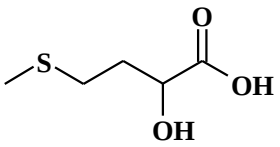
3. INTRODUCTION

HMBi is the provisional technical name of a liquid product consisting of over 90% by weight of the isopropyl ester of 2-hydroxy-4-methylthiobutanoic acid (HMB). HMB (I) is referred to by the manufacturer as a hydroxylated analogue of methionine (II) because, when fed to livestock, it can serve as a precursor of methionine.

(I)



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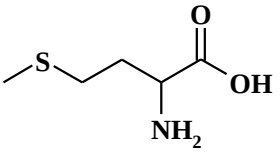

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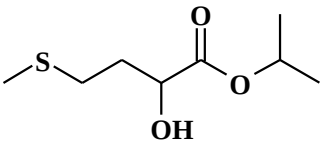


In poultry this process of conversion for both the D and L forms of HMB has been shown to occur primarily in the liver and to a lesser extent in kidneys. Conversion is *via* oxidation to an intermediate 2-keto-4-methylthiobutanoic acid which in turn yields L-methionine by transamination (Saunderson, 1985; Dupuis *et al.*, 1989). In ruminants however, infusion of HMB into the mesenteric vein of lambs suggested that HMB is more extensively metabolised by tissues other than the liver where it is able to meet directly tissue requirements (Wester *et al.*, 2000a,b).

Methionine and lysine are frequently identified as limiting amino acids for milk protein production in high yielding dairy cows. The challenge in ration formulation for dairy cows is to supply both amino acids at their apparent optimum concentrations. One approach is to introduce a rumen-protected source of methionine or precursor of methionine.

HMB is an approved feed ingredient in the European Union for use with all animal species. However, in practice, it is extensively used as a substitute for methionine only in diets for poultry and, to a lesser extent, pigs. Although HMB has been shown to exert some beneficial effects on rumen fermentation including increased cellulolytic activity and fibre degradation, and increased acetate production (Patterson and Kung, 1988, Robert *et al.*, 1998; Sloan *et al.*, 2000.), it is extensively degraded/utilised by the rumen microflora. Consequently, any productive benefits are the result of ruminal rather than post-ruminal mediated effects and are seen primarily in terms of milk yield and milk fat content.

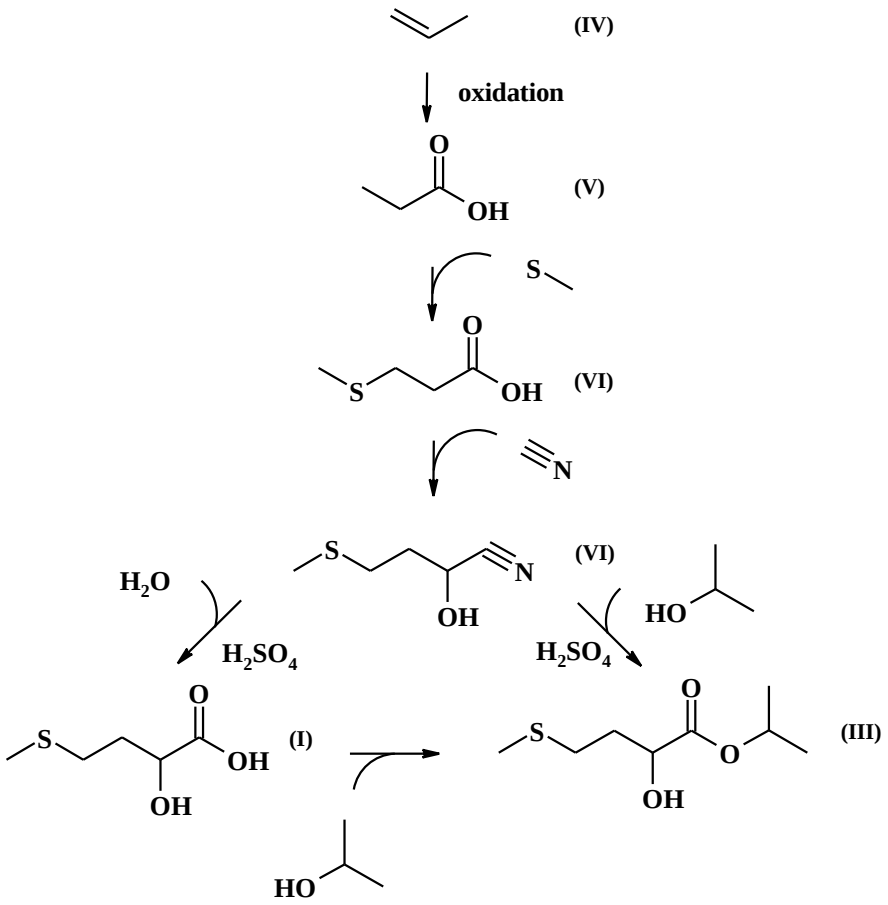
The isopropyl derivative of HMB (III) is intended to serve the same function as HMB, but the ester group is added to provide additional protection against microbial degradation in the rumen.

