I M A A asbl

International Methionine Analogue Association[1]

Relative bioequivalence of HMTBA (Hydroxy Analogue of Methionine) and DL-Methionine

BOOKLET

[1] IMAA is a non-profit Association, set up in 2012 by Methionine Analogue producers, for the promotion of the reputation, utilization and dissemination of HMTBA (Hydroxy Analogue of Methionine).

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ref. HMTBA/BO/EXT/1/0916/WO - 25 October 2016

INTRODUCTION

Methionine is an essential amino acid in all animal species. Methionine is clearly recognized as the first limiting amino acid in poultry, and probably also in high-yielding cows, and as the second or third limiting amino acid in pigs fed conventional diets.

Therefore, additives containing DL-methionine or DL-hydroxy analogue of methionine (HMTBA) as the active substance are frequently used in the feed industry to adjust dietary methionine to meet the requirements of target animals in order to maximize production performance and reduce nitrogen emission.

Hydroxy analog of methionine is commercially available in two different forms:
- as aqueous solution with 88 % HMTBA activity;
- as dry calcium salt with 84 % HMTBA activity.

The molecule 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) is an L-methionine precursor like DL-Methionine (DL-Met). Both materials are absorbed, metabolized, converted and used to provide L-Methionine (L-Met) to the animal; however, because of the chemical differences between the two molecules, the mechanisms of their absorption, metabolism and conversion to L-Met are quite different.

Given these chemical differences, animals supplemented with either of the two compounds do not follow the same form of dose response due in part to differences in feed intake, particularly at the extremes of the dose responses curves. At methionine deficient levels of the response curve, HMTBA fed animals may exhibit lower growth than DL-Met while at requirement levels and above they may have greater growth. Given the fact of different forms of dose response to graded levels of each molecule, it is clear that relative differences in growth between the two molecules at one part of the dose response curve are not predictive of growth in other portions of the same curves.

Field nutritionists supply commercial animal feed with doses of HMTBA or DL-Met allowing achieving maximum performance. At these commercial levels, the full relative bioequivalence of HMTBA over DL-Met has been well proven in the literature and generally reported to be 100 %.

This booklet is intended to be used by feed manufacturers to scientifically substantiate the bioequivalence factor they use to calculate the methionine equivalent value of HMTBA following the guidance of the “EU code of good labelling practice for compound feed for food producing animals”.

SCIENTIFIC SUBSTANTIATION PAPER

Relative bioequivalence of 2-hydroxy-4-(methylthio) butanoic acid and DL-methionine

A review of the literature with relation to nutrition, metabolism and statistical aspects of bioequivalence of the two Methionine sources

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This report summarizes the published work involved in the relative bioequivalence of 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) and DL-methionine (DL-Met) which includes nutritional, metabolic and statistical aspects of the bioequivalence. The differences between these two products are explained and the evidence and reasons for the full bioequivalence of HTMBA in monogastrics are discussed. In addition, appropriate statistical methods for comparing the bioequivalence of these two products for successful use of each product are provided.

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Contents

Executive summary

The controversy regarding the relative bioequivalence value of 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) and DL-methionine (DL-Met) has been continued for over 50 years. This report summarizes the body of published work involved in the relative bioequivalence of HMTBA and DL-Met which includes nutritional, metabolic and statistical aspects of its bioequivalence. The differences between these two products are explained and the evidence and reasons for the full bioequivalence of HMTBA in monogastric animals are discussed. In addition, appropriate methods for comparing the bioequivalence of these two products for successful use of each product are provided.

HMTBA is an organic acid precursor of L-met. The report summarizes the chemical structure differences between HMTBA and DL-Met that leads to differences in how and where the two materials are absorbed, metabolized, converted and used to provide L-met to the animal. Because of these differences, when the two compounds are supplemented into animal feeds in graded doses, they do not produce dose response curves of the same form due in part to differences in intake and metabolism at the extremes of the dose response curves. At methionine deficient levels of the response curve, HMTBA fed animals may exhibit lower feed consumption and growth than DL-Met while at requirement levels they may have greater feed consumption and growth. This report provides evidence for why these differences in growth response occur in poultry and swine. It also demonstrates that lower growth, whether for DL-Met or HMTBA, does not mean that either product is being converted to methionine inefficiently. Since both products have different dose response curves, statistically valid methods are provided for unbiased determination of relative biopotency across tested dose ranges. Evidence is provided that proves the assumption for the same form of dose response between the two methionine sources cannot be accepted and the use of linear or exponential slope ratio techniques well below the intended level of use to determine a single relative bioequivalence value for HMTBA for the entire dose range are inappropriate and inaccurate. Field nutritionists will be feeding commercial doses of HMTBA or DL-Met at a total sulfur amino acid dietary level capable of achieving maximum performance. At these commercial levels, and based on the evidence found in the literature and summarized in this report, the full relative bioequivalence of HMTBA over DL-Met has been well proven.

