European Food Safety Authority

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SCIENTIFIC OPINION

Safety and efficacy of guanidinoacetic acid as feed additive for chickens for fattening[1]

Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed

(Question No EFSA-Q-2007-050)

Adopted on 3 March 2009

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SUMMARY

Following a request from European Commission, the European Food Safety Authority (EFSA) was asked to deliver a scientific opinion on guanidinoacetic acid. The additive (trade name: CreAmino^{TM}) is described by the applicant as a nutritional additive under the functional group amino acids, their salts and derivatives.

CreAmino^{TM}, a granulated product, contains a minimum of 96 % chemically synthesised guanidinoacetic acid (GAA) as active substance. Cyanamide (≤ 0.03 %) and dicyandiamide (≤ 0.5 %) are impurities arising from the production process.

GAA, a glycine derivative, occurs naturally in the body of animals (and humans) and acts as a precursor of creatine. The metabolic step from GAA to creatine requires methyl groups (from methyl donators) and methyl transferring enzymes.

Based on data from five efficacy trials, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concludes that 600 mg GAA kg^{-1} feed for chickens for fattening can be considered as the minimum dose improving performance characteristics. However, the FEEDAP Panel notes that the effects are most consistently seen at 800 mg GAA kg^{-1} feed, which is in line with the dose level recommended by the applicant.

[1] For citation purposes: Scientific Opinion of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on a request from the European Commision on the safety and efficacy of CreAmino^{TM} (guanidinoacetic acid) as feed additive for chickens for fattening. *The EFSA Journal* (2009) 988, 1-30

* One member of the Panel did not participate in the discussion on the subject referred to above because of possible conflicts of interest.

The FEEDAP Panel considers that the safety for the target animal at the applied dose range 600–1200 mg kg^{-1} feed has not been demonstrated. The lowest proposed dose, 600 mg GAA kg^{-1} feed, could be considered safe. The highest recommended dose (1200 mg GAA) was not tested but 1500 mg showed adverse effects on blood parameters.

The product did not show mutagenic or genotoxic properties. The effects reported in the 28- and 90-day studies in laboratory animals generally reflect physiological responses to high exposures to a metabolic intermediate and do not identify any novel or unexpected toxicity.

The FEEDAP Panel considers that the use of CreAmino™ as a feed additive for chickens for fattening at the levels proposed would not give rise to residues of concern to the consumer, neither from the active substance or the identified impurities.

The FEEDAP panel has no concerns regarding user safety for CreAmino™, provided that the recommendations of the safety data sheet are followed.

The FEEDAP Panel concludes that the use of CreAmino™ as a feed additive would not pose a risk to the environment.

The FEEDAP Panel commented on the proposed category of the additive and made a recommendation for the product's specifications.

Key words: nutritional additives, amino acids, CreAmino™, safety, efficacy, guanidinoacetic acid, creatine, homocysteine, cyanamide, dicyandiamide, chickens for fattening

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BACKGROUND

Regulation (EC) No 1831/2003[2] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lies down that any person seeking an authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from the company AlzChem Trostberg Gmbh[3] for authorisation of the product CreAmino™ to be used as a feed additive for broilers (category: nutritional additive; functional group: amino acids, their salts and analogues) under the conditions mentioned under Table 1.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4.1 (authorisation of a feed additive or a new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application.[4] According to Article 8 of that Regulation, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. The particulars and documents in support of the application were considered valid by EFSA as of 28 May 2007.

The additive CreAmino™ is a preparation of guanidinoacetic acid produced by chemical synthesis. This product has not been previously authorised in the Community.

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003 EFSA shall determine whether the feed additive complies with the conditions laid down in Article 5. Therefore, EFSA shall deliver an opinion on the efficacy and the safety for the target animals, user and consumer and the environment of the product CreAmino™, which is a preparation of guanidinoacetic acid produced by AlzChem Trostberg Gmbh when used under the conditions described in Table 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Working Group on Amino Acids as well as Annette Schuhmacher for the preparation of this opinion.

[2] OJ L268, 18.10.2003, p.29
[3] AlzChem Trostberg Gmbh, Albert-Frank-Strasse 32, D-83308 Trostberg, Germany
[4] Dossier reference: FAD-2007-0003

Table 1. **Register entry as proposed by the applicant**

Additive	Guanidinoacetic acid (GAA)
Registration number/EC No/No (if appropriate)	
Category of additive	Nutritional additives
Functional group of additive	Aminoacids, salts and analogues

Description			
Composition, description	**Chemical formula**	**Purity criteria (if appropriate)**	**Method of analysis (if appropriate)**
GAA min. 96 %; water max. 1 %	$C_3H_7N_3O_2$	Chemical pure product	Ion chromatography

Trade name (if appropriate)	CreAmino™
Name of the holder of authorisation (if appropriate)	AlzChem Trostberg GmbH

Conditions of use				
Species or category of animal	**Maximum Age**	**Minimum content**	**Maximum content**	**Withdrawal period (if appropriate)**
		mg kg^{-1} of complete feedingstuffs		
Broilers	n.a	600 mg/kg	1200 mg/kg	n.a

Other provisions and additional requirements for the labelling	
Specific conditions or restrictions for use (if appropriate)	Recommended dose level 800 mg/kg
Specific conditions or restrictions for handling (if appropriate)	n.a
Post market monitoring (if appropriate)	n.a
Specific conditions for use in complementary feedingstuffs (if appropriate)	

Maximum Residue Limit (MRL) (if appropriate)			
Marker residue	**Species or category of animal**	**Target tissue(s) or food products**	**Maximum content in tissues**
n.a	n.a	n.a	n.a

ASSESSMENT

1. Introduction

The applicant requested the authorisation for use of CreAmino™ as a feed additive for chickens for fattening, according to Regulation (EC) No 1831/2003; the active substance of this product is guanidinoacetic acid (GAA), a chemical derivative of the amino acid glycine. GAA is synthesised endogenously in the body of humans and animals and serves as precursor of creatine.

2. Identity, characterisation and conditions of use, methods of analysis

2.1. Identity of the additive

The additive contains (a minimum) of 96 % GAA. The applicant intends to market the additive as CreAmino™.

2.1.1. Proposal of classification

The applicant proposes that GAA, as a derivative of the amino acid glycine, should be classified as a nutritional additive belonging to the functional group of 'amino acids, their salts and analogues'.

2.1.2. Qualitative and quantitative composition

The end product of the chemical synthesis (technical GAA) contains 98% GAA on a dry matter basis (range of five batches: 98.0-99.6 %). The final granulated product CreAmino™ contains 98 % technical GAA, with a minimum GAA content of 96 % (range of five batches: 97.0-98.6 %), and contains a maximum of 1 % starch and 1 % water.

2.1.3. Purity

Technical GAA contains a maximum of 1.5 % glycine, 0.5 % dicyandiamide and 0.03 % cyanamide. Those impurities, resulting from the chemical synthesis, were routinely analysed.

Maximum concentrations for heavy metals, arsenic and fluorine in CreAmino™ are indicated by the applicant (5 mg Pb kg^{-1}, 0.1 mg Hg kg^{-1}, 0.5 mg Cd kg^{-1}, 2 mg As $kg^{-1,}$ and 30 mg F kg^{-1}) but analytical data were not submitted.

Control measures are in place for the substances and feed materials used in the chemical process and in the formulation of the additive.

2.1.4. Physical state of the product

The bulk density of CreAmino™ is about 640 kg m^{-3}. CreAmino™, as a granulated product, has a low level of dustiness (measured by optical absorption under defined conditions and reported on a relative scale from 0 to 100 %, where 0 % indicates no dust; the result for Creamino™ was 5.3 %). The product contains approximately 0.2 % of particles < 5 µm, 4 % < 63 µm, no particles > 850 µm.

2.2. Characterisation of the active substance

2.2.1. Description

GAA is the common name of N-(aminoimino-methyl)-glycine (synonyms: guanyl glycine; N-amidinoglycine, glycocyamine). (CAS No. 352-97-6; Molecular formula: $C_3H_7N_3O_2$; Molecular Weight 117.11 g mol^{-1}).

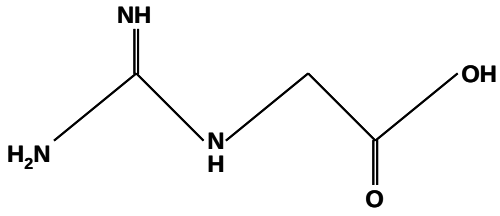

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Figure 1. **Structural formula of GAA**

2.2.2. Relevant properties

The thermal decomposition temperature is > 190 °C. Solubility in water at 15 °C is 1.5 g l^{-1}. The pH-value of a saturated solution in water at 25 °C is 8.1 (10 g of product suspended in 90 g water).