4. MANUFACTURE AND PROPERTIES OF HMBI

4.1. Manufacture

HMBi is a synthetic product prepared from the raw chemicals, propylene, methylmercaptan, cyanide, sulfuric acid and 2-propanol. Two routes of manufacture are described differing only in the final steps of the synthesis (Figure 1).

Figure 1. Steps in the manufacture of the isopropyl ester of 2-hydroxy-4-methylthiobutanoic acid (III)



In the common stages, propylene (IV) is first oxidised to acrolein (V) and then condensed with methylmercaptan to give methyl mercaptopropionaldehyde (VI). Methyl mercaptopropionaldehyde is then reacted with cyanide to give 2-hydroxy-4-(methylthio)butanenitrile (VII). In scheme A the nitrile is converted directly to the final product (III) by reacting with sulfuric acid and 2-propanol, while in scheme B acidification of the nitrile produces HMB (I) from which the ester is then produced. Both schemes make use of the same

reagents and both schemes would be expected to contain the same impurities/side products. However, it appears from an answer to a question from the Member States that HMBi is currently manufactured by esterification of HMB.

The resulting material is a colourless to slightly brown liquid with a density of 1.074 compared to water and a logP of 1.2 (octanol-water). It is soluble in water (25.1g/L at 30^{o}C) giving a pH of about 3.6 (10g/L water).

4.2. Purity and impurities

Analysis of production batches has shown that the Company can support a specification of a minimum HMBi content of 90% by weight (average of 12 batches, 92.2%, range 90.5-93.5%).

The major impurities present in the final preparation are HMB and dimers of HMB (DHMB) and HMBi (DHMBi). Limits set by the manufacture for these impurities are for HMB + DHMB, <2.5% by weight and for DHMBi, <5%. Suitable hplc-based methods for the analysis of these compounds are available. A number of other impurities have been recognised but not all have been fully identified. Some are residual amounts of the raw materials used in manufacture (2-propanol, methylmercaptan, water), while others appear to products of minor side reactions, in some cases involving loss of the thio group. The maximum acceptable concentration of these other impurities is set at <2.5 % by weight, determined by difference.

The final product is routinely monitored for heavy metal (Pb and As) and microbiological contamination (total aerobes, coliforms, *E. coli*, *Salmonella* spp., yeasts and other fungi).

4.3. Stability

Extended studies has shown the commercial product stored in an unopened container is stable for periods of at least one year at ambient temperatures (loss of <1% after 484 days) and for periods of at least 246 days at elevated temperatures (loss of 1.3% at 40^{o}C). The product can be recovered virtually intact after pelleting in both a protein-rich and energy-rich matrix. It is stable for periods of at least three months when incorporated into a mineral premix or added to a solid support (sepiolite) and for at least two months when added to soybean meal or incorporated into complete feeds.

5. METABOLISM OF HMBI IN CATTLE

5.1. Uptake and conversion to methionine

Several studies in which HMBi or protected methionine (in amounts calculated to deliver 50g methionine) were rapidly introduced directly into the rumen of non-gestating cows have shown that when HMBi was used there was a very rapid detection of HMB in the peripheral blood. Detection was possible after a few minutes and reached a peak plasma concentration within two hours. This was followed by a decline in HMB concentration and a concomitant increase in plasma methionine. Methionine in turn reached a

peak concentration after about four hours and thereafter declined. This was in marked contrast to protected methionine, which although detectable in blood soon after infusion into the rumen, took more than 24 hours to reach its peak concentration. The implication of these studies is that HMBi is predominately absorbed via the rumen wall after breakdown to HMB. This differs from protected methionine, which largely escapes the rumen intact and passes to the small intestine before absorption.