Introduction

There are two primary product forms of supplemental L-methionine (L-met) activity commercially available for supplementation of Met deficient diets; 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) most commonly available as an 88% solution with 12% water (for example [4]ALIMET® Feed Supplement or [5]Rhodimet AT-88®), or as 84% dry Ca salt ([6]MHA® Feed Supplement), and dry DL-methionine, (DL-Met, 99% powder). While these compounds both provide L-met activity to avian and mammalian species alike, they are chemically different in that HMTBA has a hydroxyl group at the asymmetric carbon whereas DL-Met has an amino group (Figure 1). This chemical difference results in substantial differences in how and where the two molecules are absorbed, metabolized and converted to provide L-met to the animal (Dibner, 2003, Lobley et al., 2006, Wester et al., 2006, Zhang et al., 2015). Both of these compounds have been commercially available and used in animal production systems for over 50 years; however, there remains controversy and confusion with respect to the relative bioequivalence of the two compounds. This situation is fueled by publication of individual product comparisons as well as compilations of previously published results with apparently conflicting conclusions (Jansman et al., 2003; Vazquez-Anon et al., 2006a; Sauer et al., 2008; Vedonov and Pesti, 2010).

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Objectives

The objective of this review is to summarize what is known concerning the similarities and differences of these compounds and how they are metabolized and used by the animal to support growth. Furthermore, these differences will be discussed in the context of the statistical methodologies used to compare their relative bioequivalence. Ultimately, the bioequivalence controversy exists today because of misunderstanding and misapplication of the statistical methods of bioequivalence determination and not because lack of efficiency of HMTBA as Met source.

HMTBA and DL-Met are different compounds

As described in Figure 1, HMTBA and DL-Met differ chemically. While DL-Met is a DL mixture of the amino acid methionine, HMTBA is a DL mixture of a naturally occurring organic acid (Dibner et al., 1990). It occurs in animals as part of normal methionine and thio-methyl metabolism but the nitrogen is added only during the process of its conversion to L-met. Many organic acids like HMTBA exhibit antimicrobial activities at low pH (Geraert et al., 2005). This has been demonstrated for HMTBA as well for a variety of bacteria including E. coli, Salmonella species and Campylobacter (Enthoven et al., 2002). The fact that HMTBA contains a hydroxyl instead of a nitrogen group influences where and how it is absorbed from the gastrointestinal tract as well as how it is transported and metabolized in the body. Since it is an organic acid it is very lipophilic and is absorbed primarily by diffusion (Knight and Dibner, 1984) that is, it follows a concentration gradient going from higher concentrations to lower concentrations. It is more lipophilic at low pH and therefore, it is absorbed primarily in the upper GIT and approximately 85% of it is absorbed before reaching the small intestine in broilers (Richards et al., 2005) and in pigs (Jendza et al., 2011). The rest of the GIT including all sections of the small intestine and hindgut have been shown to be capable of absorbing HMTBA as well (Dibner et al., 1987; Martin-Venegas et al., 2006). While diffusion is the primary means of absorption, there are reports that have demonstrated a portion of HMTBA is absorbed through a low affinity lactic acid carrier mechanism (Martin-Venegas et al., 2007) as well.

HMTBA is similar to DL-Met and L-met in that there is a redundancy of absorptive capacity. For these essential molecules, excess absorption capacity is advantageous in assuring that no methionine source escapes the upper gut. This also minimizes the loss of methionine activity to the microflora of the lower GIT. This subject has been recently reviewed (Zhang et al., 2015).

The free form of HMTBA is an aqueous solution that contains 88 % product in an equilibrium mixture of HMTBA monomer, dimer, and trimers. The esterification that results in dimer formations takes place between the carboxyl group of one monomer and the alfa-hydroxy group of another, with the liberation of water. Once the concentrated product is on feed, the equilibrium shifts in the direction of monomer (Bruyer and Vandelle, 1990) resulting in the formation of salts of HMTBA (e.g. calcium salts). After ingestion, monomer formation is favor by the availability of water. The amount of non-monomeric forms of HMTBA would be highest in the supplement and would decrease in feed and after ingestion (Dibner, 2003). Several in vitro and in vivo techniques have been used to elucidate the hydrolysis and fate of the HMTBA non-monomeric forms by the pancreatic intestinal enzymes (Lawson and Ivey, 1986), intestinal epithelial cells (Dibner 2003) and everted sacs (Martin-Venegas 2006), in vivo intestinal perfusion studies (Martin-Venegas et al., 2006), and growth performance studies (Bruyer and Vanbelle, 1990a, b). From these studies the researchers concluded that the HMTBA polymer fractions that accurately represent those found in the product are subject to hydrolysis into monomers by intestinal enzymes and mucosa and are not a limiting factor in the absorption of HMTBA.