2.3. Manufacturing process

GAA is manufactured by chemical synthesis based on the reaction of the amino acid glycine (CAS No 56-40-6) with cyanamide (CAS No 420-04-2) in aqueous solution. During the synthesis, GAA forms a sediment that is separated and isolated.

To prepare the final product (CreAmino™), the wet raw crystalline product is granulated with starch and dried.

2.4. Physico-chemical and technological properties of the additive

2.4.1. Stability

2.4.1.1. Shelf life of the additive

The stability of crystalline GAA (not the additive) has been studied under accelerated conditions (three days at 85 °C, three weeks at 60 °C and three months at 45 °C; the model packaging simulated an original paper bag with a PE foil-inlay). Under those experimental conditions, no loss of crystalline GAA could be observed (99.6, 99.7, and 99.7 % GAA after three days, three weeks and three months, respectively). Therefore, it was concluded that there would not be a limit to the stability at 20 °C. The applicant proposes a shelf life of two years.[5]

[5] Technical Dossier/ Annex II.4

2.4.1.2. Stability of the additive in premixtures and feedingstuffs

Stability studies with CreAmino™ in a premixture containing mineral and trace elements (10 % GAA) were performed in a similar manner as that described for shelf life (three days at 85 °C, three weeks at 60 °C). No loss of GAA was found.[6]

Two levels of GAA (500 and 1500 mg GAA from CreAmino kg^{-1}) were added to turkey-specific compound feed formulations, which were mixed at different intensities, conditioned and pelleted at two temperature levels (75 and 85 °C). GAA was analysed before conditioning and after three months of storage under cool and dry conditions. Despite the fact that the graph (data not provided) shows a certain loss in GAA content of the turkey feed, the applicant concluded that GAA is stable in poultry compound feed for three months.

2.4.2. Homogeneity

Poultry compound feed at a supplementation level of 600 mg GAA kg^{-1} showed a coefficient of variation (CV) of 4.1 % in ten samples. The corresponding CV for a premixture containing 50 % GAA was 1.2 %.

2.4.3. Physico-chemical incompatibilities or interactions

Physico-chemical incompatibilities or interactions with feed, carriers, other approved additives or medicinal products are not to be expected.

2.5. Conditions of use of the additive

The additive is intended to be used in chickens for fattening; the applicant proposes a dose range of 600 to 1200 mg GAA kg^{-1} complete feedingstuff.

2.6. Evaluation of the analytical methods by the Community Reference Laboratory (CRL)

EFSA has verified the CRL report as it relates to the methods used for the control of CreAmino™ in animal feed. The Executive Summary of the CRL report and its amendment can be found in the appendices.

2.7. GAA metabolism: synthesis and further derivatives

GAA is a metabolic intermediary product being synthesised (Figure 2) from the amino acids glycine and arginine, with synthesis taking place mainly in kidney and pancreas. The transfer of the amidino group of arginine to glycine is catalysed by the enzyme L-arginine:glycine amidinotransferase (AGAT). After transport to the liver, GAA is methylated to creatine with S-adenosylmethionine (SAM) as the methyl group donor and by the action of S-adenosyl-L-methionine:N-guanidino acetate methyltransferase (GAMT) as the mediating enzyme (Daly, 1985; Stead et al,. 2001; Komoto et al., 2003). The formation of GAA from arginine and glycine is the principal regulatory site and rate-limiting step in the biosynthetic pathway of creatine (Walker, 1979). Several studies indicate that creatine regulates the AGAT expression by a feedback mechanism (McGuire et al., 1984; van Pilsum et al., 1992; Guthmiller et al., 1994), whereas GAMT expression is not controlled by creatine (Walker, 1979). The formation of GAA and creatine cannot be reversed.

[6] Technical Dossier/ Annex II.5

In the course of the conversion of GAA to creatine, a further metabolite, S-adenosylhomocysteine (SAH), is formed which is reversibly hydrolysed to adenosine and homocysteine by the enzyme S-adenosylhomocysteine hydrolase (Walker, 1979). Biological methylations and homocysteine formation are closely linked. Several studies, including labile methyl group balance studies in humans, and estimates of the methylation demand indicate that the methylation of GAA to creatine by GAA methyltransferase consumes more SAM than all other methylation reactions combined (Mudd and Poole, 1975; Mudd et al., 1980; Wyss and Walliman, 1994; Wyss and Kaddurah-Daouk, 2000; Stead et al., 2001). Dietary GAA supplementation considerably increases the methylation demand (Stead et al., 2006) and is therefore an efficient tool to induce hyperhomocysteinemia in animals (Stead et al., 2001; Fukada et al., 2006; Setoue et al., 2008; Ohuchi et al., 2008).

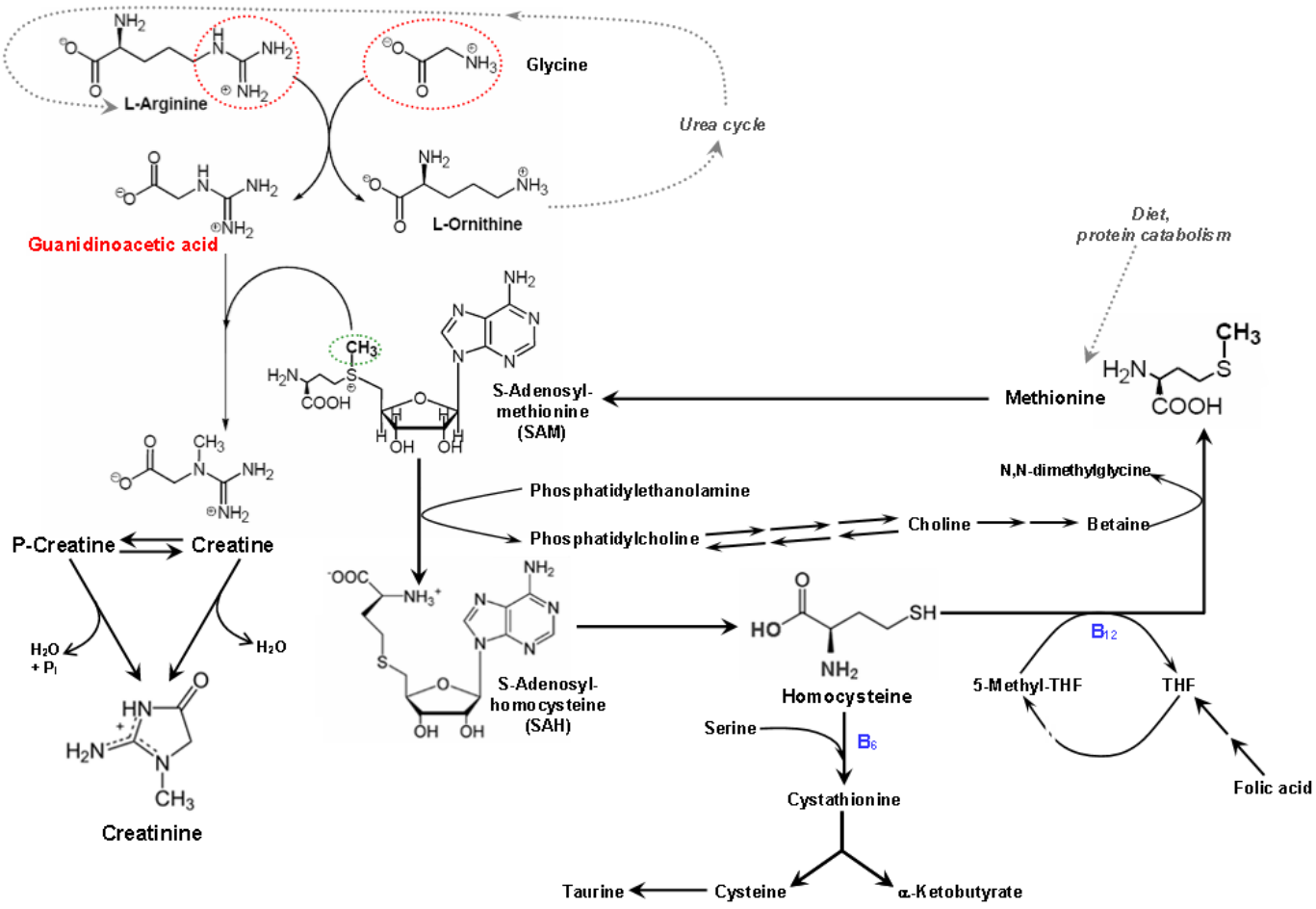

Figure 2. **Major pathways of the GAA, creatine and homocysteine metabolism**

The major pathways of GAA metabolism are shown in Figure 2. There is little available information on the efficiency of absorption of GAA in the gut.