In one study, a product containing 39% dimers (see 4.2) by weight was compared to a second HMBi product essentially free of dimeric material. This demonstrated that essentially no methionine was produced from the dimeric material; any appearance of methionine being directly proportional to the amount of the monomer present. Whether the inability of the dimers to act as methionine precursors is due to an absence of uptake and/or to the absence of cleavage of dimers back to the parent monomers in unknown.

Data from these rumen infusion experiments were used to calculate the relative "bioavailability" of methionine from HMBi - bioavailability being defined in this case as the proportion of HMBi converted to methionine and found in the blood after consumption. Calculation was by reference to the known bioavailibility of protected methionine (80%, Rulquin and Kowalczck, 2000). Two methods of calculation were used, a linear method which assumed that the blood response of the animal to dietary methionine was linear and a second method that involved feeding different amounts of protected methionine to develop a calibration curve (the curvilinear method). The results of four such studies are shown in Table 1.

Table 1. Calculated relative "bioavailability" of methionine derived from HMBi

Study	Bioavalability (%) of methionine from HMBi	
	Linear method	Curvilinear method
1	52	66
2	45	51
3	36	46
4 (dry preparation[1])	39	50

[1]Dry preparation, 30% HMBi and 70% sepiolite

5.2. Absorption, distribution and excretion studies

Tracer studies (Tables 2 and 3) have been made with HMBi labelled either at the carbonyl group (tracer for HMB) or the ester group (tracer for 2-propanol). However, it should be recognised that incorporation of label within tissues does not indicate incorporation of the parent compound or HMB. Since both HMB and 2-propanol behave as normal metabolic intermediaries, the expectation would be for part of the label to be found associated with most body tissues. Six lactating cows in total were used, three given [carbonyl-^{14}C]-HMBi and three given [isopropyl-^{14}C]-HMBi supplied in the form of gelatine capsules (10 g HMBi) given twice daily for seven consecutive days. One cow from each group was killed after 24 hours, a second pair at 72 hours and the remaining pair after 7 days.

Table 2. A summary of the distribution, excretion and recovery of radioactivity supplied in the form of [carbonyl-^{14}C]-HMBi.

Sample	Percentage of total dose supplied		
	24 hours	72 hours	168 hours
Urine	6.1	7.3	6.7
Faeces	2.5	3.2	3.0
Milk	8.1	12.0	16.1
Total excreted	16.7	22.5	25.8
Fat	0.6	0.6	0.5
Kidneys	0.3	0.2	0.1
Liver	2.1	1.8	1.4
Muscle	5.5	7.2	5.5
Rumen contents	0.3	0.1	0.1
Whole blood	trace	trace	trace
Total	25.5	32.4	33.4

Table 3. A summary of the distribution, excretion and recovery of radioactivity supplied in the form of [isopropyl-^{14}C]-HMBi.

Sample	Percentage of total dose supplied		
	24 hours	72 hours	168 hours
Urine	2	3.5	2.7
Faeces	1.6	1.7	1.9
Milk	11.7	14.6	15.1
Total excreted	16.5	19.8	19.7
Fat	1.9	1.2	0.7
Kidneys	0.1	Trace	0.2
Liver	0.3	0.5	0.4
Muscle	5.0	4.0	2.8
Rumen contents	0.2	Trace	Trace
Whole blood	0	0	0
Total	24.0	26.3	23.8

Low levels of radioactivity were found in fat muscle, rumen contents, whole blood and blood plasma for both treatment groups. The overall distribution of radioactivity within tissues was the same for both radiolabelled forms of HMBi but with a faster rate of depletion of radioactivity in the [isopropyl-^{14}C]-HMBi treated cows.

5.3. Metabolism in the rumen

From the studies made on the "relative bioavailability" of methionine from ingested HMBi, there are indications that approximately half is available to the animal and the remaining half metabolised by the rumen flora. In the rumen, de-esterification as a result of non-specific microbial esterase activity results in the release of HMB and isopropyl alcohol. *In vitro*, approximately half of the HMBi initially present disappears within 8 hours when incubated with a suspension of rumen micro-organisms and none is detectable after 48h.