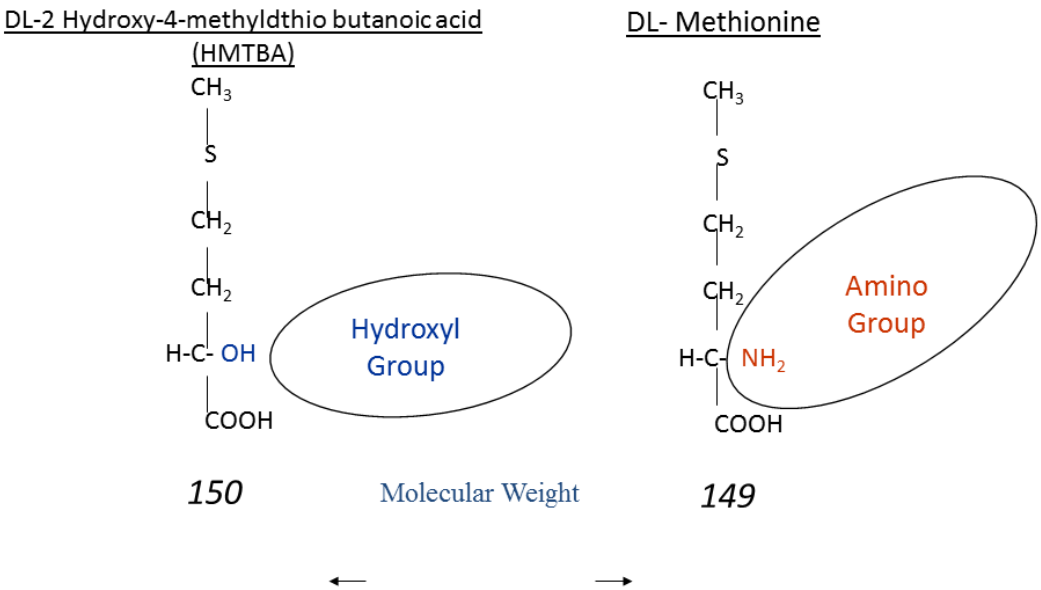

Figure 1. Chemical structure of 2-hydroxy-4-(methylthio) butanoic acid (HMTBA) and DL Methionine (DL-Met). The chemical structure of the two molecules differ in the second carbon HMTBA has a hydroxyl group and DL-Met has an amino group

Conversion of L- and D-HMTBA to L-Met

The conversion of L- and D-HMTBA and D-methionine to L-met is a two steps process, each compound being converted first to keto-methionine intermediate (chemically named keto-methyl-butanoic acid or KMB) which is then transaminated to L-met. The L isomer of HMTBA and the D isomer of methionine are both converted to keto-methionine in the peroxisomes of the cells by an L-hydroxy acid oxidase (L-HAOX) and a D-amino acid oxidase (D-AAOX), respectively (Dibner and Knight, 1984). Peroxisomes are located primarily in liver and kidney (Dibner & Knight, 1984) however; they have been shown to be present in other tissues of the gastrointestinal tract as well (McCollum et al., 2000). The D isomer of HMTBA is converted to keto-methionine by a D-hydroxy acid dehydrogenase (D-HADH) that is present in the mitochondria. This represents a substantial difference between L-HMTBA and D-methionine since the enzymes responsible for conversion of those isomers have a somewhat restricted distribution in the body, while every living cell contains active mitochondria that can convert the D-HMTBA to keto-methionine and ultimately L-met (Figure 2). The second step of the conversion is a transamination of the keto-methionine to form L-met. The enzymes needed for this step are present in all tissues and the conversion to methionine is rapid enough such that there is no measurable pool of keto-methionine (Dibner, 2003).

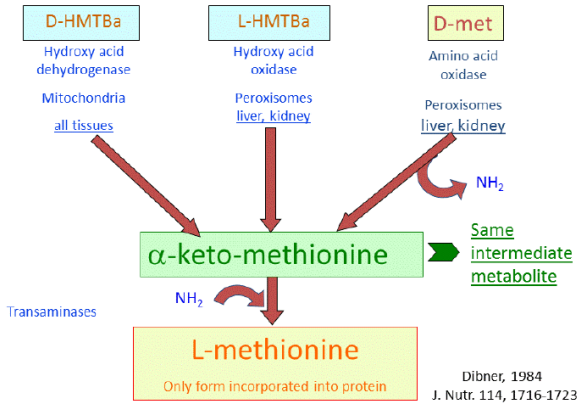

Figure 2. Schematic of the conversion of D-HMTBA, L-HMTBA and D-Met to L-Met. All of the isomers have the same general conversion pathway with an initial oxidation step to keto-methionine followed by transamination to L-Met. Adapted from Dibner (2003)

HMTBA and DL-Met have different sites of metabolism in the body

The differences in chemical, biochemical (enzymatic conversion) and biological (absorption) between HMTBA and DL-Met have some impact on how and where both sources are metabolized in the body. The location of the L-hydroxy acid oxidase enzyme in peroxisomes would suggest that the liver and the kidney would play a key role in the conversion of L-HMTBA to L-met. However, the broad distribution of the D-HMTBA dehydrogenase enzyme raises the potential for every cell in the body to be able to convert D-HMTBA to L-met. Isotope dilution infusion studies (Lobley et al., 2006; Wester et al., 2006) indicated that all tissues synthesized L-met from HMTBA as suggested by the broad distribution of the D-HADH, with the greatest enrichment obtained in the liver and kidney in agreement with the presence of peroxisomes and L-HAOX enzymes. However, with the exception of the kidney, the HMTBA-derived L-met was retained in the tissues in which it was converted. After kidney and liver, the upper small intestine exhibited the highest enrichments of the remaining tissues possibly due to the fact that it is the first tissue in contact with HMTBA. Several key aspects of HMTBA metabolism were provided by the isotope work. First, HMTBA is transported to the tissues primarily as HMTBA rather than Met. This is due to the fact that HMTBA is taken up by all body tissues and converted to L Met locally and very little of it gets secreted back into circulation. These data provide the metabolic rationale for a lower plasma free Met increase with HMTBA supplementation than DL-Met, as observed in the literature (Vazquez-Anon et al., 2003; Gonzalez-Esquerra et al., 2007). Circulating free Met levels can have significant effects on feeding behavior in animals and will be discussed later in terms of ad libitum feed consumption.