However, if the transport mechanism across gut mucosa is similar to that through muscle cell membranes, a high efficiency of absorption of GAA is likely; GAA as such can be excreted via the kidney (Hoberman et al., 1948; Wyss and Kaddurah-Daouk, 2000).

Recent findings confirm this general view also for chickens. Lemme et al. (2007), feeding diets supplemented with 600 and 6000 mg GAA kg^{-1}, found true feacal digestibilities of 99.4 % and

98.9 % for GAA in colon-fistulated chickens. Calculated utilisation of digested GAA amounted to 77.1 % and 46.4 %, respectively.

3. Efficacy

Eight trials[7] carried out at seven different locations with chickens for fattening were submitted in the dossier, some of which include measurements of end points relevant to meat quality and deposition of GAA and creatine in tissues and organs.

3.1. Zootechnical effects

3.1.1. Trial I: dose-response trial

One-day-old male chickens for fattening (n=1520; Ross 308) were allocated to six experimental treatments (negative and positive control: seven floor pens of 40 birds each; other treatments: six floor pens of 40 birds each).[8] The treatments resulted from the supplementation of a pelleted vegetable diet (wheat/maize) with 0, 314, 628, 942 and 1256 mg GAA kg^{-1}. A positive control (fish meal) was included. The experimental period of 0–42 days was divided into a starter (0–21 days) and a grower phase (21–42 days). Feed was available *ad libitum*. Body weight and feed intake of each pen were recorded on days 0, 21 and 42, and feed/gain ratio derived. The health of the birds was examined daily. All mortalities were recorded and examined for cause of death. The results of the trial are shown in Table 2.

Table 2. **Response of chickens for fattening to graded levels of GAA (0–42 days)**

GAA intended (mg kg^{-1})	**0**	**314**	**628**	**942**	**1256**	**(Fish meal)**
GAA analysed, starter (mg kg^{-1})	-	240	600	950	1,100	-
GAA analysed, grower (mg kg^{-1})	-	200	600	970	1,400	-
Weight gain (g $bird^{-1}$)	2557^{bc}	2685^{abc}	2664^{abc}	2735^{ab}	2660^{abc}	2744^{a}
Feed intake (g $bird^{-1}$)	4493	4423	4446	4518	4372	4397
Feed/gain ratio	1.75^{a}	1.65^{b}	1.67^{ab}	1.65^{b}	1.64^{b}	1.60^{b}

[a, b, c] Values in a row with different superscripts differ significantly ($P \leq 0.05$)

Mortality was low and not treatment-related. GAA generally improved feed/gain ratio at a dose of 314 mg kg^{-1} and above, although the difference at a dose of 628 mg kg^{-1} was not significant.

3.1.2. Trial II: dose-response trial

One-day-old male and female chickens for fattening (n=2112; Ross 308) were allocated according to a 6x2 factorial design to six experimental treatments and two sexes with eight replicates (floor pens) and 22 birds per experimental unit.[9] A pelleted vegetable diet (corn/soybean meal) was supplemented with 0, 400, 600, 800 or 1200 mg GAA kg^{-1}. A positive control (meat and bone meal) was included. The experimental period of 0-42 days was divided into a starter (0–21 days) and grower phase (22–42 days). Feeding was *ad libitum*. Body weight and feed intake of each pen of birds was recorded on days 21 and 42, and feed/gain ratio derived. The health status of the birds was examined daily. The results of the trial are shown in Table 3.

[7] Technical Dossier/ Annexes III.1-5; III.7, III.9-10
[8] Technical Dossier/ Annex III.1
[9] Technical Dossier/ Annex III 2

Table 3. **Response of chickens for fattening to graded levels of GAA (0–42 days)**

GAA intended, (mg kg^{-1})	**0**	**400**	**600**	**800**	**1200**	**(MBM[1])**
GAA analysed, starter (mg kg^{-1})	0	420	536	754	1191	0
GAA analysed, grower (mg kg^{-1})	0	373	592	768	1184	0
Weight gain, g bird^{-1}	2433^{c}	2486^{ab}	2476^{abc}	2516^{a}	2475^{abc}	2448^{bc}
Feed intake, g	4274	4248	4241	4309	4194	4233
Feed/gain ratio	1.76^{a}	1.71^{b}	1.71^{b}	1.71^{b}	1.69^{b}	1.73^{b}
Mortality, %	11.9	10.8	8.2	11.9	9.1	11.4

$^{a, b, c}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)

[1] MBM: meat and bone meal

GAA significantly improved weight gain at 400 and at 800 mg kg^{-1}, while feed/gain ratio was significantly improved from 400 mg kg^{-1} upwards.

3.1.3. Trial III: dose-response trial

One-day-old male chickens for fattening (n=1312; Ross 308) were allocated to four experimental treatments with eight replicates (floor pens) and 41 birds each.[10] The treatments resulted from the supplementation of a pelleted vegetable diet (corn/soybean meal) with 0, 600, 800 and 1000 mg GAA kg^{-1}. The experimental period of 0–42 days was divided into a starter (0–21 days), grower (22–38 days) and finisher phase (39–42 days). Feed was available *ad libitum*. Body weight and feed intake of each pen of birds were recorded on days 21 and 42, and feed/gain ratio derived. The health of the birds was examined daily. The results of the trial are shown in Table 4.

Table 4. **Response of chickens for fattening to graded levels of GAA (0–42 days)**

GAA intended, (mg kg^{-1})	**0**	**600**	**800**	**1000**
GAA analysed, starter (mg kg^{-1})	0	482	608	907
GAA analysed, grower (mg kg^{-1})	0	475	637	827
GAA analysed, finisher (mg kg^{-1})	0	549	708	934
Weight gain (g bird^{-1})	2902	2881	2864	2898
Weight gain (g day^{-1})	69.1	68.6	68.2	69.0
Feed intake (g day^{-1})	140.6^{a}	135.5^{b}	136.1^{b}	135.9^{b}
Feed/gain ratio	2.03^{a}	1.97^{b}	1.99^{ab}	1.97^{b}

$^{a, b,}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)

Mortality was low and not related to treatment. GAA had no effect on weight gain but significantly lowered feed intake at concentrations of 600 mg kg^{-1} and above. Feed/gain ratio was significantly improved at 600 and 1000 mg kg^{-1}.

3.1.4. Trial IV: dose-response trial

One-day-old male chickens for fattening (n=3120; Cobb 500) were randomly assigned to four dietary treatments (4 x 6 floor pens, with 130 broilers each).[11] A pelleted vegetable diet (corn/wheat/soybean meal) was supplemented with 0, 200, 400 or 600 mg GAA kg^{-1}. The experimental period of 0–41 days was divided into a starter (0–10 days), a grower (11–28 days) and a finisher phase (29–41 days). Feed was available *ad libitum.* Production performance was recorded on days 10, 28 and 41, and health and mortality were checked daily. The results are summarised in Table 5.

[10] Technical Dossier/ Annex III.3
[11] Technical Dossier/ Annex III.7

Table 5. **Response of chickens for fattening to graded levels of GAA (0–41 days)**

GAA intended (mg kg^{-1})	**0**	**200**	**400**	**600**
GAA analysed, starter (mg kg^{-1})	< 0.08	158	338	499
GAA analysed, grower (mg kg^{-1})	< 0.08	177	375	542
GAA analysed, finisher (mg kg^{-1})	< 0.08	189	394	587
Weight gain, (g day^{-1})	62.4	61.9	63.1	62.7
Feed intake, (g day^{-1})	103	102	102	102
Feed /gain ratio	1.66	1.64	1.62	1.63
Mortality (%)	7.18	7.18	7.54	5.91

There were no significant effects of GAA supplementation on growth performances or mortality.

3.1.5. Trial V: growth trial

One-day-old male and female broilers (n=192; Ross 308) were randomly assigned to two dietary treatments (2 x 8 battery cages, with 12 broilers each).[12] The treatments resulted from the supplementation of a pelleted vegetable diet (corn/wheat/soybean meal) with 0 and 800 mg GAA kg^{-1}. The experimental period of 0–42 days was divided into a starter (0–21 days) and a grower phase (22–42 days). Feeding was *ad libitum*. Production performance (days 21 and 42) was recorded. Health and mortality were checked daily. The results are summarised in Table 6.

Table 6. **Response of chickens for fattening to GAA (0–42 days)**

GAA intended (mg kg^{-1})	**0**	**800**
GAA analysed, starter (mg kg^{-1})	-	737
GAA analysed, grower (mg kg^{-1})	-	765
Weight gain (g day^{-1})	72.7	73.3
Feed intake (g day^{-1})	127.3	123.7
Feed/gain ratio	1.75^{a}	1.69^{b}
Mortality (%)	8.65	3.94

^{a,b} Values in a row with different superscripts differ significantly ($P \leq 0.05$)

GAA supplementation significantly improved feed/gain ratio, while the other performance parameters were not affected.