Some of the 2-propanol released by the flora is oxidised to acetone by alcohol dehydrogenase, but this is a reversible reaction and some acetone so produced may be reduced back to the secondary alcohol. Since radioactivity studies (see 5.2) made with HMBi labelled on the isopropyl group showed that virtually no label was seen in faeces it is likely that any 2-propanol (or acetone) present is either absorbed through the rumen wall or lost by eructation.

5.4. Metabolism of 2-propanol by cattle

In studies in which HMBi was delivered directly to the rumen as a bolus the rapid appearance of HMB in the peripheral blood of cows was accompanied by the presence of 2-propanol and acetone. In animals given 69g HMBi, plasma concentration of 2-propanol increased from zero to a peak value of 2 mg/L (study 1) and 2.65 mg L (study 2) within one hour. In the same studies acetone concentrations rose from approximately 9 mg/L before HMBi addition, in one case reaching a peak of 23mg/L after two hours and in the second study a peak of 23 mg/L after six hours. After 24 hours plasma concentrations had fallen to approximately half of the peak values. However, while 2-propanol was found in studies in which HMBi was infused directly into the rumen, none was detected in the peripheral blood of lactating cows given HMBi/day with the feed.

6. NUTRITIONAL VALUE AND PRODUCT QUALITY

The product is intended to supplement the rations of dairy cattle when there is an imbalance in methionine content. The manufacturer anticipates that the level of incorporation would be dependent on the existing methionine content of the feed and level of milk production, but would not exceed 60g/cow/day. The proposed use of HMBi is not restricted and is not subject to a period of withdrawal.

6.1. Effect of HMBi supplementation on milk protein content

Four experiments were made in which dairy cows receiving diets well provided with available lysine but deficient in methionine were supplemented with HMBi. Three trials were made using a Latin square design with periods of two weeks for a total of eight weeks after peak lactation with animals fed mixed rations based on maize silage and soybean meal (two trials) or grass/maize silage and soybean meal (one trial). The soybean meal was used to deliver the supplement.

Trial 1. Holstein cows (16) after 17 weeks lactation (producing around 32 kg milk/day) were allocated to one of four treatment groups on the basis of milk fat/protein production measured during the previous two weeks. One group received no supplementation, the remaining three received protected methionine, the HMBi product or another experimental product disregarded here. Amounts of supplements used were calculated to supply 19g methionine/day. Although responses were small, there was a significant ($P<0.05$) increase in milk protein (casein) concentration of about 0.7g/kg milk in groups receiving HMBi or the protected methionine. A numerical increase in protein production was recorded for both treatments but this did not reach

significance. No other significant effects were seen on milk production parameters.

Trial 2. As in Trial 1, 16 Holstein cows, this time in the eighth week of lactation producing around 31.5 kg milk/day, were allocated to one of four treatment groups on the basis of milk fat/protein production measured during the previous two weeks. One group received no supplementation, the remaining three either protected methionine the HMBi product (calculated to supply 10.6g methionine/day), or another experimental product again disregarded here. Milk protein concentration increased significantly ($P<0.001$) by 1g/kg milk with HMBi and 0.7g/kg milk with the protected methionine. In the case of HMBi, this increase in milk protein concentration was accompanied by a significant rise in casein concentration ($P<0.01$). Protein production also increased significantly ($P<0.05$) with HMBi (32g/day) and the protected methionine (41g/day). Treatment with HMBi caused a small but significant reduction in the concentration of short chain fatty acids and 18:2 unsaturated fatty acid and an increase in C18:0 and C15:0 saturated fatty acids. It was suggested that these changes could have arisen through effects on ruminal fermentation patterns. The consequence of this change for product quality is unknown.

Trial 3. The third trial was a dose-response study made with forty multiparous Holstein cows assigned randomly to a balanced split block 5x5 Latin square design involving two replicates of four squares. Animals assigned to the first replicate had an average days in milk of 177 days and those assigned to the second replicate 104 days. Experimental periods were 14 d with the last seven days used for measurements. Each square consisted of five concentrations of a methionine source provided in conjunction with a methionine deficient basal diet. The methionine sources and the amounts used are shown in Table 4.

Table 4. Amount of each product fed (g/day/25kg DMI) to provide the methionine equivalents selected.