Implications of HMTBA metabolism during oxidative and heat stress

Recent work has also pointed out the differential cell metabolism of HMTBA leading to its antioxidant effects and thus can improve the anti-oxidative capacity, enhance the immune system and alleviate the stress response of the animals (Zhang et al., 2015). Using Caco-2 cells, Martin-Venegas et al. (2013) reported how HMTBA partially was able to prevent inflammation and improve the antioxidant capacity of the cells whereas DL-Met was not. The protective role of HMTBA on intestinal epithelia barrier function is correlated with higher taurine and reduced glutathione, which are products of L-met conversion after transsulfuration (Zhang et al., 2015). These results suggest HMTBA might be preferentially diverted to the transsulfuration pathway (Martin Venegas et al., 2006) and the mechanism for its higher antioxidant capacity over DL-Met. Supplementation of HMTBA partially prevented the growth depressing effect of heat exposure and alleviated oxidative damage caused by heat stress on broiler chickens (Dibner et al., 1992; Willemsen et al., 2011). Dibner et al., (1992) and Knight et al., (1994) have reported benefits of HMTBA during intermittent exposure to heat stress and they related these animal growth benefits to the way HMTBA is absorbed via diffusion during a time when absorption capacity of the villus is compromised. In their studies, the rate of HMTBA uptake via diffusion increased, whereas, DL-Met active transport decreased during heat stress conditions.

The new findings associating HMTBA with antioxidant metabolism brings further light to the benefits on HMTBA under heat and oxidative stress conditions, but also under other nutritional conditions. Several studies have linked the overall improvements in antioxidant capacity observed in birds fed HMTBA with improvements in performance over DL-Met under low CP diets (Swennen et al., 2011) and when fed at adequate levels of Met (Zou et al., 2015).

Differences in metabolism leads to differences in feed intake

It is well known that dietary imbalances of amino acids can result in a very rapid reduction in voluntary feed intake in both mammals and birds and in particular, dietary Met affects feed intake such that both low and high concentrations of Met depress feed consumption (Harper et al., 1970; Edmonds and Baker 1987; Suagahara and Kubo 1992). Given the close association of circulating Met levels on voluntary feed intake and the fact that HMTBA is delivered to tissues as HMTBA rather than

Met, it would appear likely that this metabolic difference could result in different ad libitum feeding patterns between HMTBA and DL-Met supplemented animals. In addition, this effect would be most pronounced at low levels of dietary methionine since under those conditions the bulk of the HMTBA converted to L-met in the kidney and other tissues would remain in the tissues to support intracellular use and would not be secreted back into the plasma.

Several studies in the literature have examined the relationship between levels of HMTBA and DL-Met supplementation at deficient, adequate and above requirements on ad libitum feed consumption, plasma Met and performance. Gonzalez-Esquerra et al. (2007) illustrated the association between plasma Met and feed intake for HMTBA and DL-Met. Plasma Met response to increasing DL-Met was approximately three times the increase for HMTBA. Along with the more rapid increase in plasma there was a more rapid increase in feed intake with DL-Met over HMTBA at the lower levels of supplementation. In contrast, as level of supplementation increased feed intake for HMTBA overtook that of DL-Met such that feed intake at requirement levels of supplementation for HMTBA was greater than control while feed intake for the same level of DL-Met was not.

At levels of supplementation above total sulfur amino acids requirements, such as 1% or above of the diet, broiler feed intake and growth rate are significantly reduced; however, the magnitude of feed intake depression is less with HMTBA (Baker, 1977; Vazquez-Anon et al., 2003). Although plasma free Met concentrations are elevated for both Met sources at these supplementation rates, DL-Met-supplemented chickens and pigs demonstrated significantly greater plasma free Met and homocysteine than for HMTBA (Vázquez-Añón et al., 2003; Dibner, 2003), indicating a close association of differences in plasma free Met and differences in feed intake levels for HMTBA and DL-Met as described in Figure 3.