Three additional trials included in the dossier were not considered due to deficiencies in the design.[13]

3.2. Carcass and meat quality

Some data are included in five efficacy studies and in the tolerance study relating to the effect of GAA supplementation on carcass and meat quality:

Trial I: dose-response trial

At the end of trial I, samples of 114 birds (three from each pen, 7 x 3 for both control groups, 6 x 3 per treatment group) were killed for carcass analysis.[14] There was no significant difference in the breast meat yield between treatments.

[12] Technical Dossier/ Annex III.9
[13] Technical Dossier/ Annex III.4 ; III.5 and Supplementary Information July 07/ Enclosure 1- 3
[14] Technical Dossier/ Annex III.1

Trial II: dose-response trial

At the end of trial II, 288 birds (8 x 3 per sex and treatment) were killed for carcass analysis.[15] The results of the trial are shown in Table 7.

Table 7. **Response of chickens for fattening to graded levels of GAA (0–42 days)**

GAA intended (mg kg^{-1})*	**0**	**400**	**600**	**800**	**1200**	**(MBM[1])**
Carcass weight (g)	1905^{c}	1944^{ab}	1925^{bc}	1958^{a}	1919^{bc}	1912^{c}
Breast weight (g)	653^{b}	673^{ab}	669^{ab}	685^{a}	679^{a}	654^{b}
Abdominal fat (g)	32.9^{bc}	34.3^{b}	30.3^{cd}	32.5^{bc}	27.6^{d}	38.5^{a}

$^{a, b, c, d}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)
* See table 3 for analysed dietary GAA concentration
[1] MBM: meat and bone meal

While GAA at doses of 800 mg kg^{-1} and above increased breast weight and 1200 mg kg^{-1} reduced the amount of abdominal fat, there were no significant effects at the lowest doses of GAA.

Trial III: dose-response trial

At the end of trial III, 96 birds (8 x 3 per treatment) were killed for carcass analysis.[16] The results of the trial are shown in Table 8.

Table 8. **Response of chickens for fattening to graded levels of GAA (0–42 days)**

GAA intended, (mg kg^{-1})*	**0**	**600**	**800**	**1000**
Carcass weight (g)	2426^{a}	2387^{a}	2182^{b}	2327^{a}
Breast weight (g)	527^{ab}	537^{a}	478^{b}	536^{a}
Abdominal fat (g)	71.8^{a}	57.1^{b}	49.8^{b}	55.1^{b}

$^{a, b}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)
* Analysed dietary GAA concentration (see Table 4)

The results show that GAA significantly reduced the amount of abdominal fat at doses of 600 mg kg^{-1} and above, while breast weight was only increased at a dose of 800 mg kg^{-1}.

Trial IV: dose-response trial

On day 41, slaughter characteristics (meat and liver quality) were recorded for 120 birds (five birds/pen; six pens/treatment).[17] The results are summarised in Table 9.

Table 9. **Response of chickens for fattening to graded levels of GAA (0–41 days)**

GAA intended (mg kg^{-1})*	**0**	**200**	**400**	**600**
Breast muscle pH at 1h post mortem	6.22	6.25	6.30	6.22
Breast muscle pH at 4h post mortem	6.05^{a}	6.07^{a}	5.95^{b}	5.92^{b}
Drip loss, (%)**	0.37	0.38	0.44	0.47

$^{a, b}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)
* Analysed dietary GAA concentration (see Table 5)
** Drip loss determination method (Honikel, 1998)

Breast muscle pH at four hours post-mortem was significantly lower with the two highest GAA doses, but the relevance of this finding was not indicated. Drip loss was not affected by treatment.

[15] Technical Dossier/ Annex III 2
[16] Technical Dossier/ Annex III.3
[17] Technical Dossier/ Annex III.7

Trial V: growth trial

On day 42, some carcass characteristics and meat parameters were recorded for 48 birds (three birds/pen; eight pens/treatment); GAA supplementation did not affect breast muscle yield (average 18.4%) or abdominal fat (average 1.85%).[18]

Data from the tolerance study

Carcass quality data were also measured in the tolerance study, which is described in detail in Section 4.1.2.[19] The data shown in Table 10 are based on 20 birds per treatment.

Table 10. **Effect on product quality of male chickens for fattening fed diets containing GAA for 35 days**

GAA intended (mg kg^{-1} feed)*	0	600	1500	3000	6000
Carcass weight (g)	1295^{a}	1291^{a}	1293^{a}	1293^{a}	1152^{b}
Breast weight (g)	484^{a}	491^{a}	493^{a}	512^{a}	445^{b}
Abdominal fat (g)	19.4^{a}	20.0^{a}	16.0^{ab}	15.9^{ab}	12.0^{b}
Drip loss (%)	1.99^{b}	2.17^{ab}	2.61^{a}	2.10^{ab}	2.50^{ab}
Muscle L **	47.1^{b}	46.8^{b}	49.9^{a}	49.9^{a}	49.0^{ab}

* Analysed dietary GAA concentration (see Table 11)
** L: brightness (L*a*b system, McLaren, 1976)
$^{a, b}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)

Drip loss was increased at all dietary GAA concentrations but the increase was significant only at a dose of 1500 mg GAA kg^{-1} feed. This may be an indicator of reduced product quality in chickens treated with GAA, although abdominal fat was reduced at high GAA levels (significant at 6000 mg GAA kg^{-1} feed). Brightness of meat was increased at 1500 mg GAA kg^{-1} feed and higher, whereas a (redness) and b (yellowness) remained unaffected.

3.3. Conclusion on efficacy and product quality

CreAmino^{TM} showed a significant positive effect on the performance of chickens for fattening in one trial (Trial I) at 314 mg GAA kg^{-1} complete feed and in another trial (Trial II) at 400 mg GAA kg^{-1}. The minimum recommended level, 600 mg GAA kg^{-1} complete feed has been examined in four trials, two of which (II and III) showed a significant positive response while the other two (I and IV) did not.

The FEEDAP Panel concludes that 600 mg GAA kg^{-1} feed for chickens for fattening can be considered as the minimum dose improving performance characteristics. However, the Panel notes that the effects are most consistently seen at 800 mg GAA kg^{-1} feed, which is in line with the dose level recommended by the applicant.

Significantly reduced abdominal fat in chickens for fattening was noted in three trials (II, III and the tolerance trial) when feed was supplemented with doses of 1000 mg GAA kg^{-1} complete feed and above.

At the minimum recommended level, no consistent effects were noted for breast muscle yield (trials II, III, V and tolerance trial) and no conclusions could be drawn regarding drip loss (trial IV, tolerance trial) or post-mortem pH (trial IV).

[18] Technical Dossier/ Annex III.9
[19] Technical Dossier/ Annex IV.5

4. Safety for the target species

4.1. Tolerance of the target animal

The FEEDAP Panel considers that tolerance studies with nutrients like the indispensable amino acids cannot be designed along the lines of conventional toxicity experiments since the appearance of amino acid imbalances at higher dosages will restrict the desired margin of safety. This view may also apply to other endogenous substances, the use of which in high doses may result in metabolic disorders.

A total of four studies were provided to investigate potential adverse effects of an overdose of GAA on the performance and health status of chickens for fattening.

The effect of a GAA overdose of around sevenfold (7.85 and 8.00 g kg^{-1} feed) the highest recommended dietary concentration (1200 mg kg^{-1} feed) was studied in three growth trials on a total of 1376 male chickens for fattening. A fourth trial investigated the effect of graded GAA levels of 0, 600, 1500, 3000 and 6000 mg kg^{-1} feed on performance, mortality, haematology and routine blood biochemistry of chickens for fattening, but the highest dose was only five times the maximum recommended dose.

4.1.1. Growth trials

In three of the trials already described in detail in the efficacy section (trials I, III, and V reported separately in the dossier), additional groups received 7850 or 8000 mg GAA kg^{-1} feed for 42 days.[20] All trials showed severe significant depression of weight gain (17 to 42 %), feed intake (11 to 27 %) and gain to feed ratio (7 to 21 %) at those high doses. Mortality was increased in two of three trials. The study design allows only limited conclusions on the tolerance of chickens to GAA but the margin of safety appears to be less than seven.

In two out of those three trials a further group received basal diet supplemented with creatine monohydrate at levels equimolar to 7850 mg GAA kg^{-1} feed. Creatine did not depress feed intake or weight gain.

4.1.2. Tolerance study

One-day-old male chickens for fattening (n=1280; Ross 308) were assigned to five dietary treatments (eight replicates (floor pens) per treatment with 32 birds each)[21] The basal pelleted diet (corn/wheat/soybean meal) was supplemented with 0, 600, 1500, 3000, and 6000 mg GAA kg^{-1} feed. The experimental period of 0–35 days was divided into a starter (0–14 days) and a grower phase (15–35 days), and feed was available *ad libitum*. Production performance (day 14 and 35) and some plasma characteristics (day 35; ten birds/treatment) were recorded. Health and mortality were checked daily. The results of the trial are shown in Table 11.