Source	Methionine equivalent (g/d per 25kg DMI)							
	0	10	15	20	25	30	35	45
Protected met.	0	13.3	20.0	26.7	33.3	-	-	-
HMB	0	-	17.0	22.7	28.4	34.1	-	-
HMBi	0		21.4	28.6	35.7	42.9	-	-
Combination of:								
1/3 HMB	0	-	5.7	-	9.5	-	13.3	17.0
2/3HMBi	0	-	14.3	-	23.8	-	33.3	42.9

Corrected percentage milk proteins for the five levels of protected methioninre were 2.99, 3.08, 3.15, 3.15 and 3.13 (quadratic effect, $P<0.01$), and for HMBi 3.05, 3.11, 3.16, 3.17 and 3.19 (linear effect, $P<0.001$). Milk yield was highest for HMBi delivering 15g/day methionine equivalent (45.8kg milk/day) and lowest for those animals receiving 25g/day methionine equivalent (43.4 kg milk/day). No effect was seen with HMB. Both protected methionine and

HMBi were effective in providing methionine for milk protein synthesis as evidenced by the increase in milk protein concentration.

Comparative responses to HMBi and protected methionine. In the above trials the data on milk protein response in animals supplemented with HMBi was compared to the response of animals provided with a rumen-protected source of methionine. Using the bioavailability figure for protected methionine (80%), the relative "bioavailability" of HMBi was variously estimated to range from 42% to slightly over 50%. These figures are in broad agreement with the approximately 50% value calculated from plasma concentrations and shown in table 1.

Trial 4. This trial, involving two groups of 18 lactating cows, one control and one given feed supplemented with HMBi (19g methionine equivalent/animal/day), was of longer duration. Cows were started on the trial two weeks post-partum when they were allocated to treatments and followed for 17 weeks of lactation. Milk production (approximately 29 kg/day), fat level and yields of fat and protein were not significantly affected by the treatment. Animals in the HMBi group had significantly higher protein ($P<0.01$) and lactose ($P<0.05$) concentrations than the control group. The protein response consisted of reduced NPN and increased nitrogen in the form of casein in the milk of cows receiving the supplemented feed. The absence of any overall effect on protein yield was ascribed, at least partially, to the differences in milk production noted between groups prior to the start of the trial.

6.2. Milk quality

Trace amounts of 2-propanol around the limit of detection (0.5mg/L) and a small increase in acetone content was seen in milk samples from trials in which HMBi was supplied with the feed (Table 5). No HMBi or HMB was detected in any milk sample examined.

Table 5. Average values for 2-propanol and acetone concentrations (mg/kg milk) in milk from cows fed HMBi

Constituent	Trial 1		Trial 2	
	Control	HMBi	Control	HMBi
2-Propanol	0.5	0.5	0.5	0.6
Acetone	3.2	9.4*	1.7	2.8*

*Differs significantly from control ($P<0.05$)

No sensory studies with panels were reported.

6.3. Conclusions

- A proportion of the HMBi ingested with feed (possibly around 50%) is available (as HMB) to dairy cattle and can serve as a precursor of methionine. In circumstances when methionine is deficient in the diet this can be of positive nutritional value.

- The dimers of HMBi and HMB present in the final product appear not to serve as precursors of methionine. Their metabolic fate is otherwise unknown.

- As no sensory studies were reported it is not possible to conclusively conclude on the consumer acceptability of milk produced from animals fed HMBi. However, the concentration of acetone is increased in the milk of animals fed HMBi. Given the volatility of acetone, this is unlikely to impair the sensory characteristics of treated (pasteurised) milk.

7. SAFETY OF HMBI

7.1. Safety for dairy cattle

7.1.1. Tolerance study.

A tolerance study was designed to determine the effect of accidental overdosing with the product. Holstein dairy cows (12 total) between the 2nd and 6th month of lactation were allocated to one of four treatment groups. The first group acted as a control group while animals in the remaining three groups received HMBi twice daily with concentrate. HMBi was given at a total of 75g, 150g or 300g/day/cow for two weeks, which approximated to x2.5, x5 or x10 the nutritional dose level (assumed by the manufacturer to be 30g/day/cow).

No problems of feed intake were recorded for the control group or for the three animals receiving the lowest dose of HMBi. There was feed refusal amongst the two groups receiving the higher doses of HMBi with approximately 6 of 8 kg concentrate consumed by cows receiving x5 the nutritional dose but only approximately 1.5kg total concentrate consumption in cows receiving ten times the nutritional dose. Since refusals were more than 50% in this group, their study was discontinued after day 6 and they were returned to a control feed free of HMBi. For the remaining animals on trial, the biological parameters measured (haematology and blood chemistry on days 1, 6 and 13) and milk yield and composition, were not affected by the administration of HMBi.