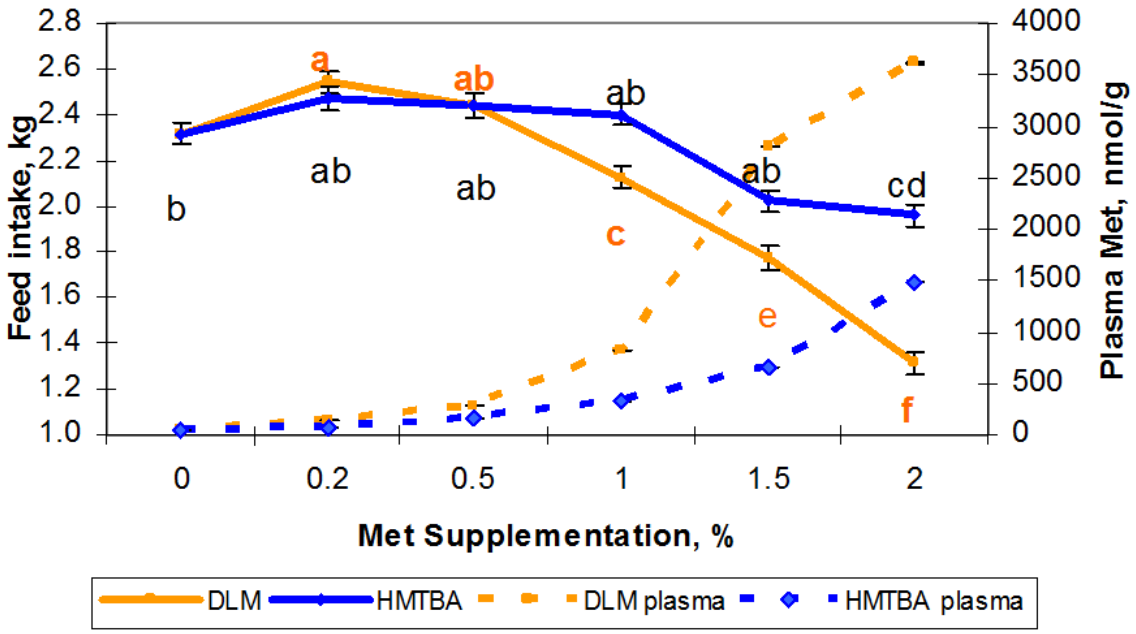

Figure 3. Differential feed intake and plasma Met concentration response to HMTBA and DL-Met in broilers beyond the maximum growth response. Adapted from Vásquez-Añón et al., 2003.

Thus, at high level of supplementation, higher plasma free methionine is associated with a reduction of feed intake while at low levels of supplementation higher plasma free methionine is associated with increasing feed intake. While the relationship between plasma free methionine and feed intake appears to be obvious, an argument can still be made that plasma free methionine is lower for HMTBA treatments because less of it is converted to L Met. To address this concern, Knight et al. (2006) used paired-feeding studies to demonstrate that differences in performance between HMTBA and DL-Met were due to differences in intake and not inefficiency of conversion of HMTBA to L Met. When fed total sulfur amino acid deficient (0.45%) diets, broilers supplemented with HMTBA consumed significantly less feed and grew more slowly than those supplemented with equimolar quantities of DL-Met. However, DL-Met-supplemented broilers pair-fed to the HMTBA ad libitum

treatments had the same growth rate as the HMTBA treatment. Likewise, when fed total sulfur amino acid adequate diets (0.70%) at high concentrations of Met supplementation (1%) demonstrated greater ad libitum consumption of HMTBA than DL-Met; however, HMTBA pair-fed to the DL-Met treatment produced growth equal to the ad libitum DL-Met treatment. Thus, these results demonstrated that the differences in gain at the extremes of the total sulfur amino acid response curve were due to differences in feed consumption, because no differences in gain between the two Met sources were observed in pair-fed treatments.

Feed consumption is a critical factor to consider when evaluating relative effectiveness of various compounds. When comparing the nutritional value of two compounds in a system where ad libitum feed consumption is different, the dose response curve of the compounds will be different, but also other nutrients consumed in the diet (total protein, energy, etc). Feed intake can play a role in the relative bioequivalence of HMTBA.

HMTBA and DL-Met have different dose response in broilers

Although DL Met and DL HMTBA are sources of methionine activity, their chemical structure, manner and site of absorption, transport in the body and conversion to L- Met by the tissues, and metabolism are quite different. Because of these differences, the two compounds do not follow the same form of dose response (Kratzer and Littell, 2006; Vazquez-Anon et al., 2006b; Gonzalez-Esquerra et al., 2007) due partially to differences in intake and metabolism at the extreme of the dose response curves (Knight et al., 2006).

There have been multiple individual studies that have demonstrated performance differences under specific conditions that have favored each compound, which has contributed to the controversy. In the last fifteen years, a significant numbers of individual broiler studies where the two methionine sources were evaluated concluded the two Met sources were not different (Daenner and Bessei, 2003; Motl et al., 2005; Agostini et al., 2015ab) or favored HMTBA over DL-Met (Vazquez-Anon et al., 2006b; Swennen et al., 2011; Willemsen et al., 2011; Montanhini Neto et al., 2013; Zou et al., 2015). However, there has been a wide range of bioequivalence estimates reported by the different meta-analysis of large number of poultry studies is partly driven by the different statistical dose response models used to determine relative bioequivalence (Jansman et al., 2003; Vázquez-Añón et al., 2006a; Sauer et al., 2008; Vedenov and Pesti, 2010).

The most common bioavailability methods used by scientist in the published literature prior to 2005 to compare relative bioequivalence for DL-Met and HMTBA were the linear or exponential slope ratio statistical methods described by Finney, (1978) and later by Littell et al. (1995). These methods were based on the a priori assumption that the nutrients being compared are the same with different concentration and therefore have the same form of dose response. When compounds being compared followed the same form of dose response comparisons made in the most deficient portion of the dose response curve are predictive of response over the entire dose response.