[20] Technical Dossier/ Annex IV.1, IV.3, IV.5
[21] Technical Dossier/ Annex IV.5

Table 11. **Effect of increasing dietary GAA fed for 35 days on performance, mortality and product quality of male broiler chicks (8 (replicates) x 32 (birds)/treatment)**

GAA intended (mg kg^{-1} feed)	**0**	**600**	**1500**	**3000**	**6000**
GAA analysed, starter feed (mg kg^{-1} feed)	< 0.05	592	1451	3496	5534
GAA analysed, grower feed (mg kg^{-1} feed)	< 0.05	586	1529	2966	5883
Weight gain (g)	2091^{a}	2094^{a}	2093^{a}	2061^{a}	1892^{b}
Feed intake (g)	3088^{a}	3110^{a}	3085^{a}	2976^{ab}	2792^{b}
Feed per gain (kg kg^{-1})	1.48^{a}	1.49^{a}	1.48^{a}	1.47^{a}	1.48^{a}
Mortality (%)	5.5	1.2	3.5	4.3	6.6
Leukocytes (G L^{-1})	7.8^{ab}	9.9^{a}	8.0^{ab}	7.6^{ab}	3.9^{b}
MCV (fL)	133.6^{c}	141.4^{bc}	169.9^{ab}	182.8^{a}	184.5^{a}
MCH (pg)	0.44^{b}	0.45^{b}	0.54^{ab}	0.58^{a}	0.59^{a}

* L: brightness (L*a*b system, McLaren,, 1976)
$^{a, b, c}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)
MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin

GAA concentrations up to 1500 mg kg^{-1} feed did not affect performance and mortality of the chickens for fattening, whereas at 3000 mg GAA kg^{-1} feed, body weight gain and feed consumption were slightly reduced, although the difference was not significant. At the highest inclusion level (6000 mg GAA kg^{-1} feed), feed consumption was significantly impaired, resulting in significantly lower weight gains. In contrast to the previous studies, an effect on feed conversion ratio was not observed and mortality was not affected by the GAA treatment.

Serum substrates (total protein, albumin, cholesterol, glucose, urea, uric acid) and enzymes (ALT, AST, γ-GT) remained essentially unchanged.

Mean corpuscular volume of erythrocytes (MCV) was significantly increased by 1500 mg GAA kg^{-1} feed. Macrocytic erythrocytes are frequently seen in anaemia, which was not observed. Alternatively, a high MCV can be a sign of deficiency of vitamin B_{12} and folic acid. The basal diet contained 7.5 µg added vitamin B12, 0.5 mg added folic acid kg^{-1} and 100 mg added choline-Cl kg^{-1}. Those supplementation levels could be considered to be marginally low, particularly for the methyl-donor choline.

4.2. Microbial safety

There are no reports of antimicrobial properties of GAA, nor can they be expected on the basis of the chemical properties of the molecule. Consequently, the possibilities of the compound to select for resistances against antimicrobials are remote. As there were no indications of harmful effects associated with intestinal disturbances in the tolerance trials, there are no reasons to expect microbial imbalance in the gut or increased shedding of pathogens as a result of GAA supplementation.

4.3. Conclusions on the safety of GAA for the target animal

Severe signs of intolerance (depression of feed intake and weight gain) were observed in chickens for fattening at dietary GAA levels of 6000 mg kg^{-1} feed or higher. Although not significant, a similar tendency could also be seen at 3000 mg GAA kg^{-1} feed. The effects at those levels are specific to GAA and not to its derivative creatine, the final metabolic end product.

The increase in the mean corpuscular volume of erythrocytes (MCV) observed at 1500 mg GAA kg^{-1} feed is considered as a sign of deficiency of vitamin B_{12} and folic acid and/or methyl donors (except methionine). The high requirement for methyl groups and methyl transferases

by endogenous creatine synthesis from GAA may have created this deficit. This conclusion is supported by a recent study in rats, where hyperhomocysteinemia by GAA could be suppressed by dietary choline or betaine (Setoue et al., 2008).

The FEEDAP panel considers that safety for the applied dose range of 600 to 1200 mg GAA kg^{-1} feed has not been demonstrated. The lowest proposed dose, 600 mg GAA kg^{-1} feed, could be considered safe, 1200 mg GAA was not tested and 1500 mg shows already adverse effects.

5. Safety for the consumer

5.1. Tissue deposition

Data on the consequences of GAA supplementation of feed on concentration of GAA and its metabolites in plasma and tissues are provided from four efficacy studies and in the tolerance study.

Trial I: dose-response trial

At the end of trial I, samples of 114 birds (three from each pen, 7 x 3 for both control groups, 6 x 3 per treatment group) were killed for meat analysis.[22] The results are shown in Table 12.

Table 12. **Response of chickens for fattening to graded levels of GAA (0–42 days)**

GAA intended (mg kg^{-1})*	**0**	**314**	**628**	**942**	**1256**	**(Fish meal)**
Creatine in muscle (mg kg^{-1})	4665^{a}	5337^{b}	5370^{b}	5322^{b}	5689^{c}	5215^{d}
GAA in muscle (mg kg^{-1})	7.59^{a}	1.30^{b}	1.78^{b}	1.21^{b}	0.91^{b}	1.41^{b}

$^{a, b, c, d}$ Values in a row with different superscripts differ significantly ($P \leq 0.05$)

* Analysed dietary GAA concentration (see Table 2)

Creatine content in breast muscle was significantly increased by GAA addition to feed at all doses and was higher than in a group given control diet containing fish meal. GAA content in breast muscle was significantly decreased by all doses of GAA in feed but was not different from the control diet containing fish meal.

Trial IV: dose-response trial

On day 41, liver and muscle tissues were analysed from 120 birds (five birds/pen; six pens/treatment). The results are summarised in Table 13.[23]

[22] Technical Dossier/ Annex III.1
[23] Technical Dossier/ Annex III.7

Table 13. **Response of chickens for fattening to graded levels of GAA (0–41 days)**

GAA intended (mg kg^{-1})*	**0**	**200**	**400**	**600**
Breast muscle GAA (mg kg^{-1})	23.7^{a}	13.7^{b}	6.2^{c}	3.7^{c}
Breast muscle creatine (mg kg^{-1})	3896^{c}	4006^{c}	4357^{b}	4560^{a}
Breast muscle creatinine (mg kg^{-1})	10.7^{b}	13.0^{a}	14.0^{a}	14.5^{a}
Breast muscle ATP (µmol g^{-1} 1h pm)	3.10^{b}	3.31^{b}	4.83^{a}	4.30^{ab}
Breast muscle AMP (µmol g^{-1}1h pm)	0.81^{a}	0.81^{a}	0.57^{b}	0.46^{b}
Breast muscle IMP (µmol g^{-1}1h pm)	4.06^{a}	3.65^{ab}	2.65^{b}	2.67^{b}
Liver GAA (mg kg^{-1})	161.2^{b}	215.0^{a}	205.0^{ab}	222.5^{a}
Liver creatine (mg kg^{-1})	45.3	52.0	48.8	54.2

* Analysed dietary GAA concentration (see Table 5)

ATP: Adenosine triphosphate; AMP: Adenosine monophosphate; IMP: Inosine monophosphate

[a, b, c] Values in a row with different superscripts differ significantly ($P \leq 0.05$)

At all doses tested, decreased GAA levels in breast muscle were accompanied by increased creatine levels. ATP levels increased with increased GAA doses in feed, at the expense of AMP and IMP. Effects on liver GAA and creatine were less marked.

Trial V: growth trial

On day 42, GAA, creatine and creatinine were analysed in breast meat from 48 birds (three birds/pen; eight pens/treatment). The results are summarised in Table 14.[24]

Table 14. **Response of chickens for fattening to GAA (0–42 days)**

GAA intended (mg kg^{-1})*	**0**	**800**
Breast meat GAA content (mg kg^{-1})	1.81	0.70
Breast meat creatine content (mg kg^{-1})	4481^{a}	5045^{b}
Breast meat creatinine content (mg kg^{-1})	6.7	8.0

[a,b] Values in a row with different superscripts differ significantly ($P \leq 0.05$)

* Analysed dietary GAA concentration (see Table 6)

GAA supplementation at 800 mg kg^{-1} feed significantly increased the creatine content of breast meat, but the reduction of GAA content was not significant and creatinine content was essentially unchanged.