7.1.2. Effect of acetone

In ruminants, most of the acetone derived from 2-propanol arises from the action of alcohol dehydrogenase in the liver (Fuller and Marucci, 1972), although other pathways may play a minor role. Acetone is a normal metabolite of lipid metabolism in mammals, derived from the β-oxidation of fatty acids. In high yielding dairy cows at the start of lactation there can be an accumulation of so-called ketonic bodies (which include acetone) associated with excess lipid metabolism with potentially severe effects for the animal. Although there was an increase in the acetone produced, the levels of acetone found in animals given HMBi in various trials rarely

exceeded 10mg/L and were always well within the normal range expected for dairy cows. They were well below the concentrations thought to give rise to sub-clinical (>50mg/L) or clinical (>200mg/L) symptoms (Bruss and Lopez, 2000).

7.1.3. Conclusion.

SCAN recognises that tolerance studies may be difficult to make when the test substance is essentially a nutrient. The responses observed in this case may simply be due to amino-acid imbalance, which often results in a reduced feed intake. In this case the target species appears protected from any adverse effects of over-dosing by feed refusal. However it should be noted that the actual reasons for this refusal are unknown, but are evident at application rates only 4-5 time the recommended nutritional dose after less than two weeks exposure.

7.2. Toxicological studies

A number of safety studies were made to determine the extent of any potential hazards that might be posed by the product, including the 10% of impurities that may be present, notably the dimers of HMB and HMBi.

7.2.1. Acute oral and sub-chronic toxicity studies in rats.

A total of six rats (three male and three female) received a single dose of 2000 mg HMBi/kg body weight by gavage. No mortalities were observed during the 15 days of the experiment and no clinical problems were observed. From this it was concluded that the lethal dose for rats was greater than 2000 mg.

The 90-day sub-chronic toxicity study was preceded by a shorter 14-day study intended to define the dose range to be used in the longer study. Groups of Wistar rats (five male and five female/group) were fed the vehicle (5ml of 0.5% methylcellulose/day) or the vehicle containing 100, 300 or 1000mg HMBi/kg body weight. No mortalities were recorded and the only observation of possible clinical significance was an increase in salivation in 2/5 males on the lowest dose tested and in all animals given the higher dose. Food consumption and live weight gain was not affected by treatment and on autopsy no change was found in the absolute or relative weights of organs or their macroscopic appearance. Microscopic examination showed some vacuolisation of hepatocytes and an accumulation of hyaline deposits in the kidney, but this was restricted to males on the highest dose.

The longer study made use of the same protocol as the 14-day study, but involved twice the number of animals (ten Wistar rats/sex/group). Clinical signs were recorded on a daily basis throughout the study and feed intake and weight gain recorded at weekly intervals. Blood samples were taken during the week preceding autopsy and urine samples collected immediately preceding autopsy. All animals were

autopsied and selected organs weighed and fixed for subsequent microscopic examination.

As was noted during the shorter study, the forced gavage caused an increase in salivation in animals receiving > 100 mg HMBi/kg body weight/day. A total of five deaths were recorded, two as a result of the anaesthesia used to collect blood and three of unknown cause (one female in the 1000 mg group and two females in the 300 mg dose group). None of the five animals showed any treatment related macroscopic or microscopic changes indicating a cause of death.

Feed consumption and live weight gain of males receiving 1000 mg HMBi/kg body weight was significantly reduced in male rats in comparison with the control group. There were also small changes in haematological parameters (reductions in numbers of red corpuscles, haemoglobin and heamocrit values) and blood chemistry, and lower urine pH. Macroscopic and histological examination found a number of treatment-related effects, principally in the liver, spleen and kidney, but only in rats exposed to the highest dose. These effects included a change in liver pigmentation and, in the spleen, an increase in the accumulation of hemosiderine and extramedullary hematopoiesis. For the male rats only, the relative and absolute kidney weight was significantly higher than that of the control animals. This was attributed to the hyaline deposits in the tubulary cells observed in this study and the previous 14-day study.

This data would suggest that consumption of HMBi has no effect at doses up to 300 mg/kg body weight/day other than causing increased salivation.