Since DL Met and HMTBA are different compounds and metabolized differently in the body once absorbed, the assumption that both should have identical dose response curves cannot be made. In 2006, Kratzer and Littell (2006) reported an in depth analysis of the application of the exponential slope ratio technique to measure HMTBA relative bioequivalence and the invalidity of the assumptions. The slope ratio analysis assumes the products compared are the same compounds, the only differences being concentration and that each follows dose response curves of the same form and approach a common plateau. The authors provided an example of the misapplication of the exponential slope ratio technique in a previously published paper by Schutte and DeJong (1996). Applying the exponential slope ratio technique and forcing the two Met sources to have equal plateau, the relative bioequivalence value for HMTBA was estimated to be 89 % rather than 100%, and while confidence limits were overlapping, the conclusions of the paper suggested lower efficacy of HMTBA across the entire dose range. However, when exponential curve was fit for each of the Met sources a better fit was observed using Bayesian Information Criterion (BIC; Sy et al., 2004) and

the predicted plateau for HMTBA trend to be higher (P<0.1) than DL-Met (Figure 4 and 5). When significantly different plateaus are found, the use of slope ratio analysis to evaluate sources becomes invalid. The authors tested and rejected the hypothesis of equal plateaus for HMTBA and DL-Met across thirteen studies published and used in previously published (Jansman et al., 2003) meta-analysis. With this analysis, Kratzer and Littell, (2006) demonstrated that HMTBA and DL-Met do not always follow the same form of dose response, and therefore, one would obtain differing bioavailability estimates depending on where in the dose response curve the estimates are made. The authors in the paper put forward a new statistical method to determine relative bioequivalence that does not require the assumption of same form of dose response and provides an unbiased comparison of the products across the entire dose response.

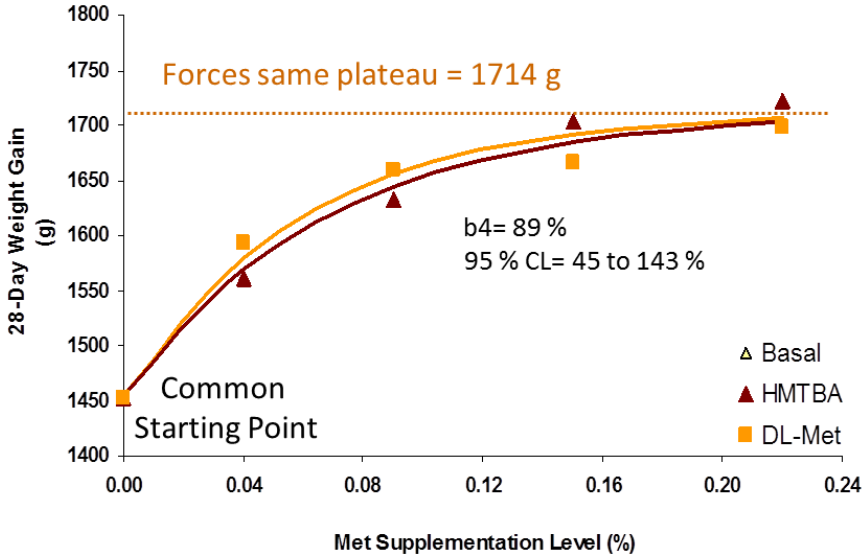

Figure 4. Mean values of 28-d body weight gains (g) in broiler chickens by Schutte and de Jong (1996). Use of the non-linear common plateau asymptotic regression model that forces a common plateau for the two sources of methionine; however, 95% confidence limits includes 100% efficacy, indicating no significant difference in relative biological efficacy. Adapted from Kratzer & Littell, 2006.

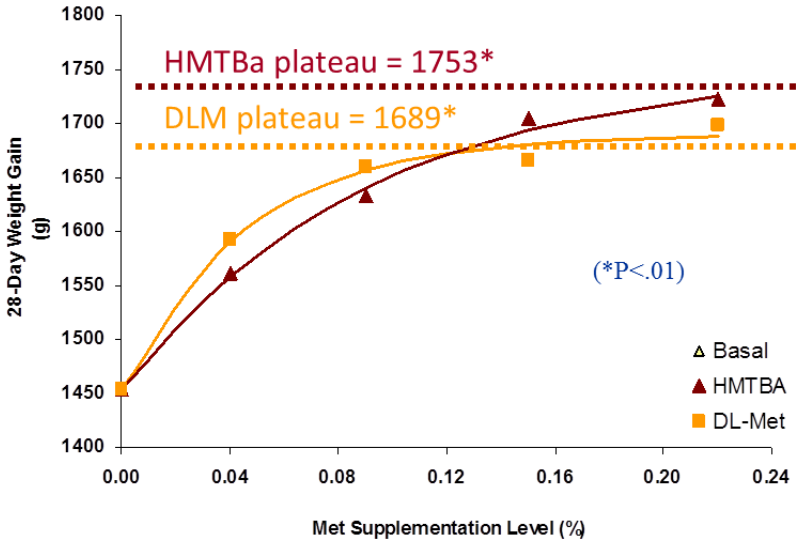

Figure 5. Mean values of 28-d body weight gains (g) in broiler chickens as reported by Schutte and de Jong (1996). At very low levels of supplementation, birds fed HMTBA had numerically lower weight gain relative to DL-Met birds; however when fed at levels closer to requirements, birds fed HMTBA had numerically higher body weights. A t-test reveals that the predicted plateau for HMTBA was significantly higher than that for DL-M (P=0.001), which demonstrated that the dose responses of the 2 products are different. Adapted from Kratzer & Litell, 2006.

Using the statistical methods outlined in Kratzer and Littell (2006), Vázquez-Añón et al. (2006b) compared different methionine sources by defining their own dose response within each source and

determining their relative performance by comparing the predictions of each model at the levels of expected use. Vazquez-Anon et al. (2006b) in four different trials imposed linear, quadratic and exponential equations to body weight gain and selected the best model using best goodness of fit to estimate the gain responses to feeding different doses of HMTBA vs DL-Met within and across four trials. They concluded the two methionine sources have different dose response with HMTBA outperforming DL-Met at commercial levels and DL-Met outperforming HTMBA at total dietary sulfur amino acids deficient levels. Similar results were observed in turkeys by Gonzalez-Esquerra et al., (2007).