Deposition data from the tolerance study

The results of the biochemical analysis of breast muscle and liver (20 per treatment) and of the blood of male broiler chickens (ten per treatment) are summarised in Table 15.[25] GAA concentration in breast muscle and liver decreased with increasing dietary GAA. There was also a dose-dependent increase of creatine and creatinine in the tissues and in plasma. A significant increase of plasma homocysteine was observed, but only in the highest GAA treatment group. High plasma homocysteine may be considered to be due to a reduced methyl group transfer capacity (either by a relative methionine/choline/betaine deficiency or a lack of vitamin B_{12} and/or folic acid, or by both). The supplementation levels of those nutrients in the diet used could be considered to be marginally low, particularly for the methyl-donor choline.

[24] Technical Dossier/ Annex III.9

[25] Technical Dossier/ Annex IV.5

Table 15. **Effect of increasing dietary GAA on GAA, creatine, creatinine and homocysteine concentrations in plasma, muscle and liver (day 36)**

GAA intended (mg kg^{-1} feed)*	**0**	**600**	**1500**	**3000**	**6000**
No of broilers (tissue/blood)	20 / 10	20 / 10	20 / 10	20 / 10	20 / 10
Breast muscle					
GAA (mg kg^{-1})	3.95^{a}	3.01^{a}	1.26^{b}	0.78^{b}	0.85^{b}
Creatine (mg kg^{-1})	4741^{c}	5157^{b}	5678^{a}	5863^{a}	5920^{a}
Creatinine (mg kg^{-1})	11.8^{c}	12.3^{c}	15.3^{bc}	17.9^{ab}	21.4^{a}
Liver					
GAA (mg kg^{-1})	45.0^{a}	49.3^{a}	47.3^{a}	13.5^{a}	6.2^{b}
Creatine (mg kg^{-1})	67.3^{c}	90.0^{bc}	113.2^{ab}	114.2^{ab}	132.4
Plasma					
Creatine (mg dL^{-1})	1.2^{c}	1.1^{c}	1.3^{bc}	1.8^{b}	2.6^{a}
Creatinine (µmol L^{-1})	3.6^{b}	3.7^{ab}	4.0^{ab}	4.2^{a}	4.2^{a}
Homocysteine (µmol L^{-1})	42.9^{b}	39.1^{b}	42.9^{b}	39.6^{b}	56.1^{a}

* Analysed dietary GAA concentration (see Table 11)
nd = not detectable
^{a,b,c} different superscripts within one row indicate significant differences ($P \leq 0.05$)

Tissue deposition data from the 90-day toxicity study in rats

Table 16 shows the mean homocysteine concentrations in the plasma of male and female rats fed graded levels of GAA for 90 days.[26] Although tissue concentration was measured in muscle, liver, kidney and brain, the data for tissue concentrations are not shown because of the small number of animals per group (two males and two females per group)[27] and hence high standard deviations and inconsistent results.

Table 16. **Effect of increasing dietary GAA on homocysteine concentrations in the plasma of groups of ten male and female rats after an overnight fast**

	GAA (mg kg^{-1} feed)					
	0	**500**	**1 000**	**3 000**	**10 000**	**50 000**
Males [day 91]						
Homocysteine (µmol L^{-1})	8.25	8.42	8.24	9.07	11.47*	49.44*
Females [day 92]						
Homocysteine (µmol L^{-1})	7.20	6.76$^{\#}$	5.95*	7.06	9.64*	25.54*

* Values differ significantly ($P \leq 0.05$) from control group
$^{\#}$ Mean based on a group of 9 animals

In non-fasted animals (day 83), a higher effect of GAA supplementation on the plasma homocysteine levels could be observed than that described for fasted rats in Table 16 (day 91/92). In non-fasted males, plasma homocysteine was raised from 17.3 µmol L^{-1} in the control group to 31.6 and 61.5 µmol L^{-1} in groups supplemented with 10000 and 50000 mg GAA kg^{-1} feed, respectively; the corresponding values for the females are 8.0 µmol homocysteine L^{-1}, 19.6 and 33.0 µmol L^{-1}, respectively. Those results are not totally in line with data recently published by a Japanese research group (Fukada et al., 2006; Setoue et al., 2008). After a feeding period of ten days, Fukada et al. (2006) found in response to 5000 and 10000 mg GAA kg^{-1} feed a considerably higher increase of plasma homocysteine in non-fasted male rats (255 and 421 % higher than in control animals, respectively; approximately 15 µmol L^{-1} homocysteine in controls). In rats fed 5000 mg GAA kg^{-1} feed, liver homocysteine concentrations increased around threefold within five to ten days (base level app. 1.2 nmol homocysteine g^{-1} liver). In another study, feeding a diet supplemented with 5000 mg GAA kg^{-1}

[26] Supplementary Information October 08/ Attachment I/ Annex I
[27] Supplementary Information October 08/ Attachment II/ Annex 15

feed and without choline addition over seven days resulted in a fourfold increase of plasma homocysteine levels and a fivefold increase of hepatic homocysteine concentrations, compared to a control group receiving 0.1 % dietary choline without GAA supplementation. However, the addition of 0.1, 0.25 and 0.5 % choline to the GAA diet gradually decreased plasma and liver homocysteine concentrations (Setoue et al., 2008).

5.2. Toxicological studies

5.2.1. Acute oral toxicity

A study was conducted in rats using the acute toxic class method (OECD 423).[28] Six rats were administered a dose of 300 mg GAA kg^{-1} bw by gavage as a suspension in deionised water; a further six rats received a dose of 2000 mg kg^{-1} bw. All animals were observed for 14 days. No adverse effects were seen, thus no classification of this substance is required regarding oral toxicity.

5.2.2. Mutagenicity and genotoxicity

Ames test

The potential mutagenicity of GAA was investigated in an Ames test conducted according to OECD guideline 471, in *Salmonella typhimurium* strains TA102, TA100, TA98, TA97a and TA1535 using the direct plate incorporation method.[29] Positive control materials demonstrated that the test was capable of detecting genotoxic effects. Test concentrations of 62, 185, 556, 1667 and 5000 µg/plate were used both with and without S-9 mix. A precipitate was noted when the test substance was added to the plates but this was no longer visible at the end of the test. The study showed no evidence of any mutagenic activity.

In vitro mammalian cytogenetics study

GAA was tested for ability to induce chromosome aberrations in human lymphocytes, in compliance with GLP and according to a protocol based on OECD guideline 473, with and without microsomal enzyme activation.[30] Exposure to concentrations up to 1.17 mg mL^{-1} lasted for three hours with metabolic activation and for 20 hours without; cells were harvested at about 20 hours. The highest dose tested is the maximum recommended dose under the guideline, being equivalent to 0.01M. There was no evidence for genotoxic effects in human lymphocytes exposed to the test article, with or without metabolic activation, at any dose.

5.2.3. Repeated dose toxicity

28-day study

Groups of five individually housed F344 rats of each sex were fed diets containing GAA at concentrations of 0, 0.05, 0.15, 0.5, 1.5 and 5.0 % for 28 days.[31] The dietary levels were calculated to equate to 0, 45, 135, 450, 1250 and 4400 mg kg^{-1} bw day^{-1} for males and 0, 46, 138, 455, 1260 and 3860 mg kg^{-1} bw day^{-1} for females. The study was conducted in compliance

[28] Technical Dossier/ IV 6
[29] Technical Dossier/ IV 10
[30] Technical Dossier/ IV 11
[31] Technical Dossier/ IV 12

with GLP and according to OECD guideline 407. Additional groups of five rats of each sex were fed either 0 or 5.0 % GAA for 28 days and then maintained for a further 14 days without treatment. The health and status of all animals was monitored daily and in detail once a week. Functional observations were made during the last week of treatment. Body weight and feed intake was monitored by weekly measurements. Peripheral blood samples were taken from all animals just prior to necropsy; a full range of haematological and clinical chemistry end points were measured on those samples. A full necropsy was carried out on all animals and tissues preserved for possible histopathological examination. Adrenal glands, brain, epididymides, heart, liver, kidneys, testes and thymus were weighed. Microscopic examination of tissues was confined to the highest dose and control animals.

Some evidence of adverse effects on clinical observations was recorded for the highest dose group. This was also reflected in slightly reduced feed intake and body weight gain in high-dose females. Clinical chemistry and haematological measurements also revealed effects of treatment at the highest dose, particularly in females. Homocysteine levels were increased in both sexes at the highest dose and in males at the 1.5 % dose. The only relevant findings recorded at necropsy were mineral deposits noted in ureters and urinary bladders of high-dose females.