7.2.2. Dermal toxicity.

Purified HMBi was applied to the skin of five males and five females rats in a single application of 2000 mg/kg body weight and the treated area covered for 24 hours. Animals were observed for a further 14 days and then autopsied. No adverse macroscopic or microscopic effects were detected.

7.2.3. Dermal and eye irritation.

HMBi (0.5 ml) was applied to the skin of each of three white NZ rabbits and covered by a patch for four hours. No harmful effects of the application were observed during the subsequent three days of observation. Instillation of HMBi (0.1 ml) into one of the conjunctival sacs of the eye of each of three white NZ rabbits also failed to show any sustained evidence of a reaction. As would be expected there was a small and transitory effect on the cornea and conjunctiva.

7.2.4. Dermal and respiratory senstisation.

The potential for individuals developing a delayed hypersensitivity to HMBi was examined using the guinea pig as the test subject. On the basis of the results obtained in a preliminary study five control and ten test animals were subject to the following sequential exposure to HMBi:

(1) Intradermal injection of 5% HMBi in peanut oil and/or adjuvant;

(2) Topical induction with HMBi alone;

(3) A triggering application of HMBi alone or a mix of equal amounts of HMBi and peanut oil.

Two animals showed a slight erythemic reaction 24 hours after the triggering application at the site of the weakest dose but not at the site of the higher dose. These results were considered artefacts of the experimental system. Only one animal showed a clear and positive response with well defined erythemia on both sites of the triggering application evident after both 24 and 48 hours. The response shown in only 1/10 animals represents only a limited hazard but is sufficient to warrant inclusion of advice to avoid contact with HMBi and to wash any area of contact.

As the product is a liquid with a low volatility, this reduces substantially the potential for respiratory exposure and sensitisation. In addition, compounds producing a respiratory sensitisation invariably produce a cutaneous response. Consequently, the results obtained for dermal sensitivity can be taken to reflect the potential for sensitisation *via* a respiratory route.

7.2.5. Mutagenicity.

HMBi was found not to induce mutations in a series of recognised in vitro assays:

- Ames test with five histidine-dependent strains of *Salmonella typhimurium* in the presence and absence of metabolic activation;
- A human peripheral blood lymphocyte chromosomal aberration assay (HMBi at 10mM in two independent experiments);
- A mouse lymphoma cell assay looking for mutations at the *tk* locus (Six doses of HMBi from 62.5 to 1930 µg/ml in the presence and absence of metabolic activation in two separate experiments).

As there were no positive results in these assays, there is no requirement for testing *in vivo*.

7.2.6. Conclusions

HMBi exhibits a relatively low acute and sub-chronic oral toxicity. It is non-toxic after dermal exposure and non-irritant for the skin and the eyes. A limited sensitization of the skin was observed. HMBi is not mutagenic.

7.3. Safety for consumers

A significant proportion (possibly 50%) of the dietary supply of HMBi is absorbed by a dairy cow and is completely metabolised, initially by de-esterification, yielding HMB and 2-propanol. The HMB produced converted to methionine to be used as any other source of methionine. Propanol also is metabolised initially by the same metabolic route used for ethanol. However, as a secondary alcohol, its affinity for alcohol dehydrogenase and its rate of oxidation is less than that observed for the primary alcohols. The acetone so produced may be excreted intact or further metabolised to carbon dioxide.

7.3.1. Exposure to acetone

Calculations made by the manufacturer based on the highest concentration of acetone detected in milk (16 mg/L) indicated that a 5 kg infant consuming 150/180 ml milk/kg body weight would consume 3.2 mg acetone/ kg body weight/day (total intake rounded to 1L/day for the calculation). This value was compared to the results of a study (reported in INRS, 1997) made with rats dosed with 1.8 ml acetone /kg body weight daily for four months. This equated to an intake of 1400 mg/kg body weight/day in the study. No mortalities were observed. The only adverse response reported was a small and reversible reduction in growth rate. Based on a supplementation of 30 g HMBi/day this represents a x400 margin of safety, or x200 if the maximum amount of HMBi envisaged by the manufacturer (60 g/day) is used.