Several meta-analysis in the literature have reported a wide range of relative bioequivalence values that ranged from 79 to 100 %. The meta- analysis that used exponential slope ratio with common plateau as statistical method concluded the lowest relative bioequivalence value for HMTBA (Jansman et al., 2003; Sauer, 2008). Whereas, the meta-analysis that allowed for each source to define its own response curve reported bioequivalence values for HMTBA that included 100% (Vazquez-Anon et al., 2006a). This illustrates the relevance of the statistical method in evaluating relative bioequivalence.

A practical approach to comparing HMTBA and DL-Met

Extensive research evaluating the relative efficiency of HMTBA and DL-Met as sources of Met activity in broilers has generated a large number of studies over the last 5 decades. Efforts have been made to provide a comprehensive summary of all existing literature in which the environmental and nutritional factors that determine the response to HMTBA and DL-Met could be evaluated and help predict the response of the two Met sources under relevant commercial conditions with broader inference than a single study. Vazquez-Anon et al., (2006a) compiled all previously published data containing 100 experiments in which HMTBA and DL-Met were evaluated and used multiple regression analysis to develop prediction models for each Met source. All the nutritional and experimental conditions identified in the multiple regression analysis contributed similarly to each Met source prediction model and both gain and feed conversion models described a quadratic dose response (Figure 6). Under the average experimental and commercial conditions, the predicted responses for gain and feed conversion models did not significantly differed between HMTBA and DL-Met, with a trend for the peak gain response for HMTBA to be numerically greater than DL-Met, suggesting benefits of HMTBA over DL-Met in the region of supplementation that is commercially relevant. The lack of differences between the two predicted models under experimental and commercial conditions supports an overall conclusion of equal performance of DL-Met and HMTBA when compared on an equal molar basis.

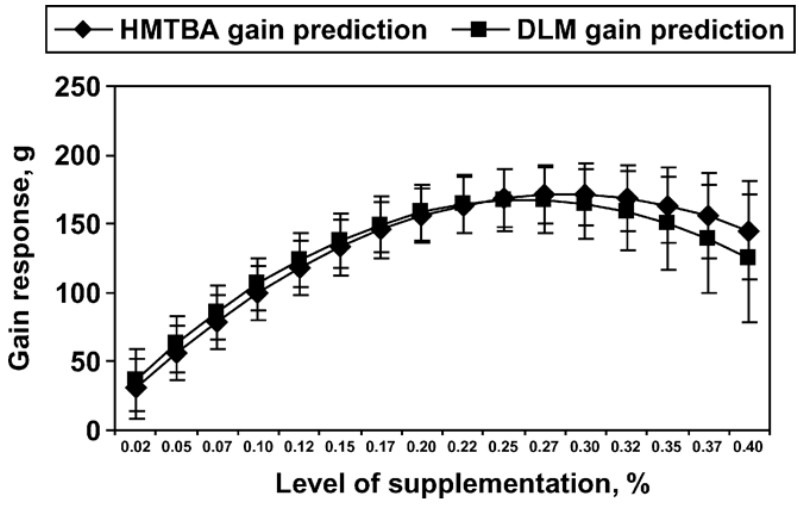

Figure 6. Comparison of the dose response predictions of HMTBA and DL-Met gain models under commercial conditions. The gain dose responses from each methionine source were compared under the feed nutrient profile and management practices reported by Agri Stats, Inc. (2004). The response curve followed a quadratic response and no differences were

observed between HMTBA and DL-Met gain predictions across levels of supplementation. Adapted from Vásquez-Añón et al., 2006.

The fact that the response to the two methionine sources follows a quadratic response and not a plateau it means that the over supplementation of either product can result in reduced performance, an important reason for properly determining the relative bioequivalence value of HMTBA and DL-Met. Statistical methods have been provided that allow for valid comparisons of HMTBA and DL-Met across a wide range of doses and it is clear from the data presented that the level of total sulfur amino acids in the diet will affect the performance of animals fed HMTBA and DL-Met differently. It is also clear that there is not a single relative bioequivalence value for two products with different dose response. The relative bioequivalence value determined in one part of the dose response will not predict the relative bioequivalence value in another. Lower growth may be obtained when feeding HMTBA vs DL-Met at deficient concentrations, whereas a greater maximum response is observed for HMTBA when fed at adequate or commercially relevant concentrations (Agostini et al., 2015a,b). Most nutritionist aim to feed growing animals at a level that allows for maximum performance. Therefore, it is most efficient to evaluate the products at a doses and diets within the range of expected use and feeding commercially relevant diets.