90-day study

Groups of ten F344 rats of each sex were fed diets containing GAA at concentrations of 0, 0.05, 0.1, 0.3, 1.0 and 5.0 % for 90 days.[32] Additional groups of ten per sex received either the control diet or the highest dose of GAA for 90 days and then were kept without treatment for a further 28 days. Two additional rats of each sex received each treatment and were used for additional biochemical and tissue studies. The health status of animals was monitored daily; body weight and water intake were monitored weekly. Ophthalmoscopy examination was made on day 0 and day 85 on all animals from the main study. Peripheral blood samples were taken after fasting just prior to necropsy; a full range of haematological and clinical chemistry end points were measured on those samples. An additional non-fasting sample was taken from all animals on day 83 for measurement of homocysteine. Urine was collected from all animals during the last week of treatment and examined for markers of adverse effects. During the same week, the animals were examined for behaviour, motor activities and sensory reactivity using standard functional observation tests. The animals from the main study were brought to necropsy at day 91 (males) and day 92 (females) while the additional groups of high dose and control were necropsied at day 119. A full necropsy was carried out on all animals and tissues preserved for possible histopathological examination. Adrenal glands, brain, epididymides, heart, liver, kidneys, ovaries, spleen, testes, uterus and thymus were weighed. Microscopic examination of tissues was confined to the highest dose and control animals and to any animal showing a macroscopic abnormality. Frozen sections of liver from all animals of the high-dose and control groups were stained with oil red O for detection of lipid material.

One female animal from the high-dose group died during the study but death was considered unlikely to be related to treatment. There were generally no treatment-related differences in observations, although it was noted that in all high-dose animals there were crusts/crystals on the fur in the anogenital region. Functional observations and ophthalmoscopy showed no treatment-related differences. Body weight was reduced in both sexes at the highest dose, although the difference was not always significant. There was no corresponding difference in food intake. There were very small differences in MCHb and MCV in the high-dose animals after 90 days. Clinical chemistry analyses showed elevated potassium levels in high-dose males

[32] Supplementary information/ October 2008/ Attachment II /Annex II.1

and reduced triglycerides and cholesterol in at least one sex for the three highest-dose groups. At the highest dose, urea, homocysteine, alkaline phosphatase, alanine aminotransferase and γ-glutamyl transferase were all increased but at the 1 % dose the only difference seen were higher homocysteine levels. Most of the differences appear to have disappeared in the animals kept for four weeks after the end of the treatment. Urine of high-dose males was of higher volume and lower osmolarity, otherwise no differences were seen in urine measurements. Renal effects were seen in the macroscopic observation of high-dose females at necropsy, with calculi/concretions present in the bladder or ureters, causing secondary changes. The calculi were analysed and found to contain 96.9 % GAA. Differences in organ weights were seen only in high-dose animals and were not present when expressed relative to body weight, apart from reduced thymus weight and increased kidney weight. Kidney weight changes were still present after the reversal period. Histopathological examination revealed thymic atrophy in high-dose females and epithelial proliferation in the renal pelvis, urinary bladder and ureters of high-dose rats of both sexes. Special examinations of tissues from supplementary groups for cardiovascular and other changes show no effects of concern on the heart and confirm the presence of moderate hepatic fatty change and renal effects at the highest dose tested.

Effects seen at the highest dose of GAA provide indicators of the target effects, and the only change seen at lower doses and relevant to risk assessment is elevated homocysteine levels. Such effect is undesirable in a product directly consumed by humans but should also be avoided in target animals at the normal levels of use. However, it is unlikely that the proposed levels of use in animal feed would lead to elevated serum homocysteine in the target species.

The results of the 90-day study would indicate a NOAEL of 0.3 % (210 or 234 mg GAA kg^{-1} bw day^{-1} for males and females, respectively) based upon the elevated homocysteine levels. The use as a feed additive does not give rise to elevated homocysteine levels in the products from animals fed with CreAmino™ and there is no significant additional ingestion of GAA *per se* by consumers as a result of use. It is therefore concluded that for a feed additive assessment the homocysteine level is not of relevance and this end point can thus be ignored. Since no other effects were seen at the 1 % dose, the NOAEL from this study may be considered to be 1.0 % in the diet (685 or 754 mg GAA kg^{-1} bw day^{-1} for males and females, respectively).

5.3. Consumer safety assessment

Considering that the metabolism of GAA and the tissue deposition data, the content of GAA and creatine in edible tissues needs specific consideration.

Homocysteine in edible tissues also requires attention since in humans elevated plasma concentrations have been established as an important independent risk factor for the development of cardiovascular and arteriosclerotic diseases (Boushey et al., 1995; Selhub, 1999). Total plasma homocysteine values of ~10 μM for men and ~ 8 μM for women are considered normal. However, an increase by 5 μM in total plasma homocysteine is associated with an increased risk of coronary artery disease by 60 % for men and 80 % for women (Stead et al., 2001).

Additionally, the impurities present in the additive require separate attention.

5.3.1. GAA and creatine

Feeding CreAmino™ to chickens in doses up to 6000 mg GAA kg^{-1} feed resulted in a dose-related decrease of GAA concentrations in muscle tissue. This may indicate an adaptation of the enzyme systems to an increased GAA availability (inhibition of de-novo synthesis of

GAA). GAA in liver followed a similar tendency (although at concentrations up to 600 mg GAA kg^{-1} feed there was some evidence of accumulation).

CreAmino™ (at doses of 600 mg kg^{-1} feed and above) significantly increased the creatine content in breast meat in all trials in which creatine content was measured (trials I, IV, V and tolerance trial). The increase in muscle creatine expected from the use of GAA in broiler diets would be approximately 1 g kg^{-1} tissue resulting in a total creatine concentration which is within the physiological range of fresh meat from different animal species (pork, beef, poultry). CreAmino™ at use level did not influence the creatine content of chicken liver.

5.3.2. Homocysteine

The data provided on homocysteine levels in plasma of GAA treated animals is of very limited value but indicates that plasma homocysteine is not elevated at the applied dietary dose range of CreAmino™ (Tables 15 and 16). The data from tissues of rats available from the 90-day toxicity study is of questionable value but appears to support the above findings.[33]

Considering the metabolic behaviour of homocysteine, the level in tissues would be in principle reflected by plasma concentrations. The FEEDAP Panel concludes that the use of CreAmino™ at the proposed levels of use in chickens for fattening would not result in elevated homocysteine in edible tissues.

5.3.3. Impurities

The product is specified to contain two impurities: dicyandiamide (max. 0.5 %) and cyanamide (max. 0.03 %). Both those impurities have been independently assessed for toxicity and the applicant states that the ADI for cyanamide is 0.002 mg kg^{-1} bw day^{-1} while that for dicyandiamide is 1 mg kg^{-1} bw day^{-1}.[34]Although brief details of the studies conducted have been provided, the precise basis of the allocated ADI is not specified.[35] Both the ADIs stated by the applicant could be considered as provisional tolerable daily intake (PTDI). Consumer exposure to cyanamide and dicyandiamide may result from use in a range of different applications in food, feed, medicaments and pesticides.

A worst case calculation on the potential residues is based on the following assumptions: for a 500 g broiler tissue/organ intake, 1 kg feed is required, containing 1.2 g GAA; GAA contains dicyandiamide and cyanamide at the highest level and the impurities consumed are not metabolised and fully retained. The intake of dicyandiamide (6 mg day^{-1}) would amount to 10 % of the PTDI. The intake of cyanamide (0.360 mg day^{-1}) would amount to 300 % of the PTDI. A 10 % retention for cyanamide (derived from a metabolic study)[36] would be more appropriate. This being the case, the intake would decline to 30 % of PTDI. Limited analytical data revealed cyanamide concentration in poultry meat less than the LOD (17 µg kg^{-1}) up to 6000 mg GAA kg $feed^{-1}$.[37]

The FEEDAP Panel concludes that a maximum content of 0.5 % dicyandiamide and of 0.03 % cyanamide in GAA would not be of concern.

[33] Supplementary information/ October 2008/ Annex II 15
[34] Technical Dossier/ Supplementary information July 07/ Enclosure 3
[35] Technical Dossier/ Supplementary information July 07/ Enclosure 3.3 and 3.2
[36] Technical Dossier/ Supplementary information July 07/ Enclosure 3-6
[37] Technical Dossier/ Supplementary information July 07/ Enclosure 3-9

5.3.4. Conclusions

The mutagenicity/genotoxicity studies provided show sufficient evidence of the lack of any effects of concern in this respect. The effects reported in the 28- and 90-day studies generally reflect physiological responses to high exposures to a metabolic intermediate and do not identify any novel or unexpected toxicity. Feeding CreAmino™ would not result in GAA, creatine or homocysteine concentrations in chicken tissues relevant to consumer safety.

The FEEDAP Panel considers that the use of CreAmino™ as a feed additive for chickens for fattening at the levels proposed does not give rise to residues of concern to the consumer, neither from the active substance or the identified impurities.

5.4. Safety for the user

5.4.1. Irritation

GAA was assessed for dermal irritancy in compliance with GLP and according to OECD guideline 404.[38] The substance was applied slightly moistened with water to the skin of three rabbits for four hours. After the exposure period there was no evidence of any irritant or corrosive effect thus GAA does not require classification for skin irritancy.