7.3.2. Conclusion.

Human exposure to the products of HMBi would be almost entirely through the consumption of milk. HMBi itself is undetectable in plasma, milk or any other sample/tissue examined. Its immediate conversion product HMB is similarly undetectable in milk, the radioactivity observed (Table 4) largely deriving from its conversion to methionine and the incorporation of methionine into milk casein. Very low amounts of 2-propanol and its oxidation product are also present, but not in concentrations of toxicological concern. Acetone is a normal metabolite of mammals. Acute and chronic toxicity studies made in rats also point to a lack of toxicological concern for HMBi and its mammalian metabolites.

7.4. Safety for users

The potential for contact with the final product is considered by the manufacturer to be minimal. Workers could become exposed during the filling of transport containers at the site of manufacture by accidental splashing of HMBi in the eyes or onto skin. There is little likelihood of exposure through an inhalatory route as the product is a non-volatile liquid (vapour pressure 0.6 Pa at 25^{o}C). Other potential points of contacts could occur during delivery of HMBi to feed manufacturers, during incorporation into feed and when handling feed to which HMBi has been added. Most operations in the feed mill are closed with minimal/no worker exposure and so the most likely route of exposure is during cleaning of the spray systems used to apply the liquid product. On-farm, the level of incorporation into concentrates (<3% by weight) or complete feeds (<1%) would reduce any potential hazards for those handling treated material.

Exposure to HMBi is likely to be limited and sporadic. Based on the available data there is no evidence to conclude that the product would be toxic under these circumstances by either a dermal or oral route. The product is also a non-irritant. There is a remote possibility of a delayed sensitisation in those regularly exposed to the product, but this risk could be managed using the normal precautions to avoid contact when handling the product.

8. ENVIRONMENTAL IMPACT

HMBi fed to dairy cows is fully metabolised and the parent compound would not enter the wider environment under the proposed conditions of use. Of the likely metabolites of HMBi, HMB is already authorised as a feed ingredient and 2-propanol and acetone are found as natural products within the wider environment, produced by a wide variety of organisms. Nonetheless, the manufacturer made a number of ecotoxicological studies that support the conclusion that HMBi would have no impact on the environment under proposed conditions of use.

9. OVERALL CONCLUSIONS

A proportion of the HMBi ingested with feed (possibly around 50%) is available (as HMB) to dairy cattle and can serve as a precursor of methionine. In circumstances when methionine is deficient in the diet this can be of positive nutritional value.

As no sensory studies were reported it is not possible to conclusively conclude on the consumer acceptability of milk produced from animals fed HMBi. However, the concentration of acetone is increased in the milk of animals fed HMBi. Given the volatility of acetone, this is unlikely to impair the sensory characteristics of treated (pasteurised) milk.

The target species, the dairy cow, appears partially protected from adverse effects of over-dosing by feed refusal. The reason for this refusal is unknown but may be a product of the animal's response to an amino acid imbalance. No uptake of HMBi itself has been detected because of the rapid de-esterification that occurs.

Consequently, the cow is exposed only to HMB (produced by the same synthetic route as used for HMBi), which is already an authorised feed ingredient.

Worker exposure to HMBi is likely to be limited and sporadic and to occur predominately through splashes and spillage or through cleaning of equipment used to distribute the product. There is no evidence to conclude that the product would be toxic under these circumstances by either an oral or dermal route. The product is also a non-irritant. It is unlikely to be inhaled due to its low volatility. There is a remote possibility of a delayed sensitisation in those regularly exposed to the product but this risk could be readily managed using the normal precautions to avoid contact when handling the product. The risk for users of the product would be similar to that of workers, but exposure would not be to the pure product and so would be correspondingly lower.

Human exposure to the products of HMBi would be almost entirely through consumption of milk. HMBi and its immediate conversion product HMB are undetectable in milk. The only metabolites to which consumers of milk from HMBi-treated cows would be exposed are methionine incorporated into milk protein and very low amounts of 2-propanol and its oxidation product acetone. These are not of toxicological concern at the concentrations present. Consequently milk from HMBi supplemented cows can be considered safe for consumers.

No data was provided on the metabolic fate of the HMB/HMBi dimers other than an indication that they did not act as precursors for methionine. Consequently it is not known whether these compounds are of any toxicological concern to humans or dairy cows. However, no adverse responses were noted in any toxicological study involving the commercial product.

HMBi would have no impact on the environment under proposed conditions of use.

Finally, SCAN considers that the safety assessment of a manufacturing process based on chemical synthesis is beyond its competence.

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