Bioequivalence of HMTBA in pigs

Total sulfur amino acids requirements in pigs have been defined as the third essential amino acid after lysine and threonine NRC (1998 and 2012). The majority of the Met studies published in the literature in which the two sources of Met are compared supported 100 % or better bioequivalence of HMTBA relative to DL-Met. Peer-reviewed articles (Chung and Baker, 1992; Knight et al., 1998; Reifsnyder et al., 1984; Urbanczyk et al., 1981; Romer and Abel, 1999; Gaines et al., 2005; Yi et al. 2007), three research institute reports (Callesen and Balle, 1997; Jansman and de Jong, 1999; van Oostrum and. Guillou, 2015) and one feature article in Feedstuffs (Stockland et al., 1992) that supported 100% or better bioequivalence of HMTBA (acid or calcium) relative to DL-Met. There were only two publications that indicated HMTBA was less effective than DL-Met (Roth and Kirchgessner, 1986; Kim et al., 2005). The discrepancy between the conclusions of these two papers and the rest of the publications relates to the misapplication of the exponential slope ratio analysis to calculate bioequivalence. The same arguments that exist in poultry are applicable to swine, in that HMTBA and DL-Met are metabolized differently leading to differences in the dose response. Using linear or exponential slope ratio analysis to compared sources in swine is also invalid. Gains et al. (2005) showed in commercial diets marginally deficient in Met (22 % deficient) the response to the two Met sources to follow a quadratic response with no differences between the two Met sources. Kim et al. (2005) fed diets very deficient in Met (49 % deficient) and using exponential slope ratio analysis reported lower bioequivalence for HMTBA. This lower bioequivalence value is driven by the influence of the lower performance of HMTBA in the deficient part of the curve when using slope ratio analysis and ignores the equal performance to DL-Met in the part of the curve approaching requirements that is commercially relevant. On the other hand, Yi et al. (2006) reported full bioequivalence value of HMTBA in diets deficient in Met (37 % deficient) and this deficiency was intermediate to the other two (Kim et al., 2005; Gaines et al., 2006). The relative bioequivalence value determined by each of these authors is consistent with what was observed in poultry. At the extremes of the dietary Met levels HMTBA and DL-Met exhibit different dose response and that these differences must be taken into account when using standard bioequivalence methods. The full bioequivalence of HMTBA is well reported in the swine literature at the supplemented levels of intended use.

Bioequivalence of HMTBA in other poultry species (turkeys and layers)

Various evaluations have been published describing the performance of laying hens fed different sources of supplemental methionine, including HMTBA (free acid or calcium salt) and DL-Met (Reid et al., 1982; van Weerden et al., 1984; Scott and Shurman, 1987; Harms and Russell, 1994; Bateman et al., 2003; Liu et al., 2004; Liu et al., 2005). These studies were conducted under controlled conditions

using practical feed ingredients, but with a wide array of experimental diets, strains of birds, production phases and cycles, age of birds and production conditions. In all studies no significant differences were reported between the two Met sources, with the exception of the studies where the two Met sources were not compared on an equal molar basis (Liu et al., 2004), and therefore cannot be consider in the evaluation. Several of the studies evaluated the dose response of the two Met sources to assess its relative bioequivalence using slope ratio analysis. Using this technique the relative bioequivalence value for HMTBA vary from above and below DL Met, but in all cases the 95% confidence interval around the mean bioequivalence value included 100 %, concluding there were no differences between the two Met sources.

In turkeys, the body of information evaluating the bioequivalence of HTMBA relative to DL-Met is not very extensive. Blair (1983) and Noll et al. (1984) were the first to report the use of exponential slope ratio analysis as a method for comparing relative bioequivalence of HMTBA and DL-M. Neither Blair (1983) or Noll et al. (1984) reported a significantly different relative bioequivalence value for HMTBA and DL-M. One study conducted by Hoehler et al. (2005) concluded the efficacy of HTMBA to be 55 to 74 %, however in this study the two Met sources were not compared on an equal molar basis and therefore no conclusions can be made over their relative bioequivalence. Later on, Gonzalez-Esquerra et al., (2007) critically evaluated dose responses of turkey poults to HMTBA and DL-Met in sorghum- and corn-based diets to determine the best-fit prediction equations to describe the response and predict the efficacy of the two Met sources at various points of the total sulfur amino acid response curve. Similarly to findings in poultry and swine, Gonzalez-Esquerra et al. (2007) reported that HMTBA and DL-Met elicit a different dose response in young turkey poults in which a lower growth may be obtained when feeding HMTBA vs. DL-Met at more deficient concentrations, whereas a greater maximum response is observed for HMTBA when fed at adequate or commercially relevant concentrations. The authors linked this effect at least partially, to the differential effect of HMTBA and DL-Met on plasma free methionine and the consequent effect on feed intake and growth. From the studies published in turkeys with appropriate comparisons it can be concluded that HMTBA has full bioequivalence value at the levels of intended use.

Conclusions and implications

Differences in the chemical structure of the two Met sources leads to differences in how the molecules are absorbed and metabolized. This differential metabolism affects the growth dose response curves of animals depending on what doses are fed. At lower levels of the response curve, below the TSAA requirement, HMTBA-fed animals may have lower growth than DL-Met while at higher levels, TSAA requirement and beyond, they may have greater growth. This report provides evidences that an assumption for same form of dose responsiveness between HMTBA and DL-Met cannot be made and that use of slope ratio techniques (either exponential slope ratio or linear slope ratio well below the intended level of use) to determine a single relative bioequivalence value for HMTBA are inappropriate. The performance response for either product at the extremes of the Met dose response curve is not representative of the relative bioequivalence value of either product at the maximum response levels. Field nutritionists will be feeding commercial doses of HMTBA or DL Met at a total sulfur amino acid dietary level capable of achieving maximum performance. At these commercial levels, and based on the evidence found in the literature and summarized in this report, the full relative bioequivalence of HMTBA over DL-Met has been well proven.

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