GAA was assessed for eye irritancy in compliance with GLP and according to OECD guideline 405.[39] Approximately 0.1 ml of the dry substance was instilled into the conjunctival sac of three rabbits. After the exposure there was no evidence of any irritant or corrosive effect, thus GAA does not require classification for eye irritancy.

5.4.2. Skin sensitisation

GAA was tested in a guinea-pig maximisation assay using a treated group of 20 animals and a control group of ten, according to OECD guideline 406 and in compliance with GLP.[40] All animals showed no positive reaction on challenge, it is thus concluded that GAA is not a dermal sensitiser, under the conditions of the test.

5.4.3. Conclusion on user safety

The applicant describes the product as virtually dust free and provides particle size data showing that the proportion of particles < 5 μm is less than 0.2 %. However, this is not the generally accepted cut-off for the respirable fraction (10 μm). The data provided also indicate that < 4 % of the product consists of particles < 63 μm. It is likely therefore that the level of respirable particles is low but this has not been demonstrated. Since no data are provided on respiratory toxicity, a risk to the user from inhalation exposure cannot be excluded. This is consistent with the advice in the safety data sheet to wear a dust-protection mask. The FEEDAP panel therefore has no concerns regarding user safety for CreAmino™ provided that the recommendations of the safety data sheet are followed.

5.5. Safety for the environment

GAA is a physiological molecule and a natural component of animals. GAA from CreAmino™ would not lead to an increased faecal excretion of molecules which are not normally present in

[38] Technical Dossier/ Annex IV 7
[39] Technical Dossier/ Annex IV 8
[40] Technical Dossier/ Annex IV 9

the faeces of the target animals. The FEEDAP Panel concludes that the use of CreAmino™ as a feed additive would not pose a risk to the environment.

6. Post-market monitoring

The applicant will follow the requirements of the Feed Hygiene Regulation[41] and the rules of Good Manufacture Practice, including full traceability system complaint and emergency procedures. This being the case, the FEEDAP Panel does not see the need for specific requirements for a post-market monitoring plan.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The FEEDAP Panel concludes that 600 mg GAA kg^{-1} feed for chickens for fattening can be considered as the minimum dose improving performance characteristics. However, the Panel notes that the effects are most consistently seen at 800 mg GAA kg^{-1} feed, which is in line with the dose level recommended by the applicant..

The FEEDAP Panel notes that abdominal fat in chickens for fattening was significantly reduced at 1000 mg GAA kg^{-1} complete feed and above. Due to inconsistent effects, no conclusions could be drawn regarding breast muscle yield, drip loss and post-mortem pH.

The FEEDAP Panel considers that safety for the applied dose range of 600 to 1200 mg kg^{-1} feed has not been demonstrated. The lowest proposed dose, 600 mg GAA kg^{-1} feed, could be considered safe, 1200 mg GAA was not tested and 1500 mg shows already adverse effects.

The mutagenicity/genotoxicity studies provided show sufficient evidence of the lack of any effects of concern in this respect. The effects reported in the 28- and 90-day studies generally reflect physiological responses to high exposures to a metabolic intermediate and do not identify any novel or unexpected toxicity.

The FEEDAP Panel considers that the use of CreAmino™ as a feed additive for chickens for fattening at the levels proposed does not give rise to residues of concern to the consumer, neither from the active substance or the identified impurities.

The FEEDAP panel has no concerns regarding user safety for CreAmino™ provided that the recommendations of the safety data sheet are followed.

The FEEDAP Panel concludes that the use of CreAmino™ as a feed additive would not pose a risk to the environment.

RECOMMENDATIONS

In the view of the FEEDAP Panel, the functional group ‘amino acids, their salts and analogues’ describes substances which finally enter the metabolism of the body as amino acids and as such take part in the protein synthesis pathways.

GAA does not play a comparable role. Although resulting from the amino acid metabolism (arginine + glycine → ornithine + GAA) GAA is exclusively converted to creatine, which serves, in its phosphorylated form, as a dynamic reservoir of high energy phosphate in exchange with ATP. Grouping GAA under ‘amino acids, their salts and analogues’ would therefore not be consistent with the logic behind this functional group.

[41] OJ L35, 12.01.2005, p. 1

The FEEDAP Panel also does not consider creatine as an essential nutrient which, itself or in the form of a direct precursor (GAA), must be contained in the diet (i.e. nutritional additive).

The FEEDAP Panel recommends to incorporate under specification in the Annex entry maximum 0.5 % dicyandiamide and 0.03 % cyanamide.

DOCUMENTATION PROVIDED TO EFSA

1. Creamino. February 2007. Submitted by AlzChem Trostberg Gmbh
2. Supplementary information.July 2007. Submitted by AlzChem Trostberg Gmbh
3. Supplementary information. November 2007. Submitted by AlzChem Trostberg Gmbh
4. Supplementary information. October 2008. Submitted by AlzChem Trostberg Gmbh
5. CRL evaluation report on the analytical methods of Creamino
6. Comments from the Member States received through the EFSAnet

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APPENDICES

APPENDIX A

Executive Summary of the Evaluation Report of the Community Reference Laboratory Feed Additives on the Method(s) of Analysis for Creamino for broilers and turkeys

In the current application authorisation is sought for guanidinoacetic acid (GAA) under the category 'nutritional additives', functional group 'amino acids, their salts and analogues', according to the classification system of Annex I of Regulation (EC) No 1831/2003.

The applicant intends to place GAA on the market as a granular product containing at least 96 % of GAA and at maximum 1 % of starch as granulation agent under the trade name CreAmino™. The product is intended being mixed into feedingstuffs for broilers and turkeys at a minimum concentration of 300 mg/kg and at a recommended concentration of 600 mg/kg of GAA in complete feedingstuffs.

For the determination of the active substance in the *product* and in *feedingstuffs,* the same single-laboratory validated method is proposed, which is based on ion chromatography (IC) method coupled to ultraviolet-visible (UV-VIS) detection operated at a wavelength of 200 nm. Method performance characteristics were determined by conducting replicate analyses of *CreAmino*™ and two types of feed samples containing the active substance at different concentrations. The limit of detection (LOD) and limit of quantification (LOQ) was 20 mg/kg and 55 mg/kg, respectively. The relative standard deviation for repeatability (RSD_r) was 0.1 % for the feed additive and varied from 0.77 % to 2.86 % for the feed samples depending on the concentration of the active substance. The values for the recovery rate were in all cases above 97 %. Also the ruggedness of the method was confirmed by analysing feed samples of different composition on different instruments and days. The performance characteristics are considered acceptable and therefore the method is suitable for official control purposes within the frame of the authorisation.

For the determination of the active substance in *premixtures,* the applicant proposed a different method which has also been single-laboratory validated and which is based on high performance liquid chromatography (HPLC) coupled to ultraviolet-visible (UV-VIS) detection operated at a wavelength of 210 nm. The validation of the method was performed on typical premixtures with supplementation levels of the active substance at 1, 5, 25 and 50 %, respectively. The obtained values for the recovery rate varied between 96 and 108 % and the obtained values for the RSD_r ranged from 0.6 to 1.4 %. All methods have also been successfully tested by a second independent analytical laboratory. The performance characteristics are considered acceptable and therefore the method is considered suitable for the intended purpose.

For the determination of GAA in *muscle tissues*, *liver* and *kidney* an IC method is proposed which is based on the same principle as the method for the determination of GAA in the *product* and in the *feedingstuffs*. The method was single-laboratory validated on the target tissues, obtaining acceptable values for the recovery rate and the precision. The limit of quantification (LOQ) was 0,8 mg/kg in *muscle tissues* and 1,6 mg/kg in *liver* and *kidney*. Since the applicant did not propose Maximum Residue Levels of GAA in the target tissues, the CRL is unable to comment on the suitability of the proposed method for official control purposes.

Further testing or validation is not considered necessary.

APPENDIX B

Amendment to the Executive Summary of the Evaluation Report of the Community Reference Laboratory Feed Additives on the Method(s) of Analysis for Creamino for broilers and turkeys

Based on the proposed register entry regarding the dosage (minimum content for the active substance in complete feedingstuffs of 300 mg/kg), the CRL-FA recommend in the above mentioned report a single-laboratory validated method which has been proposed by the applicant. The method is based on ion chromatography (IC) method coupled to ultraviolet-visible (UV-VIS) and was considered suitable for the official control of active substance (guanidinoacetic acid) in complete feedingstuffs. With the present amendment the CRL-FA confirms the validity of this statement also for the modified target content of the active substance in complete feedingstuff, with 600 mg/kg for the minimum and 1200 mg/kg for the maximum concentration.